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Dynamic content exchange between liprotides

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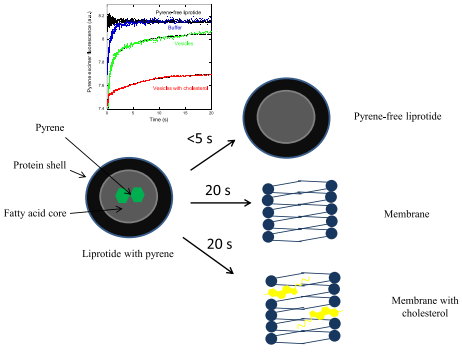
HIGHLIGHTS

- Lipids are exchanged between protein-lipid complexes (liprotides).
- Exchange of lipids between liprotides takes place within 5 s.
- Transfer of lipid from liprotides to membranes takes place within 20 s.

GRAPHICAL ABSTRACT



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ABSTRACT

Liprotides are complexes composed of partially denatured proteins and fatty acids in which the fatty acids form a micelle-like core surrounded by a shell of proteins. Liprotides, composed of α-lactalbumin (aLA) and oleic acid (OA), are similar in components and cytotoxicity to the original HAMLET protein-fatty acid complex. Liprotides composed of aLA and OA kill tumor cells by transferring the OA component to, and thus destabilizing, the cell membrane. Here we investigate liprotides' dynamics of transfer of contents between themselves and membranes using the hydrophobic fluorescent probe pyrene. We find that pyrene incorporated into liprotides is exchanged between liprotides within the dead time of a stopped-flow instrument, while the transfer to membranes occurs within 20 s. Transfer kinetics was not affected by the presence of the membrane stabilizing lipid cholesterol. Thus, transfer is a remarkably rapid process which illustrates liprotides' efficacy as transporters of hydrophobic compounds.

1. Introduction

A complex of α-lactalbumin (aLA) and oleic acid (OA), discovered in human milk in 1995 [1] and termed HAMLET (human α-lactalbumin made lethal to tumor cells), selectively kills tumor cells while leaving normal cells unaffected [1–3]. aLA is a 123-residue (14.2 kDa) milk protein whose native fold is stabilized by four disulfide bonds and one Ca^{2+} ion [4–6]. Release of Ca^{2+} favors binding of the hydrophobic monounsaturated omega 9-fatty acid OA [2,7]. HAMLET's cytotoxicity has been ascribed to the OA component, while the major function of aLA is to enhance the solubility of OA [8,9]. Protein-lipid complexes similar to HAMLET can be formed with a wide range of different

Abbreviations: aLA, α-lactalbumin; CMC, critical micelle concentration; DOPC, 1,2-dioleoylphosphatidylcholine; DOPG, 1,2-dioleoylphosphatidylglycerol; HAMLET, human α-lactalbumin made lethal to tumor cells; liprotides, complexes between lipids and partially denatured proteins; lip20, liprotide prepared at 20 °C; lip80, liprotide prepared at 80 °C; OA, oleic acid; SDS, sodium dodecyl sulfate

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proteins [8,10–12] and *cis* fatty acids [3,13,14] through a variety of preparation approaches [15–18]. Cytotoxicity depends on the fatty acid in question rather than the protein [14]. These complexes appear to have a common core-shell structure where the fatty acids are organized in a micelle-like core decorated with a shell of proteins [10,13] and have been termed liprotides. They are typically ~100 nm in diameter and contain 12–33 fatty acids and 2–4 proteins [10,13]. Recently we have shown that the OA component of aLA:OA liprotides is transferred to the plasma membrane of cells [13]. The incorporation of OA in membranes increases the fluidity and induces plasma membrane injuries which eventually kill the cell [13,19]. Not only OA can be transferred; liprotides can be used as encapsulation and delivery system of hydrophobic molecule such as vitamin D_3, α-tocopherol, tryptophan and retinaldehyde [20,21]. These hydrophobic molecules are transferred to membranes together with the fatty acid component [20,21].

To date there is little information available on the kinetics of these transfer processes. Here we address this aspect using the hydrophobic fluorescent probe pyrene, a fused four-ring aromatic compound whose fluorescence emission depends on the polarity of the environment. While monomeric pyrene's emission spectrum shows a collection of peaks around 370–400 nm, higher pyrene concentrations and the consequent spatial proximity between two pyrene molecules leads to a so-called excimer emission peak at 460 nm [22,23]. Conversely, dilution of pyrene will lead to abolition of this excimer peak. We exploit excimer formation to investigate exchange between liprotides and transfer of pyrene from liprotides to membranes. Pyrene is soluble in water up to ~1 μM and OA up to ~0.3 μM. Based on this parameter, pyrene and OA have comparable hydrophobicity, and we therefore assume that insights into pyrene transfer between liprotides and to membranes will also apply to OA.

We deploy liprotides prepared at 20 °C (lip20) and 80 °C (lip80). High liprotide preparation temperatures induces disulfide shuffling and a greater robustness against Ca^{2+}-induced dissolution of liprotides by refolding [20]; however, this has no effect on the dynamics of lipid content exchange. Liprotide exchange content within the dead time of a stopped-flow instrument, while transferring content to membranes within < 20 s independent of the presence of cholesterol in the membrane. This rapidity illustrates the usefulness of liprotides for transport and transfer of otherwise intractable hydrophobic compounds.

2. Materials and methods

2.1. Materials

Ca^{2+} depleted α-lactalbumin from bovine milk (aLA, ≥85%, L6010), sodium oleate (OA, ≥95% pure, O3880), pyrene (≥98%, P2146), sodium dodecyl sulfate (SDS, ≥99%, 71,725), cholesterol (≥99%, C8667) and chemicals for buffer solutions were from Sigma-Aldrich (St. Louis, MO). aLA was suspended in Milli-Q water and dialyzed against Milli-Q water for 24 h at 4 °C. 1,2-Dioleoylphosphatidylcholine (DOPC, 261584) and 1,2-dioleoylphosphatidylglycerol (DOPG, 209403) lipids were supplied as dry powder from Avanti Polar Lipids (Alabaster, AL).

2.2. Liprotide preparation

aLA and OA were mixed at a molar aLA:OA ratio of 1:12 in 50 mM PBS (50 mM $NaHPO_4$, pH 7.4, 150 mM NaCl) and incubated 1 h at 20 °C or 80 °C, leading to lip20 and lip80, respectively. At this molar ratio most of the liprotides form a simple core-shell structure [20].

2.3. Formation of liprotides in complex with pyrene

Preformed lip20 and lip80 were incubated 1 h at room temperature with different amounts of pyrene (dissolved to 5 mM in 96% EtOH).

2.4. Vesicle preparation

10 mg/ml stock solution of vesicles (either 80% DOPC and 20% DOPG or 65% DOPC, 15% DOPG and 25% cholesterol) were prepared by weighing off the lipid and dissolving them in chloroform. Chloroform was evaporated under a stream of nitrogen gas yielding a thin lipid film, which was dissolved in 50 mM PBS and mixed by vortexing. Vesicles were subjected to 10 cycles of freezing in liquid nitrogen and thawing in a 50 °C water bath, after which the lipid solution was extruded through a filter with a pore size of 100 nm. Vesicle solutions were stored at 4 °C and used within a week.

2.5. Pyrene fluorescence measurements

Pyrene fluorescence spectra were recorded on an LS-55 luminescence spectrometer (Perkin Elmer, Waltham, MA) at ambient temperature with excitation at 335 nm and emission from 360 to 500 nm using a slit width of 2.5 nm, scan speed of 200 nm/min and 2 repeats. The cuvette used was a 3.0 mm quartz cuvette. For experiments where liprotides with pyrene were mixed with different amounts of liprotides or vesicles without pyrene, samples were incubated 1 h before measurements. Concentration of lip20 and lip80 used was 35 μM (units of aLA concentration).

2.6. Poisson distribution

A Poisson distribution was used to assess the distribution of pyrene molecules in liprotides:

$$P(x;\mu) = (e^{-\mu})(\mu^x)/x! \quad (1)$$

where μ is the average number of pyrene molecules per liprotide/micelle and x is a given number of pyrene molecules per liprotide.

2.7. Stopped-flow

To determine the rate of exchange between liprotides or between liprotides and vesicles, pyrene fluorescence changes were followed by stopped-flow using a Chirascan spectrometer with stopped-flow accessory (Applied Photophysics, Leatherhead, Surrey, UK) equipped with a xenon lamp. The excitation wavelength used was 335 nm with 2 nm bandwidth and emission was measured with a 455 nm cut-off filter. Solutions were mixed at a 1:1 volume ratio. 35 μM lip20 (units of aLA concentration) containing 10 μM pyrene was mixed with either 35 μM lip20, PBS or 0.6 mM vesicles with or without cholesterol. Data were analyzed using Kaleidagraph version 4.0 (Synergy). Signals were fitted either to single (liprotides into buffer), double (liprotides into cholesterol-vesicles) or triple (liprotides into cholesterol-free vesicles) exponential decay functions: $Signal(t) = Offset + \Sigma_i Amp_i e^{-k_i} * t$ where i denotes the individual exponential decay with rate constant k_i and Amp_i is the amplitude associated with this decay.

3. Results

3.1. Pyrene is solubilized by lip20 and lip80 and forms excimers

The objective of this study was to investigate liprotides' kinetics of exchange of their hydrophobic contents and whether this is affected by the type of liprotide used. Initially we ascertained that pyrene could be incorporated into liprotides. 35 μM lip20 and lip80 (units of aLA concentration) were mixed with different pyrene concentrations ranging from 0 to 20 μM (Fig. 1ab). The intensity of the pyrene emission spectrum increases with increasing concentration of pyrene for both lip20 and lip80. At a pyrene concentration > 7 μM, a clear excimer peak at 460 nm is observed for both liprotides. At concentrations higher than 20 μM the excimer peak starts to decrease (data not shown), an effect we attribute to quenching of numerous pyrenes when