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Study of the miscibility of cholesteryl oleate in a matrix of ceramide, cholesterol and fatty acid

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ABSTRACT

Cholesteryl esters (CE) are not generally abundant but are ubiquitous in living organisms and have markedly different properties from cholesterol because of their acyl chain. The miscibility/immiscibility of CE with biological lipid structures is a key property for their functions. In this work we study the solubility of cholesteryl oleate (ChO) in a model of the stratum corneum lipid matrix composed of ceramide C16, cholesterol and palmitic acid in excess water. Experiments were done in conditions of fully ionized (pH = 9.0) and fully neutralized fatty acid (pH = 4.0), and differential scanning calorimetry of the ternary mixtures with added ChO at pH = 9.0 clearly displayed a main transition with the same maximum temperature, peak shape, and enthalpy, suggesting that ChO was excluded from the remaining lipids. This technique is not conclusive at pH = 4.0 because the transitions of the lipid matrix and ChO overlap. The insolubility of ChO at both pH values is supported by X-ray diffraction. Adding the ceramide:cholesterol:fatty acid lipid mixture to ChO did not change the X-ray pattern of the mixture nor that of the ChO. To supplement the above physical techniques, we applied ^{13}C MAS NMR spectroscopy with C-13 carbonyl-labeled ChO. A single ^{13}C carbonyl peak from the ChO at 171.5 ppm was observed, indicating exposure to only one environment. The chemical shift was identical to pure ChO below and above the temperature of isotropic liquid formation. Taken together, our results lead to the conclusion that the solubility of ChO is negligible in the ceramide:cholesterol:fatty acid lipid mixture.

1. Introduction

Cholesteryl esters, CE, are a class of lipidic molecules often found in biological systems. In fact, cholesterol is transported in the blood stream, and stored in living cells mainly in the form of esters. Most attention to the physical properties of CE has been focused on their bulk properties in the oily and fluid phase in lipoprotein cores and lipid-rich domains of plaques (Small, 1988). More recent studies have investigated whether and how these weakly polar lipids fit into membrane bilayers. Several cholesteryl esters have a very low solubility in phosphatidylcholine (Janiak et al., 1979; Salmon and Hamilton, 1995) and bovine brain sphingomyelin bilayers (Mackay et al., 1980). The CE have a precise conformation in the bilayer with the carbonyl at the aqueous interface, and their limited solubility in this conformation is diminished with high levels of cholesterol (Mackay et al., 1980; Spooner et al., 1986).

Much less attention has been paid to the interaction of cholesteryl esters with ceramides, alone or in lipid mixtures, in particular those found in the stratum corneum (SC). The SC protects mammalians from external xenobiotic aggression and restrains the loss of internal constituents. It is a composite structure mainly made of a network of interconnected dead hydrophilic cell bodies with the voids between them filled by lipids and water. The main lipid components are ceramides, cholesterol and long chain saturated fatty acids, roughly in 9:5:2 weight proportions (Wertz and Norlén, 2004).

Ceramide alone, or ceramide:cholesterol aggregates, form very rigid crystalline lamellar phases in equilibrium with low interbilayer water content (Shah et al., 1995; Souza et al., 2009). Cholesterol forms stoichiometric aggregates with C16-Cer in the

Abbreviations: C16-Cer, N-palmitoyl-D-*erythro*-sphingosine; Ch, cholesterol; ChO, cholesteryl oleate; egg-Cer, natural ceramide of egg origin; PA, palmitic acid; SC, stratum corneum.

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L_C phase and is miscible with the L_α phase until at least 75 mol% in both cases (Souza et al., 2009), which is even higher than in phosphocholine bilayers ~50–60 mol% (Huang et al., 1999). Another particularity of the ceramide–cholesterol systems is the difficulty to incorporate external components once formed, for example additional cholesterol. To incorporate Ch into Cer 16 in vitro, it is mandatory that the mixture is made prior to the bilayer-assembling step (Huang et al., 1999; Souza et al., 2009).

Cholesteryl esters are considered by some investigators to be minor components of the SC, but several laboratories have found such esters to comprise as much as 18–19% by weight of the SC lipids (the sum of *ChO*, Cer, Ch and FA) (Bonte et al., 1997; Norlen et al., 1999; Wertz et al., 1987). The origin of the CE found in the analysis of the SC lipids is not consensual. While some sustain that they are sebum contaminants, e.g. (Elias, 2005), others present experimental evidence that seem to support that they are constituents of the SC lipid matrix (Wertz et al., 1987; Pappinen et al., 2008). For the present study the origin of CE is irrelevant since, being in direct contact with the SC lipid matrix, only its solubility will dictate if it mixes with the remaining lipids.

In the present work we examine the solubility of cholesteryl oleate (*ChO*), in a system containing N-palmitoyl-D-erythro-sphingosine (C16-Cer) with cholesterol and palmitic acid, in molar proportions 44:38:18, composition hereafter designated as the Cer:Ch:FA matrix. Cholesteryl oleate was chosen because it has been identified as the most abundant cholesteryl ester in the stratum corneum (Wertz et al., 1987). The particular proportion of ceramide, cholesterol and fatty acid used is approximately that found in the SC (Wertz and Norlén, 2004) and similar ternary mixtures have often been used as models to study the physical–chemical properties of the SC lipid matrix (for a review see (Kessner et al., 2008)). Despite the importance of the information obtained with these systems they do not mimic the natural tissue due to three main reasons: (i) the ceramide is non-hydroxylated while in the SC ca. 70 wt.% of the ceramides are hydroxylated (Wertz and Norlén, 2004), (ii) the lipids in the SC are arranged in a thick phase (ca. 13 nm) (Bouwstra et al., 1991; Elias, 1991; Swartzendruber et al., 1989; White et al., 1988) that is not observed with these mixtures, and (iii) the SC, as a multicomponent system, may not have the same mixing properties of a ternary mixture, e.g. (Janssens et al., 2009). Despite the enumerated limitations, including the use of a palmitoyl ceramide, which is not the more abundant component of the CER NS class (SC nomenclature), useful information can be obtained concerning ChO solubility with the system tested. The short chain length of the ceramide is probably not a concern since their properties do not depend significantly from the hydrophobic tail length (Chen et al., 2000). Palmitic acid has been frequently used as a generic fatty acid and it is particularly adequate for our study due to the importance of using a chain length that matches the ceramide (Chen et al., 2007).

Our strategy was to use several biophysical methods to study this complex model system because one single analytical technique would be insufficient. In a ternary system, several phases may coexist and the solubility of ChO could differ from one phase to another. X-ray diffraction was used to discriminate structures and potential structural alterations with CE, DSC was used to monitor changes in the thermotropic behavior of the components upon mixing, and ^{13}C NMR to discriminate if the carbonyl group reports different environments when alone and when the Cer:Ch:FA lipid matrix is present.

Because the ionization state of the fatty acid could modify the physical–chemical properties of the lipid mixture as well as affect its capacity to incorporate ChO, we characterized the interaction of *ChO* with our model lipid system at both pH = 9.0 (fully ionized) and 4.0 (fully protonated). At pH 9.0 we took advantage of the formation of a single 2D lamellar crystalline phase for the mixture Cer:Ch:PA in the 44:38:18 molar ratio, with the consequent congruent melting, that simplifies the detection of non-dissolved ChO. However, at pH 4.0 the scenario is more complicated and the analysis of the SAXS and WAXS of the system reveals the presence of three coexisting phases at low temperature, two L_C and one L_α, as will be shown in an independent publication. Published data of equimolar ternary mixtures based on bovine brain ceramide propose two rigid phases together with non-incorporated PA and Ch (Bouwstra et al., 1997). Other studies using synthetic non-hydroxy C16 ceramide report the coexistence of rigid and possibly liquid phases at pH = 5.2 (Brief et al., 2009). The solubility is determined by the characteristics of the lipidic phases and since no other phases are observed at intermediate pH the conclusion attained for the two pH tested is valid for all the 4.0–9.0 pH range.

2. Materials and methods

2.1. General reagents

Ceramides used were obtained from Avanti Polar Lipids (Birmingham, AL, USA), a synthetic ceramide (2S, 3R, 4E)-2-hexadecanoylaminooctadec-4-ene-1,3-diol (N-palmitoyl-D-erythro-sphingosine), and a natural ceramide of egg origin (egg-Cer) which is 84% C16 non-hydroxylated ceramide according to Avanti specification. Sodium azide, succinic and palmitic acids, cholesterol and cholesteryl oleate from Sigma (Sintra, Portugal), benzene was from Panreac (Barcelona, Spain), methanol and chloroform, both HPLC grade, sodium chloride, sodium hydroxide and disodium salt of ethylenediaminetetraacetic acid (EDTA) were from Merck (Darmstadt, Germany), and boric acid from Riedel-de-Haën (Seelze, Germany). All chemicals were used without further purification. Water for buffer preparation was double distilled and further purified with an Elgastat UHQ-PS system (Marlow, U.K.). Buffers were borate for pH = 9.0 with 100 mM ionic strength (Na^+) and succinic acid for pH = 4.0 also 100 mM.

2.2. Synthesis of labeled ChO

^{13}C carbonyl-labeled ChO, was synthesized according to procedure of direct esterification developed by Sripada (1988). Following this method, cholesterol was condensed with [1-^{13}C]-oleic acid at 35–40 °C in the presence of dimethylaminopyridine and dicyclohexylcarbodiimide (reagents from Aldrich, Steinheim, Germany) in anhydrous chloroform. The reaction was stopped after 1.5 h, and the product purified by silica gel column chromatography and purity was verified by TLC. A single carbonyl carbon signal was observed by NMR for the pure ChO, as expected.

2.3. Preparation of dispersions

The molar proportion of ceramide, cholesterol and fatty acid we used, 44:38:18, was derived from the conversion of the weight percentages 57.3% Cer:29.3% Ch:13.4% FA (Norlen et al., 1999) into molar fractions based on the C24 chain ceramide and corresponding fatty acid. Among the large range of published compositions for the SC lipid matrix, we chose this composition on the basis of carefully reviewed data (Wertz and Norlén, 2004) showing that at least three independent research groups, using two different techniques, attained very similar compositions (Bonte et al., 1997; Norlen et al., 1999; Wertz et al., 1987). Determinations that appeared after the cited review that we are aware of (De Paepe et al., 2004; Pappinen et al., 2008; Merle et al., 2010), proposing relations of Cer:Ch:FA of ca. 19:49:32, 38:34:28 and 48:22:30, respectively, are very disperse and do not change the conclusions presented by Wertz and Norlén. Except for DSC, in which egg ceramide was used, the experiments were done with synthetic C16 ceramide. Lipid stock solutions in