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Digitonin does not flip across cholesterol-poor membranes

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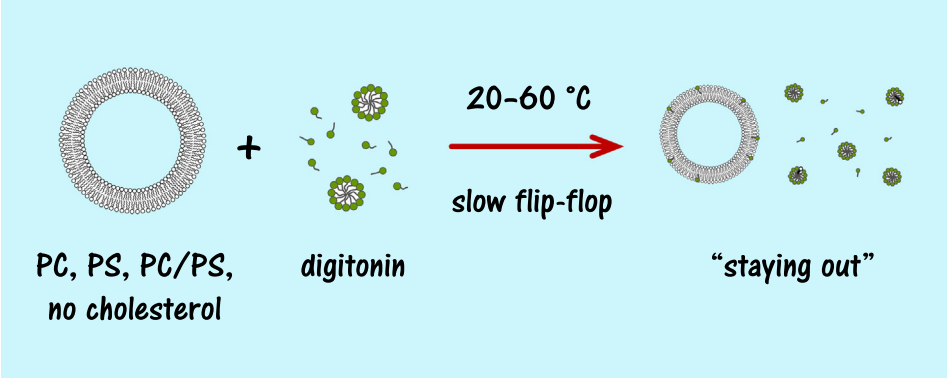
GRAPHICAL ABSTRACT



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ABSTRACT

Digitonin is commonly used to permeabilize cell membranes and solubilize membrane components. It interacts specifically with cholesterol in the membrane which leads to the formation of pores. Thus far, the mechanism by which digitonin interacts with the membrane has only been described qualitatively. We investigated this interaction in model membranes that contain little or no cholesterol with a combination of isothermal titration calorimetry, dynamic light scattering, and zeta potential measurements. Digitonin partitions fully asymmetrically into large unilamellar vesicles of phosphocholine (PC) lipid at 20 °C (remaining in the outer leaflet only), with a partition coefficient of 0.22 ± 0.04 mM^{-1} and ΔH of partitioning of 23.3 ± 1.6 kJ mol^{-1}. Beyond a digitonin/lipid ratio of ~0.1 in the outer leaflet, digitonin micelles coexist with vesicles without solubilizing them—even at high digitonin concentrations. This "staying out" of digitonin was also observed with phosphoserine (PS), PC/PS, and PC/PS/cholesterol vesicles. The mechanism by which digitonin perturbs and solubilizes the membrane is very different when the membrane contains little or no cholesterol as opposed to 20–30 mol% cholesterol. The role of digitonin should thus be carefully considered in the design of preparative protocols and experiments in studies of cellular processes and membrane proteins.

1. Introduction

Digitonin, a saponin extracted from foxglove, has long been used in biotechnology for its mild detergency. As a membrane-active compound, it has been found to act synergistically with

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doxorubicin against multi-drug resistant cancer cells [1]. Like many detergents, digitonin has a head–tail structure: its bulky sugar headgroup consists of two galactoses, two glucoses, and a xylose; its rigid sterol tail loosely resembles cholesterol. Digitonin self-assembles into micelles at low concentrations, with an aggregation number of ~60 [2]; it solubilizes rhodopsin by forming a belt of ~180 molecules around the protein [3]. Despite the long history of its usage, interesting properties are still being discovered. For example, it was recently shown that the presence of a strong base significantly enhances the fluorescence of digitonin solutions at concentrations above its critical micellar concentration (CMC) [4].

Digitonin has primarily been used to permeabilize cell membranes and extract/solubilize membrane components. It induces pores in the plasma membrane which allow for the permeation of peptides and even antibodies into cells [5,6]. Digitonin selectively permeabilizes the plasma membrane which has enabled numerous studies on protein phosphorylation [5], nuclear protein transport [7], lipid metabolism [6], and even light adaptation in photosynthetic bacteria [8]. It has also enabled the separation of cytosol from nuclear and plasma membranes [9] and studies on the budding of influenza virus in mammalian cells [10]. Although digitonin is more effective against plasma membranes, it has been used to permeabilize outer mitochondrial membranes [11] for studies on mitochondrial import of RNA [12]. Its effective permeabilization of the membrane depends to a great extent [13,14] but not exclusively [15] on the sugars in its headgroup.

Increasing the fraction of digitonin in biological membranes progressively perturbs, permeabilizes and finally solubilizes the membranes. As such, it has been used to extract tobacco cell membranes for the *in vitro* synthesis of cellulose [16]. Digitonin also efficiently extracts membrane proteins such as supercomplexes from the mitochondrial inner membrane [17–19], T-cell antigen receptors [20,21], and P2Y receptors [22]. Furthermore, it has been shown to support cell-free synthesis of membrane proteins (e.g., *Acetabularia* rhodopsin II [23]).

The main cause for digitonin's permeabilization of biological membranes is its well-known interaction with cholesterol. It has been observed that digitonin (i) partitions more extensively into cholesterol-containing lipid monolayers and bilayers [24], (ii) induces leakage in eggPC liposomes with 50 mol% cholesterol at two orders of magnitude lower concentration than in those without cholesterol [25], (iii) counteracts (somewhat) the effect of cholesterol on the phase transition of DPPC [13], (iv) forms hemitubules and co-precipitates with cholesterol [26], and (v) selectively causes cholesterol-containing POPC giant unilamellar vesicles (GUVs) to collapse [27]. Digitonin induces phase separation by replacing cholesterol-lipid contacts in the membrane [28] but does not greatly decrease membrane order as suggested by anisotropy experiments with brain cortex membrane extracts [29].

In the absence of cholesterol, digitonin has a much weaker interaction with the membrane; it has been observed to have no macroscopic effect on large unilamellar vesicles (LUVs) and GUVs [28]. Neutron reflectivity experiments with supported lipid bilayers (20 mol% cholesterol in lethicin) showed that digitonin's sterol tail does penetrate the hydrophobic portion of the membrane [28]. According to ESR studies with spin-labeled PC in eggPC liposomes, digitonin partitions with an ordering effect similar to that of cholesterol [13]; our earlier anisotropy experiments with POPC LUVs also demonstrated little membrane disordering by digitonin [30].

The current work clarifies interactions between digitonin and phospholipids *in the absence* of cholesterol. We specifically address two questions: (1) How strong is the partitioning of digitonin into a membrane? (2) Does digitonin interact with lipids like other detergents? We use isothermal titration calorimetry (ITC) to first quantify the thermodynamics of micelle formation of digitonin, and then to quantify digitonin-lipid interactions in conjunction with dynamic light scattering (DLS). Both techniques are commonly used in studies of detergent-lipid interactions [31].

2. Materials and methods

2.1. Materials

All solutions were made in HEPES buffer (20 mM HEPES, 120 mM NaCl, 0.8 mM EDTA, adjusted to pH 8.0 with KOH) using Millipore water. Cholesterol was obtained from BioShop Canada (Burlington, ON). Buffer components were purchased from BioShop Canada and Sigma Aldrich (Oakville, ON) at >99.5% purity. Digitonin was purchased from Wako Chemicals USA (Richmond, VA). Digitonin solutions were prepared gravimetrically and sonicated at low power for 5 min to ensure complete dissolution. The highest concentration prepared was 30 mM. Solutions were used within two days of preparation.

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) were purchased from Avanti Polar Lipids (Alabaster, AL). Liposomes were prepared by extrusion as previously described elsewhere [32]. Briefly, solutions of the phospholipid in chloroform were dried under nitrogen and subsequently in a vacuum chamber overnight. The resulting lipid films were rehydrated with buffer, vortexed, and subjected to 8 freeze-thaw cycles. Liposomes of 100 nm diameter were extruded over 15 passes at room temperature, through Nuclepore polycarbonate filters (Whatman, GE Healthcare) in a LIPEX extruder (Northern Lipids, Burnaby BC). The size of the extruded liposomes was confirmed by dynamic light scattering on a Nano ZS Zetasizer (Malvern Instruments Ltd., UK).

2.2. ITC demicellization experiments

Demicellization experiments were performed as previously described elsewhere [33,34]. Relevant details are briefly described here and in the Results section. For each titration experiment, a digitonin solution was loaded into the injection syringe and its matching buffer into the sample cell. Injections began at 1 μL and gradually increased to 10 μL in 1 μL increments. Two titration experiments were performed to obtain each complete demicellization profile: one at a low syringe concentration of ~6–7 mM digitonin and the other at a high syringe concentration of ~27–30 mM digitonin. Demicellization experiments were performed at 15 °C, 20 °C, 25 °C, 35 °C, 42 °C, and 50 °C. All experiments were triplicated at minimum.

2.3. ITC solubilization experiments and DLS

Various concentrations of extruded POPC LUVs (and those with other lipid compositions) were prepared gravimetrically. The samples were measured in the Zetasizer at 20 °C prior to cell filling. Digitonin solutions at ~30 mM were loaded into the injection syringe. The stirring speed was 307 rpm for all experiments. Injections began at 1 μL and gradually increased to 10 μL in small increments. Injection spacing was increased from 500 s to 900 s. Immediately after the completion of the titrations, cell contents were extracted and measured again in the Zetasizer at 20 °C. Representative samples were then subjected to 2 h of heat treatment (HT) under nitrogen at 65 °C. DLS measurements were again made, at 65 °C or 20 °C or both. Since POPC is diluted over the course of the titration experiment, post-ITC and HT derived count rates (DCR) were adjusted to reflect similar lipid concentrations as