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Review

Interfacial mechanism of lipolysis as self-regulated process

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ABSTRACT

Obesity is a serious public health concern with an increasing prevalence worldwide. The diet contributes strongly to this problem and high-fat food leads to unhealthy conditions. Fat digestion is an energy intensive process as it requires hydrolysis to allow the body to profit from this nutrient. This additional energy expenditure is also present in a highly redundant hormonal regulation system for fat storage which is converted in not a readily accessible form (therefore, an upstream is required in order to tackle fat-related diseases).

Due to the low water solubility of oils and fats, the lipase catalyzed reactions take place at a specific locus, the oil–water interface. Despite considerable efforts in the past the effects of the interfacial composition on lipase activity have been only qualitatively understood until recently. It has recently been undertaken a detailed study of the interfacial behavior of lipases, their substrates and products which have contributed to shed light into the mechanism of lipolysis [1–4]. In addition, there are strong evidences that lipase activity is a function of interfacial composition and changes concurrently with lipolytic conversion [1]. In these cases lipase "inhibition" should be attributed to substrate depletion and not to lipase desorption or denaturation as previously hypothesized [5]. This self-limiting effect through the feedback of interfacial composition to the reaction conditions of the enzyme opens a new avenue to control lipase catalysis through the interface. A set of experiments are proposed which can be performed to further characterize and gain a deeper insight into interfacial enzymology.

Contents

1. Introduction

Interfacial enzymes have evolved to deal with the biophysical realities of interfaces. About half the proteins in cells are membrane-associated and must function within the interfacial kinetic constrains for substrate accessibility, distribution, orientation, partitioning and exchange. Such processes ultimately control the processivity of the interfacial turnover cycles. Thus the binding of the enzyme to the interface, substrate accessibility and replenishment in the microenvironment of the bound enzyme determine the microscopic steady state condition for the catalytic turnover. An understanding of the interfacial processes during the reaction progress also permits an unequivocal identification of the kinetic path for the analysis of the steady state turnover events.

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Study of the interfacial rate and equilibrium processes is a discipline in its own right because the interplay of elementary processes under the dimensionally constrained conditions poses conceptual challenges that begin with the definition of the variables rooted in the model. As developed in the present paper, for the analysis of the kinetic analysis of the interfacial turnover it is necessary to consider the variables that control the interfacial processivity.

A well-grounded quantitative description of the interfacial phenomena raises issues of concern for researchers in cell biology, biochemistry, pharmacology, colloid and interface science. In this article we shed light into the fundamentals of chemistry and physics of lipases at water/oil interfaces and its biological consequences.

2. Lipases

Lipases are lipolytic enzymes that play a key role in fat metabolism [6]. They are catalysts for the hydrolysis of triacylglycerides, which contribute for major portion of calories to our daily diets [7]. Due to the apolar nature of oils and fats, the oil–water interface is where the control of lipolytic conversion and finally digestion takes place. It might come as a surprise that despite past efforts the interfacial behavior of lipases is not completely understood.

The catalytic triad of lipases with an alpha/beta hydrolase fold is composed of three amino acids (serine, histidine, aspartate/glutamate), which are far apart in the primary sequence but spatially very close in the folded protein. The interaction of the negatively charged residue Asp or Glu allows the His residue to act as a general base which can remove a proton from the hydroxyl group of the active site Ser. The thus generated nucleophilic alkoxide ion on the Ser residue is proposed to attack the carbonyl carbon of the esterified substrate forming an acyl-enzyme intermediate. Another important component of the catalytic mechanism is the oxyanion-hole which is composed of properly arranged H-bond donors (mostly main-chain NH groups). The oxyanion hole helps to stabilize a reaction intermediate during catalysis when the carbonyl oxygen carries a partial negative charge. The active serine residue is embedded in the short consensus sequence GXSXG (with X being any amino acid), a motif also found in esterases and proteases. The active site of lipases in the "closed" form is shielded from the surface by protective surface loops called the "lid". Upon activation, the lid undergoes a conformational rearrangement exposing the active site serine and creating the active, open form of the enzyme. Both the open and the closed form of lipases have been observed in the X-ray structures of lipases [8,9].

In the past, the interfacial influence on lipase activity has been described through the "quality of the interface" which determines the behavior of the lipases and the outcome of the enzymatic reaction [10]. However, determining the quality of the interface is ambiguous and has proven difficult to define [11]. Additionally, a variety of interfacial phenomena were reported to have detrimental influence on both enzymes and reactions [12]. For example, enzymes irreversibly denature at interfaces under high interfacial tension conditions (low surface pressure) or are excluded from the interface under low interfacial tension conditions, respectively [13]. On the other side, a large number of studies of lipases in microemulsions show that lipases can be part of the interface and stay fully active at even very low interfacial tension [14]. Lipase action at the interface can also be inhibited by the interaction with amphiphiles such as bile salts or fatty acid salts. Electrostatic interactions leading to conformational changes or complexation of small surface active molecules were reported to lead to enzyme inhibition [15] Furthermore the enzyme reaction might stall through competitive inhibition from small molecules [16]. An important source of inhibition was found to be the interaction of detergents with lipases [17]. However, no direct relationships were established between lipase activity and the changes of interfacial tensions due to the presence of detergents.

Lipases are activated by binding to an insoluble emulsified or aggregated substrate [18]. The extent of binding is related to the physicochemical, as well as the compositional, structure of the interface, the so called "quality of the interface". This term, which is yet undefined, is thought to contain contributions from electrostatic interactions, orientation of substrate, and hydration forces [18]. It has been clearly established that the rate of triglyceride lipolysis depends strongly on the specific area of the emulsion drops [19,20]. Since long-chain triglyceride emulsification is difficult to perform mechanically, emulsifiers have been widely used, although these increase the physico-chemical complexity of the system. As previously described, low molecular weight surfactants can expel proteins from the interface [4]. Therefore, the consequences of enzyme-surfactant interaction have an important impact on the regulation of lipase catalysis. Lipase from *Rhizomucor miehei* was shown to bind to droplets stabilized by cationic surfactants (didodecyldimethylammonium bromide and dioctadecyldimethylammonium bromide) but not to those stabilized by anionic surfactants (sodium bis(2-ethylhexyl) sulfosuccinate — AOT). It was experimentally impossible to show any adsorption of the lipase to droplet surfaces covered by non ionic surfactants [21]. However, one should not generalize these results to all lipase–surfactant interactions. These processes are highly dependent on the concentration and the type of amphiphile. The protein-ionic surfactant interactions are also very much dependent on the properties of the aqueous phase, such as pH an ionic strength.

The effect of different surfactants on lipase activity has been widely investigated [21–23]. Non ionic and cationic systems provided high reaction rate in water-in-oil emulsion while for anionic systems almost no reaction was observed [21]. However, a different scenario was published for water in oil microemulsions where cationic surfactants decreased lipolysis as compared to anionic or non ionic systems [24].

Several publications show efficient lipase catalysis in AOT stabilized microemulsions [25–29]. According to Skagerlind et al. [21], lipase can adsorb at an interface consisting of negatively charged surfactants if the surfactants are neutralized by counterions. The water pools in these microemulsions are small and the surfactant concentrations are high; therefore, the counterion concentration is also high. This effect has been considered as the reason for the high efficiency of lipase catalysis in AOT based microemulsions.

Bile salts have been shown to inhibit lipolysis of triglycerides near the critical micelle concentration [30]. This inhibition has been attributed to electrostatic repulsions between the negatively charged surfactant and the lipase. However, colipase can reverse the inhibitory effect of bile salts by helping lipase to anchor at the interface. In fact, colipase is known to reactivate surfactant-inhibited pancreatic lipase, but in all cases activation is limited to a narrow range of surfactant concentration [17].

By comparing the inhibitory curves obtained with surfactants and bile salts with their respective effects on the interfacial tension at the triolein/water interface, no direct relationship could be established between the decrease in lipase activity and the lowering of the interfacial tension [17]. Therefore, a better understanding of the effects of interfacially active molecules on lipase activity is required. Moreover, lipolysis of triglycerides generates highly interfacially active molecules that can probably compete with the enzyme for the interface and/or modify the protein via molecular interactions. Both of these potential mechanisms can have an impact on lipase catalysis.

During the past three decades, various kinetic models have been proposed to describe the interfacial mechanism of enzymatic lipolysis [10]. Verger-De Haas' model [31] is the simplest adaptation of the Michaelis–Menten–Henri kinetic model for the interfacial hydrolysis of short-and medium-chain lipids, which upon biocatalysis generate soluble products (Fig. 1). The first step in this process is the adsorption of a water soluble enzyme (E) at the lipid–water interface leading to a more favorable energy state of the enzyme (E^*). The enzyme present at the interface binds a substrate molecule (S), resulting in the

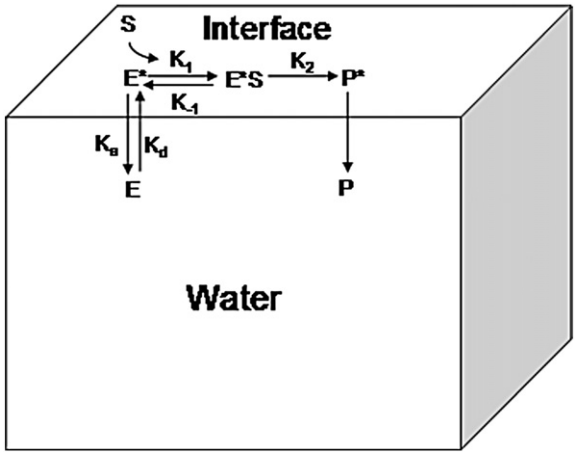

Fig. 1. Kinetic model for the action of lipase on an insoluble substrate (redrawn from Ref. [33]).

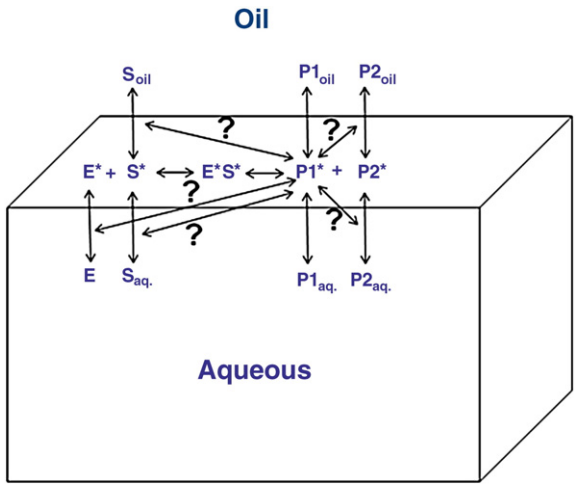

Fig. 2. Kinetic model for the action of lipase where the generated product 1 (P1) has an impact on enzyme (E), substrate (S) and product 2 (P2) interfacial accessibility.

formation of an enzyme-substrate complex (E*S). After a two dimensional catalytic process the product (P*) is generated and solubilised into the aqueous phase. Using this simple approach, many kinetic experiments have been carried out on the hydrolysis of synthetic medium chain lipids, generating water-soluble products. However, the natural substrates for lipolytic enzymes are long-chain lipids, generating water insoluble products. In general, kinetic models have to take into account the processes involved in the interfacial molecular reorganization and segregation of the insoluble products, which affect the so called "interfacial quality" of the interface. In order to remove the lipolysis products from the interface, water-soluble acceptors such as β-cyclodextrine can be used [32]. In vivo, a similar process of complexation and solubilisation of the lipolytic products into the aqueous sub-phase can also occur: micellar solubilisation of free fatty acids and monoglycerides are carried out by bile salts in the gastro-intestinal tract while serum albumin binds free fatty acids in the blood.

To take maximum advantage of these enzymes, as well as to better understand their biological roles, it is important to determine how the expression of their activities depends on the surrounding environment. In the case of lipases, this goes far beyond defining experimental conditions such as concentrations of enzymes and substrates, ionic strength, pH, and temperature, because both the reactants and the catalyst are distributed non-uniformly within the system [34]. Lipolysis is, in fact, a classic example of heterogeneous biocatalysis [35]. Hence, the reaction rate and direction are controlled by the overall composition at the interfacial microenvironment. Lipolytic reactions are therefore strongly dependent on the interfacial activity, kinetics of diffusion and partitioning of substrates, products and inhibitors. Confining a reaction to an interface can considerably speed up the substrate-enzyme association rate by decreasing the entropy through motional constrains, as well as by hydrophobic and electrostatic interactions. These latter effects are also important with respect to the diffusion of substrate and products to and from the active site. For instance, it has been proposed that an electrostatic repulsion might occur between the negatively charged active site of lipase and ionized fatty acids at alkaline pH, thus indicating a fast release of the lipolysis reaction products from the interface [36].

Berg et al. have studied in detail the impact of different variables on interfacial enzyme kinetics [37]. However, there is still no model which integrates the amphiphilic properties of lipase, substrates and products on the interfacial composition throughout the reaction progress. Fig. 2 shows a kinetic model of lipase action which takes into account the impact of the interfacial substrate (S*) and interfacial product 1 (P1*) on enzyme (E) and product two (P2*) interfacial accessibility. A more complex and realistic model could be further developed where the impact of each molecule on the interfacial composition is integrated.

In order to have a clear picture of all the parameters affecting lipolysis, it is crucial to determine the interfacial properties of each single molecule involved in the reaction and to characterize the interfacial composition in complex systems.

Lipases, which are water soluble, act at an oil–water interface during lipolysis of a triglyceride. During lipolysis, the hydrolysis products such as glycerol, free fatty acids, di- and monoglycerides, will alter the lipid self-assembled structure of the substrate and the characteristics of the lipid-aqueous interface. Lipase-catalyzed lipolysis is a complex reaction involving many steps, for example binding, orientation and activation of the lipase, binding of the substrate molecule into the active site, and the catalytic reaction itself. These steps, as well as the solubility of the products formed, will be determined by the self-assembled structure formed by the amphiphiles present. Detailed knowledge of the phase behavior of the relevant lipid-aqueous systems is necessary in order to predict the lipid self-assembly structures expected to form during lipolysis and to understand how those substrates (vesicles, micelles, emulsions, gels and liquid crystals) will affect the lipase activity. According to Borné et al. [38], the lipolysis reaction seemed to produce the same steady-state composition regardless of the initial substrate composition and self-assembled structure. It is therefore important to stress that the action of lipase only decreases the time to reach equilibrium and does not affect the equilibrium composition as such.

3. Fat digestion

The efficacy of absorption of dietary triglycerides under physiological conditions in human adults is above 95% [39]. Hydrolysis of triacylglycerides results in the formation of metabolites that have a higher tendency to interact with water than their parent compounds. Small and Carey [40] classified the lipids on the basis of their tendency to interact with water. The group of non-polar lipids do not interact with water and will be present in aqueous environment either as a lens of oil or as a crystal (e.g. paraffin). The group of polar lipids can be subdivided into three classes. Class I is also known as insoluble non-swelling amphiphiles and are exemplified by triglycerides, diglycerides and protonated long-chain fatty acids. Class II polar lipids are insoluble swelling amphiphiles, such as phospholipids and monoglycerides. In addition to forming a stable monolayer on the top of an aqueous solution, these lipids can penetrate into the bulk aqueous phase in the form of liquid crystals. Class III polar lipids are the so-called micelle forming or soluble amphiphiles (e.g. sodium or

potassium salts of long-chain fatty acids and lysophosphatidylcholine) that form unstable monolayers. Bile salts belong to the class III polar lipids that form micelles which can solubilise class II and, to a limited extent, even class I polar lipids. Through the formation of mixed micelles the total concentration of class I and class II polar lipids in the aqueous phase can be increased 100 to 1000 fold [41]. Monolayers of class III polar lipids are unstable due to their solubility in water.

Intestinal hydrolysis of triacylglycerides is largely completed in the proximal duodenum [42]. This rapid and efficient process occurs in the presence of bile salts and phosphatidylcholine. Major constituents of the bile needed to effectively disperse lipase substrates and facilitate the removal of hydrolysis products [43]. In vitro, however, triacylglycerol hydrolysis is inhibited by these surface active bile constituents [44]. Inhibition of triacylglycerol hydrolysis in emulsions by the nonsubstrate phosphatidylcholine is characterized by a "lag time" beyond which substrate is rapidly hydrolyzed. The explanation for the more efficient lipolysis in vivo lies in the stomach. Primary lipolysis products, like fatty acids and diacylglycerol, are produced in the stomach through the partial hydrolysis of triacylglycerols catalyzed by gastric lipase. These lipolysis products are known to shorten the lag phase in lipolysis, and their presence in the surface of lipid droplets entering the intestine is believed to contribute to efficient intestinal lipolysis [45]. Substantial amounts of procolipase, a protein cofactor of pancreatic lipase, but not gastric lipase, are secreted and activated in the stomach as well as in the intestine. In vitro, colipase acts synergistically with lipolysis products to shorten the lag phase of triacylglyceride hydrolysis by pancreatic lipase. Thus, gastric lipolysis, coupled with colipase addition in the stomach and at the beginning of the intestine, primes lipid droplets for attack by pancreatic lipase as they enter the intestine. Previous studies show that colipase binds preferentially to surfaces containing lipolysis products [46].

Based on model studies, 2.5 to 4% of emulsified triglycerides are present at the oil–water interface, which can be accessed by pancreatic lipase [47]. At least during the initial stages, the rate of lipolysis significantly exceeds the capacity to incorporate the resulting fatty acids and monoglycerides into mixed bile salt micelles [48]. Rather, these class II polar lipids accumulate at their site of origin, the oil–water interface forming a liquid crystalline phase [34].

Free fatty acids and Sn-2 monoglycerides are the final products of fat digestion due to the Sn-1,3 regiospecificity of the gastro-intestinal lipases towards triglyceride hydrolysis. Product accumulation can occur at the interface, provided that the *in-situ* generation of amphiphiles is faster than their solubilisation into the aqueous phase. Moreover, the solubilisation of the reaction products depends also on their partitioning into the adjacent bulk phases. Short and medium chain glycerides have a rather high solubility in the aqueous phase and do not depend on bile salts to facilitate their solubilisation [49]. These free fatty acids and Sn-2 monoglycerides can be absorbed directly into the blood via intestine capillaries [50] and travel through the portal vein just as other absorbed nutrients do. For more apolar lipids, bile salt emulsification into mixed micelles is mandatory for increasing their bioavailability. These emulsified lipids are transported close to the brush border of the gastrointestinal villi. The acidic microenvironment of the unstirred interfacial layer of enterocytes induces destabilization of the mixed micelles and their consequent delivery [51]. Long chain Sn-2 monoglycerides are then re-esterified with free fatty acids into triglycerides and complexed with transported to the adipose tissue or muscle cells.

The scenario around a fat droplet is schematically depicted in Fig. 3. While triglycerides (TAG) and diglycerides (DAG) are essentially oil soluble and stay in the droplet, the more hydrophilic monoglycerides (MAG) and the free fatty acids (FFA) can cross the interface and distribute between the oil and water phase. Depending on the interfacial activity and water solubility, fatty acids and monoglycerides can accumulate at the interface if no transporter (e.g. bile) is present in the solution. Therefore, an interfacial accumulation of Sn-2 monoglycerides which cannot be cleaved by the enzyme, can lead to interfacial exclusion of substrate di- and triglycerides), having an impact on the extension of lipolysis [4].

4. Interfacial properties of lipase and polar lipids

Emulsions and bilayer dispersions frequently exhibit size heterogeneity and coalesce with time. Moreover, other uncontrolled interfacial interactions may occur, which interfere with lipase catalysis. Therefore complex systems are inadequate for a fine assessment of lipolysis. For studying lipase adsorption, a useful approach has been to eliminate the bulk lipid phase and to use air–water systems [52–54]. Experiments with monolayers have the unique advantage that the arrangement and packing of the molecules can be easily measured and controlled [55]. Previous work carried out with lipase has shown that the plots of enzyme activity vs. film pressure are bell-shaped. The initial increase in lipase activity with the surface pressure was attributed to an improvement of enzyme-substrate binding [56]. The decrease of the enzymatic activity at high surface pressure was recognized as poor penetration capacity of the lipase into the surface. However, there is a lack of understanding of the interfacial properties of lipase in water/oil systems. It is difficult to discriminate between the adsorption behavior of lipases and that of their products of catalysis. In order to overcome this problem, a buffer/inert oil (decane) system with lipase at the interface where the reactants are presented in a controlled way has been studied [3]. This approach enables a quantitative analysis of the interfacial properties of substrates and products formed during the lipolysis and their interaction with lipase. By using the pendant drop technique the amphiphilic character of fatty acid salts, monoglycerides and diglycerides were assessed, as individual surfactants and as mixtures. Medium chain glycerides were used as their derivatives are more interfacially active than short chain glycerides (mainly hydrophilic) or long chain ones (essentially hydrophobic). Lipase from *Rhizomucor miehei*, was used for interfacial tension measurements as it is deprived from undesirable interfacially active contaminants (like most preparations of pancreatic lipase).

In a recent publication it is shown that monoglycerides give considerably lower values of interfacial tension than lipases [4]. From an interfacial energy viewpoint it is therefore reasonable to assume that monoglycerides can expel a lipase from the interface, provided that this low molecular weight surfactant is not cleaved by the enzyme. Under physiological conditions monoglycerides will coexist with other amphiphilic lipolysis products, in particular diglycerides and fatty acid salts. However, the interfacial activity of the monoglyceride is shown to be retained in combination with these two hydrolysis products [3].

The interfacial properties of lipase from *Rhizomucor miehei* has been compared with that of BLG, which is also a globular protein [2]. The results showed that lipases have unique interfacial properties. Despite the two proteins having similar diffusion coefficients, lipase from *Rhizomucor miehei* was shown to be more interfacially active than BLG [2]. Such high interfacial activity provides the capacity to perform fat metabolism at low enzymatic concentrations. Both proteins undergo conformational rearrangement at the interface as could be observed by skin formation upon aging [2]. Nevertheless, lipase activity is not affected by this process. Therefore, it is reasonable to state that lipases remain active under starvation conditions, provided that no biochemical degradation occurs (e.g. proteolysis). This preserved lipase activity may be explained by their interfacial rheological properties which are dramatically different from those of BLG [2]. The observed slow increase in the dynamic elasticity during adsorption and the low interfacial viscoelasticity upon shearing stress has been detected for lipases, which most likely form weak bounds

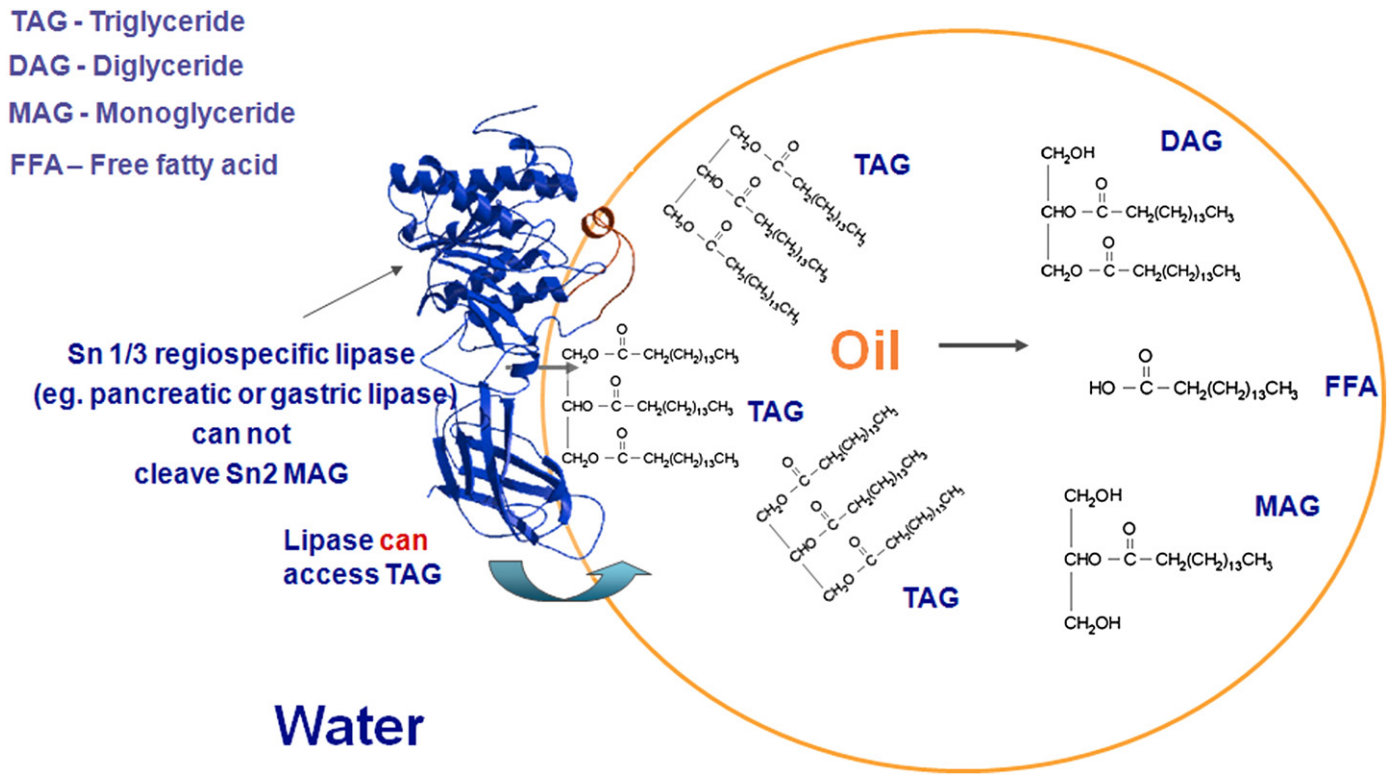

Fig. 3. Scheme of the enzymatic reaction of pancreatic lipase with triglycerides, leading to diglycerides (DAG), which are mainly oil-soluble, monoglycerides (MAG) and free fatty acids (FFA), which are mainly water-soluble.

with the interface [2]. Despite the high interfacial activity of lipase, its weak interaction with the interface would allow a better protein adaptation to changes at the interface during lipolysis, as previously reported by Haiker et al. [57] on the rapid exchange of pancreatic lipase between triacylglycerol droplets.

It has only recently been proven that low molecular weight surfactants can displace proteins from interfaces [58]. Lipase catalyzed hydrolysis of triglycerides yields diglycerides and fatty acids (or fatty acid salts). The diglycerides are further degraded to monoglycerides and fatty acids. Provided that the enzyme is Sn-1,3 regiospecific, which is the case for the normal gastric and pancreatic lipases, the formed monoglyceride is the ester of the secondary hydroxyl group of glycerol and this species is relatively stable towards further degradation. The adsorption behavior of lipases and BLG in a model water/oil system in the presence of monoglycerides has been studied [59]. Tensiometry and rheology results confirm that BLG is desorbed from the interface at well defined concentrations of monoglycerides [2,4]. This interfacial behavior was shown to be valid for both the Sn-1/3 and the Sn-2 isomers [2,4]. However, when added to an interface already covered by a lipase having Sn-1,3 regiospecificity, there is a major difference in the time dependency with respect to the interfacial tension [2,4]. This is illustrated in Fig. 4a and b for monocaprylin and monopalmitin, respectively. The monoglyceride is injected into the oil phase 1000 s after addition of the lipase. As can be seen, the Sn-2 monoglyceride reduces the interfacial tension to very low values, which do not change with time. The situation is different with the Sn-1/3 monoglyceride. Immediately after addition the interfacial tension drops below the value of that of pure lipase but then rapidly recovers (corresponding to a lipase activity of 3,95 E-5 mol/min) . The only reasonable explanation of this behavior is that the Sn-1/3 monoglyceride, but not the Sn-2 monoglyceride, is rapidly hydrolyzed to the less interfacially active degradation products fatty acid and glycerol, which cannot compete with the lipase for a place at the interface. By comparing Fig. 4a and b one can see that the dip in interfacial tension occurs during a shorter time scale for Sn-1/3 monopalmitin than for Sn-1/3 monocaprylin. Evidently, the lipase catalyzed hydrolysis of monopalmitin occurs faster than that of monocaprylin. This is noteworthy in light of the fact that monocaprylin is the most interfacially active of the two and should therefore have a stronger driving force for the interface than monopalmitin. The latter is a relatively hydrophobic amphiphile that to a considerable extent partitions into the oil phase. The difference in hydrolysis rate found for the monoglycerides seems to indicate that monopalmitin is a better substrate for the lipase than monocaprylin.

Similar results were obtained for lipoprotein lipase and pancreatic lipase in the presence of co-lipase [4]. Therefore one may conclude that Sn-2 monoglycerides reduce the interfacial tension of lipase saturated water/oil interfaces, independently of the enzyme source and presence of colipase.

Freshly distilled monoglycerides used in food industry normally contain an equilibrium ratio of 95% Sn-1/3 isomer and 5% Sn-2 isomer [60]. Provided that monoglycerides are incorporated in a normal meal, lipase will have to compete with these amphiphiles for the interface. Fig. 5 shows the effect on the interfacial tension of adding lipase after 1000 s to the phosphate buffer/decane system to which either Sn-1/3 or Sn-2 monopalmitin has been pre-added. As is seen from the Fig. 5, the interfacial tension attains approximately the same low value with both regio-isomers. When the Sn-2 monoglyceride is covering the interface, the interfacial tension value is unaffected by the lipase addition. With the Sn-1/3 monoglyceride at the interface, on the other hand, there is a steady increase in interfacial tension. This can only be explained by a lipase catalyzed hydrolysis of the monoglyceride into palmitic acid and glycerol.

With tensiometry one follows the change in surface free energy but the technique does not *per se* give information about what molecules are present at the interface. Interfacial shear rheology gives complementary information because it provides information on the stiffness of the interface. The kinetic irreversibility of protein adsorption in pure systems normally leads to the formation of very stiff layers while the reversibility of low molecular weight surfactants adsorption usually leads to flexible

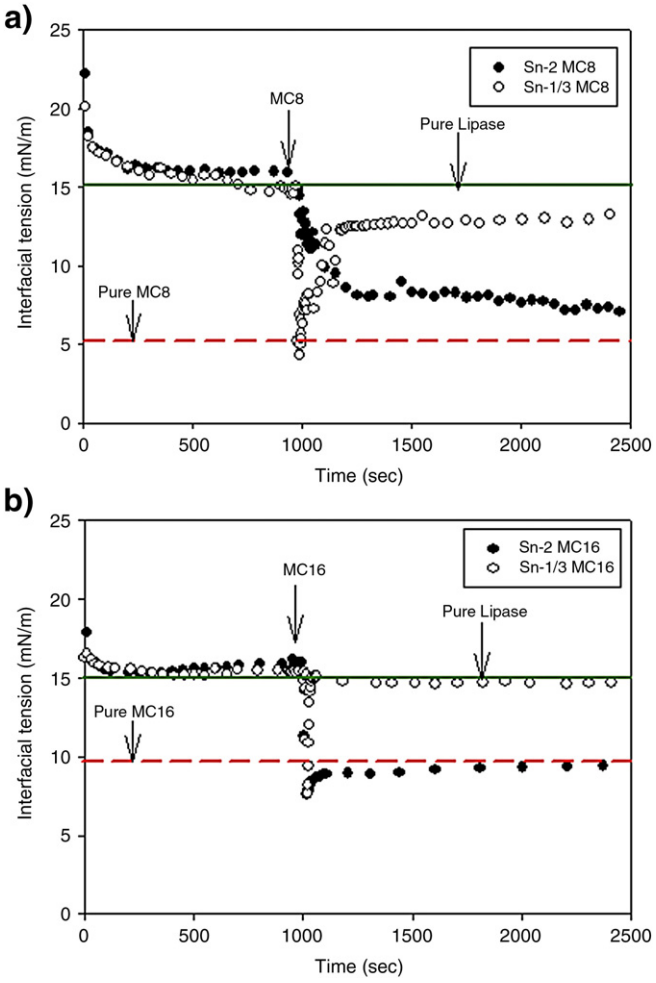

Fig. 4. Interfacial tension of a buffer/decane system with 3.3 E-5M lipase from *Rhizomucor miehei* added followed by injection into the oil phase of (a): 5.0 E-3M of either Sn-1/3 monocaprylin (Sn-1/3 MC8) or Sn-2 monocaprylin (Sn-2 MC8), or (b): 6.7 E-4 M of either Sn-1/3 monopalmitin (Sn-1/3 MC16) or Sn-2 monopalmitin (Sn-2 MC16) (redrawn from Ref. [4]).

ones. The torsion pendulum technique gives information about the stiffness of the interfacial layer. By measuring the variation of oscillation amplitude with time, a change in rheological properties of this layer can

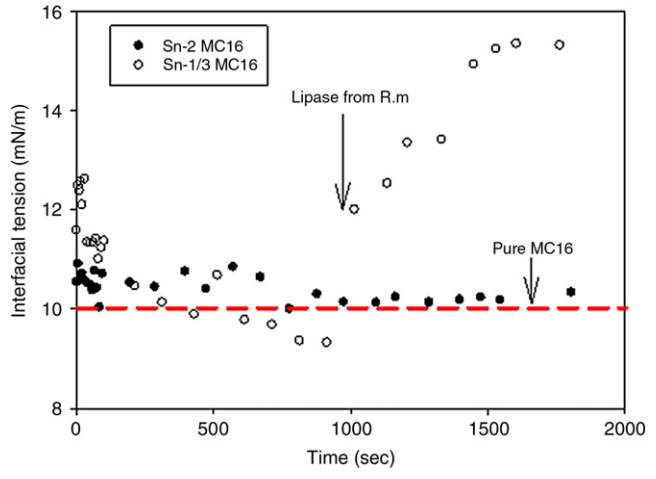

Fig. 5. Interfacial tension of a buffer/decane system with 6.7 E-4 M of either Sn-1/3 monopalmitin (Sn-1/3 MC16) or Sn-2 monopalmitin (Sn-2 MC16) added followed by injection after 1000 s of 3.3 E-5 M lipase from *Rhizomucor miehei* into the aqueous phase (redrawn from Ref. [1]).

be detected. Previous results have confirmed that Sn-2 monoglycerides exclude the lipase from the interface as the rheological properties of mixed system correspond to the one of the low molecular weight surfactant [1–4]. However the enzyme remains in an adjacent sublayer [4]. The idea that proteins can remain underneath a monoglyceride film either through hydrophobic interactions between the protein and the lipid or by local anchoring through the monoglyceride layer has been suggested before [4,61]. A different model has highlighted that proteins can still be associated with the hydrophilic side of the surfactant monolayer after the reaching the collapse pressure has been reached [62]. Actually, it has been shown that lipase can also adsorb at hydrophilic surfaces, although to a lesser extent than at hydrophobic ones [63].

From the adsorption isotherms of the pure molecules it is possible to determine the key parameters (e.g. molecular area, adsorption constant, interaction parameter, compressibility) that allow modulation of the interfacial composition of mixed systems. The minimum concentration of Sn-2 monoglycerides required to desorb lipase from the interface can be estimated. Reis et al. have showed that lipases and tricaprylin can easily be excluded from the interface by the more interfacially active monoglyceride [4]. In fact, the concentration of monoglyceride needed to desorb 50% of tricaprylin from the interface is about 2 orders of magnitude lower than that required for desorbing the lipase.

During digestion, lipases are exposed to a wide variety of interfacially active molecules, which either occur naturally in the gastrointestinal tract, are added into the food, or are generated upon hydrolysis. The fact that lipase is exposed to high interfacial pressures (as in the case in the presence of Sn-2 monoglycerides) does not *per se* mean that the enzymatic activity is completely blocked.

It is known that the interfacial quality of lipid emulsion droplets influences the rate and extent of lipolysis. It was found that emulsions prepared with digalactosyldiacylglycerol (DGDG) had a longer lag phase prior to lipase activation with a decrease in lipolysis rate [64]. In contrast, no inhibitory effect on lipase kinetics was observed in emulsions prepared with monogalactosyldiacylglycerol (MGDG) [64]. It was postulated that the larger headgroup and the more tightly packed molecular organization of DGDG at the interface gave rise to steric hindrance that retarded colipase and lipase adsorption onto the substrate surfaces and hence delayed and reduced lipolysis. Moreover, bile salts did not completely displace DGDG from the interface, explaining the reason why DGDG still possessed lipase inhibitory activity in the presence of bile salts at physiologically relevant concentrations [64].

Previous reports have shown that in vitro lipolysis of triglycerides can be decreased by lecithin-coated emulsions in the presence of chitosan [65] This result was attributed to the fact that chitosan formed a protective layer around the droplets and that it promoted extensive droplet flocculation leading to a decrease in the total surface area and consequence lipase kinetics In order to test the hypothesis of self-regulation of the enzymatic reaction through accumulation of Sn-2 monoglycerides, a model biphasic system was studied where the substrate (p-nitrophenylpalmitate, pNPP) is converted into a yellowish product (nitrophenol) upon lipase catalyzed hydrolysis. It has clearly been demonstrated that the presence of Sn-2 monolaurin reduced the hydrolysis of pNPP as compared to reaction in the presence of the Sn-1/3 isomer [1,4]. The experiment showed that the Sn-2 monoglyceride exerts a control of the lipolytic activity.

Micellar solubilisation in the duodenal lipolysis has long been proposed as an important step in fat absorption [66]. Hofmann and Borgström [67] have demonstrated a micellar phase composed of bile salts, fatty acids and monoglycerides during fat digestion in the human gastro-intestinal tract. Therefore, the solubilisation by bile salts of lipolytic products from the interface into the aqueous solution may affect the self-regulation mechanism induced by Sn-2 monoglycerides. However, the solubilisation capacity of bile is limited [68]

and controlled by the delivery kinetics of mixed micelles to the brush border epithelial cells of the upper intestine. As a consequence, lipolysis should be slowed down due to an interfacial accumulation of Sn-2 monoglycerides when the solubilisation limit of bile is exceeded. Recent studies confirmed that Sn-2 monoglycerides can decrease lipase catalyzed hydrolysis of pNPP in a biphasic system when bile salts are used at physiological concentrations [69]. Despite the presence of bile (as verified by an increased turbidity of the apolar phase), Sn-1/3 monoglycerides were not efficient in preventing hydrolysis of pNPP. In this case the presence of nitrophenol (hydrophilic yellowish product of biocatalysis) can be easily detected in both the polar and the apolar phase. Spectrophotometry results confirm that 95% of the initial substrate was consumed in the biphasic system enriched with Sn-1/3 monoglyceride while no hydrolysis was detected in the presence of the Sn-2 isomer [69]. These encouraging results were taken a step further by studying the influence of surfactants on lipolysis in a model gastro-intestinal system [70]. This model is frequently used in nutritional studies and mimics digestive processes occurring in the upper gastro-intestinal tract. Studying the processes with added enzymes and bile salt micelles at biological concentrations and pH allows monitoring the digestion in a stepwise fashion at various positions along the different compartments that simulate the upper digestive system.

The impact of three different species (Sn-2 monopalmitin, β-lactoglobulin and 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine) on the lipolysis of tricaprylin was assessed throughout the model gastro-intestinal tract from TNO [70]. The presence of high interfacially active molecules could have an impact on the accessibility of lipase to their substrates in mixed micelles. The results from digestion trials of tricaprylin (TC8) with and without Sn-2 monopalmitin (Sn-2 MC16) were collected. The results revealed that a large and significant difference in the liberation of fatty acids and in the extent of the lipolytic reactions. The strong suppression of fat hydrolysis induced by Sn-2 monopalmitin can be attributed to exclusion of both tricaprylin and lipase from the interface. In fact, the TIM experiments showed that gastric lipolysis is reduced in the presence of all the tested interfacially active molecules [70]. This result could be explained by a reduced accessibility of gastric lipase to the triglyceride. However, an increased lipolysis in all the other compartments of the gastrointestinal tract was found for the tested meals enriched with either lysophosphatidylcholine or β-lactoglobulin.

Previous reports [71] have described that lysophosphatidylcholine causes an increase in the K_m and a decrease in the V_{max} values for the interaction between pancreatic lipase and triacylglycerol. This indicates that the substrate affinity and the catalytic activity are inhibited by lysophosphatidylcholine. The TIM experiments also show that gastric lipolysis of tricaprylin is decreased by lysophosphatidylcholine [70]. This regulatory effect can most likely be attributed to decreased substrate accessibility to the enzyme. Lysophosphatidylcholine, which is naturally formed by phospholipase A2 catalyzed hydrolysis of phosphotidylcholine, is more interfacially active than both lipase and tricaprylin (data not shown). Therefore, at the model stomach environment, the lysophospholipid will expel triglycerides from the interface. However, in the duodenum, lysophospholipids will most likely be solubilised into bile salts micelles. Addition of taurodeoxycholic acid salts or phospholipids has previously been shown to restore the inhibitory effect of lysophospholipids on pancreatic lipase [68]. An increased hydrolysis of tricaprylin in the duodenum as compared to the control has been observed for test meals enriched with lysophospholipids. This result has been attributed to an increased interfacial area induced by lysophosphatidylcholine micelles which increases the dispersion of the test meal. However, the interfacial adsorption of lysophosphatidylcholine on the oil droplets did not hamper lipase accessibility to its substrate (probably due to a low packing of the surfactant at the interface).

BLG can either be replaced from the interface by more interfacially active molecules or be cleaved by proteases and converted into peptides, which are not very surface active and which allow the lipase to access the substrate without major restrictions. Since the proteolytic activity in the stomach is not maximized, then the presence of interfacially active protein blocks gastric lipase accessibility to their substrates having an impact on fat digestion.

5. Tools to be applied

Despite the current advances in characterizing the interfacial events occurring during digestion, there are still some tools which can be applied for clarifying points which have not yet been addressed.

Drop profile analysis with drop volume exchange is a good tool to have a clear picture on the impact of system perturbation (either in the aqueous or oil phase) in the interfacial behavior of lipolysis molecules. This unique methodology allows to perform adsorption experiments with different protocols, i.e. sequential adsorption of enzyme and other surface active compounds. Adding a flow cell to the instrumentation, even competitive adsorption from the oil and water phases, simultaneously, is feasible [72]. In this way, the enzyme could be injected as component 1, a water soluble surfactant (fatty acid) as component 2, and an oil soluble surfactant (diglyceride) as component 3. These experiments, based on the dynamic behavior of the liquid interface, will provide information on the interfacial composition, as each adsorbed compound has its own "fingerprint", i.e. adsorbs according to respective mechanisms and reacts to external perturbations at respective frequencies [73]. Hence, it should be possible to learn about the location of the enzyme in the interfacial region. This approach would allow for example to evaluate the impact of bile (which could be introduced in a later stage in the aqueous phase) on the interfacial properties of Sn-2 monoglycerides and lipase (Fig. 6).

Circular Dichroism (CD) refers to the differential adsorption of the left and right circularly polarised radiation. This effect occurs when a chemical group is chiral (optically active) either intrinsically due to its structure, or covalently linked to a chiral centre, or being placed in an asymmetric environment. This technique gives structural information for peptides, proteins or nucleic acids, i.e. the secondary structures such as α helical or β sheets. Typical CD studies of proteins are realised in bulk solutions, however it is also possible to study the conformation of proteins at an interface (Fig. 7) [74]. It has been shown that accumulated proteins at and near the air/water interface can be detected by using External Reflection Circular Dichroïsm (ERCD) and that the signals obtained contain information on the conformation properties and concentration of the proteins residing at the interface. This tool would allow the quantification of lipase at the oil/water interface in the presence of emulsifiers. Moreover, eventual changes in the enzyme could be monitored and related to the measured activity.

We propose to apply infrared external reflection spectroscopy, also known as infrared reflection/absorption spectroscopy (IRRAS, Fig. 8) to probe the adsorption layers at the oil/water interface. IRRAS is a powerful technique using IR light to obtain structural information about monomolecular thin interfacial films [75]. For this application, a special instrument must be set up, trying to get access to the respective signals at the liquid/liquid interface. As shown schematically in Fig. 8, an IR external reflection spectrum of a monomolecular film adsorbed on a reflective substrate is obtained by reflecting the incoming radiation from the three-phase ambient — thin film — substrate system and measuring the reflected intensity as a function of the wavelength. Because this is a reflection experiment, the reflection–absorbance spectrum is created by rationing the sample reflectance (R) against the reflectance of the film-free substrate ($R0$) as $A = -\log(R/R0)$. The reflection spectrum obtained by this process is a function of the wavelength, the state of polarization, the thin film

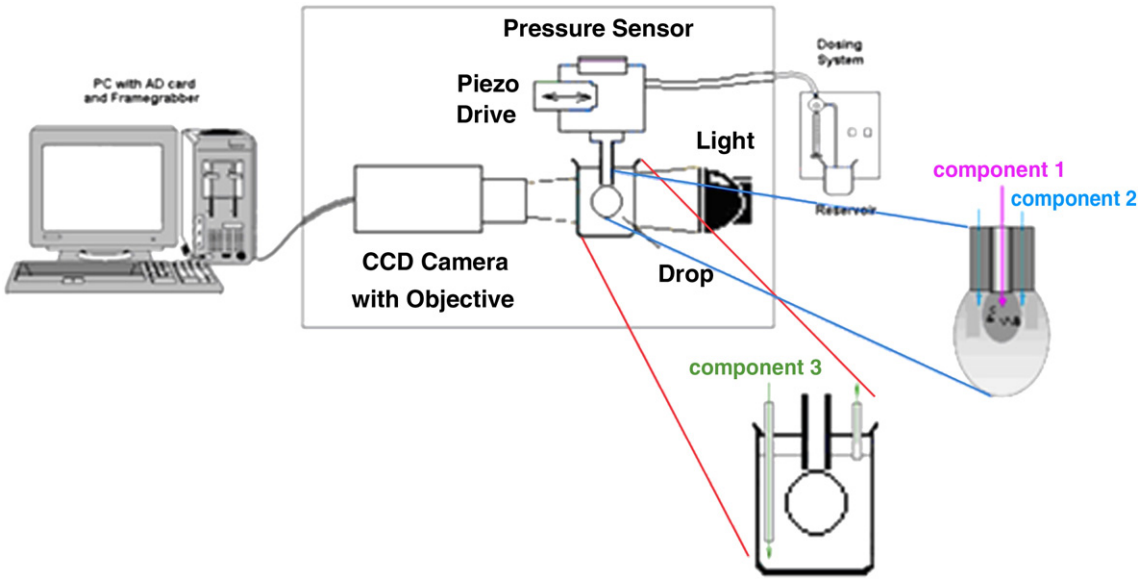

Fig. 6. Drop profile analysis combined with double capillary, flow cell and capillary pressure/piezo drive.

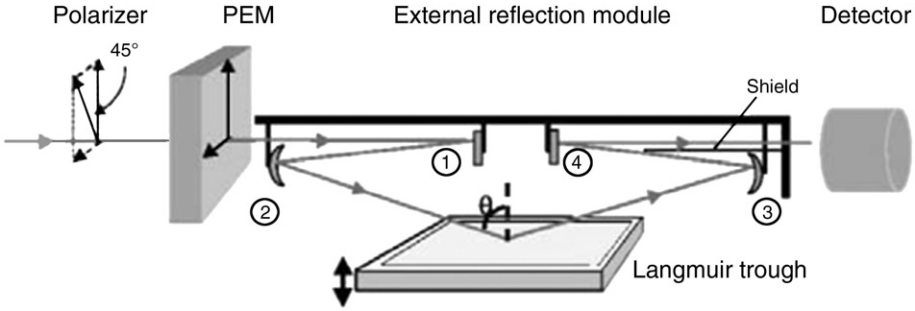

Fig. 7. Principle of circular dichroism at liquid interfaces in the ERCD mode, composed by 4 mirrors (1 and 4 are fixed and 2 and 3 can be rotated allowing different incident angles (θ) from 90° to 50°) and connected at the position of the detector to a CD spectropolarimeter; right — spectra showing the amid group transitions in the backbone of a protein.

thickness, the angle of incidence of the reflected light and the optical constants of the three phases involved.

IR spectroscopy at the air/water interface has been especially applied for studying the interactions between lipid model systems and biopolymers, such as proteins and peptides [76]. One can predict the conformation and orientation of lipid-chains or the interactions between the lipid-head groups (the lipid orientation could be related to the capacity lipase substrate to be also part of the interface). In addition, the secondary structure of proteins and peptides can be obtained and also their orientation at the air/water interface. To the best of our knowledge, there is no work so far performed at the interface between two immiscible liquids.

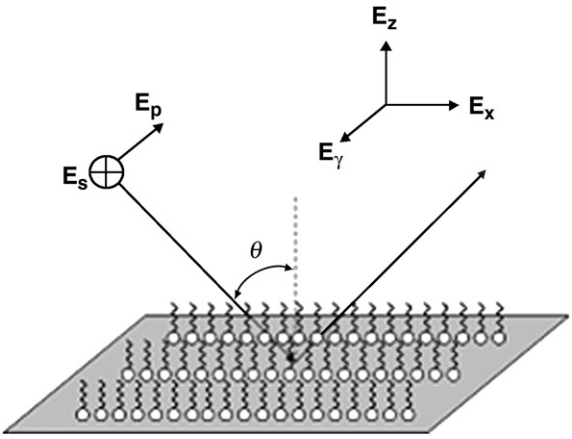

Fig. 8. Principle of IRRAS at a liquid interface.

Enzymes form complexes with surfactants in the solution bulk, due to ionic (for molecules with a charged head group) and hydrophobic interaction [77]. Using quasi-elastic light scattering the size distribution of the formed aggregates and their zeta potential can be measured. For such investigations, a Zetasizer Nano ZS should be available. The principle of this technique is based on that fact, that particles or molecules illuminated with a laser, is scattered so that the reflected light fluctuates at a rate that is dependent upon the size of the particles. The analysis of these intensity fluctuations yields the velocity of the Brownian motion and hence the particle size. CD and IRRAS experiments are planned to gain important information on the structure changes of enzymes at the interface due to adsorption, due to competition with low molecular weight surfactants of different origin, but also other proteins (and also enzymes). With the help of Brewster Angle Microscopy (BAM, see Fig. 9), we can study and visualise structures in thin films on flat solid or liquid surfaces. BAM is based on the properties of reflectivity of laser light at interfaces. When a *p*-polarised light is incident on a flat sample under a certain angle, the so-called Brewster angle (θ), no light is reflected, and the detector does not measure any signal. The Brewster angle can be determined from the ratio of the refractive indices of the two phases (n1, n2). When a monolayer covers the surface, it changes the reflection of the light, the refractive index of the upper media is changed and this in

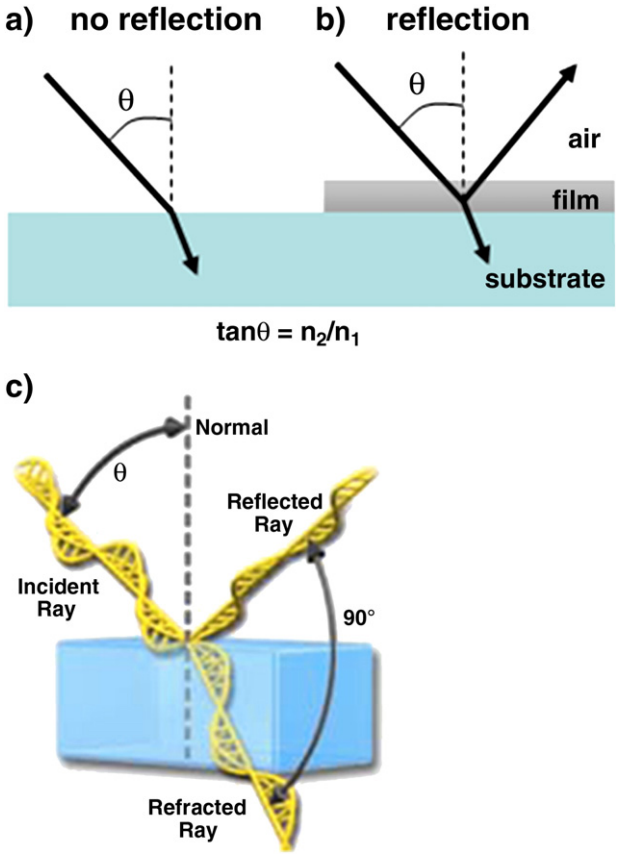

Fig. 9. Principle of Brewster Angle Microscopy.

turn changes the Brewster angle. In this situation, we obtain a reflection leading to a contrast picture (Fig. 9a). By using an analyzer before the detector, we can get more detailed information about the anisotropy of the studied layer, for example about the orientation of the molecules in a domain. In another possible configuration (Fig. 9b) the incoming light is non-polarised and under the Brewster angle only the *s*-polarised light is reflected. When the reflected beam passes now a polariser, which is transparent only for *p*-polarised light, the detector does not measure any signal.

If a layer covers the surface, it changes the Brewster angle, the reflected light is now not only *s*-polarised and a part of the light can pass through the polarizer to the detector and we get again a contrast picture. BAM experiments will allow for visualisation of the interfacial layer and gives answer on the formation of 2D or 3D structures. In contrast to methods which require a transfer of the surface layer onto a solid support, this method can be applied directly to the liquid interface. As a suitable verification of the suggested interfacial research, it is planned in vitro gastrointestinal model experiments using an instrument described recently in [70].

The application of this model system will allow to qualify the interfacial approach by mimicking the most important processes in the digestion of fat [78].

A new system has recently been developed for in-situ assembly and screening of enzyme inhibitors with surface tension microarrays [79]. Generally, microarrays are produced by attaching one of the interacting components to the array surface. Subsequent screening of the surface-displayed entities against a liquid sample enables the identification of specifically interacting partners. Unfortunately, such a heterophase assay cannot be applied accurately to the majority of (bio)chemical reactions, which normally proceed in the solution phase. Gosalia and Diamond proposed a simple, yet elegant, solution to this problem by conducting enzymatic reactions in glycerol nanodroplets printed on the surface of a glass slide [79,80]. This and related techniques have subsequently been used for profiling enzyme activities, inhibitors, and polymeric biomaterials. It looks evident that this tool would be ideal for screening other lipase interfacial tension inhibitors beyond Sn-2 monoglycerides.

6. Conclusion

According to OMS, there are currently 1 billion overweight and 300 million obese people in the world [81]. Many governments and companies react on this global situation and put much activity into the design of healthier food. However, fatty food still prevails in the market and is commonly best selected by the consumer. Many companies react on this global situation and put much activity into the design of healthier food [52]. The consequences of the alarming overweight are hypertension, type 2 diabetes, coronary heart disease, stroke, gallbladder disease, osteoarthritis and more. These problems already arrived in the fast developing countries like China and call for a general global strategy. In case prevention fails, medicinal treatment of obesity becomes necessary. In many cases obesity cannot be cured, as treatment strategies are effective only as long as they are used. Most effective appears to be combined therapies. In a recent overview [82] the functions for a drug to have significant impact on body weight are to ultimately reduce energy intake, increase energy expenditure, or both. Two main strategies have been advanced by the food and pharmaceutical industries to decrease fat absorption after its ingestion: lipase inhibitors (e.g. Orlistat) and fat replacers (e.g. Olestra). Orlistat is a lipase competitive substrate that irreversibly binds to the catalytic site of the enzyme while Olestra (despite mimicking the physical characteristics of triglycerides) cannot be digested due to steric hindrances. Unfortunately both strategies have undesirable side-effects and need significant improvement. Since obesity is recognized as a serious health problem and given the lack of long-term success of non-surgical treatments there is clearly an urgent need for efficient body weight reducing alternatives. Due to the apolar nature of oils and fats, the oil–water interface is where the control of lipolytic conversion and finally digestion takes place. By virtue of their physical properties, substrates, products and enzymes will partition themselves among the bulk and surface phases. Furthermore, this distribution is not fixed, but changes as lipolysis proceeds [4,82]. Thus, superimposed on the more classical aspects of enzyme regulation are the complications of phase heterogeneity and its temporal changes. As a consequence of the physical complexity of the environment in which lipolysis occurs, the level of understanding of the regulation of lipolysis has lagged behind that for homogeneous catalysis.

We here highlight that lipolysis is a self-regulated process which can be modulated throw interfacial engineering. Synergies between different expertise (e.g. colloid science, biophysics and biochemistry) could move forward the current boundaries on interfacial enzymology and deliver a new avenue to fight obesity.

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