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Controlling the Diameter, Monodispersity and Solubility of ApoA1 Nanolipoprotein Particles using Telodendrimer Chemistry

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Supplementary Material is provided in “supplementary_materials.docx” and contains figures presenting the native PAGE gel electrophoresis of cell-free expressed NLPs, increase in yield and solubility of nanoparticles upon telodendrimer addition, size distribution of TD-lipid particles, greater level of aggregation associated with increase in the amount of telodendrimer to lipid ratio, size exclusion chromatography (SEC) trace of TD-NLP comparing with normal NLP and cell-free BR expression with presence of TD-lipid result in production of functional yet insoluble membrane protein.

Abstract Nanolipoprotein particles (NLPs) are nanometer-scale discoidal particles that feature a phospholipid bilayer confined within an apolipoprotein “scaffold”, which are useful for solubilizing hydrophobic molecules such as drugs and membrane proteins. NLPs are synthesized either by mixing the purified apolipoprotein with phospholipids and other cofactors or by cell-free protein synthesis followed by self-assembly of the nanoparticles in the reaction mixture. Either method can be problematic regarding the production of homogeneous and monodispersed populations of NLPs, which also currently requires multiple synthesis and purification steps. Telodendrimers (TD) are branched polymers made up of a dendritic oligo-lysine core that is conjugated to linear polyethylene glycol (PEG) on one end, and the lysine “branches” are terminated with cholic acid (CA) moieties that enable the formation of nano-micelles in aqueous solution. We report herein that the addition of TD during cell-free synthesis of NLPs produces unique hybrid nanoparticles that have drastically reduced polydispersity compared to NLPs made in the absence of TD. This finding was supported by dynamic light scattering (DLS), fluorescence correlation spectroscopy (FCS), and cryo transmission electron microscopy (Cryo-EM). These techniques demonstrate the ability of TDs to modulate both the NLP size (6 to 30 nm) and polydispersity. The telodendrimer NLPs (TD-NLPs) also showed 80% less aggregation compared to NLPs alone. Furthermore, the versatility of these novel nanoparticles was shown through direct conjugation of small molecules such as fluorescent dyes directly to the TD as well as the insertion of a functional membrane protein.

Keywords: nanolipoproteins, telodendrimer, cell-free expression, apolipoprotein, nanotechnology, membrane protein, mono-dispersity

Broader statement: Nanolipoprotein particles (NLP) are nanometer-scale discoidal particles that have proven useful for biochemical studies of membrane proteins. However, NLPs have problems with aggregation, polydispersity and require extensive hands on experimentation to control the size of the particle. By adding branched polymer amphiphiles called telodendrimers, we have developed a process for controlling the size, monodispersity and versatility of NLPs for direct conjugation of other biologically active molecules such as imaging dyes.

Introduction

NLPs are discoidal nanoparticles that form when apolipoproteins and phospholipids (DMPC: 1,3-bis(sn-3'-phosphatidyl)-sn-glycero-2'-phosphocholine; POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, etc) self-assemble to form water-soluble particles featuring a phospholipid bilayer that effectively mimics the cell membrane¹⁻³. NLPs can offer distinct advantages over other membrane model systems in terms of particle size and solubility when compared to liposomes⁴. The presence of a circular apolipoprotein scaffolding or “belt”⁵ constrains the dimensions of the bilayer, helping to maintain particle diameters^{6,7}. The protein belt may also contribute to improved NLP thermal stability compared with micelles or liposomes. The size and stability of NLPs has been shown to be useful for a broad range of biotechnology applications^{8,9}, mainly involving the solubilization of functional membrane proteins^{4,10-14}. The NLP lipid bilayer provides a hydrophobic patch for the incorporation of membrane proteins^{5,12,15}, as well as the formulation of hydrophobic drugs^{9,16}.

We recently developed an approach for the synthesis of NLPs via cell-free protein expression in the presence of phospholipids¹². This convenient method results in the spontaneous formation of NLPs as the apolipoprotein is translated in the reaction mixture. The cell-free method is potentially useful for incorporating unnatural amino acids, labels and tags on NLPs, a strategy not readily available with whole cell systems, and is fast – requiring only a few hours from start to finish¹⁷⁻²⁰. Cell-free methods have allowed one to go from gene to protein to structure in a single day²¹, and accommodates a wide range of additives that augment protein expression, including chaperonins²², lipids²³, redox factors^{20,24}, detergents, and protease inhibitors^{23,25}. Additionally, varying the lipid selection in a

cell-free reaction may affect membrane protein function, as has been demonstrated in recent studies with G-protein coupled receptors (GPCR) reconstituted into NLPs using DMPC alone ¹⁰, POPC alone ²⁶, or a mixture of POPC /POPG (palmitoyl-oleoyl-phosphoglycerol) ¹¹.

Amphiphilic PEG-dendritic block copolymers (Telodendrimer, TD) have favorable properties for both cancer imaging and nanoparticle-mediated drug therapy ^{27,28}. The presence of the hydrophilic PEG chain serves to prevent immunogenicity, increase blood circulation time, and minimize biological degradation of nanoparticles *in vivo*. The presence of cholic acid (CA) moieties imparts amphiphilicity to TDs, enabling them to form nano-micelles upon exposure to the appropriate solvent conditions. In addition, TDs may contain additional functional covalent attachment sites that act as handles for biologically active targeting ligands and cellular uptake molecules. Because TDs are excellent at dissolving hydrophobic compounds for drug formulation applications, they may be amenable to the solubilization of functional membrane proteins. It has been reported that detergent amphipols could impart solubility to membrane proteins depending on their charge, size and aqueous solubility ^{29,30}. This effect has never been shown with TD-lipid particles and will be tested. We hypothesize that combining the favorable attributes of TD with NLPs may result in a novel nanoparticle with improved capabilities and broader application potential compared to either nanoparticle, TD or NLP, composition alone.

In this study, we used cell-free protein expression to generate TD and NLP hybrid nanoparticles. The TD-containing NLPs (TD-NLPs) demonstrated improved nanoparticle homogeneity and the size was tunable by varying the fraction of TD incorporated into the NLPs. We also assessed the versatility of TD-NLPs as a platform technology through the

addition of fluorescent molecules and the insertion of a model membrane protein. This assembled nanoparticle represents a novel entity with unique capabilities when compared with either nano-micelles made of TD-lipids or NLP nanocarriers alone.

Results and discussion

Cell free production of TD-NLPs

We have used a cell free protein expression method to synthesize TD-NLPs that were different in size and solubility when compared to NLPs or TD-lipid particles. The process to produce NLPs in the presence of additional additives is outlined in **Figure 1**. The process outlined in Figure 1 represents a novel path for generating NLPs. Typically, the entire process can be completed in only 8 hours, compared to potentially taking days to prepare when using traditional methods to form NLPs³. Several types of TDs were utilized as depicted in **Table 1** and these molecules varied according to the molecular weight of the PEG in Daltons and the number of CA per TD monomer unit. This study used the following molecules: PEG^{2k}-CA₄, PEG^{2k}-CA₈, PEG^{5k}-CA₈ and PEG^{5k}-CF₄, which contains 4 cholesterol molecules per TD molecule. Plasmids coding for a His-tagged ApoA1 derivative, $\Delta 49$ ApoA1, and TD monomers were premixed with DMPC lipids, and incubated with cell free reaction mixtures to produce fully assembled nanoparticles. As controls we mixed TD monomers with DMPC to form TD-lipid particle. NLP and TD-NLP particle formation were first confirmed by native poly acrylamide electrophoresis (PAGE) and then purified by nickel affinity purification, which removed TD-lipid particles. Samples were then analyzed using native gel and size exclusion chromatography (SEC). The 10% PEG^{5k}-CA₈ TD-NLP has a size of approximately 350 kDa compared to approximately 242 kDa of normal NLP (**Supplemental Figure 1**).

Incorporation of TD enhances soluble NLP yield

We first analyzed the purified yield of the soluble TD-NLP nanoparticles. A total of 1 ml of cell-free reaction mixture was used for particle synthesis with and without TD to compare protein yields and solubility (**Figure 1**). A 4-12% SDS-PAGE gel was used to separate the products during and after nickel affinity chromatography (**Supplemental Figure 2**). We noted a 2-4 fold increase in production of soluble TD-NLPs produced when compared to NLPs alone. As shown in **Supplemental Figure 2**, the PEG^{5k}-CA₈ TD reaction yielded approximately 750 µg/mL of TD-NLP, compared to 190 µg/mL of NLP alone, which was a 2.95 fold improvement. The difference in solubility appeared to be a function of TD incorporation. It is fully plausible that the increased yield may have been caused by increased accessibility to the His-tag, which may have been due to changes in the His-tag accessibility that were induced by the TD. This result is particularly interesting because an increase in soluble NLP yield would aid in making cell free methods more cost effective. Moreover, the increase in NLP solubility could potentially help enhance the solubility and yield of a membrane protein that is supported by TD-NLPs by just increasing the efficiency of the purification, which leads to sample enrichment.

The size of TD-NLP is controlled by the type of TD used

We next studied how telodendrimers impacted the size of NLP-based nanoparticles. Dynamic light scattering (DLS) was used to evaluate the size and monodispersity of the TD-lipid particles, NLPs and TD-NLPs. All three particle types had distinct size differences. The TD-NLP particle sizes were significantly dependent upon the length of the incorporated PEG molecule (**Figure 2**). The PEG^{2K} TD-NLPs ranged in mean size from 6 to 13 nm, while the PEG^{5K} TD-NLPs were 23 to 30 nm in diameter based on DLS traces (**Figure 2, Panel C**). The p-value between the mean sizes of PEG^{2K} and the PEG^{5K} TD-NLPs was 0.0001, while it

was 0.32 between PEG^{2K}-CA₄ and PEG^{2K}-CA₈ NLPs, and 0.43 between PEG^{5K}-CA₈ and PEG^{5K}-CA₄ NLPs. The TD-lipid particles formed larger nanoparticles compared to those made in the presence of the apolipoproteins (TD-NLPs) and the size of the particles was dependent on the type of telodendrimer used (**Supplemental Figure 3**). In general the more telodendrimer added to the DMPC lipid solution would result in greater TD-lipid particle size increases as well as particle aggregation (data not shown). Previously reported studies have shown that NLPs alone measure approximately 8 nm, depending on the apolipoprotein, in solution when properly dispersed through multiple rounds of purification ¹⁴. Changing the amount of TD added to the NLP assembly process over a range of 1 – 10% of total lipid did not significantly alter the overall size of the TD-NLP particles (**Figure 2, Panel A, C**). This study demonstrates a new approach to rapidly design and assemble TD-NLPs of desired size to meet specific needs, for example, to potentially support or limit membrane protein complexes of varying sizes that could be carried within the lipid bilayer.

TD-NLP shows decreased level of aggregation

Next we assessed the aggregation of TD-NLPs based on DLS measurements. Although not addressed in previous publications, aggregation could complicate experimentation using NLPs. TD-NLP aggregation appears to be size dependent, with larger TD molecules exhibiting higher levels of aggregation. Interestingly, the overall level TD-NLPs aggregation rate was reduced 10-100 times compared to NLPs assembled only in the presence of DMPC alone (**Figure 2, Panel A, B**). Increases in the amount of TD to lipid ratio (>10%) were associated with greater levels of aggregation (**Supplemental Figure 4**).

There were no significant changes in NLP size or aggregation when adjusting for TDs containing cholic acid or cholesterol head groups over the 1 – 10% TD to lipid ratio. Monodispersity of TD-NLPs was also confirmed by size exclusion chromatography **(Supplemental Figure 5)**. The much larger TD-lipid particles when isolated were found within the void volume of the SEC fractions (Data not shown). It is clear that the addition of cholic acid or cholesterol based TDs to the cell free method can disaggregate NLP-based nanoparticles resulting in a more monodispersed entity.

To further study the shape and aggregation properties of TD-NLP complexes, we performed Cryo-EM experiments. Cryo-EM images illustrated that NLPs are discoidal in shape with height dimensions consistent with previously published images of 10 nm diameter nanodiscs with phospholipid bilayers **(Figure 3)**. Three assemblies are shown, with DMPC alone (panel A) and with two different types of TDs (panel B, C). Unlike previous reports, NLPs alone (panel A) in our study exhibited large clustering rather than stacked particles, or “rouleaux,” which may be attributable to the inclusion of tobacco virus particles in this study. All of the TD-NLPs showed minimum aggregation that is in accordance with the above DLS results. The disc-like shape of TD-NLPs allows for accessibility from both side of the membrane when supporting membrane protein, which may be an advantage over the traditional liposome and micelle methods.

TD provides a convenient approach for NLP labeling

To investigate the feasibility of using the telodendrimer in NLP labeling as well as measure the solution-based associations between the different molecules, we covalently

conjugated FITC fluorophore to the PEG tail of the TD. FITC-TD was then added to the cell-free reactions containing TexasRed-POPC dissolved in DMPC to generate FITC/TexasRed dual labeled TD-NLPs. In comparison, cell-free reactions were used to assemble NLPs tagged with Bodipy®-FL and TexasRed labels. We then performed Fluorescent Correlation Spectroscopy (FCS) analysis ¹⁴ to topologically confirm TD association and NLP labeling. As seen in **Figure 4**, both NLP (identified by cross-correlating Bodipy and TexasRed in the complex) and TD-NLP (identified by cross-correlating FITC and Texas Red in the complex) diffused significantly faster than DMPC vesicles alone, but slower than the protein $\Delta 49$ ApoA1 (apolipoprotein without any DMPC). These results are a clear indication of TD-NLP formation and successful nanoparticle labelling. In theory, our TD-NLP could also allow for the rapid design and production of *in vivo* targeting, imaging, and therapeutic delivery of biologically active molecules through direct surface conjugation to the telodendrimers. Novel labeling and modification strategies as shown by the FITC labeling method used in this study, exhibit the versatility of these vehicles.

TD-NLP supports functional membrane protein

Self-assembled TD-NLPs will support incorporation of a functional integral membrane protein. It has been previously shown, that bacteriorhodopsin (bR; a seven transmembrane helical protein, from *Halobacterium salinarium*) can be robustly co-expressed and assembled into NLPs for biophysical characterization ^{12,14,15}. In this study, assembly of the soluble bR-NLP complex was observed within 4 h after addition of plasmids to an *E. coli* cell-free lysate (**Figure 5, panel A**). Addition of TDs to the cell-free reaction did not affect bR function as indicated by the observable pink coloration of the

tubes, which is an indication of proper folding and function ¹². Production of similar amounts of total bR protein with and without TDs was also observed (**Figure 5, panel B**). For control, we expressed bR in the presence of TD-lipids without apolipoprotein. This also produced functional bR (**Supplemental Figure 7, panel A**), but the particles were not soluble when tested for solubility (**Supplemental Figure 7, panel B**). This solubility difference was distinct, when compared to co-expressed bR TD-NLP. Our data demonstrated that inclusion of the TDs do not inhibit the production of functionally inserted bR protein. This is important because it paves the way for inserting additional receptors for *in vivo* use. TD-NLPs may be used to deliver nearly any type of membrane protein/receptor or other small molecules. Combined with the labeling technique described above, our soluble TD-NLP complexes could be exploited for multiple applications in cellular targeting, delivery of therapeutics, and membrane protein biochemistry. Moreover, the production of the TD-NLP which only requires minimum steps to achieve usable material for these biotechnology applications, represents the first unique technique to infer multifunctionality to NLPs. Our study is limited by the small number of biophysical and biochemical endpoints used to characterize the TD-NLP molecules. We also limited our experiments to cell-free reactions generated from *E. coli* lysates. There are other amphiphilic particles (so-called amphipols) that are well known to form soluble particles and can also act as supports for membrane proteins ³¹. In fact the use of amphiphiles has been found to be an important approach for determining the structure of some membrane proteins ³⁰. We only used one class of telodendrimers in this study, which can form TD-lipid particles and closely resemble other amphipols. When compared to TD NLPs, this TD-lipid particle has distinct size distribution as well as lacked water solubility

when associated with membrane proteins. We focused on demonstrating both the production and potential capabilities of TD combined NLP nanoparticles in what is considered a low cost system for lysate-based expression, which is uniquely different from what has been demonstrated previously with amphipols. It would be of interest to see how other facile molecules interact or modify apolipoproteins to generate novel nano-scaffolds. This data all indicates that we are obtaining a novel form of nanodisc, which is monodispersed based on the interaction with the telodendrimers. Our data also did not identify the molecular mechanism of how telodendrimers modify the apolipoprotein to lipid interaction. It is well known that varying the protein to lipid ratio can directly affect NLP diameter ⁷. It is possible that the branching of TDs presents a steric structure, which could limit the apolipoprotein to lipid interaction. However, it is clear that the TDs have a favorable impact on the NLP aggregation levels. Also, the TDs clearly facilitate unique labeling strategies for direct application to the NLP as demonstrated by the fluorescent labeling. Future studies will be required to evaluate the specific molecular mechanisms by which TDs offer size tunability.

In summary, we present a novel nanoparticle using apolipoproteins and lipids combined with telodendrimers. The end product is rapidly produced and results in homogeneous NLPs that are monodispersed. In addition, varying telodendrimers can be selected to tune disc size between 6 nm and 25 nm, thus obviating the need for optimizing the lipid to protein ratio and conducting multiple rounds of size exclusion chromatography. Lastly, telodendrimers add to the versatility of the NLP as they can serve as chemical handles for direct surface conjugation of multiple biologically active molecules. Future

studies will be performed to evaluate the utility of this vehicle for the delivery of imaging, targeting, or therapeutic agents.

Materials and Methods

Plasmids

The truncated form of Apo A1 ($\Delta 1-49$) or $\Delta 49$ ApoA1 was cloned into pIVEX2.4d using NdeI and SmaI restriction sites. This vector contained a His-tag used for nickel affinity purification as previously described ¹².

Preparation of the telodendrimers

We have previously published on the use of telodendrimers for as a nano-delivery tool ²⁷. PEG^{5k}-CA₈ and related cholesterol or cholic acid based amphiphilic polymers, were prepared according to the published methods. For the TD-lipid mixtures a total of 25 mg/mL DMPC and each individual polymer were mixed at different molar ratios at an approximate 0.5-10%. The mixtures of DMPC/polymer were then sonicated for 15 min, or until optical clarity was achieved. Samples were kept on ice during the entire sonication process. After the sonication, samples were centrifuged at 13000 RCF for 1-2 min to remove any metal contamination from the probe tip.

DMPC/polymer preparation

Small unilamellar vesicles of DMPC (Avanti Polar Lipids, Alabaster, Alabama) were prepared by probe sonication of a 25 mg/mL aqueous solution of DMPC until optical clarity was achieved; typically 15 min was sufficient. Samples were kept on ice during the sonication process. After the sonication, the samples were centrifuged at 13000 RCF for 2 minutes to remove metal contamination from the probe tip. TDs lipid mixtures (TD-lipid) were created with the above method with a total of 25 mg/mL DMPC and each individual

polymer mixed at varying molar ratios between approximately 0.5-10%. If a fluorescent label on the NLP complex was desired, a 0.1% Texas Red-POPC (Life Technologies Corporation, Carlsbad, California) was added to the DMPC solution before sonication. TD-lipid mixtures were purified by spin column size fractionation and SEC fractionation as described below.

Cell-free Reaction

Small-scale reactions (100 μ L) or large scale (1 mL) were carried out using Expressway Maxi kit (Life Technologies Corporation, Carlsbad, California). Reaction components (lysate, reaction buffer, T7 polymerase, amino acid mix, and methionine) were combined as specified by the manufacturer. 10 μ g of Δ 49ApoA1 DNA was added to each 1 mL reaction. A total of 2mg DMPC/polymer mixture was then added. The reactions were incubated at 30 °C, with shaking at 990rpm for 2-8 hrs or over night in a thermomixer.

Affinity purification of NLP complexes

Immobilized metal affinity chromatography was used to isolate the protein of interest (Δ 49ApoA1) from the cell-free reaction mixture. 2ml of 50% slurry nickel-nitrilotriacetic acid Superflow resin (Qiagen, Hilden, Germany) was equilibrated with PBS (50 mM Na₂HPO₄, 300 mM NaCl, pH 8.0) under native conditions in a 10 mL capped column. The total cell free reaction (1ml) was mixed with the equilibrated resin, and was incubated/nutated on ice for 2hr. 1ml of slurry and 5mL capped column were used for the purification from small-scale reactions. The column was then washed with increasing concentrations of imidazole (10, 20 and 50mM) in the mentioned PBS buffer. Two column

volumes (CV) of each wash buffer were used for a total of 6 CVs of washing. The His-tagged proteins of interest were eluted in six 1mL fractions of 400 mM imidazole, PBS buffer. All elution were combined, dialyzed against PBS for 18 hrs at 4°C with stirring. After that, the combined elution was concentrated using a Vivaspin 6 100K MWCO molecular weight sieve filters (GE Healthcare, Waukesha, Wisconsin) to a volume of ~200 μ L.

SDS PAGE

10 μ L aliquots of the purified NLPs or lipid micelles were mixed with 10 μ L 2x LDS Sample buffer with reducing agents (Life Technologies Corporation, Carlsbad, California), heat denatured and loaded on to a 4-12% gradient pre-made Bis-Tris gel (Life Technologies Corporation, Carlsbad, California) along with the molecular weight standard NovexSharp (Life Technologies Corporation, Carlsbad, California). The running buffer was 1X MES-SDS (Life Technologies Corporation, Carlsbad, California). Samples were electrophoresed for 38 minutes at 200V. Gels were stained with Coomassie Brilliant Blue.

Native PAGE

10 μ L aliquots of the purified NLPs or lipid micelles were mixed with 2x native gel sample buffer (Life Technologies Corporation, Carlsbad, California) and loaded onto 4-12% gradient pre-made Tris-glycine gels (Life Technologies Corporation, Carlsbad, California). Samples were electrophoresed for 2 hours at 125 V. After electrophoresis, gels were imaged using the laser (488 nm) of a Typhoon 9410 (GE Healthcare, Waukesha, Wisconsin) with a 520 nm bandpass 30 filter for the detection of the produced NLPs with incorporated FITC labeled polymer. For detection of the produced NLPs with incorporated TexasRed-

POPC, the laser (532 nm) with a 610nm bandpass 30 filter is used. Molecular weights were determined by comparing migration vs. log molecular weight of standard proteins found in the NativeMark standard (Life Technologies Corporation, Carlsbad, California).

Dynamic light scattering (DLS)

The measurements were performed on a Nanotrak Particle Size Analyzer (Microtrac, Montgomeryville, Pennsylvania) following the manufacturer's instruction menu.

Cryo Transmission Electron Microscopy (Cryo-EM)

All the samples are preserved as frozen hydrated specimen in the presence of saturated ammonium molybdate and examined with a JEOL JEM-2100F transmission electron microscope (JOEL USA, Peabody, Massachusetts) at magnification of 80,000x under liquid nitrogen temperature. Clusters of NLPs were found with plain NLP sample, while only a few clusters of NLPs were found with TD-NLPs. The majority of the NLPs are shown inside views. A few of them appeared in their top view. Tobacco mosaic virus (TMV) was added as reference to indicate the quality of Cryo-EM preparation, as well as the internal calibration of microscope magnification.

Solution Phase Characterization using Fluorescent Correlation Spectroscopy (FCS)

The methods in this study have been described in detail previously¹⁴. Characterization of nano-particles and their dynamic shape and association in solution remains a challenge, which we have addressed using FCS performed on a MicroTime 200 single molecule fluorescence lifetime measurement system (PicoQuant GmbH, Berlin, Germany). FCS is

capable of measuring molecular diffusion statistics in solution with sensitivity for single molecule fluorescence. This allows us to rapidly and accurately determine the hydrodynamic radii of the newly formed nano-complexes in an aqueous environment. Complimentary techniques were performed, such as size exclusion chromatography (SEC) using a Superdex 200 HR 10/300 column (GE Healthcare, Waukesha, Wisconsin) that was expected to efficiently separate different sized NLP constructs.

Electronic Supplementary Material

Supplemental data shows Native PAGE gel electrophoresis of cell-free expressed NLPs, an increase in yield and solubility of nanoparticles upon telodendrimer addition, size distribution of TD-lipid particles, increased levels of aggregation associated with increasing amounts of telodendrimer to lipid, size exclusion chromatography (SEC) trace of TD-NLP and empty NLP and cell-free BR expression with presence of TD-lipid result in production of functional yet insoluble membrane protein and are provided in Figures 1, 2, 3, 4, 5 as well as 6, respectively, in supplementary material.

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Figure Legends

Figure 1. Schematic of TD-NLP assembly process using telodendrimer-based chemistry. Cell-free synthesis of telodendrimer conjugated NLPs. (A) The chemical structure of a telodendrimer comprised of an octamer of cholic acid (CA₈) linked to the terminal end of a linear 5kDa PEG molecule (PEG^{5K}), which is solubilized with DMPC lipid. (B) The TD-lipid mixture is then added to a general cell free reaction, resulting in the self-assembly of TD-NLPs.

Figure 2. Telodendrimer addition to nanoparticle formation changed aggregation levels and size tenability. Dynamic Light Scattering (DLS) was used to assess several biophysical properties of the TD-NLP particles. (A) The size distribution of TD-NLPs measured by Dynamic Light Scattering. TD-NLPs demonstrated an improved homogeneity compared to normal DMPC-NLP. (B) Total aggregations of various TD-NLPs are significantly less compared to normal DMPC-NLP ($p < 0.01$) (C) PEG tail length and size of the TD-NLPs are significantly correlated (PEG^{2K} vs. PEG^{5K}, $p < 0.01$).

Figure 3. Imaging of TD-NLPs with decreased aggregation compared to NLP. Cryo-EM visualization of NLP preparations with and without telodendrimers is shown to validate reduced aggregation levels of TD-NLPs. (A) NLP assembly using DMPC lipids alone. (B) NLP assembly using DMPC lipids with 10% telodendrimer PEG^{2K}-CA₄. (C) NLP assembly using DMPC lipids with 10%

telodendrimer PEG^{2K}-CA₈. (D) Magnification of NLP with 10% PEG^{2K}-CA₈ (arrowhead) from its top view. Tubular structures (white arrow) represent Tobacco Mosaic Viruses for reference.

Figure 4. Telodendrimers are associated with nanoparticles as a complex. Diffusion curves of proteins and NLP/TD-NLP complexes as measured by FCS. Green, black, blue, and red curves correspond to $\Delta 49A1$, TD-NLP, NLP and DMPC vesicles in 1X PBS, respectively.

Figure 5. Telodendrimers are compatible with membrane protein production. The cell free reactions are setup with 1 μ g/mL pIVEX-2.4d- $\Delta 49ApoA1$ and 10 μ g/mL pIVEX-2.4d-boP and 2mg/mL lipid (100% DMPC or 99.5% DMPC and 0.5% TD, molar ratio, etc), BODIPY-FL and 50 μ M all trans retinal, 30°C at 990rpm for 4 hours. (A) The pictures of the tubes were taken after the reaction tubes were centrifuged at 14,000 rpm for 10 mins. The purple colored solution indicated soluble production of correctly-folded bR. (B) Denaturing SDS PAGE gel electrophoresis of cell-free expressed proteins. All samples were loaded along with a molecular weight standard (M.W.). The pictures were taken with GE-TYPHOON 9410 using laser/filter 488nm/520nm. The non-specific bands below 20kDa are free BODIPY-FL. The tubes and lanes are as follows: (1) Co-expression of BR and $\Delta 49ApoA1$ with DMPC (BR-NLP), (2) Cell free expression of $\Delta 49ApoA1$ with DMPC (empty NLP), (3) Co-expression of BR and $\Delta 49ApoA1$ with DMPC and 0.5% telodendrimer PEG^{2k}-CA₄, (4) Co-expression of BR and

ApoA1 with DMPC and 0.5% telodendrimer PEG^{2k}-CA₈, (5) Co-expression of BR and Δ49ApoA1 with DMPC and 0.5% telodendrimer PEG^{5k}-CF₄, (6) Co-expression of BR and Δ49ApoA1 with DMPC and 0.5% telodendrimer PEG^{5k}-CA₈.