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(54) **METHODS AND SYSTEMS FOR MONITORING PRODUCTION OF A TARGET PROTEIN IN A NANOLIPOPROTEIN PARTICLE**

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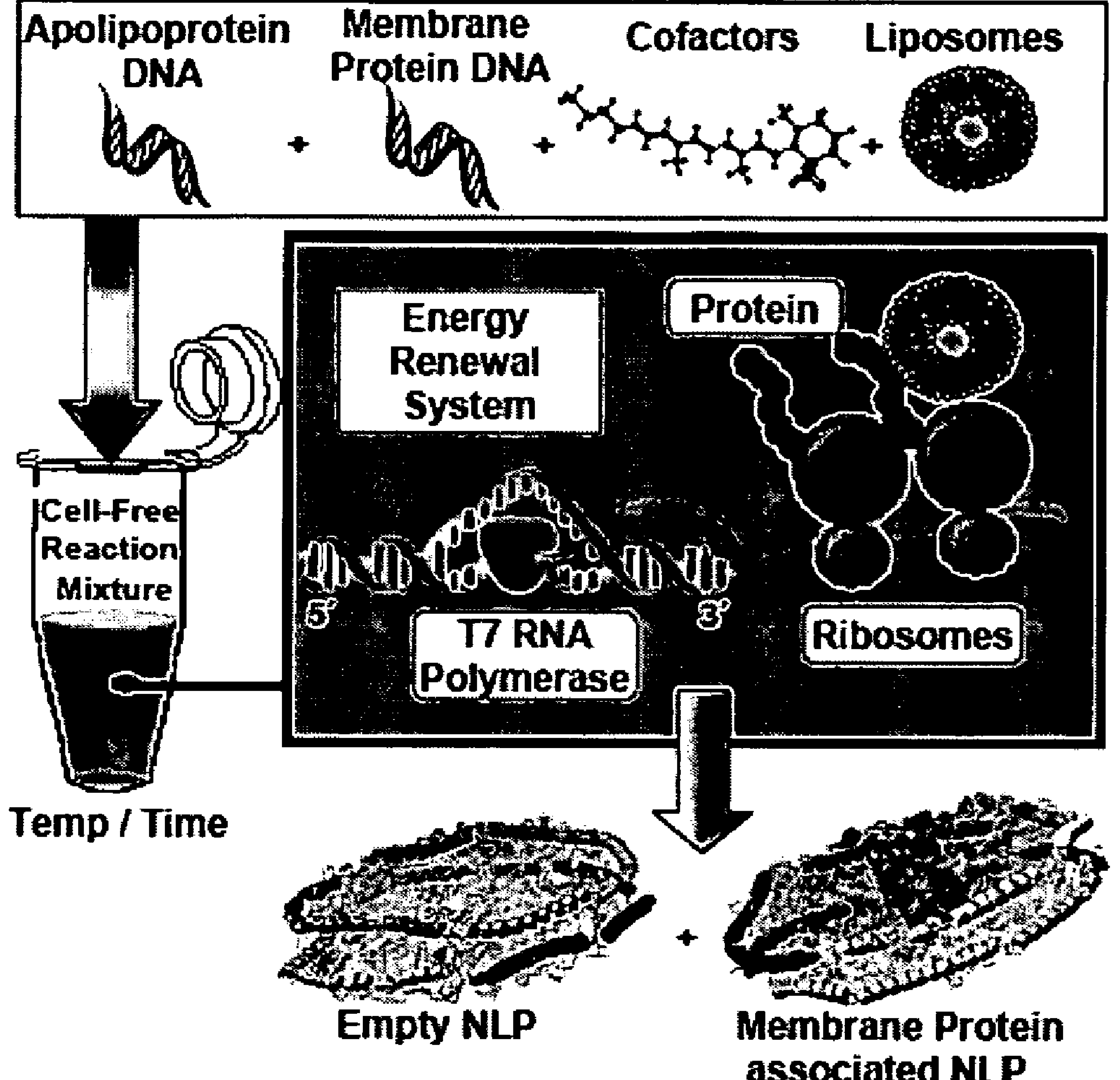
(57) **ABSTRACT**

Provided herein are methods and systems for the monitoring production of a target protein in of a nanolipoprotein particle (NLP) that also includes a scaffold protein and a membrane forming lipid. The target protein is capable of assuming an active form and an inactive form. Monitoring is performed by an indicator protein that is capable of assuming an active form and an inactive form, the active form associated with a detectable activity of the indicator protein, the detectable activity further associated with the active form of the target protein.



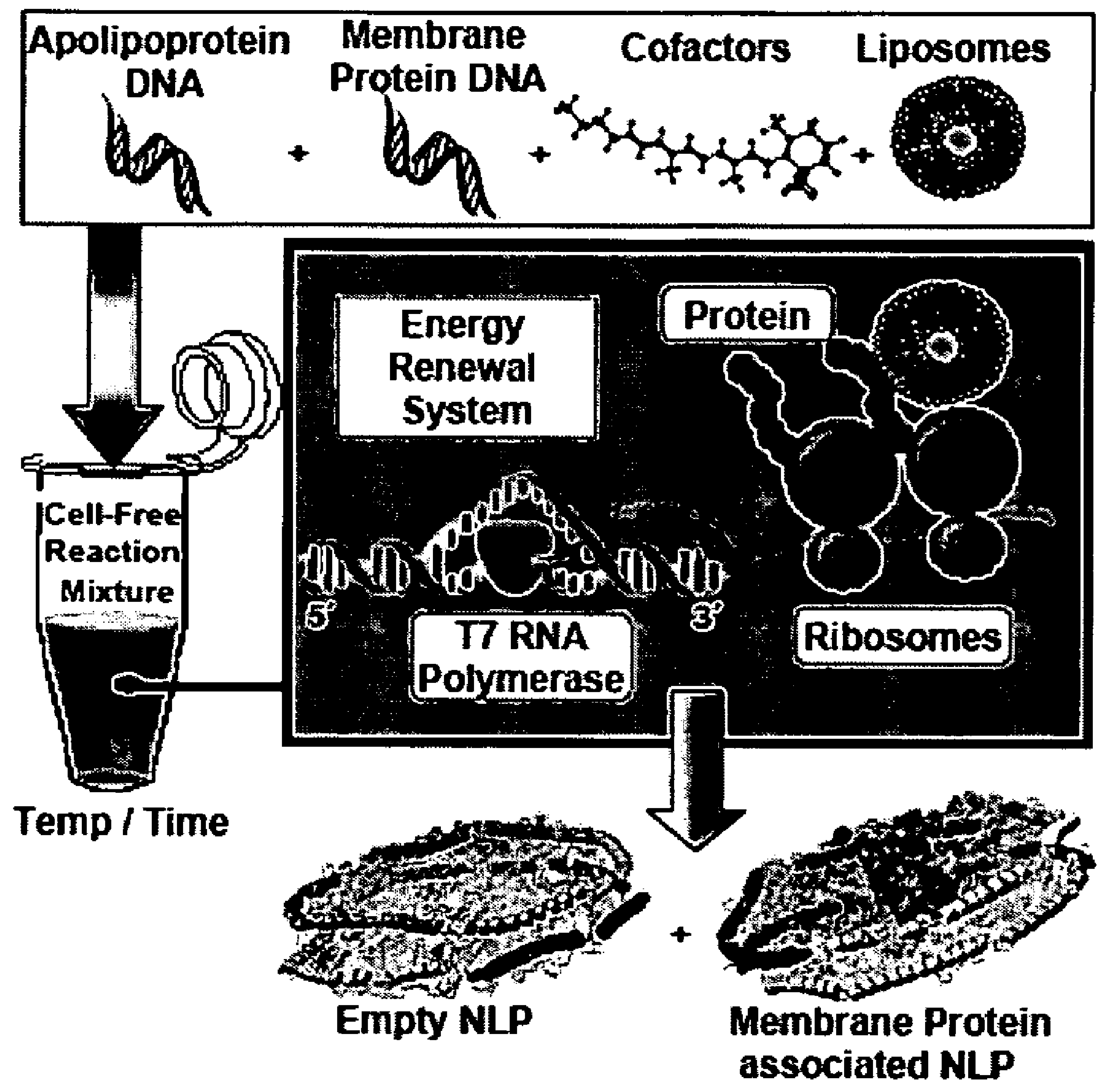


FIG. 1

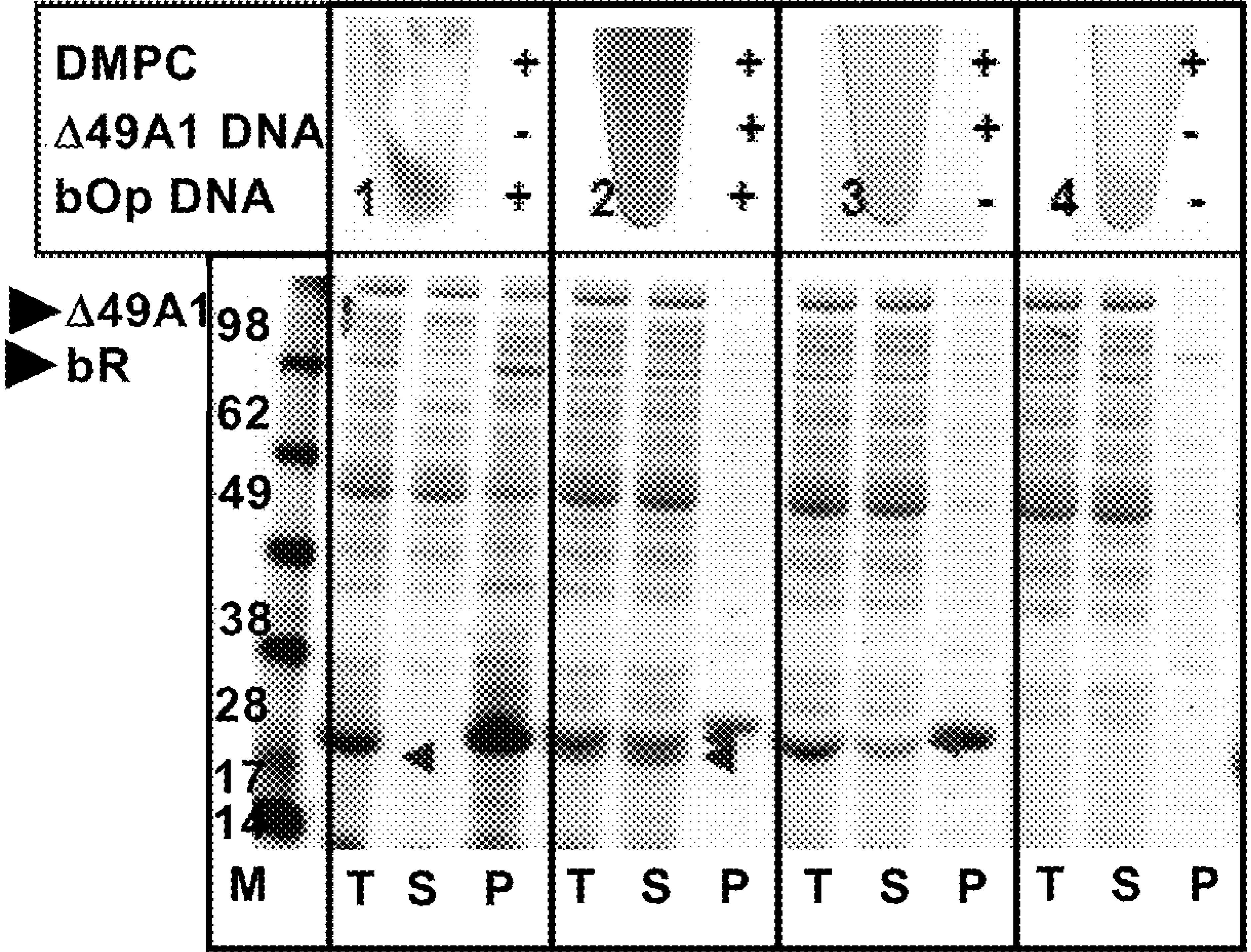


FIG. 2

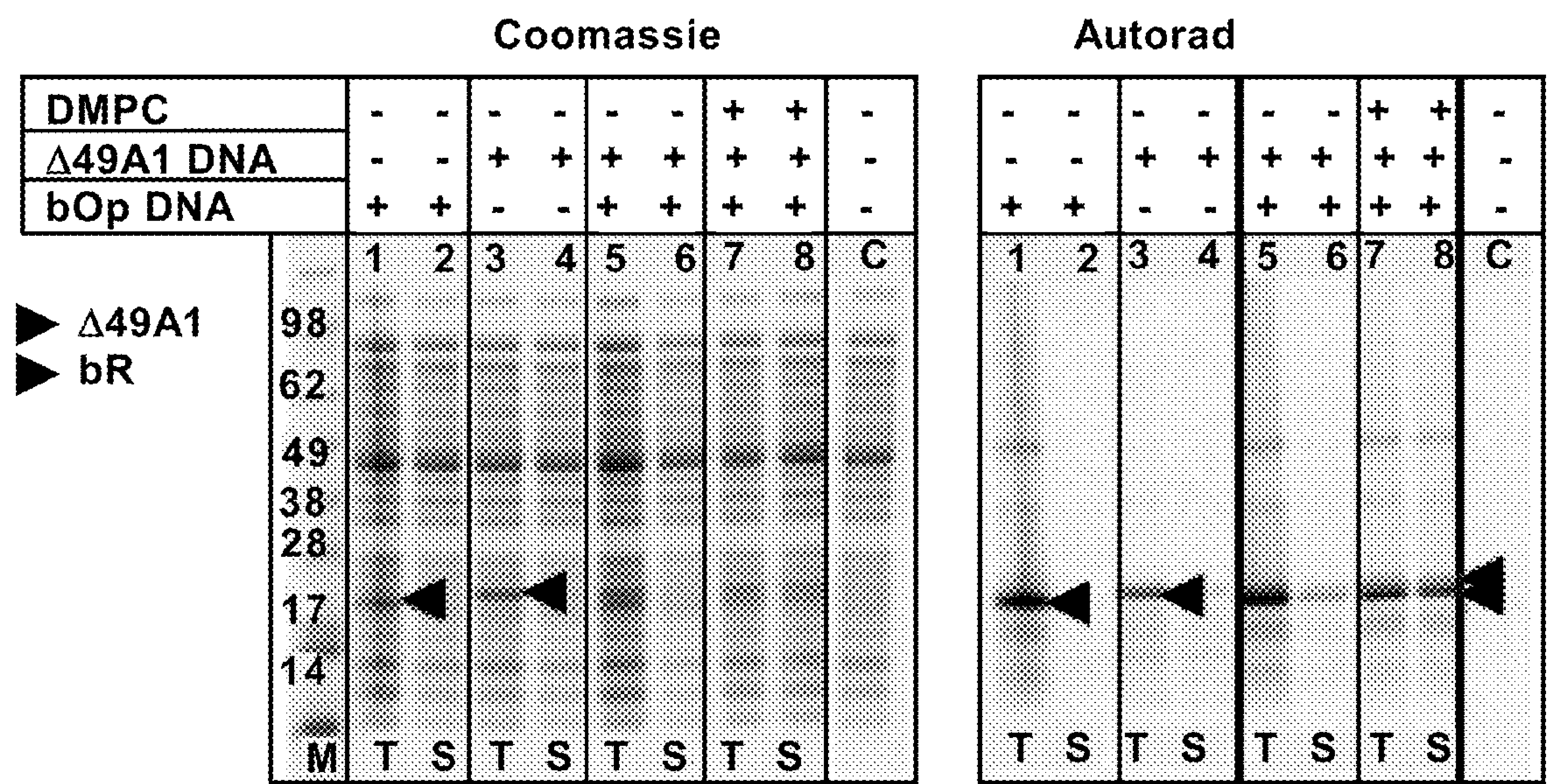


FIG. 3

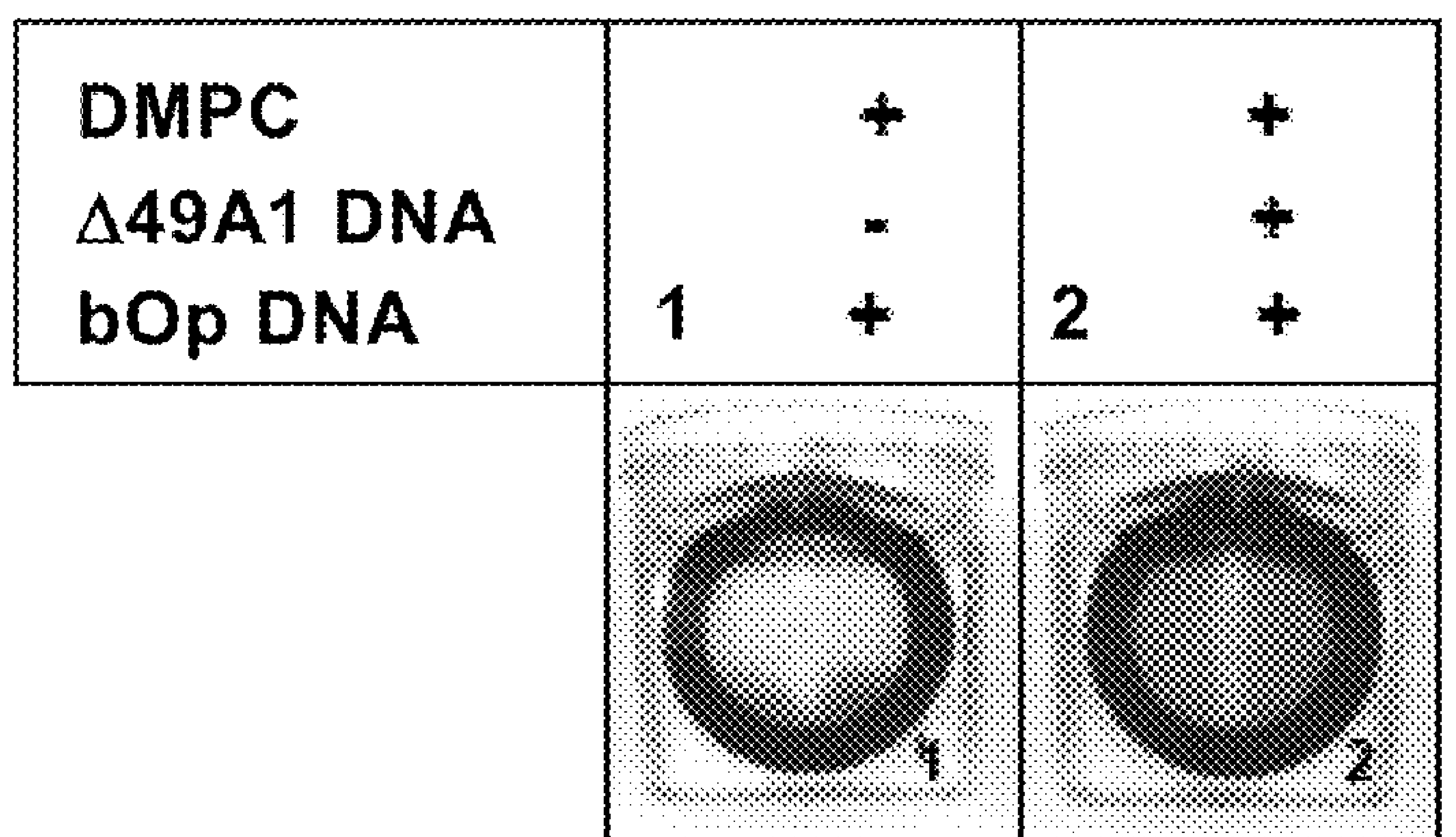


FIG. 4

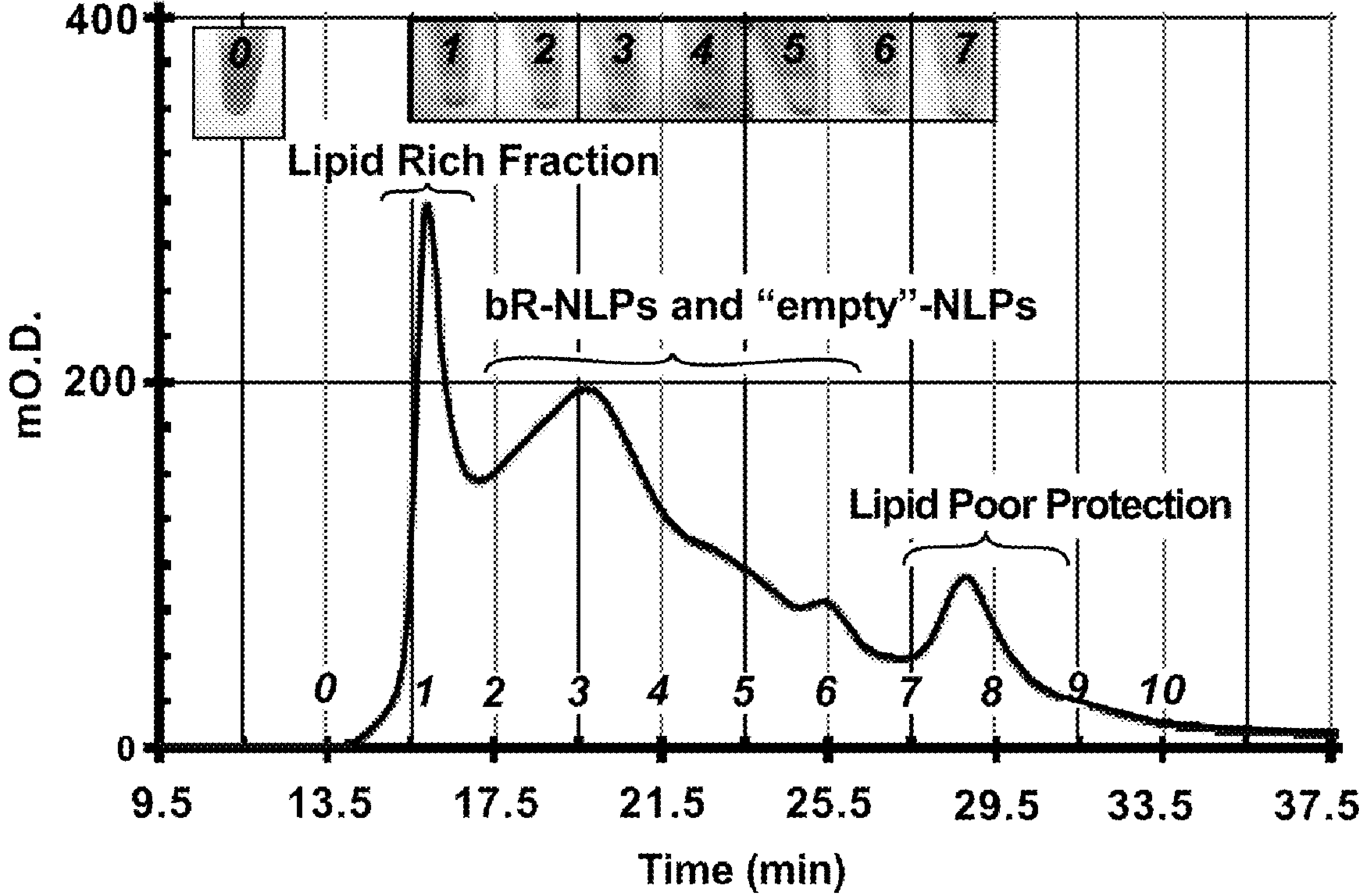


FIG. 5

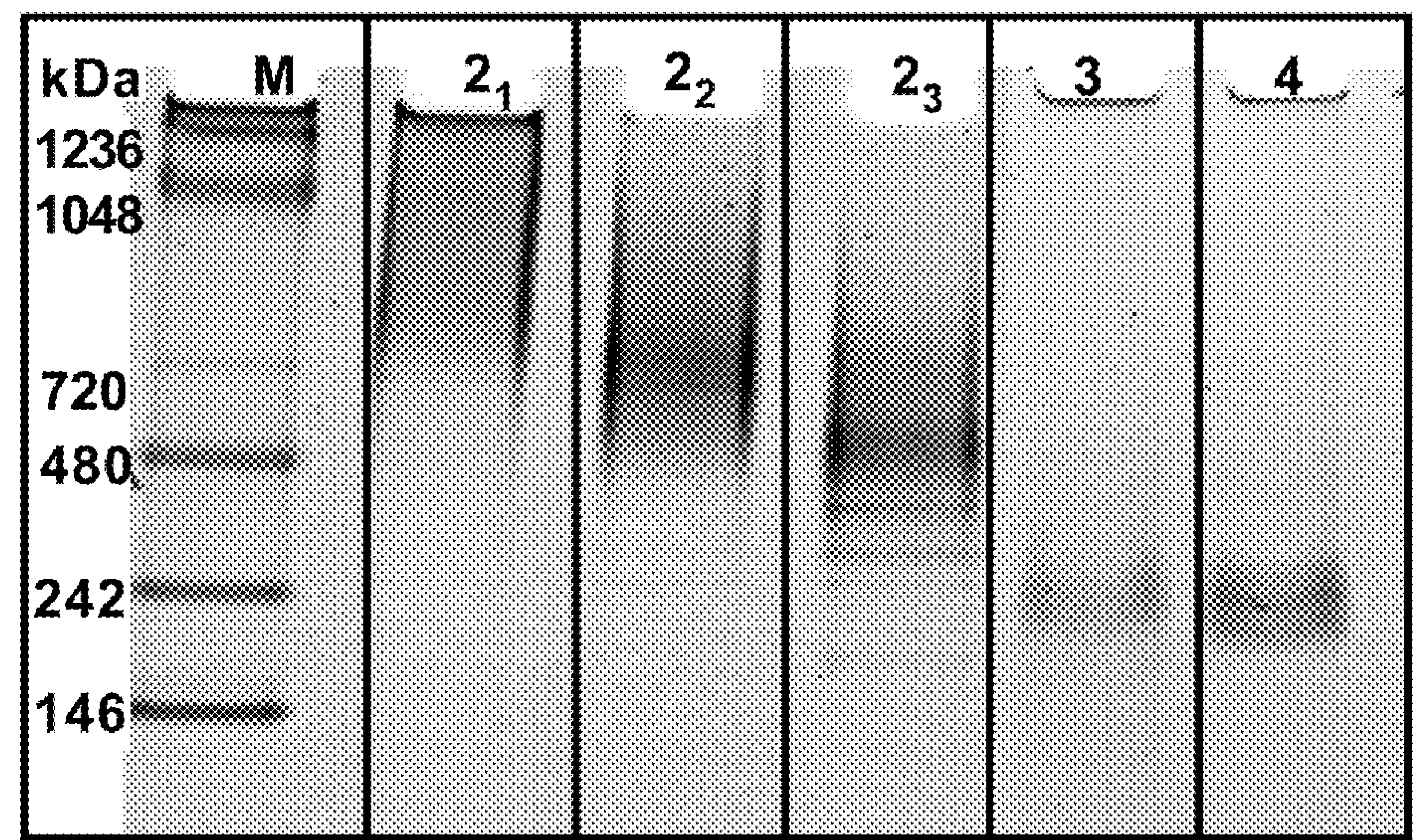


FIG. 6

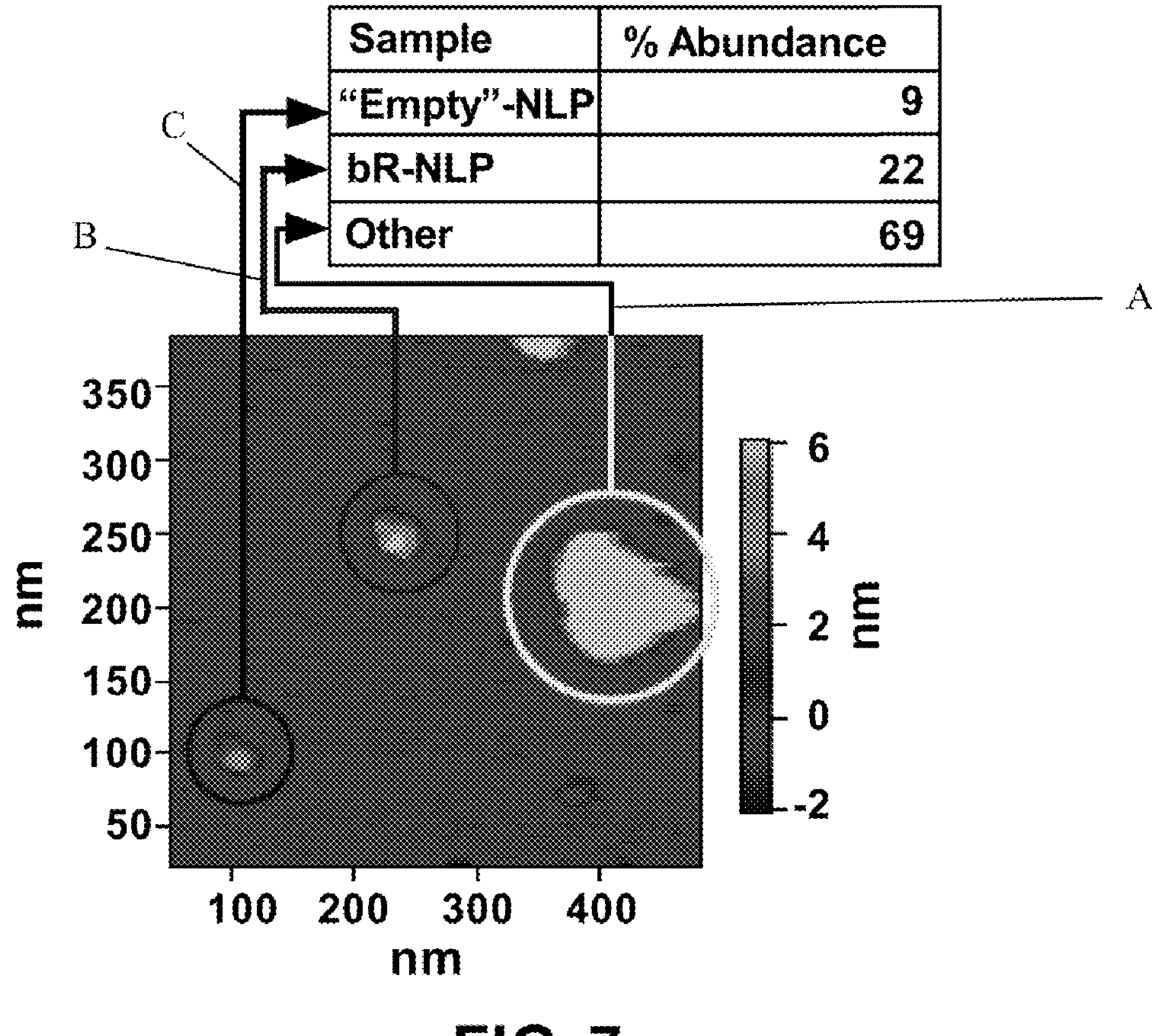


FIG. 7

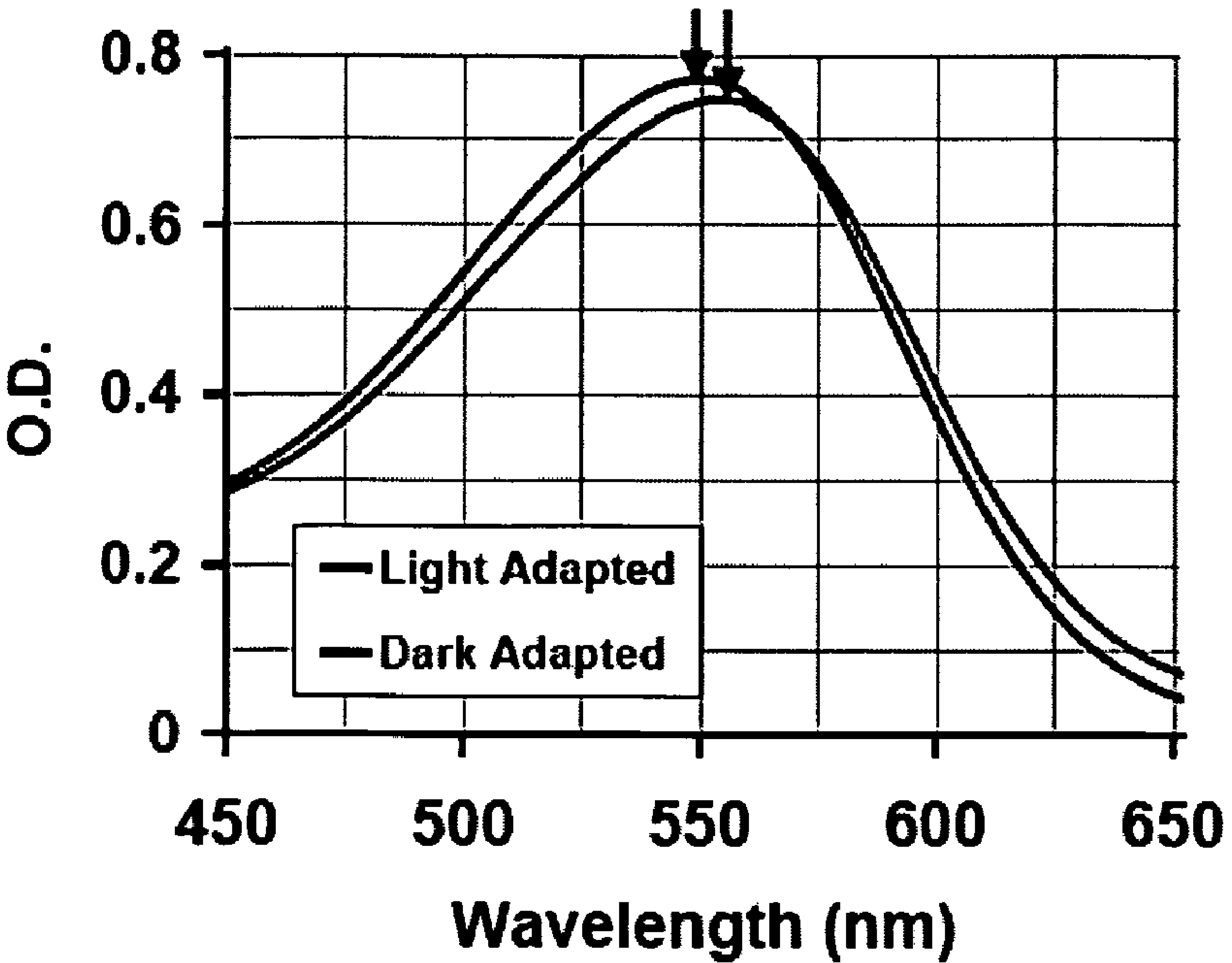


FIG. 8

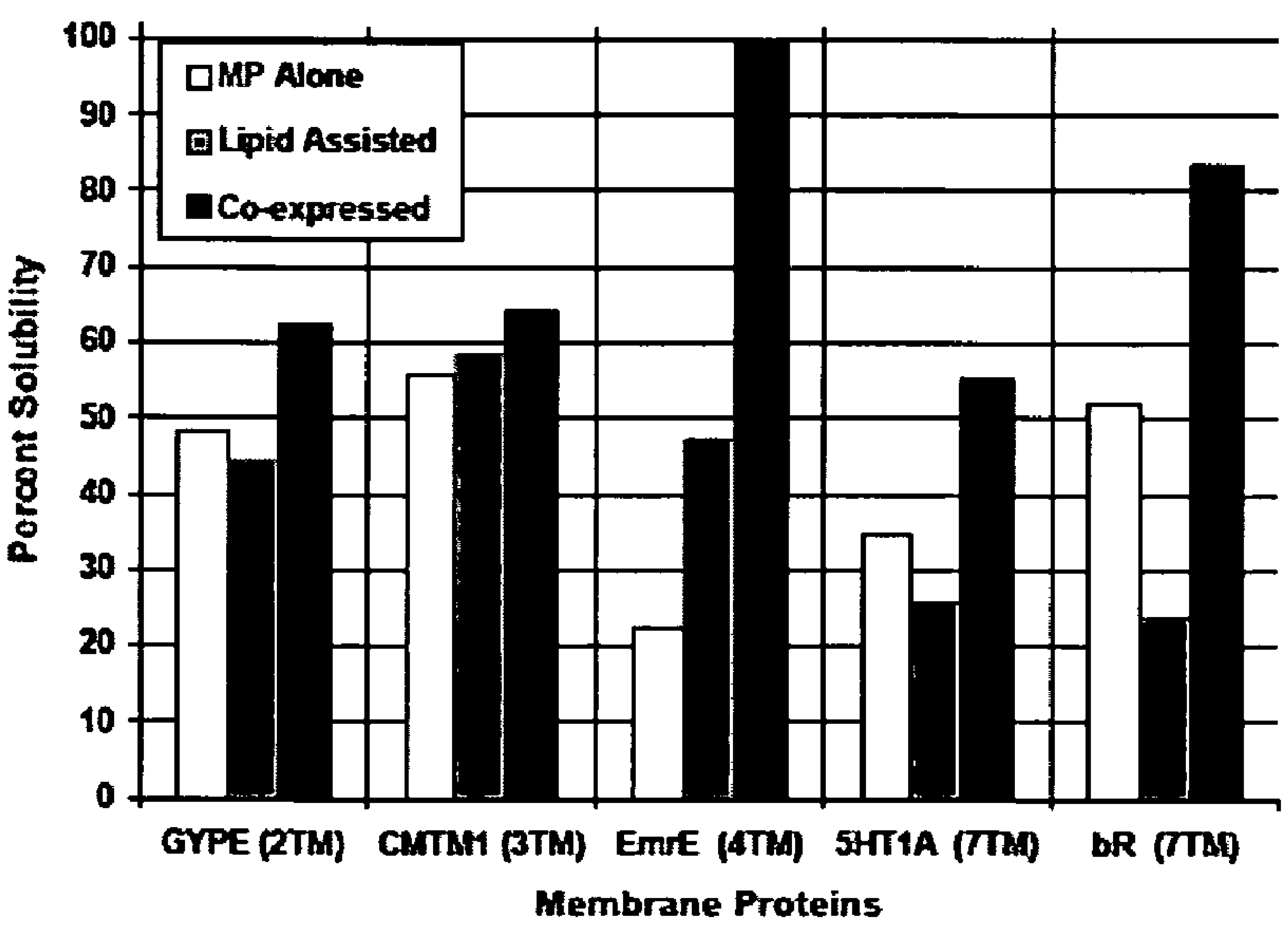


FIG. 9

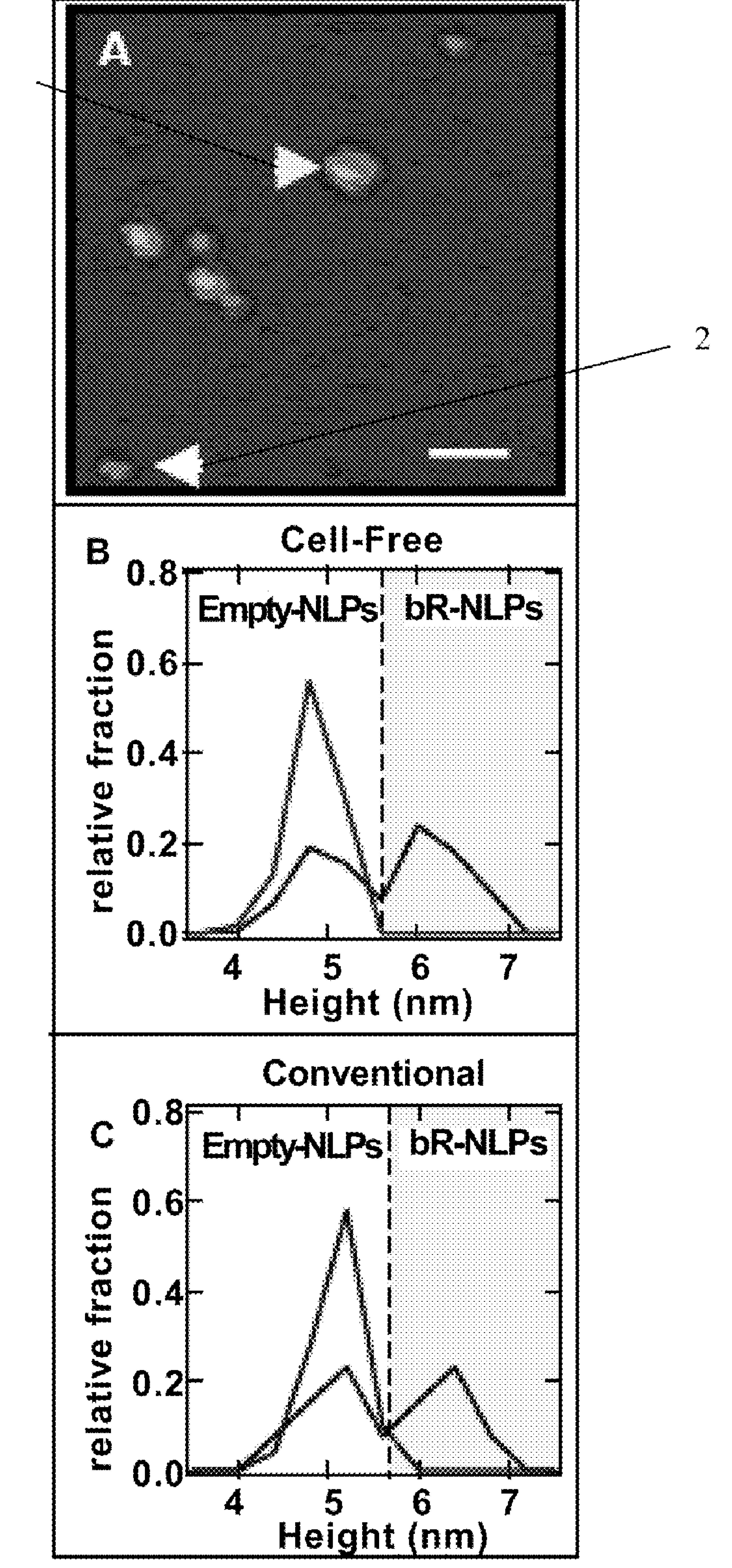


FIG 10

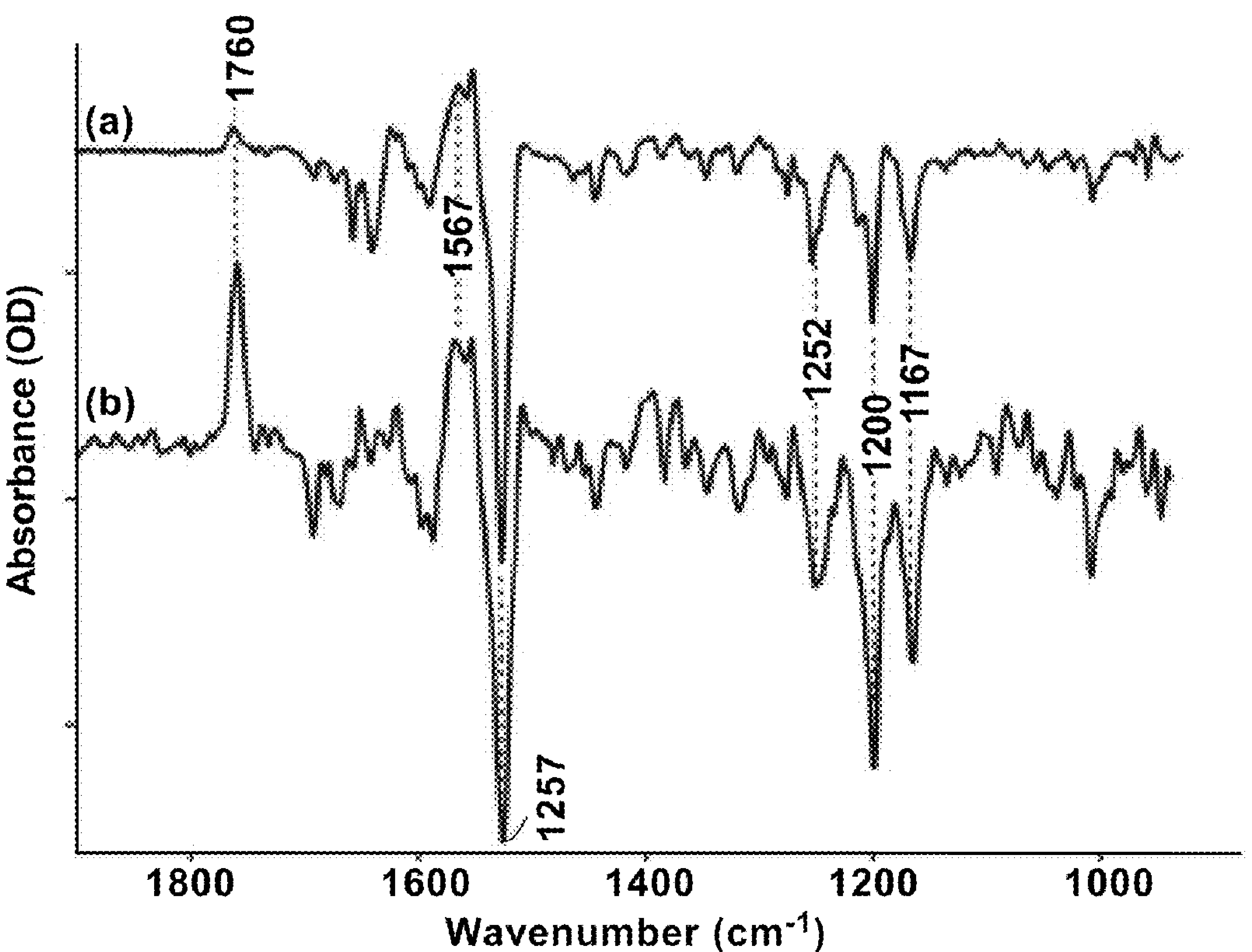


FIG 11

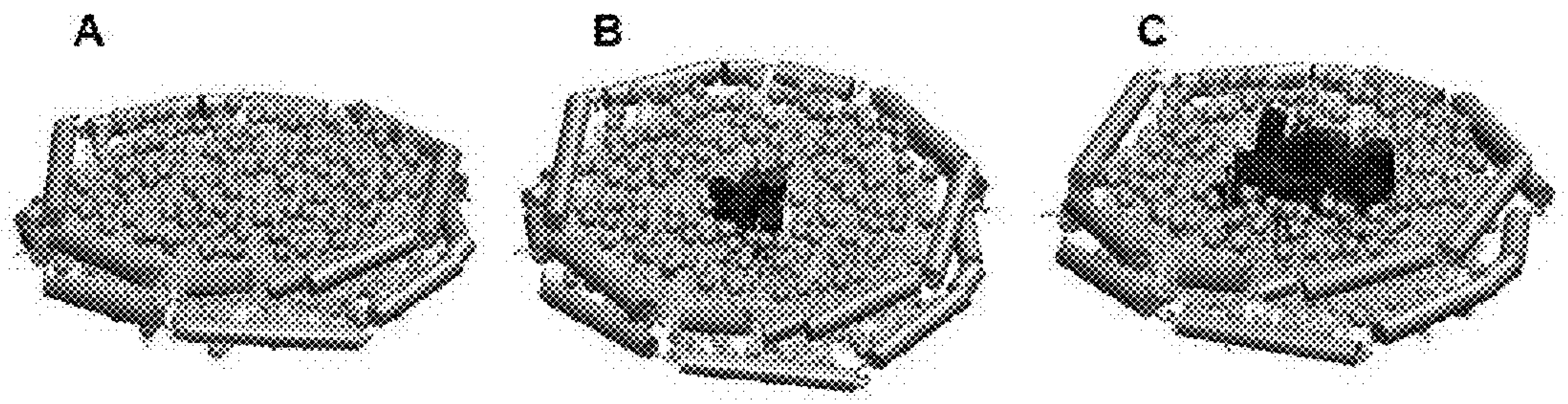


FIG 12

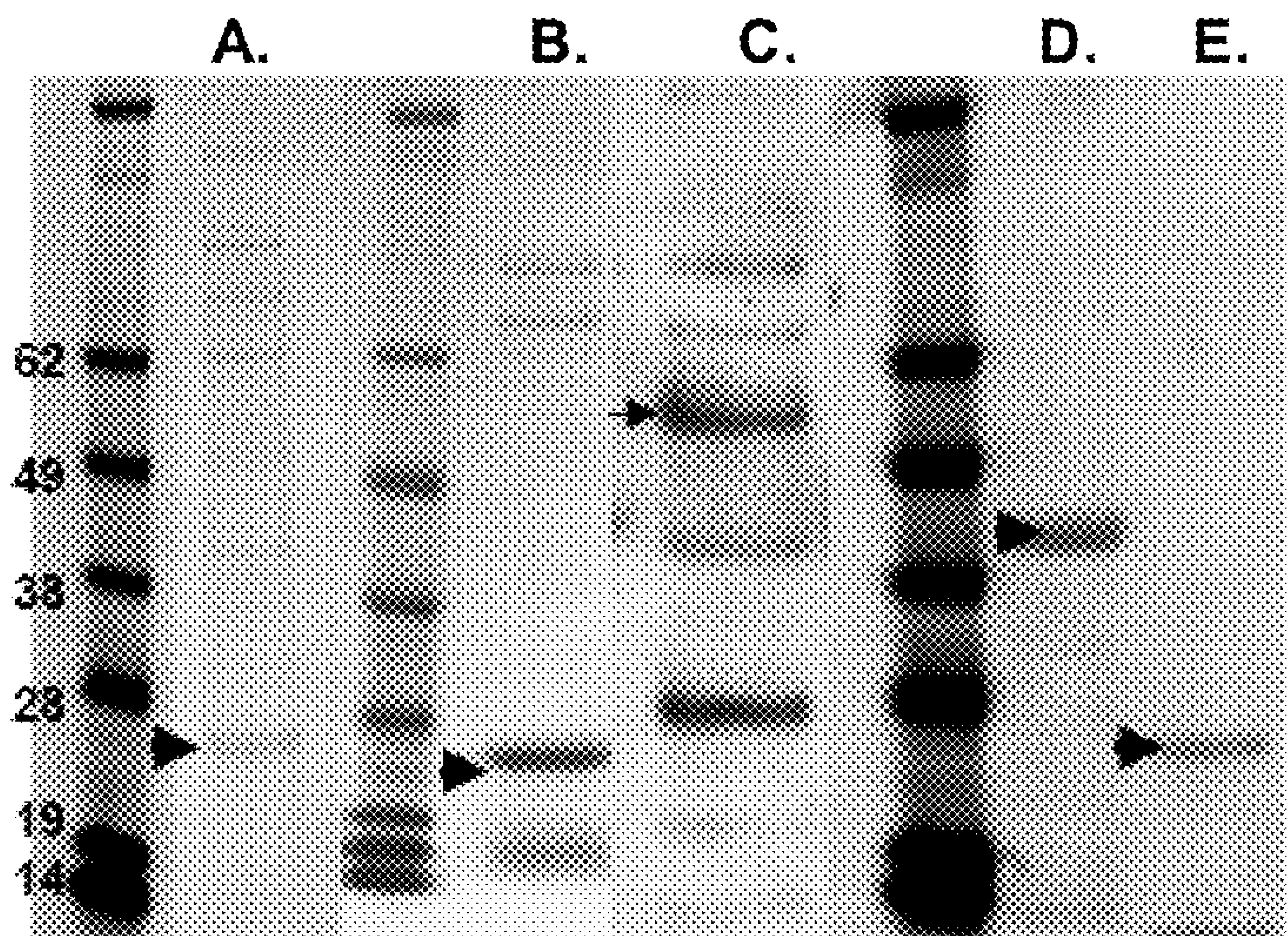


FIG 13

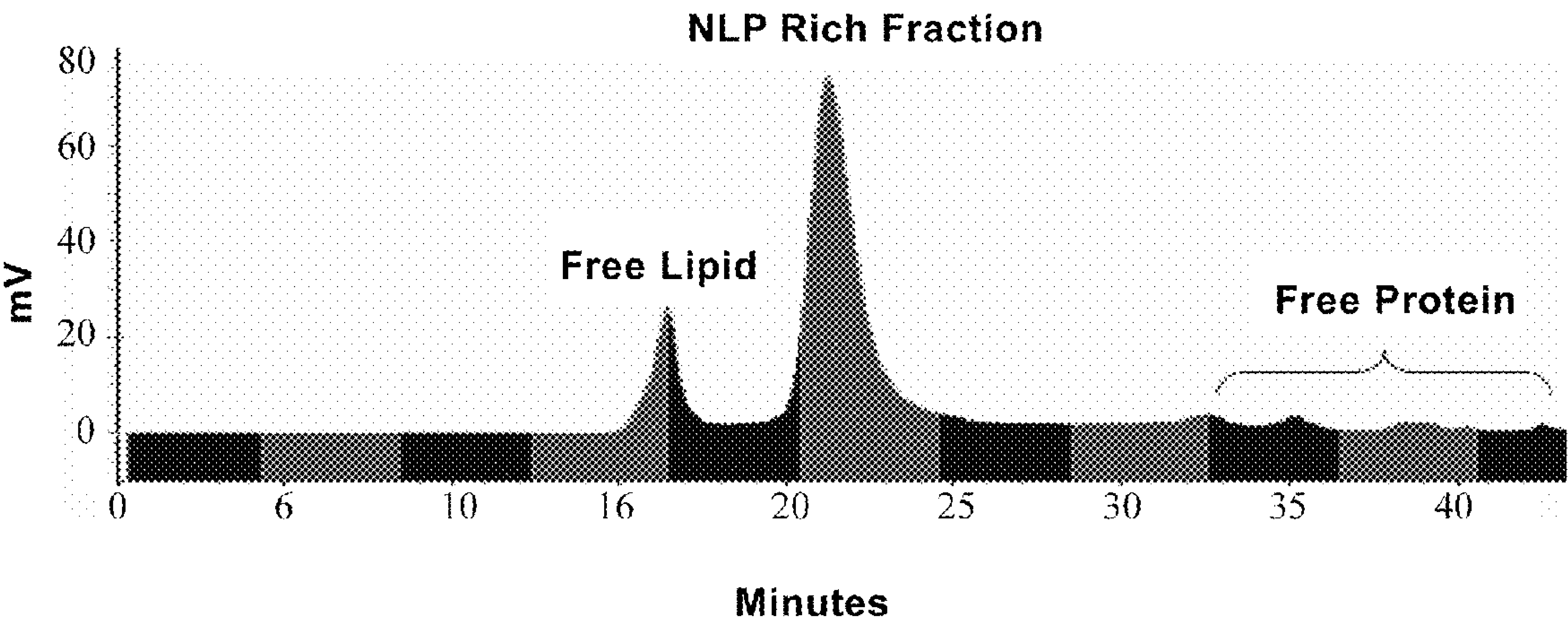


FIG 14

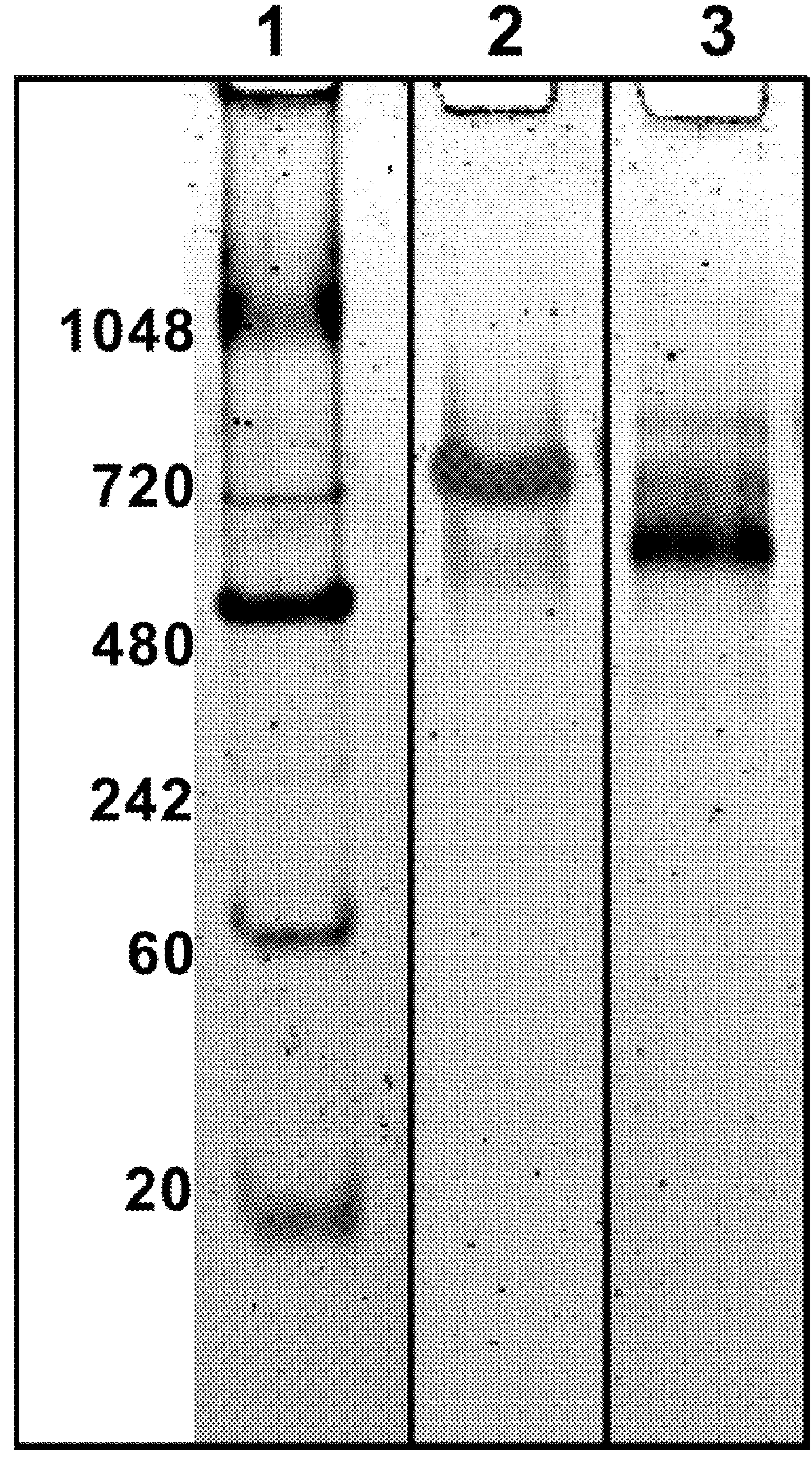


FIG 15

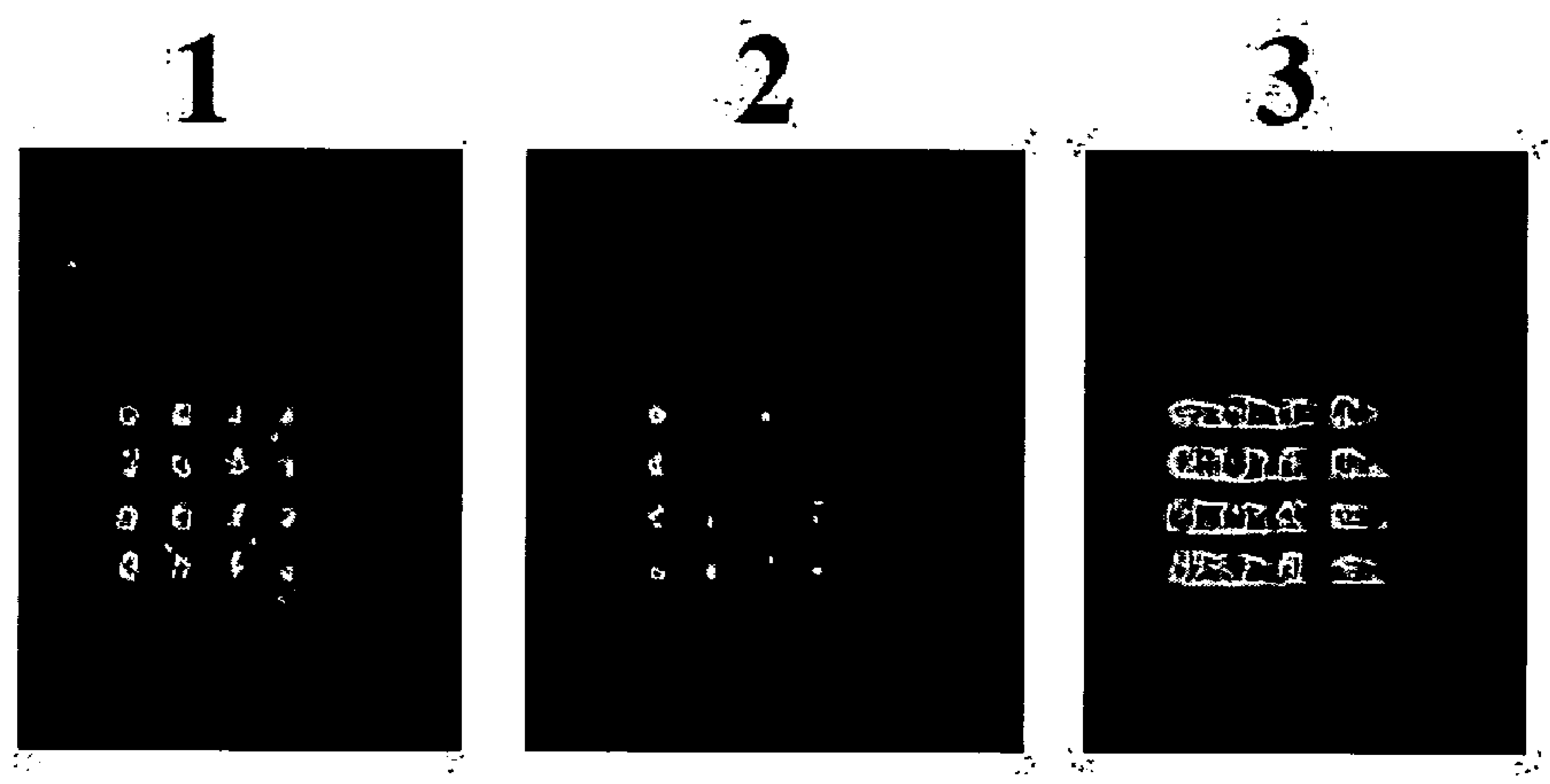


FIG. 16

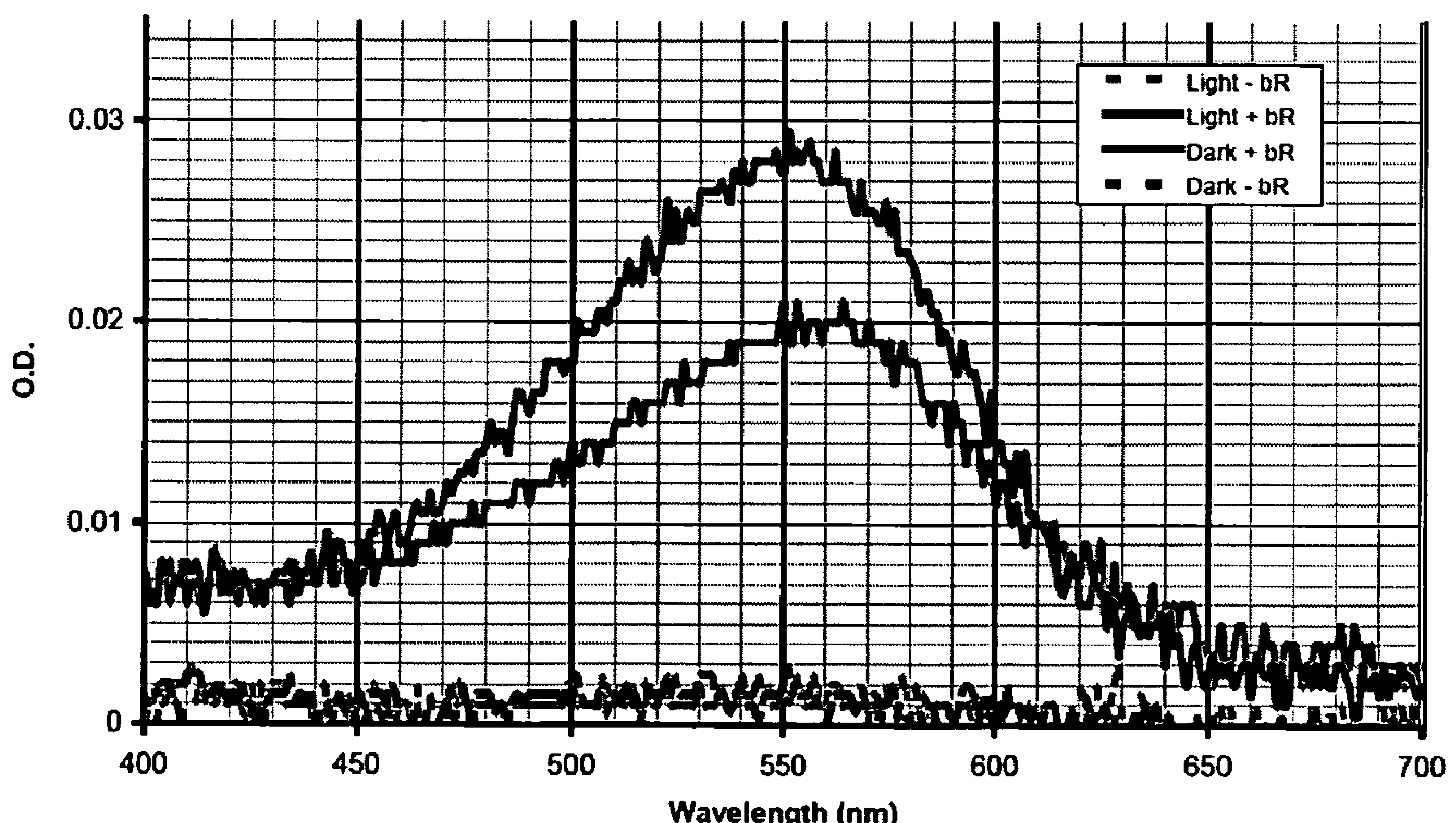


FIG. 17

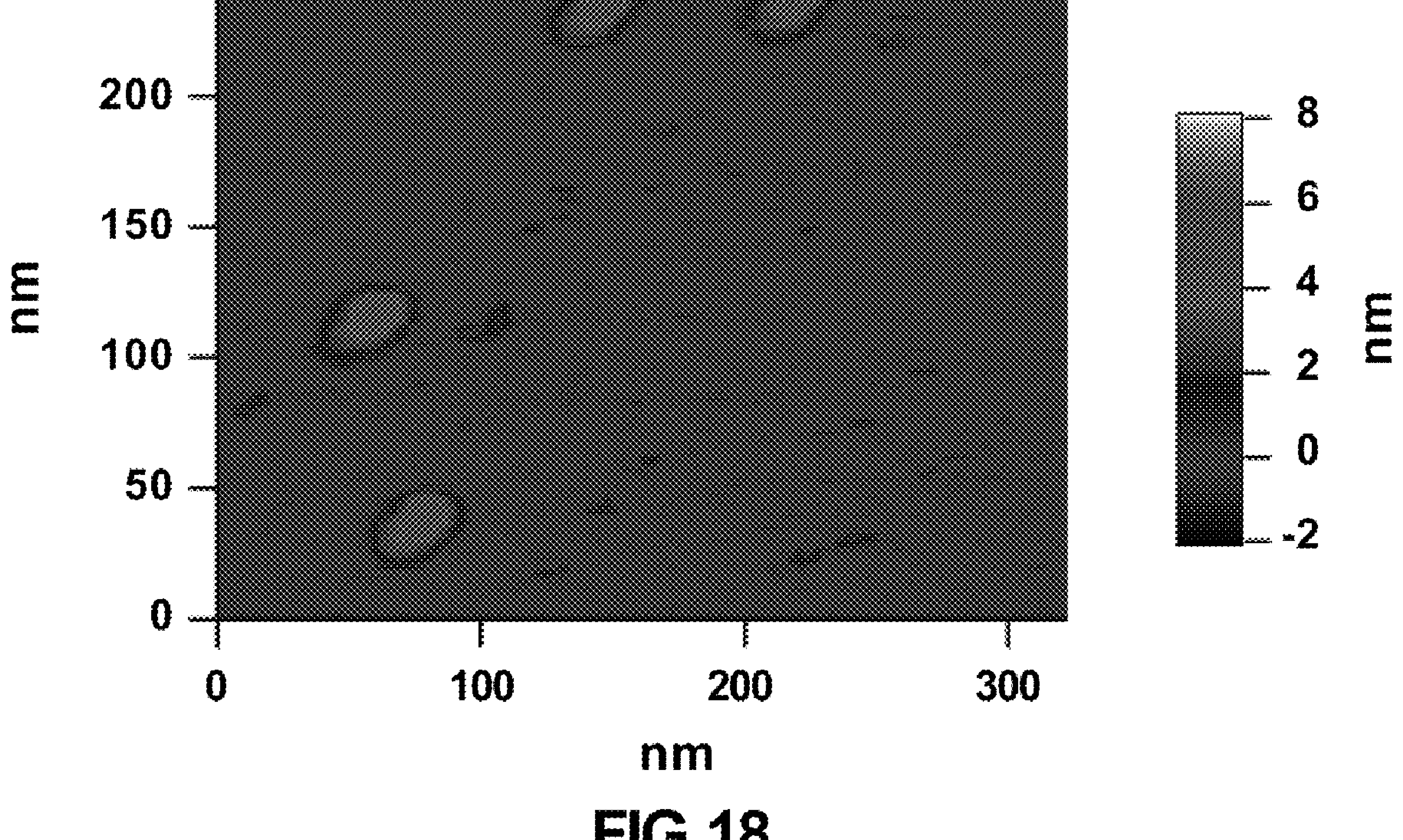


FIG 18

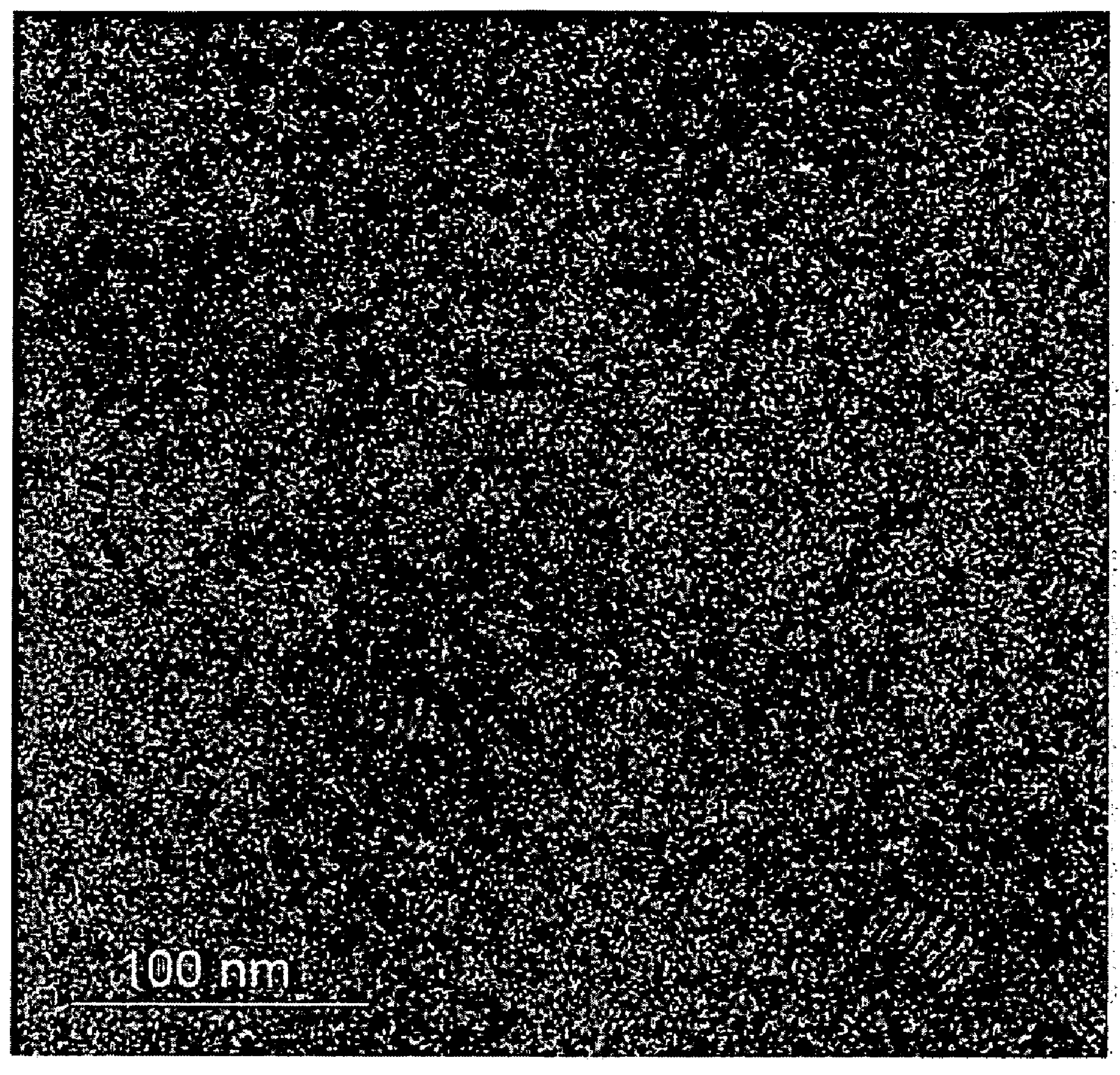


FIG. 19

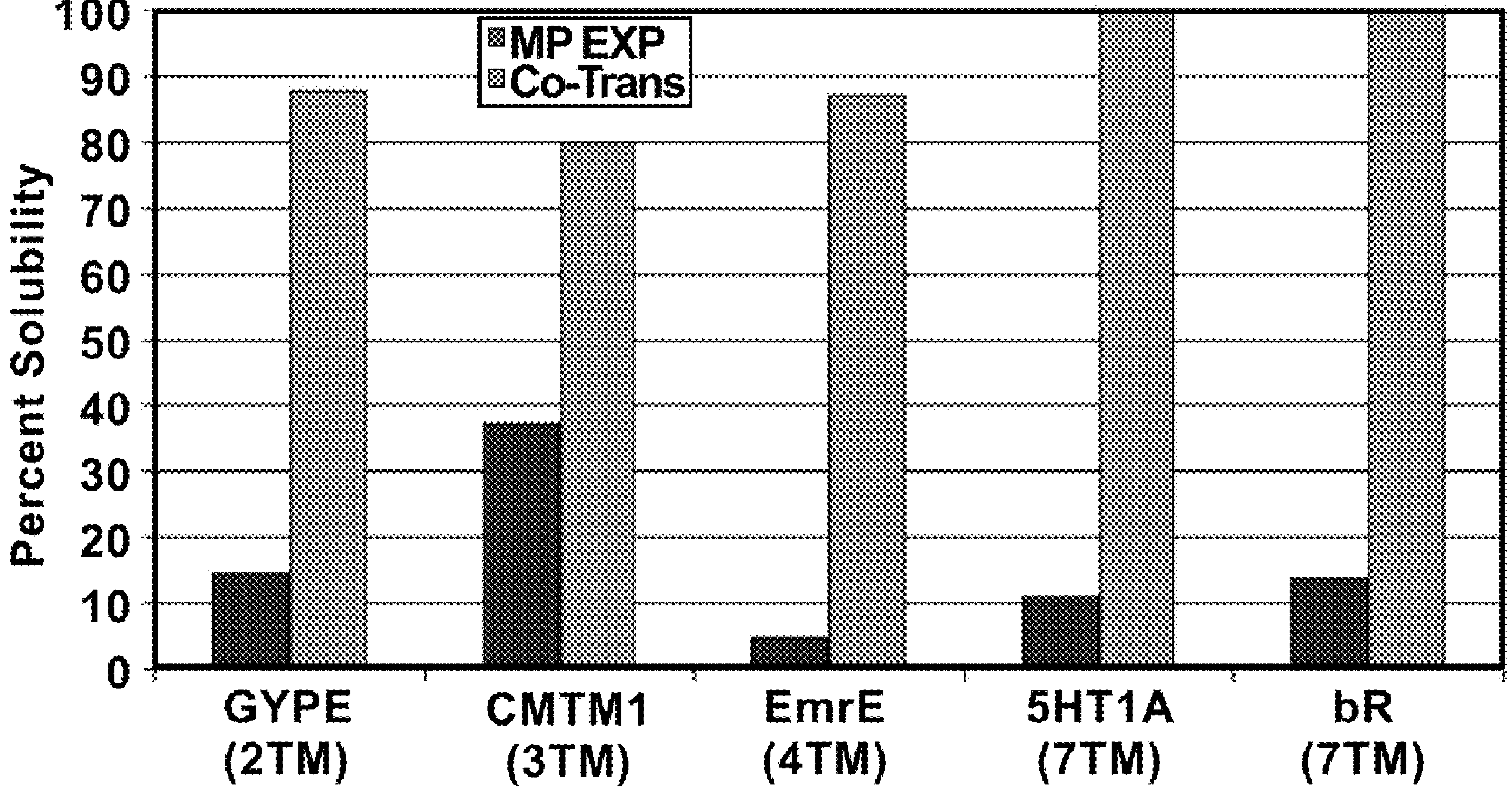


FIG. 20

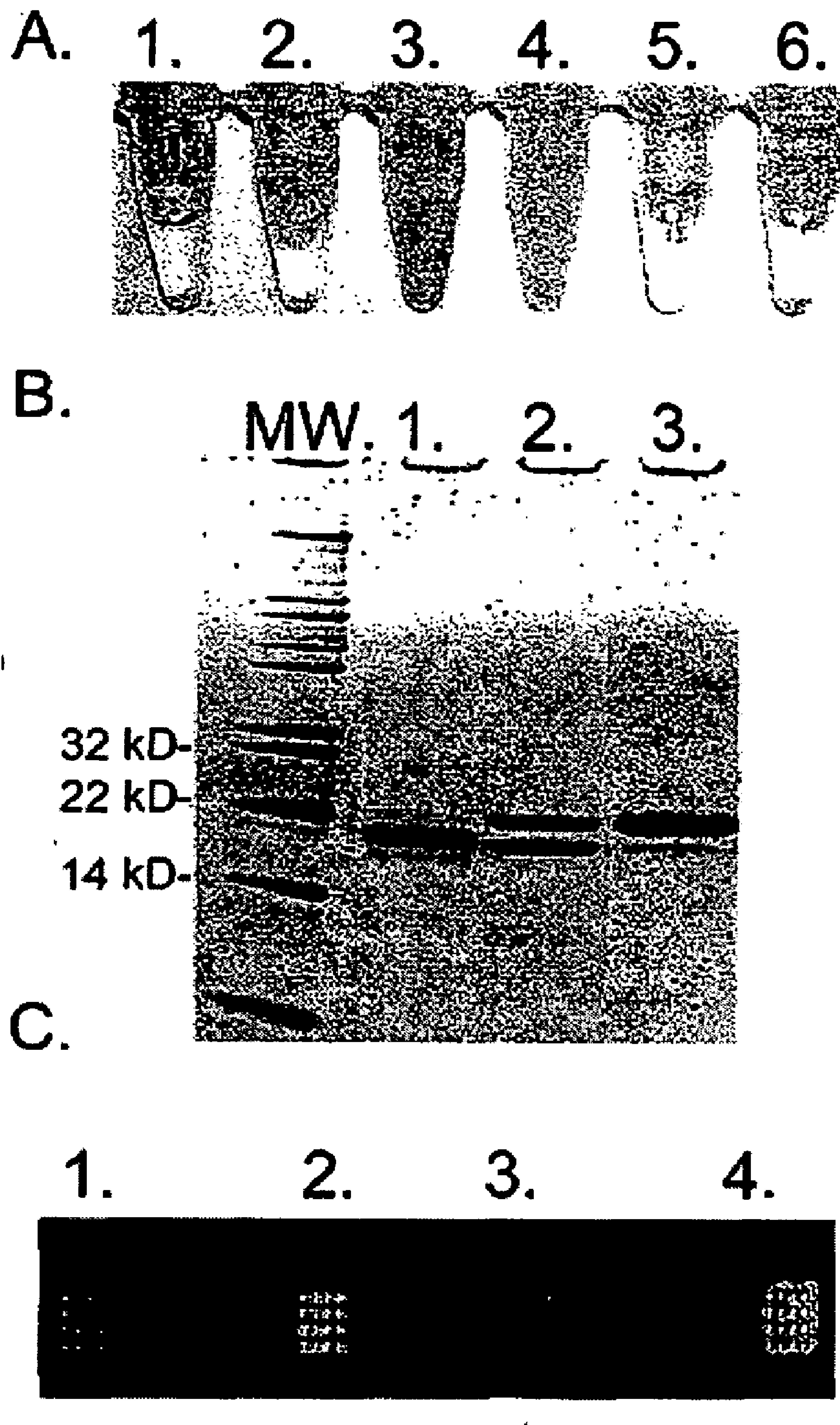
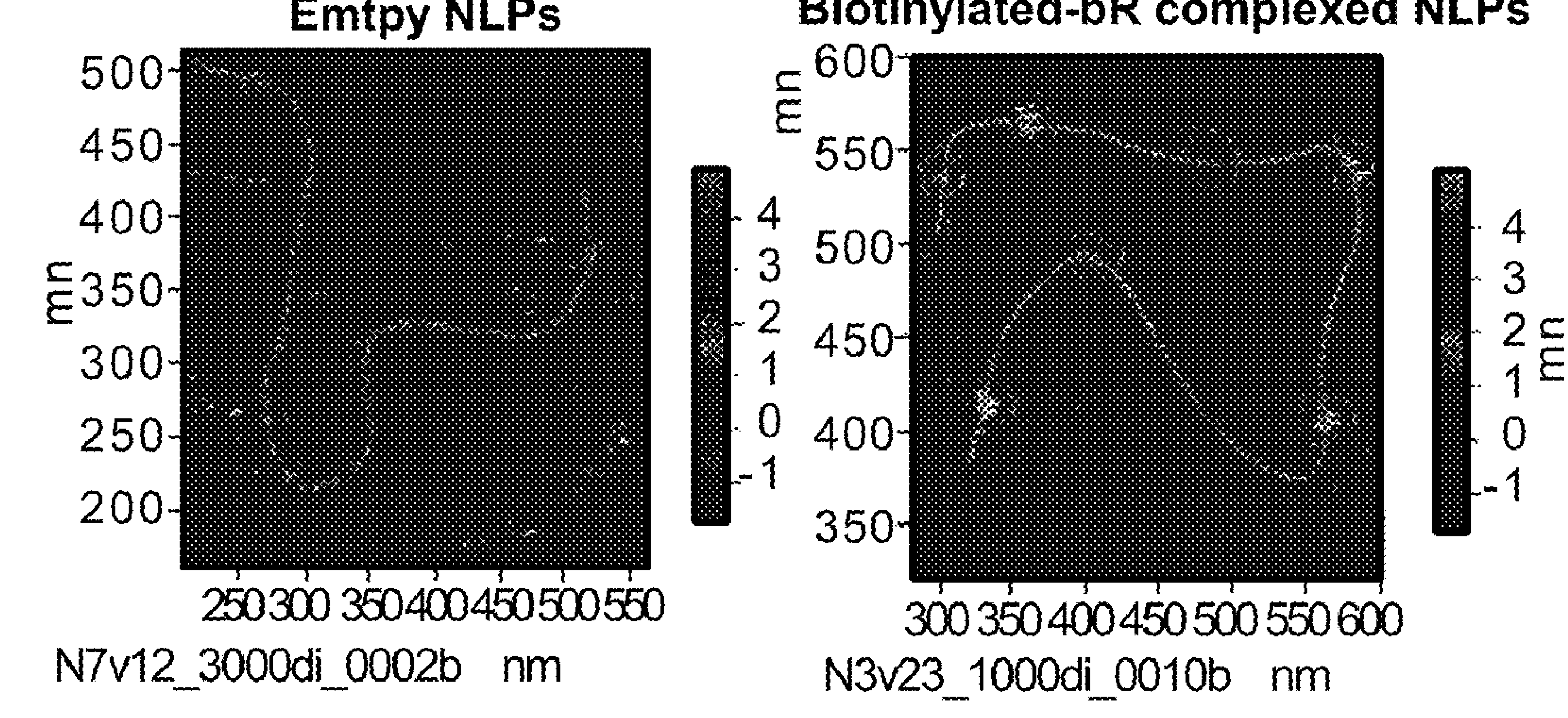


FIG 21



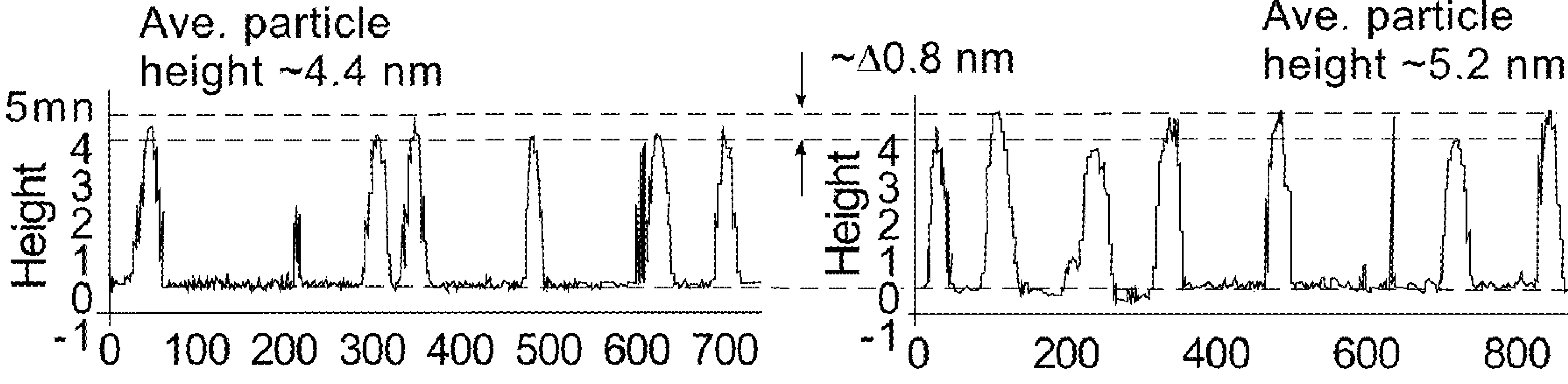


FIG. 22

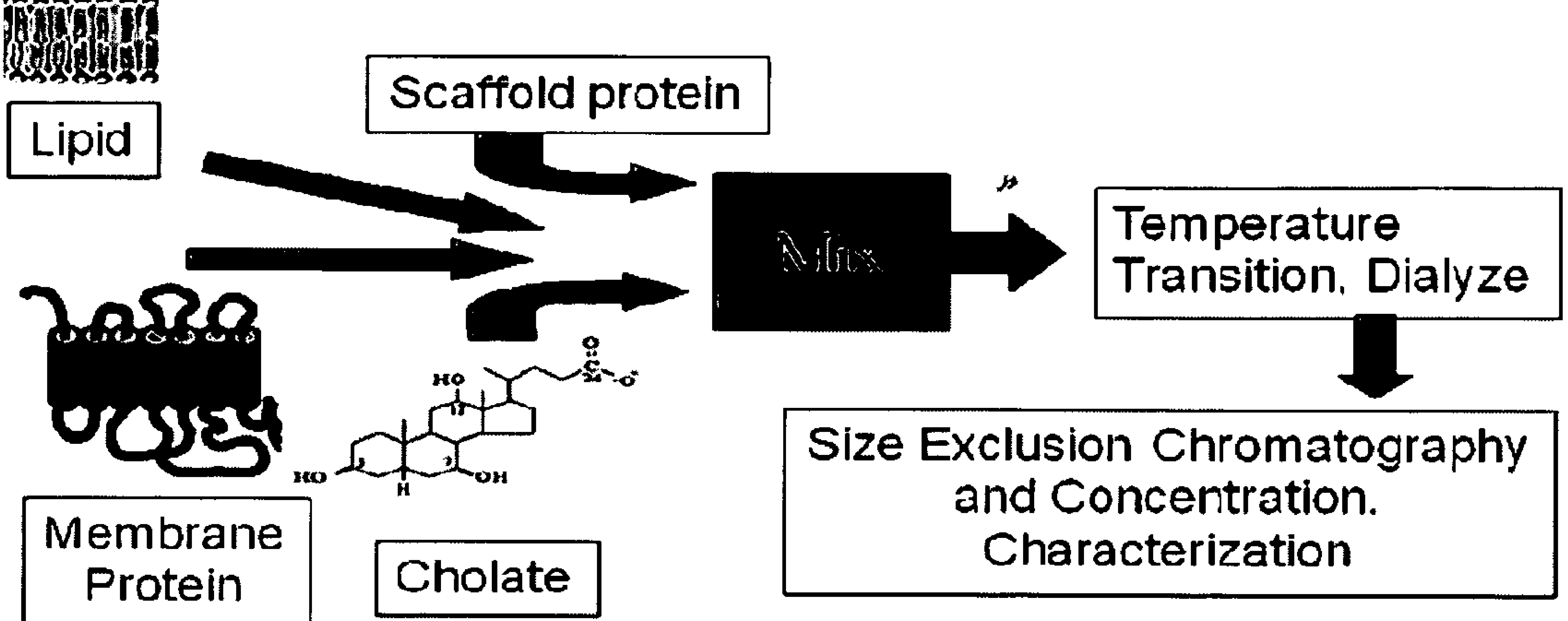


FIG. 23

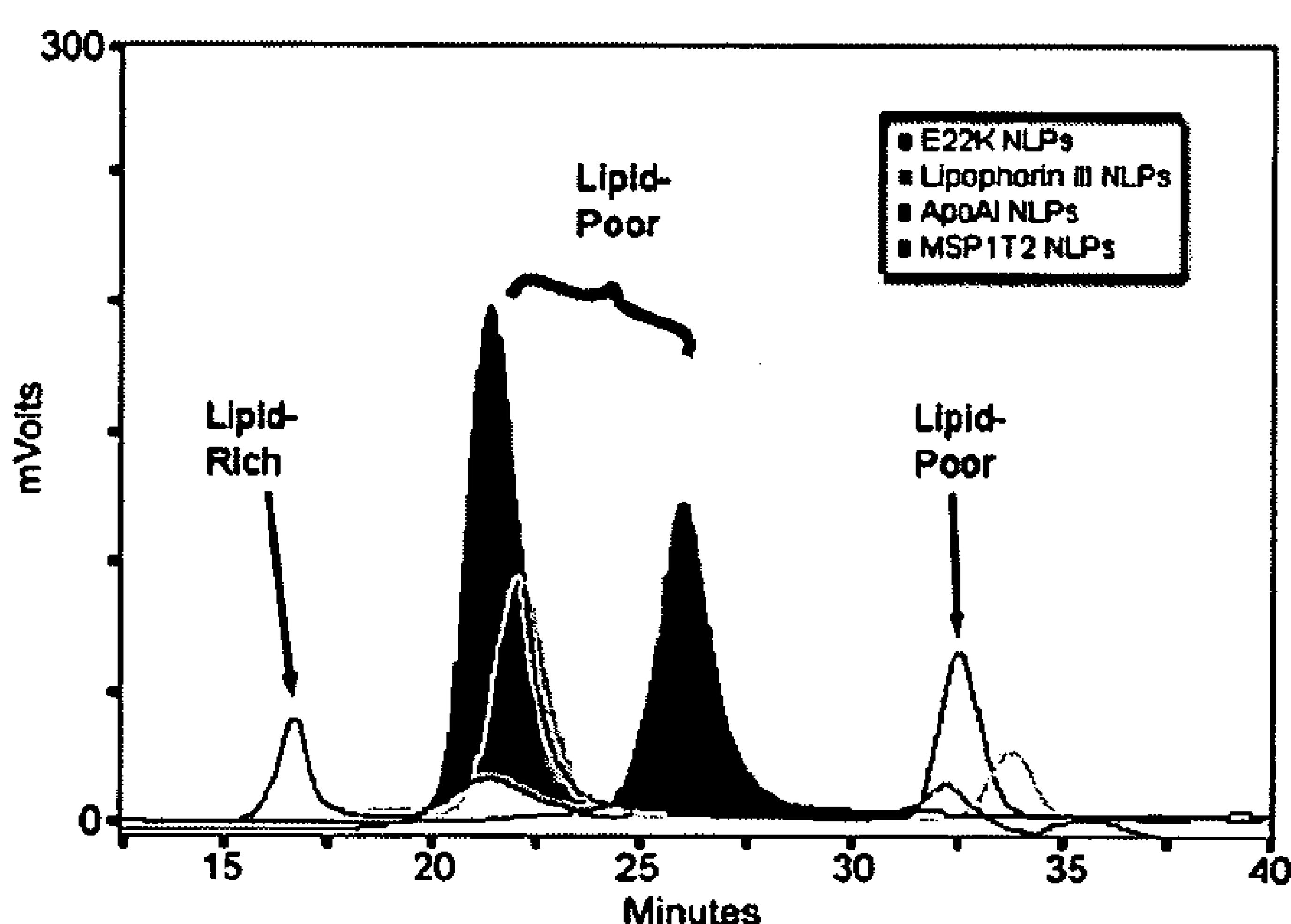


FIG. 24

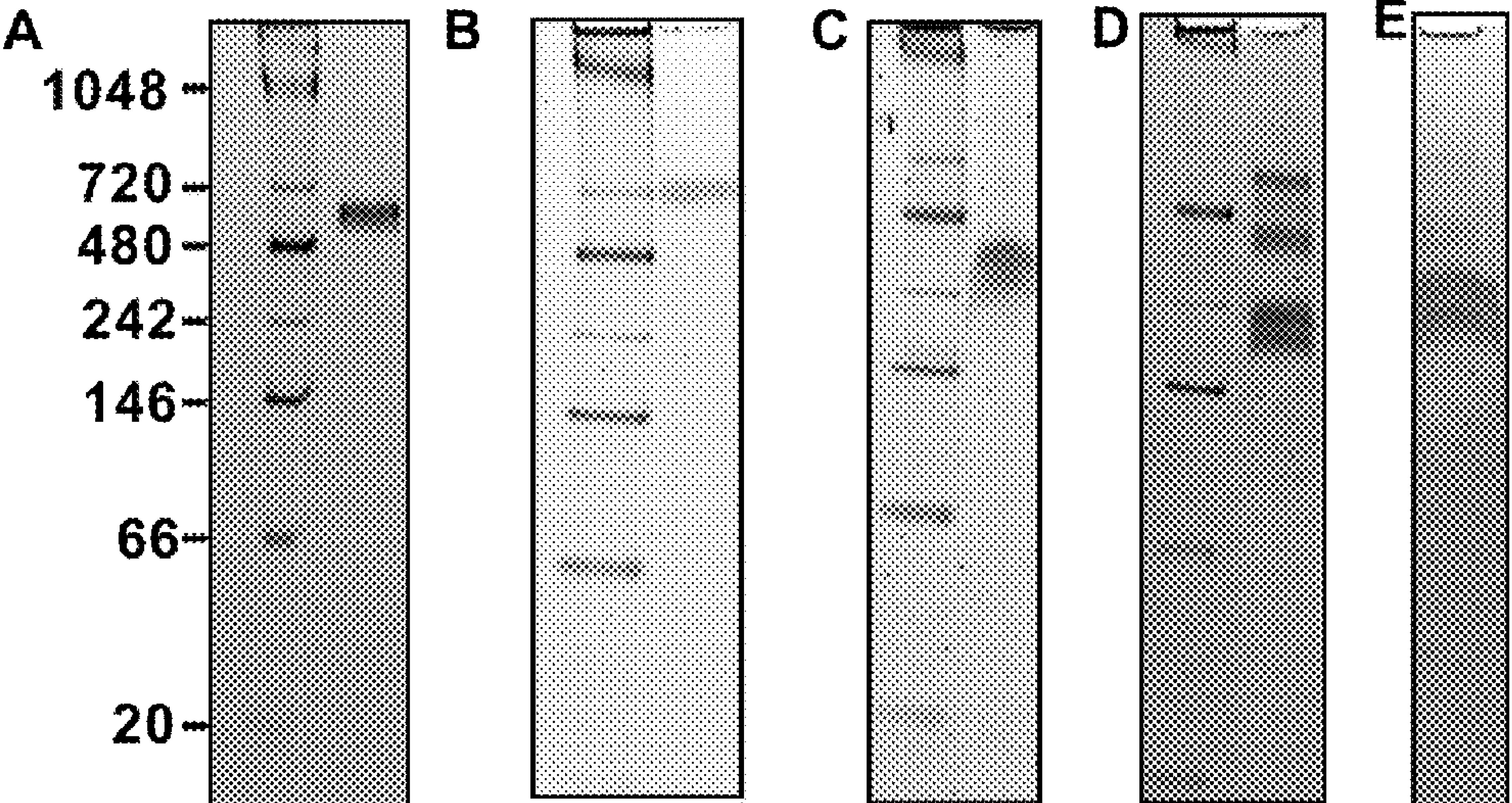


FIG 25

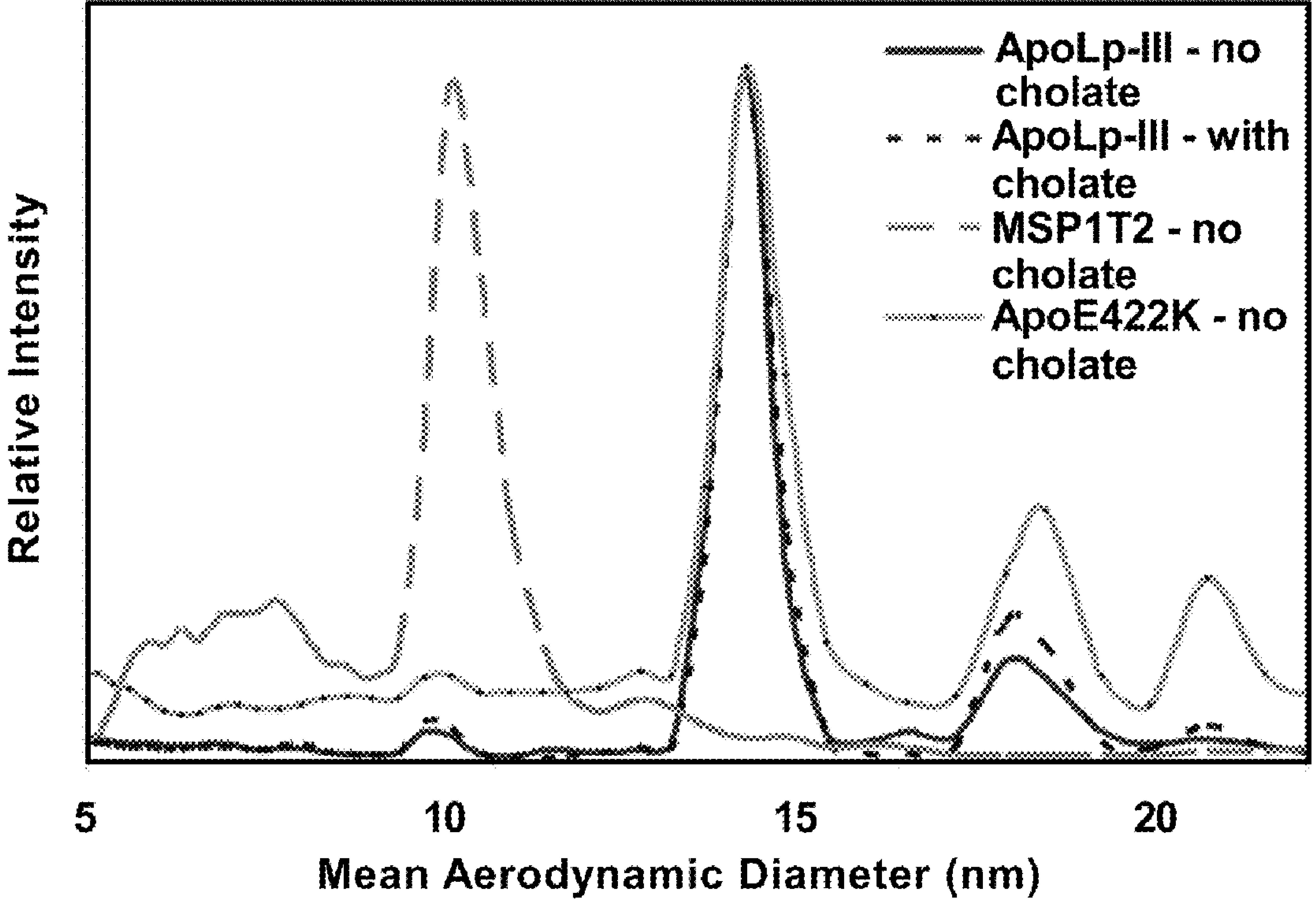


FIG 26

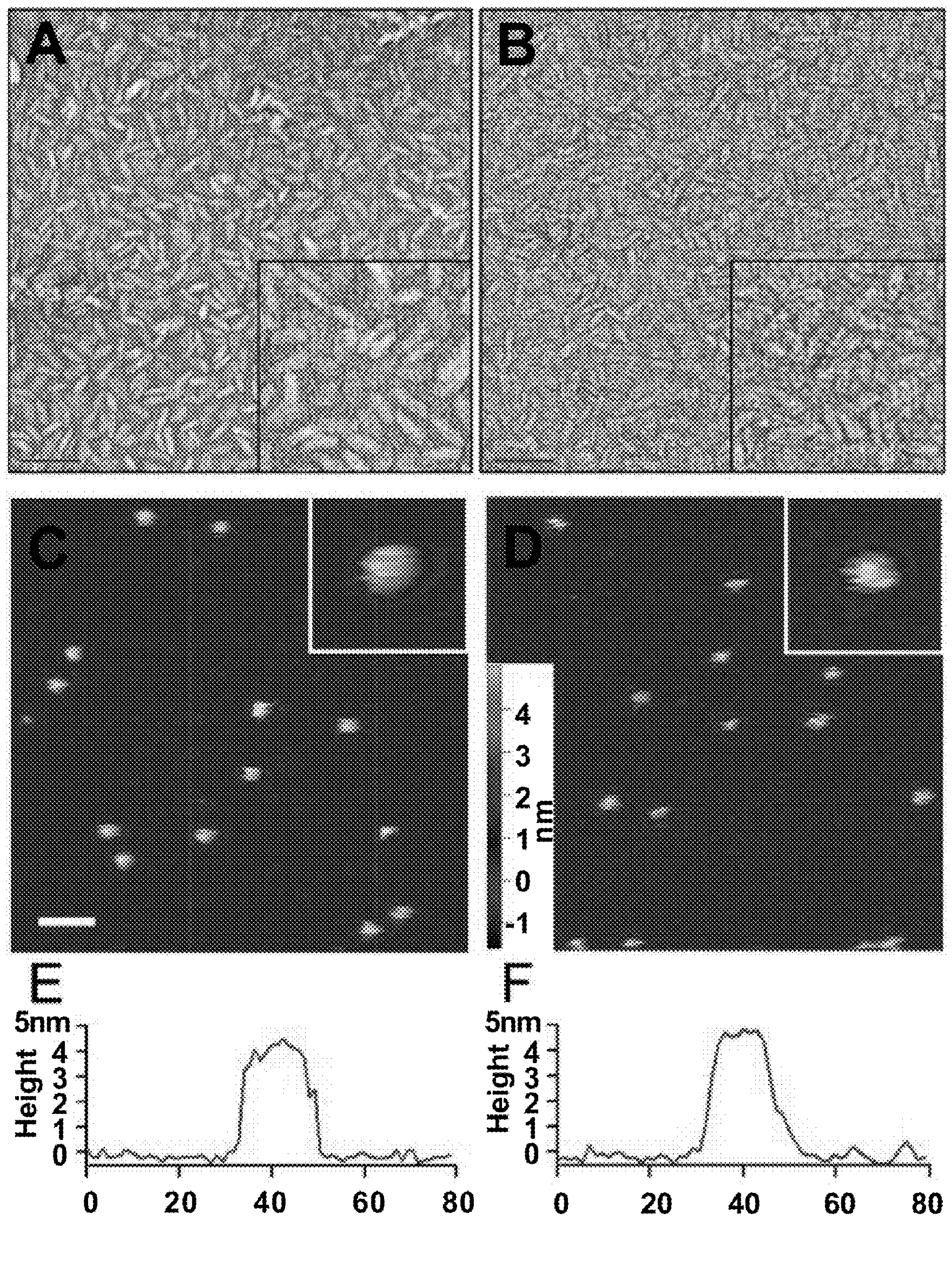


FIG. 27

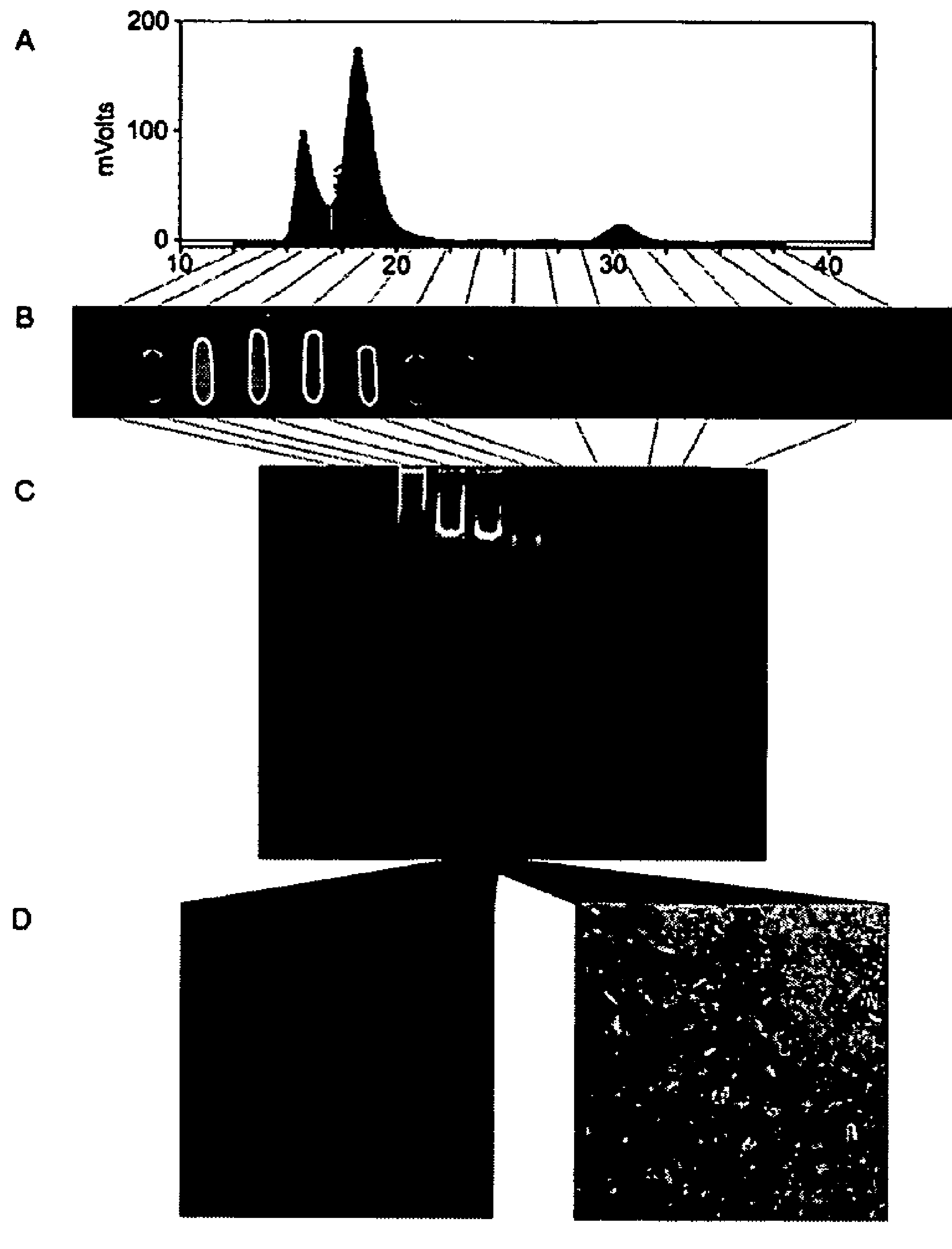


FIG. 28

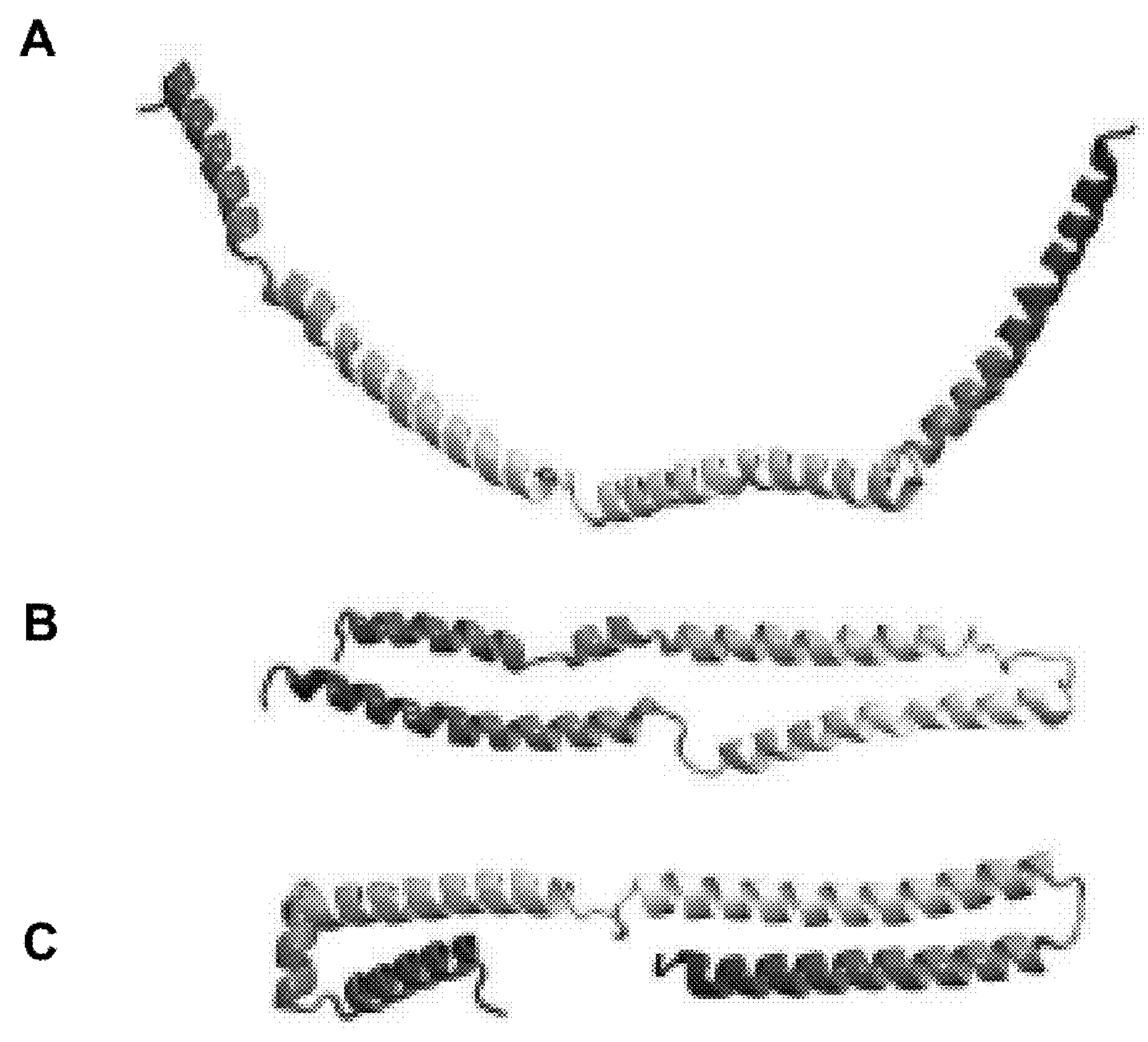


FIG. 29

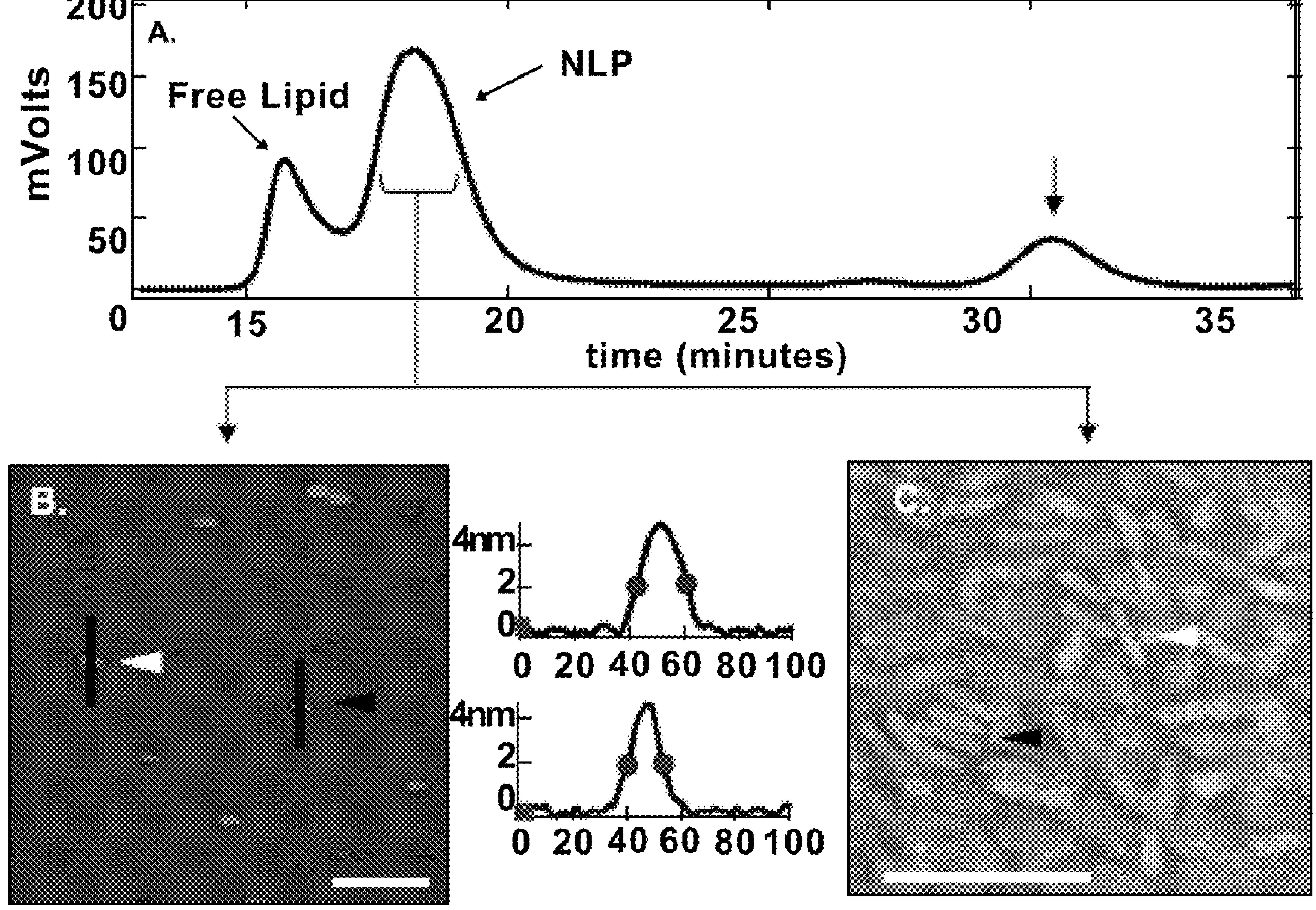


FIG. 30

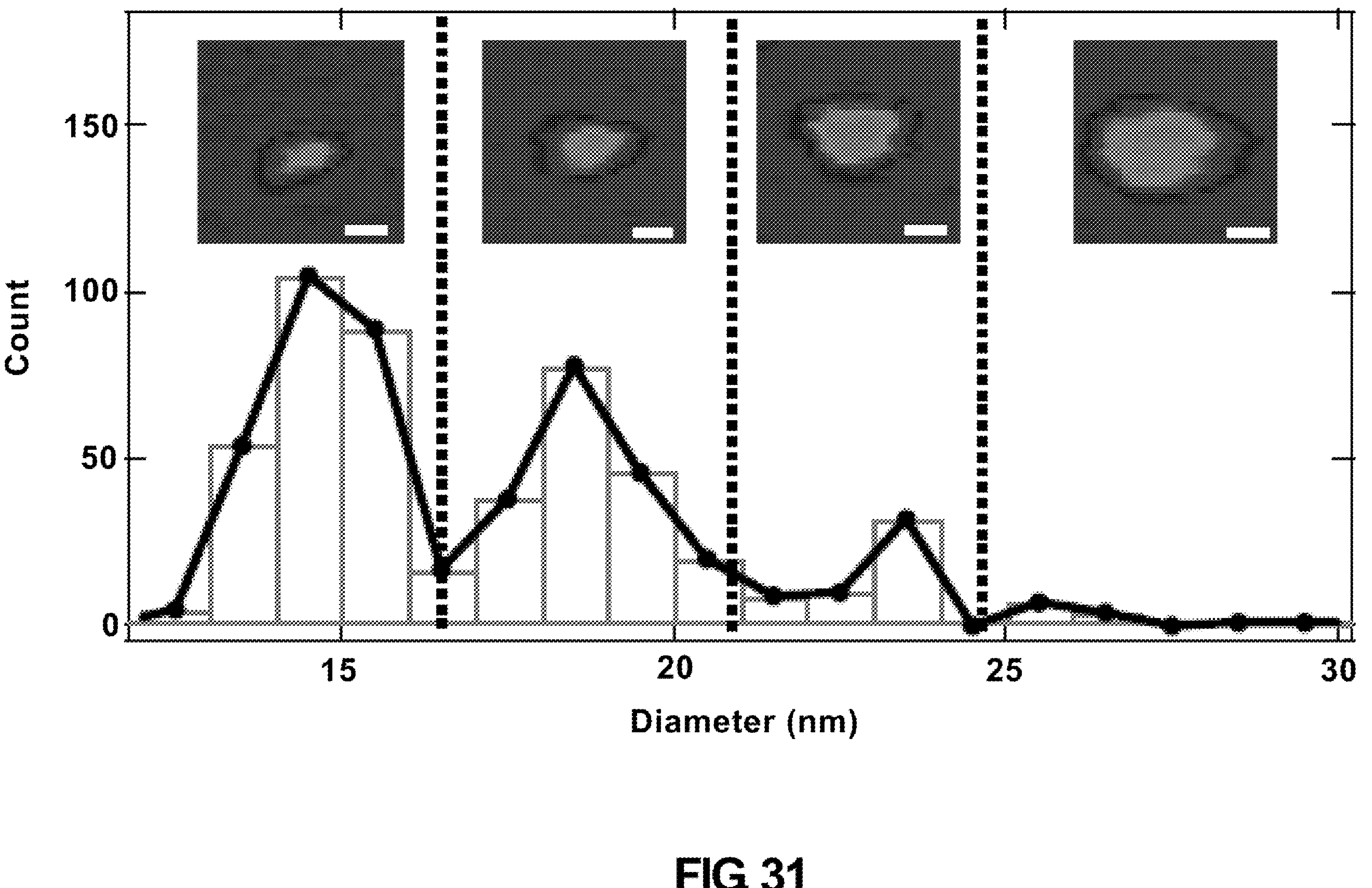


FIG 31

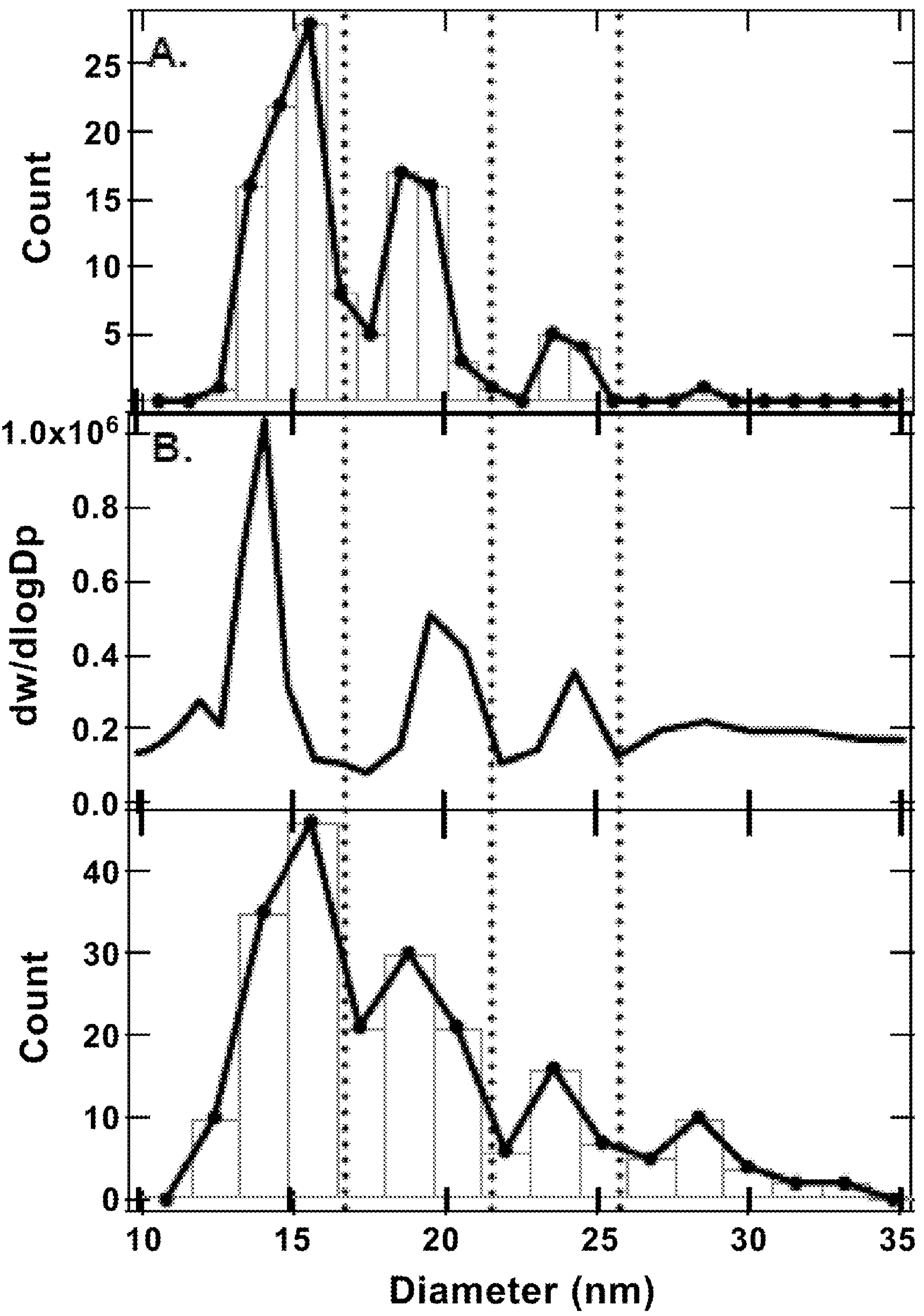


FIG 32

METHODS AND SYSTEMS FOR MONITORING PRODUCTION OF A TARGET PROTEIN IN A NANOLIPOPROTEIN PARTICLE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application entitled "Cell-free Self Assembly of Nano-lipoprotein Particles as a Platform for Co-expressed Membrane Proteins" Ser. No. 60/928,579, filed on May 9, 2007 Docket No. IL-11841, and to U.S. Provisional Application entitled "Monitoring IVT or Cell-free Membrane Protein Expressions, Folding and Functional Using In Situ Expression of Bacteriorhodopsin as an Internal Colorimetric" Ser. No. 60/928,573 filed on May 9, 2007 Docket No. IL-11842, the disclosures of which are incorporated herein by reference in their entirety. This application may also be related to U.S. application entitled "Methods and Systems for Producing Nanolipoprotein Particle" filed on the same day of the present application with Docket No. P197-US, the disclosure of which is also incorporated herein by reference in its entirety.

STATEMENT OF GOVERNMENT GRANT

[0002] The United States Government has rights in this invention pursuant to Contract No. DE-AC52-07NA27344 between the U.S. Department of Energy and Lawrence Livermore National Security, LLC, for the operation of Lawrence Livermore National Security."

TECHNICAL FIELD

[0003] The present disclosure relates to membranes and membrane associated proteins and to complexes mimicking said membranes and membrane associated proteins.

BACKGROUND

[0004] Membrane-associated proteins and protein complexes account for—30% or more of the cellular proteins. Membrane proteins are held within a bilayer structure. The basic membrane bilayer construct consists of two opposing layers of amphiphilic molecules know as phospholipids; each molecule has a hydrophilic moiety, i.e., a polar phosphate group/derivative, and a hydrophobic moiety, i.e., a long hydrocarbon chain. These molecules self-assemble in a biological (largely aqueous) environment according to thermodynamics associated with water exclusion or hydrophobic association.

[0005] In order to facilitate the myriad functions of biological membranes including the passage of nutrients, signaling molecules and other molecules into and out of the cell, membrane proteins are arrayed in the bilayer structure as depicted below. Note that some proteins span the bilayer, others are anchored within the bilayer, and still others organize "peripheral" proteins into complexes. Many membrane bound protein complexes mediate essential cellular processes e.g. signal transduction, transport, recognition, and cell-cell communication. In general, this class of proteins is challenging to study because of their insolubility and tendency to aggregate when removed from their protein lipid bilayer environment.

[0006] Membrane proteins are optimally folded and functional when in a lipid bilayer, but standard protein purification methods often remove lipids, invariably altering protein conformation and function.

[0007] Furthermore, also non-membrane proteins (i.e. proteins that do not exercise a biological activity in connection with a location on a membrane) may still be desirably associated with a membrane for the purpose of solubilization and/or transporting and delivering to a cell.

[0008] To overcome these problems, fully functional integral membrane proteins and additional proteins can be assembled in lipid/protein-based particulate structures called nanolipoprotein particles (NLPs) usually comprising membrane forming lipids and apolipoproteins.

[0009] NLP assembly and function usually involves the association of specific apolipoprotein and lipid molecules leading to formation of proteolipid complexes; the latter are used to transport a diverse array of lipid molecules within organisms.

[0010] NLPs made in the presence of a solubilized membrane protein (target) result in a membrane protein NLP construct Accordingly, NLP assembly can also be used for stabilization and characterization of membrane proteins.

SUMMARY

[0011] Provided herein, are methods and systems for monitoring production of a target protein in a NLP nanostructure. In particular, the methods and systems herein disclosed allow monitoring of synthesis, correct folding and incorporation of the target protein in a NLP, following assembly of the NLP, which in some embodiments can also occur in a single reaction.

[0012] According to a first aspect, a method for monitoring production of a target protein in a nanolipoprotein particle is disclosed. The nanolipoprotein particle comprises the target protein a membrane forming lipid and a scaffold protein. The target protein is capable of assuming a target protein active form and a target protein inactive form. The method comprises: providing an indicator protein, the indicator protein capable of assuming an indicator protein active form and an indicator protein inactive form, the indicator protein active form associated to an indicator protein detectable activity, the indicator protein detectable activity associated to the target protein active form. The method further comprises contacting the indicator protein with the target protein, the membrane forming lipid and the scaffold protein for a time and under conditions to allow assembly of the indicator protein, the target protein, the membrane forming lipid and the scaffold protein in the nanolipoprotein particle. The method also comprises detecting the indicator protein detectable activity from the nanolipoprotein particle.

[0013] According to a second aspect, a method for monitoring production of a target protein in a nanolipoprotein particle is disclosed. The nanolipoprotein particle comprises the target protein a membrane forming lipid and a scaffold protein. The target protein is capable of assuming a target protein active form and a target protein inactive form. The method comprises: providing a first polynucleotide encoding for the target protein; providing a second polynucleotide encoding for the indicator protein, the indicator protein capable of assuming an indicator protein active form and an indicator protein inactive form, the indicator protein active form associated to an indicator protein detectable activity, the indicator protein detectable activity associated to the target

protein active form. The method further comprises: contacting the first and second polynucleotides with the membrane forming lipid and the scaffold protein for a time and under conditions to allow assembly of the indicator protein, the target protein, the membrane forming lipid and the scaffold protein in the nanolipoprotein particle. The method also comprises: detecting the indicator protein detectable activity from the nanolipoprotein particle.

[0014] According to a third aspect, a method for monitoring production of a target protein in a nanolipoprotein particle is disclosed. The nanolipoprotein particle comprises the target protein a membrane forming lipid and a scaffold protein. The target protein is capable of assuming a target protein active form and a target protein inactive form. The method comprises: providing a first polynucleotide encoding for the target protein; providing a second polynucleotide encoding for the indicator protein, the indicator protein capable of assuming an indicator protein active form and an indicator protein inactive form, the indicator protein active form associated to an indicator protein detectable activity, the indicator protein detectable activity associated to the target protein active form; and providing a third polynucleotide encoding for the scaffold protein. The method further comprises: contacting the first, second and third polynucleotides with the membrane forming lipid and the scaffold protein for a time and under conditions to allow assembly of the indicator protein, the target protein, the membrane forming lipid and the scaffold protein in the nanolipoprotein particle. The method also comprises: detecting the indicator protein detectable activity from the nanolipoprotein particle.

[0015] According to a fourth aspect, a system for monitoring production of a target protein in a nanolipoprotein particle is disclosed. The nanolipoprotein particle comprises the target protein a membrane forming lipid and a scaffold protein. The target protein is capable of assuming a target protein active form and a target protein inactive form. The system comprises: an indicator protein capable of assuming an indicator protein active form and an indicator protein inactive form, the indicator protein active form associated to an indicator protein detectable activity, the indicator protein detectable activity associated to the target protein active form. The system further comprises at least one of the target proteins the membrane forming lipid and the scaffold protein.

[0016] According to a fifth aspect, a system for monitoring production of a target protein in a nanolipoprotein particle is disclosed. The nanolipoprotein particle comprises the target protein a membrane forming lipid and a scaffold protein. The target protein is capable of assuming a target protein active form and a target protein inactive form. The system comprises: the system comprising: a first polynucleotide encoding for the target protein; and a second polynucleotide encoding for the indicator protein, the indicator protein capable of assuming an indicator protein active form and an indicator protein inactive form, the indicator protein active form associated to an indicator protein detectable activity, the indicator protein detectable activity associated to the target protein active form.

[0017] According to a sixth aspect, a system for monitoring production of a target protein in a nanolipoprotein particle is disclosed. The nanolipoprotein particle comprises the target protein a membrane forming lipid and a scaffold protein. The target protein is capable of assuming a target protein active form and a target protein inactive form. The system comprises: a first polynucleotide encoding for the target protein; a second polynucleotide encoding for the indicator protein, the indicator protein capable of assuming an indicator protein active form and an indicator protein inactive form, the indicator protein active form associated to an indicator protein detectable activity, the indicator protein detectable activity associated to the target protein active form; and a third polynucleotide encoding for the scaffold protein.

[0018] The methods and systems herein described can be used in connection with the characterization and in particular the optimization of the reaction conditions related to the production of a target protein of interest

[0019] The methods and systems herein described can be also used to identify proper folding parameters for general/active membrane protein production.

[0020] The methods and systems herein described can be further used in processes wherein the indicator protein is used as an additive to cell free expression systems regardless of organism/system extracts.

[0021] The methods and systems herein described can additionally be used for the correct and efficient production of membrane proteins, with particular reference to the membrane proteins difficult to produce from native systems.

[0022] The methods and systems herein described can also be used in processes for screening parameters for evaluation of production of membrane proteins.

[0023] The methods and systems herein described can also be used in processes for identification of novel membrane protein folding conditions

[0024] The methods and systems herein described can also be used in a manufacturing quality control assay for cell-free lysates.

[0025] The methods and systems herein described can also be used in experimental quality control assay for cell-free lysates.

[0026] The methods and systems herein described can also be used in processes screening for production parameters and successful membrane protein folding evaluation.

[0027] The methods and systems herein described can be applied in several fields including basic biology research, applied biology, bio-engineering, bio-energy, medical research, medical diagnostics, therapeutics, and bio-fuels.

[0028] The details of one or more embodiments of the disclosure are set forth in the accompanying drawings and the description below. Other features, objects, and advantages will be apparent from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0029] The accompanying drawings, which are incorporated into and constitute a part of this specification, illustrate one or more embodiments of the present disclosure and, together with the detailed description, serve to explain the principles and implementations of the disclosure.

[0030] FIG. **1** shows a schematic illustration of single step cell-free co-expression and stabilization of integral membrane proteins using an apolipoprotein scaffold according to an embodiment herein described.

[0031] FIG. **2** shows single-step production, purification and characterization of MP-NLP complexes, according to an embodiment herein described. a. Coomassie stained SDS-PAGE gel image of Total (T), Soluble (S) and Pellet (P) fractions from cell-free produced bacteriorhodopsin (bR) in the presence and absence of co-expressed apolipoprotein (Δ49A1). A (+) indicates the addition of either DMPC,

Δ49A1 DNA or bacteriOpsin DNA (bOp) to the cell-free reaction, (−) denotes absence of additive. Grey arrows indicate Δ49A1 (lane 2S upper arrow and lane 3S), and black arrows indicate bR (lane 1T and lane 2S lower arrow). Sample 1 indicates bOp & DMPC; Sample 2 indicates bOp, Δ49A1 co-expressed in the presence of DMPC; Sample 3 indicates Δ49A1 & DMPC; Sample 4 indicates the control cell-free reaction (No DNA) in the presence of DMPC only.

[0032] FIG. **3** shows solubility of cell-free produced bR-NLPs. The (+) indicates the addition of either DMPC, Δ49A1 DNA or bacteriOpsin DNA (bOp) to the cell-free reaction, (−) denotes absences of additive, all were expressed in the presence of 50 μM all-trans-retinal. a. Solubility of bR is increased in the presence of Δ49A1 DNA. Cell-free reactions were carried out in the presence of ^{35}S-Methionine. Grey arrows indicate Δ49A1 (Coomassie: lane 3; Autorad: lanes 3 and 8 upper arrow), and black arrows indicate bR (Coomassie: lane 1; Autorad: lanes 1 and 8 lower arrow—in Autorad). Left Panel, Coomassie stained SDS-PAGE gel image of total (T) and soluble (S) fractions from cell-free produced bR in the presence and absence of co-expressed apolipoprotein (Δ49A1) and DMPC. Right Panel, Autoradiogram of the gel shown in left panel, illustrating the benefit of adding the apolipoprotein for increasing the solubility of bR.

[0033] FIG. **4** shows solubility of bacteriorhodopsin observed directly in reaction vessel. The solubility of expressed bR is increased by Δ49A1 co-expression. A (+) indicates the addition of either DMPC, Δ49A1 DNA or bacterioOpsin DNA (bOp) to the cell-free reaction, (−) denotes absence of additive. All were expressed in the presence of —30-50 μM all-trans-retinal. Reaction 1, expression of bR in the presence of DMPC alone, purple color (shown in the figure as a dark gray) has settled to bottom of vessel. Reaction 2, expression of bR in the presence of Δ49A1 co-expression, purple color (also shown as dark gray) remains dispersed through out vessel indicating the formation of soluble bR-NLPs.

[0034] FIG. **5** shows co-expressed Ni-NTA purified bR-NLPs have several distinct sizes. Size exclusion chromatography of affinity purified co-expressed bR and Δ49A1. Tube 0, color of sample before SEC. Numbered fractions collected are indicated at the bottom of the trace. The color of corresponding concentrated fractions (top) is shown in the numbered tubes. Purple color (shown in the figure as various shades if gray) indicates the presence of properly folded bR. Fraction(s) determined to contain lipid rich constructs (vesicles or sheets), bR-NLPs, empty-NLPs and lipid poor protein are indicated on the trace. Samples were collected using a VP HPLC (Shimadzu) with a Superdex 200 10/300 GL column (GE Healthcare) at a flow rate of 0.5 mL/min of 10 mM Tris pH 7.4; 0.15M NaCl; 0.25 mM EDTA, 0.005% NaN_3. Fractions were concentrated using a molecular sieve cut off filter (MWCO 50 k) Vivaspin 2 (Sartorius) or MWCO 10 k for the free protein fractions

[0035] FIG. **6** shows the results of an experiments related to single-step production, purification and characterization of MP-NLP complexes according to an embodiment herein described. Native gel of size exclusion purified nanolipoprotein particles (NLPs) prepared with Δ49A1 with and without bR. Lane M, Molecular weight marker; Lane 2_{1-3}, Fractions from SEC purified cell-free co-expressed bR-NLPs; Lane 2_1, lipid rich first fraction; Lane 2_2 bR-NLPs second fraction; Lane 2_3, bR-NLPs third fraction; Lane 3, Cell-free produced "empty"-NLPs. Lane 4, conventional assembly of "empty"-NLPs with purchased Δ1-55 apolipoprotein A1 (Δ55A1).

[0036] FIG. **7** shows AFM image of lipid rich SEC fraction. An AFM image representing the first fraction collected by size exclusion chromatography of the cell-free co-expression of Δ49A1 and bR in the presence of DMPC. AFM image of the lipid rich fraction displaying all three populations depicted in the top table. Observed diameter and height measurements for the lipid rich fraction displays three populations; Line A indicates, large lipid complexes, liposomes or membrane sheets; Line B indicates bR-NLPs and Line C indicates "empty"-NLPs. This analysis clearly indicates the majority of structures in this fraction were lipid vesicles or membrane sheets with much larger diameters and heights.

[0037] FIG. **8** shows Light/Dark adaptation of bR-NLPs. Top Dark adapted bR-NLPs with a λ_{max}=549 nm; Bottom, light adapted bR-NLP with a λ_{max}=554 nm. Arrows indicate the maximum peak heights which differ by a 5 nm shift between light and dark adapted.

[0038] FIG. **9** shows diagram illustrating co-expression of membrane proteins with apolipoprotein Δ49A1 in the presence of lipid increases solubility of multiple membrane proteins according to an embodiment herein described. The membrane protein expressed alone is indicated in white blocks; the membrane protein expressed in presence of DMPC vesicles is indicated in gray blocks, and the membrane protein co-expressed with apolipoprotein (Δ49ApoA1) in the presence of DMPC vesicles are indicated with black blocks. Membrane proteins with the number of transmembrane domain in parentheses are (GYPE) glycophorin B (MNS blood group) (2™), (CMTM1) CKLF-like MARVEL transmembrane domain (3TM), (EmrE) *E. coli* SMR efflux transporter (4TM) (5HT1A) 5-hydroxytryptamine (serotonin) receptor (7 TM), (bR) bacteriorhodopsin (7TM).

[0039] FIG. **10** shows the result of AFM analysis confirming the association between NLPs and bR according to an embodiment herein disclosed. Panel A shows the AFM image of NLPs produced through cell-free co-expression of Δ49A1 and bR in the presence of DMPC. The brighter green regions (show in the figure as light grey) are NLPs with a higher height indicating the insertion and plausible location of bR in the lipid bilayer. Scale bars are 50 nm; Arrow 1, indicates expression of "empty"-NLP's, while arrow 2, indicates the bR-NLP complex. Panel B shows a height histogram of NLPs produced through conventional assembly of (top trace) Δ49A1 with DMPC alone or (bottom trace) in the presence of purple membrane bR and DMPC. The shaded areas indicate populations with an increased height. Panel C shows a eight histogram of NLPs produced through cell-free expression of (top trace) Δ49A1 with DMPC alone or (bottom trace) co-expression of bR and Δ49A1 in the presence of DMPC. The shaded areas indicate populations with an increased height. NLPs heights were analyzed through cross-sectional analysis

[0040] FIG. **11** shows the FTIR difference spectra for the bR→M transition FTIR difference spectra of (A) bR WT and (B) bR-NLP. The largest peaks are 9.4 and 0.34 mOD, respectively. The positive bands represent vibrations in the M state and negative bands represent the ground state. Despite the smaller signal, the spectrum of BR NLP clearly indicates functional protein that is stable over ~10^4 laser flashes.

[0041] FIG. **12** shows a schematic illustration of some models of NLPs with (Panels B and C) and without (Panel A) bacteriorhodopsin (shown in black) according to an embodiment herein described.

[0042] FIG. 13 shows lipoproteins expressed in cell-free extracts according to an embodiment herein described. Lipoprotein were purified using Ni-NTA affinity chromatography and run on a SDS-PAGE gel, stained with Coomassie Brilliant Blue (A-B and D-E) or detected by fluorescent scanning of labeled lysine residues (C). Arrows indicate apolipoprotein of interest. Proteins A-D and F-G are shown with SeeBlue MW marker (Invitrogen) (A) Full-length apolipoprotein A1 (B) MSP1 truncated form of ApoA1 (C) Full-length Apolipoprotein E4 (D) 22kD truncated ApoE4-fusion protein (H) Thrombin cleaved truncated ApoE422k. Other Lipoproteins produced (not shown) include Apolipophorin III *B. mori*, Apolipophorin III, *M. Sexta*.

[0043] FIG. 14 shows a diagram illustrating the results of size exclusion chromatography separation of ApoE422k Nanolipoprotein particles (NLPs) according to an embodiment herein disclosed. Free lipid, and free protein denoted on graph are separated from the NLP rich fraction.

[0044] FIG. 15 shows native gel electrophoresis of NLPs according to an embodiment herein disclosed. 1) Native Mark molecular weight marker. 2) "Empty"-NLPs 3) Membrane protein (bacteriorhodopsin) bR-NLPs. 4-20% Tris-glycine gel, with Tris-glycine running buffer, stained with Sypro-Ruby Stain (BioRad) Imaged with a Typhoon scanner.

[0045] FIG. 16 shows a protein microarray of biotinylated bR-NLPs according to an embodiment herein disclosed. (1) biotinylated-bacteriorhodopsin (2) negative control, native bacteriorhodopsin (3) biotinylated-bacteriorhodopsin associated NLPs

[0046] FIG. 17 shows a diagram illustrating the light and dark adapted visible spectra of bacteriorhodopsin associated NLPs, according to an embodiment herein disclosed. (Top traces) Light and dark adapted visible spectra of bacteriorhodopsin associated NLPs and (bottom traces) NLPs without membrane protein. Black) Dark adapted spectra (bR Xmax=550 nm). Grey) Light adapted spectra (bR Xmax=560 nm)

[0047] FIG. 18 shows a diagram illustrating the Atomic Force Microscopy of nanolipoprotein particles (NLPs) according to an embodiment herein disclosed. NLPs consisting of cell-free produced apoE4 22K lipoprotein and DMPC. Particle dimensions are as follows; Height: 4.94 nm, std dev: 0.369 nm Width of top: 9.72 nm, std dev: 1.50 nm, Full width at half max: 20.4 nm std dev: 3.5 nm

[0048] FIG. 19 shows the results of the electron microscopy of nanolipoprotein particles (NLPs) according to an embodiment herein disclosed wherein the NLPs show a discoidal shape. The magnification is 40K

[0049] FIG. 20 shows a diagram illustrating cell-free expression of membrane proteins in the presence of NLPs (Co-translation) according to an embodiment herein disclosed. The membrane protein expressed alone is indicated with black bars; the membrane protein expressed in the presence of pre-formed ApoE4 22k NLPs (Co-Translation) is indicated with grey bars. Membrane proteins with the number of trans membrane domain in parentheses are (GYPE) glycophorin B (MNS blood group) (2™), (CMTM1) CKLF-like MARVEL transmembrane domain (3™), (EmrE) *E. coli* SMR efflux transporter (4™) (5HT1A) 5-hydroxytryptamine (serotonin) receptor (7 ™), (bR) bacteriorhodopsin (7™).

[0050] FIG. 21 shows the results of experiments related to cell free production of a membrane protein in the presence of NLP scaffold according to an embodiment herein disclosed. Panel A shows the cell free production of bR with a cell free extract, Lane 1. bOp+retinal, Lane 2. bOp alone Lane 3. bOp+Lipid+retinal, Lane 4. Apolipoprotein+bOp+Lipid+Retinal 5. Apolipoprotein+bOp+Lipid, 6. Apolipoprotein alone, 7. Apolipoprotein+bOp+Retinal. Panel B shows the results of PAGE analysis of size exclusion purified proteins or NLP associated with bR. Lane 1. bR, Lane 2. bR+NLP, Lane 3. NLP. Panel C shows microarray analysis of purified bR associated with NLPs using an anti-biotin antibody. Lane 1. bR only, Lane 2. Size exclusion purified biotinylated bR-NLPs. Lane 3. NLPs only. Lane 4. Biotinylated IgG.

[0051] FIG. 22 shows the results of experiments related to the cell free production of NLPs according to an embodiment herein disclosed illustrated by Atomic force microscopy (AFM). The AFM can resolve membrane surface features at a lateral resolution of 0.6-1 nm and a vertical resolution of 0.1 nm, under physiological conditions without the need of a crystalline system. AFM of scaffolded NLPs with and without biotinylated bR are shown. Note, homogeneous globular structures about 5 nm in height and 20-60 nm in diameter.

[0052] FIG. 23 shows the schematic of NLP assembly according to an embodiment herein disclosed.

[0053] FIG. 24 is a diagram illustrating size exclusion chromatography of a NLPs assembly reaction mixture, according to an embodiment herein disclosed.

[0054] FIG. 25 shows a sypro RUBY-stained native PAGE indicating molecular size and relative homogeneity of NLPs prepared from four different apolipoproteins without cholate according to an embodiment herein disclosed.

[0055] FIG. 26 shows a diagram illustrating ion mobility traces of mean aerodynamic diameter size distributions for four NLP preparations obtained according to an embodiment herein disclosed.

[0056] FIG. 27 shows a negative stain TEM and AFM of apoE422K-NLP preparations with (panel B, D and F) and without cholate (Panel A, C and E) according to an embodiment herein disclosed. The scale bar in each panels is 50 nm; insets are a higher magnification.

[0057] FIG. 28 illustrates characterization of fluorescently-labeled NLPs showing similar structure and in comparison with unlabeled NLPs, according to an embodiment herein disclosed. Cy_3-labeled apoE422K (50%) and DMPC containing 1% NBD-DMPC were used to form labeled NLPs. Panel A shows the SEC fractionation of labeled NLPs. Early eluting peaks correspond to large DMPC vesicles and NLP fractions, later eluting peak contains unreacted apoE422K. Panel B shows a fluorescent scan of SEC fractions: that contain NBD-DMPC (green), Cy_3-apoE422K(red), and fluorescent NLPs (yellow). Panel C shows the native PAGE of SEC fractions highlighting the migration and homogeneity of the NLP peak. Panel D shows a topographical AFM image (left) and TEM image (right) of the main NLP fraction vial, highlighting the homogeneity, size, and structure of the fluorescent NLPs

[0058] FIG. 29 shows a schematic illustration of refolded apoE422K proteins generated in three different forms according to an embodiment herein disclosed. Panel A. shows a fully extended form. Panel B shows a doubled-back/"hairpin". and Panel C shows semi-extended/"double-hairpin" folds.

[0059] FIG. 30 shows a method of purification and characterization of self-assembled NLPs according to an embodiment herein disclosed. Panel A shows the size exclusion chromatography (SEC) trace of apoE422K/DMPC NLPs. Panels B and C show AFM (Panel B) and TEM (Panel C) images of apoE422K/DMPC NLPs after formation and purification. Cross-sectional analysis of AFM images (white and

black arrowed line in image corresponds to top and bottom cross-sectional traces shown to the left, respectively) was used to measure NLP height and diameter. The circles in the traces correspond to the FWHM points used to determine particle diameter. Arrow heads in the AFM and TEM images (C and D) point to NLPs of differing diameter indicating size heterogeneity (black arrow head points to smaller NLPs and white arrow head larger NLPs). AFM scale bar 100 nm, TEM scale bar 50 nm.

[0060] FIG. **31** shows the NLP diameter distribution for 1000 NLPs with a bin width of 1.0 nm according to an embodiment herein disclosed. The inset shows the AFM image of the four different sized NLPs. The scale bar is 10 nm.

[0061] FIG. **32** shows a diagram illustrating the comparison of diameter distributions according to an embodiment herein disclosed, wherein the diameter is measured by AFM, IMS and TEM. A. NLP diameters determined through AFM binned at 1 nm. B. NLP diameters determined through IMS. C. NLP diameters determined through TEM binned at 1.6 nm

DETAILED DESCRIPTION

[0062] Methods and systems for monitoring the production of a target protein in a NLP are disclosed. In particular, the methods and systems herein disclosed allow monitoring the synthesis, correct folding and incorporation of a target protein in a NLP. The term "nanolipoprotein particle" "nanodisc" "rHDL" or "NLP" as used herein indicates a supramolecular complex formed by a membrane forming lipid and a scaffold protein, that following assembly in presence of a target protein also include the target protein. The scaffold protein and target protein constitute protein components of the NLP. The membrane forming lipid constitutes a lipid component of the NLP. The term "protein" as used herein indicates a polypeptide with a particular secondary and tertiary structure that can participate in, but not limited to, interactions with other biomolecules including other proteins, DNA, RNA, lipids, metabolites, hormones, chemokines, and small molecules.

[0063] The term "polypeptide" as used herein indicates an organic polymer composed of two or more amino acid monomers and/or analogs thereof. Accordingly, the term "polypeptide" includes amino acid polymers of any length including full length proteins and peptides, as well as analogs and fragments thereof. A polypeptide of three or more amino acids can be a protein oligomer or oligopeptide.

[0064] As used herein the term "amino acid", "amino acidic monomer", or "amino acid residue" refers to any of the twenty naturally occurring amino acids including synthetic amino acids with unnatural side chains and including both D and L optical isomers. The term "amino acid analog" refers to an amino acid in which one or more individual atoms have been replaced, either with a different atom, isotope, or with a different functional group but is otherwise identical to its natural amino acid analog. The term "scaffold protein" as used herein indicates any protein that is capable of self assembly with an amphipatic lipid in an aqueous environment, organizing the amphipatic lipid into a bilayer, and include but are not limited to apolipoproteins, lipophorines, derivatives thereof (such as truncated and tandemly arrayed sequences) and fragments thereof (e.g. peptides), such as apolipoprotein E4, 22K fragment, liphorin III, apolipoprotein A-1 and the like. In particular, in some embodiments rationally designed amphipathic peptides can serve as a protein component of the NLP. In some embodiment, the peptides are amphipatic helical peptides that mimic the alpha helices of an apolipoprotein component that are oriented with the long axis perpendicular to the fatty acyl chains of the amphipatic lipid and in particular of the phosphoplipid.

[0065] The term "target protein" as used herein indicates any protein having a structure that is suitable for attachment to or association with a biological membrane or biomembrane (i.e. an enclosing or separating amphipathic layer that acts as a barrier within or around a cell). In particular, target proteins include proteins that contain large regions or structural domains that are hydrophobic (the regions that are embedded in or bound to the membrane); those proteins can be extremely difficult to work with in aqueous systems, since when removed from their normal lipid bilayer environment those proteins tend to aggregate and become insoluble. Accordingly, target proteins are protein that typically can assume an active form wherein the target protein exhibits one or more functions or activities, and an inactive form wherein the target protein doe not exhibit those functions/activities. Exemplary target proteins include but are not limited to membrane proteins, i.e. proteins that can be attached to, or associated with the membrane of a cell or an organelle, such as integral membrane proteins (i.e. proteins (or assembly of proteins) that are permanently attached to the biological membrane.), or peripheral membrane proteins (i.e. proteins that adhere only temporarily to the biological membrane with which they are associated). Integral membrane proteins can be separated from the biological membranes only using detergents, nonpolar solvents, or sometimes denaturing agents. Peripheral membrane proteins are proteins that attach to integral membrane proteins, or penetrate the peripheral regions of the lipid bilayer with an attachment that is reversible.

[0066] The term "membrane forming lipid" or "amphipatic lipid" as used herein indicates a lipid possessing both hydrophilic and hydrophobic properties that in an aqueous environment assemble in a lipid bilayer structure that consists of two opposing layers of amphipathic molecules know as polar lipids. Each polar lipid has a hydrophilic moiety, i.e., a polar group such as, a derivatized phosphate or a saccharide group, and a hydrophobic moiety, i.e., a long hydrocarbon chain. Exemplary polar lipids include phospholipids, sphingolipids, glycolipids, ether lipids, sterols and alkylphosphocholins. Amphipatic lipids include but are not limited to membrane lipids, i.e. amphipatic lipids that are constituents of a biological membrane, such as phospholipids like dimyrisoylphosphatidylcholine (DMPC) or Dioleoylphosphoethanolamine (DOPE) or dioleoylphosphatidylcholine (DOPC).

[0067] The membrane forming lipid and the protein components of the NLP are generally able to self-assemble in a biological (largely aqueous) environment according to the thermodynamics associated with water exclusion (increasing entropy) during hydrophobic association. In the methods and systems herein provided, the amphipatic lipid and the protein components of the NLP are allowed to assembly in a cell free expression system.

[0068] In the methods and systems herein disclosed, the production of the target protein in a NLP is monitored by way of an indicator protein. The term "indicator protein" as used herein refers to a protein that is capable of assuming an active form wherein the indicator protein is capable of exhibiting a detectable activity and a non active form wherein the indicator protein is not capable of exhibiting the detectable activity. In the methods and systems herein disclosed, the indicator protein is selected so that the detectable activity exhibited by the indicator protein of choice is predictive and can be asso-

ciated with the active form of the target protein. In particular, in some embodiments the indicator protein is a target protein itself and/or is structurally related to the target protein of interest so that the production of the indicator protein in an active form can be associated and considered predictive of the production of the target protein in an active form.

[0069] In some embodiments, the indicator protein and the target protein are contacted with the membrane forming lipid and the scaffold protein for a time and under conditions that allow self-assembly of the indicator protein, target protein, scaffold protein and membrane forming lipid in an NLP.

[0070] In some embodiments, the indicator protein and the target protein expressed in a cell free expression system in presence of the membrane forming lipid and the scaffold protein for a time and under condition that allow the expression of the indicator protein and the target protein and self-assembly of the indicator protein, target protein, scaffold protein and membrane forming lipid in an NLP.

[0071] In some embodiments, the indicator protein, target protein and scaffold protein are expressed in a cell-free expression system in presence of the membrane forming lipid that allow the expression of the indicator protein, the target protein and the scaffold protein and self-assembly of the indicator protein, target protein, scaffold protein and membrane forming lipid in an NLP

[0072] As used herein, "the wording cell free expression" "cell free translation", "in vitro translation" or "IVT" refer to at least one compound or reagent that, when combined with a polynucleotide encoding a polypeptide of interest, allows in vitro translation of said polypeptide/protein of interest.

[0073] The term "polynucleotide" as used herein indicates an organic polymer composed of two or more monomers including nucleotides, nucleosides or analogs thereof. The term "nucleotide" refers to any of several compounds that consist of a ribose (ribonucleotide) or deoxyribose (deoxyribonucleotides) sugar joined to a purine or pyrimidine base and to a phosphate group, and that are the basic structural units of nucleic acids. The term "nucleoside" refers to a compound (as guanosine or adenosine) that consists of a purine or pyrimidine base combined with deoxyribose or ribose and is found especially in nucleic acids. The term "nucleotide analog" or "nucleoside analog" refers respectively to a nucleotide or nucleoside in which one or more individual atoms have been replaced with a different atom or a with a different functional group.

[0074] Accordingly, the term polynucleotide includes nucleic acids of any length DNA RNA analogs and fragments thereof. A polynucleotide of three or more nucleotides is also called nucleotidic oligomers or oligonucleotide.

[0075] In some embodiments, the polynucleotide is an engineered polynucleotide designed such that the resulting protein may be expressed as a full-length protein. In some embodiments the polynucleotide is an engineered polynucleotide designed to encode a protein fragment. Protein fragments include one or more portions of the protein, e.g. protein domains or subdomains. In some embodiments the polynucleotide is an engineered polynucleotide designed to encode a mutated proteins. In particular, in some embodiments the polynucleotide can also be designed such that the resulting protein, protein fragment or mutated protein is expressed as a fusion, or chimeric protein product (i.e. it is joined via a peptide bond to a heterologous protein sequence of a different protein), for example to facilitate purification or detection. A chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other using standard methods and expressing the chimeric product. In particular, in some embodiments wherein the polynucleotide encodes for a target protein, the polynucleotide can be engineered so that target protein are labeled or tagged. Labeling or tagging can be performed with methods that include, for example, FRET pairs, NHS-labeling, fluorescent dyes, and biotin, as well as coding for a "His-tag" to enable protein isolation and purification via established Ni-affinity chromatography. In some embodiments herein disclosed, the polynucleotide is a DNA molecule that can be in a linear or circular form, and encodes the desired polypeptide under the control of a promoter specific to an enzyme such as an RNA polymerase, that is capable of transcribing the encoded portion of the DNA.

[0076] In embodiments where the polynucleotide is DNA, the DNA may be transcribed as part of the cell free reactions or system. In those embodiments the DNA contains appropriate regulatory elements, including but not limited to ribosome binding site, T7 promoter, and T7 terminator, and the reagents or compounds include appropriate elements for both transcription and translation reactions. In other embodiments, the polynucleotide can be prepared prior to addition to the cell free reactions/system, wherein the polypeptide of interest is produced, and the reagents or compounds include appropriate elements for and translation reactions only.

[0077] Accordingly, as used herein, the term "cell free expression", "cell free translation", "in vitro translation" or "IVT" refer to methods and systems wherein the transcription and translation reactions are carried out independently, and to systems in which the transcription and translation reactions are carried out simultaneously in a non-cellular compartment, e.g. glass vial. In each of these methods and systems, the reagents or compounds typically include a cell extract capable of supporting in vitro transcription and/or translation as appropriate. In any case the cell extracts must contain all the enzymes and factors to carry out the intended reactions, and in addition, be supplemented with amino acids, an energy regenerating component (e.g. ATP), and cofactors, including factors and additives that support the solubilization of the protein of interest.

[0078] These methods and systems are known in the art and can be identified by the skilled person upon reading of the present disclosure, and exist for eukaryotics, yeast, plants and prokaryotic applications. Exemplary cell free expression systems that can be used in connection with the methods and systems of the present disclosure includes but are not limited to commercial kits for various species such as extracts available from Invitrogen Ambion, Qiagen and Roche Molecular Diagnostics, cellular extracts made from *E. coli* or wheat germ or rabbit reticulocytes or prepared following protocols, such as published laboratory protocols, identifiable by a skilled person upon reading of the present disclosure.

[0079] In some embodiments, the cell free system can operate in batch mode or in a continuous mode. In the batch mode the reaction products remain in the system and the starting materials are not continuously introduced. Therefore, in batch mode, the system produces a limited quantity of protein. In a continuous mode instead, the reaction products are continuously removed from the system, and the starting materials are continuously restored to improve the yield of the protein products and therefore the system produces a significantly greater amount of product.

[0080] In some embodiments, the cell free expression system is a high-throughput expression system, where an array (i.e., at least two) of polynucleotides (coding for the same or different polypeptides) is processed simultaneously in multi-well reaction plates, where each polynucleotide is in a well of the plate. The reaction plate can typically have at least 2 wells, and typically has 12-, 24-, 96-, 384-, or 1536-wells; other sizes may also be used.

[0081] In some of those embodiments the array is carried out to explore the function and potential relationships of proteins encoded within any genome. In some of those embodiments the array is carried out for parallel analysis of multiple binary interactions between proteins and other molecules. In addition, in some embodiments engineering and tagging techniques allows the orientation of proteins of interest and expands the capabilities and use of protein microarrays. Some of those embodiments wherein cell-free expression is combined with array-based proteomics are applicable in particular in protein biochemistry, molecular diagnostics and therapeutics. In some embodiments array-based methods and systems provide a high-throughput format with which to investigate protein-protein, protein-DNA, and protein-small molecule interactions on the NLP.

[0082] In some embodiments, the target protein and the indicator protein are expressed in the cell free reaction system where a preformed NLP is included. In those embodiments addition of pre-formed NLPs to an actively expressing cell-free protein synthesis reaction is performed for a time and under condition to allow direct insertion of the target protein and indicator protein as they are synthesized in the cell-free system, into the NLP.

[0083] In some embodiments, the target protein, indicator protein and scaffold protein are co-expressed in a cell free system wherein both kinds of proteins can be expressed in a single reaction in a system that can include the appropriate additives directed to facilitate reactant solubilization. In those embodiments, the co-expressed target protein, indicator protein and scaffold protein are then allowed to assembly of membrane proteins into NLP nanostructures; possibly within the same reaction mixture. Some of those embodiments allow providing NLPs overcoming the requirement for the purification and reassembly of the NLP complex. Some of those embodiments also provides a single-step process for the production of soluble membrane proteins that eliminates the need for cell growth, cell lysis, and subsequent purification, refolding. Some of those embodiments allow avoiding use of detergents while allowing single-step addition of lipids and other molecules important to protein function.

[0084] In particular, co-expression of both scaffold protein and target membrane protein in presence of phospholipids and surfactant/detergents can be performed in a single reaction mixture, wherein a "one-pot" reaction generates, in situ, both scaffold protein and target membrane protein; NLP self-assembly will ensue using phospholipid already in the reaction mixture. Some of those embodiments are exemplified by the co-expression of a truncated apolipoprotein (Ll-ApoA1) and the bacteriorhodopsin gene, which results in the functionally active seven transmembrane helix bacteriorhodopsin protein (bR) upon addition of retinal cofactor as illustrated in Examples 1-22 and in FIGS. **1-32**.

[0085] In some embodiments, each of the target protein, indicator protein and/or the scaffold protein expressed in the IVT system is comprised of more than one protein, thus resulting in NLP including two or more target proteins and/or two or more scaffold proteins. In particular, in some of those embodiments cell-free co-expression of membrane proteins in NLP complexes enable production in a same NLP of multiple classes of membrane associated proteins previously not conveniently obtainable.

[0086] In some embodiments the target proteins are membrane proteins such as protein coupled receptors (GPCRs), which include, for example acetylcholine receptors (AChRs) and rhodopsin. GPCRs conform to a shared common structure that is believed to traverse the cell surface membrane seven times forming a helical structure encompassing a ligand binding site. Of the three cytoplasmic dominions the C-loop has a C-terminal tail that recognized and activates specific hetero-trimeric GTP binding proteins (G proteins) in an agonist dependent manner.

[0087] Further exemplary target proteins include Ion channels (IC) and small multidrug resistance transporter (SMR), and additional membrane proteins that mediate essential cellular processes including signal transduction, transport, recognition, bioenergetics, and cell-cell communication. This would include G-coupled receptors, Toll receptors and various kinases that important for the aforementioned processes. Additional examples include targeting whole family of proteins within any species such as membrane proteins from Thiobacillus denitrificans that contain unusual membrane associated [NiFe]hydrogenase complex, a group of highly expressed membrane bound c-type cytochromes and a group of highly upregulate membrane proteins of unknown functions that contribute to the bioenergetics of an organism.

[0088] Exemplary proteins suitable to be used as target proteins in the methods and systems herein disclosed are indicated in Table 1.

TABLE 1

Exemplary target proteins

	CB	FL	CF	Target	Endogenous ligand	Family	Fluorescent ligand	Availability
1	X	X	X	V2R	Vasopressin	GPCR	FAM-Vasopressin	commercial
2	X	X	X	CRF	Corticotropin RF I (CRF)	GPCR	FAM/Rhod-CRF	commercial
3	X	X	X	ETB	Endothelin	GPCR	FAM/Rhod-Endothelin	commercial
4	X	X	X	MC5R	Melanocortin	GPCR	BODIPY-TMR NDP-α-MSH	commercial
5	V	X	X	NTR1	Neurotensin	GPCR	FAM-neurotensin	commercial
6	X	S		5HT1A	Serotonin	GPCR		synthesize
7	X	S		D1	Dopamine	GPCR		synthesize
8	X	S		H2	Histamine	GPCR		synthesize
9	X	X		M1	Acetylcholine/Muscarine	GPCR	FITC-pirenzapine	commercial
10	X	X		hERG	Voltage	IC	IVGN-0107	synthesized

TABLE 1-continued

	CB	FL	CF	Target	Endogenous ligand	Family	Fluorescent ligand	Availability
				Exemplary target proteins				
11		X		α1AR	Epinephrine	GPCR	BODIPY FL prazosin	commercial
12		X		β1AR	Epinephnne	GPCR	BT-CGP12177	commercial
13	V	X		OP1R	Opiods	GPCR	FL-naltrexone/naloxone	commercial
14			X	β2AR	Epinephrine	GPCR		synthesize
15	V		X	M2	Acetylcholine/Muscarine	GPCR		synthesize

CB = Cell Based Assay available (X = CBA exists, V = vector exists for assay in development)
FL = Fluorescent Ligand available (X = Yes, S = requires synthesis)
CF = Expressed in Cell-Free system

[0089] In general, target proteins that can advantageously be included in NLP using methods and systems herein disclosed, comprise all the proteins and in particular membrane protein, whose over-expression results in cell toxicity (in vivo), protein aggregation, mis-folding, and low yield and that are instead expressed in a cell free system that includes appropriate additives.

[0090] In some embodiments, the additives used in the cell free reaction systems include any substance that improves the solubilization of the protein of interest and/or of any other protein components that are present in the reaction mixtures, any substance that may augment protein production and any substance that improves protein functions. Those additives include but are not limited to cofactors (e.g. retinal, heme) other proteins that facilitate modification (e.g. glycosylases, phosphatases, chaperonins) lipids, redox factors, detergents and protease inhibitors, and in particular, phospholipids such as dimyristoylphosphatidyl choline (DMPC) and the like, and surfactants/detergents such as cholate, triton X-100 and the likes. Exemplary detergents that can be used for protein solubilization in the methods and systems herein disclosed, include Heptanoyl-N-methyl-glucamide, Octanoyl-N-methyl-glucamide, Nonanoyl-N-methyl-glucamide, n-Nonyl-b-D-gluco-pyranoside, N-Octyl-b-D-glucopyranoside, Octyl-b-D-thiogluco-pyranoside, N,N-Dimethyldodecylamine-N-oxide and Glycerol.

[0091] Suitable indicators include Bacteriorhodopsin (bR) from *H. salinarium*, which is also an exemplary target protein having 7 transmembrane spanning regions that can be produced, purified and regenerated by exogenously adding all-trans retinal. bR was used in a series of experiments exemplified in the Examples section as a model target protein to be included in NLPs according to methods and systems herein disclosed. bR is in particular suitable as an indicator for monitoring the production in an NLP of a GPCR, a heme containing protein or other chromospheres containing membrane protein. Additional exemplary indicator proteins that can be used in the methods and systems herein disclosed and in particular for in situ monitoring of cell-free membrane protein synthesis with concomitant NLP formation, include GFP, GFP-fused to a membrane protein, cytochromes and dye labeled proteins such as sensory rhodopsin, proteorhodopsin, and phytochromes. Like bR, these proteins can be produced by the cell-free transcription/translation technology in the presence of detergents, lipids and phospholipids, as well as other reaction additives such as chromophores and coupled to NLP formation. The ability of the protein to turn color through the binding of the chromophore provides a direct calorimetric measure assay for a properly folded and active protein. This allows the inference to assay conditions that are favorable to proper conditions for folding and obtaining functional activity of any target protein such as GPCRs. Additional target proteins that can be monitored using the indicator proteins herein described can be identified by a skilled person upon reading of the present disclosure and will not be further discuss in details.

[0092] In some embodiments, the detectable activity of the indicator protein in its active form is the ability of the indicator protein to directly or indirectly bind a label or labeled molecule wherein binding of the indicator protein to the labeled molecule is associated with the emission of a labeling signal.

[0093] The terms "label" and "labeled molecule" as used herein refer to a molecule capable of detection, including but not limited to radioactive isotopes, fluorophores, chemiluminescent dyes, chromophores, enzymes, enzymes substrates, enzyme cofactors, enzyme inhibitors, dyes, metal ions, nanoparticles, metal sols, ligands (such as biotin, avidin, streptavidin or haptens) and the like. The term "fluorophore" refers to a substance or a portion thereof which is capable of exhibiting fluorescence in a detectable image. As a consequence the wording and "labeling signal" as used herein indicates the signal emitted from the label that allows detection of the label, including but not limited to radioactivity, fluorescence, chemolumiescence, production of a compound in outcome of an enzymatic reaction and the likes.

[0094] In some embodiments, detecting the indicator protein detectable activity can be performed by providing a labeled molecule that specifically binds to the indicator protein, the labeled molecule providing a labeling signal. The labeled molecule is in particular contacted with the nanolipoprotein particle for a time and under condition to allow binding of the labeled molecule with the indicator protein in the nanolipoprotein particle. The labeling signal is then detected from the labeled molecule bound to the indicator protein in the nanolipoprotein particle.

[0095] In some embodiments, the labeled molecule comprises a molecule that specifically binds to the indicator protein (or to another polypeptide or protein to be detected) and a label attached to the molecule, with the label providing the labeling signal.

[0096] In some embodiments, (e.g. when the label or labeled molecule is a chromophore), the signal can be a visual signal (e.g. calorimetric) detectable by naked eye and/or with the aid of appropriate equipment.

[0097] In particular, indicators able to provide a detectable visual signal, such as calorimetric indicators e.g. bacteriorhodopsin will only incorporate the chromophore when prop-

erly folded. UV-visible spectroscopy is an example of a detection method that could be used to confirm proper folding of the indicator protein. Empirical observation of color development is the primary detection method. Visualization can take place before, during and after a cell-free reaction is complete. The measurement/visualization of color can also be used to follow purification/isolation steps. Visualization is primarily by eye or UV-visible spectroscopy, and is compatible/possible using other technologies such as IR, and radiolabeling and additional technologies identifiable by the skilled person upon reading of the present disclosure.

[0098] Additional indicators able to provide a detectable visual signal include proteins able to bind a specific antibody labeled with a fluorophore only when properly folded. In those embodiments the antibody can be labeled for detection, which can occur by visualization of the antibody bound to the indicator protein or by other methods identifiable by a skilled person upon reading of the present disclosure.

[0099] In the methods and systems herein disclosed, detection can be performed without visualization. For example, in some embodiments, (e.g. wherein binding of the indicator protein to the label results in the production of a compound in outcome of an enzymatic reaction), the labeling signal can be provided by the measured amounts of compound produced in outcome of the enzymatic reaction or other detecting techniques identifiable by a skilled person upon reading of the present disclosure.

[0100] In some embodiments, the methods and systems herein disclosed are used to produce proteins for structural studies, including for example NMR and X-ray crystallography. In particular, these cell free methods can be applied to integral membrane proteins in a high-throughput manner, as a variety of conditions can be rapidly tested to identify optimal expression parameters.

[0101] In some embodiments, the methods and systems herein disclosed are used to produce NLPs suitable as drug delivery vehicles, wherein the particles are formed by taking advantage of the ability of amphipathic apolipoproteins to solubilize certain phospholipid vesicle substrates, transforming them into a relatively homogeneous population of disk-shaped bilayers whose perimeter is circumscribed by apolipoprotein molecules.

[0102] In some embodiments, NLPs are provided by the methods and systems herein disclosed by using different scaffold proteins, which allow to tailor the average size of the particles, e.g. from 10 to 60 nm (+/−3%), and in particular 10 to 30 nm (+/−3%), at an average height of 5.0 nm. The nanoscale bilayers so obtained can be used to investigate and control assembly of oligomeric integral membrane proteins critical to macromolecular recognition and cellular signaling. Those embodiments can be performed using any apolipoprotein-like molecules as potential structures for solubilizing the membrane proteins via NLP formation. Examples include, but are not limited to ApoE4, ApoA1, MSP1 (ApoA1 truncations), synthetic peptides and insect lipophorins.

[0103] In some embodiments, the methods and systems herein disclosed are performed at predefined lipid protein ratio, assembly conditions and/or with the use of preselected protein component and amphipatic lipid so to increase the yield, control the size of the resulting NLP and/or provide an NLP of pre-determined dimensions so to include a predetermined target protein.

[0104] In particular, in some embodiments the scaffold protein is selected to define the size of NLPs. Lipophorin III lipoproteins make assemble into larger NLPs with diameters 10-30 nm range, apolipoprotein A1 NLPs range in size from 10-25 nm, truncated Δ(1-49)Apolipoprotein Δ115-35 nm. Adjustment of protein to lipid ratios increasing lipid will also increase the size of the NLP.

[0105] In some embodiments the amphipatic lipid is tested to provide the most stable and native-like environment. For example a target protein that is naturally found in the inner mitochondrial membrane would contain lipids specific to that region of the cell. In particular the protein of the inner mitochondrial membrane requires a membrane compose of 20% cardiolipin for proper function. A protein that requires more flexibility in it function may require lipids with a higher degree of unsaturation creating a bilayer with more fluidity. While incorporating a target protein the stability of the protein may be improve by using a detergent that has been proven to allow the protein to retain native activity as measured/monitored by our indicator protein.

[0106] In some embodiments the amphipatic lipid is selected to resemble the native lipid composition in which the membrane protein is known to function.

[0107] In some embodiments the lipid to scaffold protein ratio: is selected to optimize and maximize the yield leading to NLP formation.

[0108] In some embodiments, the assembly parameters are selected to allow the constituents reach maximum NLP formation reflective of a thermodynamic endpoint.

[0109] In some embodiments, the methods and systems herein disclosed can be used to monitor the efficiency of selected cell-free reagents in producing a predetermined target protein.

[0110] In some embodiments, the methods and systems herein disclosed can be used to monitor the efficiency of refolding of a denatured target protein.

[0111] In some embodiments, the indicator protein can be used be used as a marker for various gel and column separations methods and system, which are identifiable by a skilled person upon reading of the present disclosure.

[0112] In some embodiments, the indicator protein can be used as a marker for protein microarrays, in various applications identifiable by a skilled person upon reading of the present disclosure.

[0113] In some embodiments, the indicator protein can be used as sizing calibrators for microfluidics, in applications identifiable by a skilled person upon reading of the present disclosure.

[0114] The systems herein disclosed can be provided in the form of kits of parts. For example the protein can be provided as embedded in the NLP to follow process controls. The indicator protein can be included as a protein alone or in the presence of lipids/detergents for transition in to nano-particles. The indicator protein can be included as a plasmid or PCR DNA product for transcription/translation. The indicator protein may be included as encoded RNA for translation.

[0115] In a kit of parts, a polynucleotide, amphipatic lipid, target protein, indicator protein and/or scaffold protein are comprised in the kit independently possibly included in a composition together with suitable vehicle carrier or auxiliary agents. For example a polynucleotide—can be included in one or more compositions alone and/or included in a suitable vector. Also each polynucleotide can be included in a composition together with a suitable vehicle carrier or auxiliary agent. Furthermore, the indicator protein can be included in various forms suitable for appropriate incorporation into

the NPL. For example, in embodiments wherein the indicator protein is bR, the cofactor all-trans-retinal would be included in a kit that also contained the encoded genetic information for the production of bacteriorhodopsin as the calorimetric indicator.

[0116] In some embodiments, the labeling molecule can also be included in the kit herein disclosed, including but not limited to labeled polynucleotides, labeled antibodies, other labels identifiable by the skilled person upon reading of the present disclosure.

[0117] . Additional components can also be included and comprise microfluidic chip, reference standards, and additional components identifiable by a skilled person upon reading of the present disclosure.

[0118] In the kit of parts herein disclosed, the components of the kit can be provided, with suitable instructions and other necessary reagents, in order to perform the methods here disclosed. In some embodiments, the kit can contain the compositions in separate containers. Instructions, for example written or audio instructions, on paper or electronic support such as tapes or CD-ROMs, for carrying out the assay, can also be included in the kit. The kit can also contain, depending on the particular method used, other packaged reagents and materials (i.e. wash buffers and the like).

[0119] Further details concerning the identification of the suitable carrier agent or auxiliary agent of the compositions, and generally manufacturing and packaging of the kit, can be identified by the person skilled in the art upon reading of the present disclosure.

EXAMPLES

[0120] The methods and system herein disclosed are further illustrated in the following examples, which are provided by way of illustration and are not intended to be limiting.

[0121] In particular, in the following examples, the methods and systems herein disclosed are exemplified by a calorimetric assay that indicates production, correct folding, and incorporation of bacteriorhodopsin (bR)— a 7-transmembrane (TM) protein and prototypical of the 7-TM class of membrane receptors—into a soluble nanolipoprotein particle. In particular, in the following examples NLP are membrane bilayer mimetics resulting from self-assembly of an apolipoprotein, (including related fragments, peptides and derivative thereof) and phospholipid.

[0122] The results of the experiments illustrated in the following examples show that synthesis of bR membrane proteins in cell-free reactions was functional by UW/V is and that the bR proteins associate directly with the NLPs. The bR protein is only purple when retinal ligand is present allowing proper folding. In particular, as illustrated in some of the examples below, when bR and apolipoprotein are co-expressed in a cell-free system, assembly into bR-NLP constructs follows; if retinal is present in the reaction mixture, a clear pink-purple colored solution results indicating formation of bR-NLPs.

[0123] In some experiments herein exemplified the approach did provide both structural and functional results within minutes. This assay approach is demonstrated' for example, by the expression of bacterioOpsin gene in the presence and absence of different additives, which results in properly folded and functionally active seven transmembrane helices bacteriorhodopsin protein (bR) contained within an apo-AI apolipoprotein NLP construct.

Example 1

Cell-Free Production of NLPs bR Protein

[0124] The experimental strategy for cell-free membrane protein-NLP self-assembly was based on the ability of membrane proteins to insert into lipid bilayers during cell-free synthesis, the apolipoprotein ability to sequester lipid bilayer patches, and the demonstrated ability of NLPs to than solubilize membrane proteins. Individual plasmid DNAs encoding the membrane protein and the apolipoprotein are added to the cell-free reaction with the addition of phospholipids and cofactors to produce membrane protein associated discoidal nanolipoprotein particles (NLPs) in a single reaction. In particular, as shown in FIG. **1** constituents (DNA, lipid vesicles, cofactors and cell-free lysates) are added together in a single reaction vial. The cell-free lysates take advantage of the T7 coupled transcription and translation system to produce a mixed population of self-assembled NLPs with and without associated integral membrane protein.

[0125] In a first series of experiments, two plasmids were used to generate the integral membrane protein and the lipoprotein NLP support, one encoding membrane protein bacterioOpsin (bOp) and the second encoding a Δ1-49 apolipoprotein A-1 fragment (Δ49A1). The plasmids were co-expressed, in the presence of all-trans-retinal and the phospholipid dimyristoyl-phosphatidylcholine (DMPC), resulting in functional bacteriorhodopsin (bR) protein solubilized in a discoidal bR-NLP.

[0126] In particular, the truncated form of Apo A1 (Δ1-49) or Δ49A1 was cloned using the following primers: forward, 5'-atgctaaagctccttgacaactgg-3' (SEQ ID NO: 1) and reverse, 5'-ttactgggtgttgagcttcttagtg-3' (SEQ ID NO: 2). This construct is six amino acids shorter than our truncated form of Apo A1 (Δ1-49) or Δ49A1, and was expected to perform similarly in NLP assembly and characterization.

[0127] The resulting PCR product was cloned into the vector pIVEX2.4d using NdeI and SmaI restriction sites. This vector also contains a His-tag for nickel affinity purification. The bacterioOpsin sequence (bOp), which encodes the bacteriorhodopsin protein, was amplified from a plasmid p72bop (Sonar et al., 1993; obtained from Kenneth Rothschild) using the following primers: 5'-ggggcatatgcaagctcaaat-3' (SEQ ID NO: 3) and 5'-ggggatccaaaaaaaacgggcc-3' (SEQ ID NO: 4). The gene represents a synthetic form of bOp that was designed for *E. coli*-based expression (1). The resulting PCR product was cloned directionally into the HIS-tagged pIVEX 2.4b vector using the NdeI and BamHI restriction enzyme sites. All constructs were verified by DNA sequencing.

[0128] Cell-free reactions were then performed. In particular, preparative reactions are carried out using the Invitrogen's Expressway Maxi kit or Roche's RTS 500 ProteoMaster Kit. Basically, lyophilized reaction components (Lysate, Reaction Mix, Amino Acid Mix, and Methionine) are dissolved in Reconstitution Buffer and combined as specified by the manufacturer. For co-expression a total of 5 μg of each plasmid DNA (bOp and Δ49A1) was added to the lysate mixture with added DMPC vesicles and retinal cofactor (see below). The reactions were incubated at 30° C. or 37° C. for 4-24 h. For membrane protein survey studies co-expression of 0.2 ug Δ49A1 DNA and 1 ug of each membrane protein DNA was added to the cell-free mixture where [^{35}S]Met (135 mCi/

mmol final) (Perkin Elmer, Waltham, Mass.) was added in place of methionine. The soluble fraction was obtained by centrifuging the reactions at 14000×g for 5 min. Autoradiograms were generated by overnight exposures to proteins separated by SDS-PAGE (data not shown). Percent solubility was determined using ImageJ software (U.S, National Institutes of Health) to quantize autoradiogram bands for the soluble fractions in the presence and absence of apolipoprotein Δ49A1.

[0129] In some reactions a retinal cofactor was added. To this purpose, an all trans-retinal (Sigma) solution was prepared with 100% ethanol at a stock concentration of 0.586 or 10 mM. The stock solution was diluted to achieve a final working concentration of 30-50 μM in cell-free reactions.

[0130] The lipid component of the NLPs was also prepared. Small unilamellar vesicles of DMPC (liposomes) were prepared by probe sonicating a 68 mg/mL aqueous solution of DMPC until optical clarity is achieved; typically 15 min on ice is sufficient. A 2 min. centrifugation step at 13700 RCF was used to remove any metal contamination from the probe tip. The individual lipid component was added to the cell-free reaction at a concentration of 2 mg/mL.

[0131] Soluble fractions were purified. In particular, NLP complexes were then purified through Affinity purification. In particular, immobilized metal affinity chromatography was used to isolate the proteins of interests (truncated 49A1 and bOp) from the cell-free reaction mixture based on affinity of the N-terminal poly-His tag. The soluble fraction was separated from precipitated protein by centrifugation for 5 min at 18K RCF at 4° C. The soluble fraction was mixed with Ni-NTA Superflow resin (Qiagen) according to the manufacturer's protocol using native purification conditions with the following modifications; 5 mM imidazole in PBS buffer was used for washing and 400 mM imidazole PBS buffer was used for elution of the His-tagged proteins. All elutions were combined, concentrated and buffer exchanged into TBS using a 100K MWCO molecular weight sieve filters (Vivascience) in a volume of 200 μL.

[0132] The samples were also characterized by SDS-PAGE, Native PAGE, UV-visible spectroscopy, and atomic force microscopy (AFM) as illustrated in the following examples. A survey study of other membrane proteins co-expressed with the truncated apolipoprotein also significantly increased solubility all of the membrane proteins surveyed

Example 2

Characterization of NLPs Produced By Cell-Free System

Solubilization of the bR-NLP Complex

[0133] The experimental design outlined in Example 1 of cell-free co-expression for refolding and incorporation into NLPs was also demonstrated using bR from Halobacterium salinarium, and truncated apolipoprotein A-1 (A 1-49) or Δ49A1. The bR protein is a seven transmembrane (TM) helical protein and serves as a structural model protein for rhodopsin and other 7-TM proteins such as GPCR family members.

[0134] Simultaneous cell-free protein expression of both bR and Δ49A1 in the presence of DMPC in a single reaction produces a functional bR-NLP complex (FIG. **2** and FIG. **3**).

[0135] In particular, as illustrated in FIG. **2**, bOp & DMPC (sample 1) shows bR is insoluble in the absence of co-expression of Δ49A1; bOp, Δ49A1 co-expressed in the presence of DMPC (sample 2) shows bR remains in the soluble fraction with co-expressed Δ49A1; Δ49A1 & DMPC (sample 3) shows production of "empty"-NLPs; the control (sample 4) shows cell-free reaction (No DNA) in the presence of DMPC only. All were expressed in the presence of 30-50 μM all-trans-retinal and 2 mg/mL DMPC. Purple color development observed in sample 1 and 2 indicates incorporation of retinal into the bOp transcript representing proper folding of bR.

[0136] Single-step co-expression, assembly and purification of the soluble bR-NLP complex was completed within 4 hours giving analogous yields and functions comparable to previous published findings (2). This extremely rapid approach was also applicable to a wide variety of other transmembrane proteins (FIG. **4**).

[0137] Although bR coloration was observed in the presence of DMPC without Δ49A1, very little of the material was soluble compared to when the Δ49A1 was co-expressed in the reaction mixture (FIG. **2** and FIG. **5**), as indicated in the soluble (S) and pelleted (P) lanes with and without Δ49A1 co-expression). Two methods for refolding of cell-free expressed bR into lipid vesicles have been previously reported by Sonar et al., and Kalmbach et al (2, 3). However, these two approaches required multiple steps over a lengthy period of time and were further encumbered by limited membrane protein accessibility due to the nature of liposomes (2, 3). Similar results, cell-free synthesis of bR in the presence of liposomes and cofactor alone, produced functional membrane protein (purple color) that was insoluble (FIG. **2**, and FIG. **5**). In contrast the co-expressed bR-NLP complexes were functional, stable and soluble using our procedure.

[0138] Demonstration using bacteriorhodopsin (bR) and truncated apolipoprotein A1 (Δ49A1) produced bR-NLP complexes that were shown to be soluble, discoidal in shape and light active FIGS. **2**, **4**, **8**, **10**, and **11**. Distinct purple coloration, an indication of properly folded functional bR protein, was observed when all-trans retinal and phospholipid were included in the reaction mixtures (see FIGS. **2**, **3**, **4**, and **21**). Solubility survey results indicate this rapid approach may also be applicable to a wide variety of other transmembrane proteins (FIG. **9**).

Example 3

Characterization of NLPs produced by cell-free system

SDS Page,

Native Page SEC and AFM

[0139] bR-NLP complex heterogeneity was also observed by both native gel electrophoresis and SEC.

[0140] In particular, NLPs produced as described in Example 2 were first analyzed by SEC, to detect the separation of NLPs from larger lipid-rich material. Size exclusion chromatography identified a size shift in the bR-NLP complex compared to empty NLPs or liposomes. The bR-NLP complexes eluted primarily before empty NLPs and after liposomes (FIG. **5**). A size range of approximately 470-680 kDa was observed for bR-NLP complexes, which was 160-370 kDa larger than the empty self-assembled NLPs (FIG. **6**).

[0141] The NLPs were also analyzed by SDS Page. In particular, a 1 μL aliquot of the total (T) cell-free reaction, soluble (S) fraction and resuspended pellet (P) were diluted with 1×LDS Sample buffer with reducing agents (Invitrogen), heat denatured and loaded on to a 4-12% gradient pre-

made Bis-Tris gel (Invitrogen) along with the molecular weight standard SeeBlue plus2 (Invitrogen). The running buffer was 1× MES-SDS (Invitrogen). Samples were electrophoresed for 38 minutes at 200V. Gels were stained with coomassie brilliant blue.

[0142] The particles were also analyzed by native PAGE. Equal amounts of NLP samples (0.5-1.0 μg) were diluted with 2× native gel sample buffer (Invitrogen) and loaded onto 4-20% gradient pre-made Tris-glycine gels (Invitrogen). Samples were electrophoresed for 2 hrs. at a constant 125 V. After electrophoresis, gels were incubated with SYPRO Ruby protein gel stain (Bio-Rad) for 2 hours and then destained using 10% MeOH, 7% Acetic acid. Following a brief wash with ddH_2O, gels were imaged using the green laser (532 nm) of a Typhoon 9410 (GE Healthcare) with a 610 nm bandpass 30 filter. Molecular weights were determined by comparing migration vs. log molecular weight of standard proteins found in the NativeMark standard (Invitrogen).

[0143] This heterogeneity may have been due to multiple factors such as number of lipids per NLP, bR oligomerization within the NLPs and/or generation of NLPs with varying diameters. Particle diameters measured by atomic force microscopy AFM (data not shown) supports the latter.

[0144] To this extent NLPs were imaged using and Asylum MFP-3D-CF atomic force microscope. Images were captured in tapping mode with minimal contact force and scan rates of 1 Hz. Asylum software was used for cross-sectional analysis to measure NLP height and diameter. For experimental analysis, the heights and diameters were measured on 182 NLPs produced by cell-free expression in the absence of bR and 430 total NLPs (empty-NLPs 185 and 255 bR-NLPs) produced by cell-free co-expression. Two-tailed student T-tests were run to compare both the height and diameter of the "empty"-NLP population in the sample co-expressed with bR compared to the sample with no bR expressed. A p-value of <0.01 was considered significant. A student T-test compares two populations of data and can determine if the difference between the two sets is statically significant or insignificant.

[0145] Size and shape of the NLPs determined by AFM showed a height of the "empty"-NLP to be 5.0+/−0.3 while the height of bR-NLPs was 6.4+/−0.3. UV-visible spectroscopy identified a 5 nm shift upon light adaption indicating functionality

Example 4

Characterization of NLPs Including Membrane Protein Indicator

Demonstration of Membrane Protein Activity

[0146] Functional activity of the soluble, self-assembled, co-expressed bR-NLP complex was determined by light-dark adaptation (FIG. **8**).

[0147] The light-dark adaptation yielded a 5 nm shift with a dark absorption maximum of 549 nm and light absorption maxima of 554 nm. These results indicated that the majority of active bR was in a monomeric form (4). This is in agreement with other studies that used pre-purified apolipoprotein scaffolds to solubilize native forms of bR (5). The major advantage of our approach is that it allows to obtain folded light active bR-NLP assemblies in less than four hours that have been self-assembled in a single step, thereby eliminating the need for isolation of membrane protein, protein purification, dialysis and refolding protocols prior to the formation of NLP-membrane protein complexes.

Example 5

Characterization of NLPs

Co-Expression Survey of Membrane Proteins

[0148] In order to determine if the co-expression method increased solubility a series of membrane proteins with varying numbers of transmembrane domains were expressed using cell-free methods. Cell-free expression of the membrane protein alone and lipid-assisted expression of the membrane protein were used for comparison. The reactions included ^{35}S-methionine in order to quantify the protein. Autoradiograms (not shown) were generated from total and soluble fractions separated by SDS-PAGE. Densitometry using ImageJ software (National Institutes of Health) was used to analyze the autoradiograms. The percent solubilized membrane protein compared to the total membrane protein was plotted in FIG. **9**.

[0149] In particular in FIG. **9** a comparison was made between (MP alone—Grey) the membrane protein expressed alone, (Lipid Assisted-striped) expression of the membrane protein in the presence of DMPC vesicles, and (Co-expressed—Black) membrane protein co-expressed with apolipoprotein (Δ49ApoA1) in the presence of DMPC vesicles. The data was generated from autoradiograms by the incorporation of ^{35}S-Methionine in to the cell-free reaction (data not shown) that were quantified using ImageJ software (U.S. National Institutes of Health).of autoradiograms generated from SDS-PAGE.

[0150] In all cases co-expression with the truncated apolipoprotein Δ49A1 was greater that the expression of the membrane protein alone or lipid-assisted membrane protein expression (FIG. **9**). Also in all cases the solubility of the membrane protein increased with co-expression of Δ49ApoA1 with added (FIG. **9**).

Example 6

Compared Self-Assembly of Cell-Free Produced and Conventionally Produced NLPs

[0151] NLPs produced by cell-free methods and NLPs assembled by conventional means (6) were both examined by AFM to assess NLP size and shape and to demonstrate the association between bR with NLPs. For comparison to cell-free produced bR-NLP complexes, both bR-NLPs and "empty"-NLPs were also prepared using previously described methods (6, 7).

[0152] Conventional assembly of NLPs is described herein in Examples 13 to 22. Briefly, the truncated form of Apo A1 (Δ1-55) called MSP1T2 or Δ55A1 was purchased from Nanodisc Inc. For "empty"-NLPs Δ55A1 was combined with DMPC liposomes in a ratio of 1:4 by mass in TBS buffer. The mixture was then incubated at room temp for 2 hours. The NLPs were then purified by size exclusion chromatography. Assembly of bR-NLPs: Δ55A1 was mixed with DMPC in a ratio of 1:4 by mass in TBS buffer. Sodium cholate solution was then added to a final concentration of 20 mM. Purple membrane bacteriorhodopsin was then added in a 0.67 mass ratio to the Δ55A1 apolipoprotein. Incubation proceeded as described above, followed by dialysis in TBS for detergent removal. The NLPs were then purified by size exclusion chromatography.

[0153] In particular, the size exclusion chromatography was performed as follows. The NLPs made with and without incorporated membrane protein were purified from 'free pro-

tein' and 'free lipid' by HPLC (Shimadzu) using a Superdex 200 10/300 GL column (GE Healthcare), with TBS at a flow rate of 0.5 ml/min. The column was calibrated with four protein standards HMW Gel filtration calibration kit (GE Healthcare), of known molecular weight and Stokes diameter that span the separation range of the column and the NLP samples. The void volume was established with blue dextran. The NLP fraction is concentrated about 10-fold to approximately 1.0 mg/ml using molecular weight sieve filters (Vivascience) having molecular weight cutoffs of 50 kDa. Protein concentration was determined using the ADV01 protein concentration kit (Cytoskeleton), which is based on Coomassie dye binding.

[0154] These NLPs and bR-NLP complexes were made with a similarly truncated form of Apolipoprotein A1 (Δ1-55) or Δ55A1 (MSP1T2, Nanodisc Inc.), purple membrane bR and DMPC liposomes (6, 7). Both the co-expressed and conventionally assembled bR-NLPs showed similar increases in particle height relative to an "empty" NLP indicating likely association of bR protein within the NLPs (FIG. **10**).

[0155] Empty NLPs produced either by conventional methods or cell-free displayed heights of approximately 5.0±0.3 nm (s.d.) as determined by AFM (FIGS. **10**B and **10**C respectively). The NLPs produced by either conventional assembly of Δ49A1 and bR (FIG. **10**B) or co-expression of Δ49A1 and bR (FIG. **10**C) appeared as two distinct discoidal populations when examined by AFM cross-sectional height analysis. The first population is approximately 5.1±0.3 nm (s.d.) in height, analogous to "empty"-NLPs (FIG. **10**C). The second population, which was not observed in control experiments lacking bR, was approximately 6.4±0.3 nm (s.d.) in height (FIG. **10**C). The increased height observed in the presence of bR is located in the center region of the NLP (bright green dot, pseudo color) is consistent with the bR being contained within the NLP lipid bilayer (FIG. **10**A). Additionally, the increased height particles produced in the presence of bR also had an associated increase mean diameter (27.8±5.8 nm (s.d.)) relative to the "empty" Δ49A1-NLPs (22.0±5.1 nm) (Table 2).

[0156] Using solely the increase in height as a basis for distinguishing bR-NLPs from "empty" Δ49A1-NLPs, an overall yield of protein incorporation of 58% was determined (Table 2). Two-tailed student T-tests indicated that there was no statistically significant difference between the diameter and height of the "empty" Δ49A1-NLPs produced by cell-free methods in the presence (n=185; 2a) and absence (n=182; 1) of bR (Table 2) with p-values of 0.94 and 0.04 respectively. However, a statistically significant increase in diameter and height was observed between the bR-NLPs (n=255; 2b) and "empty" Δ49A1-NLPs (n=185, 2a) with p-values of 1.8 E^{-24} and 3.9 E^{-155} respectively (Table 2). Those results are illustrated in Table 2 that includes a summary of analysis of cell-free expressed NLPs with and without co-expressed bR

TABLE 2

	Sample	Height (nm) +/− s.d.	Relative % Increase Diameter	% NLP
1	"empty" Δ49A1 NLPs	5.0 +/− 0.3	1	100
2a	Co-expressed "empty"-Δ49A1 NLPs	5.1 +/− 0.3	1	42
2b	Co-expressed Δ49A1/bR-NLPs	6.4 +/− 0.3 *	1.3 *	58

* Statistically significant, see text for specific values.

[0157] AFM was also used to visualize the first SEC fraction, where high molecular weight lipid complexes were observed consistent with results reported in Chromy et al (6) in which was described lipid-based macro-molecular formations unable to enter a native-PAGE and had the appearance of liposom-like material (FIG. **7**). The majority of this material was distinctly different in size, ranging 35-60 nm in diameter and 6.5-20 nm in height indicating the majority of the material was large lipid complexes such as liposomes or membrane patches (FIG. **7**).

Example 7

Cell-Free Expression and Purification of Apolipoproteins

[0158] The methods described below outline cell-free expression and purification of apolipoproteins. In particular, it is described the cell-free production of a selected N-terminal truncation of human apolipoprotein E4 which does not require post-translational modification.

[0159] The following materials and instruments were used: Apolipoprotein (ApoA1, MSP, Apo E4, lipophorin III, or truncations Δ49ApoA1 and ApoE4 22k) clones of interest from the LLNL-IMAGE Consortium cDNA collection or as a gift from collaborating labs, subcloned in to an expression vector such as, pET32a thioredoxin (Novagen) (8, 9), pIVEX-2.4b (Roche), or pEXP4 (Invitrogen); Spectrophotometer UV-visible A_{260}/A_{280} quantification or PicoGreen dsDNA Quantification Kit (Invitrogen/Molecular Probes); Cell-Free Expression System: Expressway™ Maxi Cell-Free *E. coli* Expression System (Invitrogen) or RTS 500 ProteoMaster *E. coli* HY Kit (Roche); Thermomixer, Eppendorf Thermomixer R (for Roche lysates) or Incubator shaker for example New Brunswick C24 (for Invitrogen lysates); Disposable fritted columns 3 mL capacity (Bio-Rad); Ni-NTA Superflow resin (Qiagen); Ni-NTA buffers (modified Qiagen recipes) Binding buffer: 50 mM NaH_2PO_4, 300 mM NaCl; pH 8.0; Wash Buffer: 50 mM NaH_2PO_4; 300 mM NaCl; 10 mM Imidazole; pH 8.0; Elution Buffer: 50 mM NaH_2PO_4; 300 mM NaCl; 400 mM Imidazole; pH 8.0; Gel electrophoresis equipment; NuPAGE 4-12% Bis-Tris SDS-PAGE gel with 1×MES-SDS running buffer (Invitrogen), Protein Quantification Kit and standards, such as Bio-Rad Protein Assay (Bio-Rad) Vivaspin6, ultrafiltration Devices, 10 k MWCO (Sartorius Biotech); Centrifuge such as Eppendorf 5804R (Needs to fit 15 mL Falcon tubes); Thrombin (Novagen); DMPC; 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (Avanti Polar Lipids); Probe or bath sonicator; β-mercaptoethanol; TBS Buffer: 10 mM Tris-HCl; 0.15 M NaCl; 0.25 mM EDTA; 0.005% NaN_3 (sodium azide) adjust to pH 7.4; and FPLC Instrument (Shimadzu SCL-10A), size exclusion column (Superdex 200 10/300 GL (GE Healthcare Life Sciences).

[0160] In particular, Lipophorin III DNA clones (*M. sexta* and *B. mori*) were obtained from the lab of Robert Ryan at Children's Hospital Oakland Research Institute (CHORI). Truncated Apolipoprotein E4 22kDa N-terminal thioredoxin fusion plasmid was obtained from Karl Weisgraber at the University of California, San Francisco. The 193 amino acid protein sequence of the 22 kD Apolipoprotein E4 construct is as follows, with the two initial amino acids, Gly-Ser, are left over from the thrombin cleavage site in pET32a. Midi or Maxi prepped plasmid DNA was prepared according to the Qiagen protocol.

(SEQ ID NO: 5)

GSKVEQAVETEPEPELRQQTEWQSGQRWELALGRFWDYLRWVQTLSEQVQ

EELLSSQVTQELRALMDETMKELKAYKSELEEFQLTPVAEETRARLSKEL

QAAQARLGADMEDVRGRLVQYRGEVQAMLGQSTEELRVRLASHLRKLRKR

LLRDADDLQKRLAVYQAGAREGAERGLSAIRERLGPLVEQGRVR

[0161] The cDNAs for apolipoprotein were selected and cloned into expression vector of interest such as pIVEX-2.4b (Roche Applied Science), GFP folder or pETBlue-2 (Novagen.), pET32a (thioredoxin fusion vector). The plasmids were propagated by transforming into Top10 or DH5α chemically competent cells (Invitrogen) and isolate DNA using HiSpeed Plasmid Maxi or Midi Kits (Qiagen). The N-terminal truncated apolipoprotein E4-22kD (ApoE422k) thioredoxin (trx) fusion protein construct in pET32a (ApoE422k-trx) is illustrated here (FIG. **13**).

[0162] The Midi or Maxi prepped plasmid DNA concentrations were determined by PicoGreen dsDNA Quantification Kit (Molecular Probes) or by UV-visible spectroscopy A_{260}/A_{280}. The cell-Free protein production reactions were performed using either the Expressway™ Maxi Cell-Free *E. coli* Expression System (Invitrogen) or RTS 500 ProteoMaster *E. coli* HY Kit (Roche) using ~15 μg of midi or maxi prepped DNA in a 1 mL reaction size.

[0163] The reactions were incubated at 30° C. shaking at 990 rpm in a thermomixer (Roche RTS ProteoMaster or Eppendorf Thermomixer R)-(Roche Lysates) or 37° C. shaking at 225 rpm in a shaker incubator (New Brunswick)-(Invitrogen Lysates). All reactions were run overnight (although 4 hours is sufficient). A 5-10 μl sample was collected for further analysis.

[0164] The His-tagged apolipoprotein (ApoE422k-trx) was purified by using Ni-NTA native affinity chromatography, and 1 mL of the Ni-NTA slurry, equivalent to 500 μL column bed volume (Qiagen) was equilibrated with binding buffer and resuspend the resin to form a 50% slurry again. The equilibrated slurry was added to the cell-free post-reaction mixture and mix at 4° C. for 1-2 hours. The mixture was added to a 3-mL fritted plastic column and collected the flow through for SDS-PAGE analysis.

[0165] The column was washed with eight column volumes (500 μL) of native wash buffer. Fractions are collected for SDS-PAGE analysis.

[0166] The bound apolipoprotein was eluted with six column volumes of native elution buffer.

[0167] All collected fractions were analyzed by denatured gel electrophoresis using a NuPAGE 4-12% Bis-Tris SDS-PAGE gel with 1× MES-SDS running buffer for 38 minutes at 200V (Invitrogen). The load buffer is LDS Sample Buffer (Invitrogen). Volumes to load for SDS-Page gels were as follows: 1 μL of total reaction and non-bound flow through, 5 μL wash fractions 1-2, 20 μL of remaining washes and all elutions. Gels were stained with Coomassie brilliant blue.

[0168] Elution fraction of interest determined by gel electrophoresis were combined and concentrated and buffer exchanged into TBS using an ultrafiltration device vivaspin6. In particular, concentration from 6 mL to 100 μL was easily achieved in ~15 min at 5000 RCF in an Eppendorf 5804R centrifuge with a fixed angle rotor check each 3-5 min. Buffer exchange into TBS pH 7.4 required at least 3 dilutions and re-concentration steps. Alternatively eluted protein could were dialyzed (spectrapor 1 MWCO 3500) against TBS buffer overnight and concentrated by immersion of the dialysis membrane in PEG 8000 (polyethylene glycol).

[0169] The final protein concentration was determined by Bradford total protein concentration following the manufacturer's protocol.

[0170] Small unilamellar vesicles of DMPC were then prepared by probe sonicating 20 mg DMPC lipid into 1 mL TBS at 6 amps for approximately 15 minutes or until optical clarity is achieved. Typically fifteen minutes is sufficient to achieve optical clarity. An appropriate container choice was a thick walled 3 mL glass conical vial. In particular, lipid solution were vortexed lightly before sonication to help get to lipid into the buffer. Lipid should be stored at −20° C. when not in use, and protected from water absorption. When sonicating lipid overheating of the lipids was avoided by either sonicating in a beaker of ice or cooling the sample every few minutes. The solution was practically water clear at the end of the sonication. If the probe hits the side of the glass vessel metal will be sloughed off into the solution and the solution will become grayish. The metal can be removed by a short centrifugation at 13,700 RCF for two minutes after transferring to a 1.5 ml Eppendorf tube. Remove the supernatant and use. Any white pellet indicated DMPC that is not in vesicle form. Alternatively, sonicate in bath sonicator to optical clarity and skip the centrifugation step.

[0171] The sample was transferred to an 1.5 mL tube. Any contaminant metal was removed from the probe by centrifugation at 13700 RCF for 2 minutes in a 1.5 mL tube.

[0172] Thioredoxin fusion protein tags were removed by incubating 2-4 mg of the produced protein with 100 μg/mL of the sonicated DMPC overnight at 24° C. Thrombin was added at 1:500 w/w ratio (thrombin:apolipoprotein) and incubated at 37° C. for one hour. The reaction was halted by the addition of β-mercaptoethanol to a final concentration of 1%. 5 μg of the product were analyzed by SDS-PAGE as described above. The results are shown in FIG. **13**.

[0173] Contaminant thioredoxin (trx), thrombin and β-mercaptoethanol were then removed from the apolipoprotein, ApoE422k by size exclusion chromatography using a FPLC Instrument (Akta, GE Healthcare and Life Sciences or Shimadzu SCL-10A), and size exclusion column (Superdex 200 10/300 GL) with a TBS buffer at a flow rate of 0.5 mL/min. The fractions of interest were determined by gel electrophoresis combine and concentrate as above.

Example 8

Nanolipoprotein Particle (NLP) Formation and Purification

[0174] The methods described below outlines nanolipoprotein Particle (NLP) formation and purification. The following materials and equipments were used: DMPC: 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (Avanti Polar Lipids); Purified apolipoprotein protein or truncation (ApoE422k construct); TBS Buffer: 10 mM Tris-HCl; 0.15 M NaCl; 0.25 mM EDTA; 0.005% NaN3 (sodium azide); adjust to pH 7.4. 30° C. and 20° C. water baths; Probe or bath sonicator; Spin filter, 0.45 μm; Concentrator 50 kD MWCO, Vivaspin 2 (Sartorius Inc.) (Other concentrator brands that are angled are also acceptable such as Agilent, because the nanolipoprotein particle produced will be larger than 200 kD, a 100 kD filter may

be useful); FPLC Instrument (Shimadzu SCL-10A), size exclusion column (Superdex 200 10/300 GL (GE Healthcare Life Sciences).

[0175] Nanolipoprotein particles (NLPs) form in a self assembly process in the correct mass ratio of apolipoprotein to lipid. This ratio needs to be optimized for each different apolipoprotein. The ratio described below is for ApoE422k (6). Other ratios can be found in the literature (7, 10, 11).

[0176] The water bath incubators were started with temperatures at 30° C. and 20° C. 34 mg of DMPC were probe sonicated into 1 mL of TBS at 6 amps for approximately 15 minutes or until optical clarity is achieved. DMPC solution was centrifuged at 13700 RCF for 2.5 min to remove residual metal from probe sonicator. The supernatant was transferred into a new tube. Apo E422K was combined with DMPC in a ratio of 1:4 by mass in TBS buffer in a 1.5 mL Eppendorf tube. Typically batches are of the 250 μL size.

[0177] Transition temperature procedure was performed as follows: the tube was immersed in water bath for 10 minutes each 30° C. (above DMPC transition temp.) followed by 20° C. (below DMPC transition temp.). The procedure was repeated three times then the tube was incubated at 23.8° C. overnight.

[0178] Filter preparation was performed through a 0.45 μm spin filter at 13700 RCF for 1 min. Purify NLPs using size exclusion chromatography. A Shimadzu SCL-10A FPLC was used that was equipped with a Superdex 200 10/300 GL column with TBS buffer, a 200 μL sample injection volume, and a flow rate of 0.5 mL/min. Collect 0.5 mL fractions see FIG. **14**.

[0179] Fractions were concentrated using a Vivaspin 2 ultrafiltration device with a 50k MWCO as described in Example 7.

Example 9

Biotinylation of Membrane Protein

[0180] The methods described below outline the biotinylation of membrane bound proteins, and in particular of Bacteriorhodopsin (bR). The following materials and equipments were used: EZ-Link Sulfo-NHS-LC-Biotin (Pierce); Bacteriorhodopsin (Sigma); Bath sonicator; Ultracentrifuge (Beckman-Coulter Optima TLX, TLA-120.2 fixed angle rotor); 1× BupH PBS buffer (Pierce): 0.1 M NaH2PO4, 0.15 M NaCl; pH 7.0. Bacteriorhodopsin can also be produced in a cell-free manner and purified in the denatured state. A re-folding procedure is then employed to incorporate the retinal according to the methods of Rothschild et al (2, 12).

[0181] Biotinylation of the membrane protein (MP) provides a tool for investigating the incorporation of the MP with the NLP. Biotinylation using of bacteriorhodopsin supplied in membrane sheets from Sigma selectively labels only the solvent exposed lysine residues when using EZ-Link Sulfo-NHS-LC-Biotin (Pierce) which is impermeable to membranes. Bacteriorhodopsin in membrane sheets is easily separated from the aqueous phase by centrifugation. For other membrane proteins that may be solubilized in detergent micelles removal of excess biotin solution will need to be accomplished using a desalting column or other means. Membrane proteins including bR may be expressed in a cell free manner and biotinylated (2, 13-16).

[0182] In particular, bacteriorhodopsin (bR) purchased from Sigma and stored as a lyophilized powder at 4° C. was resuspended in BupH PBS buffer in the original bottle. Amine containing buffers such as TBS, were avoided due to the interaction with the biotinylation reagent. The sample was bath sonicated eight times for 1 min. each chilling the bottle on ice for one minute in between each burst. UV-visible spectra were recorded to confirm the concentration of bR in solution using the molar extinction coefficient at 568 nm of 63,000 M-1-cm-1.

[0183] A freshly made 10 mM solution of EZ-Link Sulfo-NHS-LC-Biotin (Pierce) was prepared according to the manufactures recommendation in ddH2O.

[0184] The biotin solution was added to the bacteriorhodopsin solution in a 20-fold molar excess, and incubated on ice for two hours.

[0185] The excess biotin was removed by centrifugation of the solution in an ultracentrifuge at an RCF of 89,000 (although 50,000 should be sufficient) for 20 minutes at 4° C. The supernatant was removed and the bR pellet resuspended in TBS buffer. This process was repeated two times total. In particular, Bacteriorhodopsin in membrane sheets was extremely sticky, and did pellet well at the RCF listed. 85-90% recovery of bR was achieved with careful resuspension and washing of tips and tubes. Resuspension should be in the TBS buffer used for assembly (or other buffer of interest that will be used for assembly).

[0186] UV-visible spectra were collected as described above to calculate the concentration of the solution and the percent recovery typically around 85-90% with careful resuspension.

Example 10

Membrane Protein Incorporation into Nanolipoprotein Particles (MP-NLPs)

[0187] The methods described below outline incorporation of a membrane protein into nanolipoprotein particles (NLPs). The following materials and equipments were used: DMPC [1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine] (Avanti Polar Lipids); Purified apolipoprotein or truncation (ApoE4 22kD construct); TBS Buffer: 10 mM Tris-HCl; 0.15 M NaCl; 0.25 mM EDTA; 0.005% NaN_3 (sodium azide), adjusted to pH 7.4; Sodium Cholate (Sigma) 500 mM solution in TBS; Biotinylated Bacteriorhodopsin (bR) (Sigma) from Example 21; 30° C. and 20° C. and 23.8° C. water baths; Probe Sonicator; Dialysis cups 10,000 MWCO (Pierce) or D-Tube Dialyzers, mini (Novagen); Spin filter, 0.45 μm; FPLC Instrument (Shimadzu SCL-10A); size exclusion column (Superdex 200 10/300 GL (GE Healthcare Life Sciences); Concentrator 50 kD MWCO, Vivaspin 2 (Sartorius Inc.).

[0188] The water bath incubators were started at temperatures at 30° C. and 20° C. 34 mg of DMPC were probe sonicated into 1 mL of TBS at 6 amps for approximately 15 minutes or until optical clarity was achieved. Alternatively, the DPMC can be sonicated in bath sonicator to optical clarity (see Example 7).

[0189] The solution was centrifuged at 13K for 2 minutes to remove residual metal sloughed off from probe sonicator. 250 μL were batched in a 1.5 mL Eppendorf tube. Combine Apo E422K with DMPC in a ratio of 1:4 by mass in TBS buffer. Sodium cholate solution was then added to a final concentration of 20 mM. The biotinylated bacteriorhodopsin membrane protein was then added in a 0.67 mass ratio to the Apo E422k apolipoprotein.

[0190] The transition temperature procedure was performed as follows: the tube was immersed in water bath for 10 minutes each 30° C. (above DMPC transition temp.) followed by 20° C. (below DMPC transition temp.). The procedure was repeated three times and the tube was then incubated at 23.8° C. overnight.

[0191] The cholate detergent was removed and MP-NLPs (bR-NLPs) were allowed to self-assembly; the sample was loaded into a pre-soaked D-Tube Dialyzers, mini (Novagen). The sample was then dialyzed against 3 changes each of IL TBS buffer over a 2-3 day period at room temperature. In particular, dialysis at 4° C. was used for unstable membrane proteins. Detergent use was compatible with the membrane protein of interest. Adsorbent beads (Bio beads, Bio-Rad) were also used to remove the detergent. If dialysis cups were used (Pierce) the sample was split into three pre-soaked dialysis cups. Care was taken not to create bubbles or droplets on the sides of the cups.

[0192] The sample was then concentrated using an ultrafiltration device, Vivaspin 2 (Sartorius) MWCO 50K to 200 μL.

[0193] The supernatant was transferred into new tube. Size exclusion chromatography was performed using a Shimadzu SCL-10A FPLC, equipped with a Superdex 200 10/300 GL column (GE Healthcare Life Sciences). The buffer was TBS with a 200 μL sample injection volume, a 0.5 mL/min flow rate and 0.5 mL-1.0 mL fraction size.

[0194] The fractions of interest were concentrated using an ultrafiltration device, Vivaspin 2 (Sartorius) MWCO 50K for NLP peaks.

Example 11

Validating Nlp Formation by Native Gel Electrophoresis and Confirmation of Membrane Protein Association and Functionality with NLPs by Microarray, UV Visible Spectroscopy, AFM and EM

[0195] The methods described below outlines a procedure to validate protein association by microarray and UV visible spectroscopy. The following materials and equipments were used: 4-20% Tris-Glycine polyacrylamide gel, 1× Tris-Glycine native running buffer, 2× Native Sample buffer, Native Mark molecular weight marker (Invitrogen); Sypro Ruby Stain (Bio-Rad) light sensitive, Aqueous destain solution: 10% Methanol; 7% acetic acid; Fluoroimager with appropriate filter for SyproRuby stain; Biotinylated positive control protein such as biotinylated-bR; Bovine serum albumin 1 mg/ml solution; PBS-Tween buffer: 1.06 mM KH2PO4; 2.97 mM Na2HPO4; NaCl 1551.72 mM, 0.05% tween-20 (v/v) pH 7.4; 1×PBS buffer, (Gibco): 1.06 mM KH2PO4; 2.97 mM Na2HPO4; NaCl 1551.72 mM, pH 7.4; Cyanine-5-Strepavidin (Rockland) solution (5 μg/mL); Barcoded γ-Aminopropylsilane coated glass slides (GAPS-II; Corning); Robotic arrayer; Hybridization Chamber (Grace Bio-Labs); Blocking buffer: 1 mg/mL BSA in 1×PBS, Wash Buffer: 1×PBS; Laser-based confocal scanner (ScanArray 5000 XL; Perkin-Elmer); UV-visible plate reader (Bio-TEK Synergy HT); 96 well flat bottom UV plate (Corning Costar UV Plate).

Validation Through Native Polyacrylamide Gel Electrophoresis

[0196] Native polyacrylamide gel electrophoresis is used to validate the association of proteins of interest (apolipoprotein and/or membrane protein) with NLP fractions eluted from the size exclusion column. Protein identification is confirmed with mass spectrometry.

[0197] Native-PAGE gels, 4-20% Tris-glycine, were run with 0.75 μg total loaded protein estimated by A_{280} absorbance. 10 μL of molecular weight standards, Native mark (Invitrogen) diluted 20× in native sample buffer were loaded on the gels. The gels were run at 125V for approximately 2 hours.

[0198] The gels were stained with ~150 mL of SyproRuby protein stain (Bio-Rad) following the microwave staining method: 30 sec. microwave, 30 sec. mixing on shaker table, 30 sec microwave, 5 min. shake, 30 sec. microwave, finally 23 min. on shaker table at room temperature. The gels were destained for 1.5 hours on a shaker table at room temperature.

[0199] The gels were imaged using a Typhoon Imager with appropriate filters selected for the SyproRuby fluorescence.

[0200] The results are illustrated in FIG. **15**.

Confirmation of Membrane Protein Association and Functionality with NLPs by Microarray

[0201] Microarray spotting technology was used to attach NLPs to an amino-silane coated glass slide in an array format for streptavidin binding studies (17-19). Biotinylated bacteriorhodopsin (bR) was used to validate the incorporation of bR into nanolipoprotein particle fractions eluted from size exclusion chromatography. Cyanine-5-Strepavidin was used for fluorescence detection of biotinylated bacteriorhodopsin.

[0202] Microarray single print head was used to deposit approximately 1 mL of diluted protein solution on the slide. It was determined that robotic spotting is best when the humidity is greater than 30%. Proteins were spotted in 4×4 squares with 16 replicates of each sample, generating ~300 μm diameter spots with a spot-to-spot distance of 350 μm.

[0203] Protein microarrays were spotted on GAPSII amino silane glass slides (Corning) with bacteriorhodopsin bR (non-biotinylated), biotinylated-bR, biotinylated-bR-NLPs, using a robotic arrayer. Non-biotinylated bR was used as a negative control, and biotinylated-bR was used as a positive control.

[0204] Bacteriorhodopsin (bR) concentrations of 10 mM, as determined by UV-visible spectroscopy as described above were used for all samples.

[0205] Proteins were cross-linked to the glass slides by exposure to UV light for five minutes. Unused slides were stored at 4° C. without UV cross-linking.

[0206] The hybridization chamber was applied with a volume capacity of 950 μL to the slide carefully as to not disrupt the array. Carefully add reagents below without injecting bubbles.

[0207] The slides were blocked with BSA (1 mg/mL) for 30 minutes. The slides were washed with 1×PBS for 15 minutes. Cyanine-5-streptavidin (5 μg/mL) was bound for 15 minutes. The slides were washed in 1×PBS then nanopure water each for 15 min. The slides were dried by centrifugation or air dry.

[0208] Protein microarrays of bR, biotinylated-bR and bR-NLPs were imaged with a laser-based confocal scanner (ScanArray 5000 XL; Perkin Elmer) using the VheNe 594 nm laser for detection of any bound Cyanine-5-streptavidin.

[0209] Images were collected and analyzed using the mean pixel intensities with Scan Array software (Perkin Elmer) (data analysis not shown).

[0210] The results are illustrated in FIG. **16**.

Confirmation of Membrane Protein Association and Functionality with NLPs by UV-Visible Spectroscopy, AFM and EM

[0211] UV-visible spectroscopy of light and dark adapted bacteriorhodopsin can be used to determine the functionality of the protein and relates information regarding the conformation of the protein (4).

[0212] UV-visible spectra were collected in 96-well plate reader using 100 μL of sample in a UV detectable flat bottom plate. Dark adapted spectra were collected after keeping the sample wrapped in foil overnight taking care not to expose the sample prior to spectral collection. Light adapted spectra were collected after exposure to a full spectrum bright lamp for 15 min. The results are illustrated in FIG. **17**. A 5-10 nm visible shift between light and dark adapted spectra indicates a functional protein (4).

[0213] Further in-depth physical characterization of these particles was used to demonstrate functional protein insertion/association. Combined with the biochemical evidence methods such as Atomic force microscopy (AFM) and Electron microscopy (EM) addresses whether the end product of self-assembly/association was successful by determining physical parameters to identify insertion and localization of membrane proteins. Atomic force microscopy (AFM) (FIG. **18**), and Electron microscopy (FIG. **19**) although not fully described here, but are used to image the prepared discs and determine diameter and height measurements as well as sample heterogeneity.

Example 12

Membrane Protein Synthesis and Purification in a Single Step Using Cell-Free Synthesis in Conjunction with Pre-Formed Nanolipoprotein Particles (NLPs)

[0214] The methods described below allow membrane protein synthesis and determination of solubility in a single step using cell-free synthesis in conjunction with pre-formed nanolipoprotein particles (NLPs).

[0215] Cell-free expression of membrane proteins has usually employed either of two possible methods; one: expression and purification in a denatured state followed by refolding in the presence detergents and/or lipids as well as any cofactors such as all trans-retinal for bacteriorhodopsin or two: expression in the presence of detergents or lipids (2, 13-15). Solubilization of the membrane protein with detergent is generally followed by a dialysis step to return the membrane protein to a lipid bilayer vesicle. The method described here utilizes preformed NLPs as an additive to increase the membrane protein production, solubility and stabilization by incorporation into a NLP lipid bilayer (Co-translation). The procedure uses commercially available cell-free extracts with the addition of membrane protein plasmid DNA (pEXP4 expression vector (Invitrogen)), and pre-formed NLPs to synthesize folded functional membrane protein in one step.

[0216] Cloned membrane protein cDNAs of interest were into the expression plasmid pEXP4 (Invitrogen) and were propagated by transforming into Top10 or DH5α chemically competent cells (Invitrogen). Isolate plasmid DNA using a HiSpeed Plasmid Maxi or Midi Kits (Qiagen).

[0217] Cell-Free expression reactions were carried out using the Expressway™ Maxi Cell-Free *E. coli* Expression System (Invitrogen) protocols with the addition of 15 μg of membrane protein DNA, for a 1 mL reaction, 300 μg of purified NLPs (ApoE4 22k assembled with DMPC see above section). For scintillation counting the manufacturer protocol for the incorporation of 35S-Methionine was followed. Reactions were scalable to other volumes following the same ratios. Control experiments were carried out without the addition of NLPs using the same lysate batch.

[0218] The reactions were incubated at 37° C. shaking at 225 rpm in a shaker incubator (New Brunswick). The reactions were continued for 1.5-2 hours.

[0219] A 5 μL aliquot of the total (T) reaction was retained for SDS-PAGE and autoradiograms (not shown), the reaction was then centrifuged for 5 min. at 4° C., and 18000 RCF. The supernatant was collected and a 5 μL aliquot of the soluble (S) fraction placed into a 12×75 mm glass tube.

[0220] 100 μl of 1N NaOH was added and the resulting mixture was incubated at room temperature for 5 minutes. 2 ml of cold 10% TCA (trichloroacetic acid) were further added to the 12×75 mm tube. Place at 4° C. for 10 minutes.

[0221] The precipitate was collected via vacuum filtration through a Whatman GF/C glass fiber filter (or equivalent). The filter was pre-wetted with a small amount of 10% TCA prior to adding the sample.

[0222] The tube was rinsed twice with 1 ml of 10% TCA and then rinsed once with 3-5 ml of 95% ethanol. Each of the rinses was passed through the GF/C filter.

[0223] The filter was placed in a scintillation vial, aqueous scintillation cocktail was added, and counted in a scintillation counter. The cpm did reflect the amount of radiolabel that was incorporated.

[0224] FIG. **20** shows protein yield for the soluble (S) fraction based on scintillation counting of incorporated 35S-Methionine in the presence and absence of added NLPs.

[0225] In particular, in FIG. **20**, a comparison was made between the membrane protein expressed alone (Black bars) or in presence of pre-formed ApoE4 22k NLPs (Co-Translation) (Grey bars).

[0226] In all cases the expression in the presence of NLPs increased membrane protein solubility. Solubility is determined by removing a 5 ul of the reaction supernatant after a 10 minute centrifugation at 14000 rpm and determining yield by TCA precipitation and scintillation counting as described in section 3.6.

[0227] A survey of several membrane proteins with various numbers of transmembrane (TM) segments are expressed using this method. Solubility of the membrane protein is clearly increased in the presence of pre-formed NLPs indicating association with the NLP.

Example 13

Cell-Free Production of NLPs

[0228] In a further series of experiments performed following the approach outlined in Example 1 and illustrated in FIG. **1**, preparative reactions were carried out using the Invitrogen's Expressway Maxi kit and/or for comparison the RTS High Yield Kits as outlined below.

[0229] Basically, lyophilized reaction components (Lysate, Reaction Mix, Amino Acid Mix, Methionine) were dissolved in Reconstitution Buffer and combined as specified by the manufacturer. Then, 1-5 μg of each plasmid DNA were added and the reactions are incubated at 30 DC-37 DC for 14-24 h. Small-scale reactions, can make use of PCR products. This is especially convenient for conducting screening experiments in volumes as low as 2 μL. PCR products are quantified using

a fluorescence-based TpicoGreen assay Then 0.1 μg of linear template DNA is added to initiate the reaction, which is incubated at 30 DC for 4 h.

[0230] For expression screening, reactions were performed in 12-25 μL volumes and the resulting products were analyzed by immunoblotting or using a 96-sample format dot blot or array using previously described techniques adapted to NLP-GPCRs.

[0231] The DNA constructs to produce the scaffold proteins E422K, E22K, and apoLp-III from *B. mori* were provided. The ApoAI and MSPI (truncated form of ApoAI) were also cloned (see Table 3 of Example 15 below).

[0232] In general, the bacterial overexpression of these scaffold proteins was started by transferring 20 ml of a bacterial overnight culture into I LM9 minimal medium supplemented with 50 μg/l ampicillin. The expression was induced with 2 M isopropyl-thio-galactopyranoside (IPTG) at an OD600 nm of 0.55. Four hours later the bacteria were centrifuged (10 min, 4500 rpm, Beckman JA 10), the supernatant filtrated (0.8 urn) and subsequently concentrated to a volume of 2 ml by ultrafiltration through a 10 kDa membrane (Amicon). The concentrate was heated for 5 min at 100° C., centrifuged (15 min, 13,000 rpm, Eppendorf5415 C), and the supernatant was exchanged against 20 mM BisTris (pH 6.5) by 3-kDa ultrafiltration (Centriprep, Amicon). The prepared sample as applied onto a DEAE-Sepharose CL-6B anion exchange column (bed volume 20 ml, Sigma) connected with a Gradifrac-system (Pharmacia). Flow rate was I ml/min, and I.-ml fractions were collected. The late fractions of the flow-through containing apoLp-III were pooled, exchanged against physiological saline (172 mM KCI, 68 M NaCI, 5 mM NaHCO3, pH 6.1) and applied in a volume of 2 ml onto a gel filtration column (HiLoad 16/60, Superdex 75, Pharmacia) operated with an FPLC system (Pharmacia). Protein purity was checked by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

[0233] Lipid (20 mg) was weighed out and combined in a glass, round bottom tube. Chloroform (200 μl) was added to dissolve lipid. Chloroform is evaporated in a stream of nitrogen gas, rotating rapidly to distribute the lipid evenly. Samples are placed under vacuum for 30 min. to assure removal of solvent. The individual components are placed into an assembly solution with the appropriate ratios of lipid (800 μg), scaffold protein (200 μg), detergent (21 mM), creating a mass ratio of 4:I for lipid to protein and maintaining the cholate above the critical micellar concentration. The self-assembly process is started with 3 repeated sets of transition temperature incubations, bracketing the transition temperature of DMPC (23.80 C), by incubating at 30° C. for 10 minutes, then at 20° C. for 10 minutes with light hand mixing between incubations.

[0234] Following these transitions, the samples are incubated at 23.8° C. overnight. Following assembly, samples with cholate are dialyzed against 1OOO× volume of TBS buffer using 3 changes in 24 hrs. The NLPs are purified from 'free protein' and 'free lipid' by a VP HPLC (Shimadzu) with a Superdex 200 HR 10/30 column (GE healthcare), using TBS at a flow rate of 0.5 ml/min. The column was calibrated with four protein standards of known molecular weight and stokes diameter that span the separation range of the column and the NLP samples.

[0235] The void volume was established with blue dextran. The NLP fraction is concentrated about 10-fold to ~approximately 0.1 mg/ml using molecular weight sieve filters (Vivascience) having molecular weight cutoffs of 50 kDa. Protein concentration was determined using the ADVOI protein concentration kit (Cytoskeleton), which is based on Coomassie dye binding.

[0236] To performed Native PAGE validation, equal amounts of NLP samples (0.5-2 μg) were diluted with 2× native gel sample buffer (Invitrogen) and loaded onto 4-20% gradient pre-made Tris-glycine HCI gels (Invitrogen). Samples were electrophoresed for 250 V/hrs at a constant 125 V. After electrophoresis, gels are incubated with Sypro Ruby for 2 hours and then de-stained using 10% MeOH, 7% Acetic acid. Following a brief wash with ddH20, gels are imaged using the green laser (532 nm) of a Typhoon 9410 (GE Healthcare) with a 610 nm bandpass 30 filter.

[0237] Molecular weights were determined by comparing migration vs. log molecular weight of standard proteins found in the NativeMark standard (Invitrogen). The Stokes diameter of the NLPs is calculated from the known Stokes diameter of the same proteins in the standard sample.

Example 14

Characterization of NLPs Produced by Cell-Free Systems

Solubilization of the bR-NLP Complex

[0238] The experimental approach described in Example 13 was applied to obtain cell free expression for single step production and refolding of the membrane protein bacteriorhodopsin (bR) from *Halobacterium alinarium*, which serves as model protein for G-protein coupled receptors (GPCRs), an important membrane protein family.

[0239] Cell-free expression of bR in the presence of NLPs is shown in FIGS. **21** and **22**. Preliminary results show that cell-free synthesis of bR in the presence of NLPs yields nanoparticles containing bR, i.e. NLP-bR.

[0240] Additionally, these NLP-bR constructs are functional as assessed by UV/Vis spectrometry (data not shown). The bR protein is only purple when co-factors are present to allow proper protein folding. The figure also demonstrates that the bR protein is incorporated within the NLP following size exclusion chromatography to purify and isolate the complex (FIG. **21**, Panel B). The bR containing NLPs were also assayed using a Western microarray format as preliminary development of rapid fluorescent screening techniques (FIG. **21**, Panel C) and by Atomic Force Microscopy, which showed an increase in particle height (FIG. **22**). Interestingly, co-expression in RTS or Expressway) cell-free reactions of an apolipoprotein (scaffold protein) with two other membrane protein targets showed significantly enhanced soluble expression levels (FIG. **22**). These results, suggesting similar incorporation of membrane protein into NLPs, support the claim of a single step reaction process yielding soluble NLP/membrane protein constructs. These results therefore establish cell-free co-protein expression as a viable expression strategy for membrane proteins in general, which comprise a significant fraction of any genome and are notoriously difficult to isolate and characterize.

Example 15

apoE422K and apoLp-III Protein Production

[0241] Apolipoproteins apo E422K and apoLp-111 where selected from the ones illustrated in Table 3 below.

TABLE 3

Apolipoprotein	Mol. Wt.	Lipid	Cholate	Key REFs
ApoAI	28.1 kDa	DMPC	No	Jonas et al., 1980[8]
MSP1T2 (Δ1-55 ApoAI)	24.8 kDa	DMPC	No	Sligar et al., 2005[9]; Denisov et al., 2005[10]
ApoE422K	22.3 kDa	DMPC	No	Lu et al, 2000[11]
ApoLp-III	18 kDa	DMPC	No	Weintzek, M. et al. 1994[12]; Weers and Ryan, 2003[13]
ApoAI	28.1 kDa	DMPC	Yes	Jonas et al., 1980[8]
MSP1T2 (Δ1-55 ApoAI)	24.8 kDa	DMPC	Yes	Shaw et al., 2004[14], Bayburt et al., 2006[15]
ApoE422K	22.3 kDa	DMPC	Yes	This work
ApoLp-III	18 kDa	DMPC	Yes	Garda HA et al., 2002[16]
Cy3-apoE422K	22.3 kDa	DMPC	Yes &No	This work
Cy3-apoE422K	22.3 kDa	DMPC + 1% NBD-DMPC	Yes &No	This work

[0242] The expression clone to produce apoE422K, the N-terminal 22 kDa fragment of apolipoprotein E4 (apoE4), as a 6His and thyrodoxin tagged construct was kindly provided by Dr. Karl Weisgraber. ApoE422K was over-expressed and in *E. coli* as previously reported. Pelletted *E. coli* cells expressing apoE422K were re-suspended in lysis buffer (50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole, pH 8.0) and lysed with an Emulsiflex-05 homogenizer (Avestin Inc., Ottawa, Canada) at 4° C. Following centrifugation, the clarified supernatant was first partially purified by nickel affinity chromatography using a 5 ml His Trap FF crude nickel column (GE Healthcare) on an Akta FPLC (GE Healthcare) then further purified with a 320 ml Superdex 75 HiLoad 26/60 column (GE Healthcare) using TBS running buffer (10 mM Tris, pH 7.4, 0.15 M sodium chloride, 0.25 mM EDTA, 0.005% sodium azide) giving one predominant peak.

[0243] The collected material was cleaved with bovine α-Thrombin (Haematologic Technologies) 1/500 enzyme/protein for 1 hour at 37° C. Resulting products were separated by SEC on a 320 ml Superdex 75 HiLoad 26/60 FPLC column with one column volume of TBS. Protein fractions were analyzed by SDS-PAGE gels stained with Sypro Ruby (Bio-Rad), gels were imaged with a Typhooon 9410 (GE Healthcare). Relative purity of the proteins was determined to be greater than 95% by densitometry and overall yields are on the order of 6 mg/L bacterial culture.

[0244] The *B. mori* apoLp-111 expression clone was a kind gift from Dr. Rob Ryan. ApoLp-III was over-expressed in *E. coli* as described. The protein was expressed with a PEL leader sequence, targeting the protein to the periplasm, where the leader sequence is cleaved and protein secreted in to the media. Expression was induced for four hours, bacteria were pelleted and the supernatant was collected, filtered (0.8 μm), and subsequently concentrated to a volume of ~20 ml using a Vivaflow 200 (Sartorius) with a 5-kDa MW cutoff PES membrane. The concentrated protein was exchanged against 20 mM Tris pH 8.0 over a HiPrep 26/10 desalting column on an Akta FPLC (GE Healthcare). The protein was then purified to homogeneity by HPLC (Shimadzu) using a ProPac WAX-10 column (Dionex) and eluted as follows: 0-100% gradient between 20 mM Tris pH 8.0 and 20 mM Tris pH 8.0 with 0.5M NaCl. Fractions showing highest protein content by A_{280} were pooled.

[0245] Protein purity was checked by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectroscopy analysis. The protein was >90% pure by gel electrophoresis, MS analysis gave expected molecular ion peaks; overall yield was 40%.

[0246] Fluorescently labeled apoE422K was obtained by using a Cy3 labeling kit and following the manufacturer's instructions (GE Healthcare). Dye:protein ratio was determined by comparing the absorbance of the protein at 280 nm and the absorbance of the CyDye at 532 nm. The ratio provided a 1:1 correlation suggesting that a single Cy3 molecule is present on each apoE422K protein.

Example 16

Nanolipoprotein Particle (NLP) Formation Through Self-Assembly of Lipids and Apolipoproteins

[0247] In order to better understand the self-assembly process and the range of attributes of NLPs extensive comparison of particles from a number of self-assembly conditions was performed, using four different apoliporoteins, and a battery of characterization techniques, was applied. In particular NLPs from each of the four apolipoproteins apoA-I, Δ-apoA-I fragment, apoE4 fragment, and apolipophorin III (apoLp-III) assembled and characterized in combination with DMPC, with and without cholate, with and without fluorescent labels on the apolipoprotein and DMPC molecules

[0248] Phospholipids (DMPC and NBD-DMPC) were purchased from Avanti Polar Lipids. Inc (Alabaster, Ala.). Full-length apoA-I was purchased from Fitzgerald, Inc. (Concord, Mass.), apoA-I, Δ 1-55 (MSP1T2) and Nanodisc™ particles were purchased from Nanodisc, Inc., (Urbana, Ill.). The latter particles were made from DMPC and apoA-I, Δ1-55 protein fragment (MSP1T2); this fragment has a modified NH 2-terminus containing a His tag and a tobacco etch virus (TEV) cleavage site.

[0249] FIG. **23** schematically shows the assembly process while Table 4 details the NLP preparations undertaken in this example. Individual reactants are combined, mixed and subjected to a series of temperature transitions before overnight incubation. NLPs are separated from the reaction mixture by chromatography, concentrated and characterized.

[0250] DMPC (20 mg) is weighed out, added to a glass, round bottom tube followed by chloroform (200 μl) to dis-

solve lipid. Chloroform is evaporated in a stream of nitrogen with constant rotation to distribute the lipid evenly along the tube wall and placed under vacuum overnight. DMPC is either re-suspended in TBS with probe sonication or with TBS/cholate and gentle vortexing; the final concentration of cholate (20 mM) is above its critical micellar concentration (CMC). Apolipoproteins (200-250 μg) are added to the TBS/DMPC solution+/−cholate at a mass ratio of 4:1 for apoE422K and 3:1 of apoLp-III. The particle formation process is started with 3 repeated sets of transition temperature incubations, above and below the transition temperature of DMPC (23.8° C.), i.e. 10 minutes at 30° C., then 10 minutes at 20° C., with light hand mixing between incubations. After 3 heating and cooling transitions, the samples are incubated at 23.8° C. overnight.

[0251] Following assembly, samples containing cholate are dialyzed against 1000× volume of TBS buffer using 3 changes in 24 hrs. The NLPs are purified from 'free protein' and 'free lipid' by size-exclusion chromatography (VP HPLC, Shimadzu) using a Superdex 200 HR 10/30 column (GE Healthcare), in TBS at a flow rate of 0.5 ml/min. The column was calibrated with four protein standards of known molecular weight and Stokes diameter that span the separation range of the column and the NLP samples. The void volume was established with Blue dextran. The NLP fractions are concentrated to approximately 0.1 mg/ml using molecular weight sieve filters (Vivascience) with molecular weight cut-offs of 50 kDa. Protein concentration was determined using the ADVOI protein concentration kit (Cytoskeleton, Inc.).

Example 17

NLPs Purification from Free Lipid and Free Protein Starting Reactants

[0252] Comparison of Size Exclusion Chromatography (SEC) traces from NLP assemblies illustrated in FIG. **24** provides insight on particle molecular size and homogeneity. In particular the chromatogram of the ApoE422K-, Lipophorin III-, ApoAI-, and MSP1T2-derived NLPs shows one to three peaks at A280 nm. The NLP peak eluted as the predominant peak in the chromatogram, well separated from lipid-rich and lipid-poor fractions and was isolated for further analysis. The larger E422K and apoLpIII peaks are eluted at about 21 min, while the smaller ApoAI derived NLPs elute at about 26 min. Molecular size information from SEC is shown in Table 4 below.

[0253] ApoE422K and apoLp-III derived NLPs eluted a few minutes after the void volume, whereas the two apoA1-based NLPs eluted 3-4 minutes after the others. These data suggest larger particles are formed from apoE422K and lipophorin apolipoproteins versus particles derived from apoA-I proteins. The SEC profiles of apoE422K and lipophorin-NLPs were quite similar eluting in nearly the same position showing a diameter of ~14-15 nm. Each had a small 'free lipid' peak and a larger 'free protein' peak surrounding the single predominant NLP peak; this elution pattern for E422K is similar to previous results (20). Interestingly, altering the lipid:protein ratio for apoLp-111 assembled NLPs enhanced the NLP peak, while diminishing the free component peaks consistent with previous, work (21). When cholate was used to solubilize lipid films deposited by chloroform evaporation, the 'free lipid' peak is diminished or completely disappears suggesting that altering lipid:protein ratio affects apparent yield of lipophorin-based NLPs (data not shown).

Example 18

NLPs' Size and Heterogeneities Associated with Individual Apolipoproteins

[0254] Equal amounts of NLP samples (0.5-2 μg) are diluted with 2× native gel sample buffer (Invitrogen) and loaded onto 4-20% gradient pre-made Tris-HCl gels (Invitrogen). Samples are electrophoresed for 250 Vhrs at a constant 125V. After electrophoresis, gels are incubated with Sypro Ruby for 2 hours and then destained using 10% MeOH, 7% Acetic acid. Following a brief wash with ddH_2O, gels are imaged using a Typhoon 9410 (GE Healthcare) at 532 nm (green laser) with a 610 nm bandpass 30 filter. Molecular weights are determined by comparing migration vs. log molecular weight of standard proteins found in the Native-Mark standard (Invitrogen). The Stokes diameter of the NLPs is calculated from the known Stokes diameter of the same proteins in the standard sample.

[0255] The results illustrated in FIG. **25** show predominant single bands<700 kDa for E422K-NLPs (A) and apoLp-111 NLPs (B). The apoAI-derived (Fitzgerald, Inc.) NLPs show five major species when full-length ApoAI (C) or MSP1T2 (D) proteins (Nanodisc, Inc.) are used for making NLPs. Panel E shows the commercially available NLP sample from Nanodisc, Inc. Native gels of NLP fractions obtained from cholate containing preparations were qualitatively similar to those shown here. Each gel was stained with Sypro RUBY which has a wider dynamic range than Coomassie stain and is more sensitive; it is possible that, some protein species may not be detected.

[0256] Accordingly, native gel electrophoresis reveals (FIG. **25**) that apoE422K and apoLp-111 NLPs appear predominantly as single bands. On the contrary NLP preparations using apoAI and MSP1T2 (Δ1-55 apoAI) show multiple bands. Five bands on the gel corresponding to putative NLPs were observed using purchased MSP1T2; the three larger molecular weight bands constitute less than 10% of the total protein amount. Overloading apoE422K NLPs on native gels show minor larger molecular weight bands; AFM analysis of the latter show NLP species of larger diameter. These larger species likely do not affect size characterization shown in Table 4. Our MSP1T2-derived NLPs averaged 260 kDa, consistent with the molecular weight obtained from purchased MSP1T2-based 'nanodiscs', of 255 kDa. The Stokes diameter of all NLP assemblies was determined by migration comparison to protein standards with known Stokes diameters and shown in Table 4. The calculated Stokes diameter of the apoAI-derived NLPs was approximately 11 nm, while the apoE422K- and apoLp-III-derived NLPs showed around 13 nm diameters. The apoLpIII-NLPs were slightly larger by native PAGE when compared to apoE422K-NLPs; this observation is not consistent with the SEC data that suggested apoE422K-derived particles were larger. This discrepancy might be due to differences in protein shape, charge and/or bound DMPC molecules. Size of NLPs determined by SEC and native PAGE are based on calibration standards used for soluble proteins. As such, these standards may not be appropriate for calibrating lipid-containing NLPs by native PAGE.

Example 19

NLPs Characterization by Mass Spectrometry and Ion Mobility Spectrometry (IMS)

[0257] In addition to using previously reported analytical methods for examining NLP, ion mobility spectrometry

(IMS) was also used, a very sensitive and precise technique for measuring particle size Mass determination was performed using Bruker APEX II 9.4 T FTICR mass spectrometer through a homebuilt nanospray interface on an Apollo (Bruker Daltonics, Billerica, Mass., USA) ESI source. Protein solution concentrations were 1-10 μM or 1 nM in 10 mM ammonium acetate, pH 7.5. Solutions were desalted and concentrated by centrifugal filtration using Microcon or Amicon Ultra-4 filters (Millipore, Bedford, Mass.).

[0258] The aerodynamic diameter of NLPs was determined with a Macroion Mobility Spectrometer (Model 3890, TSI Inc., Shoreview, Minn.). The details of the instrumentation and a method for measuring protein sizes have been described elsewhere (4,5). Interestingly, this method has been used to measure the size distribution for HDL, LDL and VLDL taken directly from serum (6). Briefly, the instrument consists of an electrospray ionization source with a charge-neutralizing chamber, a differential mobility analyzer (DMA) and a condensation particle counter (CPC). Multiply charged droplets generated by electrospray are charge-reduced by interaction with air ions formed by α-radiation (^{210}Po). NLP samples are exchanged via dialysis (3× buffer exchange) into a volatile buffer and then pumped into the electrospray source at 100 mL/min. These conditions were chosen so primary electrospray droplets contain, on average, less than one individual NLP in 25 mM ammonium acetate. The droplets ultimately evaporate, leaving individual NLPs in the gas phase carrying, predominantly, a single charge (7). Charged NLPs pass through a scanning differential mobility analyzer and are counted by a condensation particle counter. The size distribution of a population of NLPs is determined from the scanning parameters; mobility measurements are used to infer NLP mean aerodynamic diameter.

[0259] FIG. **26** shows differential ion mobility spectra for four representative NLP preparations. MSP1T2 scaffold without cholate, apoLp-III scaffold with cholate, apoLp-III scaffold without cholate, and apoE422K without cholate. The centroid and full width at half maximum (FWHM) of the highest abundance peak within a trace is used to represent the average mean aerodynamic diameter of the particles within a sample. The lower abundance peaks at ~17 and 19 nm in the apoLp-III and ApoE422K traces are respectively likely due to slightly larger particles of lower abundance that are not detected by native gel. The MSP1T2-NLPs appear significantly smaller than the apoLp-III and apoE422K-NLPs while the addition of cholate during formation of NLPs utilizing apoLp-III as the scaffold has no significant effect on the ion mobility trace and the average mean aerodynamic particle diameter. Ion mobility traces of mean aerodynamic diameter size distributions for the other NLP preparations shown in Table 4 were qualitatively and quantitatively similar to those shown here.

[0260] Together with the ion mobility data summarized in Table 4 below, the spectra illustrated in FIG. **27** indicate that IMS can resolve size differences in NLP arising from the use of differing apolipoproteins. Moreover, these spectra also illustrate that, for a given apolipoprotein, cholate addition and removal does not alter particle size. The full-width half maximum of the predominant peak in each spectrum is similar suggesting that, at least for the predominant IMS peak, NLP size heterogeneity may not be strongly dependent on choice of scaffold protein. Heterogeneity observed in the native gel electrophoresis data for apoA1 and MSP1T2 preparations is not reflected in the ion mobility FWHM data. This likely arises as different bands on a gel correspond to different peak diameters within an IMS spectrum and consequently, the IMS FWHM data are only assessing the heterogeneity within a single gel band.

Example 20

NLPs Characterization by Transmission Electron Microscopy (TMS) and Atomic Force Microscopy (AFM)

[0261] Samples were diluted using TBS to achieve a final concentration of 0.02 mg/ml. Three μl of each sample was pipetted onto a carbon coated 400 mesh copper EM grid (Ted Pella). After sitting for 1 minute, the sample was blotted with Whatman filter paper. Three μl of 2% Uranyl Acetate (Electron Microscopy Sciences) was applied for one minute and then blotted. Grids were dried for 30 minutes before use in the EM. Negative stain images were recorded using a Philips CM300 FEG transmission electron microscope operating at an accelerating voltage of 300 keV. Images were recorded as 8-bit and 16-bit tiff at varying magnifications onto a Gatan digital CCD and stored as jpegs and Gatan image format files. Images were then analyzed using Gatan Digital Micrograph software.

[0262] Micrographs in FIG. **27** show NLPs whose dimensions are consistent with previously described observations (see Table 4 below). In particular, the micrographs of FIG. **27** show a negative stain TEM and AFM of apoE422K-NLP preparations with and without cholate. For TEM, each sample was stained with a 2% solution of uranyl acetate, as described herein. Samples for AFM were measured in solution, using non-contact mode. Both electron micrographs were taken at 65,000× magnification, panel B shows sample prepared with cholate. Panels C and D show 400×400 nm topographical AFM images of apo4E22K-NLPs having similar shape and height; they were prepared without cholate (C) or with cholate (D). The scale bar represents 50 nm. A color bar scale identifies NLP height and insets show zoomed in regions (50×50 nm), showing single particles. A section line trace below each image show both the typical heights and diameters in the slow scan direction for the respective AFM images, Panel E (no cholate) and F (cholate). The average height for NLPs with cholate is 4.8 nm+/−0.2 nm and the heights of NLPs without cholate 4.8 nm+/−0.3 nm, which is consistent with the theoretical size of the lipid bilayer. The data suggest discoidal structures with a diameter of about 10-20 nm and height consistent with the thickness of a bilayer.

[0263] Two of these assemblies made from apoE422K and DMPC are shown, with (panel B) and without cholate (panel A). The lower right-hand corner of each panel shows a region at higher magnification to highlight the presence of discoidal structures. Cholate has no effect on the size and structure of apoE422K-derived NLPs. Like previous reports, images of stacked particles were observed—described as "rouleaux"—but not in all samples. Others have described these formations as artifacts of sample preparation and concentration (22).

[0264] Atomically flat Muscovite mica disks were glued to metal substrates to secure them to the scanner of a stand-alone MFP-3D AFM (Asylum Research, Santa Barbara, Calif.). 2 uL of solution was incubated for two minutes on the mica surface in imaging buffer (10 mM MgCl2, 10 mM Tris-HCL, and 0.1 M NaCl, adjusted to pH 8.0) and then lightly rinsed. The AFM has a closed loop in the x, y, and z axes. The

topographical images were obtained with "Biolevers" (Olympus, Tokyo, Japan) with a spring constant of 0.03 N/m. Images were taken in alternate contact (AC) mode in liquid, with amplitudes below 20 nm and an amplitude setpoint at 50% tapping amplitude. Scan rates were below 1.5 Hz. Height, amplitude, and phase images were recorded. Heights of features in images were determined by histogram analysis of contiguous particles. Experiments were carried out in a temperature controlled room at 23+/−1° C.

[0265] All four apolipoprotein assemblies show a common discoidal bilayer structure. In particular, it was observed that NLPs made from different apolipoproteins examined by AFM showed discrete bracketed structures even at high concentration, indicative of individual particles. When DMPC without apolipoprotein is examined, planar fusible features, ~4-5 nm in thickness are observed consistent with the presence of a lipid bilayer (data not shown). Also, when apolipoprotein is examined alone, globular features on the order of 2-3 nm are seen. NLPs have diameters ranging from 10 to 20 nm and heights of approximately 5 nm; these observations are consistent with diameters measured by other techniques described above. Particle size and structure is unaffected by cholate as shown in FIG. **27** with apoE422K-derived NLPs. These AFM data indicate that the particles are just less than 5 nm high with diameters of ~20 nm. This diameter size is larger than was derived from TEM, but AFM is known to increase the size of x, y resolution due to tip convolution effects. Combined, AFM and TEM data suggests discoidal structures with height dimensions consistent with a phospholipid bilayer and a diameter of about 10-20 nm; cholate addition during assembly does not appreciably change the heights of the apoE422K and apoLp-III assemblies (see Table 4).

Example 21

Monitoring NLPs Assembly Process by Fluorescent Labeling

[0266] FIG. **24** shows analyses of a labeled NLP using a Cy3-labeled apoE422K mixed with 7-nitrobenz-2-oxa-1,3-diazol-4-yl labeled DMPC (DMPC-NBD).

[0267] In the analysis illustrated in FIG. **28** fractions collected from SEC were characterized by native PAGE (panel C), AFM (panel D, right) and TEM (panel D, left). Importantly, it appears that labeled NLP reactants do not affect NLP formation. The SEC trace (panel A) shows similar lipid-rich, NLP, and lipid-poor peaks that co-elute with those species in non-fluorescent samples. The SEC fractions were analyzed for fluorescence (panel B) and the fluorescent lipid (green pseudo color), fluorescent protein (red pseudo color), and NLPs (yellow pseudo color) show up in the expected vials based on the SEC trace. The results illustrated in FIG. **28** indicate that fluorescently-labeled NLPs show similar structure and size compared to unlabeled NLPs.

[0268] Table 4 shows the size characteristics of NLPs made using fluorescently-labeled reactants do not appreciably change from the unlabeled reactants. Moreover, the size and shape are maintained as observed AFM and EM analyses show similar discoidal structures (panel D). These data suggest that fluorescent dye attachment to lipid and protein reagents can be used to track NLP assembly as well as provide means to detect individual reactants within the particle.

Example 22

NLPs Combined Characterization

[0269] Table 4 summarizes results from combined characterization approaches and highlights particle size parameters of NLPs assembled from each of four apolipoproteins in combination with a single phospholipid, dimyristoylphosphatidylcholine (DMPC). Apoliprotein can assume three different forms: fully extended, doubled-back/"hairpin", and semi-extended/"double-hairpin" folds as illustrated in FIG. **29**.

[0270] Reaction of each protein with DMPC yields NLPs with unique overall structural/shape characteristics. In general, particles produced were found to be discoidal in shape with diameters ranging from 10-20 nm dependent on the apolipoprotein or derivative used in assembly; a height of ~5 nm was determined for all NLP preparations, consistent with a membrane bilayer formed by DMPC (23).

[0271] The fundamental observations are that the apolipoprotein is the primary determinant of NLP size and that a discoidal shape was consistent among the four assemblies. These characterization results, irrespective of the method or apolipoprotein used, show remarkable consistency in measuring overall NLP size and shape for any given apolipoprotein. Moreover, measured sizes and shapes did not differ appreciably when formed in the presence of cholate and when using fluorophore labeled reactants. The following sections summarize results from each of the specific characterization techniques.

TABLE 4

			Native Gel		SEC				
Apolipo-protein	Lipid	Cholate	Mol. Wt. (kDa)	Stokes D (nm)	Mol. Wt. (kDa)	Stokes D (nm)	Ion Mobility AMAD ± FWHM	AFM (nm) Height	TEM (nm) Diameter
Nanodiscs (MSP1T2)	100% DMPC	No	290 ± 10	10.8 ± 0.1	190 ± 15	9.3 ± 0.3	10.6 ± 1.4	6.1 ± 0.2	10.2 ± 3.1
apoAI	100% DMPC	No	360 ± 10	11.4 ± 0.1	270 ± 30	12.6 ± 0.4	10.5 ± 1.1	4.3 ± 0.5	13.0 ± 1.4
MSP1T2 (ΔapoAI)	100% DMPC	No	260 ± 30	10.1 ± 0.8	300 ± 120	12.8 ± 1.4	9.5 ± 0.9	4.8 ± 0.2	12.7 ± 3.0
apoE422K	100% DMPC	No	505 ± 60	12.6 ± 0.3	560 ± 15	15.1 ± 0.1	13.2 ± 0.9	4.9 ± 0.2	17.6 ± 2.7
apoLp-III	100% DMPC	No	620 ± 60	12.8 ± 0.4	480 ± 25	14.5 ± 0.2	13.1 ± 0.7	4.4 ± 0.3	17.6 ± 2.6
apoE422K	100% DMPC	Yes	680 ± 40	13.2 ± 0.1	600 ± 15	15.3 ± 0.1	13.6 ± 1.0	4.9 ± 0.3	16.2 ± 2.4
apoLp-III	100% DMPC	Yes	530 ± 10	12.6 ± 0.1	425 ± 12	14.1 ± 0.5	13.1 ± 0.7	4.0 ± 0.4	18.0 ± 2.6
Cy3-apoE422K	100% DMPC	Yes &No	510 ± 40	12.4 ± 0.4	660 ± 10	15.1 ± 0.1	14.0 ± 1.5	4.6 ± 0.3	20.8 ± 2.9
Cy3-apoE422K	100% DMPC (1% NBD)	Yes &No	630 ± 70	13.0 ± 0.2	670 ± 15	15.1 ± 0.1	14.1 ± 1.5	5.1 ± 0.3	17.4 ± 2.7

[0272] Table 4 illustrates the results of the physical characterization of NLPs by native gel electrophoresis, SEC, Ion mobility spectrometry, AFM and negative stain TEM performed according to Examples 1 to 7. Molecular weights and Stokes diameters of the NLPs from native gels and SEC were determined using known protein standards and are shown in kDa and nm, respectively. The average mean aerodynamic diameter (AMAD) corresponds to the centroid and full width at half maximum (FWHM) of the most abundance peak within an ion mobility trace. The centroid provides a robust measurement of the average mean aerodynamic diameter of the particles within a sample while the FWHM provides a comparative estimate of sample heterogeneity. AFM derived measurements of height and TEM derived measurements of diameter are reported as the mean+/−standard deviation of individual measurements from typically 100 NLPs within a sample. ApoAI and MSP1T2 assembled NLPs were noticeably smaller than E422K and apoLp-III assembled NLPs, ranging in size from 10-13 nm in diameter as compared to 12-20 nm in diameter for the E and apoLp-III assemblies. Cholate addition during assembly did not appear to appreciably change the size of any of the structures. The addition of fluorescently labeled assembly components also had little affect on the molecular size, but likely affects the homogeneity of the assembled structures since fluorescent components were unlikely to be uniformly distributed throughout the NLP population. Characterization data using purchased empty Nanodiscs™ are also shown for comparative purposes.

[0273] Additional experiments and related results concerning monitoring of NLP assembly and related characterization are illustrated in FIGS. **30** to **32**.

[0274] The system exemplified in the preceding examples is useful to monitor/indicate successful in situ cell-free protein synthesis of membrane proteins, in general and their incorporation into NLPs. Preceding examples although performed with indicator bR are also indicative of the application of the methods and systems herein disclosed with other such indicator proteins that could be used for in situ monitoring of cell-free membrane protein synthesis with concomitant NLP formation; these include, but are not limited to, sesory rhodopsin, proteorhodopsin and phytochromes.

[0275] Like bR, these proteins can be produced by the cell-free transcription/translation technology in the presence of detergents, lipids and phospholipids as well as other reaction additives and couples to NLP formation.

[0276] The examples set forth above are provided to give those of ordinary skill in the art a complete disclosure and description of how to make and use the embodiments of the devices, systems and methods of the disclosure, and are not intended to limit the scope of what the inventors regard as their disclosure. Modifications of the above-described modes for carrying out the disclosure that are obvious to persons of skill in the art are intended to be within the scope of the following claims. All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the disclosure pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

[0277] The entire disclosure of each document cited (including patents, patent applications, journal articles, abstracts, laboratory manuals, books, or other disclosures) in the Background, Summary, Detailed Description, and Examples is hereby incorporated herein by reference. Further, the hard copy of the sequence listing submitted herewith and the corresponding computer readable form are both incorporated herein by reference in their entireties.

[0278] It is to be understood that the disclosures are not limited to particular compositions or biological systems, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise. The term "plurality" includes two or more referents unless the content clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosure pertains.

[0279] Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the specific examples of appropriate materials and methods are described herein.

[0280] A number of embodiments of the disclosure have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the present disclosure. Accordingly, other embodiments are within the scope of the following claims.

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Val Gln Glu Glu Leu Leu Ser Ser Gln Val Thr Gln Glu Leu Arg Ala
50                  55                  60

Leu Met Asp Glu Thr Met Lys Glu Leu Lys Ala Tyr Lys Ser Glu Leu
65                  70                  75                  80

Glu Glu Gln Leu Thr Pro Val Ala Glu Glu Thr Arg Ala Arg Leu Ser
85                  90                  95

Lys Glu Leu Gln Ala Ala Gln Ala Arg Leu Gly Ala Asp Met Glu Asp
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Lys Leu Arg Lys Arg Leu Leu Arg Asp Ala Asp Asp Leu Gln Lys Arg
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Ser Ala Ile Arg Glu Arg Leu Gly Pro Leu Val Glu Gln Gly Arg Val
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Arg
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What is claimed is:

1. A method for monitoring production of a target protein in a nanolipoprotein particle,

the nanolipoprotein particle comprising the target protein, a membrane forming lipid and a scaffold protein,

the target protein capable of assuming a target protein active form and a target protein inactive form,

the method comprising:

providing an indicator protein, the indicator protein capable of assuming an indicator protein active form and an indicator protein inactive form, the indicator protein active form associated to an indicator protein detectable activity, the indicator protein detectable activity associated to the target protein active form;

contacting the indicator protein with the target protein, the membrane forming lipid and the scaffold protein for a time and under conditions to allow assembly of the indicator protein, the target protein, the membrane forming lipid and the scaffold protein in the nanolipoprotein particle; and

detecting the indicator protein detectable activity from the nanolipoprotein particle.

2. The method of claim **1**, wherein the target protein is a membrane protein and the membrane forming lipid is selected from the group consisting of phospholipids, sphingolipids, glycolipids, ether lipids, sterols and alkylphosphocholins.

3. The method of claim **1**, wherein the target protein is selected from the group consisting of a protein coupled receptor (GPCR), an ion channel protein (IC) and a small multidrug resistance transporter (SMR).

4. The method of claim **1**, wherein the target protein is selected from the group consisting of V2R, CRF, ETB, MC5R, NTR1, 5HT1A, H2, M1, herg, α1AR, β1AR, OP1R, β2AR and M2.

5. The method of claim **1**, wherein the indicator protein is structurally related to the target protein so that the production of the indicator protein in an active form can be associated with the production of the target protein in an active form.

6. The method of claim **1** wherein the indicator protein is selected from the group consisting of GFP, GFP-fused to a membrane protein, cytochromes and dye labeled proteins.

7. The method of claim **1**, wherein the indicator protein is selected from the group consisting of sensory rhodopsin, proteorhodopsin, and phytochromes.

8. The method of claim **1**, wherein the indicator protein is bacteriorhodopsin.

9. The method of claim **1**, wherein detecting the indicator protein detectable activity is performed by

providing a labeled molecule that specifically binds to the indicator protein the labeled molecule providing a labeling signal;

contacting the labeled molecule with the nanolipoprotein particle for a time and under condition to allow binding of the labeled molecule with the indicator protein in the nanolipoprotein particle; and

detecting the labeling signal from the labeled molecule bound to the indicator protein in the nanolipoprotein particle.

10. The method of claim **9**, wherein the labeled molecule is selected from the group consisting of radioactive isotopes, chemiluminescent dyes, fluorophores, chromophores, enzymes, enzymes substrates, enzyme cofactor, enzyme inhibitors, dyes, metal ions, nanoparticles, metal ions and ligands.

11. A method for monitoring production of a target protein in a nanolipoprotein particle,

the nanolipoprotein particle comprising the target protein a membrane forming lipid and a scaffold protein,

the target protein capable of assuming a target protein active form and a target protein inactive form,

the method comprising:

providing a first polynucleotide encoding for the target protein;

providing a second polynucleotide encoding for an indicator protein, the indicator protein capable of assuming an indicator protein active form and an indicator protein inactive form, the indicator protein active form associated to an indicator protein detectable activity, the indicator protein detectable activity associated to the target protein active form;

contacting the first and second polynucleotides with the membrane forming lipid and the scaffold protein for a time and under conditions to allow assembly of the indicator protein, the target protein, the membrane forming lipid and the scaffold protein in the nanolipoprotein particle; and

detecting the indicator protein detectable activity from the nanolipoprotein particle.

12. The method of claim **11**, wherein contacting the first and second polynucleotide with the membrane forming lipid and the scaffold protein is performed in a single reaction mixture.

13. The method of claim **11**, wherein the target protein is a membrane protein and the membrane forming lipid is selected from the group consisting of phospholipids, sphingolipids, glycolipids, ether lipids, sterols and alkylphosphocholins.

14. The method of claim **11**, wherein the target protein is selected from the group consisting of a protein coupled receptor (GPCR), an ion channel protein (IC) and a small multidrug resistance transporter (SMR).

15. The method of claim **11**, wherein at least one of the first and the second polynucleotide is an engineered polynucleotide encoding for a chimeric product.

16. The method of claim **11**, wherein the target protein is selected from the group consisting of V2R, CRF, ETB, MC5R, NTR1, 5HT1A, H2, M1, herg, α1AR, β1AR, OP1R, β2AR and M2.

17. The method of claim **11**, wherein the indicator protein is structurally related to the target protein of interest so that the production of the indicator protein in an active form can be associated with the production of the target protein in an active form.

18. The method of claim **11** wherein the indicator protein is selected from the group consisting of GFP, GFP-fused to a membrane protein, cytochromes and dye labeled proteins.

19. The method of claim **11**, wherein the indicator protein is selected from the group consisting of sensory rhodopsin, proteorhodopsin, and phytochromes.

20. The method of claim **11**, wherein the indicator protein is bacteriorhodopsin.

21. The method of claim **11**, wherein detecting the indicator protein detectable activity is performed by

providing a labeled molecule that specifically binds to the indicator protein the labeled molecule providing a labeling signal;

contacting the labeled molecule with the nanolipoprotein particle for a time and under condition to allow binding of the labeled molecule with the indicator protein in the nanolipoprotein particle; and

detecting the labeling signal from the labeled molecule bound to the indicator protein in the nanolipoprotein particle.

22. The method of claim **21**, wherein the labeled molecule is selected from the group consisting of radioactive isotopes, chemiluminescent dyes, fluorophores, chromophores, enzymes, enzymes substrates, enzyme cofactor, enzyme inhibitors, dyes, metal ions, nanoparticles, metal ions and ligands.

23. A method for monitoring production of a target protein in a nanolipoprotein particle, the nanolipoprotein particle comprising the target protein, a membrane forming lipid and a scaffold protein,
the target protein capable of assuming a target protein active form and a target protein inactive form,
the method comprising:
providing a first polynucleotide encoding for the target protein;
providing a second polynucleotide encoding for an indicator protein, the indicator protein capable of assuming an indicator protein active form and an indicator protein inactive form, the indicator protein active form associated to an indicator protein detectable activity, the indicator protein detectable activity associated to the target protein active form;
providing a third polynucleotide encoding for the scaffold protein;
contacting the first, second and third polynucleotides with the membrane forming lipid and the scaffold protein for a time and under conditions to allow assembly of the indicator protein, the target protein, the membrane forming lipid and the scaffold protein in the nanolipoprotein particle; and
detecting the indicator protein detectable activity from the nanolipoprotein particle.

24. The method of claim **23**, wherein contacting the first, second and third polynucleotided with the membrane forming lipid and the scaffold protein is performed in a single reaction mixture.

25. The method of claim **23**, wherein the target protein is a membrane protein and the membrane forming lipid is selected from the group consisting of phospholipids, sphingolipids, glycolipids, ether lipids, sterols and alkylphosphocholins.

26. The method of claim **23**, wherein the target protein is selected from the group consisting of a protein coupled receptor (GPCR), an ion channel protein (IC) and a small multidrug resistance transporter (SMR).

27. The method of claim **23**, wherein at least one of the first, the second polynucleotide and the third polynucleotide is an engineered polynucleotide encoding for a chimeric product.

28. The method of claim **23**, wherein the target protein is selected from the group consisting of V2R, CRF, ETB, MC5R, NTR1, 5HT1A, H2, M1, herg, α1AR, β1AR, OP1R, β2AR and M2.

29. The method of claim **23**, wherein the indicator protein is structurally related to the target protein of interest so that the production of the indicator protein in an active form can be associated with the production of the target protein in an active form.

30. The method of claim **23** wherein the indicator protein is selected from the group consisting of GFP, GFP-fused to a membrane protein, cytochromes and dye labeled proteins.

31. The method of claim **23**, wherein the indicator protein is selected from the group consisting of sensory rhodopsin, proteorhodopsin, and phytochromes.

32. The method of claim **23**, wherein the indicator protein is bacteriorhodopsin.

33. The method of claim **23**, wherein detecting the indicator protein detectable activity is performed by
providing a labeled molecule that specifically binds to the indicator protein the labeled molecule providing a labeling signal;
contacting the labeled molecule with the nanolipoprotein particle for a time and under condition to allow binding of the labeled molecule with the indicator protein in the nanolipoprotein particle; and
detecting the labeling signal from the labeled molecule bound to the indicator protein in the nanolipoprotein particle.

34. The method of claim **33**, wherein the labeled molecule is selected from the group consisting of radioactive isotopes, chemiluminescent dyes, fluorophores, chromophores, enzymes, enzymes substrates, enzyme cofactor, enzyme inhibitors, dyes, metal ions, nanoparticles, metal ions and ligands.

35. A system for monitoring production of a target protein in a nanolipoprotein particle,
the nanolipoprotein particle comprising the target protein, a membrane forming lipid and a scaffold protein,
the target protein capable of assuming a target protein active form and a target protein inactive form,
the system comprising:
an indicator protein capable of assuming an indicator protein active form and an indicator protein inactive form, the indicator protein active form associated to an indicator protein detectable activity, the indicator protein detectable activity associated to the target protein active form, and
at least one of the target protein, the membrane forming lipid and the scaffold protein.

36. The system of claim **35**, wherein the target protein is a membrane protein and the membrane forming lipid is selected from the group consisting of phospholipids, sphingolipids, glycolipids, ether lipids, sterols and alkylphosphocholins.

37. The system of claim **35**, wherein the target protein is selected from the group consisting of a protein coupled receptor (GPCR), an ion channel protein (IC) or a small multidrug resistance transporter (SMR).

38. The system of claim **35**, wherein the target protein is selected from the group consisting of V2R, CRF, ETB, MC5R, NTR1, 5HT1A, H2, M1, herg, α1AR, β1AR, OP1R, β2AR and M2.

39. The system of claim **35**, wherein the indicator protein is structurally related to the target protein of interest so that the production of the indicator protein in an active form can be associated with the production of the target protein in an active form.

40. The system of claim **35**, wherein the indicator protein is selected from the group consisting of GFP, GFP-fused to a membrane protein, cytochromes and dye labeled proteins.

41. The system of claim **35**, wherein the indicator protein is selected from the group consisting of sensory rhodopsin, proteorhodopsin, and phytochromes.

42. The system of claim **35**, wherein the indicator protein is bacteriorhodopsin.

43. The system of claim **35**, further comprising a labeled molecule that specifically binds to the indicator protein, the labeled molecule providing a labeling signal.

44. The system of claim **43**, wherein the labeled molecule is selected from the group consisting of radioactive isotopes, chemioluminescent dyes, fluorophores, chromophores, enzymes, enzymes substrates, enzyme cofactor, enzyme inhibitors, dyes, metal ions, nanoparticles, metal ions and ligands.

45. A system for monitoring production of a target protein in a nanolipoprotein particle,

the nanolipoprotein particle comprising the target protein, a membrane forming lipid and a scaffold protein,

the target protein capable of assuming a target protein active form and a target protein inactive form,

the system comprising:

a first polynucleotide encoding for the target protein; and

a second polynucleotide encoding for an indicator protein, the indicator protein capable of assuming an indicator protein active form and an indicator protein inactive form, the indicator protein active form associated to an indicator protein detectable activity, the indicator protein detectable activity associated to the target protein active form.

46. The system of claim **45**, wherein the target protein is a membrane protein and the membrane forming lipid is selected from the group consisting of phospholipids, sphingolipids, glycolipids, ether lipids, sterols and alkylphosphocholins.

47. The system of claim **45**, wherein the target protein is selected from the group consisting of a protein coupled receptor (GPCR), an ion channel protein (IC) or a small multidrug resistance transporter (SMR).

48. The system of claim **45**, wherein the target protein is selected from the group consisting of V2R, CRF, ETB, MC5R, NTR1, 5HT1A, H2, M1, herg, α1AR, β1AR, OP1R, β2AR and M2.

49. The system of claim **45**, wherein the indicator protein is structurally related to the target protein of interest so that the production of the indicator protein in an active form can be associated with the production of the target protein in an active form.

50. The system of claim **45**, wherein the indicator protein is selected from the group consisting of GFP, GFP-fused to a membrane protein, cytochromes and dye labeled proteins.

51. The system of claim **45**, wherein the indicator protein is selected from the group consisting of sensory rhodopsin, proteorhodopsin, and phytochromes.

52. The system of claim **45**, wherein the indicator protein is bacteriorhodopsin.

53. The system of claim **45**, further comprising a labeled molecule that specifically binds to the indicator protein, the labeled molecule providing a labeling signal.

54. The system of claim **53**, wherein the labeled molecule is selected from the group consisting of radioactive isotopes, chemiluminescent dyes, fluorophores, chromophores, enzymes, enzymes substrates, enzyme cofactor, enzyme inhibitors, dyes, metal ions, nanoparticles, metal ions and ligands.

55. A system for monitoring production of a target protein in a nanolipoprotein particle,

the nanolipoprotein particle comprising the target protein, a membrane forming lipid and a scaffold protein,

the target protein capable of assuming a target protein active form and a target protein inactive form,

the system comprising:

a first polynucleotide encoding for the target protein;

a second polynucleotide encoding for an indicator protein, the indicator protein capable of assuming an indicator protein active form and an indicator protein inactive form, the indicator protein active form associated to an indicator protein detectable activity, the indicator protein detectable activity associated to the target protein active form; and

a third polynucleotide encoding for the scaffold protein.

56. The system of claim **55**, wherein the target protein is a membrane protein and the membrane forming lipid is selected from the group consisting of phospholipids, sphingolipids, glycolipids, ether lipids, sterols and alkylphosphocholins.

57. The system of claim **55**, wherein the target protein is selected from the group consisting of a protein coupled receptor (GPCR), an ion channel protein (IC) or a small multidrug resistance transporter (SMR).

58. The system of claim **55**, wherein the target protein is selected from the group consisting of V2R, CRF, ETB, MC5R, NTR1, 5HT1A, H2, M1, herg, α1AR, β1AR, OP1R, β2AR and M2.

59. The system of claim **55**, wherein the indicator protein is structurally related to the target protein of interest so that the production of the indicator protein in an active form can be associated with the production of the target protein in an active form.

60. The system of claim **55**, wherein the indicator protein is selected from the group consisting of GFP, GFP-fused to a membrane protein, cytochromes and dye labeled proteins.

61. The system of claim **55**, wherein the indicator protein is selected from the group consisting of sensory rhodopsin, proteorhodopsin, and phytochromes.

62. The system of claim **55**, wherein the indicator protein is bacteriorhodopsin.

63. The system of claim **55**, further comprising a labeled molecule that specifically binds to the indicator protein, the labeled molecule providing a labeling signal.

64. The system of claim **63**, wherein the labeled molecule is selected from the group consisting of radioactive isotopes, chemiluminescent dyes, fluorophores, chromophores, enzymes, enzymes substrates, enzyme cofactor, enzyme inhibitors, dyes, metal ions, nanoparticles, metal ions and ligands.

* * * * *