University of Ioannina
School of Health Sciences
Department of Biological Applications and Technology
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Development of microemulsions for the administration of bioactive compounds

Doctoral Thesis

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"If you see someone without a smile give him one of yours"

Evgenia

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This is the wonder of devotion
I see the torch
We all must hold
This is the mystery of the quotient
Upon us all a little rain must fall

Led Zeppelin, The Rain Song, Houses of the Holy, 1973

Publications

Parts of the present thesis have been published (or submitted) in peer-reviewed scientific journals.

(The publications can be found attached at the **Results/Publications** section)

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1. **33rd Conference of European Colloid and Interface Society** (ECIS 2019), 8-13 September 2019, University of Leuven, Belgium, Participation with poster presentation, Title: Development of biocompatible microemulsions for intestinal and nasal delivery of natural bioactive compounds

2. **8th Panhellenic Greek Lipid Forum "New Trends in the field of Lipids"**, 21 July 2019, National Hellenic Research Foundation, Participation with poster presentation Title: Microemulsion as systems for administration of bioactive compounds

3. **32nd Conference of European Colloid and Interface Society** (ECIS 2018), 2-7 September 2018, University of Ljubljana, Slovenia, Participation with poster presentation, Title: Novel water-in-oil (W/O) microemulsion as gallic acid carrier for pharmaceutical applications. A structural and efficacy study.

4. **European Federation for Pharmaceutical Sciences** (EUFEPS) **Annual Meeting**, 24-26 May, 2018, Titania Hotel, Athens. Participation with poster presentation, Topic: Formulation of biocompatible colloidal nanodispersion as appropriate vehicle for enzyme drug delivery: the model case of horseradish peroxidase (HRP).

5. **7th Panhellenic Greek Lipid Forum "New Trends in the field of Lipids",** 5 October 2017, Technological Institute of Thessaloniki, Participation with oral presentation Title: Biocompatible non-ionic water-in-oil microemulsions, based on Extra Virgin Olive Oil, as carriers of bioactive compounds and enzymes.

6. **31st Conference of European Colloid and Interface Society** (ECIS 2017)**,** 3-8 September 2017, Universidad Complutense de Madrid, Faculty of Medicine, Madrid, Spain, Participation with poster presentation, Title: Non-ionic water-in-oil microemulsions as vehicles for enzyme drug delivery: the model of Horseradish Peroxidase (HRP)

7. **30th Conference of European Colloid and Interface Society** (ECIS 2016), 4-9 September 2016, University of Rome "La Sapienza", Participation with poster presentation, Title: Development and in vitro evaluation of Water-in-Oil microemulsions for the intestinal delivery of Hydroxytyrosol.

Abbreviations

W/O	Water-in-oil	**G**	Gibbs Energy	**A_o**	Micropolarity
DLS	Dynamic Light Scattering	**ΔH**	Enthalpy change	**EPT**	Enzyme-prodrug stratefy
EPR/ESR	Electron Paramagnetic Resonance	**CPP, p**	Critical Package Parameter	**BBB**	Blood Brain Barrier
DIT	Dynamic Interfacial Tension	**CMC**	Critical Micellar Concentration	**EFSA**	European Food and Safety Authority
SAXS	Small Angle X-ray Scattering	**HLD**	Hydrophilic Lipophilic Difference	**LDL**	Low-density lipoprotein
HRP	Horseradish peroxidase	**CTAB**	Cetyltrimethyl ammonium bromide	**HDL**	High-density lipoprotein
HT	Hydroxytyrosol	**DDAB**	Distearyldimethyl ammonium bromide	**MBGs**	Microemulsion Based Gels
GA	Gallic acid	**AOT**	Bis-(2-ethylhexyl) sulfosuccinate sodium salt	**GI**	Gastrointestinal
DMG	Distilled monoglycerides	**GRAS**	Generally Regarded as Safe	**DC**	Direct current
EVOO	Extra Virgin Olive Oil	**Rh**	Hydrodynamic diameter	**CLDN**	Claudin
SO	Sunflower Oil	**PdI**	Polydispersity index	**OCLN**	Occludin
IPM	Isopropyl myristate	**NO**	Nitric oxide		
PG	Propylene glycol	**HLB**	Hydrophilic Lipophilic Balance		
IFT, γ	Interfacial Tension	**5-DSA**	5-doxyl stearic acid		
O/W	Oil-in-Water	**τ_R**	Rotational correlation time		
H	Interfacial curvature	**S**	Parameter S		

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Abstract

Microemulsions, as liquid colloidal systems, have attracted the scientific interest mainly in the field of pharmaceuticals and foods, as they serve as excellent hosts for compound encapsulation increasing their final bioavailability. The aim of the present thesis was the formulation of biocompatible microemulsions based mainly on natural oils and biocompatible surfactants in order to serve as hosts for the encapsulation of natural bioactive compounds of therapeutic interest. Two (2) different routes of administration of high user compliance were studied in the present work in order to exploit the multi-functional nature of the formulated colloidal dispersions.

Different microemulsions were formulated, for the encapsulation of bioactive compounds, belonging to the category of water-in-oil (W/O) systems. The structural characterization of the microemulsions was conducted by means of Dynamic Light Scattering (DLS), Electron Paramagnetic Resonance (EPR), Dynamic Interfacial Tension (DIT) and Small Angle X-ray Scattering (SAXS) techniques. In order to study their future application as pharmaceutical formulations two (2) different types of bioactive compounds (enzymes and antioxidants) were encapsulated in the novel systems. Due to the polar nature of the dispersed phase of a W/O system the following hydrophilic compounds were chosen for encapsulation: the enzyme Horseradish peroxidase (HRP) and the natural antioxidants hydroxytyrosol (HT) and gallic acid (GA). Structural characterization shed light to the dimensions of the systems but also revealed information about the localization of the bioactive compounds in the complex microstructure.

In order to study the efficacy of the formulations regarding their ability to protect the encapsulated compounds and to increase their bioavailability, the proposed studied systems were examined with proper techniques. Kinetic studies were conducted in the case of HRP encapsulation and antioxidant activity was investigated with the use of EPR following antioxidants' encapsulation. The studies revealed that the encapsulated compounds retained their activity after the encapsulation, enhancing the suitability of the microemulsions for pharmaceutical applications, as they are able to maintain the properties of their hosts.

For the biological assessment of the proposed formulations two (2) different routes of administration were studied namely oral and nasal. In detail, the study focused on the absorption of the encapsulated bioactives after oral and nasal administration in the intestine and nasal cavity, respectively. As a result, *in vitro* techniques were used in order to understand the microemulsions' effect on the absorption of bioactive compounds following

administration. For both administration routes the appropriate cancer cell lines were used for *in vitro* studies, including cell viability and epithelial permeability assays. In order to exploit the absorption after oral administration of the microemulsions the intestinal co-culture model Caco-2/TC7 with HT29-MTX was used whereas, for nasal *in vitro* studies RPMI 2650 cell line was selected as the most appropriate. The *in vitro* assessments revealed the appropriate concentrations of the systems in order not to provoke any cytotoxic effect while the permeability assays in constructed epithelia indicated the strong participation of the microemulsion's ingredients in the transport profile of the bioactive compounds.

To conclude with, novel W/O microemulsions with the use of biocompatible ingredients were formulated serving as excellent hosts for bioactive compounds with therapeutic interest such as enzymes and antioxidants. The suitability of the proposed systems for oral and nasal delivery demonstrated the significant benefits of liquid-in-liquid nanodispersions arising from their microstructure and ingredients. The present study forms the basis for the development of pharmaceutical products which will combine a cost-effective strategy with high patient compliance.

Keywords: microemulsions; biocompatible; encapsulation; antioxidants; enzymes; Dynamic Light Scattering (DLS); Electron Paramagnetic Resonance (EPR); Small Angle X-ray Scattering (SAXS); cytotoxicity, epithelial permeability

Περίληψη

Τα μικρογαλακτώματα, ως υγρά κολλοειδή συστήματα, έχουν συγκεντρώσει το επιστημονικό ενδιαφέρον, ιδιαίτερα του τομέα φαρμάκων και τροφίμων, καθώς αποτελούν άριστο περιβάλλον εγκλωβισμού ουσιών αυξάνοντας ταυτόχρονα τη βιοδιαθεσιμότητά τους. Σκοπό της παρούσας διδακτορικής διατριβής αποτελεί η ανάπτυξη βιοσυμβατών μικρογαλακτωμάτων βασισμένων, κυρίως, σε φυσικά έλαια και επιφανειοενεργά με σκοπό τη χρήση τους στον εγκλωβισμό ουσιών με θεραπευτικό ενδιαφέρον. Δύο (2) διαφορετικές οδοί χορήγησης βιοδραστικών ουσιών μελετήθηκαν στο πλαίσιο της συγκεκριμένης διατριβής με σκοπό τη μελέτη της πολυ-λειτουργικής φύσης των υγρών νανοδιασπορών σε φαρμακευτικές εφαρμογές.

Διαφορετικά μικρογαλακτώματα, που ανήκαν στην κατηγορία τύπου νερού-σε-έλαιο (N/E) αναπτύχθηκαν στο πλαίσιο της παρούσας μελέτης και τελικά χρησιμοποιήθηκαν για τον εγκλωβισμό βιοδραστικών ουσιών. Ο δομικός χαρακτηρισμός των μικρογαλακτωμάτων, πραγματοποιήθηκε με τη χρήση των τεχνικών της Δυναμικής Σκέδασης Φωτός (DLS), του Ηλεκτρονικού Παραμαγνητικού Συντονισμού (EPR), της Δυναμικής Επιφανειακής Τάσης (DIT) και της Σκέδασης ακτινών-Χ υπό μικρή γωνία (SAXS). Στα αναπτυχθέντα συστήματα εγκλωβίστηκαν δύο (2) διαφορετικοί τύποι βιοδραστικών ουσιών (ένζυμα και αντιοξειδωτικά). Λόγω της πολικής φύσης της διεσπαρμένης φάσης των συστημάτων εγκλωβίστηκαν το ένζυμο Υπεροξειδάση του χρένου (Horseradish peroxidase, HRP) και τα φυσικά αντιοξειδωτικά υδροξυτυροσόλη (hydroxytyrosol, HT) και γαλλικό οξύ (gallic acid, GA). Η μελέτη των δομικών χαρακτηριστικών των συστημάτων απουσία και παρουσία των βιοδραστικών ουσιών, καθώς και οι αλλαγές αυτών, έδωσαν πληροφορίες σχετικά με το μέγεθος των διασπορών των αναπτυχθέντων μικρογαλακτωμάτων αλλά και συντέλεσαν στον προσδιορισμό της θέσης των ουσιών στην πολύπλοκη μικροδομή τους.

Για τον έλεγχο της αποτελεσματικότητας των συστημάτων, τα προτεινόμενα μικρογαλακτώματα ελέγχθηκαν είτε ως προς την ικανότητά τους να καταλύουν γνωστές αντιδράσεις (στην περίπτωση εγκλωβισμού ενζύμου) είτε να διατηρούν την αντιοξειδωτική ικανότητα των εγκλωβισμένων ουσιών (στην περίπτωση της υδροξυτυροσόλης και του γαλλικού οξέος). Διαπιστώθηκε ότι οι εγκλωβισμένες ουσίες διατήρησαν τις αρχικές τους ιδιότητες μετά τον εγκλωβισμό τους, ενώ τα ίδια τα μικρογαλακτώματα λόγω της φύσης τους εμφάνισαν ισχυρή αντιοξειδωτική δράση, αυξάνοντας την καταλληλότητα τους για χρήση σε φαρμακευτικές εφαρμογές.

Για τη βιολογική αποτίμηση των προτεινόμενων συστημάτων δύο (2) διαφορετικοί οδοί χορήγησης μελετήθηκαν: η από του στόματος και η ρινική. Συγκεκριμένα, η μελέτη εστιάστηκε στην συμπεριφορά των μικρογαλακτωμάτων και στην συμβολή τους στην απορρόφηση των εγκλωβισμένων βιοδραστικών ουσιών, μέσω *in vitro* τεχνικών, στις περιοχές απορρόφησης που συνδέονται με την από του στόματος και τη ρινική χορήγηση. Τόσο για την από του στόματος όσο και για τη ρινική χορήγηση πραγματοποιήθηκαν *in vitro* μελέτες κυτταροτοξικότητας και διαπερατότητας από κατασκευασμένα επιθήλια με τη χρήση κατάλληλων καρκινικών σειρών. Συγκεκριμένα, στην πρώτη περίπτωση χρησιμοποιήθηκε η συγκαλλιέργεια των εντερικών επιθηλιακών καρκινικών κυτταρικών σειρών Caco-2/TC7 και HT29-MTX, ενώ στη δεύτερη περίπτωση η καρκινική κυτταρική σειρά ρινικού επιθηλίου RPMI 2650. Μέσω των *in vitro* μελετών προσδιορίστηκε το εύρος των μη τοξικών συγκεντρώσεων των συστημάτων, σε κάθε περίπτωση, ενώ η μελέτη επιθηλιακής διαπερατότητας των βιοδραστικών ουσιών ανέδειξε την ισχυρή συμμετοχή των συστατικών των μικρογαλακτωμάτων στην συγκεκριμένη διαδικασία.

Εν κατακλείδι, στο πλαίσιο της συγκεκριμένης διατριβής αναπτύχθηκαν πρωτότυπα βιοσυμβατά μικρογαλακτώματα τα οποία αποτέλεσαν εξαιρετικό μικροπεριβάλλον για τον εγκλωβισμό βιοδραστικών ουσιών όπως ένζυμα και αντιοξειδωτικά. Η καταλληλότητα των παραπάνω συστημάτων για από του στόματος και ρινική χορήγηση ανέδειξε τα πλεονεκτήματα αυτών των συστημάτων. Τα αναπτυχθέντα μικρογαλακτώματα λόγω του μικροπεριβάλλοντός τους, των συστατικών αλλά και των ιδιοτήτων τους μελλοντικά μπορούν να μορφοποιηθούν περαιτέρω με σκοπό την εφαρμογή τους στη φαρμακευτική βιομηχανία ως προϊόντα που θα συνδυάζουν το χαμηλό κόστος και την αποτελεσματικότητα προσφέροντας παράλληλα στον χρήστη έναν εύκολο τρόπο χορήγησης ιδιαίτερα για μακράς διάρκειας θεραπείες.

Λέξεις κλειδιά: μικρογαλακτώματα, βιοσυμβατό, εγκλωβισμός, αντιοξειδωτικά, ένζυμα, Δυναμική Σκέδαση Φωτός (DLS), Ηλεκτρονικός Παραμαγνητικός Συντονισμός (EPR), Σκέδαση ακτινών Χ υπό μικρή γωνία (SAXS), κυτταροτοξικότητα, διαπερατότητα επιθηλίων

1.
Introduction

1. Introduction

Microemulsions are thermodynamically stable, transparent, isotropic, low-viscosity colloidal dispersions consisting of microdomains of oil and/or water stabilized by an interfacial film consisting of surfactant (and co-surfactant) molecules as Danielsson and Lindman first defined[1]. Microemulsions emerged as an area of scientific research with a broad range of applications from the decade of 1960. Strong research efforts were directed to this type of colloidal systems long before the term microemulsion was established. During a long period, there was no agreed definition on what should constitute a microemulsion, but the term was used broadly to include several types of surfactant systems. However, these initial confusions did not work as a hurdle to the creation of the strong research field of nanotechnology with great impact in the scientific community and in different industries. Interestingly, the attention has been given to those systems in a petroleum crisis back in 60s and 70s. Their unique properties have been the reason for exploiting all the possible uses of these systems. Food and pharmaceutical industries have been given a lot of effort, money and research in order to understand their properties. Cosmetics[2], detergent[3], paints[4] even degreasing of leathers[5] are some of the fields were microemulsions were and are still used, offering their low-cost effective formula for improving the multifaceted state of life. In the case of pharmaceutical applications, microemulsions are studied as effective drug delivery systems as they are able to increase the bioavailability of an encapsulated compound while offering a safe, quick and low-cost solution. Oral, transdermal, buccal, nasal, ocular, pulmonary and other delivery strategies have been studied for the development of final efficient products.

The main purpose of the present thesis was the formulation of biocompatible, non-toxic microemulsions in order to be used as carriers of bioactive compounds appropriate for oral and nasal administration. The study was focused on the development of systems based on polysorbate 80 (Tween 80) and distilled mono- and diglycerides (DMG, E471) in the presence of natural and pharmaceutically grade oils such as extra virgin olive oil (EVOO), sunflower oil (SO), isopropyl myristate (IPM) and their mixtures. Co-surfactants were used in extremely low concentration or excluded totally from the formulation strategy due to their ability to induce allergic and irritancy effects after application. Two (2) different categories of bioactive compounds were studied in microencapsulated state: enzymes and natural antioxidants.

For enzyme encapsulation, a biocompatible non-ionic water-in-oil (W/O) microemulsion was formulated with the use of IPM as the oil phase, Tween 80 and DMG as surfactants and water with propylene glycol (PG) as the dispersed aqueous phase. The multi-component system was successfully used as a host system for HRP while its structural study in the presence of different enzyme concentrations shed light on the localization of the macromolecule with the use of Dynamic Light Scattering (DLS) and Electron Paramagnetic Resonance (EPR) spectroscopy techniques. The effect of PG content was studied in correlation with the microemulsion's structural characteristics but also with the efficiency of HRP to catalyze the oxidation of 2,2′-azino-bis [3-ethylbenzo-thiazoline-6-sulfonic acid] (ABTS). Additionally, the enzyme release rate was studied with the use of a biphasic system. Due to its biocompatibility, the proposed system could be used as an effective carrier of therapeutic enzymes and peptides in treatment of various diseases.

In the case of the natural hydrophilic antioxidants, four different microemulsions were formulated in the presence of Tween 80, DMG and mixtures of IPM, EVOO and SO. The antioxidants chosen were hydroxytyrosol (HT) and gallic acid (GA) –belonging in the group of "biophenols"[6]- both known for their high scavenging activity. In the last years there has been an increasing interest in the study of these natural polyphenols due to their claimed health benefits for neuroprotective, anti-depressant and anti-proliferative actions. For that reason, the formulated systems were studied as antioxidant carriers for two different routes of administration: by oral and nasal route. The systems' biocompatibility was tested *in vitro* with the use of Caco-2/TC7 and HT29-MTX coculture for oral delivery and RPMI 2650 cell line for nasal delivery. After the determination of the concentrations that did not provoke any cytotoxic effect, the above cell lines were used to create epithelia in special semipermeable filter inserts. The permeability of the encapsulated compounds through the constructed intestinal and nasal epithelia were studied and correlated with microemulsions' composition.

The present thesis is structured in the following sections: ***Theoretical background***, ***Purpose of the study***, ***Methods***, ***Conclusion and Results***. The first part- ***Theoretical background***- of the study is a brief introduction in the field of microemulsions. The aim of that part is the elucidation of the properties of the thermodynamically stable colloidal microstructures. The wide range of applications are discussed with emphasis in those applying in the pharmaceutical industry. Supplementary, in this section are described the main techniques used for the structural characterization of the systems including Dynamic Light Scattering (DLS), Electron Paramagnetic Resonance (EPR), Small Angle X-ray Scattering (SAXS) and other activity assays. In addition, the theoretical background is referring to the two (2) categories of bioactive compounds studied in the present thesis. In particular,

enzymes and antioxidants are described with an emphasis on their use in the nanotechnology field, especially in cases were microemulsions serve as hosts. The last part of the section presents the different ways of bioactive compound administration studied in the present study while reporting their advantages, disadvantages and a mini literature review focused on the use of microemulsions in the field. Oral and nasal administration are analyzed in the present study as easy, common and with great patient compliance administration routes.

In the second part of the thesis- ***Purpose of the study-*** the main aim and objectives of the present study are highlighted while it is presented how the PhD thesis has been subcategorized in three (3) different subprojects corresponding to the encapsulated bioactive compound and the administration route. The section includes a schematic representation of the projects and a description of each subproject.

Thereinafter, in the section of ***Methods*** all the followed methods are reported in detail with specific reference to every subproject. In the ***Conclusion*** section a commentary of the results obtained from the thesis is presented, revealing the utility of the novel biocompatible microemulsions in a wide range of pharmaceutical applications.

The last section of the thesis, ***Results/ Publications,*** includes the findings of the study which were presented as the published and submitted research manuscripts in order to provide to the reader a clear demonstration of the aim, the followed strategies and the final results of every subproject.

2.
Theoretical background

2. Theoretical background

2.1 Microemulsions

The introduction of microemulsions in the scientific literature is ascribed to Schulman[7] and others [8,9], although such systems had appeared in patents earlier. In 1943, Hoar and Schulman prepared the first microemulsion by dispersing oil in an aqueous surfactant solution and adding an alcohol as a co-surfactant, leading to a transparent, thermodynamically stable formulation[10]. Other major contributors in the early period of microemulsions were Winsor [11], Friberg [12] and Shinoda [13].

The history behind the microemulsions is interestingly correlated with two oil crises in 1973 and 1979. In order to optimize oil recovery, water-surfactant mixtures were used in order to entrap -due to the ultra-low surface tension- the oil which was allocated in rock cavities. As a result, the 70s and 80s were the decades of extensive investigation of the microemulsions. It was then that Danielsson and Lindman[1] defined the term microemulsion as *a thermodynamically stable, transparent, isotropic, low-viscosity mixture of oil, water and surfactant*, a definition used until today. Microemulsions are optically transparent and thermodynamically stable as the surfactant molecules reduce drastically the interfacial tension (IFT) between the oil and the aqueous phase allowing the dispersion of one phase into the other. In some cases, in order to achieve a lower interfacial tension value, small alcohols and polyols, such as ethanol and PG, are used as co-surfactant molecules. Interestingly, the last decades categories of non-conventional microemulsions have been described such as the surfactantless microemulsions[14] (formulated with the presence of co-surfactants only) and the water-in-ionic liquids microemulsions[15] (an ionic liquid as the continuous phase of the system). This signifies the scientific evolution made from the first formulations back in the early 60s until today in the field.

Microemulsions are categorized in water-in-oil (W/O), bicontinuous and oil-in-water (O/W), depending on their dispersed and continuous phase. In the first category, an aqueous phase is dispersed in excess of oil whereas in the latter one the conformation is reversed. An intermediate state, described as bicontinuous microemulsions, is when the aqueous and oil phases are in comparable volumes creating channels (Fig.1). The interfacial curvature (H) of the systems is a factor of high importance in the formulation. Depending on the surfactant molecules, interfacial curvature alters as the type of microemulsion changes. Structure, temperature and pressure are some of the factors that provoke interfacial curvature changes as Strey et al. [16] clearly demonstrates.

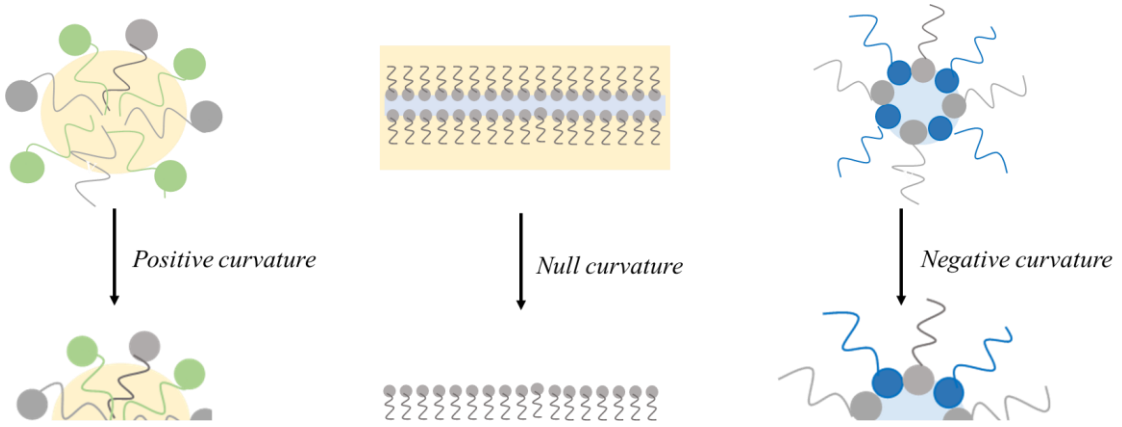

Figure 1. Graphic representation of different microemulsion types and corresponding interfacial curvature.

One of the main characteristics of the microemulsions, easily identified macroscopically making them appealing for many applications, is their optical transparency. This arises from the dimensions of the microenvironment formulated by the dispersed phase. In general, the dimension's range of the dispersed phase is between 5 and 50 nm (100 nm also have been reported in the literature) smaller than the light's wavelength. The system is not able to scatter light making the mixtures transparent even though they are not homogenous. It has to be mentioned here, that the term microemulsion is not in accordance with its dimensions, which belong in the nanoscale, leading to confusions with the term nanoemulsion. This terminology misconception is a result of the historical evolution of the field of colloidal science as both terms were used before being clarified, as McClements has commented on a review article[17].

Another, and probably the most important feature of a microemulsion is its thermodynamic stability, under specific temperature and pressure conditions. This property distinguishes microemulsions from the kinetically stable nanoemulsions. The thermodynamic stability ensures the preservation of the efficacy of the system and also creates a stable microenvironment for the encapsulation of even unstable compounds. The industries (pharmaceutical, food, cosmeceutical) are keen on the thermodynamic stability of the systems as this offers a low-cost procedure for the formulation of the final products since the energy requirements are almost zero, in contrast to energetically costly macro- and nano-emulsions. As it is known, water and oil, due to the very high Interfacial Tension (IFT, γ) value are immiscible. From the Eq.1, when γ is positive, the Gibbs energy (G) is also positive and the mixing fails. As a result, in order to make the free energy change negative, to achieve mixing, the γ requires to be drastically reduced. This reduction can be achieved by the addition of surfactant and co-surfactant molecules.

$$\gamma = (\frac{\delta G}{\delta A})T, P \qquad \text{(Eq. 1)}^{18}$$

Eq. 2 is the relation correlating the interfacial tension and the free energy. The enthalpy change (ΔH) is negligible for the mixing of water and oil. As the droplet size decreases, in a water/oil system in the presence of surfactants/co-surfactants, there is a positive change in entropy causing negative ΔG for the system. As a result, the dispersion of O/W or W/O becomes spontaneous and stable (thermodynamic stability). For a microemulsion, the free energy of the colloidal dispersion is lower than the free energy of the separate phases, which means that a microemulsion is thermodynamically stable.

$$\Delta G = \Delta H - T\Delta S + \gamma\Delta A \qquad \text{(Eq. 2)}$$

where ΔG is the Gibbs free energy, ΔH the enthalpy change, ΔS the entropy change, T temperature in Kelvin and ΔA change in interfacial area[19].

Surfactant and **co-surfactants** are molecules of amphiphilic nature with a polar head and a non-polar tail oriented to the aqueous and oil phase, respectively[20]. These two different functional groups with different affinity on the surfactant molecule create its unique properties. Surfactants are substances that create self-assembled molecular clusters called micelles in a solution (polar or non-polar) and adsorb to the interface between two different phases. A characteristic value for surfactants, which can empirically categorize them, is the Hydrophilic Lipophilic Balance value (HLB). The concept of HLB was presented by Griffin[21] in order to classify the non-ionic surfactants according to their solubility in water and oil. Many studies have proven the gaps in the theory of the HLB value pointing out that this value differs even for the same surfactant due to batch to batch variations[22]. In general, HLB is a numerical representation between the hydrophobic and hydrophilic part of the surfactant. Surfactants with low HLB values tend to form W/O microemulsions and those with high HLB values O/W. The formation of a microemulsion and especially the geometry of the microdomains the surfactant layer creates, depends on the concentration and the shape of the amphiphile molecules. Critical Packing Parameter (*CPP or p*) expresses these geometrical considerations. If *CPP* is between 0 and 1 O/W microemulsions are formed whereas the W/O ones with values higher than 1. Also, the possible shape of micelles can be speculated with the use of Eq. 3. For example, when $0 < CPP \leq 1/3$ only spherical micelles exist in solution. If $1/3 < CPP \leq 1/2$ aggregations with a rod-like shape or hexagonal are most likely[23]. Hydrophilic Lipophilic Difference (HLD) is another parameter, which expresses how the salinity, the temperature, the nature of oil and surfactant affect the final formulation. HLD<0

indicates the formation of an O/W system whereas in the case of positive values the formulation of W/O microemulsions is favored[24].

$$CCP=\frac{v}{al} \qquad \text{(Eq. 3)}$$

Where *v* is the tail volume, *l* the tail length and *a the* head area of the surfactant.

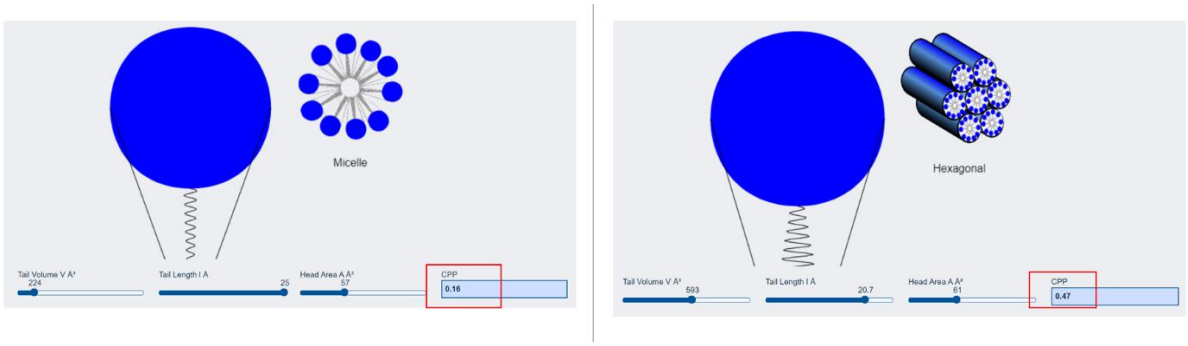

Figure 2. Micelle conformation depending on CCP. Illustration of CPP effects obtained from the online tool: https://www.stevenabbott.co.uk/practical-surfactants/cpp.php

Surfactants are generally categorized by the charge of their polar head to:

- ***Non-ionic (Non charged surfactants)***

Non-ionic surfactants, such as sorbitan esters and ethoxylated alkyl ethers are generally less irritating and toxic than ionic surfactants. Interestingly, non-ionic surfactants in contrast to the ionic ones can form microemulsions without the use of co-surfactants[25]. The most commonly used non-ionic surfactants include sorbitan monoleate (Span 80®), polyoxyethylene sorbitan monoleate (Tween 80®), polyoxyethylene sorbitan monolaurate (Tween 20®) and other polyoxyethylene surfactants such as (Brij 35®). The above surfactants have been extensively used in topical applications such as cosmetics[26] and dermal drug formulations[27], parenteral[28] administrated forms and food products[29]. Their biocompatibility and reduced need for co-surfactants make them ideal candidates for safe, non-irritant and non-toxic systems. In addition, their insensitivity towards pH and electrolytes makes such surfactants preferable compared to the other categories of surfactants [30].

Tween 80 (E433) is a surfactant derived from polyethoxylated sorbitan and oleic acid with a Critical Micellar Concentration (CMC)- concentration above which micelles are formed- of 0.12 mM and an HLB value around 18. It is one of the most widely used surfactants in pharmaceutical applications. It can be found as a component of creams,

ointments, lotions, and multiple medical preparations (e.g. vitamin oils, vaccines and anticancer agents) while it has been used in more sophisticated formulations as a coating ingredient in nanoparticles for brain delivery[31]. It is a well-known penetration and absorption enhancer with relatively low toxicity (compared to other Tweens). The low toxicity levels of Tween 80 have been correlated with its low HLB values compared to other polysorbates. Noudeh et al. have mentioned that the increased hydrophobic content of a surfactant leads to decreased hemolytic effects due to the lower cell membrane permeability[32]. Many microemulsions have been reported, mainly O/W, with Tween 80 as a surfactant as it induces a wider monophasic area and provides a range of beneficial properties.

DMG (E471, Mono- and diglycerides of fatty acids) is a surfactant well known in the food industry. It is often used in bakery products, beverages, ice creams, chewing gums, shortening, whipped toppings and margarines. DMG has been used except foods also in cosmetic, pharmaceutical, plasticizer and detergent formulations. The wide use of DMG has led to the use of enzymes as catalysts for their production from oils and fats[33]. The HLB value of DMG is around 3, making them an oil-soluble surfactant in contrast to Tween 80. DMG has been used for the formulation of microemulsions. The majority of the microemulsions consisted of DMG are applicable to food products whereas limited is the number of studies with DMG-based microemulsions in drug applications[34,35]

- ***Anionic, cationic and zwitterionic (Charged surfactants)***

Ionic surfactants find limited use in pharmaceutical formulations due to their toxicity which increases by the addition of co-surfactants. There are two types of ionic surfactants, namely, cationic and anionic. Surfactants having both negative and positive charges are called zwitterionic. Cationic, anionic and zwitterionic surfactants belong in the category of charged surfactants. When the polar head of the surfactant consists of a positively charged group, the surfactants are characterized as a **cationic** one. A few examples of cationic surfactants are cetyltrimethyl ammonium bromide (CTAB), distearyldimethyl ammonium bromide (DDAB) and dialkylmethylimidazolium methylsulfate. Toxicity is reported for many surfactants of this category with mouth and nasopharynx related side effects after oral ingestion even in concentrations up to 1%. In case of a negatively charged polar head, the molecule is characterized as an **anionic** surfactant. Alkylbenzene sulfonates, ester sulfonates, sulfate esters, phosphate esters, fluorinated surfactants, fatty acid isethionates and sulfosuccinate esters are the most widely used anionic surfactants. Bis-(2-

ethylhexyl)sulfosuccinate sodium salt (AOT) is an extensively used surfactant in biotechnology applications for the formulation of microemulsions[36], nanoparticles[37] and microemulsion based gels (MBGs)[38]. Interestingly has been used in drug applications such as formulations against psoriasis or photodynamic therapies, even though it is known as an irritant agent[39]. Cationic and anionic surfactants are not extensively used for humans as in their majority are skin irritants and can cause membrane perturbation.

Zwitterionic surfactants (also known as amphoteric) are amphiphiles with both positive and negative charged head groups. The cationic part is based on primary, secondary or tertiary amines or quaternary ammonium acids. The anionic part can include sulfonates, as in CHAPS. The most representative and widely used zwitterionic surfactants are phospholipids and particularly lecithin (E322) but also synthetic ones such as betaines. Lecithin is a naturally occurring, non-toxic and generally regarded as a safe (GRAS) material. As it is one of the constituents of the biological membranes it is used mainly in microemulsions for pharmaceutical and cosmetic applications in order to increase the absorption of different drugs/compounds without causing any side effects[40,41].

- ***Other surfactants and co-surfactants***

Catanionic, bio-surfactants and gemini surfactants are relatively new categories of surfactants that have not been studied so extensively as the molecules previously described. Catanionic ones have been studied in drug delivery systems[42] whereas gemini[43] are used with more emphasis as agents for non-viral gene delivery[44]. Bio-surfactants[45] are surfactants produced by bacterial organisms facing different problems of purity and high cost.

However, in the most of the studies found in literature, charged surfactants are usually accompanied by **co-surfactants** as the surfactant molecules alone cannot lower the oil-water interfacial tension sufficiently to form a microemulsion. Co-surfactants affect the packing of surfactant molecules at the interface, because of their short chain amphiphilic nature (with a length of the carbon chain ranging from C2 to C10). The microemulsion structure and the curvature of the interface are influenced by the co-surfactant chain length. Long chain alcohols swell the tail region more than the head region (negative curvature), whereas short chain alcohols swell the head region more than the tail region (positive curvature)[46]. Apart from the interfacial curvature, the fluidity of the interfacial film has also been found to be modified by co-surfactants. In detail, when co-surfactant molecules penetrate into the surfactant monolayer additional flexibility is provided to the interfacial film. PG[47] and Transcutol®[48] are some of the most representative co-surfactant molecules applicable in

pharmaceutical excipients. They are able to alter the properties of the final formulation, making possible an increased solubility for the encapsulated compound, a more flexible environment in terms of release and a possible increased penetration rate through physiological barriers.

Advantages and disadvantages of surfactants

The reduced requirements of energy for microemulsions preparation is not the only **advantage** offered by the surfactants. The presence of surfactants is reported to increase the solubility of the encapsulated compounds, making the microemulsions appropriate delivery systems for increased concentrations of bioactive compounds to the targets. This behavior is a consequence, in the case of oppositely charged surfactants and compounds, of the electrostatic interactions between the molecules that cause a decrease in the repulsive forces between the head groups of the surfactant molecules[49]. Also, in the pharmaceutical sector, the ability of the surfactants to penetrate the deeper layers of epithelia and tissues is of paramount importance, as they work as channel designators for the hosted bioactive compounds. Tweens[50], Transcutol[51] and Cremophor[52] are some of the surfactants working as penetration enhancers in different applications such as in transdermal and oral delivery. The mechanism of the opening of "channels" is quite complicated as it involves enzyme degradation and other mechanisms that will be discussed later.

However, surfactants do not only act as advantageous molecules as they also create big obstacles in their applications. A major **disadvantage** of microemulsions that contain high concentrations (or concentrations above a specific limit depending on the application) of amphiphiles is the increased cytotoxic or irritant effects that may be induced after their application. As a result, their use requires extensive analysis of their potent toxic/irritant nature with the use of adequate *in vitro* and *in vivo* studies. All the above, are the result of the lytic activity of surfactants, below or close to CMC values, on the cell membrane[53]. For example, Triton X-100 is one of the most common surfactants used for cell lysis and is prohibited in pharmaceutical formulations. Tweens and Spans are some of the most common safe (in specific concentrations) surfactants for pharmaceutical use[53]. Another disadvantage, of the microemulsions with co-surfactants is the instability upon dilution with aqueous biological fluids, which generally occurs after *in vivo* administration (through the majority of routes) leading to decreased efficiency of the system.

Except for surfactants and co-surfactants, the non-polar phase of the system (oil phase) is important and must be chosen regarding the application. Many non-polar ingredients have

been used for the formulation of microemulsions from organic solvents such as isooctane and hexane, especially in biocatalytic applications[54], to edible oils such as coconut oil for the necessities of the food industry[55]. Regardless if it is used as the external or as dispersed phase, the oil must increase solubility of the bioactive compound while acting also as beneficial ingredient for the site of administration. Overall, the final formulation should be of low surfactant concentration ensuring the absence of irritancy even in repeated uses.

Table 1. Commonly used surfactants and oils applied in pharmaceutical industry.

Oils	Surfactants	Co-surfactants
Fatty acids (Oleic acid, Palmitic acid, Stearic acid, Linoleic acid)	Tweens (80 and 20)	Propylene glycol
	Poloxamers (231, 182)	Ethanol
	Pluronics (F127)	Glycerin
Long chain triglycerides (Corn oil, Soybean oil)	Brijs (35)	Polyethylene glycol
Medium chain triglycerides (Capric/caprylic)	Spans (20, 80,85)	
	Lecithin	
Propylene glycol esters (Propylene glycol monolaurate)	Transcutol P	
	Labrasol	
	Cremophor RH40	
	Labrafil M1944 Cs	

Advantages of microemulsions

Microemulsions have a plethora of advantages which make them appropriate systems for various applications. First of all, the **ease of preparation**, in the absence of external energy makes these formulations appropriate for application at industrial scale as cost-effective formulations. Their **thermodynamic stability** creates a final product with extended stability and shelf life. In addition, their **tunable nature**, as they can be formulated from a variety of oils and surfactants, make them appropriate systems for different applications varying from food supplements to coloring ingredients. The scientific community and the industries have focused their attention on the unique properties of microemulsions. Interestingly, the microenvironment of a microemulsion system (i.e. the dispersed phase) can incorporate

drugs and other compounds and simultaneously is able to increase their penetration through biological membranes for efficient delivery. Their dispersed phase (water or oil) is able to encapsulate a wide range of compounds including enzymes[56], drugs[57] and even DNA[58]. Microemulsions are able not only to increase the solubility of poorly soluble drugs (hydrophilic or lipophilic) but also to protect them against chemical and enzymatic degradation. The presence of surfactants and/or co-surfactants increase the solubility of the compound while offering opportunities for its enhanced bioavailability. Importantly, microemulsions can be administered by any route of administration depending on the nature of the hosted compound and its site of action. Oral, transdermal, nasal, buccal, sublingual, ocular, intravenous, vaginal and pulmonary administration routes have been studied for the delivery of microemulsion encapsulated drugs. However, only products related to oral and transdermal delivery have been marketed, until today.

Microemulsions are characterized as the "**ultimate enzymatic microreactors**" due to their unique properties applicable in biocatalysis[59]. In details, their microenvironment enables the coexistence of compounds of different polarities in a colloidal compartmentalized solution. Moreover, the microemulsions provide a low-water media where, interestingly, the enzymes retain their catalytic activity, or even present superactivity, in contrast to the denaturation effect of organic solvents used in the past decades. Microemulsions provide in the same formulation an aqueous phase for hydrophilic enzymes, an interface for surface-active enzymes (e.g. lipases[60]), and an organic phase for hydrophobic substrates or products. The formulated microstructures communicate due to their dynamic nature which permits the content exchange of their water pools and surfactant interfaces with one another and with bulk solvent (in the case of hydrophobic components)[56]. Enzymatic reactions in microemulsions and microemulsion related systems gained interest on the grounds that through these systems the correlation between the *in vitro* and the *in vivo* behavior of enzymes can be studied, as the micro-domain structure of these systems simulates cell compartmentalization. Microemulsions provide a larger surface area to the enzymatic reaction and also protect the biomolecules—because of the presence of water molecules in the reverse micelle—from denaturation caused by the organic solvent. The determination of the properties of enzymes in a microemulsion (catalytic activity, kinetic parameters and mechanism) can be achieved by the same techniques applied in aqueous reaction media. This is due to the microemulsion's transparency allowing the application of photometric methods, which gives these formulations a big advantage over other non-conventional systems. Nevertheless, microemulsions present a disadvantage as the isolation of the products from the reaction medium may be hindered by the presence of surfactants. A solution to this

problem is the formulation of stable systems, which are able to incorporate enzymes in the absence of surfactants such as surfactant-free microemulsions[61].

2.2 Structural characterization

One of the main challenges regarding the development and the study of microemulsions over the years was their structural characterization using the appropriate techniques. The main objectives were the study of the dimensions and the characteristic shape of the dispersed phase in combination with the properties of the surfactant membrane. These techniques were evolved over the years from simple optical techniques to sophisticated and large facilities techniques in order to understand how the microemulsions' properties affect their scientific or industrial applications.

Dynamic Light Scattering (DLS), Electron Paramagnetic Resonance (EPR) spectroscopy, Interfacial Tension (ITF) measurements and Small Angle X-ray Scattering (SAXS) are some of the commonly used techniques. The combination of the above described techniques with phase behavior studies are the tools used for the characterization of nanosized systems which are discussed in the present section.

2.2.1 Phase Behavior

The thermodynamic stability of microemulsions is depended on the nature and the ratio of the ingredients used in their fabrication. From phase behavior studies, it is possible to identify the conditions under which the surfactant solubilizes the maximum amount of water and oil in case of W/O and O/W microemulsions respectively. In order to examine the different states of mixtures of the same components the use of phase diagrams is needed. In the case where the ingredients are three (water, oil, surfactant) the phase diagram is described as ternary. When the ingredients are more than three, due to the presence of co-surfactants or mixtures of oil or surfactants the phase diagram is described as pseudo-ternary phase diagram.

A typical ternary phase diagram is represented in the equilateral triangle of Fig. 3. Every edge represents the 100 % pure component whereas the axes represent binary mixtures of the relative components. Inside the phase diagram, every point represents mixtures of the three components (water, oil and surfactant). Depending on the composition, extremely different structures can be obtained both in monophasic and non-monophasic area ranging from microemulsions to gels and liquid crystals. The addition of co-surfactants increases the area (1φ) of the phase diagram corresponding to monophasic systems. As a phase diagram

describes the phase behavior of different mixtures of oil/water/surfactant, could be characterized as the "fingerprint" of a system.

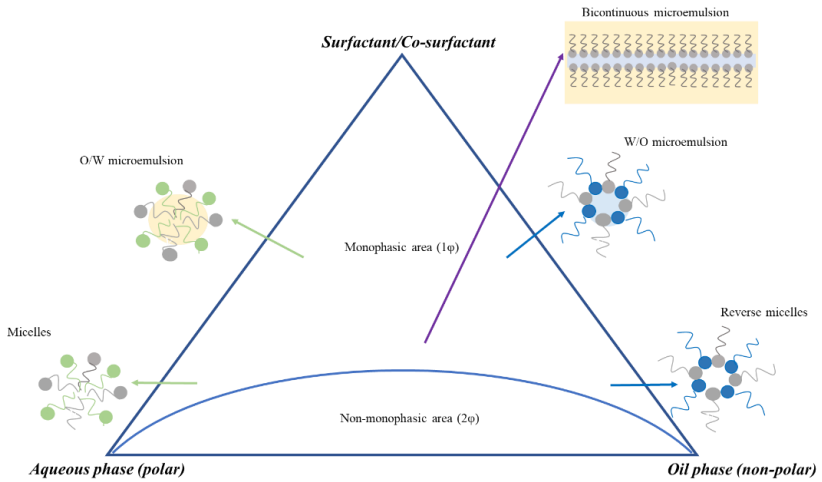

Figure 3. Schematic representation of a typical phase diagram of a three components system.

2.2.2 Interfacial Tension (IFT)

Ultra-low interfacial tension play a major role in the microemulsion preparation. Adsorption of the amphiphilic molecules at the water-oil interface reduces drastically the interfacial tension from 30-50 mN m^{-1} to 10^{-3}-10^{-4} mN m^{-1}. Many studies have described this phenomenon and how it is affected by different factors such as the chain length of the surfactant. For example, Leitäio et al.[62] mentioned that as the chain length of the surfactant in a water-n-octane-CiEj microemulsion increases the minimum of the interfacial tension decreases. Suitable methods to measure interfacial tensions as low as 10^{-3} mN m^{-1} are the sessile or pendant drop technique. Ultra-low interfacial tensions (as low as 10^{-5} mN m^{-1}) can be determined with the surface light scattering and the spinning drop technique. As the latter is comparative simple to use it can be regarded as the most suitable technique to measure ultra-low interfacial tensions in the described systems.

2.2.3 Dynamic Light Scattering (DLS)

During the last 30 years, a lot of knowledge about structure and self-assembly processes in systems containing amphiphiles was gained using scattering techniques. Among them Dynamic Light Scattering (DLS) is the most common non-invasive and non-destructive technique for measurements in the nanoscale. DLS is a technique based on the Brownian motion of dispersed particles. When particles are dispersed in a liquid they move randomly in all directions. For suspensions consisting of a continuous phase and a dispersed phase

(particles, droplets, clusters, or macromolecules) the scattering of light is caused by the inhomogeneities of the system creating a grainy random diffraction pattern. The principle of Brownian motion is that particles are constantly colliding with solvent molecules. Those collisions cause a certain amount of energy to be transferred, which induces particle movement. The energy transfer is more or less constant and therefore has a greater effect on smaller particles. As a result, smaller particles move at higher speed than larger particles[63]. If the other parameters that influence particle movement (such as solvent viscosity) are known, the hydrodynamic diameter of the particles can be determined by measuring their speed. In the case that an inhomogeneous system contains spherical particles, the hydrodynamic radius *Rh*, is calculated using the Stokes-Einstein equation (Eq. 4):

$$D = kT/(6\pi\eta Rh) \qquad \text{(Eq. 4)}\ [64]$$

where: D is the diffusion coefficient, k the Boltzmann's constant, T the absolute temperature and η the solvent's viscosity. The term hydrodynamic diameter refers to the particle size of smooth, spherical particles diffusing at the same speed as the particles of the sample. In the case of non-spherical particles, *Rh* is considered as the apparent hydrodynamic radius or equivalent sphere radius. Except *Rh*, polydispersity index (PdI) is also obtained by DLS measurements. PdI is a value which describes the size distribution of particles in the colloidal system. In other words, the smaller the PdI (closer to 0) the higher the homogeneity of the system.

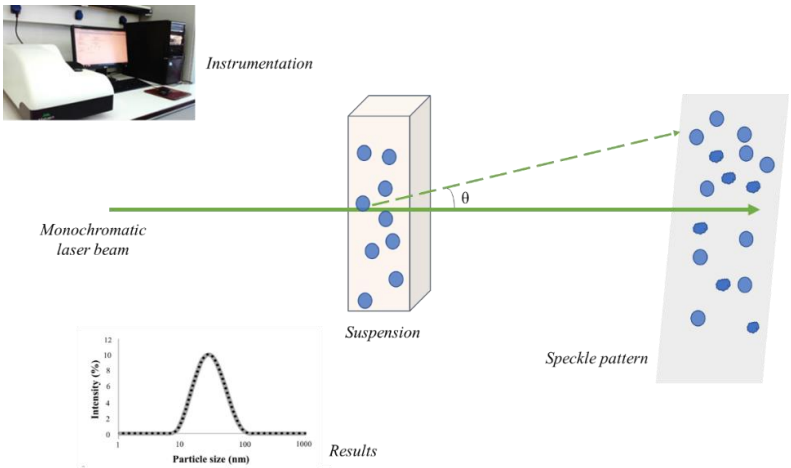

Figure 4. (a) DLS instrumentation, Zetasizer Nano ZS (ZEN3600) from Malvern Instruments (U.K.) (b) Schematic representation of the basic DLS concept (laser light scattered by a random medium such as a suspension with its speckle pattern in the far field and (c) DLS results expressed as intensity distribution of a W/O microemulsion.

A light scattering instrumentation is equipped with a light source, optics, a detector and signal processing electronics. Two different instrumentations are available regarding the angle of scattering. The devices with goniometer contain a cylindrical scattering cell allowing the scattering angle to be changed easily. On the other hand, there are also more automated DLS apparatus with fixed angles of measuring (Fig.4). The DLS measurement can take place in different angles (typically at 15°, 90°, and 173°). If the measurement is performed at 173° (back scattering) the scattering volume (the volume where the incident laser beam and the detected light overlap) is near the front cuvette wall. That means the path length of the laser within the sample is very short. This serves as an ideal setup for highly concentrated and turbid samples as it minimizes the effect of multiple scattering.

Generally, DLS is a fast and easy technique to obtain information about the size and size distribution of particles or self-assembled structures in highly diluted suspensions.

2.2.4 Small Angle X-ray Scattering (SAXS)

Small Angle X-ray Scattering was discovered by A. Guinier in 1938[65]. SAXS is another scattering technique widely used for the structural characterization of a wide range of formulations. This analytical method is able to determine the structure in terms of particle shape, size and distribution based on the different electron densities in the sample. Is an accurate, non-destructive method based on observing the scattering intensity of an X-ray beam scattered by a sample with inhomogeneities in the nanometer scale recorded at small angles (0.1-10º). Not only particles, but also the structure of ordered systems like lamellae and fractal-like materials can also be studied. Its broad range of applications includes colloids[66], polymers[67], plastics[68], proteins[69] and others. SAXS technique possesses many advantages compared to other techniques as it is non-destructive, can be applied in different sample states (solids, liquids) and in situ transition studies can be conducted. SAXS experiments can be conducted either in synchrotrons or in laboratory-made devices.

2.2.5 Electron Paramagnetic Resonance or Electron Spin Resonance (EPR or ESR) Spectroscopy

Electron Paramagnetic Resonance (EPR) is a sophisticated spectroscopic technique which deals with the interaction between electromagnetic radiation and magnetic moments of electrons. With this technique species that have unpaired electrons, namely free radicals, can be detected. The term electron paramagnetic resonance was introduced as a designation taking into account contributions from electron orbital as well as spin angular momentum[70]. EPR has been facilitated in different fields from chemistry, physics, biology, material sciences

and others as unpaired electrons can be found in a wide range of materials (free radicals, transition metal ions etc.).

In the field of microemulsions, this spectroscopic technique permits the indirect study of the colloid's microenvironment with the use of free radicals. More specifically, the microwave radiation that the EPR equipment produces is absorbed by the free electrons of the free radicals (paramagnetic molecules). Measuring the energy splitting of the electrons excited by magnetic field makes EPR a non-invasive and non-destructive technique which has been used in various applications from the structural study of microemulsions[71], to enzyme kinetics[72] and structural characterization of biological membranes[73].

Nitroxides is a large class of stable free radicals, deriving from nitric oxide (NO). The nitroxide free radicals are molecules containing the paramagnetic moiety shown in Fig. 5. In the present study doxyl stearic acids (DSAs) and especially 5-DSA were used to study the membrane dynamics in microemulsions. The EPR spectra of a nitroxide radical is characterized by three lines resulting from the coupling of the spin of the unpaired with the nitrogen nuclear spin. The EPR spectra of the 5-DSA (Fig. 5) is a sum of three different movements in three axes xx', yy' and zz'.

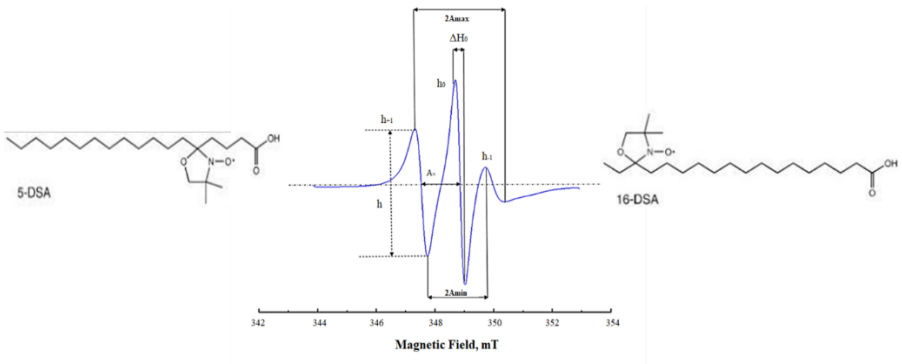

Figure 5. The structural formula of 5- and 16- doxyl stearic acids (5- and 16- DSA) and 5-DSA characteristic EPR spectrum in isooctane.

An EPR spectrometer is equipped with a magnet system, a microwave bridge (contains microwave source and the detector) and the cavity. The sample is placed with a cuvette in the cavity, which amplifies the weak signals from the sample. The detector, with the help of a circulator, equipped in the microwave bridge, recognizes the signal (microwave radiation) coming back from the cavity due to spectroscopic transitions. Following, the microwave power is converted to an electrical current producing the characteristic spectrum.

Figure 6. Bruker EMX EPR spectrometer (X band). National Hellenic Research Foundation, Greece.

The information obtained from the analysis of EPR spectra of a spin probe are: a. rotational correlation time (τ_R), b. order parameter (S) and c. micropolarity (A_o)[74]. In the present study, all the above parameters, are referring to the spin probe which, due to its amphiphilic nature, is located at the surfactant interface of the microemulsions, "probing" indirectly any alterations in that location. It has to be mentioned that spin probes with different solubilities are available for interface studies (amphiphilic doxyl stearic acids)[75], the polar (hydrophilic hydroxy-TEMPO)[76] or the non-polar phase (lipophilic doxyl methyl stearates)[77]. The τ_R, S and A_o values are characteristic for a given microemulsion and can be altered in any modification in its ingredients or after the incorporation of bioactive compounds. The rotational correlation time τ_R of the spin probe is calculated from the EPR spectra using the Eq. 5:

$$\tau_R = 6 \times 10^{-10} [(h_0 / h_{+1})^{1/2} + (h_0 / h_{-1})^{1/2} - 2] \Delta H_0, (s) \qquad \text{(Eq. 5)}$$

where, ΔH_0 is the width of the central field and h_{+1}, h_0, h_{-1} are the intensities of the low, center and high field peaks of the spectrum respectively (Fig. 5). The values of τ_R can be categorized in two groups, the slow-motion area (τ_R >3 ns) and the fast regime area (τ_R<3ns). As the τ_R increases the EPR spectra changes drastically with bigger alteration in the heights and the widths of the peaks and also with the appearance of new peaks. When τ_R value is higher than 3ns the above equation is not valid and special simulation tools must be used.

In addition, the order parameter S is calculated from the EPR spectra using the equation:

$$S = (A_{II} - A_{\perp}) / [A_{ZZ} - 1/2(A_{XX} + A_{YY})]\, k \qquad \text{(Eq. 6)}$$

where, A_{XX}= 6.3 x 10^{-4}, A_{YY}= 5.8 x 10^{-4} and A_{ZZ}= 33.6 x 10^{-4} T the single crystal values of 5-DSA. A_{II} and $A_{\perp}$the hyperfine splitting constants. A_{II} is the half distance of the outermost EPR lines ($2A_{max}$) and $A_{\perp}$ is the half distance of the inner EPR lines. The ratio $k = A_0/A'_0$ represents the polarity correction factor, where, $A'_0 = 1/3(A_{XX} + A_{YY} + A_{ZZ})$ is the hyperfine splitting constant for the nitroxide in the crystal state and $A_0 = 1/3(A\,II + 2A\perp)$ the isotropic hyperfine splitting constant for the spin probe in the membrane. A_0 values depend on the polarity of the spin probe's environment and increase with increasing polarity near the paramagnetic moiety.

The spin probing technique has been widely used by different research groups in order to study structurally different microemulsions (O/W, W/O and bicontinuous) with different free radicals. The membrane dynamics of a microemulsion system is of high importance as they are able to structurally characterize a system providing information related to its membrane dynamics. For example, if the rigidity of the membrane (expressed among others by high S value) is increased this may attribute to alterations in the enzymatic activity. Also, the effect of the addition of different compounds such as enzymes[78], antioxidants[79] and drugs[80] has been investigated with the use of EPR with interesting results, which permits defining even the specific depth of the membrane where a bioactive molecule is located.

3.

Microemulsions and encapsulation of bioactive compounds

3. Microemulsions and encapsulation of bioactive compounds

Microemulsions are suitable candidates for encapsulating molecules such as enzymes, drugs, antioxidants etc. as those molecules find a favored environment in the microdroplets of the dispersed phase of these systems. Both hydrophobic and hydrophilic compounds can be encapsulated in O/W and W/O microemulsions, respectively. The microencapsulation of different compounds finds application in different sections varying from the pharmaceutical[81] and food sector[82] to industrial processes like corrosion of metals [83] and the production of detergents[84]. In the present section we overview two different categories of encapsulated bioactive compounds and their applications in the pharmaceutical sector namely: 1. Enzymes and 2. Antioxidants. They were chosen for this study as they correspond to the pharmaceutical industry's requirements covering an area from therapeutic enzymes to natural products as precursor molecules for the synthesis of new drugs.

3.1 Enzymes

Apart from synthetic drugs, other compounds such as proteins and peptides have been exploited for medical use to treat various diseases[85]. The most important feature of enzymes which makes them potent drug candidates, is that they bind and act on their targets with great affinity and specificity while being able to convert multiple target molecules to the desired products[86], The concept of "therapeutic enzyme" was introduced back in the 1960s and since then Food and Drug Administration (FDA) has approved many recombinant enzymes for the treatment of various diseases. For example, superoxide dismutase has been used for the protection of donor organ tissue from damage or injury mediated by oxygen-derived free radicals that are generated during septic shock incident [87]. In 2000, recombinant urate oxidase (by Sanofi SA) has been entered the market for prophylaxis of chemotherapy-induced hyperuricemia. Other enzymes are used for the treatment of infectious diseases[88], for the treatment of cancer[89] and treating of damaged tissues[90]. Asparaginase, dornase alfa, PEGylated arginine deiminase, alpha- galactosidase A and more are some of the characteristic FDA approved therapeutic enzymes. The advances of biotechnology over the past years have allowed the pharmaceutical companies to offer safer and cheaper enzymes with enhanced specificity and efficacy. These enzymes are able to partially replace commercial drugs offering an innovative and effective strategy for dealing with diseases.

Even though the pharmaceutical industry has focused on these enzymes the problem of their low permeability in the human body still exists[91,92]. Therefore, many groups have used a plethora of systems in order to overcome this hurdle, delivering the macromolecules to their target. As enzymes are proteins, they are water soluble and as a consequence W/O microemulsions have been used in order to encapsulate, protect and deliver enzymes to their therapeutic target. Chang et al.[91] have used a microemulsion system composed of the oil Labrafac CC, a mixture of surfactants namely Labrasol, Plurol Oleique CC 497 and saline for the oral delivery of earthworm fibrinolytic enzymes. The study revealed higher intestinal absorption in both *in vitro* and *in vivo* studies for the orally administrated enzyme encapsulated in the W/O microemulsion, indicating high efficiency in the treatment of cardiovascular diseases. A U.S patent (US9249424B2)[93] published in 2016, claims that a lipid-based microemulsion prevents, inhibits or treats neurological deterioration by the nasal administration of enzymes associated with a lysosomal storage disease. The encapsulation of enzymes in microemulsions and its nasal administration offers circumvention of the low oral absorption of proteins by avoiding the presystemic enzymatic degradation and the poor membrane penetration of gastrointestinal mucosa. In general, literature focuses in the encapsulation of peptides and proteins in W/O microemulsions rather than enzymes for therapeutic applications.

The ingredients of the microemulsion could lead to induced structural and fluidity changes in the mucosal membrane increasing the intracellular and paracellular permeability of the biomolecule. The careful choice of the system's ingredients as the avoidance of possible enzyme substrates or toxic compounds, is of major importance in order to create an environment of protection and a carrier appropriate for *in vivo* administration. As the literature focuses on applications of microemulsions containing enzymes for biocatalytic processes intensive work must be done in the field of the delivery of potent therapeutic enzymes.

Enzymes are also implicated in the biocatalytic process of drugs synthesis. For example, ibuprofen, ketoprofen and ketorolac are some of the most commonly used analgesics. Lipase and protease- catalyzed hydrolysis of esters have been used for the production of the above described drugs. Interestingly higher yields have been observed in comparison to conventional techniques, making biocatalysis an important part of drug production[94]. Hedstrom et al. have studied the enantioselective esterification of racemic ibuprofen, catalyzed by a *Candida cylindracea* lipase, in an isooctane/AOT/n-propanol/water microemulsion[95]. Microemulsions, also in that case appeared to be appropriate hosts offering the advantages of enzyme catalysis over the chemical one. Microemulsions provide an

environment for the solubilization of both polar and non-polar molecules. The coexistence of a continuous organic phase and a dispersed aqueous one overcomes the hurdle of enzyme's exposure in the organic media. The dispersed phase offers the appropriate environment for the enzyme while permits the communication with the external environment. Also, they provide an extensive interface for surface active enzymes such as lipases. Also, many studies have reported that the enzyme encapsulation in microemulsions leads to more efficient (lower concentration of enzyme needed) and of high specificity biocatalysts.

3.1.1 Horseradish peroxidase

Horseradish peroxidase (HRP, EC 1.11.1.7) was selected as the model enzyme in the present thesis. HRP is a heme-containing enzyme, obtained from a plant source, that uses hydrogen peroxide to oxidize a wide variety of organic and inorganic compounds[96]. It is a metalloenzyme with many isoforms, which is active over a great range of pH values and has the ability to catalyze various substrates such as phenols, biphenols, benzidines etc. Due to its commercial use (clinical diagnostic kits and immunoassays) it is a widely studied enzyme. HRP is one of the most versatile biocatalysts available, relatively stable and with wide substrate specificity. HRP has already been used either immobilized or encapsulated in a variety of systems such as Sol-Gel Glass and Ionic Liquids[97,98]. HRP encapsulation in environments with non-ionic surfactants, which are generally characterized as mild, is not widely studied. The main substrates used for the investigation of HRP activity in microemulsions and microemulsion-related systems are ABTS (2,20-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), pyrogallol (1,2,3-trihydroxybenzene) and guaiacol (2-methoxyphenol). Oxidation of ABTS by HRP generates the ABTS radical cation, whereas oxidation of pyrogallol leads to the formation of purpurogallin and that of guaiacol gives 3,30–dimethoxy-4,4'-biphenoquinone.

HRP has been used in different biomedical applications. It has been used as anatomic marker in studies of vascular permeability allowing the visualization of brain tumors[99]. Throne et al. have quantified HRP across olfactory sensory neurons after intranasal administration. Biologically significant protein concentrations were achieved in the brain making nasal administration a promising route for enzyme theraies[100]. HRP was also used for gene cancer therapy[101] and in enzyme-prodrug strategy therapy (EPT)[102] seeking to apply enzymes able to catalyze the activation of non-toxic prodrugs to produce a toxic drug at targeted locations. HRP has been used in such therapy for the activation of the prodrug indole-3-acetic acid [103]. Also, HRP has been coupled to a drug against Herpes simplex virus and the protein is readily transported retrogradely in corneal sensory axons to ganglion cell

somata in the trigeminal ganglion[104]. The enzyme has also been served as a model molecule for jejunum and arterial wall transport studies [105].

All the above, have established HRP as one of the most used model enzymes for *in vitro* and *in vivo* studies in pharmaceutical sector. The formulation of an appropriate microemulsion to serve as an enzyme-carrier is a great challenge as all the excipients must be chosen with caution for a final non-toxic, non-irritant, with enhanced bioavailability carrier which will simultaneously offer the suitable mild environment for the encapsulated enzyme.

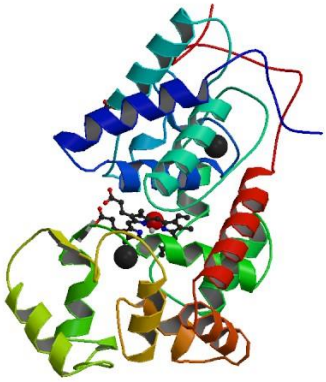

Figure 7. Three-dimensional representation of the crystal X-ray structure of Horseradish peroxidase (HRP). The heme group is located at the center of the molecule. Obtained from Protein Data Bank 1HCH.

3.2 Antioxidants

The second category of natural compounds with beneficial properties we have focused on in the present thesis are the antioxidants. An antioxidant molecule can be defined as "*substance that when present in low concentrations compared to those of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate*" The antioxidants prevent damage of cellular components caused by chemical reactions involving free radicals. They act in several ways, such as decreasing oxygen concentration, intercepting singlet oxygen, preventing first-chain initiation by scavenging initial radicals (hydroxyl radicals), binding metal ion catalysts, decomposing primary products to non-radical compounds, and chain-breaking to prevent continued hydrogen abstraction from substrates[106]. Common diseases such as atherosclerosis, chronic renal failure, diabetes mellitus and certain cancers are related with extensive oxidative stress and increased amounts of free radical damage products, particularly markers of lipid peroxidation, in body fluids. Biochemists and health

professionals are interested in antioxidants because of their important role in protecting the body from severe damage caused by reactive oxygen species

Antioxidants behave as radical terminators either by directly reacting with the radicals, like phenolic antioxidants do, or using other paths such as oxygen scavenging. Many groups have reported the beneficial effects of antioxidants and their use in the protection and adjuvant therapy of common diseases[107]. Consumption of dietary antioxidants that are present in various foods is associated with a lowered risk of degenerative diseases in addition to the protective effect of endogenous enzymatic antioxidant defenses. Small molecule dietary antioxidants (ascorbate, tocopherol) have attracted particular interest for their action as anticarcinogens and against degenerative diseases[108]. Antioxidants are not only used for oral consumption but also for topical application in anti-aging cosmetic products or for cutaneous delivery. Interestingly, brain targeted delivery strategies have been recently exploited for the delivery of phenolic antioxidants for age-related disorders such as Alzheimer's and Parkinson's diseases[109]. Attempts are made for the study of possible direct neuroprotective potential of polyphenolic compounds. Figueira et al.[110] have reported that antioxidants are able to cross the Blood Brain Barrier (BBB) endothelium, metabolized then into novel components with beneficial effects in different neuronal systems.

Major concern of the scientific community regarding the use of antioxidants is their unspecific activity without targeting a particular pathway based on the pathological mechanism of a specific illness. In addition, targeted delivery in specific regions of the human body is another issue that has to be solved with specific strategies (such as carriers) in order to exploit the full potentials of the natural compounds.

The polar paradox is a phenomenon associated with antioxidants. The theory of polar paradox is summarized in the next words "primary antioxidants that are polar or are amphiphiles of high hydrophilic-lipophilic balance (HLB) tend to be more active in bulk oils, a nonpolar medium, whereas nonpolar or amphiphilic antioxidants with low HLB tend to be more active in polar emulsions and polar lipids"[111]. The localization of the antioxidant molecule seems to be connected to activity/effectiveness. This makes microemulsions (and in general the system where two phases of different polarity coexist), appropriate for encapsulation. Both food and pharmaceutical industries are keen on the development of biocompatible systems, especially microemulsions, for the encapsulation of bioactive compounds with high radical scavenging activity. One of the main aims in food research is to introduce novel carriers for natural compounds and produce functional foods that have positive health benefits when consumed in specific concentrations. However, the major challenge is the limited bioavailability or the poor solubility of these bioactive compounds

inside the human body. Microemulsions, like food delivery systems, can overcome these problems in a satisfactory level due to their unique structural characteristics. The presence of surfactants and co-surfactants is able to increase the solubilization capacity of the antioxidants. wherein this environment the antioxidants are more protected and also more efficient due to their enhanced bioavailability.

In the present thesis we focused on two different natural antioxidants found in extra virgin olive oil (EVOO) and tea leaves which have been reported to have therapeutic properties. Hydroxytyrosol (HT) and gallic acid (GA) were encapsulated in different microemulsions in order to protect their activity and also to increase their bioavailability.

3.2.1 Hydroxytyrosol

Hydroxytyrosol (HT, 3,4-dihydroxyphenylethanol) is a multi-functional natural compound. It is a secoiridoid of amphiphilic nature, deriving from the hydrolysis of oleuropein, with a great antioxidant capacity [112]. HT is naturally present in high concentrations in the leaves of the Olea europea tree (common olive) and is recognized as one of the principal bioactive compounds of EVOO. Owing to its structural and molecular features, HT provides many beneficial effects after consumption[113]. HT may act as a controller of lipid oxidation of natural oils,[114] and as an antioxidant in films used for active food packaging [115]. Many studies give evidence for health benefits including neuroprotective[116], antimicrobial[117], anti-cancer[118,119] and anti-inflammatory[120] properties. The European Food and Safety Authority (EFSA) has endorsed a daily intake of 5 mg HT per day as HT acts as a protector of the cardiovascular system avoiding oxidation of low-density lipoprotein (LDL) cholesterol by free radicals, maintaining normal blood high-density lipoprotein (HDL) cholesterol concentrations preventing thus atherosclerosis[121]. Another reported interesting aspect of HT is its antiproliferative [122] effect on various cell types, supporting the claim of LDL protection against oxidative damage. Even though the biological properties of HT are extensively studied, its bioavailability has been rarely investigated, mainly with two single exceptions: one *in vivo* absorption study in rats[123] and an *in vitro* with the use of Caco-2 cell line[124].

3.2.2 Gallic acid

Gallic acid (3,4,5-trihydroxybenzoic acid, GA), a natural phenolic compound mostly found in grapes [125] and black tea [126], it is known for its multi-purpose activity. It has strong antioxidant [127] and antimicrobial activity [128], anti-inflammatory [129] and even antidepressant properties [130] have been reported. Interestingly, it has been reported that regular

consumption of GA may inhibit the amyloid fibril formation which characterizes many protein misfolding diseases[131]. However, its therapeutic potential is reduced by its pharmacokinetic drawbacks. For example, oral administration of GA in animals showed low bioavailability with low maximum drug concentration in plasma[132]. Gallic acid and its derivatives have been used to treat respiratory, dermal, cardiovascular diseases and others. Regarding neurodegenerative diseases such as Alzheimer's disease, GA has been reported as an efficient compound against cerebral oxidative stress[133]. Interestingly, the compound has also been also studied for its anxiolytic-like properties[134].

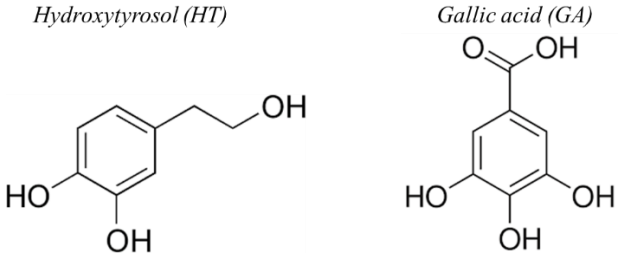

Figure 8. The molecular formula of hydroxytyrosol (HT) and gallic acid (GA).

4.

Microemulsions as delivery systems for administration of bioactive compounds

4. Microemulsions as delivery systems for administration of bioactive compounds

The increasing knowledge of the properties of bioactive compounds and the development of new drugs has generated a lot of interest in the study of possible delivery strategies for exploiting their beneficial properties. However, challenges such as low solubility, low bioavailability and susceptibility in enzymatic oxidation, possible toxicity and irritancy, need to be addressed. Nanotechnology is able to provide new solutions in many of these obstacles. Depending on the nature and the target of the bioactive compound, microemulsions, among others, can be designed in order to serve as an appropriate delivery system either for a functional food or as a pharmaceutical formulation.

The term functional food refers to foods or food components that may provide health benefits beyond nutrition. In other words, functional foods contain a variety of components, nutrients and non-nutrients that affect a range of body functions that are relevant to a state of well-being and health and/or reduce the risk of a disease[135]. In general, microemulsions with GRAS components and low concentration of surfactants can be used for the delivery of bioactive compounds through food intake. Antioxidants[127], antibacterial peptides[136], vitamins[137,138] and others have been successfully encapsulated in different microemulsion systems appropriate for consumption offering the opportunity to deliver the compounds of interest under a protected environment in the human body via the route of the highest compliance.

In the pharmaceutical sector, extensive research has been conducted on the use of microemulsions as delivery systems. In recent years, however, protein and peptide drugs have attracted a considering amount of attention and research in delivery systems has shifted to address this new area. A number of studies in the pharmaceutical field have reported enhanced solubilization of poorly soluble compounds and improved bioavailability following incorporation into microemulsions. It has to be mentioned that due to the increased interest in microemulsions, various microemulsion based systems have also been formulated for drug delivery such as Microemulsion Based Gels (MBGs)[139], solid lipid nanospheres composed from O/W systems[140], nanocapsules[141] and others. It is clear that the liquid colloidal structure of microemulsions offers great advantages in drug delivery as summarized in Table 2.

Table 2. Advantages of microemulsions as pharmaceutical products in relation to their physicochemical properties.

	Advantage	Property
1	Increased solubility of compounds	Presence of Surfactants and co-surfactants
2	Increased bioavailability of compounds	Surfactants interact with cell membranes
3	Appropriate for delivery of peptides and enzymes	Protective microenvironment
4	Ease of preparation and cost-effective approach	Thermodynamic stability
5	Ease of compliance	Liquid, auto-administration

In the present thesis different microemulsions have been prepared and characterized applying advanced techniques for structural studies. For the biological assessment of oral and nasal delivery, *in vitro* techniques using cell lines were applied. In addition, the correlation between structure and their *in vitro* behavior was exploited. In the present section the two different routes of administration and the existing literature referring to microemulsions as delivery systems are analyzed.

4.1 Oral route

Oral delivery of drugs and bioactive compounds has been the most widely used form of drug administration. This fact is attributed to the presence of microvilli in the human intestinal epithelium (small intestine) offering an increased absorption surface area in the gastrointestinal tract (GI)[142]. Across the GI tract, different types of cells are present with most representative enterocytes (absorptive) and goblet cells (mucus secreting) both playing a crucial role in the absorption of nutrients and drugs.

Administration by oral route has some disadvantages such as (i) poor stability of the drug due to the acidic gastric environment (ii) low solubility and as subsequence low bioavailability and (iii) low absorption rates due to the hurdle that mucus layers create. The mucus layer of the intestinal epithelium protects the exposed epithelial surface acting as a significant barrier to permeation of drugs or nanodispersions[143]. To overcome these challenges nanodispersions have been used extensively in order to protect the bioactive compounds from the unfriendly environmental conditions along the GI tract. In addition, they increase their solubility and offer higher absorption rates by the presence of penetration enhancers and mucus adhesive compounds. For the above described reasons, nanotechnology has been focusing its interest, the last 30 years, on the formulation of

appropriate carriers for the oral delivery of drugs (nanoparticles[144], gelled emulsions[145], cubosomes[146] etc.). The nanotechnology-based systems have been used not only for conventional drugs but also for the oral administration of therapeutic enzymes and bioactive compounds with potential therapeutic effects and health benefits.

Literature concerning microemulsions for oral drug delivery has revealed a preference in preparation of O/W microemulsions, appropriate for encapsulation of lipophilic drugs offering the wanted increased solubility in an aqueous environment. An extensive list of drugs has been encapsulated in microemulsions including drugs against cancers, viruses, liver diseases and other. For example, cyclosporin is a cyclic undecapeptide with immunosuppressive activity which dramatically improves the prevention of transplant rejection[147]. The main obstacle for its bioavailability is that is only absorbed in the upper part of the GI tract and needs to be dissolved in bile to be absorbed. Microemulsions with varying surfactant to co-surfactant weight ratios were prepared by Gao et al.[148], using caprylic/capric triglyceride (Captex 355®) as an oil, polyoxyethylated castor oil (Cremophor EL®) as a surfactant, Transcutol® as a co-surfactant and saline as reported. The study indicated a higher drug bioavailability in comparison with a commercial product especially in the case of microemulsions with lower diameter of the dispersed phase. Silva et al. formulated O/W microemulsions as carriers for amphotericin B, a drug against leishmaniasis. *In vitro* cytotoxicity tests against macrophage-like cells revealed the suitability of such formulation in oral delivery[149].

The microemulsions are of growing interest to the food industry as vehicles for delivering and enhancing solubilization of natural food supplements with nutritional and health benefits. In the food sector the research group of Garti showed in various publications the excellent solubilization effect of microemulsions for lipophilic nutrients, such as β-carotene, lycopene[150], lutein[151] or phytosterol[152], in aqueous environment. Feng et al. studied vitamin E (present in its stable acetate form) containing microemulsions based on nonionic emulsifiers[153]. The results show that the vitamin E-containing microemulsion system induces a slight sustaining release effect when compared to vitamin E release from the ethanol reference system. Moreover, cell toxicity of the microemulsion was lower than that of the single components. Curcumin, one of the most studied antioxidants in recent years, has been encapsulated in a microemulsion consisting of Capryol 90 (oil), Cremophor RH40 (surfactant), and Transcutol P aqueous solution (co-surfactant) which increased the oral bioavailability *in vivo* 22.6-fold than that of a curcumin suspension[154].

W/O microemulsions have been described by Constantinides et al. in the late 90s for the delivery of different enzymes but also of insulin and fibrinogen receptor antagonist[155,156].

Insulin loaded microemulsions offered 10-fold enhancement in bioavailability compared with plain insulin solution administered orally indicating the promising character of microemulsions in diseases with repeated and long-period dosages[157]. Many groups have focused their attention in the encapsulation of bioactive compounds such as antioxidants in W/O microemulsions in order to increase their bioavailability after oral uptake and their final health benefits. Chatzidaki et al. encapsulated four different antioxidants of natural origin in a biocompatible MCT/ Tween80/DMG/ethanol/water system to serve as a functional food[127]. To our knowledge, bicontinuous microemulsions, have not been studied for oral delivery, as they are generally used in topical applications[158–161] for the delivery of compounds such as testosterone, aceclofenac and curcumin. This may be attributed to the high surfactant concentration which may induce toxicity and irritation side-effects in the sensitive gastrointestinal (GI) tract in comparison to stratum corneum. Last but not least O/W microemulsions are the most studied systems for oral administration of different bioactive compounds from vitamins[137,162] to even antitumor drugs[163].

As far as our knowledge is concerned, few articles have focused on the intestinal absorption of polyphenolic molecules encapsulated in nanosystems after their consumption. Trying to elucidate the processes concerning the fate of novel formulations in the GI tract, Xenakis group focuses on the digestion and absorption of oil-based nanodispersions and their encapsulated compounds. The gastric and intestinal digestion of W/O emulsions and microemulsions composed of medium chain triglycerides (MCT) and a variety of surfactants with encapsulated HT have been tested with a two-step digestion model using gastric and pancreatic lipases[164]. As a subsequent step, the present study aims at characterizing the intestinal absorption profile of HT encapsulated in W/O microemulsions. The systems proposed in the present thesis are biocompatible and could be used as functional food formulations to enrich our diet in polyphenols with health benefits as described in section 3.2.

So far, the systems proposed in the literature are composed mainly of amphiphiles such as Cremophor, Labrasol, Plurol, Oleique and Labrafac. These non-ionic surfactants have low toxicity and are known to be less affected by pH or ionic strength changes. In addition, some of them, such as Cremophor and Transcutol can enhance the intestinal permeability of drugs.

4.1.1 Oral route - In vitro studies

In order to examine a microemulsion for oral uptake the first step is to examine its properties with *in vitro* techniques. It has to be underlined that several *ex vivo* and *in vivo* models have been described for the study of compounds' permeability, such as excised

animal tissue, but those models are time consuming and do not involve human cells. As a result, in order to study the toxicity of a possible delivery system and the bioavailability of its encapsulated compound(s), *in vitro* models are generally used prior to *in vivo* experiments. Cell-based models offer an intermediate level of complexity, with multiple transport systems that reflect the *in vivo* conditions and importantly offer the ability for different labs to reproduce easily the experiments.

The use of cancer cell lines for the study of intestinal permeability of bioactive compounds has been developed in the last decade and is the most applied technique. The cells are cultivated in order to show morphological and functional characteristics same with those of the intestinal epithelial cells. The cells in the appropriate stage are cultivated in specific semipermeable membranes in order to create monolayers, which will facilitate forming tight junctions and enzyme exression[165]. The inserts are constructed by polymers and divided into compartments, the apical and the basolateral (See Fig. 9). The cells are placed on the apical side of the insert which has appropriate pore dimensions for supporting the cell layer and facilitating the transport of the bioactive compounds. The studied compound is solubilized in the appropriate transport medium (in its free or encapsulated form) and it is placed on the apical side of the compartment. In the basolateral compartment the same transport medium is present in the absence of the studied compound. The above described configuration is designed to allow access to both apical and basolateral compartments. The most commonly used filters are Transwell® and Thnicerts™, providing a variety of filters regarding the pore size and the constructed material. Depending on the cell line, the culture period time varies. After total differentiation and monolayer construction, the monolayer's integrity must be studied for reliable results.

In order to study the integrity of the constructed monolayer different approaches are used before proceeding with drug testing: freeze-fracture electron microscopy, radiolabeled compounds, dyes and enzymatic markers are the most used among them. As the use of tracer compounds can interfere with the transport process and affect the barrier integrity, non-invasive techniques such as Transepithelial Electrical Resistance (TEER) were developed. TEER is a widely accepted quantitative, sensitive and reliable technique to measure the integrity of tight junction dynamics in cell culture models of endothelial and epithelial monolayers. Specifically, measuring the electrical resistance across a cellular monolayer offers the advantage of monitoring live cells during their various stages of growth and differentiation. Two electrodes are used, with one electrode placed in the upper compartment and the other in the lower compartment. The electric resistance is calculated based on Ohm's law as the ratio of the voltage to current. As direct current (DC) can damage both the cells and

the electrodes, an alternating current (AC) voltage signal with a square waveform is applied. The measurement procedure includes measuring the blank resistance (R_{Blank}) of the semipermeable membrane only (in the absence of cells) and the resistance across the cell layer on the semipermeable membrane (R_{Total}). The monolayer resistance ($R_{Monolayer}$) in units of Ω, can be obtained from Eq. 7. TEER values of cellular monolayers were expressed in Ω x cm^2 and calculated by Eq. (8):

$$R_{Monolayer} = R_{Total} - R_{Blank} \quad \text{(Eq. 7)}$$

$$TEER = R_{Monolayer}(\Omega) \times M_{AREA}(cm^2) \quad \text{(Eq. 8)}\ ^{166}$$

where $R_{Monolayer}$ is the monolayer resistance and M the effective area of the semi-permeable culture filter.

In the literature there are numerous cell lines and their cocultures serving as *in vitro* models for GI tract absorption studies. The following cell models are widely used for representation of the intestinal functioning: the Caco-2 monolayer[165]; Caco-2 and HT29-MTX co-culture[167]; Caco-2 and Raji co-culture[168] and Raji B co-culture[169]. Among them, Caco-2 cell line is one of the most used for intestinal studies. It is well accepted as a model to investigate the relationship between the molecular structure, physicochemical properties, absorption mechanism and rates of bioactive compounds[170]. Caco-2/TC7 clone, introduced in intestinal transport studies, has shown to consist of a more homogeneous population with respect to the most representative functions of the small intestinal enterocytes, with more developed intercellular junctions[171]. However, this cell line (as all the absorptive cell lines) is not able to product mucus layer, the main obstacle for permeation of drugs and bioactive molecules in mucosal epithelia[172] . Mucin that is continuously released from the goblet cells forms a viscoelastic layer that covers the GI tract constituting thus, a diffusion barrier to nutriments, drugs, ions, toxins, heavy metals, and macromolecules in physiological conditions. The mucus layer in the human intestine ranges from 10 to 200 μm thickness and consists of an outer, loosely adherent layer, and an inner, thinner, and more strongly adherent one[173]. The main representative of goblet intestinal cell lines is HT29. Different clones have been produced with reported differences. For example, Karlson et al. have shown that the mucus produced by the HT29-H was a significant barrier to the lipophilic compound testosterone[143], indicating different behaviors of the clones. To further investigate the role of mucus on drug transport across the intestinal barrier, the human adenocarcinoma HT29-MTX model has been

developed. This cell line is derived from the parental cell line HT29 and adapted to a medium containing methotrexate for 6 months to acquire the morphological and mucin producing characteristics of goblet cells[174].

In 2001, a co-culture model of the Caco-2/TC7 and the HT29-MTX clones was proposed for the intestinal permeation studies[167]. In order to differentiate and produce an appropriate epithelium for permeation assays the cells were cultured in a specific ratio (9:1). This specific proportion is of high importance as the HT29-MTX cells have the potential to overgrow compared to Caco-2 cells. Among all the cells in intestinal epithelium, the goblet cells and enterocytes represent the two major phenotypes in the intestinal epithelium in the proportion of 10 and 90%, respectively[175].

The above described model offers the ability to recognize the transport mechanism of a compound and also its absorption rate. A compound can be transported through the intestinal epithelium through passive diffusion either paracellularly or transcellularly or by active transport and especially transcytosis or by carrier mediated transport. This depends on the nature of the compound and especially its hydrophilicity, size, chemical composition and charge[176]. Some molecules, water for instance, are transported by both routes. In contrast, the tight junctions are impermeable to large organic molecules such as amino acids, glucose and drugs. These types of molecules are transported exclusively by the transcellular route, and only because the plasma membrane of the absorptive enterocytes is equipped with transporter molecules that facilitate entry into and out of the cells. Tight junctions are seals between adjacent epithelial cells restricting paracellular flux of water, ions and solutes. The tight junctions are localized in a ring towards the apical pole of the cell. They are composed of branching strands which are themselves composed of rows of transmembrane proteins with extracellular domains, predominantly of the 27-member claudin (CLDN) family and occludin (OCLN). Interestingly, tight junctions can be modified and regulated by different compounds from food constituents and factors such as Tumor necrosis factor a (TNF-a) that may open the tight junction[177]. As a result, many studies, are focusing on the opening of the tight junctions which will facilitate the paracellular permeation of compounds without permanent effect on the integrity of the epithelium and also with the less possible alteration of the compound of interest (intracellular metabolism). Labrasol, one of the main representatives of surfactants used in microemulsions for pharmaceutical purposes, was shown to increase the permeability of a paracellular marker by 33.8-fold and the mechanism of opening of tight junctions was found to involve F-actin-related changes and redistribution of ZO-1 in Caco-2 monolayer[178]. Also, Tween 80 (and in general, surfactants with high HLB values) is considered to can rapidly and reversibly open paracellular tight junctions[179]. Interestingly, a

transport study of barbiturates across goldfish membrane showed that the nonionic surfactant Tween 80 with a concentration below the CMC increased drug absorption, while it decreased absorption at concentrations above the CMC[180].

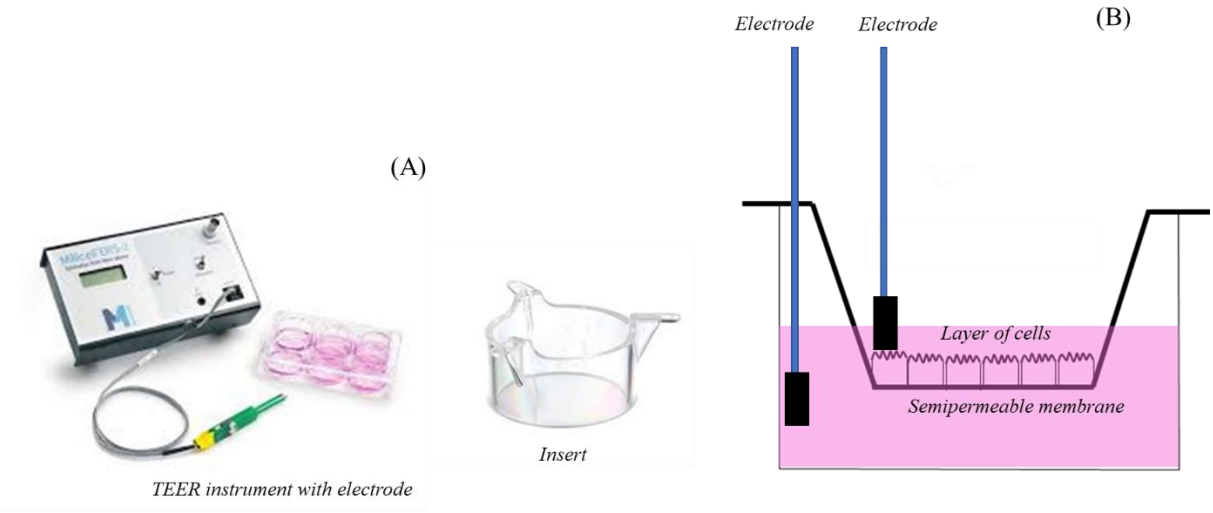

Figure 9. TEER device, semi-permeable filters and schematic representation of TEER measurement in constructed epithelium.

Table 3. *In vitro* models for GI tract absorption and their reported TEER values.

***In vitro* model**	**TEER values (Ω.cm²)**
Caco-2	1100 - 1350
Caco-2/TC7	711 ±79
(HCEC)	200
Caco-2 and HT29-MTX	122±19, 100 – 300, 110 - 185
HT29-MTX	25
Caco-2 and Raji B	88 ± 27
Caco-2, HT29 and Raji B	60

4.2 Nasal route

Nasal administration of drugs is one of the most convenient and reliable routes, not only for systemic and local delivery but also for targeting the Central Neurons System (CNS)[57,181]. The administration through nose avoids the first-pass hepatic metabolism, in contrast to oral uptake while offering rapid absorption of drugs due to the high vascularization of its epithelium. It is a convenient route for users-patients especially in long-term therapies. The limitations of this specific route, on the other side, are possible toxicity and irritation, caused by the ingredients of the formulation and the repeatable doses, in combination with low absorption surface in comparison with GI tract.

Nasal local delivery of compounds has been studied extensively for the treatment of nasal allergy and nasal congestion but the last few years this route has been, interestingly, exploited for the delivery of compounds in different targets across the human body. Nasal delivery is crucial in situations where a rapid or prolonged delivery of a drug is needed as in epilepsy crisis treatment or in long-term therapy (i.e. diabetes). There are commercially available nasal formulations for systemic delivery such as sumatriptan, ergotamine and butorphanol, all of them for the treatment of migraine. Pharmaceutical companies focusing their interest in nasal formulations appropriate for the delivery of morphine, midazolam, fentanyl, non-steroid anti-inflammatory drugs but also for larger compounds such as parathyroid hormone, insulin, interferon and others. Interestingly, in 2001 a nasal vaccine for influenza was launched but it was withdrawn from the market because of possible nasal toxicological problems, whereas others are in the stage of clinical trials[182]. The interest around nasal administration can be evidenced by the increased publications regarding this route of administration and the plethora of different relative delivery systems such as emulsions[183], powders[184], nanoparticles,[185] liposomes[186], gel suspensions[187] and others.

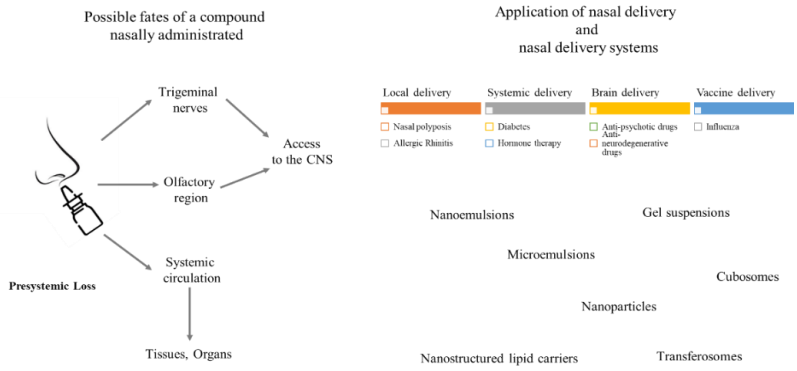

Figure 10. (a) Diagram of possible fates of a compound following nasal administration, (b) nasal delivery applications and (c) systems.

The nasal route is considered to be permeable by lipophilic compounds (with absorption rates close to intravenous administration) but seems to be a hurdle for more polar compounds including low molecular weight compounds, peptides and proteins. For the first case the rates are of 10% whereas for molecules with higher molecular weight, such as peptides and proteins, around 1%. The problems of the above described absorption profile are (1) low nasal membrane permeability, (2) quick clearance from mucosa and (3) enzyme degradation. The molecular size of the drug influences absorption of the drug through the nasal route. For lipophilic drugs, MW shows a direct relation to drug permeation whereas water soluble compounds depict an inverse relation. The rate of permeation is highly sensitive to molecular size for compounds with MW ≥ 300. Mucociliary clearance is one of the functions of the upper respiratory tract which also limits the nasal transport of compounds. The function of this mechanism is the prevention of reaching the lungs substances such as allergens, bacteria, viruses, toxins etc. When such materials adhere to, or dissolve in, the mucus lining of the nasal cavity, they are transported towards the nasopharynx for eventual discharge into the gastrointestinal tract[188]. It is reported that the half-life of clearance is in the order of 15-20 min. For that reason, different approaches are used in order to avoid the nasal mucus clearance. In order to diminish the effect of mucociliary clearance the administration is preferred in the anterior part of the nasal cavity where the clearance is decreased and different mucoadhesive agents are used in the delivery systems in order to increase the retention time in the area.

Drugs can cross the epithelial cell membrane either by transcellular route (concentration gradients, receptor mediated transport) or by paracellular route through the tight junctions of the cells. In order to improve the nasal absorption of bioactives, promoting enhancers are used. These enhancers act by different mechanisms: they change the permeability of the epithelial layer by modifying the phospholipid layer, leaching out enzymes or the outer layer of mucus, or by affecting the tight junctions present in the nasal epithelium. Surfactants, bile salts, chelators, fatty acid salts, cyclodextrins and glycols are some of the most commonly used penetration enhancers[189]. The majority of absorption enhancers belong in the category of surfactants with laureth-9[190], phospholipids[191] and others being the most representative. Natural occurring compounds, such as chitosan, have also been described as penetration enhancers.

In literature, many chitosan solutions of drugs have been examined as alternatives for increased nasal permeability[192]. Chitosan is produced, by a process of deacetylation, from the chitin found in crustacean shells. Different studies have been conducted in *in vivo* studies with morphine[193] and insulin[194], all indicated at least 3 times higher absorption of the

compound when administrated with different chitosan forms. The mechanism of action of chitosan in improving the transport of polar drugs across the epithelial membrane is believed to be a combination of bioadhesion with the transient opening of the tight junctions in the cell membrane to enable the passage of polar drugs[195,196]. Extensive toxicological and tolerance studies in animals and humans have shown chitosan to be non-toxic and non-irritant to the nasal membrane[197]. Its positive charge helps to interact with the negatively charged mucin and offers prolonged residence time and simultaneously acts as a penetration-enhancer in nasal formulations inducing the opening of the tight junctions. It has been previously reported, that chitosan increases cell permeability by affecting the tight junctions [196]. Recent work has studied the interactions of nasal mucus layer with an O/W nanoemulsion with the use of SAXS. Interestingly, chitosan seems to disrupt the mucus gel conformation, while creating channels and facilitating mucosal penetration of carriers[198]. More specifically, chitosan in acidic environment is positively charged and its amine groups interact with the mucus layer which, as a consequence, affects the permeability of the epithelial membrane by the transient opening of tight junctions. Chitosan induces changes in cellular actin which is one of the major factors affecting the regulation of paracellular flow across epithelia.

Microemulsions are able to encapsulate polar compounds while offering a protective environment against enzymatic degradation in the presence of ingredients acting as mucoadhesive and permeability enhancers. Shah et al. formulated microemulsions of rivastigmine for nasal brain delivery and performed *in vivo*[199] and *ex vivo*[200] studies to investigate its efficacy against Alzheimer's disease. In this context, other groups focused on Alzheimer's disease treatment by encapsulating compounds against neurological disorders in microemulsions for intranasal application. Epileptic and anxiety crisis, schizophrenia, bipolar disorder and other related diseases are also in the center of interest for this specific route of administration. The nasal absorption of diazepam was found to be fairly rapid with maximum drug plasma concentration reached within 2-3 min with the use of ethyl laurate/Tween80/propylene glycol/ethanol/water microemulsion[47]. Nasal delivery was also used as possible route of administration for insulin, as it is able to offer an easy way of administration for this chronic treatment. This needle-free and self-administration strategy was exploited with the use a non-alcoholic GRAS microemulsion composed of isopropyl palmitate/glyceryl oleate/ Labrasol/ propylene carbonate/ water, that increased the bioavailability of insulin without the presence of any penetration enhancer[201]. Mucoadhesive microemulsions, with the use of polycarbophil in low concentrations (0.5%) resulted in induced sleep onset in contrast to non-mucoadhesive microemulsions in the case of delivery of diazepamines for insomnia indicating the crucial role of mucoadhesion agents in higher

retention times in nasal mucosa[202]. Chitosan has been studied several times as mucoadhesion enhancer especially in the case of microemulsions for the delivery of different compounds to brain such as buspirone hydrochloride[203], carbamazepine[204] and others.

It has to be underlined, that as in all cases, microemulsions that are candidates for nasal administration must be decorated adequately as their ingredients may cause different side effects. Especially, the surfactant molecules must be used in concentrations which will be able to increase the bioavailability of the delivered compound without disrupting the nasal membrane. For that reason, it is of high importance to study all the formulated systems extensively with the use of *in vitro* models in order to find the range of non-toxic and non-irritant concentrations.

4.2.1 Nasal route - In vitro models

In order to evaluate the toxicity and also the permeability efficiency of drugs in free or encapsulated state in the nasal epithelium a specific model was needed. In the first studies regarding nasal delivery, many toxicity and permeability studies were conducted with the use of excised mucosa tissue and the use of *ex vivo* techniques by the Franz Diffusion Cell. In general, three strategies have been used for nasal absorption studies: (i) primary cell cultures of human nasal cells (ii) human nasal cell lines and (iii) excised nasal epithelium (rabbit, bovine, ovine, canine, human). In the case of excised nasal epithelium, arising problems include differences between the species in enzyme activities or in cell type distribution. Rabbit, bovine and porcine mucosa have been widely used for permeation studies and examination of permeation enhancers. Also, the correlation with human nasal absorption has been satisfactorily confirmed[205]. Human nasal cell cultures, used *in vitro* are not preferred due to their dependency on repeated sampling of cells, the complex isolation, limited lifespan, questionable reproducibility due to differences between the donors and possible selective isolation and cultivation of tight junction forming cells[206].

The nasal cell lines most often used in nasal transport studies are RPMI 2650 (human origin), BT (bovine origin) and NAS 2BL (rat origin). The former one, derived from septum carcinoma is closely related to normal human nasal epithelium concerning its karyotype, its cytokeratin polypeptide pattern and the presence of mucus on the cell surface[207]. The majority of studies indicate the creation of monolayers in membranes with the formation of tight junctions and TEER values typical for leaky-type epithelia (50-150 Ωcm^2). This cell line has been extensively used in the studies of nasal permeability as it is considered a superior model to the human primary cell cultures due to the low cost and high reproducibility of the results. As the conditions for appropriate manipulation of the RPMI 2650 cell line have been

well established, the cell line is also used in more sophisticated models such as 3D printed apparatus in order to resemble the conditions affecting the transport from the delivery device to the nose[208].

For transport studies, RPMI 2650 cells are cultivated in semi-permeable filter inserts as described previously for the intestinal cell line. Variations in TEER values and permeability rates of different compounds have been reported for a wide range of semi-permeable inserts making the model the most standardized nasal model for valid in vitro studies[209]. In 2008 Bai et al. studied the culture of RPMI 2650 cell line under Air-Liquid Interface (ALI) conditions in contrast to the convenient Liquid-Liquid Interface (LLI). The microscopic and protein examination revealed that the cell line grows as a confluent monolayer and forms a tight nasal epithelial barrier with appropriate tight junctions[210]. The RPMI 2650 cell line requires a shorter culture time to reach confluence than human primary nasal cell cultures and is therefore considered superior[211]. As a result, the cells are cultivated in semi-permeable filters for approximately 21 days (appropriate TEER values), while the culture medium has been removed from the insert 8-days post seeding for a reliable *in vitro* model.

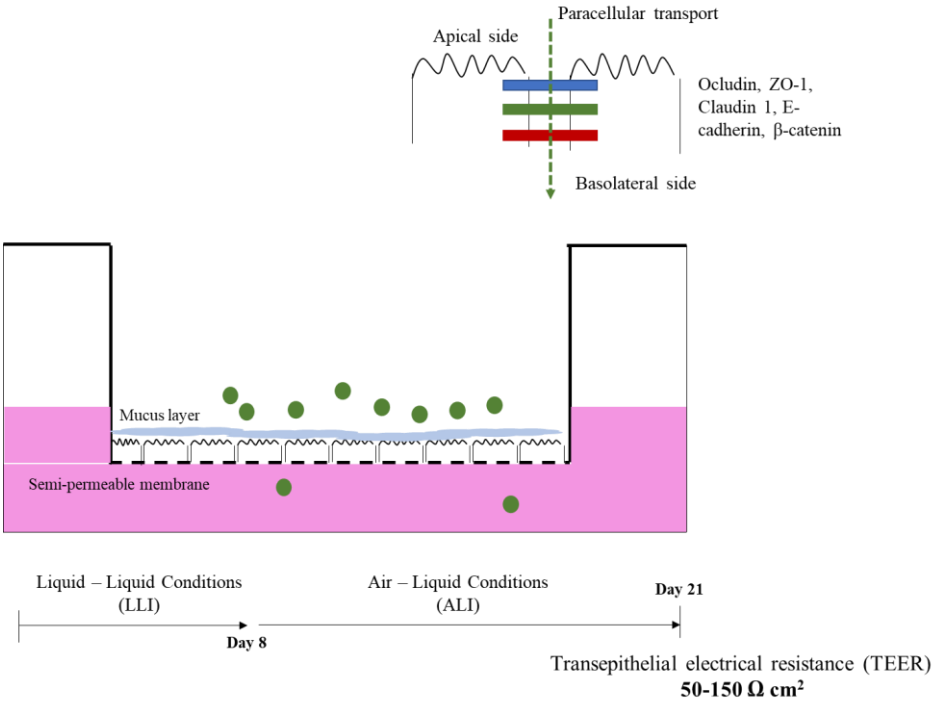

Figure 11. Schematic representation of RPMI-2650 cell line cultured in semi-permeable filter inserts.

To conclude with, microemulsions as drug and bioactive delivery systems, have gained researchers' interest and during last decade many groups have reviewed their biomedical

applications, related to different routes of administration. Examples of microemulsions designed for different administration routes are summarized below.

Table 4. List of drugs encapsulated in microemulsion systems and their administration route.

Administration route	Drug
Oral	Cyclosporin, Pitavastatin, acyclovir, Amphotericin B, Glipizide, Paclitaxel, rifampicin, isoniazid, HIV protease inhibitors
Nasal	Clonazepam, Carbamazepine, Clobazam, Levetiracetam, Diazepam, Asenapine maleate, Sulpride, Insulin, Nimodpine, Zolmitriptan, Sumatripan succinate, Progesterone, Indomethacine,
Transdermal	Ketoprofen, Tacrolimus, Aceclofenac, Indomethacin, Diclofena, Retinoic acid, Scopolamine, Broxaterol, Methyl nicotinate, Meloxicam, Hydrocortisone, 5-fluorouracil, testosterone
Occular	Rivoflabin phosphate, Timolol, Levobunolol, Pilocarpine nitrate, Dexamethasone, Pilocarpine hydrochloride, Chloramphenicol, Everolimus, Prostagladin, Diclofena, Tarolimus
Vaginal	Clotrimazole, Fluconazole, UC-781 (antiretrociral)
Buccal	Carvedilol, triamcinolone acetonide, fluocinole acetonide
Sublingual	Insulin, Sildenafil,

5.

Purpose of the thesis

5. Purpose of the thesis

The main purpose of the present study was the development of novel W/O microemulsions to serve as hosts for a wide range of bioactive compounds with the aim to be further used as delivery systems of the encapsulated compound by different routes of administration.

As the scientific community has focused the attention in the production of efficient therapeutic enzymes and the synthesis of drugs based on natural compounds the objective of the present thesis was the formulation of appropriate systems which will be able to encapsulate hydrophilic compounds such as enzymes and antioxidants for potent pharmaceutical applications. Microemulsions were considered as the most appropriate enzyme and antioxidant carriers for their efficient protection and administration. As the formulation must comply with the pharmaceutical regulations all the ingredients were chosen in order to be natural and GRAS (Generally Recognized as Safe). In order to avoid any potent toxic and irritant effects, the study focused on the use of minimum co-surfactant concentration (up to 2% v/v) or their total absence from the final systems. Furthermore, the oil phase of the systems was exclusively composed of natural oils, namely IPM, EVOO and SO. EVOO and SO were used in order to increase the antioxidant efficacy of the systems while IPM, as a widely studied pharmaceutical oil, to amplify the beneficial properties of the developed microemulsions.

As a) oral administration is one of the most common routes of drug administration and b) nasal administration is a self-medication route for rapid onset, the use of microemulsions could lead to a low-cost and efficient alternative to other existing formulations. All ingredients, and especially surfactants, were investigated for the ability to enhance penetration of the bioactive compounds through epithelia (intestinal and nasal). In the case of enzymes, the presence of surfactants in the systems is able to increase their bioavailability which is relatively low due to their high molecular weight.

In addition, a widely studied antioxidant such as hydroxytyrosol was encapsulated in the microemulsions and the intestinal permeability was simulated using cell lines. In the case, of nasal administration of antioxidants, the aim of the thesis was the finding of an appropriate formulation, with no cytotoxicity, which will increase the permeability of a hydrophilic bioactive. As a result, increased systemic concentration or access to the CNS could be achieved.

As shown in the next page the present thesis was divided in three (3) different subprojects, for reasons of coherence and clarity, that will be further analyzed in section 6.

Main objectives of the thesis

The main objectives of the present thesis were:

- Formulation of biocompatible W/O microemulsions appropriate for human use.
- Encapsulation of enzymes and antioxidants in microemulsions, with aim to retain their activity
- The cytotoxicity evaluation of the proposed microemulsion systems with the use of appropriate *in vitro* models.
- The study of the factors that affect the epithelial permeability of the encapsulated compounds with the use of the appropriate *in vitro* models in the cases of intestinal and nasal epithelia.
- Investigation of nasal epithelial permeability of hydrophilic antioxidant with the use of natural penetration enhancer.

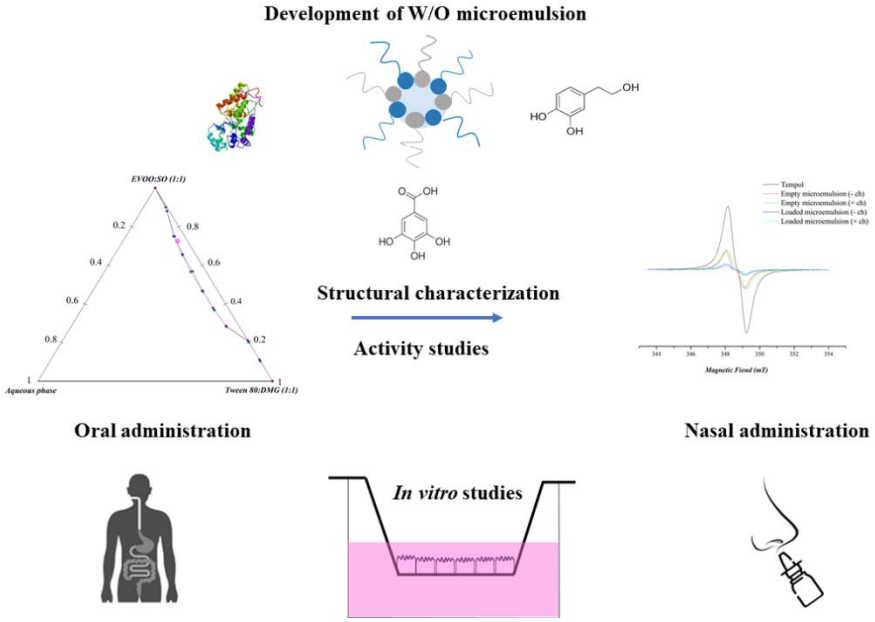

Figure 12. Schematic representation of the PhD objectives

6.

Description of the subprojects of the thesis

6. Description of the subprojects of the thesis

Subproject 1. Encapsulation of enzymes in microemulsions (Publications 1 & 2)

A major advantage of microemulsions, as described in the previous sections, is their ability to solubilize products with different polarities, a fact that makes them appropriate hosts for both enzymes and their substrates irrespectively to their solubility. As the interest for therapeutic proteins, against various diseases or as activators of prodrugs increases, many groups have investigated their delivery efficiently using a variety of formulations. The development of an appropriate microemulsion for pharmaceutical purposes is a great challenge as all the excipients must be chosen with caution for a final non-toxic, non-irritant formulation which will enhance the absorption and bioavailability of the encapsulated compound. The major problems of those versatile formulations are the high concentration of surfactants and the increased difficulty in penetrating the complex and numerous barriers of the target organism.

Publication *1* is a literature review on the use of microemulsions as "microreactors" for oxidation reactions. The review deals with the use of different oxidative enzymes such as heme peroxidases, chloroperoxidases, laccases and others in the non-conventional media. The review presents an overview of the work made on biotransformations catalyzed by oxidative enzymes. Also, the review comments on how surfactantless microemulsions have been applied in these reactions in order to overcome problems raised from the microemulsion's nature.

For that reason, in **Publication *2***, HRP was encapsulated for the first time in a biocompatible W/O microemulsion in the presence of exclusively non-ionic surfactants and the absence of co-surfactant molecules. A novel biocompatible W/O microemulsion was developed composed of IPM/Tween 80/DMG/water/PG and was investigated as a potential enzyme delivery system for pharmaceutical applications. The system had a low total surfactant concentration (8.3% w/w) and was able to incorporate approximately 3% w/w aqueous phase containing HRP. Structural and activity aspects of the system were studied using a variety of techniques such as DLS, EPR and DIT. Different enzyme concentrations were used, indicating the high loading capacity of the system despite its low water content. According to the results the participation of HRP in the surfactant monolayer of the

microemulsion is evident. In order to assure that the system allows the enzyme to retain its catalytic activity, an oxidative reaction catalyzed by HRP was successfully carried out with the use of the model substrate ABTS. The influence of several parameters such as temperature, pH and PG concentration were examined in order to optimize the reaction conditions and a kinetic study was conducted revealing an ordered-bi-bi mechanism. As a final step, the release of the encapsulated enzyme was studied using an adequate receiver phase formulated based on Winsor systems. The two-phase conformation (W/O microemulsion in coexistence with a surfactant rich aqueous solution) revealed the effectiveness of the proposed microemulsion not only as a microreactor but also as carrier for therapeutic biomolecules.

Subproject 2.
Oral delivery of natural antioxidant encapsulated in a microemulsion (Publication 3)

Over the last years, the incorporation of natural antioxidants in food and pharmaceutical formulations has gained attention, delaying or preventing oxidation phenomena in the final products. Also, recent studies have revealed many of the health-benefit properties of natural compounds exhibiting antioxidant, anti-proliferative and anti-depressant activities leading to a growing interest for natural substances serving as lead compounds. Moreover, in the food industry, in order to obviate the oxidation of products during storage and to design high added-value products, companies tend to replace synthetic compounds by naturally occurring ones. In order to take full advantage of their properties, protection in special microenvironments is of great importance.

The main aim of the project described in **Publication *3*** was the development of novel microemulsion systems, able to be used in both pharmaceutical and food applications. In the present study biocompatible non-ionic W/O microemulsions composed of IPM and/or EVOO as the oil phase and Tween 80 in combination with, the less studied, DMG as surfactants. All the ingredients are widely used in the food and pharmaceutical industries. The formulated W/O microemulsions were used as hosts for the encapsulation of HT, a natural polyphenol which has raised the attention the last decades. HT may act as a controller of lipid oxidation of natural oils, and as an antioxidant in films used for active food packaging. Many studies have evidenced health benefits including neuroprotective, antiproliferative and antimicrobial properties. EFSA has claimed that HT acts as a protector of the cardiovascular system avoiding oxidation of LDL cholesterol by free radicals, maintaining normal blood HDL cholesterol concentrations and preventing atherosclerosis.

The colloidal liquid nanodispersions were studied structurally before and after the encapsulation with the use of DLS and EPR technique. The combined findings from the above techniques indicated the involvement of the antioxidant in the surfactant monolayer. Also, as the surfactant concentration of the system was increased and the oil phase became more complex (presence of EVOO) the rigidity of the surfactant monolayer increased creating a more restrictive environment for the encapsulated compound.

EPR technique was also utilized for the investigation of the antioxidant scavenging effect of HT after its encapsulation in the reverse micelles. Antioxidant assessment, with the use of

the lipophilic free radical galvinoxyl, revealed that the incorporation of HT in the microemulsions increased the antioxidant activity of the system the first 2 minutes of the reaction indicating its retention of activity upon encapsulation. However, the correlation of the membrane's rigidity with the antioxidant assessment revealed the existence of a possible hurdle for the reaction.

Additionally, the aim of the present study was to evaluate the intestinal absorption of free HT, following oral administration, in comparison with encapsulated HT in biocompatible microemulsion systems. As only few studies have reported the use of W/O systems for oral bioavailability of natural hydrophilic compounds and none of them has used natural oils such as EVOO, *in vitro* cytotoxicity and intestinal epithelial permeability studies were conducted with the use of the Caco2-TC7/HT29-MTX model. The microemulsions, as the MTT assay revealed, could be used in a high concentration of 1% v/v in the culture media without affecting the viability of the cells. The HT intestinal permeability of HT was different between the microemulsion systems, with lower rates in the case of the microemulsion with higher surfactant concentration indicating a possible implication between the surfactant and the mucus layer produced by the HT29-MTX goblet cells.

Subproject 3.
Nasal delivery of natural antioxidant encapsulated in a microemulsion
(Publication 4)

Nasal administration of bioactive compounds has gained the interest of the scientific community as a promising administration route for bypassing the first-pass metabolism conducting to increased concentration of bioactives in the system circulation while offering a way to reach the brain. In **Publication *4*** the formulation of a novel biocompatible W/O microemulsion is described, with the use of natural oils for the nasal administration of the natural hydrophilic antioxidant GA.

The work presents evidence that the microemulsion can be used as a nasal pharmaceutical formulation for the delivery of polar antioxidants. In order to improve the proposed formulation, the microemulsion was modified by the addition of chitosan in its aqueous phase. The microemulsion was studied by DLS, EPR and SAXS techniques. The DLS measurements revealed that the incorporation of a polyelectrolyte like chitosan, did not provoke changes in the size of the system's dispersed phase but the encapsulation of GA resulted in small but significant changes in the apparent hydrodynamic diameter. In the case of EPR experiments, regarding the structural conformation of the system, the participation of both molecules (GA and chitosan) in the interface was proved, fundamental fact for two reasons: i) the chitosan molecules interact with the mucus of the nasal epithelium exploiting its mucoadhesive properties and ii) the encapsulated-GA is more likely to be released. EPR was also used in order to assess the antioxidant efficiency of the system and the encapsulated compound, where both found to be high against the stable free radical galvinoxyl.

RPMI-2650 cell line was used as the *in vitro* model for cell viability and for GA nasal epithelial transport studies after microemulsion administration. The results suggested that the nasal epithelial permeation of GA was enhanced, 3h post administration by the presence of the microemulsion. The system was found non-cytotoxic for the cell culture in concentrations up to 0.2% v/v. However, the rate of the transported antioxidant in the presence of chitosan was higher indicating the polymer's effect in the paracellular transport of the GA.

Overall, this study demonstrated that biocompatible microemulsion systems, formulated by edible oils, can be used offering protection to the sensitive antioxidant molecules with potent pharmaceutical activity, until the time of administration. Moreover, their ingredients are able to affect the permeability profile of the bioactive molecules through constructed

epithelia making it a promising absorption enhancing system for nasal delivery of antioxidants for reaching the systemic circulation or the brain.

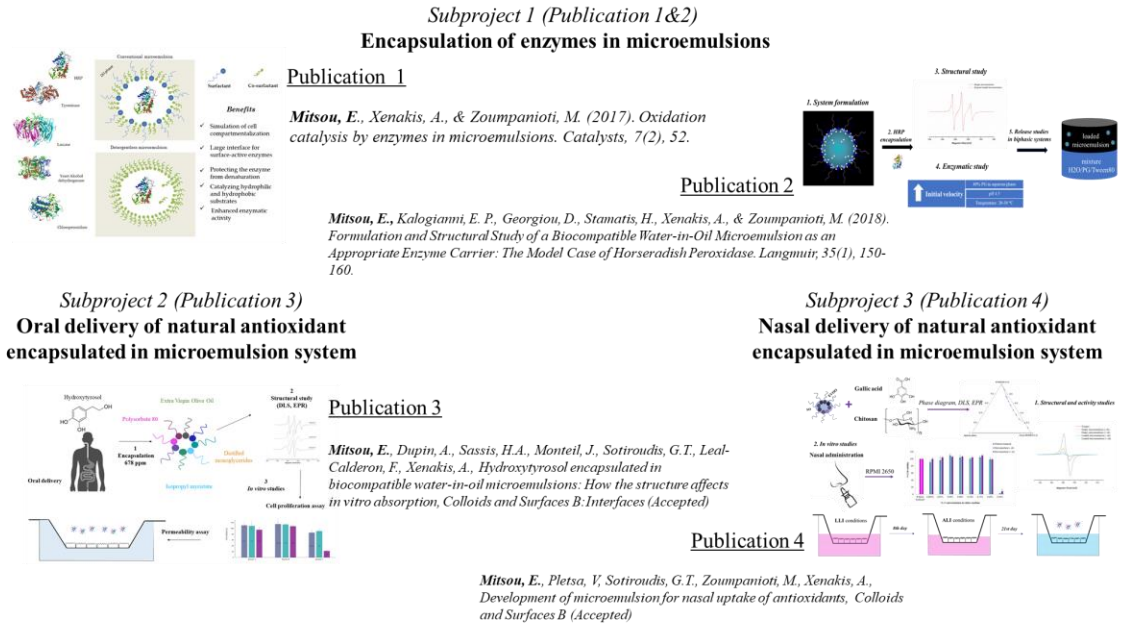

Figure 13. Subprojects and related publications.

7.
Methods

7. Methods

In the present section all the methods used in the thesis are analyzed. The methods are divided into categories referring to those used for: **a.** the study of **the phase behavior of the systems**, **b.** ***Structural studies*** of the microemulsions, **c.** ***Enzymatic studies*** of encapsulated HRP, **d. Release studies, e. A*ctivity assessment*** of the encapsulated bioactive compounds, **f.** ***Biological assessment*** of the microemulsions and all the **g.** ***Analytical methods*** used.

7.1 (Pseudo-) Ternary phase diagrams

In all the subprojects of the present thesis pseudo-ternary phase diagrams have been constructed due to presence of more than three components in every system. The W/O microemulsions (**Publications 2, 3 and 4**) consisting of oils as the continuous phases, Tween 80 and DMG as surfactants and ultra-pure water as the dispersed phase, were prepared by adding the appropriate amounts of aqueous phase to a mixture of oil and surfactants followed by gentle shaking. Mixtures of surfactants and oils with weight ratios varying from 1:9 to 9:1 were prepared in glass vials and incubated overnight at 25 °C. The mixtures were then titrated with water (5 μL/24 h) until the solubilization limit was reached. The appearance of turbidity followed by phase separation indicated the phase transition from monophasic to biphasic systems. Clear and isotropic oil-rich samples correspond to the monophasic (1φ) area of the phase diagram. Pseudo-ternary phase diagrams were drawn from experimental data using the ProSim Ternary Diagram plotting tool or Origin 6.1 Scientific Graphing and Data Analysis Software. In **Publication 2** the oil phase was IPM and the surfactants Tween 80 and DMG in a 1:1 ratio while the aqueous phase was composed by water and PG at a 1.5:1 weight ratio. Similarly, a pseudo-ternary phase diagram was constructed replacing water with 50mM acetate buffer, pH 4.5, following the same procedure, in order to investigate the effect of salts on the extent of the monophasic area of the phase diagram. In **Publication 4** for the preparation of a potent mucoadhesive microemulsions a chitosan solution (0.1 % chitosan in 1% acetic acid solution) was used as aqueous phase.

Table 5. Composition of the microemulsions developed in the present thesis expressed as % w/w.

System	Oil phase	Surfactants	Aqueous phase	Corresponding Publication
A	IPM 89%	Tween80:DMG 8%	Water:PG 3%	2
B	IPM 93.8%	Tween80:DMG 4.2%	Water:PG 2%	3
C	EVOO:IPM 89.6%	Tween80:DMG 8.4%	Water 2	3
D	EVOO 79.7%	Tween80:DMG 17.8%	Water 2.5%	3
E	EVOO:SO 72.4%	Tween80:DMG 23.4%	Water 4.2%	4
F	EVOO:SO 72.4%	Tween80:DMG 23.4%	Chitosan aqueous solution 4.2%	4

7.2 Structural studies

7.2.1 Dynamic Light Scattering measurements (DLS)

The DLS technique was used not only to shed light on the dimensions of the microemulsions but also helped in combination with other techniques to investigate the location of an encapsulated molecule. In all subprojects a Zetasizer Nano ZS (ZEN3600) from Malvern Instruments (UK) equipped with a He-Ne laser (632.8 nm) with a non-invasive back scatter (NIBS) technology was used for the DLS measurements. A scattering angle of 173° was used. The droplets' apparent hydrodynamic mean diameter was deduced from the Stokes-Einstein (Eq. 4) . The average droplet size and the polydispersity index (PdI) of the empty and loaded microemulsions were measured at the temperature of the scattering cell adjusted at 25 °C, in all cases. Sample preparation involved filtering through 0.45 μm regenerated cellulose filters and the experiments were carried out in a quartz type cuvette under dust-free conditions. Data were processed using the Malvern Zetasizer Nano software, which fits a spherical model of diffusivities with low polydispersity. The diameters of the

microemulsions' dispersed phases were measured with the use of viscosities and refractive indexes obtained by measurements in DVI-Prime viscometer and refractometer.

Table 6. Refractive indices and viscosities of the ingredients used in the DLS experiments.

Ingredient	Refractive index	Viscosity (cP)
IPM	1.433	4.50
EVOO	1.468	83.00
SO	1.471	48.98
EVOO: IPM (1:1)	1.449	9.700
EVOO: SO (1:1)	1.472	54.80
Water	1.330	0.89

7.2.2 Small Angle X-ray Scattering (SAXS)

In **Publication 4,** Small angle X-ray Scattering (SAXS) experiments, for microemulsions in the presence and absence chitosan solution, were carried out on a Nano-InXider vertical SAXS/WAXS system of Xenocs SA, France equipped with a Cu k-a source of a two detectors setup for SAXS/WAXS measurements. The wavelength of the X-ray radiation was 0.154 nm and the sample-to-detector distance was 937.5 mm. The exposure time for each scattering frame was 600s (in the VHS mode) in all vacuum environment.

7.2.3 Electron Paramagnetic Resonance Spectroscopy (EPR)

In order to obtain information about the interfacial properties of the surfactant monolayer in empty and loaded microemulsions 5-DSA, spin labeled fatty acid was incorporated in the studied microemulsions. In all cases, EPR spectra were recorded at constant room temperature (25 °C), using a Bruker EMX EPR spectrometer operating at the X-Band (microwave frequency range 8-12 GHz, typical frequency 9.5 GHz). Samples were contained in a WG-813-Q Wilmad (Buena, NJ) Suprasil flat cell. Typical instrument settings were: Center field: 0.349 T, scan range: 0.01 T, gain: 5.64 x 10^3, time constant: 5.12 ms, modulation amplitude: 0.4 mT and frequency: 9.78 GHz. Data collection and analysis were performed using the BrukerWinEPR acquisition and processing program. In **Publications 3 and 4** spectral simulations were performed with the *EasySpin toolbox* for EPR spectroscopy based on the MATLAB® platform (The MathWorks, Natick, USA). EasySpin is a computational

package for simulating and fitting a wide range of Electron Paramagnetic Resonance (EPR) spectra. It is based on Matlab, a commercial technical computation software. EasySpin was developed in ETH Zurich (Laboratory of Physical Chemistry) and published by Stoll & Schweiger in 2006[212].

In order to obtain the desired concentrations of the spin probes in the microemulsions the following procedure was followed. The microemulsions (empty or loaded) were added to vials where the appropriate amount of the spin probe had been formerly deposited as ethanol solution which has been evaporated. In detail, stock solutions of 5-DSA in ethanol were prepared (7.8 x 10^{-3} M each). Then 15 μL of each solution was placed in test tubes. After ethanol evaporation, 1 mL of the respective microemulsion was added. The samples were incubated overnight in water bath (25 ºC) in order to reach equilibrium and to have the clearest EPR spectra. Spin probe concentration in the microemulsions in all cases was 10^{-4} mol.L^{-1}. Experimental results were analyzed in terms of rotational correlation time (τ_R), order parameter (S) and isotropic hyperfine splitting constant (α'_o). The τ_R, S and α'_o values were calculated with the use of Eq. 5 (see section 2.2.4). The above-mentioned equation of τ_R is applicable in the fast motion region ($\tau_R < 3 \times 10^{-9}$ s). For the slow-motion region ($\tau_R > 3 \times 10^{-9}$ s) the values were calculated using computer simulations assuming average rotational correlation times.

7.2.4 Dynamic Interfacial Tension (DIT)

Dynamic interfacial tension measurements were performed, only in the case of **Publication 2**, using the pendant drop/axisymmetric drop shape analysis method. CAM200 (KSV) was used and analysis was performed via the Young Laplace equation using One-Attension Software (version 1.8 Biolin Scientific). First, measurements of the aqueous and oil phases against air were performed followed by measurements of the aqueous/oil interface in the presence and absence of Tween 80. The latter measurements were performed after forming an aqueous phase pendant drop in the oil phase contained in a quartz cell (Hellma Analytics, Müllheim, Germany). All measurements were taken at 25 ± 1 °C.

Figure 14. Dynamic Interfacial Tension equipment**.**

7.3 Enzymatic activity and kinetic study

Enzymatic activity and kinetic study were conducted in **Publication 2** in order to study enzyme's properties, especially HRP's affinity with the substrate, when encapsulated in the IPM/Tween80/DMG/PG/water microemulsion on the enzymatic properties. For this reason, the model reaction of enzymatic oxidation of ABTS towards $ABTS^{\cdot+}$ catalyzed by HRP was used. The reaction was started by adding 6 μL of concentrated H_2O_2 solution (30% w/w) in 1 g of microemulsion containing ABTS and enzyme in the aqueous phase. The reaction took place at w_o (water-to-surfactant molar ratio) was 5.4. For enzymatic activity studies the ABTS, H_2O_2 and HRP concentrations were 0.250 mM, 0.125 mM and 0.42 μg/ml respectively. In detail, stock solutions of ABTS and HRP have been produced and appropriate quantities were added at oil/surfactants mixture in order to produce the final microemulsion. The progress of ABTS oxidation was followed spectrophotometrically using a Cary 3E spectrophotometer (Varian) at 414 nm (the λ_{max} of the oxidized product of ABTS) for several minutes. The initial velocities of the reaction were taken into account, assuring the absence of significant amounts of products. They were calculated by the linear part of the absorption intensity. The effect of the PG content, the pH and the temperature on enzymatic activity was studied.

For kinetic studies, final concentrations ranged between 0.05-0.15 mM for ABTS and 0.03-0.15 mM for H_2O_2 was used. The chemical reactions catalyzed by enzymes generally follow Michaelis-Menten kinetics. Considering the enzyme-catalyzed conversion of substrate (S) to product (P) the equation that describes the kinetic behavior is:

$$V_{\max=} \frac{V_{max}[S]}{[S]+K_m} \qquad \text{(Eq.9)}$$

where V is the reaction's velocity, Vmax the reaction rate when the enzyme is fully saturated by the substrate (all the binding sites are occupied), [S] is the concentration of the substrate and K_m is the Michaelis-Menten constant.

The catalysis of ABTS by HRP is a Bi Bi reaction in which two different substrates (A and B, in our case ABTS and H_20_2) are involved, leading to two different products (P and Q, in our case $ABTS^{\bullet+}$ and water). In order to study the influence of the two substrates on the rate of the oxidation reaction, different concentrations of one substrate were tested at constant concentration of the second substrate and vice versa. The graphs of initial velocity towards substrate concentration and the respective Lineweaver-Burk plots were plotted. The double reciprocal plots (1/v versus 1/[A] at several fixed concentrations of B) indicated an ordered Bi Bi mechanism as the lines are not parallels but intercept at the same point. The two different point of interception in the Lineweaver-Burk plots when H_20_2 concentration changed indicated inhibition induced by the second substrate.

Using the data available from the double reciprocal plot for fixed H_20_2 concentrations, two replots were constructed, using the inverse slope of the line corresponding to each fixed H_20_2 concentration. Firstly, the inversed slopes were replotted against the inversed H_20_2 concentration. The plot gives a curve, which is in accordance to a dead-end substrate inhibition in an ordered bi-reactant system. Upon H_20_2 concentration variation the plot is linear only at high values of 1/ [H_20_2], that is for low H_20_2 concentrations (H_20_2 acts mainly as substrate).

Secondly, the inversed slopes were replotted against H_20_2 concentration. The plot gives again a curve, which is in accordance to a dead-end substrate inhibition in an ordered bi-reactant system. Upon H_20_2 concentration variation the plot is linear only at high values of H_20_2concentration (H_2O_2 acts mainly as inhibitor). In the case of an ordered bireactant system with two products (ordered bi bi), where substrate B (in our case H_2O_2) reacts with the free enzyme to yield a dead-end EB complex, the velocity equation is:

$$\frac{V}{V_{max}} = \frac{[A][B]}{K_{ia}K_{mB}\left(1+\frac{[B]}{K_i}\right)+K_{mA}[B]\left(1+\frac{[B]}{K_i}\right)+K_{mB}[A]+[A][B]} \qquad \text{(Eq. 10)}$$

where, v is the initial rate of reaction; Vmax is the maximum velocity; [A] and [B] are the initial concentrations of ABTS and H_2O_2 respectively; K_{mA} is Michaelis constant for ABTS; K_{mB} is Michaelis constant for H_2O_2; Ki is the dissociation constant for EB and K_{ia} is the dissociation constant for A.

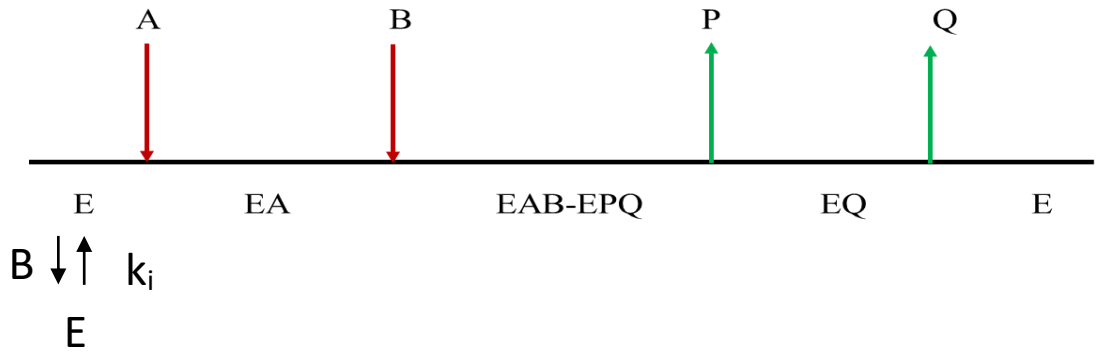

Figure 15. Schematic representation of ordered bi-bi mechanism with dead-end inhibition by substrate B.

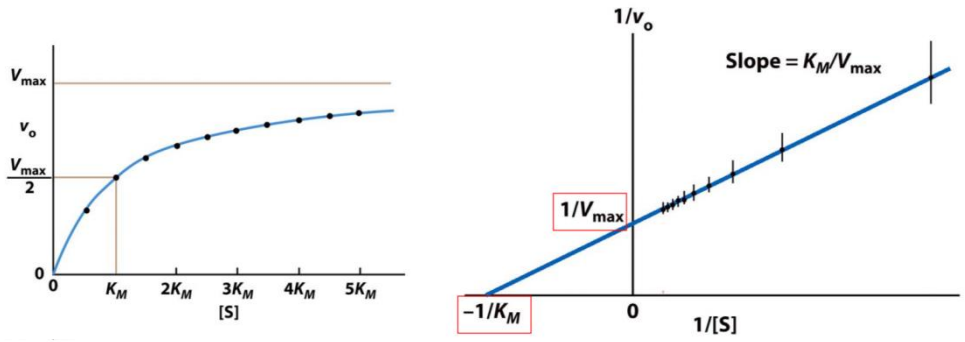

Figure 16. Plots used for the reactions mechanism study. (a) Michaelis-Menten plot and (b) Lineweaver-Burk.

7.4 Release studies

In order to study the release rate of an encapsulated compound from a microemulsion system different methods are used with the most common among them the Franz Diffusion Cell technique, firstly described in 1975[213]. In this technique a device divided in two compartments (acceptor and donor) with a semi-permeable membrane between them is used in order to study the release and permeation rate of different compounds. The under-study system is placed in the donor compartment and the released compound is measured, with the adequate technique, in the acceptor compartment. Franz Diffusion Cell is generally used, among others, for compounds encapsulated in O/W systems as the acceptor compartment is loaded with aqueous solution. However, in the case of W/O systems, as the

contact between the two phases (microemulsion and aqueous solution) lead to system separation due to the osmotic phenomena, this technique is not applicable.

As a result, a different approach, described for the first time, was used for the release studies in the case of the encapsulated enzyme in W/O microemulsions (Publication 2). In general, in order to obtain equilibrium between the microemulsion and the receiver phase, two strategies can be followed: (i) prepare a Winsor II system yielding after final equilibrium a W/O microemulsion (source phase) in thermodynamic equilibrium with an excess aqueous phase (receiver phase) or (ii) prepare a dilute surfactant/co-surfactant aqueous solution as a receiver phase. According, to Winsor there are three types of Winsor systems: Winsor I where an O/W system is in equilibrium with an excess of oil, Winsor II where a W/O system is in equilibrium with water and Winsor III where a surfactant rich system co-exists with an excess phase of water and oil[11]. As the formulation of a Winsor II system in some cases is a time-consuming procedure the approach based on surfactant/co-surfactant solution was used in the Subproject 1.

In order to obtain an acceptor compartment, where it could be feasible to measure photometrically the concentration of the released bioactive compound, an aqueous solution of surfactants and co-surfactants was prepared at different molar ratios of surfactant/co-surfactant. The acceptor compartment contained 29, 58.8, and 12.2% w/w ultra-pure water, PG and Tween 80 respectively. In the donor compartment we added 10mL of the proposed W/O microemulsion loaded with 160 μg of HRP. The microemulsion was added carefully on top of the receiver phase to avoid mixing of the two layers and the subsequent destruction of the system. The constructed biphasic system was mildly stirred at 1 rpm at room temperature. At defined time intervals, samples of 0.75 mL were withdrawn from the receiver phase and analyzed spectroscopically at 419 nm, where the heme group of the HRP absorbs [96]. A standard curve of HRP in the receiver phase was constructed with the different enzyme contents in the mixture ranging from 1 to 15 μg/mL. The percentage of HRP released from the apical to the receiver phase was presented as a function of time.

7.5 Antioxidant Activity assessments

In order to assess the antioxidant activity of the compounds encapsulated in the microemulsions, a technique based on EPR spectroscopy was used. In **Publications 3 and 4** the hydrophobic stable galvinoxyl free radical was used. All microemulsions added at 10% v/v in a solution of galvinoxyl free radical in isooctane (0.25×10^{-3} mol.L^{-1}). The solution was kept at -4 ºC and was brought at room temperature 30 minutes prior the experiment. Each

microemulsion, empty or loaded with HT or GA was incorporated at 10% v/v in a solution of galvinoxyl with final volume 1mL. The mixture was stirred well and transferred immediately in the EPR cuvette. EPR spectra were taken at room temperature for 30 min with a 5 min time interval among them. EPR spectrum of galvinoxyl stable free radical consists of one broad peak. EPR spectra were recorded with a center field of 0.348 T, scan range 0.01 T, gain of 5.64 × 10^3, time constant of 164 ms, conversion time of 5 ms, modulation amplitude of 0.4 mT, frequency of 9.8 GHz. The percentage of inhibition (% Inhibition) of the corresponding EPR spectrum was calculated from Eq. 11, where A_o is the integrated intensity of the control sample (free radical solution) at a specific time point and A the integrated intensity of the sample containing the antioxidants at the same time point.

$$\%\text{Inhibition} = \left(1-\frac{A}{Ao}\right) \times 100 \qquad \text{(Eq. 11)}$$

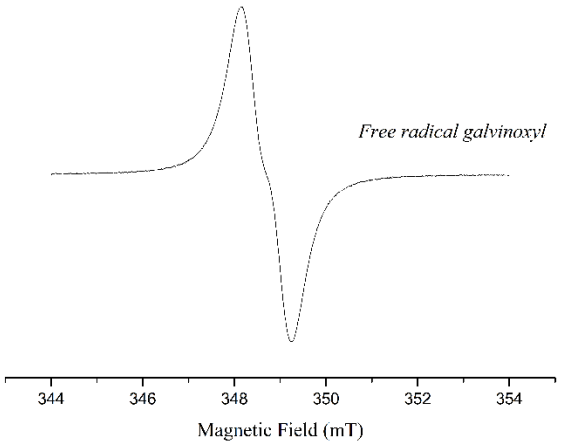

Figure 17. Characteristic spectrum of galvinoxyl free radical in isopropanol (25mM). Center field of 0.348 T, scan range 0.01 T, gain of 5.64 × 10^3, time constant of 164 ms, conversion time of 5 ms, modulation amplitude of 0.4 mT, frequency of 9.8 GHz.

7.6 Biological assessments

7.6.1 Cell proliferation assay

Cells in the exponential phase of growth are exposed to a possible cytotoxic compound. The duration of exposure is determined by different facts such as the stability of the drug or the future exposure to the studied compound. After removal of compound, surviving cells are determined indirectly by the MTT dye reduction. MTT is a yellow water-soluble tetrazolium dye that is reduced by live cells to a purple formazan product that is

insoluble in aqueous solutions. The amount of MTT-formazan produced can be determined spectrophotometrically once solubilized in a suitable solvent. A drawback of the assay is that it does not distinguish between a cytotoxic (cell kill) and a cytostatic (reduced growth rate) effect[214]. As a result, the assay can be used as a first step for in vitro studies in order to determine the non-toxic concentrations of a compound or generally of a formulation.

Cytotoxicity of microemulsions was tested with the MTT assay. In **Publication 3,** the cytotoxicity of microemulsions was assessed 6h after treatment by MTT assay according to the manufacturer's standard protocol in the co-culture model of Caco-2/TC7 and HT29-MTX model. In more detail, 100 μL of medium containing cells were added to the 96-well plate at a density of 2.7 × 10^4 per well and incubated for 24 h at 37° C under 5% CO2. Cells were exposed to microemulsions at different final ratio of 0.1%, 0.5% and 1% v/v in the culture medium. After the incubation period, the cells were washed with PBS once and 50 μL of MTT solution were added and incubated for 1 h. At the end of the incubation period, the medium was removed and the converted dye was solubilized in isopropanol in order to dilute the insoluble purple formazan. Cultural supernatant was discarded, and 50 μL isopropanol was placed in each well and stirred for 1 h.

In the case of **Publication 4**, a slightly different protocol was used. RPMI-2650 nasal cells were seeded in 96-well plate at a density of 2.7 × 10^4 per well and incubated for 24 h at 37° C. Cells were exposed to microemulsions at different final ratio ranging from 0.01% to 0.5 % v/v in the culture medium for 48 and 72. MTT stock solution (5mg/mL) was added to each culture being assayed to equal one tenth of the original culture volume and incubated for 3h. At the end of the incubation the solution was removed and the converted dye was solubilized in isopropanol: DMSO solution in ratio 1 to 1 in order to dilute the insoluble purple formazan. Absorbance of converted dye was measured at 570nm.

The absorbance of the converted dye was measured, at both cases, at a wavelength of 570nm with a Safire II Microplate Luminometer (TECAN, Switzerland) device. Eq. 12 was used to determine the cell viability.

$$\text{Cell viability (\%)} = \left(\frac{OD\ of\ treated\ cells}{OD\ of\ control}\right)x100 \qquad \text{(Eq. 12)}$$

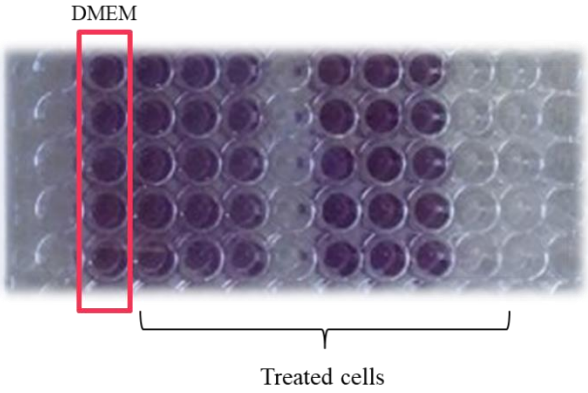

Figure 18. 96-well plate with RPMI-2650 cells after 72h of treatment with EVOO/ SO/ DMG/ Tween80/water microemulsion.

7.6.2 Permeability assay

For permeability experiments, in **Publications 3 and 4**, cells were seeded on 12-well ThinCertrs™ permeable polyester filters of 0.4 μm pore size, at a density of 9.1 x 10^4 cells/cm^2 and 4 ×10^5 cells/cm^2 for intestinal and nasal epithelial model respectively. In **Publication 3**, a 9:1 Caco-2/TC7 to HT29-MTX ratio was used, and grown to confluence in a complete medium. Cells were then cultured for 21 days in the complete medium which was changed in both compartments every two days until total differentiation. In **Publication 4**, due to the different nature and needs of the nasal epithelial cells RPMI-2650, the medium on the apical compartment was removed 8 days post-seeding and the medium in the basolateral chamber was replaced every two days. Cell layers were allowed to grow and differentiate under ALI (Air-Liquid Interface) conditions up to 21 days, in contrast to LLI (Liquid-Liquid Interface) conditions needed for intestinal epithelial cell lines.

Transepithelial electrical resistance (TEER) measurements were carried out the last 7 days of the experiment, prior to media change, using a Millicell-ERS2 apparatus equipped with a TEER Electrode MERSSTX01 (Millipore Corp, USA) in order to examine the procedure of epithelial formation in both cases. In addition, TEER was measured on the day of experiments, before and after microemulsion administration in order to evaluate their effect on the barrier function of the constructed epithelium in both cases. The measurements were taken in ambient temperature after 15 minutes of equilibration out of the incubator. To determine the resistance across the cell monolayer, Eq. (7) (see section 4.4.1) was used.

In **subproject 2 (Publication 3)** in order to test the permeability of free and encapsulated HT from the constructed intestinal epithelium, on the experiment day, monolayers were incubated in the apical compartment containing the HT aqueous solution (678 ppm), empty

or loaded microemulsions at 1% v/v in medium without FBS. Each plate contained a control sample with the medium only. The apical compartment received 0.5 mL of medium while the basolateral 1.5 mL. The basolateral media were collected after 6 h of incubation and were immediately used for LC-MS/MS analysis for the detection and quantification of HT. A calibration curve of HT in the free-serum medium was constructed with different HT, and CA (internal standard, IS) concentrations ranging from 0.1 to 20 μg/mL. For the detection of HT in the mucus layer of the coculture the same number of cells was seeded and after lysis analyzed by LC-MS/MS. For the removal of mucus, cells were washed by agitation on a plate-shaker at 135 rpm for 10 min with 2 mL HBSS. The HBSS was replaced with fresh and the agitation was repeated twice. For the determination of HT in the cells of the constructed epithelial model, the cells where trypsinized, frozen and thawed 3 times and were centrifuged in order to obtain the pellet and the supernatant which were stored at −20 ºC until analysis. Prior to LC-MS/MS analysis protein precipitation protocol was followed:100 μL from the samples were mixed with 450 μL ice cold acetonitrile and they were centrifuged at 4 °C for 12 minutes in 12.5rpm. The pellet was discarded and the supernatant was evaporated. The residue was reconstituted in 100 μL HBSS and injected into the LC–MS/MS system (see section 7.7.2).

In **subproject 3 (Publication 4),** a different procedure was followed for the nasal permeability studies of GA in its free and encapsulated form in the absence and presence of chitosan. On the 21st day post-seeding, monolayers were incubated in the apical compartment containing a GA aqueous solution, empty or loaded microemulsions at 0.2 % v/v in HBSS. Each plate contained a control sample with the HBSS only. The apical compartment received 0.6 mL of medium while the basolateral compartment received 1.2 mL. The basolateral media were collected at time intervals of 10, 30, 60, 120, 180, 240 min of incubation and were immediately analyzed by LC-MS/MS for the detection and quantification of GA. A calibration curve of GA in the transport medium was constructed with different GA and PC (internal standard, IS) concentrations ranging from 0.05 to 5 μg/mL. For sample preparation before HPLC-MS/MS analysis 100 μL of the basolateral side was transferred into a 1.5 mL Eppendorf tube and 450 μL of ice-cold acetonitrile were added. The sample was then centrifuged for 10 minutes in 10.000 rpm at 4 °C for protein precipitation. The supernatant was transferred into a clean HPLC appropriate vial and evaporated to dryness. The pellet was redissolved in 100 μL of H20: ACN (1:1) and 10 μL IS solution (1 μg/mL) were individually added in the sample. The mixture was vortexed and a 5 μL aliquot was injected into HPLC–MS/MS for analysis

7.7 Analytical methods

7.7.1 Photometric method

The progress of the oxidation of ABTS, in **Publication 2**, was followed spectrophotometrically using a Cary 3E spectrophotometer (Varian) at 414 nm (λ_{max} of the oxidized product of ABTS) for several minutes. Immediately, after the addition of the 6 μL of H_2O_2 the microemulsion containing ABTS was gently shaken and placed in Helma Semi-Micro-cuvette 114-QS, 10mm layer for measurement. The initial velocity of the reaction was calculated by the linear part of the absorption intensity versus time. Short reaction times were kept to ensure ABTS•+ being the only product of the reaction, as later a pale-yellow product forms indicating overoxidation.

7.7.2 LC-MS/MS analysis

The LC-MS/MS analysis facilitates the identification and quantification of compounds with the use of an HPLC system in combination with mass spectrometry (MS). The HPLC part of the apparatus is the separation tool whereas the mass spectrometer is responsible for the ionization of the separated peak solution and for providing a molecular weight for each peak component[215]. The LC-MS/MS fragments the parent ion and separates the daughter ions for identification and quantification. The apparatus used in the present thesis was consisted of a 3200 Q TRAP triple-quadrupole linear ion trap mass spectrometer fitted with a TurboIon Spray interface (SCIEX, USA) and an Agilent 1200 HPLC system (consisting of a G1379B micro vacuum degasser, a G1312A binary pump, a G1329 autosampler and a G1316A column compartment) (Agilent, USA). The data were processed using the Analyst Software program (version 1.4.2) (SCIEX, USA). In all cases, separation was performed with an Agilent Eclipse Plus C-18 column (50 mm × 2.1 mm inner diameter, 3.5 um particle size) with a RRLC in-line filter kit (2.1 mm, 0.2μm filter) (Agilent, USA).

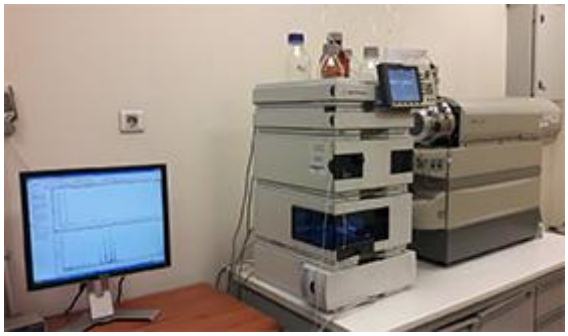

Figure 19. LC-MS/MS apparatus. National Hellenic Research Foundation.

In Subproject 2 (Publication 3) a variation of the method by Del Boccio et al. [216] was applied. The gradient mobile phase consisted of solvent A (water- 0.5% (v/v) formic acid) and solvent B (acetonitrile- 0.5% (v/v) formic acid). The flow rate was 350 μL/min and the gradient elution program was initial 10% B for 0.97 min, to 35% B at 3.07 min, to 100% B at 3.42 min and hold to 4.47 min, finally to 10% B at 5 min and hold with re-equilibration of the column for another 5 min. The total run time was 10 min. The injection volume was 5 μL. Electrospray Ionization (ESI) operating in negative mode was used for both HT and CA. Compound-specific optimization of MS/MS parameters was performed via direct infusion of a mixture of standard reference solutions (2 μg/mL each, in methanol) using a syringe pump. Source parameters were set to optimal values after flow injection analysis (FIA) source optimization. Quadrupoles one and three were set to unit resolution. Data acquisition was performed in the MRM mode monitoring the following transitions (m/z): hydroxytyrosol 152.9→123.9 (quantitative), 152.9→108.7 (qualitative) and caffeic acid (internal standard, IS) 178.8→134.9 (quantitative) and 178.8→78.8 (qualitative). Dwell time for each transition was 150 ms. Tandem mass spectrometry parameters are shown in Table 5. Source parameters were set as follows: Curtain Gas (CUR), 20; Temperature (TEM), 600; CAD gas, medium; Gas 1 (GS1), 50; Gas 2 (GS2), 50; Ion Spray (IS), -4500.

Table 6. LC/MS/MS parameters used in Subproject 2.

Compound	Q1 Mass (amu)	Q3 Mass (amu)	DP/V	EP/V	CEP/V	CE/V	CXP/V
Hydroxytyrosol	152.9	123.9	-35	-12	-10	-20	-2
	152.9	108.7	-30	-12	-10	-10	-2
Caffeic acid (IS)	178.8	134.9	-35	-12	-10	-22	-2
	178.8	78.8	-35	-12	-10	-32	0

DP: declustering potential, **EP**: entrance potential, **CEP**: collision entrance potential, **CE**: collision energy, **CXP**: cell exit potential and **V**: voltage. All are voltage dependent parameters of the instrument.

In subproject 3 (Publication 4) a variation of the method by Basu et al.[217] was applied. Electrospray Ionization (ESI) operating in negative mode was used for both GA and PC (IS). The gradient mobile phase consisted of solvent A (5 mM ammonium acetate in water- 0.5% (v/v) formic acid) and solvent B (acetonitrile- 0.5% (v/v) formic acid). The gradient elution

program was initial 50% A for 1 min, 5% A at 2 min, 50% A at 3.5 min and hold to 7 min. The flow rate was also variable, up to 4 min at a flow of 0.2 ml/min, from 4.1 min to 6.2 min it was 0.25 ml/min and again at 0.2 ml/min up to 7 min. There was a minute of equilibration at initial conditions before each injection. The injection volume was 5 μL.

Table 7. LC-MS/MS parameters used in Subproject 3.

Compound	Q1 Mass (amu)	Q3 Mass (amu)	DP/V	EP/V	CEP/V	CE/V	CXP/V
Gallic acid	169.0	125.0	-40	-12	-10	-20	-2
	169.0	79.0	-40	-12	-10	-30	-2
Protocatechuic acid	153.0	109.0	-30	-12	-10	-26	0
	153.0	91.0	-30	-12	-10	-32	0

Compound-specific optimization of MS/MS parameters was performed via direct infusion of a mixture of standard reference solutions (2 μg/mL each, in methanol) using a syringe pump. Source parameters were set to optimal values after flow injection analysis (FIA) source optimization. Quadrupoles one and three were set to unit resolution. Data acquisition was performed in the MRM mode monitoring the following transitions (m/z): gallic acid 169.0 → 125.0 (quantitative), 169.0 → 79.0 (qualitative) and protocatechuic acid (internal standard, IS) 153.0 → 109.00 (quantitative) and 153.0 → 91.0 (qualitative). Dwell time for each transition was 150 ms. Source parameters were set as follows: Curtain Gas (CUR), 10; Temperature (TEM), 500; CAD gas, medium; Gas 1 (GS1), 50; Gas 2 (GS2), 50; Ion Spray (IS), -4500.

8.

Conclusions

8. Conclusions

In the present thesis biocompatible W/O microemulsions were formulated with the use of natural oils and exclusively non-ionic surfactants. For their development the use of co-surfactants was limited or totally avoided. An enzyme (Horseradish peroxidase, HRP) and hydrophilic antioxidants (Hydroxytyrosol, HT and Gallic acid, GA) were successfully encapsulated in the systems. In the case of HRP encapsulation (subproject 1) scattering and spectroscopic techniques revealed that the W/O microemulsion consisted of IPM/Tween 80/DMG/PG/water was able to host a wide range of enzyme concentrations. The encapsulated bioactive was able to retain its biocatalytic activity and kinetic study was conducted for the catalysis of ABTS by HRP in the non-ionic microemulsion. Release studies revealed that 70% of the encapsulated biomolecule is able to be released from the microenvironment of the biocompatible system in a time period of 24 h. As a result, both structure and efficacy studies revealed the potential of the biocompatible microemulsion to be used as a carrier for therapeutic enzymes with possible applications in the treatment of various diseases.

Following (subproject 2), non-toxic microemulsions were formulated for the encapsulation of the hydrophilic antioxidant HT and its intestinal absorption was studied *in vitro.* The effect of the studied microemulsions, composed of natural oils and their mixtures, on HT intestinal absorption was studied in correlation with their composition. Successfully, HT was encapsulated and induced alterations in the carriers regarding its dimensions and membrane dynamics, indicating the participation of the molecule in the surfactant monolayer. The antioxidant efficacy of the microemulsions was measured and revealed that the systems, due to the nature of their components are high scavengers of free radicals. Most importantly HT retained its antioxidant activity after encapsulation in microemulsions, however the different membrane dynamics affected its efficacy. Caco-2/TC7 and HT29-MTX cell lines were used for the construction of an appropriate *in vitro* intestinal epithelium model which subsequently was used as the model for transport studies. The systems were tested *in vitro* for their cytotoxicity, which was extremely low even for concentration up to 1% v/v in the culture media. The systems' effect in the intestinal permeability efficiency of HT was assessed and interestingly, the increased surfactant concentration indicated a decreased HT permeability which can be explained by the interactions of Tween 80 and DMG with the mucus layer produced by goblet cells. This subproject, provided experimental evidence about the lower bioavailability of HT through intestine in comparison to previous

studies where the use of one cell culture model was used. Overall, the key finding of the work is the use of microemulsions with low concentration of surfactants in order to increase the intestinal permeability and obtain a biocompatible formulation.

In the last subproject (subproject 3) of the present thesis, another biocompatible microemulsion was formulated for the nasal administration of the hydrophilic antioxidant GA. Chitosan was used as a mucopenetrating agent and was incorporated in the aqueous phase of the developed system. The structural study revealed the participation of both molecules (GA and chitosan) in the surfactant monolayer of the system. The system's cytotoxicity was measured with the classic MTT cell proliferation method in the RPMI 2650 cell line. The developed system did not exhibit cytotoxic effect, in the absence and in the presence of chitosan, up to the threshold of 0.2% v/v in the cell culture medium. Our study confirmed that chitosan, even in low concentration, increased the GA permeability through nasal epithelium by opening the tight junctions of the cell layer. The proposed microemulsion could be used as a carrier for hydrophilic antioxidants with potent therapeutic effects for systemic or brain delivery.

Overall, the present thesis demonstrates that biocompatible microemulsions, formulated by natural oils, can be used in order to protect the sensitive enzyme and antioxidant molecules with therapeutic activity, until the time of administration. Moreover, this study demonstrates that mucosal epithelial (intestinal and nasal) have different behavior towards similar microemulsion systems due to difference in their mucosal layer. In addition, natural mucopenetrating agents can be also incorporated in microemulsions while affecting positively the absorption of bioactive compounds.

9.
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9. References

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10.

Results-Publications

catalysts

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Review

Oxidation Catalysis by Enzymes in Microemulsions

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Abstract: Microemulsions are regarded as "the ultimate enzyme microreactors" for liquid oxidations. Their structure, composed of water nanodroplets dispersed in a non-polar medium, provides several benefits for their use as media for enzymatic transformations. They have the ability to overcome the solubility limitations of hydrophobic substrates, enhance the enzymatic activity (superactivity phenomenon) and stability, while providing an interface for surface-active enzymes. Of particular interest is the use of such systems to study biotransformations catalyzed by oxidative enzymes. Nanodispersed biocatalytic media are perfect hosts for liquid oxidation reactions catalyzed by many enzymes such as heme peroxidases, phenoloxidases, cholesterol oxidase, and dehydrogenases. The system's composition and structural properties are important for better understanding of nanodispersion-biocatalyst interactions.

Keywords: biocatalysis; detergentless microemulsions; liquid oxidations; heme peroxidases; phenoloxidases; cholesterol oxidase; dehydrogenases

1. Introduction

Enzymes are macromolecular biological catalysts evolved in nature to catalyze and accelerate chemical reactions necessary to develop and maintain life. Biocatalysis can be defined as the use of enzymes as biocatalysts for industrial synthetic chemistry, under controlled conditions in a bioreactor. Enzyme catalysis has several advantages over chemical catalysis. Enzymes are active under mild reaction conditions (low temperature, pressure, and pH values), more efficient (lower concentration of enzyme needed) and have high specificity which indicates minimum or no production of by-products. As a result, the use of enzymes reduces the energy requirements for an industrial scale process [1]. The use of enzymes has been applied in oxidation reactions, among others. Biocatalysts have traditionally been used in aqueous media with detailed studies on kinetics and stereoselectivity of bioorganic reactions [2,3]. However, some groups have studied the use of enzymes in anhydrous organic solvents for the synthesis of products insoluble in water [4,5], as aqueous environments frequently give rise to unwanted side reactions making the product recovery difficult. Alcohol dehydrogenases [6] and horseradish peroxidase [7] are some of the oxidoreductases that have been used in such solvents. However, the interest during past decades has turned from aqueous or organic to low water media (non-conventional systems) because most of the enzymes retain their catalytic activity in low water content systems in contrast to organic solvents. Additionally, these systems have the ability to host substrates of different polarities. One of the most intensively studied approaches has been the use of water-in-oil (W/O) microemulsions as "microreactors".

The most widely accepted definition for microemulsions is that proposed by Danielson and Lindman: a microemulsion is a system of oil, water, and an amphiphile, which is a single optically isotropic and thermodynamically stable liquid solution [8]. The presence of surfactant and co-surfactant

molecules (amphiphiles) decreases the surface tension between oil and water to very low values, thus formulating a microemulsion with an extensive interface area.

Enzymatic reactions in microemulsions and microemulsion related systems gained interest on the grounds that through those systems the correlation between the in vitro and the in vivo behavior of enzymes can be studied, as the micro-domain structure of these systems simulates cell compartmentalization. In addition, they provide a larger surface area due to their conformation, protecting the molecule—because of the presence of water molecules in the micelle—from the denaturation effect of the organic solvent. In addition, the biocatalysts are able to react with substrates of different polarities. Microemulsions provide an aqueous phase for hydrophilic enzymes, an interface for surface-active enzymes, and an organic phase for hydrophobic substrates or products [9]. The determination of the properties of enzymes in a microemulsion system (catalytic activity, kinetic parameters, and mechanism) can be achieved by the techniques applied in aqueous solutions. This is due to the microemulsion's transparency allowing the application of photometric methods, which gives to these formulations a big advantage over other non-conventional systems. Since the late 1980s microemulsions have been extensively studied, on account of their microstructure properties, using different methods such as NMR (Nuclear Magnetic Resonance) [10], TEM (Transmission Electron Microscopy) [11], SAXS (Small-angle X-ray scattering) [12], SANS (Small-angle neutron scattering) [13], DLS (Dynamic Light Scattering) [14], and EPR (Electron Paramagnetic Resonance) [15].

Nevertheless, microemulsions present a disadvantage as the isolation of the products from the reaction medium may be hindered by the presence of surfactants. This problem leads to the formulation of stable systems, which are able to incorporate enzymes in the absence of surfactants [16].

W/O microemulsions are capable of hosting proteins in their dispersed water-pools such as lipases [17,18], oxidases [19], phosphatases [20] etc. The studies of enzyme behavior in microemulsions have been focused in altering several parameters including water content [21–25], pH [26], ionic strength [27,28], size of the reverse micelles [29], and nature of the surfactant [21,28,30]. From the parameters above the most widely studied is the water amount in the microemulsions which can alter the equilibrium of the reactions. Early studies indicated that in W/O microemulsions the properties of water are different from those of bulk water [31,32]. More recent studies revealed that water in reverse micelles exists in four hydration states [33]. Free water is defined as out-core water, because it is isolated from the in-core water by the oriented monolayers formed by surfactant molecules. The other three types are defined as in-core water, namely, anion-bound, cation-bound, and bulk-like water. This diversity of the water state can influence the behavior of the encapsulated enzyme. The water/surfactant molar ratio is abbreviated as w_o, and refers to the number of water molecules per surfactant molecule.

A particular case of enzymatic biocatalysis in microemulsions is that involving oxidative enzymes, which will be presented in this review. Extensive studies have been performed on peroxidases (with main representative horseradish peroxidase (HRP)), polyphenol oxidases (tyrosinase, laccase), and hydrogenases.

2. Oxidative Enzymes in Microemulsions

2.1. Heme Peroxidases

Peroxidases are hydrogen peroxide oxidoreductases that catalyze the oxidation of various substrates (such as phenols, aromatic amines, non-aromatic compounds etc.) [34,35] using hydrogen peroxide or organic peroxides as oxidants (reaction 1). Products of such reactions are insoluble polymers of free radicals, which can be removed from the solution by sedimentation and filtration.

$$2AH + H_2O_2 \rightarrow 2A + 2H_2O \quad (1)$$

2.1.1. Horseradish Peroxidase

Horseradish peroxidase (HRP, EC 1.11.1.7) is a heme containing enzyme of paramount importance obtained from plant sources. It is a metalloenzyme of many isoforms, which is active over a great range of pH and has the ability to catalyze various substrates such as phenols, biphenols, benzidines etc. [36]. HRP in biocatalysis has already been used in a variety of systems, ranging from biphasic [37] and sol-gel [38] supports to microemulsions and ionic liquids [39]. It is generally known that one of the main factors necessary for HRP to retain its activity in a reverse micellar system is the appropriate hydration of the enzyme molecule. Additionally, an outstanding "superactivity" has been observed and many research groups have tried to modulate different factors in order to achieve this state [40,41]. The main substrates used for the investigation of HRP activity in microemulsions and microemulsion-related systems are ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), pyrogallol (1,2,3-trihydroxybenzene) and guaiacol (2-methoxyphenol). Oxidation of ABTS by HRP generates the ABTS radical cation, whereas oxidation of pyrogallol leads to the formation of purpurogallin and that of guaiacol gives 3,3′–dimethoxy-4,4′-biphenoquinone.

Effect of Surfactant Nature and Concentration

The properties of W/O microemulsions can affect the structure of an enzyme and as a consequence its catalytic activity. The first reports obtained were with systems based on anionic surfactants, such as AOT (Bis(2-ethylhexyl) sulfosuccinate sodium salt) and SDS (Sodium dodecyl sulfate). Chen et al. [42] investigated by FTIR (Fourier Transform Infrared Spectroscopy) the alterations in the secondary structure of HRP in isooctane/surfactant/water microemulsions and concluded that AOT has the least influence on the structure of the molecule and as a result on its activity, followed by SDS. It is generally observed, that in microemulsions with SDS as surfactant, increased concentration of this component leads to enhanced activity of HRP. The above observation can be explained by the fact that SDS leads to a decrease in the buffer pH that is favorable for the enzyme [43]. Parida et al. [44] enhanced the catalytic activity of the enzyme at higher temperatures and pH values with the addition of cholesterol molecules which interacted with the head group of AOT in an isooctane/AOT/cholesterol/water system and hardened the membrane (substrates: *o*-phenylenediamine, pyrogallol). HRP activity has been studied, also, in microemulsions formulated with mixed surfactants, AOT and a nonionic one such as lauryl alcohol ethoxylate ($C_{12}E_3$). An enhanced activity of the enzyme was observed by reducing the charge on the surface where the enzyme is located with a concurrent increase in the droplet diameter of the dispersed phase [44].

Since microemulsions are used to provide a biomimetic system for hosting enzymes, it is also of great importance to study cationic surfactants because living cells also contain positively charged molecules. DTAB (dodecyltrimethylammonium bromide) and CTAB (cetyltrimethyl ammonium bromide) are the main representatives of this group of surfactants. Mahiuddin et al. [41] studied the oxidation of ABTS by hydrogen peroxide catalyzed by HRP in *n*-dodecane/SDS/DTAB/*n*-hexanol/buffer system where the low DTAB weight fraction in combination with high buffer content presented higher enzymatic activity than in the *n*-dodecane/SDS/*n*-hexanol/buffer microemulsion, even though DTAB acts as an enzyme inhibitor. Increase of CTAB concentration in microemulsions reduced dramatically the HRP activity, a fact that confirms that for surface-active enzymes, enzyme efficiency increases with a decrease in surfactant concentration. Roy et al. [45] investigated the improvement of HRP activity towards pyrogallol in cationic microemulsions by varying the CTAB concentration in an isooctane/CTAB/*n*-hexanol/water system, with constant values of pH = 7 and w_o = 24. The activity of the enzyme was found to increase with an increase in surfactant concentration (higher activity at 20–25 mM) but declined for higher surfactant concentrations (30–50 mM). Debnah et al. [46] studied the activity of HRP in isooctane/cationic surfactant/1-hexanol/water systems where the cationic surfactants vary depending on the degree of saturation of their head groups. The use of surfactants with unsaturated head groups leads to a decreased HRP activity towards pyrogallol oxidation, in contrast to the use of

surfactants with acyclic saturated polar heads. Many studies have investigated the effect of the mixture of anionic and cationic surfactants on HRP activity, revealing that anionic surfactants suppress the inhibition effect caused by cationic surfactants. Moreover, Biswas et al. [40] measured the activity of HRP towards the amount of SDS in the system dodecane/DTAB/SDS/*n*-hexanol/citrate buffer. The highest activity of HRP was observed in the case of the highest SDS concentration. These observations can be attributed to the changes to the pH values induced by the nature and amount of surfactant ions (Debye–Hückel effect), strongly affecting the affinity of the substrate to the biomolecule, as was demonstrated by Bauduin et al. [47]. As a result, the presence of charged surfactants (anionic or cationic) in a microemulsion affects the catalytic activity of a biomolecule.

Although nonionic surfactants are not charged and they are generally characterized as "mild", the studies regarding this particular type of microemulsion lag behind the amount of work done in other categories. In such a report, Gébicka and Jurkas [48] studied the effect of polyethylene lauryl ethers (Brij35 and Brij30) towards the oxidation of ABTS and guaiacol by H_2O_2. In isooctane/Brij/phosphate buffer microemulsions, the activity of HRP was comparable with that in aqueous solution but after replacing isooctane with cyclohexane and dodecane the enzymatic activity was lost, a fact that indicates the dependence of the activity upon the oily phase in such systems. The small amount of water in the last two systems was not able to protect the enzyme from denaturation. Motlekar and Bhagwat [49] observed that the activity of HRP towards the oxidation of guaiacol by H_2O_2 was slightly enhanced in the presence of Tween 40 (polyoxyethylenesorbitan monopalmitate) and suppressed by Tween 80 (polyoxyethylenesorbitan monooleate) at concentrations above their CMC (Critical Micellar Concentration) values. Kumar [50] observed that the spectral characteristics of HRP in cyclohexane/TritonX-100 (4-(1,1,3,3-Tetramethylbutyl) phenyl-polyethylene glycol)/1-hexanol/water system within a pH range from 5 to 11.8 are similar to those in aqueous solution, indicating no enzyme conformational changes. Nonionic surfactants combined with cationic ones enhance the activity of HRP by modification of the anisotropic interface of the microemulsion (Figure 1). Brij30 or 90 in combination with appropriate amounts of CTAB reduce the positive charge at the interface of the micro-domain, inducing thus, enhanced HRP activity [51].

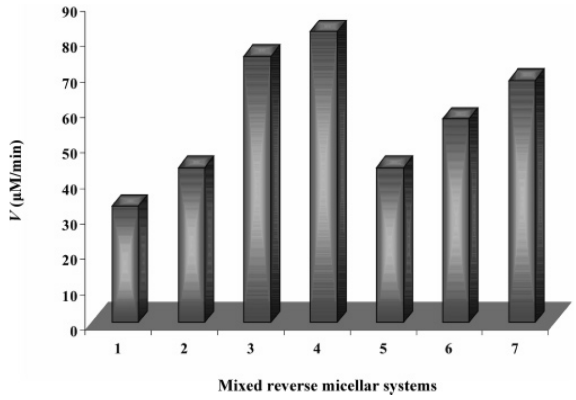

Figure 1. Dependence of horseradish peroxidase (HRP) activity on the composition of mixed reverse micelles at z = 8, 25 °C, pH = 7 (25 mM phosphate buffer). [HRP] = 1 μg/mL, [Pyrogallol] = 0.3 mM. Numbers 1–7 referred to different surfactants in surfactant/*n*-hexanol/isooctane/water system. (1: cetyltrimethyl ammonium bromide (CTAB), 2–4: CTAB + Brij30, 5–7: CTAB + Brij92); z = [co-surfactant]/[surfactant] [51]. Reprinted with permission from Shome, A.; Roy, S.; Das, P.K. Nonionic surfactants: A key to enhance the enzyme activity at cationic reverse micellar interface. *Langmuir*, **2007**, *23*, 4130–4136. Copyright 2007, American Chemical Society.

Effect of Co-Surfactant

In microemulsion systems, usually, small molecules such as alcohols and short chain polyols are used in order to "assist" the amphiphilic surfactants to reduce the surface tension of the interface

between the immiscible components of the system. These molecules are called co-surfactants and affect both microemulsion size and structure as their short alkyl chains strongly influence the interfacial composition [52–55]. In some cases the partition of the co-surfactant between the oil and water phases may disturb the encapsulated enzyme functionality [56]. In the case of microemulsions loaded with oxidative enzymes, increase of alcohol chain length leads to a bell shaped curve versus the catalytic activity, with the lowest reaction rates in dodecane/SDS/*n*-alcohol/buffer system recorded for 1-octanol and 1-butanol, respectively (Figure 2). This was attributed to the solubility of each alcohol in the buffer. The study underlies the importance of a quick thermodynamic equilibrium for the microemulsion because in the opposite case (as happens with the addition of long-chain alcohols) the enzyme is exposed to denaturation factors [43]. The above conclusion supports the fact that enzymatic activity in microemulsions based on SDS is lower than in systems based on the more hydrophobic AOT, where the equilibrium is immediately reached. It should be mentioned that AOT does not need a co-surfactant to formulate a microemulsion (feature of the surfactants with two hydrocarbon chains) while SDS needs the presence of a co-surfactant [57]. In the majority of the W/O microemulsions used for HRP encapsulation, *n*-hexanol is used as co-surfactant. Variations in its concentration can influence HRP activity. Increase of *n*-hexanol in microemulsions [40,41] generally leads to a decrease in the catalytic activity due to the fact that more alcohol is in contact with the surface-active enzyme. Surprisingly, Roy et al. [45] demonstrated that increase of *n*-hexanol in isooctane/CTAB/*n*-hexanol/water system enhances the activity of HRP towards the oxidation of pyrogallol at pH = 7, even though alcohols are inhibitors for this enzyme.

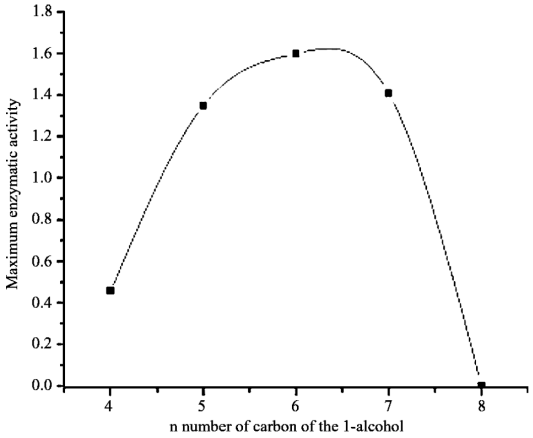

Figure 2. Maximum enzymatic activity, defined as $A = V/V_0$ (with V initial velocity of the enzymatic reaction in the microemulsion and V_0 initial velocity of the same reaction in the standard buffer solution), observed in microemulsion vs. n, the number of carbon atom of the chain of 1-alcohol used as co-surfactant in dodecane/sodium dodecyl sulfate (SDS)/*n*-alcohol/buffer microemulsion [43]. Reprinted from *J. Colloid Interface Sci.*, *292*, Bauduin, P.; Touraud, D.; Kunz, W.; Savelli, M.P.; Pulvin, S.; Ninham, B.W., The influence of structure and composition of a reverse SDS microemulsion on enzymatic activities and electrical conductivities, 244–254. Copyright 2005, with permission from Elsevier.

Effect of the System's Water Content

The activity of enzymes in microemulsions has been extensively studied towards w_o. This parameter, being an indicator of the biomolecule's hydration rate and the protection that it has towards the organic solvent, is crucial for the enzymatic activity in W/O microemulsions. At low water content, denaturation of the enzyme occurs because part of the water is spent on solvation of the surfactant molecules. The consequent dehydration can affect the conformation of the protein and decrease its catalytic activity. As water content increases the conformation of the enzyme reverts, but with a further water content increase, decrease of the enzymatic activity takes again place due to the

conformational rearrangements in the micelles. This leads to the characteristic bell-shaped curve of enzymatic activity towards w_o which also applies in the case of HRP.

Bauduin et al. [43] demonstrated that for w_o values of approximately 20, the system dodecane/SDS/*n*-alcohol/water (with different alcohols as co-surfactants) offers a properly hydrated environment for the enzyme and as a result its maximum activity occurs when tested towards the oxidation of ABTS. In the same study, it was shown that when using 1-butanol and 1-heptanol, enzymatic activity presents two different linear parts when plotted versus w_o, with a sharp increase in the first part and a slight decrease in the second part, whereas, when 1-hexanol and 1-pentanol were used, the plot exhibits three distinctive regions. In another study by Biswas et al. [40] on *n*-dodecane/SDS/DTAB/*n*-hexanol/water system, as the water content increased an increased activity was observed, although the droplet size of the microemulsion decreased. This was attributed to the reduction of *n*-hexanol concentration on the surface of the droplets, which further reduces the denaturation effect of the alcohol on the biocatalyst. Mahiuddin et al. [41] calculated an optimum percentage of buffer concentration of approximately 15%, for the same system used for the oxidation of ABTS. In addition, Pietikainen and Adlercreutz [58] changed the buffer strength of the aqueous phase of the microemulsion and observed a shift in the optimum w_o. According to their study, Tris-HCl buffer with five times bigger concentration shifts the optimum w_o from 23 to 29. Roy et al. [45] also asserted that buffer solutions with lower strength cause the enzyme to show a higher enzymatic activity, as with higher buffer strengths CTAB microemulsions are not sufficiently stable to carry out the enzymatic reaction. An exception in the above pattern was reported for microemulsions based on synthesized surfactants with unsaturation at the head group. In isooctane/surfactant/hexanol/water system [46], increase in the water content did not affect the activity of HRP towards pyrogallol oxidation. The polarity of the surfactant's polar head seems to play a key role in the enzymatic activity towards enzymes located on the interface.

Effect of pH

pH alterations in the microemulsion can change dramatically HRP activity. Decrease in pH values (lower than 4) has been shown to induce enhanced activity of HRP towards different substrates. Even changes of 0.5 in pH values, e.g., pH change from 5 to 4.5, can lead to 100% increase in the activity of HRP in *n*-dodecane/SDS/DTAB/*n*-hexanol/buffer microemulsion [59]. It is crucial to underline that the properties of water in a microemulsion are different to those of the aqueous solution. However, in microemulsions based on cationic surfactants, especially CTAB-based systems, alterations in pH do not affect the activity of the enzyme, with the only reported exception of a system was that based on monohydroxylated surfactants [46].

In recent years, interest has shifted to biocatalysis in biocompatible and edible W/O microemulsions, which are appropriate for future biotechnological applications. Microemulsions with olive oil as organic phase have successfully incorporated HRP. Even with a low amount of water (0.8% *w*/*w*) these systems can serve as microreactors, but the absence of free water in the enzyme microenvironment can induce changes to its activity and substrate specificity [60,61].

HRP has also been used in water-in-ionic liquid microemulsions (W/IL) in a study by Moniruzzaman et al. [62]. These systems feature some unique properties such as negligible vapor pressure, high thermal stability etc., therefore, they are used in synthetic reactions as "green" solvents. In the above study, HRP was solubilized in the aqueous droplets of [C8mim][Tf2N] (1-octyl-3-methylimidazolium bis(trifluromethylsulfonyl)amide)/AOT/1-hexanol system and used for the oxidation of pyrogallol by H_2O_2. An enhanced activity was observed. The insolubility of the oxidation product in the aqueous phase with the subsequent reduction of product inhibition and the change in enzyme microenvironment were some of the possible explanations for the observed enhanced activity. Moreover, in this study [62], the plot of enzymatic activity versus w_o reported for pyrogallol oxidation does not follow the bell shaped pattern expected for enzymes in microemulsions, but shows a saturation curve (Figure 3).

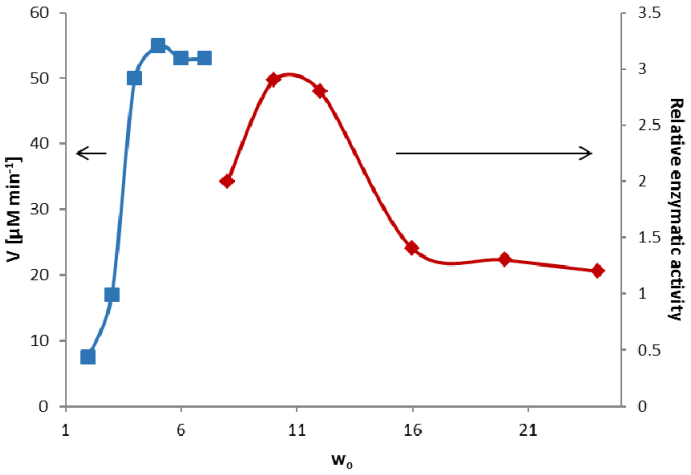

Figure 3. Variation in the enzymatic activity for the HRP-catalysed oxidation of pyrogallol in (♦) W/O microemulsion consisted of isooctane/AOT/*n*-hexanol/buffer (25 mM phosphate buffer, pH = 7.0, z = 9.6) [46] and (■) a W/IL microemulsion consisted of [C8mim][Tf2N] (1-octyl-3-methylimidazolium bis(trifluromethylsulfonyl) amide)/AOT/1-hexanol/buffer (50 mM Tris buffer, pH = 8.0) [62]. AOT: Bis(2-ethylhexyl) sulfosuccinate sodium salt; IL: ionic liquid; z = [*n*-hexanol]/[surfactant].

2.1.2. Chloroperoxidases

Chloroperoxidases (CPO, EC 1.11.1.10) are the most versatile enzymes in the heme peroxidase family. Morris and Hager discovered CPOs in the marine fungus *Calduriomyces fumago* [63]. Although CPO is a member of the heme peroxidase family, the proximal ligand is cysteine, which is identical to that of cytochromes P450. Chloroperoxidase catalyzes a variety of reactions, such as halogenation, peroxidation, oxygen insertion reactions, and decomposition of hydrogen peroxide to oxygen and water, and has been considered as a potential candidate for industrial applications [64]. CPO from *C. fumago* has not been so extensively studied in microemulsions or microemulsion-based systems, as HRP. The biggest problems when using this enzyme are the formation of side products, the limited stability, and the low aqueous solubility of the substrates. The use of microemulsions as a solution to these problems started back in 1988 when Franssen et al. [65] used the peroxidase in octane/CTAB or CTAC/pentanol/phosphate buffer system. The use of these surfactants leads to stabilized reversed micelles but also provides counter ions that are used as a substrate. The compounds 2-monochlorodimedon and 1,3-dihydrobenzene were halogenated within the system, giving the 2-halo and 4-halo derivatives, respectively, in the presence of hydrogen peroxide. Both reactions were characterized by about twice higher rates compared to aqueous media. Of exceptional importance was the enzyme's enhanced stability due to the labile behavior of the enzyme in aqueous media. The CTAC system provides a more efficient environment for enzyme stabilization in comparison with CTAB, a fact that has been explained by the oxidation of the bromide ions, which results in enzyme inactivation. For the above CTAC system, the study by Chen et al. [66] revealed that the specific activity of CPO increased significantly with the addition of CO_2 in the microemulsion and was 10 times higher than in the "conventional" microemulsion. This study was the first indicator that the enzyme catalytic reaction can be effectively controlled by CO_2. This was attributed to the reduction of viscosity (due to the addition of CO_2) which enhanced the diffusion of the substrates. The same enzyme was used for the peroxidation of ABTS and oxidation of indole in isooctane/CTAB or DTAB/pentanol/water system [67]. Because the anionic surfactant AOT inhibits the enzyme activity [68], the above system was used as an alternative and proved an excellent host for the enzyme as it causes a favorable strengthening of a-helix structure that results in enhanced activity for both reactions. Due to the extremely enhanced activity and thermostability exhibited by the enzyme, the group studied the influence of surfactant, water, and organic solution concentration towards the activity of the enzyme

in the peroxidation reaction, obtaining a bell-shaped pattern in each case. The decrease in the activity of CPO as a result of the high concentration of surfactants was explained by the increase of the micro-interface net charges, resulting in weakly electrostatic interactions of the surfactant with CPO, by interaction of the enzyme with the micellar matrix and by the thicker interfacial film, which becomes a barrier between the enzyme and its substrates. High concentrations of water can lead to increased activity due to the position of the enzyme in the structured water, near the polar head, that affects its activity. Finally, kinetic studies showed that k_{cat} increased and K_m decreased in comparison to that obtained in aqueous solution, which confirms that encapsulation of the enzyme into reverse micelles may induce a favorable conformational change on the enzyme molecule improving both the catalytic activity and stability of CPO [67].

VCPO (vanadium chloroperoxidase) from *Curvularia inaequalis* was active towards chlorination and single oxygenation in microemulsions of octane/$C_{10}E_4$/water, where $C_{10}E_4$ is a nonionic surfactant [69]. The use of ethoxylated fatty alcohols for the formulation of microemulsions leads to systems that can host the oxygenation of 9,10-dimethylanthracene (DMA) giving similar rates as in aqueous buffer, a result that is very interesting as very few studies have dealt with biocatalysis in microemulsions based on nonionic surfactants although the results were not encouraging.

2.1.3. Lignin Peroxidases (LiP)

LiP belongs to the family of oxidoreductases, specifically those acting on peroxide as acceptor and can be included in the broad category of ligninases [70]. LiP (EC 1.11.1.14) is a heme peroxidase that catalyzes the one-electron oxidation of non-phenolic aromatic compounds with high redox potentials via the formation of a substrate cation-radical [71]. It has the potential to oxidize a wide range of environmentally persistent compounds, such as polyaromatic hydrocarbons etc. Due to the fact that these substrates are hydrophobic, in 2004 Kimura et al. [72] used an isooctane/AOT/water microemulsion in order to formulate an appropriate environment for the oxidation of aromatic pollutants such as *p*-nonylphenol, Bisphenol A, and 2,4-dichlorophenol. It has to be mentioned here that LiP did not show any activity in organic solvents, so the use of microemulsion was one-way for a solution. Alterations in the organic phase of the microemulsion indicated that decane as external phase creates an environment more appropriate for the enzyme as the initial rate was increased four times compared to the one observed in isooctane, but it has the disadvantage that at higher temperatures phase separation occurs. The general opinion that the enzymatic activity is the highest when the size of the micelle is equal to the diameter of the enzyme is verified in this study, with the w_o effect towards the reaction's initial rate displaying a bell-shaped curve. Zhang et al. [73] compared the above system with a novel reverse micellar system in order to enhance the catalytic activity of LiP. They synthesized a two-tail nonionic surfactant, namely GGDE (*N*-glutamic acid didecyl ester) which they used to prepare a cyclohexane/GGDE/Triton X-100/water system. They observed, towards the oxidation of veratryl alcohol, a 40 times higher catalytic efficiency than the one observed in AOT microemulsions. LiP was studied also in W/IL microemulsions and more specifically in a [BMIM][PF6]/Triton X-100/water microemulsion [74]. The correlation of w_o with the initial rate of the enzyme catalyzed reaction showed a bell-shaped curve, and compared to the pure or water saturated [BMIM][PF6] the catalytic activity of the enzyme increased, probably due to the protective effect of Triton X-100.

2.2. Cholesterol Oxidase

The use of cholesterol oxidase (EC 1.1.3.6) in W/O microemulsions has been studied with a great deal of interest since 1988 because in these systems the water insoluble substrates can be dissolved. Cholesterol oxidase is an enzyme produced by several microorganisms such as: *Arthrobacter*, *Nocardia erythropolis*, *Rhodococcus erythropolis*, *Mycobacterium* etc. [75].

Cholesterol oxidase catalyzes the oxidation by dioxygen of the 3β-hydroxyl group of cholesterol and related steroids to a keto group and the isomerization of the Δ5 double bond, which leads to the formulation of a Δ4 cholestone [76]. The above reaction is of great importance as the cholestone is

a precursor of androst-1,4-diene-3,17-dione which can be chemically modified to manufacture oral contraceptives. In 1988 Lee and Biellmann [76] studied this reaction in the presence of cholesterol oxidase from *Streptomyces* and *Nocordia* in W/O microemulsions using cyclohexane and butanol as organic media with different surfactants. The kinetic studies showed K_m values of 10–14 mM whereas Laane et al. [77] for the same reaction in heptane/CTAB/octanol microemulsion found the K_m value decreased about 10 times. The above difference supports the opinion that the nature of the microemulsion's ingredients plays a crucial role to the catalytic efficiency of the entrapped enzyme. Laane et al. [77] observed that as the a_o values increase (with a_o being the molar ratio of hexanol to CTAB in the interface) the activity of the enzyme increases too, as the substrate has a higher solubility in those solvents and more substrate is available for the interface-active enzyme. Hedström et al. [78] studied the same reaction in an isooctane/AOT/water system, where the reaction rate in the presence of cholesterol oxidase from *Brevibacterium* sp., increased linearly versus w_o in discordance to the "classic" bell-shaped profile. Cholesterol oxidase from *N. erythropolis* showed a similar pattern [79]. In this study, Bru and co-workers studied the dependence of the enzymatic reaction on the micelle size concluding that the activity in the reverse micellar system approaches that in the aqueous medium as the micelle size increases [79]. Backlund et al. [19] used cholesterol oxidase from *Brevibacterium* sp. in a bicontinuous microemulsion composed of hexadecane/soybean lecithin/ethanol/water, in which they observed higher enzymatic activity with increasing ethanol. They assumed, due to the nature of the system, the majority of the enzyme and substrate molecules are distributed in different phases, which explains the low reaction yields. Lee & Biellmann [80] studied the oxidation of cholesterol in the presence of cholesterol oxidase from *N. erythropolis* in three different types of microemulsions composed of nonionic (cyclohexane/Triton X-100/*n*-butanol/water), anionic (cyclohexane/SDS/*n*-butanol/water) or cationic (cyclohexane/CTAB/*n*-butanol/water) surfactants. In the microemulsions based on SDS and CTAB the activity of the enzyme decreased rapidly to 25% and 75% with respect to the initial ones, whereas in the presence of Triton X-100 the enzymatic activity remained stable for a long period of time. The enzyme, remarkably, retained its activity in an extremely low water content microemulsion with nonionic surfactants. A more enlightened study by Gupte et al. [81] focused on different microemulsions based on the same surfactants (AOT, CTAB, Triton X-100) in isooctane. The study revealed that the surfactant of the microemulsion plays a crucial role in the optimum pH conditions for the enzymatic reaction. More specifically, in an isooctane/AOT microemulsion the optimum pH moved to lower values than in aqueous solution, a result that is in contrast to the study by Bru et al. [79]. In isooctane/CTAB/buffer microemulsion, the optimum pH is more basic whereas in microemulsions with nonionic surfactants, such as Triton X-100, the optimum pH is the same as in aqueous buffer. In the first two cases, the profile of the enzymatic activity versus w_o gives a bell-shaped curve, in contrast to the third case where the activity increases linearly with the molar ratio. The enzymatic reaction in those systems follows the classical Michaelis–Menten kinetics, with 100–1000 fold higher kinetic constant $k_{cat,app}$ and $K_{m,app}$ values, in comparison to those obtained in aqueous solution.

2.3. Phenoloxidases

Laccase (EC 1.10.3.2) and tyrosinase (EC 1.14.18.1) are two groups of phenoloxidases that catalyze the transformation of a large number of phenolic and non-phenolic aromatic compounds.

2.3.1. Laccases

The laccase molecule is a dimeric or tetrameric glycoprotein, which usually contains four copper atoms per monomer distributed in three redox sites [82]. This enzyme catalyzes the oxidation of *ortho*- and *para*-diphenols, aminophenols, polyphenols, polyamines, lignins and aryl diamines, as well as some inorganic ions coupled to the reduction of molecular dioxygen to water [83]. In a typical laccase reaction, a phenolic substrate is subjected to a one-electron oxidation giving rise to an aryloxyradical. This active species can be converted to a quinone in the second stage of the oxidation.

Laccases, which use molecular oxygen as electron acceptor, are attractive catalysts especially for waste treatment. There is increasing interest in their use to treat textile wastewaters which also contain auxiliary chemicals such as surfactants and salts [84,85]. Environmental pollutants (dioxins, polycyclic aromatic hydrocarbons, chlorophenols) which are some of the substrates of laccases, are not water soluble so the use of microemulsions was unavoidable [86].

Yellow laccases catalyze the oxidation of non-phenolic aromatic compounds but they lose their activity in apolar organic solvents. As a result, the use of microemulsions for the extension of their applications is essential. Rodakiewicz-Nowak et al. [87] demonstrated that yellow lacasse from *Pleurotus ostreatus* in isooctane/AOT/water microemulsions retained its catalytic activity, however, it was markedly reduced in comparison to that obtained in aqueous solution. This observation was extensively investigated with respect to the microemulsion's w_o and the conclusion was that w_o versus the oxidation rates for both yellow laccases and blue laccases have a linear dependence [88]. Moreover, laccase from *Coriolus versicolor* was active when immobilized in isooctane/AOT/water microemulsions towards Bisphenol A (BPA) oxidation and the addition of mediator 1-hydroxybenzotriazole (HBT) was not necessary, unlike in aqueous solutions. The above system can be used not only for the oxidation of BPA but also for *p*-nonylphenol and chlorophenols [89]. The main advantage of these systems is their ability to solubilize higher concentrations of substrates with low solubility in water. Nevertheless, the stability of the enzyme in the micellar system is decreased, showing a lower initial reaction rate and a significant loss of activity.

Laccase is a phenoloxidase that was also solubilized in W/IL microemulsions showing a higher catalytic activity when compared to aqueous solution [87]. Xue et al. [90] constructed a W/IL microemulsion consisting of [BMIM][PF6]/AOT/Triton X-100/water and studied the activity of laccase. In their previous work [74] they observed that in a system with the same ingredients but without AOT the enzyme was active, so they studied the effect of a different surfactant on the activity of the enzyme. In fact the addition of AOT (the solubility of which in the ionic liquid is increased by the presence of Triton X-100) creates a microemulsion in which the activity of laccase is higher, in contrast to the one without AOT. In addition, they observed that the catalytic activity versus w_o in both cases gives a linear pattern, which indicates that the specific interface has an inhibitory effect on the enzyme and specifically, the increase of Triton X-100 decreases the enzymatic activity.

2.3.2. Tyrosinase

Tyrosinase (EC 1.14.18.1, monophenol monoxygenase) is widely distributed throughout the phylogenetic scale from bacteria to mammals. Tyrosinase is an enzyme that has drawn attention due to its application in enzyme biosensors for the detection of phenolic pollutants. This enzyme is one of the first that has been proven to function in reversed micelles, such as those formed by AOT surfactant in isooctane [91], cyclohexane [92], and hexane [93] and others composed of nonionic Brij96 (poly-(10)-oxyethylene oleyl ether) in cyclohexane [94]. Yang and Robb [95] studied two different microemulsions composed of AOT and isooctane and CTAB, hexane and chloroform, where mushroom tyrosinase was immobilized and effectively used to catalyze the formation of *o*-quinones by oxidation of *o*-diphenols and presented superactivity in AOT microemulsion. These results are in disagreement with the findings by Rojo et al. [92], where the same enzyme in cyclohexane/AOT reverse micelles demonstrates equal activity towards the oxidation of 4-methylcatechol to 4-methylquinone in comparison with the one obtained in aqueous solution. In addition, Yang and Robb [95] observed that the kinetic constant, K_m, showed a small increase in reverse micelles based on AOT due to substrate partitioning, an observation that was also made by Bru et al. [91] for AOT microemulsions towards the oxidation of 4-methylcatechol. Moreover, K_m was observed to be higher in microemulsions than in aqueous solutions, while the higher V_{max} value was calculated for the AOT system. The previous conclusion that the activity is preserved in chloroform but is rapidly lost in hexane was confirmed by the above study [96].

Studies on the dependence of enzymatic activity towards temperature showed that surfactant affects the enzymatic activity. In AOT microemulsions enzymatic activity shows a peak on the increase of temperature, whereas in CTAB microemulsions there is no optimum found [95]. Rodakiewicz-Nowak et al. [87] investigated the oxidation of 4-*t*-butylcatechol by tyrosinase in the same system and showed that w_o affected the enzyme activity, with the enzyme being active in highly concentrated AOT microemulsions even at a low water content. Nevertheless, the effect of water content of the microemulsion towards the initial rate of the reaction did not demonstrate a bell-shaped dependence, but showed an increase followed by a plateau. Shipovsov et al. [97], in contrast to the previously reported data, found that when using isooctane and cyclohexane the profile of specific enzymatic activity ($\log(V_{max}/[E])$) versus w_o displayed two peaks, at w_o = 12 and w_o = 25. Estimation of the volume of reverse micelles and correlation with the molecular volume of tyrosinase monomers and multimers provided evidence that tyrosinase is likely to exist in two different catalytically active forms when entrapped in reverse micelles, specifically, a monomeric form at w_o = 12 and a tetrameric form at w_o = 25.

Furthermore, Papadimitriou et al. [98] constructed biocompatible W/O microemulsions with lecithin as surfactant and extra virgin olive oil, in which the oxidation of oleuropein by tyrosinase successfully took place. The same systems based on different oils (refined olive oil and isooctane) disabled the activity of tyrosinase. This result leads to the conclusion that the minor components of extra virgin olive oil influence the accessibility of enzyme by oleuropein. In contrast to other studies, in such systems substrate inhibition was very intense, probably due to the eventual accumulation of oleuropein and the corresponding *o*-quinone products around the protein molecule and within the amphiphile monolayer [99].

2.4. Dehydrogenases

Dehydrogenases are another group of oxidoreductases. They oxidize a substrate by transferring hydrogen to an electron acceptor, with NAD^+ (nicotinamide adenine dinucleotide) or FAD (flavin adenine dinucleotide) being common electron acceptors. Alcohol dehydrogenases (ADHs, EC 1.1.1.1) are oxidoreductases that catalyze the reversible oxidation of alcohols to aldehydes or ketones, with the concomitant reduction of NAD^+ or $NADP^+$ (nicotinamide adenine dinucleotide phosphate) [100]. These enzymes all have the same catalytic center but the size and the number of the active groups is different depending on the enzyme's source.

Meier and Luisi studied horse liver alcohol dehydrogenase (HLADH) successfully incorporated in a simple isooctane/AOT/water microemulsion following acetaldehyde reduction [101]. Martinek et al. [102] studied the oxidation of aliphatic alcohol in reverse micelles. The specificity (expressed in terms of k_{cat}/K_m) was higher for butanol when HLADH was encapsulated in the microemulsion (saturated with NAD^+) in contrast to the aqueous buffer solution where the enzyme had higher specificity towards octanol. The above finding is an example of alteration in substrate specificity of the enzyme in the environment of a reverse micelle. Samama et al. [103] saw the same alteration in substrate specificity in AOT micelles towards the oxidation of cinnamyl alcohol. Berezin et al. [104] studied the oxidation of isobutanol to isobutyraldehyde and particularly the equilibrium constants of the reaction in an isooctane/AOT/water system. In 1987 Larsson et al. [105] studied the activity and the stability of the above enzyme in a cyclohexane/AOT/water system for the oxidation of ethanol and the reduction of cyclohexanone. The two reactions are part of a reaction system in which the co-factor (NAD^+) was regenerated by the enzyme itself. The apparent K_m for ethanol in reverse micelles was about eight times lower as compared to the one in buffer, in contrast to the K_m of cyclohexanone that was almost unaltered. For the storage stability, the enzymatic activity decreased quickly in the first 24 h, but it further remained stable for two weeks.

Vos and coworkers [106] studied the catalytic properties of the enzyme in two different microemulsions in order to investigate the effect of the surfactant on the enzymatic behavior. AOT and CTAB were used in isooctane/surfactant/water microemulsions. For both systems the enzymatic

activity in the highest values of w_o (w_o = 40) was lower than in aqueous solution with the lowest activity value for CTAB microemulsion. They claimed that the incorporation of the enzyme in the reverse micellar system barely affects the secondary/tertiary structure, as revealed by CD (Circular Dichroism) studies. These results disagree with the results of phosphorescence lifetimes of LADH that revealed significant alterations in the conformational state of the macromolecule, as Strambini and Gonnelli [107] claimed in the most studied isooctane/AOT/water system. Samama et al. [103] studied different microemulsions composed of anionic surfactant-SDS and cationic-CTAB in cyclohexane with or without octane as oil phase and 1-butanol, 1-pentanol, 1-hexanol, *t*-butanol as co-surfactants. In SDS microemulsions, the enzyme quickly lost its activity, which was explained by the subunit dissociation of the enzyme by the SDS in aqueous solutions. On the contrary, in CTAB microemulsions the enzyme activity seemed to be more stable especially in the presence of higher alcohols. The difference between the two systems was the dependence of the velocity towards the water content. In the case of SDS microemulsion, the velocity increased as the water content increased but in CTAB microemulsion, the velocity did not show a clear dependence on the water content. The same group studied the behavior of HLADH in a cyclohexane/Triton X-100/1-butanol/water system where 1-butanol worked as both co-surfactant and a substrate [108]. They concluded that the enzyme in the above microemulsion has a higher stability over time in comparison to other microemulsions with anionic surfactants.

Another enzyme from the family of ADHs is the yeast alcohol dehydrogenase (YADH) which is a tetramer whereas HLADH is a dimer. Sarcar et al. [109] focused on the activity and stability of the enzyme in an isooctane/AOT/water system, when used for the oxidation of ethanol. The enzyme showed a maximum activity at w_o = 28 and pH = 8.1. The plot of k_{cat} of the reaction versus w_o and pH showed a bell shaped curve. In the micellar system, the activity of the enzyme sharply increased at 30 °C (double as in aqueous buffer) and then decreased rapidly. The enzyme's stability in the microemulsions decreased rapidly in comparison to that in aqueous buffer solution, probably due to the inactivation of the enzyme caused by the ionic interaction between the surfactant head group and the enzyme. The same group studied the same reaction and system by CD and concluded that the inactivation probably can be explained by the transformation of α-helix into either β-sheet structures or random coil conformations after the entrapment of the enzyme in the micelles [110]. Chen and Liao [111] examined the effect of nonionic surfactants in the activity of YADH. They studied a reduction reaction in isooctane/AOT/Brij30/water microemulsion and they concluded that the presence of Brij30 in concentrations lower than 0.1 M would be helpful for the enhancement of the steady residual activity of YADH in AOT reverse micelles.

YADH was also used in W/IL microemulsions. As alcohol dehydrogenase was inactive in [BMIN][PF6] due to the presence of immidiazole group which was a competitor of NAD^+ for the binding site, [BMIN][PF6] was used in microemulsion in the presence of Tritox X-100. The nonionic surfactant constructed a protective interfacial membrane for the enzyme. This is a very interesting approach which can lead to the replacement of pure ionic liquids by the related microemulsions [112].

3. Superactivity

Some enzymes are more active in nanodispersions than in reference buffered solutions. This behavior is called superactivity and is defined as a significant increase in the activity of a specific enzyme [113]. The three explanations that can be found in the literature for this phenomenon are: (i) conformational changes in the enzymes after the encapsulation in the microemulsions; (ii) state of the micellar water and (iii) ionic effect of the surfactants head group.

Mahiuddin et al. [41] studied HRP in *n*-dodecane/SDS/*n*-hexanol/buffer system, where the presence of a low DTAB weight fraction led to higher enzymatic activity, even though DTAB acts as an inhibitor of the enzyme. Gébicka et al. [48] studied the effect of different w_o values in a *n*-heptane/AOT/water system where HRP was entrapped and used for the oxidation of the hydrophilic substrate ABTS. The activity of HRP indicated a case of superactivity from which they assumed that the incorporation in the reverse micelles changed the secondary structure of HRP and as a

result, the mechanism of the catalyzed reaction was influenced. On the other hand, the rate of oxidation of guaiacol (2-methoxyphenol), which is better solubilized in organic phase than in water, by HRP encapsulated in AOT reverse micelles, appeared lower than in homogeneous aqueous solution. Biswas et al. [40] investigated the effect of the addition of SDS on the HRP structure. Due to the fact that very small changes in the pH can have a drastic effect on the initial velocity of the enzyme catalyzed reaction, the addition of the above surfactant to the citric buffer led to a decrease of the pH, as does the addition of conventional inorganic electrolytes.

The strong dependence of the enzymatic activity on pH in systems with ionic surfactants can be attributed to the Debye–Hückel effect [47], which also affects the partition of the substrates in the different microemulsion domains [114]. Therefore, the addition of different alcohols, such as pentanol, hexanol, and heptanol cause superactivity due to the high ionic strength in the nanodroplets and the pH decrease. Biswas et al. also reported that the superactivity of HRP encapsulated in cationic microemulsions was correlated with the slowest solvent dynamical modes of the confined water pool [40]. The application of W/IL microemulsions (composed of [C8mim][Tf2N] (1-octyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide)/AOT/1-hexanol/ water) as the reaction medium for the enzymatic oxidation of pyrogallol catalyzed by HRP was also investigated. The results demonstrated that the rate of HRP-catalyzed reactions in these microemulsions in the presence of imidazolium-based IL was significantly increased when compared with the results obtained in the classic microemulsions (without IL) [62].

Another peroxidase, the Iraqui Turnip peroxidase, was studied in a W/O microemulsion composed of chloroform, buffer, SDS, and the alcohols 1-propanol, 1-butanol, 1-pentanol, and 1-hexanol as co-surfactants. The enzymatic activity of the enzyme entrapped in the microemulsions was increased in the presence of alcohols with longer chain lengths. The above result was combined with the alterations in the size of the water pool [115].

Chloroperoxidase from *C. fumago* showed two-fold reaction rates in octane/CTAC/pentanol/water microemulsion in comparison to aqueous media towards the conversion of apolar compounds. Chen et al. [66] revealed that the specific activity of CPO increased significantly by 10 times with the addition of CO_2 in the CTAC-based microemulsion.

Laccase from *C. versicolor* also presented superactivity in AOT/isooctane microemulsion in BPA oxidation. This was probably due to an interaction of the surfactant with the enzyme inducing some renaturation or refolding of the partly inactivated enzyme when incorporated in the microemulsion systems. The correlation of w_o with the initial rate showed two optimal values for w_o, 15 (higher activity) and 30, a fact that can be explained by the size of the protein which corresponds with the one of the reverse micelle, as Martinek et al. [59] also observed for other enzymes. In water-in [BMIM][PF6] microemulsions LiP and laccase showed an increased catalytic activity, as well [74]. This was due to the carefully chosen amounts of Triton X-100 added to create a membrane to separate the enzyme from the ionic liquid that inhibits the enzyme.

Tyrosinase from *Agaricus bisporus* [96] also presented superactivity toward the conversion of *o*-disphenols to *o*-quinones entrapped in AOT-based microemulsion.

4. Surfactantless Microemulsions

The high cost of biocatalysis procedures based on microemulsions, especially at large scale, due to the high concentration of surfactants and the difficulty of product isolation led the researchers to a new approach. The use of detergentless (also called surfactantless or surfactant-free) microemulsions is a challenging perspective. To begin with, detergentless W/O microemulsions, as well as the conventional ones, are capable of simultaneously solubilizing water-soluble and water-insoluble reactants. They consist of a hydrocarbon, a short chain alcohol (hydrotrope) and water. The hydrotrope is not a typical surfactant but an amphiphilic substance, which is partially miscible with the aqueous and the oil phases. The combination of these three categories of components results in the formulation of two distinct domains (water-rich and oil-rich) where the hydrotrope is partitioned between the

coexisting pseudo-phases [116]. This was recently demonstrated by Diat et al. [117] using the powerful techniques of SANS and SWAXS (small- and wide-angle X-ray scattering). Water pools are stabilized by alcohol molecules absorbed at their surface [118]. The result is a stable and of low viscosity micro-heterogeneous dispersion of water in an organic solvent, optically transparent and thermodynamically stable.

The presence of the hydrotrope in a surfactantless microemulsion does not induce the formation of a similar monolayer at the interface as in the case of conventional microemulsions. This results in the interaction between the solubilized biomolecule and the organic solvent, a fact that does not take place in surfactant-based microemulsions where the surfactant monolayer provides a "barrier" between the aqueous and the oil phase [119]. A typical example is given in Figure 4.

Since this environment provides a low-cost "reactor" which permits easy product isolation, several surfactant-free systems were investigated in respect to their use as biocatalysis media. The first report in the literature appears 10 years after the first studied surfactant-free microemulsion by Smith et al. [120], when Khmelnitsky et al. [121] investigated the use of trypsin in ternary systems composed of hexane, isopropanol, and water. Since then, a wide range of enzymes has been successfully solubilized in that type of microemulsion, such as different types of hydrolases with emphasis on lipases [122] and oxidoreductases such as laccases [123] and peroxidases [124].

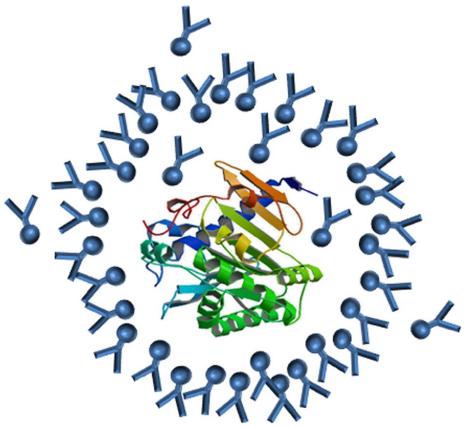

Figure 4. Model arrangement of the protein molecule within a water pool, with propanol molecules forming the interface between hexane and water [124]. Reprinted from *Curr. Opin. Colloid Interface Sci.*, 22, Xenakis, A.; Zoumpanioti, M.; Stamatis, H., Enzymatic reactions in structured surfactant-free microemulsions, 41–45. Copyright 2016, with permission from Elsevier.

These surfactantless systems have been mainly composed of non-polar solvents (as the "oil phase") with hexane [16,121–123,125–127] and toluene [126,128,129] being the most frequently used. Tziala et al. demonstrated for the first time the use of cyclic terpenes as natural organic solvents, such as α-pinene and D-limonene [130]. Such oils were efficiently used to formulate a stable surfactantless microemulsion where fungal chloroperoxidases and laccases retained their catalytic activity [131]. Furthermore, these systems provided higher bioconversion yields of hydrophobic and hydrophilic compounds in comparison to the yields obtained in aqueous-based media. The above study was of great importance because the use of surfactantless systems based on essential oils provides a new approach in bioconversions while offering an environment where the catalytic activity of oxidative enzymes can be enhanced. The stabilization of the dispersed aqueous phase in such systems occurs with the addition of short-chain alcohols such as isopropanol, 1-propanol, and *t*-butanol. As the microstructure of a surfactant-based microemulsion, in detergentless microemulsions a transparent dispersion is obtained by two immiscible liquids with the simultaneous construction of an interfacial film that separates the two liquids but also provides the appropriate compartmentation for the activation of surface active enzymes [132]. The most crucial and as a result the most frequently

studied factor is the effect of the components' ratio on the activity of the encapsulated enzymes in detergentless microemulsions.

Khmelnitsky et al. [123] studied the activity patterns for laccase and cholesterol oxidase in isopropanol/*n*-hexane/water system. In the first case lacasse revealed higher activity in the monophasic region of the ternary phase diagram that corresponds to water-in-oil microemulsions, in contrast to cholesterol oxidase and polyphenoloxidase [126], which in the same region presented the lowest activity. The results were inversed regarding the inactivation rate constant [118]. The difference is that in the case of cholesterol oxidase, hydrophobic substrates are involved whereas in the case of laccase the substrates are hydrophilic. Khmelnitsky et al. [123] revealed also that the catalytic activity of the enzyme towards the oxidation of pyrocatechol was similar to that obtained in aqueous solution, whereas the Michaelis constant was invariably much higher in media with high concentrations in organic solvents. This is due to the extremely high solubility of this substrate in the organic solvents. Water content is one of the most decisive factors that affects the catalytic activity of the entrapped enzyme. By increasing the water content of the systems a higher enzymatic activity for laccases, chloroperoxidases, and other enzymes can be observed. This is because there are sufficient water molecules for adequately hydrating the enzyme. This behavior could also be attributed to the partition and therefore the availability of the lipophilic substrates in the enzyme aqueous microenvironment [130–132]. Nevertheless, in the case of cholesterol oxidase at the optimum water content, the entrapped enzyme showed lower stability in a temperature range of 25–40 °C in comparison to aqueous solutions [118,120].

Surfactantless microemulsions were proven to be appropriate systems to host oxidative enzymes, providing an environment where the enzymes can retain their catalytic activity, even an enhanced one. In some cases, enzymes immobilized in surfactant-free microemulsions exhibit a greater stability in comparison to the one they present in surfactant-based microemulsions, a fact that makes the surfactantless microemulsions attractive for biotechnological applications.

5. Conclusion

The use of low water content media has attracted the interest of researchers for various enzymatic reactions. The use of microemulsions provides a medium for the solubilization of hydrophobic compounds and a region for activation of surface active enzymes. They can shift reaction equilibrium, may improve the thermal stability of the enzymes thereby enabling reactions to be carried out at higher temperatures, and can enhance enzymatic activities. The problem of product isolation or the reuse of the enzymes that arises due to the high concentration of surfactants can be solved by the formulation of surfactant-free microemulsions, which maintain the properties of conventional microemulsions. The use of microemulsions both conventional and non-conventional in different aspects of life is undeniable. The understanding of the enzymes' behavior within the micelles, as well as the interactions of different surfactants in the protein's molecule structure has to be further clarified. Of great importance is the application of the acquired knowledge on oxidation catalysis in microemulsions to the large scale in order to optimize the appropriate procedures. The replacement of "classic" organic solvents with more environmentally friendly ones, such as essential and edible oils, can be a very promising aspect in the use of microemulsions. Additionally, future enzymatic studies on water-in-ionic liquid microemulsions need to focus on the reuse of the enzyme, crucial for large-scale reactions.

Conflicts of Interest: The authors declare no conflict of interest.

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Formulation and Structural Study of a Biocompatible Water-in-Oil Microemulsion as an Appropriate Enzyme Carrier: The Model Case of Horseradish Peroxidase

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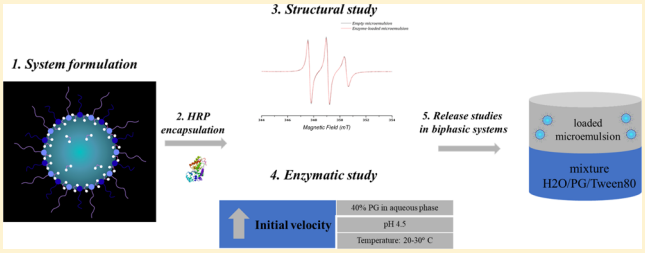
Supporting Information

ABSTRACT: A novel biocompatible water-in-oil microemulsion was developed using nonionic surfactants and was investigated as a potential enzyme delivery system for pharmaceutical applications. The system was composed of isopropyl myristate/polysorbate 80 (Tween 80)/distilled monoglycerides/water/propylene glycol (PG), had a low total surfactant concentration (8.3% w/w), and was able to incorporate approximately 3% w/w aqueous phase containing horseradish peroxidase (HRP). Structural and activity aspects of the system were studied using a variety of techniques such as dynamic light scattering (DLS), electron paramagnetic resonance (EPR), and dynamic interfacial tension. The apparent hydrodynamic diameter of the empty droplets was calculated at about 37 nm. Different enzyme concentrations, ranging from 0.01 to 1.39 μM, were used for both DLS and EPR studies to effectively determine the localization of the macromolecule in the microemulsion. According to the results, for high enzyme concentrations, a participation of HRP in the surfactant monolayer of the microemulsion is evident. The number of reverse micelles in the microemulsion was defined by a theoretical model and was used to clarify how the enzyme concentration affects the number of empty and loaded reverse micelles. To assure that the system allows the enzyme to retain its catalytic activity, an oxidative reaction catalyzed by HRP was successfully carried out with the use of the model substrate 2,2′-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid]. The influence of several parameters such as temperature, pH, and PG concentration was examined to optimize the reaction conditions, and a kinetic study was conducted revealing an ordered-Bi−Bi mechanism. Values of all kinetic parameters were determined. The release of the encapsulated enzyme was studied using an adequate receiver phase, revealing the effectiveness of the proposed microemulsion not only as a microreactor but also as a carrier for therapeutic biomolecules.



INTRODUCTION

Microemulsions are systems that during the past years have been extensively investigated not only as the ultimate "microreactors" but also as drug delivery formulations.[1] They were successfully used for, among others, oral,[2] transdermal,[3] intestinal,[4] and ocular[5] delivery as they are able to penetrate the epithelial barriers because of the properties of their components and the small size of their micelles. A major advantage is their ability to solubilize products with different polarities. Apart from drugs, other bioactive compounds such as proteins and peptides have been exploited for medical use to treat various diseases.[6] Permeation efficiencies of enzymes and peptides for pharmaceutical use through different epithelia are low;[2,7] therefore, many groups have investigated their delivery efficiently using a variety of formulations acting as "Trojan horses".[8−10] The formulation of an appropriate microemulsion for pharmaceutical purposes is a great challenge as all of the excipients must be chosen with caution for a final nontoxic nonirritant, enhancing the penetration and bioavailable carrier.

Surfactants play a crucial role because of their effectiveness in formulation of stable nanodispersions with low cost and ease of handling. They are amphiphilic molecules that reduce the surface tension and enhance bioavailability.[11] Nonionic surfactants are considered as less toxic toward biological membranes than the ionic ones.[12] The main representative

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nonionic surfactants are as follows: polysorbates (Tweens), sorbitan esters (Spans), polyoxyethylene ethers (Brijs), etc.

In the present study, mixtures of two nonionic surfactants, namely, distilled monoglycerides (DMG) and polysorbate 80 (Tween 80), were selected as water/oil (w/o) solubilization increases when surfactants with different hydrophilic−lipophilic balance values are mixed.[13−15] Tween 80 has a polar part consisting of polyoxyethylene and a lipophilic one of oleic hydrocarbon chain, and it works as a very good permeation enhancer with excellent solubilization efficiency.[16] It has also lower toxicity than that of the most commonly used Brijs[17] and is reported to enhance enzymatic activity.[18] Monoglycerides are composed of a glycerol molecule linked to a fatty acid via an ester bond. They are not soluble in water but can form stable hydrated dispersions and when heated beyond their melting point with water a gel is formed.[19] Both surfactants are widely used as emulsifiers in the food and pharmaceutical fields.[20,21]

Peroxidase of horseradish (HRP, EC 1.11.1.7) was selected as the model enzyme in the present study. HRP is a heme-containing enzyme that uses hydrogen peroxide to oxidize a wide variety of organic and inorganic compounds.[22] It is a widely studied enzyme[23] because of its commercial use (clinical diagnostic kits and immunoassays). HRP is one of the most versatile biocatalysts available to the biotechnology industry, is relatively stable with a wide substrate specificity,[24] and has already been used immobilized or encapsulated in a variety of systems.[25−29] HRP encapsulation in environments with nonionic surfactants, which are generally characterized as mild, is not widely studied, and, to the best of our knowledge, the present study is the first that reports on HRP activity in a biocompatible w/o microemulsion in the presence of exclusively nonionic surfactants and especially distilled monoglycerides.

Microemulsions have been used for the delivery of different therapeutic molecules as they are able to incorporate a wide range of drugs and improve the bioavailability while increasing the release rates of the molecule.[30] Also, they can provide protection against oxidation and enzymatic degradation and improve the solubilization of the incorporated compound while providing sustained and targeted delivery. The major problems of those versatile formulations are the high concentration of surfactants, the increased difficulty in penetrating the complex, and numerous barriers of the target organism.

The aim of the present study was the development of a novel nonionic biocompatible w/o microemulsion appropriate for enzymatic encapsulation, to serve as a potent drug carrier.

EXPERIMENTAL SECTION

Materials. Propylene glycol (1,2-propanediol) (PG), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), peroxidase from horseradish (HRP, 382 units/mg solid), and 5-doxyl-stearic acid (5-DSA) spin probe were purchased from Sigma-Aldrich, Germany. Isopropyl myristate (IPM) was from Fluka, Switzerland. Polyoxyethylene sorbitan monoolate (Tween 80) was obtained from Sharlau, Spain. Hydrogen peroxide 30% was purchased from Merck, Darmstadt, Germany. Distilled monoglycerides of vegetable fatty acids (DMG 0295) were a kind gift from Palsgaard, Denmark.

Preparation of a w/o Microemulsion and Pseudoternary Phase Diagram. W/o microemulsions consisting of IPM as the oil (continuous) phase, Tween 80 and DMG as surfactants, and propylene glycol in ultrapure water as the aqueous (dispersed) phase were prepared by adding the aqueous phase to a solution of oil and surfactants and gentle shaking.

For the determination of the system's monophasic area, the corresponding pseudoternary phase diagram was constructed. A mixture of water and PG, 1.5:1 w/w, was used as the aqueous phase. Mixtures of surfactants and oils with weight ratios varying from 1:9 to 9:1 were prepared in glass vials and incubated overnight at 25 °C. The mixtures were then titrated with the water/PG mixture (5 μL/24 h) until the solubilization limit was reached. Phase transition from the monophasic area to the two-phase region was visually observed by turbidity appearance. The pseudoternary phase diagram was constructed using ProSim Ternary Diagram Software. Clear and isotropic oil-rich samples were chosen. No attempts were made yet to completely identify the other regions of the pseudoternary phase diagram as they are beyond the scope of the present study. The composition of the system used in the present structural and enzymatic study corresponds to the point A of the pseudoternary phase diagram. This was chosen as a system with low total surfactant concentration, which is essential for pharmaceutical applications, in combination with the highest amount of aqueous phase corresponding to the surfactant concentration to be able to host the highest amount of loaded bioactives. Similarly to the phase diagram constructed with water, a second one was constructed replacing water with buffer (50 mM acetate buffer, pH 4.5). Similarly, a pseudoternary phase diagram was constructed replacing water with this buffer.

Enzyme Encapsulation in the Microemulsion System. Enzyme encapsulation in a microemulsion system can be achieved by injection, dissolution, or phase transfer. In the present study, the injection procedure was used, where an aliquot of concentrated enzyme solution in buffer is added to the solution of surfactants in the nonpolar solvent. Solubilization was achieved by gentle shaking for less than 10 s. The microemulsion was kept at ambient temperature with exception of the temperature-dependent measurements.

Dynamic Light Scattering (DLS). Zetasizer Nano ZS (ZEN3600) from Malvern Instruments (U.K.) equipped with a He−Ne laser (632.8 nm) using a noninvasive back-scatter technology was used for the DLS study of the microemulsions. A scattering angle of 173° was used. The droplet's apparent hydrodynamic mean diameter was computed by Stokes−Einstein eq 1

$$R_H = \frac{k_B T}{6\pi\eta D} \quad (1)$$

where R_H, k_B, T, η, and D are the hydrodynamic radius of the droplet, Boltzmann constant, temperature (in Kelvin), viscosity, and diffusion coefficient, respectively. Data were processed using Malvern Zetasizer Nano software, which fits a spherical model of diffusivities with low polydispersity. The empty and loaded w/o nanodispersions were measured in terms of droplet size and droplet size distribution evaluation. Sample preparation involved filtering through 0.45 μm cellulose filters. A quartz-type cuvette was used under dust-free conditions, and the temperature of the scattering cell was 25 °C. Each sample was measured in triplicate.

Estimation of the Number of Enzyme Molecules per Micelle. In w/o microemulsions, water pools are formed in the continuous organic phase separated by an interface of surfactant molecules, creating reverse micelles. The water-to-surfactant molar ratio, w_o, and the size of the dispersed micelles are the main factors that affect the micellar concentration in a system. The micellar concentration of the nonionic microemulsion was calculated by eq 2[31] using the micelle diameter obtained by DLS. Both eq 2 and DLS software assume a spherical monodispersed model. The values calculated from eq 2 were used to estimate the number of HRP molecules per micelle in the proposed system

$$[M] = \frac{V_w[S]w_o}{4/3\pi R^3} \quad (2)$$

where [M] is the micellar concentration, V_w is the volume of one water molecule (30 Å^3), [S] is the total surfactant concentration, w_o is

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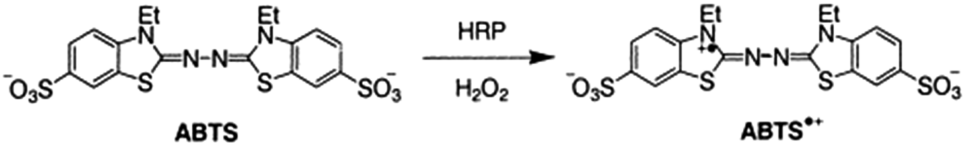
Scheme 1. Oxidation of 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) into the Corresponding Radical-Cation, $ABTS^{\bullet+}$



the water/surfactant molar ratio, and R is micelle's radius for the specific w_o (in Å).

Physicochemical Properties of the System. *Viscosity.* Viscosity of the oil and w/o microemulsions was measured using a DV-I Prime viscometer, Brookfield Engineering Laboratories. The samples were measured at a shear rate of 10 rpm at 25 °C.

Dynamic Interfacial Tension. Dynamic interfacial tension measurements were performed using the pendant drop/axisymmetric drop shape analysis method. CAM200 (KSV) was used, and analysis was performed via the Young Laplace equation using One-Attension software (version 1.8 Biolin Scientific). First, measurements of the aqueous and oil phases against air were performed, followed by measurements of the aqueous/oil interface in the presence and absence of Tween 80. The latter measurements were performed after forming an aqueous phase pendant drop in the oil phase contained in a quartz cell (Hellma Analytics, Müllheim, Germany). All measurements were taken at 25 ± 1 °C.

Electron Paramagnetic Resonance (EPR). To obtain information about the interfacial properties of the surfactant monolayer in enzyme-free and loaded microemulsions as well as microemulsions with different PG content, the spin-probing technique was used.[32,33] To obtain the desired concentration of the spin probe in the microemulsions, 1 mL of the formulation was added to a vial where the appropriate amount of the 5-DSA spin probe had been formerly deposited, as described in a previous study.[34] More specifically, 15 μL of stock 5-DSA solution in ethanol (7.8×10^{-3} M) was placed in the vial, followed by ethanol evaporation. The final concentration of the spin probe in the microemulsion was 1.2×10^{-4} M, and the samples were incubated overnight in a water bath (25 °C) to reach equilibrium. EPR spectra were recorded at constant room temperature (25 °C), using a Bruker EMX EPR spectrometer operating at the X-band, Bruker. A WG-813 Q-Wilmand (Buena, NJ) Suprasil flat cell was used. Typical settings were as follows: center field: 0.349 T, scan range: 0.01 T, receiver gain: 5.63×10^3, time constant: 5.12 ms, modulation amplitude: 0.4 mT, and frequency: 9.78 GHz. Data collection and analysis were performed using the Bruker WinEPR acquisition and processing program. Experimental results related to the mobility of the probe and the rigidity of the interface were expressed by the rotational correlation time (τ_R) and the order parameter (S), whereas micropolarity near the paramagnetic moiety of the 5-DSA molecule was expressed using the isotropic hyperfine splitting constant (a'_o). All parameters were calculated from the EPR experimental spectra as described in detail in previous works of our group.[34,35] In the present study, the parameters τ_R, S, and a'_o were calculated for empty and loaded microemulsions with concentrations of HRP ranging from 0.2 to 44.5 μg/mL microemulsion.

Enzymatic Activity. Enzymatic oxidation of ABTS by HRP toward $ABTS^{\bullet+}$ (Scheme 1) started by adding 6 μL of concentrated H_2O_2 solution in 1 g of microemulsion containing ABTS and enzyme, at room temperature, achieving a microemulsion with w_o = 5.4. Final concentrations ranged between 0.21 and 44.50 μg/mL for HRP, 0.05 and 0.15 mM for ABTS, and 0.03 and 0.15 mM for H_2O_2. The progress of the oxidation of ABTS was followed spectrophotometrically using a Cary 3E spectrophotometer (Varian) at 414 nm (λ_{max} of the oxidized product of ABTS) for several minutes. The initial velocity of the reaction was calculated by the linear part of the absorption intensity versus time. Short reaction times were kept to ensure $ABTS^{\bullet+}$ being the only product of the reaction, as later a pale yellow product forms indicating overoxidation.[36]

Kinetics and Mechanism of the Oxidation Reaction. Parametric identification of apparent maximum velocity, V_{max}^{app}, Michaelis–Menten constants, K_m^{app}, and inhibition constant, K_i^{app}, were used from the equation for reaction initial velocity. Kinetic constant values were calculated graphically using Lineweaver–Burk graphs as described elsewhere[37] and are presented in the Supporting Information.

Release Studies of HRP in a Biphasic System. The proposed w/o microemulsion (10 mL) was prepared and loaded with 160 μg of HRP. Macromolecule's release from the microemulsion was determined by layering an equal volume of the enzyme-containing microemulsion (referred to as source phase) on top of a mixture of ultrapure water, PG, and Tween 80 (referred to as receiver phase) in following ratios: 29, 58.8, and 12.2% w/w, respectively. The layer of source phase was added carefully on top of the receiver phase to avoid mixing of the two layers and the subsequent destruction of the system. In the literature, various release studies have reported the use of w/o microemulsions on top of aqueous solutions (such as phosphate-buffered saline) as receiver phases, a procedure that disturbs the thermodynamic equilibrium of the microemulsion because of osmosis phenomena. More specifically, water from the receiver phase (high water concentration) is transferred to the apical side (low water concentration), altering the system's water composition. This results into a transition from the monophasic area to a multiphasic one, making the obtained results controversial. To obtain equilibrium between the microemulsion and the receiver phase, two strategies can be followed: (i) prepare a Winsor II system yielding after final equilibrium a w/o microemulsion (source phase) in thermodynamic equilibrium with an excess aqueous phase (receiver phase)[38] or (ii) prepare a dilute surfactant/cosurfactant aqueous solution as a receiver phase. In the present study, we have chosen the second approach (preparing a mixture of ultrapure water, PG, and Tween 80) as the formulation of a Winsor II system in our case occurs after approximately 6 months (Figure S1 and Table S1). The constructed biphasic system was mildly stirred at 1 rpm at room temperature. At defined time intervals, samples of 0.75 mL were withdrawn from the receiver phase and analyzed spectroscopically at 419 nm, where the heme group of the HRP absorbs.[39] A standard curve of HRP in the receiver phase was constructed with the different enzyme contents in the mixture ranging from 1 to 15 μg/mL. The percentage of HRP released from the apical to the receiver phase was presented as a function of time.

RESULTS AND DISCUSSION

Pseudoternary Phase Diagram. The phase behavior of multicomponent systems such as microemulsions can be studied by pseudoternary (for complex systems with more than three components) phase diagrams. In the present study that focuses on a system with possible pharmaceutical applications, the choice of biocompatible constituents is of high importance. As a result, IPM, Tween 80, DMG, water, and PG were chosen as the appropriate ingredients of the proposed microemulsion. The composition of the aqueous phase was chosen to be water/PG 1.5:1 as this ratio was the most appropriate one for the oxidation of ABTS (see section "Effect of PG on Reverse Micelle's Diameter And Enzymatic Activity" and Figure 5). Likewise, a DMG/Tween 80 ratio of 1:1 was chosen as more adequate for the formulation of a nonionic system with low

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toxicity and viscosity. In fact, Tween 80 can induce toxicity effects in high concentration and DMG can increase the viscosity, leading thus to a system which could not be appropriate for pharmaceutical applications. Figure 1 represents the pseudoternary phase diagram of the system, where two distinct areas can be seen. More specifically, a narrow isotropic region can be observed (1φ) that corresponds to the monophasic, rich-in-oil phase and a broad multiphase region. When the ratio of emulsifiers was increased, increasing amounts of the dispersed aqueous phase were incorporated in the system, thus expanding the boundaries of the monophasic region. However, although a wider monophasic area could be obtained with increased surfactant concentrations, it would only lead to a system appropriate for enzymatic reactions, as a microemulsion suitable for pharmaceutical applications should have low surfactant concentration. Also, it should be mentioned here that for weight ratios of oil/surfactants above 3:7, a stable microemulsion could not be formed because of precipitation of the distilled monoglycerides. A similar monophasic area was also obtained when water was replaced by acetate buffer that was also used for the enzyme kinetic studies. The fact that the phase diagram remains similar can be explained by the nonionic nature of the surfactants, which makes the microemulsion stable against the ionic strength of the dispersed aqueous phase.[15] Given the demand for biocompatibility and low toxicity in the case of pharmaceutical applications, the concentration of emulsifiers was maintained low. Surfactants, at higher concentrations, may cause negative effects such as hemolysis on parenteral administration or irritation to a mucosal epithelium or skin.[40] The composition of the proposed microemulsion that was chosen for further studies was as follows: IPM 88.7% w/w, DMG/Tween 80 (1:1) 8.3% w/w, and H_2O/PG (1.5:1) 3% w/w (point A, Figure 1).

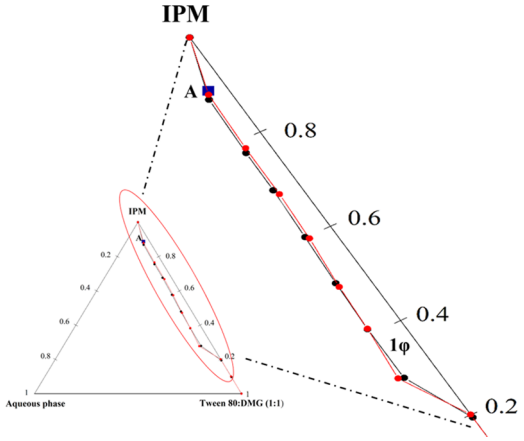

Figure 1. Pseudoternary phase diagram and enlargement of the monophasic area (1φ) of the system consisted of IPM as the oil phase, Tween 80/DMG (1:1) as surfactants, and water/PG (1.5:1) as the aqueous phase. Temperature was kept constant at 25 °C. Point A represents the microemulsion chosen for further structural and activity studies: 0.89 IPM, 0.08 DMG/Tween 80 (1:1), and 0.03 aqueous phase. The black line confines the monophasic area using water/PG as the aqueous phase; the red line confines the monophasic area using acetate buffer/PG instead.

Dynamic Light Scattering (DLS). The hydrodynamic diameter of a dispersed phase and droplet size distribution are important features for the structural characterization of a microemulsion. They can both be estimated by DLS, a fast, simple, and noninvasive technique. This method not only provides information about the size and the uniformity of the dispersed phase of a system but also can help estimating the effect of an encapsulated additive on the structure, as well as determine its localization in the nanodroplets.[41] In the present study, the term additive refers to the macromolecule of HRP. The chosen biocompatible w/o microemulsion (point A, Figure 1) presents a hydrodynamic diameter of approximately 40 nm and a polydispersity index of 0.30 ± 0.03. Figure 2 shows the droplet diameter distribution of the system in the absence and presence of 0.42 μg HRP/mL of microemulsion.

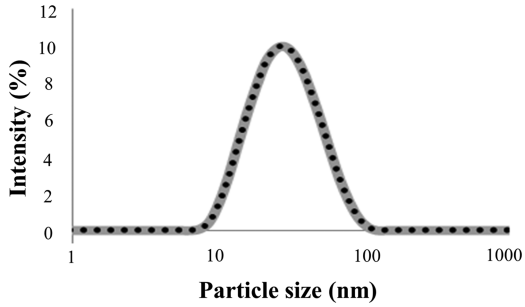

Figure 2. DLS results (droplet diameter distribution) of a w/o microemulsion consisting of 88.7% w/w oil phase, 8.3% w/w surfactants, and 3% w/w aqueous phase. The continuous line represents the empty microemulsion, and the dotted one represents that loaded with HRP microemulsion (0.42 μg enzyme/mL of microemulsion). Measurements were performed using a backscattering DLS method (detection angle 173°).

HRP is a protein with dimensions of about 4.0 × 6.8 × 11.7 nm^3 according to X-ray crystallography (Protein Data Bank ID code 1H5A),[42] molecular mass of 40 kDa, and Einstein−Stoke diameter of 4 nm.[43] After enzyme encapsulation, neither the hydrodynamic diameter of the dispersed aqueous droplets nor the polydispersity index changes significantly. This could possibly be attributed to the assumption that enzyme molecules are incorporated in the interior of the dispersed phase not interfering with the interface or to the assumption that the majority of the micelles are empty and only a few micelles are enzyme-loaded.[44]

To clarify the effect of HRP on the size of the micelles, the diameter of the dispersed phase was measured for increased HRP concentration. As can be seen in Table 1, the micelle diameter was not affected by the addition of HRP up to 44.5 μg/mL microemulsion. A possible explanation could be that for low enzyme concentrations the system shows a behavior

Table 1. Droplet Diameter (nm) and Polydispersity Index for the Microemulsions in the Presence of Different HRP Concentrations

μg HRP/mL microemulsion	diameter (nm)	PdI
0	36.8 ± 1.3	0.31 ± 0.03
0.42	38.7 ± 0.8	0.30 ± 0.03
0.84	39.9 ± 1.7	0.33 ± 0.02
1.89	39.5 ± 0.8	0.32 ± 0.03
44.50	41.9 ± 0.3	0.32 ± 0.05
55.40	59.3 ± 1.2	0.33 ± 0.02
62.80	66.0 ± 1.0	0.30 ± 0.10
71.80	73.0 ± 2.0	0.40 ± 0.10

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similar to the one shown by the empty microemulsion. However, for higher enzyme concentrations, more micelles appear to be loaded and this is reflected by the increased hydrodynamic radii recorded by DLS. Another explanation could be the localization of the macromolecule in the core of the microemulsion's droplets. As Pileni et al. have mentioned, if a protein molecule is located in the water pool but strongly interacts with the inner part of the surfactant interface, the micelle's radius increases because of an increase in the polar volume, with a constant interfacial area.[45] To explain the changes induced by the enzyme concentration, conductivity measurements were conducted. However, due to the low water content of the system, conductivity values were under the limit of detection.

Estimation of the Number of Enzyme Molecules per Micelle. Every host molecule encapsulated in the dispersed phase of a microemulsion is potent to provoke conformational changes in the nanodispersion. Even at very low enzyme concentrations, a change of the microemulsion's droplet radius is possible to occur.[46] Therefore, the calculation of micellar concentration and the further estimation of the ratio of enzyme molecules per micelle can shed more light on the topic. Micellar concentration was estimated using eq 2, and the diameter was obtained by DLS (Table 1). It was found that approximately 0.1 mM micelles are present in the empty microemulsion with w_o 5.4; however, loaded systems present lower micellar concentrations because of the rearrangement of the structure (increased micellar diameter and decreased population). The results presented in Table 2 are in accordance with other systems referred in the literature.[31,47] The micellar concentration drastically changed after the encapsulation of 55.4 μg HRP/mL of microemulsion. In lower enzyme concentrations, the majority of the micelles are empty in contrast to the higher concentrations, where the full micelles increased drastically. The results obtained by DLS for low enzyme content correspond to an empty system where changing the amount of enzyme does not significantly alter the diameter. On the other hand, for higher enzyme content, DLS results correspond to a loaded system, where changing the amount of enzyme highly affects the diameter.[48]

Table 2. Number of HRP Molecules per Reverse Micelles As Calculated by the Theoretical Model of Equation 2 Using Data from Table 1 [a]

μg HRP/mL microemulsion	micellar concentration (mM)	enzyme molecule/number of micelles
0	0.104	
0.42	0.089	1/8154
0.84	0.081	1/3861
1.89	0.084	1/1788
44.5	0.076	1/68
55.4	0.025	1/18
62.8	0.018	1/11
71.8	0.014	1/8

[a] Total surfactant concentration: [S] = 0.167 mM, w_o = 5.4.

Physicochemical Properties of the System. Viscosity, like all of the rheological properties of a liquid system, is an important feature, especially when it is proposed as a drug delivery system. In many pharmaceutical applications, such as oral and ocular administration, low viscosity is preferred as it offers higher bioavailability and ease of application. It has been reported[49,50] that the increased viscosity is able to decrease the delivery of drugs. The viscosity of the studied microemulsion at 25 °C was found to be 6.34 mPa s, a desired value for the above applications.

The equilibrium interfacial tension of the water/PG system in the absence of Tween 80 against air was 49.8 ± 0.1 mN/m, which is very close to the values reported in the literature.[51] The equilibrium interfacial tension against air of the IPM/DMG system was 29.0 ± 0.1 mN/m. The dynamic interfacial tension of the water/PG system against the IPM/DMG system in the absence of Tween 80 is presented in Figure 3. It is well known that PG, which was present in the aqueous phase, and DMG (dissolved in the oil phase) are surface-active and adsorb at the interface. Their presence induces a fast decrease in interfacial tension in the system. The addition of Tween 80 in the mentioned system led to ultralow values of dynamic interfacial tension, which were impossible to measure, a characteristic of the spontaneous formulation of microemulsions.

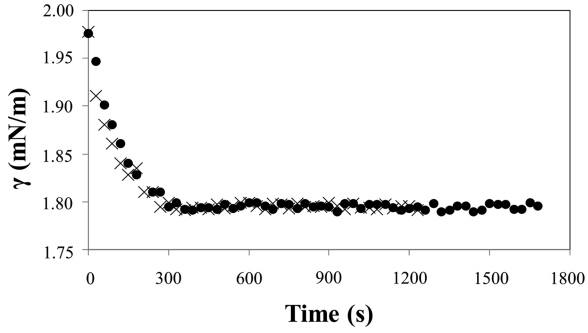

Figure 3. Dynamic interfacial tension of the IPM/DMG/PG/water system. The two sets of data correspond to two repetitions of the measurement.

Electron Paramagnetic Resonance (EPR). Interface dynamics of the novel w/o microemulsion was investigated by EPR spectroscopy using the spin-probe technique. In the present study, a known spin-labeled fatty acid was used, namely, 5-DSA, a long amphiphilic molecule with a nitroxide ring on the fifth carbon atom of its chain that has the tendency to align with the surfactant molecules. This interface-located fatty acid probe can give data on the mobility and the rigidity of the environment near its paramagnetic moiety. The three-line EPR spectra were obtained for the empty as well as enzyme-loaded w/o microemulsion and no important changes were observed upon the addition of 0.42 μg of HRP/mL of microemulsion (Figure 4). Rotational correlation time (τ_R), order parameter (S), and isotropic hyperfine splitting constant (α'_o) were calculated from the experimental spectrum. The τ_R value can monitor the dynamics of the spin probe located at the surfactant interface. The order parameter, S, provides a measure of the spin probe's arrangement in a supramolecular assembly and varies from 0 (random state) to 1 (ordered state). Changes in the polarity of the spin probe's environment can be determined by the hyperfine splitting constant value, α'_o, which increases when the polarity of the medium is increased.

To clarify the effect of enzyme addition on the surfactant monolayer of the reverse micelles, we encapsulated different concentrations of HRP. As it can be observed from Table 3, τ_R was the only parameter that has been affected. Specifically, a

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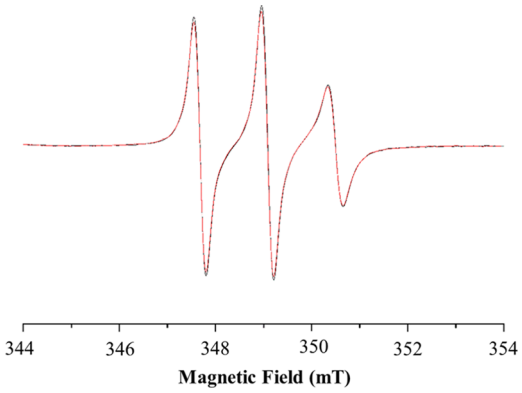

Figure 4. EPR spectra of 5-DSA in IPM/DMG/Tween 80/water/PG in enzyme-free (black line) and enzyme-loaded microemulsions 0.42 μg/mL microemulsion (red line).

decrease in the spin probe mobility can be detected but the order parameter, S, remains constant. When solubilized in reverse micelles, HRP molecules are likely to interact with the inner part of the surfactant molecules as they are located at the anisotropic interface like other surface-active enzymes such as lipase, lactate dehydrogenase, and so forth.[52] As can be seen from Table 3, τ_R values increased upon increasing the enzyme concentration, which is in agreement with the findings by DLS. The addition of enzymes such as trypsin and alkaline phosphatase in w/o microemulsions also increased the τ_R values of 5-DSA but slightly as the concentration of the enzymes was relatively low.[53] Our findings can be attributed to the increased number of loaded micelles. For low enzyme concentrations where most of the micelles are empty, the membrane seems to be unaffected by the changes in the amount of enzyme as Avramiotis et al. have mentioned in the case of lipase from *Pseudomonas cepacia* in lecithin microemulsion.[32]

EPR spectroscopy was also used to study the interfacial properties of the surfactant monolayer as well as the participation of PG in it. The study was performed with w/o microemulsions having different weight ratios of PG in the aqueous phase, keeping w_o constant (5.4). τ_R, S, and α'_o calculated from the experimental EPR spectra are shown in Table 4. As it can be observed, S values remain unaltered in all systems, meaning that the presence of PG does not result in different membrane fluidity. In contrast, the τ_R values increase, which correspond to decreased mobility of the spin probe. Nevertheless, from the values of Table 4, we can conclude that the addition of PG in the aqueous phase results in changes in the surfactant monolayer and confirms the polyol's participation in it. This was expected as PG can work as a cosurfactant and assist the stabilization of the microemulsion.

Enzymatic Activity and Kinetic Study. *Effect of PG on Reverse Micelles' Diameter and Enzymatic Activity.* As the use of low surfactant concentration is a challenge for the construction of biocompatible w/o microemulsions, the use of linkers, such as polyols that can influence the micellization process and the formation of thermodynamically stable systems, is indispensable. With the addition of polyols, we avoid rigid structures such as gels, precipitates, and liquid crystals, whereas the microemulsion phase in the phase diagram is extended.[54,55] It is obvious from the EPR results of the present study that the added PG participates into the surfactant layer, affecting, thus, its interfacial fluidity (Table 4). Moreover, the increase in the PG content affects not only the monophasic area in the pseudoternary phase diagram (Figure 1) but also the profile of the reaction that takes place in the microenvironment (Figure 5). Conformational changes in the surfactant monolayer are able to create a different profile of substrate exchange in a microemulsion system, affecting drastically the velocity of a reaction.[52]

As can be seen from Figure 5 for the initial velocity of the oxidation reaction of ABTS in the presence of HRP, the systems with higher PG content favor the reaction. As mentioned in the literature,[56,57] the increase in the fluidity of the membrane can increase the efficiency of intermicellar exchange. According to the EPR results shown above, the increase of the PG content in the present system leads to systems with higher membrane rigidity, a fact that cannot explain the increased reaction velocity presented in the system.[58] One possible explanation is the increased w_o value of the system upon addition of PG because of its highly hydroscopic nature. Khmelnitsky et al.[59] described an increase in the catalytic activity of a-chymotrypsin in systems rich in PG and attributed this behavior to the nature of PG, being the weakest denaturant of proteins compared with other nonaqueous solvents. They claimed that the protein molecules dissolved in polyol−water mixtures are hydrated much more strongly, retaining a more or less undisturbed hydration shell. As a result, 40% v/v PG in the aqueous phase was chosen as the most appropriate system for conducting enzymatic activity studies. It should be mentioned here that higher PG contents lead to systems with increased polydispersity.

It is worth mentioning that there is an at least 5 min time lag when the oxidation reaction takes place in the proposed microemulsion, a fact that has been observed in other systems too.[60,61] This could be attributed to the low micellar concentration and the relatively long time that the micelles need to exchange their encapsulated molecules.[62]

Effect of pH. Enzyme activity is influenced by a variety of parameters, such as pH. An enzymatic reaction may have a different pH optimum in an aqueous environment than in a restricted one. Even between different microemulsion systems, the optimum pH value can differ. In the literature, a variety of examples confirm the above observation. The catalytic activity of HRP in hydroxylated cationic surfactant nanodispersions is pH-sensitive, whereas when HRP is encapsulated in a nonhydroxylated cationic surfactant system, its activity is independent of pH.[52]

Table 3. Rotational Correlation Time (τ_R), Order Parameter (S), and Hyperfine Splitting Constant (α'_o) of 5-DSA in the Studied w/o Microemulsions with Different HRP Concentrations

μg HRP/mL microemulsion	0	0.42	0.84	1.89	44.5	55.4	62.8
τ_R (ns)	0.82 ± 0.01	0.81 ± 0.01	0.81 ± 0.01	0.82 ± 0.01	0.85 ± 0.01	0.88 ± ≤0.01	0.96 ± 0.02
S	0.06	0.06	0.06	0.06	0.06	0.06	0.06
α'_o	14.45 ± ≤0.01	14.46 ± ≤0.01	14.46 ± ≤0.01	14.45 ± ≤0.01	14.46 ± ≤0.01	14.46 ± ≤0.01	14.39 ± ≤0.01

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Table 4. Rotational Correlation Time (τ_R), Order Parameter (S), and Hyperfine Splitting Constant (α'_o) of 5-DSA in the Studied w/o Microemulsions with Different PG Concentrations in the Aqueous Phase

PG concentration in aqueous phase (% v/v)	0	20	25	35	40
τ_R (ns)	0.76 ± 0.01	0.79 ± 0.01	0.80 ± 0.01	0.82 ± ≤0.01	0.82 ± 0.01
S	0.06	0.06	0.06	0.06	0.06
α'_o	14.43 ± ≤0.01	14.42 ± 0.03	14.48 ± 0.01	14.44 ± 0.04	14.45 ± ≤0.01

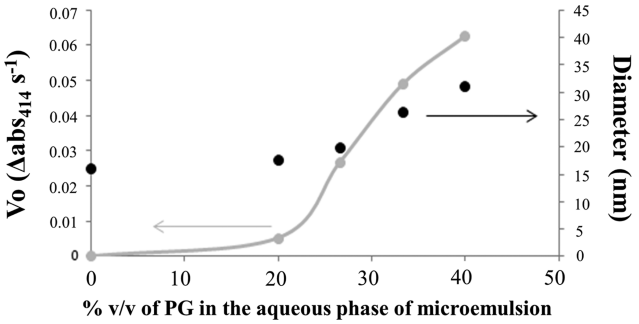

Figure 5. Oxidation rate of ABTS (black circles) and apparent hydrodynamic diameter (gray circles) at different propylene glycol (PG) concentrations in the aqueous phase of the microemulsion. Conditions: [ABTS] = 0.250 mM, [H_2O_2] = 0.125 mM, and HRP = 0.42 μg/mL, 50 mM acetate buffer, pH = 4.5. The aqueous phase was kept constant at 3% w/w.

In the present study, the oxidation of ABTS catalyzed by HRP in the microemulsion was studied as a function of pH in the range 4.0−7.0. The results are presented in Figure 6. The

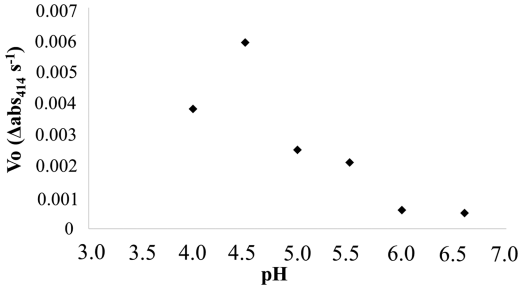

Figure 6. Oxidation rate of ABTS in IPM microemulsion at different pH values. pH adjusted using acetate buffer (50 mM, pH = 4−5.5) or phosphate buffer (50 mM, pH = 6−6.5) were used. Reaction conditions were as described in Figure 5.

maximum activity of the encapsulated enzyme was observed at pH 4.5, whereas the free enzyme exhibits the maximum activity at pH 4.0.[63] Similar results involving a shift of the optimum pH value to more alkaline values have been reported in the literature for HRP after immobilization.[52] On the contrary, in CTAB w/o microemulsions, oxidation of pyrogallol by H_2O_2 catalyzed by HRP is independent of pH.[52] We have to take into consideration that the pH of the intermicellar water does not change significantly because of the noncharged surfactants.[64] This different pH profile may be due to conformational changes of the enzyme after encapsulation. We have to mention that the present study is the only one, as far as we know, that has studied the effect of pH in the specific reaction in a nonionic microemulsion system.

Effect of Temperature. The effect of temperature on the activity of the encapsulated HRP was investigated by following ABTS oxidation at different temperatures. Figure 7 shows the results obtained for temperature values from 18 to 37 °C. As it

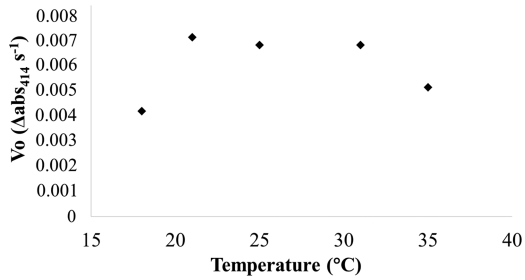

Figure 7. Effect of temperature on enzymatic activity. Reaction conditions were as described in Figure 5 and 6.

can be seen, an increased temperature does not significantly affect the reaction rate and as a consequence all of the kinetic studies were conducted at ambient temperature. A mild increase was observed at 21 °C. As Chattopadhyay et al.[65] have mentioned, at a temperature near 40 °C, we have the formation of an intermediate structure of HRP in which the tertiary structure around the heme group was melted, whereas the overall secondary structure remained almost unchanged. The effect of temperature on the catalytic activity of enzymes encapsulated in w/o microemulsions is a complex phenomenon because of the number of factors that affect the rate of the process. In most of the cases, a bell-shaped curve is observed, with an increase of enzyme activity at low temperatures followed by a decrease at higher temperatures, probably due to the potent enzyme denaturation.[65]

Kinetic Study. The oxidation reaction of ABTS catalyzed by HRP is usually studied in buffer.[66−68] In the present study, however, the reaction is catalyzed by HRP encapsulated in the proposed microemulsion and the initial velocity was measured as a function of H_2O_2 concentration (0.025−0.150 mM), for fixed concentrations of ABTS (0.05−0.15 mM). The initial velocities were taken into account, assuring the absence of significant amounts of products. Figure 8A,B shows how the initial velocity of the oxidation reaction varies as a function of substrate concentration, whereas the respective Lineweaver−Burk plots are shown in Figure 8C,D.

As can be seen from Figure 8, at fixed H_2O_2 concentrations, the classic pattern is followed. However, at fixed ABTS concentrations, a negative cooperativity is obtained, which is more intense for higher H_2O_2 concentrations. This means that binding of the first substrate lowers the affinity of the enzyme for binding of the second substrate, which leads to the conclusion of inhibition by H_2O_2. The reciprocal plots of Figure 8C,D are indicative of an ordered-Bi−Bi mechanism with inhibition by the second substrate.[37]

Apparent K_m values for both substrates, apparent inhibition constant, and maximum velocity are calculated and presented in Table 5. The values calculated are in the range of values reported for other peroxidases.[69,70]

The HRP activity recorded in the present nonionic microemulsion was lower than the one presented in the aqueous buffer solution under the same pH, temperature, and

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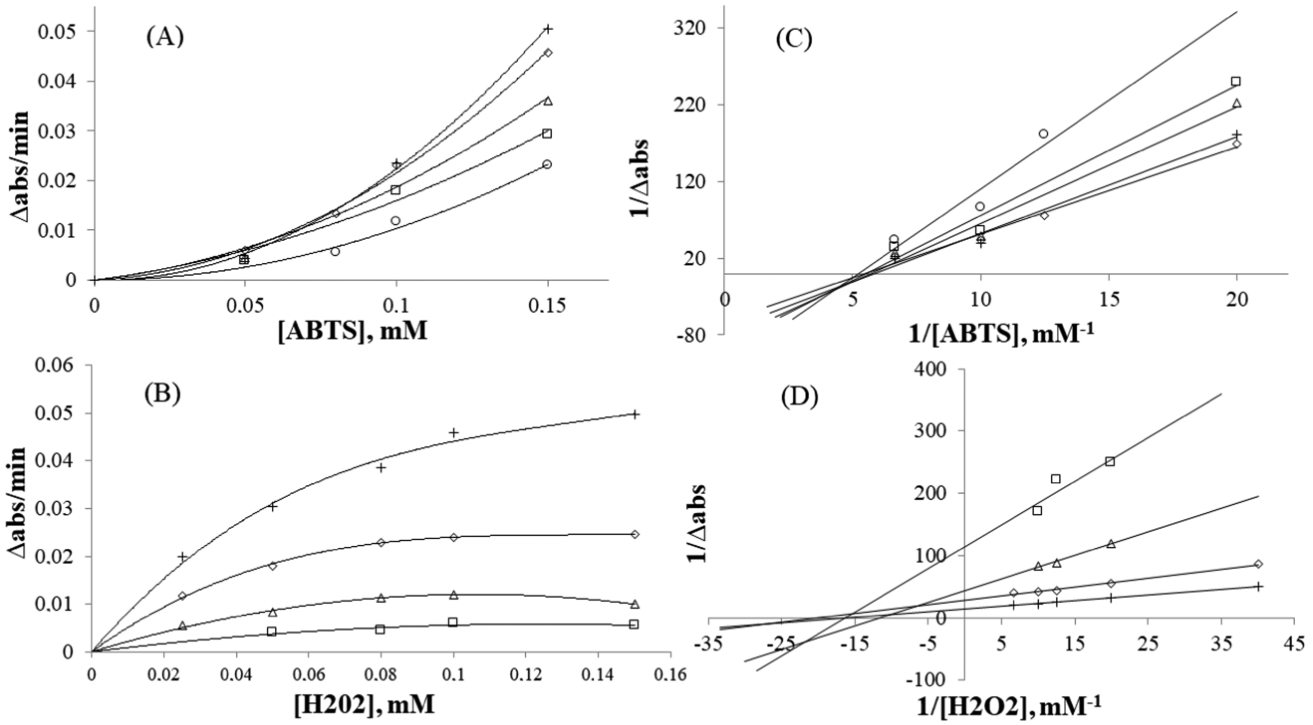

Figure 8. (A) Effect of ABTS concentration on the initial velocity of the oxidation reaction and (C) respective reciprocal plot. (B) Effect of H_2O_2 concentration on the reaction velocity and (D) respective reciprocal plot. HRP: 0.42 μg/mL. In all plots, (○): 0.025 mM; (□): 0.05 mM; (Δ): 0.08 mM; (◊): 0.10 mM; and (+): 0.15 mM.

Table 5. Values of Kinetic Constants by the Experimental Data[a]

parameter	values
V_{max}	0.015
K_{mA}	0.12
K_{mB}	0.021
K_i	0.347

[a]A: ABTS; B: H_2O_2.

substrate conditions. Nevertheless, the enzyme retains its activity in the restricted microenvironment of the studied microemulsion, and a kinetic study was conducted successfully. Similar behavior of HRP has been reported by Noritomi et al.,[71] where HRP activity toward hydroquinone oxidation in AOT−*n*-octane reverse micelles was studied. The V_{max} value in reverse micelles was half the one observed in aqueous solution. In addition, circular dichroism measurements of HRP both in aqueous and reverse micellar environments indicated conformational changes of the protein as the water content of the reverse micelles decreased. HRP was also entrapped in Igepal reverse micelles[72] at different water contents and its activity toward ABTS oxidation was measured. When the water content of the nonionic system was quite low, peroxidase activity was lower in reverse micelles than in aqueous buffer solution. Tzika et al.[73] have observed the same behavior of HRP after its encapsulation in multicomponent systems in olive oil-based microemulsions.

Release Studies of HRP in a Biphasic System. HRP is a monomeric glycoprotein with a single polypeptide chain consisting of 308 residues. The complete amino acid sequence was determined by Welinder.[74] From the 308 residues, 26 are aromatic. When the UV−vis absorption spectrum of HRP in buffer was compared with the one obtained in the receiver phase, it was observed that the absorption band attributed to the prosthetic heme shifts from 404 to 419 nm because of the addition of Tween 80 and PG in the solution, which interact with the heme group.

To evaluate the ability of the proposed microemulsion to serve as a novel carrier of biomolecules, permeation studies with the use of biphasic systems were carried out. Two compartments (the one containing the enzyme-loaded microemulsion (source phase) and the other being the receiver phase) were combined to form a biphasic system to avoid the destruction of the microemulsion's structure taking place in experiments where an oil-based microemulsion is in contact with a classic buffer solution (e.g., w/o microemulsion used in a Franz diffusion cell). Enzyme-loaded microemulsion was prepared as described in the Experimental Section with an HRP concentration of 16 μg/mL. Figure 9 shows the release of HRP from the nanodispersion as detected in the receiver phase. The results were calculated using a standard curve at 419 nm (Figure S2). As it can be observed (Figure 9), the release of HRP from the microemulsion was continuous for up

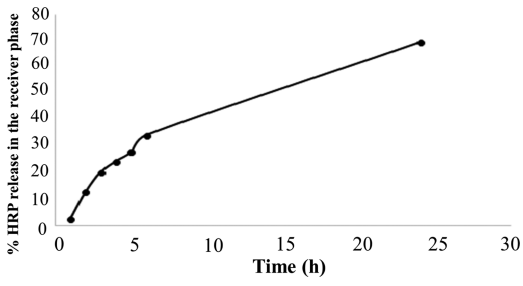

Figure 9. Released HRP profile from the w/o microemulsion in biphasic system microemulsion-aqueous solution of surfactants.

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to at least 24 h, although the release rate was lower after the first hours. The amount of the enzyme released from the microemulsion, at the end of a 24 h period, was 69.6%. A similar release profile with a plateau after several hours but with slower rates is described by Nornoo et al. for paclitaxel encapsulated in a w/o microemulsion.[75]

It therefore seems that the use of an appropriate biphasic system can be applied for release studies. In the present case, its application demonstrated that the microemulsion can be effectively used as a potent delivery system of proteins and generally enzyme-based drugs.

SUMMARY AND DISCUSSION

A biocompatible nonionic water-in-oil microemulsion can be obtained using isopropyl myristate as the oil phase, polysorbate 80 and distilled monoglycerides as surfactants, and propylene glycol and water as the aqueous phase. The present study clearly demonstrates that the proposed multicomponent system can be successfully used as a host system for horseradish peroxidase. The structural study of the proposed system in the presence of different enzyme concentrations shed light on the localization of HRP in the described microemulsion. Dynamic light scattering (DLS) and electron paramagnetic resonance (EPR) techniques revealed that the system is able to incorporate a high range of HRP concentrations, affecting the diameter of the reverse micelles while inducing changes in the inner part of the surfactant's monolayer where the enzyme is localized. The addition of propylene glycol (PG) in the aqueous phase of the system was also investigated. The increasing PG content leads to an increased hydrodynamic diameter and unaltered fluidity of the surfactant membrane but revealed interactions with the inner part of the nanodroplets' membrane. In addition, the increase of the PG content induced an increased efficiency of HRP to catalyze the oxidation of ABTS. The optimization of the parameters that affect the enzymatic activity was studied, and a kinetic study was conducted proving the ability of the microemulsion to serve as an appropriate host for HRP. Additionally, the system can be effectively used for enzyme delivery as almost 70% of the enzyme incorporated is released from the system in 24 h.

Overall, by studying the system regarding both structure and efficacy, we are able to shed light on systems with high complexity, which are reported more and more nowadays in the literature and in patents. By studying the structural changes upon enzyme concentration, we are able to predict these changes and formulate an effective carrier with appropriate characteristics such as diameter, rigidity of surfactant membrane, etc.

The proposed system could be suitable for potential applications of biocompatible microemulsions as carriers of therapeutic enzymes and peptides in treatment of various diseases.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.8b03124.

Preparation of a Winsor II system (Section S1); composition of the initial mixture (Table S1); Winsor II system (Figure S1); standard curve of absorbance versus HRP concentration (Figure S2); calculation of kinetic constants (Section S2); slope$_{1/[ABTS]}$ replot against the reversed concentration of H_2O_2 and against high range of H_2O_2 concentrations (Figure S3); 1/V-axis intercept replot against the inversed H_2O_2 concentrations (Figure S4) (PDF)

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Notes

The authors declare no competing financial interest.

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Hydroxytyrosol encapsulated in biocompatible water-in-oil microemulsions: How the structure affects *in vitro* absorption

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ABSTRACT

Over the last years, the incorporation of natural antioxidants in food and pharmaceutical formulations has gained attention, delaying or preventing oxidation phenomena in the final products. In order to take full advantage of their properties, protection in special microenvironments is of great importance. The unique features of the natural phenolic compound hydroxytyrosol (HT) - including antioxidant, anti-inflammatory, anti-proliferative and cardioprotective properties - have been studied to clarify its mechanism of action. In the present study novel biocompatible water-in-oil (W/O) microemulsions were developed as hosts for HT and subsequently examined for their absorption profile following their oral uptake. The absorption of HT in solution was compared with the encapsulated one *in vitro,* using a coculture model (Caco-2/TC7 and HT29-MTX cell lines). The systems were structurally characterized by means of Dynamic Light Scattering (DLS) and Electron Paramagnetic Resonance (EPR) techniques. The diameter of the micelles remained unaltered after the incorporation of 678 ppm of HT but the interfacial properties were slightly affected, indicating the involvement of the HT molecules in the surfactant monolayer. EPR was used towards a lipophilic stable free radial, namely galvinoxyl, indicating a high scavenging activity of the systems and encapsulated HT. Finally, after the biocompatibility study of the microemulsions the intestinal absorption of the encapsulated HT was compared with its aqueous solution *in vitro*. The higher the surfactants' concentration in the system the lower the HT concentration that penetrated the constructed epithelium, indicating the involvement of the amphiphiles in the antioxidant's absorption and its entrapment in the mucus layer.

1. Introduction

Through the last years, both food and pharmaceutical industries have shifted their focus to natural products by exploiting their beneficial properties. This trend is motivated by the avoidance of the high-cost procedures and the harmful side effects that synthetic bioactive molecules may exhibit. Recent studies have revealed many of the health-benefit properties of natural compounds including antioxidant [1], anti-proliferative [2] and anti-depressive [3] activities. Interestingly, in the pharmaceutical sector there is a growing interest for natural substances serving as lead compounds. Numerous drugs in the market are based on natural compounds from plants, whereas others are in clinical trials. Moreover, in the food industry, in order to obviate the oxidation of products during storage and to design high added-value products, companies tend to replace synthetic compounds by naturally occurring ones. Antioxidants may be defined as substances that, when present in foods, delay, control, or inhibit oxidation and deterioration of food quality [4]. The replacement of synthetic antioxidants, with natural ones, limits the necessary high-cost and time-consuming testing procedures.

Hydroxytyrosol (HT, 3,4-dihydroxyphenylethanol) is a secoiridoid of amphiphilic nature, deriving from the hydrolysis of oleuropein, with a great antioxidant capacity [5]. HT is naturally present in high concentrations in the leaves of the *Olea europea* tree and is recognized as one of the principal bioactive compounds of Extra Virgin Olive Oil (EVOO). Owing to its structural and molecular features, HT provides many beneficial effects after consumption. HT may act as a controller of lipid oxidation of natural oils [6] and as an antioxidant in films used for active food packaging [7]. Many studies have evidenced health benefits including neuroprotective [8], antiproliferative [9] and antimicrobial

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[10] properties. European Food and Safety Authority (EFSA) has claimed that HT acts as a protector of the cardiovascular system avoiding oxidation of LDL cholesterol by free radicals, maintaining normal blood HDL cholesterol concentrations and preventing atherosclerosis [11].

In order to take full advantage of the health benefits of molecules such as HT, both pharmaceutical and food industries are trying to develop strategies to incorporate them in products so that they are more soluble, stable against oxidation and degradation phenomena, with extended shelf-life, while possessing an increased bioavailability profile. To undertake these challenges, a variety of non-toxic systems such as nanoparticles [12], nanodispersions [13,14] and gelled emulsions [15], has been proposed for the incorporation of the functional compounds. Much attention has been paid to liquid-in-liquid colloidal systems, and especially nanoemulsions [16] and microemulsions [17]. Both oil-in-water (O/W) and water-in-oil (W/O) nanodispersions have been studied: the first type for the delivery of poorly water-soluble bioactive compounds and the second for antioxidants, peptides, *etc.* These colloidal formulations allow overcoming hurdles related to unpleasant taste, product instability and poor bioavailability. Especially, microemulsions, which are thermodynamically stable and isotropic mixtures of two immiscible phases stabilized by surfactants (and potentially co-surfactants), have been extensively studied for their ability to improve the bioavailability of peptides, drugs and other compounds [18,19]. In addition, microemulsions represent an interesting and potentially powerful alternative system for drug delivery because of their high solubilization capacity, transparency, ease of preparation, and high diffusion and absorption rates when compared with solvents without surfactant molecules. W/O microemulsions have been tested for the delivery and absorption of highly water-soluble compounds in the treatment of pathologies, such as diabetes, revealing increased intestinal bioavailability of the active molecules after their intake [20]. Nanoencapsulation ensures protection of antioxidants, controlled release and increased bioavailability. As part of the daily diet, a nutraceutical and potentially therapeutic agent, HT and its encapsulation in nanocarriers has attracted increasing attention over the last years [21]. As far as our knowledge is concerned, few references have focused on the intestinal absorption of polyphenolic molecules encapsulated in nanosystems after their consumption. Trying to elucidate the processes concerning the fate of novel formulations in the gastrointestinal tract (GIT), our group focuses on the digestion and absorption of oil-based nanodispersions and their encapsulated compounds. The gastric and intestinal digestion of W/O emulsions and microemulsions composed of medium chain triglycerides (MCT) and a variety of surfactants with encapsulated HT has been tested with a two-step digestion model using gastric and pancreatic lipases [22]. As a subsequent step, the present study aims at characterizing the intestinal absorption profile of HT encapsulated in W/O microemulsions.

Following the oral uptake of lipid-based formulations with encapsulated bioactive compounds, absorption studies allow to evaluate their bioavailability and their fate in the intestine. During the development of new products, epithelium permeability studies are required to assess the efficacy of the final product. *In vitro* cell culture models provide an approach to predict permeability by utilizing cell monolayers cultivated in permeable filter inserts to mimic the transport of a compound from the intestinal lumen. The cell culture models are widely used as they provide simplicity and reproducibility allowing inter-laboratory comparison of results. For the described reasons, different well-characterized cancer cell lines are used. Cancer cell lines are an auto-replication source of high homogeneity which can be easily manipulated while simultaneously providing reproducible results for drug delivery studies. Although, isolation of primary enterocytes from the human small intestine has been intended, their application remain limited due to obstacles such as poor viability and short life span [23]. Caco-2 cell line, one the most widely used and studied culture model, is able to construct a monolayer with morphological and functional characteristics similar to enterocytes. The permeability of drugs across Caco-2 monolayers has been shown to correlate well with the percentage of drug absorbed by humans for both passively absorbed and actively transported compounds [24].

However, many studies propose the use of coculture models in order to mimic the human intestinal epithelium more realistically by combining absorptive cells and mucus secreting goblet ones. The coculture of Caco-2/TC7 and HT29-MTX cell lines in a 9:1 proportion is the model closest to the physiological conditions. The mucus layer mimics the physiological barrier for the absorption of molecules and of high molecular weight compounds. Their culture on specific filters leads to the formation of a monolayer which permits rapid evaluation of bioactive absorption [25]. The above described co-culture model was chosen as it represents the epithelium of small intestine, the mainly absorption site of bioactives into systemic circulation after oral administration. As the present study deals with the HT absorption and the microemulsion's effect on it, we focused on the intestinal epithelium without studying the fate of the system along the whole GIT.

In the present study we designed biocompatible non-ionic W/O microemulsions composed of Isopropyl myristate (IPM) and/or Extra Virgin Olive Oil (EVOO) as the oil phase and Polysorbate 80 (Tween 80™) in combination with distilled monoglycerides (DMG) as surfactants. As the formulated microemulsions are intended to be used either to enrich food products or to stand alone as functional formulations, the choice of the ingredients was made based on their safety [26] as approved ingredients in the food industry. For that reason, EVOO was used as one edible natural oil with beneficial health properties. Nevertheless, due to its complex nature, and its endogenous humidity, high surfactant amounts are needed for microemulsion formation [27,28]. Therefore, a less complex oil should be used in order to increase the dispersed aqueous phase. The natural occurring IPM [29], the isopropyl ester of myristic acid, is widely used in the pharmaceutical industry due to its penetration enhancing properties in topical drug delivery [30] but is also used as flavoring agent in food products. In addition, in the present work the food-grade Tween 80 and DMG were used as surfactants. The formulated W/O microemulsions were used as hosts for the encapsulation of HT. The colloidal nanodispersions were studied structurally before and after the encapsulation of HT with the use of Dynamic Light Scattering (DLS) and Electron Paramagnetic Resonance (EPR) technique. EPR technique was also used for the investigation of the antioxidant scavenging effect of HT after its encapsulation in the reverse micelles. Additionally, the aim of the present study was to evaluate the intestinal absorption of free HT in comparison with the encapsulated in biocompatible microemulsion systems. As far as we know, only few studies have reported the use of W/O systems for oral bioavailability of natural hydrophilic compounds and none of them has used natural oils such as EVOO in combination with absorption *in vitro* studies across the intestinal epithelium.

2. Materials and methods

2.1. Chemicals

5-Doxyl-stearic acid (5-DSA) spin probe, Galvinoxyl free radical and caffeic acid (CA) were purchased from Sigma-Aldrich, Germany. Isopropyl myristate (IPM) and 2-propanol (isopropanol) were from Fluka, Switzerland. Polyoxyethylene sorbitan monoolate (Tween 80™) was obtained from Sharlau, Spain. Distilled monoglycerides of vegetable fatty acids (DMG 0295) were a kind gift from Palsgaard, Denmark. Extra Virgin Olive Oil (EVOO) was obtained from the local market. 3-Hydroxytyrosol (98% purity) was from Extrasynthèse (France). Formic acid obtained from Merck (Darmstadt, Germany) and Acetonitrile for HPLC-GOLD ultragradient from Carlo Erba Reagents (Cornaredo MI, Italy). All chemicals were used without any further purification. Highly purified water was obtained from a Millipore Milli Q Plus device.

2.2. Cell lines

Human colon carcinoma Caco-2/TC7 were kindly provided by Dr. Monique Rousset (INSERM UMR 505, Paris, France) [31] and mucus producing HT29-MTX cell line by Dr. Thécla Lesuffleur (INSERM UMR S 938, Paris, France) [32]. The cells were used at passages 3–20 and 14–30 respectively. Dubelccoo's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), L-glutamine, non-essential amino acids (NEAA), 100 U/mL penicillin and 100 mg/mL streptomycin, phosphate-buffered saline (PBS) and trypsin-EDTA were purchased from Gibco (Invitrogen Corporation, Life Technologies, UK). 12-well ThinCert™ polycarbonate inserts, 0.4 µm pore size and growth surface 4.67 cm^2 were purchased from Greiner Bio-One GmbH (Frickenhausen, Germany).

2.3. Preparation of W/O microemulsions and phase behavior

W/O microemulsions consisting of IPM or/and EVOO as the continuous phases, Tween 80 and DMG as surfactants and ultra-pure water as the dispersed phase, were prepared by adding the appropriate amounts of aqueous phase to a mixture of oil and surfactants, and gentle shaking. For the determination of monophasic domains, the corresponding pseudo-ternary phase diagrams were constructed. The phase diagram corresponding to system composed of IPM, Tween 80, DMG and water (referred as system A) has been previously described [33] but is also presented here for the sake of comparison. Mixtures of surfactants and oils with weight ratios varying from 1:9 to 9:1 were prepared in glass vials and incubated overnight at 25 °C. The mixtures were then titrated with water (5 µL/24 h) until the solubilization limit was reached. The appearance of turbidity followed by phase separation indicated the phase transition from monophasic to biphasic systems. Clear and isotropic oil-rich samples were selected for further studies. The compositions of the systems used for HT encapsulation, structural and permeability studies correspond to points A, B and C, respectively, in the pseudo-ternary phase diagrams (Fig. 1, Table 2).

2.4. HT encapsulation

Microemulsions loaded with HT were prepared by dissolving HT in ultra-pure water at the desired concentration and by adding this solution in the oil(s)-surfactants mixture corresponding to the selected A, B and C systems of Table 1. The final concentration of HT in the systems was 678 ppm. This concentration was chosen based on the solubility of HT in water and kept constant in all systems for means of comparison.

Table 1
Composition of the microemulsions expressed as % w/w.

	Oil phase (% w/w)		Surfactants (% w/w)		Aqueous phase (% w/w)
System	IPM	EVOO	Tween80	DMG	Water
A	93.8	–	2.1	2.1	2
B	44.8	44.8	4.2	4.2	2
C	–	79.7	8.9	8.9	2.5

2.5. Dynamic light scattering

A Zetasizer Nano ZS (ZEN3600) from Malvern Instruments (UK) equipped with a He-Ne laser (632.8 nm) with a non-invasive back scatter (NIBS) technology was used for the DLS study of the microemulsions. A scattering angle of 173° was used. The droplets' apparent hydrodynamic mean diameter was deduced from the Stokes-Einstein equation [34]. The average droplet size and the polydispersity index (PdI) of the empty and HT-loaded microemulsions were measured at the temperature of the scattering cell adjusted at 25 °C. Sample preparation involved filtering through 0.45 µm cellulose filter and the measurements were carried out in a quartz type cuvette under dust-free conditions. Data were processed using the Malvern Zetasizer Nano software, which fits a spherical model of diffusivities with low polydispersity.

2.6. Electron paramagnetic resonance

2.6.1. Structural study

In order to obtain information about the interfacial properties of the surfactant monolayer in HT-free and loaded microemulsions 5-DSA, a spin labeled fatty acid, was incorporated in the studied microemulsions as described in a previous study [35]. 5-DSA concentration in the microemulsion was 10^{-4} $mol.L^{-1}$ and the samples were incubated overnight in a water bath (25 °C). EPR spectra were recorded at room temperature, using a Bruker EMX EPR spectrometer operating at the X-

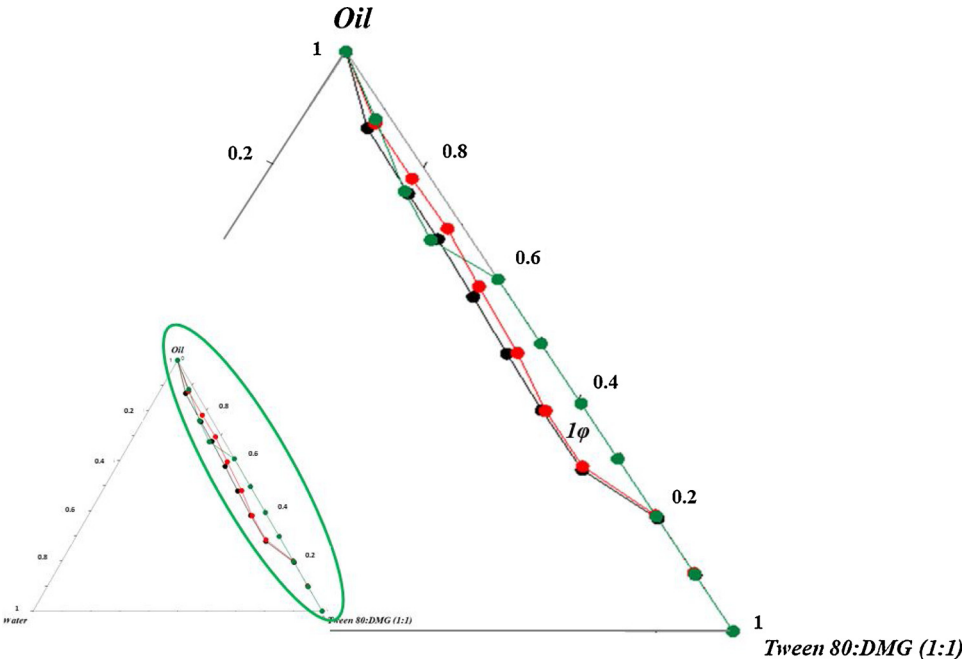

Fig. 1. Pseudo-ternary phase diagram and enlargement of the monophasic area (1φ) of the systems consisting of IPM (System A, black line), IPM: EVOO (1:1) (System B, red line) and EVOO (System C, green line) as the oil phase, Tween 80/DMG (1:1) as surfactants and water. Temperature was kept constant at 25 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Band, Bruker USA. A WG-813 Q-Wilmand (Buena, NJ) Suprasil flat cell was used. The typical settings and the experimental spectra analysis were the same as in the previous mentioned work. Spectral simulations were performed with home-written programs in MATLAB (The MathWorks) employing the Easy Spin toolbox for EPR spectroscopy [36].

2.6.2. Antioxidant activity

For the study of antiradical properties of natural antioxidants, the procedure reported in our previous study [37] was modified regarding the different final concentrations of the antioxidant in the free radical solution. In the present study, each microemulsion was incorporated at 10% v/v in a solution of galvinoxyl free radical in isooctane (0.25×10^{-3} mol.L^{-1}). According to Chatzidaki et al. [38] the addition of the microemulsions in the free radical solution does not affect the structure of the reverse micelles but only dilutes the dispersed droplets. The concentration of HT in the investigated microemulsions was kept constant at 678 ppm. EPR spectrum of galvinoxyl free radical had a characteristic single broad peak and its intensity decreased in the presence of molecules with scavenging activity. The percentage of inhibition of the corresponding EPR spectrum was calculated from Eq. (1) where A_o is the integrated intensity of the control sample (solution of galvinoxyl in isopropanol) at a specific time point and A is the integrated intensity of the sample containing the antioxidant at the same time.

$$\%\text{Inhibition} = (1 - \frac{A}{Ao})\text{x}100 \quad (1)$$

2.7. In vitro studies

2.7.1. Cell lines

Caco-2/TC7 and HT29-MTX cell lines were routinely cultured in 75 cm^2 flasks in DMEM medium without pyruvate containing 4.5 g L^{-1} glucose supplemented with 10% FBS, 1% NEAA and 0.5% antibiotics and maintained under a 5% CO_2 atmosphere at 37 °C.

2.7.2. Cell viability

Cytotoxicity of systems was initially assessed 6 h after treatment by MTT assay according to the manufacturer's standard protocol [39]. In more detail, 100 μL of medium containing cells were added to the 96-well plate at a density of 2.7×10^4 per well and incubated for 24 h at 37 °C under 5% CO2. Cells were exposed to microemulsions at different final ratio of 0.1%, 0.5% and 1% v/v in the culture medium. After the incubation period, the cells were washed with PBS once and 50 μL of MTT solution were added and incubated for 1 h. Routinely, MTT stock solution (5 mg/mL) was added to each culture being assayed to equal one tenth of the original culture volume and incubated for 3 h. At the end of the incubation period, the medium was removed and the converted dye was solubilized in isopropanol in order to dilute the insoluble purple formazan. Cultural supernatant was discarded, and 50 μL isopropanol was placed in each well and stirred for 1 h. The absorbance of the converted dye was measured at a wavelength of 570 nm with a Safire II Microplate Luminometer (TECAN, Switzerland) device. Eq. (2) was used to determine the cell viability.

$$\text{Cell viability}\,(\%) = (\frac{OD\ of\ treated\ cells}{OD\ of\ control})\text{x}100 \quad (2)$$

2.7.3. Permeability studies

For permeability experiments, cells were seeded on 12-well ThinCertrs™ permeable polyester filters of 0.4 μm pore size, at a density of 9.1×10^4 cells per cm^2, at a 9:1 Caco-2/TC7 to HT29-MTX ratio, and grown to confluence in a complete medium. Cells were then cultured for approximately 21 days in the complete medium which was changed in both compartments every two days until total differentiation. Transepithelial electrical resistance (TEER) was measured the last 7 days of the experiment, prior to media change, using a Millicell-ERS2 apparatus equipped with a TEER Electrode MERSSTX01 (Millipore Corp, USA) in order to examine the procedure of epithelial formation. In addition, TEER was measured on the day of experiments, before and after microemulsion administration in order to evaluate their effect on the barrier function of the constructed epithelium. The measurements were taken in ambient temperature after 15 min of equilibration. To determine the resistance across the cell monolayer, Eq. (3) was used:

$$R = R_{monolayer} - R_{blank} \quad (3)$$

where $R_{monolayer}$ is the experimentally measured resistance and R_{blank} is the resistance of the insert without cells. TEER values of cellular monolayers were expressed in Ω x cm^2 and calculated by Eq. (4):

$$TEER = R \text{ x } A_{membrane} \quad (4)$$

On the experiment day, monolayers were incubated apically with the HT aqueous solution, empty or loaded microemulsions at 1% v/v in medium without FBS. Each plate contained a control sample with the medium only. The apical compartment received 0.5 mL of medium while the basolateral 1.5 mL. The basolateral media were collected after 6 h of incubation and were immediately used for LC–MS/MS analysis for the detection and quantification of HT. A calibration curve of HT in the free-serum medium was constructed with different HT, and CA (internal standard, IS) concentrations ranging from 0.1 to 20 μg/mL (Fig. S1).

In order to identify the presence of HT in the mucus layer of the coculture the same number of cells was seeded and after lysis analyzed by LC–MS/MS. The mucus layers were removed from half of the cells, following the protocol of Wikman et al. [40]. Briefly, cells were washed by agitation on a plate-shaker at 135 rpm for 10 min with 2 mL HBSS. The HBSS was replaced with fresh and the agitation was repeated twice. For the determination of HT in the cells of the constructed epithelial model, the cells where trypsinized, frozen and thawed 3 times and were centrifuged in order to obtain the pellet and the supernatant which were stored at −20 °C until analysis, following the protocol of Chen et al. [41]. Prior to LC–MS/MS analysis protein precipitation protocol was followed:100 μL from the samples were mixed with 450 μL ice cold acetonitrile and they were centrifuged at 4 °C for 12 min in 12.5 rpm. The pellet was discarded and the supernatant was evaporated. The residue was reconstituted in 100 μL HBSS and injected into the LC–MS/MS system.

2.8. Liquid chromatography–mass spectroscopy analysis

2.8.1. Instrumentation

The LC–MS/MS system consisted of a 3200 Q TRAP triple-quadrupole linear ion trap mass spectrometer fitted with a TurboIon Spray interface (SCIEX, USA) and an Agilent 1200 HPLC system (consisting of a G1379B micro vacuum degasser, a G1312A binary pump, a G1329 autosampler and a G1316A column compartment) (Agilent, USA). The data were processed using the Analyst Software program (version 1.4.2) (SCIEX, USA).

2.8.2. Chromatographic conditions

A variation of the method by Del Boccio et al. [42] was applied. Separation was performed with an Agilent Eclipse Plus C-18 column (50 mm × 2.1 mm inner diameter, 3.5 um particle size) with a RRLC in-line filter kit (2.1 mm, 0.2 μm filter) (Agilent, USA). The gradient mobile phase consisted of solvent A (water- 0.5% (v/v) formic acid) and solvent B (acetonitrile- 0.5% (v/v) formic acid). The flow rate was 350 μL/min and the gradient elution program was initial 10% B for 0.97 min, to 35% B at 3.07 min, to 100% B at 3.42 min and hold to 4.47 min, finally to 10% B at 5 min and hold with re-equilibration of the column for another 5 min. The total run time was 10 min. The injection volume was 5 μL.

2.8.3. Mass spectrometry analysis

Electrospray Ionization (ESI) operating in negative mode was used for both HT and *CA*. Compound-specific optimization of MS/MS parameters was performed *via* direct infusion of a mixture of standard reference solutions (2 μg/mL each, in methanol) using a syringe pump. Source parameters were set to optimal values after flow injection analysis (FIA) source optimization. Quadrupoles one and three were set to unit resolution. Data acquisition was performed in the MRM mode monitoring the following transitions (*m/z*): hydroxytyrosol 152.9→123.9 (quantitative), 152.9→108.7 (qualitative) and caffeic acid (internal standard, IS) 178.8→134.9 (quantitative) and 178.8→78.8 (qualitative). Dwell time for each transition was 150 ms. Tandem mass spectrometry parameters are shown in Table S2. Source parameters were set as follows: Curtain Gas (CUR), 20; Temperature (TEM), 600; CAD gas, medium; Gas 1 (GS1), 50; Gas 2 (GS2), 50; Ion Spray (IS), -4500.

3. Results and discussion

3.1. Pseudo-ternary phase diagram

The pseudo-ternary phase diagrams of the studied systems containing IPM, EVOO and their mixture are shown in Fig. 1. In all systems the monophasic region (1φ region) is a narrow domain which does not cover the whole surfactant concentration range due to solidification of the emulsifiers at room temperature. Distilled monoglycerides were heated at 40 °C before mixing with the oil and Tween 80. The obvious difference between the three phase diagrams is related to the 1φ area which is wider in the case of IPM and narrower with the incorporation of EVOO. This observation can be explained considering the endogenous humidity of EVOO. DLS measurements have revealed that EVOO contains reverse micelles, potentially hosting compounds of hydrophilic nature [27]. The composition of the microemulsions which were selected for further studies can be found in Table 1. The selection criteria were low surfactant concentration (biocompatibility) and a sufficient water-dispersed phase for the encapsulation of hydrophilic compounds such as HT.

3.2. Dynamic light scattering

DLS not only sheds light on the dimensions, in the nanoscale, but also helps investigating the position of an encapsulated molecule, such as HT. Table 2 shows the droplet diameters and the PdI of the three systems in the absence and in the presence of 678 ppm of HT. Empty W/O microemulsions present a range of hydrodynamic diameters from 7 nm to approximately 20 nm and a polydispersity index from 0.10 to 0.30. These parameters did not change after the encapsulation of HT in the reverse micelles in accordance with previous results from Chatzidaki et al. [38,43].

3.3. Electron paramagnetic resonance

3.3.1. Structural study

EPR technique has already been used for the structural characterization of microemulsions for food [44] and pharmaceutical applications [45]. Measurements with the use of the amphiphilic spin probe 5-DSA were undertaken in order to investigate the possible structural alterations at a specific depth of the surfactant membrane that HT may provoke. 5-DSA can provide information about the mobility and the rigidity of the environment near its paramagnetic moiety. The rotational correlation time (τ_R) and the order parameter (*S*) were calculated from the experimental spectra of systems A and B. The spectrum of system C was simulated with a simulation software as the movement of 5-DSA belonged to slow motion regime $\tau_R > 3$ ns. The three-line EPR spectra were obtained for the empty and HT-loaded W/O microemulsions and revealed a slight increase in the mobility of the amphiphilic probe upon addition of HT (Table 2). These findings, in agreement with previous studies [38] and the DLS results, indicate the involvement of the antioxidant in the surfactant layer at the specific depth where the paramagnetic moiety of the probe is located. As the surfactant concentration in the nanodispersion increases, the movement of the fatty acid spin-probe becomes slower and the time that the probe needs to rotate increases. We can assume that at the highest concentration, surfactants are “packed” in the membrane of the microemulsion and therefore the rigidity of the membrane increases. Moreover, unlike IPM, EVOO contains a variety of minor amphiphilic compounds such as free fatty acids, phospholipids *etc.* that may have an effect on the interfacial membrane of the microemulsion. In all systems, the parameter *S* remained unaltered after the encapsulation of HT indicating no alterations in rigidity of the surfactant monolayer. The above-described alterations are reflected in Fig. S3 where the distances in the experimental spectra and heights were altered, with no sharp peaks in the systems A and B in comparison to System C.

3.3.2. Antioxidant activity

The EPR technique is a powerful method to determine the scavenging activity of various antioxidants as well as the kinetics of the reaction between the antioxidant and the appropriate free radical. The antioxidant activity is the most studied property of HT in free state or encapsulated in different systems as assessed by different methods, proving the antioxidant potency of the molecule attributed to the o-dihydroxyphenyl moiety. In the present study, we focused not only in the antioxidant activity of the encapsulated HT but also to the one referring to the empty microemulsion systems as EVOO [46] has a high antioxidant activity itself and Tween80 has also been reported as a molecule with antioxidant potencies [47]. In Fig. 2 empty microemulsions, especially those composed of EVOO, were found to be good scavengers towards the galvinoxyl free radical. A plethora of compounds with antioxidant activity has been identified in EVOO with most representatives hydroxytyrosol, p-coumaric and oleuropein. Their composition and concentration in the final product vary due to different variety, region and processing procedures. It is evident from Fig. 2 that the increased concentration of EVOO in the continuous phase of the systems contributes decisively in the scavenging profile of the system. The percent of scavenging activity increased rapidly in the first 2 min, and then increased slowly until reaching a plateau value. Consequently, all systems can be incorporated in products providing protection against free radicals before and after consumption. Reducing the autooxidation of a product conducts to longer shelf life and its ingestion increases the potential health benefits the product may have.

The encapsulation of HT drastically increased the scavenging effect towards the stable free radical and led to values close to 90% (Fig. 3) for

Table 2
Structural characterization parameters obtained from DLS an EPR techniques (size of reverse micelles, PdI, τ_R and S) in the absence and presence of HT.

	Diameter (nm)		PdI		τ_R (ns)		S	
System	Empty	Loaded	Empty	Loaded	Empty	Loaded	Empty	Loaded
A	6.8 ± 0.8	7.1 ± 0.5	0.15 ± 0.05	0.12 ± 0.03	0.76 ± 0.01	0.71 ± 0.01	0.06 ± 0.01	0.06 ± < 0.01
B	17.8 ± 1.4	18.9 ± 0.9	0.27 ± 0.06	0.19 ± 0.08	1.68 ± 0.01	1.64 ± 0.03	0.10 ± 0.01	0.10 ± < 0.01
C	13.9 ± 1.7	14.0 ± 1.2	0.13 ± 0.01	0.02 ± 0.06	5.05 ± 0.04	4.85 ± 0.04	0.39 ± < 0.01	0.37 ± < 0.01

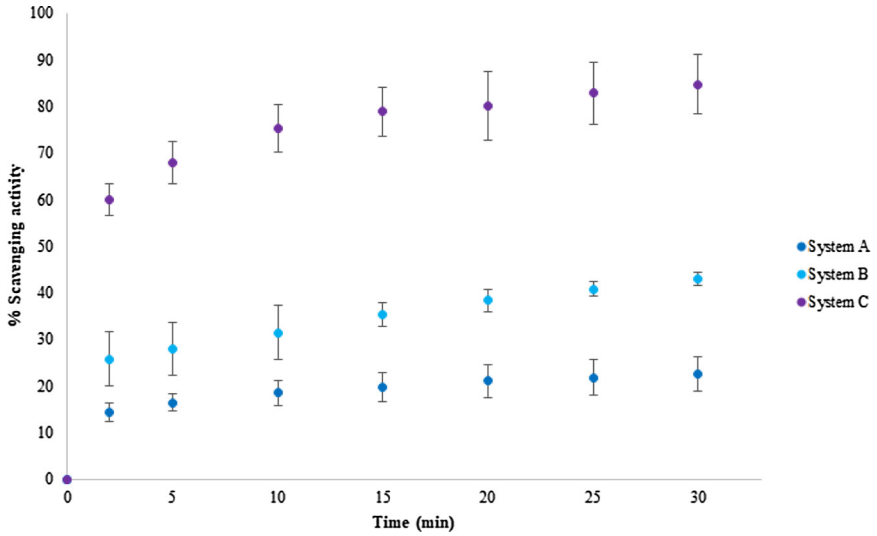

Fig. 2. Scavenging effect on galvinoxyl free radical as function of incubation time for the empty microemulsions. In all the experiments, 10% v/v of microemulsions was added in free radical solution.

the systems A and B. The majority of HT molecules react rapidly with the molecules of the free radical. To conclude, the total antioxidant activity of the systems is enhanced and therefore makes them appropriate carriers for antioxidants. The addition of HT in the system C did not provoke the levels of the free radical scavenging recorded for the systems A and B phenomenon which could be explained by the increased rigidity of the system C (increased parameter *S*) which creates a hurdle for the reaction between the antioxidant and the radical. Therefore, considering that all the microemulsions contain the same concentration of encapsulated HT, one can assume that systems A and B are slightly better candidates when used as HT vehicles due to their "flexible" surfactant monolayer.

3.4. In vitro studies

3.4.1. Cell viability

In the last few years, the coculture of Caco-2/TC7 and HT29-MTX cells has been proposed as a suitable model of intestinal epithelium for *in vitro* studies simulating intestinal transport phenomena. In order to use it for absorption studies, the coculture was incubated with microemulsions at a final ratio of 0.5%–5% v/v for 6 h. Cell viability, using the MTT approach, was recorded and plotted as a percentage in comparison to untreated cells. As it can be seen in Fig. 4, the microemulsions did not exhibit significant cytotoxic effect except in the case of System C at the 5% v/v ratio, which is probably due to the high concentration of surfactants. For the 1% v/v concentration, cell viabilities after the administration of the systems A and B were found to be around 100% whereas for system C was around 90%. Results revealed that microemulsions has a dose-dependent cytotoxicity effect.

3.4.2. Permeability studies

Because the *in vivo* activity of bioactive compounds is very dependent on their intestinal absorption, transport studies through *in vitro* simulated epithelia were conducted in the present study. The coculture used in the permeability studies of the present work is representative of the two major cell types found in the small intestinal epithelium. Caco-2/TC7 represents the absorptive type cells and HT29-MTX the goblet ones. Mucus secreting cell line HT29-MTX is a subpopulation of HT29 human colonic adenocarcinoma cells selected for resistance to methotrexate [48]. Studies have shown that their coculture creates

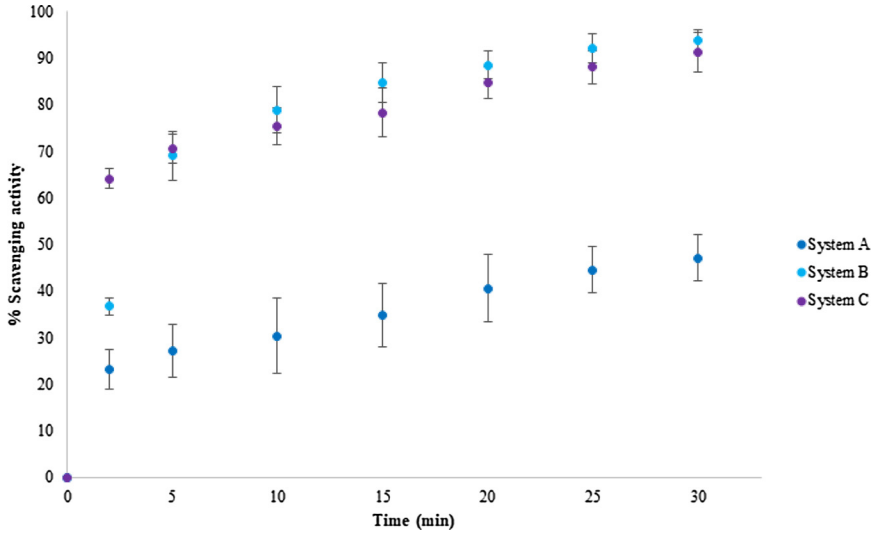

Fig. 3. Scavenging effect of encapsulated HT on galvinoxyl free radical as function of incubation time. In all the experiments, 10% v/v of microemulsions was added in free radical solution.

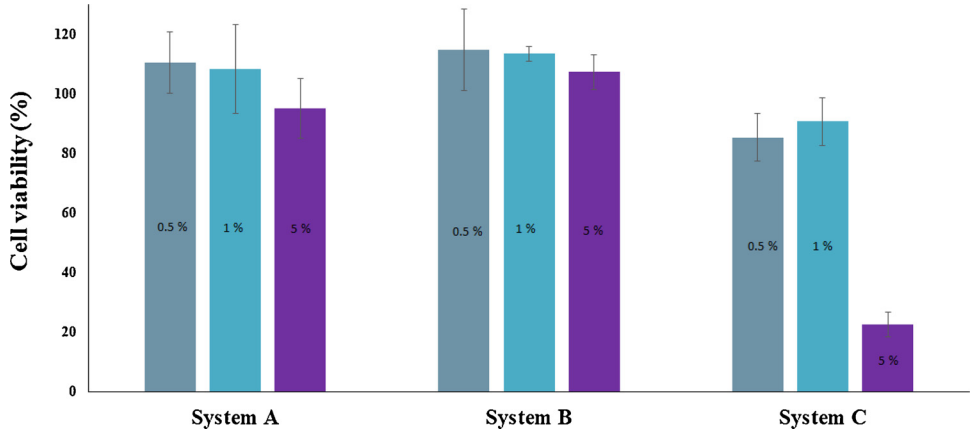

Fig. 4. Effect of the microemulsions in cell viability, at three different concentrations in the culture media of the Caco2/TC7 and HT29-MTX coculture (incubation period 6 h).

monolayers with appropriate formulated tight junctions. Mucus, which is largely composed of heavily glycosylated proteins (mucins), is secreted by goblet cells.

For the permeability of the free and encapsulated HT the coculture was seeded in appropriate filters and prior to each transport experiment, the confluency of the coculture in the inserts was checked by measuring TEER values. Monolayers exhibited TEER values before treatment averaging 450 Ω cm^2. The values did not vary after the 6 h incubation indicating no disruption of the monolayer's tight junctions (Fig. S4). So far, even if the interest around polyphenols and natural antioxidants is increasing, only few data exist concerning their bioavailability and metabolism with limited and inconsistent results [49–51]. HT bioavailability and metabolism were examined in Caco-2 cells and it was reported that the molecule was absorbed by passive diffusion [52]. To investigate the transport of free HT in comparison with encapsulated one, in different microemulsion systems, Caco-2/TC7 and HT29-MTX monolayers, were apically incubated with the formulations (1% v/v in the total medium). The final concentration of HT in the apical compartment of the monolayer (mimicking the gut lumen), free or encapsulated, was approximately 7 ppm. Analysis by LC–MS/MS of apical and basolateral medium after 6 h incubation period demonstrated cell and mucus absorption. Fig. S5 shows a typical multiple reaction monitoring (MRM) spectrum obtained after analysis of the incubated medium in the presence of HT and CA (IS).

Fig. 5 shows the distribution profile of HT in the apical and basolateral compartments while other referring to the percentage of HT that was not detected. When HT was administrated in an aqueous solution (free HT), after 6 h of experiment, about 54% of the incubated compound was still found in the apical compartment and 37% was detected in the basolateral. Whereas, when HT was administrated in the microemulsions, the profile changed, especially in the case of systems B and C. Only in the case of the system A, HT followed a similar profile with free HT. Increased concentrations of surfactants in microemulsion systems lead to the detection of HT inside the epithelial monolayer and absorbed by the mucus layer produced by the HT29-MTX cells. This result is not in accordance with the findings of Corona et al. [51] who used the Caco-2 model and concluded that the majority of HT appeared on the basolateral side, probably due to the absence of the critical barrier created by mucus. It has to be mentioned, that empty systems A, B and C were, also, examined as blanks and the HT (in cases of system B and C) was below the limits of detection, indicating that the EVOO, consisting the oil phase of the systems, did not affect the measurements in apical and basolateral compartments.

According to a previous study [22] surfactants play a critical role in the digestion process. The increased concentrations affect the activities of various enzymes, mainly lipases, leading to a slower digestion process. In agreement with these finding, in the present work, the presence of the surfactant molecules also affected the absorption rate of HT drastically. Likewise, the localization of the antioxidant in the intestinal epithelium is affected. The luminal surface of mucosal tissues, in general, is protected by a layer of highly viscoelastic and adhesive mucus. As is apparent from the values from Table 3, the analysis of the mucus layer and cell lysate, in all cases an amount of HT is entrapped in the mucus layer with higher values in the case of microemulsions. As a

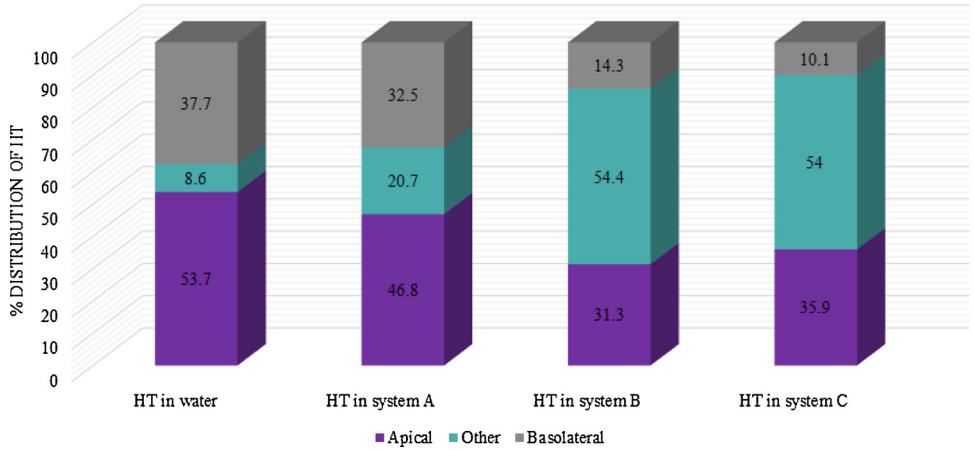

Fig. 5. % distribution of HT in the two compartments (apical and basolateral) and in the cells and mucus barrier of the Thincerts™ after 6 h incubation with HT solution and HT-loaded microemulsions.

Table 3
Ratio of [HT]/[IS] in cell cocultures in the absence and presence of mucus layer after the administration of HT solution and loaded systems.

Ratio [HT]/[IS]	HT solution	System A	System B	System C
Cell lysate and mucus	0.149	0.265	0.264	0.399
Cell lysate	0.057	0.171	0.065	0.143

result, the addition of HT29-MTX cell line and its producing mucus could explain the different absorption behavior of the hydrophilic compound.

Emulsifiers are of great importance in the development of new delivery systems, functioning as cell permeation enhancers. Among the two surfactants, Tween 80 has been extensively studied for its absorption enhancing properties in Caco-2 cells, by inhibiting the P-gp efflux transporter [53,54]. On the other hand, the role of DMG in absorption has not been described yet. Especially, Tween-80 increased passive transcellular transport [55] and inhibited P-gp activity [56] whereas, for paracellularly transport compounds, such as HT, Tween 80 did not exhibit any effect of such behavior [57]. It is obvious, that the mucus plays a crucial role in the permeability due to its complex nature, described as a hydrogel with mucin as the gelling agent. As Lafitte et al. [58] described, the diffusion of Tween 80 molecules in the presence of intestine mucus was affected by mucin obstruction and also tend to interact strongly with lipid depots. The polymer-rich nature of mucus hurdles the HT distribution creating an entrapment environment in comparison with simpler *in vitro* models. As monoglycerides have a low HLB value they have, also, a significantly smaller effect on tight junction than ones with higher HLB [59].

In addition, we have to underline that the polyphenol molecule itself interacts with the mucin molecules creating aggregations, depending on the polyphenol concentration, which affects its final bioavailability. Although cinnamic acid and p-coumaric acid are the polyphenols which interact stronger with mucin, HT has also this ability as reported previously [60]. As a result, it can be assumed that the interaction of surfactants with the mucus layer and the nature of the compound are both affecting the permeability profile and the subsequent bioavailability of a hydrophilic compound, such as HT. The careful choice of the nature, but also, of the concentration of the surfactant molecules of potent delivery systems is of high importance not only in terms of biocompatibility but also of effective transport of the encapsulated compounds. In order to achieve the formulation of an efficient system for the delivery of hydrophilic compounds with health benefits many factors must be considered. In the present study, it is clearly demonstrated that the surfactant concentration range is of great importance. This fact must be taken into account in combination with other strategies in order to exploit the maximum of the oral administration of nanotechnology-based systems. Therefore, specific design considerations must be taken into account for the formulation to have the highest possible absorption rate, depending on the site of absorption as it has been recently described in great detail [61]. As Pereira de Sousa et al. have proposed, the addition of proteolytic enzymes could be an interesting strategy to improve the mucus penetration [62]. This strategy could be combined with the proposed formulations, as microemulsions are appropriate carriers for enzyme encapsulation. Enteric polymers, or agents for degrading mucus may offer the ability to even microemulsions with higher surfactant concentration to be used, leading to an efficient oral administration product. The careful design of new delivery systems in combination with agents for targeting specific sites of the gastrointestinal tract and mucus penetration compounds can lead to promising nanotechnology-based carriers *in vivo*.

4. Conclusions

W/O microemulsions based on biocompatible ingredients and natural edible oil, EVOO, were formulated as appropriate hosts for the natural antioxidant HT. Structural characterization of the systems in the absence and the presence of 678 ppm HT was conducted and revealed interactions between the encapsulated molecule and the surfactant monolayer. Additionally, the systems were effective scavengers of the free radical galvinoxyl, examined by EPR technique, due to the presence of EVOO, activity that increased after the encapsulation of HT. The rigidity of the surfactant monolayer of the system seems to affect the reaction's kinetics in the case of microemulsion with increased membrane rigidity. Finally, the systems were assessed and examined for their effect in the permeability efficiency of HT through an *in vitro* intestinal epithelium model constructed by Caco-2/TC7 and HT29-MTX cell lines. The increased surfactant concentration indicated a decreased HT permeability which can be explained by the interactions of Tween 80 and DMG with the mucus layer produced by goblet cells.

The information generated in this study could potentially facilitate the understanding of intestinal distribution of hydrophilic molecules encapsulated in nanodispersions with the use of a valuable tool mimicking the permeabilities of human intestine. In addition, it provides experimental evidence for a lower bioavailability HT profile through intestine with the use of a coculture mode. Overall, the key finding of the present work is the use of reverse micelle systems with low concentration of surfactants for terms of biocompatibility and intestinal permeability. Further experimentation of similar systems with different nature and concentration of surfactants, would also be interesting in order to identify a profile of systems appropriate for oral uptake.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.colsurfb.2019.110482.

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Development of a Microemulsion for Nasal Uptake of Antioxidants

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Highlights

- Water-in-oil (W/O) microemulsion was formulated with biocompatible oils and surfactants for nasal administration.
- Encapsulation of chitosan and gallic acid (GA) was achieved in the microemulsion and the system was structurally characterized.
- The cytotoxicity of the system was tested *in vitro* with the use of RPMI 2650 cell line.
- The addition of chitosan increased the transport of GA through the constructed nasal epithelium confirming its mucopenetrating properties.

Abstract

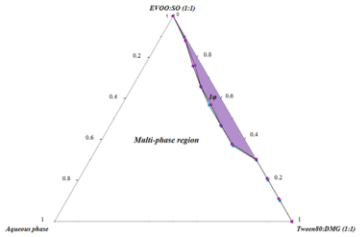
A novel water-in-oil (W/O) microemulsion based on natural oils, namely extra virgin olive oil (EVOO) and sunflower oil (SO), in the presence of non-ionic surfactants was successfully formulated without co-surfactants. The novel microemulsion was used as a carrier for gallic acid (GA) to assure its protection and efficacy upon nasal administration. The work presents evidence that this microemulsion can be used as a nasal pharmaceutical formulation for the delivery of polar antioxidants, especially, after incorporation of chitosan in its aqueous phase. The structure of the microemulsion was studied by Small Angle X-ray Scattering, Dynamic Light Scattering, and Electron Paramagnetic Resonance techniques. By the addition of chitosan, the diameter of the microemulsion remained unaltered at 47 nm whereas after the incorporation of GA, micelles with 51 nm diameter were detected. The dynamic properties of the surfactant monolayer were affected by both the incorporation of chitosan and GA. Moreover, the antioxidant activity of the latter remained unaltered (99%). RPMI 2650 cell line was used as the *in vitro* model for cell viability and for GA nasal epithelial transport studies after microemulsion administration. The results suggested that the nasal epithelial permeation of GA was enhanced, 3h post administration, by the presence of 0.2% v/v microemulsion in the culture medium. However, the rate of the transported antioxidant in the presence of chitosan was higher indicating the polymer's effect on the transport of the GA. The study revealed that nasal administration of hydrophilic antioxidants could be used as an alternative route besides oral administration.

Keywords

Extra Virgin Olive Oil; Sunflower Oil; Dynamic Light Scattering; Small Angle X-ray Scattering, Electron Paramagnetic Resonance; Chitosan; RPMI 2650

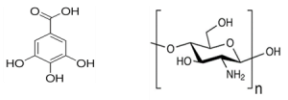
Graphical abstract

Structural studies



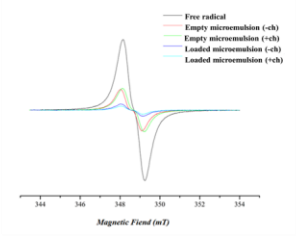
Pseudo-ternary phase diagram
DLS
EPR (spin probe technique)

Encapsulation of Gallic acid (GA)
Addition of chitosan



- Extra Virgin Olive Oil (EVOO)
- Sunflower Oil (SO)
- Tween 80
- Distilled monoglycerides (DMG)

Activity studies

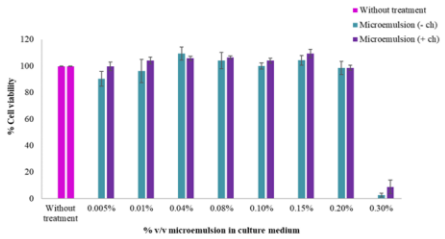


Antioxidant efficacy
EPR
(Stable free radical galvinoxyl)

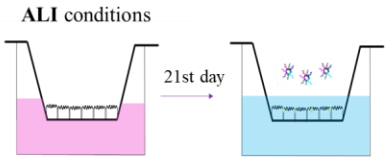
Nasal administration

Cell culture model: RPMI-2650
Semi-permeable culture filters
21days

In vitro viability assays



In vitro transport studies



1. Introduction

The delivery of bioactive compounds with drug-like activities to their targets is a challenging field, as the carrier needs to protect the hosted molecules while overcoming the limitations of the selected route of administration. Nanotechnology based carriers such as nanoparticles [1], cubosomes [2], liposomes [3], polymer-based formulations [4], nanoemulsions [5], microemulsions [6] and pickering emulsions [7] have already been successfully used for the delivery of bioactive compounds via different routes of administration offering protection of the encapsulated molecule, higher solubility and bioavailabity, targeted and sustained release and also user compliance [8]. Microemulsions are thermodynamically stable, transparent nanodispersions of oil, surfactant, co-surfactant (occasionally) and water [9]. In the literature, there are many examples of microemulsions successfully used for the delivery of bioactive compounds and the treatment of pathological conditions. These include O/W (oil-in-water) and W/O (water-in-oil) microemulsions based mainly on non-ionic or in a less extent on ionic surfactants, with mucoadhesive or penetration enhancers [10,11]. Nonetheless, the high concentration of emulsifiers and the occurrence of phase separation in physiological conditions (temperature, pH, high salinity) are the drawbacks that these formulations may exhibit.

Administration of bioactive compounds hosted in nanosystems can be achieved by different routes such as oral [12], dermal [13], ocular [14], intravenous [15], buccal [16], nasal [17] etc. Oral administration is the most well-studied one but exhibits many disadvantages regarding the first-pass effect [18], the slow onset of action and the high metabolic profile due to the presence of digestive and proteolytic enzymes [19]. As a result, different approaches must be used for oral administration such as the development of specific capsules and coatings for targeting different parts of the Gastrointestinal (GI) tract [20]. A recent study of our group showed the relative low transport of the hydrophilic antioxidant hydroxytyrosol, encapsulated in microemulsions, across intestinal epithelium [21], indicating the need of an alternative administration route for antioxidant uptake and modification of the used nanocarriers. The nasal route has gained attention the last decade, since it was proven to be a suitable “entrance” for both systemic and brain delivery [22], It is a non-invasive route, with high vascularization, high systemic drug absorption, able to avoid first-pass hepatic metabolism, with lower enzymatic metabolism, while offering the possibility for direct brain delivery avoiding the Blood Brain Barrier (BBB) [23]. The ease of application makes nasal administration an ideal route for treating chronic diseases due to the compliance of the patients [24], substituting oral administration. Many products can be found in the market as nasal formulations with sumatriptan [25], zolmitriptan [26], oxytocin [27] etc. being representative example drugs.

Microemulsions, as liquid-in-liquid colloidal systems with a wide range of applications, have been already studied in nasal delivery of different compounds and especially antipsychotic [28] and anti-neurodegenerative drugs [29]. The majority of the reported systems include high co-surfactant concentration (10%-30%), which may lead to side effects such as irritation and allergy especially when repeated doses are necessary. In addition, the literature focuses on O/W systems

due to the low bioavailability of the hydrophobic and the high molecular weight synthetic drugs. However, in nasal cavity, hydrophilic compounds are also facing absorption problems and the development of appropriate W/O systems is of paramount importance. For that reason, in the present study we focused on the formulation of an adequate system in the absence of co-surfactants and in the presence of natural oils in order to avoid any undesirable side effects. Non-ionic surfactants were exclusively used for the formulation of the novel system and, as far to our knowledge, it is the first time that distilled monoglycerides (DMG) were used in a microemulsion for nasal application. In addition, chitosan was used as penetration enhancer, after its incorporation in the dispersed phase of the system and its structural effect was studied by Small Angle X-ray Scattering (SAXS), Dynamic Light Scattering (DLS) and Electron Paramagnetic Resonance (EPR). The size range of microemulsions enables the use of X-rays and light scattering methods (SAXS and DLS respectively) to investigate their structure, size and polydispersity profile. EPR technique, with the use of amphiphilic spin probes, is adequate for the study of structural alterations in the surfactant monolayer of a microemulsion probing alterations upon addition of compounds.

Gallic acid (3,4,5-trihydroxybenzoic acid, GA) was used in the present study as a model bioactive to prove the effectiveness of the novel system in nasal administration, especially due to its therapeutic potency and low bioavailability after oral administration [30]. GA is a natural phenolic compound mostly found in grapes [31] and black tea [32] and it has been in the center of interest for its multi-purpose activity. More specifically, strong antioxidant [33] and antimicrobial activity [34], anti-inflammatory [35] and even antidepressant properties [36] have been reported. Interestingly, it has been reported that regular consumption of GA may inhibit the amyloid fibril formation which characterizes many protein misfolding diseases [37]. However, its therapeutic potential is reduced by its pharmacokinetic drawbacks. As has been reported, oral administration of GA to animals showed low bioavailability with low maximum drug concentration in plasma [38] leading to the need of alternative route of administration. The low metabolic environment of nasal mucosa and the leaky nature of the epithelium makes this route ideal for antioxidant delivery in comparison to intestinal absorption [39]. The strategy of encapsulation should assure the protection of the phenolic acid against possible enzymatic degradation or oxidation during the storage and consumption process until its release in the nasal cavity, ensuring its bioavailability and efficacy. Moreover, the use of mucoadhesive ingredients in the formulation can increase the residence time in the mucosal epithelium overcoming the rapid mucociliary clearance mechanism. In recent years, the natural polymer, chitosan has attracted interest as a mucus adherent agent in nasal delivery systems [40]. Chitosan is produced by deacetylation of chitin found in crustacean shells [41]. Its positive charge helps to interact with the negatively charged mucin and offer prolonged residence time and simultaneously acts as a penetration-enhancer in nasal formulations creating pores in the mucus layer while simultaneously [41] opening the tight junctions [43–45].

The human nasal epithelial cell line RPMI 2650, derived from septum carcinoma, was used in order to evaluate *in vitro* the proposed microemulsion as a nasal drug delivery system. This cell

line has been extensively used in the field of nasal administration (toxicity, transport etc.) as it closely resembles cells of the normal human nasal epithelium with respect to karyotype and the presence of mucus on the cell surface [46]. In addition, it is widely used due to the absence of standardized methods for primary cell cultures, which lack due to limited passaging, repeated sampling, inhomogeneity of tight junctions etc. [39,42]. Interestingly, the most adequate conditions for the culture of this cell line in order to construct a cell layer for drug absorption studies, is the air-liquid interface conditions (ALI) in contrast to intestinal and other cell lines [47,48].

The aim of the present study was the development of a new biocompatible W/O microemulsion based on biocompatible ingredients for the effective encapsulation of GA and its potential use as nasal carrier. Our objective was the formulation of a non-toxic carrier, in the absence of any co-surfactant molecule, which effectively protects the natural antioxidant while increasing its transport with the use of a natural mucopenetrating agent. For this purpose, Dynamic Light Scattering (DLS), Electron Paramagnetic Resonance (EPR) and *in vitro* methods were applied to characterize the structure and the biological efficacy of the novel system.

2. Materials and Methods

2.1 Materials.

5-Doxyl-stearic acid (5-DSA) spin probe, galvinoxyl free radical, gallic and protocatechuic (PC) acids were purchased from Sigma-Aldrich, Germany. Polyoxyethylene sorbitan monoolate (Tween 80™) was obtained from Sharlau, Spain. Distilled monoglycerides of vegetable fatty acids (DMG 0295) were a kind gift from Palsgaard, Denmark. Chitosan (viscosity 200–600 mPa.s, 0.1 % in 0.5 % Acetic acid, 20 °C; Deacetylation value: 80%) was purchased from TCI, Belgium. Extra virgin olive oil (EVOO) and Sunflower oil (SO) were commercial products purchased from a local market. High-purity water was obtained from a Millipore Milli Q Plus water purification system. All other chemicals used in the study were of analytical grade.

2.2 Cell line.

The cell line RPMI 2650 (CCL-30) was kindly provided by Dr. Fabio Sonvico (University of Parma, Parma, Italy). Cells between passage 16–30 were grown in 75 cm^2 flasks in complete Minimum Essential Medium (MEM) containing 10% (v/v) fetal bovine serum (FBS), 1% (v/v) non-essential amino acid (NEAA) solution and maintained in a humidified atmosphere of 95% air 5% CO_2 at 37°C. Cells were sub-cultured according to the ATCC protocol. MEM, FBS, 100 U/mL penicillin and 100 mg/mL streptomycin, phosphate-buffered saline (PBS) and trypsin-EDTA were purchased from Gibco (Invitrogen Corporation, Life Technologies, UK). 12-well ThinCert™ polycarbonate inserts, 0.4 μm pore size and growth surface 4.67 cm^2 were purchased from Greiner Bio-One GmbH (Frickenhausen, Germany).

2.3 Formulation and phase behavior study.

W/O microemulsion consisting of EVOO and SO as the oil (continuous) phase, Tween80 and DMG as biocompatible surfactants and ultra-pure water as the aqueous (dispersed) phase, were prepared by adding the aqueous phase to a solution of oil and surfactants. Gentle shaking led to an isotropic mixture. For the preparation of a mucoadhesive microemulsion the ultra-pure water was replaced by a chitosan solution (0.1 % chitosan in 1% acetic acid solution). For the determination of the systems' monophasic areas the corresponding pseudo-ternary phase diagrams were constructed as described previously [49]. ProSim software was used for the construction of the pseudo-ternary phase diagrams. In the present study for the encapsulation of GA in the microemulsions an aliquot of the phenolic acid was added in the mixture of surfactants and the non-polar solvents. Solubilization was achieved by gentle shaking. For all experiments, the concentration of GA was kept constant in the microemulsion at 2.8 mM.

2.4 Structural study.

2.4.1. Small Angle X-ray Scattering (SAXS)

Small angle X-ray Scattering (SAXS) experiments, for microemulsions in the presence and absence chitosan solution, were carried out on a Nano-InXider vertical SAXS/WAXS system of Xenocs SA, France equipped with a Cu k-a source of a two detectors setup for SAXS/WAXS measurements. The wavelength of the X-ray radiation was 0.154 nm and the sample-to-detector distance was 937.5 mm. The exposure time for each scattering frame was 600s (in the VHS mode) in all vacuum environment.

2.4.2 Dynamic Light Scattering (DLS)

A Zetasizer Nano ZS (ZEN3600) from Malvern Instruments (UK) equipped with a He-Ne laser (632.8 nm) using a non-invasive back scatter (NIBS) technology was used for the DLS study of the microemulsions. A scattering angle of 173° and a quartz type cuvette were used. Each sample was measured in triplicate after filtering through 0.45 μm cellulose for dust-free conditions at 25 ºC. Data were processed using the Malvern Zetasizer Nano software. The empty and loaded W/O microemulsions were measured in terms of droplet size and droplet size distribution (PdI) evaluation.

2.4.3 Electron Paramagnetic Resonance (EPR)-Membrane dynamics

EPR spectra, were recorded at constant room temperature (25 °C), using a Bruker EMX EPR spectrometer operating at the X-Band, Bruker USA with the use of a WG-813 Q-Wilmand

(Buena, NJ) Suprasil flat cell. Spin probe technique was used to obtain information about the interfacial properties of the surfactant monolayer in GA-free and loaded microemulsions. The 5-DSA as a fatty acid has an amphiphilic nature and its unpaired electron is located closer to the polar head and consequently closer to the surfactant polar head group area. This interface-located fatty acid spin probe reflects on EPR spectra the alterations, which take place in the specific depth of the membrane where the doxyl-ring of the spin probe is located. In order to obtain the desired concentration of the spin probe ([5-DSA] = 1.2 x 10^{-4} M) in the microemulsions the formulations were added to vials where the appropriate amount of the spin probe had been formerly deposited. Experimental results where expressed by means of rotational correlation time (τ_R) reflecting the mobility of the probe, and the order parameter (S) expressing the rigidity of the surfactant membrane. In the present study, the parameters were calculated for empty and loaded with GA microemulsions in the absence and the presence of chitosan. In addition, the parameters τ_R and S were determined for a reference microemulsion with 1% acetic acid solution as aqueous phase. The decreased pH in the microenvironment of the microemulsion core affects the localization of the spin-probe reflecting an environment that corresponds to a position closer to the oil phase [50] Therefore, the calculation of τ_R and S in the acetic acid containing system is of high importance as they will be used as reference values for the systems in the presence of chitosan. Simulations for all the spectra were performed with home-written programs in MATLAB (The MathWorks) employing the Easy Spin toolbox for EPR spectroscopy [51].

Viscosity measurements were conducted with the use of a DV-I Prime Digital Viscometer (Brookfield Engineering Laboratories, USA), equipped with a cone spindle (CPA-40Z). Experiments were performed in triplicate for each sample under constant temperature (25 °C), and results were presented as average ± S.D.

2.5. Activity assessment.

For the study of the antioxidant activity of the novel microemulsion (empty and loaded) the stable galvinoxyl free radical was used in the above described EPR spectrometer. The experimental procedure followed is described in a previous study of our group.[21] In the present investigation, 0.1 mL of each W/O microemulsion (empty or loaded with GA and chitosan) was added to 0.9 mL of galvinoxyl (0.25 mM) solution in isooctane. EPR spectra were recorded at room temperature for 30 min. The reduction of the intensity of the EPR signal of galvinoxyl radical was calculated and expressed as % inhibition in specific time points (Eq. 1).

$$\%\text{Inhibition} = \left(1-\frac{A}{Ao}\right) \times 100 \quad (1)$$

where, A_o is the integrated intensity of the control sample at a specific time point and A the integrated intensity of the sample containing the microemulsion at the same time point.

2.6 Biological assessment.

2.6.1 Cell proliferation assay.

Cell proliferation was assessed 48h after treatment by the MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay according to the manufacturer's standard protocol. MTT stock solution (5mg/mL) was added to each culture being assayed to equal one tenth of the original culture volume and incubated for 3h. At the end of the incubation the solution was removed and the converted dye was solubilized in isopropanol: DMSO solution in ratio 1 to 1 in order to dilute the insoluble purple formazan. Absorbance of converted dye was measured at 570nm. Eq. 2 was used to determine the cell viability.

$$\text{Cell viability (\%)} = \frac{(OD\ of\ treated\ cells)}{(OD\ of\ control)} \text{ x } 100 \qquad (2)$$

where, OD is the optical density.

2.6.2 *In vitro* transport studies.

For permeability experiments, cells were seeded on 12-well ThinCertrs™ permeable polyester filters of 0.4 µm pore size. In order to establish the ALI model, cell suspension was seeded onto the permeable filters at a seeding concentration of 4 $\times 10^5$ cells/cm^2 per filter. The media on the apical compartment were removed 8 days post-seeding. Media in the basolateral chamber were replaced every two days. Cell layers were allowed to grow and differentiate under ALI conditions up to 21 days. Transepithelial electrical resistance (TEER) was measured as described previously [21].

On the 21st day post seeding, monolayers were incubated in the apical compartment containing a GA aqueous solution, empty or loaded microemulsions at 0.2 % v/v in HBSS. Each plate contained a control sample with the HBSS only. The apical compartment received 0.6 mL of medium while the basolateral compartment received 1.2 mL. The basolateral media were collected at time intervals of 10, 30, 60, 120, 180, 240 min of incubation and were immediately analyzed by LC-MS/MS for the detection and quantification of GA. A calibration curve of GA in the transport medium was constructed with different GA and PC (internal standard, IS) concentrations ranging from 0.05 to 5 µg/mL. In addition, as oxidation phenomena occur during the incubation time, the oxidation profile of GA during the studied time points was also measured.

2.7 Quantitative analysis.

For the quantification of the transported GA, analysis was performed with the LC-MS/MS system described in a previous study [21]. A variation of the method by Basu et al. [52] was applied. Separation was performed with an Agilent Eclipse Plus C-18 column (50 mm × 2.1 mm inner diameter, 3.5 um particle size) with a RRLC in-line filter kit (2.1 mm, 0.2μm filter) (Agilent, USA). Electrospray Ionization (ESI) operating in negative mode was used for both gallic acid and PC (IS). More details for chromatographic conditions and mass spectrometry analysis can be found in supplementary material. (see Section S1 and Table S1)

For sample preparation, 100 μL of the basolateral side were transferred into a 1.5 mL Eppendorf tube and 450 μL of ice-cold acetonitrile were added. The sample was then centrifuged for 10 minutes in 10.000 rpm at 4 °C for protein precipitation. The supernatant was transferred into a clean HPLC appropriate vial and evaporated to dryness. The pellet was redissolved in 100 μL of H_20: ACN (1:1) and 10 μL IS solution (1 μg/mL) were individually added in the sample. The mixture was vortexed and a 5 μL aliquot was injected into HPLC–MS/MS for analysis.

3. Results and Discussion

3.1 Formulation and phase behavior study.

Phase behavior of multi-component systems such as microemulsions can be studied by pseudo-ternary phase diagrams. In order to formulate a system adequate for nasal delivery of hydrophilic compounds, ingredients of natural and non-toxic origin were chosen. EVOO and SO were used as biocompatible components of the continuous phase. The use of EVOO can give an added value to the final formulation due to its anti-inflammatory, antioxidant and neuroprotective reported properties [53,54]. Similarly, surfactants play a crucial role in the stability, the size and the efficacy of the microemulsion as a delivery system. In the present study, DMG and Tween 80 were used in equal amounts for the formulation of the system. As Constantinides et al. have reported, the use of a combination of two amphiphilic molecules with totally different HLB values is able to create a thermodynamically stable nanodispersion [55]. In previous studies our group has successfully formulated W/O microemulsions with the same surfactants and different biocompatible oils for the encapsulation of enzymes and antioxidants adequate for pharmaceutical applications with emphasis in oral administration [21,49]. In the literature, Tween 80 has been extensively studied in nasal applications due to its membrane penetration efficacy and its positive results in the absorption of many hormones (progesterone, testosterone) and drugs [56]. Also, recently, this non-ionic surfactant was shown to increase the drug systemic absorption and the passive transport to the BBB after intranasal administration [57]. In order to avoid the involvement of another synthetic surfactant the non-toxic DMG was used. DMG, also known in the food industry as E471, is a mixture of mono- and di-glycerides of fatty acids. This low-HLB surfactant (approximately HLB=3) has been widely used in systems

for food applications but also in oral delivery systems [58] and is Generally Recognized as Safe (GRAS). Water was the dispersed phase of the system. In addition, chitosan solution was used for the construction of a system with mucoadhesive/mucopenetrating properties. Fig.1 represents the pseudo-ternary phase diagram of the two systems, where a narrow isotropic region corresponding to the monophasic area (1 ϕ) and a broad multi-phase region can be observed. As the ratio of emulsifiers was increased, increasing amounts of the dispersed aqueous phase were incorporated in the system expanding the boundaries of the monophasic region. Interestingly, an almost identical monophasic area was obtained when water was replaced with chitosan solution. It has to be underlined that after the addition of chitosan solution in the oil-surfactant mixtures longer period of time was needed for the systems to reach equilibrium. The incorporation of chitosan in a microemulsion system, without affecting drastically its thermodynamic properties, can extend easily the formulation's properties by creating a final effective carrier especial for application in mucosal epithelia. To strengthen the biocompatibility of the system, referring to a future application in the nasal delivery of hydrophilic compounds, a relatively low surfactant concentration was selected. More specifically, the system was formulated with 23.4% w/w of surfactants, a low ratio comparatively to other nanosized formulations [59,60]. Additionally, natural oils are a challenging option for the formulation of new systems without the use of co-surfactants, due to their increased humidity and the complex structure [61]. Finally, the composition of the microemulsion chosen for further studies was: EVOO: SO (1:1) 72.4 % w/w, Tween80: DMG (1:1) 23.4% w/w and 4.2% w/w aqueous phase (in the absence or presence of chitosan).

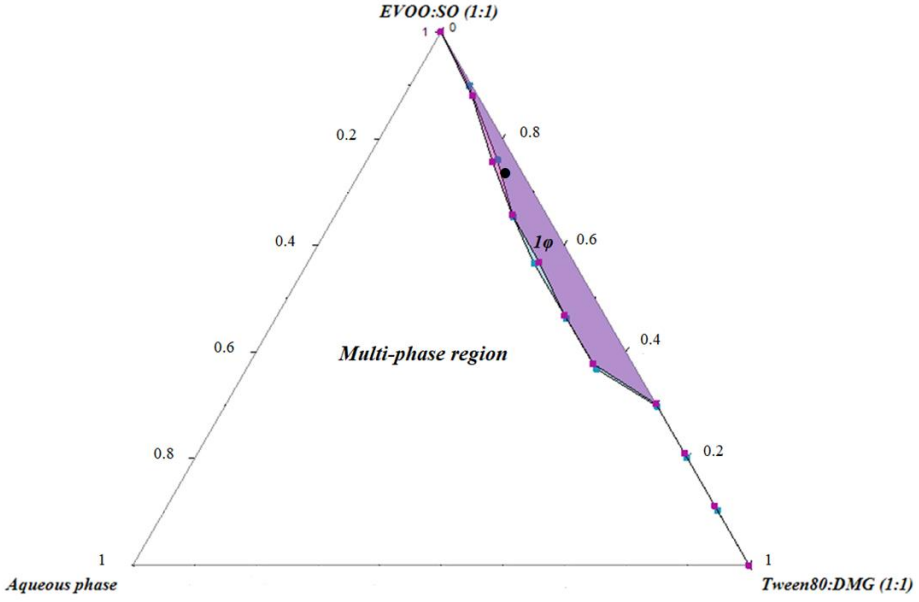


Figure 1. Pseudo-ternary phase diagram of the system consisted of EVOO and SO as the oil phase, Tween 80 and DMG as surfactants. Water and chitosan solution were used as dispersed phases of the systems. The monophasic region of the system containing water is defined by the light blue (—●—) while the one formulated with chitosan solution by the purple line (—●—).

Temperature was kept constant at 25°C. Point (●) corresponds to the following composition: EVOO: SO 72.4 % w/w, Tween 80: DMG (1:1) 23.4% w/w and 4.2% w/w aqueous phase (in the absence and presence of chitosan) and was used for further studies.

3.2 Structural study

3.2.1 Small Angle X-ray Scattering (SAXS)

SAXS is an analytical and non-destructive method for investigating nanostructures in liquids and solids. SAXS is able to probe the colloidal length scales of 10–1000 Å [62] and therefore is an appropriate method for determining the size and the structure of colloidal systems such as microemulsions. In the present study, the method was used in order to clarify the possible alterations that may be provoked in the nanodispersion's structure after the incorporation of the chitosan polymer in the dispersed phase. Fig. 2 represents the intensity profile of microemulsions in the absence (blue circles) and presence (green circles) of chitosan. The scattering curves were characterized by a monotonically decayed pattern without discernible peaks. Interestingly, the intensity profile is identical for both cases indicating that the participation of the polymer did not affect neither the size nor the structure of the system. It is assumed that the scattering pattern does not change probably due to the low concentration of incorporated chitosan (0.1% w/w in the aqueous solution). Those preliminary results clearly indicate that the reverse micelles do not have the classic intensity curve of the spherical conformation. This is an anticipated result due to the nature of the surfactant molecules Tween 80 and DMG. It is well known that the Critical Packing Parameter (cpp) of Tween 80 is below 0.2 [63] creating spherical micelles whereas DMG is able to create a wide range of structures such as lamellar, cubic and reversed hexagonal depending on temperature and other factors [64].

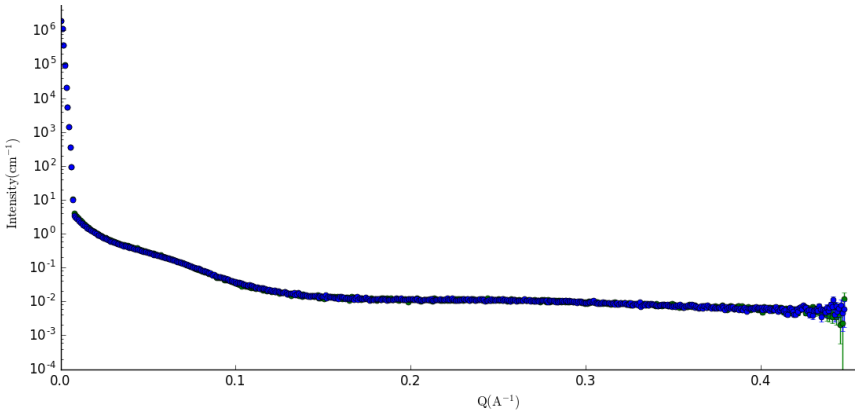


Figure 2. SAXS intensity profiles of microemulsion in the absence of chitosan solution (blue circles) and in the presence of chitosan solution (green circles).

3.2.2 Dynamic light scattering (DLS)

As the microstructure of the reverse micelles of the microemulsions does not change after incorporation of chitosan, DLS was used in order to confirm the above observation and see also how GA may affect the structure. The main application of DLS is the particle sizing and the distribution of sizes, by measuring the diffusion constants of particles undergoing Brownian motion in dilute suspensions applicable in spherical hard particles. However, if the particles are not spherical the hydrodynamic radius is often taken as the apparent hydrodynamic radius or equivalent sphere radius [65]. As observed from the SAXS study (Fig. 2) the microemulsions in the present study do not have a spherical conformation. Nevertheless, DLS can be used in order identify any alterations after the addition of different compounds regarding the apparent hydrodynamic radius. As a result, measurements were carried out to evaluate the apparent hydrodynamic diameter and size distribution and the polydispersity index (PdI) of the dispersed aqueous phase of the W/O microemulsions in the absence and in the presence of GA and chitosan. Table 1 shows the apparent hydrodynamic diameter and the PdI of the systems. As it can be observed, when GA was added in the microemulsion (2.8 mM/mL of microemulsion) the diameter slightly increased whereas the polydispersity index remained unaltered. This behavior could be possibly explained by the small molecular size of GA in combination with its partition coefficient (logPow:0.7). A similar increase in the diameter of the micelles has been also reported by our group in a system consisting of (R)-(+)-limonene/ethanol/Tween 40/water/propylene glycol [66]. The incorporation of chitosan solution (+ ch) in the aqueous phase of the system did not affect the diameter of the microemulsion, in agreement with the SAXS results, and the addition of GA followed the same profile as in the case of the absence of chitosan (- ch). To conclude with, the incorporation of a polyelectrolyte like chitosan, did not provoke changes in the size of the system's dispersed phase but the encapsulation of GA resulted in small but significant changes in the apparent hydrodynamic diameter of the aqueous droplets.

Table 1. Droplet diameter (nm) and polydispersity index (PdI) of the empty and GA-loaded microemulsions.

	Diameter (nm)	**PdI**
Empty (- *ch*)	46.7±0.6	0.101±0.01
Loaded (- *ch*)	50.1±0.8	0.110±0.02
Empty (+ *ch*)	47.4±1.4	0.109±0.01
Loaded (+ *ch*)	52.5±0.9	0.120±0.02

3.2.3 Electron Paramagnetic Resonance (EPR)-Membrane dynamics

In order to obtain information about microemulsion's surfactant membrane conformation, the use of the EPR spin probe technique was applied. The success of this technique derives from the ability of the environment close to the probe to influence the spin probe and thus it's EPR spectrum [67]. The spectrum of a spin-probe in an anisotropic environment (such as the microenvironment of a colloidal system) can reflect the changes that occur in the surfactant membrane after the addition of any molecule. These properties make the spin probe technique essential in order to identify the rigidity of the systems' membrane and the localization of an encapsulated molecule-drug, information fundamental for the prediction of the carrier's delivery efficacy.

In the present study, the encapsulation of GA and chitosan induced alterations in the surfactant monolayer that reflect on the increased τ_R values of the system as can be seen in Table 2 and Fig.3. The restrictive motion of the N-O moiety of the 5-DSA indicates the interference of both additives in the surfactant layer of the system. In a previous study, the encapsulation of GA has induced the same alterations in the mobility of the spin probe and in the rigidity of the membrane [68]. In a different system studied by our group [69] applying a molecular dynamics approach, indicated that GA does not participate in the surfactant membrane. Nevertheless, in the first case [68] the presence of Tween 40 and ethanol increased the solubility of the GA in contrast to the latter case of Chatzidaki et al., where the microemulsion system was consisted of surfactants with low HLB values (lecithin and DMG), environment limiting the solubility of GA. These apparently controversial results indicate that the nature of the system's ingredients affect the localization of the bioactive compound regarding its solubility. In the present study, Tween 80 increased the solubility of GA and as a result, the bioactive appears to be in higher concentration closer to the polar heads of the surfactant where Tween 80 and water coexist.

The pH of the microenvironment may influence the state of the fatty acid-based spin probe. In the case of the chitosan-free systems the 5-DSA exists in its ionized form whereas in the case of chitosan-loaded systems the pH decreased as a consequence of the addition of acetic acid. This change results to a different localization of the 5-DSA in the interface closer to the surfactants' chains (less polar environment). For this reason, for the microemulsions containing chitosan, we used as a reference system a microemulsion formed with 1% acetic acid solution instead of ultra pure (u.p.) water. The increased τ_R value of 5-DSA in the system with 1 % acetic acid in comparison with the one with u.p. water, reflects a more packed environment, closer to the surfactants chains. The rigidity of the membrane seems also to be increased in the localization area of the 5-DSA. On the other hand, comparing the parameters for the system with acetic acid solution and the respective one with chitosan, the interaction of chitosan with the surfactant membrane is confirmed by the decreased mobility of the spin probe.

To conclude with, the addition of acetic acid in the aqueous phase provoked changes in the localization of the 5-DSA in the surfactant layer, while the addition of GA and chitosan induced changes in the spin probe's movement indicating their involvement in the interface. The participation of both molecules (GA and chitosan) in the interface is fundamental for two reasons: i) the chitosan molecules interact with the mucus of the nasal epithelium exploiting its

mucoadhesive properties and ii) the encapsulated-GA is more likely to be released. From the values presented in Table 2 we can see a slightly altered rigidity of the membrane. No alteration in viscosities was detected in all cases (Table S2). However, the proposed system has τ_R and S values close to the ones of the microemulsion with only EVOO as the oil phase and lower concentration of surfactant mixture [21]. This could be attributed to the decreased viscosity of the system due to the partially substitution of EVOO with SO which affected the mobility of the spin probe.

Table 2. Rotational correlation time (τ_R) and order parameter (S) of 5-DSA in the studied systems.

	τ_R (ns)	**S**
Empty (- ch)	4.15±0.01	0.32±0.01
Loaded (- ch)	4.31±≤0.01	0.34±0.01
Empty (acetic acid)	4.55±0.02	0.36±≤0.01
Empty (+ ch)	4.23±0.02	0.34±0.02
Loaded (+ ch)	4.50±0.01	0.35±0.01

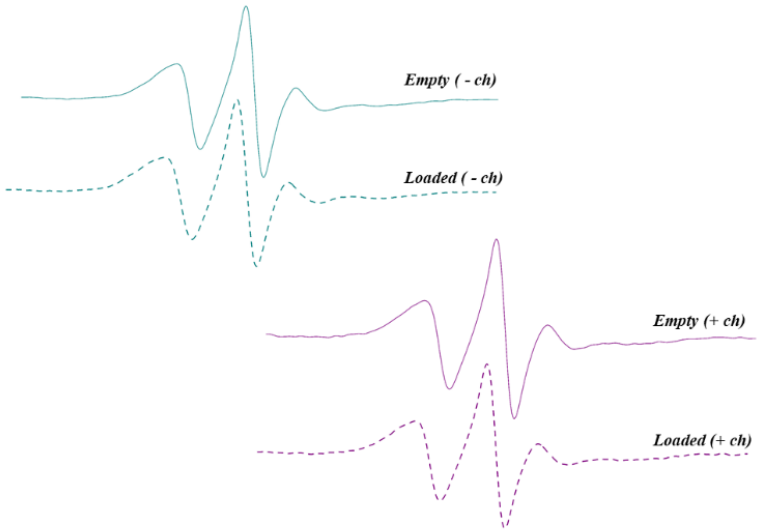


Figure 3. EPR spectra of empty and loaded system in the absence (- ch) and presence (+ ch) of chitosan. Instrumental settings were: center field 349 mT; receiver gain 5.64 x 10^3; time constant 5.12 s; conversion time 5 ms; modulation amplitude 0.4 mT; frequency 9.78 GHz.

3.3. Activity assessment.

Studying the antioxidant profile of the system with the encapsulated GA is of high importance as it proves the retention of GA's activity after the encapsulation in the microemulsion's inner phase. In addition, the antioxidant capacity of the novel system is crucial as it can serve as a useful radical scavenger in nasal cavity. Radical scavengers in nasal cavity can act as protective agents against various diseases due to inflammation responses (polyposis) [70] caused by environmental pollutants (oxidants). These responses can be mediated by the topical application of antioxidants. As Gao et al. [71] have studied, the topical application of natural oils can reduce inflammation by activating intracellular antioxidant pathways or by simple scavenging the reactive oxygen species (ROS). In the present study, the scavenging activity of the systems was studied by following the intensity of the EPR signal of the stable free radical galvinoxyl versus time. Table 3 shows the effect of the incubation time on the percentage of free radical's inhibition. The GA loaded microemulsion shows a high antioxidant activity as it is able to scavenge the total amount of the free radical after 10 minutes of reaction. Additionally, the empty microemulsion also presents a high antioxidant capacity calculated at 69%, at the first minute of reaction. EVOO is known for its high concentration in antioxidants such as hydroxytyrosol, caffeic acid, syringic acid etc. [72] The addition of chitosan in the aqueous phase of the system affects neither the total antioxidant activity of the microemulsion nor the activity of the GA. The above observations make the novel biocompatible system an excellent carrier of natural antioxidants in the nasal cavity in order to diminish any free radicals topically or transport them via nasal route. The addition of chitosan does not provoke changes in the antioxidant activity of the encapsulated molecule making the system appropriate for the co-existence of a hydrophilic antioxidant and the polymer with the mucopenetrating properties. Fig. S3 shows the EPR spectra of galvinoxyl stable free radical after 1 min incubation with the systems of Table 3.

Table 3. % Scavenging activity of empty and loaded system in the absence (- ch) and presence (+ ch) of chitosan towards galvinoxyl free radical versus time.

	% Scavenging activity			
Time (min)	Empty (- ch)	Loaded (- ch)	Empty (+ ch)	Loaded (+ch)
1	69.2±5.2	92.2±0.8	62.1±4.3	93.4±0.8
5	67.4±6.1	97.3±0.9	69.1±3.2	96.7±0.7
10	81.1±5.5	98.6±0.1	73.7±3.1	98.5±0.1
15	86.2±6.8	99.1±0.1	76.7±3.0	99.1±0.2
20	87.6±6.5	99.1±0.1	76.4±3.1	99.1±0.2

3.4 Biological assessment.

3.4.1 Cell proliferation assay.

Cell proliferation assay, in the RPMI 2650 culture, was performed using the MTT assay, a method for sensitive quantification of viable cells. Cells were treated with the empty microemulsions at a range ratio of 0.005% to 0.3% v/v in the culture medium in order to check the viability profile. As can be seen from the Fig.4 the nanocarriers did not exhibit any cytotoxic effect up to the concentration of 0.2% v/v ratio. In the case of 0.3% v/v of the microemulsion in the culture medium the cytoxicity was extremely increased. This increased cytotoxicity can be explained by the presence of the high concentration of surfactants and especially of Tween 80 in cell culture medium, as others have also observed. As Kürti et al. [73] have mentioned, after a 4-h MTT dye conversion cell viability test, Tween 80 above the concentration of 5 mg/mL significantly reduced the viability of RPMI 2650 cells in a dose-dependent way, confirming our findings. In comparison to the most studied Caco-2 cell line, RPMI 2650 is more sensitive to Tween 80, as literature indicates. O'Sullivan has mentioned that incubation of Caco-2 cell culture with 0.8mM of Tween 80 for 24 h showed a viability of 98.3% [74]. The cytotoxicity of a microemulsion with Tween 80 and DMG in the co-culture Caco-2/TC7 HT29-MTX has been previously tested and indicated that even in higher concentrations of the surfactant in the microemulsion system the co-culture can tolerate microemulsion concentrations up to 1% v/v. The above findings indicate that the nasal cell line RPMI-2650 is more sensitive than the commonly used intestinal cell lines. However, the leaky nature of the mucosal epithelium balanced the surfactant sensitivity of the cell culture especially in the presence of carefully selected excipients minimizing the quantities needed. The cytotoxicity profile between microemulsion without and with chitosan towards the RPMI 2650 cells did not change confirming its biocompatible nature and explaining the intensive tries to use this molecule in drug transport. Both exhibited significant cytotoxicity at 0.3% v/v. As a result, the formulated microemulsion can be safely used as local or systemic nasal delivery systems in the mentioned concentrations.

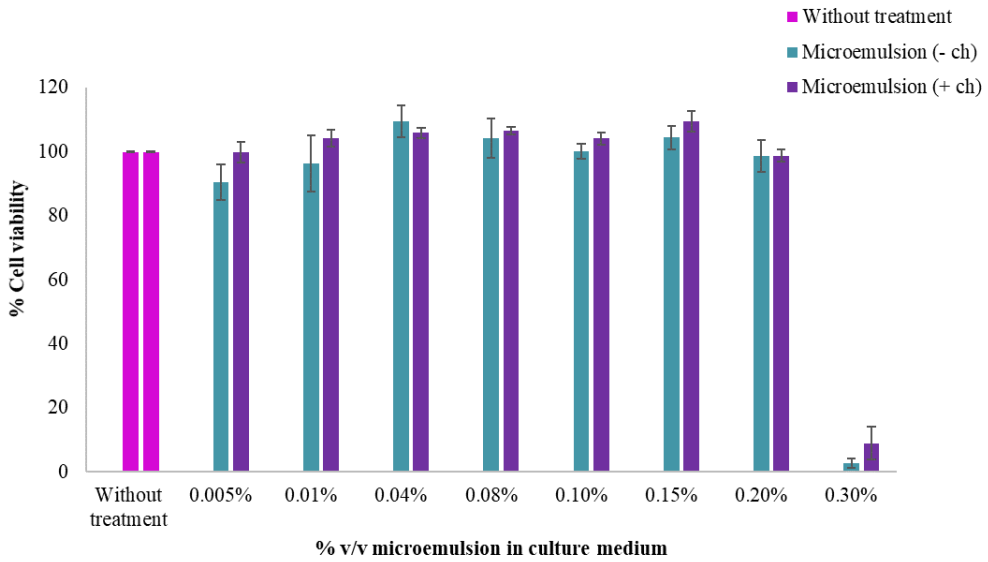


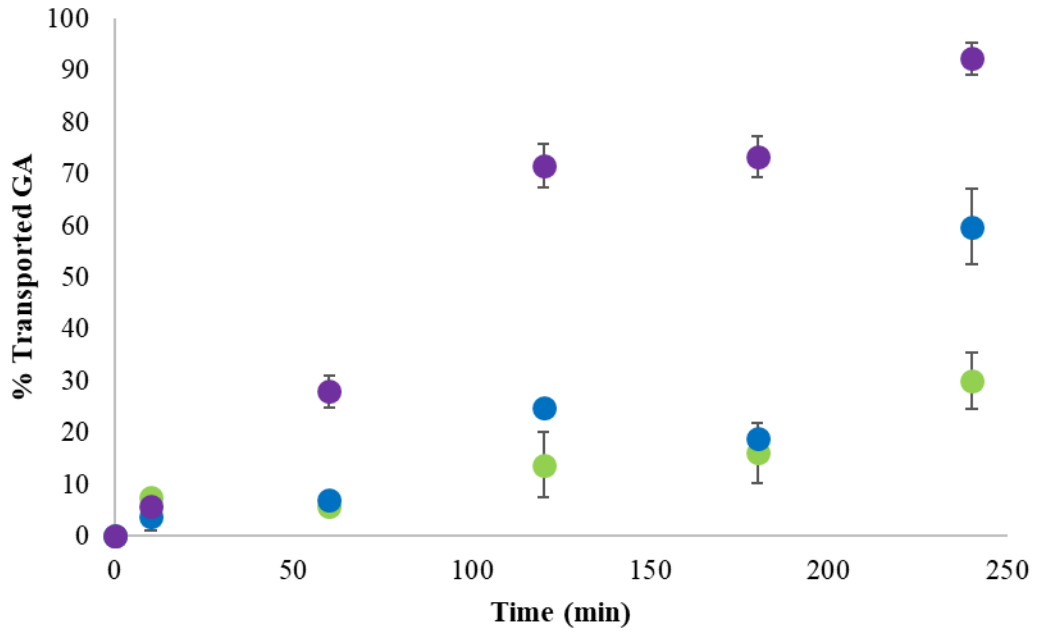
Figure 4. Effect of the empty microemulsions, in a range of concentrations 0.005% - 0.3% v/v in the culture media of the RPMI 2650 using MTT assay. The incubation period of the microemulsion in the culture media was 48 hours. Each column represents the mean ±SD. Green bars represent the empty system without chitosan and the purple the empty system with chitosan in the aqueous phase.

3.4.2 *In vitro* transport studies.

The problem of low nasal permeability rates for polar molecules, including low molecular weight drugs and compounds is reflected in their low bioavailability (from 1% to 10%) [74]. For that reason, many strategies have been used in order to overcome this hurdle. In general, polar compounds with low molecular weight (MW<1000) are able to be transported through the nasal epithelial barrier transcellularly (concentration gradient, vesicular transport mechanisms and receptor mediated transport) or paracellularly (through tight junctions). To confirm the potential permeability enhancement effect of the formulated microemulsion, the total amount of GA that was transported across nasal mucosa model was measured. For this purpose, RPMI 2650 human nasal epithelial cells were cultivated in special filters under ALI conditions. The percentages of GA, in free and encapsulated state, that has been transported through the constructed nasal epithelium towards time of incubation are presented in Fig.5. A calibration curve of GA in the transport medium (HBSS) was constructed with different GA and PC (internal standard, IS) concentrations ranging from 0.05 to 1 μg/mL (see Fig.S1). Also, as GA is susceptible to oxidation phenomena, the oxidation profile of 0.5 μg/mL was studied towards incubation time in the transport medium by LC-MS/MS analysis (see Fig. S2).

The nasal epithelial transport of encapsulated GA follows a similar pattern with the GA aqueous solution. However, after 160 min, the amount of the permeated GA shows substantial differences between the formulations, confirming the greater permeation in the presence of the

574 microemulsion's ingredient. This behavior could be attributed in the presence of different
575 penetration enhancers existing in the microemulsion, such as the phospholipids of the edible oils
576 and the surfactants. Tween 80, fatty acids and phospholipids are some of the most representative
577 ingredients which affect the permeability of the epithelial cell layer by modifying the
578 phospholipid layer, membrane proteins or the outer layer of the mucosa. Some of these
579 enhancers also have an effect on the tight junctions and work as enzymatic inhibitors [24]. This
580 interesting result, amplify the use of nasal route as the most appropriate route for hydrophilic
581 antioxidants encapsulated in microemulsions in comparison to the oral administration. In the
582 latter case, the ingredients of the microemulsion system (EVOO, IPM or mixtures of oils), and
583 especially the surfactants, did not possess any enhancing effect on the transport of
584 hydroxytyrosol, behavior attributed to the interactions of the DMG and the molecule itself with
585 the mucus layer. However, this behavior is not reproduced here as the microemulsion system
586 increased the permeability of GA even 3 h post administration. This may be attributed to the
587 different properties of the mucus layer produced by the nasal and intestinal cell lines. The nasal
588 mucus layer is generally described as less viscous and thicker than the intestinal, environment
589 that facilitates the nasal transport of GA [75].



590
591 **Figure 5.** Transport study of GA acid through the nasal constructed epithelial barrier in
592 solution (●) and encapsulated in microemulsion in the absence (●) and the presence (●) of
593 chitosan solution.
594
595 In the case of the microemulsion with chitosan, the profile changes with a significant increase
596 of polyphenol's transport through nasal epithelium. It has been previously reported, that chitosan
597 increases cell permeability by affecting the tight junctions [75] a fact which is confirmed by the
598 lower TEER values (Fig.6) after the administration of the system. In addition, chitosan in acidic
599 environment is positively charged and its amine groups interact with the mucus layer, affecting

thus, the adhesiveness in the mucosal epithelium. Chitosan induces changes in cellular actin which is one of the major factors affecting the paracellular transport across epithelia. The last years, chitosan has been incorporated in different systems from simple solutions to complex nanoparticles in order to increase the bioavailability of small molecular weight drugs and proteins [43]. The paracellular barrier of epithelial tissue can be evaluated using TEER measurements. TEER values for human nasal mucosa, of approximately 75–100 Ω ·cm^2, have been determined. Thus, an appropriate *in vitro* model of nasal mucosa which has TEER values in the same range as those determined for excised tissue is required. As recently described, by means of Small Angle X-ray Scattering, chitosan also affects the integrity of the mucus layer (gel-nature) by binding with glycoproteins and creating channels for the easier diffusion of the transported molecule [45]. As a result, the encapsulation of chitosan in the microemulsion systems and its subsequent release in the mucus layer facilitates the transport of GA. This strategy could be more efficient in delivery of compounds in comparison to chitosan nanoparticles where the carrier could strongly interact with the mucins, leading to the entrapment of the system in the complex glycoprotein layer. The incorporated chitosan in the case of microemulsions may act as a channel inducer for the transport of the encapsulated compound after its release fact that has been previously reported for another liquid-in-liquid nanodispersion [45].

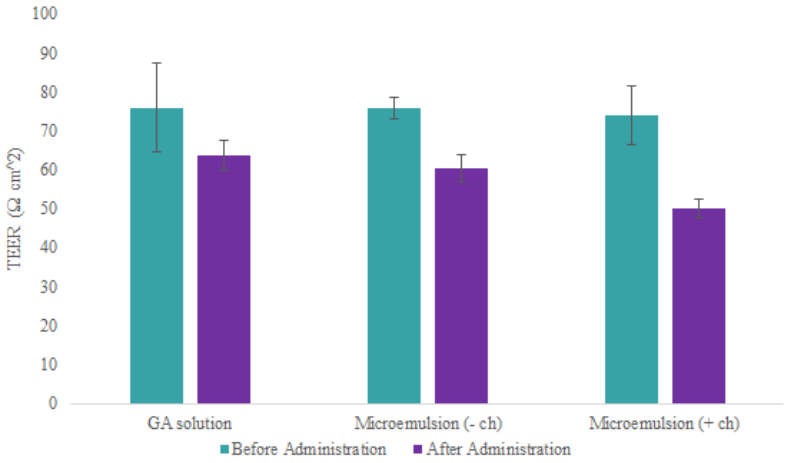


Figure 6. Transepithelial electrical resistance TEER values before and after the 4h apical incubation of the nasal cell lines with free and encapsulated GA. The measurements were taken after 15 minutes of equilibration with HBSS (transport media) in room temperature.

In the case of intestinal epithelial barrier, distribution of GA has been characterized by low transport rate with the most bioactive to be found in the apical side of a constructed in semi-permeable filters cell monolayer indicating paracellular transport [30]. It is known, that the nasal epithelium possesses higher membrane permeability than the intestinal, having a 10 times higher absorption of GA in comparison with an *in vitro* Caco-2 monolayer. As can be seen from Fig. 5 the presence of different enhancers in the culture medium provoked drastic changes in the nasal

transport of GA. Surfactants, less drastically, alter the GA distribution whereas chitosan increases its concentration in the basolateral compartment immediately [76].

Concluding this study, it should be considered that the formulation of appropriate biocompatible carriers such as microemulsions for administration of bioactive compounds is a strategy for both their protection and their efficient epithelial transport (and subsequent bioavailability). Thus, the proposed microemulsion, composed of edible oils and biocompatible surfactants, in the absence of co-surfactants, could be used as carrier of GA, and in general of polar compounds of low molecular weight for pharmaceutical applications. The ability to form a biocompatible microemulsion system with penetration enhancers which can be easily modified with the addition of biopolymers is important. The structural investigation of the proposed system revealed the participation of both GA and chitosan in the surfactant layer of the system which subsequently may affect its availability after nasal administration. The system showed high antioxidant activity and as a result can be used, also, for topical nasal inflammation conditions. The *in vitro* results of the present work, also provide strong evidence about the biocompatibility of the used oils and surfactants but also about their effect on the nasal absorption of small polar compounds. Further experimentation with the proposed system would be useful by introducing different polyphenols and derivatives of chitosan with pharmaceutical interest. A possible application of the GA loaded microemulsion could be a nasal spray for increasing systemic concentration of antioxidants while having a topical anti-inflammatory activity.

4. Conclusions

A non-toxic water-in-oil (W/O) microemulsion was successfully formulated to serve as a nasal carrier for hydrophilic antioxidants. The present system combines: exclusively natural oils, penetration (Tween 80) and mucoadhesion enhancers (chitosan) in a thermodynamically stable system with relatively low surfactant concentration. Gallic acid (GA) was successfully encapsulated while retained its antioxidant activity. The structural study revealed the participation of both molecules (GA and chitosan) in the surfactant monolayer of the system. The system's cytotoxicity was measured with the classic MTT cell proliferation method in the RPMI 2650 cell line and the system did not exhibit cytotoxic effect, in the absence and in the presence of chitosan, up to the threshold of 0.2% v/v in the cell culture medium. Our study confirmed that chitosan, even in low concentration, increased the GA permeability through nasal epithelium while affecting the tight junctions of the cell layer.

Overall, this study demonstrates that biocompatible microemulsion systems, formulated by edible oils, can be used in order to protect the sensitive antioxidant molecules with reported pharmaceutical activity, until the time of administration. Moreover, their ingredients are able to affect, in a positive way, the permeability profile of the bioactive molecules through constructed epithelia making it a promising absorption enhancing system for nasal delivery of antioxidants for reaching the systemic circulation or the brain. Even though, RPMI 2650 is one of the most

studied cell lines for nasal *in vitro* assays, the study, especially of cytotoxicity must be conducted with the use of primary physiological cell lines in order to ensure that the microemulsion will not affect the normal cells. The differences observed regarding the intestinal and nasal absorption of antioxidants encapsulated in microemulsions indicate that the mucosal epithelium of nose is more sensitive to microemulsion's ingredients in contrast to the intestinal, making the nasal administration appropriate for the delivery of those compounds. Future *in vivo* data will quantify the distribution of GA in the systemic circulation and its possible localization in brain.

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