Application of interfacial properties of polymeric surfactants in physiological processes for biomedical and nutraceutic purposes

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La doctoranda Amelia Torcello Gómez y los directores de la tesis Dr. Antonio Martín Rodríguez, Dr. Julia Maldonado Valderrama y Dr. Ana Belén Jódar Reyes, Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por la doctoranda bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

Granada, Septiembre de 2012

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SUMMARY

In recent years, the fields of food, medicine and pharmacology have met other fields more often related with chemistry and physics, such as those of colloidal and interfacial science. As a result, new colloidal-based delivery systems are being developed in order to solve the complications related with the traditional administration of drugs and bioactive compounds. Many of these problems arise when the compounds to be administered are poorly water-soluble. For that reason colloids based on a hydrophobic core are designed to dissolve and protect the compounds. However, other obstacles emerge related to the biocompatibility with the immune system when they are intravenously administered or the digestibility when they are orally administered. These issues take place at the interfaces of the colloidal carriers. Nevertheless, despite, the relevance of interfacial properties towards the rational design of delivery or therapeutic systems, very few specific works have been carried out specifically dealing with interfaces. Accordingly, the main objective of this thesis is the in-depth study of fluid-liquid interfaces under simulated physiological conditions relevant for application of colloidal carriers and the correlation between interfacial and material science and the performance of complex biotechnological systems.

The memory of this thesis is presented as a collection of research articles that have already been published or submitted for publication, in peer-reviewed journals all belonging to the first quartile. These papers are interrelated, providing consistency to the Results section which is divided in four parts:

Summary

Part 1. Interfacial characterization of model air-water interfaces covered by Pluronic F68 and correlation with the behavior of Pluronic F68-coated nanoparticles under intravenous conditions.

The aim of this work was to understand why drug nanocarriers coated by polymeric surfactants from the family of Pluronics improve their long-circulating properties in comparison with uncoated particles. The interfacial characterization was performed by means of interfacial tension and interfacial dilatational rheology where Pluronic F68-covered air-water interfaces were subjected to physiological media containing a model antibody, immunoglobulin G (IgG). To this end, we used a pendant drop film balance which allows penetration studies by subphase exchange of the bulk solution which has been designed and assembled at the University of Granada (UGR). Comparison with the adsorption of pure IgG at bare air-water interface allows interpretation of the data concluding that Pluronic F68 affects the adsorption of IgG altering its original conformation at air-water interface. This modification of adsorbed IgG conformation triggers the subsequent decrease in the immunoactivity of Pluronic F68-coated nanoparticles by supporting the partial protein denaturation suggested by the loss of immunoreactivity in the nanoparticles coated in sequence by Pluronic and IgG. The use of surface tension to obtain structural and mechanical information about the coating procedure is a novel approach to understand generic features of the biocompatibility of colloidal systems. This work validates the methodology of the studied systems that will serve as a basis for the next works and is described in the following publication:

- Torcello-Gómez, A., Santander-Ortega, M. J., Peula-García, J. M., Maldonado-Valderrama, J., Gálvez-Ruiz, M. J., Ortega-Vinuesa, J. L., & Martín-Rodríguez, A. (2011). Adsorption of antibody onto Pluronic F68-covered nanoparticles: link with surface properties. *Soft Matter*, *7*, 8450-8461.

Part 2. Physicochemical properties and interfacial characteristics of Pluronic F68 in simulated intestinal fluids.

The objective of this part was to probe the advantages of stabilizing oil-in-water emulsions with Pluronic F68 in order to delay the rate of lipid digestion, as compared with traditional phospholipids. The use of interfacial techniques to tackle this issue, as in Part 1, is original and provides new outcomes with respect to the differences observed when traditional surfactants are compared with more innovative steric surfactants such as the family of Pluronics. Oil-water interfaces that were pre-covered by Pluronic F68 or phospholipids were tested under duodenal media containing the enzyme lipase and/or bile salts by means of the subphase exchange technique. It was shown that Pluronic F68 inhibits the adsorption of lipase alone or in the presence of bile salts, subsequently limiting the rate of lipid digestion in Pluronic-stabilized emulsions, in contrast to oil-water interfaces or emulsions stabilized by phospholipids. Specifically, duodenal components decreased the interfacial tension of a Pluronic-covered interface to a lesser extent than the interface covered by phospholipids. The interfacial characterization carried out at the UGR was importantly complemented by experiments performed at the *Biopolymers and Colloids Research Laboratory*, at the University of Massachusetts (Amherst, Massachusetts, USA) under the supervision of Prof. D. J. McClements, as part of a short stay. Droplet charge, droplet size, microstructure and titrimetry measurements were used to complement the interfacial tension study. As a result, we prove that Pluronic F68-stabilized emulsions are more resistant to lipid digestion than phospholipids-stabilized emulsions. These results demonstrate that the properties of the interfacial layer surrounding lipid droplets can be designed to modulate the lipid digestion process. This chapter is explained in detail in the following articles:

Summary

- Torcello-Gómez, A., Maldonado-Valderrama, J., de Vicente, J., Cabrerizo-Vílchez, M. A., Gálvez-Ruiz, M. J., & Martín-Rodríguez, A. (2011). Investigating the effect of surfactants on lipase interfacial behaviour in the presence of bile salts. *Food Hydrocolloids*, *25*, 809-816.
- Torcello-Gómez, A., Maldonado-Valderrama, J., Martín-Rodríguez, A., & McClements, D. J. (2011). Physicochemical properties and digestibility of emulsified lipids in simulated intestinal fluids: influence of interfacial characteristics. *Soft Matter*, *7*, 6167-6177.

Part 3. Characterization of Pluronic F68, phospholipids and a model bile salt at the oil-water interface: link of Pluronic or phospholipid-covered interfaces with the stability of oil-in-water emulsions stabilized by Pluronic or phospholipids under the action of bile salts.

Bile salts play a crucial role in lipid digestion by desorbing original emulsifier from emulsion interfaces. Hence, this part was devoted to study these systems in more detail. Here, emulsifiers and a bile salt are exhaustively characterized at the oil-water interface with interfacial tension and dilatational rheology experiments, as well as by means of theoretical models. Theoretical predictions and experiments agree satisfactorily, and provide structural explanation for the interaction of the considered surfactants with duodenal components. Namely, Pluronic F68 better resists the inclusion of bile salt onto the oil-water interface due to its interfacial conformation providing steric hindrance, as compared to phospholipids. These results are again importantly complemented with emulsion behavior, correlating the higher stability showed by Pluronic-stabilized emulsions in the presence of bile salt unlike emulsions stabilized by phospholipids with the interfacial study. The detailed discussion is contained in the following papers:

- Torcello-Gómez, A., Jódar-Reyes, A. B., Maldonado-Valderrama, J., & Martín-Rodríguez, A. (2012). Effect of emulsifier type against the action of bile salts at oil–water interfaces. *Food Research International, 48*, 140-147.
- Jódar-Reyes, A. B., Torcello-Gómez, A., Wulff-Pérez, M., Gálvez-Ruiz, M. J., & Martín-Rodríguez, A. (2010). Different stability regimes of oil-in-water emulsions in the presence of bile salts. *Food Research International, 43*, 1634-1641.

Part 4. How interfacial and bulk properties of Pluronic F68 and F127 influence interaction with bile salts an affect the rate of lipid digestion.

Once it has been demonstrated the efficiency of Pluronic F68 to delay lipid digestion in contrast to phospholipids, this chapter compares the effectiveness of different Pluronics. In particular, we evaluate the effect of size by using Pluronic F127, which is a larger molecule than F68. We shall focus on studying the differences in the structure of these polymeric surfactants, which influence the final rate of lipid digestion in emulsions. A complete combination of state-of-the-art techniques such as micro-calorimetry and electron microscopy, apart from the interfacial techniques abovementioned, were used. This study was accomplished in a short stay at the *School of Biosciences*, at the University of Nottingham (Sutton Bonington, UK) under the supervision of Dr. T. J. Foster. As a result, Pluronic F127 showed to be more resistant to displacement by bile salt than F68 at the oil-water interface due to the larger steric hindrance and interfacial coverage provided. In addition, Pluronic F127 seems to interact with more molecules of bile salt, preventing them from adsorbing at the oil-water interface. Hence, Pluronic F127 affects to a larger extent the ability of bile salt to promote the further cascade of lipolysis in the presence of lipase. Once again, this colloidal characterization is complemented with interfacial tension studies. Now, we use a new set up designed at the UGR to simulate *in-vitro* digestion in a single droplet by upgrading the

subphase exchange methodology to a multi-exchange device (The OCTOPUS). This study provides a correlation between molecular size and digestion rate which importantly complements the behavior of emulsions. The research work presented in this chapter led to the preparation of two manuscripts that are submitted for publication:

- Interactions between Pluronics and bile salts in aqueous phase, at interfaces and in emulsions.
- Pluronic-covered oil-water interfaces under simulated duodenal conditions.

Accordingly, findings from this thesis prove that the new approach to look at fluid-liquid interfaces in order to understand the application of interfacial properties of Pluronics to control the biodegradation of colloidal carriers is notably innovative and will attract the attention of the scientists from pharmacology and food research.

RESUMEN

En los últimos años, los campos de investigación pertenecientes a la medicina y farmacia, y aquellos otros concernientes a la nutrición y alimentación, se han acercado a otras áreas de investigación más bien relacionadas con la química y la física, como por ejemplo la ciencia coloidal e interfacial. Como fruto de este avance, se están desarrollando nuevos sistemas coloidales transportadores de sustancias que solucionen las complicaciones que surgen cuando los fármacos o compuestos biológicamente activos son administrados en el organismo. La mayor parte de estos inconvenientes están asociados a la poca solubilidad que dichos compuestos presentan en fase acuosa. Por ello, se diseñan transportadores con núcleo hidrófobo que permitan disolver y proteger las sustancias que van a ser administradas. Sin embargo, surgen también otros obstáculos relacionados con su compatibilidad biológica con el sistema inmune, cuando la vía de administración es la intravenosa, o con su digestibilidad si la administración es vía oral. Todos estos problemas se originan en las interfases de los transportadores coloidales. A pesar de la importancia que representan las propiedades interfaciales en el diseño de estos sistemas terapéuticos o liberadores de sustancias, se han llevado a cabo muy pocos trabajos relacionados específicamente con las interfases de estos sistemas. Por lo tanto, el principal objetivo de esta tesis es el estudio en profundidad de interfases líquido-fluido bajo condiciones fisiológicas simuladas, muy importante para su aplicación en sistemas transportadores coloidales. También se persigue la correlación entre la ciencia de materiales e interfacial y el rendimiento de sistemas biotecnológicos complejos.

La memoria de esta tesis se presenta como una agrupación de artículos de investigación que se han publicado en (o enviado para su publicación a) revistas científicas indexadas pertenecientes al primer cuartil. Estos artículos están

relacionados entre sí proporcionando consistencia a la sección de Resultados, que se divide en cuatro partes:

1ª Parte. Caracterización interfacial de interfases modelo aire-agua recubiertas de Pluronic F68, y correlación con el comportamiento de nanopartículas recubiertas de Pluronic F68 bajo condiciones intravenosas.

El objetivo de este trabajo es comprender por qué los nanotransportadores de fármacos recubiertos de surfactantes poliméricos de la familia de los Pluronics mejoran sus propiedades de larga circulación en el torrente sanguíneo, a diferencia de las nanopartículas sin recubrir. La caracterización interfacial se llevó a cabo con estudios de tensión interfacial y reología dilatacional interfacial en los que interfases aire-agua recubiertas de Pluronic F68 son sometidas a un medio fisiológico que contiene un anticuerpo modelo, inmunoglobulina G (IgG). Para ello, usamos una balanza de gota pendiente que permite realizar estudios de penetración mediante la técnica del intercambio de la subfase, y que ha sido diseñada y ensamblada en la Universidad de Granada (UGR). La comparación con la adsorción de IgG sobre la interfase aire-agua sin recubrir permite interpretar los resultados, concluyendo que el Pluronic F68 afecta la adsorción de IgG en el sentido de que altera su conformación original sobre la interfase aire-agua. Esta modificación en la conformación de la IgG adsorbida desencadena la disminución de actividad inmune en las nanopartículas recubiertas de Pluronic F68. Esto se corrobora con la desnaturalización parcial que sugiere la pérdida de inmunoreactividad en partículas recubiertas secuencialmente de Pluronic e IgG. El uso de la tensión interfacial para obtener información mecánica y estructural sobre el comportamiento del recubrimiento es un enfoque novedoso con el que se puede comprender las características genéricas de la biocompatibilidad de sistemas coloidales. Este estudio da validez a la metodología empleada que servirá como base para los siguientes trabajos y se encuentra descrito en la siguiente publicación:

- Torcello-Gómez, A., Santander-Ortega, M. J., Peula-García, J. M., Maldonado-Valderrama, J., Gálvez-Ruiz, M. J., Ortega-Vinuesa, J. L., & Martín-Rodríguez, A. (2011). Adsorption of antibody onto Pluronic F68-covered nanoparticles: link with surface properties. *Soft Matter*, *7*, 8450-8461.

2ª Parte. Propiedades físico-químicas y características interfaciales de Pluronic F68 en condiciones intestinales simuladas.

El objetivo de esta parte consiste en evaluar las ventajas que presenta la estabilización de emulsiones de aceite en agua con Pluronic F68 para controlar la digestión de lípidos, en comparación con los fosfolípidos tradicionales. El uso de técnicas de tensión interfacial para abordar este tema, como en la primera parte, es original y proporciona resultados novedosos con respecto a las diferencias observadas cuando se comparan surfactantes tradicionales con surfactantes estéricos más innovadores en este campo, como los Pluronics. Se sometieron interfases aceite-agua recubiertas de Pluronic F68 o fosfolípidos a un medio fisiológico simulando las condiciones del duodeno, que contenía una enzima lipasa y/o sales biliares, mediante la técnica del intercambio de la subfase. Se mostró que la presencia de Pluronic F68 inhibe la adsorción de la lipasa pancreática, en ausencia o presencia de las sales biliares, limitando por tanto, la velocidad con la que se digieren los lípidos para el caso de emulsiones de aceite en agua estabilizadas con Pluronic, a diferencia de la presencia de fosfolípidos. Específicamente, los componentes duodenales disminuyen la tensión interfacial de una interfase aceite-agua recubierta de Pluronic en menor medida que aquélla recubierta por fosfolípidos. La caracterización interfacial que se llevó a cabo en la UGR se complementó en gran medida con los experimentos desarrollados en el *Biopolymers and Colloids Research Laboratory*, en la Universidad de Massachusetts (Amherst, Massachusetts, EEUU) bajo la supervisión del

Catedrático D. J. McClements, como parte de una estancia pre-doctoral. Se hicieron medidas de tamaño de gota, carga superficial de gota, micro-estructura y valoración de pH, para complementar el estudio de tensión interfacial. Finalmente demostramos que las emulsiones estabilizadas con Pluronic F68 son más resistentes a la digestión de lípidos que las estabilizadas por fosfolípidos. Estos resultados justifican que las propiedades de la capa interfacial que estabiliza las gotas de aceite se pueden diseñar a medida para modular el proceso de digestión de lípidos. Este trabajo se explica en detalle en los siguientes artículos publicados:

- Torcello-Gómez, A., Maldonado-Valderrama, J., de Vicente, J., Cabrerizo-Vílchez, M. A., Gálvez-Ruiz, M. J., & Martín-Rodríguez, A. (2011). Investigating the effect of surfactants on lipase interfacial behaviour in the presence of bile salts. *Food Hydrocolloids*, *25*, 809-816.
- Torcello-Gómez, A., Maldonado-Valderrama, J., Martín-Rodríguez, A., & McClements, D. J. (2011). Physicochemical properties and digestibility of emulsified lipids in simulated intestinal fluids: influence of interfacial characteristics. *Soft Matter*, *7*, 6167-6177.

3ª Parte. Caracterización de Pluronic F68, fosfolípidos y una sal biliar modelo en la interfase aceite-agua: correlación de interfases recubiertas de Pluronic o fosfolípidos con la estabilidad de emulsiones de aceite en agua estabilizadas con Pluronic o fosfolípidos bajo la acción de sales biliares.

Las sales biliares desempeñan un papel muy importante en la digestión de lípidos porque desplazan el emulsionante original de las interfases de las emulsiones. Por lo tanto, esta parte está dedicada a estudiar estos sistemas con más detalle. Los emulsionantes y un tipo de sal biliar son caracterizados exhaustivamente en la interfase aceite-agua mediante experimentos de tensión interfacial y reología dilatacional, así como la aplicación de modelos teóricos. Las

predicciones teóricas y los experimentos concuerdan satisfactoriamente, proporcionando una explicación estructural a las interacciones que tienen lugar en la interfase entre los surfactantes en estudio con las sales biliares. Concretamente, el Pluronic F68 resiste mejor la inclusión de la sal biliar en la interfase aceite-agua debido a su conformación interfacial que confiere una barrera estérica, a diferencia de los fosfolípidos. Estos resultados se complementan de nuevo con el comportamiento de emulsiones estabilizadas con los mismos surfactantes en presencia de la misma sal biliar. Este estudio interfacial se correlaciona con la mayor estabilidad que presentan las emulsiones estabilizadas con Pluronic en presencia de la sal biliar, en comparación con las estabilizadas con fosfolípidos. La discusión detallada de este trabajo se encuentra en los siguientes artículos de investigación:

- Torcello-Gómez, A., Jódar-Reyes, A. B., Maldonado-Valderrama, J., & Martín-Rodríguez, A. (2012). Effect of emulsifier type against the action of bile salts at oil–water interfaces. *Food Research International*, *48*, 140-147.
- Jódar-Reyes, A. B., Torcello-Gómez, A., Wulff-Pérez, M., Gálvez-Ruiz, M. J., & Martín-Rodríguez, A. (2010). Different stability regimes of oil-in-water emulsions in the presence of bile salts. *Food Research International*, *43*, 1634-1641.

4ª Parte. Cómo influyen las propiedades interfaciales y del seno de la disolución del Pluronic F68 y F127 en las interacciones con las sales biliares y cómo afectan a la digestión de lípidos.

Después de haber probado la eficiencia del Pluronic F68 para frenar la digestión de lípidos, en contraste con los fosfolípidos, en este capítulo se compara la eficacia de dos Pluronics. En particular evaluamos el efecto del tamaño estudiando el Pluronic F127, que es una molécula más grande que la de F68. Nos

centraremos en estudiar cómo influyen las diferencias que existen en la estructura de estos surfactantes poliméricos en la velocidad de digestión de lípidos en emulsiones. Para ello se usó una combinación muy completa de técnicas de última generación, como micro-calorimetría y microscopía electrónica, además de las mencionadas técnicas interfaciales. Este estudio se desarrolló durante una estancia pre-doctoral en *The School of Biosciences*, en la Universidad de Nottingham (Sutton Bonington, UK) dirigida por Dr. T. J. Foster. Como resultado, el Pluronic F127 mostró ser más resistente que el F68 a ser desplazado por las sales biliares sobre la interfase aceite-agua, debido a la barrera estérica más grande y al mayor recubrimiento interfacial que proporciona. Además, el Pluronic F127 parece interaccionar con más moléculas de sal biliar, evitando su adsorción sobre la interfase aceite-agua. Por lo tanto, el Pluronic F127 afecta en mayor medida la capacidad de la sal biliar de potenciar la activación de lipólisis en presencia de lipasa. Una vez más, esta caracterización coloidal se complementa con estudios de tensión interfacial. En esta parte usamos un nuevo dispositivo diseñado en la UGR para simular la digestión *in vitro* en una sola gota, mejorando la técnica del intercambio de la subfase con un dispositivo que permite realizar múltiple intercambio de la subfase (The OCTOPUS). Este estudio correlaciona el tamaño molecular del Pluronic en la interfase con la velocidad de digestión de lípidos y complementa también el comportamiento de emulsiones. El trabajo de investigación presentado en esta parte ha dado lugar a la preparación de dos manuscritos que han sido enviados para su publicación:

- Interactions between Pluronics and bile salts in aqueous phase, at interfaces and in emulsions.
- Pluronic-covered oil-water interfaces under simulated duodenal conditions.

Por consiguiente, los resultados que se obtienen de esta tesis demuestran que el nuevo enfoque de estudio de interfases líquido-fluido permite comprender la aplicación de las propiedades interfaciales de los Pluronics para controlar la biodegradación de los transportadores coloidales. Este enfoque es innovador y atraerá la atención de científicos procedentes de diversas áreas de investigación como la farmacológica y alimentaria.

1. INTRODUCTION

All life processes take place in a colloidal system. At the same time, colloids function in all body fluids. Therefore the concept of a colloidal system is relevant to physiological processes. A colloidal system can be defined as an intimate mixture of two substances, one of which, called the dispersed phase (or colloid), is uniformly distributed in a finely divided state through the second substance, called the dispersing phase. Both phases, dispersed and dispersing phase, may be a gas, liquid, or solid. Likewise, colloidal systems can be administered in the organism by different routes, for instance intravenously injected or by oral administration, as carriers of bioactive compounds for biomedical or therapeutic purposes. Hence, the use of colloidal-based delivery systems in biological processes mainly implies to study: stability, biocompatibility[1] and degradation, taking into account the interactions with the physiological media. Physicochemical characterization of the colloidal systems under physiological conditions, represent a suitable method from the empirical point of view to understand the *in-vivo* situation. Nevertheless, in order to comprehend these complex processes it is also necessary to cover a wide range of length scales so the underlying mechanisms can be fully understood. In these sense, the interfacial characterization of the colloidal systems plays an important role in the understanding of their bulk behavior. However, scarce literature is available dealing with specific work on interfaces in order to correlate with the physicochemical properties exhibited by colloidal systems under physiological conditions. Figure 1 displays two pairs of graphs comparing the research devoted to the most common colloidal systems used to study biocompatibility in blood and digestion, respectively, with the scarce research carried out directly on interfaces.

[1]The property of being biologically compatible by not producing a toxic, injurious, or immunological response in living tissue

1. Introduction

Furthermore, working in this field requires a multi/interdisciplinary approach, bringing together physiological expertise, in order to define and develop accurate *in-vitro* models representative of the *in-vivo* situation, and interfacial and colloidal skills and expertise, to characterize properties of the interface where key stages of biological processes occur, and the correlation with the bulk properties affected by the physiological conditions. The author of this thesis is aware of the complexity arising when modeling a biological situation, for that reason the compromise between simplicity and realism has been always carefully undertaken. Bearing this in mind, it is possible now to start a brief description of the fundamental concepts and ideas focused on fluid-liquid interfaces that will appear throughout this dissertation.

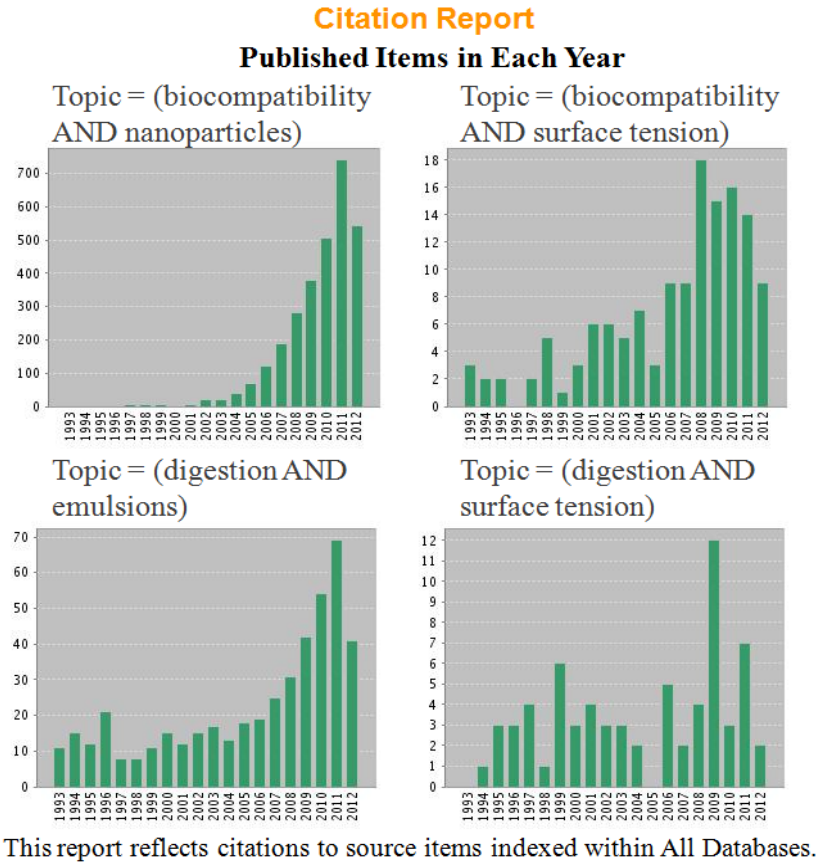

Figure 1. Results of searching in Web of Knowledge the topics biocompatibility and digestion, in combination with nanoparticles and emulsions, respectively, as well as with surface tension.

1.1. Hydrophobic interfaces in colloidal-based carriers

Interface is the region between the two different immiscible phases in a colloidal system that can be approximated to a bidimensional space, through which the properties of one phase are changing continuously to the one of the other phase. Colloidal-based delivery systems are developed in order to solve the complications related with the traditional administration of drugs or bioactive compounds and many of these problems arise when these compounds are poorly water-soluble. For that reason the systems of interest would be composed of a hydrophobic phase (constituting a hydrophobic core to solubilize the lipophilic compounds and to protect them from degradation) dispersed in a hydrophilic phase, such as aqueous solution. Hence, interfaces would be water-hydrophobic phase. When the area of interface is created, this produces an excess of potential energy in the system that tends to decrease reducing the interfacial area. Any attempt to increase the interfacial area by deforming or dividing the interface results in the appearance of new molecules from both phases at this interface. This means that the molecules already present at the interface must separate to let the new molecules to enter. The force, tangent to the interface, necessary to separate two molecules a unit of distance is called interfacial tension, γ, and its units in the International System are N/m. This term is the most commonly used at fluid-fluid interfaces in order to manifest the potential mechanic nature of their changes.

Hence, delivery systems as colloidal disperse systems whose primary characteristic is a large interfacial area, causes thermodynamic instability. Therefore, the foremost challenge in dispersion design is the reduction of interface instability and an understanding of the behavior of interfaces (Pelipenko et al., 2012). To this end surfactants or tensioactives are necessary for the formation and/or stabilization of these systems, since they are able to decrease the interfacial tension. Also, the carriers must possess a protective shell, providing biocompatibility with the physiological media. Therefore, when surfactants are

present to stabilize and protect colloidal particles, the concept of interfacial tension can be used as well to characterize their adsorption onto the hydrophobic interfaces. This adsorption occurs due to their amphiphilic[2] character reducing the interfacial tension when occupying the interfacial area available (MacRitchie, 1990). The higher the concentration of surfactant molecules onto the interface, the larger the decrease in the interfacial tension. The term adsorption refers to the change in the concentration of one compound at an interface.

The surfactant to be used in the formulation of such colloidal carriers strongly depends on the application. Surfactant selection is therefore crucial and must be based on physicochemical properties, stability of the interfacial film, mobility of the molecules in the interfacial film, hydrophilic-lipophillic balance (HLB), film formation kinetics, compatibility and interactions between molecules on the surface, critical micelle concentration (CMC), *etc.* (Rodríguez-Patino, Carrera-Sánchez, & Rodríguez-Niño, 2008). Most surfactants have an ionic character (anionic, cationic or zwitterionic) that provides colloids with a certain surface charge, preventing them from aggregation by means of ionic repulsion. However, in many practical cases where colloidal systems are applied under high ionic strength conditions, such as physiological media, this stabilization mechanism fails. For this reason, much attention has been paid in the past on surfactants that do not provide the colloid surface with a significant charge, but with steric-stabilizing groups protruding from the interface into the dispersion medium and forming a bulky layer with thicknesses of several nanometers, reducing direct contact between particles and hence destabilization (Lankveld & Lyklema, 1972; Grigoriev & Miller, 2009). This steric effect can be also applied to provide biocompatibility, for instance under intravenous conditions (Higuchi et al., 2003). Concretely, polymeric surfactants of the family of Pluronics have been extensively used to form shells for stealthiness of colloidal drug carriers (Jackson, Springate, Hunter, & Burt, 2000; Wulff-Pérez, de Vicente, Martín-Rodríguez, & Gálvez-Ruiz, 2012).

[2] simultaneous hydrophilic and hydrophobic character

One of the reasons is because Pluronics are non-toxic and several members of this family have already been approved for human formulations and medical application (FDA, 2012). Acknowledgement of the importance of polymers and their extraordinary range of possible applications, led to the emergence of polymer science as a new field of scientific activities in 20th century.

Given these facts, familiarity with the methods that allow determination of interfacial characteristics, and thus predict system behaviors, is critical.

1.2. Interfacial characterization of fluid-liquid interfaces: adsorption of surfactants and mechanical properties of adsorbed layers

1.2.1. Adsorption of surfactants: basic concepts

The adsorption of surfactants onto an interface is a dynamic process which kinetics is very important, for instance in industrial processes. For that reason the kinetics of adsorption is always studied before the equilibrium properties are characterized. The process of adsorption of surfactant molecules at an interface takes place in two processes: a diffusive one where the concentration gradient in the bulk transports the surfactant molecules towards the interface, and the adsorption itself where the surfactant molecules reach the interface. Concretely, the transference of molecules between the subphase and the area immediately close to the interface (subsurface) and between this subsurface and the interface takes place. The first is a mass transfer process controlled by diffusion whereas the second is pure adsorption/desorption process. This adsorption reduces surfactant concentration at the subsurface that is restored by the diffusion in the subphase. As the interface becomes saturated with surfactant molecules, the transfer of mass decreases approaching the equilibrium. The analysis of the evolution of the

interfacial tension during the adsorption process of surfactants, that is the interfacial tension vs. time $\gamma(t)$, provides structural information about the formed interfacial layer.

However, generally the process of adsorption of surfactants is studied in terms of the interfacial tension isotherm vs. bulk concentration, $\gamma(c)$. This curve provides the relationship between the initial surfactant bulk concentration and the final interfacial tension attained after the considered time of adsorption. At low bulk concentrations, surfactants will favor the arrangements onto the interface forming an adsorbed layer, eventually reaching a diffusive equilibrium between the molecules in the bulk and onto the interface. Upon increasing the surfactant bulk concentration, the final interfacial tension decreases as the interface gradually becomes saturated. Once the interface is completely saturated due to the formation of a surfactant adsorbed layer, no more changes are observed in the interfacial tension upon further addition of surfactant that arranges in micelles. The surfactant bulk concentration from which micelles start to form is the CMC. Its value can be determined from $\gamma(c)$ isotherms as observed in Figure 2.

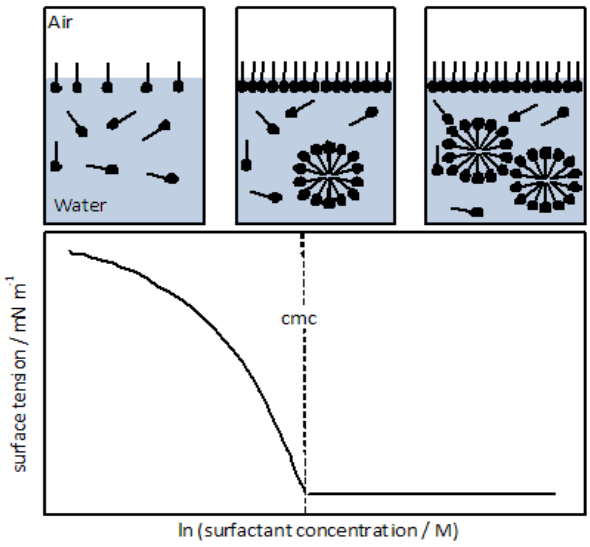

Figure 2. Structural regions in the equilibrium adsorption process of conventional surfactants: determination of the CMC.

We shall see later on that in the case of some surfactants, such as Pluronics, interfacial tension isotherms show a more complicated shape, such as stepwise trend. This occurs because the molecular structure of Pluronics differs from that of conventional surfactants. Commonly, a surfactant molecule presents a polar head and a lipophilic tail. Differently, Pluronics show a triblock structure, with a central hydrophobic part and two lateral symmetric hydrophilic parts. The staircase shape in the adsorption isotherm reflects different conformations of the adsorbed Pluronic depending on the bulk concentration regime, and hence the interfacial coverage (Muñoz, Monroy, Ortega, Rubio, & Langevin, 2000).

Usually air-water interface is considered as a model to study adsorption kinetics, structure of the adsorbed interfacial layer and interactions between molecules at the interface. In this case, the term surface tension is commonly used as a particular case of interfacial tension in which one phase is in equilibrium with its vapor. However, oil-water interface is more realistic when considering lipid-based delivery systems, such as oil-in-water emulsions, to study the transit of oil droplets through the gastrointestinal tract (Maldonado-Valderrama, 2008).

1.2.2. Thermodynamic model of adsorption of surfactants

In this thesis we have applied a theoretical model to some of the adsorption data in order to obtain further structural information of the interfacial layers. The application of this thermodynamic model allows obtaining the molecular area of the adsorbed surfactant molecule, as well as the affinity for the interface. The theory of adsorption behavior used described in detail elsewhere (Frumkin, 1925; Fainerman, Miller, & Möhwald, 2002). Therefore, only the main equations for each applied model will be given here.

First, the Frumkin equations of state and adsorption isotherm describe the adsorption behavior of surfactants:

$$\pi = -\frac{RT}{\omega}\left[\ln(1-\theta) + a\theta^2\right] \tag{1.1}$$

$$bc = \frac{\theta}{(1-\theta)}\exp(-2a\theta). \tag{1.2}$$

Here π is the interfacial pressure of the solution ($\pi \equiv \gamma_0 - \gamma$, where γ_0 is the interfacial tension of the clean oil-water interface), R is the gas law constant, T is the temperature, c is the surfactant bulk concentration, ω represents the molar area of the surfactant and θ is the surface coverage. The adsorption constant b provides information about the strength of the interaction between the adsorbing species and the surface, and the Frumkin interaction parameter a indicates whether the adsorbing molecules exhibit attractive (positive) or repulsive (negative) lateral interactions (Karakashev, Manev, & Nguyen, 2004). The Frumkin interaction parameter can be defined as $a = -U/(RT)$, providing the relationship between the intermolecular interaction energy in the adsorbed layer and the thermal energy (RT). This model fits well the experimental adsorption isotherm of common surfactants.

Then, a modification of this model includes two orientations of adsorbed surfactant molecules coexisting at the interface, with different molar areas ω_1 and ω_2 (for definiteness we assume $\omega_1 > \omega_2$). This model improves the experimental data fitting when considering surfactants adopting different conformations at the interface as the interfacial pressure increases. The equations of state and adsorption isotherm for this reorientation model read:

$$\pi = -\frac{RT}{\omega}\ln(1-\theta) \tag{1.3}$$

$$bc = \frac{\Gamma_2\omega}{(1-\theta)^{\omega_2/\omega}} \tag{1.4}$$

Where $b = b_2$ is the adsorption equilibrium constant in state 2. The total adsorption Γ and mean molar area ω are defined by

$$\Gamma = \Gamma_1 + \Gamma_2 \tag{1.5}$$

$$\omega\Gamma = \theta = \omega_1\Gamma_1 + \omega_2\Gamma_2 \tag{1.6}$$

and the ratio of adsorptions in the two possible states of the adsorbed molecules is given by

$$\frac{\Gamma_1}{\Gamma_2} = \exp\left(\frac{\omega_1 - \omega_2}{\omega}\right)\left(\frac{\omega_1}{\omega_2}\right)^{\alpha} \exp\left[-\frac{\Pi(\omega_1 - \omega_2)}{RT}\right]. \tag{1.7}$$

The constant α accounts for the fact that the adsorption equilibrium constant b_1 for surfactant molecules adsorbed in state 1 (with larger area) can exceed that in state 2, which results in an additional increase of the fraction of states of larger area.

1.2.3. Mechanical properties of adsorbed layers: interfacial rheology

The technological applications of surfactants cannot be exclusively explained in terms of the equilibrium values of the interfacial tension. When considering for instance the formation of emulsions, the interfacial response to a perturbation to reach again the equilibrium is in general more important than the equilibrium itself, since the formation and stabilization of emulsions strongly depend on the mechanical properties of the emulsifier film surrounding the droplets.

Interfacial rheology is one of the most powerful tools for observing occurrences at the interface. Interfacial characteristics are generally dependent on the behavior of both phases. Moreover, these characteristics exhibit even greater

dependency on the behavior of the molecules positioned at the interface, particularly on their chemical composition, concentration and interactions. Rheology was defined by E. C. Bingham in 1929 as the science of deformation and flow under controlled testing conditions. The term "rheology" originates from the Greek word "rheos" meaning "flowing" or "streaming", thus rheology is actually "flow science", which is based on the fundamental physical relationships concerning how materials respond to applied forces or deformations (Mezger, 2006). Since interfacial rheology deals with the response of mobile interfaces to deformation, it is usually subdivided into the areas of dilatational rheology and shear rheology because in most systems both deformation modes coexist (Edwards, Brenner, & Wasan, 1991). These modes of interfacial deformation are related to changes in area and shape that are illustrated in Figure 3. In dilatational deformation, the area of the liquid interface is changed, whereas the shape of the interfacial area remains the same. These two methods are complementary, as shown in a previous work performed at UGR that is not included in this thesis, but importantly compared both deformation forms on structurally different food systems (Torcello-Gómez et al., 2011). The two types of interfacial deformation focus on different aspects of the interfacial layer. Interfacial dilatational rheology is therefore a two-dimensional bulk elongational rheology, very sensitive to the kinetics of adsorption/desorption of surfactants. On the contrary, in a shearing deformation the shape of the liquid interface is changed while the interfacial area is kept constant. It is a two-dimensional bulk shear rheology, without changes in interfacial film composition during deformation (constant interfacial concentration) (Murray, 2002). Thus, we distinguish shear and dilatational interfacial rheological parameters.

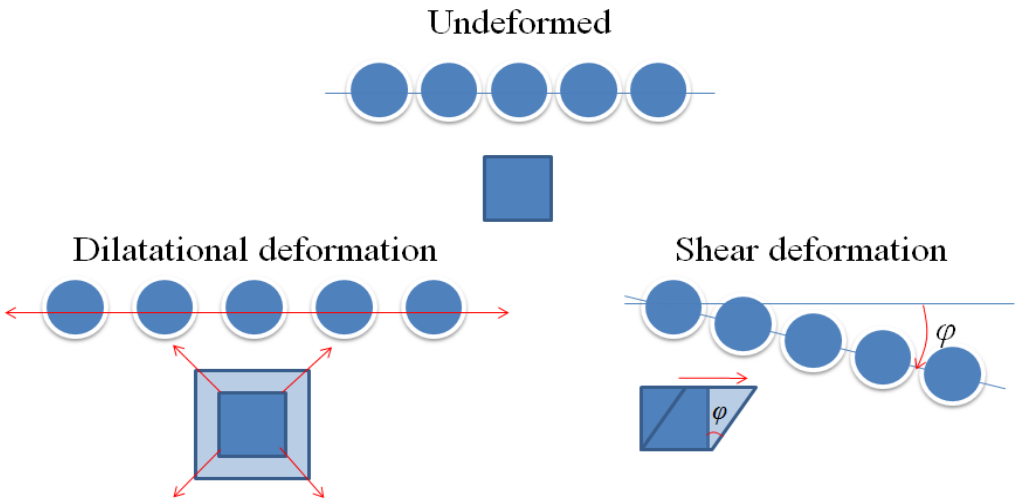

Figure 3. Schematic representation of both interfacial deformations.

When defining interfacial rheological material functions it is useful to distinguish two categories of definitions and related measuring techniques: close to equilibrium of the microstructure at the interface, within the linear regime, using dynamic experiments; and far from equilibrium of the interfacial microstructure, within the non-linear regime, in steady state and transient tests. In this dissertation, we will focus on the linear rheological aspects using small-amplitude dynamic oscillatory experiments. In this case, the material functions are obtained from the definition of interfacial elasticity and viscosity.

For a dilatational deformation the interfacial elasticity follows the definition given by Gibbs:

$$E = \mathrm{d}\gamma / \mathrm{d}\ln A = \mathrm{d}\gamma / (\mathrm{d}A/A) \tag{1.8}$$

where $\mathrm{d}A/A = \mathrm{d}\ln A$ is the relative change in interfacial area and $\mathrm{d}\gamma$ is the change in interfacial tension as a response to the area deformation. Therefore, E gives a measure of the stiffness of the interface against a dilatational compression and

expansion. For a shear deformation, the interfacial elasticity is described by Hooke's law:

$$G = \tau / \varphi \tag{1.9}$$

where φ is the strain or deformation produced when a stress τ is applied. On the other hand, interfacial viscosity for both types of deformation can be defined as (Murray, 2002):

$$\eta = d\gamma / (d \ln A/dt) \tag{1.10}$$

$$\mu = \tau / (d\varphi/dt) \tag{1.11}$$

Nevertheless, in a general case, materials exhibit both, elastic and viscous behavior, having the name of "viscoelastic". In this case, the change of interfacial tension or the stress can be rewritten as the sum of the elastic and viscous contribution for sufficiently small deformations:

$$\Delta\gamma = E' \Delta A/A + \eta' d(\Delta A/A)/dt \tag{1.12}$$

$$\tau = G' \varphi + \mu' (d\varphi/dt) \tag{1.13}$$

where A is the original interfacial area before deformation. If the deformation is sufficiently small and slow the coefficients E', η', G', μ', are constant. In a small-amplitude oscillatory experiment, the considered deformation results in a sinusoidal input signal as a function of time, t, and the response results in a sinusoidal output signal that is out of phase ($\delta \neq 0^o$) (Figure 4). For $\delta = 0^o$, the interfacial layer is purely elastic, if on the contrary $\delta = 90^o$ the interfacial structure is purely viscous and in an intermediate case $0^o < \delta < 90^o$ the interfacial layer is viscoelastic.

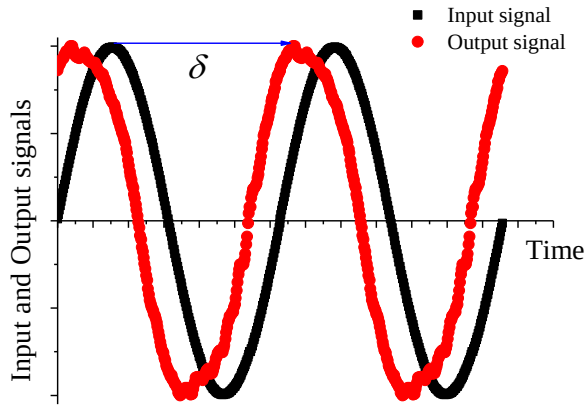

Figure 4. Output signal as response to an input deformation signal in a small-amplitude oscillatory experiment.

We first develop the basic equations for a dilatational deformation. For a small-amplitude oscillatory test, the change in interfacial area can be expressed as:

$$\Delta A(t) = A_a \sin(\omega t) \tag{1.14}$$

where ω is the angular frequency (in this case ω does not refer to the molar area from previous section anymore) and A_a is the area amplitude. The response in the interfacial tension variation can be described by the function:

$$\Delta\gamma(t) = \gamma_a \sin(\omega t + \delta) \tag{1.15}$$

where γ_a is the measured amplitude and δ is the phase angle. The material functions for small-amplitude oscillatory deformation are defined based on the interfacial tension by using trigonometric identities:

$$\gamma(t) = \gamma_a [\sin(\omega t) \cos(\delta) + \sin(\delta) \cos(\omega t)] =$$

$$= [\gamma_a \cos(\delta)] \sin(\omega t) + [\gamma_a \sin(\delta)] \cos(\omega t). \tag{1.16}$$

By substituting equation (1.14) in (1.12) and operating, we obtain:

$$\Delta\gamma(t) = E'(A_a/A)\sin(\omega t) + \eta'\omega(A_a/A)\cos(\omega t) \quad (1.17)$$

and identifying terms from equations (1.16) and (1.17), we finally have:

$$E' = \gamma_a/(A_a/A)\cos(\delta) \quad (1.18)$$

$$\eta'\omega = \gamma_a/(A_a/A)\sin(\delta) \quad (1.19)$$

If we compare now with the general complex quantity:

$$E^* = E' + iE'' \quad (1.20)$$

known as interfacial dilatational modulus, expressed as a function of the interfacial tension and area waves:

$$E^* = \gamma_a/(A_a/A)\, e^{i\delta} = |E|[\cos(\delta) + i\cos(\delta)] \quad (1.21)$$

we can identify the real part or storage modulus with the elasticity of the interfacial layer, and the imaginary part or loss modulus as proportional to the viscosity of the interfacial microstructure. These are known as the interfacial material functions:

$$E'(\omega) = \gamma_a/(A_a/A)\cos(\delta) = |E|\cos(\delta) = E' \quad (1.22)$$

$$E''(\omega) = \gamma_a/(A_a/A)\sin(\delta) = |E|\sin(\delta) = \eta'\omega \quad (1.23)$$

Following the same formalism for a shear deformation, the material functions are:

$$G'(\omega) = \tau_a/\varphi_a\cos(\delta) = |G|\cos(\delta) \quad (1.24)$$

$$G''(\omega) = \tau_a/\varphi_a\sin(\delta) = |G|\sin(\delta) \quad (1.25)$$

In this case, a small-amplitude sinusoidal strain φ is applied and φ_a is the oscillation amplitude. For linear viscoelastic materials, the response function is a sinusoidally changing shear stress τ that is out of phase with the strain, and τ_a is the stress amplitude.

The material functions quantify the viscoelastic response of the interfacial film to a dilatational or shear deformation. Specifically, the interfacial storage modulus accounts for the resistance of the interfacial layer to a deformation, whereas the interfacial loss modulus accounts for the relaxation processes. As we shall see later, such interfacial rheological material functions provide fundamental understanding of the structure, intermolecular interactions and rearrangement of the interfacial film during deformation processes (Maldonado-Valderrama & Rodríguez-Patino, 2010), complementing the information interpreted from interfacial tension data.

1.3. Biological processes as an interfacial reaction

Colloidal carriers in the form of liposomes, emulsions and microspheres have been used as a means of delivering compounds to selected sites in the body (Illum & Davis, 1982). Since the majority of natural biochemical reactions occur at interfaces of these colloidal carriers, only by understanding interfacial characteristics, we are able to explain these processes and successfully mimic biological systems.

Biocompatibility in blood stream

The main impediment of intravenously administered nanoparticles (referring to particles with a size of de order of 100 nm) in the controlled release of drug in the blood stream for extended periods of time is the biocompatibility of their surfaces with blood or plasma-derived fluids. They must be able to escape sequestration by the reticuloendothelial system (RES) (Poznansky & Juliano, 1984). The RES, also commonly known as mononuclear phagocyte system (MPS), is a part of the immune system. Phagocytic cells from MPS may recognize the nanoparticles surfaces as foreign material and may eliminate it from the body

by phagocytosis, with some degree of local inflammation (Tang, Lucas, & Eaton, 1993). Phagocytic cells are also known as white blood cells, because after centrifuging a blood sample, the white cells are found in a thin, typically white layer of nucleated cells between the sedimented red blood cells and the blood plasma. This process requires the activation of the phagocytic cells by the nanoparticle surface, through the adsorption of opsonic proteins (antibodies) from plasma, such as immunoglobulin (Ig) or complement, onto the surfaces (Norman, Williams, & Illum, 1993). Immunoglobulin G (IgG) provides the majority of antibody-based immunity against invading pathogens, so can be used as a model of antibody. In this way, phagocytic cells may recognize opsonic-adsorbed proteins and nanoparticles may be rapidly cleared from the blood (Verrecchia et al., 1995; Tan, Butterfield, Voycheck, Caldwell, & Li, 1993). A schematic representation of the whole process of opsonization is illustrated in Figure 5. A prime consideration is the initial rapid adsorption of plasma proteins when colloidal particles are

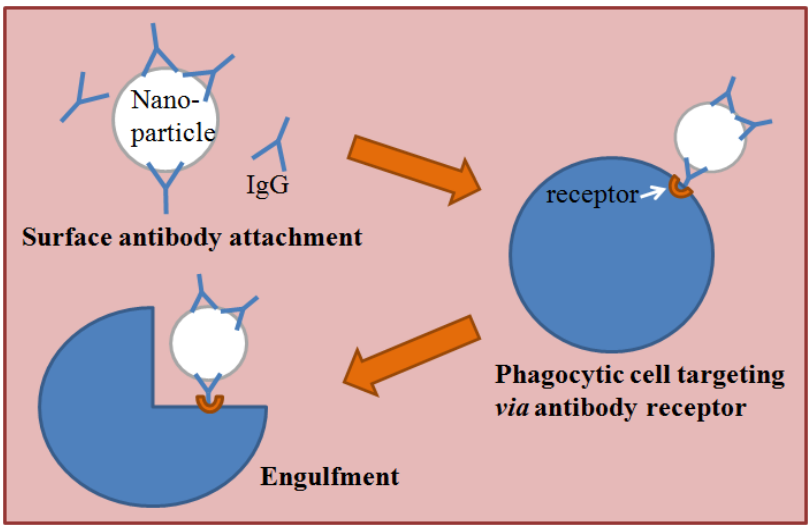

Figure 5. Schematic representation of the opsonization and phagocytosis processes.

injected intravenously, since this opsonization process is known to influence recognition by cells of the RES (Moghimi & Patel, 1989). Hence, the rapid adsorption of plasma proteins onto the nanoparticle surface is regarded as preceding and controlling subsequent phenomena such as platelet aggregation or phagocyte activation (Norman, Williams, & Illum, 1993).

Efforts to prevent recognition and phagocytosis of colloidal carriers have centered on modifying the nanoparticle surface to prevent the adsorption of opsonic proteins or the close approach of cells to the nanoparticle. This modification is termed "steric stabilization" (Jackson et al., 2000). The steric stabilization is supposed to inhibit plasma protein adsorption in order to avoid recognition by RES. Therefore, the interfacial mechanisms taking place onto sterically modified surfaces interacting with antibodies is worthy of study.

Lipid digestion in the gastrointestinal tract

It is necessary to understand the fundamental processes underlying lipid digestion. During digestion, the body secretes bio-surfactants including phospholipids and bile salts that replace the interfacial layer on emulsion lipid droplets to prepare them for enzymatic digestion (lipolysis). Lipolysis is the enzymatic hydrolysis by lipase of lipids mainly composed by triglycerides (Lowe, 1997), that is schematized in Figure 6.

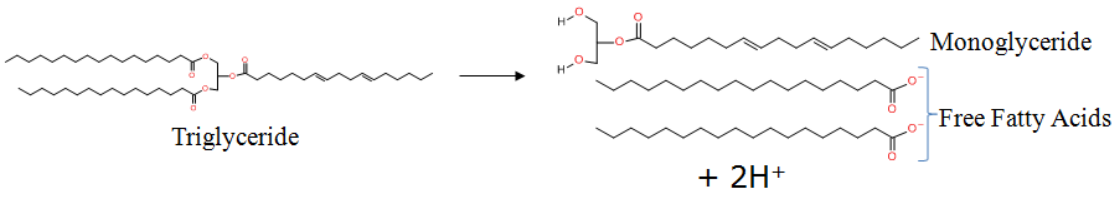

Figure 6. Schematic representation of the hydrolysis reaction of triglycerides by lipase.

1. Introduction

Although some lipolysis (c.a. 10%) occurs in the stomach, the majority occurs in the duodenum through the action of pancreatic lipase, directly after the stomach begins to empty. Because lipid is insoluble in water, lipase has to adsorb onto the lipid droplet surface in order to hydrolyze the triglycerides from the lipid substrate into fatty acids and monoglycerides. In order to promote lipolysis, surface-active bile salts are secreted through the bile duct which adsorb to the surface of the lipid droplets. Their adsorption bestows a negative charge on the interface that attracts the positively charged coenzyme colipase to the surface, which then attaches to the ester bond region of the triglyceride by hydrogen bonding. It is thought that lipase then couples tightly with the adsorbed colipase by electrostatic binding in order to adopt a suitable configuration for lipolysis (Figure 7).

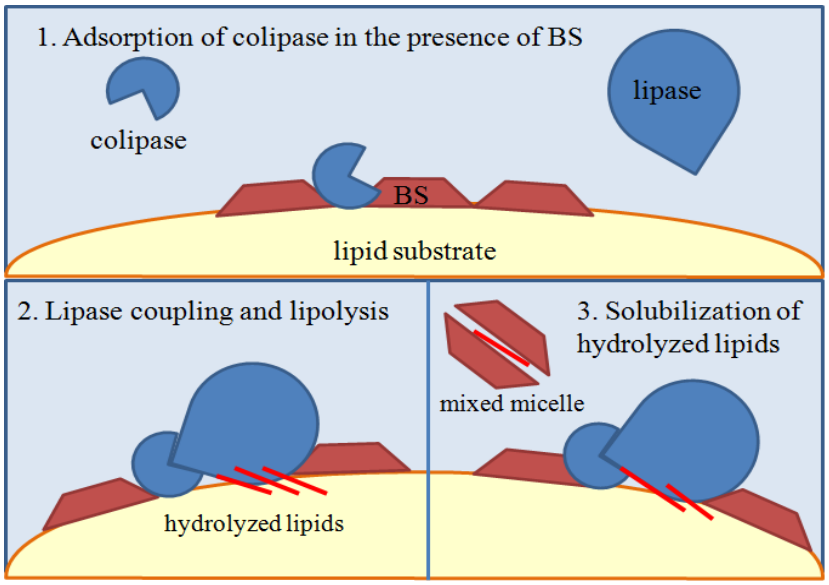

Figure 7. Schematic representation of the lipolysis onto the oil-water interface.

Thus it is the interfacial binding which is a key rate-limiting step that controls the concentration of lipase at the interface, and hence the rate of lipolysis,

and this is very sensitive to interfacial composition. Lipolysis typically has a lag period prior to the establishment of steady state hydrolysis, which appears to be due to slow interfacial penetration of the enzyme that is highly dependent on the nature and composition of the interface (Wickham, Garrood, Leney, Wilson, & Fillery-Travis, 1998; Carrière et al., 1998). Lipase action is very sensitive to interfacial composition, but the relationship is very complex, and no simple dependency has been determined (Bauer, Jakob, & Mosenthin, 2005). The extent to which the original interfacial structures are degraded during their passage through the stomach and into the duodenum will determine the ease with which bile salts can displace them. This in turn will determine the ultimate molecular composition and structure at the interface, the extent of occupation by bile salts and the adsorption of co-lipase, lipase and lipolysis. Although the basic principles and mechanisms underlying the process of lipolysis are known, there is a huge gap in knowledge of the specific interfacial mechanisms and the role of interfacial structure on the ability of lipase and co-lipase to adsorb and hydrolyze triglycerides (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011).

2. METHODOLOGY

Since all the experimental details will be described later on in each individual work, this section focuses on introducing the surfactants used and explaining the key design of the experiments that allows comparing the study at fluid-liquid interfaces with the corresponding colloidal system under physiological conditions.

2.1. Materials: Pluronics vs. Phospholipids

This subsection aims to introduce the polymeric surfactants Pluronics that were tested under *in-vitro* experiments mimicking the processes of biocompatibility in the blood and digestion in the duodenum. In addition, as a model of conventional surfactant, phospholipids were also studied in order to compare with the effect of Pluronics on lipid digestion. A brief description of their molecular architecture is presented to understand the influence of their interfacial properties on the final behavior in biological reactions.

Polymers are macromolecules consisting of repeating chemical entities (monomers) connected to each other through covalent bonds. The first and most important attribute of a polymer are the constituent monomers that it is made of. The simplest case is a homopolymer which is comprised of a sequence of only one type of repeating unit. In addition there are copolymers containing two or more types of constituent units. Depending on their distribution and arrangement along the polymer chain several main classes of copolymers can be distinguished. The molecular architecture is another important distinctive attribute. Linear polymers possibly are the most abundant in terms of type of architecture. Surfactants are low molecular weight amphiphiles. Larger representatives of the family of amphiphilic

molecules are the block copolymers, which consist of sequences of homopolymer (blocks) attached to each other to form a molecule. The simplest configuration is linear AB diblock copolymer, where here we adopt the nomenclature that A is the hydrophilic block and B is the hydrophobic one. In fact, considering their structure diblock copolymers could be regarded as surfactants but with a bigger hydrophilic head. Another type is linear triblock copolymers of two different blocks. They essentially come in two types. In the first group the hydrophobic block is situated in the middle of the molecule (ABA) flanked by two hydrophilic blocks, whereas in the second group the situation is reversed and the hydrophobic blocks are on the flanks (BAB). One family of water-soluble block copolymers that has been subjected to extensive systematic research (and used in the present study) is the polyethylene oxide (PEO) and polypropylene oxide (PPO) block copolymers often abbreviated as PEO_a - PPO_b - PEO_a. They are also known under the commercial names of Poloxamers and Pluronics. As it can be seen from their composition formula, they belong to the ABA type triblocks where the PEO segments play the role of the hydrophilic moieties in the sequence, whereas the PPO block represents the hydrophobic part of the molecule. Being commercial products, it is not a surprise that these polymers are intrinsically polydisperse, meaning that a sample is a mixture of similar species that vary with respect to the length of the PO and EO parts. This unfortunate property not necessarily frustrates all systematic studies, but undoubtedly complicates issues and needs to be taken into account.

The molecular structure influences the arrangement of the Pluronic molecules when adsorbing onto a fluid-liquid interface forming an interfacial layer, as it can be seen in Figure 8. The central PPO block adsorbs onto hydrophobic interfaces, whereas the two lateral PEO chains protrude into the hydrophilic phase, providing a steric bulky layer. It is possible to play with the length of the hydrophobic and hydrophilic blocks in order to obtain adsorbed layers with longer steric barriers, by choosing Pluronics with larger number of subunits in the PPO

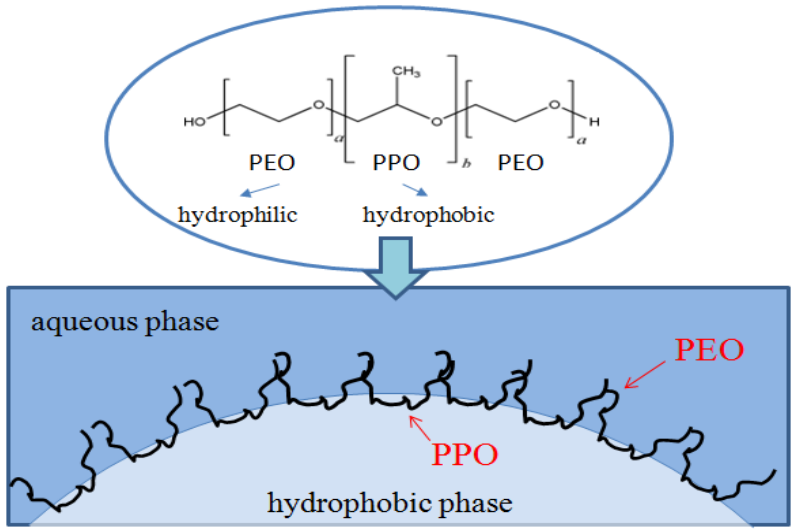

Figure 8. Scheme of the molecular structure of Pluronic and architecture of a Pluronic adsorbed layer onto a fluid-liquid interface.

and PEO segments. In this thesis the Pluronics used were F127 and F68. They are also commercially known as Poloxamer 407 and 188, respectively. The differences between them are the number of subunits in the PEO and PPO blocks and the HLB. F127 has longer PPO and PEO segments ($PEO_{100}PPO_{65}PEO_{100}$, MW = 12600 g/mol) with lower HLB (22), whereas F68 has shorter PPO and PEO blocks ($PEO_{75}PPO_{29}PEO_{75}$, MW = 8400 g/mol) which result in a greater average HLB (29) and therefore less hydrophobicity.

On the other hand, phospholipids were also studied to compare the effect of Pluronics on digestibility of surfactant-stabilized emulsions with that of conventional surfactants. A typical phospholipid molecule presents a hydrophilic polar head and a hydrophobic nonpolar tail. This molecular structure gives rise to a more compact interfacial layer when phospholipid molecules adsorb onto a fluid-liquid interface, in contrast to Pluronics (Figure 9).

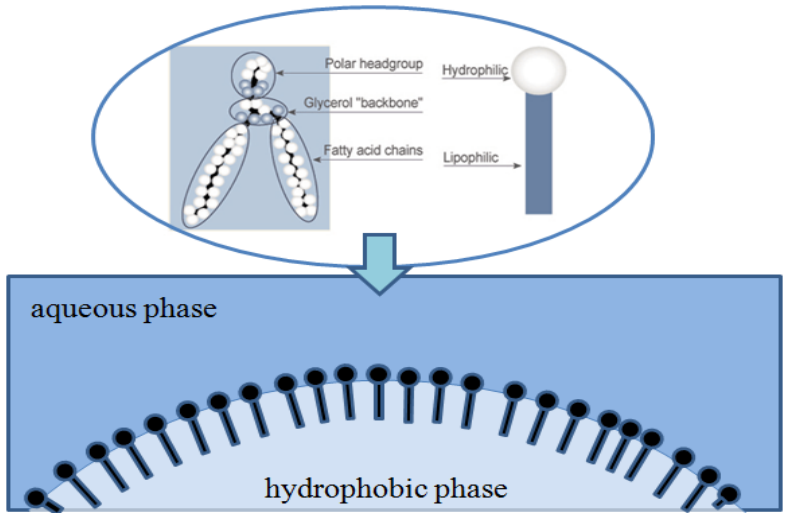

Figure 9. Scheme of the molecular structure of phospholipids and architecture of a phospholipid adsorbed layer onto a fluid-liquid interface.

Concretely, we used phospholipids from soybean lecithin enriched in phosphatidylcholine (PC). They present negative charge at pH 7. Hence, another important difference with respect to Pluronics is that the polar head of these phospholipids provide the interfacial layer with ionic repulsion at sufficiently low ionic strength conditions.

2.2. Experiments with colloidal systems

Two colloidal systems were used: solid nanoparticles under intravenous conditions and oil-in-water emulsions under duodenal conditions. These are representative colloidal-based delivery systems for parenteral and oral administration, respectively. Each system was prepared and stabilized using the considered surfactant, and then subjected to the corresponding physiological media.

In the case of solid nanoparticles, these were coated by Pluronic and then immersed in an IgG solution with physiological ionic strength and pH. The colloidal behavior was compared to that of uncoated nanoparticles, that is, in the absence of Pluronic. A combination of techniques such as colloidal stability,

electrophoretic mobility, as well as immunoassays, allows the complete characterization of Pluronic-coated nanoparticles under the action of the antibody.

Regarding oil-in-water emulsions, olive oil droplets were stabilized either by Pluronic or phospholipids, and then subjected to duodenal juice containing basically a mixture of pancreatic lipase, bile salts, calcium chloride and sodium chloride at physiological concentrations and at pH 7. In some cases it will be considered the study of individual components, such as lipase or bile salts alone, in order to understand their contribution to the final behavior in the presence of all duodenal components. Emulsions under duodenal conditions were characterized by means of droplet size distribution measurements, determination of droplet surface charge and different microscopies, and lipolysis was measured by titrimetry of free fatty acids. An original experimental approach is presented in this thesis when studying interactions of Pluronic-stabilized emulsions with duodenal components by micro-calorimetry. Micro-calorimetry is differentiated from "conventional" calorimetry by the amplified sensitivity of the machine, larger sample volumes and lower scan rates. The amplified sensitivity is attained by a design optimised to collect and calculate heat radiating from the cells. It is generally the favoured method when examining hydrocolloid solutions where the concentration is of the order of less than 1%.

2.3. Experiments at fluid-liquid interfaces: subphase exchange accessory

All the interfacial tension measurements were made in a Pendant Drop Surface Film Balance equipped with a subphase exchange device (Spanish Patent, registration number P9801626) which has been fully designed and assembled at the University of Granada. The normal capillary tip was substituted by an arrangement of two coaxial capillaries, connected to one of the channels of a double micro-

injector, which can operate independently (Cabrerizo-Vílchez, Wege, Holgado-Terriza, & Neumann, 1999). Recently, it has been built up this double capillary technique to achieve a fully automated subphase multi-exchange device by modifying the injection system and the computer program. The whole equipment, known as The OCTOPUS is schematized in Figure 10.

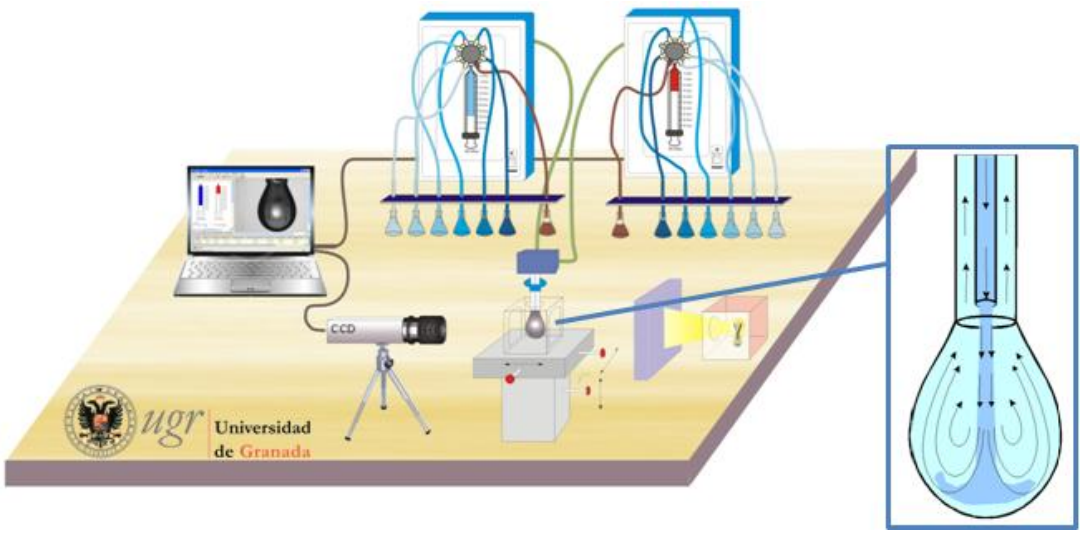

Figure 10. Schematic representation of The OCTOPUS set-up.

It comprises the following components. The subphase multi-exchange device consists of two micro-injection system (PSD/3 syringe pumps, Hamilton Company) with 9 vias valves where each of the two syringes are connected to 8 channels: 7 solutions and the double capillary. The two coaxial capillaries are connected to both syringes by two channels of the 8 port-valve micro-injectors. Both syringes operate independently and enable an automatic, non-invasive and complete exchange of the subphase of the drop maintaining intact the pendant drop volume and the surface area trough the subphase exchange. The OCTOPUS computer software has been fully programmed at the University of Granada: DINATEN©. The detection and calculation of interfacial area and interfacial tension is based on Axisymmetric Drop Shape Analysis (ADSA). The whole set-up

is fully computer controlled and DINATEN© allows planning, control and monitoring of the whole experiment. The pendant drop is placed on a three axis micro-positioner and is immersed in a glass cuvette (Hellma) which is kept in an externally-thermostatized cell.

Drop images are captured by a CCD camera (Pixelink®) connected to an optical microscope (Edmund Optics®). The computer program DINATEN© fits experimental drop profiles, extracted from digital drop micrographs, to the Young–Laplace equation of capillarity by using ADSA, and provides as outputs the volume (V), the interfacial tension (γ), and the interfacial area (A) of the pendant drop. The adsorption process is recorded at constant interfacial area through a modulated fuzzy logic PID algorithm (proportional, integral, and derivative control). The dilatational rheology of the interfacial layers is measured by applying an oscillatory perturbation to the interface at the end of each adsorption step by injecting and extracting volume to the drop. The system records the response of the surface tension to this area deformation, and the dilatational modulus (E) of the interfacial layer is calculated from this response from equation $E = \gamma_a/(A_a/A)$, as it was shown in section 1.2.3. In some cases the storage and loss modulus will be also calculated from the dilatational modulus and the phase angle as in equations (1.22) and (1.23). The applied oscillations in interfacial area were maintained at amplitude values of < 10% and the measurement frequency (f) was set to 0.1 Hz.

This device allows customization of the *in-vitro* model used, depending on the specific requirements of the experiments. The different solutions are placed in *eppendorfs* which are connected directly with the pendant drop by each of the valves (Figure 10). Initially, a surfactant layer is pre-formed at control conditions and then subjected to physiological conditions by subphase exchange of the original bulk solution with the solution which is contained in next *eppendorf*, mimicking the passage through blood stream or duodenum, as required. Subphase exchange process is designed to assure complete replacement by the new subphase

(Figure 11) (Maldonado-Valderrama, Holgado-Terriza, Torcello-Gómez, & Cabrerizo-Vílchez, 2012). The interfacial tension of the system under the new conditions is recorded, until it attains a steady state and then, the drop is subjected to 10 cycles of deformation at 0.1 Hz which provide the dilatational modulus of the interfacial layer under the new conditions.

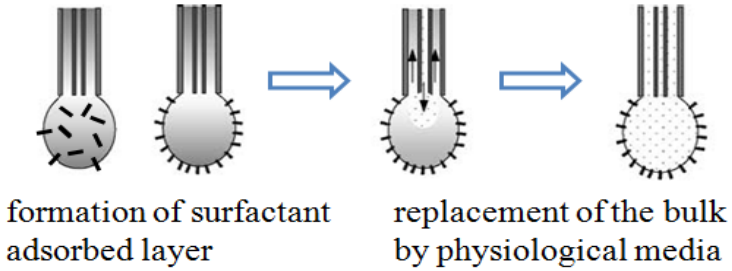

Figure 11. Schematic representation of subphase exchange.

Concretely, studies at the model air-water interface were carried out to compare with the experiments with solid nanoparticles. The interface was pre-covered by Pluronic and then, the subphase was replaced by IgG solution in physiological media. When comparing to oil-in-water emulsions, the experiments were performed at the olive oil-water interface. Similarly, the interface was either pre-covered by Pluronic or phospholipids and then subjected to the duodenal juice by means of the subphase exchange accessory. The interfacial tension was monitored throughout the whole process and the dilatational rheology measured at the end of each step of adsorption.

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3. MAIN OBJECTIVES

According to the Introduction section, interfaces are presented as the main scenario determining the adsorption of proteins that triggers the final cascade or reactions controlling biocompatibility and lipolysis. It was also presented a promising kind of polymeric surfactants that is biocompatible and able to protect colloidal carriers by steric stabilization against undesired effects in biological processes. Bearing this in mind and the lack of work specifically dealing with interfaces, the main objectives of this thesis are:

- To characterize the adsorption of Pluronics, phospholipids and physiological components at fluid-liquid interfaces by means of the combination of interfacial (surface) tension and interfacial dilatational rheology.
- To design experiments with the subphase exchange technique in order to simulate several biological situations, such as the passage through the blood stream or the duodenum, onto the considered emulsifier adsorbed layers.
- To evaluate the interactions between components in the blood or the duodenum with the Pluronic adsorbed layers by comparing with the adsorption of such components onto the bare interface, that is in the absence of Pluronic. In some cases regarding lipid digestion it will be compared with the effect of phospholipids.
- To correlate the interfacial studies with the physicochemical behavior of colloidal systems. Namely, nanoparticles for the biocompatibility study and oil-in-water emulsions for the digestibility study were subjected to the same physiological conditions as on interfaces.

3. Main Objectives

- To understand interfacial mechanisms underlying opsonization and lipolysis in the presence of Pluronic interfacial layers that might influence the final behavior in colloidal systems. Concretely:
    - To comprehend why Pluronic coatings confer stealth properties to nanoparticles prolonging their circulating time in the blood stream.
    - To discern how Pluronic-covered oil-water interfaces influence the rate of lipid digestion in emulsions, in contrast to common surfactants such as phospholipids.

4. RESULTS

4. Results

4.1. Interfacial characterization of model air-water interfaces covered by Pluronic F68 and correlation with the behavior of Pluronic F68-coated nanoparticles under intravenous conditions.

4. Results

Soft Matter

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PAPER

Adsorption of antibody onto Pluronic F68-covered nanoparticles: link with surface properties

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ABSTRACT

The use of nanoparticles as drug delivery systems is an emerging application to improve intravenous therapy. Controlling the biocompatibility of the nanoparticles is a crucial step towards the optimal implementation of these systems. Adsorption of serum components onto the nanoparticles is driven mainly by hydrophobic forces. Thus, incorporation of hydrophilic polymers such as polyethylene oxide (PEO) derivatives to the nanostructure surface reduces the interaction of nanoparticles with blood stream components (IgG). The effectiveness of the poloxamer for reducing protein adsorption depends on the resistance of this coating layer. A fundamental understanding of the properties of this surface coating is crucial towards the rational design of these systems. Here, we have used an innovative combination of experimental techniques to evaluate the properties of the nanoparticles and more specifically, the mechanical properties of the coating. Electrophoretic mobility and colloidal stability data suggest similar surface characteristics between IgG-Pluronic-polystyrene (PS) and IgG-PS complexes, indicating that the protein adsorption is just slightly reduced by the presence of poloxamer. Nevertheless, the biological activity of the adhered antibodies suggests that the Pluronic F68 significantly altered their immunoactivity. The decrease in the activity might indicate a partial denaturation of the protein and/or changes in the preferential orientation when adsorbing caused by the surfactant-protein interactions. The surface characterisation of the IgG layers adsorbed onto a Pluronic covered surface importantly provides evidence of the conformational change undergone by the protein, supporting the partial protein denaturation suggested by the loss of immunoreactivity in the IgG-Pluronic-PS particles. The use of surface tension to obtain structural and mechanical information about the coating procedure is a novel approach to understand generic features of the biocompatibility of colloidal systems. These results may help to understand why drug nanocarriers coated by poloxamers improve their long-circulating properties in comparison with uncoated particles.

1. Introduction

Administration of nanoparticles through intravenous route is the most rapid and simplest method for delivering drugs into the systemic circulation.[1] However, just after systemic injection nanoparticles are immediately covered by the proteins present in the blood stream, leading to a protein "corona"[2-4] that determines the pharmacokinetics and the pharmacodynamics of the administered nanoparticles.[5-7] At the same time, the composition of the protein corona depends on the physicochemical properties of nanoparticle formulation, such as size, surface hydrophobicity, surface charge, *etc*., as well as the concentration and affinities of the surrounding serum components.[8] Some components of the protein corona are opsonins, such as immunoglobulin G (IgG) or complement, inducing the rapid blood stream clearance of the nanoparticles by cells of the reticuloendothelial system (RES).[9,10] This short blood circulating time is one of the main impediments in the development of new therapeutic treatments based on nanoparticles technology.[11]

It is well known that surface hydrophobicity, among others, plays a relevant role on serum components adsorption.[12,13] For this reason, incorporation of hydrophilic polymers such as polyethylene oxide (PEO) derivatives to the nanostructure surface has became a classical alternative to reduce the interaction of nanoparticles with blood stream components and cellular membranes, producing long-circulating or "stealth" formulations.[11,14-23] It has been reported that adsorption of poloxamers, a family of PEO – polypropylene oxide (PPO) – PEO triblock copolymers, inhibits protein adsorption and platelet adhesion on hydrophobic surfaces.[24-26] The poloxamer effectiveness for reducing protein adsorption depends on the PEO block length, the coating concentration, as well as on the surface configuration of the adsorbed poloxamer.[27,28] It should be noted that very thin hydrophilic coatings cannot impart the necessary steric hindrance, as occurs when working with Pluronics L62 or L64 (that have short hydrophilic

moieties of 5 and 13 PEO units, respectively). For that reason, Pluronics with larger PEO content has been often used to prevent protein adsorption. Concretely, Pluronic F68, as well as F108 or F127, have been extensively used to form shells for stealthiness of colloidal drug carriers.[26] Regarding the surface configuration, the brushlike conformation, obtained at high surface coverage and when the poloxamer is adsorbed on highly hydrophobic surfaces,[28,29] has been described as the most efficient to reduce the deposition of proteins and microorganisms.

Although these results indicate that incorporation of PEO derivatives avoids undesirable interactions of proteins with nanoparticles by entropically driven steric hindrance,[30] an accelerated blood clearance (ABC) process of particles modified with PEO derivatives upon repeated *in vivo* injections in mouse, rats and rhesus monkeys has been recently reported.[31-36] While the appearance of this ABC process is not totally clarified yet, it seems that antibodies produced in the spleen as immunoresponse to the first particles injection could overcome the steric hindrance and bind to the particles modified with PEO derivatives.[37-39] This reduction of the stealth properties of the formulation enhances the clearance of the second dose by the Kupffer cells, increasing the liver accumulation of the formulation.[40] Therefore, although PEO modification of nanoparticles has been reported as a successful way to reduce the recognition by the RES, this suggests that hydrophilic polymers cannot fully prevent the interaction of the nanostructure with the blood components. Further understanding of the fundamental mechanisms controlling the interaction of PEO derivatives covered surfaces with antibodies would be very important so as to rationally improve the biocompatibility.

Bearing this in mind, the present work aims to investigate the surface modification of colloidal particles with Pluronic F68 against antibodies deposition. We have used IgG as an antibody model and we have investigated two hydrophobic interfaces: polystyrene (PS)-water and air-water interfaces. This experimental procedure enables to obtain generic information of the specific

surface properties of the poloxamer as in both cases the hydrophobic interaction is the main driving force.[41] The use of surface tension techniques to gain further insight into surface modification of colloidal particles is a novel and emerging area of research. The potential inhibitory effect of the surfactant against antibody deposition was firstly evaluated at the PS-water interface by comparing the electrokinetic behaviour and colloidal stability of Pluronic and IgG coated PS nanoparticles (denoted as Pluronic-PS and IgG-PS, respectively) with those obtained by sequential adsorption of the poloxamer followed by the antibody (denoted as IgG-Pluronic-PS). In addition, an analysis of the immunoreactivity of the possibly adhered IgG molecules of IgG-Pluronic-PS nanoparticles can be performed by means of latex immunoaggregation assays (LIA)[42] in order to investigate the role of the surfactant on the conformation and/or the preferential orientation of the co-adsorbed protein. That is, whether the interaction with the surfactant layer denaturalises and/or alters the mean orientation of the adsorbed protein (or not).

Secondly, we have simulated this situation at the air-water interface with the aim of obtaining further information on the specific surface properties of the system. In order to reproduce this physically relevant situation we take advantage of a specially designed pendant drop film balance equipped with a subphase exchange technique.[43] With this device we evaluated the effect of IgG on a surface covered with Pluronic mimicking the previous study. Through the surface characterisation we obtained structural information about the surface layer and the composition of the interface.[44] Furthermore, this experimental procedure allows us to quantify the potential inhibitory effect as a function of the surface coverage.

The design of the experiments provides generic information of the surface properties of the nanoparticles with direct biomedical applications. The combination of these techniques provides new information about the effect caused on the adsorption of antibodies by PEO derivatives at a molecular scale. This

fundamental understanding is crucial not only to understand why drug nanocarriers coated by PEO derivatives can improve their long-circulating properties in comparison with uncoated particles, but also to clarify why some systems can lose their stealth properties and suffer an accelerated blood clearance after repeated doses.

2. Experimental section

2.1. Materials

The sulfonated PS latex particles were synthesised, purified and characterised in our labs following the methods described previously.[45] The particle size of this PS sample is (203 ± 6) nm, its polydispersity index is equal to 1.0055, and its surface charge density is -(7.2 ± 0.6) μC/cm^2.

The poloxamer Pluronic F68 was obtained from Sigma-Aldrich. It is a triblock copolymer based on PEO*a*-PPO*b*-PEO*a* structure, being a = 75 and b = 30. The molecular weight of this non-ionic polymeric surfactant is 8400 Da. The critical micelle concentration (CMC) provided by the manufacturer is 0.04 mM (0.34 mg/mL).

Different proteins have been used in this work. For the colloidal characterisation experiments with PS nanoparticles, human C-Reactive Protein (CRP) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, and polyclonal antiCRP-IgG was obtained, purified and kindly donated by Biokit S.A. (Spain). The isoelectric point (IEP) of the polyclonal IgG pool was determined by isoelectric focusing, and it is in the 6.0 - 7.9 range. For the air-water interface study, polyclonal IgG from goat serum (reagent grade, 95%) was purchased from Sigma-Aldrich. This protein has a molecular weight of approximately 160 KDa.

Milli-Q purified water (0.054μS) was used for buffer preparation and all other purposes. Only freshly prepared dispersions were used for each experiment. All the glassware was cleaned in sulphuric acid and then repeatedly rinsed with ultrapure water. All other solvents and chemicals used were of the highest grade commercially available.

2.2. Adsorption onto PS nanoparticles

Adsorption of poloxamer onto the PS nanoparticles was carried out according to the protocol described in Santander-Ortega *et al.*.[29] A small amount of the PS stock (total surface area of 0.2 m^2) was added to 8 mL of 2mM phosphate buffer at pH 7 containing poloxamer concentrations below its CMC. Incubation was performed in vessels gently agitated in a rotating plate at constant temperature (25 ºC) for 20 h. This time was enough to reach a steady state. Quantification of the non-adsorbed poloxamer molecules was carried out by analysing the supernatants (after centrifugation) with the molybdophosphoric acid reagent.[46] The poloxamer concentration was spectrophotometrically determined (Beckman DU 7000 Spectrophotometer) using calibration curves constructed in the 0 - 400 μg/mL range, where the errors are usually within the 5 - 10% range.

The adsorption of IgG was carried out similarly: 0.2 m^2 of PS nanoparticles immersed in 8 mL of 2mM phosphate buffer at pH 7 were incubated with IgG molecules with a concentration within the 0 - 8 mg/m^2 range. Incubation was carried out in a thermostatic bath where samples were gently agitated at 25 ºC for 15 h. After incubation, samples were centrifuged and the supernatant filtered using a polytetrafluoroethylene filter (Millipore, pore diameter equal to 100 nm). The protein concentration in solution was determined, before and after adsorption, by direct UV spectrophotometry at λ = 280 nm (Σ_{IgG} = 1.40 mL mg^{-1} cm^{-1}), and then, the adsorbed amounts were calculated by subtracting the final concentration values from the initial ones.

4. Results

Sequential adsorption of Pluronic F68 and IgG (in that order) was performed as follows. According to the data obtained from the plateau of the poloxamer adsorption isotherm, the PS nanoparticles were coated with a saturated poloxamer monolayer of 1.1 mg/m^2. Then, the same IgG adsorption procedure described above was carried out onto the Pluronic-PS complexes (2mM phosphate buffer, pH 7).

Electrophoretic mobility

The electrophoretic mobility measurements were carried out with a Zeta-Sizer IV (Malvern Instruments). The particles were diluted in the desired buffered solutions (2mM phosphate buffer, pH 7) for 10 min just before measuring. The final particle concentration was equal to 3×10^9 mL^{-1}. The electrophoretic mobility data were taken from the average of six measurements on the same sample at the stationary level in a cylindrical cell.

Colloidal stability

The aggregation of the latex particles immersed in saline media (2mM phosphate buffer, pH 7) was measured by analysing the light scattered by the sample towards a low angle (nephelometry). In these experiments NaCl was used as aggregating salt. The nephelometric apparatus worked with a He/Ne laser, using a rectangular scattering cell with a 2 mm path length, and measured the light scattered at an angle equal to 10º. Equal volumes (1 mL) of salt and latex dispersions were introduced into the cell and mixed by an automatic mixing device. The initial particle concentration was set at 5×10^{10} mL^{-1}, and the intensity (I) of the scattered light during aggregation was analysed for 120 s. The linearity in the aggregation kinetics was relatively good at the beginning, and the initial slopes (dI/dt) were easily obtained for each experiment. This allowed us to estimate the

stability ratio *W* (also called Fuchs factor), which can be calculated from the following expression:

$$W = \frac{\left(dI / dt\right)_r}{\left(dI / dt\right)_s} \quad (1)$$

where $(dI/dt)_r$ corresponds to the initial slope of rapid coagulation kinetics, whereas $(dI/dt)_s$ is the same parameter for a slow coagulation regime. The Fuchs factor is related to the number of collisions that must suffer two colliding particles before they keep definitively stuck. Therefore, when $W = 1$ the system is completely unstable, while $W = \infty$ means total stability. The critical coagulation concentration (CCC) can be easily determined by plotting the logarithm of *W* versus the logarithm of the salt concentration and locating that point where *log W* reduces to zero. In addition, in hydrophilic colloids, it is possible to determine the critical stabilisation concentration (CSC), which is that point where *W* increases from 1 to ∞ when salt concentration increases above the CCC value. This restabilisation process found at moderate and/or high salinity values is produced by short-range repulsive hydration forces.[47-51] The CSC value is associated with the surface hydrophilicity of the colloidal particles, so that the lower the CSC value the higher the surface hydrophilicity.

Immunoassays

The optimum experimental conditions for the immunoassays were established in Ortega-Vinuesa *et al.*.[42] All the assays were carried out in a standard medium, namely, BSA saline buffer (pH 8.0, 13 mM borate, 150 mM NaCl, 1 mg/mL NaN_3 as preservative, and 1 mg/mL BSA), which approximately simulates the pH and ionic strength values usually found in physiological fluids. The role played by the BSA molecules is to cover hypothetical PS bare patches to avoid particle bridging produced by an unspecific adsorption of the antigen (CRP)

molecules. In addition, the adsorption of these albumin molecules (if it took place, which is not almost probable, as shown in Ortega-Vinuesa *et al.*[42]) would help to increase the colloidal stability of our IgG-PS complexes at pH 8, avoiding any potential aggregation caused by saline effects in the immunoreaction medium. Furthermore, it is well established that no desorption of IgG protein from surface was detected caused by the direct action of the BSA saline buffer.[42] Immunoagglutination was detected by turbidimetry, working with a spectrophotometer (DU 7400, Beckman) at a λ = 570 nm. 950 μL of an antiCRP-IgG-PS (or antiCRP-IgG-Pluronic-PS) suspension in BSA saline buffer were quickly mixed with 50 μL of a CRP solution, ranging a final CRP concentration from 0.010 μg/mL to 2.5 μg/mL. The initial particle concentration was 2.3×10^{10} mL^{-1}. It should be noted that samples were colloidally stable in the BSA saline buffer, and thus, only when the antibody-antigen recognition took place, the aggregation of the particles occurred. The increase in turbidimetry was then monitored for 5 minutes that is the practical period for monitoring the anti CRP-IgG/CRP reaction with this technique.[52]

2.3. Adsorption onto air-water interface

Surface tension

The surface tension measurements have been performed in a Pendant Drop Film balance fully assembled and developed at the University of Granada and is described in detail elsewhere.[43] A solution droplet is formed at the tip of a coaxial double capillary, connected independently to a double microinjector. The computer program fits experimental drop profiles, extracted from digital drop micrographs, to the Young-Laplace equation of capillarity by using Axisymmetric Drop Shape Analysis (ADSA), and provides as outputs the drop volume *V*, the surface tension

γ, and the interfacial area *A*. The adsorption process is recorded at constant interfacial area through a modulated fuzzy logic PID algorithm (proportional, integral, and derivative control).[53] The drop is immersed in a glass cuvette (Hellma) and is kept in a thermostatised cell at 25 °C. The surface pressure values π are obtained from the relationship $\pi \equiv \gamma_0 - \gamma$, where γ_0 is the surface tension of pure air-water interface, and γ is the surface tension of the solution. The surface tension of the clean air-water interface (γ_0) was measured before each experiment to ensure the absence of surface-active contaminants obtaining values of 72.0 mJ/m^2 at 25 °C. All solutions were prepared by successive dilution in a 150 mM Hanks balanced solution (HBS) buffer at pH 7.4 in order to mimic physiological conditions, and negligible differences were found in interfacial tension data when using 2 mM phosphate buffer. The reproducibility of the experiments was verified through at least three replicate measurements.

The surface pressure is first recorded for the adsorption of individual systems, Pluronic F68 and IgG, at the bare air-water interface. Next, the subphase exchange accessory is used in order to simulate the transit through the systemic circulation of the previously poloxamer-covered interface. A coaxial double capillary enables to substitute the poloxamer bulk solution once a stable layer has been formed at the air-water interface at constant interfacial area. The stability of the Pluronic F68 film adsorbed at the interface was confirmed prior to the penetration studies by checking the surface tension after the exchange by buffer on an adsorbed layer of Pluronic.[44] Then, the subphase is exchanged by extracting the poloxamer solution through the outer capillary, and injecting simultaneously through the inner one the IgG solution. Finally, the interfacial behaviour of IgG can be monitored on a poloxamer-covered interface by evaluating the surface pressure of the system.

4. Results

Dilatational rheology

The dilatational rheology of the poloxamer and protein adsorbed layers and the sequentially adsorbed IgG onto the pre-adsorbed poloxamer layer was measured with the pendant drop technique apparatus described above. An oscillatory perturbation was applied to the interface by injecting and extracting volume to the drop. The system records the response of the surface tension to the area deformation, and the dilatational modulus (E) of the interfacial layer can be inferred from this response. In a general case, the dilatational modulus is a complex quantity that contains a real and an imaginary part:

$$E^* = E' + iE'' = \varepsilon + i2\pi f\eta \quad (2)$$

where E' is the storage modulus and accounts for the elasticity (ε) of the interfacial layer and E'' is the loss modulus and accounts for the viscosity (η) of the interfacial layer.

In our case, the applied interfacial area oscillations were maintained below 1% of amplitude to avoid excessive perturbation of the interfacial layer, as this technique requires a quasi-equilibrium drop shape for the calculation of the interfacial tension, and to avoid departure from the linear viscoelastic region. The oscillation frequency (f) was set to 0.1 Hz.

3. Results and discussion

3.1. Pluronic F68 and IgG adsorption onto PS nanoparticles

Fig. 1 shows the adsorption isotherms of Pluronic F68 and IgG onto the PS nanoparticles. The adsorbed amount (Γ_{ads}) is plotted versus the poloxamer equilibrium concentration or the protein added concentration. As expected from the work of Kayes *et al.*,[54] the specific adsorption values for the poloxamer reach a

clear plateau at bulk concentrations below its CMC. It is instructive to compare the plateau surface coverage determined in the present work with those reported in literature. Tadros *et al.*[55] and Baker *et al.*[56] obtained plateau adsorption values onto PS surfaces of around 0.9 and 1.0 mg/m^2, respectively, which approximately coincide with our results: 1.1 mg/m^2. As the adsorption was carried out at Pluronic concentrations below its CMC, micellar-like aggregates are not expected on the PS surfaces.[29] Therefore, we assume that PS complexes are coated by a single monolayer of poloxamer. Regarding the IgG adsorption, the results obtained are also in concordance with usual antibody adsorption isotherms onto hydrophobic materials, in which the slope of the initial part of the isotherm reflects a very high affinity for the surface, and a clear plateau is reached once the PS-water interface has been saturated by the protein molecules.[57,58] Finally, the adsorption isotherm of IgG onto PS particles previously coated by a monolayer of Pluronic F68 (1.1 mg/m^2 surface coverage) is also depicted in Fig. 1. It can be observed that the

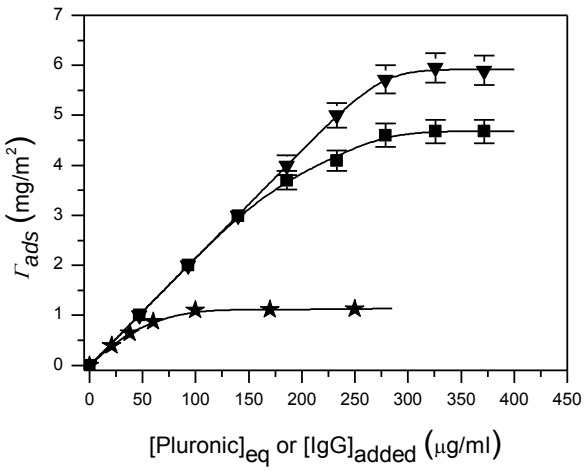

Fig. 1 Adsorption isotherms of Pluronic F68 (stars) and IgG (down triangles) onto bare PS system. Adsorption isotherm of IgG onto Pluronic-PS sample with a 1.1 mg/m^2 poloxamer concentration (squares). (2 mM phosphate buffer, pH 7)

adsorption profile also indicates high affinity, but the maximum amount adsorbed at the plateau is clearly lower than that obtained onto bare PS particles. This result proves the great influence that hydrophobic forces exert on protein adsorption phenomena.[12,59,60] At neutral pH, in which our polyclonal IgG is practically uncharged, the main driving force for adsorption is the hydrophobic one. The hydrophobic interaction is characterised by a large entropy increase and a relatively small enthalpy effect. In protein adsorption this entropic increase is not only due to the release of structured water molecules from the hydrophobic areas to the bulk, but also related to changes in the structure of the protein molecule. Our results suggest an adsorption mechanism led by hydrophobic interactions between the protein and the hydrophobic patches of the PS surfaces and/or the PPO fragments of the adsorbed poloxamer. In both cases, changes on the protein structure – that is, a partial denaturation – are expected, as shown by Molina-Bolívar *et al.*[50,51] when working with pure PS particles. The presence of PEO chains extended towards the aqueous phase could be responsible for the lower plateau value observed when the IgG is adsorbed onto the Pluronic-PS complexes as compared to that adsorbed onto the bare PS particles. These results agree quantitatively with those reported by Higuchi *et al.*,[27] who found that the original adsorption of globulin (from a mixed protein solution) on bare polysulfone (PSf) membranes was reduced when the adsorption was carried out on Pluronic F68-coated PSf membranes, remaining an adsorbed amount of globulin equal to 70-80%.

The electrophoretic mobility (μ_e) as a function of pH was evaluated in low ionic strength conditions. The experimental results are shown in Fig. 2. The electrophoretic mobility of bare PS particles is the typical for PS latex with sulfonate groups on its surface.[61] This strong acid group remains negatively charged within the whole pH range tested (from pH 4 to pH 9), and consequently the μ_e remains practically constant, being only slightly reduced at acid pH (pH 4-5). The presence of a Pluronic F68 layer on the surface does not alter the electrical

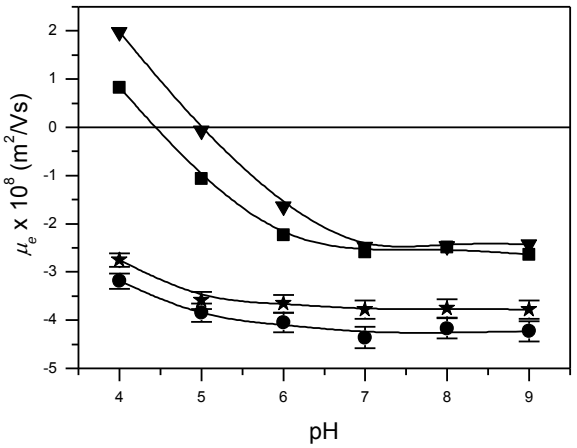

Fig. 2 Electrophoretic mobility versus pH: bare PS system (circles), Pluronic-PS sample with a 1.1 mg/m^2 poloxamer concentration (stars), IgG-PS sample with a 6.1 mg/m^2 antibody concentration (down triangles) and IgG-Pluronic-PS sample with a 1.1 mg/m^2 poloxamer concentration and a 4.9 mg/m^2 antibody concentration (squares). (2 mM phosphate buffer, pH 7)

state of the surface, as this poloxamer is a non-ionic surfactant. This is why the Pluronic-PS complex shows exactly the same μ_e trend of the bare PS particles. Still, the absolute values are slightly shifted towards lower mobility values. It should be noted that the adsorption of this surfactant modifies a relatively smooth PS surface into a rough one with extended PEO chains towards the aqueous phase. This could well shift the shear plane outward, and consequently diminish the ζ-potential. Nevertheless, the presence of IgG molecules adsorbed onto the particles (see data of the IgG-PS complex in Fig. 2) causes a more significant alteration of the μ_e. In this case, the electrophoretic mobility even inverts its sign at pH 4, finding the IEP of the complex at pH 5. This was expected since usually, when colloidal particles are coated by a protein, the original IEP of the bare particles moves towards the IEP of the pure protein.[58,61] The system in which the IgG was adsorbed onto particles previously coated by Pluronic (see the IgG-Pluronic-PS

data in Fig. 2) behaves quite similar to the IgG-PS complex, providing an IEP of 4.5. It should be noted that this complex was formed at a concentration of protein located at the plateau of the adsorption isotherm so that theoretically it has a protein coverage of 4.9 mg/m^2 (Fig. 1). Its electrokinetic behaviour is located between those shown by the Pluronic-PS system and the IgG-PS one, although it is clearly shifted toward the latter. This could mean that IgG is able to adsorb to a great extent onto a Pluronic F68-covered PS surface, corroborating data shown in Fig. 1.

The next set of experiments was based on colloidal aggregation studies performed at pH 7. Fig. 3 shows the stability factor (*W*) of several systems versus the NaCl concentration added. The stability of the bare PS latex shows a CCC value of around 800 mM. This is a value typically associated with highly stable systems in which the stability is supplied by the electrostatic forces due to the high surface charge densities. The lack of restabilisation mechanisms given by hydration forces at high salt concentrations is a clear indication of the hydrophobic nature of the PS surface. As it can be seen, the behaviour of *W* drastically changed when the bare PS particles were fully coated by Pluronic F68 or by IgG. In the Pluronic-PS complex, a completely stable system was found, as it was impossible to coagulate at any NaCl salt concentration. This feature is characteristic of sterically stabilised colloids, and it clearly indicates that the Pluronic F68 molecules were effectively adsorbed onto the surface particle. Santander-Ortega *et al.* reported a similar behaviour when working with hydrophobic polylactic-co-glycolic acid (PLGA) particles coated by Pluronic F68.[29] On the other hand, a less stable system was obtained when the bare PS particles were coated by IgG at a 6.1 mg/m^2 surface concentration. The CCC value (50 mM) shows that the high surface charge density of the bare latex has been screened by a poorly charged protein layer. This decrease in stability agrees with the μ_e reduction shown in Fig. 2. In addition, restabilisation at high salinity by hydration forces was now observed (with a CSC = 180 mM),

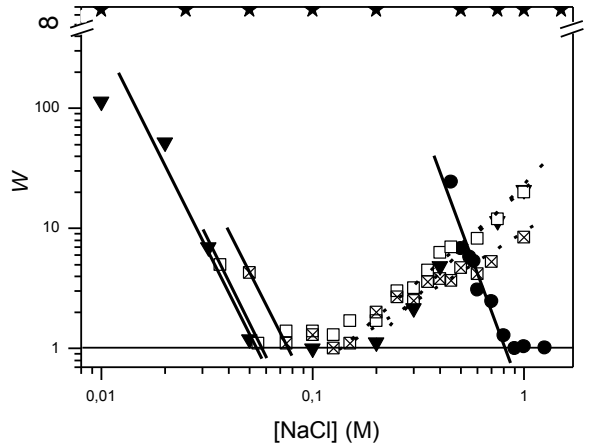

Fig. 3 Dependence of the stability factor on NaCl concentration: bare PS (circles), Pluronic-PS (stars), IgG-PS (down triangles), IgG-Pluronic(50)-PS (squares) and IgG-Pluronic(100)-PS (crossed squares) samples. (2mM phosphate buffer, pH 7)

which also demonstrates that the IgG coating has effectively converted the original hydrophobic surface into a hydrophilic one.

Santander-Ortega *et al.* also found that the stability behaviour of Pluronic F68-PLGA complexes was completely different from those exclusively coated by IgG.[29,52] Owing to the very different stabilities of these three systems (PS, Pluronic-PS and IgG-PS) it is possible to probe whether a Pluronic F68 layer placed onto a hydrophobic interface avoids (or not) a subsequent adsorption of IgG. Two complexes, referred to as IgG-Pluronic(100)-PS and IgG-Pluronic(50)-PS, were evaluated. The number in brackets in the previous notation refers to the percentage PS area theoretically coated by the Pluronic F68 in the first step, so that the effect of the surface coverage of poloxamer can be also analysed. In both cases, the usual sequential adsorption of poloxamer and IgG was carried out performing the adsorption of the surfactant in a first step and subsequently adding the protein in excess to assess that the particle surface was completely coated by the IgG in case this second adsorption takes place. If the adsorbed poloxamer was able to

prevent the IgG deposition, the stability of these new complexes might tend to the behaviour of the initial Pluronic-PS complex, which was completely stable. Interestingly, Fig. 3 shows that the stability pattern of the two IgG-Pluronic-PS systems differed absolutely from that expected for complexes in which the IgG cannot be adsorbed. Instead of finding completely stable systems, both IgG-Pluronic-PS complexes practically matched the stability curve of the IgG-PS system, being almost coincident with that shown by the IgG-Pluronic(50)-PS one. It is worth remarking that colloidal stability is a property that depends almost exclusively on the surface characteristics of the particles. Since the stability of the complexes where the sequential adsorption took place behaves as that of the IgG-PS complex, it can be inferred that the IgG molecules added in the second step are able to adsorb onto the previously adsorbed Pluronic F68 layer, confirming not only the co-adsorption data shown in Fig. 1, but also the mobility results in Fig. 2.

The last set of experiments was designed to evaluate the possible denaturation and/or changes in the preferential orientation of the IgG molecules adsorbed onto a PS surface previously coated by a Pluronic F68 monolayer. The extent of the agglutination caused by the presence of the corresponding antigen (CRP) gives quantitative information about the antibody immunoactivity. Fig. 4 shows such immunoactivity results, which reflects the typical bell-shape curve (or *precipitin curve*) with a maximum separating the low antigen concentration regime from that in which the antigen are in excess with regard to the antibody molecules.[62] Despite the differences between the IgG-PS and IgG-Pluronic-PS complexes related to the main colloidal properties were almost negligible (see Fig. 2 and 3), the immunoagglutination extent clearly differs between both systems. If the adsorption of IgG is preceded by the poloxamer deposition on the PS, the immunoaggregation behaviour becomes three times lower than that of the IgG-PS sample (Fig. 4). Taking into account that the IgG load was fairly similar in both samples (6.1 and 4.9 mg/m^2), one can infer that this significant immunoactivity reduction might be due to two processes that are not mutually exclusive: i) on one

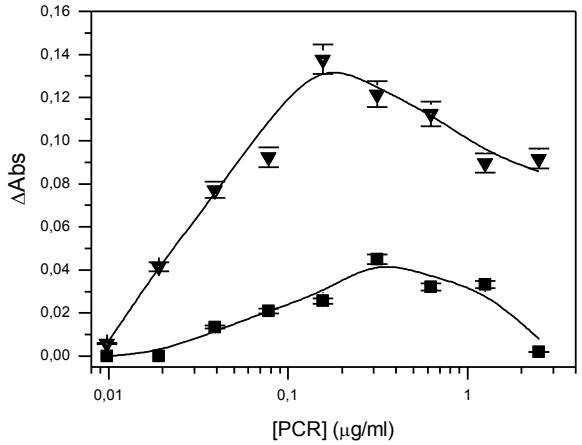

Fig. 4 Absorbance change after 300 s (λ = 570 nm) due to aggregation induced by different CRP concentrations: antiCRP-IgG-PS (down triangles) and antiCRP-IgG-Pluronic-PS (squares) samples. (13 mM borate, 150 mM NaCl, 1 mg/mL NaN_3, 1 mg/mL BSA, pH 8.0)

hand, the denaturation of the antibody molecules in contact with the surfactant layer, and *ii)* on the other hand, a change in the mean orientation of the adsorbed IgG. If the surface of the Pluronic-PS nanoparticles was considered hydrophilic, it would be possible that antibodies adsorbed with the F_{ab} region (hydrophilic fragment) facing toward the nanoparticle orientation, and thus, leading to the decrease of the immunoagglutination efficiency due to the shielding of antigen recognition region from CRP. However, it should be noted that a 1.1 mg/m^2 layer of Pluronic F68 does not represent a hydrophilic surface, as shown in Santander-Ortega *et al*.,[29] since repulsive hydration forces typical of hydrophilic materials do not almost exist in this type of surfactant layer. Therefore, it is likely that antibodies were preferentially oriented with the hydrophobic F_c region to the surface in both cases (pure PS and Pluronic-PS particles). With regard to the other possibility, that is, an additional denaturation compared with the PS surface, it is well known that there exist different types of interactions between proteins and

surfactants,[63] being capable to modify the tertiary or quaternary structure of the formers depending on the nature of both, protein and surfactant.[64,65] Therefore, our immunoagglutination assays would suggest a loss of activity in the adsorbed IgG molecules, ascribed to a partial denaturation caused by the Pluronic F68 layer. This denaturation process might alter the usual pathways of the complement system in *in vivo* media. The stealthiness of colloidal drug carriers coated by Pluronic F68 molecules would not come exclusively from the ability of the surfactant to inhibit protein adsorption (as this adsorption takes place according with our results), but it would be also caused by alterations in the tertiary structure of the adsorbed antibodies that would slow down the activation of the complement system to retire foreign nanoparticles from the circulating blood. This is a very important result and it is consistent with the studies of Higuchi *et al.* on the adsorption of fibrinogen and platelets onto Pluronic F68-covered surfaces.[27] In blood coagulation, adsorption of platelets is also mediated by a given cascade of reactions in which the adsorption of fibrinogen plays a key role.[66-69] These authors showed that a PluronicF68-coated PSf membrane was not able to totally avoid the fibrinogen adsorption from plasma, although it was reduced. The fibrinogen deposition was 25% of the total amount adsorbed on unmodified PSf membranes. However, the platelet deposition was completely inhibited (less than 0.1% comparing to the original PSf material). Therefore, a Pluronic F68 layer was not able to prevent the deposition of a key protein in the blood coagulation pathways (although this deposition was hindered to some extent), but it was able to block the final part of this cascade of reactions: the platelet adhesion. Hence, something similar could be extrapolated to the antibody adsorption and its role in the activation of the complement system. The Pluronic F68 layer in our PS particles can reduce (but not inhibit) the IgG adsorption. This reduction is so low – 80% of the protein remains – that even the final colloidal properties of the IgG-Pluronic-PS particles are coincident with those of the IgG-PS ones. However, interactions between IgG and surfactant produce denaturation of the antibody molecules that significantly changes their

immunoactivity, and therefore might alter the final product of the cascade of immunoreactions in *in vivo* systems, which is the retirement of the colloidal particles from the blood.

It should be noted that recent studies aim in the same direction. Nonckreman *et al.*[70] also supported the concept that blood compatibility of materials is primarily governed by proteins (mainly fibrinogen) in the adsorbed phase, being the final adsorption extent strongly influenced by the surface hydrophobicity. They concluded that turning a hydrophobic surface into hydrophilic by adsorbing a Pluronic F68 layer diminishes, but does not avoid, fibrinogen adsorption. However, they suggested that conformational changes as well as molecular reorientations are strongly dependent on the surface hydrophobicity/philicity. Conformation and denaturation of a given protein probably differ from one surface to the other, which would be responsible for changes in subsequent blood-materials interactions (*i.e.* platelet adsorption). Besheer *et al.*[71] also stated that the prevention of plasma protein adsorption may not be the only mechanism by which PEO chains coatings generate stealthy properties in drug nanocarriers. They also discussed about "differential protein adsorption": the presence of PEO layers does not prevent adsorption, but they would produce a selective adsorption different to that occurring in the un-coated surface, that alters the pharmacokinetics and fate of nanoparticles.

3.2. Pluronic F68 and IgG adsorption onto air-water interface

In order to further analyse the poloxamer-protein surface interactions and the conformational change induced in the IgG, we study specifically the structural and mechanical properties of the surface layers. In order to reproduce the study on PS nanoparticles, we firstly present the properties of the surface layers of Pluronic F68 and IgG and then the surface properties of IgG adsorbed onto a Pluronic-covered interface by means of the subphase exchange technique. Moreover, a

surface pressure control in the set-up enables us to do the exchange at different surface pressures, so that the effect of poloxamer surface coverage on the adsorption of IgG can be studied. Additionally, this effect is evaluated after 3 and 20 h of adsorption.

The surface pressure isotherms of Pluronic F68 and IgG are shown in Fig. 5. These experimental curves are obtained by plotting the steady state surface pressure (± 0.2 mJ/m^2) values obtained for each of the bulk concentrations of Pluronic F68 (after 30 min of adsorption) and IgG (after 20 hours of adsorption). Regarding IgG isotherm, it can be seen in Fig. 5 that the protein adsorption proceeds within a range of concentrations from 10^{-5} mg/mL to 0.1 mg/mL. Also, the surface pressure isotherm shows a plateau at a bulk concentration of 0.1 mg/mL with a saturation surface pressure value of 16 mJ/m^2, in agreement with literature results under similar conditions of pH and temperature.[72] The "T"- or "Y"-shaped structure of the IgG molecule is characterised by three fragments: two hydrophilic F_{ab} and one hydrophopic F_c. According to Baszkin *et al.*,[72] such saturation behaviour in the surface pressure could correspond to the adsorption of surface-active macromolecules with their hydrophobic fragments oriented vertically towards the interface (end-on orientation), so that further adsorption or reorientation of molecules, above the steady-state surface pressure value, practically unaccounts for the surface pressure of the film. Differently, the surface pressure isotherm of Pluronic F68 exhibits a complicated staircase shape (Fig. 5). This stepwise trend has been reported previously in the literature for Pluronic layers adsorbed at air-water interface,[73-75] corresponding to surface phase transitions from a dilute regime at low surface pressures to a "brush" organization at high surface pressures.[74,75] From the view that the CMC corresponds to the high-concentration breakpoint of surface tension isotherm,[74] the value obtained in Fig. 5a for Pluronic is 0.1 mg/mL. Pornsunthorntawee *et al.* found an estimated CMC

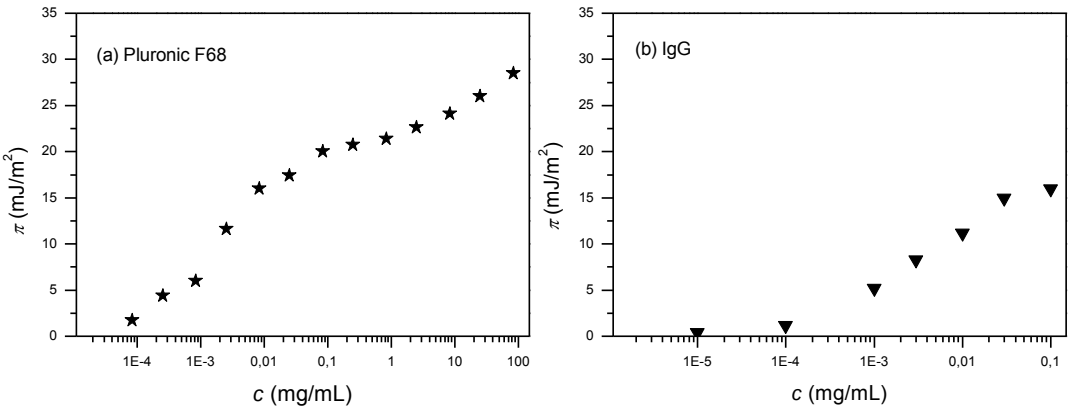

Fig. 5 (a) Adsorption isotherm of Pluronic F68 at the bare air-water interface. Adsorption time 30 min. (b) Adsorption isotherm of IgG at the bare air-water interface. Adsorption time 20 h. (150 mM HBS buffer, pH 7.4) Error bars are within the size of the symbols.

value of 0.35 mg/mL from the breakpoint of surface tension versus log of concentration curve.[76] This is in agreement with Fig. 3 and with the CMC value provided by the manufacturer (0.34 mg/mL) taking into account that for Pluronic block copolymers it is generally considered acceptable that the CMC value will vary 3-10 times, depending on the method of measurement employed and the temperature at which is evaluated.[77,78] However, the surface pressure of adsorbed Pluronic does not seem to reach a plateau within the range of concentrations considered here. The largest concentration used is limited by the rupture of the droplet due to the fast drop in the interfacial tension. Hence, it is probably that in the range of higher concentrations, a constant value of the surface pressure is achieved. Actually, the CMC reported by Muñoz *et al.* is 10 mM (84 mg/mL),[75] corresponding to the last bulk concentration studied here. Given the number of

publications dealing with the aqueous solutions of Pluronic copolymers, there are surprisingly few reliable CMC data for these copolymers in the literature.[74] For that reason, we will not consider it in this work, but just bear in mind the surface pressure isotherm displayed in Fig. 5.

In order to study the adsorption of IgG onto a surface that has been previously covered with Pluronic F68 we used the subphase exchange accessory, which allows doing sequential adsorption experiments at water-fluid interfaces.[44,79] Therefore, we can first adsorb the poloxamer until it reaches a certain surface coverage, which is controlled by the surface pressure. We have chosen a concentration of Pluronic of $2.5 \cdot 10^{-3}$ mg/mL that is well below the CMC range, as in adsorption experiments onto PS nanoparticles. As seen in Fig. 5a, this poloxamer concentration covers a wide range of surface pressure varying from 0 to 13 mJ/m^2, so that the adsorption process can be stopped at different surface coverage. Then, we can exchange the subphase and add the protein into the bulk solution with minimal perturbation of the interface. Regarding the protein, we used a high concentration (0.1 mg/mL) which ensures a saturated surface layer in case this adsorption took place. The subphase exchange experiments are shown in Fig. 6. The poloxamer is firstly adsorbed onto the air-water interface. Once the desired surface pressure is attained, the poloxamer subphase is substituted by the protein solution. This moment is marked in Fig. 6 by the lines at different surface pressures values of 5, 8, 10 and 12 mJ/m^2. Then, the adsorption of IgG (0.1 mg/mL) onto the interface covered by Pluronic F68 was monitored by following the evolution of the surface pressure up to 3 h, in order to evaluate the effect of the poloxamer on the subsequent adsorption of IgG at short times. The rate of change of surface pressure after the exchange might be an indication of the adsorption of IgG. Specifically, after the subphase exchange at the lowest surface coverage of Pluronic (π = 5 mJ/m^2) the surface pressure rapidly increased up to 7.5 mJ/m^2 and then kept gradually increasing with time. The irreversibility of the Pluronic F68 adsorption onto water-fluid interfaces after washout with water has been previously reported

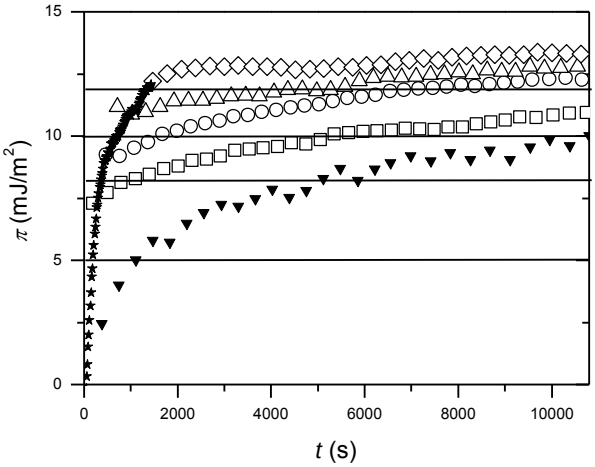

Fig. 6 Adsorption of IgG (0.1 mg/mL) onto a surface covered with Pluronic F68: effect of poloxamer surface coverage at short times (3 h). Adsorption of Pluronic F68 ($2.5 \cdot 10^{-3}$ mg/mL) at the bare air-water interface before the subphase exchange (stars). Lines mark the surface pressures attained by the poloxamer at the subphase exchange moment: adsorption of IgG after subphase exchange at 5 mJ/m^2 (squares), 8 mJ/m^2 (circles), 10 mJ/m^2 (up triangles) and 12 mJ/m^2 (diamonds). Adsorption of IgG (0.1 mg/mL) at the bare air-water interface (down triangles). (150 mM HBS buffer, pH 7.4)

in literature[44,80] and attributed to a strong desorption barrier. Therefore, after the subphase washout the changes observed in surface pressure will contain information on the possible interactions of the protein with the poloxamer. The increase in the surface pressure (Fig. 6) suggests that the protein is adsorbing onto the interface, probably penetrating but not completely displacing Pluronic F68. The surface pressure is higher than those of pure systems at the bare air-water interface possibly due to the formation of complexes of poloxamer and protein with enhanced hydrophobicity. Increasing the poloxamer surface coverage (exchange at

π = 8 and 10 mJ/m^2) the initial fast rise in the surface pressure is reduced and is practically negligible at the largest coverage of Pluronic (exchange at π = 12 mJ/m^2). In addition, a closer look into the experimental data reveals that the rate of change in the surface pressure after the exchange slightly decreases with increasing surface coverage of the poloxamer. The surface concentration seems to play a crucial role in the effect on the IgG adsorption at short times, indicating that lower surface coverage of the poloxamer is more susceptible to the presence of IgG.

On the other hand, the effect of the Pluronic-covered interface on the subsequent adsorption of IgG at longer times is shown in Fig. 7, where the adsorption is recorded for 20 h after the subphase washout. Also, a reference curve of the protein adsorption onto the bare air-water interface is included. It is very interesting to note that the final surface pressure is the same in all the sequential adsorption experiments regardless of the surface pressure at which the exchange was done, *i. e.* of the poloxamer surface coverage. Hence, the protein adsorption onto the surface covered by the poloxamer proceeds independently of the degree of surface coverage attained, in terms of surface pressure. Moreover, the final value is slightly higher than that corresponding to the adsorption of IgG at the bare air-water interface (16 mJ/m^2) and also different to that of pure Pluronic (Fig. 5). This feature suggests that after 20 h more protein molecules could have finally reached the interface affecting the surface pressure. But, it also indicates that the protein has not just completely displaced the poloxamer from the interface since the value of surface pressure does not correspond to that of the pure protein, leading to the synergistic effects as we mentioned before. In order to obtain further information on the composition of this surface layer, we shall look into the dilatational properties of the surface layers below.

Given that the surface layer seemed to change over time (Fig. 6 and 7), we have evaluated the dilatational modulus of the adsorbed IgG onto Pluronic-covered interface after 3 and 20 h of adsorption after the subphase exchange. In this manner

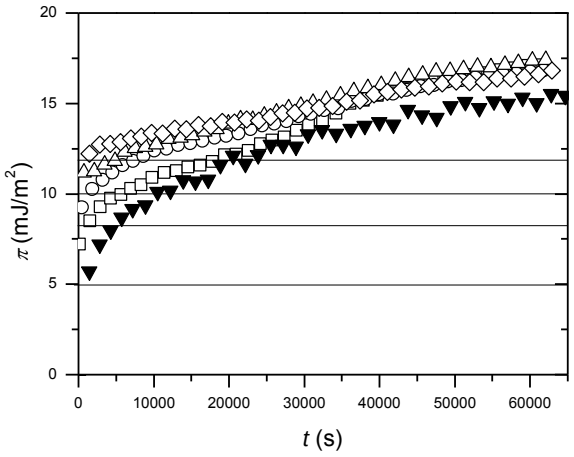

Fig. 7 Adsorption of IgG (0.1 mg/mL) onto a surface covered with Pluronic F68: effect of poloxamer surface coverage at long times (20 h). Lines mark the surface pressures attained by the poloxamer at the subphase exchange moment: adsorption of IgG after subphase exchange at 5 mJ/m^2 (squares), 8 mJ/m^2 (circles), 10 mJ/m^2 (up triangles) and 12 mJ/m^2 (diamonds). Adsorption of IgG (0.1 mg/mL) at the bare air-water interface (down triangles). (150 mM HBS buffer, pH 7.4)

we can study the time evolution of the interface as the protein tries to reach the surface through the adsorbed poloxamer. As it was done in previous sections, let us first characterise the surface structure of pure poloxamer and pure IgG in terms of their surface dilatational modulus. Table 1 shows the surface pressure and dilatational modulus of adsorbed layers of IgG and Pluronic F68 at the same concentrations used in the subphase exchange experiments. The dilatational rheology was measured at a fixed frequency of 0.1 Hz. At this relatively high frequency, the viscous component of the dilatational modulus is very small for Pluronic and IgG (data not shown) and the adsorbed layers appear predominantly elastic.[81] Accordingly, the values presented in this study correspond to the

dilatational modulus, equation (2), and the viscous component is not reported here. Regarding the protein adsorbed layer, the IgG has a very high dilatational modulus. Interestingly, the dilatational modulus of the adsorbed protein layer at 3 h is nearly the same than that obtained after 20 h of adsorption (120 ± 5 mJ/m^2). This contrasts with the visible increase of the surface pressure with time (from 10 mJ/m^2 at 3 h to 16 mJ/m^2 at 20 h of adsorption) and suggests that the protein forms very rapidly an elastic network and further changes in the adsorbed protein, such as surface denaturation or multilayer formation, do not have an effect on the dilatational modulus. The compact "T"-shaped tertiary structure[82,83] could explain this feature.[84] As regards the surface dilatational modulus of the poloxamer, the value obtained (25 ± 5 mJ/m^2) is in agreement with literature results[81,85] for similar bulk concentrations of the Pluronic F68 and appears much lower to that of protein, meaning a lower level of structure at the interface as compared to that of IgG. This different dilatational response of the two systems allows us to probe the effect of the poloxamer adsorbed at the air-water interface on the subsequent adsorption of the protein, as it was done in colloidal characterisation experiments with coated PS nanoparticles.

Let us now evaluate the surface dilatational modulus of IgG adsorbed onto a Pluronic-covered interface in the same conditions described in Fig. 6 and 7. Since the adsorbed layer of Pluronic is predominantly elastic at this frequency that was

Table 1. Surface pressure and dilatational modulus measured at different adsorption times for adsorbed layers of Pluronic F68 ($2.5 \cdot 10^{-3}$ mg/mL) and IgG (0.1 mg/mL) at the bare air-water interface. (150 mM HBS buffer, pH 7.4)

	π (± 0.5 mJ/m^2)	E (± 5 mJ/m^2)
Pluronic F68 (1.5 h adsorption)	14	25
IgG (3 h adsorption)	10	120
IgG (20 h adsorption)	16.5	125

too fast to allow any exchange of the poloxamer with the bulk phase,[81] any observed change in the dilatational modulus relative to that of pure poloxamer will be attributed to adsorption of the protein.[44] Table 2 displays the surface pressure and the surface dilatational modulus of the subphase exchange experiments of Fig. 6 and 7 at the different surface pressures, *i. e.* surface coverage of poloxamer. The dilatational moduli of the IgG adsorbed onto a Pluronic-covered interface after 3 h of adsorption (Table 2) are very different (20-30 mJ/m^2) to that of pure IgG at the same adsorption time (120 mJ/m^2) in Table 1. Furthermore, the values correlate with that obtained for the adsorbed layer of Pluronic F68 (25 mJ/m^2) in Table 1. However, the surface pressure values (Fig. 6 and Table 2) increased after 3 h of adsorption after the subphase exchange, exceeding the value of the pure systems at the bare air-water interface (Fig. 6 and Table 1). As we mentioned before, this feature might indicate a co-adsorption of the protein onto the adsorbed layer of the poloxamer, even for the largest surface coverage of Pluronic F68. IgG penetrates the interfacial layer of poloxamer, leading to the formation of interfacial complexes

Table 2. Surface pressure and dilatational modulus measured at different adsorption times for adsorbed IgG (0.1 mg/mL) onto different Pluronic F68 ($2.5 \cdot 10^{-3}$ mg/mL) covered interfaces. (150 mM HBS buffer, pH 7.4)

Surface coverage of Pluronic F68	**3 h of IgG adsorption after subphase exchange**		**20 h of IgG adsorption after subphase exchange**	
Surface pressure at which is done the subphase exchange	$\boldsymbol{\pi}$ (± 0.5 mJ/m^2)	$\boldsymbol{E}$ (± 5 mJ/m^2)	$\boldsymbol{\pi}$ (± 0.5 mJ/m^2)	$\boldsymbol{E}$ (± 5 mJ/m^2)
5 mJ/m^2	11.0	30	17.5	35
8 mJ/m^2	13.0	20	17.0	30
10 mJ/m^2	13.5	20	17.5	30
12 mJ/m^2	13.5	25	17.0	30

which accounts for the increase in the surface pressure. Therefore, the values of the dilatational modulus indicate that the adsorbed poloxamer has somehow inhibited the subsequent adsorption of IgG molecules avoiding the formation of a very cohesive protein network like that formed by IgG molecules at the bare air-water interface. Even the lowest surface coverage of poloxamer considered here, given by a surface pressure of 5 mJ/m^2, has a remarkable effect on the dilatational modulus of the surface layer. The slightly higher value of the dilatational modulus (30 mJ/m^2) obtained for this surface coverage could indicate a residual effect of the protein on the surface layer.

In order to probe the effect of time on the adsorption of IgG onto the poloxamer surface layer, we evaluated the dilatational properties of the surface layers after 20 h of adsorption (Table 2). As seen in Fig. 7, the surface pressure values have increased in all cases to exceed the value of the surface pressure attained by the pure IgG adsorbed layer at the bare air-water interface (Fig. 7 and Table 1). In addition, the dilatational modulus of the IgG adsorbed onto a Pluronic-covered interface just slightly increased after 20 h (30-35 mJ/m^2), still remaining very different to that corresponding to a pure IgG adsorbed layer at the bare air-water interface (120 mJ/m^2) in Table 1. This is a very interesting result since it indicates that the poloxamer prevents completely the formation of an elastic network at the interface though the protein might have entered into the surface. It is also interesting to note that the values of the dilatational moduli follow a similar trend to those at 3 h. That is, at the lowest poloxamer surface coverage, the value of the dilatational modulus is slightly higher, suggesting that is more susceptible to the presence of protein, in agreement with surface tension data.

We can conclude from the surface properties of the IgG adsorbed onto Pluronic interfaces that the poloxamer does not fully prevent the adsorption of IgG, although it does affect the conformation of the IgG molecules, due to the interaction with the surfactant, affecting the mechanical properties of the interface.

This is consistent with the results from the immunoassays with coated PS nanoparticles and provides further insight into the intrinsic properties of these systems. Studies at the air-water interface provide evidence of the presence of protein at the surface despite the presence of Pluronic. Still, the conformation and possibly the functionality of the protein is importantly affected by the presence of Pluronic. This is consistent with the findings at the PS nanoparticles and extremely important in the understanding of these systems. Basic information about the nature of the interaction between IgG and poloxamer can be crucial in the rational modification of surface to improve biocompatibility of nanoparticles.

4. Conclusions

Stealth properties of nanoparticles modified with PEO derivatives have usually been ascribed to the ability of the PEO chains to prevent protein adsorption from plasma by imparting an entropically driven steric hindrance. The results presented here demonstrate that the poloxamer is not able to fully inhibit the adsorption of an antibody, IgG, but it rather affects the conformation of this protein, possibly affecting its functionality and definitely affecting its mechanical properties. We have obtained these results by an innovative approach to the problem, combining the study of colloidal properties of nanoparticles and mechanical properties of the surface layers by surface tension techniques.

Both electrophoretic mobility and colloidal stability data suggest similar surface characteristics between IgG-Pluronic-PS and IgG-PS complexes, indicating that IgG adsorption is just slightly reduced, remaining around 80% of the original adsorption onto bare PS particles. Nevertheless, the biological activity of the adhered antibodies suggests that the Pluronic F68 significantly altered their immunoactivity. The decrease in the activity might indicate a partial denaturation of the IgG caused by the surfactant-protein interactions. However, it might be also caused by a change in the preferential orientation of the IgG, although experiments

at the air/water interface importantly provides evidence of the conformational change undergone by this protein. In addition, we can conclude from the discussion that IgG adsorption, driven by hydrophobic forces at neutral pH, was significant not only onto the pure PS surface, but also onto the Pluronic-PS system. Comparison of the dilatational moduli of the interfaces demonstrates that the Pluronic F68-covered interface prevented the formation of a cohesive IgG network, altering the original structure of the IgG layer at the bare air-water interface and having a long lasting effect (20 h).

Therefore, we have shown that the presence of Pluronic F68 on a hydrophobic surface does not fully prevent IgG adsorption (although it is slightly reduced), but it does affect the protein conformation. This structural alteration might be crucial to modify subsequent steps in the complement system pathways when working with *in vivo* fluids, which in turn would prolong the circulating time of poloxamer-coated nanocarriers.

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4. Results

4.2. Physicochemical properties and interfacial characteristics of Pluronic F68 in simulated intestinal fluids.

4. Results

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Investigating the effect of surfactants on lipase interfacial behaviour in the presence of bile salts

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ABSTRACT

It is known that non-ionic surfactants and phospholipids provide large protection in emulsions against lipase-induced destabilization as compared to proteins, even in the presence of bile salts. In relation to this, the aim of this study is to probe the ability of two surfactants of industrial interest, poloxamer Pluronic F68 (non-ionic) and Epikuron 145V (phospholipid), to modify the adsorption of lipases at an oil-water interface under the physiological conditions existing in the duodenum. We have designed an experimental procedure by means of a pendant drop film balance equipped with a subphase exchange technique, which allows sequential adsorption of the compounds. This allows the investigation of the interfacial behaviour of lipase in the presence and absence of surfactant. According to this experimental approach, the lipase is added directly into the subphase only after the surfactant has been adsorbed onto the oil-water interface. We have used interfacial dilatational and shear rheology techniques to characterise the interfacial layers. The results suggest that Pluronic F68 reduces the interfacial activity of lipase more efficiently than Epikuron 145V. Furthermore, it seems that Pluronic F68 affects the accessibility of the lipase to the oil-water interface, even in the presence of the bile salts. These results may have applications in the development of novel strategies to rationally control lipid digestion in the diet.

1. Introduction

The design of food emulsions that allow controlled digestion of lipids has been a subject of interest in recent years (Dickinson, 2008; Singh, Ye, & Horne, 2009; Golding & Wooster, 2010). In some cases, such as obesity or cardiovascular diseases, it may be important to decrease the bioavailability of these lipids. During the digestion process, most lipids ingested, that are non-homogenized, are converted into oil-in-water (O/W) emulsions due to the mechanical stresses they experience and the role of different stabilizing agents. In addition, there are normally appreciable changes in the interfacial properties that may influence bioavailability of the fats (Golding & Wooster, 2010). On the one hand, there may be changes in the total area of the oil-water interface in the system, which may promote either adsorption or desorption of surface-active substances. On the other hand, many different types of surfactants, such as emulsifiers originally located at the surfaces of the lipid droplets in the food and various other surface-active substances arising from the food or the human body (free fatty acids, phospholipids, proteins…), compete for the available area and the nature of the resulting interfacial structure may influence bioavailability of the fats. Around 70-90% of fat digestion occurs in the small intestine (Fave, Coste, & Armand, 2004; Mun, Decker, & McClements, 2007). In this process, enzyme lipase has to adsorb onto the droplet surface, with the help of its cofactor co-lipase and in the presence of bile salts, before it can hydrolyse the lipids into a form that can be adsorbed by the human body (lipolysis) (Wickham, Wilde, & Fillery-Travis, 2002; Bauer, Jacob, & Monsenthin, 2005; Mun et al., 2007). Therefore, interfacial properties, like the composition of the interfacial layer surrounding the lipid droplets, will affect the rate and extent of lipid digestion (Fillery-Travis, Foster, & Robins, 1995; Mun, Decker, Park, Weiss, & McClements, 2006). Due to the apolar nature of oils and fats, the oil-water interface in emulsified fats is where eventually digestion

takes place. Therefore, bioavailability of fat could be ultimately controlled by the interfacial layer that stabilises the emulsion.

The use of fundamental interfacial measurements such as interfacial tension and interfacial rheology in combination with other techniques in O/W emulsions are necessary to investigate the molecular changes at interfaces under digestion conditions, giving additional information to this subject. In this sense, several interfacial tension studies were developed by Verger and Carrière group (Gargouri, Julien, Bois, Verger, & Sarda, 1983; de La Fournière, Ivanova, Blond, Carrière, & Verger, 1994; Labourdenne, Brass, Ivanova, Cagna, & Verger, 1997; Tiss, Carrière, & Verger, 2001), as well as by Reis et al. (Reis et al., 2008; Reis, Miller, Leser, Watzke, Fainerman, & Holmberg, 2008; Reis et al., 2008; Reis, Holmberg, Miller, Leser, Raab, & Watzke, 2009) to evaluate the measurement of fatty acid formation. Regarding interfacial rheology of adsorbed layers, it also contains vital information regarding the mechanical properties of the interfacial films (Krägel, O'Neill, Makievski, Michel, Leser, & Miller, 2003; Maldonado-Valderrama et al., 2008). In addition, neutron and X-ray scattering techniques have been used to establish the role of specific micelles (bile and surfactant micelles) on the activation of the lipase-colipase complex (van Tilbeurgh, Egloff, Martinez, Rugani, Verger, & Cambillau, 1993; Hermoso, Pignol, Penel, Roth, Chapus, & Fontecilla-Camps, 1997). The use of an inhibitor that irreversibly binds to the active site of the lipase in its lid-open conformation is also known (Borgström, 1988). Furthermore, atomic force microscopy has been used in the study of interfacial structuring (Maldonado-Valderrama et al., 2008; Chu et al., 2010), as well as electron microscopy has been used in the study of the inactivation by lipolytic products (Pafumi et al., 2002) and zeta-potential measurements to evaluate the lipolytic activity (Mun et al., 2006). It has been recently demonstrated that non-ionic surfactants and phospholipids provide larger protection in emulsions against lipase-induced destabilization in the presence of bile salts if compared to proteins (Mun et al., 2007). The goal of this study was to explore this protective

functionality in more detail. For that reason, we investigated the ability of two surfactants of industrial interest, poloxamer Pluronic F68 (non-ionic) and Epikuron 145V (phospholipid), to affect the adsorption of lipases at an oil-water interface under the physiological conditions of the duodenum. Another objective of the present study was the design of a novel experimental procedure that allows the simulation of the digestion process based on a pendant drop film balance equipped with a subphase exchange technique (Cabrerizo-Vílchez, Wege, Holgado-Terriza, & Neumann, 1999).

In this study we aim to focus on the fundamental processes underlying lipid digestion. A basic understanding of this complex phenomenon is crucial if we are to engineer food emulsions to control lipid digestion. This work provides a new insight into the interfacial processes affecting lipase activity at a fundamental level, being a useful tool in order to complement the existing techniques.

2. Materials and methods

2.1. Materials

As non-ionic surfactant we used the poloxamer Pluronic F68 from Sigma-Aldrich. It is a triblock copolymer based on poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) structure which is also typically expressed as $PEO_{75}PPO_{30}PEO_{75}$ (8350 g/mol). The central block has an hydrophobic character and hence adsorbs onto the oil-water interface, whereas the two chains of poly(ethylene oxide) remain in the aqueous phase. As phospholipid we used Epikuron 145V (around 800 g/mol), a deoiled, wax-like phosphatidylcholine (PC) enriched soybean lecithin (min. 45% PC) from Cargill Ibérica S. L. Highly refined olive oil was purchased from Sigma-Aldrich, and purified with activated magnesium silicate (Florisil, Fluka) to eliminate free fatty

acids and surface active impurities. The oil was kept under mild agitation with the resins for 3 h and centrifuged at 12000 rpm for 30 min in a bench centrifuge. It was then filtered and stored away from light.

For the duodenal juice preparation we used lipase from porcine pancreas L3126, Type II (100-400 units/mg protein, using olive oil -30 min incubation-), and bile extract porcine B8631, both of them purchased from Sigma-Aldrich, as this combination has been previously used for *in vitro* duodenal digestion models (Mun et al., 2007). The lipase sample hydrolyses tri-, di-, and monoglycerides (in decreasing order of rate) and contains amylase and protease activity as well. The composition of the bile extract has previously been analyzed: total bile salt content = 49 wt%; with 10-15% glycodeoxycholic acid, 3-9% taurodeoxycholic acid, 0.5-7% deoxycholic acid; PC 5 wt% (Zangenberg, Mullertz, Kristensen, & Hovgaard, 2001a). $CaCl_2 \cdot 2H_2O$ of analytical-grade, manufactured by Sigma-Aldrich, was also used in a final concentration of 3 mM, which is in the range of concentration found in the fasted state of the duodenum (Hofmann & Mysels, 1992; Lindahl, Ungell, Knutson, & Lennernas, 1997).

All the samples were prepared in a 2 mM Trizma Maleate (Fluka) buffer with 150 mM NaCl (Scharlau Chemie S.A.). The pH was adjusted to 6.5 with 1 M NaOH (Panreac Quimica S.A.). All chemicals used were of analytical grade. Milli-Q purified water 0.054 μS was used for buffer preparation and all other purposes. All the glassware was cleaned with Micro-90 and ethanol, and repeatedly rinsed with distilled and ultrapure water. Only freshly prepared solutions were used for each experiment. The interfacial tension of the clean oil-water interface (γ_0) was measured before every experiment to ensure the absence of surface-active contaminants obtaining values of (26 ± 0.5) mN/m at 37 ºC. All the experiments were performed at the physiological temperature of T = 37 ºC and their reproducibility was verified through at least three replicate measurements.

2.2. Interfacial Tension Set-up and Subphase Exchange Technique

The interfacial tension measurements have been performed in a Pendant Drop Film balance fully assembled and developed at the University of Granada and is described in detail elsewhere (Cabrerizo-Vílchez et al., 1999). A solution droplet is formed at the tip of a coaxial double capillary, connected independently to a double microinjector. The computer program fits experimental drop profiles, extracted from digital drop micrographs, to the Young-Laplace equation of capillarity by using Axisymmetric Drop Shape Analysis (ADSA), and provides as outputs the drop volume *V*, the interfacial tension γ, and the interfacial area *A*. The adsorption process is recorded at constant interfacial area through a modulated fuzzy logic PID algorithm (proportional, integral, and derivative control) (Wege, Holgado-Terriza, & Cabrerizo-Vílchez, 2002). The drop is immersed in a glass cuvette (Hellma), which contains the oil phase and is kept in a thermostatized cell at 37 °C. The interfacial pressure values π are obtained from the relationship $\pi \equiv \gamma_0 - \gamma$, where γ_0 is the interfacial tension of pure oil-water interface, and γ is the interfacial tension of the solution.

The interfacial pressure is first recorded for the pure systems at the bare oil-water interface. Next, the subphase exchange accessory is used in order to basically simulate the transit through the duodenum of the previously covered interface. A coaxial double capillary enables to substitute the surfactant bulk solution once a stable layer has been formed at the oil-water interface at constant interfacial area. The subphase is exchanged by extracting the surfactant solution through the outer capillary, and injecting simultaneously through the inner one. Then, the interfacial behaviour of pancreatic lipase, in the absence and presence of bile salts, can be also monitored on a previously covered interface.

2.3. Interfacial Dilatational Rheology

The dilatational rheology of the surfactant layer and the sequentially adsorbed lipase and bile salts onto the pre-adsorbed surfactant layer was measured with the pendant drop technique apparatus described above. An oscillatory perturbation was applied to the interface by injecting and extracting volume to the drop. The system records the response of the interfacial tension to the area deformation, and the dilatational modulus (E) of the interfacial layer can be inferred from this response. The applied interfacial area oscillations were maintained below 1% of amplitude to avoid excessive perturbation of the interfacial layer, as this technique requires a quasi-equilibrium drop shape for the calculation of the interfacial tension, and to avoid departure from the linear viscoelastic region. The oscillation frequency (f) was set to 0.1 Hz, which roughly corresponds to the peristaltic frequency in the intestine during the digestive processes (11.7 per minute in the duodenum) (Kellow, Borody, Phillips, Tucker, & Haddad, 1986).

In a general case, the dilatational modulus is a complex quantity that contains a real and an imaginary part:

$$E^* = E' + iE'' = \varepsilon + i2\pi f\eta \quad (\pi=3.14) \tag{1}$$

where E' is the storage modulus and accounts for the elasticity (ε) of the interfacial layer and E'' is the loss modulus and accounts for the viscosity (η) of the interfacial layer.

2.4. Interfacial Shear Rheology

Shear rheological measurements for the bare oil-water interface were carried out using an interfacial rheology system (IRS). This consists of a torsional rheometer (Physica MCR from Anton Paar) working, in this case, in strain control

mode, with an interfacial rheology cell based on bicone geometry. Temperature was maintained at 37 ºC using a peltier. To avoid evaporation and interfacial film perturbation from external agents, the cell was covered using a solvent trap during the measurements. Raw viscoelastic data obtained were then numerically analyzed to determine the interfacial moduli (G_i' and G_i''). This numerical treatment consists in a full hydrodynamic analysis of the flow field and is used to account for the influence of bulk phase contribution (Oh & Slattery, 1978).

The extent of the viscoelastic linear region was determined by means of strain amplitude sweep tests at a constant frequency of 0.1 Hz by varying the strain amplitude from φ_0 = 0.01 % to φ_0 = 100 % (results not shown here for brevity). Then, a small-amplitude oscillatory shear test was carried out to follow the interfacial film build up during the 2 hours. The strain amplitude was fixed to 0.1 % (well in the linear regime). A frequency of 0.1 Hz was chosen in order to compare the shear deformation results with those obtained for dilatational rheometry.

3. Results and discussion

We have investigated the effect of simulated duodenal digestion on interfacial layers at the olive oil-water interface. In order to discriminate the possible effect on lipase adsorption we first characterise the effect of simulated digestion on the bare olive oil-water interface. We then compare with the effect of lipase on previously covered interfaces. The duodenal digestion is simulated by solution containing 150 mM NaCl, 3 mM $CaCl_2$, pH 6.5, T 37 ºC and a mixture of lipase and bile extract at physiological concentrations. We used the concentration typically found in the human small intestine for pancreatic lipase, 1.6 mg/mL, (Wickham, Garrood, Leney, Wilson, & Fillery-Travis, 1998). The final concentration of bile extract was 1 mg/mL, which is the maximum concentration limited by the rupture of the droplet due to the fast drop in the interfacial tension.

This concentration is well above the critical micelle concentration (cmc) found by Zangenberg, Mullertz, Kristensen and Hovgaard (2001b): (0.07 ± 0.04) mM, and close to the bile salt concentrations reported during the fasted stated in the duodenum (3-7mM) (Sjövall, 1959; Hernell, Staggers, & Carey, 1990; Lindahl et al., 1997). No co-lipase was added into the system since test experiments were carried out in the presence of co-lipase (results not shown) and gave no differences as regards the interfacial behaviour (Mun et al. 2007).

3.1. Lipase and bile extract at the bare olive oil-water interface

3.1.1. Interfacial tension

Figure 1 shows the evolution of the interfacial tension of bile extract (1 mg/mL), pancreatic lipase (1.6 mg/mL) and the mixture at the bare oil-water interface under duodenal conditions. The time evolution of the interfacial pressure is indicative of the rate of adsorption while the final value of the interfacial pressure provides information about the amount of material adsorbed. Figure 1 shows that the lipase extract increases rapidly the interfacial pressure, reaching a saturation value of 17 mN/m after 30 minutes. This is due to the rapid adsorption of the enzyme combined with the adsorption of other components contained in the sample such as amylase and protease. Also, the final interface might contain hydrolysis products (Flipsen, van der Hijden, Egmond, & Verheij, 1996). In case of hydrolysis of long-chain triacylglycerols at the olive oil-water interface, the lipolytic products would remain transiently at the interface where either a reorganization process takes place and/or the lipolytic products desorb slowly into the aqueous or the oil phase (Labourdenne et al., 1997). However, this experiment alone does not allow us to elucidate the influence of the adsorption of the lipase and the effect from the adsorption of the lipolysis products at the oil-water

interface. In this sense, recent works have postulated the way to look at the mechanism involved in lipolytic reactions during lipid digestion at oil-water interfaces (Reis et al., 2008; Reis, Miller, Leser, Watzke, Fainerman, & Holmberg, 2008; Reis et al., 2008; Reis, Holmberg, Miller, Leser, Raab, & Watzke, 2009).

As observed in Figure 1, the adsorption of bile extract alone is very steep. Bile extract adsorbs onto the oil-water interface clearly faster than lipase, reaching a practically constant value of the interfacial pressure in less than 10 minutes (18 mN/m) which is slightly higher than that of lipase. Bile salts are extremely surface active molecules that reach relatively low saturation values when compared to conventional surfactants (Maldonado-Valderrama et al., 2008).

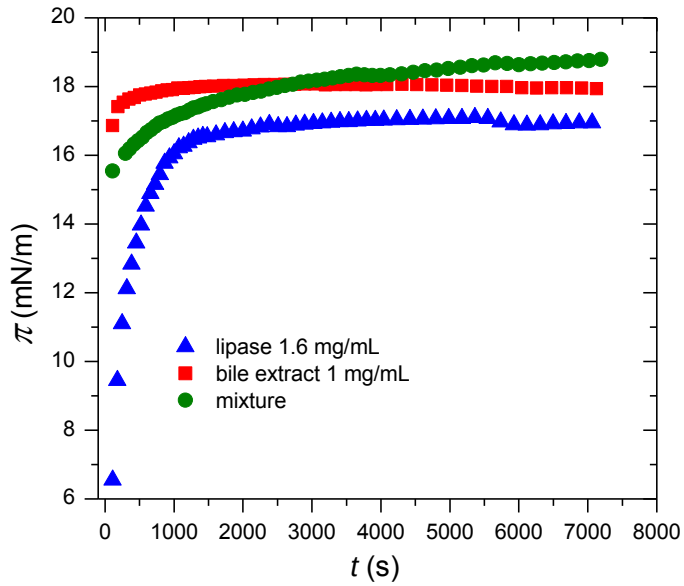

Figure 1: Variation as a function of time in the interfacial pressure at the olive oil-water interface T=37 ºC, pH 6.5, 150 mM NaCl, 3 mM $CaCl_2$: (triangles) pancreatic lipase 1.6 mg/mL, (squares) bile extract 1mg/mL, (circles) mixture. Error bars are within the size of the symbols.

Finally, Figure 1 also shows the behaviour of the mixed system composed by pancreatic lipase/bile extract at the olive oil-water interface. The evolution of the interfacial pressure of the mixture is complex. At short times the curve lies between that of bile extract and lipase alone whereas the final interfacial pressure of the mixture overcomes that of the individual systems. This suggests that, at short times, the bile extract occupies very quickly the interface and the lipase adsorbs in a competitive way, having a smaller interfacial area available. At longer times, the final interfacial pressure is higher than that obtained for each of the individual components. Furthermore, it does not seem to reach a saturation value but continuous to increase after two hours. This fact could be due to some degree of unfolding of lipase as well as the formation of lipolytic reaction products at the interface due to an enhancement of the hydrolysis of the oil owing to the presence of bile extract.

3.1.2. Interfacial dilatational rheology

We have monitored the behaviour of pancreatic lipase and bile extract at the bare oil-water interface by recording the interfacial dilatational storage and loss moduli as a function of time (2 h) during the interfacial tension measurements performed in the previous section. In this way, the dilatational rheology for individual components, as well as for the mixture, is represented in Figure 2. As a general feature all of the systems presented a predominantly elastic behaviour, since at this relatively high frequency (0.1 Hz) the viscous component of the dilatational modulus was very small.

Starting our analysis for the lipase, it is interesting to note the initial maximum reached for both, the interfacial storage (E') and the loss (E'') moduli, in the first stages of the adsorption process (17 min). This behaviour is not normally found in interfacial layers of proteins and surfactants. Nevertheless, as it is observed in Figure 2, the dilatational elasticity $E´$ dramatically decreased after

reaching a maximum and then kept constant up to 2 hours. This maximum coincides with the onset of the saturation of the interface given by the plateau in the interfacial pressure in Figure 1. Accordingly, the drop in the dilatational elasticity might indicate the appearance of hydrolysis products at the interface, reducing the intermolecular interactions between lipase molecules and other components in the lipase extract, as well as the interfacial restructuring of the hydrolysis products. In addition, the low interfacial viscoelasticity corresponding to the assumed lipase extract adsorption (the elastic modulus is around 15 mN/m) indicates that the molecules of the components present in the lipase extract most likely do not unfold significantly at the interface in agreement with (Reis, Holmberg, Watzke, Leser, & Miller, 2009). Despite the high interfacial activity of lipase extract, its little

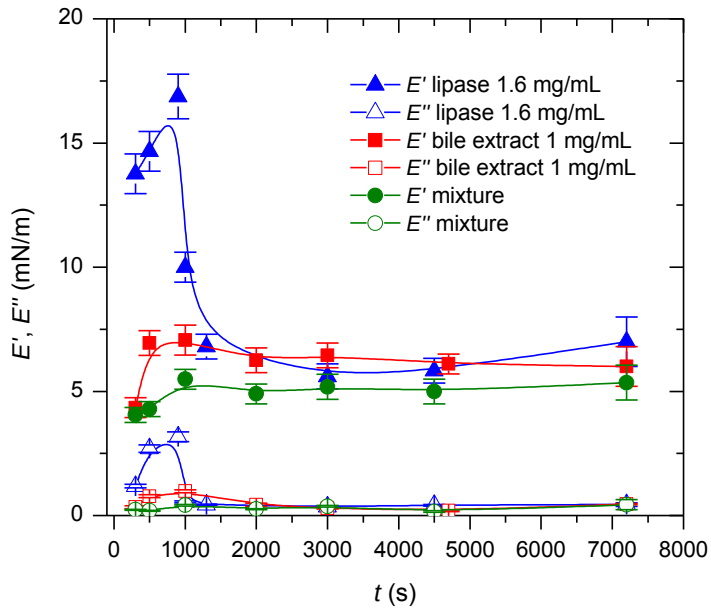

Figure 2: Dilatational storage (*E'*) and loss (*E''*) moduli as a function of time; closed symbol, *E´*; open symbol, *E´´*; (triangles) pancreatic lipase 1.6 mg/mL, (squares) bile extract 1mg/mL, (circles) mixture. Lines are plotted as a guide for the eye. Measurements are made at the olive oil-water interface T=37 ºC, pH 6.5, 150 mM NaCl, 3 mM $CaCl_2$ and frequency 0.1Hz.

unfolding at the interface would allow a better protein adaptation to changes at the interface during lipolysis, as previously reported by Haiker, Lengsfeld, Hadváry and Carrière (2004).

Regarding the dilatational response of bile extract, the storage modulus increased slightly during initial adsorption, and remained constant after the saturation of the interface reaching a value of 7.5 mN/m (see Figure 2). The dilatational elastic modulus of bile salts at this oscillation frequency is very low due to the extremely fast adsorption rates of these compounds as reported by Maldonado-Valderrama et al. (2008) under similar conditions. However, the minor dilatational response shown in Figure 2 is possibly due to the presence of phospholipids and other surface active impurities in the bile extract.

Finally, the dilatational response of the mixture shows a very similar behaviour to the bile extract alone. This suggests that the adsorption is controlled by the bile extract in agreement with Figure 1. Nevertheless, the dilatational storage modulus appears to be even smaller than those moduli reported for individual components at long times. This fact might be due to an enhanced presence of lipolysis products in the case of the mixture as compared with lipase alone probably owing to a larger hydrolysis of the oil in the presence of bile extract. This fact is in agreement with the results of the interfacial tension measurements presented in Figure 1.

3.1.3. Interfacial shear rheology

We have also measured the interfacial shear rheology of bile extract (1 mg/mL), pancreatic lipase (1.6 mg/mL) and the mixture at the bare oil-water interface under duodenal conditions. We have used the same concentrations as in the experiments with the pendant drop technique and the adsorption process was also recorded for 2 hours. The interfacial shear storage (G'_i) and loss (G''_i) moduli

versus time are shown in Figure 3. The response to the oscillatory shear deformation was also predominantly elastic at the same frequency as in dilatational rheology (0.1 Hz). Interestingly, a maximum is found in the rheological parameters for the lipase extract. This is an exceptional feature which importantly corroborates the dilatational response. Again, we can infer from this curve that lipase extract (including amylase and protease) adsorbs onto the oil-water interface forming an interfacial network which is disrupted as hydrolysis products appear. These products could reduce the intermolecular interactions between lipase, amylase and protease molecules in the lipase extract that have to adapt to changes at the

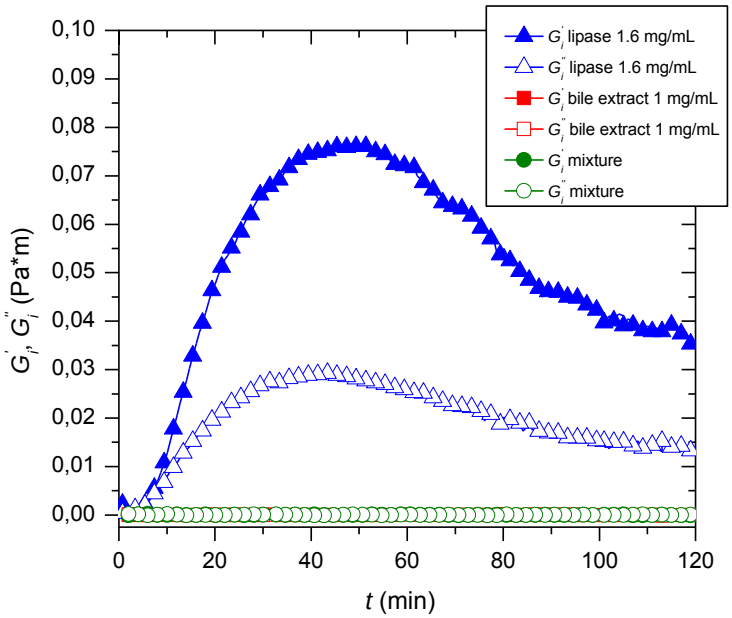

Figure 3: Small-amplitude oscillatory shear time sweep tests at a constant frequency of f = 0.1 Hz and a constant strain of φ = 0.1 %; closed symbol, G_i'; open symbol, G_i''; (triangles) pancreatic lipase 1.6 mg/mL, (squares) bile extract 1mg/mL, (circles) mixture. Measurements are made at the olive oil-water interface T=37 ºC, pH 6.5, 150 mM NaCl, 3 mM $CaCl_2$.

interface during the possible lipolysis, accounting for low interfacial shear viscoelasticity.

Bile extract provided negligible values for the interfacial shear moduli in agreement with the liquid-like interfacial layers created by these types of surfactants as reported in the literature (Georgieva, Schmitt, Leal-Calderon, & Langevin, 2009). Mixture of lipase and bile extract provided a similar negligible shear response. These results agree with the fact that the adsorption is initially controlled by the bile extract and the interface is possibly populated with bile salt and phospholipids molecules in the bile extract, as well as lipolysis products behaving as surfactants at the interface. Further work should be done in order to fully determine the composition of the interface including chemical analysis of the lipolytic reaction products.

3.2. Lipase and bile extract at surfactant-covered interfaces

3.2.1. Interfacial tension

In this section, we consider the effect of two types of surfactants, which have been previously adsorbed onto the oil-water interface, on the interfacial properties of lipase behaviour in the absence and presence of bile salts. Namely, Pluronic F68 (non-ionic) and Epikuron 145V (phospholipid) were studied.

The experimental procedure was designed as follows: firstly, the surfactant is adsorbed at a constant interfacial area (30 mm^2) for 30 min, and then the subphase is exchanged by substituting the remaining surfactant solution by that of lipase, bile extract or the mixture, in all cases at the same concentrations used in section 3.1 for the bare oil-water interface. Accordingly, the interfacial behaviour of pancreatic lipase and bile extract can be monitored by recording the change of

interfacial pressure as a function of time, after the sequential adsorption of the duodenal juice (or individual components) onto the pre-adsorbed surfactant layer.

3.2.1.1. Effect of Pluronic F68 covered interface on interfacial behaviour of lipase and bile extract

The experiments of sequential adsorption of the duodenal juice (mixture) and individual components onto the pre-adsorbed interfacial layer of Pluronic F68 at the concentration of 10 mg/mL, are plotted in Figure 4.

Figure 4 shows first the adsorption of Pluronic F68 (10 mg/ml). After 30 minutes the subphase is exchanged by buffer, as a reference, showing that the interfacial layer is stable at the interface at an interfacial pressure of 14.5 mN/m. This process is repeated but the surfactant bulk solution was exchanged by that of lipase (1.6 mg/mL), bile extract (1mg/mL) or the mixture. This experiment mimics the interfacial processes undergone by a surfactant stabilised emulsion as it passes through the duodenum. Exchange by bile extract severely disrupted the poloxamer interfacial layer increasing the interfacial pressure up to the value reached by the bile extract at the bare oil-water interface (18 mN/m). This suggests that the bile salts penetrate into and displace the Pluronic F68 from the oil-water interface. Differently, exchange by lipase extract had little effect on the interfacial pressure, as the dynamic curve of the interfacial pressure collapsed with that of the exchange with buffer (14.5 mN/m). This suggests that the Pluronic F68 inhibits the adsorption of lipase that should reach a value of 17 mN/m at the bare oil-water interface (c.f. Figure 1). Furthermore, this inhibition of the surface activity occurs even in the presence of bile extract (Figure 4). Exchange by the mixture lipase/bile extract neither achieved the value attained at the bare oil-water interface (19 mN/m). In fact, the increase in the interfacial pressure might be caused by the bile salt molecules and phospholipids in the bile extract, rather than the lipase itself, as we observed in their individual behaviour onto the surfactant layer. At any rate, it is

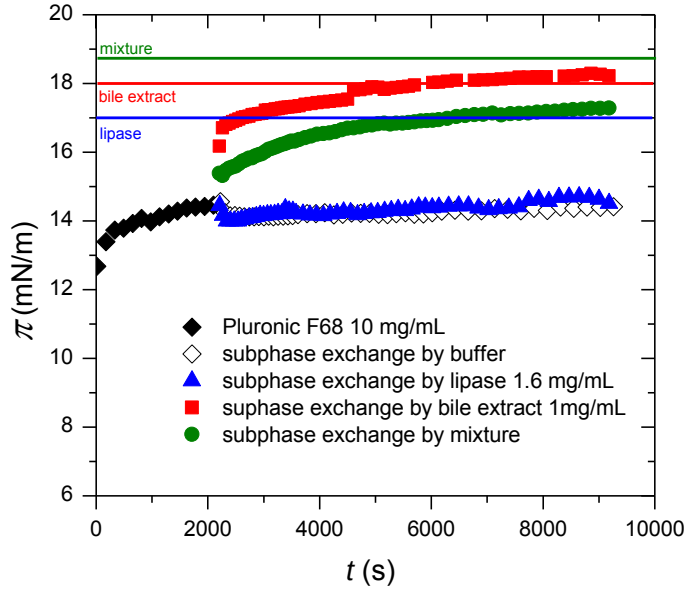

Figure 4: Variation as a function of time in the interfacial pressure at the olive oil-water interface and at constant interfacial area (30 mm^2), T=37 ºC, pH 6.5, 150 mM NaCl, 3 mM $CaCl_2$: (diamonds) Pluronic F68 10 mg/mL, (open diamonds) subphase exchange by buffer, (triangles) subphase exchange by pancreatic lipase 1.6 mg/mL, (squares) subphase exchange by bile extract 1mg/mL, (circles) subphase exchange by mixture. Lines represent the final interfacial pressure reached by lipase, bile extract and mixture at the bare oil-water interface.

clear that using this surfactant concentration, the accessibility of lipase onto the oil-water interface is importantly hindered (Figure 4). It is thought that the poloxamer PEO chains, which remain in the aqueous phase, form a steric bulky layer with thicknesses of several nanometers, preventing the adsorption and penetration of the protein into the interface, similarly as this mechanism stabilises the O/W emulsions avoiding direct contact between droplets and hence coalescence (Grigoriev & Miller, 2009; Wulff-Pérez, Torcello-Gómez, Gálvez-Ruiz, & Martín-Rodríguez, 2009). Apart from the steric hindrance, the inhibition of lipase action could be due

to direct binding of the lipase to the poloxamer. This feature should be studied in more detail and could be potentially used in the control of the lipolysis in future experiments in O/W emulsions.

3.2.1.2. Effect of Epikuron 145V covered interface on interfacial behaviour of lipase and bile extract

Consider now the effect of surfactant Epikuron 145V on the interfacial behaviour of lipase in presence and absence of bile salts. We have used a phospholipid concentration of 0.1 mg/mL which led to similar interfacial pressures to those attained by the poloxamer concentration used in this work. Figure 5 shows the time evolution of the surface pressure after the subphase exchange by each of the duodenal components, once the Epikuron 145V was adsorbed for 30 min under the same conditions carried out for Pluronic F68. It is evident the greater interfacial activity of phospholipids in comparison to that of the poloxamer, since a phospholipid concentration one order of magnitude lower than that used for poloxamer led to similar interfacial pressure values (13 mN/m). Due to their amphiphatic character, phospholipids have a hydrophilic-lipophilic balance (HLB) value much smaller -Lecithins have HLB numbers of 8-14 depending on their exact composition (Malik, Washington, & Purewal, 1999)- than that for poloxamer -29 in the case of Pluronic F68 (Kabanov, Batrakova, & Alakhov, 2002)-; and hence, Epikuron 145V is more surface-active than Pluronic F68. Figure 5 shows that the subphase exchange with buffer provided a stable interfacial layer reaching a final interfacial pressure of 13 mN/m. Exchange with lipase extract caused an abrupt drop followed by a continuous rise in the interfacial pressure. This result might suggest that the lipase extract has displaced and removed Epikuron 145V from the interface, given that the interfacial pressure approached the value obtained by the

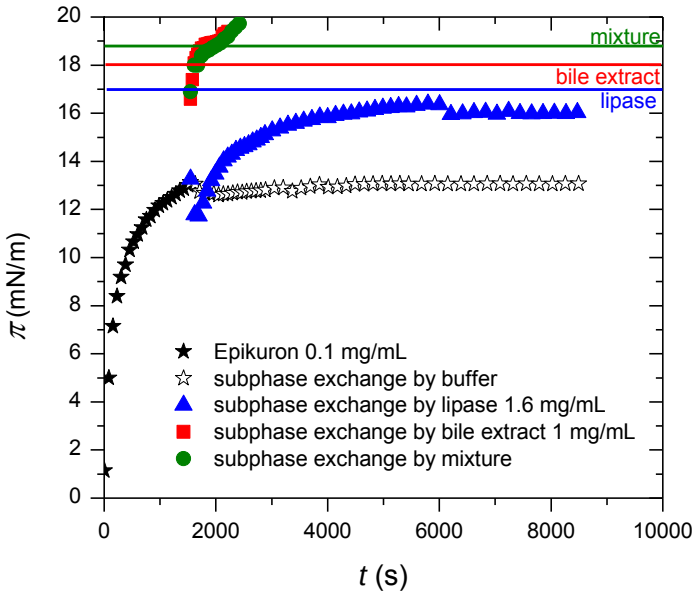

Figure 5: Variation as a function of time in the interfacial pressure at the olive oil-water interface and at constant interfacial area (30 mm^2), T=37 ºC, pH 6.5, 150 mM NaCl, 3 mM $CaCl_2$: (stars) Epikuron 145V 0.1 mg/mL, (open stars) subphase exchange by buffer, (triangles) subphase exchange by pancreatic lipase 1.6 mg/mL, (squares) subphase exchange by bile extract 1mg/mL, (circles) subphase exchange by mixture. Lines represent the final interfacial pressure reached by lipase, bile extract and mixture at the bare oil-water interface.

lipase extract at the bare oil-water interface (16 mN/m). It might be also possible the presence of phospholipase-A2 in the lipase extract that could hydrolyse the phospholipids. Exchange by bile extract and mixture had a dramatic effect on the phospholipids covered interface. After the subphase exchange both systems rapidly increased the interfacial pressure, exceeding the values attained by the bile extract and mixture at the bare oil-water interface and yielding to the droplet fall in both cases. This feature suggests a rapid penetration of the bile extract and the mixture into the interface, and in the case of the mixture, the hydrolysis of the Epikuron

145V by the phospholipase-A2 in case it is present in the sample. A closer look at the time scale shows that, probably due to hydrolysis products, the mixture takes about 4 min more to fall the droplet. Accordingly, despite its greater surface-activity compared with Pluronic F68, Epikuron 145V does not seem to provide any protection against adsorption of lipase and bile extract at the oil-water interface. These results are in agreement with previous studies that have shown that lipase can adsorb to oil-water interfaces in both the absence and presence of bile salts (Gargouri et al., 1983; Gargouri, Pieroni, Lowe, Sarda, & Verger, 1986; Mun et al., 2007). Indeed, the addition of the bile extract (mixture) lead to an interface initially comprised of surfactants has been shown to promote the lipase adsorption (Gargouri et al., 1983; Mun et al., 2007), even for Pluronic F68 that inhibited adsorption of the lipase extract in the absence of bile salts. The fact that the non-ionic surfactant hindered the access of pancreatic lipase to the interface to a larger extent when compared with phospholipids agrees with recent works carried out in emulsions that showed the impact of surfactant type on the digestion of emulsions (Mun et al., 2007; Hur, Decker, & McClements, 2009).

3.2.2. Interfacial dilatational rheology

The characteristics of the adsorbed layers formed in the experiments of previous section can be further investigated by means of dilatational viscoelasticity which in fact, describes the response of the interfacial tension to dilatational deformations acting on the interface. Unfortunately it was not possible to characterize the shear rheological properties of the interfaces by means of the interfacial rheometer equipped with the biconical bob device, because of the extremely low values measured for surfactants (data not shown), as we mentioned before in the case of pure bile extract (Georgieva et al., 2009). In this way, as it was done in the interfacial dilatational rheology of duodenal juice components at the bare oil-water interface, we present the dilatational moduli for all the systems

studied in the previous section at a fixed frequency of 0.1 Hz. Due to the fact that at this frequency, all the systems including the surfactants show a predominantly elastic behaviour and the viscous component is very small, the interfacial dilatational modulus *E* (equation (1)) is reported for brevity, rather than the storage *E'* and loss *E''* modulus. In addition, given that at the bare oil-water interface the dilatational parameters of duodenal juice remained constant at the end of the process, we report the values of the *E* before and after 2 hours of lipase adsorption in the absence and presence of bile salts onto the interface previously covered with the surfactant (Table 1).

Table 1 shows the interfacial dilatational modulus for the sequential adsorption of duodenal juice and individual components onto the pre-adsorbed poloxamer layer. The dilatational response of Pluronic F68 is also measured after 30 min of adsorption and just before carrying out the subphase exchange, in order to analyse the evolution of the adsorbed layer with time. Literature values of the elastic modulus of Pluronic are slightly higher (around 8.5 mN/m) for this concentration of poloxamer at an oil-water interface, at the same frequency of 0.1 Hz and at room temperature (Georgieva et al., 2009). This slight difference is probably due to the use of body temperature that might result in a more fluid, less compact interfacial film (Burgess, & Sahin, 1997).

The dilatational moduli measured after the subphase exchange with each of the duodenal components were higher than those of pure poloxamer and individual components of the duodenal juice at the bare oil-water interface. This fact agrees with the interfacial measurements reported in Figure 4. Moreover it strongly suggests that although bile extract and mixture penetrated the interface, the poloxamer was not completely displaced and might have formed complexes at the interface that would have increased the rheological properties. In the case of the lipase, the increase of the rheological properties might be due to the co-adsorption

Table 1: Interfacial dilatational modulus measured before and after the subphase exchange (2 hours) by duodenal juice components at a Pluronic F68 and an Epikuron 145V covered interfaces. Interfacial dilatational modulus of lipase, bile extract and mixture at the bare olive oil-water interface (2 hours) are shown as a reference. (T=37 ºC, pH 6.5, 150 mM NaCl, 3 mM $CaCl_2$).

***E* surfactant interfacial coverage** (mN/m)	***E* lipase addition** (mN/m)	***E* bile extract addition** (mN/m)	***E* mixture addition** (mN/m)
10 mg/mL Pluronic F68 7.2 ± 0.3	11.5 ± 0.5	8.7 ± 0.7	9.0 ± 0.7
0.1 mg/mL Epikuron 145V 24 ± 4	8.6 ± 0.5	-	-
Bare O/W interface	**7 ± 1**	**6.0 ± 0.7**	**5.4 ± 0.7**

of lipase and other components in the lipase extract onto the Pluronic layer, forming some type of complexes which we could not infer by interfacial tension measurements. The interfacial pressure after the subphase exchange might only suggest that the poloxamer prevented the access of the lipase extract to the interface. Therefore, the results obtained by these techniques (interfacial tension and dilatational rheology) suggest that Pluronic F68 could reduce the accessibility of pancreatic lipase to the interface even in the presence of bile extract.

Table 1 also displays the dilatational modulus obtained for pure phospholipids at the concentration of 0.1 mg/mL, in order to compare with the effect of the poloxamer. In this sense, the phospholipids gave rise to a dilatational modulus being around 24 mN/m. This numerical value was higher than that obtained for poloxamer. This reflects the greater surface activity of Epikuron 145V if compared to that of Pluronic F68, which gives rise to a higher degree of accumulation at the oil-water interface. Phospholipids form elastic networks of

interacting adsorbed molecules at the interface, leading to a closed-packed layer, due to the double tail anchoring group of the phospholipids (de Vleeschauwer & Van der Meeren, 1999). However, despite this higher strength of phospholipids film, the low dilatational modulus of the interfacial layer after adding the lipase extract into the bulk (8.6 mN/m) suggested that the Epikuron 145V adsorbed layer was dramatically disrupted by the lipase extract. In addition, this value coincides with that of the lipase film (within the margin of error) at the bare oil-water interface. These results are in agreement with the interfacial tension data (Figure 5). It seems possible that the lipase extract accessed, and/or even hydrolysed, the Epikuron covered interface reducing the dilatational properties and increasing the interfacial pressure. In the case of adding the bile extract and the mixture, the droplet fell and we were not able to measure the dilatational rheology. Nevertheless, this fact was further evidence that bile extract and mixture rapidly penetrated the interface, increasing the interfacial pressure. From these results we can safely conclude that Epikuron 145 V did not reduce the accessibility of pancreatic lipase to the interface, either in the absence or presence or bile salts. It is clear that the type of structure formed by different nature surfactants onto the oil-water interface will differently affect the access of the pancreatic lipase to the interface.

4. Conclusions

We have used fundamental interfacial techniques, such as interfacial tension and interfacial rheology, to investigate the molecular changes occurring during adsorption of pancreatic lipase and bile extract at the bare oil-water interface and at the surfactant-covered interfaces, under duodenal conditions. We have then evaluated the effect of two different surfactants: Pluronic F68 (non-ionic) and Epikuron 145V (phospholipids), on the adsorption of duodenal components. The goal was to evaluate the possible effect of previously adsorbed surfactants on the

interfacial behaviour of lipase with controlling lipid hydrolysis. The lipase extract in the absence of the bile extract adsorbed very fast onto the bare oil-water interface. Both, the dilatational and the shear response of lipase covered interfaces show that a cohesive network is formed in the first instants, which is then possibly disrupted by the appearance of the hydrolysis products at the oil-water interface. These products probably reduce the intermolecular interactions between lipase molecules and other components in the lipase extract, thus decreasing the dilatational and shear viscoelasticity. Finally, the interface would be saturated by a mixture of lipase and lipolysis products. Interfacial tension techniques showed that the presence of bile extract at the interface affects the initial adsorption of the lipase that eventually reaches the interface undergoing complex molecular rearrangements, while the dilatational and shear rheology was mainly controlled by the mobility of bile extract. Regarding the effect of surfactant on lipase interfacial behaviour, both interfacial tension and interfacial dilatational rheology suggest that Pluronic F68 reduces, to a larger extent than Epikuron 145V, the accessibility of lipase to the oil-water interface, even in the presence of bile extract. Accordingly, the type of structure formed by the surfactants onto the oil-water interface might affect the rate of lipase adsorption. Further research work will be carried out in O/W emulsions to perform the lipolysis on the same systems and correlate the results obtained with our findings in interfacial measurements. In this sense, Pluronic F68 could be potentially used to control lipolysis. Moreover, the addition of the bile extract to an interface initially comprised of surfactants seems to promote the lipase adsorption, even for Pluronic F68 that inhibited adsorption of the lipase extract in the absence of bile salts.

The combination of interfacial tension and interfacial rheology provides new insight towards the interfacial process of lipase and bile salts under duodenal conditions. This study reveals the usefulness of surface tension techniques to approach physiological issues at a molecular scale and provides additional

information in the applicability of these systems to engineer food emulsions to rationally control lipid digestion.

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4. Results

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PAPER

Physicochemical properties and digestibility of emulsified lipids in simulated intestinal fluids: influence of interfacial characteristics

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ABSTRACT

An improved understanding of the behaviour of lipids within the gastrointestinal tract will facilitate the structural design of foods that provide specific physiological responses. In this work, we studied the influence of interfacial characteristics (emulsifier type and surface area) on the behaviour of emulsified lipids within a simulated small intestine (duodenum). Pluronic F68-stabilized emulsions were more resistant to lipid digestion than lecithin-stabilized emulsions. Interfacial tension, droplet charge (ζ-potential), and microstructure measurements were used to identify the physicochemical origin of this effect. Pluronic F68 was shown to be more difficult than lecithin to displace from lipid droplet surfaces by bile salts and lipase arising from the duodenal juice. Specifically, duodenal components decreased the interfacial tension of a Pluronic-covered interface to a lesser extent, as compared to the interface covered by lecithin. These results demonstrate that the properties of the interfacial layer surrounding lipid droplets can be designed to modulate the lipid digestion process. This knowledge is critical for the rational development of delivery systems for food and pharmaceutical applications that can control the uptake of lipids and lipid-soluble components under physiological conditions.

1. Introduction

The ability to rationally control lipid digestion profiles within the gastrointestinal (GI) tract would facilitate the development of functional foods with tailored biological activities, *e.g.*, increased bioavailability, controlled release, or targeted release.[1-4] The successful development of these functional food materials relies on the application of the structural design principles of soft matter physics.[5] The lipids in many processed foods are present as emulsions, which can be end products in themselves or part of a more complex food system. A number of recent review articles have shown that emulsion structure and stability can affect lipid digestion and absorption.[3,4,6,7] In particular, studies have shown that the initial properties of oil-in-water (O/W) emulsions can alter the rate and extent of lipid digestion.[8-14]

Lipid digestion is affected by emulsion structure and composition because it is largely an interfacial reaction: lipase must adsorb to the lipid droplet surfaces and come into close proximity to the lipid substrate (usually triacylglycerols) before any digestion can occur. Hence, it should be possible to tailor the lipid digestion process by controlling interfacial characteristics, such as interfacial tension, thickness, rheology, composition, and cross-linking. Some studies have shown that initial emulsifier type does impact the behaviour of lipid droplets within simulated GI conditions,[15-17] whereas other studies have noted only a limited effect.[18] Despite the crucial role played by interfaces in lipid digestion, there are still relatively few studies that have specifically related interfacial characteristics to the rate and extent of lipid digestion. At present, details about the specific interaction between digestion compounds and emulsifiers at the interface are still unclear.[19] Maldonado-Valderrama and co-workers recently studied some interfacial aspects of the digestion process.[20-22] Chu *et al.*[15,16] found that highly surface active surfactants (galactolipids) could retard the rate and extent of lipid digestion by preventing

other surface active components within the GI tract (*e.g.*, bile and lipase) from adsorbing to the lipid droplet surfaces.

Even if one controls the initial interfacial properties of lipid droplets, it should be stressed that these properties may change appreciably as they pass through the gastrointestinal tract due to their interactions with biological fluids and their exposure to complex flow profiles and mechanical forces.[4,23] The droplet size distribution may change due to droplet disruption, coalescence, and/or digestion processes. The interfacial composition may change due to competitive adsorption, co-adsorption, or digestion processes.[20-23] The rational design and production of emulsion-based delivery systems to control the release of bioactive components in the human GI tract therefore depends on developing a better understanding of these processes in well-defined emulsion systems.[17]

The purpose of the current study was to examine the behaviour of O/W emulsions within an *in vitro* digestion model, and to correlate their interfacial, physicochemical, and structural properties to the lipid digestion process. The influence of initial emulsifier type was examined by comparing the behaviour of lipid droplets coated with a non-ionic synthetic surfactant (Pluronic F68) with those coated by a zwitterionic biological surfactant (lecithin). Pluronic F68 is a non-ionic triblock copolymer capable of forming a bulky adsorbed layer that prevents droplet coalescence through steric repulsion.[24] This surfactant has been approved by the United States Food and Drug Administration for oral intake. In previous studies, we have shown that this surfactant can stabilize O/W emulsions,[25] and that Pluronic-coated droplets have a higher resistance to destabilization by bile salts than phospholipid-coated droplets.[26] In addition, studies at oil-water interfaces showed that Pluronic F68 was more effective at inhibiting the adsorption of lipase than phospholipids, in both the absence and presence of bile salts.[27] One aim of the current study was therefore to build on this knowledge so that we could relate the

rate and extent of lipid digestion to the interfacial properties of a well-characterized system.

A mechanistic understanding of the influence of emulsifier type on the lipid digestion process was obtained by examining the effects of individual components within the simulated duodenal juice (*i.e.*, bile salts and lipase) on interfacial properties, physical stability, emulsion microstructure, and lipid digestion. We also studied the impact of initial droplet size on lipid digestion, since the rate of lipid digestion (fatty acids released per minute) increases with decreasing droplet size (increasing droplet surface area).[8,9,28,29] Accordingly, in a final section, the behaviour of emulsions with higher initial droplet sizes was also tested, since literature works suggested that lipid droplets would always be relatively large in the small intestine.[10,12,30]

In summary, a major innovation of this work is the correlation found between microemulsion structure, lipolysis and interfacial tension measurements that helps to understand the physical chemistry underlying lipid digestion. The information generated by this study should facilitate the rational design of colloidal delivery systems to control the rate and extent of lipid digestion within the gastrointestinal tract. These delivery systems could be incorporated into foods that are designed to combat chronic human diseases, such as obesity, cancer, diabetes, hypertension and heart disease. For example, they could be used to increase the bioavailability of highly lipophilic bioactive components, or they could be used to deliver bioactive components to specific regions in the GI tract (such as the colon). As this knowledge improves, rational strategies can be developed to improve the biological impact of foods and pharmaceuticals. A better knowledge of lipase structure and mode of action will help the development of new products. These strategies will need to be validated *in-vivo*,[31] hence colloid scientists working in this field will be working increasingly in partnership with biologists, physiologists

and clinicians to understand precisely how these processes can directly impact on human health.

2. Materials and methods

2.1. Materials

A non-ionic synthetic surfactant (Pluronic F68) was purchased from Sigma-Aldrich (St Louis, MO). Pluronic F68 is a triblock copolymer based on a poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) structure, which can be expressed as $PEO_{75}PPO_{30}PEO_{75}$. The central PPO block has a hydrophobic character so it adsorbs to lipid droplet surfaces, whereas the two lateral PEO blocks have a hydrophilic character and therefore tend to remain in the aqueous phase. A zwitterionic biological surfactant (soybean lecithin) was also obtained from Sigma-Aldrich. This surfactant (Asolectin) comprises roughly equal proportions of lecithin, cephalin and phosphatidylinositol along with minor amounts of other phospholipids and polar lipids (~25% phosphatidylcholine (PC)). Highly refined olive oil was obtained from Sigma-Aldrich, and purified with activated magnesium silicate (Florisil, Fluka) to eliminate free fatty acids prior to utilization following the protocol described previously.[20]

For the duodenal juice preparation we used lipase from porcine pancreas (L3126), Type II (100-400 units/mg protein, using olive oil with 30 min incubation), and porcine bile extract (B8631), both of them purchased from Sigma-Aldrich. The lipase sample hydrolyses tri-, di-, and monoglycerides (in decreasing order of rate) and contains amylase and protease activity. The composition of the bile extract has been previously analyzed: total bile salt content = 49 wt%; with 10-15% glycodeoxycholic acid, 3-9% taurodeoxycholic acid, 0.5-7% deoxycholic

acid; PC 5 w%.[32] $CaCl_2 \cdot 2H_2O$ (Sigma-Aldrich) of analytical-grade was also used in the preparation of the duodenal juice.

All the samples were prepared in a 2 mM Trizma Maleate (Fluka) buffer with 150 mM NaCl (Sigma-Aldrich). The pH was adjusted to 7.0 with 1 M NaOH (Fisher Scientific Chemical Company). All chemicals used were of analytical grade. Double distilled and deionized water was used for buffer preparation and for all other purposes.

2.2. Interfacial tension

The interfacial tension study was carried out using a pendant drop film balance developed at the University of Granada and described in detail elsewhere.[33] A computer program fitted experimental drop profiles, extracted from digital drop micrographs, to the Young-Laplace equation of capillarity using Axisymmetric Drop Shape Analysis (ADSA), and provides as outputs the drop volume *V*, the interfacial tension γ, and the interfacial area *A*. This set-up is also equipped with a subphase exchange device which allows non-invasive penetration studies. The drop is immersed in a glass cuvette (Hellma), which contains the oil phase and is kept in a temperature controlled cell at 37 °C. The interfacial tension was first recorded for the pure systems (Pluronic F68 and lecithin) at the bare oil-water interface. This simulates the control emulsion (blank). Next, pancreatic lipase, bile extract or their mixture was added into the subphase using a subphase exchange accessory. Information about changes in interfacial composition were obtained by measuring the evolution of the interfacial tension under conditions simulating the duodenum. The duodenal conditions of the aqueous media (T=37 ºC, pH 6.5, 150 mM NaCl, 3 mM $CaCl_2$), as well as the concentration of the pancreatic lipase and bile extract were chosen similar to that used in the emulsion hydrolysis experiments (see below), taking into account the specifications for the pendant drop technique. In this sense, the bile extract concentration (1 mg/mL) was limited by the droplet fall

due to the rapid decrease of the interfacial tension. The concentrations of the surfactants (10 mg/mL Pluronic F68 and 0.1 mg/mL lecithin) were chosen to lead similar interfacial tension values, in order to compare their susceptibility to the simulated duodenal juice.

2.3. Emulsion preparation

The purpose of the emulsion preparation procedure was to produce lipid droplets coated with different emulsifiers that were large enough to observe with optical microscopy, and within the size range found in many food products (d = 1-100 μm). An aqueous emulsifier solution was prepared by dissolving emulsifier into buffer solution and stirring for 2 hours. The final emulsifier concentration was 1.03 wt% (10.3 mg/mL) in buffer. A pre-emulsion was prepared by homogenizing 1.648 mL of purified olive oil and 48.352 mL of emulsifier solution (50 mL final volume), in a high-speed blender (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland) for 2 minutes. Therefore, the final composition of the emulsion was 3 wt% olive oil, 1 wt% Pluronic F68 or lecithin (taking into account the oil volume). Some of the coarse emulsions were then passed five times through a membrane homogenizer (pore diameter = 4 μm) at 2 psi (external pressure type micro kit from SPG Technology Co., LTD in Japan) to reduce the mean droplet diameter to around 4 μm so as to evaluate the effect of droplet size in the final section of our study: hydrolysis of emulsions.

2.4. Pancreatic lipase-catalyzed hydrolysis of emulsions

The release of free fatty acids from emulsified lipids due to pancreatic lipase activity was monitored using a titration method that was a modification of that described previously.[13,32] A 30 mL sample of emulsion (pH = 7.0) was transferred into a clean glass beaker that was placed in a water bath at 37.0 ºC for

10 min. Then 1 mL of $CaCl_2$ solution and 4 mL of bile extract solution (110 mg $CaCl_2$ and 187.5 mg bile extract dissolved in buffer solution, pH 7, 37 ºC) were added to the emulsion under stirring (speed 4) and then the system was adjusted back to pH 7 if required. Next, 2.5 mL of freshly prepared lipase suspension (60 mg lipase powder dispersed in buffer solution, pH 7, 37 ºC) was added to the above mixture. The final concentration of pancreatic lipase was 1.6 mg/mL to simulate that typically found in the human small intestine.[34] The final concentration of bile extract was 5 mg/mL, which is well above the critical micelle concentration (cmc) found by Zangenberg *et al.*[35]: (0.07 ± 0.04) mM, and close to the BS concentrations reported during the fasted stated in the duodenum (3-7mM).[36-38] $CaCl_2$ had a final concentration of 20 mM. For the control experiments we added 7.5 mL of buffer to keep the same final volume of the test sample (37.5 mL). Finally, the lipolysis was measured by a pH-stat automatic titration unit (Metrohm, USA Inc.) by titrating appropriate amounts of NaOH solution (1.5 M) to maintain the pH at 7.0. The concentration of free fatty acids (FFAs) generated by lipolysis was calculated from the volume of NaOH added. The percentage of FFAs released by the lipolysis was calculated from the number of moles of NaOH required to neutralize the FFA divided by the number of moles of FFA that could be produced from the triacylglycerols if they were all digested (assuming 2 FFA produced per 1 triacylglycerol molecule):

$$\%FFA = 100 \times \left(\frac{V_{NaOH} \times m_{NaOH} \times M_{Lipid}}{w_{Lipid} \times 2} \right) \quad (1)$$

Here V_{NaOH} is the volume of sodium hydroxide required to neutralize the FFA produce (in mL), m_{NaOH} is the molarity of the sodium hydroxide solution used (in M), w_{Lipid} is the total weight of oil initially present in the reaction vessel (0.9 g), and M_{Lipid} is the molecular weight of the oil (assumed to be 800 g/mol).

The percentage of total free fatty acids released (Φ) as a function of time (t) measured by the pH-stat method can be characterized by the following equation[29]:

$$\Phi = \phi_{\max}\left(1-\left(1+\frac{3kMt}{2d_0\rho_0}\right)^{-2}\right) \quad (2)$$

Here, ϕ_{max} provides a measure of the extent of digestion (*i.e.,* the maximum percentage of the total FFA present that is released at the end of the reaction), k provides a measure of the rate of digestion (*i.e.,* μmoles of FFA released per unit droplet surface area per unit time), d_0 is the initial droplet diameter (mean droplet diameter), ρ_0 is the oil droplet density (910 kg/m^3 for olive oil), and M is the molar mass of the oil (0.8 Kg/mol for olive oil). A pH-stat profile can then be characterized in terms of just two parameters: ϕ_{max} and k, which can be determined by finding the values which give the best fit between the experimental data and the mathematical model.

2.5. ζ-Potential measurements

The ζ-potential was determined after 600-fold dilution with buffer solution at room temperature. Diluted samples were then placed in a disposable cuvette that acted as the measurement chamber of the particle electrophoresis instrument (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, UK), and the ζ-potential was determined by measuring the direction and velocity that the droplets moved in the applied electric field. The Smoluchowski mathematical model was used by the software to convert the electrophoretic mobility measurements into ζ-potential values.

2.6. Particle size measurements

The mean particle diameter and particle size distribution of the emulsions were measured using a laser light scattering instrument (Mastersizer 2000, Malvern Instruments Ltd., Malvern, UK). A few drops of emulsions were dispersed in approximately 125 mL buffer in the same chamber with agitation until

approximately 15% obscuration was obtained, in order to avoid multiple scattering effects. The particle size measurements are reported as the volume-weighted mean diameter ($d_{43} = \Sigma n_i d_i^4 / \Sigma n_i d_i^3$), where n_i is the number of particles with diameter d_i. The instrument finds the particle size distribution that gives the best fit to the experimental measurements and predictions based on light scattering theory (Mie theory). A refractive index of 1.47 for olive oil and 1.33 for water was used to calculate the particle size distributions. Measurements were conducted at room temperature.

2.7. Optical microscopy

Emulsions were gently agitated in a glass test tube before analysis to ensure that they were homogeneous. A drop of emulsion was then placed on a microscope slide and covered by a cover slip. The microstructure of selected emulsions was determined using optical microscopy (Nikon microscope Eclipse E400, Nikon Corporation, Japan). The images were acquired using a CCD camera (CCD-300-RC, DAGE-MTI, Michigan City, IN) connected to a Digital Image Processing system (Micro Video Instruments Inc., Avon, MA).

2.8. Creaming stability measurement

Ten milliliters of sample were transferred into a test tube (internal diameter 15 mm, height 125 mm), tightly sealed with a plastic cap, and then stored for one day at room temperature, after which appreciable phase separation was observed in some of the systems. A photograph of the emulsions was recorded during this time using a digital camera.

2.9. Data analysis

All measurements are reported as the average and standard deviation of measurements made on at least two freshly prepared samples.

3. Results and discussion

Initially, we investigated the effects of lipase, bile salts and their combination on the physicochemical properties of the two emulsions stabilized by different surfactants: Pluronic F68 and soybean lecithin. The experiments were designed so as to discern the different roles played by lipase and bile salts in the digestion process. An improved understanding of the fundamental mechanisms underlying lipid digestion is important if we are to design new strategies to rationally control lipid bioavailability.

First, the role of interfacial composition was evaluated by measuring the effects of lipase, bile salts and their mixture on the interfacial tension of oil-water interfaces in the presence of either Pluronic or lecithin. Second, the influence of the same digestion components on the physicochemical properties of lipid droplets coated by Pluronic or lecithin was studied under simulated duodenal conditions. Third, the influence of emulsifier type and droplet size on the digestion profiles of emulsified lipid was examined. Finally, the digestion results are discussed in terms of the basic interfacial phenomena involved.

3.1 Interfacial tension

Fig. 1 shows the change in interfacial tension over time for oil-water systems containing the two different emulsifiers after different duodenal components were added: buffer (control), bile, lipase, or bile + lipase. Digestion conditions were changed at a fixed interfacial coverage as measured by the interfacial tension. Emulsifier type clearly influenced the ability of lipase to adsorb

to the oil-water interface. Initially, the interfacial tension decreased steeply after the emulsifiers were added, which indicated that they adsorbed to the oil-water interface. After about 20 minutes, a relatively constant value was attained, which suggested that the interface had become saturated with emulsifier or reached a steady-state interfacial composition. There was no change in interfacial tension when buffer was added to either system, which suggested that the original emulsifiers remained adsorbed to the oil-water interfaces. On the other hand, there were appreciable decreases in interfacial tension when some of the duodenal components were added. For the system containing Pluronic F68, there was little

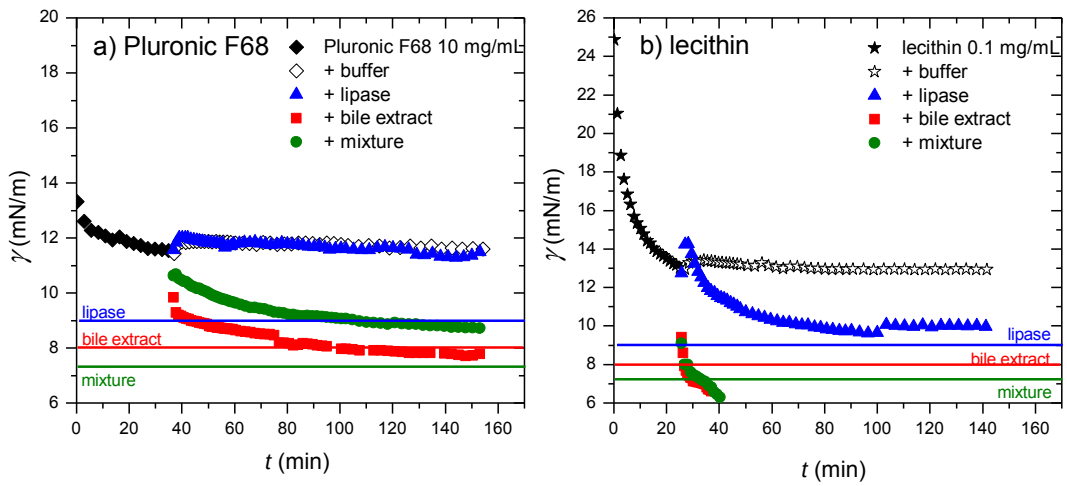

Fig. 1 Variation as a function of time in the interfacial tension at the olive oil-water interface and at constant interfacial area (30 mm^2). Horizontal lines represent the final interfacial tension reached by lipase, bile extract and mixture at the bare oil-water interface. Addition of duodenal components was performed at a fixed interfacial tension: 12 mN/m for Pluronic and 13 mN/m for lecithin. (T = 37 °C, 150 mM NaCl, 3 mM $CaCl_2$, pH 6.5).

change in the interfacial tension with time after addition of lipase alone (Fig. 1a), which suggested that the enzyme could not displace the non-ionic surfactant from the oil-water interface. On the other hand, there was an appreciable decrease in interfacial tension when bile extract or bile extract + lipase were added to this system, which suggested that they could replace the Pluronic from the oil-water interface. However, in the presence of the mixture, the interfacial tension did not reach the value attained by lipase in the presence of bile extract at the bare oil-water interface (indicated by a horizontal line), suggesting that some Pluronic remains at the interface and was not completely replaced.

Our results suggest that lecithin was much more susceptible to replacement from the oil-water interface by duodenal components than Pluronic F68. For the system containing lecithin, there was a very steep decrease in interfacial tension when lipase, bile extract, or bile extract + lipase were added (Fig. 1b), in contrast to the Pluronic-covered interface that gave a steady value even in the presence of the mixture. A more detailed investigation of these effects has recently been published.[27]

This experiment already proves the increased resistance of Pluronic to lipase accessibility at least at the interfacial level. Whether this increased resistance can be also applied to more realistic emulsion systems and then to quantitative lipolysis assays, is a question that will be answered below.

3.2. General appearance of the emulsions

In this section, we subjected O/W emulsions initially stabilized by the two kinds of emulsifiers to similar conditions as used in the interfacial tension studies. Firstly, we evaluated the general appearance of the emulsions studied. Fig. 2 shows the control emulsion (with added buffer) and the effect of lipase, bile salts, and the lipase/bile extract mixture on olive O/W emulsions stabilized by Pluronic (Fig. 2a)

and lecithin (Fig. 2b). Both blanks stabilized by Pluronic F68 or lecithin initially had a creamy white appearance, which can be attributed to light scattering by the lipid droplets.[39] However, both systems separated into an optically opaque layer of cream at the top, and a clear or slightly cloudy serum layer at the bottom 2 hours after adding buffer. The relatively poor creaming stability of the emulsions can be attributed to their relatively large mean particle diameter. The fact that the serum layer of the lecithin-stabilized emulsions was turbid suggests that there was a significant population of smaller emulsion droplets ($d \leq 1$ μm) in this system.

Both emulsions become clearer after 2 hours in the presence of lipase, bile salts or their mixture. In the case of bile extract alone this is attributed to the destabilization of the system leading to a thick cream layer on top of the sample. This suggests that bile extract similarly affects the emulsion stability in both systems (Pluronic and lecithin) destabilizing the emulsions. When the lipase was added alone, this is attributed to digestion of the lipid phase by the lipase and, in case of the mixture, also due to incorporation of the lipid digestion products into micelles or mixed micelles.[18] However, the remaining cream layer observed on top of the emulsions after the addition of the mixture is thinner than that observed when adding bile extract alone. This also suggests that at least some of the lipid droplets had not been digested. The remaining cream layer is thicker in the case of the emulsion initially stabilized by Pluronic F68 (Fig. 2a) suggesting the increased resistance of the Pluronic-stabilized emulsions to lipase in agreement with the interfacial tension studies.

Another effect of lipase, bile salts and their mixture in the emulsified lipid is the appearance of a thin layer of free oil on the top of each sample (Fig. 2). This layer appeared in both emulsions (stabilized by Pluronic or lecithin) but it was more noticeable in the case of adding bile salts and mixture, probably due to the bile extract colour (yellow/orange). The free oil layer could be due to the digestion

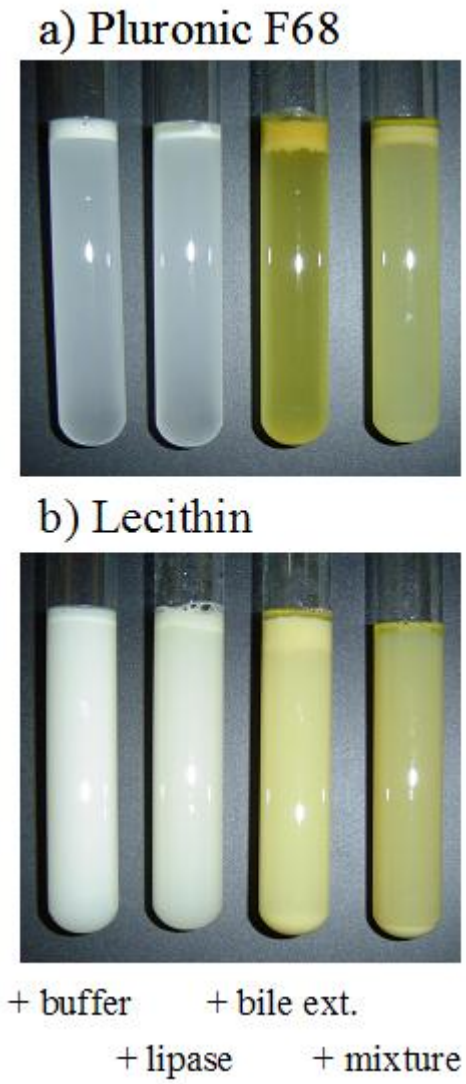

Fig. 2 General appearance of 3 wt% olive O/W emulsions (fine emulsions) stabilized with 1 wt% a) Pluronic F68 or b) lecithin after 2 hours of adding buffer solution (blank), 1.6 mg/mL pancreatic lipase, 5 mg/mL bile extract, or the mixture of lipase and bile extract (T = 37 ºC, 150 mM NaCl, 20 mM $CaCl_2$, pH 7.0).

of the lipid phase by the lipase, in which case it would be mainly composed of free fatty acids. However, since the layer appears also in the presence of bile salts alone, it is more probably originated by the destabilization of the emulsion droplets by the

bile salts. Bile salts are very surface active molecules, able to solubilize phospholipids[19] and replace the interface of emulsifier leading to the appearance of free oil. This correlates with the interfacial tension results in which the bile salts alone decreased the interfacial tension of Pluronic and lecithin covered interfaces (Fig. 1).

Another important effect observed from the general aspect of the emulsions was the appearance of precipitates, especially in the lecithin-stabilized emulsions. These precipitates appeared only after adding bile extract alone or the mixture of lipase and bile extract (Fig. 2b) and did not appear when lipase was added alone. We hypothesize that calcium present in the bile extract and the digestion medium promoted the precipitation of anionic components, such as bile salts, lecithin, and free fatty acids, leading to the formation of sediment. The formation of this precipitate could also be the reason for the extreme effect of bile salts and mixture on the interfacial tension of lecithin in Fig. 1b. After the subphase exchange both systems rapidly decreased the interfacial tension, exceeding the values attained by the bile extract and mixture at the bare oil-water interface (indicated by horizontal lines) and yielding to the droplet fall in both cases. This feature suggests a rapid penetration of the bile extract and the mixture into the interface[27].

3.3. Emulsion droplet surface charge : interfacial composition

The ζ-potential of the lipid droplets provides indirect information about changes in interfacial composition due to *in vitro* duodenal digestion. The effects of lipase, bile extract and their mixture on the droplet charge of olive O/W emulsions stabilized by Pluronic and lecithin was measured. The ζ-potential of the initial emulsions stabilized by Pluronic F68 or lecithin (blanks), were -4 ± 1 mV and -24.0 ± 1.5 mV, respectively (Fig. 3). The negative charge on the emulsions stabilized by lecithin was presumably due to the presence of anionic phospholipids from the lecithin. However, the negative charge measured for the non-ionic Pluronic F68

stabilized emulsions can be attributed to the adsorption of OH^- species from the water or free fatty acid impurities from the oil to the oil-water interface.[40] This result agrees with previous works on lipid droplets stabilized by Pluronic F68, which are slightly negatively charged at neutral pH.[26]

In both systems, addition of pancreatic lipase, bile extract or their mixture resulted in an increase of the negative surface charge of the emulsion droplets (Fig. 3). These results suggest that at least some lipase and bile extract molecules adsorbed onto the oil droplet surfaces stabilized by both emulsifiers. These molecules could have displaced, interpenetrated or adsorbed on top of the existing surface-active molecules at the interface. However, the results follow the same trend as the evolution of the interfacial tension in Fig. 1.[27] In the case of Pluronic-stabilized emulsions, the addition of lipase alone caused only a slight increase in negative charge (-8 mV), whereas the addition of bile extract (alone or in combination with lipase) caused an appreciable increase in negative charge

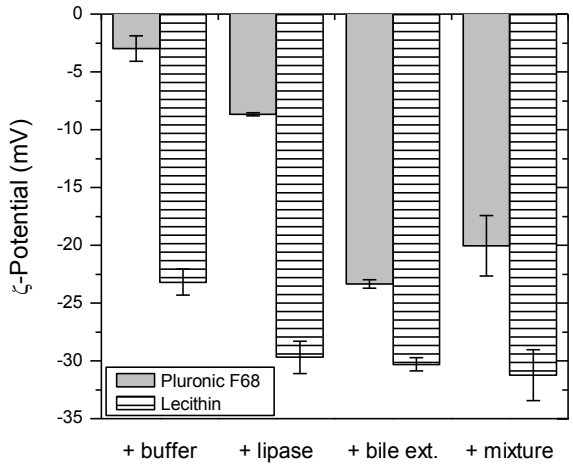

Fig. 3 ζ-potentials of 3 wt% olive O/W emulsions (fine emulsions) stabilized with 1 wt% Pluronic F68 or lecithin after 2 hours of adding buffer solution (blank), 1.6 mg/mL pancreatic lipase, 5 mg/mL bile extract, or the mixture of lipase and bile extract (T = 37 ºC, 150 mM NaCl, 20 mM $CaCl_2$, pH 7.0).

(-23 mV and -20 mV, respectively). In the case of lecithin-stabilized emulsions, the droplets become appreciably more negative in all cases, with similar change being observed when either lipase, bile extract or the mixture was added to the system (Fig. 3). The interfacial tension of Pluronic or lecithin covered interfaces follows similar trends when lipase, bile salts, or their mixture was added into the subphase (Fig. 1), providing evidence of the correspondence between ζ-potential and interfacial composition.

Finally, since the bile extract seemed to have a pronounced effect on the changes in interfacial composition, the ζ-potential of lipid droplets saturated with pure bile extract was determined as -42 ± 1 mV at pH 7 by preparing olive O/W emulsions stabilized by bile extract using the method described in Section 2.3. This value did not change with increasing concentration of bile salts assuring the saturation of the interface. The final concentration of bile extract in emulsion was equivalent to that in digestion experiments. This value (-42 ± 1 mV) differs from that obtained after addition of bile extract to the emulsions and hence, could indicate that the bile extract was unable to completely displace all of the original surface-active molecules from the droplet surfaces at the concentration used in this study. Especially for the Pluronic F68 stabilized emulsion, whose ζ-potential remains well below this value in all cases. This again supports the improved resistance of Pluronic-stabilized emulsions to emulsifier displacement by duodenal components.[27]

3.4. Structural changes

The mean particle diameter (d_{43}) of the emulsions was determined from laser diffraction measurements (Fig. 4 and 5), while the overall microstructure of the emulsions was assessed by optical microscopy (Fig. 6). The mean particle diameter was reported as d_{43} rather than d_{32} (data not shown) since the former is more sensitive to droplet aggregation.[40]

4. Results

Fig. 4 and 5 show the mean particle diameter of lecithin and Pluronic stabilized emulsions after the simulated duodenal digestion process. Again, the effects of pancreatic lipase, bile salts and their mixture on the structural properties of the emulsions were examined separately with the aim of understanding the fundamental interactions occurring in the system. The particle size appeared to be smaller in the control emulsions containing Pluronic-coated droplets than in the ones containing lecithin-coated droplets (Fig. 4 and 5). This result suggests that the non-ionic surfactant was more effective at producing small droplets during homogenization and/or preventing droplet aggregation after homogenization than the lecithin.[40] One reason for this could be the much lower elasticity of a Pluronic interfacial layer compared to a lecithin interfacial layer as reported in previous work.[27] This lower elasticity of the interfacial layer of Pluronic would explain the highly deformable droplets that are easily broken down through the membrane.

When pancreatic lipase, bile extract or a mixture of lipase and bile extract was added to the emulsions, an increase in the mean particle size was observed in all cases (Fig. 4 and 5). This increase was more noticeable in the presence of bile salts and mixture. In fact, the largest change in mean droplet size occurred in the presence of bile extract alone regardless of the emulsifier type. This agrees with the ζ-potential measurements which indicated that the bile extract partially displaced the original emulsifier from the lipid droplet surfaces (Fig. 3). Moreover, the increase in mean particle diameter suggests that these bile covered droplets have poor stability to droplet coalescence, creaming and oiling off. This might even also account for the free oil layer observed on the top of the emulsions (Fig. 2).

Incorporating either bile extract or lipase/bile extract into the lecithin-stabilized emulsions had similar effects on the increase in mean droplet size (Fig. 4 and 5). The physicochemical origin of this effect may be attributed to displacement of lecithin from the oil-water interface, as suggested by the ζ-potential measurements (Fig. 3), and followed the same trend found in the interfacial tension

(Fig. 1). Specific interactions between lipase, phospholipids and bile salts have been reported previously in the literature and attributed to the formation of a new

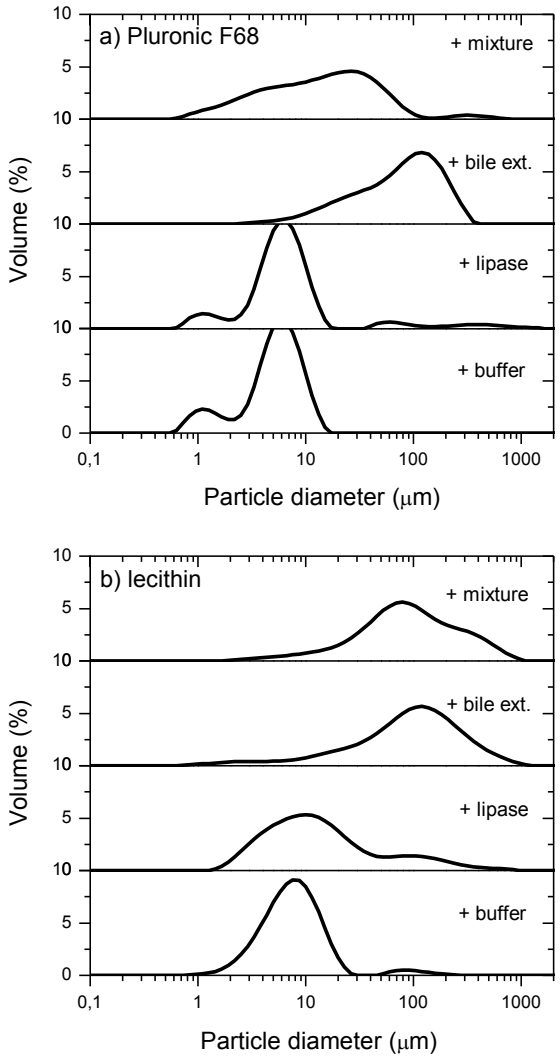

Fig. 4 Particle size distribution of 3 wt% olive O/W emulsions (fine emulsions) stabilized with 1 wt% a) Pluronic F68 or b) lecithin after 2 hours of adding buffer solution (blank), 1.6 mg/mL pancreatic lipase, 5 mg/mL bile extract, or the mixture of lipase and bile extract (T = 37 ºC, 150 mM NaCl, 20 mM $CaCl_2$, pH 7.0).

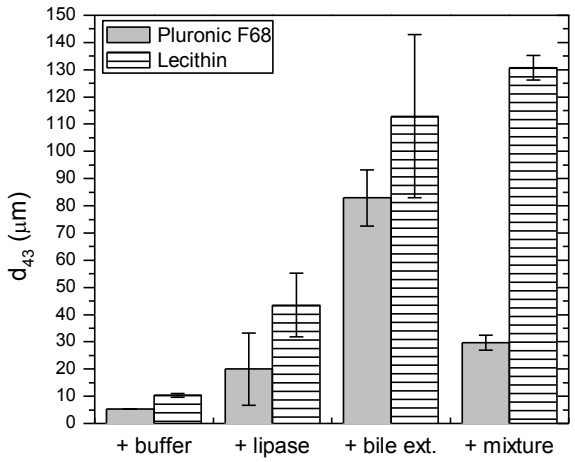

Fig. 5 Mean particle diameter (d_{43}) of 3 wt% olive O/W emulsions (fine emulsions) stabilized with 1 wt% Pluronic F68 or lecithin after 2 hours of adding buffer solution (blank), 1.6 mg/mL pancreatic lipase, 5 mg/mL bile extract, or the mixture of lipase and bile extract (T = 37 ºC, 150 mM NaCl, 20 mM $CaCl_2$, pH 7.0).

catalytically active enzyme complex, (enzyme)–(mixed micelle)–(calcium ion).[34]

The largest differences in the stability of the emulsions for the two different surfactants were seen when lipase and bile extract were added in combination (Fig. 5). There was a large increase in droplet size when lecithin was used as an emulsifier, whereas a large population of small droplets still remained when Pluronic was used. This result is consistent with the interfacial tension measurements (Fig. 1), which indicated that lecithin was almost completely displaced from the oil-water interface by lipase/bile extract leading to a low interfacial tension. Low interfacial tensions are known to promote droplet coalescence and emulsion de-stabilization. In contrast, the interfacial tension measurements suggested that Pluronic remained at the oil-water interface even in

the presence of lipase/bile extract, thus leading to a more physically stable emulsion (Fig. 4 and 5).

Optical microscopy images of the lipid droplets after the simulated duodenal digestion process provided further information about the structural changes occurring within the emulsions (Fig. 6). In the control emulsions, the lecithin-coated lipid droplets have a much higher degree of polydispersity than the Pluronic-coated droplets, which supports the light scattering data (Fig. 4). After addition of lipase alone, there was little change in the size of the individual droplets in the emulsions stabilized by Pluronic (Fig. 6), but there was some evidence of increased flocculation. Droplet aggregation may have occurred due to bridging flocculation caused by some component in the lipase extract that adsorbed to the surfaces of more than one lipid droplet (Fig. 3). On the other hand, when lipase was added to the emulsions containing lecithin-coated droplets, there appeared to be a noticeable decrease in lipid droplet concentration, which is probably due to some digestion of the lipid phase by pancreatic lipase. This behaviour correlates with the interfacial tension results shown in Fig. 1.

After addition of bile extract alone, there were also distinct surfactant-dependent changes in the microstructure of the emulsions (Fig. 6). There appeared to be an appreciable increase in the number of larger droplets present in the emulsions initially containing lecithin-coated droplets, which suggested that the presence of bile salts had promoted droplet coalescence. There appeared to be a decrease in the total amount of droplets present in the emulsions containing droplets initially coated by Pluronic, which we attributed to a sampling error. The majority of the particles in this emulsion were so large that they rapidly creamed to the top of the sample (Fig. 2), which made it difficult to collect a representative sample for analysis. The increase in negative charge on oil droplets when the bile extract was added (Fig. 3) would have been expected to increase the electrostatic

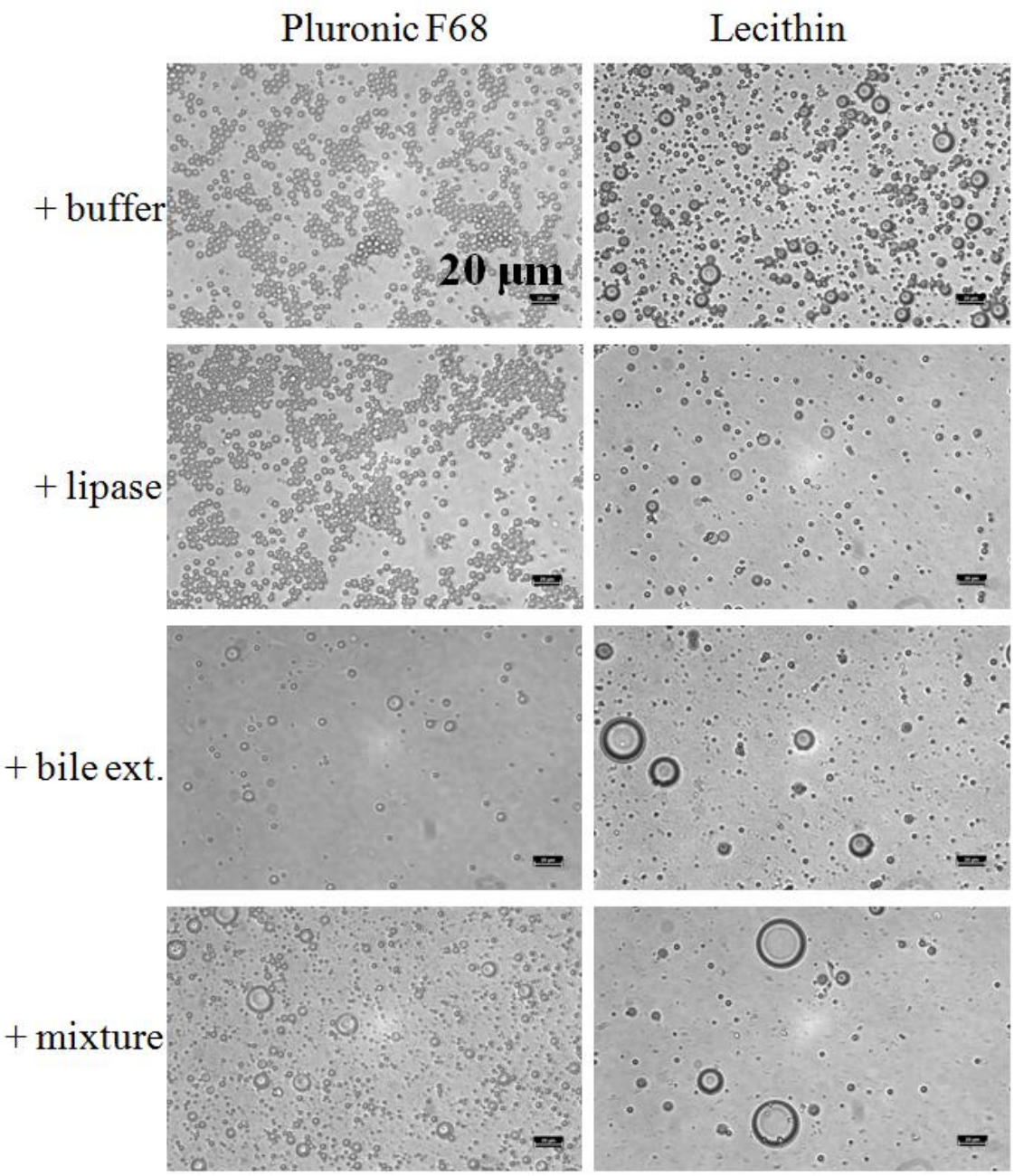

Fig. 6 Microstructure of 3 wt% olive O/W emulsions (fine emulsions) stabilized with 1 wt% Pluronic F68 or lecithin after adding buffer solution (blank), 1.6 mg/mL pancreatic lipase, 5 mg/mL bile extract, or the mixture of lipase and bile extract (T = 37 °C, 150 mM NaCl, 20 mM $CaCl_2$, pH 7.0).

repulsion between the oil droplets, and thus reduce the tendency for droplet aggregation and coalescence to occur. However, bile extract may have partially displaced the original emulsifier molecules from the lipid droplet surfaces, and then the cationic sodium (Na^+) and calcium (Ca^{2+}) ions may have promoted droplet flocculation through charge neutralization effects and thereby coalescence.[40]

Addition of lipase/bile extract to the lecithin-stabilized emulsions had a similar impact on their microstructure as addition of bile extract alone (Fig. 6): there was an increase in the number of large lipid droplets present, which is consistent with coalescence. On the other hand, there appeared to be far more smaller droplets remaining in the emulsions stabilized by Pluronic after addition of lipase/bile extract, which suggests that less lipid digestion had occurred. This is an important result since some previous studies have shown that changes in emulsion microstructure during digestion were fairly similar regardless of the initial emulsifier type (Tween 20, WPI or caseinate) at the oil–water interface.[18] Indeed, the emulsions stabilized by these emulsifiers had microstructures that resembled that of lecithin (Fig. 6). Our study therefore shows that Pluronic-stabilized lipid droplets may behave quite differently from other types of emulsifiers. The relatively high concentration of lipid droplets remaining after digestion suggests that this emulsion was more resistant to the duodenal digestion. This fact, in addition to the features observed above, suggested that the interfacial layer formed by Pluronic is more resistant to being disrupted by lipase and bile extract molecules, which may therefore influence the subsequent digestibility of lipids.

3.5. Enzyme hydrolysis of lipid droplets

Finally, we examined the impact of emulsifier type and initial droplet size on lipase activity in olive O/W emulsions. The activity of pancreatic lipase in the presence of bile salts was quantified in terms of the percentage of free fatty acids (FFA) released from the emulsions during 2 hours of hydrolysis using a pH-stat

method (Fig. 7). The best fit between the experimental digestion data (FFA *versus* time) and the mathematical digestion model (equation 2) was established for each sample, and the corresponding fitting parameters are reported in Table 1. For the initial droplet diameter d_0 we used the mean droplet diameter (d_{43}) of the original emulsions (blanks).

Initially, we focus on the results for fine emulsions stabilized by either Pluronic F68 or lecithin so as to compare the effects of emulsifier type (Fig. 7). As a general trend, there was an initially steep increase in the percentage of FFA released after which a fairly constant value was reached. The initial rate of FFA release was slightly faster for lecithin-stabilized emulsions than for Pluronic-stabilized emulsions. In addition, the total amount of triglyceride hydrolysis after 2 hours was also slightly higher for lecithin-stabilized emulsions (~80 %) than for Pluronic-stabilized emulsions (~70 %). The reason why a fairly constant percentage of FFAs released was reached after a certain time, even though all of the lipids had not been digested, may have been because of the inhibition of lipase activity by the free fatty acids and intermediate products present at the droplet surfaces.[41] Due to their surface activity, monoglycerides and fatty acids compete for the surface of lipid droplets displacing lipase molecules from the oil-water interface at sufficiently high concentrations. Alternatively, they may form a liquid crystalline phase around the lipid droplets, which prevents the lipase from accessing the undigested lipid inside.[42] This means that the final extent of hydrolysis of lipid droplets coated by either Pluronic F68 or lecithin might still increase after longer times. The reason for obtaining a slightly lower rate and extent of lipid hydrolysis for the emulsions stabilized by Pluronic F68 might be due to the ability of this surfactant to hinder the access of lipase molecules, even in the presence of bile extract, to the oil-water interface, as was shown in the interfacial tension (Fig. 1) and ζ-potential (Fig. 3) measurements. Nevertheless, the differences found in the hydrolysis profiles for the emulsions stabilized by each

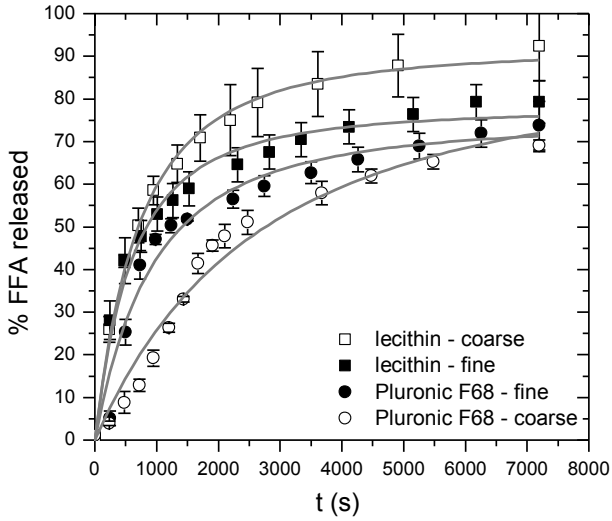

Fig. 7 The time dependence of the fatty acid release (%) from 3 wt% olive O/W emulsions (fine vs. coarse emulsions) stabilized with 1 wt% Pluronic F68 or lecithin, after adding the mixture of lipase (1.6 mg/mL) and bile extract (5 mg/mL) (T = 37 ºC, 150 mM NaCl, 20 mM $CaCl_2$, pH 7.0). Lines represent the mathematical model fit using equation (2).

type of emulsifier were not very pronounced (Fig. 7, Table 1). The interfacial area of an emulsion is inversely related to its droplet size.[40] In a fine emulsion, there will be a large interfacial area and so most of the original emulsifier molecules will be adsorbed to the oil-water interface rather than being free in the aqueous phase. This may have meant that it was easier for the surface active components in the lipase/bile extract to compete for the oil-water interface, thereby making emulsifier type less important.

For this reason, we have performed an additional study on coarse emulsions. We measured the rate and extent of lipid digestion in coarse emulsions containing relatively large droplets so as to examine the influence of interfacial

area (Fig. 7). In this case, there were major differences between the digestion rates of the lipid droplets initially coated by different types of emulsifier. The droplets coated by Pluronic F68 were digested at an appreciably slower rate than those coated by lecithin (Fig. 7, Table 1). This effect can be attributed to the ability of the Pluronic F68 to retard lipase adsorption to the droplet surfaces, and thereby inhibit the lipid digestion reaction. In coarse emulsions containing relatively large droplets, the total interfacial area will be relatively small. Consequently, a considerable fraction of the original emulsifier will be present in the aqueous phase, rather than adsorbed to the droplet surfaces. This surfactant may be able to better compete with the surface active components in the bile extract and lipase, so that surfactant type had a bigger impact on lipid digestion.

The influence of droplet size on the rate and extent of lipid digestion appeared to be surfactant dependent (Fig. 7). The rate of lipid digestion was faster in the coarse emulsion than in the fine emulsion for the lecithin stabilized system, but the opposite was true for the Pluronic stabilized systems. There may be a number of possible explanations for this behaviour. First, one would expect the rate of lipid digestion to increase with decreasing droplet size, since there would be

Table 1: Parameters describing the rate (k) and extent (ϕ_{max}) of digestion in 3 wt% olive O/W emulsions stabilized with 1 wt% Pluronic F68 or lecithin after adding the mixture of lipase (1.6 mg/mL) and bile extract (5 mg/mL). d_0 is the initial mean droplet diameter. (T = 37 ºC, 150 mM NaCl, 20 mM $CaCl_2$, pH 7.0).

Emulsifier type	d_0 (μm)	k (μmol s^{-1} m^{-2})	ϕ_{max} (%)
Pluronic F68	20 (coarse)	3.0 ± 0.4	88 ± 6
	5 (fine)	2.0 ± 0.2	74 ± 3
Lecithin	15 (coarse)	7.8 ± 0.8	92 ± 1
	10 (fine)	6.0 ± 0.5	78 ± 1

a greater number of triacylglycerol molecules exposed to the lipase, *i.e.*, a higher interfacial area.[8,29] Second, the rate of lipid digestion depends on interfacial composition, which will change with droplet size due to competitive adsorption effects between the different surface active components present within the system. Third, the droplet size (and therefore interfacial area) changes during the digestion due to droplet aggregation and digestion processes and hence differences in initial droplet sizes may be less important.

For emulsions stabilized by Pluronic F68, the rate of lipid digestion was lower in the coarse emulsions (d_0 = 20 μm) than in the fine emulsions (d_0 = 5 μm) (Fig. 7). Earlier *in vitro* digestion studies also found that the rate of lipid hydrolysis decreased with increasing droplet size.[8,29] Interestingly, when the digestion rate was normalized to the droplet surface area (k), it actually increased with increasing droplet size (Table 1).[29] The concentration of lipase within all of the emulsions was the same, and so the increase in k with increasing the mean droplet diameter may have occurred because the amount of lipase available per unit droplet surface area increased as the droplet size increased. Thus, there may have been more lipase molecules adsorbed per unit surface area in the emulsions containing the large oil droplets than those containing smaller ones.[43] On the other hand, the final extent was fairly similar (~70 %) in both coarse and fine emulsions (Fig. 7), which is in agreement with *in vivo* digestion studies in which the overall extent of lipid hydrolysis was not affected by the initial droplet diameter.[10] The extent of digestion ϕ_{max} obtained for fine emulsions stabilized by Pluronic in table 1 (74 ± 3 %) fits well with the value of 70 % observed in Fig. 7. Nevertheless, the fitting parameter for coarse emulsions (88 ± 6 %) differs from this value (70 %). This is due to the change in the initial rate observed in the hydrolysis profile, which is not well fitted by equation (2). Indeed, it appeared that there was a lag time in these systems, which may have been due to the time taken for the surface active components in the bile extract and lipase to adsorb to the droplet surfaces and displace the initial surfactant layer.

For lecithin-stabilized emulsions, we observed a different situation. A similar hydrolysis profile was found in both, coarse and fine emulsions (within the margin of error) (Fig. 7). This fact agrees with the negligible differences found in ζ-potential values after adding lipase and/or bile extract between fine and coarse emulsions (data not shown). This probably is due to the instability of the lipid droplets coated by lecithin during the digestion process. For that reason, the mean droplet diameters in initial coarse (d_0 = 15 μm) and fine (d_0 = 10 μm) emulsions were very similar.

In any case, the rate and extent of lipid digestion for Pluronic-stabilized emulsions were always lower than that obtained in lecithin-stabilized emulsions (Fig. 7 and Table 1). It is likely that Pluronic molecules formed an interfacial layer around the droplets that prevented the enzyme from coming into close proximity to the emulsified lipids. It is thought that the poloxamer PEO chains, which remain in the aqueous phase, form a steric bulky layer with a thickness of several nanometers, preventing the adsorption and penetration of the enzyme into the interface.[24,25] This steric mechanism has been proposed before in the literature by Chu *et al.*[15] for galactolipids. It should be noted that free emulsifier may have also interacted with the lipase, such as binding directly with lipase molecules in the bulk or inhibiting the formation of lipase-colipase complex, hindering their adsorption at the lipid droplet surfaces and thereby diminishing the lipase activity. These hypotheses are supported by the fact that there was a relatively small change in ζ-potential (Fig. 3), particle size and microstructure (Fig. 4-6) when lipase was added to Pluronic-stabilized emulsions.

Our proposed hypothesis for the impact of the initial emulsifier type on the lipid digestion process is summarized schematically in Fig. 8, where it has been suggested that the main difference between the two emulsifiers is the ability in hindering the access of the lipase to the emulsified lipid.

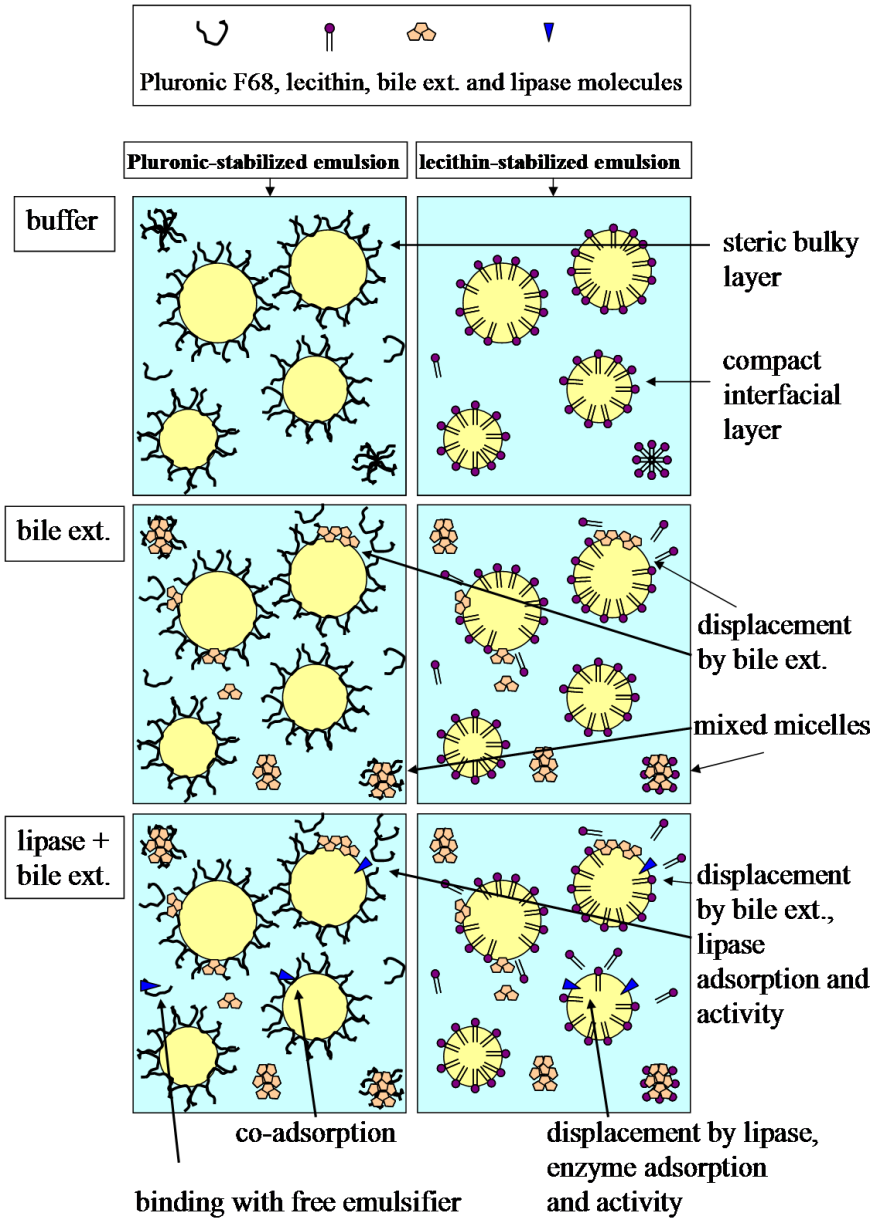

Fig. 8 Schematic diagram of the mechanisms involved in lipid digestion of olive O/W emulsions stabilized by Pluronic F68 or lecithin.

4. Conclusions

This study demonstrates the impact of the nature of the initial emulsifier on the digestibility of emulsified lipids. We have shown that olive oil emulsions stabilized by Pluronic are more resistant to lipolysis than lecithin stabilized emulsions. We have also shown that there is a correlation between changes in interfacial composition (as determined by interfacial tension and ζ-potential measurements), emulsion microstructure (as determined by particle size and microscopy measurements), and lipid digestibility (as determined by pH-stat measurements). We showed that Pluronic is more resistant than lecithin to displacement from oil-water interfaces by bile extract and/or lipase. This difference had important consequences for the subsequent digestibility of the lipid droplets within a simulated small intestine environment. Lipid droplets coated with Pluronic were digested at a slower rate than those coated with lecithin, which was attributed to the ability of the non-ionic surfactant to inhibit the ability of lipase to come into contact with the emulsified triacylglycerol molecules within the droplets. This is a very promising result that shows the potential of poloxamers for the design of food with tailored properties. The different interfacial structures formed by these two types of surfactants could be used in the rational design of emulsion-based delivery systems. In this sense, the steric hindrance provided by a Pluronic interfacial layer might be used for delaying lipid digestion and consequently delivering bioactive compounds more gradually over time, whereas a compact interfacial layer of lecithin might facilitate rapid lipid digestion and thereby a quick release of bioactive compounds.

Another important aspect of this work is the mechanistic insights into the physicochemical phenomena occurring during lipid digestion. We studied the action of lipase, in the presence and absence of bile salts, on emulsions using several complementary analytical methods. We were thus able to relate differences in lipid digestion to interfacial, physicochemical, and structural properties of the

emulsions. A basic understanding of the phenomena underlying lipid digestion is crucial if we are to rationally design food emulsions for controlled or targeted release applications.

Acknowledgments

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4. Results

4.3. Characterization of Pluronic F68, phospholipids and a model bile salt at the oil-water interface: link of Pluronic or phospholipid-covered interfaces with the stability of oil-in-water emulsions stabilized by Pluronic or phospholipids under the action of bile salts.

4. Results

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Effect of emulsifier type against the action of bile salts at oil-water interfaces

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ABSTRACT

Bile salts (BS) are important agents in lipid digestion and absorption. This biological process involves high amounts of BS micelles and this could induce depletion flocculation of oil-in-water (O/W) emulsions by non-adsorbed micelles that are excluded from the interstitial space. In a previous work we observed that the emulsifier type - Pluronic F68 (non-ionic) and phospholipids Epikuron 145V (anionic) - provided different emulsion stability in the presence of a bile salt (sodium taurodeoxycholate, NaTDC). Namely, Pluronic provided higher stability than Epikuron against the action of BS. In order to elucidate these results observed in O/W emulsions, the aim of the current study is to probe the effect of NaTDC on the interfacial behavior of such emulsifiers. The interfacial properties were measured with a pendant drop film balance equipped with a subphase exchange technique, which allows adding the bile salt directly into the subphase once the surfactant has been pre-adsorbed onto the oil-water interface. We can hence monitor *in-situ* the effect of the BS on the interfacial layer by comparing with the behavior of the individual systems. Interfacial tension showed lower adsorption rates for BS onto Pluronic-covered interface, as compared to pre-adsorbed Epikuron layer. Finally, in order to gain structural information we have fitted the experimental data with the Frumkin adsorption Isotherm by using software package IsoFit. As a result, we get that the molecular area and molecular interaction decrease in the following order: Pluronic > NaTDC > Epikuron within the interfacial layer. Therefore, we prove that the Pluronic adsorbed layer is more resistant to bile salt adsorption than the Epikuron interfacial layer. This correlates with the physicochemical properties of O/W emulsions. The use of interfacial techniques provides new insight into the action of BS on O/W emulsion, which constitutes one of the main challenges in order to clarify the mechanisms involved in lipid digestion and absorption.

1. Introduction

Bile salts (BS) are important agents in lipid digestion and absorption (Wickham, Garrood, Leney, Wilson, & Fillery-Travis, 1998; Maldonado-Valderrama, Wilde, Mazierzanka & Mackie, 2011). In the literature, it is possible to find different descriptions of the effect of BS on such processes. For instance, bile salts are considered to displace lipase at oil-water interfaces in the absence of co-lipase; however, they also promote desorption of other surfactants from the interface, so that lipase/co-lipase complexes can adsorb onto the interface promoting lipid digestion (Mun, Decker, & McClements, 2007). Other authors show that bile salts reduce the penetration of the lipase due to the electrostatic repulsion between the negatively charged BS adsorbed at the interface and the lipase (Wickham et al., 1998). The interfacial properties of BS will certainly play a crucial role in these phenomena. The importance of this aspect has boosted the publications dealing with this topic recently (Euston, Bellstedt, Schillbach, & Hughes, 2011; He et al., 2011; Fernández-Leyes, Messina, & Schulz, 2011) However, specific work on the fundamental surface properties of BS is still very scarce in the literature as reviewed recently (Maldonado-Valderrama et al., 2011). Bearing this in mind, improving the current understanding of the behavior of BS at oil-water interfaces is one of the main challenges in order to clarify the mechanisms involved in lipid digestion and absorption.

In a previous work (Jódar-Reyes, Torcello-Gómez, Wulff-Pérez, Gálvez-Ruiz & Martín-Rodríguez, 2010), we studied the stability, the electrokinetic behavior and droplet size of surfactant-stabilized olive oil-in-water (O/W) emulsions after the addition of different bile salt concentrations. Adsorption of BS onto lipid droplet surfaces allowed us to explain the higher stability at low bile salt concentrations as well as the more negative electrophoretic mobility when comparing with the emulsion in the absence of BS. Differently, BS micelles induced depletion flocculation of O/W emulsions at high bile salt concentrations.

Effects of the type of emulsifier present at the oil-water interface were also observed. However, further work became necessary in order to ascertain which interfacial mechanism (i.e. displacement of the emulsifier by the bile salt and/or co-adsorption at the emulsion interface) was taking place.

In this sense, the importance of the interfacial mechanisms underlying the behavior of emulsions is well-known. However, details about the specific interaction between digestion compounds and emulsifiers at the interface are still unclear (Maldonado-Valderrama et al., 2011). Accordingly, the aim of this work was to analyze the behavior of a bile salt at an oil-water interface in the presence of two types of surfactants, which were used as emulsifiers in our previous study (Jódar-Reyes et al., 2010), and to correlate it with the physicochemical properties observed in emulsions. Concretely, we elucidate the interfacial interactions taking place between bile salt and surfactant molecules, providing a better understanding of the different emulsion stability induced by each emulsifier. This will be achieved by using state of the art surface tension techniques. Hence, the evolution of the interfacial composition was monitored by looking at its mechanical properties (interfacial dilatational rheology) (Torcello-Gómez et al., 2011a). Despite the variety of BS, they all behave in a qualitative similar manner (Maldonado-Valderrama et al., 2011). Sodium taurodeoxycholate (NaTDC) is one of the main components in human bile salt (Arleth et al., 2003; Friesen et al., 2008). For this reason, we chose NaTDC as a BS model in our study. Two different surfactants were used to study the effect of the emulsifier type on BS adsorption. Namely, one synthetic, polymeric, uncharged (Pluronic F68), and one natural, charged small molecule (Epikuron 145V). These, instead of proteins, were chosen as emulsifiers because they provide greater protection against lipase-induced destabilization in the presence of bile salts (Mun et al., 2007; Wulff-Pérez, Gálvez-Ruiz, de Vicente, & Martín-Rodríguez, 2010; Torcello-Gómez, Maldonado-Valderrama, Martín-Rodríguez, & McClements, 2011b).

Dilatational rheology and interfacial tension are sensitive tools to the intermolecular interactions and adsorption providing information about the interfacial structure (Maldonado-Valderrama, & Rodríguez, 2010a). However, it is sometimes difficult to interpret at the molecular level. In this work we have applied a theoretical model to the adsorption data in order to explain the data, so as to obtain further information about the structure of the interfacial layers. This same procedure has been recently applied to study interfacial properties of protein layers under *in-vitro* gastric conditions (Maldonado-Valderrama, Miller, Fainerman, Wilde, & Morris, 2010b). The application of a theoretical model to study this biological system provides a comprehensive analysis and new information of the role of molecular interactions on the properties of the interfacial layers formed by different emulsifiers. Moreover, by combining experiments and theory original insights have been gleaned into the role of interfacial structuring against the action of bile salts, relevant to physiological studies. Despite the fact that this system does not reflect realistic conditions in the gastrointestinal tract, the generic information obtained in this study can be extrapolated to duodenal conditions (Torcello-Gómez et al., 2011a, 2011b). As this knowledge improves, rational strategies can be developed to improve the biological impact of foods and pharmaceuticals.

2. Materials and methods

2.1. Materials

As non-ionic surfactant we used poloxamer Pluronic F68 from Sigma-Aldrich. It is a triblock copolymer based on poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) structure which is also typically expressed as $PEO_{75}PPO_{30}PEO_{75}$ (MW=8350 g/mol). The central block has a hydrophobic character and hence adsorbs onto the oil-water interface, whereas the

two chains of poly(ethylene oxide) remain in the aqueous phase. As ionic surfactant we used Epikuron 145V (around 800 g/mol), a deoiled, wax-like phosphatidylcholine (PC) enriched soybean lecithin (min. 45% PC) from Cargill Ibérica S. L. This phospholipid presents negative charge at pH 7. Sodium taurodeoxycholate (NaTDC, 97% purity) was purchased from Sigma-Aldrich. This bile salt is negatively charged and its molecular weight is 521.7 g/mol. Highly refined olive oil was also purchased from Sigma-Aldrich, and purified with activated magnesium silicate (Florisil, Fluka) to eliminate free fatty acids and surface active impurities. The oil was kept under mild agitation with the resins for 3 h and centrifuged at 12000 rpm for 30 min in a bench centrifuge. It was then filtered and stored away from light.

The Pluronic and NaTDC solutions were prepared in a 1.13 mM phosphate buffer (pH 7), whereas Epikuron samples were dissolved in purified olive oil under stirring and gentle warming to guarantee complete dissolution. All chemicals used were of analytical grade. Milli-Q purified water 0.054 μS was used for buffer preparation and all other purposes. All glassware was washed with 10% Micro-90 cleaning solution and exhaustively rinsed with tap water, ethanol, distilled and ultrapure water in this sequence. Only freshly prepared solutions were made by successive dilution from a concentrated stock and used for each experiment.

2.2. Interfacial Tension Set-up and Subphase Exchange Technique

The interfacial tension measurements were carried out in a Pendant Drop Film Balance equipped with subphase exchange technique, which has been fully assembled and developed at the University of Granada. This device is described in detail elsewhere (Cabrerizo-Vílchez, Wege, Holgado-Terriza, & Neumann, 1999). A solution droplet is formed at the tip of a coaxial double capillary, connected independently to a double microinjector. The whole set-up is computer controlled through the software DINATEN© developed by Dr. Holgado Terriza. DINATEN©

fits the experimental drop profiles, extracted from digital drop micrographs, to the Young-Laplace equation of capillarity by using Axisymmetric Drop Shape Analysis (ADSA), providing as outputs the drop volume *V*, the interfacial tension γ, and the interfacial area *A*. The adsorption process is recorded at constant interfacial area (30 mm^2) through a modulated fuzzy logic PID algorithm (proportional, integral, and derivative control) by changing the volume (Wege, Holgado-Terriza, & Cabrerizo-Vílchez, 2002). The drop is immersed in a glass cuvette (Hellma), which contains the oil phase and is kept in a thermostatized cell. The interfacial tension of the clean interface was measured before every experiment to ensure the absence of surface-active contaminants obtaining values of (29.5 ± 0.5) mN/m at 20 ºC. All the experiments were performed at room temperature and their reproducibility was verified from the standard deviation of at least three replicate measurements.

The experiments were designed following this sequence. First, the interfacial tension was recorded for the pure systems at the bare oil-water interface at constant interfacial area in order to pre-form the emulsifier interfacial layer (12 mM Pluronic, 1 mM Epikuron). Next, the subphase was exchanged by means of the coaxial double capillary, which enables to substitute the surfactant bulk solution by that of BS, hence mimicking the passage through the intestine. The subphase is exchanged by extracting the surfactant solution through the outer capillary, and injecting simultaneously the BS through the inner one. Hence, the effect of bile salt on a previously covered interface was monitored by recording the change in interfacial tension and dilatational elastic modulus as detailed below.

2.3. Interfacial Dilatational Rheology

The dilatational rheology of the different adsorbed layers was measured with the same pendant drop device. An oscillatory perturbation was applied to the interface by injecting and extracting volume to the drop. The system records the

response of the interfacial tension to the area deformation, and the dilatational modulus (E) of the interfacial layer can be calculated from this response by following the method described by Myrvold and Hansen (1998). The applied interfacial area oscillations were maintained below 5% of amplitude to avoid excessive perturbation of the interfacial layer and the departure from the linear viscoelastic region, which was checked in preliminary experiments. The oscillation frequency (f) was set to 0.1 Hz.

In a general case, the dilatational modulus is a complex quantity that contains a real and an imaginary part: $E^* = E' + iE'' = \varepsilon + i2\pi f\eta$, where E' is the storage modulus and accounts for the elasticity (ε) of the interfacial layer and E'' is the loss modulus and accounts for the viscosity (η) of the interfacial layer.

3. Theoretical background

In order to fit the experimental data to a theoretical model, we have used IsoFit. This is an easy-to-use windows based software for the analysis and prediction of the adsorption behavior of interfacial layer as part of AdSo project developed by Dr. Aksenenko (2010).

The theory of adsorption behavior used in this work is described in detail elsewhere (Frumkin, 1925; Fainerman, Miller, & Möhwald, 2002). Therefore, only the main equations for each applied model will be given here.

The Frumkin equations of state and adsorption isotherm describe the adsorption behavior of surfactants:

$$\pi = -\frac{RT}{\omega}\left[\ln(1-\theta) + a\theta^2\right] \quad (1)$$

$$bc = \frac{\theta}{(1-\theta)} \exp(-2a\theta). \quad (2)$$

Here π is the interfacial pressure of the solution ($\pi \equiv \gamma_0 - \gamma$, where γ_0 is the interfacial tension of the clean oil-water interface), R is the gas law constant, T is the temperature, c is the surfactant bulk concentration, ω represents the molar area of the surfactant and θ is the surface coverage, given by $\Gamma\omega$, where Γ is the adsorbed amount. The adsorption constant b provides information about the strength of the interaction between the adsorbing species and the surface, and the Frumkin interaction parameter a indicates whether the adsorbing molecules exhibit attractive or repulsive (lateral) interactions (Karakashev, Manev, & Nguyen, 2004).

A modification of this model includes two orientations of adsorbed surfactant molecules coexisting at the interface, with different molar areas ω_1 and ω_2 (for definiteness we assume $\omega_1 > \omega_2$). The equations of state and adsorption isotherm for this reorientation model read:

$$\pi = -\frac{RT}{\omega} \ln(1-\theta) \quad (3)$$

$$bc = \frac{\Gamma_2 \omega}{(1-\theta)^{\omega_2/\omega}} \quad (4)$$

Where $b = b_2$ is the adsorption equilibrium constant in state 2. The total adsorption Γ and mean molar area ω are defined by

$$\Gamma = \Gamma_1 + \Gamma_2 \quad (5)$$

$$\omega\Gamma = \theta = \omega_1\Gamma_1 + \omega_2\Gamma_2 \quad (6)$$

and the ratio of adsorptions in the two possible states of the adsorbed molecules is given by

$$\frac{\Gamma_1}{\Gamma_2} = \exp\left(\frac{\omega_1 - \omega_2}{\omega}\right)\left(\frac{\omega_1}{\omega_2}\right)^{\alpha} \exp\left[-\frac{\Pi(\omega_1 - \omega_2)}{RT}\right]. \quad (7)$$

The constant α accounts for the fact that the adsorption equilibrium constant b_1 for surfactant molecules adsorbed in state 1 (with larger area) can exceed that in state 2, which results in an additional increase of the fraction of states of larger area.

The authors of this model have recently developed a set of software tools for the analysis and prediction of the behavior of adsorbed monolayers which is distributed on a non-profit basis through a web-page (Aksenenko, 2010). In this work, we have used the software package IsoFit, developed by E. V. Aksenenko to fit the models to experimental data (Fainerman et al., 2009). The software also provides the target function ε, which is the weighted (over π) average of the relative deviations between the experimental and the calculated c_i values.

4. Results and discussion

4.1. Adsorption of BS onto surfactant-covered interfaces

In a previous work (Jódar-Reyes et al., 2010), we dealt with the behavior of O/W emulsions stabilized by two different surfactants in the presence of NaTDC at different concentrations. Interestingly, the emulsifiers provided different emulsion stability against BS action. In order to deepen into the effect of the emulsifier type, we have evaluated in this section the interfacial behavior of NaTDC in the presence of the two types of surfactants, Pluronic F68 (non-ionic) and Epikuron 145V (phospholipids), which have been previously adsorbed onto the oil-water interface.

Figures 1 and 2 display the evolution of the interfacial tension for these systems. Firstly, the surfactant is adsorbed at a constant interfacial area (30 mm^2) for at least 30 min until the interfacial tension reaches a certain value, (9.0 ± 0.2)

mN/m for Pluronic F68 (Figure 1) and (10.0 ± 0.2) mN/m for Epikuron (Figure 2). These interfacial tension values are similar for both emulsifiers in order to make direct comparisons between the behaviors. Only after the interfacial layer has been formed, the subphase is exchanged by substituting the aqueous subphase by BS solution. In the case of Pluronic we exchanged the remaining surfactant solution (Figure 1) and in the case of Epikuron we exchanged the buffer as it is diluted in the oil phase (Figure 2). In order to further understand the behavior of emulsions, the effect of NaTDC on previously surfactant covered interfaces was evaluated at bile salt concentrations below and above the critical micelle concentration (cmc), as in our previous work (Jódar-Reyes et al., 2010). Namely, we used different concentrations of NaTDC: 30 μM (0.015×cmc), 300 μM (0.15×cmc), 3 mM (1.5×cmc), 10 mM (5×cmc) and 30 mM (15×cmc). In this way, the effect of NaTDC on the surface properties of each emulsifier can be monitored by recording the change of interfacial tension as a function of time due to adsorption onto the pre-adsorbed surfactant layer. This experiment mimics the effect of addition of bile salts onto different surfactant stabilized emulsions. The interfacial coverage chosen for the surfactants prior to the addition of BS (i.e. the interfacial tension at which the subphase exchange was done) warrants a saturated interface, as we will observe below in the interfacial tension isotherms, mimicking the poloxamer and lipid packing at the oil emulsion interface.

Figure 1 shows the last 15 min of the adsorption process of Pluronic F68 (100 mg/ml-12 mM) onto the olive oil-water interface. After a total adsorption time of 30 min, the subphase was exchanged. Exchange by buffer is shown as a reference proving that the adsorbed layer remains adsorbed at the interface at an interfacial tension of 9 mN/m. Figure 1 also shows the exchange of the aqueous subphase by NaTDC at different concentrations (30 μM, 300 μM, 3 mM, 10 mM and 30 mM). Addition of the lowest bulk concentration had little effect on the

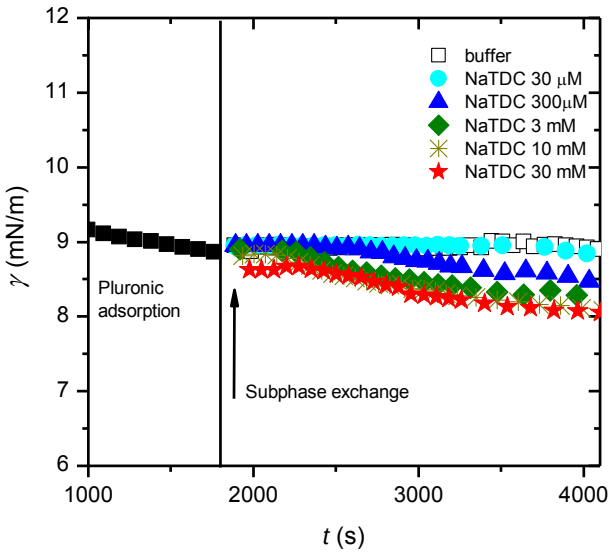

Figure 1: Variation as a function of time of the interfacial tension of Pluronic F68 adsorbed at the olive oil-water interface (T = 25 ºC, 1.13 mM phosphate buffer, pH 7). Subphase exchange with different NaTDC bulk concentrations at a fixed interfacial tension for Pluronic F68 of 9 mN/m. Error bars are within the size of the symbols.

interfacial tension, as the dynamic curve of the interfacial tension collapsed with that of the exchange with buffer (9 mN/m). Increasing bulk concentration of NaTDC resulted in further decrease of the interfacial tension until a limiting value. Addition of the two higher concentrations of BS (10 and 30 mM) provided a similar interfacial response. This could be due to the bile salt having difficulties to adsorb into the more packed interfacial layers. Accordingly, the adsorption of NaTDC into the Pluronic-covered interface resulted in a net decrease of the interfacial tension of the system at sufficiently high bulk concentrations of BS (Figure 1) owing to the saturation of the interfacial layer by the bile salt. Similar results were obtained by Chu and co-workers for interfacial layers of galactolipids in the presence of bile salts (Chu et al. 2010). The ability of BS to displace protein

from interfaces has been also studied recently (Euston et al., 2011; Maldonado-Valderrama et al., 2008). The displacement is determined mainly as a consequence of clustering of the bile salts at the interface. This saturation also correlates with the behavior of O/W emulsions stabilized by Pluronic (Jódar-Reyes et al., 2010). The increase of the electrophoretic mobility (data not shown) upon increasing the BS concentration suggested some interfacial composition changes due to the adsorption of NaTDC onto the oil droplet surface. Interestingly, electrophoretic mobility did not change above a BS concentration of approximately 20 mM, indicating the saturation of the emulsion interface.

Consider now the effect of NaTDC on the interfacial behavior of surfactant Epikuron 145V. We have used a phospholipid concentration of 1 mg/mL (1 mM) which provided similar interfacial tensions to those attained by the Pluronic F68 after 30 min of adsorption onto the olive oil-water interface. Figure 2 shows first the time evolution of the interfacial tension during the last 15 min of Epikuron adsorption. In order to assure that the interfacial layer is not disturbed by the subphase exchange, we exchanged the aqueous subphase by buffer, obtaining a monotonous decrease of the interfacial tension. This curve provided a suitable reference to be compared to the effect of exchanging the aqueous subphase by NaTDC (30 μM, 300 μM, 3 mM). Addition of the lowest concentration of NaTDC (30 μM) slightly increased the interfacial tension just after the subphase exchange. This could be attributed to some displacement of adsorbed phospholipids by the bile salt but the interface is rapidly filled again and the interfacial tension curve collapsed with the reference curve obtained after the addition of buffer (Figure 2). Differently, increasing BS bulk concentration resulted in a rapid decrease of the interfacial tension (± 0.2 mN/m) upon addition of NaTDC into the subphase. This behavior contrasts with the adsorption of BS onto a Pluronic-covered interface, which was more gradual. In fact, addition of bile salt had a dramatic effect on the phospholipids covered interface: BS rapidly decreased the interfacial tension, yielding to the droplet fall in 1 min (3 mM NaTDC). For this reason we only show

bulk concentrations up to 3 mM. This feature suggests a rapid penetration of the bile salt into the interface (Torcello-Gómez et al., 2011a) and possibly a synergistic effect with the remaining phospholipids, such as the formation of BS-phospholipids complexes. It is important to note here that the situation is slightly different in each case. Figure 1 shows a sequential adsorption of Pluronic and BS onto the oil-water interface in which the subphase is depleted of excess of surfactant. Conversely, in Figure 2 we are dealing with a competitive adsorption of BS and Epikuron onto the olive oil-water interface. In any case, comparison of Figures 1 and 2 shows clearly that the rate and extent of bile salt adsorption are higher at the Epikuron-covered interface as compared to the Pluronic system. This can be related to the stability regimes observed in O/W emulsions (Jódar-Reyes et al, 2010). For Pluronic- and Epikuron-stabilized emulsions, the systems were more stable in the presence of

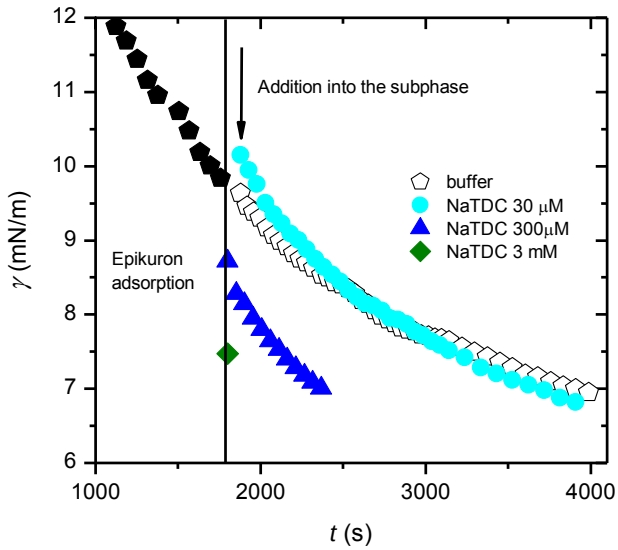

Figure 2: Variation as a function of time of the interfacial tension of Epikuron 145V adsorbed at the olive oil-water interface (T = 25 ºC, 1.13 mM phosphate buffer, pH 7). Subphase exchange with different NaTDC bulk concentrations at a fixed interfacial tension for Epikuron 145V of 10 mN/m. Error bars are within the size of the symbols.

NaTDC up to a BS concentration of 19.2 mM and 1.92 mM (results not shown here), respectively, than in the absence of BS. These BS concentration ranges agree with those studied with interfacial tension measurements (Figures 1 and 2). Namely, Pluronic-covered interface reached a BS saturation at a concentration between 10 and 30 mM (Figure 1), which can explain the stability of Pluronic-stabilized emulsions up to a BS concentration of approximately 20 mM. On the other hand, the Epikuron-covered interface was clearly disrupted at a BS concentration of 3 mM (Figure 2), in agreement with the stability regime of Epikuron-stabilized emulsions up to a BS concentration of approximately 2 mM.

The characteristics of the adsorbed layers formed in these experiments were further investigated by means of the dilatational viscoelasticities of the interfacial layers. The oscillating frequency was fixed at 0.1 Hz. Table 1 shows the interfacial dilatational moduli *(E*)* after addition of NaTDC onto the pre-adsorbed surfactant layer. These values are compared to those of the Pluronic F68 (11.0 mN/m) and the Epikuron (53mN/m) which were measured after 30 min of adsorption and just before carrying out the subphase exchange. Table 1 also displays the dilatational moduli of NaTDC (for different bulk concentrations) at the bare olive oil-water interface as a reference. Regarding the Pluronic adsorbed layer (E^* = 11 mN/m), it can be seen how the dilatational modulus of the sequentially adsorbed layers remains practically unmodified upon addition of bile salts. Only at bulk concentrations of BS equal or higher than 10 mM the dilatational moduli slightly decreased. Still, the values differ from those of the pure NaTDC interfacial layer (Table 1) at these bulk concentrations (10 and 30 mM). As regards Epikuron (E^* = 53 mN/m), the BS importantly affected the dilatational modulus of the interfacial layer. The lower BS concentration (300 μM) already reduces 30% the dilatational modulus (E^* = 37 mN/m). Further increasing the NaTDC bulk concentration decreased to half (25 mN/m) the original dilatational modulus of the pure Epikuron interfacial layer. These results importantly complement the interfacial tension data (Figures 1 and 2), providing further evidence of the greater susceptibility of the

4. Results

Table 1: Interfacial dilatational modulus measured before and after the subphase exchange by NaTDC at a Pluronic F68 and an Epikuron 145V covered interfaces. Interfacial dilatational moduli of systems alone at the bare olive oil-water interface are shown as a reference. (T = 25 ºC, 1.13 mM phosphate buffer, pH 7).

E* **surfactant interfacial coverage** (mN/m)	***E**** **NaTDC addition** (mN/m)				
	30 µM	**300 µM**	**3 mM**	**10 mM**	**30 mM**
Pluronic 11.0 ± 0.5	12 ± 1	12 ± 1	11.0 ± 0.5	9.0 ± 0.5	7.5 ± 0.5
Epikuron 53 ± 3	54 ± 3	37 ± 2	25 ± 2	-	-
Bare O/W interface	9 ± 1	11 ± 1	8.0 ± 0.5	5.0 ± 0.5	3.0 ± 0.5

Epikuron interfacial layer to the action of bile salts. Moreover in both systems (Pluronic and Epikuron) the dilatational moduli show intermediate values between those of pure systems at clean interface, which suggests the formation of a mixed interfacial layer, being more noticeable the change recorded at the Epikuron interfacial layer. Similar results have been reported recently by He and co-workers regarding the surface interaction of NaDC and aminoacids (He et al., 2011). The formation of a mixed surface layer is proven by increasing values of the viscoelasticity as the concentration of aminoacids increases in the surface layer. This finding agrees with the effects recorded on the interfacial tension in Figures 1 and 2. Namely, the resistance of the interfacial layer of Pluronic to accommodate bile salts, given by the constant interfacial tension, and the positive synergism between NaTDC and Epikuron at the olive oil-water interface. The interfacial characterization presented here also importantly correlates with the kinetics of phase separation observed in O/W emulsions (Jódar-Reyes et al, 2010), that is the destabilization of emulsions in the presence of bile salt versus time (results not shown here). This destabilization was greater and faster for Epikuron-stabilized emulsions than for Pluronic-stabilized emulsion. According to a recent work by

Euston and co-workers, the hydrophobicity of the bile salt molecule makes the difference (Euston et al., 2011). Euston and co-workers state that bile salts behave very much like amphiphilic surfactants being the overall hydrophile:lipophile balance of the molecule that is important in the interaction within the interfacial layer. In agreement with our work, these authors show a correlation between the interfacial behavior of bile salts and the displacement of protein from emulsions.

4.2. Adsorption of systems alone at the bare oil-water interface

In order to gain more information about the structure of the interfacial layers formed above, we measured the individual adsorption of each system onto the bare olive oil-water interface.

Consider first the interfacial layer of NaTDC. The interfacial tension profiles of bile salt are often not reported in the literature, especially, regarding dynamic adsorption properties (Maldonado-Valderrama et al., 2011) and therefore little is known about the interfacial properties of bile salts. Figure 3 shows the dynamic adsorption of NaTDC at the olive oil-water interface for several bulk concentrations. Figure 4 shows the interfacial tension isotherm after 2 h of adsorption onto the olive oil-water interface at constant interfacial area. Line represents the theoretical curve calculated from the Frumkin model which will be discussed later on. Figure 3 shows that NaTDC adsorbs very rapidly attaining a steady value in less than 5 min even for the lowest concentrations. This behavior has been previously reported for other bile salts: sodium taurocholate (NaTC) and sodium glycodeoxycholate (NaGDC) (Maldonado-Valderrama et al., 2008, 2011). Maldonado-Valderrama and co-workers explained this extremely high interfacial activity owing to the flat conformation of the BS; just one molecule would occupy a large area of interface hence affecting importantly the interfacial tension.

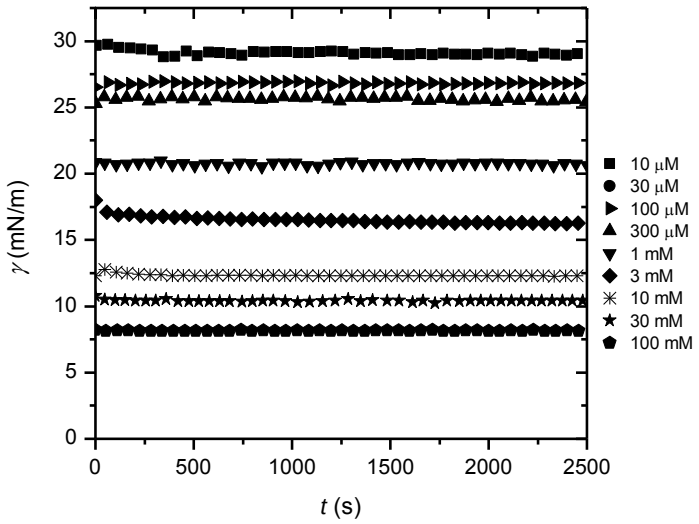

Figure 3: Adsorption dynamics of NaTDC at different bulk concentrations at the olive-oil water interface. (T = 20 ºC, 1.13 mM phosphate buffer, pH 7).

According also to these authors, despite this extreme interfacial activity, bile salts reach relatively low saturation values when compared to conventional surfactants (Maldonado-Valderrama et al., 2008, 2011). Figures 3 and 4 corroborate this latter observation for NaTDC showing a saturation interfacial tension of 10 mN/m. This value is similar than those reported by Maldonado-Valderrama and co-workers for NaTC and NaGDC. This fact also agrees with Fernandez-Leyes and co-workers who also point out the influence of the hydration of the molecule on the surface behavior encountered (Fernandez-Leyes et al., 2011). From Figure 4 we can also calculate the critical micelle concentration (cmc) of the NaTDC from the break point of the interfacial tension versus its log of bulk concentration curve. The value obtained here for NaTDC was (2 ± 1) mM, in good agreement with those reported in literature using tensiometry (Wickham, Wilde, & Fillery-Travis, 2002; Tejera-García, 2008) or spectrophotometry (Carey & Small, 1969).

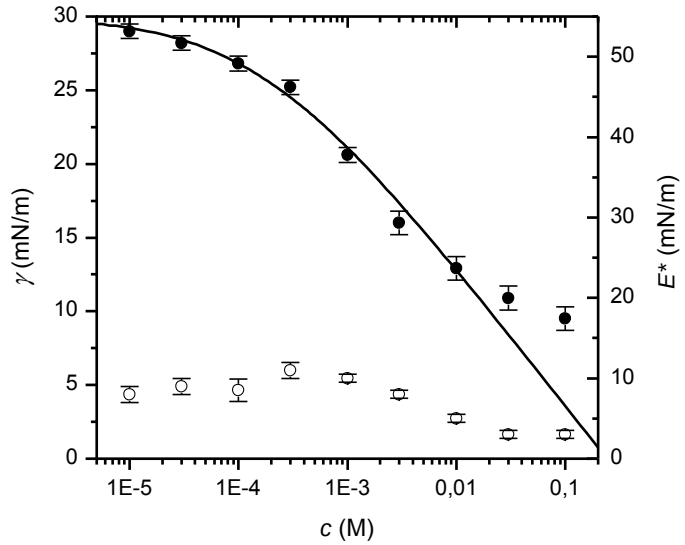

Figure 4: Interfacial tension isotherm (closed symbols) and dilatational modulus (open symbols) of NaTDC at the olive-oil water interface. (T = 20 ºC, 1.13 mM phosphate buffer, pH 7). Error bars represent standard deviations from three independent measurements. Line represents the theoretical curve calculated from the Frumkin model using parameters displayed in Table 2.

The concentrations above the cmc considered in this study are close to the bile salt concentrations reported during the fasted stated in the duodenum (3-7mM) (Sjövall, 1959; Hernell, Staggers, & Carey, 1990; Lindahl, Ungell, Knutson, & Lennernas, 1997). In this sense, the generic information provided in this work will be useful for developing studies of *in-vitro* duodenal digestion as already done in a previous work (Torcello-Gómez et al., 2011b). Regarding the discussion in previous section, it is interesting to note that the steady interfacial tension for the bile salt was actually higher than that of the surfactant layers it was adsorbing into (Figures 1 and 2). Therefore, the displacement of Pluronic and Epikuron by NaTDC is unlikely to go to completion as the interfacial tension would have increased toward

the value seen for the BS-only system (Figure 4). Hence, it was most likely that the bile salt formed a mixed surfactant-bile salt interfacial layer and compressed the adsorbed layer as indicated by the decrease in interfacial tension (Chu et al., 2010).

Figure 4 also shows the dilatational modulus E^* of the adsorbed layers of NaTDC as a function of the bulk concentration. The oscillations were performed once the steady interfacial tension had been attained for each concentration. For NaTDC, this system presented a predominantly elastic behavior, since at this relatively high frequency (0.1 Hz) the viscous component of the dilatational modulus was practically zero (results not shown) (Torcello-Gómez et al., 2011a). Still, it can be seen how there was practically no response of the interfacial films at this oscillating frequency, having very low values of the viscoelastic modulus at concentrations close and above the cmc ($E^* \leq 10$ mN/m). The reason for this is again the rapid adsorption rate of NaTDC at the oil-water interface with the exchange of molecules between the interface and the bulk occurring within the time-scale of the oscillation. This correlates with the rapid diffusion observed in the dynamic interfacial tension curves (Figure 3) and agrees with literature works (Maldonado-Valderrama et al., 2011; He et al., 2011). Overall, the observed low dilatational modulus of the adsorbed bile salt films confirms the mobile nature of these natural bio-surfactants and their great affinity for the interface, having extremely fast adsorption and low packing density.

Figures 5 and 6 show the interfacial characterization of the two emulsifiers used: Pluronic F68 and Epikuron 145V, respectively. This was earlier studied under similar conditions (T = 25 ºC, pH 7, olive oil-water interface) in a previous work in our laboratory (Wulff-Pérez, Torcello-Gómez, Martín-Rodríguez, Gálvez-Ruiz, & de Vicente, 2011). The interfacial tension isotherms of these surfactants prove that the bulk concentrations used for Pluronic (12 mM) and Epikuron (1mM) in previous section provided a saturated interface, mimicking the interfacial packing of emulsifier in emulsion lipid droplets in order to correlate with the

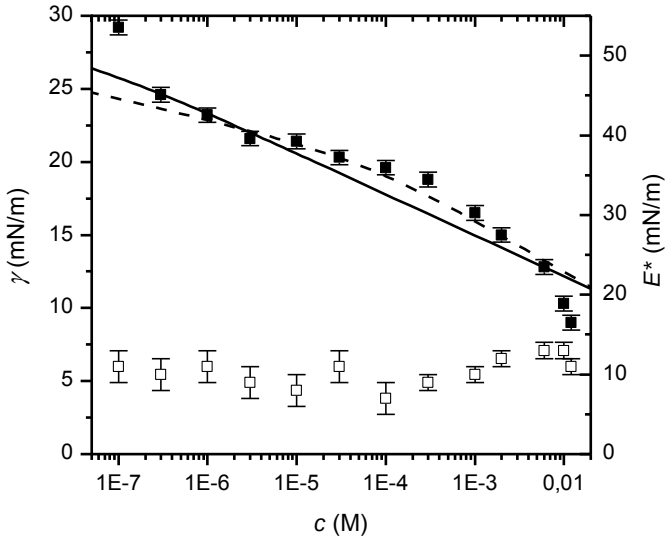

Figure 5: Interfacial tension isotherm (closed symbols) and dilatational modulus (open symbols) of Pluronic F68 at the olive-oil water interface. (T = 20 ºC, 1.13 mM phosphate buffer, pH 7). Error bars represent standard deviations from three independent measurements. Lines represent the theoretical curves calculated from the Frumkin model (solid line) and reorientation model (dashed line) using parameters displayed in Tables 2 and 3, respectively.

results obtained by Jódar-Reyes and co-workers in olive O/W emulsions (Jódar-Reyes et al., 2010). Slight differences with the interfacial tension reported by Wulff-Pérez et al. (2011) are due to different oil purification methods used. Figures 5 and 6 also display the dilatational modulus *E** of the adsorbed layers of Pluronic and Epikuron, respectively. The oscillating frequency was fast enough to assure that the viscous component of the dilatational modulus was negligible (keeping constant with surface concentration) (results not shown) and the adsorbed layers appear predominantly elastic (Wulff-Pérez et al., 2011). The interfacial layer formed by Pluronic F68 (Figure 5) is clearly less elastic than that of Epikuron

145V (Figure 6) at an interfacial coverage corresponding to the saturated interface studied in Figures 1 and 2. Pluronic F68 molecules as a whole are mostly irreversibly adsorbed (Svitova & Radke, 2005), as proved by the practically constant value of the interfacial tension after depleting the bulk solution of molecules when exchanging the subphase by buffer (Figure 1). However, that may not involve that all segments of the molecules are. Segments protruding into the bulk (PEO chains) can adsorb and desorb reversibly, leading to low values of the dilatational modulus. Differently, the Epikuron adsorbed layer (Figure 6) displays a higher dilatational elastic modulus within the whole range of interfacial tension (within the error margin). This value could be an indication of the formation of a network of interacting adsorbed phospholipid molecules at the interface, forming a

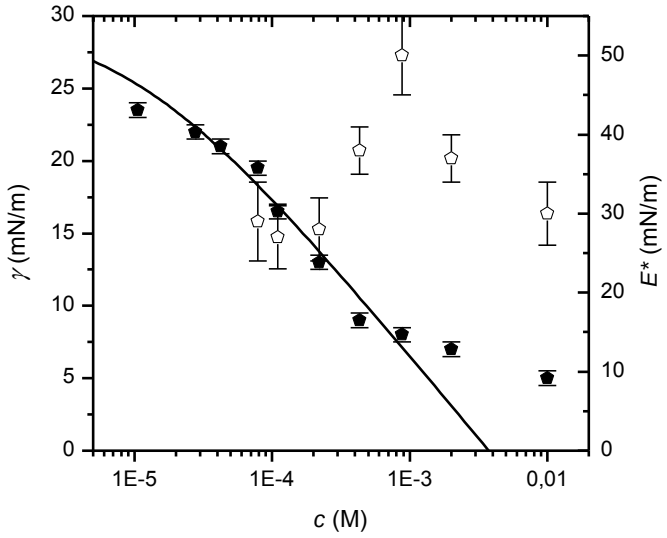

Figure 6: Interfacial tension isotherm (closed symbols) and dilatational modulus (open symbols) of Epikuron 145V at the olive-oil water interface. (T = 25 ºC, 1.13 mM phosphate buffer, pH 7). Error bars represent standard deviations from three independent measurements. Line represents the theoretical curve calculated from the Frumkin model using parameters displayed in Table 2.

closed-packed layer. Possibly, the double tail anchoring group of the phospholipid originates a higher degree of accumulation at the oil-water interface (Vleeschauwer & Van der Meeren, 1999).

In order to gain further information on the interfacial structure of the surfactant layers, we have applied the Frumkin model (equations 1 and 2) to fit the experimental curves shown in Figures 4-6 with the input (ω, a) and fitting (b) parameters displayed in Table 2. The correlation between theory and experiment is satisfactory (given by the parameter ε in Table 2) within the range of applicability of the model, which excludes the interfacial saturation interval (Fainerman et al., 2002). It is important to note that the values of the molecular area (ω) for NaTDC, Pluronic and Epikuron shown in Table 2 are in agreement with values reported in literature (Small, 1971; Guay & Bisaillon, 1983; Van der Meeren, Vanderdeelen, & Baert, 1989; Souza, Oliveira, Scarpa, & Oliveira, 2004). In fact, the interfacial area of NaTDC given in Table 2 agrees with the area reported in a very recent work for NaDC at the air-water interface (He et al., 2011). These values enlighten the interfacial behavior encountered in Figures 1 and 2. According to the theoretical fittings, the interfacial area of the Pluronic is larger than that of Epikuron. Apart from being larger molecules per se, poloxamer molecules form a steric bulky layer due to the PEO chains which remain in the aqueous phase whereas the hydrophobic portion PPO adsorbs onto the interface, contributing to the interfacial area occupied at the interfacial layer. On the contrary and in agreement with the values reported

Table 2: Input (ω, a) and fitting (b) parameters used to fit the experimental data in Figures 4-6 with the Frumkin model (equations (1) and (2)).

Surfactant	**ω** (m^2/mol)	(nm^2/molecule)	***a***	***b*** (l/mol)	***ε***
NaTDC	6×10^5	0.99	-1	1.73×10^4	0.1
Pluronic	2×10^6	3.32	-3	4.59×10^9	0.5
Epikuron	5×10^5	0.83	-0.5	1.68×10^5	0.1

in Table 2, phospholipids form a compact interfacial layer with small headgroups adsorbed onto the interface. Regarding the bile salt molecules, they have an intermediate molecular area between that of Pluronic and Epikuron. NaTDC clearly disrupts the phospholipid layer, as compared to poloxamer adsorbed layer (Figures 1 and 2). This could be due to increased packing at the interface by binding into the spaces among the phospholipid molecules as reported by Chu and co-workers for a similar system (Chu et al., 2010). The steric barrier of Pluronic molecules, which is reflected in the large interfacial area, could be responsible for the improved resistance of Pluronic to accommodate bile salts as suggested in Figure 1.

Regarding the values obtained for the Frumkin interaction parameter *a* in Table 2, we observe negative values in all cases. This indicates a repulsion between adsorbed molecules, which is characteristic of solutions of ionic surfactants (Fainerman et al., 2002) at low ionic strength, as in the case of NaTDC and Epikuron. However, being Pluronic a non-ionic emulsifier, this implies a sort of repulsion between the adsorbed species (Karakashev et al., 2004). Interestingly, this repulsion corroborates the steric bulky layer and the large interfacial area. The absolute value of the Frumkin parameter is a measure of the intermolecular interaction. Comparing *a* for all the surfactants, we conclude that Pluronic shows a stronger intermolecular interaction than NaTDC and Epikuron. This fact again could explain the improved resistance of the poloxamer layer to accommodate NaTDC molecules. In the same line, phospholipids show a weak repulsion between molecules as reported by the lowest value of *a* (Table 2) and are therefore susceptible to the presence of BS.

Finally, we also observe in Table 2 that the adsorption constant *b* increases when increasing the molecular weight of surfactant molecules. This parameter is related to the interfacial activity of the surfactants.

In order to improve the fitting of the poloxamer isotherm, we applied another theory that might provide a more realistic approach to the adsorption behavior

considering the conformation of the poloxamer. Figure 5 shows the theoretical curve obtained with the reorientation model given by equations (3-7) and parameters displayed in Table 3. We observe an appreciable advantage of the reorientation model as compared to the Frumkin model for Pluronic adsorption behavior. Similar to oxyethylated alcohols and Tritons (Fainerman et al., 2009), the adsorption behavior of Pluronic agrees better with the two-state reorientation model. It has been shown that PEO-PPO-PEO triblock copolymers adopt different conformations as adsorption increases. The layer structure changes from a flat structure with all the segments lying at the interface at higher interfacial tension values to a brush-like structure where PEO segments are protruding into the bulk solution at lower interfacial tension values (Muñoz, Monroy, Ortega, Rubio, & Langevin, 2000). The molar areas of adsorbed molecules in states 1 and 2 (ω_1 and ω_2, $\omega_1 > \omega_2$) reported in Table 3, agree well with the value reported for the molar area (ω) of Frumkin model (Table 2). In turn, these parameters reflect the fact that the Pluronic molecules adopt an extended configuration at lower interfacial coverage (higher interfacial tension values) and a compact configuration at higher interfacial coverage (lower interfacial tension values). The parameter α, which characterizes the extra adsorption activity of the molecules in the unfolded state 1, is essentially the effect of the PEO chains adsorbed onto the interface at lower interfacial coverage (Fainerman et al., 2002). The parameter *b* is now related to the adsorption activity in state 2 with minimum specific interfacial area. That is the reason for obtaining a lower value as compared to that observed in Table 2 for Frumkin model (Fainerman et al., 2009).

Table 3: Input (ω_1, ω_2, α) and fitting (*b*) parameters used to fit the experimental data in Figure 5 with the reorentation model (equations (3)-(7)).

Surfactant	ω_1 (m^2/mol)	ω_2 (m^2/mol)	α	*b* (l/mol)	ε
Pluronic	4×10^6	1.6×10^6	9.5	9.96×10^6	0.4

4.3. Link with oil-in-water emulsion properties (summary)

We have shown that for Pluronic and Epikuron stabilized O/W emulsions, the system was more stable in the presence of NaTDC up to a BS concentration of 19.2 mM and 1.92 mM, respectively, than in the absence of BS (Jódar-Reyes et al, 2010). This indicated that bile salts were present at the oil-water interface, providing an electrosteric effect, which would increase the colloidal stability of the emulsion with respect to the blank (in the absence of NaTDC). These stability regimes agree well with the BS concentrations studied in interfacial tension measurements. In this sense, Pluronic-covered interface reached a BS saturation at a concentration between 10 and 30 mM (Figure 1) and Epikuron-covered interface is clearly disrupted at a BS concentration of 3mM (Figure 2). From the electrokinetic analysis of the emulsions, we also observed that the droplet surfaces became more negatively charged for both emulsions stabilized by either Pluronic or Epikuron when BS was added to the system (Jódar-Reyes et al, 2010; Torcello-Gómez et al., 2011b), indicating changes of the interfacial composition. The formation of a mixed surfactant-bile salt interfacial layer, as concluded from the interfacial tension study described above, could explain such results. For Pluronic-stabilized emulsion there was a significant increase in mobility (absolute values) at 19.2 mM NaTDC. However, the mobility did not significantly changed when increasing NaTDC concentration (64.2 and 95.8 mM), probably indicating a BS saturation of the interface. Hence, the saturation concentration of NaTDC is around 20 mM. These findings agree with the interfacial tension results, as it became increasingly difficult for the bile salt to adsorb into the more packed interfacial layers of Pluronic at 10 and 30 mM NaTDC.

At NaTDC concentrations well above its cmc, the destabilization of the system took place owing to micelle depletion (Jódar-Reyes et al, 2010). This destabilization occurred at lower concentrations of NaTDC for Epikuron-stabilized emulsion (13 mM NaTDC) than for Pluronic-stabilized emulsion (44 mM NaTDC).

In addition, the kinetics of phase separation was faster for Epikuron-stabilized emulsion in contrast to emulsion stabilized by Pluronic. This can be also explained by interfacial tension measurements, since the rate and extent of bile salt adsorption are higher at the Epikuron-covered interface as compared to the Pluronic system. In turn, this can be understood at the molecular level. In this sense, Pluronic molecules adopt a brush-like conformation at high interfacial coverage providing a steric hindrance, which is responsible for the improved resistance of Pluronic interfacial layer to accommodate bile salts. On the contrary, the more compact interfacial layer formed by Epikuron, due to the smaller molecular area of the phospholipids and the weak electrostatic repulsion between Epikuron molecules at the interface, make this phospholipids adsorbed layer more susceptible to disruption by bile salts. Furthermore, the combined effect of the greater affinity of NaTDC for the interface as well as the electrostatic repulsion between BS and Epikuron molecules at the interface also might contribute to this observation. Thus, Pluronic provides a higher stability to the emulsion in the presence of NaTDC than Epikuron corroborating the interfacial findings.

5. Conclusions

We have shown that the analysis of the interfacial behavior of a bile salt (NaTDC) in the presence of two different surfactants (Pluronic F68 and Epikuron 145V) at an oil-water interface provides a better understanding of the physicochemical properties observed in oil-in-water emulsions stabilized by the same surfactants after the addition of the same bile salt. The interfacial tension and dilatational rheology data suggested the formation of a mixed surfactant-bile salt interfacial layer after the addition of NaTDC onto the pre-adsorbed surfactant layer. However, Pluronic F68 appears more resistant to bile salt adsorption than Epikuron 145V at higher concentrations of NaTDC. In order to gain more information about interfacial structure, we also analyzed the interfacial tension isotherm of these

systems alone at the bare oil-water interface by means of a thermodynamic model (Frumkin isotherm). The theoretical fitting provides a larger interfacial molecular area of Pluronic F68 as compared to NaTDC. This agrees with the steric hindrance provided by its molecular conformation at the interface and might explain the higher resistance against the action of bile salt. Differently, theoretical fitting of the Epikuron isotherm gives a smaller molecular area than NaTDC. It seems that the compact interfacial layer of phospholipid molecules is readily disrupted by bile salt molecules. The use of a two-state reorientation model importantly improves the correlation with experimental data of Pluronic. This finding allows understanding the molecular organization of the interfacial layer. Interfacial conformation of poloxamer prevents the inclusion of NaTDC molecules into the layer. This interfacial behavior provides a plausible explanation to the higher stability found in Pluronic-stabilized emulsion under bile salt addition at higher concentrations of the bile salt, as compared to Epikuron-stabilized emulsion. This study demonstrates the crucial role played by interfaces in emulsion stability and hence, in control of lipid digestion.

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Different stability regimes of oil-in-water emulsions in the presence of bile salts

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ABSTRACT

In this work, we study the stability of emulsified olive oil-in-water emulsions at different bile salt (BS) concentrations. The effect of the interfacial properties of the emulsion is analyzed by using different emulsifiers (Epikuron 145V and Pluronic F68). Emulsion characteristics (electrophoretic mobility, average droplet size) are measured under the different conditions, and the stability of these systems is characterized by monitoring backscattering, using a Turbiscan. Adsorption of BSs at the emulsion interface would explain the higher stability at low bile salt concentrations as well as the more negative electrophoretic mobility when comparing with the emulsion in the absence of BS. The stability of the emulsion decreases above a critical BS concentration, which is higher when the emulsifier is Pluronic, and is much higher than the critical micelle concentration of the BS. Depletion flocculation induced by BS micelles is the destabilizing mechanism proposed at high BS concentrations.

1. **Introduction**

The mechanisms of lipid digestion and absorption are of great importance in the human diet (Singh, Ye, & Horne, 2009). During digestion, most lipids are present as oil-in-water (O/W) emulsions due to the mechanical stress they undergo and the effects of different stabilizing agents. Lipid digestion takes place in the stomach and in the small intestine. When the partially digested food moves from the stomach into the small intestine, it is mixed with bile and pancreatic secretions in the duodenum, which contain a variety of surface-active substances, e.g. bile salts (BSs), enzymes, proteins, and phospholipids (Mun, Decker, & McClements, 2007). It is well known that bile salts are important for lipid hydrolysis by lipases as well as for lipid absorption in the small intestine (Wickham, Garrood, Leney, Wilson, & Fillery-Travis, 1998). Different effects of BSs on such processes have been described (Mun et al., 2007): on the one hand, they inhibit the activity of lipases at O/W interfaces as the salts displace lipase molecules in the absence of colipase; on the other hand, high BS levels promote desorption of any other surface active material from the surface of emulsified lipids, and lipase/colipase can adsorb onto the interfacial layer of BS and promote digestion. In addition, the adsorption of negatively charged BS enhances the repulsion between the lipase and the interface, reducing the penetration of the lipase (Wickham et al., 1998).

The BS concentration in the above-mentioned processes is far above the critical micelle concentration (cmc), so that a great amount of micelles would be expected in the system. These micelles are important for the solubility of hydrolysis products. Several experimental studies on model O/W emulsions in the presence of different surfactants have shown that depletion flocculation of the emulsion can be induced by non-adsorbed surfactant micelles that are excluded from the interstitial space (Aronson, 1991; Bibette, 1991; McClements, 1994; Dimitrova & Leal-Calderón, 1999; Shields, Ellis, & Saunders, 2001; Dickinson & Ritzoulis, 2000; Radford & Dickinson, 2004; Wulff-Pérez, Torcello-Gómez,

Gálvez-Ruiz, & Martín-Rodríguez, 2009). For instance, certain dietary fiber is found to promote droplet flocculation through a depletion mechanism (Beysseriat, Decker, & McClements, 2006), and this may restrict the access of digestive enzymes to the lipids, and in turn depress lipid digestion. This depletion phenomenon has been found at very high surfactant concentrations in the case of colloidal systems (Jódar-Reyes, Martín-Rodríguez, & Ortega-Vinuesa, 2006).

In this work, we show that depletion flocculation of olive O/W emulsions can be induced by bile salt micelles, when high concentrations of BSs are involved, as is the case under the physiological conditions of the duodenum. For this, we have studied the colloidal stability of the emulsions at different bile salt concentrations. As we need to control the cmc of the bile salt, we have used sodium taurodeoxycholate (NaTDC), one of the main components in human bile salt (Arleth, Bauer, Gendal, Egelhaaf, Schurtenberger; & Pedersen, 2003; Friesen, Shanker, Crew, Smithey, Curatolo, & Nightingale, 2008) and its cmc has been well determined in the literature under different conditions. Also, we have studied the way in which the addition of BS affects the electrokinetic behavior and droplet size of the olive O/W emulsions and the way in which the type of emulsifier (anionic and non-ionic) alters such an effect. The ionic emulsifier (Epikuron 145V) is a natural phospholipid-based emulsifier, and the non-ionic emulsifier (Pluronic F68) is a synthetic block copolymer. These, instead of proteins, have been chosen as emulsifiers because they provide greater protection against lipase-induced destabilization in the presence of bile salts (Mun et al., 2007).

Results from the different techniques point to adsorption of the bile salt at the emulsion interface, making it difficult to control the amount of BS micelles in the system. In addition, there are reports on the formation of BS and emulsifier mixed micelles (Arleth et al., 2003). Therefore, it is not appropriate to make a theoretical analysis of the depletion phenomenon (Wulff-Pérez et al., 2009) for this system.

2. Materials and methods

2.1. Materials

All chemicals used were of analytical grade. The water was purified by a Milli-Q Academic Millipore system. The pH was controlled using buffered solutions (phosphate, pH 7), maintaining the ionic strength constant at a value of 1.13 mM. The olive oil (Sigma) was purified with activated magnesium silicate (Florisil, Fluka). The ionic emulsifier, which presents a negative charge at pH 7, was Epikuron 145V (around 800 g/mol), a deoiled, wax-like phosphatidylcholine (PC) enriched soybean lecithin (min. 45% PC) from Cargill. The non-ionic emulsifier used was Pluronic F68 ($PEO_{75}PPO_{30}PEO_{75}$, 8350 g/mol) from Sigma. The bile salt used, sodium taurodeoxycholate (NaTDC, 521.69 g/mol) at 97% purity (Sigma), is negatively charged and presents a cmc of 0.05 wt% in water, 25 ºC, 150 mM NaCl , by tensiometry (Tejera-García, 2008), and by spectrophotometry (Carey & Small, 1969). In pure water, the cmc is 0.08 wt% at 20 ºC and 0.09 wt% at 30 ºC (Carey et al., 1969). To gain data at BS concentrations above and below the cmc, we used the following BS concentrations: 0, 0.025, 0.1, 1, 3.35 and 5 wt%.

2.2. Preparation of emulsions

We needed O/W emulsions that would remain stable before adding the BS solution. When Epikuron was used, this anionic emulsifier was previously dissolved in the buffered solution with 150 mM NaCl, at 50 ºC and 700 rpm during 2 h. The corresponding Epikuron concentration was 2.07 wt%. A pre-emulsion was prepared with 1.648 ml of purified olive oil and the buffered solution of emulsifier up to a final volume of 50 ml, and mixed with a Diax 900 homogenizer for 5 min at 18,800 rpm. Then, it was homogenized in an EmulsiFlex-C3 (11 cycles,

15,000psi). The final composition of the emulsion was 3 wt% oil, 2 wt% Epikuron. The average droplet diameter (D = 250 nm) and the polydispersity index (PDI = 0.10) did not significantly change after 4 h. The destabilization of the system took place versus the time, but it was reversible (flocculation) by shaking. Negligible differences were found when NaCl was absent from the preparation, and only a slightly larger droplet size resulted when the Epikuron concentration was reduced by half. The preparation protocol we finally chose was the one previously described (2 wt% Epikuron) as it guaranteed that the interface was completely covered by emulsifier. When Pluronic F68 was used, this emulsifier was previously dissolved in the buffered solution without heating, at a concentration of 0.65 wt%. NaCl was not used. The rest of the procedure was similar to that followed for Epikuron and the final composition of the emulsion was 3 wt% oil, 0.63 wt% Pluronic. Again, the average droplet diameter (D = 160 nm) and the polydispersity index (0.06) did not significantly change, even after 24 h, as the system remained stable. The reproducibility of these results for the two types of emulsions was tested.

2.3. Colloidal stability

The colloidal stability of the emulsion was evaluated from the diffuse reflectance, $R(\theta = 135^{o})$, as a function of the height , h, of a vertical sample tube by using a Turbiscan MA 2000 (Formulaction, l'Union, France, incident wavelength λ = 850 nm). This device enables the detection of two kinds of destabilization phenomena: particle migration (creaming, sedimentation) which are often reversible by mechanical agitation, and particle size variations (coalescence, aggregation) (Mengual, Meunier, Cayre', Puech, & Snabre, 1999). Both the transmitted and backscattered light from the emulsion depend on the droplet size and concentration. However, such dependence differs when the system is above or below certain critical size and particle concentration.

Backscattering increases with the volume fraction (ϕ) for $\phi_c < \phi < \phi_s$ where ϕ_c is the critical volume fraction and ϕ_s the saturation volume fraction. The critical volume fraction corresponds to the concentration at which photons begin to be transmitted. For $\phi < \phi_c$, only the signal in transmission of the Turbiscan should be used. The transmission flux (T) exponentially decreases with the particle volume fraction until it reaches the value $T = 0$. In our experiments, the transmitted signal was zero, and thus the volume fraction of droplets was above the critical volume fraction and therefore only the reflectance diffuse (backscattering) was analyzed.

The backscattering flux (diffusive reflectance) increases with the mean particle diameter when the particles are smaller than the incident wavelength and decreases with the mean diameter for particles larger than λ. Therefore, it is necessary to examine all the three parts of the graph: top, middle, and bottom. Changes at the top and bottom of the graph are associated with particle migration whereas changes in the middle correspond to particle size variation and/or changes in particle concentration.

For a better visualization of the change in the colloidal stability of the emulsion, we will present the graphs in the reference mode, i.e. the residual diffuse reflectance at time t, $R_t - R_{t=0}$, as a function of the height, h, of the vertical sample tube.

The kinetics of phase separation was also analyzed from the residual diffuse reflectance at the center of the sample tube versus time.

To determine how the colloidal stability of the emulsion changes by increasing the concentration of bile salt added to the system, the samples were prepared by mixing 5 ml of emulsion with 5 ml of a buffered solution with NaTDC and 150 mM NaCl. A blank was also made without adding NaTDC. A volume of 7 ml of each sample was introduced in the Turbiscan tube.

2.4. Emulsion droplet size

The mean hydrodynamic diameter (*D*) of the droplets, and the polydispersity index (PDI) of the emulsion were determined at 25 ºC from dynamic light scattering measurements using ALV®-NIBS/HPPS (ALV-Laser VertriebsgesellschaftmbH, Langen, Germany). This device has been designed to analyze highly concentrated samples. A combination of high performance particle sizer technology and a non-invasive backscattering method, minimized multiple scattering. The samples were prepared by taking 0.5 ml of the samples used in the stability analysis and diluting them in a buffered solution up to a final volume of 2.5 ml.

2.5. Electrokinetic characterization

The electrophoretic mobility and the solution conductivity were measured by using Nanozeta dynamic light scattering analyzer (Malvern Instruments, UK).

To determine how the presence of bile salt in the system affected the interfacial properties of the emulsion, we measured the electrophoretic mobility (μ_e) of the samples at the different NaTDC concentrations, since one of the reasons for system destabilization at high bile salt concentrations could be related to a loss of charge of the emulsion under these extreme conditions. We chose a particle concentration of 0.02 wt% as it gave good quality measurements in the Nanozeta device.

3. Results and discussion

In the following, the results on the colloidal stability of O/W emulsions in the presence of different concentrations of the bile salt NaTDC will be presented

and discussed. The explanations for the different behaviors of the systems will be supported by data from droplet size and electrokinetic analysis.

3.1. Colloidal stability

In this section we explain how the colloidal stability of the emulsion changes by increasing the concentration of bile salt added to the system. In addition, we analyze the effect of the electrolyte (NaCl) concentration and the type of emulsifier (ionic, non-ionic) on these results. First, we show the results by fixing time and by observing the change in backscattering along the vertical tube (stability curves). In this way, we can identify the destabilization phenomena (creaming, sedimentation, aggregation). Secondly, we fix a position along the tube and study the change in the backscattering versus time. We then record information on the kinetics of the phase separation.

3.1.1. Stability curves

3.1.1.1. Epikuron-emulsified emulsions

Starting with the Epikuron-emulsified O/W emulsion, just after mixing the emulsion and the buffered solutions at the different NaTDC concentrations, we could see by the naked eye that the sample at a final concentration of 5 wt% NaTDC phase-separated (creamed), as it can be seen in Figure 1.

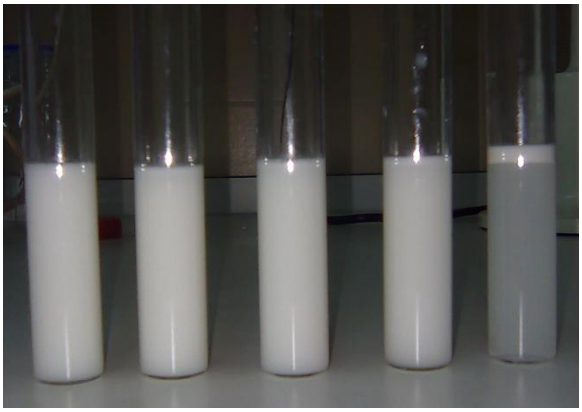

Figure 1: Oil-in-water emulsion (emulsified with Epikuron) mixed with bile salt NaTDC. NaCl 150 mM. From left to right: [NaTDC] = 0, 0.025, 0.1, 1, 5 wt%. The volume of the sample in the tube is 7 ml.

We shook the samples to get the reference curve at time t = 0 s in Turbiscan ($R_{t=0}$) for each NaTDC concentration, as shown in Figure 2. The curves were similar at low bile salt concentrations, but clearly differed when a high amount of NaTDC is present in the system (5 wt%). The slight decrease in the middle part of the graph found at 0.1 and 1 wt% could only be due to small differences in the particle concentration when the samples were prepared, as, on the one hand, we will see that the mean droplet size was below the wavelength, and therefore, backscattering increased with the mean particle diameter. On the other hand, there was no increase in other parts of the graph, indicating particle migration (creaming or sedimentation). On the contrary, there was a decrease in the diffuse reflectance at the bottom (left part of the graph) and the top (right part of the graph) corresponding to the lower and upper limits of the sample, respectively. An increase at the top was observed, however, when the highest BS concentration was considered (5 wt%), even though the sample was shaken. Then, a quick phase separation occurred at this NaTDC concentration.

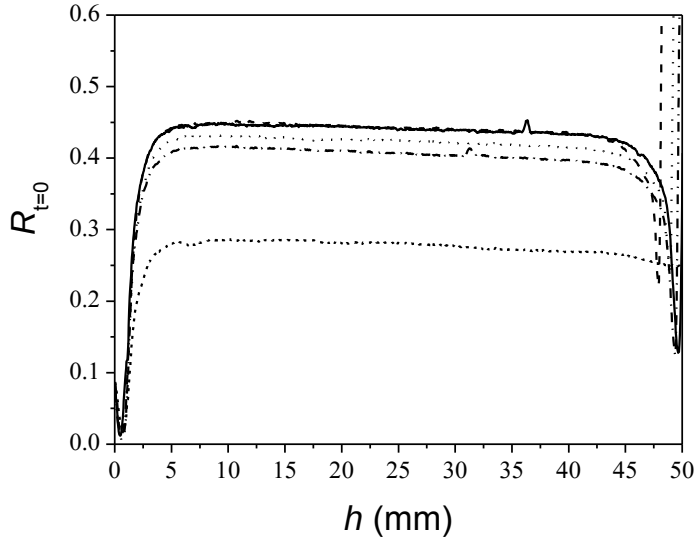

Figure 2: Reference stability curves (at t = 0 s) for the O/W emulsion (emulsified with Epikuron) at the different NaTDC concentrations (in %wt): 0 (solid), 0.025 (dash), 0.1 (dot), 1 (dash dot), 5 (short dash). NaCl 150 mM

At half an hour after mixing, all samples showed a creaming phenomenon. The curves are presented in the reference mode (Figure 3). For NaTDC concentrations up to 1 wt%, the backscattering increased with respect to the reference curve at the top (increase of the dispersed phase) and decreased at the bottom (the concentration of particles diminished), remaining constant in the middle part, which corresponds to a creaming process. It bears noting that the curve for the blank (solid) presents higher values at the top than those in the presence of NaTDC up to 1 wt%. Thus, the system can be considered more stable in the presence of these NaTDC concentrations. This result could indicate that bile salt was present at the O/W interface, providing an electrosteric effect, which would increase the colloidal stability of the emulsion with respect to the blank. As the emulsifier was thought to cover the interface completely, a displacement of such an emulsifier by the bile salt (as reported for other surfactants (Beysseriat et al., 2006)

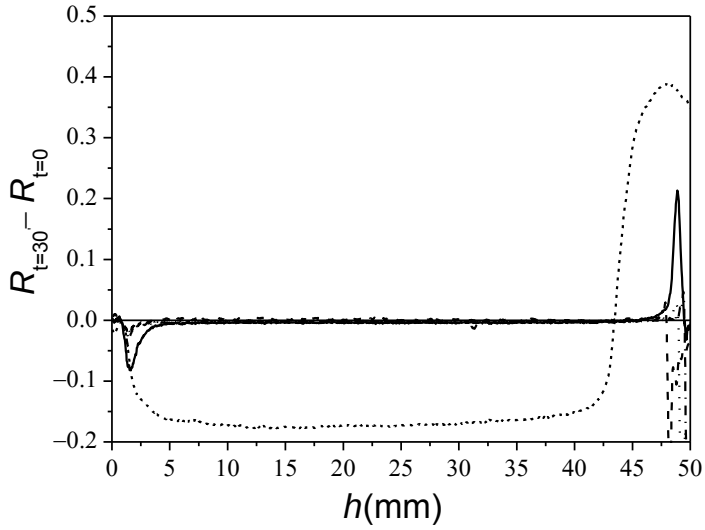

Figure 3: Stability curves in reference mode for the O/W emulsion (emulsified with Epikuron) 30 min after mixing at the different NaTDC concentrations (in %wt): 0 (solid), 0.025 (dash), 0.1 (dot), 1 (dash dot), 5 (short dash). NaCl 150 mM

and phospholipids (Mun et al., 2007; Wickham et al., 1998)) or a co-adsorption are the two mechanisms proposed. We will return to this point after presenting electrokinetic data. When the BS concentration was 5 wt%, the backscattering sharply increased with respect to the reference curve at the top of the sample due to a higher particle concentration, and decreased in the rest of the curve, reflecting a lower particle concentration. At this NaTDC concentration, the system presents a lower colloidal stability than in the absence of bile salt. The high amount of BS micelles involved could provoke the flocculation of the emulsion by micelle depletion. This phenomenon should be reversible by micelle dilution (Jódar-Reyes et al., 2006).

Creaming can be coupled with coalescence or flocculation. Both phenomena lead to larger droplet size, and therefore, an increase in the backscattering (when the droplet size is lower than 850 nm). However, coalescence

is irreversible and leads to a fusion of the interfaces, whereas flocculation is a reversible aggregation of the particles. In order to ascertain whether our samples had coalesced, we shook them gently after 4 h from the mixing and plotted their stability curves. For all bile salt concentrations, except for 5 wt%, the curves resembled the reference curve, demonstrating the reversibility of the destabilizing mechanism. At 5 wt% NaTDC, greater backscattering at the top and less in the rest with respect to the reference curve appear to indicate irreversible destabilization. However, this reasoning could not be valid, as the high amount of micelles is kept under shaking, and the system could again quickly destabilize.

We designed the following set of experiments in order to test the hypothesis of flocculation of the system by depletion of bile salt micelles at 5 wt% NaTDC. This consisted of checking the reversibility of the system by micelle dilution. We prepared three samples. For sample 1, we shook the emulsion in the presence of 5 wt% NaTDC, took 0.2 ml and mixed them with buffer 150 mM NaCl up to 10 ml. The final bile salt concentration was 0.1 wt%. For sample 2, we also shook the emulsion in the presence of 5 wt% NaTDC, took 0.2 ml and mixed them with a buffered solution 150 mM NaCl containing NaTDC up to 10 ml and a final bile salt concentration of 5 wt%. Sample 3 was prepared by mixing 0.2 ml of the blank with buffer 150 mM NaCl up to 10 ml, and thus, the final NaTDC concentration was 0. Reference curves (t = 0 s) in the three cases were approximately similar. Therefore, aggregates that had formed at 5 wt% broke, even when maintaining the concentration of micelles in the system. As the particle concentration was much lower than in previous experiments, destabilization of the system should take place more slowly. The stability curves 4 h after dilution in the reference mode for the three samples are presented in Figure 4. Reversibility by micelle dilution was thus demonstrated, since, unlike the sample at 5 wt% NaTDC, the sample at a final bile salt concentration of 0.1 wt% remained stable after 4 h.

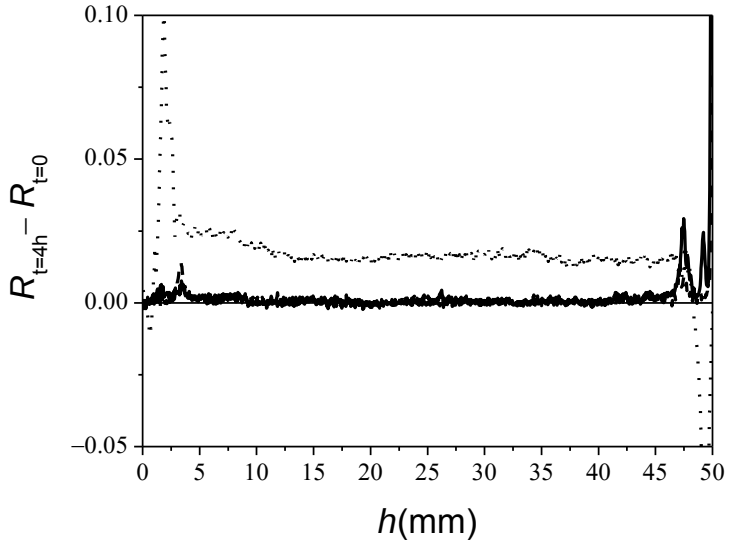

Figure 4: Stability curves in reference mode for the O/W emulsion (emulsified with Epikuron) 4 h after dilution at the different NaTDC concentrations (in %wt): 0 (solid), 0.1 (dash), 5 (dot). NaCl 150 mM.

It would be worth analyzing how the above results (made at 150 mM NaCl) changed at low electrolyte concentrations: on the one hand, the emulsion should present a more negative interfacial charge, and, thus higher colloidal stability; on the other hand, the cmc of the bile salt would increase (Carey et al., 1969). Therefore, we would expect that the system to become more resistant to the destabilization by micelle depletion.

There will constantly be a certain amount of NaCl coming from the original emulsion. To minimize such an amount in our experiments, we prepared the samples by mixing 3.3 ml of emulsion with 6.7 ml of a buffered solution with NaTDC, and thus, the final NaCl concentration was 49.5 mM. A blank was also made by not adding NaTDC. As the emulsion particle concentration was reduced, we repeated the experiments done at 150 mM NaCl by using such a particle

concentration. A new bile salt concentration of 3.35 wt% was also tested. At this NaTDC concentration, the destabilization of the emulsion was also observed at 150 mM NaCl. However, at any time, the system was less unstable than at 5 wt% NaTDC. Dilution experiments showed that the sample was reversible by micelle dilution, and therefore, flocculation by bile salt micelle depletion was also the destabilizing mechanism proposed at 3.35 wt% NaTDC.

At low electrolyte concentrations, the reference curves at low bile salt concentrations were similar, but differed when the NaTDC concentration was equal or higher than 3.35 wt%. In agreement with the results for 150 mM NaCl, at 3.35 wt% NaTDC the emulsion quickly became unstable. Figure 5 presents the stability curves corresponding to 45 min after mixing in the reference mode for the blank and the samples at the different NaTDC concentrations. For NaTDC concentrations up to 0.1 wt%, the backscattering increased slightly with respect to the reference curve at the top and a decreased at the bottom, remaining constant in the middle part, which corresponded to a creaming process. The curve for the blank (solid) presents a slightly higher value at the top than those ones in the presence NaTDC. Then, we could say that the system is slightly more stable in the presence of NaTDC concentrations up to 0.1 wt%. At 1 wt% NaTDC there was also a slight decrease in the middle part of the curve, while the top part showed higher values than for the blank. Therefore, the emulsion was less stable in the presence of this bile salt concentration. When the NaTDC concentration was 3.35 wt%, the backscattering strongly increased with respect to the reference curve at the top of the sample, and decreased in the rest of the curve. The system showed lower colloidal stability than when bile salts are absent, and lower also than that corresponding to 1 wt%, which is consistent with the hypothesis of flocculation of the emulsion by micelle depletion as this effect intensifies when the number of micelles increases. We can see such dependence in the pair potential between two

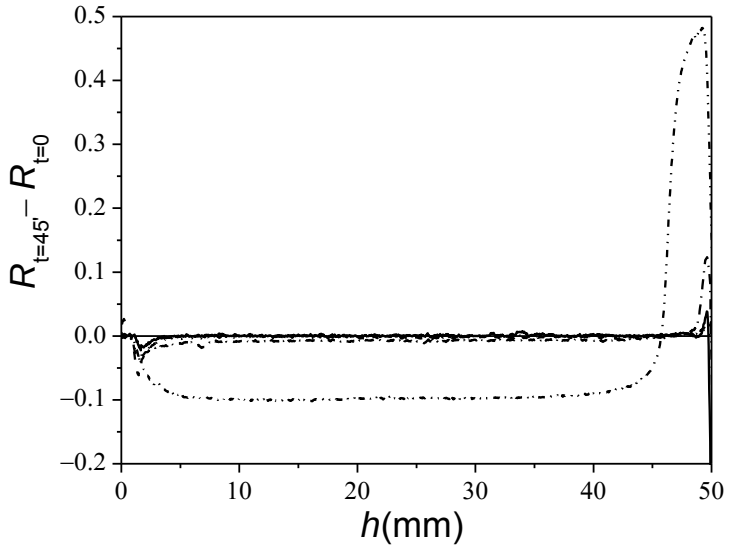

Figure 5: Stability curves in the reference mode for the O/W emulsion (emulsified with Epikuron) 45 min after mixing at the different NaTDC concentrations (in %wt): 0 (solid), 0.025 (dash), 0.1 (dot), 1 (dash dot), 3.35 (dash dot dot). NaCl 49.5 mM.

identical spherical particles resulting from spherical micelle depletion (equation 1), which is a rough model for the system analyzed in the present work. This potential was adapted by Jódar-Reyes et al. (Jódar-Reyes et al., 2006) from the model of Walz and Sharma (Walz & Sharma, 1994) for the interaction potential between two spherical particles due to the presence of spherical macromolecules. The way it was adapted, together with the limitations of the model in the case of surfactant micelles, are described in (Jódar-Reyes et al., 2006).

$$\frac{V_{dep}(H)}{k_B T} = \begin{cases} +\infty, & H < 0, \\ -\rho_\infty \pi \left[\frac{4}{3} r^3 + 2r^2 a - r^2 H - 2raH + \frac{aH^2}{2} + \frac{H^3}{12} \right], & 0 \le H < 2r, \\ 0, & H \ge 2r \end{cases} \quad (1)$$

where H is the shortest distance between the particle surfaces, a is the radius of the particle, and r is the radius of the micelle, ρ_∞ is the number of micelles per volume unit in the system, k_B is the Boltzmann's constant, and T is the temperature.

This sample was again reversible by micelle dilution.

To analyze the effect of the electrolyte concentration on the stability behavior of the emulsion, we compared the stability curves 45 min after mixing in the reference mode for the blank, and the sample at 3.35 wt% NaTDC, at low and high electrolyte concentrations (Figure 6). Significant differences were not detected. In the absence of bile salt, the emulsion at the lower electrolyte concentration is only slightly more stable. We will discuss the differences in electrokinetic charge below. At 3.35 wt%, the emulsion was less stable at low NaCl concentration, as discussed above for 1 wt%. Therefore, contrary to what might be expected, the emulsion became less resistant to the destabilization by micelle depletion at low electrolyte concentrations. This can be explained by taking into account that with a decreased electrolyte concentration in the system, the "effective

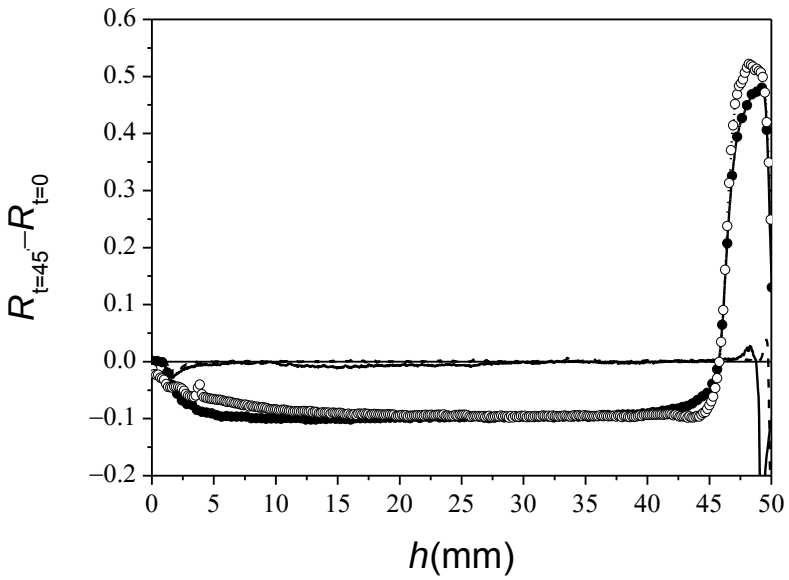

Figure 6: Effect of the electrolyte concentration on the stability curves in the reference mode for the O/W emulsion (emulsified with Epikuron) 45 min after mixing at the different NaTDC concentrations (in %wt): 0 (solid line), 3.35(solid circles) at 150 mM NaCl; 0 (dashed line), 3.35 (open circles) at 49.5 mM NaCl.

size" of charged micelles, which would be equal to the physical size plus the electrostatic screening length or Debye length (this is, $r + \kappa^{-1}$), increases. With Eq. (2), we show the Debye length for a monovalent salt, where ε_o is the vacuum permittivity, ε the dielectric constant of the medium, c the salt concentration in mol/L, and e the charge of an electron

$$\kappa^{-1} = \sqrt{\frac{\varepsilon\varepsilon_o k_B T}{2ce^2}} \,. \tag{2}$$

The insertion of the "effective size" of the micelle in Eq. (1) would enhance the depletion effect at low electrolyte concentrations (Jódar-Reyes et al., 2006).

3.1.1.2. Pluronic-emulsified emulsions

Up to now, we have seen that the O/W emulsions synthesized with the ionic emulsifier Epikuron 145V show a dependence of the colloidal stability with the concentration of an anionic bile salt (NaTDC). Such stability increases at low NaTDC concentrations, but diminishes at bile salt concentrations well above its cmc due to flocculation by depletion of BS micelles. To ascertain whether these results are affected by the type of emulsifier used in the preparation, we repeated the experiments with the non-ionic emulsifier Pluronic F68. The particle concentration was that used for Epikuron-emulsified emulsions at 150 mM NaCl. The stability curves 45 min after mixing in the reference mode for the blank and the samples at the different NaTDC concentrations are shown in Figure 7. For NaTDC concentrations up to 1 wt%, the backscattering increased at the top and decreased at the bottom, remaining constant in the middle part, as in a creaming process. Again, the system was more stable in the presence of NaTDC concentrations up to 1 wt%, which could indicate that bile salt is present at the O/W interface. At a BS concentration of 3.35 wt% the emulsion is just slightly less stable than in the absence of bile salt. The backscattering increased with respect to the reference curve at the top, and decreased in the rest of the curve. At 5 wt% NaTDC such a destabilization was more notable. Again, the high amount of BS micelles involved in the two last situations could have provoked the flocculation of the emulsion by micelle depletion. This phenomenon was reversible by micelle dilution. When we compare with results of Epikuron-emulsified emulsions (Figures 3 and 5), we find that the emulsions made by using Pluronic are more resistant to the destabilization by micelle depletion, as is evident at 3.35 and 5 wt% NaTDC.

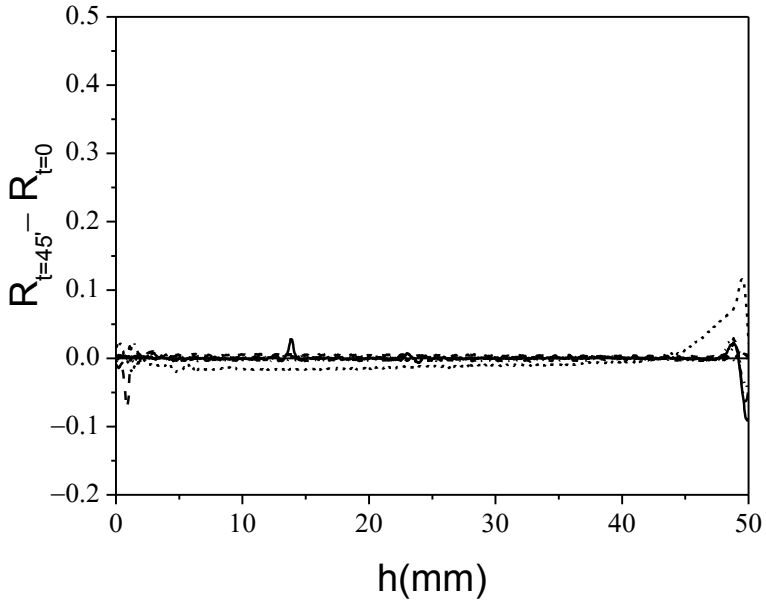

Figure 7: Stability curves in reference mode for the O/W emulsion (emulsified with Pluronic) 45 minutes after mixing at the different NaTDC concentrations (in %wt): 0 (solid), 0.025 (dash), 0.1 (dot), 1 (dash dot), 3.35 (dash dot dot), 5 (short dash).

3.1.2. Kinetics of phase separation

Changes in the middle part of the vertical tube may have been due to particle migration and to particle size variations. The stability curves (by fixing time) indicated that these changes took place at bile salt concentrations that make the system less stable than in the absence of NaTDC. By analyzing this change versus time, we can gain information on the kinetics of destabilization, i.e. how fast the emulsion loses its colloidal stability, at the different bile salt concentrations.

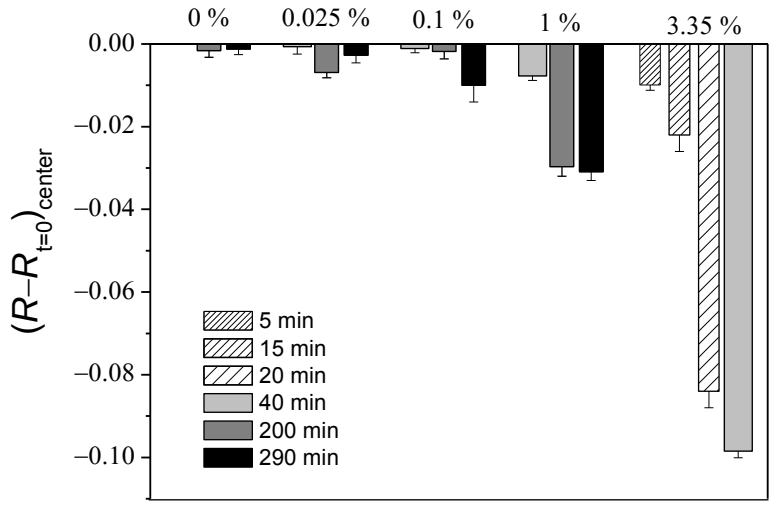

Figure 8: Residual diffuse reflectance at the center of the sample tube for the O/W emulsion (emulsified with Epikuron) at the different NaTDC concentrations (in %wt), at different times.

3.1.2.1. Epikuron-emulsified emulsions

Results for the Epikuron-emulsified emulsion at low ionic strength are shown in Figure 8. There was no significant change in backscattering up to 0.1 wt% NaTDC. At 1 wt% bile salt, diffuse reflectance decreases at the beginning due to particle migration to the top of the sample, and then remains constant, perhaps due to competition between the decreased particle concentration (*R* decreases), and the increased particle size (*R* increases). At 3.35 wt% NaTDC, the decrease in *R* at the beginning becomes stronger as the destabilization of the system is more important and takes place quickly than at lower bile salt concentrations.

3.1.2.2. Pluronic-emulsified emulsions

Figure 9 shows the kinetics of phase separation for the Pluronic-emulsified emulsion. In this case, backscattering up to 1 wt% NaTDC does not significantly change for more than 20 h. At 3.35 wt% bile salt, diffuse reflectance decreases slowly due to particle migration to the top of the sample tube. At 5 wt% NaTDC, *R* decreases at the beginning and increases after more than 2 h as the increase in particle size becomes stronger. Clearly, the destabilization of the system in the presence of bile salt is greater and takes place more quickly for the Epikuron-emulsified emulsion.

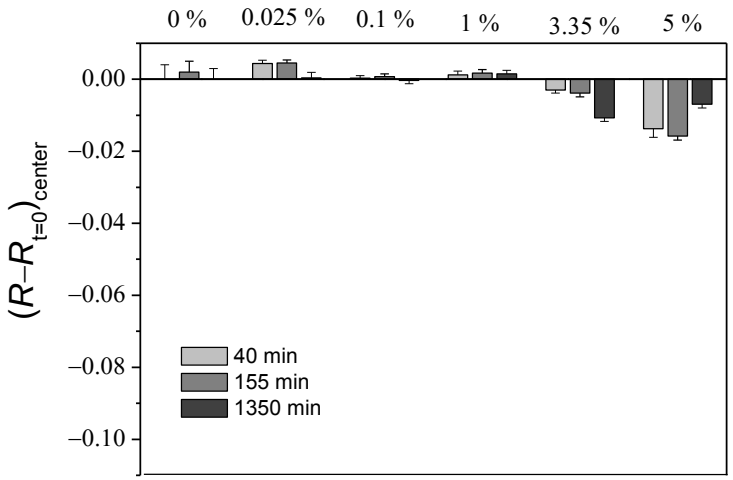

Figure 9: Residual diffuse reflectance at the center of the sample tube for the O/W emulsion (emulsified with Pluronic) at the different NaTDC concentrations (in %wt), at different times.

3.2. Emulsion droplet size and polydispersity

Dilutions of the samples used in the stability analysis in the buffered solution were made, on one hand, without bile salt, and, on the other hand, by maintaining the corresponding NaTDC concentration. With these two sets of

experiments, we can determine whether the NaTDC concentration affects the size distribution of the emulsion droplets, and also whether the dilution of the amount of micelles in the system influences such a distribution.

3.2.1. Epikuron-emulsified emulsions

When Epikuron was used as emulsifier (D = 250 nm, PDI = 0.10), data recorded around 190 min after the samples for Turbiscan measurements were mixed, showed that only for the samples at or above 3.35 wt% NaTDC and only by maintaining the original bile salt concentration did the mean diameter of the droplet and the polydispersity index (D = 570 nm and PDI = 0.15) significantly change. When dilution was made with a buffered solution in the absence of bile salt, the sample at 5 wt% NaTDC in the Turbiscan experiments presented a final BS concentration of 1 wt%, and the corresponding mean diameter and PDI were 220 nm and 0.07, respectively. These results support the hypothesis of a destabilization of the system at 5 wt% NaTDC due to a flocculation mechanism that is reversible by the dilution of the micelles. The same was checked for 3.35 wt% NaTDC.

Four hours after mixing, we shook the samples and again measured the size and polydispersity. For the samples diluted in buffer without bile salt, the results were similar to those found previously. Thus, reversibility by shaking was demonstrated. However, by keeping the NaTDC concentration, large aggregates were detected in the system at 5 wt% (D = 8000 nm, PDI = 0.25). This is consistent with the results found with Turbiscan.

There were no significant differences when these experiments were repeated at low electrolyte concentrations.

3.2.2. Pluronic-emulsified emulsions

To analyze the effect, exerted on the above results, by using another type of emulsifier in the preparation of the emulsion, we repeated the experiments with Pluronic F68 (D = 160 nm, PDI = 0.06). One hour after mixing, no differences in the mean size were detected, taking into account the device error (D = (180 ± 40) nm at 3.35 wt% NaTDC and D = (190 ± 40) nm at 5 wt% NaTDC).

Again, these results show that Pluronic-emulsified emulsions were more resistant to flocculation by micelle depletion than were the Epikuron-emulsified emulsions.

3.3. Electrokinetic behavior

3.3.1. Epikuron-emulsified emulsions

For the emulsions prepared with Epikuron, major differences were found when the dilution was made in buffer (μ_e = (–5.37 ± 0.06) μm cm/Vs, conductivity = 0.229 mS/cm) or in a buffered solution 150 mM NaCl (μ_e = (–0.906 ± 0.016) μm cm/Vs, conductivity = 16.7 mS/cm). The droplets are negatively charged due to the presence of Epikuron at the interface.

We also measured the electrophoretic mobility of the three samples used in the experiments described above to check the reversibility by dilution with Turbiscan, at 150 mM and 49.5 mM NaCl. The results are shown in Table 1. It bears noting that for the sample with the lowest stability (5 wt%), the system presents strongly negative mobility. Thus, the destabilization of the emulsion is not due to the loss of charge of the system. On the other hand, in the presence of bile salt, the emulsion becomes more negative, indicating changes at the interface by displacement of the emulsifier by NaTDC or by co-adsorption. There are reports on

Table 1: Electrokinetic behavior of Epikuron-emulsified O/W emulsions at different bile salt concentrations. Effect of NaCl concentration. μe: electrophoretic mobility.

[NaCl] (mM)	**[NaTDC]** (%wt)	μ_e (μm cm/Vs)	**Conductivity** (mS/cm)
49.5	0	–5.86 ± 0.07	0.229
	0.1	–8.55 ± 0.03	0.330
	5	–7.9 ± 0.3	4.5
150	0	–0.89 ± 0.08	17.1
	0.1	–3.83 ± 0.14	16.9
	5	–4.80 ± 0.15	19.4

the displacement of Tween80 adsorbed onto O/W emulsions by bile extract on using zeta potential data (Beysseriat et al., 2006). When the data were compared at the two electrolyte concentrations, the emulsion presented a significantly higher charge (in absolute value) at the lower electrolyte concentration. However, these differences were not evident in the stability results.

3.3.2. Pluronic-emulsified emulsions

We found that O/W emulsions emulsified with Epikuron had a strongly negative electrokinetic charge in the presence of the bile salt NaTDC, which was higher (in absolute value) than the corresponding to the original emulsion. The results for using a non-ionic emulsifier in the preparation of the emulsion, particularly Pluronic F68, are shown in Table 2. In the absence of bile salt, the emulsion showed a slightly negative charge. When BS was added to the system, the interface became more negative. Again, displacement and/or co-adsorption of the emulsifier by the bile salt could explain such results. There was a marked increase in mobility at 1 wt% NaTDC as the displacement was favored at high bile salt concentrations (Beysseriat et al., 2006). At 5 wt% the mobility was similar to that

4. Results

Table 2: Electrokinetic behavior of Pluronic-emulsified emulsions at different bile salt concentrations.

[NaTDC] (%wt)	μ_e (μm cm/Vs)	**Conductivity** (mS/cm)
0	–0.36 ± 0.03	0.105
0.025	–3.03 ± 0.04	0.142
0.1	–2.631 ± 0.015	0.426
1	–6.34 ± 0.24	1.61
3.35	–7.76 ± 0.19	4.86
5	–6.464 ± 0.017	6.3

found at lower NaTDC concentrations, perhaps indicating that BS saturation at the interface was reached. The fact that the emulsions made by using Pluronic were more resistant to destabilization at high NaTDC concentrations than those made with Epikuron cannot be explained by using these electrokinetic results.

4. Conclusions

Different stability regimes were found for olive oil-in-water emulsions as a function of the concentration of bile salt (NaTDC) added to the system. The colloidal stability of these emulsions increased in the presence of bile salt up to a critical concentration. Above this concentration, which is higher than the cmc of the bile salt, the system became less stable. Depletion flocculation induced by bile salt micelles was the destabilizing mechanism proposed at high BS concentrations: unstable emulsions were re-stabilized by decreasing the micelle concentration in the system.

The emulsion became less resistant to the destabilization by micelle depletion at low electrolyte concentrations, as the effective size of charged micelles increased and, consequently, the depletion effect augmented. For Epikuron-emulsified emulsions, the system was more stable in the presence of NaTDC up to

(at least) 20×cmc at 150 mM NaCl. However, at 49.5 mM NaCl, the system was already less stable at a bile salt concentration of 12.5×cmc than in the absence of NaTDC.

The critical bile salt concentration at which the stability regime of the emulsion changed from more stable to less stable than in the absence of BS, depended on the type of emulsifier used in the preparation. This BS concentration was higher when the non-ionic emulsifier Pluronic F68 was used instead of the ionic emulsifier Epikuron 145V. At 49.5 mM NaCl, the system was more stable in the presence of NaTDC up to 12.5×cmc, and even at 42×cmc, the emulsion was only slightly less stable than in the absence of bile salt. The kinetics of phase separation of the emulsion was slower in the first case.

Displacement of the emulsifier by the bile salt and/or co-adsorption at the emulsion interface would explain the increase in the colloidal stability at low bile salt concentrations as well as the electrokinetic behavior of these emulsions. Further work needs to be done in order to determine which of these processes take place.

As a final conclusion, this work presents novel results on the effects of bile salts on the colloidal stability of O/W emulsions, and these findings should be considered in order to understand the mechanisms involved in lipid digestion.

Acknowledgements

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4.4. How interfacial and bulk properties of Pluronic F68 and F127 influence interaction with bile salts an affect the rate of lipid digestion.

4. Results

Interactions between Pluronics and bile salts in aqueous phase, at interfaces and in emulsions (Submitted)

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ABSTRACT

The use of triblock copolymers from the family of Pluronics to bestow stealth properties to colloidal-based nanocarriers is well known in the pharmaceutical field. Also, Pluronics are being introduced in food research in order to delay lipid digestion, owing to the steric hindrance provided by the adsorbed interfacial layer stabilizing oil-in-water emulsions. It has been shown recently that differences in the molecular structure of different Pluronics, such as length of hydrophilic and hydrophobic chains, influence the rate of lipid digestion. Since bile salts play a crucial role in the lipid digestion process, the aim of this work is to analyze the interactions between the emulsifier (Pluronic F127 or F68) and a model bile salt (NaTDC) when the latter is added at physiological concentrations. These interactions are studied at the Pluronic-covered oil-water interface, in Pluronic-stabilized emulsions, and in the continuous (aqueous) phase of such emulsions. This work has been carried out with a complete and original combination of state-of-the-art techniques such as micro-differential scanning calorimetry, interfacial tension, dilatational rheology, and scanning electron microscopy. As a result, Pluronic F127 showed to be more resistant to displacement by bile salt than F68 at the oil-water interface due to the larger steric hindrance and interfacial coverage provided by this larger molecule. In addition, Pluronics have the ability to compete for the oil-water interface and interact in the bulk with the bile salt. Concretely, Pluronic F127 seems to interact with more molecules of bile salt in the bulk, thus hindering their adsorption onto the oil-water interface. As a conclusion, Pluronic F127 affects to a larger extent the ability of bile salt to promote the further cascade of lipolysis in the presence of lipase owing to a combination of interfacial and bulk events. These findings are relevant towards the rational design of functional foods controlling lipid digestion or having specific physiological response.

1. Introduction

Biocompatibility of triblock copolymers with plasma-derived fluids is one of the main reasons why these materials, also known under their commercial names as Pluronics or Poloxamers, have been extensively used in the biomedical field for drug delivery, among other applications (Kabanov, Batrakova, & Alakhov, 2002; Sezgin, Yüksel, & Baykara, 2006; Ma, Xu, Wang, Nie, & Pan, 2008). Their molecular structure provides them with an amphiphilic character: a hydrophobic poly-propylene oxide (PPO) central block linked to hydrophilic poly-ethylene oxide (PEO) end blocks. Thus, monomers can aggregate in aqueous solution upon heating at a fixed concentration or upon increasing concentration at a fixed temperature, building up micelles with a PPO core and a PEO corona. In addition, they are also surface active, thus they have been used as protective coating for nanocarriers (Harper et al., 1991; Tan, Butterfield, Voycheck, Caldwell, & Li, 1993; Jackson, Springate, Hunter, & Burt, 2000; Jain et al., 2008), with the central PPO block adsorbed onto hydrophobic interfaces, whereas the two lateral PEO chains remain in the hydrophilic phase, forming a steric bulky layer. It seems that the protective effect under intravenous conditions is ascribed to this interfacial structure adopted when adsorbed onto nanoparticles (Rudt & Müller, 1993; Torcello-Gómez et al., 2011a). This protective effect can be also applied for drug delivery through oral route, since Pluronic micelles solubilize drugs and enhance drug transport across the intestinal barriers (Batrakova & Kabanov, 2008).

The idea now is to extend the functionality of Pluronics to stabilize oil-in-water emulsions for controlling lipid digestion in order to deliver bioactive compounds to specific locations in the digestive tract (Wulff-Pérez, de Vicente, Martín-Rodríguez, & Gálvez-Ruiz, 2012). Therefore, it is necessary to understand the fundamental processes underlying lipid digestion. Lipid digestion mainly takes place in the duodenum (upper small intestine) where different surface active systems exist: the enzyme pancreatic lipase and its co-factor co-lipase, and bile

salts (BS). BS are natural surfactants which adsorb onto oil–water interfaces in food emulsions, displacing any other surface active system and hence preparing the interface for the adsorption of the lipase–co-lipase complex. Once lipase reaches the oil–water interface, the lipid digestion or lipolysis starts. In this process, lipase hydrolyses the triglycerides from the lipid substrate into a simpler form, such as free fatty acids and monoglycerides (Chu et al., 2009). Then, these lipolysis products are removed from the oil–water interface and solubilized by mixing with BS micelles, ready to be assimilated by the human body. The release of lipid-soluble compounds (nutrients, drugs…) would occur during this digestion process. Therefore, BS play a key role in the lipolysis, solubilization and transport of lipophilic compounds to the mucosa of the small intestine (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). Thus, a comprehensive study of the behavior of oil-in-water emulsions stabilized by different Pluronics under the action of BS would provide the basis for accurately deriving the details of the molecular interactions occurring during the gastrointestinal transit. These biological functions of BS are closely related to their amphiphilic nature, having the ability of adsorbing at hydrophobic interfaces and/or forming micelles in solution. The unusual surface activity of BS is due to a planar molecular structure with a facial amphiphilic nature, given by a rigid steroid backbone containing methyl groups that are oriented to a hydrophobic face, whereas the hydroxyl groups and the amino group are oriented to a hydrophilic face (Hofmann & Mysels, 1988). The amino group can be conjugated with taurine, glycine or other amino acids. BS differ in the number, position and stereochemistry of the hydroxyl groups as well as on the conjugated amino acid, but behave in a qualitative similar manner. For that reason only one type of BS was chosen for the current study: sodium taurodeoxycholate (NaTDC), which is one of the main components in human BS (Arleth et al., 2003; Friesen et al., 2008). The Pluronics used for this work, Pluronic F68 and Pluronic F127, have been approved as an inactive ingredient for oral intake by U. S. Food and Drug Administration (FDA). Pluronic F68 has been

shown to be resistant to the displacement by BS (Torcello-Gómez, Jódar-Reyes, Maldonado-Valderrama, & Martín-Rodríguez, 2012), hindering the access of pancreatic lipase to the oil–water interface as compared to model surfactants, such as soybean lecithin (Torcello-Gómez et al., 2011b). This is a very important result that could have implications in delaying the rate of lipid digestion (Wulff-Pérez, Gálvez-Ruiz, de Vicente, & Martín-Rodríguez, 2010; Torcello-Gómez, Maldonado-Valderrama, Martín-Rodríguez, & McClements, 2011c). Furthermore, it was shown that oil droplets covered by larger Pluronics (F127) were digested at a slower rate and extent than those covered by smaller Pluronics (F68) (Wulff-Pérez et al., 2012), with both systems being largely unaffected by gastric conditions.

With these premises, the goal of the current study is to look into the interactions between a model bile salt (NaTDC) and two different types of Pluronic (F68 or F127), not only at the lipid droplet surface of oil-in-water emulsions, but also in the aqueous phase (continuous phase in these emulsions). This work is based fundamentally in micro-differential scanning calorimetry (mDSC), by comparing the thermograms of Pluronic-stabilized emulsions with those of Pluronic aqueous solutions, in the absence and presence of NaTDC. Calorimetric techniques have been previously used for the investigation of thermal transitions of different Pluronics in aqueous solution (Hecht & Hoffmann, 1994; Alexandridis, Nivaggioli, & Hatton, 1995; Cabana, Aït-Kadi, & Juhász, 1997; Barba et al., 2009; Álvarez-Ramírez et al., 2009; Boucenna, Royon, Colinart, Guedeau-Boudeville, & Mourchid, 2010) but the interactions with BS had not been reported previously with this technique. Hence, this is a good background that allows us to discuss our calorimetric results. As a complement to calorimetric studies in bulk and in emulsion another innovation of this work is the use of interfacial tension measurements and dilatational rheology to further study the interactions of Pluronic and BS in sequential and competitive adsorption, now at oil-water interface. The use of surface science to study digestion processes is a relatively new area of research (Wilde & Chu, 2011) and has proven useful determining interfacial

mechanisms underlying physiological aspects of lipid digestion owing to the correlation with emulsion behavior. Finally, cryogenic scanning electron microscopy is used to visualize Pluronic-stabilized emulsions in the absence and presence of BS providing important clarification of the molecular mechanisms.

The ability of Pluronics to resist the interfacial displacement by BS or compete with BS for the oil–water interface constitutes a major mechanism contributing to the delay of lipid digestion, as BS are natural surfactants that prepare the oil–water interfaces in emulsions for the adsorption of the enzymes in order for lipolysis to take place. These new findings, based on the combination of bulk, emulsion and interfacial aspects of Pluronic-BS interactions can be exploited in tailoring novel food matrices with improved functional properties such as greater control of digestibility for specific physiological responses.

2. Materials and methods

2.1. Materials

As emulsifiers we used Pluronic F127 and Pluronic F68 purchased from Sigma-Aldrich without further purification. They are also commercially known as Poloxamer 407 and 188, respectively. These non-ionic triblock copolymers are based on a $PEO_aPPO_bPEO_a$ structure. The main difference between them is the number of subunits in the PEO (*a*) and PPO (*b*) blocks and therefore, in the hydrophilic–lipophilic balance HLB (Table 1). The bile salt used in this study is sodium taurodeoxycholate (NaTDC, 97% purity) from Sigma-Aldrich. It is negatively charged and its molecular weight is 521.7 g/mol.

The aqueous phase was 1.13 mM phosphate buffer (pH 7) prepared with ultrapure water purified in a Pur1te Select system. Highly refined olive oil was also purchased from Sigma-Aldrich, and purified with activated magnesium silicate

(Florisil®, Fluka) to eliminate free fatty acids and surface active impurities. Namely, a mixture of oil and Florisil® in proportion 2:1 w/w was shaken mildly for 3 h and centrifuged at 4000 rpm for 30 min in a bench centrifuge. It was then filtered through Whatman filter paper #1 under vacuum and stored away from light.

Table 1: Physicochemical characteristics of the Pluronics used to stabilize the oil-in-water emulsions.

Emulsifier	**MW** (g/mol)	***a*** (PEO subunits)	***b*** (PPO subunits)	**HLB**
F127	12600	100	65	22
F68	8400	75	29	29

2.2. Solution preparation

For the experiments in aqueous solution we dissolve F68 or F127 in the aqueous phase at room temperature. NaTDC is dissolved in the aqueous phase at different concentrations by successive dilution from a concentrated stock. Then, different NaTDC aqueous solutions are added to aliquots of Pluronic aqueous solution at room temperature, so that the final concentration of F68 and F127 is fixed at 1 wt% and the final concentration of NaTDC ranges from 0 mM to 50 mM.

2.3. Emulsion preparation

We emulsify 1 wt% F68 or F127 aqueous phase with 10 wt% oil phase using a high speed ULTRA-TURRAX® homogenizer (IKA® T18 basic), by dropwise addition of oil into the aqueous phase under stirring (speed 3).

Some of the experiments were carried out in washed emulsions, that is, without non-adsorbed emulsifier in the aqueous phase. For washing these Pluronic-stabilized emulsions, the original emulsion sample was centrifuged at 4000 rpm for

15 min. Then the subnatant (aqueous phase containing the non-adsorbed Pluronic) was carefully removed and replaced by the same volume of pure buffer to redisperse the supernatant (cream). The subnatant was also stored at 4 ºC for further analysis.

For the experiments in the presence of bile salt, 300 µl of a NaTDC aqueous solution was added to 5 mL of emulsion sample or subnatant at room temperature, being the final concentration 10 mM NaTDC (physiological conditions).

The morphology of emulsion droplets was observed with an optical microscope (Leitz Diaplan, Germany) obtaining for all the samples a wide droplet size distribution comprising 1–100 µm.

2.4. Micro-Differential Scanning Calorimetry (mDSC)

Scanning calorimetry experiments were performed in a Micro-Differential Scanning Calorimeter (DSC III Setaram, Caluire, France) using cells made from Hastalloy, capable of holding ~0.8 mL and sealed with "O" rings on Hastalloy screw tops. Samples were initially cooled to a starting temperature of 10°C to equilibrate. Samples were run at a scanning rate of 1 °C min^{-1} from 10 to 90 °C, cooled and rerun while all steps were recorded. The reference cell was filled with ultrapure water and coordinated for heat capacity with the sample. Enthalpy values were calculated using Setaram software with a linear interpolated baseline, based on an extension of the trace before and after the thermal event. Heat and temperature calibrations can be checked using the transition in naphthalene.

2.5. Interfacial tension

The interfacial tension measurements were carried out in a Pendant Drop Film Balance equipped with a subphase multi-exchange device: The OCTOPUS, fully assembled and developed at the University of Granada (Maldonado-

Valderrama, Holgado-Terriza, Torcello-Gómez, & Cabrerizo-Vílchez, 2012a). The subphase multi-exchange device upgrades the technology of the single subphase exchange device that is described in detail elsewhere (Cabrerizo-Vílchez, Wege, Holgado-Terriza, & Neumann, 1999). This accessory consists of two coaxial capillaries connected independently to two syringes which are in turn connected to different solutions by 8 channels of both 8 port-valve micro-injectors (known as The OCTOPUS). Thus, several subphase exchanges of the bulk solution of the pendant drop can be performed preserving the interfacial area and the volume of the droplet, by extracting the bulk solution in the droplet through the outer capillary, and injecting simultaneously the next solution through the inner one. The whole set-up is computer controlled through the software DINATEN© developed by Dr. Holgado Terriza. DINATEN© fits the experimental drop profiles, extracted from digital drop micrographs, to the Young-Laplace equation of capillarity by using Axisymmetric Drop Shape Analysis (ADSA), providing as outputs the drop volume *V*, the interfacial tension γ, and the interfacial area *A*. The adsorption process is recorded at constant interfacial area through a modulated fuzzy logic PID algorithm (proportional, integral, and derivative control) by changing the volume (Wege, Holgado-Terriza, & Cabrerizo-Vílchez, 2002). The drop is immersed in a glass cuvette (Hellma), which contains the oil phase and is kept in a thermostatized cell. The interfacial tension of the clean interface was measured before every experiment to ensure the absence of surface-active contaminants obtaining values of (29.5 ± 0.5) mN/m at 20 ºC. All the experiments were performed at 20 ºC and their reproducibility was verified from the standard deviation of at least three replicate measurements.

The dilatational rheology of the different adsorbed layers was measured by applying an oscillatory perturbation to the interface by injecting and extracting volume to the drop. The system records the response of the interfacial tension to the area deformation, and the dilatational modulus (*E*) of the interfacial layer is

calculated from this response by following the method described by Myrvold and Hansen (Myrvold, & Hansen, 1998) implemented in the computer program CONTACTO©. In a general case, the dilatational modulus is a complex quantity that contains a real and an imaginary part:

$$E = E' + iE'' = \varepsilon + i2\pi f\eta \quad (1)$$

where E' is the storage modulus and accounts for the elasticity (ε) of the interfacial layer and E'' is the loss modulus and accounts for the viscosity (η) of the interfacial layer. The applied interfacial area oscillations were maintained below 10 % of amplitude to avoid excessive perturbation of the interfacial layer and the departure from the linear viscoelastic region, which was checked in preliminary experiments. The oscillation frequency (f) was set to 0.1 Hz. At this frequency the viscous component of the dilatational modulus (E'') is negligible in all the systems, and the adsorbed layers are predominantly elastic, hence, we will report values of the dilatational modulus E as given by equation (1).

2.6. Cryogenic scanning electron microscopy (cryo-SEM)

A FEI Quanta 200 3D SEM (FEI, Hillsboro, Oregon, USA), featuring a Quorum Technologies PP2000T cryotransfer unit (Quorum Technologies Ltd, Hailsham, UK) and an Omniprobe micromanipulator (Omniprobe Inc., Dallas, Texas, USA), was used to perform cryogenic scanning electron microscopy (cryo-SEM) allowing the internal structure of the emulsions to be visualized. It was operated at 15 kV and 3×10^{-10} A. The sample was mounted on the sample holder and plunged into "slushy" nitrogen (i.e. N_2 cooled to just above its melting point) and transferred into the evacuated cryotransfer unit antechamber held at –140°C. The sample was fractured using a cold knife to expose the internal structure. Then, the freshly exposed emulsions surface was sputter-coated with a thin conductive layer of platinum to allow high resolution imaging in the SEM. The samples were

finally transferred under vacuum from the antechamber into the SEM chamber, also held at −140°C.

3. Results and discussion

3.1. Interactions of Pluronic aqueous solutions with bile salts

If the aim of the current work is to study the interactions between Pluronic and BS in oil-in-water emulsions stabilized by the copolymers, then it is necessary first to analyze the interactions of Pluronic with BS in the continuous phase of the emulsion, that is, in aqueous solution. In this manner, we can separate the contribution from the interactions taking place onto the oil–water interface in emulsion, so that the later comparison with data in real emulsions would be easier to interpret. In addition, we will first analyze the thermal behavior of neat Pluronic aqueous solutions before adding the BS to study the interaction between them.

Figure 1 displays the m-DSC traces for neat Pluronic aqueous solutions at different concentrations (Figure 1a for F127 and Figure 1b for F68). Due to the reproducibility and reversibility of the heating and cooling traces, only the heat flow on heating is reported in all the results. We can see that both systems (Figure 1a and 1b) give rise to an endothermic transition peak with increasing temperature. This peak is related to the formation of micelles, that is, the aggregation of polymer single-chain "unimers", with a central core built up of PPO hydrophobic segments and an external corona built up of PEO hydrophilic segments. The micellization of Pluronics in water is an endothermic phenomenon driven by a decrease in the polarity of PEO and PPO segments as the temperature increases and by the entropy increase in water when unimers aggregate to form micelles (Wanka, Hoffmann, & Ulbricht, 1990). Hence, it can be detected by properly designed DSC tests, as it has been previously reported in literature for several Pluronics (Hecht & Hoffmann,

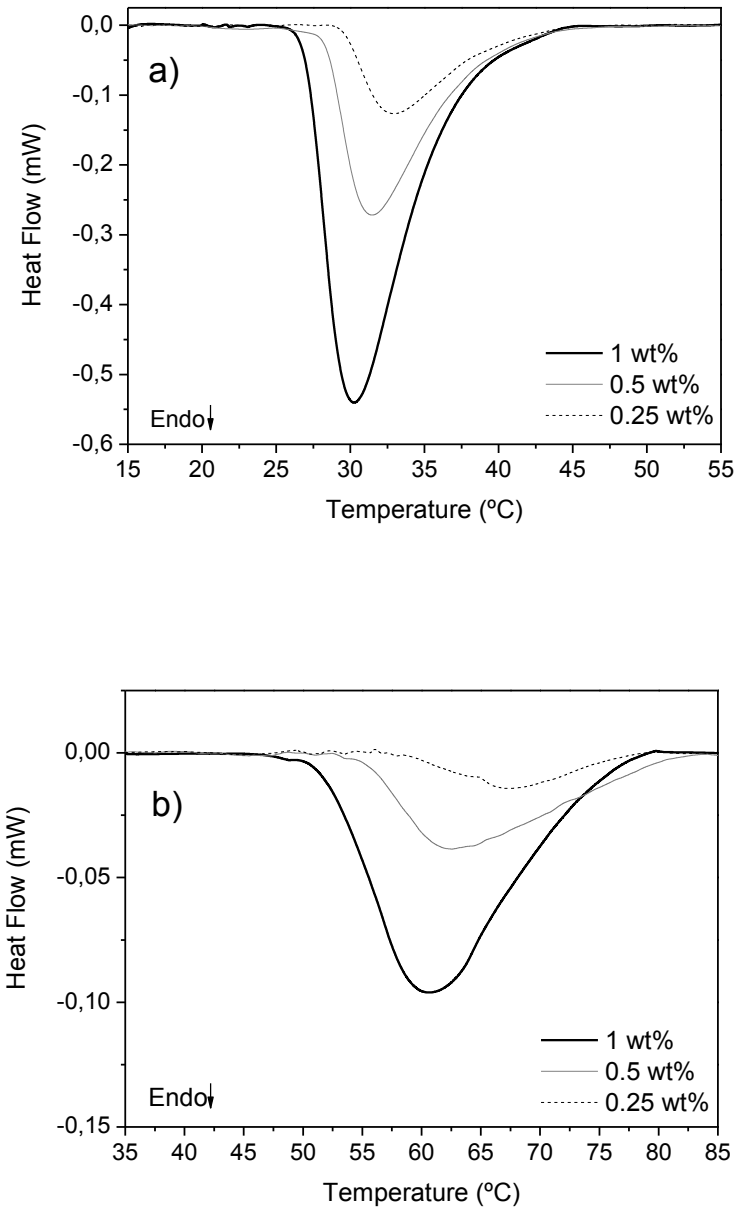


Figure 1: Micro-DSC: endothermic peak on heating aqueous solutions of a) F127 and b) F68 at several concentrations.

1994; Alexandridis, Nivaggioli, & Hatton, 1995; Cabana, Aït-Kadi, & Juhász, 1997; Barba et al., 2009; Álvarez-Ramírez et al., 2009; Boucenna et al., 2010). The process can be explained at as the melting of the hydration water from the PPO groups in the monomer state (Hecht & Hoffmann, 1994). The micellization process can be characterized by either the position of the transition peak, T_{peak}, or the onset temperature, T_{onset}, which corresponds to the temperature where micelles start to form, that is the critical micellization temperature (CMT) (Alexandridis et al., 1995). We can observe in Figure 1 that the micellization of F127 occurs at lower temperatures (T_{onset} = 27 °C) than the micellization of F68 (T_{onset} = 52 °C). This should be due to the higher content of hydrophobic segments of F127 (25% of PPO) as compared to F68 (16% of PPO), similar to the lower gelation temperature found for cellulose derivatives biopolymers with higher content of hydrophobic substituents (Haque, Richardson, Morris, Gidley, & Caswell, 1993; Sun et al., 2009). As a general trend, the peaks are rather broad, which is attributed to the fact that the copolymers show a broad molecular weight distribution (Wanka et al., 1990), or additionally due to a gradual increase in the number of micelles in solution as temperature increases (Alexandridis et al., 1995). We can observe for both Pluronics that the peak maximum decreases towards lower heat flow values and shifts to higher temperatures (T_{peak} increases) upon decreasing Pluronic concentration, as documented by DSC for different Pluronic systems (Wanka et al., 1990; Alexandridis et al., 1995; Cabana et al., 1997; Barba et al., 2009; Feitosa & Winnik, 2010). However, the enthalpy of micellization, obtained from the integral of the DSC peak with respect to the baseline, normalized to unit mass of polymer is independent of Pluronic concentration (Alexandridis et al. 1995), being 27.6 ± 0.6 J/g of polymer (350 kJ/mol) for F127, in agreement with the value reported by Boucenna and co-workers (27.9 J/g of polymer), and 8 ± 2 J/g of polymer (70 kJ/mol) for F68. Measurements on different systems have shown that the total heat flow is related to the amount of PPO in solution (Hecht & Hoffmann, 1994). This makes sense as the enthalpy of micellization for F127 is similar to that reported by

Alexandridis and co-workers for Pluronic P104 (370 kJ/mol) that has a similar content of PPO blocks. Thus, this explains the decrease in intensity of the endothermic peak when decreasing the concentration for both Pluronics, as well as the lower value of the enthalpy of micellization obtained for F68 than that for F127. These thermograms at different concentrations of Pluronic will be useful to compare later on with the experiments of Pluronic-stabilized emulsions in order to roughly quantify the concentration of non-adsorbed Pluronic in the aqueous phase. A last observation about these results is that no endothermic peak related to gelation, that is, close packing of the micelles with further temperature increase, was found for both Pluronics at temperature higher than the micellization. The reason for this is that the gelation energy is very small, less than micellization energy, and it is only observed at sufficiently high concentrations of the copolymers (Cabana et al., 1997; Barba et al., 2009).

Next, we plot the micro-DSC traces for 1 wt% Pluronic aqueous solutions (Figure 2a for F127 and Figure 2b for F68) upon addition of bile salt. Before discussing these results and as reference data, note that a relatively high concentration of NaTDC (10 mM) above the critical micelle concentration at room temperature (cmc = 1–2 mM) (Torcello-Gómez et al., 2012) does not exhibit a visible thermal transition within the range of temperature studied (results not shown). Figure 2 shows that the endothermic peak corresponding to Pluronic micellization (0 mM NaTDC in Figures 2a and 2b), gradually vanishes with increasing NaTDC concentration. In Figure 3a, the enthalpy of micellization of 1 wt% Pluronic aqueous solution is plotted as a function of the NaTDC concentration. This figure shows the decrease of the enthalpy of micellization as the bile salt concentration is increased. It seems that the BS interact with Pluronic molecules suppressing the micelle formation. A similar suppression of micelle formation was reported for F127 in the presence of ionic surfactants such as sodium dodecyl sulfate (SDS) (Hecht and Hoffmann, 1994). In order to make

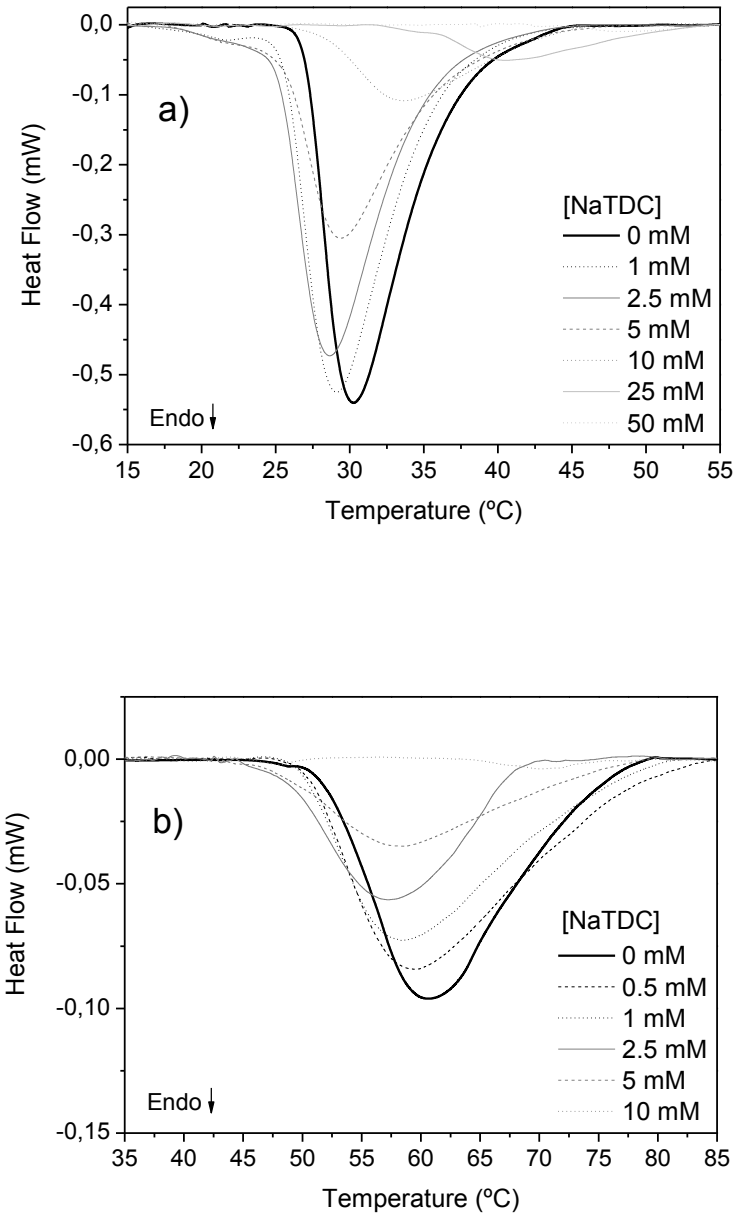


Figure 2: Micro-DSC: endothermic peak on heating 1 wt% aqueous solutions of a) F127 and b) F68 as a function of the added NaTDC concentration.

direct comparisons for both Pluronics, the relative change of micellization enthalpy with respect to the micellization enthalpy of each Pluronic in the absence of bile salt (ΔH_0) upon increasing NaTDC concentration, is plotted in Figure 3b. We can now see clearly that the BS concentration from which $|\Delta H - \Delta H_0|/\Delta H_0$ starts to increase, that is, the endothermic peak corresponding to Pluronic micellization starts to decrease, is lower for F68 (1 mM) than for F127 (2.5–5 mM), suggesting that F68 is more susceptible to interact with bile salts. Since the interaction begins at concentrations below or around the cmc of NaTDC, it seems that this interaction occurs similarly between BS molecules and copolymers and between BS micelles and copolymers. It is likely that the monomeric copolymers bind to NaTDC molecules and/or micelles, forming some type of complex, similar to the mixed micelles of bile salt and emulsifier reported by Arleth and co-workers (Arleth et al., 2003). Also, the bile salt concentration at which the endothermic peak completely vanishes ($|\Delta H - \Delta H_0|/\Delta H_0$ reaches the unit in Figure 3b) is appreciably lower for F68 (10 mM NaTDC) than for F127 (50 mM NaTDC). The molar concentrations of F127 and F68 are 0.79 mM and 1 mM, respectively. Therefore the molar concentration ratio NaTDC/Pluronic at which the endothermic peak completely disappears is at least higher than 32:1 for F127 (considering the curve corresponding to 25 mM NaTDC the one presenting a minimum peak before disappearing at 50 mM NaTDC) and around 10:1 for F68 (Figures 2 and 3). That means that each molecule of F127 would be able to bind with a larger number of NaTDC molecules (or more micelles) as compared to F68. Therefore, F127 might prevent more BS molecules from adsorbing onto the oil droplets than F68, as it has been previously shown that the complexes formed by these block copolymers and ionic surfactants are no longer surface active and remain in the bulk phase (Hecht and Hoffmann, 1994).

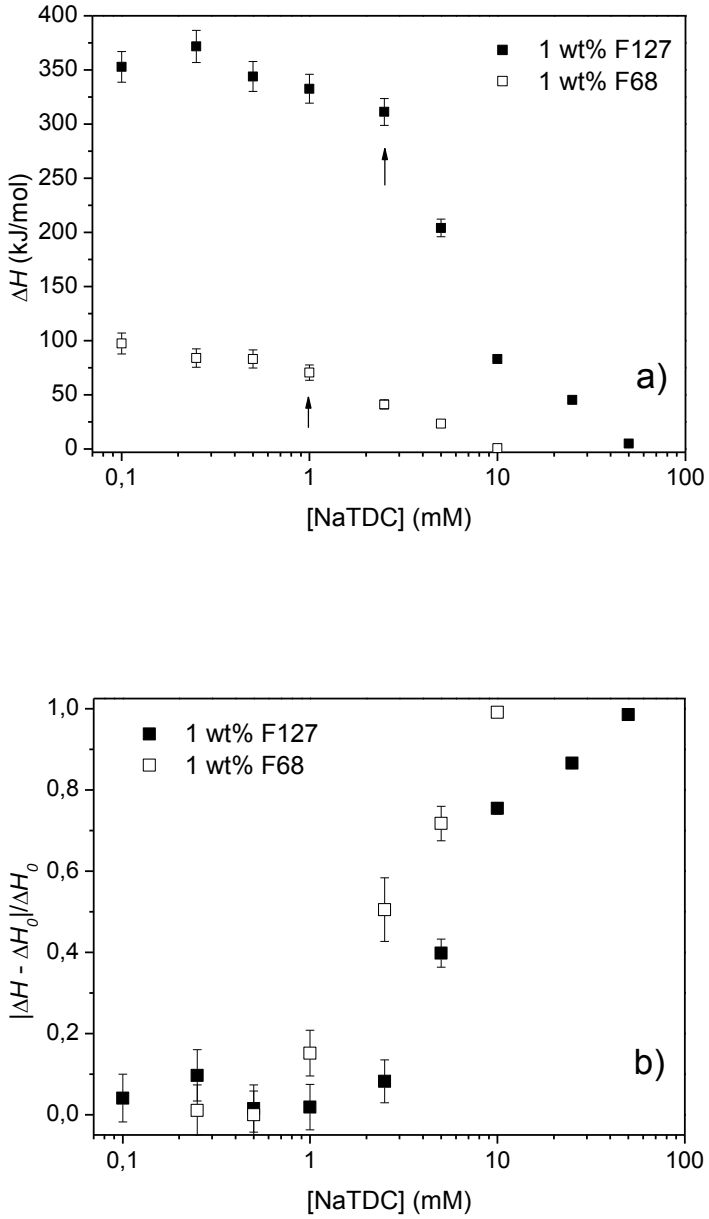


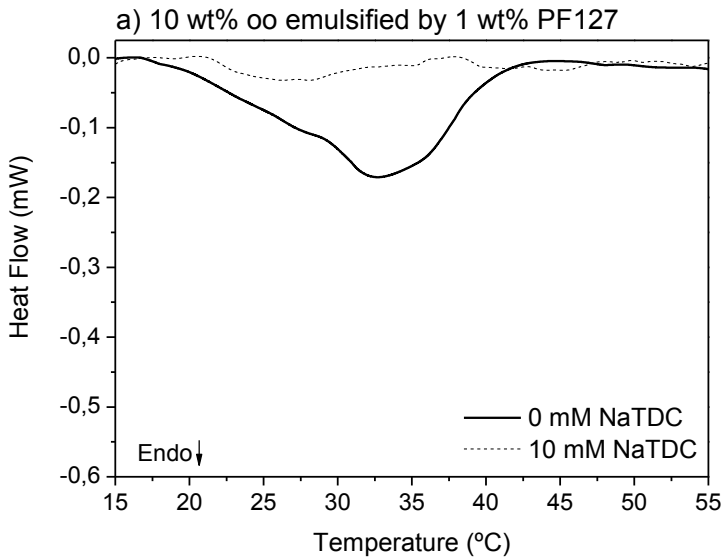
Figure 3: Micro-DSC: a) micellization enthalpy and b) relative change of micellization enthalpy of 1 wt% aqueous solutions of F127 and F68 as a function of the added NaTDC concentration.

3.2. Interactions of Pluronic-stabilized oil-in-water emulsions with bile salts

After studying the interactions between Pluronics and bile salt in aqueous solution, we will now compare with the thermograms of 10 wt% olive oil (oo) in water emulsions stabilized by 1 wt% Pluronic in the absence and presence of 10 mM NaTDC, which is within the range of physiological concentrations found in the duodenum (Kalantzi et al., 2006). This section is divided in two different parts, each one corresponding to the results for F127 or F68-stabilized emulsions.

3.2.1. F127-stabilized emulsions

Figure 4a displays the micro-DSC traces for 10 wt% oo in water emulsion stabilized by 1 wt% F127, in the absence and presence of bile salt. In the absence of BS (0 mM NaTDC), we can observe a broad endothermic peak within the range of temperatures corresponding to micellization of F127 (Figure 1a). However, the peak maximum appears now towards lower heat flow values and at around 32.5 °C,



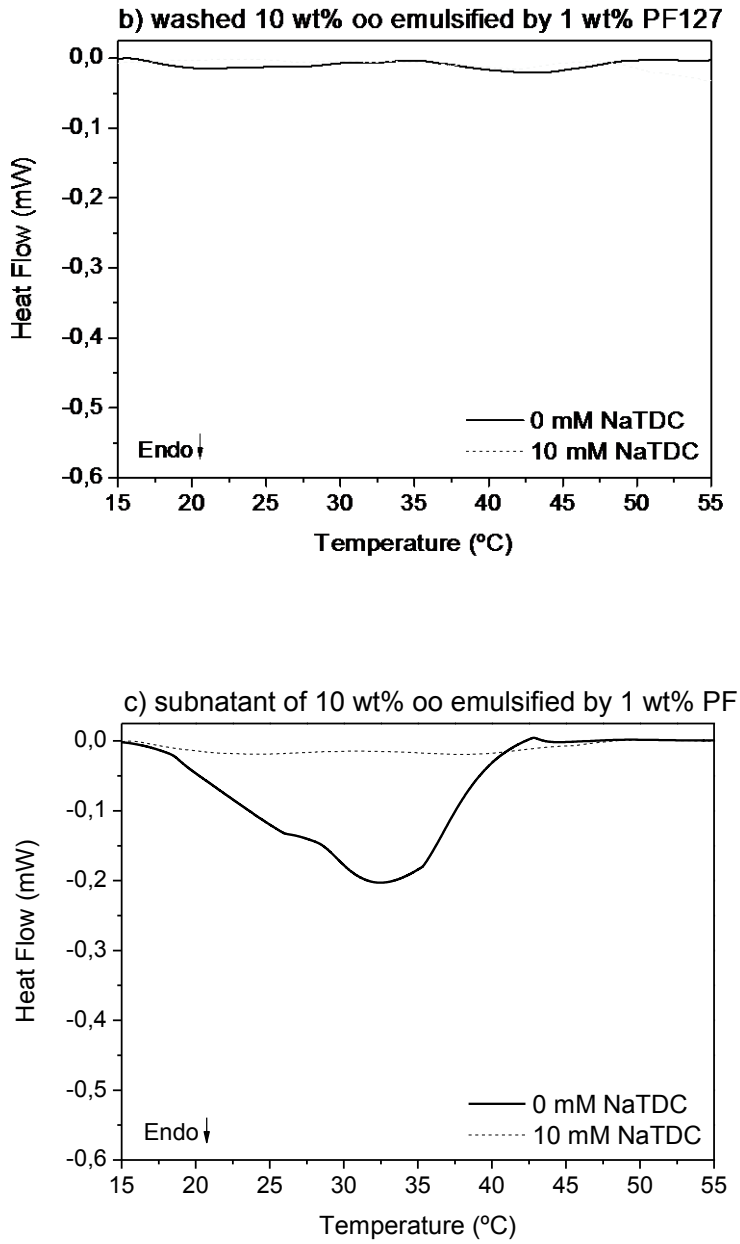


Figure 4: Micro-DSC traces for a) 10 wt% olive oil in water emulsion stabilized by 1 wt% F127, b) corresponding washed emulsion and c) corresponding subnatant, in the absence and presence of bile salt (10 mM).

instead of 30 °C as compared to 1 wt% F127 aqueous solution. If we compare with the thermograms of neat F127 solutions at different concentrations from Figure 1a, it is clear that the concentration of F127 in the emulsion aqueous phase is lower than 1 wt%, as a considerable fraction was adsorbed onto the lipid droplets surface to stabilize the emulsion. Comparison of the heat flow value in the peak maximum from emulsion and solution (Figures 1a and 4a) suggests that the actual F127 concentration in the emulsion aqueous phase might be between 0.25 and 0.5 wt%. On the other hand, the endothermic peak appears broader in emulsion than in aqueous solution, implying a contribution from the lipid droplet interactions. Then, in the presence of 10 mM NaTDC, the endothermic peak vanishes, indicating that the bile salt interacts with remaining non-adsorbed F127 molecules, preventing them from forming micelles, in agreement with the results in aqueous solution.

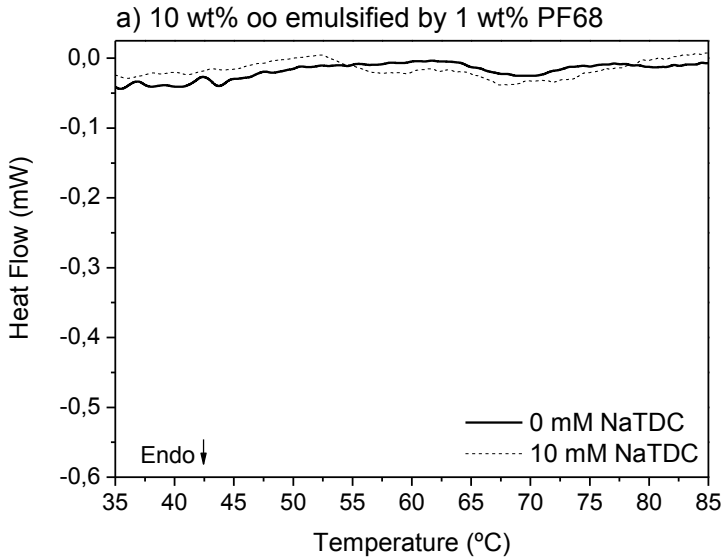
We analyze now the micro-DSC traces of washed emulsions stabilized by 1 wt% F127 in the absence and presence of BS, which are presented in Figure 4b. In washed emulsions, the continuous (aqueous) phase was removed after centrifuging and replaced by pure buffer. After this procedure the presence of non-adsorbed F127 molecules forming micelles is not expected. We can observe in Figure 4b that in the absence of NaTDC, the endothermic peak is no longer present, corroborating that the origin of the peak observed for non-washed emulsions (Figure 4a) indeed corresponds to the micellization of F127. In the presence of 10 mM BS the effect of NaTDC on F127 molecules would come from the oil–water interface, as there is no remaining F127 in the aqueous phase. In this case, the absence of an endothermic peak suggests that the molar concentration ratio NaTDC/Pluronic in the aqueous phase is at least higher than 32:1, as we have seen in the thermograms of 1 wt% F127 aqueous solution upon increasing NaTDC concentration (Figure 2a). This indicates that the possible F127 molecules displaced from the oil–water interface by NaTDC are not enough to form micelles, owing to the saturation of BS adsorbed onto Pluronic molecules. To recall, a similar behavior was found in non-

washed emulsions (Figure 4a), where in addition, there were already non-adsorbed F127 molecules interacting with BS.

Next, in order to gain further insight into these results we also measured the micro-DSC traces for the subnatant removed from the washed emulsions, in the absence and presence of NaTDC (Figure 4c). We can observe either in the absence or presence of BS similar thermograms than those obtained in non-washed emulsions (Figure 4a). In particular, we find once more in the absence of NaTDC, a broader endothermic peak than that found in F127 aqueous solutions (Figure 1a). The presence of oil droplets is not expected in the subnatant, which leads to this broad peak as in non-washed emulsions. It is likely the presence of some fraction of smaller oil droplets in the subnatant after centrifuging the emulsion. This was checked by observing the subnatant samples with an optical microscope and indeed, we found a slight fraction of lipid droplets of the order of 1 μm (results not shown). On the other hand, we can determine the concentration of F127 in the subnatant by interpolating the heat flow value in the peak maximum vs. Pluronic concentration from the thermograms of neat Pluronic aqueous solutions (Figure 1a), obtaining a value of 0.38 wt% (0.3 mM). When 10 mM NaTDC is added to the subnatant, the ratio NaTDC/Pluronic can be estimated assuming that there is no interfacial area available to adsorb onto, and that the concentration of F127 is the abovementioned. In this way, the ratio NaTDC/F127 would be 33:1. This value is in agreement with that estimated in the aqueous phase of emulsions, either washed or non-washed (Figure 4a, 4b). Namely, this concentration might be at least higher than 32:1. Bearing in mind that in the case of emulsions the final NaTDC/Pluronic ratio results from the balance between the adsorption of BS onto lipid droplets surface and the possible displacement of Pluronic molecules, a comparison with the ratio calculated in the subnatant suggests little displacement of F127 from the oil–water interface. This will be further checked later by measuring the sequential adsorption of Pluronic and NaTDC onto the oo–water interface.

3.2.2. F68-stabilized emulsions

In this subsection we will discuss the calorimetric results for the same set of experiments of 10 wt% oo in water emulsions stabilized now by 1 wt% F68. Figure 5a shows the micro-DSC traces for non-washed emulsions, in the absence and presence of NaTDC. Firstly, in the absence of NaTDC, the appearance of a small endothermic peak with the maximum located at 68 °C suggests that some fraction of non-adsorbed F68 still remains in the aqueous phase forming micelles as we have seen for F127-stabilized emulsions. This is corroborated by the absence of the endothermic peak in washed emulsions (Figure 5b). Then, a remaining peak after the addition of 10 mM NaTDC (Figure 5a), indicates an appreciable displacement of F68 from the lipid droplets surface. However, the thermogram of washed emulsions stabilized by F68 (Figure 5b) in the presence of NaTDC does not show such a peak, indicating that the possible fraction of F68 molecules displaced from the oil–water interface is not enough to form micelles as they interact with the



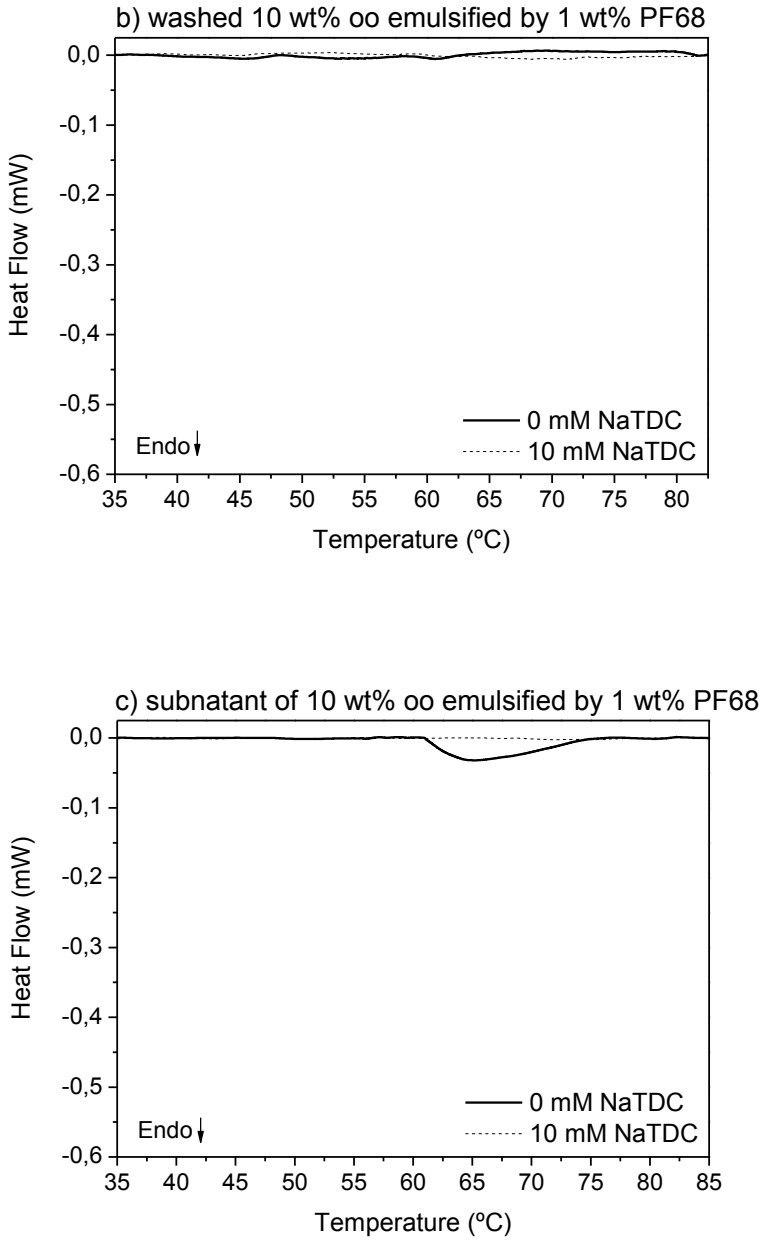


Figure 5: Micro-DSC traces for a) 10 wt% olive oil in water emulsion stabilized by 1 wt% F68, b) corresponding washed emulsion and c) corresponding subnatant, in the absence and presence of bile salt (10 mM).

micelles of BS. In this case (Figure 5b), the ratio NaTDC/Pluronic in the aqueous phase will be at least 10:1 or higher, if we compare with the thermograms of 1 wt% F68 aqueous solution upon increasing NaTDC concentration (Figure 2b) when the peak is absent.

Further, we will look into the micro-DSC traces for the subnatant removed from the washed emulsions stabilized by F68, in the absence and presence of NaTDC (Figure 5c). Firstly, a comparison of the heat flow value of the peak maximum in the absence of NaTDC from the subnatant sample and neat F68 aqueous solutions from Figure 1b, suggests that the remaining F68 concentration in the emulsion aqueous phase is between 0.25 and 0.5 wt%, with an interpolation indicating a concentration of 0.42 wt% (0.5 mM). Next, the presence of bile salt (10 mM NaTDC) leads to the disappearance of the endothermic peak as we observed for aqueous solutions (Figure 2b) and for F127-stabilized emulsions. Therefore, it seems that the origin of the endothermic peak found in non-washed emulsions in the presence of NaTDC (Figure 5a), with an intensity similar to that in the absence of the bile salt, might be the contribution of the non-adsorbed emulsifier along with the displaced fraction from the oil-water interface and also the lipid droplets interactions. The ratio NaTDC/F68 in the subnatant (Figure 5c) indicates a value of 20:1. This value is also in agreement with that estimated in the aqueous phase of washed emulsions. Despite the fact that we cannot calculate more precisely the ratio NaTDC/Pluronic in emulsions from the calorimetric results, the minimum estimated for F68-stabilized emulsion (10:1) still gives rise to a marginal F68 interfacial displacement by NaTDC if we compared with the value calculated in the subnatant (20:1). This contrasts with the minimum ratio determined in F127-stabilized emulsion (32:1) as compared with that in subnatant (33:1). The possible greater displacement of PF68 by BS is now probed with interfacial tension measurements.

3.3. Interactions of Pluronic and bile salts at oo-water interface

In order to further study the role played by the surface of oil droplets in Pluronic-stabilized emulsions, interfacial tension measurements were carried out at the oo-water interface in a single droplet. Two different studies were designed: on one hand sequential adsorption studies of NaTDC onto Pluronic-covered interfaces, mimicking the behavior of washed emulsions stabilized by Pluronic after the addition of BS (Figure 6). On the other hand, competitive adsorption studies of both species at the oo–water interface (Figure 7).

The experiments in Figure 6 were designed as follows. First, a solution droplet of 1 wt% Pluronic is formed into the oil phase and the polymer adsorbs onto the oo–water interface at constant interfacial area simulating the emulsification process. After 30 min a steady state of the interfacial tension is reached and the dilatational modulus is measured at 0.1 Hz. Next, the bulk subphase of the drop is exchanged by a solution containing 10 mM NaTDC. Hence, during this exchange the bulk is depleted of non-adsorbed Pluronic, comparable to the process for washing the emulsions, whereas the new solution of NaTDC is introduced into the droplet to study the effect on the pre-formed layer of Pluronic. It is important to mention here that a stable interfacial layer of Pluronic remains adsorbed after the subphase exchange, as previously checked by monitoring the interfacial tension during and after an exchange with pure buffer (Torcello-Gómez et al., 2012). Once the interfacial tension becomes stable after the complete removal of the original solution, the dilatational modulus is measured again. Finally, the subphase is exchanged thoroughly with pure buffer, in order to desorb all the reversibly adsorbed species, and gain information about the final composition of the irreversibly adsorbed material remaining at the interfacial layer. Once more the dilatational modulus is measured at the end of the experiment. All the values characterizing the interfacial layer obtained at the end of each of these three steps are reported in Table 2. Figure 6 shows that the adsorption of 1 wt% of

both Pluronics gives rise to different rate and extent of the interfacial tension. Namely, F127 adsorbs faster, reaching a stable and lower value of the interfacial tension (10 mN/m) earlier than F68 (13 mN/m). This is due to the more hydrophobic character and higher molecular weight of F127 as compared to F68 (Ramírez et al., 2011). However, despite having different adsorption rates, both Pluronics show similar value of the dilatational modulus at this concentration (Table 2). When the subphase is exchanged by the bile salt solution (NaTDC 10 mM), the extent of change in the interfacial tension is different for each Pluronic-adsorbed layer (Figure 6). BS reduces the interfacial tension of the F68 pre-covered interface, whereas the same concentration of bile salt slightly increases the interfacial tension for a F127 pre-formed layer. This opposite behavior is attributed

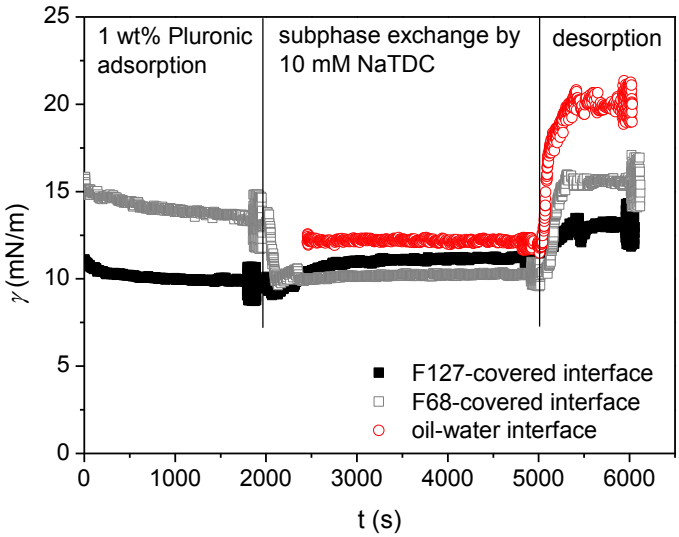


Figure 6: Time evolution of the interfacial tension during the sequential steps of Pluronic (1 wt%) adsorption, NaTDC (10 mM) adsorption and desorption at the olive oil–water interface at 20 ºC. Also, the steps of NaTDC adsorption and desorption at the bare olive oil–water interface (in the absence of Pluronic) is included as a reference.

to the interfacial tension reached by the pure bile salt at the oo-water interface (12 mN/m), which is intermediate between that attained by pure F68 (13 mN/m) and pure F127 (10 mN/m). Therefore, when the bile salt is introduced into the bulk, the interfacial tension of the Pluronic-adsorbed layer tends to match the interfacial tension of the pure NaTDC at the oo–water interface, indicating the penetration/displacement of BS molecules into the interfacial layer. This feature is corroborated by the decrease in the dilatational modulus after the adsorption of BS (Table 2), which also approaches the value of NaTDC in the absence of Pluronic (4 mN/m, result not shown in Table 2). However, the interfacial tension values after BS adsorption onto both Pluronic-covered interfaces are always lower than that in the absence of Pluronic, suggesting that both species, Pluronic and NaTDC, coexist at the oo–water interface. Finally, after washing the subphase with pure buffer, all the reversibly adsorbed molecules are removed from the interface increasing the interfacial tension. This process is mainly due to the desorption of bile salt (see red curve in Figure 6). Nevertheless, the desorption profiles in the presence of Pluronic give rise to interfacial tension values much lower (13 and 15 mN/m) than that of pure NaTDC at the oo–water interface (21 mN/m). These results suggest that at the end of the experiment a high fraction of material is still adsorbed onto the interface and arise from the remaining adsorbed Pluronic and/or possible interfacial

Table 2: Interfacial tension and dilatational modulus after sequential steps of Pluronic adsorption, NaTDC adsorption and desorption at the olive oil–water interface as in Figure 6. Values are obtained as mean of at least three replicate measurements.

STEPS	**Pluronic adsorption**		**NaTDC adsorption**		**Desorption**	
Pluronic	γ (mN/m)	E (mN/m)	γ (mN/m)	E (mN/m)	γ (mN/m)	E (mN/m)
F68	13.0 ± 0.5	12 ± 1	10.0 ± 0.5	5 ± 1	15.0 ± 0.5	14 ± 1
F127	10.0 ± 0.5	12 ± 1	11.0 ± 0.5	6 ± 1	13.0 ± 0.5	14 ± 1

complexes formed by the poloxamer and NaTDC. Furthermore, the dilatational modulus also recovers after desorbing the bile salt (Table 2), hence indicating that Pluronic-adsorbed layers are resistant to the displacement by bile salts. On the other hand, we can observe some differences in the desorption profiles displayed in Figure 6. In the presence of a F68 pre-formed layer, the interfacial tension increases more than in the presence of F127, suggesting that Pluronic F68 is more susceptible to bile salt penetration and/or displacement than Pluronic F127. These findings importantly agree with the calorimetric results of emulsions that also suggested a greater disruption of the F68 interfacial layer by BS, as compared to F127, providing evidence of the interfacial mechanisms underlying the behavior of emulsions. In addition, these interfacial results clarify the fact that even in the case of F68 adsorbed layer, the emulsifier is not completely displaced by the bile salt.

Next, we will study the competitive adsorption of Pluronic and NaTDC at the oo–water interface in both cases, F68 and F127. Adsorption isotherms of the mixture Pluronic (0.5 wt%)/NaTDC after 1 h of adsorption are represented in Figure 7a. The dilatational rheology was also measured after this time and the dilatational modulus is represented in Figure 7b. The Pluronic concentration chosen for these experiments (0.5 wt%) is equivalent to the concentration of non-adsorbed Pluronic calculated in non-washed emulsions. Figures 7a and 7b also include the curves for adsorption of pure NaTDC as a reference, illustrating a sigmoidal interfacial tension isotherm and a maximum in the dilatational modulus vs. bulk concentration as reported for other bile salts (Maldonado-Valderrama, Muros-Cobos, Holgado-Terriza, & Cabrerizo-Vílchez, 2012b). By comparing these reference curves with those of Pluronic/NaTDC mixture, we can elucidate the adsorption process as a function of the BS concentration. As a general trend, it can be seen that at low NaTDC concentrations there exists a strong deviation of both, interfacial tension isotherms and dilatational moduli of mixed systems from pure bile salt curves, indicating that the adsorption is mainly controlled by Pluronics. Accordingly, within this interval, it might be assumed that the final adsorbed layer

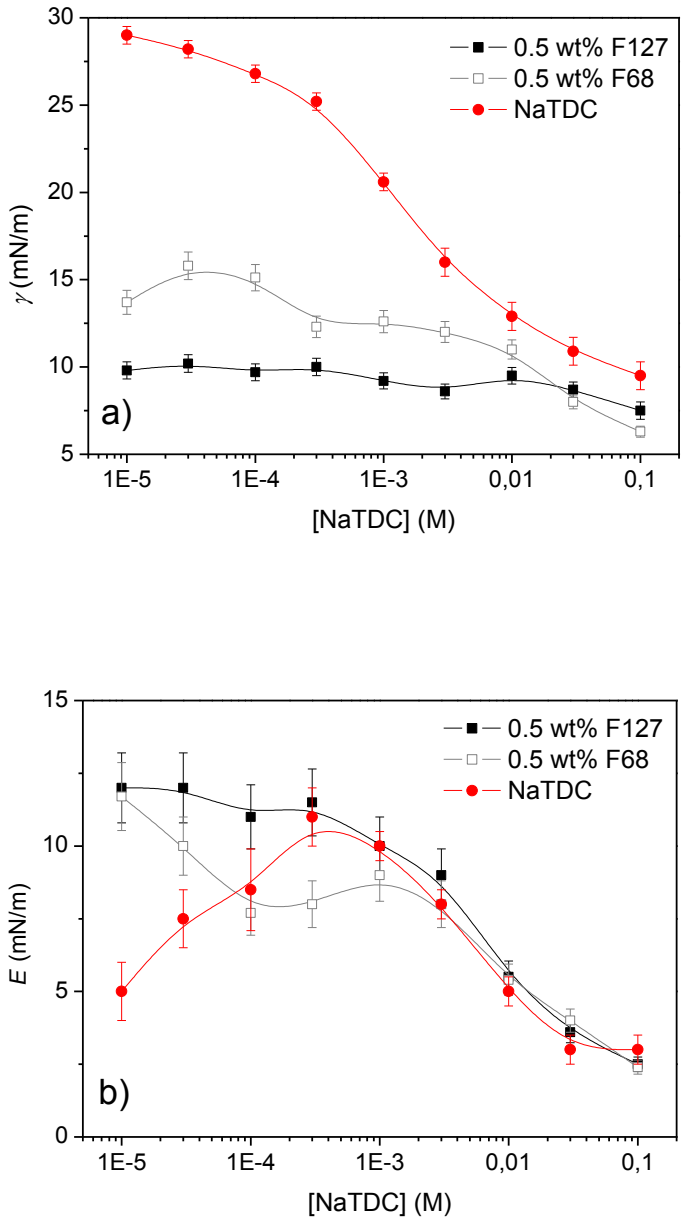


Figure 7: a) Interfacial tension and b) dilatational modulus of competitive adsorption of 0.5 wt% Pluronic as a function of bile salt concentration after 1 h of adsorption at 20 ºC. A reference curve of pure NaTDC in the absence of Pluronic is also included.

is composed entirely of Pluronic. Conversely, at higher NaTDC concentrations, bile salt seems to dominate the adsorption process since the mixed systems curves approach those of pure NaTDC (Figure 7a, 7b) suggesting that the interfacial layer at this high concentration of NaTDC is composed essentially (but not solely) of bile salt. Nevertheless, still at the highest NaTDC concentration, having excess of bile salt with respect to Pluronic, the interfacial tension is lower than that for pure bile salt. This is a very important observation that indicates the presence of Pluronic coexisting with NaTDC onto the interface. This reflects a great ability of Pluronic to compete with the bile salt for the available interfacial area in oil-in-water emulsions, in addition to the demonstrated resistance of the Pluronic adsorbed layer stabilizing the lipid droplets. Interestingly, we can also observe some differences between F68 and F127 mixed curves at low and intermediate NaTDC concentration. Bile salt seems to affect the adsorption process controlled by F68 at a very low concentration of BS as compared to the mixed system dominated by F127. This is reflected in the appearance of a maximum in the interfacial tension isotherm and a local minimum in the dilatational modulus of mixed F68–NaTDC system. This again agrees with the greater susceptibility of F68 to interact in the bulk with bile salt, as previously detected by micro-DSC measurements. The slight maximum observed in the interfacial tension, might be due to the less surface activity of the complexes formed by Pluronic and NaTDC, as detected by thermal analysis, and as it was previously reported for Pluronic and ionic surfactants (Hecht & Hoffmann, 1994). This maximum in the interfacial tension also corresponds to the local minimum found in the dilatational modulus. However, this pattern of interfacial tension with increasing NaTDC concentration is not reflected in the F127 curve, probably due to the lower interfacial tension provided by the polymer. For lower concentrations of Pluronics is easier to observe the interfacial tension vs. surfactant concentration (in this case NaTDC) profile shown by F68 (Hecht & Hoffmann, 1994).

3.4. Microstructure of the emulsions

Images obtained by cryo-SEM enable the visualization of the microstructure of the emulsions used in this study. Figure 8 shows a set of images for 10 wt% oo in water emulsions stabilized by 1 wt% F127 or F68 in the absence and presence of the bile salt (10 mM NaTDC). In the absence of BS, we can observe lipid droplets with a relatively smooth surface, surrounded by a homogeneous aqueous phase. In the image of a F68-stabilized emulsion, we also find the hollows where lipid droplets were located before fracturing the sample to expose the internal structure.

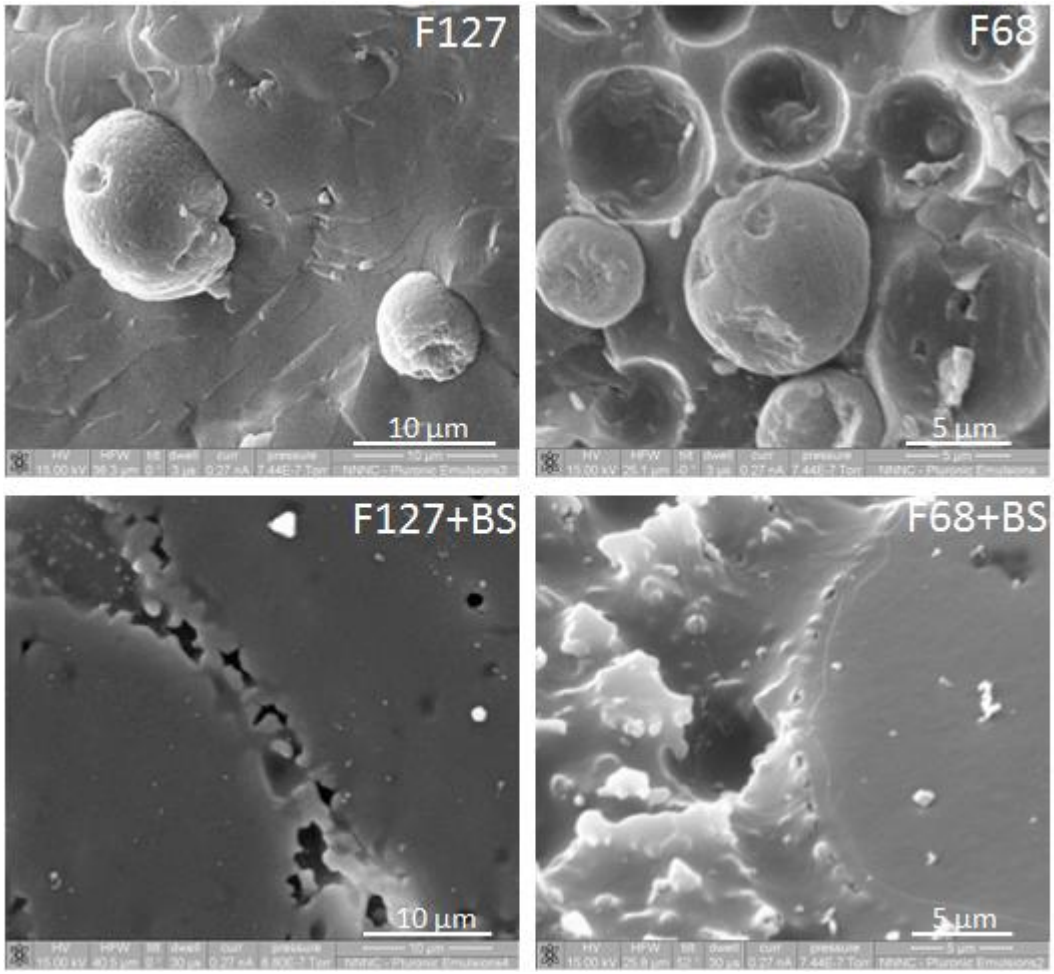


Figure 8: Images from cryo-SEM of 10 wt% olive oil in water emulsions stabilized by 1 wt% F127 or F68 in the absence and presence of the bile salt (10 mM).

The uniform texture is also reflected in these cavities. Interestingly, cryo-SEM reveals the presence of lumps in the aqueous phase upon addition of NaTDC. The image of the system stabilized by F127 shows the surface of two lipid droplets, whereas the image corresponding to the sample stabilized by F68 illustrates the interface between a lipid droplet and the aqueous phase in the bulk (Figure 8). It can be seen how this granular texture also affects the surface of lipid droplets that now appear much larger than in the absence of BS. The interfacial tension and mDSC results demonstrated a great resistance of Pluronics to be displaced by NaTDC, however it is likely that the bile salt co-adsorbs, penetrating the interfacial layer of Pluronic. The disruption caused by the BS also decreases the elasticity of the interfacial layer, as it was shown in Table 2, hence turning it more susceptible to some destabilization phenomena such as coalescence (Torcello-Gómez et al., 2011c).

4. Conclusions

The results from the thermal analysis suggest that there is more probability of displacement of F68 by the model bile salt NaTDC from the lipid droplets surface, as compared to F127. Interfacial tension and dilatational rheology studies corroborate this fact. Therefore, we demonstrate that the access of BS to the oil–water interface might be easier in the presence of a F68 interfacial layer (smaller molecular area) than in the presence of adsorbed F127 (larger molecular area). We propose that the differences found in the ability of these Pluronics to delay lipid digestion in emulsions, are due to the differences in the interfacial structure of the lipid droplets (not only different length of the steric bulky layer provided by the PEO blocks, but also different interfacial coverage provided by the PPO blocks). Additionally, it is also important to take into account the ability of Pluronics to compete for the oil-water interface, also influenced by the degree of interaction with NaTDC in the bulk. Pluronic F68 seems to be more susceptible to interact

with bile salt although eventually, each molecule of F127 could bind to more molecules/micelles of NaTDC, preventing them from adsorbing onto the oil-water interface, affecting their ability to promote the further cascade of lipolysis in the presence of co-lipase and lipase enzymes. The combination of bulk, emulsion and interfacial events provides a new perspective into the interaction of BS with Pluronics. Findings from this study underscore the importance of accurately combining colloidal and interfacial knowledge in order to promote reliable advances.

Acknowledgments

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4. Results

Pluronic-covered oil-water interfaces under simulated duodenal conditions (Submitted)

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ABSTRACT

An emerging application of colloidal science is the design of foods that provide specific physiological responses. This includes the development of biotechnologically remodeled lipids with tailored properties, such as control of satiety and delivery of bioactive components to specific locations. Pluronics are known to hinder the access of lipase in the presence of bile salts to the lipid droplets owing to the formation of a steric barrier, thus delaying the rate of lipid digestion. The aim of this work is to advance into the understanding of the impact of Pluronics on lipid digestion. We have studied the effect of the lengths of the hydrophobic/hydrophilic chains on *in-vitro* digestion of interfacial layers of Pluronics adsorbed at the olive oil-water interface. Pluronics F127 and F68 were tested. F127 has longer hydrophobic and hydrophilic chains than F68, providing higher interfacial coverage at the same molar concentration, due to the hydrophobic chains adsorbed to the oil–water interface and greater steric bulky layer provided by the hydrophilic chains remaining in the aqueous phase. F127 and F68 interfacial layers were selected to provide same interfacial coverage, i.e. same interfacial tension, but differing in the length of the steric bulky layer. The experiments have been carried out in a novel pendant drop film balance developed at the University of Granada (The OCTOPUS), which allows the simulation of the digestion process on a single droplet. A correlation between molecular size and digestion rate is obtained from the interfacial response importantly complementing the behavior of emulsions. The chosen Pluronic adsorbed layers showed similar resistance to lipase activity in the presence of bile salts, indicating the importance of the interfacial coverage when controlling lipid digestion rate. These fundamental studies can have enormous implications towards the rational design of functional biointerfaces.

1. Introduction

Pluronics are non-ionic triblock copolymers widely used as stabilizer for suspensions and emulsions in industrial applications, such as cosmetics and pharmaceuticals (Batrakova & Kabanov, 2008). Their applications are mainly related to their surface properties and micelle formation (Alexandridis & Lindman, 2000; Nakashima & Bahadur, 2006), due to the amphiphilic character provided by their molecular structure. A poly-propylene oxide (PPO) central block constitutes the hydrophobic part, whereas two lateral poly-ethylene oxide (PEO) blocks constitute the hydrophilic part. These polymers may act as steric barriers at solid surfaces in medical devices to avoid undesired adhesion of proteins (Li & Caldwell, 1996; Moghimi & Hunter, 2000; Jackson, Springate, Hunter, & Burt, 2000; Torcello-Gómez et al., 2011a) and stabilize the liquid interfaces of emulsions to prevent coalescence and flocculation (Jumaa & Müller, 1998; Wulff-Pérez, Torcello-Gómez, Gálvez-Ruiz, & Martín-Rodríguez, 2009; Gotchev, Kolarov, Khristov, & Exerowa, 2011). The application of Pluronics in physiological processes is a promising scenario opening up new horizons in food research for control of satiety and delivery of bioactive components to specific locations (Wulff-Pérez, de Vicente, Martín-Rodríguez, & Gálvez-Ruiz, 2012a). Their ability to rationally control lipid digestion profiles within the gastrointestinal tract would facilitate the development of functional emulsion-based delivery systems with tailored biological activities. During digestion, the body secretes bio-surfactants such as bile salts that replace the interfacial layer on emulsion droplets to prepare them for enzymatic digestion: lipolysis. Lipolysis is the enzymatic hydrolysis of lipids by lipase. Although some lipolysis (c.a. 10%) occurs in the stomach, the majority occurs in the duodenum through the action of pancreatic lipase (Fave, Coste, & Armand, 2004), directly after the stomach begins to empty. Since lipids are insoluble in water, lipase must adsorb onto the lipid droplet surface, adopting a suitable configuration for lipolysis with the help of its co-factor colipase in the

presence of bile salts (Reis, Holmberg, Watzke, Leser, & Miller, 2008), in order to come into close proximity to the lipid substrate to hydrolyse the triglycerides into free fatty acids and monoglycerides. These lipolysis products are removed from the lipid droplet surface and solubilized by mixing with micelles of bile salts, which also transport the nutrients to the mucosa of the small intestine in order to be absorbed by the human body. Hence, lipid digestion is affected by emulsion structure and composition because it is largely an interfacial reaction. Therefore, it should be possible to tailor the lipid digestion process by controlling interfacial characteristics, for instance, the interfacial layer structure of the original emulsifier stabilizing the oil droplets in emulsions (McClements & Li, 2010). In this sense, it has been proven the effectiveness of the steric barrier formed by Pluronic F68 on lipase interfacial activity in the presence of bile salts, hindering the access of lipase to the oil–water interface (Torcello-Gómez et al., 2011b). This is a very important result that has been correlated with delaying the rate of lipid digestion (Torcello-Gómez, Maldonado-Valderrama, Martín-Rodríguez, & McClements, 2011c). Furthermore, it was shown that oil droplets covered by larger Pluronics (F127) were digested at a lower rate and extent than those covered by smaller Pluronics (F68) (Wulff-Pérez et al., 2012a).

Bearing this in mind, the aim of this work is to deepen the understanding of the impact of polymeric surfactants on lipid digestion. Studies dealing with adsorption properties of triblock copolymers at the oil–water interface are very scarce in the literature (Ramírez et al., 2011; Ramírez, Stocco, Muñoz, & Miller, 2012). For that reason, the first part of this work shows the interfacial characterization of Pluronic F127 and F68 at the olive oil–water interface. These systems have been approved as inactive ingredient for oral intake by U. S. Food and Drug Administration (FDA). Then, we show the effect of the lengths of the hydrophobic/hydrophilic chains on *in-vitro* digestion of interfacial layers in order to further understand the behavior observed in emulsions (Wulff-Pérez et al., 2012a). The experiments have been carried out in a new multisubphase exchange

equipment (The OCTOPUS) designed and fully assembled at the University of Granada, which allows the simulation of a customized digestion process on a pendant drop film balance (Maldonado-Valderrama, Holgado-Terriza, Torcello-Gómez, & Cabrerizo-Vílchez, 2012). A previous work showed that gastric media barely affected oil droplets covered by Pluronics (Wulff-Pérez et al., 2012a). Hence, the digestion protocol used here considers only the passage through the duodenum. *In-vitro* digestion is tested on pre-formed interfacial structures by addition of artificial digestive media as required by means of the subphase exchange technique (Cabrerizo-Vílchez, Wege, Holgado-Terriza, & Neumann, 1999) and monitoring *in-situ* changes in interfacial tension and dilatational modulus throughout the simulated digestion process. These two magnitudes can reveal molecular mechanisms for the digestion and metabolism of lipids (Chu et al., 2009; Torcello-Gómez et al., 2011b; Torcello-Gómez et al., 2011c; Maldonado-Valderrama, Torcello-Gomez, Holgado-Terriza, Cabrerizo-Vílchez, 2012). There are still relatively few studies that have specifically related interfacial characteristics to the rate and extent of lipid digestion. These fundamental studies can have enormous implications towards the rational design of functional biointerfaces in emulsion-based systems with specific physiological responses.

2. Materials and methods

2.1. Materials

The Pluronics used were F127 and F68 purchased from Sigma-Aldrich and used without further purification. These non-ionic triblock copolymers are also commercially known as Poloxamer 407 and 188, respectively and are based on a $PEO_aPPO_bPEO_a$ structure. The difference between them is the number of subunits in the PEO and PPO blocks and the hydrophilic–lipophilic balance (HLB). F127 has longer PPO and PEO segments ($PEO_{100}PPO_{65}PEO_{100}$, MW = 12600 g/mol)

with lower HLB (22), whereas F68 has shorter PPO and PEO blocks ($PEO_{75}PPO_{29}PEO_{75}$, MW = 8400 g/mol) which results in a greater average HLB (29).

For the duodenal juice preparation we used lipase from porcine pancreas L3126 (Sigma-Aldrich), Type II (100-400 units/mg protein, using olive oil -30 min incubation-). The lipase sample hydrolyses tri-, di-, and monoglycerides (in decreasing order of rate) and contains amylase and protease activity as well. Lipase samples were prepared immediately before use and filtered with Millex® filters (0.1μm PDVF). As a model bile salt we used sodium taurodeoxycholate (NaTDC, 97% purity) from Sigma-Aldrich. It is negatively charged and its molecular weight is 521.7 g/mol. NaCl (150 mM) and $CaCl_2$ (3 mM) manufactured by Sigma-Aldrich, were also used.

All the samples were dissolved in a 1.13 mM phosphate buffer (pH 7) prepared with Milli-Q purified water 0.054 μS. Highly refined olive oil was also purchased from Sigma-Aldrich, and purified with activated magnesium silicate (Florisil, Fluka) to eliminate free fatty acids and surface active impurities. The oil was kept under mild agitation with the resins in proportion 2:1 w/w for 3 h and centrifuged at 12000 rpm for 30 min in a bench centrifuge from Kronton instruments (Centrikon T-124). It was then filtered and stored under nitrogen away from light. All the glassware was cleaned with 10% Micro-90 solution and then sequentially rinsed with tap water, distilled water, isopropanol and ultrapure water. Only freshly prepared solutions were used for each experiment.

2.2. Set-up: The OCTOPUS

The interfacial tension measurements were carried out in The OCTOPUS: a Pendant Drop Film Balance equipped with a subphase multi-exchange device, fully assembled and developed at the University of Granada (Maldonado-Valderrama et

al., 2012). This new subphase multi-exchange accessory is based on a previous single subphase exchange device (Spanish Patent, registration number P9801626), where the normal capillary tip was substituted by an arrangement of two coaxial capillaries, connected each one to the channels on a double micro-injector, which can operate independently (Cabrerizo-Vílchez et al., 1999). The OCTOPUS upgrades this technology to achieve a multi-exchange device. Further details are described by Maldonado-Valderrama and co-workers (Maldonado-Valderrama et al., 2012). The whole set-up is computer controlled through the modified software DINATEN© developed by Dr. Holgado Terriza. DINATEN© fits the experimental drop profiles, extracted from digital drop micrographs, to the Young-Laplace equation of capillarity by using Axisymmetric Drop Shape Analysis (ADSA), providing as outputs the drop volume *V*, the interfacial tension γ, and the interfacial area *A*. The adsorption process is recorded at constant interfacial area through a modulated fuzzy logic PID algorithm (proportional, integral, and derivative control) by changing the volume (Wege, Holgado-Terriza, & Cabrerizo-Vílchez, 2002). The drop is immersed in a glass cuvette (Hellma), which contains the oil phase and is kept in a thermostatized cell. The interfacial tension of the clean interface was measured before every experiment to ensure the absence of surface-active contaminants obtaining values of (29.5 ± 0.5) mN/m at 20 ºC. All the experiments were performed starting at 20 ºC and their reproducibility was verified from the standard deviation of at least three replicate measurements.

The dilatational rheology of the different adsorbed layers was measured by applying an oscillatory perturbation to the interface by injecting and extracting volume to the drop. The system records the response of the interfacial tension to the area deformation, and the dilatational modulus (*E*) of the interfacial layer can be calculated from this response. The applied interfacial area oscillations were maintained below 5 % of amplitude to avoid excessive perturbation of the interfacial layer and the departure from the linear viscoelastic region, which was

checked in preliminary experiments. The oscillation frequency (f) was set to 0.1 Hz. In a general case, the dilatational modulus is a complex quantity that contains a real and an imaginary part: $E = E' + iE'' = \varepsilon + i2\pi f\eta$, where E' is the storage modulus and accounts for the elasticity (ε) of the interfacial layer and E'' is the loss modulus and accounts for the viscosity (η) of the interfacial layer.

2.3. Experimental design of Pluronic-covered oil–water interfaces under duodenal conditions

The OCTOPUS allows customization of the digestion model, depending on the specific requirements of the experiments. We have designed an *in-vitro* digestion model focusing on the events taking place within the duodenum, since gastric conditions barely affect oil-in-water emulsions stabilized by Pluronics F68 and F127 (Wulff-Pérez et al., 2012a). Initially, a Pluronic layer is pre-formed at control conditions and then subjected to conditions in the duodenum by subphase exchange of the original bulk solution with the next solution, thus mimicking the passage through the gut. Subphase exchange consists of changing 70 times the volume of the drop with the new solution, hence assuring complete replacement by the new subphase (Maldonado-Valderrama et al., 2012). The interfacial tension of the system under the new conditions is recorded, until it attains a steady state and then, the drop is subjected to 10 cycles of deformation at 0.1 Hz which provide the dilatational modulus of the interfacial layer under the new conditions. Briefly, the experiments were designed following this sequence of steps.

Pluronic adsorption at oil–water interface: the interfacial tension of the considered Pluronic (6 mM F68, 0.03 mM F127) is recorded for 30 min at constant interfacial area (30 mm^2) and at 20 ºC.

Washout: the subphase is exchanged by pure buffer in order to deplete the excess of Pluronic from the bulk and form an adsorbed layer. Then the interfacial tension is recorded for 30 min.

Duodenal conditions: the subphase is exchanged by buffer at pH 7 containing 150 mM NaCl and 3 mM $CaCl_2$, which are concentrations relevant to physiological studies and the temperature is increased to 37 ºC. The temperature remains at 37°C for the rest of the experiment. The interfacial tension is monitored for 30 min.

Lipolysis: the subphase is exchanged by a mixture of 0.16 mg/mL lipase and 1mM NaTDC dissolved in the duodenum media specified in the previous step. The concentrations of lipase and bile salt are at a physiological ratio. The interfacial tension is recorded for 40 min.

Desorption: the subphase is exchanged by pure buffer in order to desorb reversible adsorbed species such as bile salt or lipolysis products.

The interfacial tension is recorded *in-situ* during the whole process and the dilatational modulus is measured at the end of each step at 0.1 Hz.

3. RESULTS AND DISCUSSION

This section is divided in two parts. The first one aims to characterize the adsorption of the different Pluronics onto the oil–water interface. In the second part, we will select Pluronic-covered oil–water interfaces with key differences to be compared under duodenal conditions. Thus, once Pluronic adsorption is characterized, we will choose a concentration of each Pluronic leading to similar values of the interfacial tension. In this manner, the duodenal conditions will be applied onto pre-formed interfacial layers of Pluronic with the same interfacial coverage, being possible to focus on the effect of interfacial conformation rather than interfacial density. Finally, these results will be compared with the behavior

observed previously in emulsions in order to discern the interfacial aspects of lipid digestion.

3.1. Pluronic adsorption at oil–water interface

In order to choose the more suitable poloxamer-covered interfaces to test under the *in-vitro* digestion model, we need first to characterize the adsorbed layers of Pluronic F68 and F127 at the olive oil–water interface. This part comprises dynamic adsorption curves of the two types of Pluronic, as well as adsorption isotherms and dilatational moduli after 1 h of adsorption at 20 ºC.

Figure 1 shows the change in interfacial tension over time for the adsorption of F68 (Figure 1a) and F127 (Figure 1b) at constant interfacial area onto the olive oil–water interface for several bulk concentrations, at 20 ºC. As a general trend we can observe some similarities in the dynamic curves of both systems, such as fast initial adsorption rates at each bulk concentration, reaching a steady state of the interfacial tension in less than 1 h, even for the lower bulk concentrations. Also, we can see that higher bulk concentrations reach earlier a steady state of the interfacial tension. Nevertheless, the true equilibrium state is not attained at any concentration for any of the polymers within 1 h of adsorption. The interfacial tension continues to decrease very slowly with time, although almost constant surface tension values were reached at higher bulk concentrations (Blomqvist, Wärnheim, & Claesson, 2005). To reach a final value would take a very long time indeed, a range from 3 h for higher concentrations to more than 20 h for the lowest concentration (Ramírez et al., 2011). This feature is quite common for the adsorption of polymers and is probably due to very slow conformational changes that take place in a densely packed surface layer (Hansen, 2008). The shapes of the curves vary with the solution concentration, which reflects that the surface layer structure and dynamics change with surface coverage (Blomqvist et al., 2005). However, in any case, at each bulk concentration, F127 decreases the interfacial tension to a larger extent

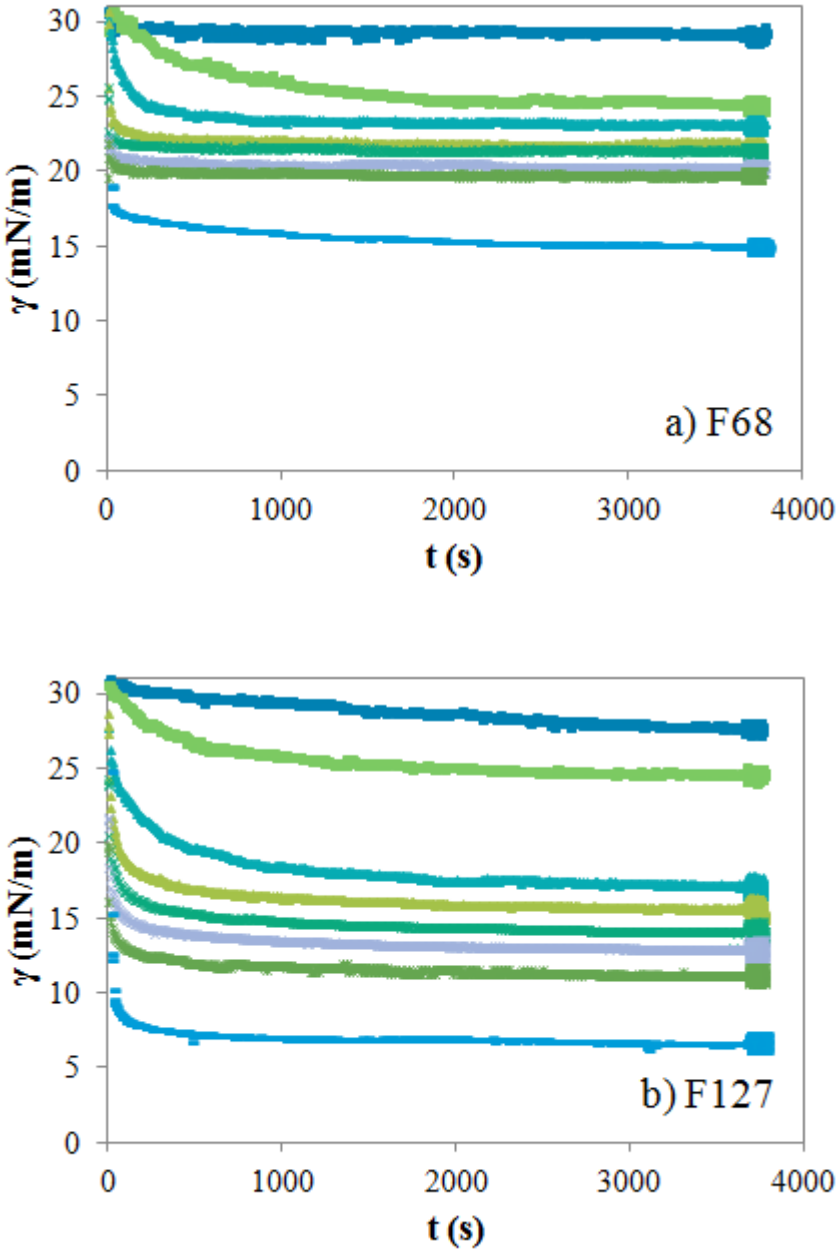

Figure 1: Dynamic adsorption curves of a) F68 and b) F127 at the olive oil–water interface at 20 ºC for different bulk concentrations (from upper side): 0.1 µM, 0.3 µM, 1 µM, 3 µM, 10 µM, 30 µM, 0.1 mM, 2 mM.

than F68. This is owing to the larger size of F127 molecule, as well as its higher hydrophobicity as compared to F68 molecule (Ramírez et al., 2011).

Next, Figure 2 shows the interfacial tension of both systems, after 1 h of adsorption at 20 ºC, vs. the bulk concentration, in order to compare the adsorption isotherms of the two Pluronics. As we have seen in Figure 1, F127 decreases the interfacial tension to a larger extent than F68 owing to its lower HLB or higher hydrophobicity, and also due to the larger molecular area occupied by one molecule of F127 as compared to F68. The area per molecule reported for PF68 lies between 3 and 3.4 nm^2/molecule (Chang, Lin, Kuo, & Gau, 2005; Noskov, Lin, Loglio, Rubio, & Miller, 2006; Torcello-Gómez, Jódar-Reyes, Maldonado-Valderrama, & Martín-Rodríguez, 2012), while this value increases to 6.5–7.21 nm^2/molecule for PF127 (Phipps, Richardson, Cosgrove, & Eaglesham, 1993; Blomqvist et al., 2005). Accordingly, a larger PPO block results in a larger surface

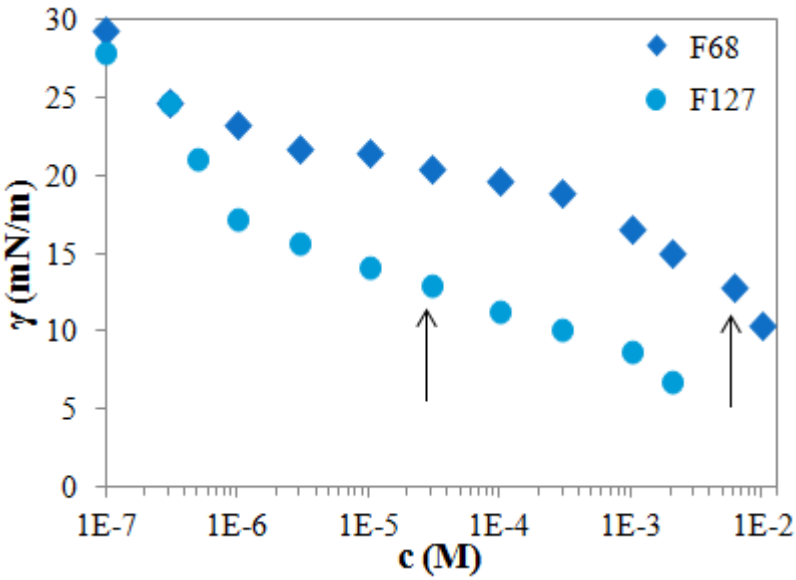

Figure 2: Interfacial tension isotherms of F68 (diamonds) and F127 (circles) at the olive oil–water interface at 20 ºC after 1 h of adsorption. Arrows indicate the Pluronic bulk concentrations chosen for *in-vitro* digestion.

tension decrease also because PPO is more surface active than PEO (Linse & Hatton, 1997). Similarly, a larger PEO block results in a larger surface tension decrease, which it is attributed to an increased repulsion between the PEO chains that decreases the interfacial tension (Blomqvist et al., 2005). The staircase shape exhibited by the adsorption isotherms is related to different conformations that PEO-PPO-PEO triblock copolymers adopt as adsorption increases (Prasad et al., 1979; Alexandridis, Athanassiou, Fukuda, & Hatton, 1994). The layer structure changes from a flat structure with all the segments lying flat at the interface at higher interfacial tension (lower interfacial coverage) to a brush-like structure where PPO blocks remain at the interface and PEO segments are protruding into the aqueous phase at lower interfacial tension (higher interfacial coverage) (Muñoz, Monroy, Ortega, Rubio, & Langevin, 2000).

Figure 3 displays the dilatational modulus measured at 0.1 Hz vs. the interfacial tension attained after 1 h of adsorption. In this way the dilatational modulus is represented as a function of the same interfacial coverage for both Pluronics. Since the modulus is a unique function of interfacial pressure, without large variations depending on adsorption time or solution concentration (Blomqvist et al., 2005), it is possible to make direct comparisons. At this frequency, the storage modulus or real component of the dilatational modulus is larger than the loss modulus or imaginary component for the two polymers over the whole concentration range (data not shown). Thus, the elastic contribution clearly dominates the interfacial dilatational behavior. We observe that the dilatational modulus as a function of the interfacial tension (Figure 3) goes through at least one maximum indicating the conformational changes occurring within the interfacial layer upon adsorption (Hansen, 2008). Concretely, the maximum observed for each Pluronic corresponds to interfacial tension values in the adsorption isotherm where the polymer adsorbed layer has already a brush conformation (Ramírez et al., 2011; Ramírez et al., 2012), with the PPO blocks adsorbed at the oil–water interface and PEO blocks stretched into the water phase (inverted "U"). As the interfacial tension

decreases from ca. 20 mN/m, that is the interfacial pressure increases, the dilatational modulus increases suggesting the formation of a more concentrated three dimensional structure. After reaching the maximum, the dilatational modulus is reduced indicating that the interfacial structure is less compact due to a mixture of polymer conformations. This feature is clearly observed for the more hydrophobic Pluronic, F127 (Ramírez et al., 2012). Once the maximum is reached, the repulsion within the interfacial layer becomes significantly stronger facilitating desorption of the more hydrophobic PPO segments, and the relaxation mechanism involving desorption of PPO segments becomes progressively more important as the interfacial coverage increases (Blomqvist et al., 2005). PPO blocks might protrude into the aqueous phase disordering the PEO interfacial structure. However, at the oil–water interface another possible scenario is that PPO protrudes into the oil phase reducing its contacts with the interface (Ramírez et al., 2012).

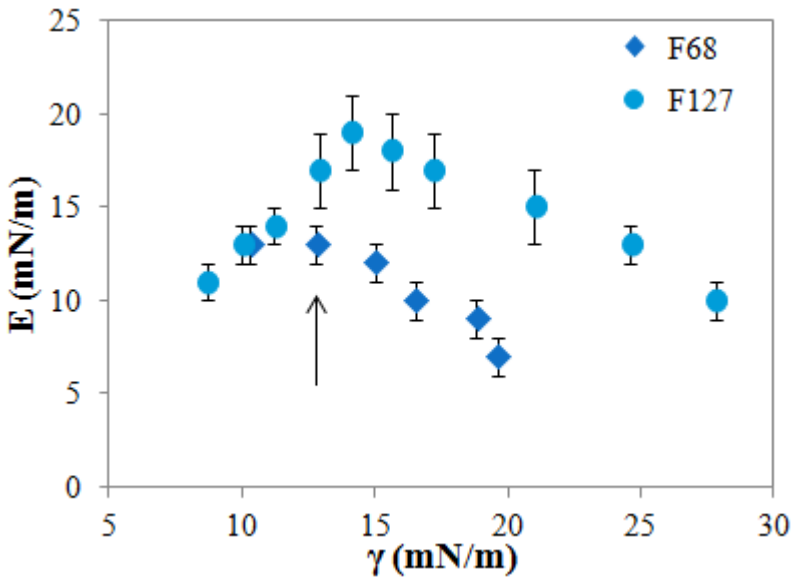

Figure 3: Dilatational modulus of F68 (diamonds) and F127 (circles) vs. interfacial tension at the olive oil–water interface at 20 ºC after 1 h of adsorption. Arrow indicates interfacial tension value chosen for *in-vitro* digestion.

In summary, an increase of loops and tails leads to a redistribution between the upper and lower regions of the layer. Concerning the differences between Pluronics, in general, F127 shows higher dilatational modulus indicating stronger intermolecular interactions as compared to F68, which could be explained by the differences in the length of PEO and PPO blocks and hydrophobicity between the two polymers. When interfacial tension decreases, the modulus rises higher for F127, due to increasing repulsion between larger PPO segments in the top layer and larger PEO segments in the sublayer, as compared to F68. This feature can be correlated with a previous work dealing with bulk shear rheology on concentrated oil-in-water emulsions stabilized by the same Pluronics (Wulff-Pérez, Martín-Rodríguez, Gálvez-Ruiz, & de Vicente, 2012b). In this study, Wulff-Pérez and co-workers show that F127 produces emulsions with higher viscoelasticity due to longer hydrophilic tails and longer hydrophobic central part.

3.2. In-vitro digestion of Pluronic-covered oil–water interfaces

In this section Pluronic-covered interfaces selected from the previous study are subjected to *in-vitro* digestion by pancreatic lipase in the presence of bile salts. We have chosen a bulk concentration for each Pluronic giving rise to the same interfacial tension and also corresponding to the maximum in the dilatational modulus, namely 0.03 mM F127 and 6 mM F68, marked by arrows in Figures 2 and 3. In this manner, we can test the *in-vitro* digestion experiments onto Pluronic-covered oil–water interfaces with a similar interfacial structure and interfacial coverage, being the main difference the length of the bulky layer provided by the PEO blocks and the molecular interactions specific to each Pluronic. In this case, F127 adsorbed interfacial layer has a longer steric barrier provided by longer PEO segments as compared to F68.

Figures 4 and 5 show the changes of the interfacial tension over time during each digestion step as designed in section 2.3. Table 1 displays the dilatational

moduli and interfacial tension values obtained for the interfacial layers after each digestion step. First, Pluronic adsorption is recorded at constant interfacial area for both systems at 20 ºC. This starting temperature takes into account the fact that, in general, food products are ingested at room temperature, so it will be possible to study the effect of change of temperature on the interfacial tension of Pluronic adsorbed layers as temperature increases up to 37 ºC. As we mentioned before, the two different polymer concentrations attain the same value of the interfacial tension, 13 mN/m (Figures 4 and 5) but different dilatational moduli, 12 mN/m for F68 and 17 mN/m for F127 (Table 1) accounting for the differences in molecular interactions as discussed in Figure 3. This control interfacial layer is saturated with the poloxamer, as measured by the steady state of the interfacial tension after 30 min of adsorption. In step 2 the subphase is exchanged by buffer in order to deplete the excess of polymer from the bulk (Figures 4 and 5). In this manner, we will

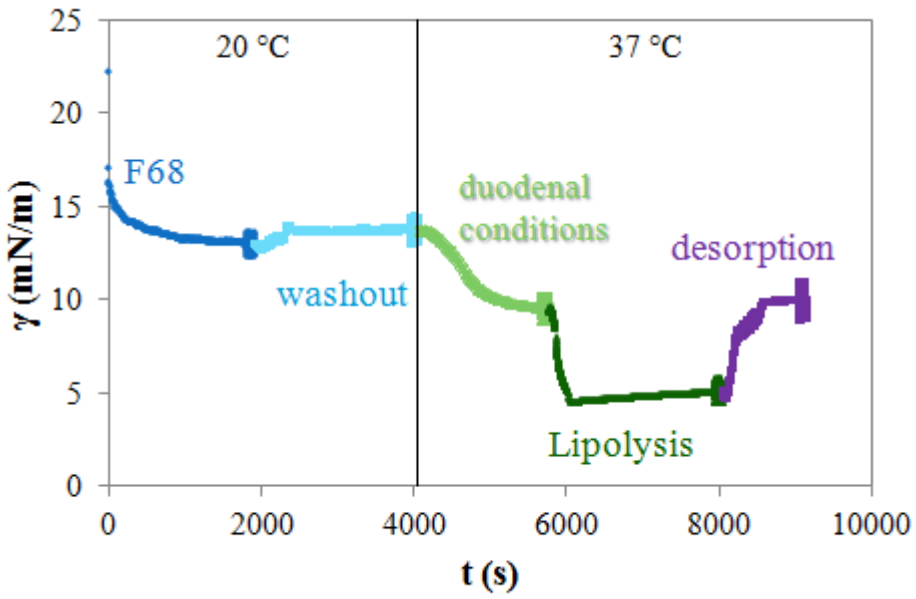

Figure 4: *In-vitro* digestion profile of F68 adsorbed layer at the olive oil–water interface. Conditions of each step are met through subphase exchange of solutions detailed in section 2.3.

consider only the contribution from the polymer adsorbed layer, but not the effect of non-adsorbed poloxamer in the bulk. Interestingly, this washout gives rise to a slight desorption of both Pluronics from the oil–water interface, as indicated by the slight increase in the interfacial tension during the subphase exchange, reaching 14 mN/m in both systems (Figures 4 and 5, Table 1). This indicates that Pluronics exhibit a strong desorption barrier in agreement with previously reported work in literature at air–water and oil–water interfaces (Svitova, Wetherbee, & Radke, 2003; Svitova & Radke, 2005). After subphase exchange, the interfacial tension keeps constant over time, hence providing a stable interfacial layer adsorbed at the oil–water interface which has similar dilatational elasticity as before the exchange (Table 1). Step 3 of simulated digestion applies duodenal conditions on the interfacial layer by increasing the temperature up to 37 ºC and by adding $CaCl_2$ (3 mM) and NaCl (150 mM) into the subphase. The interfacial tension is appreciably

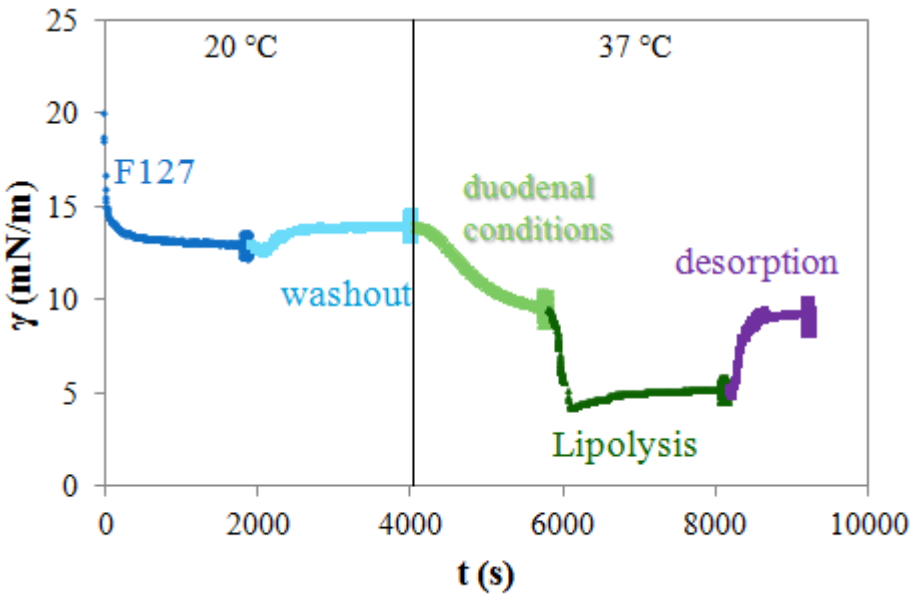

Figure 5: *In-vitro* digestion profile of F127 adsorbed layer at the olive oil–water interface. Conditions of each step are met through subphase exchange of solutions detailed in section 2.3.

4. Results

Table 1: Interfacial tension and dilatational modulus of F68 (6 mM) and F127 (0.03 mM) adsorbed layers at the olive oil–water interface after each step as designed in Figures 4 and 5. Values are obtained as mean of at least three replicate measurements.

Step	**E** (±1 mN/m)	**γ** (±1 mN/m)
F68/F127 adsorption	12/17	13
washout	14/15	14
duodenal conditions	14/17	10
Lipolysis	11	5
desorption	16/15	10/9

reduced in step 3, reaching a value of 10 mN/m in both cases after 30 min. The decrease in the interfacial tension is mainly caused by the increase of the temperature since the polymers are non-ionic and hence, ionic strength does not affect the interfacial tension on its own. Despite the changes recorded in the interfacial tension, the dilatational moduli are not affected by the duodenal conditions, as reflected in Table 1, meaning that the conformation of the polymers are not modified. In step 4 we measure the lipolysis by adding a mixture of pancreatic lipase (0.16 mg/mL) and BS (1 mM) into the subphase under duodenal conditions. This causes an abrupt decrease in the interfacial tension for both systems during the subphase exchange process, indicating BS adsorption and possible lipase onto the Pluronic-covered oil–water interfaces. This steep decrease in the interfacial tension might suggest that BS displaces Pluronic from the oil–water interface. Interestingly, after 5 min under the action of BS and lipase, the interfacial tension reaches a steady value in both systems, 5 mN/m (Figures 4 and 5), suggesting the saturation of the interface by the bile salt, adsorbed lipase and possible lipolytic products, such as free fatty acids and monoglycerides, which continues during 30 min. This decrease in the interfacial tension is accompanied by a slight decrease in the dilatational modulus, obtaining 11 mN/m for both systems (Table 1), which would corroborate the presence of duodenal components at the

interface which disrupt the interfacial network (Torcello-Gómez et al., 2011b; Torcello-Gómez et al., 2012). This will be discussed further later on in this section. Finally, step 5 of the simulated digestion protocol records the desorption of reversibly adsorbed species from the oil–water interface due to subphase exchange by buffer and hence, depletion of any bulk material from the subphase. This enables us to gain more information about the composition of the remaining adsorbed layer. The inclusion of this final step represents an improvement with respect to previous works (Torcello-Gómez et al., 2011b). For F68 (Figure 4) and for F127 (Figure 5), the desorption process occurs very rapidly, giving rise to the same interfacial tension previous to the lipolysis process (9-10 mN/m). Also, after the desorption step, the dilatational moduli increase obtaining values similar to those previous to the lipolysis process in both cases (Table 1), corroborating the interfacial tension results. This indeed indicates the presence of reversibly adsorbed material and traces of remaining material adsorbed after the exchange with buffer.

In order to further analyze the lipolysis and desorption processes, Figure 6 focuses on the two last steps of the experiments for both polymers, and for the bare olive oil–water interface. On the one hand, the above mentioned curves overlap for both Pluronic-covered oil–water interfaces, which suggests that the mixture of lipase and BS has similar effect on both Pluronic interfacial layers. In addition, the duodenal components have similar effect on the elastic modulus for both Pluronic adsorbed layers, in agreement with the interfacial tension results. Since the Pluronic-covered interfaces tested under *in-vitro* digestion mainly differ in the length of the steric barrier provided by the PEO blocks remaining in the bulk phase, the similarity observed in the changes of interfacial tension and dilatational modulus suggests the importance of the interfacial coverage provided by the PPO blocks adsorbed onto the interface. This is a very important result which corroborates the behavior of oil-in-water emulsions stabilized by the same polymers under duodenal conditions (Wulff-Pérez et al., 2012a). Wulff-Pérez and

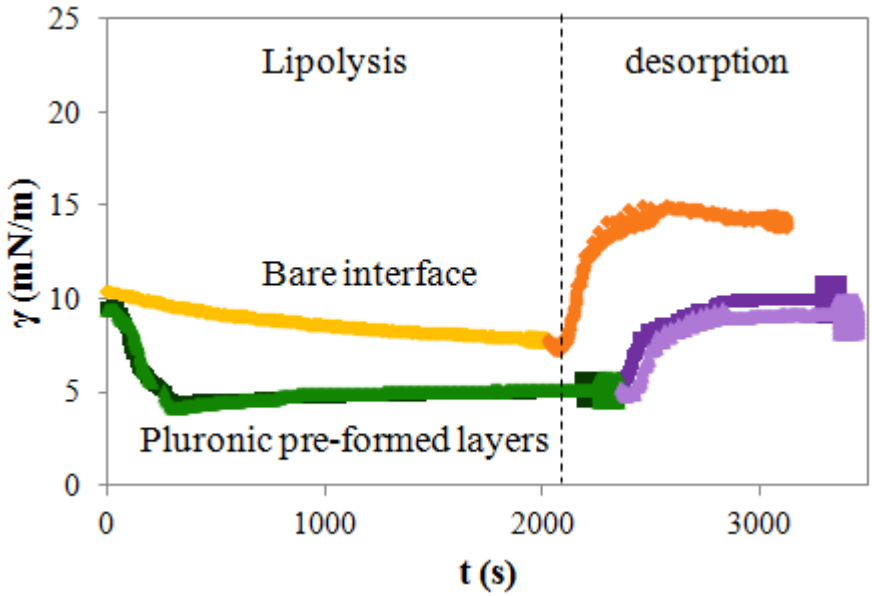

Figure 6: Lipolysis and desorption steps onto Pluronic-covered interfaces from Figures 4 and 5 and onto bare olive oil–water interface.

co-workers showed that emulsions stabilized by F127 were digested by pancreatic lipase in the presence of bile salts at a lower rate and extent as compared to emulsions stabilized by F68. However, the polymer concentration used to prepare the emulsions was the same for both Pluronics. Hence, at a fixed Pluronic bulk concentration, F127 provides not only higher steric barrier, but also greater interfacial coverage, as we have seen in section 3.1. Also, by comparing with the interfacial tension curve and dilatational modulus of the mixture of lipase and BS in the absence of Pluronic, that is, at the bare oil–water interface (Figure 6 and Table 2), we gain insight into the final composition of these interfacial layers. At a first glance, this comparison suggests that at least some Pluronic still remains adsorbed onto the oil–water interface, as the interfacial tension in the presence of Pluronic is appreciably lower than the value attained in the absence of the polymer throughout the whole lipolysis and desorption process, thus indicating the presence of more material adsorbed onto the interface. The dynamics during the lipolysis

process is also significantly different in the absence and presence of Pluronic (Figure 6). In the absence of Pluronic, the interfacial tension continuously decreases whereas the presence of Pluronic-adsorbed layer leads to a steady interfacial tension just after the subphase exchange by bile salt and lipase. Bile salt adsorbs very rapidly onto the interface governing the adsorption process at short times (Torcello-Gómez et al., 2011b). This agrees with the steep decrease of the interfacial tension during the subphase exchange in Pluronic-covered interfaces. Then, the adsorption of pancreatic lipase and possible lipolysis products contributes to the continuous decrease in the interfacial tension in the absence of adsorbed polymer. However, the lower and stable interfacial tension in the presence of previously adsorbed Pluronic suggests that the polymer is not completely displaced by the bile salt and the interface is saturated possibly limiting the lipolytic activity. The dilatational modulus values after the lipolysis in the presence (Table1) and absence of Pluronic adsorbed layer (Table 2) support the fact that Pluronic still coexists at the interface with some duodenal components. The dilatational modulus measured for the mixture of lipase and BS at the bare oil–water interface is appreciably lower, 2 mN/m (Table 2), than the modulus measured onto Pluronic-covered interfaces, 11 mN/m (Table 1). In fact, the latter value is intermediate between the modulus of pure duodenal components and that of pure Pluronic adsorbed layers, suggesting a mixed composition of the interfacial layer. The final values of the interfacial tension after the desorption process resulting from the

Table 2: Interfacial tension and dilatational modulus after lipolysis and desorption steps onto the bare olive oil–water interface as in Figure 6. Values are obtained as mean of at least three replicate measurements.

Step	**E** (±1 mN/m)	**γ** (±1 mN/m)
Lipolysis	2	8
desorption	6	15

remaining species adsorbed at the oil–water interface provide information of the digestion products. The desorption profile is mainly governed by the bile salt desorption, which is fully reversible (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011; Maldonado-Valderrama et al., 2012). In the case of the mixture of lipase and bile salt adsorbed onto the bare oil–water interface, the desorption profile gives rise to a lower value of the interfacial tension, 15 mN/m, than that corresponding to pure oil-water interface (Figure 6), indicating the presence of irreversibly adsorbed lipase and/or traces of possible lipolysis products, which were not solubilized and removed by bile salt. Interestingly, when the duodenal components were adsorbed onto Pluronic-covered oil–water interfaces, the desorption profiles give rise to even lower values of the interfacial tension (Figure 6). This proves again the presence of polymer onto the interface. Finally, comparison of the dilatational modulus after the desorption in the absence and presence of Pluronic provides again evidence of final interfacial layers mainly composed of Pluronic. The final dilatational modulus values after the desorption in the presence of Pluronic, 15-16 mN/m (Table 1), are closer to those previous to the lipolysis process in the presence of Pluronic adsorbed layers, 14-17 mN/m (Table 1), than those after desorption in the absence of the polymer, 6 mN/m (Table 2). The combination of these interfacial techniques has shown the vital importance of the interfacial coverage, apart from the steric barrier provided by these polymeric surfactants, in the resistance of Pluronics to the displacement by bile salts under duodenal conditions, with the subsequent limited rate of lipolysis.

4. Conclusions

The combination of interfacial tension and dilatational rheology has been used to characterize the adsorption and mechanical properties of Pluronic F127 and F68 at the olive oil–water interface and their *in-vitro* digestion profiles. Pluronic F127 lowers the interfacial tension to a larger extent and provides a more elastic

adsorbed layer due to stronger molecular interactions between longer PPO and PEO blocks, as compared to F68. Two different bulk concentrations of Pluronics ([PF127] < [PF68]) leading to the same interfacial tension, i.e. similar interfacial coverage, and comparable interfacial structure, but different in the elastic moduli were chosen to subject them to our simulated *in-vitro* digestion protocol. The results demonstrate that both Pluronics present a strong desorption barrier when the subphase is washed with pure buffer and similar resistance to the displacement of bile salts, subsequently retarding or limiting lipase activity. Since the two Pluronic adsorbed layers have similar interfacial coverage provided by the PPO blocks adsorbed at the oil–water interface, given by the same interfacial tension, the main difference hence resides in the length of the bulky layer provided by the number of units of the hydrophilic PEO blocks. Therefore, *in-vitro* digestion of Pluronic layers suggests that in the presence of a steric barrier, the susceptibility to lipolysis depends on the interfacial coverage controlled by the interfacial tension, rather than the steric bulky layer remaining in the aqueous phase. Obtaining the same extent of lipolysis with two different systems owing to the same interfacial tension value proves that interfacial tension is a crucial magnitude to control lipid digestion with these polymeric surfactants. The OCTOPUS provides generic information on interfacial structures, which importantly complements the behavior of emulsions. These fundamental studies can have enormous implications towards the rational design of functional biointerfaces with important applications when designing lipid digestion profiles for control of satiety and drug delivery.

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5. CONCLUSIONS

According to the objectives proposed, the conclusions of the more relevant results from this thesis are:

- The combination of interfacial tension and interfacial dilatational rheology allowed the characterization of Pluronics, phospholipids and physiological components at fluid-liquid interfaces. It was shown interesting differences between interfacial behavior of Pluronics and phospholipids. Phospholipids form a compact interfacial layer with higher elasticity than poloxamer, whereas Pluronic adsorbed layers show transitions upon increasing interfacial coverage. Larger Pluronics lower the interfacial tension to a larger extent and show higher elasticity than smaller Pluronics.

- The subphase exchange technique was successful to perform innovative *in-vitro* models, subjecting surfactant-covered interfaces to simulated physiological media. The sequential adsorption of the considered surfactant and physiological components allows interpretation of the key interactions taking place at the interfaces of colloidal systems in biological processes.

- Pluronic-covered air-water interfaces do not fully inhibit IgG adsorption, in agreement with nanoparticles coated in sequence by Pluronic and IgG that showed similar characteristics to those coated solely by IgG. Interestingly, adsorbed Pluronic affects the antibody adsorbed conformation as compared to the bare interface, correlating with the antibody denaturation suggested by the decrease in the immunoreactivity tests of solid nanoparticles coated in sequence by Pluronic and IgG. We propose that this structural alteration might be an essential mechanism decreasing immunoactivity and subsequent recognition by MPS.

5. Conclusions

- Pluronic-covered oil-water interfaces showed to be more resistant to displacement by bile salt, and inhibited the access of lipase, in contrast to phospholipids-covered oil-water interfaces. Thermodynamic adsorption models applied to individual systems provided structural explanation of interactions between emulsifiers and bile salts. Despite the lower elasticity of Pluronic interfacial layers, the steric hindrance provided demonstrated to be more effective than phospholipid adsorbed layers to reduce duodenal components adsorption. This correlates with the lower rate and extent of lipid digestion in Pluronic-stabilized emulsions than that obtained in phospholipids-stabilized emulsions.

- On the other hand, Pluronics with larger hydrophilic and hydrophobic chains showed to be more resistant to displacement from the oil-water interface by bile salts and prevent their adsorption onto the interface by interacting in the bulk. These bulk events have been firstly studied between Pluronics and bile salts, constituting another mechanism explaining the ability of Pluronics to delay lipid digestion. It was also demonstrated the relevance of the hydrophobic portion adsorbed onto the interface to limit the inclusion of bile salt and lipase and the subsequent rate of lipid digestion, importantly complementing digestibility of emulsions stabilized by these Pluronics.

- As a summary, fundamental interfacial studies satisfactorily reveal underlying mechanisms controlling biological processes such as opsonization and lipolysis.

5. CONCLUSIONES

De acuerdo a los objetivos planteados, se obtienen las siguientes conclusiones de los resultados más importantes de esta tesis:

- La combinación de tensión interfacial y reología dilatacional interfacial ha permitido caracterizar Pluronics, fosfolípidos, y componentes fisiológicos en interfases líquido-fluido. Se muestran contrastes entre el comportamiento interfacial de los Pluronics y los fosfolípidos. Los fosfolípidos forman una capa interfacial compacta y más elástica, a diferencia de los Pluronics, que presentan diferente conformación en función del recubrimiento interfacial y forman una capa interfacial menos elástica. Los Pluronics de mayor tamaño reducen en mayor medida la tensión interfacial y proporcionan más elasticidad a la capa interfacial, que los Pluronics de menor tamaño.

- Se ha logrado llevar a cabo modelos *in-vitro* muy innovadores mediante la técnica del intercambio de la subfase, sometiendo interfases recubiertas de surfactante a un medio en condiciones fisiológicas. La adsorción secuencial del surfactante en estudio y los componentes fisiológicos permite interpretar interacciones fundamentales que tienen lugar en las interfases de los sistemas coloidales durante los procesos biológicos.

- Las interfases aire-agua recubiertas de Pluronic no inhiben la adsorción de IgG, de acuerdo con las nanopartículas recubiertas secuencialmente de Pluronic e IgG que muestras características similares a las que presentan las nanopartículas recubiertas sólo de IgG. Sin embargo, el Pluronic adsorbido afecta la conformación de la IgG adsorbida en comparación con su adsorción sobre la interfase sin recubrir previamente de Pluronic. Esto se correlaciona con la desnaturalización de la IgG, como sugiere la pérdida de actividad

inmune en nanopartículas recubiertas secuencialmente de Pluronic y de IgG. En esta tesis se propone que esta alteración estructural podría ser un mecanismo esencial en la pérdida de inmunoactividad que afecta al posterior reconocimiento por el sistema mononuclear fagocítico.

- Las interfases aceite-agua recubiertas de Pluronic muestran ser más resistentes al desplazamiento de las sales biliares, e inhiben el acceso de la lipasa, a diferencia de las interfases recubiertas de fosfolípidos. La aplicación de modelos termodinámicos de adsorción a los sistemas estudiados individualmente proporciona información estructural sobre las interacciones entre emulsionantes y las sales biliares. A pesar de que la capa interfacial de Pluronic presenta una elasticidad más baja, la barrera estérica proporcionada es más eficaz para reducir la adsorción de los componentes del duodeno, que las capas interfaciales de fosfolípidos. Esto se correlaciona con la digestión de emulsiones estabilizadas con Pluronic, más lenta y de menor alcance que la de emulsiones estabilizadas con fosfolípidos.

- Los Pluronics formados por cadenas hidrofílicas e hidrofóbicas más grandes son más resistentes a ser desplazados por las sales biliares y pueden evitar la adsorción de éstas sobre la interfase aceite-agua mediante interacciones en el seno de la disolución. Es la primera vez que se estudia estas interacciones entre Pluronics y sales biliares en el seno de la disolución, proponiéndose como otro mecanismo adicional por el cual los Pluronics son capaces de frenar la digestión de lípidos. Se ha mostrado también la importancia de las cadenas hidrófobas, que una vez adsorbidas sobre la interfase, limitan el acceso de las sales biliares y la lipasa y por tanto, la digestión de lípidos. Este estudio complementa la digestión de emulsiones estabilizadas con los mismos Pluronics.

- Finalmente y a modo de resumen, los estudios interfaciales básicos ponen de manifiesto mecanismos esenciales que controlan los procesos biológicos como la opsonización y la lipólisis.

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Courses

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Publications in Peer-Reviewed Journals

1. Wulff-Pérez, M.; **Torcello-Gómez, A.**; Gálvez-Ruiz, M. J.; Martín-Rodríguez, A. "Stability of emulsions for parenteral feeding: Preparation and characterization of o/w nanoemulsions with natural oils and Pluronic F68 as surfactant". *Food Hydrocolloids*, **2009**, *23*, 1096-1102.

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3. Wulff-Pérez, M.; **Torcello-Gómez, A.**; Martín-Rodríguez, A.; Gálvez-Ruiz, M. J.; de Vicente, J. "Bulk and interfacial viscoelasticity in concentrated emulsions: The role of the surfactant". *Food Hydrocolloids*, **2011**, *25*, 677-686.

4. Torcello-Gómez, A.; Maldonado-Valderrama, J.; de Vicente, J.; Cabrerizo-Vílchez, M. A.; Gálvez-Ruiz, M. J.; Martín-Rodríguez, A. "Investigating the effect of surfactants on lipase interfacial behaviour in the presence of bile salts". *Food Hydrocolloids*, **2011**, *25*, 809-816.

5. Torcello-Gómez, A.; Maldonado-Valderrama, J.; Gálvez-Ruiz, M. J.; Martín-Rodríguez, A.; Cabrerizo-Vílchez, M. A., de Vicente, J. "Surface rheology of sorbitan tristearate and β-lactoglobulin: shear and dilatational behavior". *Journal of Non-Newtonian Fluid Mechanics*, **2011**, *166*, 713-722.

6. Torcello-Gómez, A.; Maldonado-Valderrama, J.; Martín-Rodríguez, A.; McClements, D. J. "Physicochemical properties and digestibility of emulsified lipids in simulated intestinal fluids: influence of interfacial characteristics". *Soft Matter*, **2011**, *7*, 6167-6177.

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8. Torcello-Gómez, A.; Jódar-Reyes, A. B.; Maldonado-Valderrama, J.; Martín-Rodríguez, A. "Effect of emulsifier type against the action of bile salts at oil–water interfaces". *Food Research International*, **2012**, *48*, 140-147.

Publications in Proceeding Books

1. Torcello-Gómez A., Maldonado-Valderrama J., Martín-Rodríguez A., Gálvez-Ruiz M. J. "Poloxamers as a solution for the biocompatibility of polymeric surfaces with plasma proteins: a surface tension study". In *Proceedings of the 5th International Symposium on Food Rheology and Structure*, 2009 (ISBN: 978-3-905609-43-1).

2. Wulff-Pérez M., de Vicente J., **Torcello-Gómez A.**, Martín-Rodríguez A., Gálvez-Ruiz M. J. "Stability and rheological properties of nanoemulsions based on natural oils". In *Proceedings of the 5th International Symposium on Food Rheology and Structure*, 2009 (ISBN: 978-3-905609-43-1).

3. Torcello-Gómez A., Maldonado-Valderrama J., Martín-Rodríguez A., McClements D. J. "Digestibility of emulsified lipids: influence of interfacial characteristics". In *Proceedings of the 4th Iberian Meeting on Colloids and Interfaces*, 2011 (ISBN: 978-989-97397-2-7).

Contributions to Conferences and Workshops

Oral Contributions (presenting author underlined)

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7. Maldonado-Valderrama, J.; Holgado-Terriza, J. A.; **Torcello-Gómez, A.**; Cabrerizo-Vílchez, M. A. "Development of a new subphase multi-exchange pendant drop tensiometer for *in-vitro* digestion studies". 19th International Symposium on Surfactants in Solution. June, 24-28, 2012. Alberta, Canada.

Poster Contributions

1. Torcello-Gómez, A.; Wulff-Pérez, M.; Maldonado-Valderrama, J.; Martín-Rodríguez, A.; Gálvez-Ruiz, M. J. "Inhibitory effect of Pluronic F68 on the adsorption of IgG onto fluid-liquid interfaces". 12th Food Colloids. April, 6-9, 2008. Le Mans, France.

2. Wulff-Pérez, M.; **Torcello-Gómez, A.**; Gálvez-Ruiz, M. J.; Martín-Rodríguez, A. "Stability of Emulsions for Parenteral Feeding: Preparation and Characterization of O/W Nanoemulsions with Natural Oils and Pluronic-F68© as Surfactant". 12th Food Colloids. April, 6-9, 2008. Le Mans, France.

3. Torcello-Gómez, A.; Maldonado-Valderrama, J.; Martín-Rodríguez, A.; Gálvez-Ruiz, M. J. "Poloxamers as a solution for the biocompatibility of polymeric surfaces with plasma proteins: a surface tension study". 5th International Symposium on Food Rheology and Structure. June, 15-18, 2009. Zürich, Switzerland.

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13. Torcello-Gómez, A.; Maldonado-Valderrama, J.; Jódar-Reyes, A. B.; Gálvez-Ruiz, M. J.; Martín-Rodríguez, A. "The effect of bile salt on lipolysis of emulsions stabilised with different emulsifiers". 5th World Congress on Emulsion. October, 12-14, 2010. Lyon, France.

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