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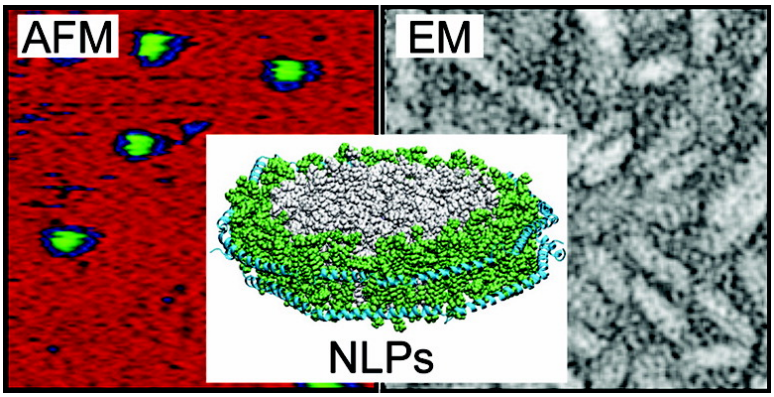

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Abstract: Spontaneous interaction of purified apolipoproteins and phospholipids results in formation of lipoprotein particles with nanometer-sized dimensions; we refer to these assemblies as nanolipoprotein particles or NLPs. These bilayer constructs can serve as suitable mimetics of biological membranes and are fully soluble in aqueous environments. We made NLPs from dimyristoylphospatidylcholine (DMPC) in combination with each of four different apolipoproteins: apoA-I, Δ-apoA-I fragment, apoE4 fragment, and apolipophorin III (apoLp-III) from the silk moth *B. mori.* Predominately discoidal in shape, these particles have diameters between 10 and 20 nm, share uniform heights between 4.5 and 5 nm, and can be produced in yields ranging between 40 and 60%. The particular lipoprotein, the lipid to lipoprotein ratio, and the assembly parameters determine the size and homogeneity of nanolipoprotein particles and indicate that apoA-I NLP preparations are smaller than the larger apoE422K and apoLp-III NLP preparations.

Introduction

The assembly and function of lipoprotein particles have been studied since they were first isolated from human plasma in the 1920s.[1] In the past 20−30 years, attention has focused on plasma lipoproteins and the correlation between cholesterol levels and coronary artery disease (CAD) of the heart, especially the inverse correlation between the concentration of high-density lipoprotein (HDL) and CAD.[2] The latter health concern has led to molecular level investigations of protein−lipid interaction, including detailed studies focusing on reconstitution of HDL-like particles. Apolipoproteins (A-I, E, and C) have been shown to associate with phospholipids *in vitro*, spontaneously self-assembling into discoidal-shaped complexes similar to nascent HDL particles produced by the liver.[3−6] More recently, Sligar and co-workers have reported that a number of recombinantly derived apolipoprotein AI (apoA-I) variants self-assemble in the presence of phospholipid, forming discoidal particles, which they call nanodiscs.[7,8] These NLPs were based on a series of recombinant apoA-I proteins, some larger than the native protein and some smaller; all were reported to form nanodisc-like structures.[9] Working with recombinant apolipoprotein E variants, most notably an N-terminal 22 kDa fragment, apoE422K, Weisgraber and co-workers also report the formation of discoidal particles.[10,11] Two independent groups have also reported similar type particles being formed using lipophorins, a class of lipoproteins from insect flight muscles, combined with phospholipids.[12,13]

To date, only limited characterization of in vitro reconstituted NLPs has been reported. Size-exclusion chromatography (SEC) and native polyacrylamide electrophoresis (PAGE) have been the predominant techniques used to determine the molecular size and relative homogeneity of these structures. Transmission electron microscopy (TEM) and atomic force microscopy (AFM) have also been used to examine shape features in some studies.[14] In an effort to better understand the self-assembly process and the range of attributes of NLPs, we have undertaken an extensive comparison of particles from a number of self-assembly conditions, using four different apoliporoteins, and applied a battery of characterization techniques. We have assembled and characterized NLPs from each of the four apolipoproteins in combination with dimyristoylphospatidyl-

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Table 1. Physical Characterization of NLPs by Native Gel Electrophoresis, SEC, Ion Mobility Spectrometry, AFM, and Negative Stain TEM[a]

			native gel		SEC			AFM	TEM
apolipoprotein	lipid	cholate	mol wt (kDa)	Stokes *D* (nm)	mol wt (kDa)	Stokes *D* (nm)	ion mobility AMAD ± fwhm	height (nm)	diameter (nm)
Nanodiscs (MSP1T2)	100% DMPC	no	290 ± 10	10.8 ± 0.1	190 ± 15	9.3 ± 0.3	10.6 ± 1.4	5.1 ± 0.2	10.2 ± 3.1
ApoA-I	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 (ΔApoA-I)	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	605 ± 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 and 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 and 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

[a] 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 abundant 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. ApoA-I and MSP1T2 assembled NLPs were noticeably smaller than E422K and apoLp-III assembled NLPs, ranging in size from 10 to 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 effect on the molecular size but likely effects 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.

choline (DMPC), with and without cholate, with and without fluorescent labels on the apolipoprotein and DMPC molecules.

In addition to using the four previously reported analytical methods for examining NLPs, we also used ion mobility spectrometry (IMS), a very sensitive and precise technique for measuring particle size.[15] The combination of different apolipoproteins used for assembly followed by extensive characterization of particles for size and shape represents a platform for which further research can follow to characterize membranes, apolipoproteins, and integral membrane proteins. This work presents the first study showing single particle characterization of these structures using multiple apolipoproteins for assembly.

Experimental Section

Materials and methods are shown in the Supporting Information.

Results

Nanolipoprotein Particle (NLP) Formation. Table 1 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). Reaction of each protein with DMPC yields NLPs with unique overall structural/shape characteristics. In general, the particles produced were found to be discoidal in shape with diameters ranging from 10 to 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.[16] Our 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,

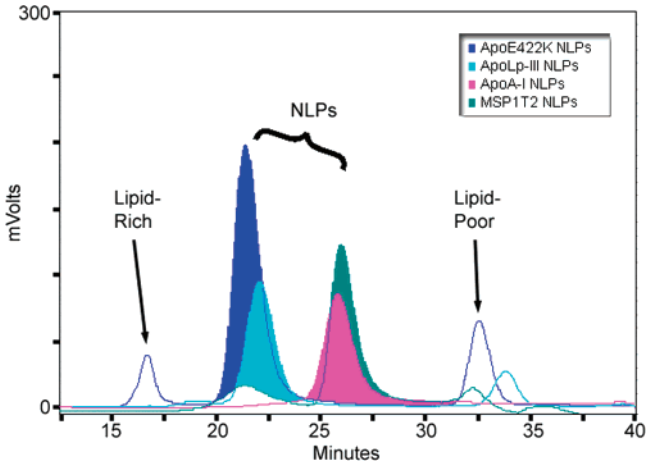

Figure 1. Size exclusion chromatography of NLP assembly from a typical reaction mixture. The chromatogram of the apoE422K-, LipophorinIII-, apoA-I-, and MSP1T2-derived NLPs shows one to three peaks at 280 nm. The NLP peak eluted as the predominant peak in the chromatogram, well separated from lipid-rich 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 apoA-I derived NLPs elute at about 26 min. Molecular size information from SEC is shown in Table 1.

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.

NLPs can be purified from free lipid and free protein starting reactants. Comparison of SEC traces from NLP assemblies (Figure 1) provides insight on particle molecular size and homogeneity. ApoE422K and apoLp-III derived NLPs eluted a few minutes after the void volume, whereas the two apoA-I-based NLPs eluted 3−4 min 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 apoE422K is

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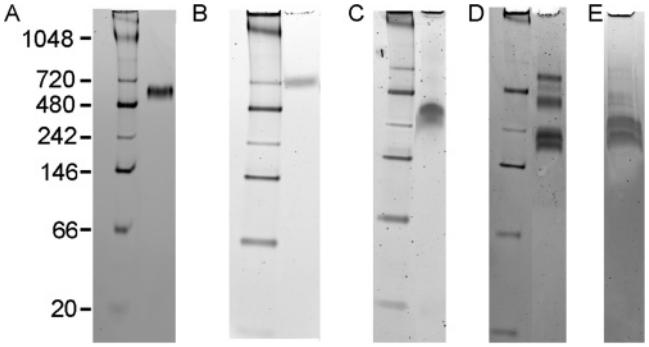

Figure 2. Sypro RUBY-stained native PAGE shows molecular size and relative homogeneity of NLPs prepared from four different apolipoproteins without cholate. Predominant single bands <700 kDa are shown for E422K-NLPs (A) and apoLp-III NLPs (B). The apoA-I-derived (Fitzgerald, Inc.) NLPs show five major species when full-length apoA-I (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.

similar to previous results.[10] Interestingly, altering the lipid/protein ratio for apoLp-III assembled NLPs enhanced the NLP peak, while diminishing the free component peaks consistent with previous work.[17] When cholate was used to solubilize lipid films deposited by chloroform evaporation (see Supporting Information, Experimental Section), the "free lipid" peak is diminished or completely disappears suggesting that altering the lipid/protein ratio affects apparent yield of lipophorin-based NLPs (data not shown).

Individual apolipoprotein assemblies give rise to different sizes and heterogeneities of NLPs. Native gel electrophoresis reveals (Figure 2) that apoE422K and apoLp-III NLPs appear predominantly as single bands; NLP preparations using apoA-I and MSP1T2 (Δ1−55 apoA-I) 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 1. 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 1. The calculated Stokes diameter of the apoA-I-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 is based on calibration standards used for soluble proteins. As such, these standards may not be appropriate for calibrating lipid-containing NLPs by native PAGE.

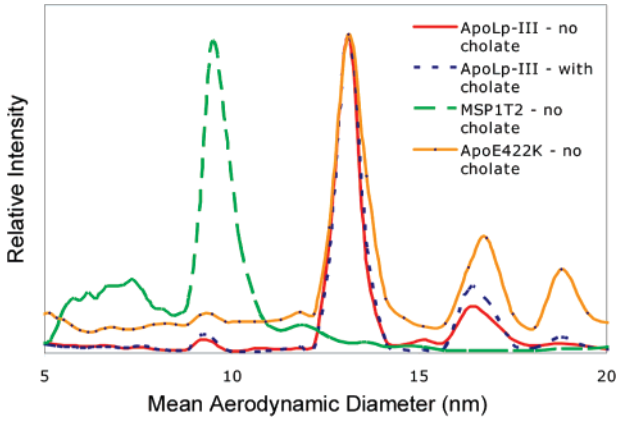

Figure 3. Ion mobility traces of mean aerodynamic diameter size distributions for four NLP preparations shown in Table 1: MSP1T2 scaffold without cholate, apoLp-III scaffold with cholate, apoLp-III scaffold without cholate, and apoE422K without cholate. The centroid and full width at half-maximum (fwhm) 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 1 were qualitatively and quantitatively similar to those shown here.

Ion Mobility Spectrometry (IMS) shows unique structural signatures for different NLPs. Figure 3 shows differential ion mobility spectra for four representative NLP preparations. Together with the ion mobility data summarized in Table 1, these spectra show that IMS can resolve size differences in NLPs arising from the use of differing apolipoproteins. Moreover, these spectra also illustrate that, for a given apolipoprotein, cholate addition and removal do not alter particle size. The full widths at half-maximum of the predominant peak in each spectrum are similar suggesting that, at least for the predominant IMS peak, NLP size heterogeneity may not be strongly dependent on the choice of apolipoprotein 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 which 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.

All four apolipoprotein assemblies show discoidal shape by transmission electron microscopy (TEM). Micrographs in Figure 4 show NLPs whose dimensions are consistent with previously described observations (see Table 1). 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, we too have observed images of stacked particles, described as "rouleaux", but not in all samples. Others have described these formations as artifacts of sample preparation and concentration.[18]

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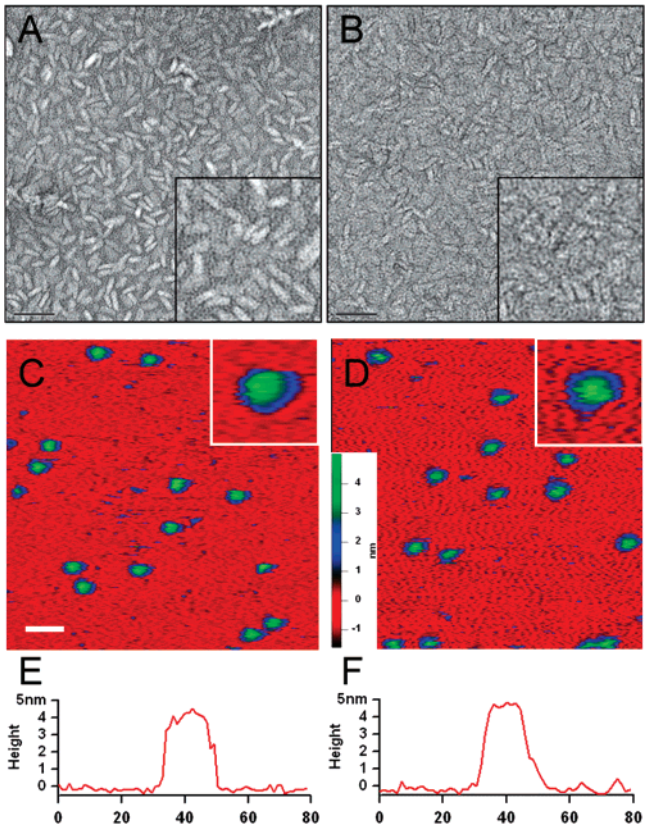

Figure 4. Negative stain TEM and AFM of apoE422K-NLP preparations with and without cholate. For TEM, each sample was stained with a 2% solution of uranyl acetate, as described in the Experimental Section. Both electron micrographs were taken at 65 000× magnification. Panel B shows sample prepared with cholate. The scale bar in both panels is 50 nm; insets are at a higher magnification. Samples for AFM were measured in solution, using a noncontact mode. 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 shows both the typical heights and diameters in the slow scan direction for the respective AFM images, panels E (no cholate) and F (cholate). The average height for NLPs with cholate is 4.8 nm ± 0.2 nm, and the height of NLPs without cholate, 4.8 nm ± 0.3 nm, which is consistent with the theoretical size of the lipid bilayer. These data suggest discoidal structures with a diameter of about 10−20 nm and a height consistent with the thickness of a bilayer.

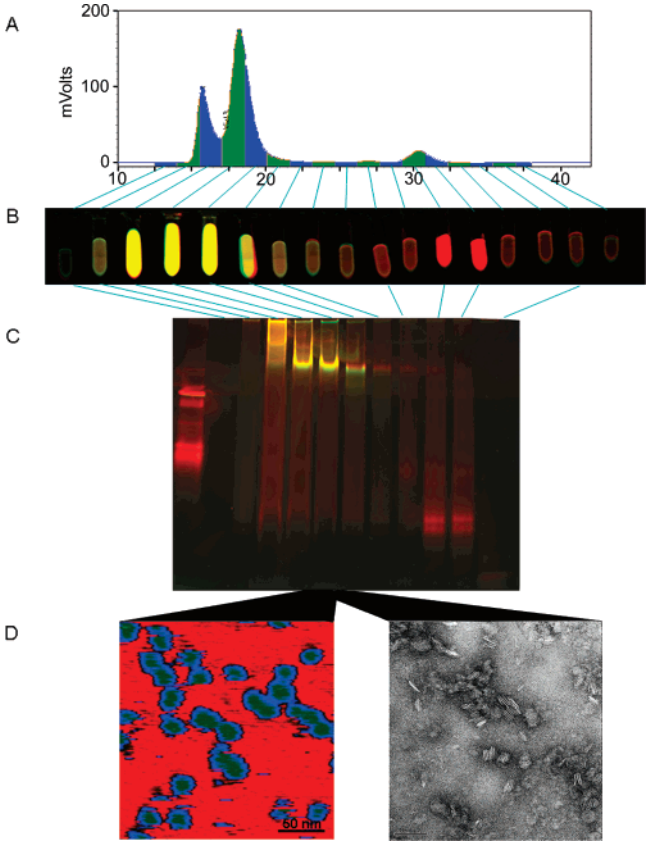

Figure 5. Characterization of fluorescently labeled NLPs show similar structure and size compared to those of unlabeled NLPs. Cy3-labeled apoE422K (50%) and DMPC containing 1% NBD-DMPC were used to form labeled NLPs. Panel A shows the SEC fractionation of labeled NLPs. Early eluting peaks correspond to large DMPC vesicles and NLP fractions, and the later eluting peak contains unreacted apoE422K. Panel B shows a fluorescent scan of SEC fractions that contain NBD-DMPC (green), Cy3-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.

All four apolipoprotein assemblies show a common discoidal bilayer structure. 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 are unaffected by cholate as shown in Figure 4 with apoE422K-derived NLPs. These AFM data indicate that the particles are just under 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 suggest 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 1).

Fluorescent labeling enables monitoring of the assembly process. Figure 5 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). 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 coelute with those species in nonfluorescent 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. Table 1 shows that the size characteristics of NLPs made using fluorescently labeled reactants do not appreciably change from those using the unlabeled reactants. Moreover, the size and shape are maintained as observed from AFM and EM analyses showing 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.

Discussion

We have shown that nanolipoprotein particles form using DMPC with one of four apolipoproteins producing assemblies that are uniquely distinguishable by size. While many factors contribute to NLP formation, three issues are prominent: the purity and preparation of NLP components, the lipid/protein ratio used in the assembly process, and the particular method used for assembly. When lower purity apoE422K and apoLp-III proteins were used for assembly, both yield and homogeneity were compromised. Besides protein purity, the lipid composition resulting from the solubilization protocol has an effect on assembly. The chloroform deposited TBS/cholate rehydrated film method allows more lipid to be available for the assembly process, providing a lower "free" lipid (or "lipid-rich") peak observed during SEC purification; alternatively "free" lipid is present in higher levels when sonication is used. Although protein and lipid purity may affect the kinetics of assembly, the purity of the starting materials will also affect the effective lipid/protein ratio during assembly. This ratio also plays a role in assembly, especially in terms of overall yield of NLP formation. Finally, the assembly conditions can greatly affect the final NLP sample. If cholate dialysis methods are employed, along with the major NLP species, smaller structures are seen by SEC and native PAGE (data not shown), containing both apolipoprotein and lipid. Importantly, however, the size and shape are identical for the main peak found in cholate dialysis assemblies (see Table 1), suggesting only yield is affected due to the presence of smaller and larger particles in these assemblies. Further work is ongoing to optimize the cholate procedure to fully characterize these other structures.

Nanometer-sized membrane bilayer mimetics have been an interest to nanobiotechnology for some time as a way to replicate or mimic high-density lipoproteins for the study of this important class of biomolecules. Since NLPs self-assemble they represent an attractive platform for studying lipoproteins, lipid bilayers, and biomimetic membranes. Notably, Sligar and co-workers have made significant progress in using recombinant apolipoproteins that act as scaffold proteins for lipid bilayers[19,20] suggesting the choice of apolipoprotein defines the size of nanodiscs. NLPs and recombinant apolipoprotein scaffold proteins used for NLP production developed by Sligar and coworkers can now be purchased from Nanodisc Inc. They have produced a number of recombinant apolipoproteins that act as scaffold proteins for lipid bilayers,[9] suggesting the choice of apolipoprotein defines the size of nanodiscs. Table 1 indicates that NLPs purchased from Nanodisc Inc. and NLPs formed from MSP1T2 purchased from Nanodisc Inc. both appeared to form discoidal-like particles with a height of ~5 nm (consistent with a bilayer) and diameter of ~10 to 11 nm which compares favorably with earlier observations.[9,20] Interestingly, native gel electrophoresis (Figure 2) indicated the MSP1T2 appeared to consist of up to five distinct molecular species which potentially could result in some heterogeneity in the size of NLPs formed from this scaffold protein. Moreover, the current report shows the characterization of single particles for each of these discs. Other earlier studies were not able to examine single particle characterization and used slightly different MSP apolipoproteins for assembly. The molecular size of the apoE422K assemblies (16−18 nm diameter) is larger than the 13.4 nm diameter obtained by Jonas and co-workers for full-length E and egg PC[21] but agrees well with previous work from Weisgraber and colleagues, which showed diameters of 16.7 ± 1.9 nm using an optimized lipid/protein ratio of 4:1.[10] Only a few studies on the composition of discoidal lipoproteins of apoLp-III exist, but these reports agree well with the shape and molecular size of the current work,[13] including the locust apoLp-III complexed with DMPC using the cholate dialysis method which was found to be 20.7 nm discs,[22] similar to the ~18 nm discs assembled here. Two additional studies concluded that the lipoproteins contain five[23] or six molecules of apoLp-III[17] per discoidal particle, suggesting similar stoichiometry for our assemblies. Moreover, the particles with six apoLp-III per disc used protein from the same organism as the one used here and gave similar results by EM and native PAGE: 18.5 ± 2.0 nm by EM and 642 kDa by native gel (17.8 ± 3.3 nm and 620 kDa here).[17] The reported diameter for Δ apoA-I assemblies is similar to ours but may differ due to the fact that the MSP1T2 purchased for this study is different than previously reported MSP (MSP1[9]). The molecular size discrepancy found using SEC may result from an average of the five species found in the gel and the Stokes diameter determined for SEC is not linear.[24] These facts suggest the molecular weight and Stokes diameter from native gels may be the more appropriate choice for comparing sizes. Although substantial alterations in the lipid to apolipoprotein ratio was used in this study and greatly affected the yield of NLPs, the single particle characterization did not change appreciably over several-fold ratios. Previous work with all of these apolipoproteins has been successful with different ratios of lipid to protein, and further investigation of this assembly parameter is needed for additional characterization such as the determination of the number of apolipoproteins and lipid molecules per NLP. Work with different apolipoproteins than the ones used here show even higher ratios of lipid may be necessary for ideal assemblies, such as for apoB[25] and apoC.[26] Discoidal particles formed with DMPC/apoB-17, 7:1 (w/w), are 23.9 ± 4.3 nm in diameter and contain ~2 molecules of apoB-17 and 2250 molecules of DMPC per disc.[25] Similarly, those formed with DMPC/apoC, 5:1 (wt/wt), are 16.9 ± 2.7 nm.[26]

The diversity of apolipoproteins has not been fully captured here, although the four different apolipoprotein choices, including those from insects and humans, cover a diverse set of proteins that expand the utility of these structures for nanobiotechnology. For example, new peptides might be synthesized that better mimic the intermolecular interactions between the aliphatic nature of the apolipoprotein molecules and the

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hydrocarbon chains of the lipid bilayer.[27] It is clear that other lipids such as DPPC and POPC can be used,[7] but the single particle comprehensive characterization has not been completed for these molecules. Clearly, changes in the apolipoprotein and lipid bilayer compositions should greatly increase the flexibility in choosing proteins and lipids to improve stability and usefulness for the particular application. When considering the incorporation of integral membrane proteins into the bilayer, these parameters take on greater importance. For example, the lipid content solubilized with different detergents at different concentrations can influence membrane stability and function.[28] Ideally, the choice of detergent, apolipoprotein, lipid, assembly ratio, and assembly conditions that best suits a particular integral membrane protein desired for incorporation will be attainable following detailed studies such as this current work. Further optimization of these will continue to improve assembly yields. As an example, Figure 1 shows SEC traces containing "free lipid" and "free protein" peaks, suggesting that the NLP formation might be able to fully utilize the assembly components. The presence of these additional peaks indicate that a more efficient assembly based on altering the lipid/protein ratio or some other factor may be necessary.

Our results have implications for diverse biotechnology applications. Membrane-associated proteins and protein complexes account for 30% or more of the proteins produced by a cell. These complexes mediate essential cellular processes such as signal transduction, transport, recognition, bioenergetics, and cell−cell signaling. As virtually all host-pathogen interactions are mediated through cell surface membrane associated proteins, they are the key to understanding detection, pathogenicity, and countermeasures. Despite their importance, membrane proteins have remained challenging to study because of their marked insolubility and tendency to aggregate/precipitate when removed from their hydrophobic protein lipid bilayer environment. Techniques and methods for isolation, purification, and analysis developed with soluble proteins are not generally transferable or applicable to membrane-associated proteins and protein complexes. We are addressing these problems by focusing on emerging methods for functional reconstitution of membrane proteins in phospholipid/protein (lipoprotein) nanostructures or NLPs. We are exploring NLP preparation methods for eventual use as a platform for stabilizing functional membrane proteins. The NLPs described herein have been characterized using a suite of advanced, analytical methods. NLPs will enable capture and presentation of cell surface protein features associated with known biothreat organisms and potentially could aid in detection of emerging biothreats. NLPs have recently been used to clarify the aggregation state of G-protein coupled receptors (GPCRs) that is necessary for G-protein activation.[29] GPCRs represent 60% of pharmaceutical targets for drug discovery. These results in combination with Sligar and co-workers' successful incorporation of membrane proteins into bilayer mimetics suggest a general approach enabling structure/function studies of this class of proteins.

The strengths of this work involve the in-depth physical characterization of these particles using different apolipoproteins that heretofore has not been addressed. Five separate analytical techniques showed similar size and structural characterization. SEC, native PAGE, and IMS provided very similar size measurements for each of the NLPs. Two structural techniques showed that the NLPs had similar sizes and shapes. The work in this manuscript expands upon the current understanding of these structures and shows their composition to be more heterogeneous than once thought. Current work in this field simply addresses whether the end product of self-assembly was successful. We suggest future methods need to be refined and improvements made to drive the self-assembly of these nanolipoprotein particles for their intended application. Not only should successful assembly be ascertained but also the self-assembly process itself should be examined for ideal formation conditions for a given set of constraints. For example, apoLp-III was used for assemblies at several different lipid to protein concentrations, from 1:1 to 12:1. Many of these ratios showed NLP formation that could be isolated and examined, but two preparations, the 5:1 and 6:1 ratios of lipid to apolipoprotein, showed the largest NLP peak by SEC, with the lowest free protein peak, providing the highest yield. Other assembly methods were attempted, including varying the time of formation and the temperature, but these did not yield nearly as high amounts of NLP assemblies. Assemblies with apoA-I also greatly varied depending on lipid/protein ratio and assembly temperature and method, as has been seen in other work.[6,7] The results of this work also highlight one means of examining single particle characterization and improving methods through the use of fluorescent labels to track the specific assembly reactants. For example, labeling apolipoproteins with fluorescent dyes and using fluorescently labeled lipid molecules can highlight the effectiveness of different assembly conditions and supply information on yield as fluorescent measurements can detect the amount of NLP formation. Moreover, FRET studies looking at the change in fluorescence in multiple fluorescent molecules will help to determine the structure of the lipid-bound form of the apolipoproteins and help to build low-resolution models of lipoproteins and potentially to examine the formation of integral membrane containing NLPs.[29]

Conclusion

This work presents the first study showing single particle characterization of these nanolipoprotein particles (NLPs) using multiple apolipoproteins for assembly. The choice of apolipoprotein used in an assembly drives the size and homogeneity of the final nanostructure. Extensive characterization of particles for size and shape is required for their use in nanobiotechnology, as represented by the biophysical techniques as described herein. This work presents a fundamental understanding of a platform technology that will enable future research to characterize functional membrane proteins.

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Supporting Information Available: Detailed materials and methods, one table describing the different apolipoproteins, and one figure describing NLP assembly process are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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