Article:
Torcello-Gómez, MA, Boudard, C and Mackie, AR orcid.org/0000-0002-5681-0593 (2018) Calcium Alters the Interfacial Organization of Hydrolyzed Lipids during Intestinal Digestion. Langmuir, 34 (25). pp. 7536-7544. ISSN 0743-7463

https://doi.org/10.1021/acs.langmuir.8b00841

LANGMUIR

Biological and Environmental Phenomena at the Interface

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Langmuir, **Just Accepted Manuscript** • DOI: 10.1021/acs.langmuir.8b00841 • Publication Date (Web): 05 Jun 2018

ACS Publications

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

CALCIUM ALTERS THE INTERFACIAL ORGANISATION OF HYDROLYSED LIPIDS DURING INTESTINAL DIGESTION

Amelia Torcello-Gómez[a,*], Chloé Boudard[a,b] and Alan R. Mackie[a]

[a]School of Food Science and Nutrition, University of Leeds, Leeds, LS2 9JT, United Kingdom.

[b]Lycée Agro-Viticole de Bordeaux-Blanquefort, 33290 Blanquefort, France.

*Corresponding author: M.A.TorcelloGomez@leeds.ac.uk (A. Torcello-Gómez)

ABSTRACT

Calcium plays an important dual role in lipid digestion: promoting removal of long-chain fatty acids from the oil–water interface by forming insoluble calcium soaps, while also limiting their bioaccessibility. This becomes more significant in food containing high calcium concentration, such as dairy products. Nevertheless, scarce attention has been paid to the effect of calcium on the interfacial properties during lipid digestion, despite this being largely an interfacial reaction. This study focussed on the dynamics of the formation of calcium soaps at the oil–water interface during lipolysis by pancreatic lipase in the absence and presence of the two primary human bile salts (sodium glycocholate or sodium glycochenodeoxycholate). The competitive adsorption of lipase, bile salts and lipolysis products, as well as the formation of calcium soaps in the presence of increasing concentrations of calcium were mainly characterised by recording the interfacial tension and dilatational modulus *in situ*. In the absence of bile salts, calcium complexes with fatty acids at the oil–water interface forming a relatively strong viscoelastic network of calcium soaps over time. The dilatational modulus of the calcium soap network is directly related to the interfacial concentration of lipolysis products and the calcium bulk concentration. Calcium soaps are also visualised forming a continuous rough layer on the surface of oil droplets immersed in simulated intestinal aqueous phase. Despite bile salts having different surface activity, they play a similar role on the interfacial competition with lipase and lipolysis products although altering their kinetics. The presence of bile salts disrupts the network of calcium soaps, as suggested by the decrease in the dilatational modulus and the formation of calcium soap islands on the surface of the oil droplets. The accelerant effect of calcium on lipolysis is probably due to fatty acid complexation and subsequent removal from the interface rather than reduced electrostatic repulsion between lipase and bile salt molecules

and promoted lipase adsorption. The work shown here has implications for the delivery of oil soluble bioactives in the presence of calcium.

Keywords: lipolysis; oil–water interface; bile salts; fatty acids, calcium soap; dilatational rheology

INTRODUCTION

Lipid-based systems can naturally encapsulate oil-soluble micronutrients in food or can be rationally designed to incorporate and deliver hydrophobic bioactive compounds in the gastrointestinal tract. The bioavailability of these micronutrients will ultimately depend on their bioaccessibility during digestion of lipids. Gastric lipase is responsible for up to 40% of triglyceride hydrolysis followed by major hydrolysis of triglycerides in the proximal small intestine by pancreatic lipase (Sn-1,3 stereospecific).[1-2] Since lipids are insoluble in water, lipase has to adsorb to the oil–water interface for the enzymatic reaction to occur. Therefore triglyceride digestion is largely an interfacial event, whereby a molecule of triglyceride yields first one fatty acid and a diglyceride, as intermediate product, and eventually one Sn-2-monoglyceride and two fatty acids. Short- and medium-chain fatty acids are relatively more water-soluble and therefore readily removed from the interface for subsequent adsorption in the intestinal mucosa. Conversely, long-chain fatty acids and monoglycerides are relatively less water-soluble and remain adsorbed at the oil–water interface until they are incorporated into mixed micelles for transport and adsorption.[3] The interfacial accumulation of these lipolysis products inhibits lipase activity and biosurfactants are required to deplete them from the interface.[4]

The interfacial process is assisted in the duodenum by bile salts, which are secreted from the gall bladder along with phospholipids and cholesterol. Bile salts are synthesised in the liver from cholesterol, leading to the primary bile acids cholic and chenodeoxycholic acid, which are conjugated with the amino acids glycine (70%) or taurine (30%).[5] Bile salts comprise a planar rigid steroid backbone with opposed hydrophilic and hydrophobic faces and the conjugating group (polar and ionic) is linked to this amphiphilic nucleus. This unusual planar polarity is the responsible of their great surface activity and their biological functions during lipid digestion: removal of surface-active components from the oil–water interface,

promotion of lipase adsorption, aiding in the removal of lipolysis products from the interface and their solubilisation into mixed micelles.[6] However, the presence of bile salts at concentrations above their critical micelle concentration (CMC), as occurs under physiological conditions, saturates the oil–water interface inhibiting lipase adsorption due to electrostatic repulsion of negatively charged species under duodenal pH conditions.[7] Colipase is a co-factor that restores lipase adsorption by binding to the interface in the presence of bile salts, and anchoring lipase in a 1:1 complex.[8]

The presence of endogenous calcium or calcium present in the food, will promote the formation of insoluble soaps with long-chain free fatty acids remaining at the oil–water interface in a 1:2 molar calcium to fatty acid ratio. These calcium-complexed fatty acids are removed from the interface by precipitation. The effect of calcium is therefore double: on the one hand it assists on the removal of free fatty acids from the interface leading to faster lipolysis rates,[9-11] but on the other hand it reduces their bioaccessibility, not only affecting their absorption and thus bioavailability,[12-13] but also the capacity of self-assembly into mixed micelles for the solubilisation of lipolysis products.[14] The formation of a crystalline phase of calcium soaps at the interface of oil droplets has been observed previously by optical microscopy,[15] and described as a rigid layer. This should have an effect on the viscoelastic parameters of the interface but to the best of our knowledge, these have not been reported. The evolution of interfacial rheology can be also used to monitor *in situ* the dynamics of the calcium soaps formation and provide more insights on the interfacial aspects of lipid digestion, such as structural reorganisation at the interface.

Indeed the interfacial tension technique was shown to be successful in the past to assess the kinetics of lipase at triglyceride–water interface in the absence[16] and presence of previously adsorbed bile salt,[17] or the interfacial activity of the individual components involved in lipid digestion: lipolysis products, such as fatty acids and monoglycerides,[18] bile salts and/or

phospholipids.[19-20] The interfacial competition was also considered for sequentially adsorbed lipase and individual fatty acids, monoglycerides or diglycerides monitoring their adsorption at an alkane–water interface,[21] or lipids, bile salts and lipase/colipase onto air–water interface.[22] Nevertheless, in these last two studies, lipase adsorption was measured without substrate and therefore the *in situ* contribution of lipolysis products to the interfacial behaviour are not accounted for. An interfacial tension and rheology approach regarding the dynamics of lipid hydrolysis on natural long-chain triglyceride–water interface by lipase in the presence of bile salts was firstly proposed[23] which was subsequently used to study the effect of surfactant/stabiliser adsorbed layers on *in vitro* lipid digestion,[24-27] and corroborated by fluorescence resonant energy transfer elsewhere.[28] However, none of these studies have separated the contribution of lipolysis products to the interfacial behaviour, nor evaluated the effect of calcium and therefore dynamics of calcium soap formation.

The aim of this study was to follow the competitive adsorption between lipase, bile salts and lipolysis products *in situ* at a sunflower oil–water interface, as an example of long-chain triglyceride, as well as the dynamics of the formation of calcium soaps, by means of interfacial tension and dilatational rheology technique. For this purpose, the individual and collective interfacial behaviour of biological compounds involved in the process of lipid digestion in the small intestine are compared. Namely, pancreatic lipase and two different bile salts, sodium glycocholate (NaGC) and sodium glycochenodeoxycholate (NaGCDC). NaGC and NaGCDC have been chosen in the current study because they are the two major forms in the human bile,[5] which are glycine-conjugated. The main difference in their molecular structure is the presence or absence, respectively, of a hydroxyl group at position C12. Therefore, NaGCDC with fewer hydroxyl groups on the concave hydrophilic face of the molecule is relatively more hydrophobic than NaGC, and might have different impact on the interfacial competition in the presence of lipase. Since pancreatic lipase starts hydrolysing the

triglycerides soon after adsorption at the oil–water interface, the presence of lipolysis products will also affect the interfacial tension and dilatational modulus measurements. In order to separate their contribution to the interfacial layer formation, separate experiments were performed where lipase was inactivated by an inhibitor (4-bromophenylboronic acid). This lipase inhibitor is known to act at the active site of the lipase molecule without affecting its surface activity at a stoichiometry of 1:1.[29] Finally, the effect of the presence of calcium in the simulated intestinal buffer on the interfacial behaviour during lipolysis was considered by recording the viscoelastic properties.

This study shows the versatility of a single interfacial tension technique to monitor in real time the interfacial aspects of lipid digestion that were not considered previously.

EXPERIMENTAL SECTION

Materials

All the chemicals were purchased from Sigma-Aldrich and used as received, unless stated otherwise. Lipase from porcine pancreas (L3126) Type II (332 units/mg protein using olive oil as substrate provided in the batch information). According to the supplier, the lipase sample hydrolyses tri-, di-, and monoglycerides (in decreasing order of rate) and contains amylase and protease activity. 4-bromophenylboronic acid (B75956, ≥ 95% purity) was used as lipase inhibitor. Sodium glycocholate (NaGC) and sodium glycochenodeoxycholate (NaGCDC) (≥ 97% purity), have a molecular weight of 487.6 Da and 471.61 Da, respectively, and are negatively charged at pH 7. $CaCl_2$ and NaCl of analytical-grade were used. BIS-TRIS (≥99.0% purity, Fisher Scientific) and Milli-Q® ultrapure water were used for buffer preparation.

Sunflower oil was purchased in a local supermarket and purified with activated magnesium silicate (Florisil®, Fluka) to eliminate free fatty acids and surface active impurities. A mixture of oil and Florisil® (2:1 wt/wt) was stirred for 3 h and centrifuged at 4000 rpm for 30 min.

Sample preparation

Samples were prepared in 2 mM BIS-TRIS buffer containing 150 mM NaCl and adjusted to pH 7 with HCl. In some cases the buffer also contained $CaCl_2$ up to a concentration of 20 mM. Lipase aqueous solutions were prepared at a stock concentration of 10 mg/mL, filtered with Agilent syringe filters (0.2 μm pore, regenerated cellulose) and diluted to the desired concentration if necessary with BIS-TRIS buffer (2 mM, 150 mM NaCl, 0-20 mM $CaCl_2$, pH 7) . To inactivate the lipase, 4-bromophenylboronic acid was first dissolved in methanol at a concentration of 1 M and then 10 μL were added to the lipase aqueous solution so that the final concentration of the lipase inhibitor was 1 mM, in order to achieve a molar inhibitor to lipase ratio of at least 10:1. This lipase inhibitor is not surface active *per se*, since it did not reduce further the interfacial tension of pure sunflower oil–water interface (results not shown). The effectiveness of this concentration of lipase inhibitor was also assessed by interfacial tension and dilatational rheology measurements. Larger concentrations of 4-bromophenylboronic acid did not affect the interfacial tension nor dilatational modulus. Bile salts were prepared as a 10 mM stock solution and then diluted with BIS-TRIS buffer or lipase aqueous solution to a concentration of 0.1 mM. Only freshly prepared solutions were used for each experiment.

Interfacial tension and dilatational rheology

Interfacial tension of pancreatic lipase aqueous solutions in the absence and presence of calcium and bile salts was measured by means of a drop tensiometer (OCA 25, DataPhysics

Instruments, Germany) at the sunflower oil–water interface. The interfacial tension was recorded at constant interfacial area (30 mm^2) during the time required to achieve a steady state value (approximately 40 min). The apparatus is computer-controlled and the software fits the experimental drop profile to the Young–Laplace equation of capillarity providing the drop volume, the interfacial area, and the interfacial tension (γ). An oil droplet was formed at the tip of a J-shaped capillary and immersed in a glass cuvette placed in a thermostatically controlled chamber at 37 °C containing the aqueous solution of pancreatic lipase and/or bile salts. The interfacial tension of the pure sunflower oil–water interface (γ_0) was measured before each experiment to ensure the absence of surface-active contaminants obtaining values of (30.00 ± 0.15) mN/m at 37 °C.

Measurements of the interfacial dilatational rheology were made during or after 40 min of interfacial layer formation by sinusoidally oscillating the drop volume while the response in the interfacial tension was recorded. The amplitude of the applied interfacial area oscillations was fixed at 5%, which is within the linear viscoelastic regime. The dilatational parameters of the interfacial layers were calculated via a Fourier transformation algorithm implemented in the software analysis. The reported dilatational modulus (E) is, in a general case, a complex quantity: $E^* = E' + iE'' = \varepsilon + i2\pi f\eta$, where the real part E' is the storage modulus or the elasticity (ε) of the interfacial layer and the imaginary part E'' is the loss modulus that accounts for the viscosity (η) of the interfacial layer. In this study the elastic contribution was predominant over the viscous contribution in all cases and therefore only the dilatational modulus is presented. The oscillation frequency (f) ranged from 0.01 to 1 Hz.

All interfacial experiments were conducted in triplicate and the data presented is the mean value of those experiments. The reproducibility was confirmed by the low standard deviations. In most cases the error bars are within the size of the symbols.

RESULTS AND DISCUSSION

Effect of lipase concentration on lipase adsorption and formation of lipolysis products

This section focuses on the lipolysis of the sunflower oil–water interface by pancreatic lipase in the absence of calcium or bile salts. For this purpose, the interfacial tension of lipase aqueous solutions at different bulk concentrations was recorded for 40 min and the dilatational modulus measured after this time at different frequencies (Figure 1a and 1b, respectively). In order to separate the adsorption of lipase from the generation of lipolysis products, the interfacial tension and dilatational modulus were compared when the lipase was either active or inactive. Thus in the absence or presence of the boronic acid-derived inhibitor, respectively. The decrease of interfacial tension over time is indicative of material adsorption at the oil–water interface, while the dilatational modulus is also related to intermolecular interactions and mobility of the adsorbed molecules. Therefore, these parameters depend on the interfacial concentration, composition and structure. Regardless of the initial lipase bulk concentration, the inactive form of lipase leads to higher interfacial tension and dilatational modulus values than the active form. When the lipase is inactive, the interface is mainly composed of adsorbed enzyme and as with all proteins, it displays a high energy adsorption barrier and is considered irreversibly adsorbed at the oil–water interface. Therefore, the lipase behaves as an insoluble monolayer during the oscillatory deformation of the interfacial area. This is reflected in a relatively high dilatational modulus as a consequence of greater intermolecular interactions. When the lipase is active, it hydrolyses triglycerides into mainly monoglycerides and free fatty acids, which are very surface active,[18] and compete for interfacial area with the adsorbed lipase.[21] The accumulation of lipolysis products at the interface gives rise to lower values of the interfacial tension. In addition, the presence of lipolysis products disrupts the lipase interfacial network in a similar manner to

low-molecular weight surfactants, reducing its viscoelasticity and a lower dilatational modulus is observed.[30]

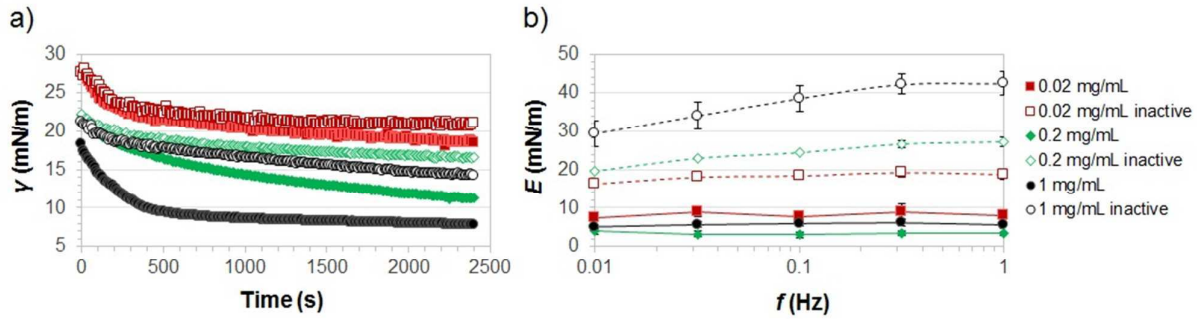

Figure 1: a) Interfacial tension versus time and b) dilatational modulus versus frequency after 40 min of interfacial layer formation, for lipase (closed symbols) and inactive lipase (open symbols) aqueous solutions (2 mM BIS-TRIS, 150 mM NaCl, pH 7, 37 °C) at different bulk concentrations (0.02, 0.2 and 1 mg/mL). Lines in b) are a guide for the eye. The data represents the mean of three replicates and the error bars the standard deviation.

Increasing the bulk concentration of either active or inactive lipase leads to further reduction of the interfacial tension due to adsorption of larger number of surface active molecules. However, the trend in the dilatational modulus depends on whether lipase is active or inactive. When lipase is inactive, the increase in the interfacial concentration of the enzyme alone produces a more compact interfacial layer of strongly absorbed protein molecules, explaining the increase in the dilatational modulus which becomes also more frequency-dependent. When the lipase is active, the initial increase of lipase bulk concentration (from 0.02 to 0.2 mg/mL), leads to the formation of larger amount of lipolysis products which seems to disrupt the adsorbed enzyme layer to a greater extent as reflected in the slight but significant decrease of the dilatational modulus. Further increasing the bulk concentration of

active lipase (up to 1 mg/mL) results in a more populated mixed interfacial layer which is inferred from the slight increase in the dilatational modulus.

The contribution of the adsorption of lipase can be separated from the formation of lipolysis products when the enzyme is active. For this purpose, the curve of the interfacial tension corresponding to the adsorption of active lipase in the presence of lipolysis products is subtracted from the interfacial tension curve of the inactive lipase. The result of this subtraction is the increase in the interfacial pressure due to the formation of lipolysis products (Figure 2).[16] It can be questioned whether the amount of adsorbed enzyme is identical in the absence and presence of hydrolysis products for inactive and active lipase, respectively. A previous study confirmed that lipase surface load was not affected by the formation of hydrolysis products from triolein and verified the validity of this subtraction method.[16] It is reasonable to assume that the initial enzyme interfacial load is not affected by the reaction products since the interfacial concentration of these is not enough to saturate the interface and become an obstacle for interface competition. However, at longer times of lipolysis, the amount of products may represent a challenge for lipase adsorption. Sn-2-Monoglycerides are known to be the most surface active lipolysis products which are able to expel lipase molecules from the oil–water interface.[21] Therefore, the subtraction of interfacial tension curves of active and inactive lipase aqueous solutions can be considered valid at early stage of lipolysis, however it may underestimate the amount lipolysis products at longer times at sufficiently high lipase bulk concentration. It can be observed in Figure 2 that the appearance of lipolysis products is immediate after oil droplet formation, and that the rate and extent of formation of lipolysis products is faster and larger, respectively, for higher bulk concentrations of active lipase. The maximum accumulation of lipolysis products occurs after 500-600 s of lipid digestion by lipase at a bulk concentration of 1 mg/mL under these experimental conditions. The gradual decrease in the calculated interfacial pressure

afterwards may be related to the underestimation of lipolysis products as explained above, due to interfacial competition between Sn-2-monoglycerides and lipase.

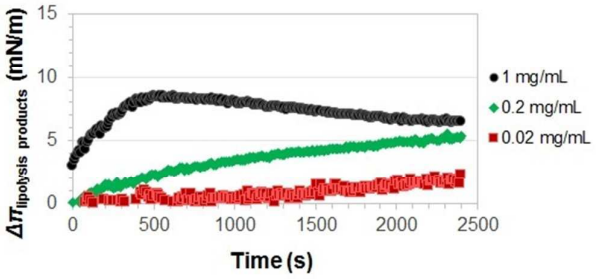

Figure 2: Increase in the interfacial pressure by lipolysis products produced by different bulk concentrations (0.02, 0.2 and 1 mg/mL) of active lipase aqueous solutions (2 mM BIS-TRIS, 150 mM NaCl, pH 7, 37 °C). The data represents the mean of three replicates and the error bars the standard deviation.

Formation of calcium soaps in the absence of bile salts

Figure 3 displays the interfacial tension recorded over time and dilatational modulus measured after 40 min of interfacial layer formation for active lipase aqueous solutions at a concentration of 1 mg/mL in the presence of increasing concentrations of $CaCl_2$. Increasing the calcium bulk concentration gives rise to lower values on the interfacial tension and higher values of the dilatational modulus up to a maximum concentration. This is presumably due to the complexation of calcium ions with the lipolysis products, more specifically long-chain fatty acids, accumulated at the oil–water interface. Sunflower oil is mainly composed of long-chain fatty acids and therefore, the free fatty acids produced during lipolysis may readily form calcium soaps. These crystalline structures are insoluble and may form a strong interfacial viscoelastic network as suggested in Figure 3b. There seems to be a saturating

effect at 10 mM $CaCl_2$ since higher calcium bulk concentrations do not result in further increase in the dilatational modulus.

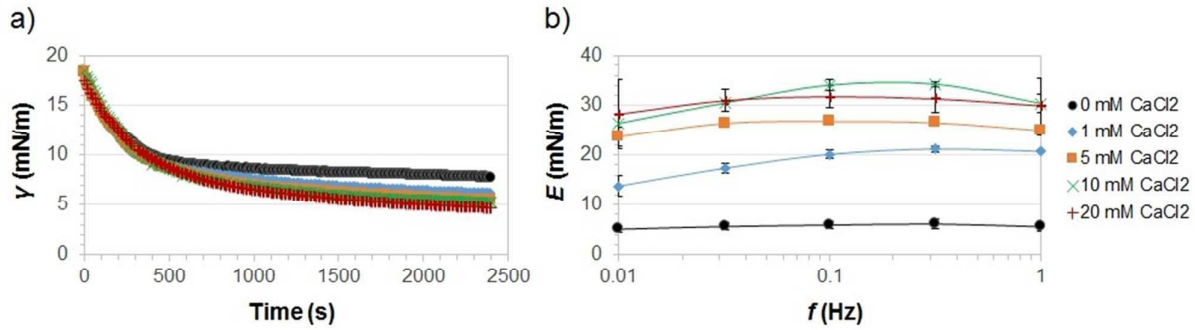

Figure 3: a) Interfacial tension versus time and b) dilatational modulus versus frequency after 40 min of interfacial layer formation, for active lipase (1 mg/mL) aqueous solutions (2 mM BIS-TRIS, 150 mM NaCl, pH 7, 37 °C) containing different $CaCl_2$ concentrations (0, 1, 5, 10 and 20 mM). Lines in b) are a guide for the eye. The data represents the mean of three replicates and the error bars the standard deviation.

The presence of calcium soaps was indeed evident on the surface of oil droplets images (Supporting Figure S1): smooth versus rough surface in the absence and presence of calcium, respectively, as previously noted.[15] In addition, when the oil droplet was detached at the end of the experiment, neck formation was only observed in the presence of calcium (Figure 4). The higher the calcium concentration, the greater this effect due to the solid-plastic behaviour.

Although saturated long-chain fatty acids are more prone to interact with calcium, which in the case of sunflower oil represent approximately 11% of the fatty acids, calcium soaps of unsaturated and polyunsaturated long-chain fatty acids can also form to a lesser extent.[31] Bearing in mind that saturated fatty acids in vegetable oils are almost exclusively located at

positions Sn-1,3 of triglycerides,[32] it is reasonable to presume that almost all the content of saturated fatty acids would be accounting for the calcium soaps.

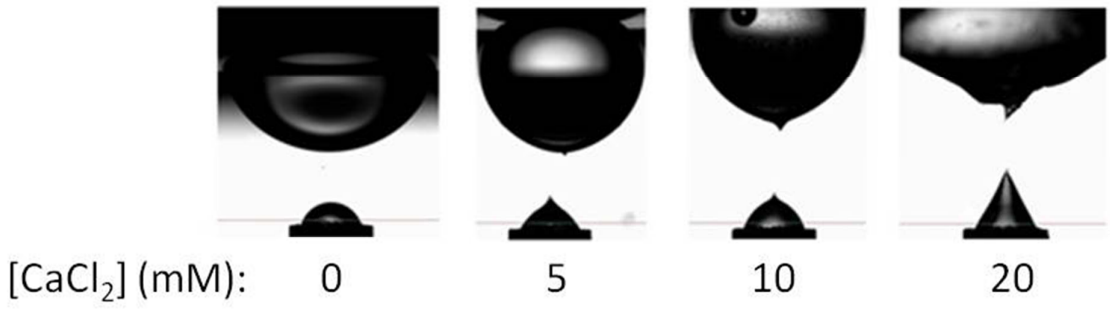

Figure 4: Detachment by extensional forces of purified sunflower oil droplets immersed in 1 mg/mL active lipase aqueous solution (2 mM BIS-TRIS, 150 mM NaCl, pH 7, 37 °C) in the presence of different concentrations of $CaCl_2$ (0, 5, 10 and 20 mM) after 40 min of interfacial layer formation.

In order to investigate the dynamics of the formation of calcium soaps, the dilatational modulus was also recorded over time at a single frequency of 0.1 Hz (Figure 5) during lipolysis. It can be observed that the dilatational modulus of the interfacial layer at each calcium bulk concentration is initially similar within the margin of error. Only after a period of approximately 600 s does the dilatational modulus start increasing in the presence of calcium and departs from the value measured in the absence of calcium. The larger the calcium bulk concentration, the steeper the initial increase in the dilatational modulus. Interestingly, this inflection point in the viscoelasticity of the interfacial layer due to the presence of calcium occurs once the interfacial accumulation of lipolysis products for this lipase concentration has reached a maximum (Figure 2). This suggests that although calcium may be complexing with produced fatty acids at the interface since early stage of lipolysis,

the strong viscoelastic calcium soap network is only formed at an interface saturated with fatty acids. Larger concentrations of calcium increase the rate and extent of the process since there are more ions available to complex with fatty acids.

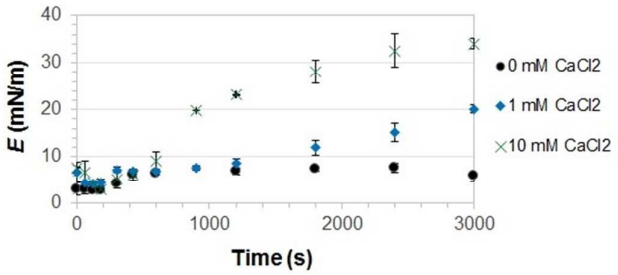

Figure 5: Dilatational modulus versus time (0.1 Hz) for active lipase (1 mg/mL) aqueous solutions (2 mM BIS-TRIS, 150 mM NaCl, pH 7, 37 °C) containing different $CaCl_2$ concentrations (0, 1 and 10 mM). The data represents the mean of three replicates and the error bars the standard deviation.

Nevertheless, the presence of calcium can also affect the interfacial behaviour of the adsorbed lipase molecules, which coexist with the lipolysis products. For that reason separate experiments were performed to ascertain the effect of calcium on the interfacial behaviour of inactive lipase (Supporting Figure S2). Indeed, the electrostatic interactions of positively charged calcium ions screening the negative charge of adsorbed lipase molecules allow a more compact reorganisation of the enzyme at the oil–water interface since intermolecular repulsion is reduced. This explains the slightly lower values of interfacial tension and slightly higher values of dilatational modulus in the presence of calcium (20 mM). However, the effect of calcium on these parameters is not as great as in the presence of lipolysis products when the enzyme is active (Figure 3), confirming that the major contribution to this

dilatational behaviour comes from the calcium soap formation rather than increased adsorption of lipase and formation of lipolysis products.

The effect of lipase bulk concentration in the presence of calcium was also evaluated, since the formation of calcium soaps will depend on the amount of lipolysis products formed at the oil–water interface, which in turn will depend on the lipase to substrate ratio that is proportional to the lipase bulk concentration. Supporting Figure S3 shows the results of the interfacial tension over time and dilatational modulus after 40 min of interfacial layer formation for lower concentrations (0.02 and 0.2 mg/mL) of active lipase aqueous solutions in the absence and presence of 100 mM $CaCl_2$. It can be seen how a relatively high concentration of calcium do not have a remarkably effect on the dilatational modulus, which can be explained by the smaller amount of lipolysis products at the oil–water interface as estimated in Figure 2. Therefore, the largest concentration of lipase studied here (1 mg/mL) is chosen for the next section since the calcium soaps formation was reflected more evidently in the dilatational behaviour.

Formation of calcium soaps in the presence of bile salts

In a more physiologically relevant scenario, the presence of bile salts contributes to the interfacial layer and needs to be considered. A bile salt concentration of 0.1 mM has been chosen because it is below their CMC at these ionic strength and pH conditions (4 mM for NaGC and 1-2 mM for NaGCDC),[33] and it does not saturate the interface inhibiting lipase adsorption.[7]

The individual and combined interfacial behaviour of bile salts and lipase (either active or inactive at 1 mg/mL) was evaluated in the absence of calcium (Figure 6). Figures 6a and 6c display the results for the bile salt NaGC, whereas Figures 6b and 6d focus on NaGCDC. There are differences in the dynamics of adsorption of the two bile salts alone (closed square

symbols Figure 6a,b). The more hydrophobic NaGCDC adsorbs faster reaching a steady value from the beginning of the experiment, as opposed to NaGC. This is in agreement with a previous study on the adsorption of different bile salts, including NaGC and NaGCDC, at solid surfaces.[34] In addition, there exist differences in the dilatational properties: NaGCDC displays lower dilatational modulus values (closed square symbols Figure 6 c,d) as compared to those for NaGC. This may be related with the reversibility of adsorption and rate of desorption from the interface upon oscillatory perturbation of the interfacial area. Chenodeoxycholate and deoxycholate bile salts have shown large reversibility of adsorption at C18-functionalised silica surface and air–water interface in contrast to cholate bile salts.[20, 34] In addition, the rate of desorption of NaGCDC when rinsing the aqueous subphase and depleting it of the bile salt, was faster than that for cholate bile salts.[34] Therefore, the faster dynamics of adsorption/desorption and complete adsorption reversibility of NaGCDC indicates higher deformability of the interfacial layer upon interfacial oscillations and leads to lower dilatational modulus as compared to NaGC. The curves corresponding to lipase alone either active or inactive (circle symbols) have been discussed above (Figure 1) and have been included here as a reference to compare the individual and collective behaviour of all the species.

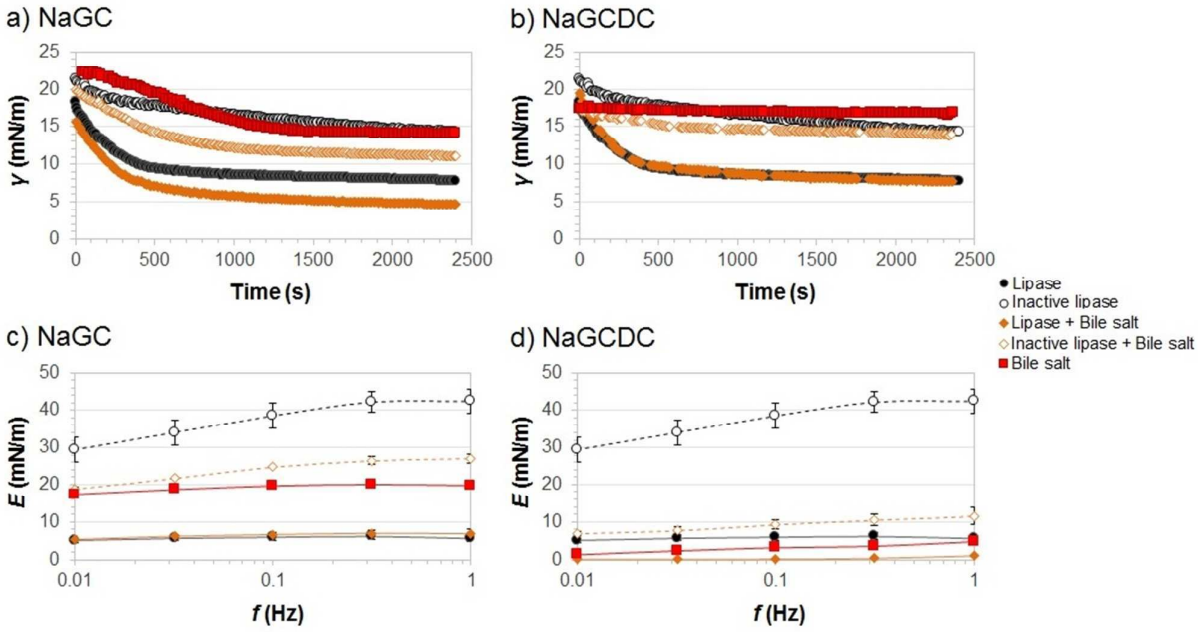

Figure 6: a), b) Interfacial tension versus time and c), d) dilatational modulus versus frequency after 40 min of interfacial layer formation, for 1 mg/mL lipase (closed symbols) and inactive lipase (open symbols) aqueous solutions (2 mM BIS-TRIS, 150 mM NaCl, pH 7, 37 °C) in the absence and presence of 0.1 mM bile salt (NaGC or NaGCDC). The curves corresponding to 0.1 mM bile salt alone (closed squares) are also included as a reference. Lines in c) and d) are a guide for the eye. The data represents the mean of three replicates and the error bars the standard deviation.

In Figure 6a, when both NaGC and lipase (either in inactive or active form) are present in the aqueous phase, the interfacial tension is reduced faster and to a larger extent than individual species, indicating a cooperative behaviour between the bile salt and the enzyme. Figure 6c shows that the dilatational moduli of the mixture of NaGC and inactive lipase are intermediate between those of individual components within this range of frequency, confirming the co-adsorption of both species in agreement with the interfacial tension results. This intermediate viscoelastic behaviour is better appreciated at higher frequency values,

when the adsorbed layer behaves as insoluble since dynamics of adsorption/desorption are slower than the rate of compression/dilation of the oscillatory deformation.[35] However, the dilatational moduli of the mixture of active lipase and NaGC overlap with those of active lipase in the absence of bile salt. For this particular case, the results of interfacial tension clarify the cooperative behaviour of NaGC and active lipase in the presence of lipolysis products.

This is not evident for the collective behaviour of NaGCDC and lipase in Figure 6b. The initial values of the interfacial tension for aqueous solutions of inactive lipase and NaGCDC is similar to that of the individual bile salt (see Supporting Figure S4), suggesting that the initial dynamics and composition of the interface is governed by NaGCDC adsorption. After 2 min of interfacial layer formation, the rate and extent of adsorption (indicated by the decrease of the interfacial tension) is faster and larger, respectively, for the mixed system as compared to individual components. The extent of the decrease in the interfacial tension is still larger for the combined inactive lipase and bile salt until 30 min of adsorption and from that time onwards the final interfacial tension values are very similar to those attained by lipase alone. This suggests that after 2 min of interfacial layer formation, the bile is gradually displaced from the interface as the lipase adsorbs into the interfacial layer but that there are significant interactions between the two species. Nevertheless, Figure 6d displays intermediate values of dilatational modulus of the mixture of inactive lipase and NaGCDC in between those of individual components clarifying that there is actual co-adsorption of NaGCDC and lipase even at the end of the interfacial layer formation, when the interfacial tension curve approached the values attained by lipase alone. The interfacial tension curve corresponding to an aqueous solution of active lipase and NaGCDC overlaps with that of active lipase in the absence of bile salt throughout the experiment within the standard deviation of the measurements. This might suggest that NaGCDC is not playing any role in

the adsorption of the mixture with active lipase, not even at the initial times when the amount of lipolysis products are more negligible, which contrasts with the cooperative behaviour observed in the presence of inactive lipase. However, the dilatational modulus of active lipase and NaGCDC is lower than the individual behaviour of the bile salt and active lipase in the presence of lipolysis products. This sheds light on the co-adsorption of NaGCDC, lipase and lipolysis products at the oil–water interface.

Despite the different adsorption dynamics and surface activity of the bile salts, both behave at least qualitatively similarly in the interfacial competition with lipase. Bearing this in mind and from the fact that the interfacial tension curves corresponding to adsorption of active lipase in combination with either bile salt are lower than those corresponding to the adsorption of inactive lipase and bile salt, it is evident that the lipase is still active in the presence of bile salts.

The relative quantification of lipolysis products at the oil–water interface in the presence of both bile salts can be estimated as above from subtraction of interfacial tension curves where lipase is either inactive or active. These results are represented in Figure 7 and compared to the interfacial pressure increase by lipolysis products in the absence of bile salts (Figure 2). It can be seen that the initial rate of formation of lipolytic products is slightly slower in the presence of NaGCDC, with a lag time of approximately 1 min, as compared to that in the presence of NaGC or the absence of bile salts. This may be related to the faster adsorption rate of NaGCDC that may compete more efficiently with lipase and retards the activation of the enzyme to start the lipolysis. Nevertheless, the relative amount of lipolysis products quantified by the interfacial pressure increase is eventually comparable in all cases. This corroborates the fact that bile salt concentrations below their CMC do not inhibit lipase adsorption, but alter its kinetics.

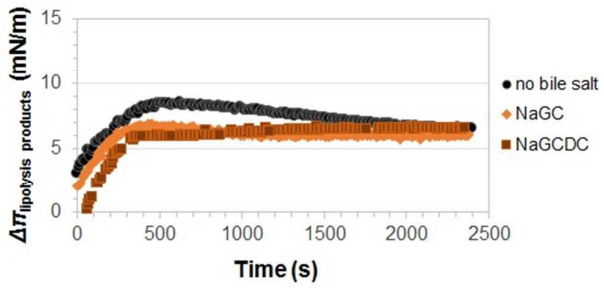

Figure 7: Increase in the interfacial pressure by lipolysis products produced by 1 mg/mL of active lipase aqueous solutions (2 mM BIS-TRIS, 150 mM NaCl, pH 7, 37 °C) in the absence and presence of 0.1 mM of either NaGC or NaGCDC. The data represents the mean of three replicates and the error bars the standard deviation.

Finally, the presence of calcium has a qualitatively similar effect on the interfacial tension and dilatational modulus curves during lipolysis in the presence of bile salt (Figure 8) as in the absence of bile salts (Figures 3 and 5). Namely, a slight decrease in the interfacial tension and an increase in the dilatational modulus compared to the systems in the absence of calcium. However, the deviation in the dilatational moduli shows a delay of approximately 17 min and the final extent is reduced about 50% at all calcium concentrations (12-14 mN/m) compared to that in the absence of bile salts (20-35 mN/m) (Figure 5). The data is similar in the presence of either NaGC or NaGCDC, likely due to comparable interfacial competition with pancreatic lipase leading to similar amount of lipolysis products adsorbed at the interface.

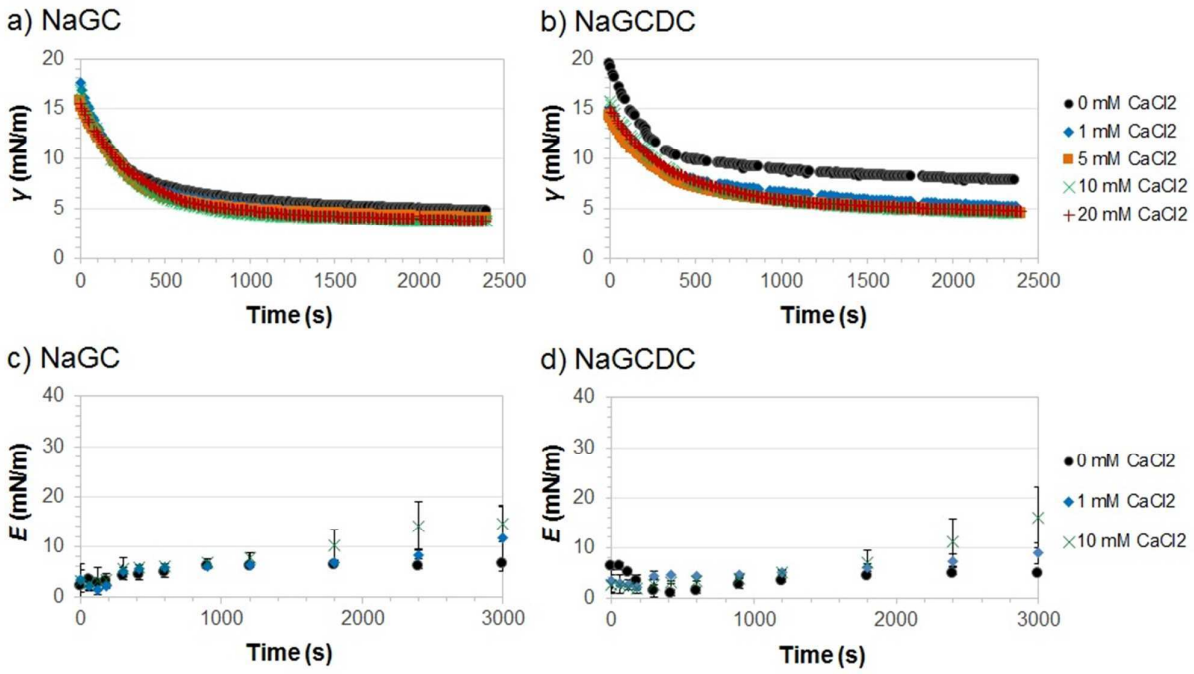

Figure 8: a), b) Interfacial tension and c), d) dilatational modulus versus time (0.1 Hz), for 1 mg/mL lipase aqueous solutions (2 mM BIS-TRIS, 150 mM NaCl, pH 7, 37 °C) containing 0.1 mM bile salt (NaGC or NaGCDC) and different $CaCl_2$ concentrations (0, 1, 5, 10 and 20 mM). The data represents the mean of three replicates and the error bars the standard deviation.

Although the adsorbed bile salts lead to lower viscoelasticity of the interfacial layer during lipolysis in the presence of calcium, the formation of calcium soaps is still evident from the necking of detached oil droplets (Figure 9). The rigid interfacial layer is most likely visible in these images due to the expulsion of more soluble components from the interfacial layer upon "snap-back" of the interface then the available surface area decreases very quickly.

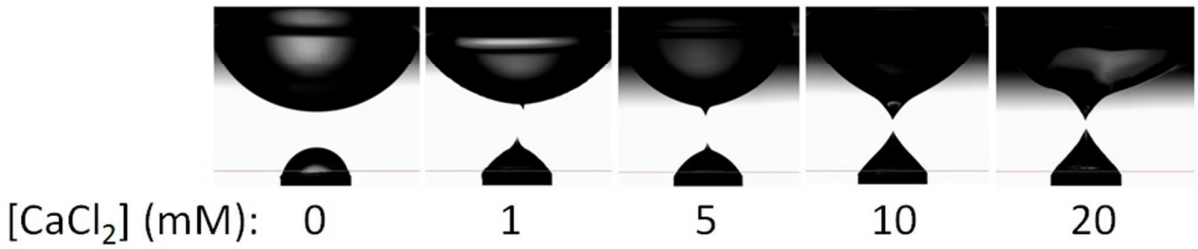

Figure 9: Detachment by extensional forces of purified sunflower oil droplets immersed in 1 mg/mL active lipase + 0.1 mM bile salt aqueous solution (2 mM BIS-TRIS, 150 mM NaCl, pH 7, 37 °C) in the presence of different concentrations of $CaCl_2$ (0, 1, 5, 10 and 20 mM) after 40 min of interfacial layer formation.

In Figure 7 the amount of lipolysis products formed in the presence of either bile salt is comparable to that in the absence of bile salts. This suggests that the lower dilatational response measured during the formation of calcium soaps in the presence of bile salts as compared to that in the absence of these, is unlikely due to a reduced amount of lipolysis products but rather to a disrupted arrangement at the interface. The calcium soaps seem to form on disconnected regions of hydrolysed lipids among patches of bile salts. The loss of birefringence in the crystalline phase formed by calcium soaps surrounding lipid droplets was reported in the presence of bile salts although it was attributed to reduced amount of calcium soaps.[9] The results found here suggest a more disordered /loose interfacial structure of calcium soaps instead. Supporting Figure S5 shows discontinuous islands of calcium soaps on the oil droplet surface that supports the disrupting effect of bile salt on the calcium soap network.

The presence of calcium can also affect the interfacial behaviour of the adsorbed bile salt and lipase molecules, which are coexisting with the lipolysis products. As in previous section, separate experiments were performed to ascertain the effect of calcium on the interfacial

behaviour of one bile salt, NaGC, and its collective behaviour with inactive lipase (Supporting Figure S6). As seen before, the electrostatic interactions of positively charged calcium ions screening the negative charge of adsorbed lipase molecules and bile salt allow a more compact reorganisation of both species at the oil–water interface. This explains the slightly lower values of interfacial tension and higher values of dilatational modulus in the presence of calcium (20 mM) both for pure bile salt and in combination with inactive lipase. Nevertheless, the slight change in interfacial tension may indicate similar adsorbed amount of enzyme/bile salt leading to comparable production of monoglycerides/fatty acids when lipase is active. Therefore, this study suggests that the known effect of calcium on increasing the lipolysis rate *in vitro* is greatly attributed to the formation and precipitation of calcium-complexed fatty acids, rather than promoted lipase adsorption because of reduced electrostatic repulsion between lipase and bile salt molecules.

Calcium soaps, which are insoluble, are removed from the oil–water interface by precipitation. This has been previously described as the extrusion of the unhydrolysed lipid droplet from the crystalline shell of calcium-complexed free fatty acids.[15] These processes might have been reflected in a sudden decrease of the viscoelastic modulus over time or fluctuations during the progress of lipolysis as a continuous removal/formation of calcium soaps. However, these features were not observed within the timeframe of the experiments. On the contrary, an average accumulation of calcium soaps is suggested from the gradual increase in the dilatational moduli (Figures 5 and 8), reaching a plateau at the end of the experiment at higher calcium concentrations indicating saturation, and progressive appearance of granulated forms at the surface of oil droplets. It is likely that the amount of fatty acids produced to complex with calcium was not large enough under these experimental conditions, due to a relatively low enzyme to substrate ratio and/or low percentage of saturated fatty acids and the small surface area available for the reaction. The concentration

of lipase was a limitation of the experimental design because of the low interfacial tension values reached that cause droplet detachment. Future work involving hydrolysis of triglycerides with higher content of saturated fatty acids might lead to larger amount of calcium soaps, and their precipitation may be better evidenced on the measured interfacial parameters.

CONCLUSIONS

During *in vitro* lipid digestion, calcium ions complexed with fatty acids produced at the oil–water interface forming a strong viscoelastic network with increasing amounts of lipolysis products over time. The strength of the network is directly related to the interfacial fatty acid and calcium bulk concentration. The presence of bile salts disrupts the calcium soap network, due to discontinuous interfacial arrangement of lipolysis products among patches of adsorbed bile salts, leading to a decrease in the dilatational modulus. Both bile salts, NaGC and NaGCDC, display similar interfacial competition with lipase during lipolysis at a long-chain triglyceride–water interface although alter its kinetics. The presence of calcium probably promotes faster lipolysis due to complexation with fatty acids and subsequent removal from the interface, rather than reduced electrostatic repulsion between lipase and bile salt molecules and promoted lipase adsorption.

Finally, regarding possible technological applications, the versatility of this approach might provide a platform to semi-quantitatively determine the saturation profile of fatty acids at position Sn-1,3 from different triglycerides sources by comparison of the dilatational moduli of the calcium soaps formed during lipolysis. Additionally, the study has implications for the delivery of hydrophobic nutrients and pharmaceuticals in the presence of foods containing high levels of calcium.

ACKOWLEDGEMENTS

Authors thank the financial support from the School of Food Science and Nutrition of the University of Leeds. C. B. would also like to thank the financial support from Erasmus Student mobility for traineeships between Programme Countries, the Department for Environment and Agriculture and the Regional Council of New Aquitaine in France.

SUPPORTING INFORMATION

Supporting information file contains additional images of calcium soaps formed at the oil droplet surface during lipolysis in the absence and presence of bile salt, results of interfacial tension and dilatational modulus on the effect of calcium on adsorption of inactive lipase and/or bile salt, as well as the effect of lipase concentration on calcium soap formation.

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