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The Use of Liprotides To Stabilize and Transport Hydrophobic Molecules

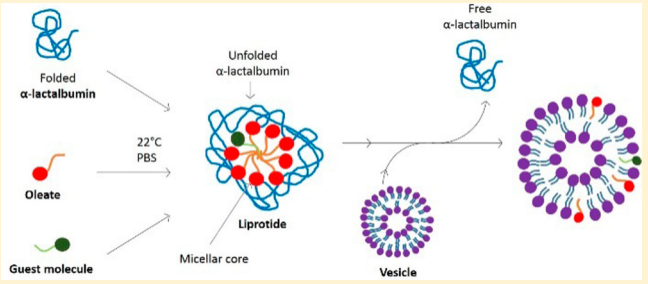
Jannik N. Pedersen,[†] Jan S. Pedersen,[‡] and Daniel E. Otzen*[,†]

[†]Interdisciplinary Nanoscience Center (iNANO), Department of Molecular Biology and Genetics, Aarhus University, Gustav Wieds Vej 14, DK-8000 Aarhus C, Denmark

[‡]Department of Chemistry and Interdisciplinary Nanoscience Center (iNANO), Aarhus University, Gustav Wieds Vej 14, DK-8000 Aarhus C, Denmark

Supporting Information

ABSTRACT: Recently, it has been shown that different complexes consisting of protein and fatty acids, which we call liprotides, have common functional and structural features. Liprotides can transfer their fatty acid content to membranes, highlighting the potential to incorporate other small molecules and help transfer them to membranes. In this study, this potential was explored with regard to the poorly water-soluble vitamin E compound α-tocopherol (Toc). Uptake into liprotides increased Toc solubility and chemical stability. The liprotide−Toc complexes retained the characteristic liprotide structure with a core of fatty acid surrounded by protein. Toc and fatty acid could be transferred to artificial vesicles upon being incorporated into the liprotide complex. Extending this work, we found that free tryptophan and the vitamin A precursor retinaldehyde could also be incorporated in the liprotides; however, other small molecules failed to be taken up, and we conclude that successful incorporation requires a hydrophobic terminal moiety that can be accommodated within the micelle interior of the liprotides. Nevertheless, our work suggests that liprotides are able to stabilize and transport a number of otherwise insoluble small molecules with significant potential health benefits.



Complexes consisting of protein and fatty acids have been known for a long time, and especially HAMLET (human α-lactalbumin made lethal to tumor cells) has been investigated intensively.[1] These complexes are cytotoxic to certain cells[2,3] and bacteria[4] and appear to work by transferring the poorly soluble fatty acid, oleic acid (OA), to cell membranes.[5,6] It is generally believed that the cytotoxicity of these complexes is due to the fatty acid and that the protein component works only as a transporter of the fatty acid.[7,8] However, α-lactalbumin appears to be unusually appropriate as a fatty acid carrier due to its high flexibility in the metal-free state and ability to assume different conformational states.[9] In the monomeric state it is able to bind oleic acid through structural changes in its C-terminal domain at neutral pH,[10] and at pH 12 the protein undergoes a series of conformational shifts with increasing molar ratio of oleic acid to accommodate the fatty acid.[11] This is not, however, the only way that oleic acid can bind to flexible proteins. Recently, a number of protein−fatty acid complexes have been found to be similar to HAMLET in terms of cellular toxicity and structure. Combinations involving different proteins[6,12−14] as well as different fatty acids[15,16] can form such complexes. Small-angle X-ray scattering (SAXS) analyses show that these complexes consist of a shell of partially denatured protein molecules bound around a core containing 10−30 fatty acids.[14] We have introduced the term liprotides (lipids and partially denatured proteins) to refer to such protein−fatty acid complexes with common structure and cytotoxicity.[14] The stabilized micellar core of the liprotide structure, together with the ability of these complexes to transfer fatty acids to phospholipid membranes,[5] immediately suggests a potential for encapsulating and transporting not only different fatty acids but also other hydrophobic molecules. This work explores this potential.

We start by testing the ability of liprotides to transport vitamin E, an essential fat-soluble family of compounds with antioxidant activity that prevents peroxidative damage of lipids in cell membranes.[17] The main form of vitamin E found in human tissue is α-tocopherol (Toc) and is, like the other vitamin E molecules, highly hydrophobic.[18] Apart from its antioxidant activity, Toc has lately also been proposed to play a role in gene regulation and cell signaling.[19] A higher level of intake of Toc has been shown to reduce the risk of cardiovascular diseases, while a low level of intake of Toc can result in vitamin E deficiency-associated ataxia.[17] Toc is sensitive to light, oxygen, and heat and at the same time highly hydrophobic, making it difficult to study.[20] Incorporation of Toc in a complex such as a liprotide could potentially increase Toc's solubility and stability and thereby improve its use in

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food and pharmaceutics. However, this must be done under gentle conditions because of Toc's chemical fragility. Liprotide formation appears to require the protein to be unfolded to allow the fatty acid to bind to the protein. Permyakov and co-workers have prepared liprotides by mixing protein and fatty acid under warm alkaline conditions. This approach has been proposed to turn virtually any natively folded protein into a carrier of OA and thus can be used to form complexes with other hydrophobic molecules.[6] However, the high pH may compromise fragile compounds, making gentler approaches more attractive. Liprotides consisting of α-lactalbumin (aLA) and OA are particularly easy to prepare, because removal of aLA's tightly bound Ca^{2+} strongly destabilizes the protein. Thus, liprotides consisting of aLA and OA can be made by simply titrating OA into a solution of apo-aLA.[21] We have therefore investigated the structure of the liprotide formed in the presence of Toc, its stability, and its ability to transfer Toc to membranes. Inspired by the success of these experiments, we have also investigated the ability of liprotides to host a number of other hydrophobic molecules, including retinaldehyde and different amino acids.

MATERIALS AND METHODS

Materials. α-Tocopherol (Toc, ≥96% pure), Ca^{2+}-depleted α-lactalbumin (apo-aLA, ≥85% pure), all-*trans*-retinal (≥98% pure), mupirocin (≥92% pure), tetracycline (≥98% pure), ofloxacin (≥99% pure), erythromycin (≥99% pure), L-tryptophan (Trp, ≥98% pure), L-histidine (His, ≥98% pure), L-tyrosine (Tyr, ≥98% pure), and sodium oleate (OA, ≥95% pure) were from Sigma-Aldrich (St. Louis, MO). All amino acids and antibiotics were used as the free acids (not salts). 1,2-Dioleoylphosphatidylglycerol (DOPG, 840475P) and 1,2-dioleoylphosphatidylcholine (DOPC, 850757P) were from Avanti Polar Lipids (Alabaster, AL). All other reagents were of high purity or HPLC grade.

Turbidity Assay. A 10 mg/mL stock of Toc was prepared in ethanol (EtOH) and diluted to 0.2 mg/mL with PBS [50 mM $NaHPO_4$ (pH 7.4) and 150 mM NaCl]. aLA (20 μM) and OA (1 mM) were mixed with different amounts of Toc in PBS in a 96-well clear bottom plate. The final EtOH concentration was <0.83%. Samples were incubated at room temperature for 15 min before absorbance at 500 nm was measured on a Varioskan Flash Multimode Reader (Thermo Scientific, Waltham, MA) to evaluate sample turbidity.

Preparation of Tocopherol–Liprotide Complexes. OA was dissolved in 20% EtOH at a concentration of 71 mg/mL, and Toc was dissolved in 96% EtOH at a concentration of 100 mM. An aliquot corresponding to a 1:50:3 aLA:OA:Toc molar ratio was prepared with a final protein concentration of 2 mg/mL in 50 mM Tris (pH 7.4). Samples were incubated at room temperature for 15 min. Samples were then loaded on a size exclusion column. All molar protein concentrations are given as monomeric protein concentrations.

Purification of Tocopherol–Liprotide Complexes. Liprotides were centrifuged at 12100*g* for 8 min prior to loading on a Superdex-200 (GE Healthcare, Little Chalfont, U.K.) size exclusion column pre-equilibrated with PBS. Samples were loaded through a 250 μL loop at a flow rate of 0.5 mL/min, and protein elution was followed at 280 nm. Fractions (1 mL) were collected, and when needed, samples were concentrated using an Amicon ultracentrifugal filter with a 3 kDa cutoff. Protein concentrations of purified and concentrated fractions was determined using the CB-X^{tm} Protein Assay (G-Biosciences, St. Louis, MO). To estimate the size of eluting species, the column was precalibrated using proteins of known molecular weight (aLA, ovalbumin, BSA, aldolase, and thyroglobulin).

Effect of UV on Tocopherol. A Stratagene UV Stratalinker 1800 (Stratagene, La Jolla, CA) was used to study the degradation of Toc caused by UV light. Then 100 μM Toc was suspended in methanol (MeOH), ethanol (EtOH), PBS, or PBS with 1 mg/mL aLA and the UV stability compared to that of liprotides containing 100 μM Toc; 200 μL of each suspension was exposed to UV light for 30 min with a wavelength of 254 nm operating at 40 W. The exposed formulation was analyzed via HPLC. The experiment was conducted in triplicate.

Physical Stability. Toc (100 μM) was suspended in MeOH, EtOH, PBS, or PBS with 1 mg/mL aLA and was together with 100 μM Toc-containing liprotides left for 16 days on the lab bench in Eppendorf tubes. Samples were taken out at different time points and analyzed via HPLC to evaluate the amount of soluble/intact Toc.

HPLC Analysis. HPLC analysis of OA, aLA, and Toc content was conducted on an UltiMate 3000 HPLC (Dionex, Sunnyvale, CA) system using a Kinetex 5u C8 100A column (150 mm × 4.6 mm) (Phenomenex, Torrance, CA). The mobile phase was a gradient of buffer A [0.1% trifluoroacetic acid (TFA) in MQ] to buffer B (0.1% TFA in acetonitrile) (20 to 100% B over 8 min followed by 5 min at 100% B). The flow rate was set to 1 mL min^{-1} and the injection volume to 100 μL for samples from ultracentrifugation and 20 μL for the rest. The calibration curves were linear with r^2 values of 0.99 for aLA, OA, and Toc.

Vesicle Preparation. A 10 mg/mL stock was prepared, consisting of 20% DOPG and 80% DOPC by weight, and dissolved in chloroform. Chloroform was evaporated and lipid resuspended in 50 mM $NaHPO_4$ (pH 7.4) and 150 mM NaCl at a concentration of 10 mg/mL. The solution was then exposed to 10 freeze–thaw cycles, with freezing conducted in liquid nitrogen and thawing in a 50 °C water bath. To prepare 200 nm large unilamellar vesicles (LUVs) for ultracentrifugation, the suspension was then extruded through a 200 nm pore filter 21 times using an Avanti Mini-extruder from Avanti Polar Lipids. Small unilamellar vesicles (SUVs) for QCM-D experiments were prepared by sonication of the frozen–thawed suspension at 10% of the maximal effect for 10 min using a QSonica Q500 instrument (QSonica, Newtown, CT).

Ultracentrifugation. The 2 mg/mL 200 nm DOPG/DOPC vesicles were mixed with liprotides in PBS, giving a final protein concentration of 50 μg/mL. Samples were spun at a g_{max} of 306000*g* for 1 h with an Optima MAX-E preparative ultracentrifuge (Beckman Coulter, Indianapolis, IN). The pellet was collected and resuspended in PBS. The Toc contents of both the pellet and the supernatant were determined by HPLC.

Quartz Crystal Microbalance with Dissipation (QCM-D). QCM-D was performed on a Q-sense E4 (Q-sense AB, Västra Frölunda, Sweden) system with 5 MHz AT-cut SiO_2-coated sensors (QSX 303). A DOPC/DOPG bilayer was formed on sensors as seen before with small changes.[22] Sensors were immersed in 2% Hellmanex III for 10 min, washed in Milli-Q, treated with ozone and UV for 25 min, and washed in Milli-Q water. Equilibration of the QCM-D was done with PBS at 20 °C followed by addition of 0.2 mg/mL DOPG/DOPC vesicles in 50 mM Tris (pH 7.4) and 2 mM $CaCl_2$. When the bilayer had been formed, sensors were equilibrated in PBS

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followed by addition of the sample with a 1 h separation between injections. The flow rate was set to 200 μL/min, while injection of samples was conducted in stagnant flow mode with injection of a 500 μL sample.

Small-Angle X-ray Scattering. Liprotides purified by gel filtration were measured on a flux- and background-optimized prototype of the NanoSTAR SAXS camera from Bruker AXS.[23] The instrument uses a rotating anode (Cu Kα), multilayer optics, and home-built "scatterless" slits. The acquisition time was 60 min, and background subtraction and conversion to absolute scale were conducted with the SUPERSAXS program package (C. L. P. Oliveira and J. S. Pedersen, unpublished) using water as a calibration standard. The intensity is presented as a function of the magnitude of the scattering vector $q = 4\pi \sin \theta/\lambda$, where λ = 1.54 Å is the wavelength and 2θ is the scattering angle.

Analysis of SAXS Data. The data were analyzed by indirect Fourier transformation (IFT), which gives the characteristic real-space distance distribution functions $p(r)$.[24,25] This function corresponds to a histogram over distances between pairs of points within the particle, weighted by the excess scattering length density at the points. The function gives direct insight into the shape and size of the particles.

The data for the smaller liprotides were modeled using a core−shell model as described previously.[14] Briefly, the core consists of the hydrocarbon chains of the OA and the "tail" of Toc, which both have negative excess scattering length densities as the electron densities are lower than that of water. The shell is expected to consist mainly of the disordered protein that has an electron density higher than that of water, and thus a positive excess scattering length density. The number of oleic acids is calculated as the core volume divided by the volume of a single hydrocarbon chain of OA, which is estimated to be 480 Å^3.[14] The core also contains the hydrocarbon chain of Toc that has a volume comparable to that of OA. Therefore, this number will approximate the total number of OA and Toc for the loaded liprotides. We note that the volume of the hydrocarbon tail of Toc can be estimated as half the volume of squalane with the addition of the volume of a CH_2 group. The squalane volume is ∼870 Å^3 as calculated from the density in ref 26, and the volume of a CH_2 group is ∼27 Å^3;[27] therefore, the volume of the Toc hydrocarbon tail is ∼462 Å^3, which is very similar to that of the OA tail. When the fit to the SAXS data is done, the relative total scattering lengths (also known as scattering mass) of the core and the shell are calculated. The estimate of the electron density of hydrocarbon is then used to convert the scattering mass of the shell into an excess number of electrons, and using the excess electron density of protein, the protein mass is finally estimated.

RESULTS

Reduced Level of Aggregation of Toc in the Presence of the Liprotide. Toc is known to be soluble in ethanol but to aggregate in aqueous solution.[28] Liprotides on the other hand are soluble in aqueous solution, so uptake of Toc into liprotides should reduce its level of aggregation. We tested uptake by monitoring the turbidity of Toc via absorbance at 500 nm. In PBS alone, increasing Toc concentrations led to a linear increase in absorbance (Figure 1). However, when Toc was incubated together with both aLA and OA at a constant aLA:OA ratio of 1:50, no turbidity could be seen when the Toc:aLA molar ratio was below ∼3.5:1, indicating that OA, aLA, and Toc formed a water-soluble complex. Higher

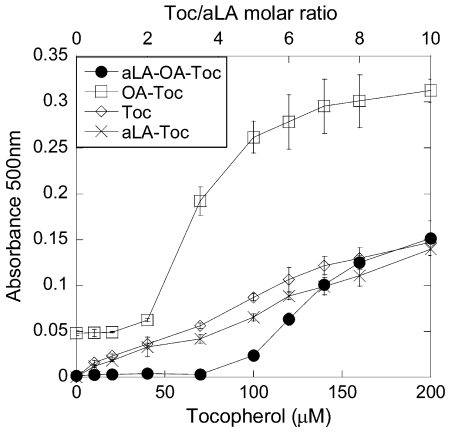

Figure 1. Turbidity of tocopherol in the presence of aLA and/or OA measured with absorbance at 500 nm.

concentrations of Toc led to an increase in turbidity, indicating formation of significantly larger aggregates. In the following studies, we therefore used an aLA:OA:Toc molar ratio of 1:50:3 to avoid larger aggregated species. Incubation of aLA alone with Toc did not prevent Toc aggregation, while OA alone actually increased the overall level of aggregation (Figure 1), probably through the formation of larger complexes between OA and Toc. This emphasizes the importance of having both OA and aLA present to form liprotides and increase the solubility of Toc.

Characterization of the Toc Complex. We used gel filtration chromatography to isolate different liprotide species. aLA/OA mixtures mainly eluted as two well-separated single peaks, indicating two major species (Figure 2). Previously, we

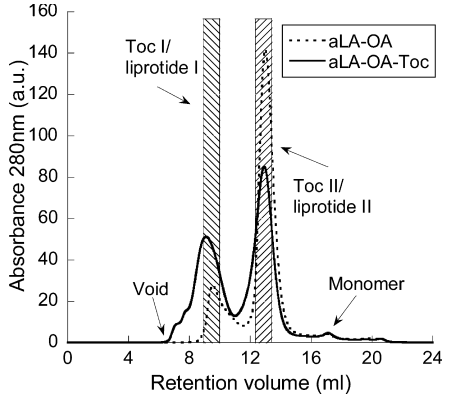

Figure 2. Size exclusion chromatogram of the aLA/OA mixture incubated with or without Toc. The shaded area indicates the fraction that was collected for further characterization.

mainly observed only one peak,[14] corresponding to the Lip II peak. The Lip I species is observed when a larger amount of OA is used to prepare the complex, while it also depends on the preparation method. On the basis of a calibration curve of standard proteins (Figure S1 of the Supporting Information), the first eluting peak (Lip I) was estimated to have an approximate weight of 607 kDa and the second (Lip II) a weight of 116 kDa. When the aLA/OA/Toc sample was mixed and subsequently separated by gel filtration chromatography, we again observed two separate peaks. The first peak (Toc I) eluted slightly earlier than Lip I, leading to an estimated weight of 741 kDa. On the basis of peak integration of A_{280}

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chromatograms from SEC, Toc I constituted 44% of the total eluting protein and accounted for a larger proportion of the total protein compared to Lip I, which accounted for only 17% of the total protein. The second peak for the aLA/OA/Toc mixture (Toc II) eluted at a volume similar to that of Lip II. The small increase in size and quantity of Toc I compared to those of Lip I suggests that Toc promotes the formation of larger species. We observed very little monomer in any of the chromatograms, likely because of the large molar ratio of OA to aLA.

Shaded areas (Figure 2) were collected for further characterization of the complexes. The stability of the complexes was tested by re-injecting the collected Lip I, Lip II, Toc I, and Toc II fractions (Figure S2 of the Supporting Information). All fractions were seen to be stable to re-injection and came out as mainly one peak. The aLA:OA:Toc stoichiometry in the liprotides with and without Toc was determined using HPLC (Table 1). Similar aLA:OA binding stoichiometry was seen for

Table 1. Stoichiometries of Complexes Determined by HPLC[a]

	OA:aLA	no. of hydrophobic molecules per aLA	RP-HPLC elution[b]
Lip II	9 ± 1	NA	NA
Lip I	21 ± 5	NA	NA
Toc II	11 ± 1	1.0 ± 0.2	100% (2 mL, isocratic)
Toc I	25 ± 3	5.4 ± 0.4	100% (2 mL, isocratic)
liprotide–retinaldehyde	13 ± 4	1.8 ± 0.1[c]	100% (5 mL, isocratic)[b]
liprotide–mupirocin	8	(ND)[d]	74% (gradient)[b]
liprotide–Trp	10 ± 1	0.8 ± 0.1	35% (gradient)[b]
liprotide–His	9	(ND)[d]	24% (gradient)[b]
liprotide–Tyr	9	(ND)[d]	23% (gradient)[b]
liprotide–tetracycline	10	(ND)[d]	20% (2.4 mL isocratic)[b]
liprotide–ofloxacin	9	(ND)[e]	20% (1.4 mL isocratic)[b]
liprotide–erythromycin	9	(ND)[d]	20% (1.2 mL isocratic)[b]

[a]Guest compounds are listed in order of decreasing hydrophobicity. [b]The value indicates the percent acetonitrile (v/v) where the compound elutes (if eluting during an isocratic step, the elution volume during this step is provided). The elution program consisted of the following sequence of acetonitrile percentages: 4 mL of isocratic 20% followed by a 20 to 100% gradient over 5 mL and then finally 10 mL at 100%. [c]Retinal concentration determined from the absorbance spectrum recorded immediately after SEC elution and assuming no degradation of retinal. [d]No binding observed within the detection limit. Fewer than one molecule per eight aLA binding. [e]No binding observed within the detection limit. Fewer than one molecule per 20 aLA binding.

Lip II (9 ± 1) versus Toc II (11 ± 1) as well as Lip I (21 ± 5) versus Toc I (25 ± 3). The binding of Toc in the liprotides was confirmed via HPLC, which indicated a higher stoichiometry of Toc in the larger Toc I (5.4 ± 0.4 Toc/protein) compared to the smaller Toc II (1.0 ± 0.2 Toc/protein). This large difference in Toc binding propensity might partly be caused by the larger OA:aLA ratio bound in Toc I.

Structure of the Toc Complexes. We have applied SAXS to obtain more information about the overall structure of the aLA–OA–Toc complexes. The "classic" aLA–OA complex alone forms a core–shell structure, in which partially unfolded aLA surrounds and stabilizes a central OA micelle.[14] The scattering data and the distance distribution function $p(r)$ are shown in panels A and B of Figure 3, respectively, for the small

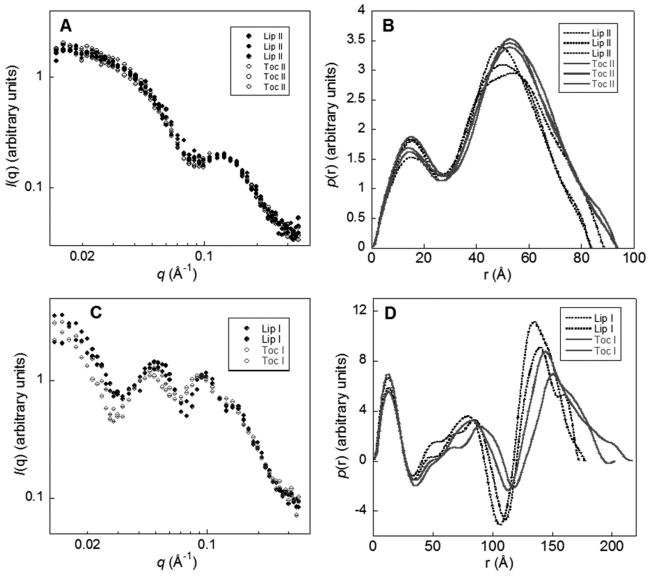

Figure 3. SAXS data of complexes formed and separated in independent experiments. (A) Scattering data for Lip II and Toc II and (B) $p(r)$ functions. (C) Scattering data for Lip I and Toc I and (D) $p(r)$ functions. All have been normalized to overlap for comparison.

particles of Lip II and the corresponding Toc-loaded liprotides, Toc II. The SAXS data look very similar and have a broad bump at $q = 0.12$ Å^{-1} originating from the negative contrast of the core and the positive contrast of the shell. These contrasts give rise to the oscillatory behavior of the $p(r)$ functions. In addition, the $p(r)$ functions show that there is a difference in the maximal diameter of the two types of particles, with the Toc-loaded ones being slightly larger and also having a maximal value for the diameter at a slightly larger distance (94 Å vs 84–89 Å for Lip II).

The scattering data and $p(r)$ functions of Lip I and Toc I are shown in panels C and D of Figure 3, respectively. The SAXS data have several oscillations, suggesting a more complex structure, and the $p(r)$ functions all have several oscillations. The particles have a maximal diameter of 173–217 Å, which is approximately twice that of Lip II and Toc II, and also for these larger particles, the Toc-loaded particles are larger than the nonloaded ones.

The SAXS data for Lip II and Toc II were fitted by the core–shell model, and the results are summarized in Table 2. The parameters for the models are very similar, with a small tendency for the core to be larger for the Toc II particles. This also results in a slightly larger number of OA molecules per particle, and in addition, there is a slightly larger amount of protein per particles for Toc II. Both particles contain 30–40 OAs per particle and around 4 aLAs per particle, i.e., ∼10 OAs per protein, in good agreement with the HPLC analysis. OA and tocopherol cannot be distinguished by SAXS, but our HPLC analysis tells us that there is only approximately one tocopherol molecule per 10 OA molecules, i.e., ∼4 tocopherol molecules per particle. Despite this, the particles are slightly larger when they contain tocopherol. The HPLC results

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Table 2. Fit Parameters from the Core−Shell Model

	R_{in} (Å)[a]	D_{shell} (Å)[b]	$N_{OA/mic}$[c]	$N_{aLA/mic}$[d]	$N_{OA/aLA}$[e]
Lip II	16 ± 1	25 ± 1	38 ± 1	3.8 ± 0.1	9.9 ± 0.4
Lip II	16 ± 1	24 ± 1	33 ± 1	3.8 ± 0.1	8.8 ± 0.3
Lip II	15 ± 1	27 ± 1	28 ± 1	3.2 ± 0.1	8.8 ± 0.4
Toc II	15 ± 1	27 ± 1	31 ± 1	3.3 ± 0.1	9.6 ± 0.4
Toc II	17 ± 1	25 ± 1	40 ± 1	4.0 ± 0.1	9.9 ± 0.3
Toc II	17 ± 1	25 ± 1	40 ± 1	4.0 ± 0.1	9.9 ± 0.4

[a]Core radius. [b]Thickness of shell. [c]Number of OA molecules per liprotide. For Toc II, this number also includes the Toc molecules. [d]Number of protein molecules per liprotide. [e]Number of OA molecules per protein in the complexes. For Toc II, this number also includes the Toc molecules.

indicate that the more complex structure of the Toc I particles accommodates a larger fraction of tocopherol molecules (one Toc for every ∼5 OAs).

Influence of Toc Complex Formation on Stability. Toc is known to be sensitive to UV light,[20] so we tested if the liprotide complex could protect Toc from degradation. In the presence of oxygen, Toc can be degraded to a tocopheroxy radical and a superoxide radical.[29] The formation of tocopheroxy radical is accelerated by increasing the temperature and is also accelerated by the formation of excited TOC molecules upon exposure to UV light irradiation. The formed superoxide radical can also react with TOC and lead to degradation and formation of hydroperoxyl that is reactive to TOC and can lead to further degradation of TOC.[29]

We used HPLC to monitor the amount of Toc in formulations exposed to UV light for 30 min (Figure 4). Toc II showed the best protection from degradation (74 ± 1%) followed by Toc I (58 ± 7%). Simply adding aLA to Toc also improved the stability of Toc significantly (36 ± 3%) compared to MeOH (17 ± 4%), PBS (21 ± 2%), and EtOH (1 ± 2%).

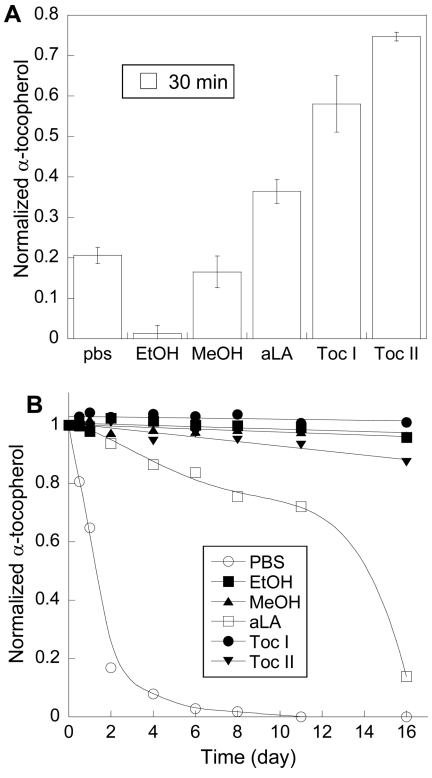

Figure 4. Stability of tocopherol in different solutions. (A) Amount of tocopherol remaining in solution after exposure to UV for 30 min. (B) Amount of tocopherol remaining in solution after several days at room temperature.

Toc was also stored in different mixtures to evaluate its stability and/or solubility over 16 days. Samples were left in Eppendorf tubes at ambient temperature (∼22 °C) and exposed to only indirect sunlight (Figure 4). Toc only had a low stability in PBS buffer and had almost completely decomposed after 6 days (3%). aLA improved Toc's stability markedly compared to PBS, and after 11 days, 72% Toc was still present. Importantly, essentially complete protection was seen for Toc I, Toc II, MeOH, and EtOH (100, 93, 98, and 99%, respectively), showing that Toc I and Toc II are stable over a long period. After 16 days, the solution containing aLA and Toc precipitated, leaving little aLA and Toc in solution. In contrast, Toc I and Toc II showed no decrease in solubility over this time period.

Transfer of Toc from Liprotides to Vesicles. We have previously proposed that the protein shell stabilizing the liprotide may function as a vehicle for transport of OA to membranes.[22] This work was based on studies with a quartz crystal microbalance, in which a liprotide consisting of OA and lysozyme (ELOA) was exposed to a supported phospholipid membrane.[22] When 3−10 μM ELOA (corresponding to 96−320 μM OA) was added to the crystal, there was an increase in energy dissipation D (indicative of a decrease in film rigidity, i.e., a more floppy membrane), while the resonance frequency f initially decreased and then increased, indicative of uptake and then release of mass. We interpreted this to mean that OA was transferred to the bilayer and rearranged it.[22] This suggests that liprotides may also transfer Toc to membranes.

Accordingly, we investigated the interaction between a lipid membrane and Lip II or Toc II using QCM-D (Figure 5). A lipid bilayer was formed from DOPC/DOPG vesicles on the crystal by spontaneous vesicle collapse as described previously.[30] The characteristic bilayer formation can be seen at 50 min, where a decrease in f and an increase in D indicate vesicle accumulation on the surface, followed by an increase in f and a decrease in D because of vesicle collapse on the surface and formation of a bilayer[30] (Figure 5). When liprotide was added to the bilayer on the crystal, 0.1−5 μM Lip II (1.18−35.4 μM OA) did not make significant changes in f and D while 5 μM Lip II (59 μM OA) resulted in the same increase in D and an initial decrease followed by an increase in f as for ELOA. The change in f could indicate rearrangements in the bilayer caused by binding of OA to the bilayer. This suggests that ELOA and Lip II interact in similar ways with lipid bilayers. The similarity between liprotides containing the same fatty acid but different (albeit homologous) proteins supports the proposal that OA is the key player in interactions with membranes, while the protein simply functions as a transporter of OA. A similar dose−response curve was seen for Toc II, where 3−5 μM Toc II (36.3−60.5 μM OA) led to changes in f and D similar to those seen with 5 μM Lip II (Figure 5). The close correspondence of these two transition concentrations suggests that the interaction of the liprotide with the lipid bilayer is not changed when Toc is added to the complex. However, the precise concentration at which the transition occurred was difficult to reproduce exactly, likely because of the very narrow concentration range where this occurred.

To establish more directly whether Toc and OA were transferred to membranes, Lip II, Toc II, and Toc I were

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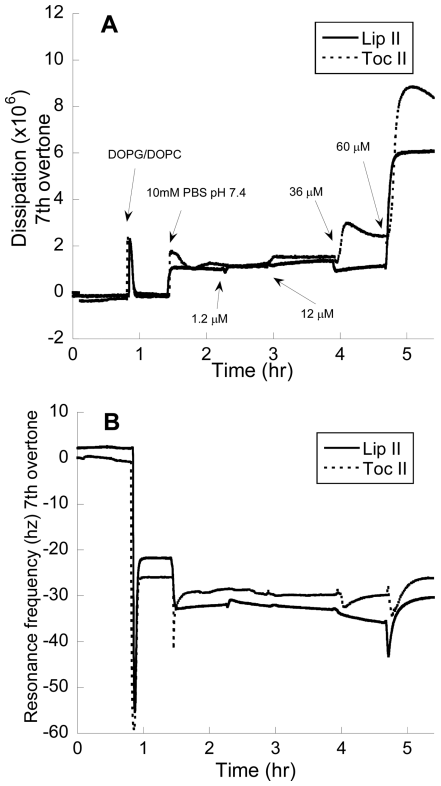

Figure 5. QCM-D of interaction of liprotides with supported lipid bilayers. Lip II or Toc II was injected onto preformed bilayers and interaction monitored through changes in (A) dissipation D or (B) resonance frequency f. Injection times and amounts of OA in the complex are marked with arrows.

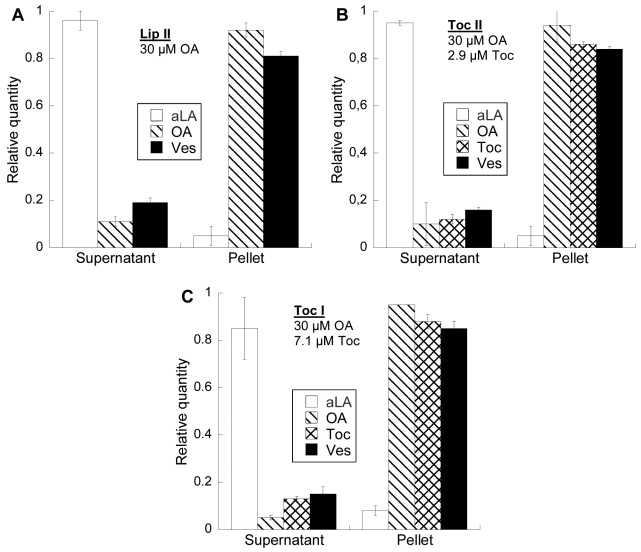

Figure 6. Amounts of aLA, OA, and Toc found in the supernatant and pellet after ultracentrifugation of vesicles incubated with (A) Lip II, (B) Toc II, or (C) Toc I. The concentration of OA in all complexes was 30 μM.

incubated with 100 nm large vesicles containing a 20:80 DOPG:DOPC molar ratio. Vesicles were spun down by ultracentrifugation, and the contents of both the pellet and the supernatant were determined by HPLC. When vesicles were spun down alone, 95% of the vesicles could be found in the pellet while only 5% remained in the supernatant (Figure S3 of the Supporting Information). Neither Toc II nor Lip II was spun down upon being centrifuged without vesicles present. aLA showed hardly any binding (3 ± 4%) upon being incubated together with the vesicles (Figure S3 of the Supporting Information), even though aLA had been depleted of Ca^{2+} and the resulting apo aLA is known to interact weakly with anionic vesicles.[31] This lack of binding by apo-aLA agrees well with the lack of interaction of apo-aLA with supported planar DOPC/DOPG bilayers by QCM-D (data not shown). Hardly any binding (<1%) was seen when Toc alone was incubated with vesicles (Figure S3 of the Supporting Information). In contrast, when Lip II was incubated with vesicles, 81 ± 2% of the vesicles were spun down, binding 92 ± 2% of OA and a negligible amount (5 ± 4%) of aLA (Figure 6). Thus, OA is transferred to the vesicles, while aLA is released after this transfer. Toc II and Toc I, like Lip II, transfer OA (94 ± 9 and 95 ± 1%, respectively) to the vesicles and are also capable of transferring Toc (86 ± 2 and 88 ± 1%, respectively) to the vesicles together with OA. The structure of Toc II and Toc I is somewhat different, and Toc I has a larger ratio of OA and Toc bound per aLA. However, this does not affect transfer of OA or Toc to vesicles.

Incorporation of Other Molecules into the Liprotide. The successful incorporation of Toc into the liprotide prompted us to attempt incorporation of other hydrophobic molecules into liprotides. Retinaldehyde (a precursor for vitamin A), the three free amino acids tryptophan, tyrosine, and histidine, and the four antibiotics erythromycin, ofloxacin, tetracyclin, and mupirocin were incubated with aLA and OA under the same conditions that were used for Toc. Separation by SEC resulted for all samples in elution profiles containing mainly two peaks eluting around the same times as Lip I and Lip II (Figure S4 of the Supporting Information). Analysis of these fractions by HPLC revealed that only retinaldehyde and tryptophan were incorporated into the complexes under these conditions (Table 1). Histidine, tyrosine, and the four antibiotics did not interact strongly enough with the liprotide to be incorporated. This reveals restrictions on what can be incorporated into liprotides. Generally, the ability to be incorporated into liprotides nicely follows the hydrophobicity of the individual compounds, as measured by their elution on an RP-HPLC column (Table 1). The only exception to the rule is mupirocin, which though significantly more hydrophobic than Trp (eluting at 74 and 35% acetonitrile, respectively) is unable to be taken up by liprotides. However, we note that mupirocin contains a long alkyl stretch sandwiched between two hydrophilic regions (a carboxylate and the polyketide monic acid). This distribution of hydrophilic and hydrophobic regions is likely to be penalized within the liprotide structure, because the hydrophobic micelle interior would favor structures with a uniformly hydrophobic terminus. In contrast, Trp has a relatively hydrophobic indole ring connected to a hydrophilic peptide backbone that easily partitions into the hydrophobic–hydrophilic interface.[32] Retinaldehyde and Toc are uniformly hydrophobic, and this is likely to promote complete insertion into the hydrophobic liprotide micelle interior.

The UV stability of retinaldehyde was increased upon incorporation into the liprotide compared to the free form (Figure S5 of the Supporting Information), showing that the liprotide in general can increase both stability and solubility of the molecules that are able to form complexes. The amount of OA bound in complexes incorporated with small molecules does not change markedly compared to that in the liprotide

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without any small molecules (Table 1). Knowing that only small changes are observed with SEC, we conclude that the structure of the liprotides is not significantly affected by the incorporation of small molecules.

DISCUSSION

We have shown that it is possible to form liprotides consisting of aLA and OA that can take up the hydrophobic vitamin E compound Toc, leading to both solubilization and stabilization of Toc. These new liprotides with Toc are similar in size to those formed without Toc (Figure 2) and share the same core–shell structure (Figure 3). This suggests that they will have similar interactions with membranes and follow similar ways of transferring OA content to membranes. Investigating the interaction of the liprotide membrane with QCM-D confirmed this to be the case, independent of whether Toc was bound in the complex (Figure 5). Furthermore, both OA and Toc were transferred to vesicles upon being incubated together, showing that the liprotide can be used to transport other molecules besides OA (Figure 6). The complex also protects Toc from exposure to light and air (Figure 4) and could thus potentially be a suitable carrier of Toc in aqueous systems, where Toc on its own shows poor solubility and stability.

The two populations of liprotides (Lip I and Lip II) differ in a number of ways, and the propensity to form one or the other is mainly determined by the amount of OA used in the preparation process. We have recently investigated the structure of Lip I in more detail and found that this liprotide consists of an additional layer of aLA and OA (Frislev et al., manuscript submitted for publication). By using an excess of OA, it seems to be possible to stabilize otherwise weak interactions that lead to a larger liprotide consisting of extra layers.

Both small and large liprotides (Toc I and Toc II) were able to bind Toc but showed different Toc binding affinity, with Toc I binding around 1 Toc per protein and Toc II around 5 Tocs per protein. This difference in binding affinity could very well be associated with the difference seen for the structure of Toc I and Toc II. Toc I has more OA per protein and potentially also contains more hydrophobic patches where Toc binding is possible. Toc is, however, also slightly less stable to UV when taken up in the Toc I complex compared to Toc II.

The structure of liprotides opens for the design of complexes that can solubilize otherwise poorly soluble hydrophobic molecules, thereby giving HAMLET and other liprotides functions that are different from their original use in cancer therapy. The successful incorporation of Toc and retinaldehyde into liprotides shows that liprotides have the potential to work as carriers of hydrophobic molecules and could potentially be useful in both the pharmaceutical and food industry. Alterations in the original liprotides have already been utilized in work within cancer therapy where aLA has been replaced with vitamin D-binding protein[33] and OA replaced with 2-hydroxyoleic acid.[34] Both alternations have been shown to improve the anticancer properties of HAMLET. Incorporation of a known anticancer molecule in such a molecule could potentially further enhance the anticancer properties originating from the liprotide. The incorporation of antibiotics in liprotides has the potential to be exploited in a similar way. The synergetic effect of using antibiotics together with HAMLET is already known[35] but might be further exploited if the antibiotics could be incorporated into the liprotide structure, thereby potentially increasing the solubility, stability, and transport of the antibiotic to a target cell. However, the antibiotics investigated in this study were not able to form a complex with aLA and OA, highlighting that not all small molecules are able to bind to liprotides. The three successful liprotide guests (retinaldehyde, Toc, and free tryptophan) are all highly hydrophobic and relatively small molecules. In addition to a high level of hydrophobicity, the poor incorporation of mupirocin suggests that a uniformly hydrophobic moiety is required to ensure uptake. These insights may provide some guidance in the selection of other compounds for uptake into liprotides in the future.

Several different small molecule-based delivery systems already exist to improve the solubility and stability of Toc, including nanoemulsions, nanostructured lipid carriers,[36] β-lactoglobulin gels,[37] monomeric β-lactoglobulin,[38] and a natural Toc binding protein called α-tocopherol transfer protein.[39] The liprotides have the advantage that they consist of molecules from simple and readily available food products (milk and oil) and can easily be prepared, making them natural candidates in nutritional food fortification. Because the components of the liprotides are natural food constituents, the liprotides are naturally very biocompatible. The liprotides protect Toc better than simple Toc binding proteins such as β-lactoglobulin[38] and α-tocopherol transfer protein[39] and combine this with a very simple preparation method. Nanoemulsions and nanostructured lipid carriers[36] show a high level of protection of Toc. These carriers show slow release of Toc in aqueous solution in contrast to the liprotides that are stable in aqueous solution but release Toc in contact with phospholipid membranes. The ability of the liprotides to transfer their content to phospholipid membranes has pros and cons. Cargo is easily delivered to membranes that are attractive targets for numerous drugs, but this transfer ability also reduces liprotide stability in complicated matrices with various interfaces.

ASSOCIATED CONTENT

Supporting Information

A calibration curve for the Superdex 200 column (Figure S1); stability of Lip I, Lip II, Toc I, and Toc II after re-injection on the column (Figure S2); quantification of the transfer of aLA, Toc, and lipids to vesicles measured by ultracentrifugation (Figure S3); size exclusion chromatogram of aLA-OA incubated with either Trp, His, Tyr, erythromycin, ofloxacin, tetracycline, mupirocin, or retinaldehyde (Figure S4); and stability of retinaldehyde when it is stabilized by liprotide (Figure S5). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00547.

AUTHOR INFORMATION

Corresponding Author

*Interdisciplinary Nanoscience Center (iNANO), Department of Molecular Biology and Genetics, Aarhus University, Gustav Wieds Vej 14, DK-8000 Aarhus C, Denmark. E-mail: dao@inano.au.dk. Telephone: (45) 20725238.

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■ ABBREVIATIONS

aLA, α-lactalbumin; apo-aLA, Ca^{2+}-depleted α-lactalbumin; *D*, energy dissipation; DOPC, 1,2-dioleoylphosphatidylcholine; DOPG, 1,2-dioleoylphosphatidylglycerol; ELOA, equine lysozyme in complex with oleic acid; *f*, resonance frequency; HAMLET, human α-lactalbumin made lethal to tumor cells; HPLC, high-performance liquid chromatography; liprotides, complexes between lipids and partially denatured proteins; LUV, large unilamellar vesicle; OA, oleate; PBS, phosphate-buffered saline; QCM-D, quartz crystal microbalance with dissipation; SAXS, small-angle X-ray scattering; SEC, size exclusion chromatography; SUV, small unilamellar vesicle; TFA, trifluoroacetic acid; Toc, α-tocopherol.

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