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The triolein/aqueous interface and lipase activity studied by spectroscopic ellipsometry and coarse grained simulations

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ABSTRACT

In spite of the importance of the triglyceride aqueous interface for processes like emulsification, surfactant interactions and lipase activity, relatively little is known about this interface compared to that between alkanes and water. Here, the contact between triolein and water was investigated in terms of water inclusion in the oil phase and orientation of the molecules at the interface. Coarse grained models of triglycerides in contact with water were constructed and correlated with experimental results of the changes in thickness and refractive index, obtained using spectroscopic ellipsometry of spin-coated triolein films. The topography of the layer was revealed by atomic force microscopy. Dry triolein and a triolein sample after equilibration with water were also compared structurally using small-angle X-ray scattering. Additionally, the kinetics of adsorption/activity of three different variants of the *Thermomyces lanuginosus* lipase (TLL) were investigated. The results show that uptake of water in the triolein phase leads to increase in thickness of the layer. The observed increase of thickness was further enhanced by an active lipase but reduced when an inactive mutant of the enzyme was applied.

1. Introduction

Triglycerides are the major component of vegetable oils as well as body fat and play an important role in energy supply (Freedman et al., 1984; St-Onge and Jones, 2002). Usually, these lipid systems are in contact with water in biological or application systems, such as food and pharmaceutical formulations. Preparation of films of such lipids and exposing them to an aqueous environment is of particular interest since it allows for mimicking of biological processes such as the action of lipases.

The molecular structure of triglyceride layers has been investigated by means of mono- and multimolecular films. Early studies on triglycerides in contact with water have proposed a model in which all molecules in a monolayer are arranged with the hydrophilic groups at the water interface and all chains point in the same direction (E-type), whereas different molecular conformations coexist in a multilayer. This multilayer was described as a combination of a monolayer and a centrosymmetric orientation of molecules in a tuning-fork form in the upper layers. As suggested, the different orientation in the upper layers is due to the large steric hindrance against the conformation of the E-type model (Bursh et al., 1968; Merker and Daubert, 1964). The orientation of triglycerides in the E-type conformation was further confirmed by (Hamilton, 1989) for tripalmitin and triolein at oil/water interfaces and by (Claesson et al., 1997) for triolein in contact with mica. Surface pressure-area curves of triolein mixtures were further analyzed by (Nakagaki and Funasaki, 1974) and showed a collapse of the triolein monolayer at a force of roughly 12 mN/m and a surface area of 100 $Å^2$/molecule.

The wetting of different solid substrates by triglycerides, i.e. tributyrin, tricaprylin and triolein, was studied by (Michalski and Saramago, 2000) to estimate the stability of triglycerides layers. The sessile drop and the captive bubble methods were used to assess the wetting behavior. On hydrophobic surfaces the triglyceride films collapsed after a certain given time, which increased with the triglyceride viscosity. Stable triglyceride films were observed on a short time scale for hydrophilic solids.

Disjoining pressure isotherms for these triglyceride films were measured by (Vazquez et al., 2006) and showed that all triglycerides form meta-stable films on both kinds of substrates. The film stability was higher on hydrophilic substrates and increased with the decrease of

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the chain lengths of the triglyceride molecules. For film rupture, two possible mechanisms were outlined: the hole nucleation and the spinodal dewetting (Brochard Wyart and Daillant, 1990; Schulze et al., 2001). For film thicknesses higher than a critical value, the rupture just occurs through nucleation of holes. Films with a thickness under the critical value are unstable against spinodal decomposition, which leads to a breakage into microscopic droplets due to a spontaneous growth of small undulations. To gain more information on triglyceride films, especially in an aqueous environment we used spectroscopic ellipsometry to study interfacial phenomena at the triolein/water interface.

Ellipsometry has turned out to be a useful tool for investigating adsorption processes at lipid/water interfaces (Benjamins et al., 2002; Benjamins et al., 2005a, b; Binks et al., 2003; Day et al., 2010; Kapilashrami et al., 2003; Reis et al., 2008; Russev et al., 2000; Wadsäter et al., 2013). We have shown qualitative and quantitative ways to measure the adsorption of nonionic surfactants at a decane/water interface (Benjamins et al., 2005a). The competitive adsorption of milk proteins and nonionic surfactants to a hexadecane/water interface was studied by (Day et al., 2010).

Triglycerides exhibit a complex behavior on a molecular level and to supplement experimental physico-chemical and analytical techniques, theoretical approaches using atomistic molecular dynamics and coarse grained model simulations have been applied (Brasiello et al., 2011; Hall et al., 2008). In order to probe time and length scales not readily available by employing molecular dynamic simulations, we used coarse grained models to simulate triglyceride layers. Molecular dynamics simulations for the system studied here would require so high computational capacity and time so it is in practice not feasible (Brasiello et al., 2012).

Earlier a coarse-grain model of a triglyceride were used for simulation of a lipid exchange mechanism of the cholesteryl ester transfer protein (Koivuniemi et al., 2012)—and in another study, for analysis of triglyceride blisters in lipid bilayers (Khandelia et al., 2010).

The aim of the present study is to reveal the changes that can occur when a triglyceride (TG), e.g. triolein, is exposed to an aqueous environment. This is achieved by combining coarse grained simulations with spectroscopic ellipsometry measurements and small angle X-ray scattering. For the ellipsometry and AFM studies, the triglyceride was coated on a substrate and placed in an aqueous solution. In the result and discussion sections we also present AFM and ellipsometry results of triglyceride layers both in air and in water. These results were used to find a model and the right conditions for our kinetic studies of neat oil and oil exposed to three different variants of the *Thermomyces lanuginosus* lipase (TLL), including active wild type, inactive mutant and a mutant for which the activity can be switched on. In order to gain further insight on the molecular mechanisms as a complement to the experimental studies, we conducted theoretical studies using coarse grained (CG) simulations.

2. Materials and methods

2.1. Sample preparation

All chemicals were used without any further purification. The water used in the experiments was ultra purified (Ω = 18.2 ohm at 25 °C) by a Millip0re water purification system. All the films were formed on hydrophilic, polished and thermally oxidized silicon wafers with a 300 Å thick SiO_2 layer. First, the substrates were cut in 1 cm^2 pieces and cleaned in a base and an acidic mixture, both at a temperature of 80 °C for 5 min (Wadsäter et al., 2013). The base mixture was made of NH_4OH (25 %, Merck), H_2O_2 (30 %, Honeywell) and H_2O (1/1/5/by volume) and the acidic mixture of HCl (37 %, Merck), H_2O_2 and H_2O (1/1/5/by volume). After the cleaning process, the wafers were rinsed with water and stored in ethanol (99.7 %, Solveco). The polystyrene layer was formed by spin-coating a toluene-polystyrene solution (99.8 %, Fluka; Mw ~ 192,000 Aldrich, received as pallets) onto the cleaned wafer using a LabSpin Spin Coater (Suss Micro Tec Lithography GmbH). An amount of 35 µL was used to cover the whole surface, followed by 5 s of rotation at 6000 rpm. The concentration of polystyrene was varied from 0.5 to 11 wt%. (See Supporting information, Fig. S1). The triolein films were prepared by spin coating as well as hexane evaporation. For spin coating, 50 µL of triolein was added on top of the polystyrene layer. The substrates were first rotated 5 s at 1000 rpm followed by 5 s at 4000 rpm. A rotation speed of 8000 rpm for different periods of time was used. For the procedure involving evaporation from hexane (99 %, Fluka) solutions of 0.1 mM and 2 mM triolein were prepared. Various amounts, ranging from 10 to 100 µL, were used to obtain lipid films of different thicknesses. To ensure a slow and controlled evaporation process, the substrate was placed in a small closed box. Before measuring kinetic of the thickness increase when incubated in water, the coated layers were stored for at least 12 h in a freezer at −20 °C. This reduced the amount of lipid removed when the frozen lipid layer was transferred through the air-water interface to be immersed in the cuvette. The lipase assay was performed in 100 mM Tris (99.9 %, SIGMA), 1 mM $CaCl_2$ (99 %, Merck) and with a lipase concentration of 2 ppm (per volume of the buffer solution). The lipase stock solution was slowly injected into the solution close to the lipid layer. Three different variants of the *Thermomyces lanuginosus* lipase, which differ only in the lid region, were used, the wild-type (WT-TLL,), the inactive form (S146A-TLL,) and the locked form (C86C255-TLL, where the lid is closed with a disulfide bond between C86 and C255(Skjold-Jørgensen et al., 2017).These enzyme variants were purified according to previous protocols using hydrophobic interaction and anion exchange chromatography methods (Hedin et al., 2002; Skjold-Jørgensen et al., 2014). The purity of the samples was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) All samples were buffer-exchanged and concentrated in 50 mM 3-propanesulfonic acid (mOPS) (pH 7.5) using centrigugal filter units (Ultracel–10 K, Millipore).

2.2. Thermal stability

A thermal stability assay was carried out as previously described (Crowther et al., 2009; Skjold-Jørgensen et al., 2014). The fluorescent dye, SYPRO Orange (SO) was diluted 250 times in 50 mM MOPS (pH 7.5). 15 µL dye solution was added to an equal volume of lipase sample (10 µM). Melting curves were determined (using StepOnePlus Real Time PCR System (Applied Biosystems)) running a temperature gradient from 25 °C to 96 °C at a scan rate of 76 °C/h with an initial 15 min reaction time at 25 °C (See Supporting information, Fig. S2).

2.3. Spectroscopic ellipsometry, SE

The ellipsometry measurements were performed on an UVISEL spectroscopic ellipsometer (HORIBA Jobin Yvon) using an angle of incident (AOI) of 70°, a modulator angle of 0° and an analyzer angle of 45°. Data was recorded at room temperature (~22 °C) in the range of 250–770 nm. Data analysis was done in the DeltaPsi2 software. For the theoretical model, software default values of the optical constants for the silicon crystal, the silicon oxide and the polystyrene layers were used. The optical constants for the lipid layer and the buffer solution were determined using a refractometer (Abbe 60/ED, Bellingham + Stanley Ltd) and Cauchy's equation was applied to account for the wavelength dependence of the refractive index. At first, the silicon oxide layer of the cleaned substrates was characterized prior to deposition of the polystyrene and the lipid film. The obtained thicknesses for the previous layers were kept fixed in the analysis of the subsequently deposited layer properties.

For the kinetic measurements, the substrates, with size of 1.0 ± 0.15 cm, were glued to the bottom of the liquid cell using double-sided tape. No release of surface-active material from the tape could be observed. The windows of the Teflon liquid cell (~7 mL) were