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(54) Title: A PRE-FABRICATED ELECTRON MICROSCOPY GRID FOR MONOLAYER PURIFICATION AND METHODS AND KITS THEREFOR

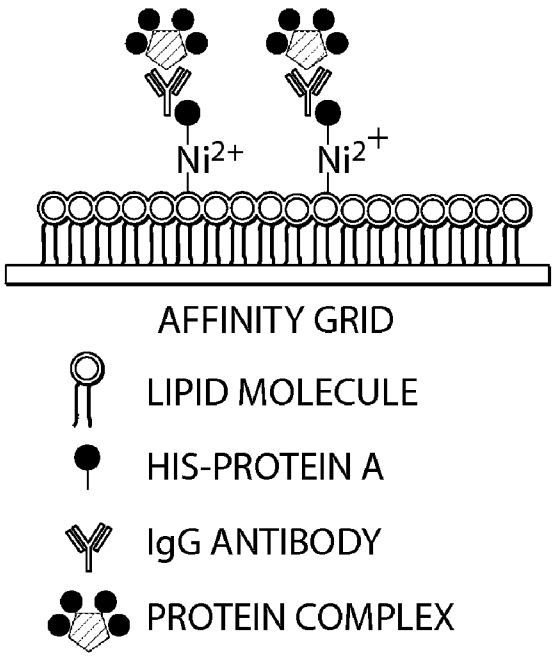

Figure 6

(57) Abstract: An apparatus, methods and kits for "monolayer purification" as a rapid and convenient technique and article of manufacture to produce specimens of His-tagged, biotin-tagged or other affinity tagged proteins or macromolecular complexes or untagged proteins for single particle electron microscopy (EM) without prior biochemical purification are provided. The pre- fabricated EM grid ("Affinity Grid") features a dried lipid monolayer that contains for example Ni-NTA lipids (lipids functionalized with a Nickel-nitrilotriacetic acid group) or biotin-lipids. The grid can be stored for several months or longer under ambient conditions, a feature that further simplifies and extends the use of monolayer purification and the convenience of EM analyses. Affinity Grids for isolating untagged complexes and for tandem affinity purification (TAP) are provided.

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A pre-fabricated electron microscopy grid for monolayer purification and methods and kits therefor

Related Application

[001] This application claims the benefit of U.S. provisional application serial number 61/130,094 filed May 28, 2008 in the U.S. Patent and Trademark Office, which is hereby incorporated by reference herein in its entirety.

Technical Field

[002] An article of manufacture that is an affinity EM grid, and methods and kits for rapid reliable electron microscopy analyses are provided.

Government Support

[003] The invention was supported in part by a grant GM62580 from the National Institutes of Health grant. The government has certain rights in the invention.

Background

[004] Biochemical purification of proteins and macromolecular complexes has become the rate-limiting step in structure determination by single particle cryo-electron microscopy (EM). A purification protocol of a recombinant protein for structural studies typically consists of an affinity chromatography step followed by size exclusion chromatography. Such a two-step purification scheme requires an appreciable amount of protein, which may not always be available, and takes several hours, during which biological complexes can disintegrate. Improved materials and methods for EM analysis are needed.

Summary

[005] An aspect of the invention provides a pre-fabricated electron microscopy (EM) grid article of manufacture, the article having a mesh; a carbon layer; and a lipid monolayer in contact with the carbon layer; and further comprising a thin layer of carbon evaporated onto the lipid monolayer. In general, a portion of the lipid monolayer includes a modified lipid molecule having an affinity moiety covalently attached, and the article is variously referred to herein as an EM grid or an "Affinity Grid" for that reason. The lipid monolayer further includes at least one neutral phospholipid. The carbon layer is either a continuous carbon film (CC) or a holey carbon film (HC).

[006] In exemplary embodiments of the article, the modified lipid molecules include a Nickel (II) Nitriloacetic acid (Ni-NTA) group or a biotin group. These affinity moieties make the article convenient for EM analysis of a wide range of complex cell structures that include a protein component modified by recombinant techniques to display an affinity ligand corresponding to the particular Affinity Grid. For example, the modified lipid containing a Ni-NTA group is 1,2-dioleoyl-*sn*-glycero-3-[N(5-amino-1-carboxypentyl)iminodi acetic acid] succinyl-nickel salt (Ni-NTA lipid).

[007] In general, the neutral phospholipid is 1,2-dilauryl-*sn*-glycero-3-phosphatidlycholine (DPLC). An exemplary proportion of Ni-NTA lipid in the lipid monolayer is about 0.5% to about 40%. The mesh includes at least one material selected from the group consisting of: copper, gold, nickel, silver, molybdenum, rhodium, titanium, steel, tungsten, nylon, and a mixture thereof. The lipid monolayer of the pre-fabricated EM grid in certain embodiments further includes a fluorinated lipid.

[008] The pre-fabricated EM grid has been found to be stable when stored under ambient conditions of room temperature and humidity for at least about six months. This property greatly simplifies and makes more efficient EM analysis, as preparative time is greatly reduced, and large complex cell structures can be obtained from cells with a preserved quarternary structure and can be visualized in an essentially pure condition.

[009] Another aspect of the invention provided herein is a method of manufacturing a pre-fabricated electron microscopy (EM) grid, the method including:

adding a solution of at least one lipid in chloroform to an aqueous solution in a container to form a lipid monolayer and contacting the lipid monolayer with a mesh, such that the mesh further comprises a carbon layer comprising a continuous carbon film or holey carbon film, whereby at least a portion of the lipid monolayer is transferred to the carbon layer on an upper surface of the mesh, and removing the mesh and associated carbon layer and lipid monolayer from the container and blotting and air drying the mesh; and

evaporating a thin layer of carbon onto the holey carbon film, thereby manufacturing the pre-fabricated EM grid.

[010] In general, at least one lipid further comprises an affinity moiety, and examples are a Nickel (II) Nitriloacetic acid (Ni-NTA) group and a biotin group. The Ni-NTA group is exemplified by 1,2-dioleoyl-*sn*-glycero-3-[N(5-amino-1-carboxypentyl)iminodi acetic acid] succinyl-nickel salt (Ni-NTA lipid). Thus, the solution of the at least one lipid is exemplified by 1,2-dioleoyl-*sn*-glycero-3-[N(5-amino-1-carboxypentyl)iminodi acetic acid] succinyl-nickel salt (Ni-NTA lipid) in chloroform.

[011] In general according to the method, the aqueous solution is a buffer. The mesh is at least one material selected from the group consisting of: copper, gold, nickel, silver, molybdenum, titanium, rhodium, steel, tungsten, nylon, and mixtures thereof. The proportion of Ni-NTA lipid in the lipid monolayer is about 0.5% to about 40%.

[012] Yet another embodiment of the invention provided herein is a method of preparing a biological sample for analysis by electron microscopy (EM), the method including the steps of:

incubating a pre-fabricated EM grid provided as described above, with a biological sample, such that at least one component of the sample binds to the pre-fabricated EM grid;

blotting to remove excess sample; and,

visualizing the at least one bound component on the grid by EM analysis.

The EM grid has a lipid monolayer further having a modified lipid wherein the modified lipid molecules comprise at least one of a Nickel (II) Nitriloacetic acid (Ni-NTA) group and a biotin group. For example, the modified lipid is 1,2-dioleoyl-*sn*-glycero-3-[N(5-amino-1-carboxypentyl)iminodi acetic acid] succinyl-nickel salt (Ni-NTA lipid).

[013] The method in various embodiments further includes, prior to incubating the pre-fabricated EM grid with the sample, adding imidazole to the sample. In certain embodiments the method includes eluting the at least one bound component of the sample from the grid with a reagent. For example, the reagent is selected from the group of: imidazole; a divalent metal ion chelator; a solution at a pH lower than about 6; and a poly-histidine containing peptide.

[014] Affinity of the EM grid for a cell component containing an affinity ligand is so substantial that the method is useful for a sample that is a cell lysate. Alternatively, the sample is a partially purified protein solution. Thus, the cell lysate (or the partially purified protein solution) includes at least one recombinant protein having an affinity ligand that binds with specificity to the modified lipid. Examples of the affinity ligand is that it is at least one genetic change encoding an insertion or addition selected from poly-histidine, Streptavidin, Avidin, Flag, Myc, Hemagglutinin, Glutathione S-transferase, Green fluorescent protein, Calmodulin, Calmodulin binding protein, tandem affinity tags, and fragments thereof.

[015] The articles of manufacture, methods and kits provided herein are suitable for visualizing and isolating proteins and protein complexes directly from a cell lysate, even non-recombinant proteins. Thus the methods herein include, after manufacturing the EM grid and prior to incubating the grid with the biological sample, first incubating the grid with His-tagged protein A, and then incubating the grid that has bound the His-tagged protein A with an IgG antibody specific for the component of interest in the biological sample. The resulting Affinity

Grid of this embodiment is universally useful for purifying from a crude extract and visualizing any protein, as long as there is an antibody specific for that protein.

[016] Alternatively, the methods herein include, after manufacturing the EM grid as above and prior to incubating the EM grid with the biological sample, first incubating the grid with Calmodulin, then incubating with a biological sample containing a TAP-tagged protein or protein complex, in which the TAP-tagged protein includes a calmodulin binding protein moiety.

[017] Yet another aspect of the invention herein provides a kit for electron microscopic analysis having a pre-fabricated EM grid as described above, a container and instructions for use in EM analysis of a biological sample. The kit in various embodiments further includes at least one reagent for adding to a cell lysate or for eluting a protein from the grid.

Brief description of the drawings

[018] Figure 1 is a set of electron micrographs showing a pre-fabricated EM grid ("Affinity Grid") and monolayer purification of a Tf-TfR complex from an extract of Sf9 cell.

[019] Figure 1 panels A and B are electron micrographs of Tf-TfR complex that was added to Sf9 cell extract containing 60 mM imidazole and further prepared by monolayer purification using either a 20% Ni-NTA lipid monolayer (A), or using a 20% HC Affinity Grid (B), respectively. The Tf-TfR complexes were observed to be more evenly distributed on the pre-fabricated EM grid. Scale bar is 30 nm.

[020] Figure 1 panel C is a photo of an SDS-PAGE gel stained by Simply Blue. Lane 1: cell extract containing Tf-TfR complex; lane 2: protein eluted from 20 monolayer purification samples; lane 3: protein eluted from 20 pre-fabricated EM mesh grids.

[021] Figure 1 panel D is a photo of a Western blot analysis of the gel shown in Figure 1 panel C developed with anti-His antibody to detect His-tagged TfR. The band at ~75 kDa represents monomeric TfR, and the band at ~150 kDa represents dimeric TfR.

[022] Figure 2 is a set of electron micrographs and drawings of the pre-fabricated EM grid preparation of ribosomal complexes.

[023] Figure 2 panel A is an electron micrograph of negatively stained ribosomal complexes adsorbed to a 2% CC pre-fabricated EM grid from an *E. coli* cell lysate containing 60 mM imidazole.

[024] Figure 2 panel B is an electron micrograph of vitrified ribosomal complexes adsorbed to a 20% HC pre-fabricated EM grid from the same *E. coli* cell lysate. The image

shows mRNA emanating from the ribosomal complexes. The arrowheads point to 30S ribosomal subunits.

[025] Figure 2 panel C is an electron micrograph of vitrified ribosomal complexes prepared by lipid monolayer purification using a 20% Ni-NTA lipid monolayer. No mRNA strands are visible.

[026] Figure 2 panel D is an electron micrograph of vitrified ribosomal complexes adsorbed to a 20% HC pre-fabricated EM grid from an *E. coli* cell lysate that was treated with RNAse A. No mRNA strands are visible. Scale bar is 30 nm.

[027] Figure 2 panels E and F are drawings of the two different methods used to prepare ribosomal complexes with pre-fabricated EM grids, respectively. Either a drop of cell extract was pipetted onto a pre-fabricated EM grid (panel E), or the pre-fabricated EM grid was placed on top of cell extract contained in a well of a teflon block (panel F).

[028] Figure 3 panels A - L are a set of electron micrographs, drawings and graphs showing three dimensional (3D) reconstructions of vitrified ribosomal complexes purified on pre-fabricated EM grids.

[029] Figure 3 panels A - D are 3D reconstructions of the 50S ribosomal subunit showing, in panel A, representative class averages, in panel B, the angular distribution plot, in panel C, the FSC curve indicating a resolution of 21 Å, and in panel D, different views of the final density map (pdb code: 1ML5, chains a – x; Klaholz BP et al. 2003 Nature 421, 90-94).

[030] Figure 3 panels E - H are 3D reconstruction of the 30S ribosomal subunit showing, in panel E, representative class averages, in panel F, the angular distribution plot, in panel G, the FSC curve indicating a resolution of 24 Å, and in panel H, different views of the final density map with fit crystal structures (pdb code: 1ML5, chains A and C – X; Klaholz BP et al. 2003 Nature 421, 90-94).

[031] Figure 3 panels I – L are 3D reconstructions of the 70S ribosome showing, in panel I, representative class averages, in panel J, the angular distribution plot, and in panel K, the FSC curve indicating a resolution of 28 Å, and in panel L, different views of the final density map with fit crystal structures (pdb code: 1ML5, all chains; Klaholz BP et al.2003 Nature 421, 90-94). The side length of individual panels in panels, A, E and I is 38 nm. The scale bar in panel D is 5 nm.

[032] Figure 4 is a set of electron micrographs and photographs of SDS gels showing pre-fabricated EM grid purification of His-tagged AQP9.

[033] Figure 4 panel A is an electron micrograph of negatively stained Sf9 membrane extract.

[034] Figure 4 panel B is an electron micrograph of negatively stained AQP9 purified by Ni-affinity and gel filtration chromatography.

[035] Figure 4 panel C is an electron micrograph of negatively stained AQP9 adsorbed to a 2% CC pre-fabricated EM grid from Sf9 membrane extract containing 60 mM imidazole. Scale bar is 30 nm.

[036] Figure 4 panels D and E are photographs of a Simply Blue stained SDS-PAGE gel and Western blot, respectively, detecting His-tagged AQP9 (lanes 1: AQP9 purified by conventional chromatographic methods; lane 2: AQP9 eluted from pre-fabricated EM grids).

[037] Figure 4 panels F and G show representative class averages of negatively stained AQP9 purified by conventional chromatographic methods or by adsorption to a pre-fabricated EM grid, respectively. Side length of individual panels is 26 nm.

[038] Figure 5 is a set of class averages of vitrified complexes containing His-tagged rpl3 on 20% HC Affinity Grids. The 200 class averages were obtained from classification of 52,507 particle images of vitrified rpl3-complexes. The side length of the individual panels is 38 nm. Class averages corresponding to projections of the 50S ribosomal subunit are marked by a white asterisk, those corresponding to projections of the 70S ribosome by a white square, and those corresponding to projections of the 30S ribosomal subunit by a white circle.

[039] Figure 6 is a drawing of an EM Affinity grid for isolating untagged protein complexes. A Ni-NTA lipid monolayer was first coated onto an EM grid. His-tagged protein A was then added to the grid followed sequentially by an IgG antibody preparation specific for the protein of interest, then a biological sample containing the protein of interest. The biological sample did not contain recombinant or tagged proteins. The antibody was observed to specifically bind to untagged proteins within minutes, and results showed that the biological complexes were purified.

[040] Figure 7 is an electron micrograph image of negatively stained mammalian ribosomal complexes purified with an antibody-bound Affinity Grid by the method shown in Figure 6. His-tagged protein A was incubated with a 2% CC affinity Grid followed by incubation with an IgG antibody which is specific for binding subunit 26 the 60S ribosomal complexes. Native complexes were observed to be purified within 5 minutes (white circles). Scale bar is 50 nm.

[041] Figure 8 is a drawing of an EM Affinity Grid used for tandem affinity purification (TAP), shown by isolating TAP-tagged protein complexes. An exemplary Ni-NTA containing Affinity Grid is incubated with and binds His-tagged calmodulin, to form a fixed calmodulin adaptor on the surface of the grid. TAP-tagged complexes containing a CBP tag are isolated as they then bind to the fixed calmodulin adaptor.

Detailed Description

[042] A pre-fabricated EM grid, the "Affinity Grid", was used in examples herein to isolate, within minutes, ribosomal complexes from *E. coli* cell extracts containing His-tagged rpl3, the human homolog of the *E. coli* 50S subunit rplC. Depending on the precise preparation technique, ribosomal complexes with or without associated mRNA were prepared. Images of vitrified specimens were used to calculate three-dimensional reconstructions of the 50S ribosomal subunit as well as the 70S ribosome and 30S ribosomal subunit. In addition, it was observed that the grids were stable in the presence of glycerol and detergents. This feature allowed isolation of His-tagged aquaporin-9 (AQP9) from detergent-solubilized membrane fractions of Sf9 insect cells. The Affinity Grid was thus used to prepare single particle EM specimens of soluble complexes and membrane proteins.

[043] Monolayer purification is a technique that combines protein purification with specimen preparation, as a fast and easy way to prepare specimens suitable for single particle EM, requiring only low expression levels of His-tagged protein (Kelly DF et al. 2008 Proc Natl Acad Sci USA 105, 4703-4708). In the monolayer purification technique, an extract of cells expressing a His-tagged protein, which can be a subunit of a larger assembly, is overlaid with a lipid monolayer that contains Ni-NTA lipid, a lipid whose head group is functionalized by Nickel-nitrilotriacetic acid (Ni-NTA). The Ni-NTA lipids recruit the His-tagged proteins to the lipid monolayer, and the proteins are then lifted off with an EM grid covered with a continuous or holey carbon support film and are prepared by negative staining or vitrification for subsequent imaging in the electron microscope.

[044] Pre-fabricated EM grids (referred to as "Affinity Grid") having a dried, Ni-NTA lipid-containing monolayer are here provided. It is shown herein using the pre-fabricated EM grids that a complex of His-tagged transferrin and transferrin receptor (Tf-TfR) was obtained, and that the specimens obtained were equivalent to or better than those obtained by monolayer purification. Although the pre-fabricated EM grids can be incubated with sample for several hours, a brief incubation time of only a few minutes is shown herein to suffice to produce suitable EM specimens. Further, the pre-fabricated grids are generally resistant to most

detergents, for incubation during a time period that depends on the detergent concentration, making them useful for isolating membrane proteins. In addition, Affinity Grids were used to isolate ribosomal complexes from an *E. coli* extract and the water channel AQP9 from a membrane extract of Sf9 insect cells. Since the pre-fabricated grids were stored under ambient conditions for several months without loss of function, they can be pre-fabricated and used whenever needed to prepare specimens for single particle EM, within minutes and with minimum effort.

[045] A portion of the examples herein have been published in two papers, Kelly DF et al. 2008 Mol Biol 382, 423-433, and Kelly DF et al. 2008 Proc. Natl. Acad. Sci. USA 105, 4703-4708, both of which are hereby incorporated herein by reference in their entireties.

[046] A skilled person will recognize that many suitable variations of the methods may be substituted for or used in addition to those described above and in the claims. It should be understood that the implementation of other variations and modifications of the embodiments of the invention and its various aspects will be apparent to one skilled in the art, and that the invention is not limited by the specific embodiments described herein and in the claims. Therefore, it is contemplated to cover the present embodiments of the invention and any and all modifications, variations, or equivalents that fall within the true spirit and scope of the basic underlying principles disclosed and claimed herein.

Examples

Example 1. Expression of each of proteins rpl3, AQP9, TfR and production of Tf-TfR complex

[047] Rpl3 was expressed in *E. coli*, TfR was expressed in 293-T cells, and the Tf-TfR complex was produced as described in Kelly DF et al. 2008 Proc Natl Acad Sci USA 105, 4703-4708 incorporated herein by reference in its entirety. AQP9 was expressed in Sf9 cells as described in Viadiu H et al. 2007 J Mol Biol 367, 80-88.

Example 2. Preparation of cell and membrane extracts

[048] Sf9 and *E. coli* cell extracts were prepared as described in Kelly DF et al. 2008 Proc Natl Acad Sci USA 105, 4703-4708. Sf9 membrane extract was produced by centrifugation of 50 ml Sf9 cell extract at 100,000g for 30 minutes at 4°C. The pellet containing the cell membranes was homogenized in 100 ml buffer (20 mM Tris, pH 8.0, 300 mM NaCl) containing 2% octyl-β,D-glucoside (OG; Anatrace, Inc., Maumee, OH). The homogenate was centrifuged at 100,000g for 30 minutes at 4°C, and the supernatant was used for pre-fabricated EM Grid examples herein.

Example 3. Conventional purification of His-tagged Tf-TfR complex

[049] Ni-affinity purification of His-tagged Tf-TfR complex was performed as described in Kelly DF et al. 2008 Proc Natl Acad Sci USA 105, 4703-4708.

Example 4. Preparation of pre-fabricated EM Affinity Grids by methods of manufacture herein

[050] Each of 1,2-dilauryl-*sn*-glycero-3-phosphatidylcholine (DLPC) and 1,2-dioleoyl-*sn*-glycero-3-[N(5-amino-1-carboxypentyl)iminodi acetic acid] succinyl-nickel salt (Ni-NTA lipid) was purchased from Avanti Polar lipids (Alabaster, AL). Each lipid was reconstituted in chloroform to a concentration of 1 mg/ml. A 25-µl buffer aliquot (20 mM Hepes, pH 7.9, 150 mM NaCl) was placed into the well of a Teflon block, and 1 µl of a lipid mixture (DLPC containing the desired percentage of Ni-NTA lipid in chloroform) was added on top of the aqueous solution to form a lipid monolayer at the air-water interface. The Teflon block was incubated in a sealed humid environment at 4°C for 15 minutes.

[051] A copper EM grid (400 mesh, Ted Pella, Redding, CA) covered with a continuous carbon film or a Quantifoil 2/1 holey carbon grid (400 mesh, Quantifoil Micro Tools GmbH, Germany) was placed onto the lipid monolayer. The grid was gently lifted off with forceps, blotted from the side (perpendicular to the grid) with Whatman #1 filter paper (Whatman International Ltd, Middlesex, England) and allowed to air-dry. In the case of Quantifoil 2/1 holey carbon grids, a thin layer of carbon was evaporated onto the carbon side of the grid for stabilization during storage.

[052] Pre-fabricated EM grids prepared according to the methods herein were stored for at least about 6 months in a grid box at ambient temperature and humidity before use. To specify the type of pre-fabricated EM grids used for each set of experiments herein, the following nomenclature is used herein: X% CC/HC pre-fabricated EM grid, wherein the percentage X denotes the proportion of Ni-NTA lipid in the monolayer, and CC and HC, respectively, denote continuous carbon film and holey carbon film, respectively.

Example 5. Pre-fabricated EM grid purification of His-tagged Tf-TfR complex, ribosomal complexes and AQP9

[053] A sample of Tf-TfR complex (2 µl of Tf-TfR complex (0.03 mg/ml) in 20 mM Hepes, pH 7.4, 150 mM NaCl) was added to 38 µl of Sf9 cell extract (6 mg/ml). This mixture was diluted 1:10 using the same buffer, and a 3 µl aliquot was applied to a 20% HC Affinity Grid. The sample was incubated on the grid for 2 minutes prior to blotting and vitrification. Similarly a, 2 µl sample of Tf-TfR complex was added to 38 µl of buffer solution (20 mM Hepes, pH 7.4, 150 mM NaCl) containing either glycerol (1 – 5% final concentration) or one of the following detergents: 1 - 5% OG (Anatrace), 0.2% n-decyl-ß,D-maltoside (DM; Anatrace),

0.02% n-dodecyl-ß,D-maltoside (DDM; Anatrace), 0.03% Triton X-100 (EMD Biosciences, San Diego, CA), 0.014% Tween 20 (EMD Biosciences), 0.5% CHAPS (Anatrace), 0.2% Fos-choline 11 (Anatrace), or 0.1% digitonin (Sigma-Aldrich, St. Louis, MO). Further, 3 µl of a 1:10 dilution of Tf-TfR complex in solutions containing either one of these detergents or glycerol was added to a 2% CC pre-fabricated EM grid and incubated for various times prior to negative staining.

[054] Ribosomal complexes were analyzed as follows: 60 mM imidazole and 20 mM $MgCl_2$ (final concentrations) were added to 1 ml of *E. coli* extract containing expressed His-tagged rpl3. Then 3 µl of this mixture was placed on a 2% CC pre-fabricated EM grid (for negative staining) or on a 20% HC pre-fabricated EM grid (for vitrification). Samples were incubated for 2 minutes, blotted from the side and either negatively stained or vitrified.

[055] In addition, 25-µl aliquots of the same mixture were placed into two tubes, and 1 µl of RNAse A (Ambion, Inc., Austin, TX; ~ 0.5 units) was added to one tube and 1 µl of buffer was added to the other tube. The samples were incubated for 30 minutes at 4°C. Two 10-µl aliquots from each tube were then placed into wells of a teflon block. One of the two wells from each sample was overlayed with a 20% Ni-NTA lipid monolayer, and 20% HC Affinity Grids were placed on top of the other two wells and later recovered. In parallel, 3 µl aliquots of the mixtures with and without RNAse A were added to two 20% HC Affinity Grids. All samples were incubated for 30 minutes. For the samples in the teflon block, monolayers were recovered using Quantifoil holey carbon grids, and the pre-fabricated EM grids were simply lifted off the wells. All samples were vitrified.

[056] AQP9: 60 mM imidazole (final concentration) was added to 1ml of the solubilized Sf9 membranes and 3 µl of this mixture was placed on a 2% CC pre-fabricated EM grid and incubated for 5 minutes. The grid was washed with 7 successive drops of MilliQ water prior to negative staining.

Example 6. Sample elution from pre-fabricated EM grids

[057] A sample was prepared on a 20% CC pre-fabricated EM grid, excess solution was blotted off with filter paper and the grid was then incubated for 2 minutes with 20 µl of 300 mM imidazole. The 20-µl drop was recovered using a pipetteman and added to the next pre-fabricated EM grid sample. In this way, proteins adsorbed to 20 pre-fabricated EM grids were eluted into the same 20-µl volume of 300 mM imidazole.

Example 7. SDS-PAGE, Western blotting and mass spectrometry

[058] SDS-PAGE: Samples were run on 10% SDS-PAGE gels and stained either with Coomassie blue or Simply Blue stain (Invitrogen Corporation, Carlsbad, CA).

[059] Western blotting: His-tagged proteins were detected with anti-His antibody (GE Healthcare, Buckinghamshire, UK) and were developed by the alkaline phosphatase method using the Sigma Fast system (Sigma-Aldrich, St. Louis, MO).

[060] Protein assay: Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL).

[061] Mass spectrometry: Entire lanes were excised from Simply Blue stained SDS-PAGE gels and were analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS) in the Taplin Biological Mass Spectrometry Facility at Harvard Medical School.

Example 8. Specimen preparation

[062] Negative staining: For conventionally purified protein samples, grids were negatively stained with 0.75% uranyl formate as described in Ohi M et al. 2004 Biol Proced Online 6, 23-34, and grids of monolayer-purified samples were stained as described in Kelly DF et al. 2008 Proc Natl Acad Sci USA 105, 4703-4708. For pre-fabricated EM grids, 3-μl sample aliquots were placed on the Affinity Grids and incubated for various times. The grids were blotted from the side, washed with one drop of 0.75% uranyl formate and stained for 20 seconds with another drop of 0.75% uranyl formate. Pre-fabricated EM grids of specimens in detergent solution were washed with 7 drops of MilliQ water before staining with 0.75% uranyl formate, and grids of specimens in glycerol solution were washed with 15 drops of MilliQ water.

[063] Vitrification: Grids of monolayer-purified samples were vitrified as described in Kelly DF et al. 2008 Proc Natl Acad Sci USA 105, 4703-4708, hereby incorporated by reference herein in its entirety. For pre-fabricated EM grids, 3-μl sample aliquots were placed on pre-fabricated EM grids, which were blotted for 3 seconds and plunged into liquid ethane using a Vitrobot (FEI Company, Hillsboro, Oregon) operating at 22°C and 65% relative humidity.

Example 9. Electron microscopy

[064] Negatively stained specimens were imaged in an FEI Tecnai 12 electron microscope (FEI, Hillsboro, OR) equipped with a LaB_6 filament and operated at an acceleration voltage of 120 kV. Images were recorded on imaging plates under low-dose conditions at a nominal magnification of 67,000x and a defocus value of about −1.5 μm. Imaging plates were scanned with a Ditabis scanner (Pforzheim, Germany) using a step size of 15 μm, a gain setting of 20,000 and a laser power setting of 30%. The images were binned over 2 x 2 pixels for a final sampling of 4.5 Å/pixel at the specimen level.

[065] Grids of vitrified specimens were transferred into an FEI F20 electron microscope equipped with a field emission gun using an Oxford cryo-specimen holder, maintaining a temperature of –180°C. Samples were examined at an acceleration voltage of 200 kV and images were recorded on Kodak SO-163 film at a nominal magnification of 50,000x using low-dose procedures and a defocus ranging from –2 to –4 μm. Film negatives were developed for 12 minutes with full-strength Kodak D-19 developer at 20°C. Micrographs were digitized with a Zeiss SCAI scanner (Carl Zeiss Inc., Oberkochen, Germany) using a step size of 7 μm and binned over 3 x 3 pixels for a final sampling of 4.2 Å/pixel at the specimen level.

Example 10. Image processing

[066] Ribosomal complexes: WEB, the display program associated with the SPIDER software package (Frank J et al. 1996 J Struct Biol 116, 190-9), was used to select 52,507 particles from 274 images of vitrified specimens of ribosomal complexes, in which mRNA could also be seen. The particles were windowed into individual images of 90 x 90 pixels. Using the SPIDER software package, the particles were low-pass filtered to 20 Å, rotationally and translationally aligned and subjected to 10 cycles of multi-reference alignment. Each round of multi-reference alignment was followed by K-means classification into 200 classes. The references used for the first multi-reference alignment were randomly chosen from the raw images.

[067] The EMAN software package (Ludtke SJ et al. 1999 J Struct Biol 128, 82-97) was used to calculate reference volumes filtered to 30 Å resolution based on the atomic model of the 70S ribosome (pdb code: 1ML5; Klaholz BP et al. 2003 Nature 421, 90-94) for the 70S ribosome (all chains) and the 50S (chains a - x) and 30S (chains A and C - X) ribosomal subunits. Re-projections from the reference volumes were calculated at 4° intervals and cross-correlated with the 200 experimental class averages. Class averages were assigned either to the 70S, 50S or 30S ribosomal complexes depending on a normalized cross-correlation coefficient of 0.8 or higher with re-projections of the respective reference volume. The particles belonging to the class averages were then used to create 3 image stacks corresponding to the 70S (3,973 particles from 10 classes), the 50S (29,600 particles from 81 classes) or the 30S (9,032 particles from 24 classes) ribosomal complexes.

[068] Three independent reconstructions were calculated with FREALIGN version 7.05 (Grigorieff N 2007 J Struct Biol 157, 117-25), which was used to determine and refine the orientation parameters for each particle and to correct for the contrast transfer function (CTF) of the microscope. The correct defocus value for each particle image was deduced from the position of each particle in the image and the tilt angles and defocus values of the images, which

were determined with CTFTILT (Mindell JA et al. 2003 J Struct Biol 142, 334-347). FREALIGN was first run for one round using mode 3 (systematic parameter search) with an angular step of 7° to determine initial orientation parameters for each particle relative to the reference model. The resulting parameters were iteratively refined over 15 additional cycles running in mode 1 (local parameter refinement) including data in the 200 – 10 Å resolution range. Only particles with a weighted cross-correlation coefficient better than 0.8 were included in the final reconstructions, which were 23,680 particles for the 50S subunit, 7,226 particles for the 30S subunit, and 3,178 particles for the 70S ribosome. The final density maps were low-pass filtered according to their respective resolutions, which were estimated by Fourier shell correlation (FSC) with the FSC = 0.5 cut-off criterion (Bottcher B et al. 1997 Nature 386, 88-91).

[069] AQP9: 9,526 particles from the conventionally purified sample and 10,292 particles from the Affinity Grid sample were selected from 15 images of each specimen and windowed into 64 x 64 pixel images. The particles in each data set were classified into 50 classes as described above.

Example 11. Characterization of function of the pre-fabricated Affinity Grid

[070] His-tagged Tf-TfR complex was used as a test specimen to characterize the pre-fabricated EM grid as a tool to prepare specimens for single particle EM. To determine whether pre-fabricated EM grids produce samples at least of the quality as those prepared with the monolayer purification technique (Kelly DF et al. 2008 Proc Natl Acad Sci USA 105, 4703-4708), His-tagged Tf-TfR complex was added to Sf9 cell extract (6 mg/ml protein, 60 mM imidazole) to a final concentration of 0.15 μg/ml. Then, 3 μl of this mixture was added to a pre-fabricated EM grid covered with a holey carbon film and a dried lipid monolayer containing 20% Ni-NTA lipid, which is referred to in tables herein as a "20% HC Affinity Grid" (a grid covered with a continuous carbon film and a lipid monolayer containing 2% Ni-NTA lipid is referred to as a "2% CC Affinity Grid").

[071] After a 2-minute incubation, the grid was blotted and vitrified. Images of the pre-fabricated EM grid sample (Figure 1 panel B) were observed to be virtually identical to images taken of Tf-TfR complexes prepared by monolayer purification (Figure 1 panel A). Further, the Tf-TfR complex was observed to cluster when prepared by monolayer purification (see Kelly DF et al. 2008 Proc Natl Acad Sci USA 105, 4703-4708, incorporated herein by reference in its entirety). However, it was observed to be more evenly distributed on the pre-fabricated EM grid. Most of the particles attached to the pre-fabricated EM grid showed the characteristic shape of the Tf-TfR complex, suggesting the absence of contaminating proteins.

[072] To determine the purity of pre-fabricated EM grid samples, 20 samples on 20% CC Affinity Grids were eluted into a single sample of 20-µl volume of 300 mM imidazole. For comparison, 20 monolayer-purified samples were eluted. Data from SDS PAGE analysis showed a smear over the entire lane for the untreated control insect cell extract used as input (Figure 1 panel C, lane 1), while the samples eluted from the monolayer purification grids (Figure 1 panel C, lane 2) and the pre-fabricated EM grids (Figure 1 panel C, lane 3) showed only two bands corresponding to Tf and TfR, which have very similar molecular weights of ~75 kDa.

[073] A Western blot of the same samples developed with an anti-His antibody (Figure 1 panel D) showed two bands for the His-tagged TfR, one at ~75 kDa and one in the higher molecular weight range of about 150 kDa, presumably representing dimeric TfR. The entire lanes 2 and 3 were excised from the gel shown in Figure 1 panel C and analyzed by mass spectrometry. The results confirmed that only Tf and TfR were present in the samples eluted from grids prepared by monolayer purification and from pre-fabricated EM grids (Table 1). Affinity Grids reproducibly gave the same results, including pre-fabricated EM grids that had been stored for at least about 6 months under ambient conditions.

Table 1. Mass spectrometry results for the His-tagged Tf-TfR complex adsorbed to a 20% Ni-NTA monolayer or to a 20% CC Affinity Grid

Preparation method	Component	I[a]	II[b]
Monolayer	Human Tf	28.2	30
	Human TfR1	50.2	52
Affinity grid	Human Tf	28.9	31
	Human TfR1	51.4	50

[a] Percentage of sequence coverage for each protein identified
[b] Number of unique tryptic peptides for each protein identified

[074] To test the potential use of pre-fabricated EM grids for preparing membrane protein samples, tolerance for detergents was tested. His-tagged Tf-TfR complex (0.15 µg/ml) in buffer containing 1% OG, 0.2% DM, 0.02% DDM, 0.03% Triton X-100, 0.014% Tween 20, 0.5% CHAPS, 0.2% Fos-choline 11 or 0.1% digitonin were each prepared and 3-µl aliquots of these samples were applied to 2% CC pre-fabricated EM grids. Samples were incubated for 30 seconds, 1, 2, 5, 10, 20, 30 and 60 minutes prior to negative staining.

[075] It was observed that pre-fabricated EM grids were stable in the presence of 1% OG, 0.2% DM and 0.02% DDM for up to 30 minutes, producing useful specimens for single particle EM. After 60 minutes of incubation, the lipid monolayer started to dissolve, resulting in specimens with little or no Tf-TfR complexes present on the grid. In the presence of 0.03%

Triton X-100 and 0.2% Fos-choline 11, the lipid monolayer was stable for up to 10 minutes before it began to dissolve. Samples in 0.5% CHAPS could be incubated for only 2 minutes and samples in 0.014% Tween-20 for 30 seconds before the monolayer degraded. The presence of 0.1% digitonin proved to be incompatible with pre-fabricated EM grids, even at incubation times as short as 30 seconds.

Table 2. Stability of pre-fabricated EM grids in the presence of detergents

Detergents	0.5 min	1 min	2 Min	5 Min	10 min	20 min	30 min	60 min
1% OG	4	4	4	4	4	3	3	2
2 % OG	4	4	4	4	4	3	2	2
3% OG	3	3	3	2	2	2	1	1
4% OG	2	2	2	1	1	1	1	1
5% OG	1	1	1	1	1	1	1	1
0.2% DM	4	4	4	4	4	4	3	2
0.02% DDM	4	4	4	4	4	4	3	2
0.03% Triton X- 100	4	4	4	3	3	2	2	1
0.014% Tween 20	3	2	2	2	1	1	1	1
0.5% CHAPS	4	3	3	2	1	1	1	1
0.2% Fos-choline 11	3	3	3	3	3	2	2	1
0.1% digitonin	2	1	1	1	1	1	1	1

4 = No apparent degradation of the lipid monolayer
3 = Minor degradation of the lipid monolayer
2 = Major degradation of the lipid monolayer
1 = Almost complete degradation of the lipid monolayer

[076] To determine whether the detergent concentration has an influence on the stability of the pre-fabricated EM grid, Tf-TfR complex was prepared in 1, 2, 3, 4 and 5% OG on 2% CC pre-fabricated EM grids and incubated for the same times prior to negative staining. It was observed that Affinity Grids tolerated up to 2% OG for 20 – 30 minutes before substantial degradation of the lipid monolayer occurred, while 3% OG already began to destabilize the grids after 2 minutes. OG concentrations of 4 – 5% were incompatible with pre-fabricated EM grids even for very short incubation times. The results of these experiments summarized in Table 2 indicate that pre-fabricated EM grids are compatible with most detergents, at least for some time at detergent concentrations not too far above the critical micelle concentration.

[077] Compatibility of pre-fabricated EM grids with glycerol was tested, as glycerol is often used to stabilize protein complexes and is employed in certain cryo-negative staining protocols. His-tagged Tf-TfR complex (0.15 μg/ml) was prepared in buffer solution containing 1, 2, 3, 4 and 5% glycerol and 3-μl aliquots of each sample were applied to 2% CC Affinity Grids. Samples were incubated for 1, 5, 10 and 15 minutes prior to extensive washing and

negative staining. Pre-fabricated EM grids were observed to remain stable in the presence of up to 5% glycerol for at least a 5-minute incubation period. After 10 minutes of incubation, only glycerol concentrations of less than or equal to 3% produced useful samples with only minor lipid layer degradation. By 15 minutes, all glycerol concentrations tested caused substantial degradation of the lipid monolayer (Table 3 shows a summary of these results).

Table 3. Stability of pre-fabricated EM grids in the presence of glycerol

% Glycerol	1 min	5 min	10 min	15 min
1	4	4	3	2
2	4	4	3	2
3	4	3	3	2
4	4	3	2	1
5	3	3	2	1

4 = No apparent degradation of the lipid monolayer
3 = Minor degradation of the lipid monolayer
2 = Major degradation of the lipid monolayer
1 = Almost complete degradation of the lipid monolayer

Example 12. Pre-fabricated EM grid purification of ribosomal complexes

[078] Native 50S ribosomal subunits containing His-tagged human rpl3 were purified from *E. coli* extracts using monolayer purification (Kelly DF et al. 2008 Proc Natl Acad Sci USA 105, 4703-4708), and the pre-fabricated EM grids were tested with the same system. *E. coli* extract (~3 mg/ml in 3-μl) was added to a 2% CC pre-fabricated EM grid (Figure 2 panel E). After an incubation of 2 minutes, the sample was negatively stained and imaged in the electron microscope (Figure 2 panel A). The images showed the same kind of complexes, 20 to 30 nm in size, that had previously been observed with specimens produced by conventional Ni-affinity chromatography and monolayer purification (Kelly DF et al. 2008 Proc Natl Acad Sci USA 105, 4703-4708). To assess the composition of the complexes seen in the images, protein was eluted from 20 samples on 20% CC pre-fabricated EM grids and analyzed by mass spectrometry. The same proteins were present as previously identified in monolayer-purified samples (Kelly DF et al. 2008 Proc Natl Acad Sci USA 105, 4703-4708); all proteins observed were known ribosomal subunits and no contaminating proteins could be identified (Table 4).

Table 4. Mass spectrometry results for the ribosomal complexes adsorbed to a 20% CC Affinity Grid

Component	I[a]	II[b]
Rpl1*	38.7	6
Rpl2*	23.6	7
Rpl3-human*	18.4	9
Rpl4*	24.6	4

Rpl5*	32.2	8
Rpl6*	17.6	6
Rpl7*	8.4	1
Rpl8*	12.7	2
Rpl9*	38.8	4
Rpl10*	12.7	2
Rpl11*	13.4	2
Rpl12*	6.0	1
Rpl13*	8.3	1
Rpl14*	16.2	2
Rpl15*	17.4	2
Rpl16*	10.7	1
Rpl17*	16.6	3
Rpl18*	21.6	4
Rpl19*	9.9	1
Rpl20*	15.6	2
Rpl21*	24.3	3
Rpl22*	64.8	12
Rpl23*	17.5	3
Rpl24*	58.6	6
Rpl25*	20.3	4
Rpl26*	11.2	2
Rpl27*	15.7	4
Rpl28*	17.3	3
Rpl29*	14.4	3
Rpl30*	17.7	2
Rps1*	9.6	3
Rps2*	3.3	2
Rps3*	14.1	3
Rps4*	46.2	10
Rps5*	12.6	4
Rps6*	7.7	3
Rps7*	4.2	1
Rps8*	3.7	1
Rps9*	12.4	2
Rps10*	5.2	1
Rps11*	27.8	4
Rps12*	15.6	2
Rps13*	63.4	8
Rps14*	8.4	1
Rps15*	6.1	1
Rps16*	7.6	1
Rps17*	4.4	1
Rps18*	7.9	1
Rps19*	4.8	1
Rps20*	14.9	2
EfTu[1]	17.8	6

[a] Percentage of sequence coverage for each protein identified
[b] Number of unique tryptic peptides for each protein identified
*Ribosomal proteins

[1]Ribosome associated proteins (Rpl = ribosomal protein large subunit; Rps = ribosomal protein small subunit; Eftu = elongation factor tu)

[079] To produce vitrified specimens, a 3-µl drop of *E. coli* extract was added to a 20% HC pre-fabricated EM grid. After a 2-minute incubation, the sample was blotted, quick-frozen in liquid ethane and imaged in the electron microscope (Figure 2 panel B). A comparison with images of vitrified samples prepared by monolayer purification (Figure 2 panel C) revealed distinct differences. As previously observed, clustering of the particles often seen in samples prepared by monolayer purification did not occur on the pre-fabricated EM grid. While both samples contained mostly particles consistent in size with the 50S ribosomal subunit and the 70S ribosome, the pre-fabricated EM grid specimen also contained smaller particles that would be consistent in size and shape with a 30S ribosomal subunit (black arrows in Figure 2 panel B).

[080] Surprisingly, the images obtained with the pre-fabricated EM grid sample showed that many of the ribosomal complexes were attached to ~15 Å thick, string-like densities, which most likely represent mRNA strands. To test this interpretation, the extract was incubated with RNAse A for 30 minutes prior to applying it to a pre-fabricated EM grid. The images from this preparation no longer showed the string-like densities associated with the ribosomal complexes (Figure 2 panel D), confirming that the string-like densities are images of mRNA. mRNA was consistently present in pre-fabricated EM grid preparations of ribosomal complexes (Figure 2 panel B), but was not observed in preparations produced by monolayer purification (Figure 2 panel C). To obtain a pre-fabricated EM grid sample in a manner more resembling the way a monolayer purification sample is prepared, 25 µl of extract was placed into a well in a Teflon block and a 20% HC pre-fabricated EM grid was placed on top of the extract (as illustrated in Figure 2 panel F). After a 30-minute incubation, the pre-fabricated EM grid was lifted off, blotted and vitrified. Images of this preparation did not include presence of mRNA, while mRNA was observed in images of a pre-fabricated EM grid specimen prepared by pippetting a drop of the same extract onto a pre-fabricated EM grid (as illustrated in Figure 2 panel E) at the same time. These results show that the presence of mRNA in pre-fabricated EM grid preparations depends on the exact way the grid is prepared.

[081] To calculate structures of ribosomal complexes, 52,507 particles were selected from 274 images of vitrified pre-fabricated EM grid specimens containing mRNA and classified them into 200 classes (Figure 5). To account for the heterogeneity in the class averages due to the presence of different ribosomal complexes, 30-Å density maps of the 30S, 50S and 70S ribosomal complexes were generated based on the crystal structure of the *E. coli* 70S ribosome (pdb code: 1ML5; Klaholz BP et al.2003 Nature 421, 90-94). These reference volumes were used to sort the class averages into groups representing the 50S ribosomal subunit

(representative class averages shown in Figure 3 panel A), the 30S ribosomal subunit (representative class averages shown in Figure 3 panel E) and the 70S ribosome (representative class averages shown in Figure 3 panel I) using the normalized cross-correlation routine implemented in SPIDER (Frank J et al. 1996 J Struct Biol 116, 190-9; see Examples 1 - 7). FREALIGN (Grigorieff N 2007 J Struct Biol 157, 117-25) was then used to calculate 3D reconstructions of the 50S subunit (Figure 3 panel D; 23,680 particles in the final map) at a resolution of 21 Å (Figure 3 panel C), the 30S subunit (Figure 3 panel H; 7,226 particles in the final map) at a resolution of 24 Å (Figure 3 panel G), and the 70S ribosome (Figure 3l panel L; 3,178 particles in the final map) at a resolution of 28 Å (Figure 3 panel K). The Euler angle distribution for each reconstruction showed that the particle orientations sampled the entire 3D space (Figure 3 panels B, F and J). Manual placement of the atomic models for each ribosomal complex into the corresponding density map demonstrated that the structural features of the density maps were consistent with the crystal structures (Figure 3 panels D, H. and I).

Example 13. Pre-fabricated EM grid purification of AQP9

[082] Since the pre-fabricated EM grid proved to be sufficiently resistant to OG, it was used to test possible isolation of AQP9 from a membrane extract of Sf9 insect cells. Sf9 cells over-expressing His-tagged AQP9 were lysed and the membrane fraction was solubilized with 2% OG. Imidazole was added to a final concentration of 60 mM and the detergent-solubilized membranes were applied to a 2% CC Affinity Grid. In parallel, a conventional purification was performed for the same membrane fraction using Ni-affinity and gel filtration chromatography. Negatively stained specimens were prepared for the two samples and examined in the electron microscope.

[083] Negatively stained specimens of the Sf9 membrane extract showed a variety of proteins, making it impossible to identify individual AQP9 tetramers (Figure 4 panel A). In contrast, specimens prepared by conventional Ni-affinity and gel filtration chromatography (Figure 4 panel B) and using a pre-fabricated EM grid (Figure 4 panel C) showed largely homogeneous particle populations. To assess the purity of the sample produced with the pre-fabricated EM grid, the protein was eluted from 20 samples on 20% CC Affinity Grids and analyzed by SDS-PAGE (Figure 4 panel D, lane 2) and Western blotting (Figure 4 panel E, lane 2) as well as by mass spectrometry. For comparison, AQP9 purified by conventional Ni-affinity chromatography was subjected to the same analyses (lane 1 in Figure 4 panels D and E). The SDS PAGE gel and Western blot of the two samples show the two bands representing unglycosylated (~32 kDa) and glycosylated AQP9 (~35 kDa) that have been previously observed (Viadiu H et al. 2007 J Mol Biol 367, 80-88) as well as dimeric AQP9 (~68 kDa). In

addition, the mass spectrometry results confirmed that AQP9 was the only protein present in the two samples (Table 5). From images of each of the chromatographically and pre-fabricated EM grid purified samples (15 Images) 9,526 and 10,292 particles were selected and each was classified into 50 classes. The class averages of OG-solubilized AQP9 obtained with the pre-fabricated EM grid specimen (Figure 4 panel G) appeared to be of the same quality as those obtained with conventionally prepared sample (Figure 4 panel F). In both cases particles about ~10 nm in size with a central stain accumulation were observed, which looked like those previously reported for single particles of AQP9 (Viadiu H et al. 2007 J Mol Biol 367, 80-88).

Table 5. Mass spectrometry results for the His-tagged AQP9 complex adsorbed to a Ni-NTA agarose column or to a 20% CC Affinity grid.

Preparation Method	Component	I[a]	II[b]
Ni-NTA column	Rat AQP9	10.3	5
Affinity grid	Rat AQP9	25.2	8

[a] Percentage of sequence coverage for each protein identified
[b] Number of unique tryptic peptides for each protein identified

Example 14. Affinity Grids in comparison to monolayer purification

[084] Affinity Grid monolayer purification as observed herein provides an easy-to-use and rapid technique to purify a His-tagged protein or macromolecular complex from cell extract while simultaneously preparing a specimen suitable for single particle EM (Kelly DF et al. 2008 Proc Natl Acad Sci USA 105, 4703-4708). While the previous technology of casting a monolayer over a cell extract and to then picking the monolayer up with an EM grid is straightforward, this multi-step procedure must be performed for every sample, potentially creating a threshold for the use of monolayer purification. Therefore it is an objective of this example to test a pre-fabricated EM grid featuring a dried, pre-deposited monolayer containing Ni-NTA lipids on a carbon-coated EM grid. If successful, the pre-fabricated EM grid would make it possible to simply apply a drop of extract to the pre-fabricated grid, which would specifically adsorb only His-tagged proteins while other proteins would be removed during washing of the grid.

[085] The results of tests with His-tagged Tf-TfR and ribosomal complexes herein showed that the pre-fabricated EM grid indeed functioned to purify proteins. Further, samples were produced that were observed to be of the same purity as those produced with monolayer purification. Further, preparing a pre-fabricated EM grid sample required much less time and was as convenient as preparing a normal conventional EM grid while not requiring any plasma cleaning or glow discharging of the grid prior to sample application. Particularly useful is the observation herein that pre-fabricated EM grids were stable and could be stored under ambient

conditions for at least six months prior to use without loss of function. Thus, pre-fabricated EM grids as articles of manufacture can be produced at any time, then stored, and used conveniently whenever needed.

Example 15. Affinity Grids extend the applicability of monolayer purification

[086] Monolayer purification depends on the formation of a lipid monolayer at the air-water interface. Since detergents and glycerol dissolve lipid monolayers, these substances are incompatible with monolayer purification. Detergents are, however, used in the standard protocols to prepare yeast extracts and they are occasionally added in low concentrations to protein solutions to minimize aggregation through hydrophobic interactions. Detergents are also a necessity for the solubilization of membrane proteins from cell membranes. Glycerol is another common additive that stabilizes proteins and complexes in solution, and it is also employed in certain cryo-negative staining procedures (Ohi M et al. 2004 Biol Proced Online 6, 23-34). The incompatibility of lipid monolayers with detergents and glycerol thus limits the use of monolayer purification. Since the lipid monolayer on the pre-fabricated EM grid is stabilized by its interaction with the carbon film, it is more resistant to detergents and glycerol. With the exception of digitonin, which is known to be particularly potent in solubilizing lipids (Moore RJ et al. 1974 Biochemistry 13, 450-456) and destroys the pre-fabricated EM grid almost immediately, the pre-fabricated EM grid is stable in most detergents for at least some period of time. The time of stability also increases with decreasing detergent concentration. Similarly, pre-fabricated EM grids are useful for only a limited time if the sample contains glycerol, with the lifetime of the pre-fabricated EM grid again increasing with decreasing glycerol concentrations. Pre-fabricated EM grids were sufficiently stable in the presence of 2% OG and could be used to directly adsorb His-tagged AQP9 from an insect cell membrane extract. For samples containing more aggressive detergents and/or a high concentration of glycerol, the best strategy would presumably be to minimize the required incubation time by using a pre-fabricated EM grid with a high percentage of Ni-NTA lipid. An alternative solution to this problem would be to prepare pre-fabricated EM grids with a lipid monolayer that contains fluorinated lipids as the filler lipid. Fluorinated lipids are more resistant to detergents (Lebeau L et al. 2001 J Mol Biol 308, 639-647) and have already been used to form two-dimensional (2D) crystals on lipid monolayers (Levy D et al. 2001 FEBS Lett 504, 187-193). Fluorinated lipids may also be more resistant to glycerol.

[087] When monolayer purification is used to prepare a specimen, the particles adsorbed to the lipid monolayer were observed to have a tendency to cluster (Figures 1 panel A and 2 panel C). Surprisingly, such clustering was not observed with samples prepared on

Affinity Grids (Figures 1b and 2b). Lipid monolayers are fluid and proteins adsorbing to them can easily diffuse, a feature that is exploited in the 2D crystallization of proteins on lipid monolayers (Uzgiris EE et al. 1983 Nature 301, 125-129) . By contrast, the lipids constituting the monolayer on the Affinity Grid are attached to the carbon film. This attachment makes the monolayer not only more resistant to detergents and glycerol, but also prevents or at least substantially reduces diffusion of the lipids. Clustering of proteins on monolayers thus appears to be due to affinity of the proteins for each other, with the lipids enabling the proteins to find each other by diffusion. Since the lipids in the monolayer are attached to the carbon film of the Affinity Grid, they are presumably prevented from diffusing and particle clustering does not occur.

[088] In summary, the Affinity Grid makes monolayer purification compatible for use with membrane proteins and protein solutions containing glycerol and it is superior to monolayer purification in most cases because it prevents particle clustering.

Example 16. Samples of ribosomal complexes differ depending on the preparation method

[089] Surprisingly, pre-fabricated EM grid samples of ribosomal complexes looked quite different from samples prepared by monolayer purification. Most notably, the ribosomal complexes were still associated with mRNA. The possibility that the RNA was simply degraded in the extract used to prepare the sample by monolayer purification was here tested. This model was ruled out by preparing monolayer and pre-fabricated EM grid samples from the same extract. mRNA was again present in the pre-fabricated EM grid sample but not in the monolayer purification sample. Since preparing a monolayer purification sample takes about 20 minutes while preparing a pre-fabricated EM grid requires only about 2 minutes, another possibility was that the RNA was degraded in the additional time it took to prepare the monolayer purification grid. However, when the pre-fabricated EM grid was prepared even after the preparation of the monolayer purification grid was completed, the observed outcomes did not change. Finally, a pre-fabricated EM grid was prepared by placing it onto the top of cell extract (Figure 2 panel F) rather than placing a drop of extract onto the pre-fabricated EM grid (Figure 2 panel E). In this preparation, mRNA was no longer visible. The reason why the two different procedures produce different samples is currently not clear, but the results were reproducible. It may be that placing extract onto the pre-fabricated EM grid is more gentle and thus allows the mRNA to remain on the monolayer while lifting the Affinity Grid from the surface of the extract may be harsher and rip the mRNA off the monolayer.

[090] Samples produced by placing cell extract on the pre-fabricated EM grid showed not only the presence of mRNA, in addition to 50S and 70S complexes, but revealed also 30S

ribosomal complexes. Since the 30S complex does not contain a His-tagged subunit (and accordingly was absent in samples prepared by monolayer purification; Kelly DF et al. 2008 Proc Natl Acad Sci USA 105, 4703-4708), the conclusion was drawn that it was present in pre-fabricated EM grid samples as bound to mRNAs that, in turn, were adsorbed to the lipid monolayer through His-tagged ribosomal complexes. The gentleness of the pre-fabricated EM grid preparation thus seems to allow the preparation of large, functional, macromolecular assemblies that usually disintegrate during specimen preparation. The pre-fabricated EM grid thus provides tools and methods for visualization of complex biological assemblies by cryo-EM or electron tomography that were not possible to isolate with previously available techniques.

Example 17. Use of Affinity Grid for the preparation of untagged complexes

[091] Antibodies bind specifically and with high affinity to their target antigens. Many biochemical purification schemes utilize this interaction to isolate protein antigens or protein complexes form various biological sources. In doing so, antibodies that have been prepared by immunizing an animal with a particular protein must first be purified from host tissue and attached to a stationary column matrix. Protein A, a surface protein found on the cell wall of S. *aureus* bacteria, strongly binds to mammalian immunoglobulin G(IgG) antibodies and has been used as an adaptor to immobilize IgGs to a stationary support matrix, creating an "antibody affinity column". A native protein of interest (lacking purification tags) can then be isolated from the other cellular components using the antibody column. However, due to the high affinity of the antibody-protein interaction, stringent conditions are often required to remove a component from the column. Elution reagents usually contain excessive quantities of salts or acid. These conditions are typically detrimental to preserving intact, active biological complexes and are not ideal for structural studies.

[092] The present example shows use of Ni-NTA containing Affinity Grids as described herein, with adaptor molecules. His-tagged protein A was bound to an Affinity Grid under ambient conditions. Following a brief incubation step, purified IgG antibodies can then be added to the system. The Affinity Grid now serves as an antibody affinity column. Once produced, these antibody-bound Affinity Grids can be used for the rapid purification of untagged macromolecular complexes from any expression system (Figure 6). An advantage is that the complexes stay attached to the Affinity Grid and are not subjected elution conditions used in conventional affinity chromatography.

[093] This universal method was tested using an IgG antibody (commercially available, Abcam Inc., Cambridge, MA) specific for the human ribosomal subunit L26 bound to recombinant His-tagged protein A. A 2% CC Affinity Grid was the used to purify native

ribosomes from a mammalian 293T cell lysate within 5 minutes. The specimen was examined using negative stain EM (Figure 7). Particles of varying sizes ranging from 30 – 50 nm were observed (Figure 7 – white circles) having features consistent with those of eukaryotic ribosomal complexes.

[094] The data show the versatility of the antibody-bound Affinity Grid, which eliminates a need for engineering purification tags. However, if other tagged constructs are readily available, this system is useful if an antibody for tag is available (i.e., an antibody specific for binding to any of Streptavidin, Flag, Myc, Hemagglutinin (HA), Glutathione S-transferase (GST), Green Fluorescent Protein (GFP), and Calmodulin. Therefore, the system is universally useful to purify and observe complexes, even non-recombinantly tagged proteins, if an antibody specific for an antigen present on the complexes is available.

Example 18. Affinity Grid usage for TAP tags

[095] To determine whether the Affinity Grid could be used with Tandem Affinity Purification (TAP) tags, the following considerations and procedures were examined. Traditionally, the TAP technique employs more than one purification tag at the C-terminus of a protein so that a protein is isolated using sequential affinity columns. Examples of fusion tags that are widely used are protein A and calmodulin binding protein (CBP). A previous purification method includes adding a cell lysate containing the tagged protein to an IgG affinity column (which binds to the protein A tag). The protein is then eluted and is added to a subsequent column with a fixed calmodulin substrate (which binds the CBP tag). The final product is a highly purified protein or protein complex.

[096] However, most biological samples contain multiple subunits, some of which dissociate during the TAP procedure. This results in sample heterogeneity that limits high-resolution structure determination. A method herein to circumvent this problem uses TAP tags in conjunction with Affinity Grids. A recombinant protein containing the protein A tag is isolated using an antibody-bound Affinity Grid (see Figure 6). Alternatively, a His-tagged calmodulin adaptor is bound directly to an Affinity Grid containing the Ni-NTA lipid layer. Recombinant proteins having a CBP tag would in turn bind the calmodulin adaptor on the grid (Figure 8). Tandem purification tags are used to formulate a TAP tag construct, likewise they are positioned at any point with the polypeptide chain.

[097] Furthermore, Affinity Grids are useful in proteomic studies. Yeast TAP-Fusion libraries are commercially available and are also constructed at university laboratories. These libraries allow for the selection and purification of any protein present in the entire yeast proteome. A library containing TAP-tagged Yeast strains contains genes engineered to produce

particular recombinant proteins. The use of genomic libraries in conjunction with Affinity Grid technology provides a high-throughput method in structure determination of eukaryotic macromolecular complexes, for analysis by EM and standard protein purification and assay methods.

What is claimed is:

1. A pre-fabricated electron microscopy (EM) grid article of manufacture comprising a mesh; a carbon layer; and a lipid monolayer in contact with the carbon layer; and further, in the case of HC Affinity Grids, comprising a thin layer of carbon evaporated onto the lipid monolayer.

2. The pre-fabricated EM grid according to claim 1, wherein a portion of the lipid monolayer comprises modified lipid molecules having an affinity moiety covalently attached.

3. The pre-fabricated EM grid according to either of claims 1 and 2, wherein the lipid monolayer further comprises at least one neutral phospholipid.

4. The pre-fabricated EM grid according to any of claims 1-3, wherein the carbon layer is a continuous carbon film or a holey carbon grid.

5. The pre-fabricated EM grid according to any of claims 2-4, wherein the modified lipid molecules comprise a Nickel (II) Nitriloacetic acid (Ni-NTA) group or a biotin group.

6. The pre-fabricated EM grid according to any of claims 2-5, wherein the modified lipid is 1,2-dioleoyl-*sn*-glycero-3-[N(5-amino-1-carboxypentyl)iminodi acetic acid] succinyl-nickel salt (Ni-NTA lipid).

7. The pre-fabricated EM grid according to any of claims 3-6, wherein the neutral phospholipid is 1,2-dilauryl-*sn*-glycero-3-phosphatidylcholine (DPLC)

8. The pre-fabricated EM grid according to any of claims 1-7, wherein the mesh comprises at least one material selected from the group consisting of: copper, gold, nickel, silver, molybdenum, rhodium, titanium, steel, tungsten, nylon, and a mixture thereof.

9. The pre-fabricated EM grid according to any of claims 5-8, wherein the proportion of Ni-NTA lipid in the lipid monolayer is about 0.5% to about 40%.

10. The pre-fabricated EM grid according to any of claims 1-9, wherein the grid is stored under ambient conditions of room temperature and humidity for at least about six months.

11. The pre-fabricated EM grid according to any of claims 1-10, wherein the lipid monolayer

further comprises a fluorinated lipid.

12. A method of manufacturing a pre-fabricated electron microscopy (EM) grid comprising:

adding a solution of at least one lipid in chloroform on top of an aqueous solution in a container to form a lipid monolayer and contacting the lipid monolayer with a mesh, wherein the mesh further comprises a carbon layer comprising a continuous carbon film or holey carbon grid, whereby at least a portion of the lipid monolayer is transferred to the carbon layer on an upper surface of the mesh, and removing the mesh and associated carbon layer and lipid monolayer from the container and blotting and air drying the mesh; and

evaporating a thin layer of carbon onto the holey carbon film, thereby manufacturing the pre-fabricated EM grid.

13. The method according to claim 12, wherein the at least one lipid further comprises modified lipid molecules with an affinity moiety selected from at least one of a Nickel (II) Nitriloacetic acid (Ni-NTA) group and a biotin group.

14. The method according to either of claims 12-13, wherein the modified lipid is 1,2-dioleoyl-*sn*-glycero-3-[N(5-amino-1-carboxypentyl)iminodi acetic acid] succinyl-nickel salt (Ni-NTA lipid).

15. The method according to any of claims 12-14, wherein the solution of the at least one lipid comprises 1,2-dioleoyl-*sn*-glycero-3-[N(5-amino-1-carboxypentyl)iminodi acetic acid] succinyl-nickel salt (Ni-NTA lipid) in chloroform.

16. The method according to any of claims 12-15, wherein the aqueous solution is a buffer.

17. The method according to any of claims 12-16, wherein the mesh is at least one material selected from the group consisting of: copper, gold, nickel, silver, molybdenum, titanium, rhodium, steel, tungsten, nylon, and mixtures thereof.

18. The method according to any of claims 14-17, wherein the proportion of Ni-NTA lipid in the lipid monolayer is about 0.5% to about 40%.

19. A method of preparing a biological sample for analysis by electron microscopy (EM) grid, the method comprising:

incubating a pre-fabricated EM grid according to any of claims 1–18 with a biological sample, wherein at least one component of the sample binds to the pre-fabricated EM grid;

blotting to remove excess sample; and,

visualizing the at least one bound component on the grid by EM analysis.

20. The method according to any of claims 12-19, wherein the EM grid comprises a lipid monolayer further having a modified lipid with an affinity moiety selected from at least one of a Nickel (II) Nitriloacetic acid (Ni-NTA) group and a biotin group.

21. The method according to any of claims 12-20, wherein the modified lipid is 1,2-dioleoyl-*sn*-glycero-3-[N(5-amino-1-carboxypentyl)iminodi acetic acid] succinyl-nickel salt (Ni-NTA lipid).

22. The method according to any of claims 12-19, further comprising, prior to incubating the pre-fabricated EM grid with the sample, adding imidazole to the sample.

23. The method according to any of claims 12-19, further comprising eluting the at least one bound component of the sample from the grid with a reagent.

24. The method according to any of claims 23, wherein the reagent is selected from the group of: imidazole; a divalent metal ion chelator; a solution at a pH lower than about 6; and a poly-histidine containing peptide.

25. The method according to any of claims 12-19, wherein the sample is a cell lysate.

26. The method according to any of claims 12-19, wherein the sample is a partially purified protein solution.

27. The method according to any of claims 12-20, wherein the cell lysate comprises at least one recombinant protein having an affinity ligand that binds with specificity to the modified lipid.

28. The method according to any of claims 12-27, wherein the affinity ligand comprises at least one genetically added or inserted tag selected from the group of: poly-histidine, Streptavidin, Avidin, Flag, Myc, Hemagglutinin, Glutathione-S-transferase, Green Fluorescent protein, Calmodulin, Calmodulin binding protein, and tandem affinity tags (TAP).

29. The method according to any of claims 12-23, further comprising after manufacturing the grid and prior to incubating with the biological sample, first incubating the grid with His-tagged protein A and then incubating with an IgG antibody that specifically binds the component in the biological sample, wherein the component is not recombinant.

30. The method according to any of claims 12-23, further comprising after manufacturing the grid and prior to incubating with the biological sample, first incubating the grid with His-tagged calmodulin, wherein the component in the biological sample comprises a calmodulin binding protein-tagged or TAP-tagged protein.

31. A kit for electron microscopic analysis comprising a pre-fabricated EM grid according to any of claims 1-11, a container and instructions for use in EM analysis of a biological sample.

32. The kit according to any of claims 1–11 and 31, further comprising at least one reagent for adding to a cell lysate or for eluting a protein from the grid.

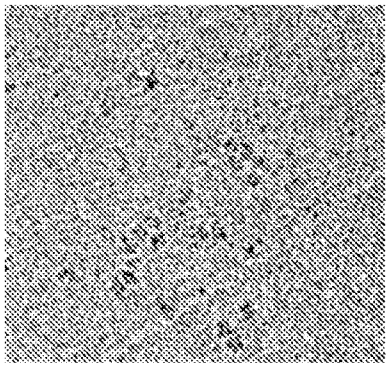

Figure 1A

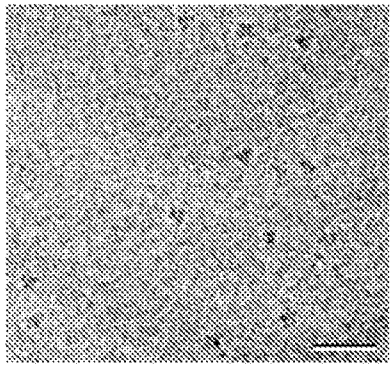

Figure 1B

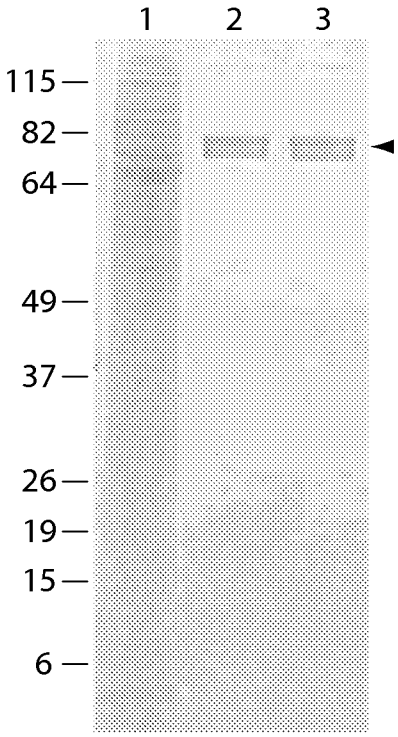

Figure 1C

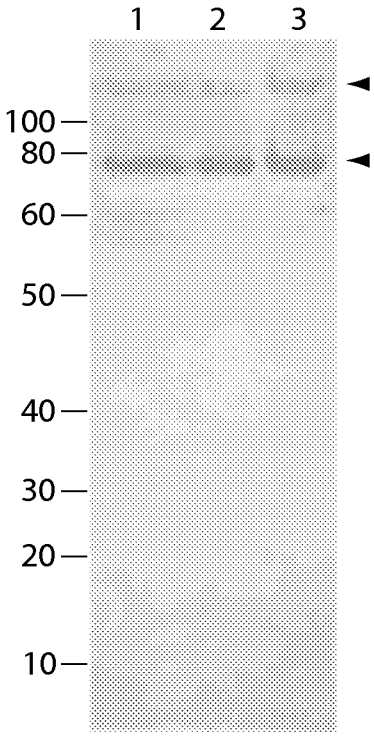

Figure 1D

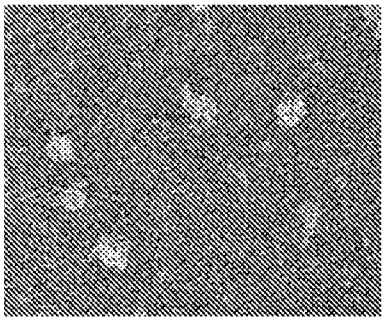

Figure 2A

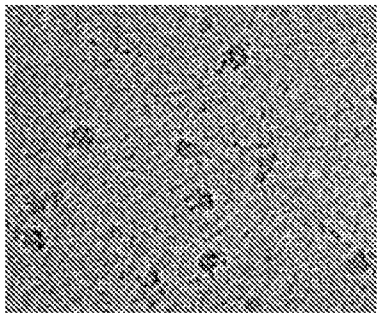

Figure 2B

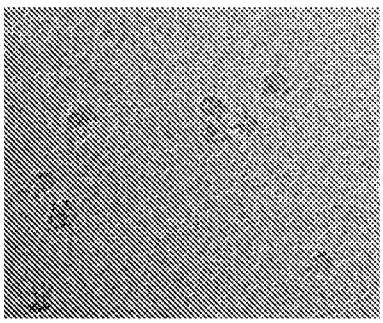

Figure 2C

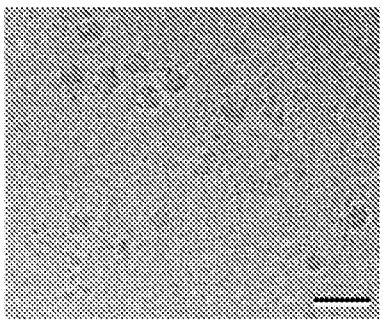

Figure 2D

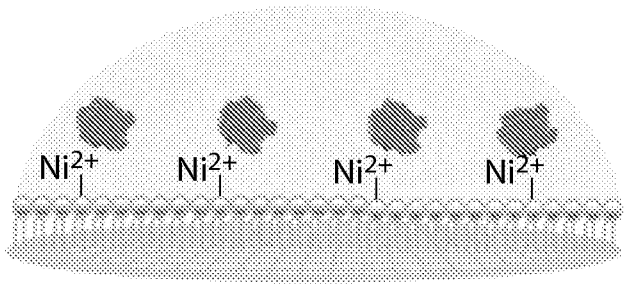

Figure 2E

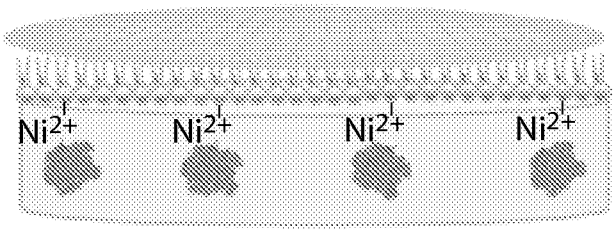

Figure 2F

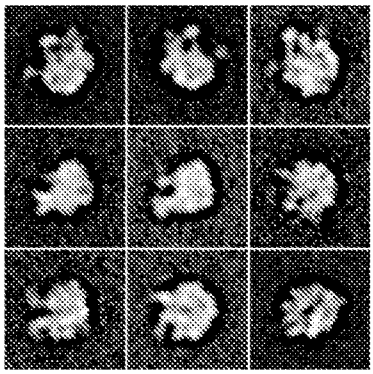

Figure 3A

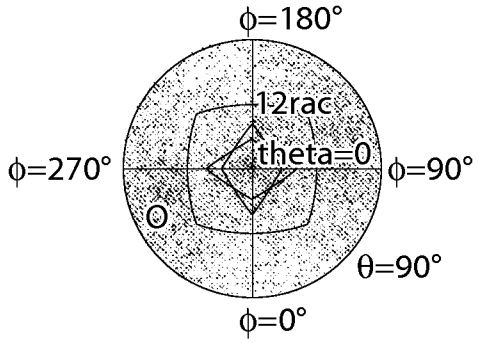

Figure 3B

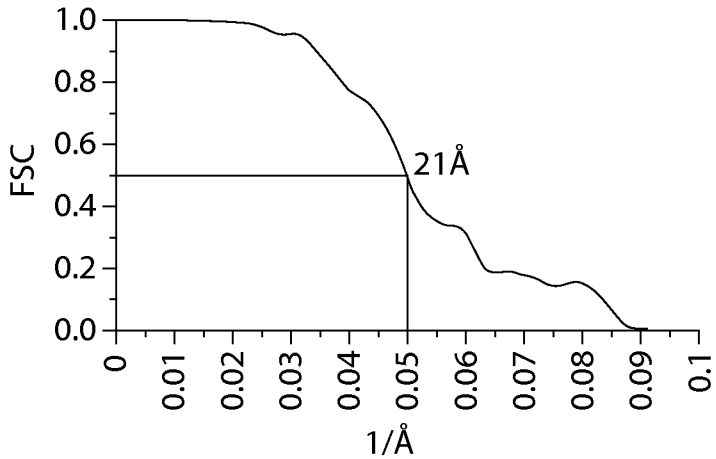

Figure 3C

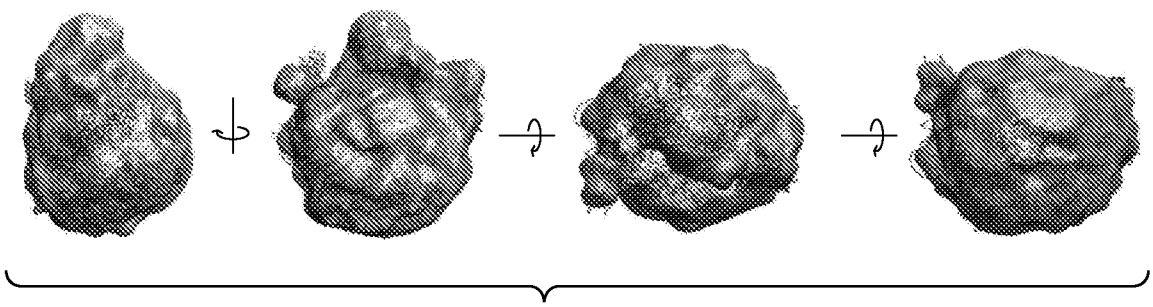

Figure 3D

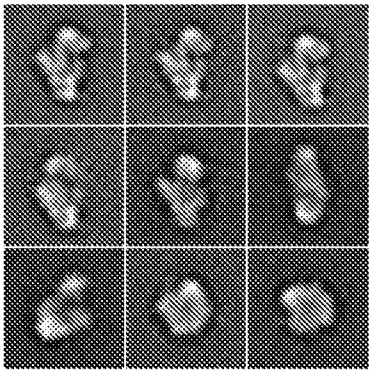

Figure 3E

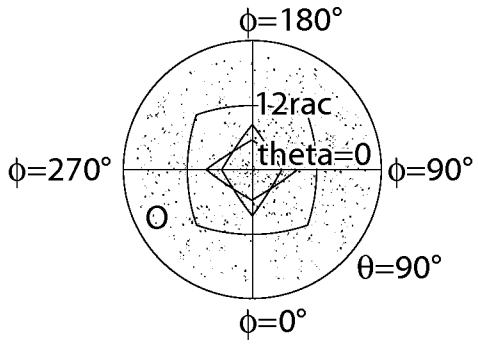

Figure 3F

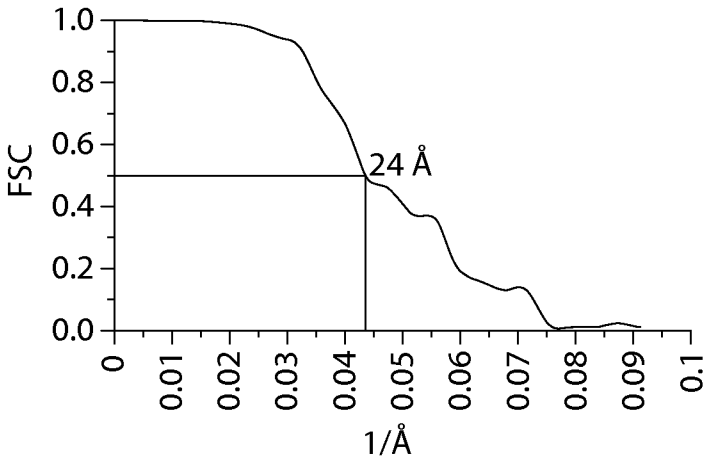

Figure 3G

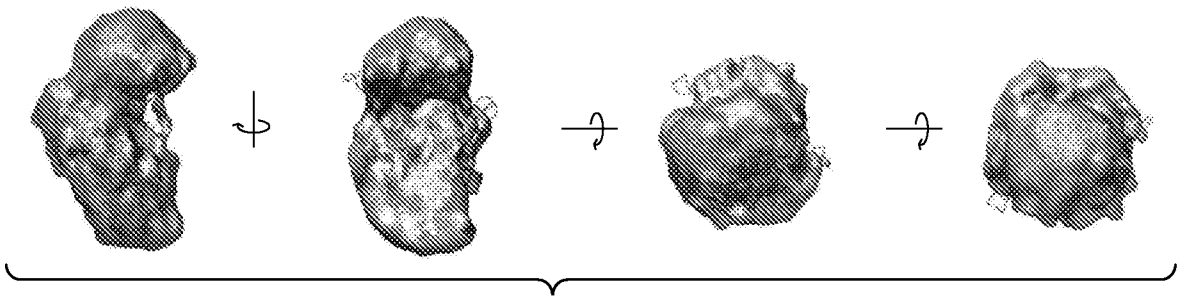

Figure 3H

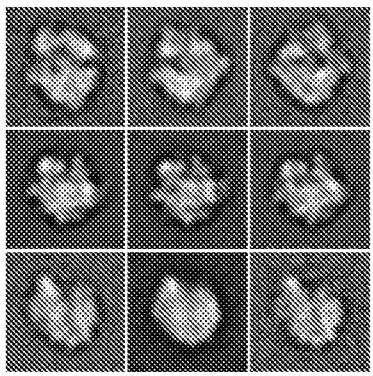

Figure 3I

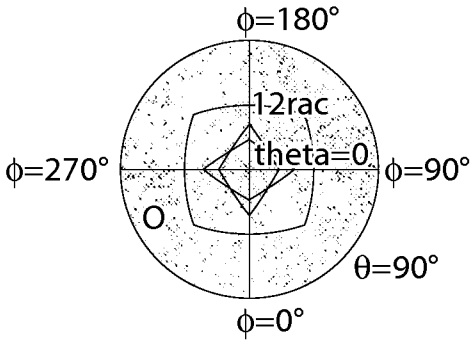

Figure 3J

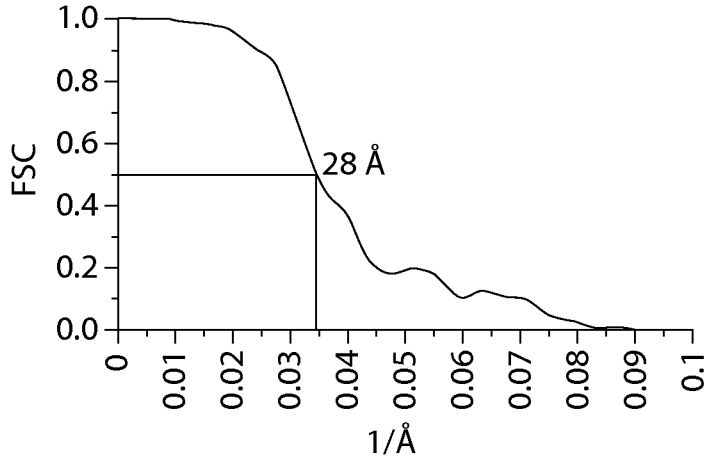

Figure 3K

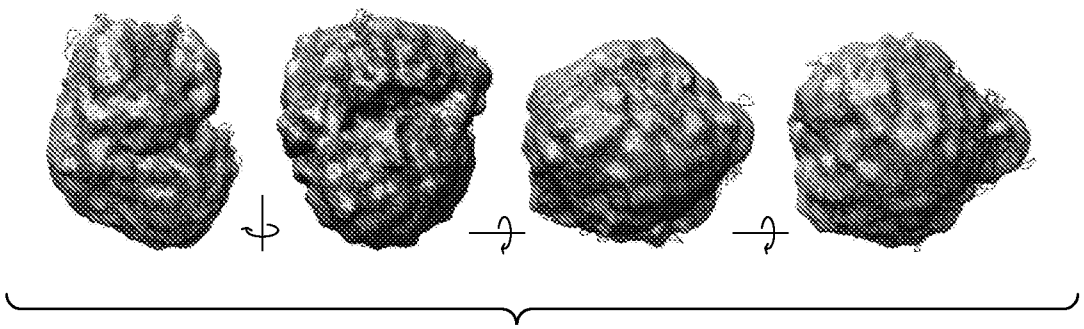

Figure 3L

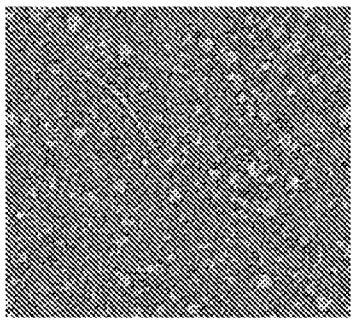

Figure 4A

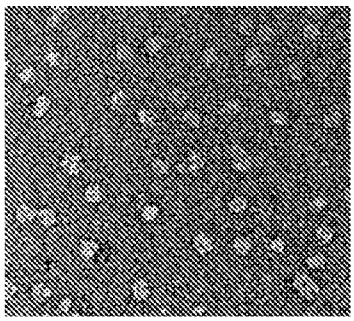

Figure 4B

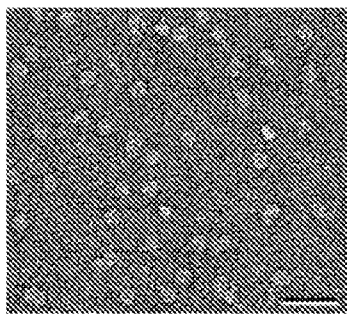

Figure 4C

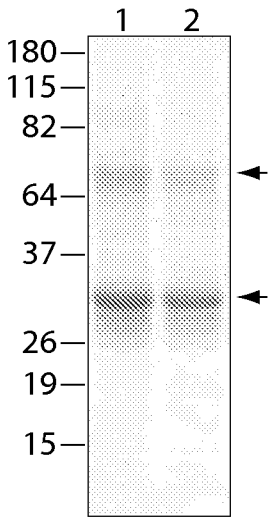

Figure 4D

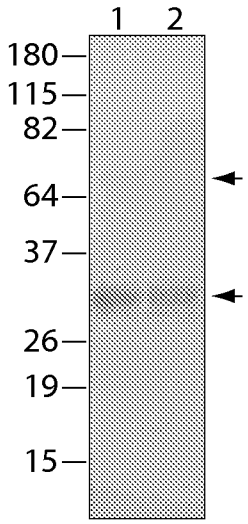

Figure 4E

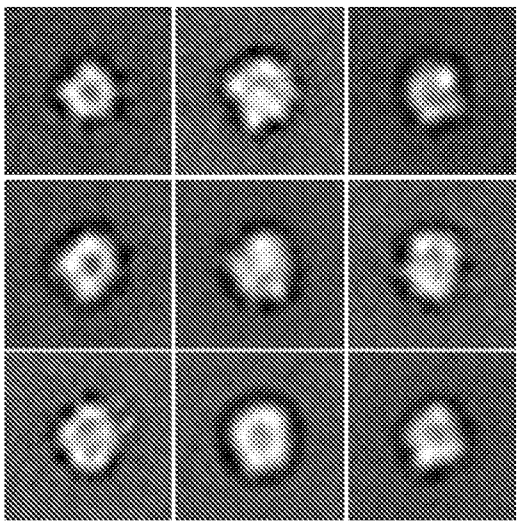

Figure 4F

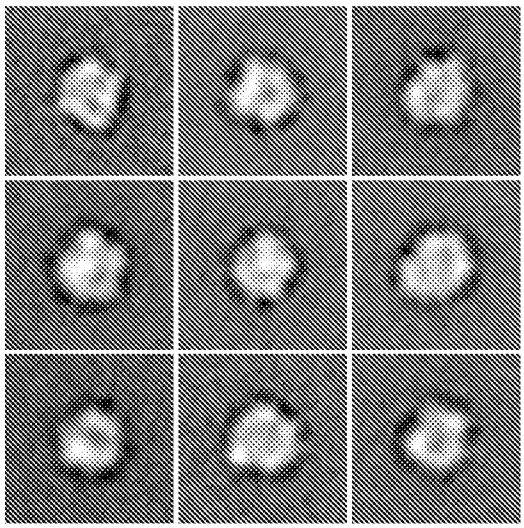

Figure 4G

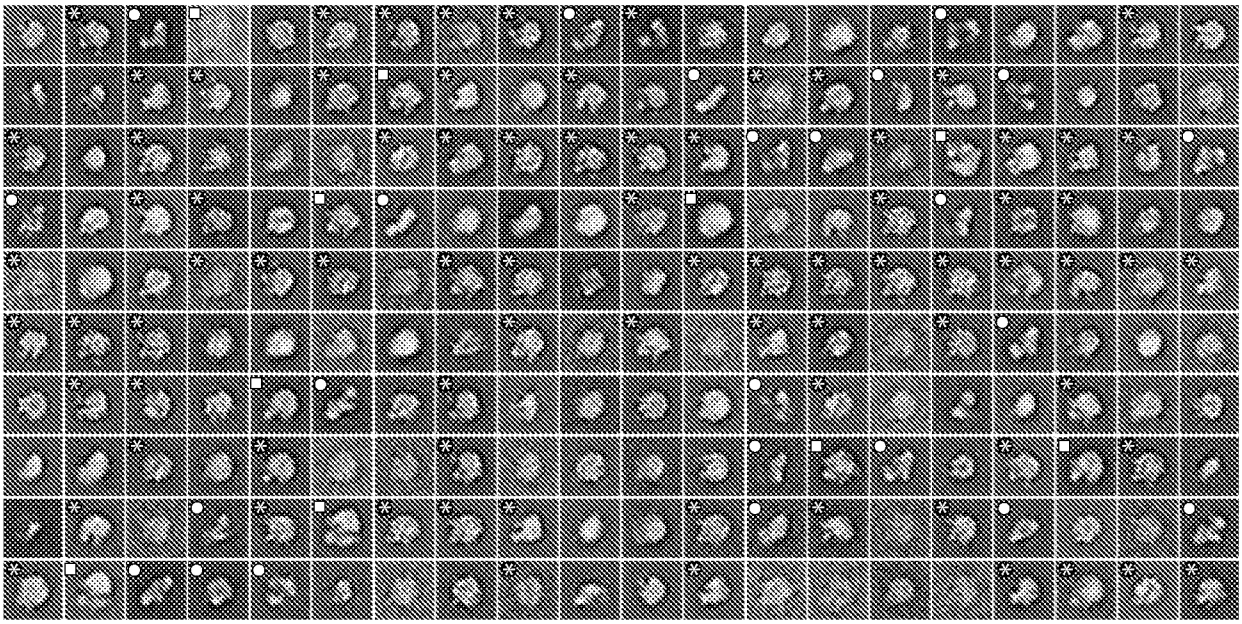

Figure 5

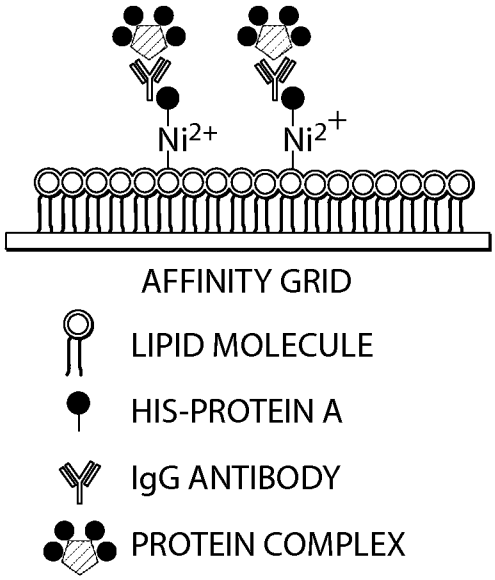

Figure 6

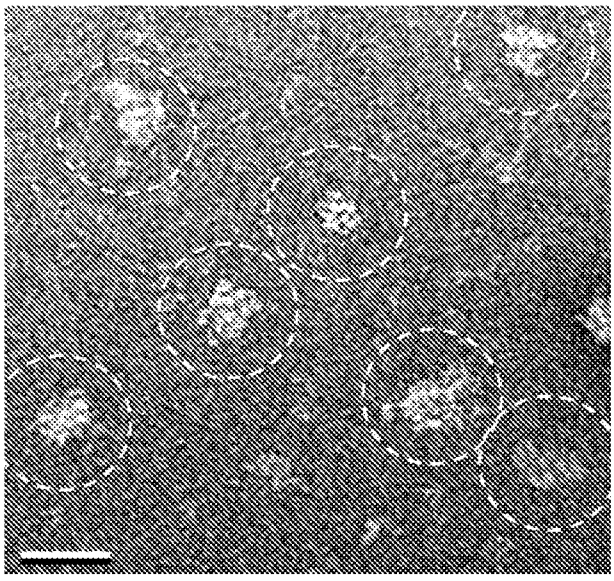

Figure 7

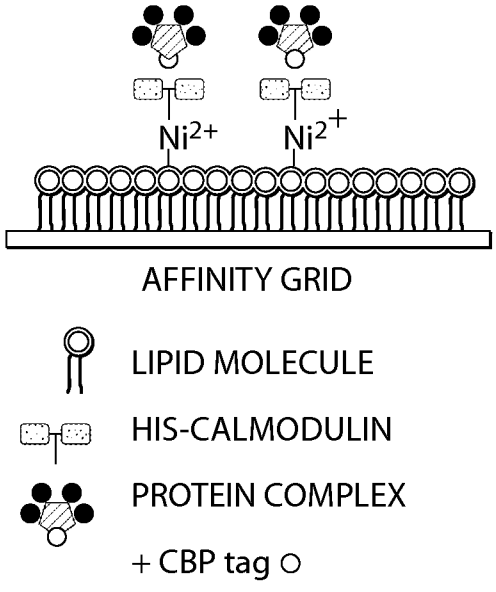
Ni2+
Ni2+
AFFINITY GRID
LIPID MOLECULE
HIS-CALMODULIN
PROTEIN COMPLEX
+ CBP tag ○

Figure 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/03253

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 23/00 (2009.01)
USPC - 250/307; 250/311
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 250/307; 250/311

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic Databases Searched: USPTO WEST (PGPUB, EPAB, JPAB, USPT), Google web. Search Terms Used: electron microscopy or EM , grid, electron microscop$ and phospholipid, carbon monolay$, nitriloacetic, nickel

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,250,127 A (Warren et al.) 10 February 1981 (10.02.1981) *entire document especially figure 1; col 6, ln 22 to col 7, ln 22*	1-3 and 12-14
Y	US 6,207,178 B1 (Westesen et al.) 27 March 2001 (27.03.2001) especially col 16, ln 66 to col 17, ln 13; col 12, ln 45-56	1-3 and 12-14
Y	US 5,891,468 A (Martin et al.) 06 April 1999 (06.04.1999) especially col 15, ln 35-41	2
Y	US 2005/0287611 A1 (Nugent IV et al.) 29 December 2005 (29.12.2005) especially para [0085]; [0101]; [0106]	13-14

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 August 2009 (15.08.2009)	27 AUG 2009
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 09/03253

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

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

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

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

3. ☒ Claims Nos.: 4-11 and 15-32
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.