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Tesi

Membrane Emulsification to develop biohybrid microstructured and multifunctional systems

Settore Scientifico Disciplinare CHIM07 – Fondamenti chimici delle tecnologie

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Sommario

L’emulsificazione a membrane è una nuova ed alternativa tecnologia introdotta nei primi anni ’80 per la preparazione di emulsioni o sospensioni attraverso l’utilizzo di strutture microporose. Grazie all’applicazione di basse forze di taglio, il processo risulta a basso consumo energetico. Inoltre, l’opportuna scelta delle proprietà e delle caratteristiche delle membrane utilizzate e dei parametri operativi permette di ottenere particelle di dimensione e distribuzione controllata, massimizzando l’uso delle risorse impiegate.

La versatilità e le potenzialità dell’emulsificazione a membrana sono state provate dal suo utilizzo nella preparazione di una vasta gamma di materiali microstrutturati con dimensioni e distribuzione altamente controllate e preparate a partire da materie prime di diverso tipo e origine tra cui proteine, oli, solventi organici, materiali polimerici. La tecnologia ha dimostrato inoltre proprietà esclusive per la preparazione di materiali contenenti molecole sensibili a stress termici o meccanici. Il processo risulta a basso impatto ambientale ed economicamente sostenibile. Queste caratteristiche rendono l’emulsificazione a membrana capace di rispondere alla crescente richiesta sia di prodotti ad alto valore aggiunto che di processi che siano nuovi, precisi, stabili, efficienti, eco-compatibili, a basso consumo energetico e con ridotti costi.

Le ricerche condotte nel presente lavoro di dottorato hanno permesso di promuovere avanzamenti tecnologici nel processo di emulsificazione a membrana (sia in quello denominato “a flusso tangenziale” che in quello condotto mediante celletta agitata) ed ha riguardato l’applicazione di tale processo alla preparazione di innovative formulazioni microstrutturate.

Il lavoro è stato supportato da una continua analisi critica del processo di emulsificazione a membrana e delle sue principali applicazioni, presentata nel **Capitolo 1**. Il grado di sviluppo del processo a livello industriale è stato inoltre valutato anche attraverso lo studio dei brevetti pubblicati ed è discusso nel **Capitolo 2**.

In particolare, gli avanzamenti tecnologici proposti dal presente lavoro di tesi nel settore dell’emulsificazione a membrana hanno riguardato:

- l’influenza delle proprietà chimico-fisiche dei materiali impiegati e dei parametri operativi nella preparazione di sistemi liquidi dispersi (**Capitolo 3**);
- l’influenza dell’utilizzo di proteine nel processo di emulsificazione a membrana (**Capitolo 4**);

- lo sviluppo di membrane con differente bagnabilità tra lo spessore e la superficie interna della membrana (**Capitolo 4**);
- nuove strategie operative per promuovere il distaccamento delle goccioline di emulsione in formazione in corrispondenza del poro della membrana (**Capitolo 5**).

Le formulazioni microstrutturate innovative sviluppate nel presente lavoro di tesi, tramite l'utilizzo del processo di emulsificazione a membrana, hanno riguardato:

- la preparazione di emulsioni multiple capaci di rispondere a stimoli esterni (**Capitolo 6**)
- la realizzazione di un bioreattore multifasico microstrutturato contenete un biocatalizzatore a trasferimento di fase all'interfaccia dell'emulsione o/w (**Capitolo 7**)
- la preparazione di emulsioni semplici complessanti il ferro per possibili applicazioni in catalisi chimica (**Capitolo 8**)
- particelle solido-liquide con dimensioni dei micron o sub-micron e gli accorgimenti tecnologici relativi necessari (**Capitolo 9**)

Le linee guida sviluppate nel presente lavoro di tesi sono riassunte nel diagramma di pag. 8 (Fig. 1) mentre i principali avanzamenti proposti , rispetto allo stato dell'arte, sono presentati nella tabella a pag. 10 (Table 1).

Complessivamente, sono stati promossi avanzamenti del processo di emulsificazione a membrana per quanto riguarda diversi aspetti tecnologici, modi operativi, composizione delle fasi, impiego in nuove formulazioni, flessibilità e versatilità per diverse applicazioni.

Summary

Membrane emulsification technology permits the preparation of high-value emulsions/suspensions via microporous architectures in which very well controlled local shear rates are obtained, leading to a process with much lower energy use and more uniform or size controlled emulsions/suspensions than conventional high-speed mixers, allowing maximization of resources use.

The robustness and flexibility of the technology has been proven by its capability to micro-manufacture a variety of products, such as microstructured multi-material droplets with target particle size and size distribution and encapsulated formulations. The technology has confirmed unique feasibility for formulations containing labile molecules and structures, and is also recognized among the most sustainable and safe manufacturing processes. These aspects are crucial to meet the demanding requirements for new, diverse, stable and efficient high value-added chemical products and plants with reduced investment cost and environmental impact.

The work presented in this thesis discusses the technological advances of cross-flow and stirred direct membrane emulsification process and its application in innovative microstructured formulations.

An overview of principles and applications of membrane emulsification process will be given in the **Chapter 1**. The patents analysis presented in the **Chapter 2** aimed at further assessing the state of the art and perspectives in the membrane emulsification process.

The technology advances of membrane emulsification process achieved in the present PhD research project are refereed in particular to:

- influence of dispersion system properties and operational parameters in the preparation of dispersed liquid materials (**Chapter 3)**
- evaluation of protein influence on membrane emulsification process (**Chapter 4)**
- development of an asymmetric membrane with different wettability properties between membrane thickness and lumen surface (**Chapter 4)**
- new operational strategies to promote droplets detachment from the pore outlet (**Chapter 5)**

The innovative microstructured formulations include:

- stimulus responsive multiple emulsions (**Chapter 6)**
- microstructered multiphase bioreactor (**Chapter 7)**
- iron-emulsion complex in chemical catalysis (**Chapter 8)**

- productions of solid lipid sub-micron particles at high temperature and related technological challenges (**Chapter 9)**

The overall strategy of work is summarized in Fig.1 and the advances promoted by the research activity are summarized in Table 1 and compared with the state of the art.

Work overall strategy

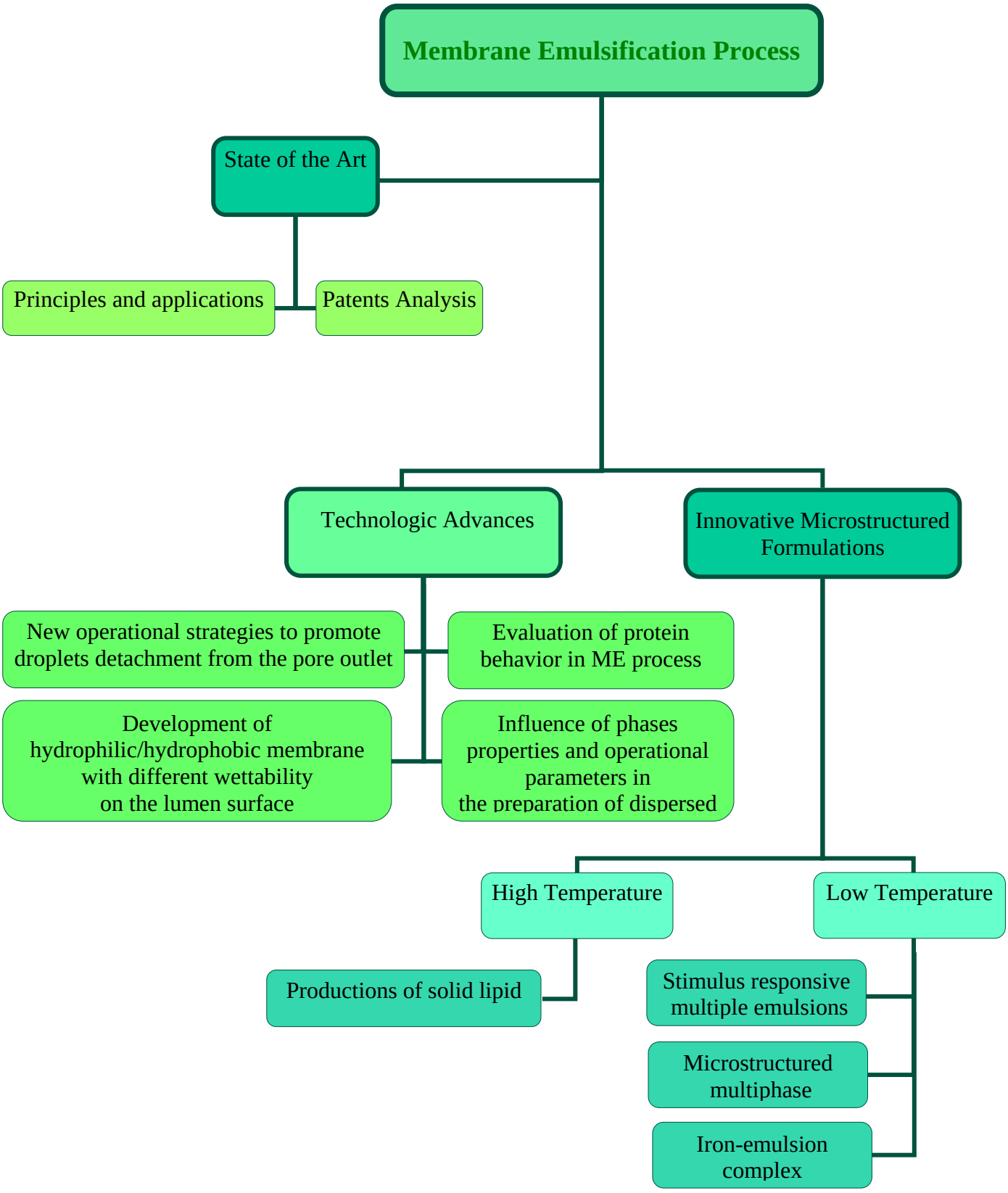


Fig.1 Flow sheet illustrating the overall strategy of the work carried out

Objectives

The research focus was on the development and characterization of membrane emulsification processes (cross-flow and stirred membrane emulsification) exploring new solutions to improve the efficiency of emulsification process with particular attention to production of micro- structures that for their specific applications and properties required low shear stress conditions. For this reason new strategies are investigated to:

- reduce shear stress condition in the conventional cross-flow membrane emulsification process
- identify new operating conditions for detachment of droplets from the pore outlet, “vibrating” or “shaking” the continuous phase during the emulsification process by inverting axial flow direction
- investigate the combined effect between shear stress conditions and continuous phase viscosity
- understand the protein-membrane interaction when protein are used as ingredients in emulsion preparation
- develop strategies to improve disperse phase flux, i.e. to improve membrane emulsification productivity reducing energy input

In this way the membrane emulsification process could be used to design droplets with specific size and size distribution for manufacturing innovative functional multi-material structures able to functions as receptor stimulus for applications in different industrial sectors (such as chemical, biomedical, biotechnology or food).

The advances of the present work beyond the-state-of the-art are illustrated in Table 1.

Table 1. Advances promoted

State-of-the-art	Advances promoted by this thesis
The influence of phase compositions and process parameters are showed in the preparation of oil-in-water emulsions but few studies have been conducted on the preparation of water-in-oil emulsions.	The correlation between emulsions phase compositions and shear stress conditions have been identified for the preparation of water-in-oil emulsion by stirred membrane emulsification. This results can be applied at the production of dispersed systems by membrane emulsifications processes in which continuous phase with high viscosity are required to reduce emulsions destabilization phenomena caused by particles' settling velocity (Stokes law).
When membrane emulsification process is used, low dispersed phase flux is usually required in order to obtain small droplets size but this determine low process productivity. The opportune choice of membrane properties permits to control dispersed phase flux during membrane emulsification.	The use of an asymmetric membrane, opportunely developed modifying wettability membrane surface only in the lumen side, permit to increase aqueous dispersed phase flux trough an hydrophilic membrane and control droplets size.
Proteins are used as components in emulsions preparations for food, biotechnological, pharmaceutical or cosmetic applications. When membrane emulsification process is used, membrane-protein interaction can be occur. This can determine membrane fouling that influence membrane process performance and availability of functional ingredients	The interaction between proteins with different properties and membranes is investigated in order to understand and prevent fouling phenomena during membrane emulsification process
For cross-flow membrane emulsification, in which shear stress is generated by the recirculation of the continuous phase, droplets breaking is observed due to the shear force caused by the flow along the circuit.	Prevent droplets breaking: selecting low shear pump, applying high axial velocity but low continuous phase flow, “vibrating” “shaking” the continuous phase along the membrane surface by inverting the axial flow direction. This strategy is expected to reduce recirculation of formed emulsion and increase disperse phase concentration for single passage along the module.
Stimulus responsive materials have been extensively investigated. Most of this are hydrogels or polymeric materials. Few examples of liquid membrane systems (such as water-in-oil-in- water emulsions) are used as stimuli responsive biomaterials.	The manufacturing of multiple emulsions as self-regulated drug delivery systems are investigated using highly controlled production of particulate materials at low shear conditions by membrane emulsification process.

Phase transfer biocatalysts are usually distributed at the interface by stirred tank reactor (STR) but some disadvantages are observed such as enzyme deactivation and non constant interfacial area.	Using membrane emulsification, the enzyme is distributed at the interface and used at the same time as catalyst and as surfactant.
The Fenton process has been typically used for the partial oxidation of organic contaminants using Fe(II) ions as homogeneous catalyst. Thus, the catalyst continuously leaves the oxidation reactor together with the treated effluent often causing environmental problems and increasing the treatment costs.	The use of Fe(II) ions confined in emulsion droplets could avoid the problems related to the homogeneous catalysis. The appropriate operating conditions for the preparation of stable oil/water droplets containing Fe(II) ions are evaluated for ensuring the adequate development of one of major interest of producing stable Fe(II) – containing emulsions as catalyst.
The preparation of solid lipid particles (SLP) using membrane emulsification has been developed.	The influence of process parameters on SLP size and size distributions at high temperature and high dispersed phase amount percentage is obtained achieving sub-micron sized droplets.

State of the art

Chapter 1

Membrane Emulsification: Principles and Applications

1.1. Introduction

Emulsions and suspensions are colloidal dispersion of two or more immiscible phases in which one phase (disperse or internal phase) is dispersed as droplets or particles into another phase (continuous or dispersant phase). Therefore, various types of colloidal systems can be obtained. For example, oil/water and water/oil single emulsions can be prepared, as well as so-called multiple emulsions, which involve the preliminary emulsification of two phases (e.g., w/o or o/w), followed by secondary emulsification into a third phase leading to a three phase mixture, such as w/o/w or o/w/o. Suspensions where a solid phase is dispersed into a liquid phase can be also obtained. In this case solid particles can be i) micro-spheres, e.g. spherical particles composed of various natural and synthetic materials with diameters in the micrometer range: solid lipid microspheres, albumin micro-spheres, polymer micro-spheres; and ii) capsules, e.g. small, coated particles loaded with a solid, a liquid, a solid-liquid dispersion or solid-gas dispersion. Aerosol, where the internal phase is constituted by a solid or a liquid phase dispersed in air as continuous phase represents another type of colloidal system.

In emulsions and suspensions, disperse phase dimensions may vary from the molecular state to the coarse (visible) dispersion. They are commonly encountered in various productions. The average droplet/microcapsules size distribution is a key feature since they determine emulsions/suspensions properties for the intended uses and stability. For large-scale emulsion production, the most commonly employed methods are based on techniques aiming at establishing a turbulent regime in the fluid mixtures. These turbulent flows cannot be controlled or generated uniformly. The consequences are that the control of the droplet sizes is difficult and wide size distributions are commonly obtained, therefore the energy is used inefficiently in these technologies. In addition, the process scale-up is extremely difficult. The

use of the ultrasonic bath yields better results with respects to the mentioned procedures, however the control of the droplet dimension is still not optimal.

For these reasons, recently much attention has been put in alternative emulsification processes, such as the membrane emulsification (ME).

Membrane emulsification is an appropriate technology for production of single and multiple emulsions and suspension. It was proposed for the first time at the 1988 Autumn Conference of the Society of Chemical Engineering, Japan. Since then, the method has continued to attract attention in particular in Japan, but also in Europe (1-10).

In the early 1990s, Nakashima T. et al. [2] introduced membrane technology in emulsions preparation by direct emulsification method whereas, in the late 1990s, Suzuki K. et al. used the pre-mix membrane emulsification to obtain production rate higher than other membrane emulsification methods [11].

The fast progress in micro engineering and semi conductor technology led at the development of micro-channels, that Nakajima M. et al. applied in emulsification technology [12].

The distinguishing feature of membrane emulsification technique is that droplets size is controlled primarily by the choice of the membrane, its micro-channel structure and few process parameters, which can be used to tune droplets and emulsion properties. Comparing to the conventional emulsification processes, the membrane emulsification permits to obtain a better control of droplet size distribution, low energy and materials consumption, modular and easy scale-up. Nevertheless, productivity (m^3/day) is much lower, and therefore the challenge in the future is the development of new membranes and modules to keep the known advantages and maximize productivity.

Considerable progress has been achieved in understanding the technology from the experimental point of view, with the establishment of many empirical correlations. On the other hand, their theoretical interpretation by means of reliable models is not accordingly advanced. The first model devoted to membrane emulsification, based on a torque balance, has been proposed in the 1998 by S.J. Peng and R.A. Williams [13], i.e. ten years later the first experimental work was published, and still nowadays, a theoretical work aiming at a specific description of the pre-mix membrane emulsification process is not available.

The non synergistic progress of the theoretical understanding with the experimental achievements, did not refrain the technology application at productive scale. In particular, membrane emulsification was successfully applied for preparation of emulsions and capsules having high degree of droplet size uniformity, obtained with low mechanical stress input [14-16]. Therefore, the application of membrane emulsification extended to various fields, such

as drug delivery, biomedicine, food, cosmetics, plastics, chemistry and some of these applications are now being developed at commercial level. Their scale vary from large plants in the food industry, to medium-scale use in the polymer industry, and to laboratories bench scale in biomedicine.

In this chapter, the experimental and theoretical bases as well as the applications of the technology will be discussed.

1.2. Membrane emulsification basic concepts

Emulsions and suspensions are key systems for advanced formulations in various industrial sectors. Membrane emulsification is a relatively new technology in which membranes are not used as selective barriers to separate substances but as microstructures to form droplets with regular dimensions, i.e. uniform or controlled droplets size distribution (Fig. 1.1).

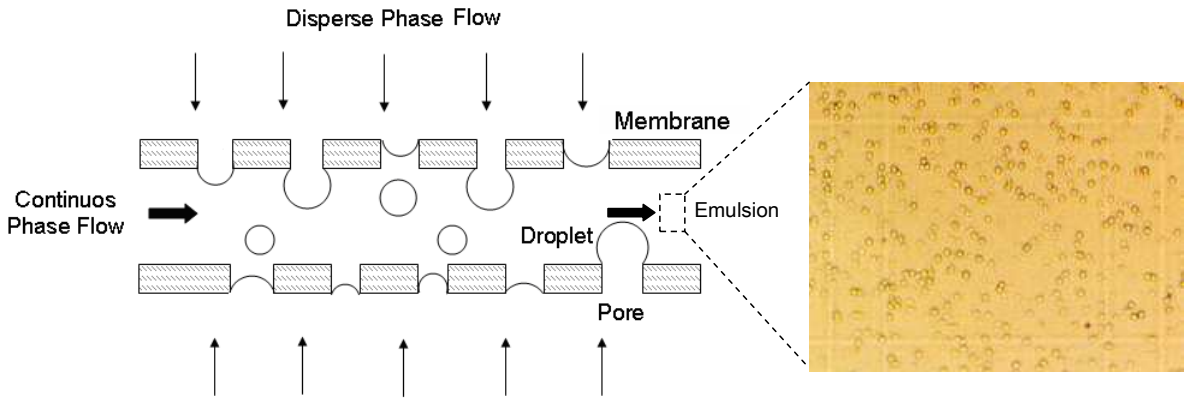


Fig. 1.1. Schematic representation *a)* of membrane emulsification, where the membrane works as a high throughput device to form droplets with regular dimensions; *b)* photo of an o/w emulsion.

Membrane emulsifications can be generally distinguished in (Fig. 1.2): (i) direct membrane emulsification (DME), in which the disperse phase is directly fed through the membrane pores to obtain the droplets. and (ii) pre-mix membrane emulsification, in which a coarse pre-mixed emulsion is pressed through the membrane pores to reduce and to control the droplet sizes.

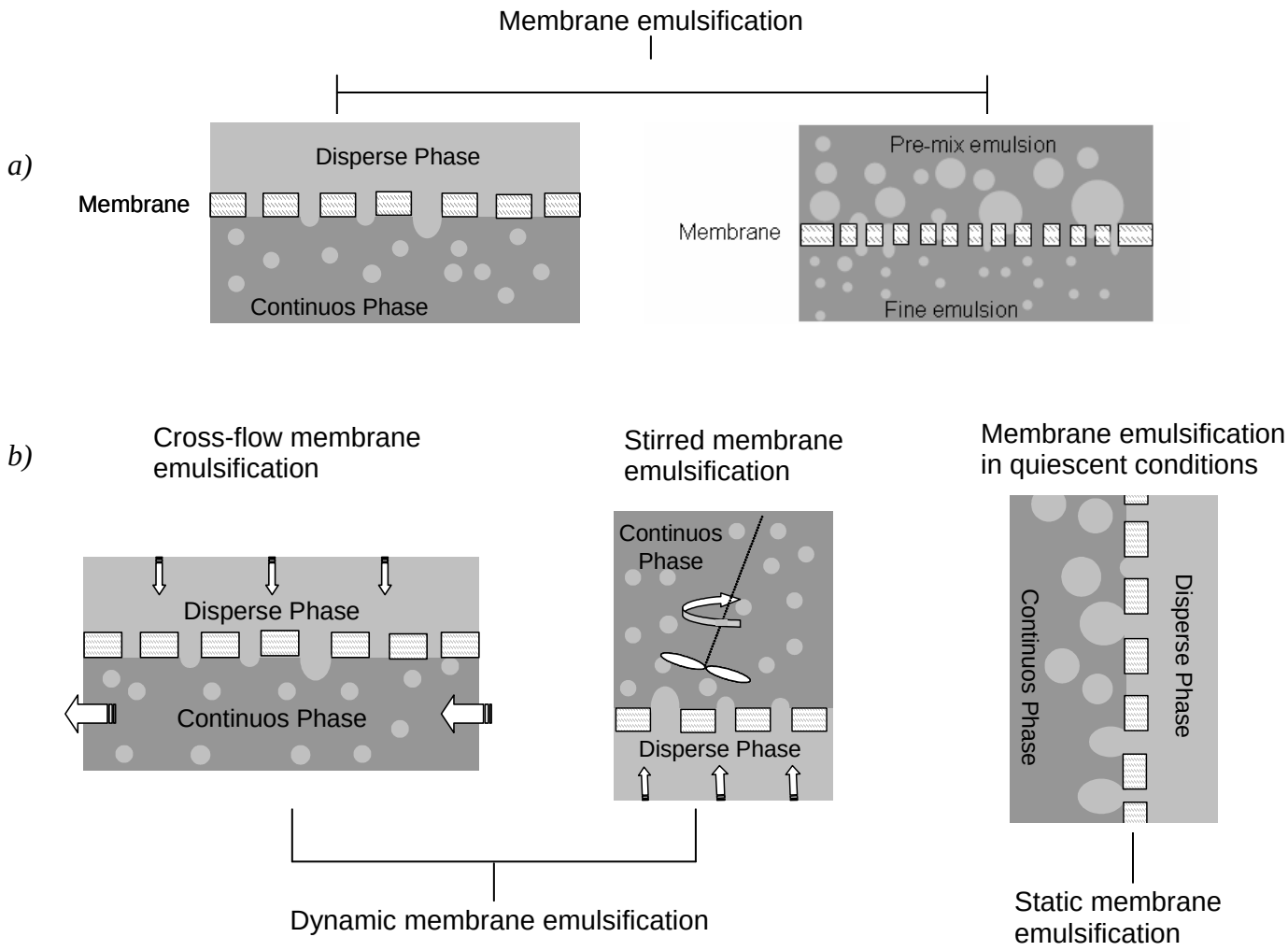


Fig. 1.2. Schematic drawing of membrane emulsification: *a)* mechanisms *b)* operation procedures.

In general, in the direct membrane emulsification, the disperse phase is pressed through a microporous membrane and droplets are formed at the opening of the pore on the other side of the membrane, which in contact with the continuous phase. Here, droplets reached a critical dimension can detach either for *spontaneous deformation* or *sheared by the continuous phase* flowing parallel to the surface. In the former case, the driving force for the droplet formation is the surface free energy minimization, that is, the droplet is formed by spontaneous deformation tending to form a sphere. For example, in quiescent conditions the droplets are formed by means of this mechanism. In the latter case, the shearing stress generated by the continuous phase is the driving force of the droplet detachment. For example, in the cross-flow membrane emulsification (CDME) and stirred membrane emulsification droplets are formed by this mechanism.

In the pre-mix emulsification the basic mechanism for the droplet formation is different from the direct emulsification. In fact, in this case the predominant formation mechanism is the droplet disruption within the pore.

Both direct and pre-mix emulsification can be obtained with a continuous phase flowing along the membrane surface (i.e. cross-flow, stirring) (Fig. 1.2b). However, it is important to distinguish between the droplet formation mechanism and the macroscopic operation procedure. In other terms, often, in the literature, the “cross-flow” term is used to indicate that the continuous phase is flowing along the surface, but this does not guarantee that the shear stress is the driving force for the droplet detachment, as long as the appropriate conditions are not verified.

The membrane emulsification can be considered as a case of micro-device emulsification process [17, 18] in which the porous membrane is used as micro-devices. Membrane emulsification carried out in quiescent conditions is also referred to as *static membrane emulsification*, while membrane emulsification carried our in moving conditions (either the membrane, i.e rotating module, or the phase, i.e cross-flow) is also referred to as *dynamic membrane emulsification* (Fig. 1.2b).

A peculiar advantage of membrane emulsification is that both droplet sizes and size distributions may be carefully and easily controlled by choosing suitable membranes and focusing on some fundamental process parameters reported below. Membrane emulsification is also an efficient process, since the energy density requirement (energy input per cubic meter of emulsion produced, in the range of 10^4-10^6 Jm^{-3}) is low with respect to other conventional mechanical methods (10^6-10^8 Jm^{-3}), especially for emulsions with droplet diameter smaller than 1 μm [1]. The lower energy density requirement also improves the

quality and functionality of labile emulsion ingredients, such as bioactive molecules. In fact, in conventional emulsification methods, the high shear rates and the resulting increase of the process temperature have negative effects on shear- or temperature-sensitive components. The shear stresses calculated for a membrane system are much less and it is possible to process shear sensitive ingredients.

The droplet size, its dispersion and the droplet formation time depend on several parameters: (i) *membrane parameters*, as pore size distribution, pore border morphology, number of active pores, porosity, wetting property of the membrane surface, (ii) *operating parameters*, as cross-flow velocity (i.e. wall shear stress), transmembrane pressure and disperse phase flow, temperature, as well as the membrane module used (tubular, flat, spiral-wound);and (iii) *phase parameters*, as dynamic interfacial tension, viscosity and density of processed phases, emulsifier types and concentration. Such quantities combine with different magnitudes, over the ranges of operating conditions, and many of them exhibit coupling effects [4]. Moreover, the production of mono-disperse emulsions is essentially related to the size distribution of membrane pores and their relative spatial distribution on the membrane surface. It is worth noting that the geometry of the module in which the membrane is located is also an important parameter since it determines in conjugation to the cross-flow velocity, the wall shear stress (Fig. 1.3).

Droplets size distribution and disperse phase percentage determine the emulsion properties characterizing the final formulation for an intended use.

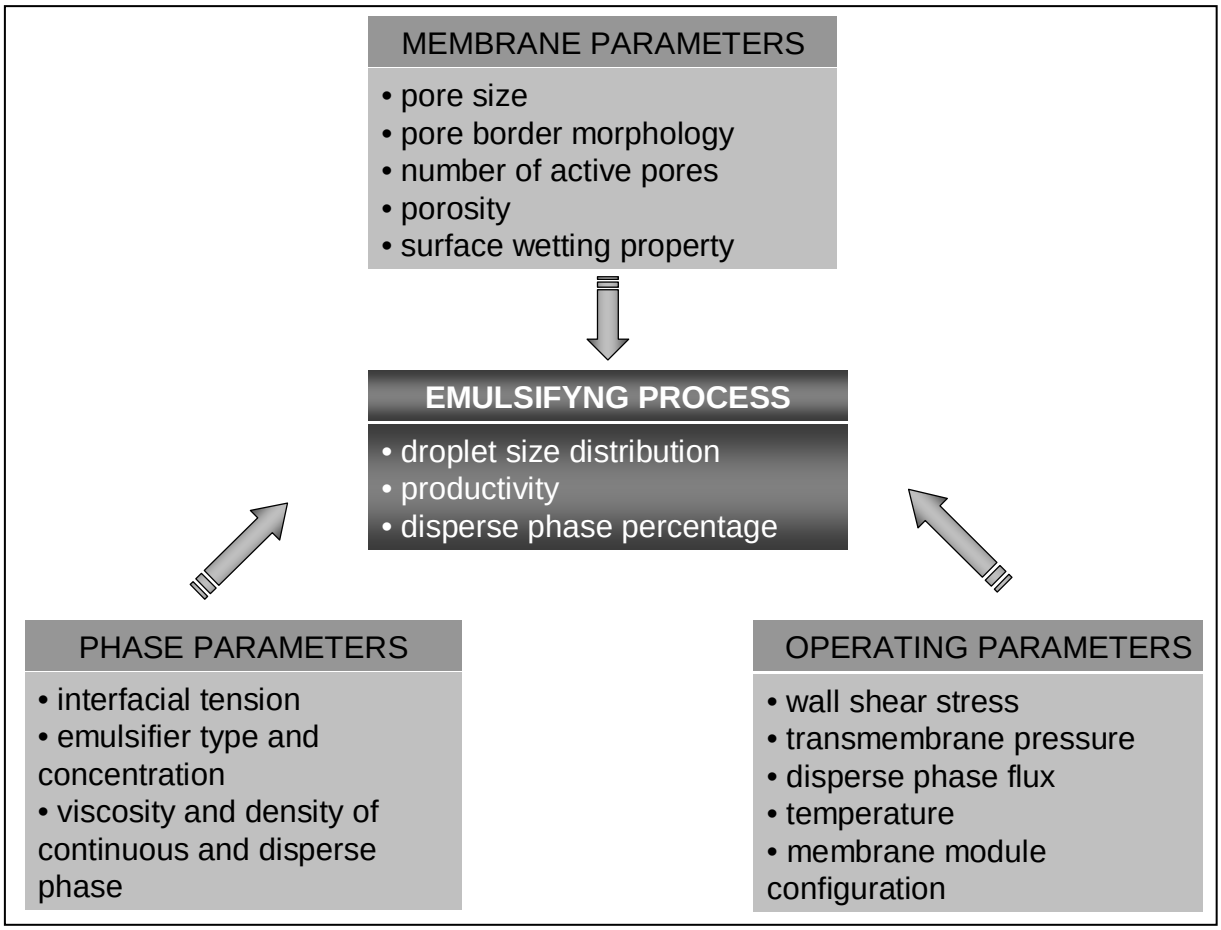


Fig. 1.3. Influence of parameters on droplet size and its formation during an emulsification process.

1.3. Experimental bases of membrane emulsification

In this section, an analysis of the experimental observations and empirical correlations related to membrane emulsification processes will be illustrated. The theoretical bases that support these results and predict membrane emulsification performance will be discussed in the next section.

As previously anticipated, the appropriate choice of the membrane dictates the droplet properties.

Membranes employed in emulsification processes are mainly of inorganic type (ceramic, glassy, metallic), but some example of polymeric membranes have also been applied. Tables 1.1 and 1.2 summarize some of the most common membranes used in direct and pre-mix membrane emulsification, respectively. Most of them have been originally developed for other membrane processes, such as microfiltration, and adapted in the emulsification technology. Nowadays, the growing interest towards membrane emulsification is promoting also research efforts in the design and development of membranes specifically devoted to membrane emulsification. Shirasu porous glassy (SPG) membranes are among the first membranes specifically developed for emulsion preparation. SPG membranes are characterized by interconnected micropores, a wide spectrum of available mean pore size (0.1-20 μm) and high porosity (50-60%). Micropore metallic membranes, developed by Micropore Technologies (United Kingdom), are characterized by cilyndrical pores, uniform and in a regular array with a significant distance between each pore. They are available with pore diameters in the range of 5 – 20 μm and exhibit very narrow pore size distribution.

Table 1.1. List of most common membranes used in direct membrane emulsification.

Membrane material / Wetting property	Membrane Configuration	Pore Geometry / Porosity (ε)	Pore diameter (μm)	Membrane producer	Application	Reference
Porous Glass Membrane						
SPG / hydrophilic or hydrophobic	Tubular or Disk	Tortuous, interconnected cylindrical pore/ 50% < ε < 60%	0.1-20	SPG Technology Co., Ltd. (Japan)	o/w, w/o emulsions	16, 19-21
MPG / hydrophilic	Tubular or disk	Cylindrical pore/ 50% < ε < 60% -	0.2 -1.36; 10.2 - 16.2	ISE Chemical Industries Co. Ltd (Japan)	o/w and w/o emulsions	21-23
Silica glass / hydrophobic	Tubular	Cylindrical pore/ ε = 61%	0.6	Lab-made	w/o emulsions	24
Ceramic Membrane						
Mullite Ceramic / hydrophilic	Disk	-	0.68	Lab-made	w/o emulsions	25
Alumina / hydrophilic	Tubular	ε = 35%	0.1-0.8	Westfalia Separator Membraflow (Germany)	o/w, w/o/w emulsions	5, 26-28
			0.5-0.2	Société des Céramiques Techniques (France)	o/w emulsions	
			0.5-0.8	Pall-Exekia (France)		
Zirconia / Hydrophilic	Tubular	ε = 60%	0.1	Pall-Exekia (France)	o/w emulsions	27, 28
				Société des Céramiques Techniques (France)		
Polymeric Membrane						
Polyamide / hydrophilic	Hollow fiber	-	10; 50 kDa (NMWCO)[1]	Forschstung Insitut Berghof, (Germany)	o/w emulsions	29
PTFE / hydrophobic	Disk	ε = 79%	0.5-50	Japan Goretex Co. (Japan)	o/w emulsions	30
Polycarbonate / hydrophilic	Disk	5% < ε < 20%	10	ISOPORE™, Nihon Millipore Co. (Japan)	o/w emulsions	31
Cellulose acetate / hydrophilic	Disk	-	0.2-03	Advantec Toyo (Japan)	w/o/w emulsions	32
Polipropilene / hydrophobic	Hollow fiber	-	0.4	Wuppertal (Germany)	w/o emulsions	14

[1] Nominal Molecular Weight Cut-Off

Table 1.2. List of most common membranes used in pre-mix membrane emulsification.

Membrane material	Membrane configuration	Operation mode	Pore diameter (μm)	Application	Membrane producer	References
Porous Glass Membrane						
SPG	Tubular	Cross flow	2.7 and 4.2	o/w emulsions	SPG Technology Co., Ltd. (Japan)	11
		Dead end, multipass	10.7	w/o/w emulsions		15
Ceramic Membrane						
Alumina / hydrophilic	Tubular	Dead end, multipass	3.2, 4 and 11	w/o/w emulsions	Lab-made	33
Polymeric Membrane						
Polycarbonate	flat	Dead end, multipass	0.6, 0.8 and 3.0	o/w emulsions	Millipore corporation (United State)	34
Cellulose acetate	flat	Dead end	0.2, 0.45, 0.8 and 3.0	w/o/w emulsions	Advantec Toyo (Japan)	35
Polyamide	flat	Dead end	0.8	o/w emulsions	Whatman Intl. Ltd., Maidstone (England)	36

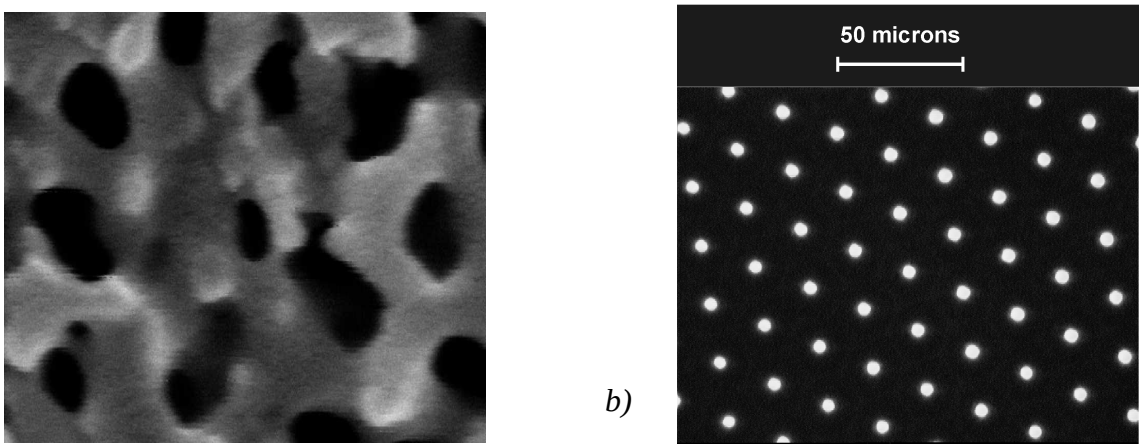


Fig. 1.4. Porous membranes developed for emulsification processes. *a)* Shirasu porous glassy membrane (from SPG Technology Co., LTD, Japan), *b)* metallic membrane (from Micropore Technologies, United Kingdom).

Membrane wetting properties may carefully be considered in the membrane selection. In general, the membrane surface where the droplet is formed should not be wetted by the disperse phase. Therefore, a w/o emulsion is prepared using a hydrophobic membrane and an o/w emulsion is prepared using a hydrophilic membrane. On the other hand, w/o and o/w emulsions were successfully prepared using pre-treated hydrophilic and hydrophobic membranes, respectively. The pre-treatment basically consisted in absorbing the continuous phase on the membrane surface so that to render the membrane non-wetted by the disperse phase [14, 23, 25]. The presence of emulsifier in the disperse phase represents another strategy that permits the preparation of emulsions with membrane wetted by the disperse phase.

The dispersion of droplet diameter mainly depends upon the membrane pore. In general, a linear relationship between membrane pore diameter (D_p) and droplet diameter (D_d) has been observed, especially for membranes with pore diameters larger than 0.1 micron. In these cases, linear coefficients varying between 2 - 10, depending on the operating conditions and emulsion composition, have been obtained [3, 23, 27]. Fig. 1.5 summarizes the behaviour of the mentioned relationships for different emulsion systems. In general, for a certain emulsion type and in comparable operating conditions, the lower the pore size the lower the droplet size.

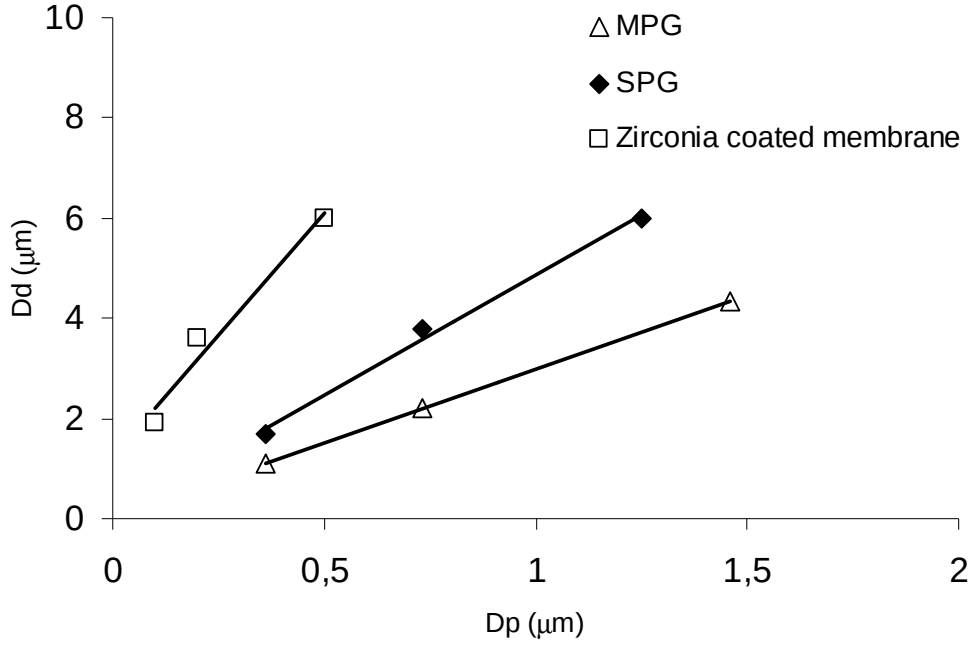


Fig. 1.5. Relationship between membrane pore diameter (D_p) and droplet diameter (D_d) (Data extrapolated from [3, 23, 27]).

Fluid dynamic operating conditions, such as axial or angular velocity (i.e. shear stress that determines drag force value) and transmembrane pressure (that determines disperse phase flux, for a given disperse phase viscosity and membrane properties), can be properly adapted to tune emulsion properties. The common observed behaviour of shear stress and disperse phase flux on D_d/D_p ratio is depicted in Figs. 1.6 and 1.7, respectively. The droplet size decreases with increasing of shear stress at the membrane surface and decreasing of disperse phase flux. However, the latter influence is less predominant and depends on the droplet formation time, which in turn is strongly affected by the interfacial dynamical tension. If the droplet formation time is larger than the complete adsorption of the emulsifier (equilibrium interfacial tension) the lower the influence of disperse phase flux. Therefore, in appropriate conditions and for emulsions with droplet size over the micron (1-50 micron, so called macroemulsions), transmembrane pressure may influence the disperse phase flux, but have little influence on changing of droplet size.

Dynamic interfacial tension, therefore emulsifier used, and related adsorption kinetics influence the emulsification process. In general, the faster an emulsifier adsorbs to the newly formed interface, the lower the interfacial tension the smaller the droplet produced. Fig. 1.8 shows a linear behaviour between D_d/D_p ratio and interfacial tension.

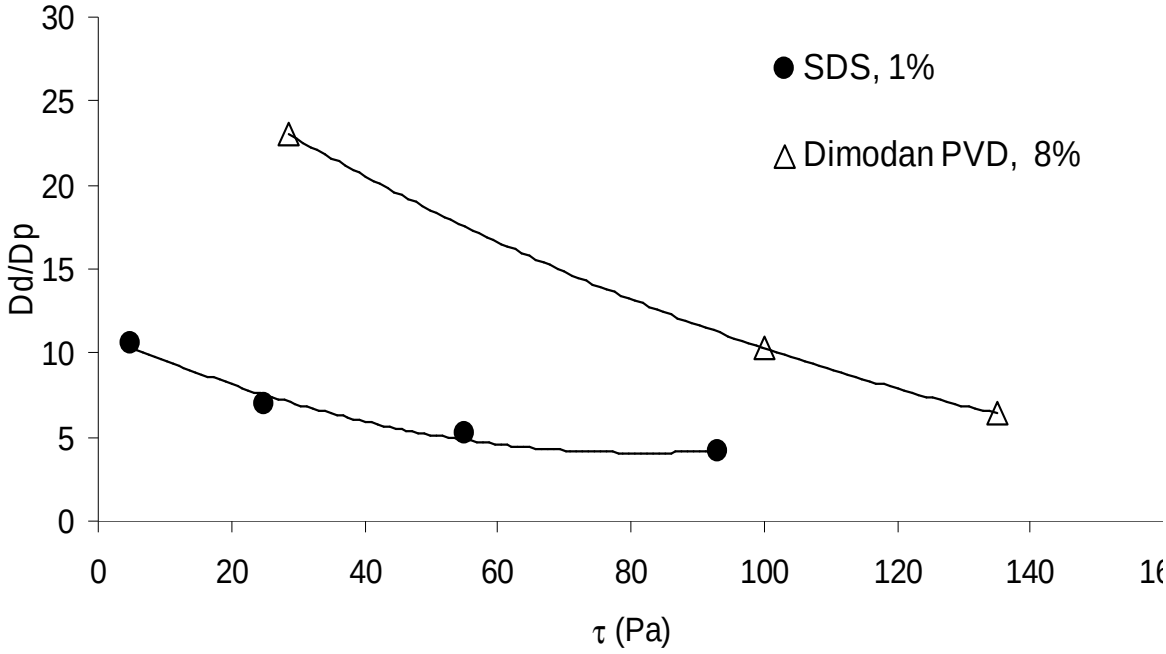


Fig. 1.6. Relationship between wall shear stress (τ) and D_d/D_p (Data extrapolated from [27, 28]).

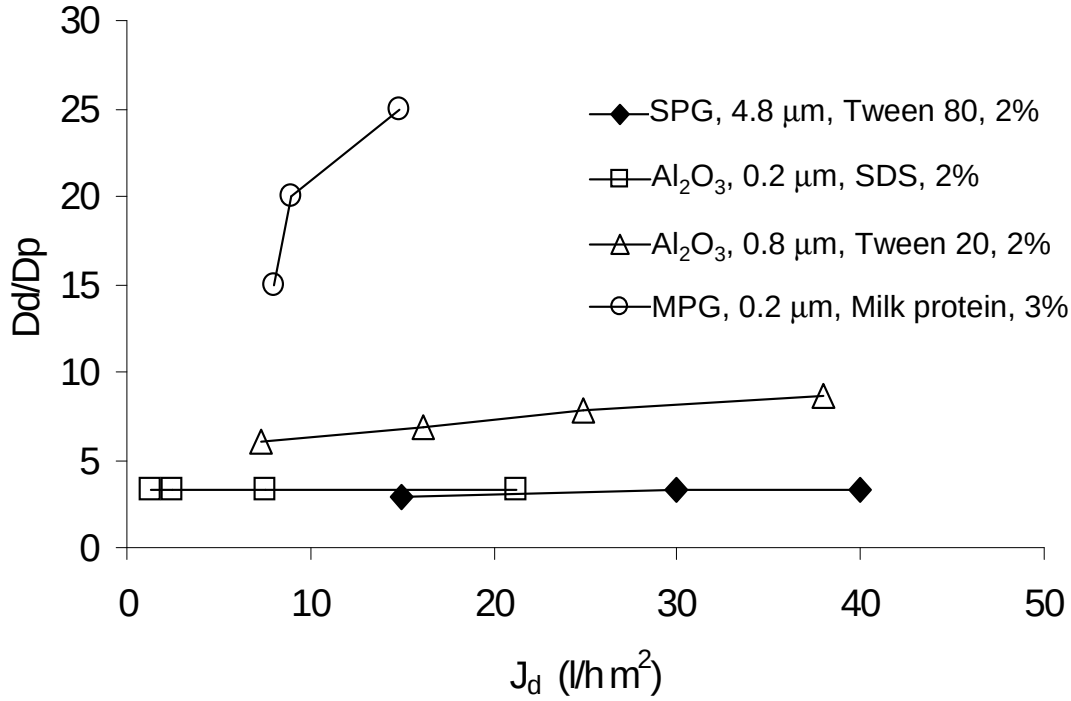


Fig. 1.7. Relationship between dispersed phase flux (J_d) and and D_d/D_p (Data extrapolated from [5, 16, 22, 26]).

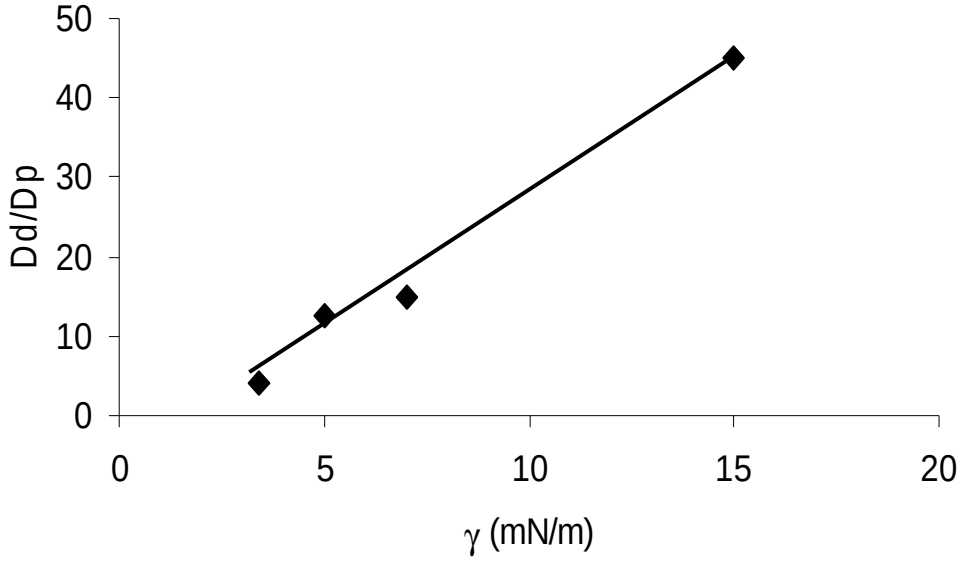


Fig. 1.8. Relationship between interfacial tension (γ) and D_d/D_p (Data extrapolated from [26]).

The axial velocity affects the droplet size by both influencing the surfactant mass transfer to the newly formed interface (that speeds up the reduction of the interfacial tension) and the drag force (that pulls droplets away from the pore mouth).

When production of submicron droplet size is aimed at, continuous phase shear stress and disperse phase flux have to match the need for small droplet (i.e. high shear stress and low disperse phase flux) with the need for a reliable system productivity (i.e. high disperse phase flux).

The physical chemical properties of the phases can influence droplet formation as well as their stability in the bulk. For example, the viscosity of the continuous phase influences both the shear stress at the membrane wall and the adsorption kinetics of the emulsifier.

Concerning thermodynamically unstable emulsions, the creation of new interface from the disruption of the disperse phase increases the free energy of the system, which tends to return to the original two separate systems. Therefore, the use of emulsifier is necessary not only to reduce the interracial tension, but also to avoid the coalescence and the formation of macro-aggregates thanks to electrostatic repulsion between adsorbed emulsifier.

1.3.1. Post-emulsification steps for microcapsules production

In this paragraph, a description of post-emulsification steps needed to complete the preparation of micro-capsules is reported. Micro-encapsulation can be described as the formation of small, coated particles loaded with a solid, a liquid, a solid-liquid dispersion, gas or solid-gas dispersion.

The concept of micro-encapsulation originated in the 1950's and provided the means by which ink formulations used in carbonless copy paper are packaged. This application has been most successful and has led to the development of other applications like the production of microcapsules for thermal printing, optical recording, photocopy toners, diazo copying, herbicides, animal repellents, pesticides, oral and injectable pharmaceuticals, cosmetics, food ingredients, adhesives, curing agents and live cell encapsulation [38].

The size of these capsules may range from 100 nm to about 1 mm. Therefore, they can be classified as nano-, micro- and macrocapsules, depending on their size. The first commercial microcapsules were made by Green with a process called complex coacervation [37]. Since then, other methods for preparing microcapsules have been developed of which some are based exclusively on physical phenomena. Some utilize polymerization reactions to produce a capsule shell. Others combine physical and chemical phenomena. But they all have three main steps in common. The steps of the microencapulation preparation are schematically depicted in Fig. 1.10. In the first step, a dispersion or emulsion has to be formed, followed by deposition of the material that forms the capsule wall (Fig. 1.9, step 2). After solidification or crosslinking (step 3) of the droplets prepared, the capsules are isolated in the last step.

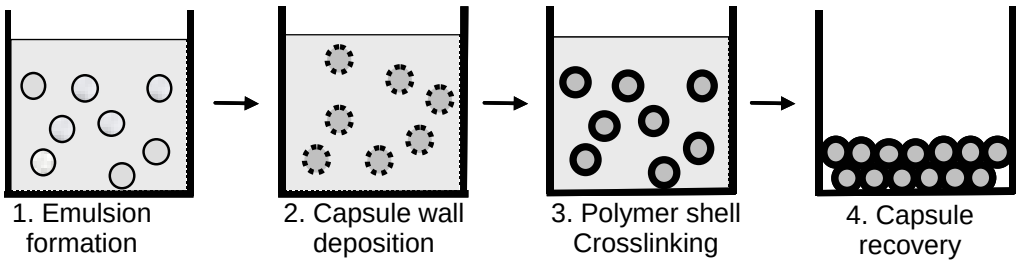


Fig. 1.9. Steps involved in the formation of microcapsules.

One of the major problem related to the capsule formation is the capsule agglomeration. It involves the irreversible or largely irreversible sticking together of microcapsules that can occur during the encapsulation process and/or during the isolation step.

The microencapsulation process can be classified in two main categories (as defined by C. Thies [38] and reported in Table 1.3: a) chemical process) and b) mechanical process.
Capsules produced by chemical process are formed entirely in a liquid-filled stirred tank or tubular reactor. Mechanical process use a gas phase at some stage of the encapsulation process.
In Table 1.4 the typical size of capsules produced is identified by a number of processes that have been commercialised.

Table 1.3. List of Encapsulation Processes (After [38])

Chemical Process	**Mechanical Process**
Complex coacervation	Spray drying
Polymer/polymer incompatibility	Spray chilling
Interfacial polymerisation in liquid media	Fluized bed
In situ polymerisation	Electrostatic deposition
In-liquid drying	Centrifugal extrusion
Thermal and ionic gelation in liquid media	Spinning disck at liquid/gas or solid gas interface
Desolvation in liquid media	Pressure extrusion or spraying into solvent extraction bath

Table 1.4. Commercial Encapsulation Processes and obtained capsule size (After [38])

Process	**Usual Capsule Size (μm)**
Spray drying	5-5000
In-Liquid drying or solvent evaporation	< 1 - 1000
Polymer phase separation (coacervation)	20-1000
Rotational Suspension Separation	>50
Fluidized Bed (Wurster)	< 100

1.3.2. Membrane emulsification devices

The various membrane emulsification procedure can be practised by using appropriate membranes and devices configuration.

The cross-flow membrane emulsification can be obtained either with tubular or flat-sheet membranes, which are fixed in appropriate housing modules connected to circuits controlling fluid dynamic conditions. A schematic drawing of a cross-flow plant is reported in Fig. 1.10. The figure also illustrates the tubular and flat-sheet membranes and modules. SPG (Japan) and Micropore (UK) are among the first companies producing plants for cross-flow membrane emulsification. Fig. 1.11 shows pictures of common marketed equipments.

Emulsification devices where the membrane is immersed in a stirred vessel containing the continuous phase, so that to obtain a batch emulsification device operating in dead-end emulsification mode, have been also developed (Fig. 1.12). Both flat-sheet and tubular membranes are used. In this membrane emulsification device, the continuous phase kept in motion creates the shear stress at the membrane surface that detaches the forming droplets. In a different operation mode, i.e. when the continuous phase is not stirred, droplet formation in quiescent conditions is obtained.

Rotating membrane emulsification is another type of batch emulsification. In this case a tubular membrane immerse in a continuous phase vessel is rotating itself and its angular velocity creates the shear stress at the membrane surface (Fig. 1.13).

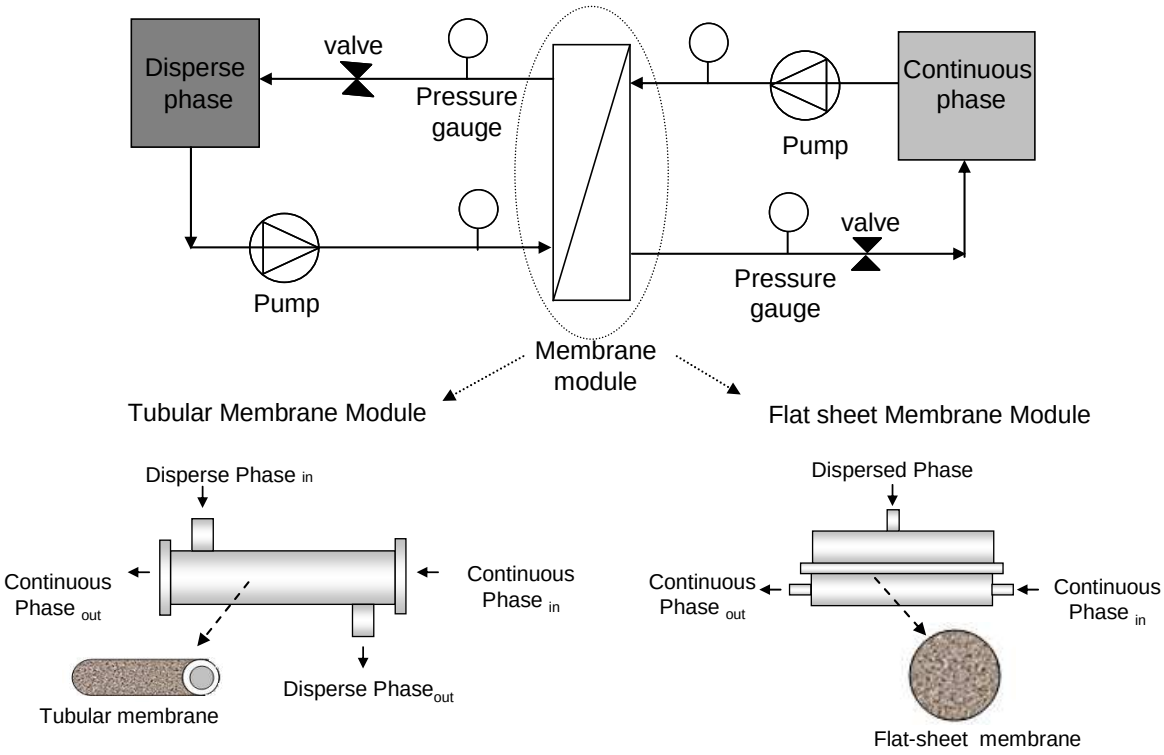
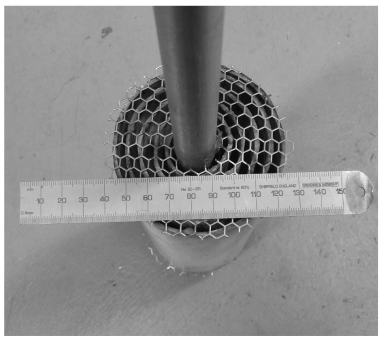


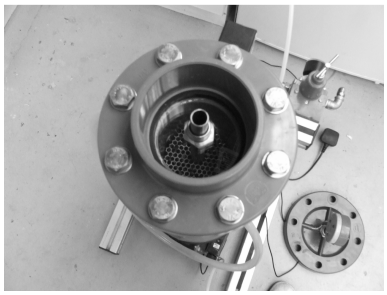
Fig. 1.10. Schematic drawing of a cross-flow plant, using either tubular or flat-sheet membranes.

Both cross-flow and dead-end systems can be used in pre-mix and direct membrane emulsification. In the cross-flow pre-mix system the coarse emulsion is diluted by permeation into pure continuous phase/diluted emulsion recirculating at the low-pressure side of the membrane. In the dead-end system the fine emulsion is withdrawn as a product after passing through the membrane, without any recirculation and/or dilution with the continuous phase. In this process, the fine emulsion can be repeatedly passed through the same membrane a number of times to achieve additional droplet size reduction and enhance size uniformity (multipass pre-mix membrane emulsification).

Each type of device has specific advantages and disadvantages. The batch emulsification is suitable for laboratory scale investigations. The construction of the device is simple and handling during emulsification as well as for cleaning. Cross-flow membrane emulsification is used when it is important a proper adjustment of all process parameters and larger amounts of emulsion have to be produced.

a)





b)

Fig. 1.11. Marketed equipments for membrane emulsification. *a)* Plant for cross-flow membrane emulsification produced by SPG Technologies Co. LTD (http://www.spg-techno.co.jp/); *b)* spiral-wound metallic membrane module produced by Micropore Technologies (http://www.micropore.co.uk/).

A potential disadvantage of cross-flow direct membrane emulsification is the relatively low maximum disperse phase flux through the membrane (0.01-0.1 $m^3/m^2 \cdot h$). Membrane, fluid properties and transmembrane pressure determine the disperse phase flux through the membrane. The opportune choice of membrane properties permits to control the flux during membrane emulsification process. Due to the low productivity, i.e. long production time, cross-flow direct membrane emulsification is more suitable for the preparation of relatively diluted emulsions with disperse phase content up to 30%. Nevertheless, this process enables to obtain very narrow droplet size distribution to be produced over a wide range of mean droplets size. Cross-flow pre-mix membrane emulsification holds several advantages over cross-flow direct membrane emulsification. In fact, disperse phase fluxes of the former emulsification process are typically above 1 $m^3/m^2 \cdot h$, which is one to two orders of magnitude higher than the latter one. In addition, the mean droplets sizes that can be achieved using the same membrane and phase compositions are smaller. Also, the experimental apparatus is generally simpler and the process is easier to control and operate since the driving pressure

and emulsifier properties are not so critical for the successful operation as in cross-flow direct membrane emulsification. One of the disadvantages of pre-mix membrane emulsification is a higher droplet polydispersity.

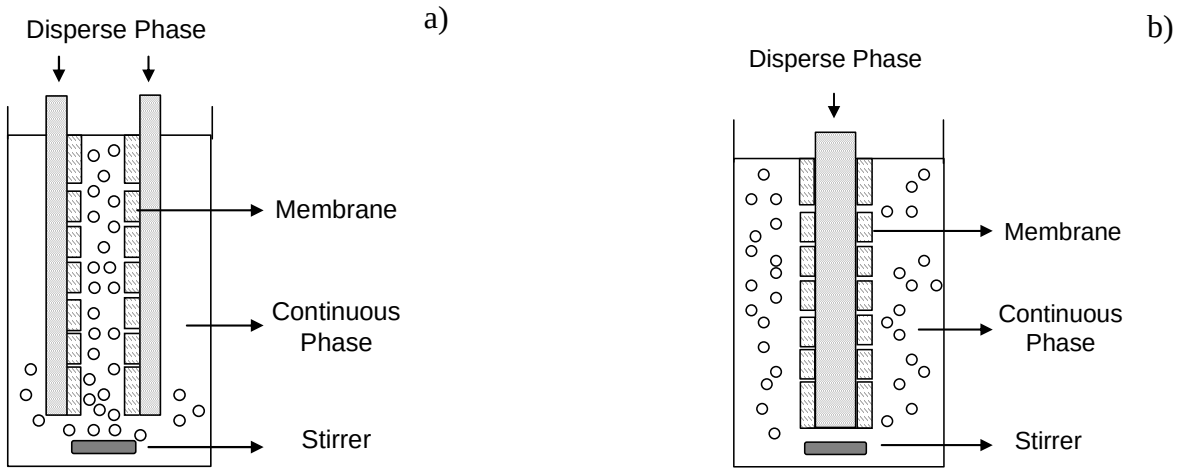


Fig. 1.12. Emulsification devices where the membrane is immersed in a stirred vessel containing the continuous phase. Transmembrane pressure applied from *a)* external or shell side, and *b)* internal or lumen side.

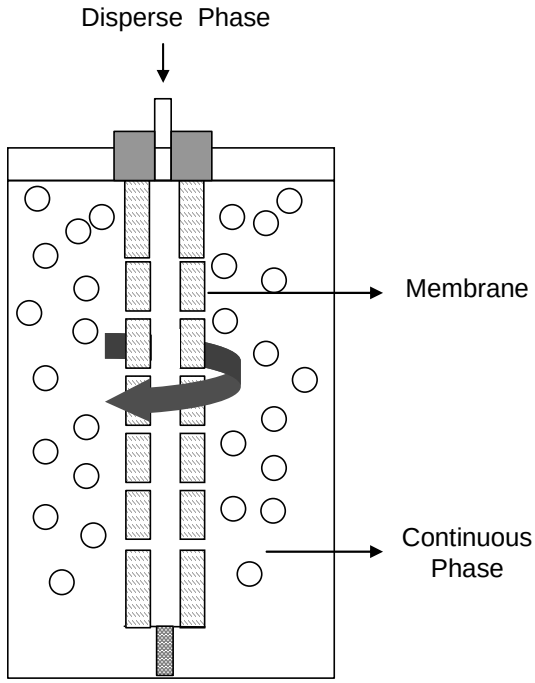


Fig. 1.13. Rotating emulsification device.

1.5. Membrane emulsification applications

1.5.1. Applications in the food industry

Emulsions play an important role in the formulation of foods, i.e., o/w emulsions are used for preparation of dressings, artificial milks, cream liqueurs, and w/o emulsions are used in the production of margarines and low fat spreads.

Food products must have appropriate texture properties. For example, it is important that mayonnaise products have thick and creamy textures, but not too high viscosity. The rheological properties depend on their composition, such as the concentration of oil droplets or the concentration of thickening agents.

The development of membrane emulsification technologies permits to produce small and uniform droplets and capsules, using mild conditions of temperature, shear stress and pressure. Furthermore, they are able to produce stable droplets with reduced stabilizers content, which will contribute to the manufacturing of improved food products with low fat content.

In this context, the Morinaga Milk Industry (Japan) developed and commercialized a very low fat spread using membrane emulsification technology [39, 40]. The advantages in the production of low fat spreads made the process one of the first large-scale applications of membrane emulsification. A w/o emulsion using a MPG hydrophilic membrane, previously treated with the oil phase, has been prepared by cross-flow membrane emulsification. The product resulted stable and free from aqueous phase separation, tasted smooth and extremely easily melt able in the mouth.

For practical applications in the food industry, where large volume production is conducted, it is especially important to obtain high disperse phase flux. Abrahamse et al. [8] reported about an industrial-scale production of culinary cream. In this study they evaluated the required membrane area for different types of membranes: an SPG membrane, an α-Al_2O_3 membrane and a microsieve filter. The requirements for culinary cream production were: a droplet size between 1 and 3 μm and a production volume of 20 m^3/h containing 30% disperse phase. They concluded that to produce large quantities of monodisperse emulsions the most suitable was a microsieve with an area requirement of around 1 m^2.

Katoh et al. [3] prepared w/o emulsions composed of salt solution, polyglycerin polyricinolate (PGPR) at 2%wt and corn oil. It has been proved that the disperse phase flux was increased 100 times using a hydrophilic membrane pre-treated by immersion in the oil phase. This made

the membrane emulsification system practical for large scale production of a w/o emulsion in food application.

Double emulsions are also very useful for food application. Sensitive food materials and flavours can be encapsulated in w/o/w emulsions. Sensory tests have indicated that there is a significant taste difference between w/o/w emulsions and o/w emulsions containing the same ingredients, and that there is a delayed release of flavour in double emulsions [41]. W/o/w or o/w/o multiple emulsions having a concentrated aqueous-soluble flavour or a concentrated oil-soluble flavour encapsulated in the internal phase can be prepared. Food products obtained with these particulates exhibit enhanced flavour perception and extended shelf-life [42].

1.5.2. Applications in the pharmaceutical industry

Among the applications of membrane emulsification, drug delivery system (DDS) is one of the most attractive field. W/o/w emulsions have been prepared to transport and deliver anticancer drug [4, 43-45]. The emulsion was directly administered into the liver using a catheter into the hepatic artery. In this way, it was possible to suppress the strong side effects of the anticancer drug and also concentrate the dosage selectively to focus on the cancer. The clinical study showed that the texture of the cancer rapidly contracted and its volume decreased to a quarter of its initial size.

Composite emulsion as carrier of hydrophilic medicine for chemotherapy was prepared by adding albumin to the internal water phase and lecithin or cholesterol to the oil phase, so that obtaining a water-in-oil emulsion. This emulsion was then pressed through Millipore membrane into an external water phase to form a w/o/w multiple emulsion. Its advantages are high size uniformity and high storage stability [46].

Nakajima et al. referred to membrane emulsification as a method to make functional ethanol-in-oil-in-water (e/o/w) emulsions. These e/o/w emulsions are suitable to encapsulate functional components that have a low water and oil solubility while being soluble in ethanol. An example is taxol which is an anticancer terpenoid [47].

Vladisavljevic et al. reported about the production of multiple w/o/w emulsions for drug delivery systems by extruding a coarse w/o/w emulsion five times through SPG membrane [48].

Several works also reported about the preparation of biodegradable polymer microcapsules to be used as drug delivery system due to their biodegradable nature and proven biocompatibility. The biopolymers employed are mainly poly(lactide) (PLA) [49], poly(lactic-co-glycolic acid) (PLGA) [50-54], chitosan [55, 56], calcium alginate [57]. Such polymers

have been applied for encapsulating proteins and peptides used as prophylactic and therapeutic agents in biomedical fields. So far, the delivery route is the injection, which not only causes distress and inconveniency to patients, but also induces unstable curative effective and side effects. This is due to the fact that the drugs have to be given frequently, resulting in rapid increase and decrease of drug concentration in blood [55]. Therefore, the sustained delivery system for proteins and peptides is necessary not only for injection administration but also for developing an oral administration system. The use of microspheres as a controlled release system is one of the prospect methods. In fact, it may prevent encapsulated drug from degradation by proteolytic enzymes, prolong its the half-life time and improve its bio-availability in vivo by controlling release rate of drug from the microspheres.

The preparation of monodisperse hydrogel microspheres, such as poly-acrylamide-co-acrylic acid, poly(N-isopropylacrylamide-co-acrylic acid), has been performed for drug devices thanks to their biocompatibility [57, 58]. The average diameters of the microspheres were dependent on the pore sizes (from 0.33 to 1.70 μm) of SPG membranes used in the preparation procedure.

Solid lipid nanoparticles (SLN) have been also introduced as alternative to solid particles, emulsions and liposomes in cosmetic and pharmaceutical preparations. Charcosset et al. reported the use of membrane emulsification for the production of SLN [60]. The lipid phase was pressed through the membrane pores into the aqueous continuous phase, at a temperature above the melting point of the lipid. The SLN are then formed by the following cooling of the preparation to room temperature. The lipids remain solid also at body temperature. The influence of process parameters on the size and the lipid phase flux was investigated. The membranes used were supplied by Kerasep ceramic membranes with an active ZrO_2 layer on an Al_2O_3-TiO_2 support. Three different microfiltration membranes were investigated: 0.1, 0.2 and 0.45 μm mean membrane pore size. It was shown that SLN nanoparticles could be prepared with a liquid phase flux between 0.15 and 0.35 $m^3/h\ m^2$ and mean SLN size between 70 and 215 nm.

1.5.3. Applications in the electronic industry

The membrane emulsification technique is also employed for the preparation of microspheres starting from monomers such as methacrylates (methylmethacrylate, cyclohexyl acrylate, etc.), polyimide prepolymer, styrene monomer [61], etc.

The occlusion of functional material such as the polyimide prepolymer (PIP) in uniform polymer particles, can find promising applications in sophisticated electronic devices such as

adhesive spacers of liquid crystal panel boards (after a minor screening process), adhesives or insulators for micro-tip circuits, and so forth. Omi et al. [62] showed that about 30% occlusion of polyimide prepolymer (diphenilmethane-4,4'-bis-allylnagiimide, BAN-I-M) was accomplished in the preparation of polymer particles composed of styrene, various acrylates and a crosslinking agent (ethyleneglycol dimethacrylate, EGDMA) via the emulsification technique with SPG membrane. Particles with a diameter of 6-12 microns were prepared. The presence of acrylates and EGDMA were essential to obtain stable lattices of styrene – based copolymers which occlude BAN-I-M. However, the presence of acrylates with longer side chains, BA and 2EHA, promoted the inclusion of BAN-I-M. In particular, the latter yielded a stable latex occluding 100% of the initial BANI-M without the crosslinking matrix and using octyl alcohol as stabilising agent. The lattices without a crosslinking network resulted in an excellent adhesive ability.

Guang Hui Ma et al. [63] prepared microcapsules with narrow size distribution, in which hexadecane (HD) was used as oily core and poly(styrene-co-dimethylamino-ethyl metahcrylate) [P(st-DMAEMA] as wall. The emulsion was first prepared using SPG membranes and a subsequent suspension polymerisation process was performed to complete the microcapsule formation. Experimental and simulated results confirmed that high monomer conversion, high HD fraction, and addition of DMAEMA hydrophilic monomer were three main factors for the complete encapsulation of HD. The droplets were polymerised at 70 °C and the obtained microcapsules have a diameter ranging from 6 to 10 μm, six times larger than the membrane pore size of 1.4 μm.

Furthermore, such monomers can be readily emulsified by dissolving in volatile solvents such as methylene chloride and chloroform. Uniform polylactide particles, and composite polystyrene (PST) and polymethyl methacrylate (PMMA) particles were produced by solvent evaporation [64-66].

1.5.4. Other applications

Membrane emulsification has been also applied for the preparation of oil-in-water emulsion to be used in cosmetics and/or dermatology, in particular for the treatment, protection, care, cleaning and make-up of the skin, mucous membranes and hair. The emulsion was composed by oil phase globules having an average size less than 20 μm; it was prepared by direct membrane emulsification through a porous hydrophilic glass membrane having an average pore size ranging from 0.1 to 5 μm and preferably from 0.3 to 3 μm [67].

The technology also represents a suitable strategy for the preparation of multiphase reaction systems that use phase transfer (bio)catalysts. Giorno et al [68] reported about the use of membrane emulsification to distribute lipase from *Candida rugosa* at the interface of stable oil-in-water emulsions. The enzyme itself was used as a surfactant. Shirasu Porous Glassy (SPG) membranes having nominal pore diameter of 0.1 μm were used to prepare emulsions. Emulsions with more than 90% of organic droplets of 1.6 (±0.40) μm were obtained. The methodology allowed to preserve the catalytic performance of the biocatalyst as well as to achieve enzyme optimal distribution at the interface of stable, uniform and small oil droplets.

Applications in the chemical field, include extrusion of an oil phase containing a photographic hydrophobic material through a microporous membrane into water [69] and emulsification of low viscosity paraffin wax in water [70].

The polyurethane (PU) can be considered an environment-friendly material because the urethane bond resembles the amide bond, which implies possible biodegradability. It can be used in various elastomer formulations, paints, adhesives for polymers and glass, and artificial leather as well as to biomedical and cosmetic fields. Polyurethane spheres were prepared from 20/40% of PU prepolymer solution in xylene [71]. PU droplets were formed in water with the SPG membrane of different pore size (1.5 to 9.5 μm) and then polymerised to form the final micro-spheres. Finally, spherical and solid PU particles of 5 μm were obtained after the removal of the solvent. In an other study, Ma et al. reported the formation of uniform polyurethane-vinylpolymer (PUU-VP) hybrid microspheres of about 20 μm, prepared using SPG membranes and a subsequent radical suspension polymerisation process [72]. The prepolymers were solubilised in xylene and pressed through the SPG membrane into the continuous phase containing a stabiliser to form uniform droplet. The droplets were left for chain extension at room temperature for some hours with di- and triamines by suspension polymerisation at 70 °C for 24h. Solid and spherical PU-VP hybrid particles with a smooth surface and a higher destructive strength were obtained.

Ha et al. [73, 74] prepared monodisperse polymer microspheres from 1 to 40 μm in diameter for medical diagnostic tests, as chromatography column packing and as calibration standards. The work deals with the synthesis of large and uniform poly(butadiene-styrene) latex. The ceramic SPG membrane, with a pore diameter of 1.6 μm, was employed. The uniform particle sizes were in the range diameter of 4-6 μm.

Westover et al. [75] prepared lightly crosslinked nitrated poly(4-hydroxystyrene) microspheres for pH sensors. The microspheres were produced using SPG membranes

followed by suspension polymerisation and they showed diameters between 1 and 2 micrometers.

Figoli et al. [76, 77] reported the preparation of polymeric capsules combining the phase inversion technique with the membrane process. Polyetheretherketone (PEEKWC) capsules of different size (300-800 micron) and morphology (asymmetric with a porous or dense layer) have been prepared. The capsules can find application both in chemical and in food packaging field [78].

Another field where emulsions are likely to become imperative is the production of fuel [79]. Simple and multiple emulsions represent alternative fuels for diesel engine to both increase combustion efficiency and reduce particulate emission. Considering the enormous volume of diesel that is being consumed today, a replacement of just a fraction of regular diesel by diesel emulsion could be of considerable interest to the surface chemistry community. Until now, diesel emulsions were prepared by conventional emulsification methods but it is expected that the membrane emulsification technique will be also become attractive for this application.

1.6. Conclusions

Membrane emulsification, a technology first appeared in the early 1990s, is gaining increasing attention with many applications being explored in various fields. Nowadays, it can be considered at a developing/exploiting stage with a significant involvement of industrial and academic research effort. Many studies have been carried, especially from the experimental point of view whereas from the theoretical point of view the knowledge is not accordingly advanced.

In this chapter, a description of membrane emulsification basic concepts, empirical correlations, theoretical studies as well as most common applications have been discussed.

Many patents have been applied, especially in Japan, which currently holds more than 60% of worldwide applications, in Europe and USA.

Main drivers for membrane emulsification development include high product quality - especially when labile molecules are involved, precise definition of droplet size distribution, low energy input, equipment modularity and easy scale-up, low equipment foot-print

Challenges in this field include the need for higher productivity, membranes and modules specifically designed for the emulsification process, modules construction standardization, and design of innovative intensified processes.

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Chapter 2

Patents analysis in Membrane Emulsification

The present report illustrates the results of an analysis of patents on membrane emulsification published from its origin to nowadays[1].The list of patents analysed is reported in Table 2.1.

The first patent regarding the membrane emulsification goes up again to 1990 (JP2095433). Fathers of the invention are the Japanese Nakajima Tadao and Shimizu Masataka of the Miyazaki Pref. Gov. The invention shows the possibility to prepare an emulsion with uniform particles through the use of a microporous membrane with diameter uniform of the pore. The droplet size and size distribution are controlled through judicious choice of the process conditions and the pore size and size distribution of the membrane. This method produces emulsions by breaking up the dispersed phase from one side to another of a well defined porous membrane. An emulsion having uniform particle size is produced by simple operation with a simple apparatus and various materials can be emulsified while remarkably improving the physical characteristics.

In the 1992 different patents are published concerning the simple and multiple emulsions production, spread, spherical silica gel and polymers through stirred-membranes emulsification or cross-flow-membranes emulsification. In 1993 a new patent (JP5220382) analyzes the production of monodisperse simple and multiple emulsions through the membrane emulsification introducing an accurate analysis of the operational parameters and the characteristics of the used materials. The fact that emulsions can be prepared by using a simple apparatus and a simple operation procedure with a reduced consumption of energy is very advantageous from the economical viewpoint.

Therefore, more specifically, the invention is very useful in the production of various materials which require emulsification treatment for their production, for example in the production of foods, medicines, cosmetics, pigments, functional plastic particles, functional inorganic material particles, raw materiala for fine ceramics and so forth as well as in solvent extraction.

In the food field, many patents results to have as applicant an important Japanese food industry, the Morinaga Milk Industry (JP4323224, JP6007085, US5279847, JP7087887,

US5417995, EP0672351). This patents respond to the request of the food industry to prepare simple (w/o and o/w) and double (w/o/w and o/w/o) emulsions having low-fat content and excellent flavour without requiring a stabilizer and a gelling agent as essential components. The present invention relates to methods for producing emulsions, a low-fat spread and multiple emulsions type spread having good taste easily , excellent in stability and preservativity on a level never achieved by any conventional methods.

The increasing attention in food field towards the production of products preserving ownerships of the natural components in terms of taste or fragrance has determined the realization of a patent (EP0737425) from the Japanese chemical company Asahi Denka Kogyo KK. The inventors of the present invention have extensively investigated the emulsified structural of natural fresh cream. As result, they have found that incorporation of an emulsifying agent and specific proteins affords and ideal oil-in-water type emulsion endowed with enhanced properties characteristics both of natural fresh cream and of vegetable cream. The invention show that the membrane emulsification process can be used also for prepare emulsions containing shear sensitive ingredients (such as proteins) preserving their property in way to get excels fresh cream in palatability.

The Japanese industry Kanegafuchi Chemical Industrial Co. Ltd. proposes the premix membranes emulsification process for the preparation of emulsions having high content of dispersed phase (water or oil) difficult to prepare with conventional (WO9731708).

The application of membrane emulsification process extended to various fields such as pharmaceutical and cosmetic products (JP11242317, WO0021491) or in the chemical industry (JP2006182890,WO2006110035).

There are various patent concern the optimization of membrane emulsification process such as apparatus and device to improve the use of this technology on productive scale.

Nakajima T. et all (JP6315617) plan at first (1994) one "stirred-membranes emulsification plant" for small volumes of dispersed and continuous phase. Subsequently (2005), to answer to the request of specific applications such as in pharmaceutical field, Nakajima N. et all (JP2005279326) propose a "disponsable membranes module" that it allows to operate under conditions of sterility and reducing the times of preparation.

Williams et all use a plant and a method to produce dispersed systems using a ceramic or metallic membrane (WO9736674) and an apparatus for the membrane emulsification endowed with a rotating membrane (WO0145830).

The research carried out evidence that Japan is the principal country in which the patent in membrane emulsification have been developed (64%) (Fig. 2.1). The data clearly show a

lower number of studies in the membrane emulsification field in the European countries. Some patents that result to be filed or granted from the EP (European Patent Office) they have as applicant some German chemical industries what the Fraunhofer ges forschung and the Basf AG.

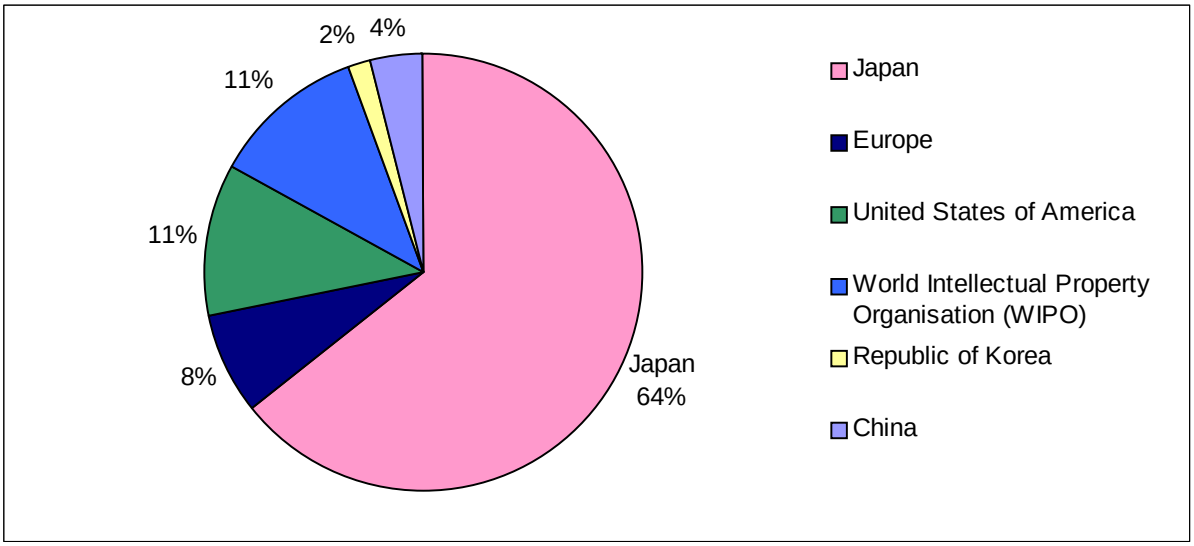


Fig. 2.1. Worldwide distribution of patents on membrane emulsification.

The percentage of patents published in the sector of the membrane emulsification during the time has been evaluated from 1990, year of the first publication, up to December 2007 (Fig. 2.2).

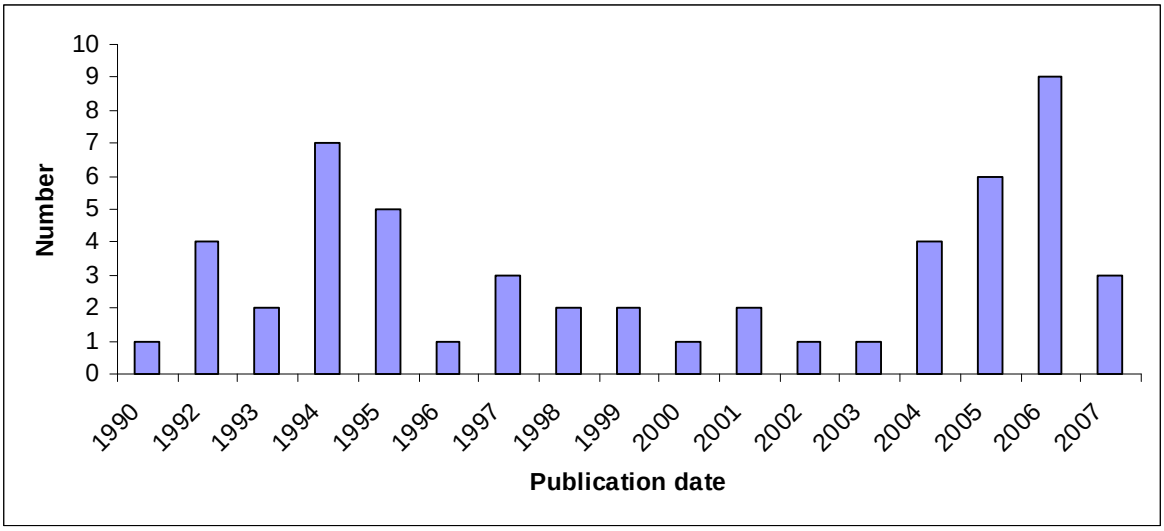


Fig. 2.2. Patents published in the time.

The distribution show two pecks, one in the first years in which the technique has been introduced and one in more recent time (2006) in which the greatest part of the published brevets concern the application of the process of membrane emulsification (photo, cosmetics, pharmaceutical, chemistry).

The patents published in the early 90 primarily concern the possibility of membrane emulsification technology application to prepare dispersed systems as alternative to the conventional mechanical methodologies. Subsequently, with the acquisition of a great number of information about the membrane emulsification methodology (such as the parameters influence) new application fields have been explored (pharmaceutical or photographic or fuels) and major attention has always been set to improve the quality of the process both in terms of plant that of device to answer better always to the productive demands.

The research carried out evidence that the field in which the membrane emulsification technology results to have the most application is the food field followed by the chemical field. In medical and cosmetic field there are only a limited number of applications.

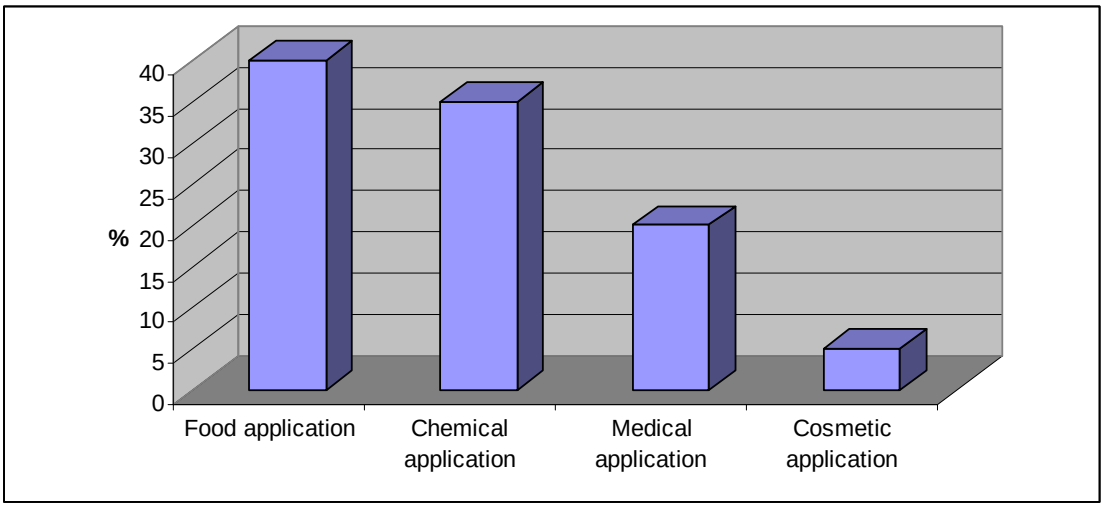


Fig. 2.3. Percentage of patents applications in different fields

2.1. Conclusions

The membrane emulsification introduced in 1990 has received in the first years a strong impulse shown by an increasing number of patent published in that period. It is however in times more recent than the potentialities of such technology have lead to the greatest number of applications in various fields. The food and chemical (for example production of fuel or material photographic) field in general results to be the fields of great interest.

Some publications concern primarily the process and the apparatus The aim result to be investigate the versatility of the membrane emulsification technology and to improve the use in more large scale

Further studies will allow to exploit to the best the advantageous opportunities that the membrane emulsification technique have in terms of product quality, costs sustained. The exploration of new applications is also attended.

Major interest from European countries is attended considering the positive perspectives and potentiality that the membrane emulsification process show in the dispersion systems production.

Table 1 List of patents developed in Membrane Emulsification

Publication date	Title	Publication number	Inventor	Applicant
1990-04-06	PRODUCTION OF EMULSION	JP2095433	NAKAJIMA TADAO; SHIMIZU MASATAKA	MIYAZAKI PREF GOV
1992-08-10	PRODUCTION OF EMULSION AND SPHERICAL SILICA GEL	JP4219131	ITO MUTSUHIRO; FUJISAKI MINORU; NAKATANI KAZUHIKO; YAMAZAKI MITSUHITO; ARIMURA MASAYUKI	FUJI DAVISON CHEMICAL
1992-09-14	PRODUCTION OF POLYMER BEAD	JP4258601	HASHIZUME KIYOKO	OHTSU TIRE & RUBBER CO LTD
1992-09-14	PRODUCTION OF DOUBLE EMULSIFIED SPREAD AND ITS PRODUCTION	JP4258251	OKONOGI SHIGEO; KUMAZAWA RENZO; TOYAMA KAZUYOSHI; KATO MAKOTO; ASANO YUZO; TAKAHASHI KIYOTAKA; FUJIMOTO MASAHISA	MORINAGA MILK INDUSTRY CO LTD
1992-11-12	PRODUCTION OF MONODISPERSE ORGANIC POLYMER BEADS	JP4323224	YAMAGOSHI TOMIO; FUJISAKI MINORU; ITO MUTSUHIRO; YAMAZAKI MURAHITO	FUJI DAVISON CHEMICAL
1993-07-27	PRODUCTION OF EMULSION	JP5184895	OTSUKA YUKIO	SEKISUI FINE CHEMICAL CO LTD
1993-08-31	MONODISPERSE SINGLE AND DOUBLE EMULSION AND ITS PRODUCTION	JP5220382	NAKAJIMA TADAO; SHIMIZU MASATAKA; KUKIZAKI MASAHITO	MIYAZAKI PREF GOV
1994-01-11	PRODUCTION OF FINE POLYMER PARTICLE AND FINE POLYMER PARTICLE PRODUCED THEREBY	JP6001854	HASEGAWA JUN; HANEDA HIDEKAZU	NIPPON ZEON CO
1994-01-18	DOUBLY EMULSIFIED SPREAD AND ITS PRODUCTION	JP6007085	TOMITA MAMORU; TOYAMA KAZUYOSHI; KATO MAKOTO; ASANO YUZO; TAKAHASHI KIYOTAKA; FUJIMOTO MASAHISA	MORINAGA MILK INDUSTRY CO LTD
1994-01-18	METHODS FOR PRODUCING EMULSIONS, LOW-FAT SPREAD AND OIL-IN-WATER-IN-OIL TYPE SPREAD	US5279847	OKONOGI SHIGEO (JP); KATO RYO (JP); ASANO YUZO (JP); YUGUCHI HIROYA (JP); KUMAZAWA RENZO (JP); SOTOYAMA KAZUYOSHI (JP); TAKAHASHI KIYOTAKA (JP); FUJIMOTO MASAHISA (JP)	MORINAGA MILK INDUSTRY CO LTD (JP)
1994-02-22	PRODUCTION OF AQUEOUS COPOLYMER RESIN DISPERSION	JP6049104	YAMAMOTO AKIHITO; YOSHINO FUMIO	DAINIPPON INK & CHEMICALS
1994-05-24	PRODUCTION OF MONODISPERSE FINE SPHERICAL PARTICLE	JP6142505	HIRAYAMA CHUICHI; IHARA HIROTAKA; IWATSUKI MAKOTO	HIRAYAMA CHUICHI; IHARA HIROTAKA; AJINOMOTO KK
1994-07-26	STABLE MULTIPLE EMULSIONS COMPRISING INTERFACIAL GELATINOUS LAYER, FLAVOR-ENCAPSULATING MULTIPLE EMULSIONS AND LOW/NO-FAT FOOD PRODUCTS COMPRISING THE SAME	US5332595	GAONKAR ANILKUMAR G (US)	GEN FOODS INC (US)
1994-08-23	PRODUCTION OF EMULSION	JP6233923	SAKA SADANORI; KITAHARA MICHIO; NAKADA SATORU	NONOGAWA SHOJI YK; FUJI SHIRISHIA KAGAKU KK
1994-11-15	EMULSIFYING METHOD AND DEVICE	JP6315617	NAKAJIMA TADAO; SHIMIZU MASATAKA; IWASAKI YOSHIHIKO; FUJIMOTO KENJI	MIYAZAKI PREF GOV; KIYOMOTO TEKKO KK
1995-04-04	MIXED EMULSIFIED SPREAD AND ITS PREPARATION	JP7087887	TOMITA MAMORU; TOYAMA KAZUYOSHI; KATO MAKOTO; ASANO YUZO; TAKAHASHI KIYOTAKA	MORINAGA MILK INDUSTRY CO LTD
1995-04-25	PRODUCTION OF EMULSION	JP7108164	SAKA SADANORI; KITAHARA MICHIO; YAMAZAKI MITSUHITO	NONOGAWA SHOJI YK; FUJI SILYSIA CHEM LTD
1995-05-23	SPREAD AND A METHOD FOR PRODUCTION OF SAID SPREAD	US5417995	TOMITA MAMORU (JP); SOTOYAMA KAZUYOSHI (JP); KATO RYO (JP); ASANO YUZO (JP); TAKAHASHI KIYOTAKA (JP)	MORINAGA MILK INDUSTRY CO LTD (JP)
1995-09-20	METHOD FOR PRODUCING EMULSIONS.	EP0672351	OKONOGI SHIGEO (JP); KATO RYO (JP); YUGUCHI HIROYA (JP); ASANO YUZO (JP)	MORINAGA MILK INDUSTRY CO LTD (JP)
1995-12-05	METHOD FOR PREPARING OIL-IN-WATER EMULSION OF EDIBLE FATTY OIL	JP7313056	YAMANO YOSHIMASA; AITANI SHOICHI; HOSOYA YASUTO	NISSHIN OIL MILLS LTD
1996-10-16	OIL-IN-WATER EMULSION CONTAINING LYSOPHOSPHOLIPO-PROTEINS	EP0737425	OKUTOMI YASUO (JP); SHIMADA TOSHIHIRO (JP)	ASAHI DENKA KOGYO KK (JP)
1997-08-05	PREPARATION OF EMULSION AND POLYMER FINE PARTICLE	JP9201526	MUKAI KATSUNORI; HISADA TAKASHI; NAITO MASANORI	SEKISUI FINE CHEMICAL CO LTD
1997-09-04	PROCESSES FOR PRODUCING EMULSIFIED FAT COMPOSITION	WO9731708	SUZUKI KANICHI (JP)	KANEGAFUCHI CHEMICAL IND (JP); SUZUKI KANICHI (JP)
1997-10-09	DISPERSION OF IMMISCIBLE PHASES	WO9736674	WILLIAMS RICHARD ANDREW (GB); WHEELER DEREK ALFRED (GB); MORLEY NEIL CHRISTOPHER (GB)	DISPERSE TECH LTD (GB); WILLIAMS RICHARD ANDREW (GB); WHEELER DEREK ALFRED (GB); MORLEY NEIL CHRISTOPHER (GB)
1998-02-17	PRODUCTION OF EMULSION	JP10043577	MUKAI KATSUNORI; HISADA TAKASHI	SEKISUI FINE CHEMICAL CO LTD
1998-08-04	SUSTAINED RELEASE EMULSION PREPARATION OF MEDICINE AND ITS PRODUCTION	JP10203962	NAKAJIMA TADAO; SHIMIZU MASATAKA; KOMATSU YOSHINORI; KATO NAOKI	MIYAZAKI PREF GOV; S P G TECHNO KK; MEIJI MILK PROD CO LTD
1999-09-07	EMULSIFYING METHOD OF PHOTOGRAPHIC HYDROPHOBIC SUBSTANCE, EMULSIFIED MATERIAL AND SILVER HALIDE PHOTOGRAPHIC SENSITIVE MATERIAL	JP11242317	ENDO KIYOSHI	KONISHIROKU PHOTO IND
1999-12-07	MEMBRANE EMULSIFYING DEVICE	JP11333271	TANIGUCHI TORU	REIKA KOGYO KK
2000-04-20	STABLE OIL-IN-WATER EMULSION, METHOD FOR PREPARING SAME AND USE IN COSMETICS AND DERMATOLOGY	WO0021491	ROULIER VERONIQUE (FR); QUEMIN ERIC (FR)	OREAL (FR); ROULIER VERONIQUE (FR); QUEMIN ERIC (FR)

2001-06-28	ROTATING MEMBRANE	WO0145830	WILLIAMS RICHARD (GB)	UNIV LEEDS (GB); WILLIAMS RICHARD (GB)
2001-07-03	POLYPHASE EMULSION	JP2001179077	GOTO MASASHI; MAEKAWA AKIO; NAKAJIMA TADAO; SHIMIZU MASATAKA	SUNSTAR INC; MIYAZAKI PREFECTURE
2002-12-04	DEVICE AND PROCESS FOR MAKING EMULSIONS	EP1262225	SCHLIESSMANN URSULA DIPL-ING (DE); STROH NORBERT DIPL-ING (DE)	FRAUNHOFER GES FORSCHUNG (DE)
2003-11-28	METHOD FOR FORMING EMULSION AND METHOD FOR FORMING RESIN PARTICLES	JP2003335804	HAYASHI SHINICHI; KOJIMA RYOJI	SONY CHEMICALS
2004-01-15	S/O SUSPENSION, S/O/W EMULSION, AND THEIR MANUFACTURING METHOD	JP2004008837	NAKAJIMA TADAO; SHIMIZU MASATAKA; KUKIZAKI MASAHITO	MIYAZAKI PREFECTURE
2004-04-15	PRODUCTION METHOD OF EMULSION	JP2004113933	KOBI YOSHIKI	KURARAY CO
2004-08-05	UNIFORM EMULSION BY MEMBRANE EMULSIFICATION	US2004152788	WU HUEY SHEN (US); OGA TAKAHIRO (JP); OMI SHINZO (JP); YAMAZAKI NAOHIRO (JP)	
2004-12-31	PROCESS FOR PRODUCING INORGANIC SPHERES HAVING UNIFORM PARTICAL SIZES AND APPARATUS THEREFOR	KR20040111082	TATEMATSU SHIN; YAMADA KAZUHIKO; YAMADA KENJI	ASAHI GLASS CO LTD
2005-02-03	METHOD AND APPARATUS FOR MANUFACTURING INORGANIC SPHERICAL BODY	JP2005028358	YAMADA KENJI; TATEMATSU SHIN; YAMADA KAZUHIKO	ASAHI GLASS CO LTD
2005-02-03	EMULSION PRODUCTION DEVICE	JP2005028254	NAGAHAMA TORU; YOSHINO TOMOAKI	TAISHO PHARMA CO LTD
2005-02-03	METHOD OF PRODUCING EMULSION	JP2005028255	YOSHINO TOMOAKI; NAGAHAMA TORU	TAISHO PHARMA CO LTD
2005-04-20	CHITOSE MICROSPHERE AND MICROCAPSULE WITH UNIFORM SIZE AND ITS PREPARATION METHOD	CN1607033	MA GUANGHUI (CN); SU ZHIGUO (CN); WANG LIANYAN (CN)	INST OF PROCESS ENGINEERING CH (CN)
2005-07-14	DEVICE AND METHOD OF PREPARING EMULSION	JP2005186026	NAKAJIMA NOBORU; FUJIWARA MITSUTERU	SPG TECHNO KK
2005-10-13	DISPOSABLE MEMBRANE MODULE FOR PREPARING EMULSION	JP2005279326	NAKAJIMA NOBORU; FUJIWARA MITSUTERU; MAEDA DAIGO	SPG TECHNO KK
2006-05-11	METHOD FOR PREPARING MICROSPHERE	US2006096715	SUZUKI TAKEHIKO (JP); MATSUKAWA YASUHISA (JP); SUZUKI AKIRA (JP)	TANABE SEIYAKU CO
2006-06-07	PROCESS FOR PRODUCING A FINE EMULSION FROM A COARSE EMULSION	EP1666130	DANNER THOMAS DR (DE); VOSS HARTWIG DR (DE); BAUDER ANDREAS (DE); VIERECK SONJA (DE)	BASF AG (DE)
2006-06-15	METHOD FOR CONTROLLING DROPLET SIZE OF AN EMULSION WHEN MIXING TWO IMMISCIBLE FLUIDS	US2006128815	CLARE HUGH J (GB); PEARSON CHRISTOPHER A (GB); SHANKS IAN A (GB)	
2006-07-13	METHOD FOR PRODUCING EMULSION FUEL AND APPARATUS FOR PRODUCING THE SAME AND APPARATUS FOR MODIFYING FUEL	JP2006182890	NAKAJIMA NOBORU; FUJIWARA MITSUTERU; MAEDA DAIGO; WATANABE KOJI	SPG TECHNO KK
2006-08-31	PROCESS FOR PREPARING AN AQUEOUS ADDITION-POLYMER DISPERSION	WO2006089939	GASCHLER WOLFGANG (DE); DANNER THOMAS (DE); BAUDER ANDREAS (DE); FUNKHAUSER STEFFEN (DE); HAMERS CHRISTOPH (DE)	BASF AG (DE); GASCHLER WOLFGANG (DE); DANNER THOMAS (DE); BAUDER ANDREAS (DE); FUNKHAUSER STEFFEN (DE); HAMERS CHRISTOPH (DE)
2006-10-19	MICROSIEVE MEMBRANE FOR EMULSIFICATION AND LITHOGRAPHIC METHOD OF MAKING THE SAME	WO2006110035	SANCHEZ-DE VRIES STEFAN (NL)	FLUXXION B V (NL); SANCHEZ-DE VRIES STEFAN (NL)
2006-12-07	METHOD OF PRODUCING METAL PARTICLE	JP2006328471	ISHIKAWA YUICHI; SON HITONORI	KRI INC
2006-12-21	METHOD FOR PRODUCING EMULSION COMPOSITION	JP2006341252	FUJIMOTO KENJI; MINAMINO TATSUO; AKAGI HIDEKUNI; IWASAKI YOSHIHIKO; SHIMIZU MASATAKA; NAKAJIMA TADAO	KIYOMOTO IRON & MACHINERY WORK; MIYAZAKI PREFECTURE
2006-12-28	METHOD FOR PREPARING EMULSION USING POROUS BODY AND ITS APPARATUS	JP2006346565	NAKAJIMA NOBORU; IWASHITA KAZUHIRO; FUJIWARA MITSUTERU; MAEDA DAIGO	SPG TECHNO KK
2007-01-17	PREPARATION OF EMULSION FOR DECREASING LIQUID-DROP DIAMETER CONTINUOUSLLY AND GRADUALLY BY POROUS FILM	CN1895763	LI NA (CN)	XI AN COMM UNIV (CN)
2007-05-24	EMULSIFICATION PROCESS AND EMULSIFICATION APPARATUS	JP2007125535	FUJIMOTO KENJI; IWASAKI YOSHIHIKO; SHIMIZU MASATAKA; TORIGOE KIYOSHI	KIYOMOTO IRON & MACHINERY WORK; SHIMIZU MASATAKA; TORIGOE KIYOSHI; MIYAZAKI PREFECTURE
2007-11-01	METHOD FOR PRODUCING MULTIPLE EMULSIONS THAT ARE STABLE IN STORAGE	US2007253986	STANGE OLAF (DE); MUTTER MARTINA (DE); OSWALD TANJA (DE); SCHMITZ MARK (DE)	BAYER TECHNOLOGY SERVICES GMBH (DE)

Technological advances

Chapter 3

Phases properties and operational parameters in ME process

3.1. Introduction

Water-in-oil (W/O) emulsification is an important process in food, cosmetic, pharmaceutical and other chemical industries [1–3]. Moreover, W/O emulsions were applied to produce multiple emulsions [4], microcapsules for capsulizing medicines, and microspheres for packing gel permeation chromatograph (GPC) and high performance liquid chromatography (HPLC) columns [5,6], immobilizing enzymes [7] and loading protein drugs [8]. The mean size and size distribution of emulsions are of especial significance for fabrications of all these "high- tech" products. In addition, the characteristics and stability of emulsions are greatly affected by their size and size distribution [9].

There is a very small number of papers dealing with the preparation of W/O emulsions by a membrane emulsification method. It can be explained by the fact that the preparation of W/O emulsions is difficult in comparison to O/W emulsions. It is because the water droplets are hard to stabilize by an electrical double-layer repulsion force in an oil phase with low dielectric constant. In addition, diffusion of surfactant molecules through the continuous oil phase is slower because of the higher viscosity of oil compared to water. Thus, stabilization of the newly formed water droplets is slower and coalescence cannot be avoided sufficiently during droplet formation. However, droplet coalescence in the prepared emulsion is slower in the case of higher continuous phase viscosity. In Table 3.1 are summarized the data of previous investigations dealing with the preparation of W/O emulsions by a membrane emulsification method. A small amount of works are presents in literature. In particular, most mainly concern the production of W/O emulsions by direct membrane emulsification in static [10-13] or cross-flow [14-19] operation procedures. In some case, when cross-flow was used, droplets size was below the mean pore size and this was attributed to the fact that: a) water which penetrates through the membrane cannot completely displace the oil phase from the

interior of the pores due to the high oil viscosity compared with water [14] or b) spontaneous emulsification occurred when the interfacial tension between the water and oil phases is less than 2 mN/m [11]. In addition, most results was referred to us of hydrocarbons not suitable for human use.

In this work, the effect of natural and biocompatible oil phase viscosity on droplets size and droplets size distribution was investigated in the preparation of W/O emulsions to applied in food, cosmetic or pharmaceutical application. The aim was to set the operations conditions to prepare W/O emulsion in which droplets size was strictly controlled from membrane pore. Stirred cell membrane emulsification was used as operations procedures. In the stirred cell membrane emulsification is achieved using a circular disc membrane with uniform pores regularly spaced, on top of which a simple paddle-blade stirrer induced shear at the membrane surface, resulting in droplet detachment during membrane emulsification [18, 20-21]. In this system, the drop size distribution of product is much better than would be expected from such a noncontrolled shear device [20]. Although the system permit to obtain only larger droplets because membrane pores available ranged from 5-40 μm, the knowledge can be used to produce W/O droplets emulsions with specified size using all membrane emulsification process in which shear induced droplet formation. Also the effect of dispersed phase composition was evaluated in order to improve W/O emulsion stability using glycerol as plasticizer or PVA as thickening.

Table 3.1. Literature data of previous investigations dealing with the preparation of W/O emulsions by a membrane emulsification method.

Method	Operation procedures	Membrane	D_p (μm)	Dispersed phase	Continuous phase	Emulsifier	Flux or P_{TM} of dispersed phase	Flour rate or shear stress of continuous phase	D_d (μm)	Ref
Pre-mix	**Static**	Hidrophobic PTFE	1	Water	Corn oil	Hexaglycerol polyricinoleate (2 wt%)	0.1-1.5 mPa		3	10
Direct	**Static**	hydrophilic SPG	0.99	Water	Toluene	PE-64 (2-10 wt%)	4 ml/min	-	0.66-0.70	11
Direct	**Static**	hydrophilic SPG	2.70	Water	Toluene	PE-64 (2-10 wt%)	4 ml/min	-	1.38-1.96	11
Direct	**Static**	hydrophilic SPG	4.70	Water	Toluene	PE-64 (2-10 wt%)	4 ml/min	-	1.59-1.87	11
Direct	**Static**	hydrophobic SPG	4.8	Water + NaCl (0.017-0.855 M)	Kerosene	PGPR (5 wt%)	3 kPa	-	13.5-15.5	12
Direct	**Static**	hydrophobic SPG	2	Water + NaCl (0.855 M)	Kerosene	PGPR (5 wt%)	7-10 kPa	-	6.5-7.5	12
Direct	**Static**	hydrophobic SPG	4.8	Water + NaCl (3% wt)	Kerosene	PGPR (5 wt%)	3 kPa	-	15	13
Direct	**Cross-flow**	Hydrophobic Polypropylene	0.4	Water	Mineral oil	PGPR (10 wt%)	0.5 l/m^{2}h	130 l/h	0.42	14
Direct	**Cross-flow**	Hydrophobic Polypropylene	0.4	Water	Mineral oil	PGPR (10 wt%)	0.12 l/m^{2}h	130 l/h	0.31	14
Direct	**Cross-flow**	Hydrophobic Polypropylene	0.4	Water	Mineral oil	PGPR (10 wt%)	0.2 l/m^{2}h	130 l/h	0.26	14
Direct	**Cross-flow**	Hydrophobic arrays of micro-orifices	2.5	Water	n-hexadecane	BolevMT (1 wt%)	250 mbar	-	125	15
Direct	**Cross-flow**	Hydrophobic arrays of micro-orifices	3.5	Water	n-hexadecane	Span 85 (1 wt%)	150 mbar	-	150	15
Direct	**Cross-flow**	Hydrophobic MPG	1	Water + glucose (5%)	soybean oil	PC + PGCR (5 wt%)	80 kPa	-	3.08	16
Direct	**Cross-flow**	hydrophilic SPG	0.99	Water	Toluene	PE-64 (2-10 wt%)	4 ml/min	23 ml/min	0.66-0.70	17
Direct	**Stirred cell**	Hidrophobic metallic	30	Water + PVA (15%)	Kerosene	Hypermer B261 (0.3 wt%) Span 80 (2 wt%)	70 l/hm^2	70 dynes/cm^2	77	18
Direct	**Stirred cell**	Hidrophobic metallic	30	Water + PVA (15%)	Kerosene/ soyabean oil(40%)	Hypermer B261 (0.3 wt%) Span 80 (2 wt%)	70 l/hm^2	126 dynes/cm^2	71	18
Direct	**Stirred cell**	Hidrophobic metallic	30	Water + PVA (15%)	Kerosene/ soyabean oil (60%)	Hypermer B261 (0.3 wt%) Span 80 (2 wt%)	70 l/hm^2	127 dynes/cm^2	65	18
Direct	**Stirred cell**	Hidrophobic metallic	30	Water + PVA (15%)	Kerosene/ soyabeab oil (80%)	Hypermer B261 (0.3 wt%) Span 80 (2 wt%)	70 l/hm^2	120 dynes/cm^2	5	18
Direct	**Rotating**	Nickel	5	Water	Sunflower oil	PGPR (1 wt%)	-	-	-	19

3.2. Materials and methods

3.2.1. Materials

Soybean oil (Sigma), Squalene and Limonene were used as continuous phase in the W/O emulsion preparation. In all experiments, 2 wt.% sorbitan monooleate (Span 80, Sigma) was used as hydrophobic surfactant. All the aqueous solutions ware prepared using ultrapure water (USF Elsa, model Purelab Classic PL5221) with a resistivity of 18.2 MΩ·cm. A 15 wt% solution of Polyvinil alcohol (PVA, 13.000-23.000 daltons) or glycerol was used as dispersed phase.

3.2.2. Membranes and membrane emulsification equipment

An hydrophobic metallic membranes, having 20 μm d_p, was used to prepare W/O emulsion in the stirred emulsification cell (Micropore Technologies Ltd) [18, 20]. The system consists in a PTFE base (named injection chamber) that hosts a flat membrane coupled with a dismountable threaded glass cylinder. A stainless steal stirrer is placed over the glass cylinder and regulated by a voltage regulator connected to the electricity. The feeding system of the cell was modified from the original version, where instead of a gravimeter cylinder a peristaltic micropump (Ismatec, model C.P. 78016-30) was connected to the injection chamber to permeate the dispersed phase at finely controlled flow rate. A schematic representation of the stirred emulsification plant is reported in the Figure 3.1.

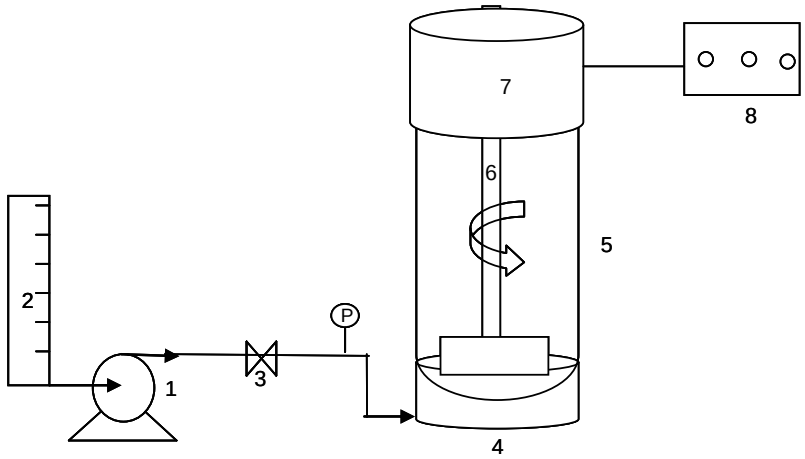


Fig.3.1. Stirred membrane emulsification equipment scheme. 1: peristaltic pump; 2: Dispersed phase graduated vessel; 3: backpressure valve; 4: injection chamber; 5: glass cylinder; 6: Stainless steal stirrer with a blade at the bottom ending; 7: motor; 8: voltage regulator

In all experiments, the dispersed phase flux was kept constant at a value of 40.7 ± 0.7 l/hm^2. The emulsification process was stopped when 3% of water in oil was obtained.

3.2.3. Dispersed drop size modelling

The equations used were introduced in the previous work [18]. For the simple paddle-bladed system used in this work, equation 1 can be used to calculate the location of the transitional radius along the paddle-blade radius. The transitional radius is the point at which the rotation changes from a forced vortex to a free vortex:

$$r = \frac{D}{2} 1.23 \left(0.57 + 0.35 \frac{D}{T} \right) \left(\frac{b}{T} \right)^{0.036} n_b^{\,0.116} \frac{\mathrm{Re}}{1000 + 1.43\,\mathrm{Re}} \qquad (1)$$

where *b* is the blade height, *T* is the tank width, *D* is the stirrer width, and *n*b is the number of blades. The Reynolds Number is defined by Re = $\rho\omega D^2/2\pi\eta$ where ρ is the continuous phase density, ω is the angular velocity, and η is the continuous phase coefficient of dynamic viscosity.

The boundary layer thickness, δ, is defined by the Landau-Lifshitz 2 equation:

$$\delta = \sqrt{\frac{\eta}{\rho\omega}} \qquad (2)$$

The shear stress in the boundary layer above the membrane surface varies according to eqs 3 and 4, for radial positions less than the transitional radius and greater than the transitional radius, respectively

$$\tau = 0.825 \eta \omega r \frac{1}{\delta} \qquad r < r_{trans} \qquad (3)$$

$$\tau = 0.825 \eta \omega r_{trans} \left(\frac{r_{trans}}{r} \right)^{0.6} \frac{1}{\delta} \qquad r > r_{trans} \qquad (4)$$

3.2.4. Droplets measurement

Droplet size was measured by Zeiss Axiovert 25 optical microscopy with a 10X lens. The images were obtained with an AxioCam (Carl Zeiss) using AxioVision 3.1 software (Carl

Zeiss). The images were processed by the “Scion Image” program. This software automatically counts identified particles and measures their diameter. For each series of experiments, standard deviation was calculated from at least three different experiments. For each experiment, three samples were taken at the same sampling time and for each one at least five photos were analysed. From these measurements, the mean droplet size reported as The mean particle size was expressed as the surface weighted mean diameter (or Sauter diameter), D[3,2]and as the volume weighted mean diameter, (or De Brouckere diameter), D[4,3]. D[3,2] and D[4,3] were determined, respectively, as follows:

$$D[3,2] = \frac{\sum D_i^3 n_i}{\sum D_i^2 n_i} \tag{5}$$

$$D[4,3] = \frac{\sum D_i^4 n_i}{\sum D_i^3 n_i} \tag{6}$$

where D_i = particle diameter of class i and n_i = number of particle in class i.

The width of droplet size distribution was expressed as a Span number, calculated by the following expression:

$$Span = \frac{D[0.9] - D[0.1]}{D[0.5]} \tag{7}$$

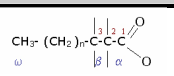
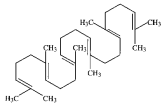
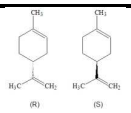
where D[x0] is the diameter corresponding to x0 vol.% on a relative cumulative droplet size curve.

3.3. Results and discussion

3.3.1. Effect of continuous phase composition

In Table 3.2 are reported the continuous phase used in the preparation of W/O emulsion, the angular velocity (ω) and the corresponding shear stress applied.

Table 3.2 Continuous phase used

Continuous phase		Viscosity (cP)	Density (g/ml)	Shear stress (dyne/cm^2)	Angular velocity (s^{-1})
 *Linoleic (18:2) ω-6 60%* *Linolenic (18:3) ω-3 8%*	Soybean oil	54	0.919	5.18	24.14
				9.82	31.45
	Squalene	11.2	0.858	18.03	34.59
				33.75	46.42
				53.54	58.03
	Limonene	1.3	0.840	44.51	68.01
				66.12	87.17
				74.68	94.14

When soyabean oil was used as continuous phase, the increase of shear stress determined droplets size decrease but droplets size below the mean pore size also obtained as showed in Fig. 3.2.

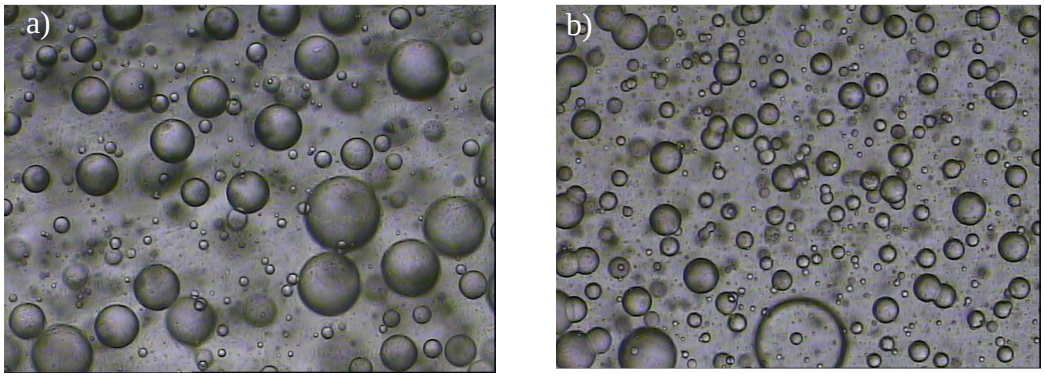


Fig. 3.2. Optical Microscopy images (10x) of emulsions produced at shear stress value of (a) 5.18 Dyne/cm^2, (b) 9.82 Dyne/cm^2. Soybean oil was used as continuous phase

This suggests that a phenomenon of breaking occurred without membrane controller. This result was also observed in the previous work and it was related to the continuous phase viscosity [18].

However, the aim of these tests was to verify the possibility to obtain droplets size controlled by membrane emulsification process, even when using a solvent with high viscosity value, selecting appropriate values of angular velocity. The tests showed that although low value of angular velocity was selected, droplets' breaking was induced. Fig. 3.3 shows that the shear stress increase reduces the emulsion droplets mean diameter but span increase. This confirms that when soybean oil was used, the contribution related to the high viscosity dominates the emulsification process also when controlling the effect of turbulences caused by the agitation speed of the continuous phase.

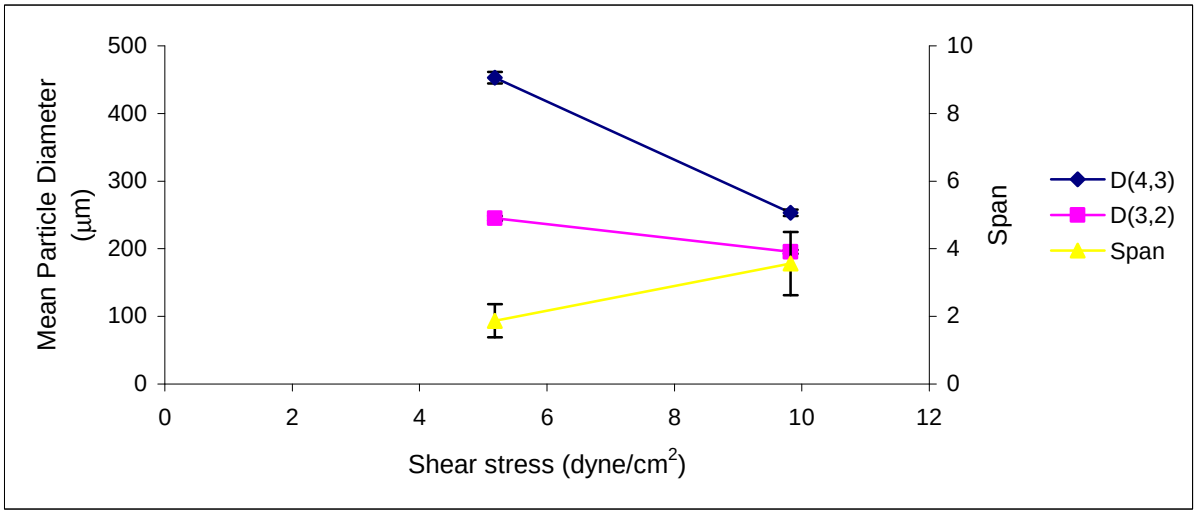


Fig. 3.3. Mean particles diameter and span of emulsions as function of shear stress. Soybean oil was used as continuous phase

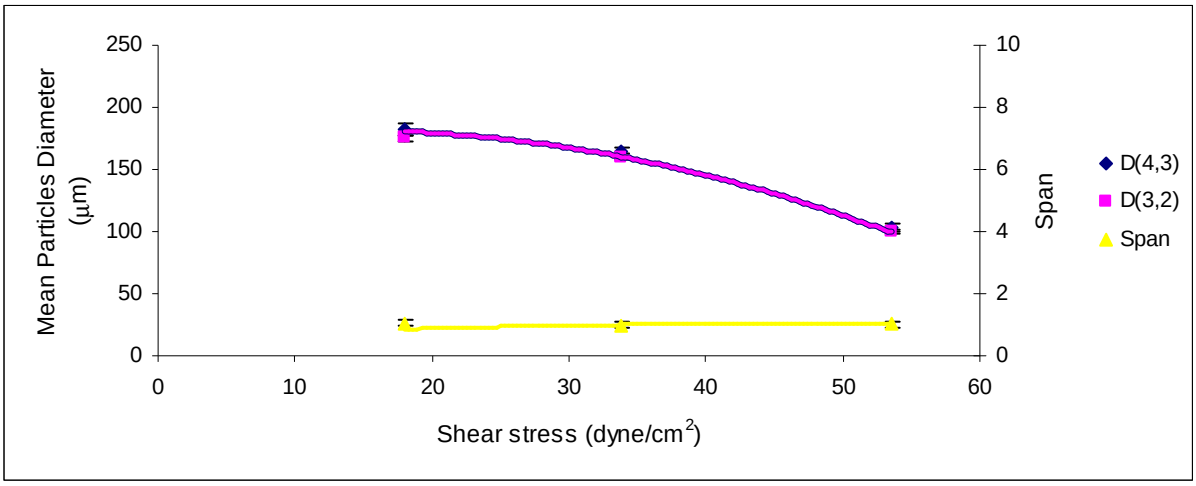


Fig. 3.4. The influence of shear stress conditions on mean particles diameter and span when squalene was used as continuous phase

It is observed that mean particles diameter decrease when shear stress increased. The droplets breaking phenomenon was observed only for shear stress values of 53.54 D/cm2 corresponding to an angular velocity of 58.03 s-1. The comparison between optical microscope images (Fig. 3.5) shows this effect.

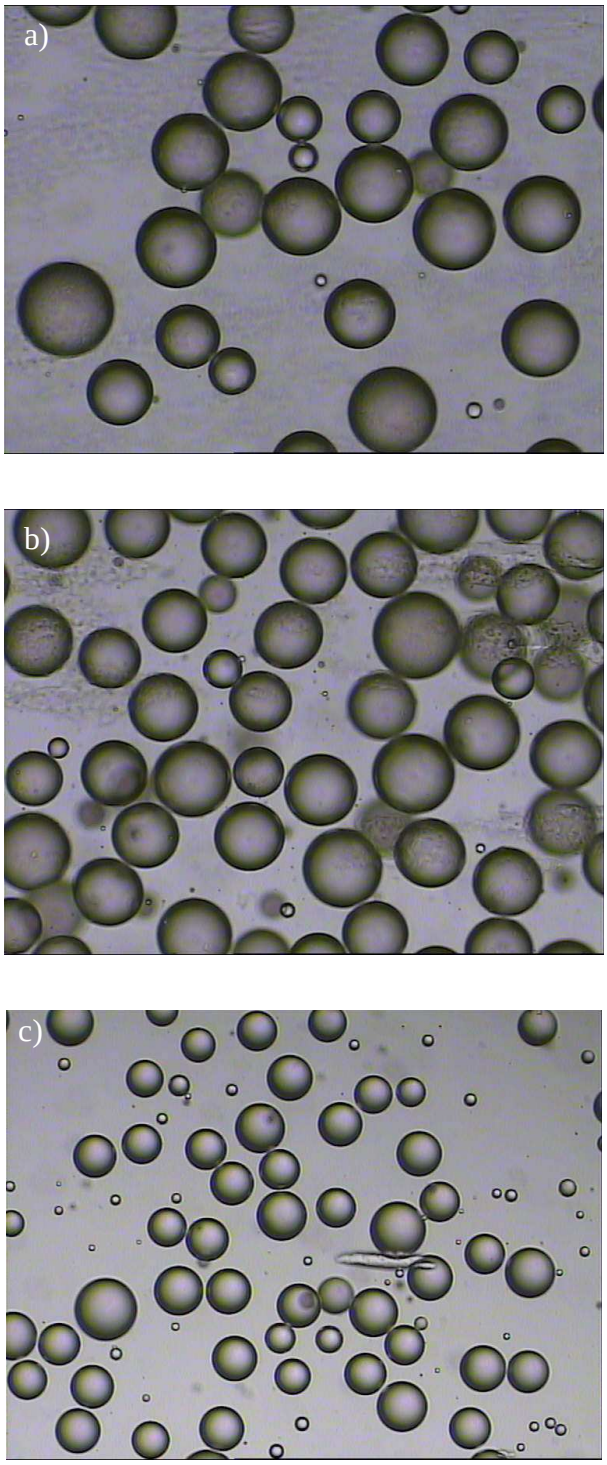


Fig. 3.5. Optical Microscopy images (10x) of emulsions produced at shear stress value of (a) 18.03 Dyne/cm^2, (b) 33.75 Dynescm^2, (c) 53.54 Dyne/cm^2. Squalene was used as continuous phase

The data shows that, when oils with a viscosity lower compared to the common vegetable oil like soybean oil (54 cP) but higher compared to the hydrocarbons such as kerosene (1.4 cP), an appropriate shear stress value through the selection of angular velocity permit to control membrane emulsification process preventing the effect of turbulence associated with the continuous phase viscosity that can be determine for droplets breaking.

The lowest viscosity value of limonene has permission to use higher values of angular velocity and thus shear stress. Fig. 3.6 shows the influence of shear stress conditions on mean particles diameter and span when limonene was used. Droplets size and droplets size distribution decrease when shear stress increase. In this case, no breaking phenomenon was observed also at very high values of angular velocity (Fig 3.7).

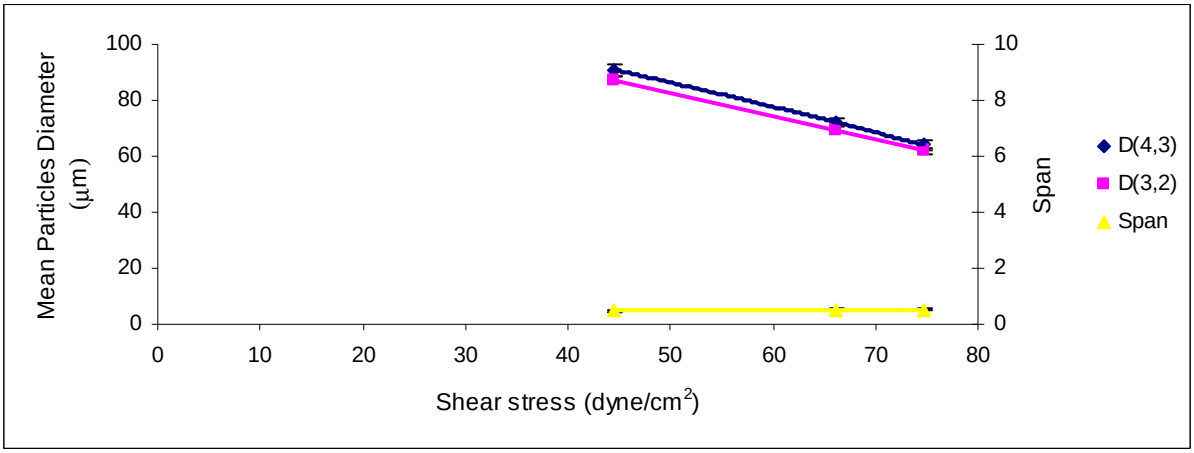


Fig. 3.6. The influence of shear stress conditions on mean particles diameter and span when limonene was used as continuous phase

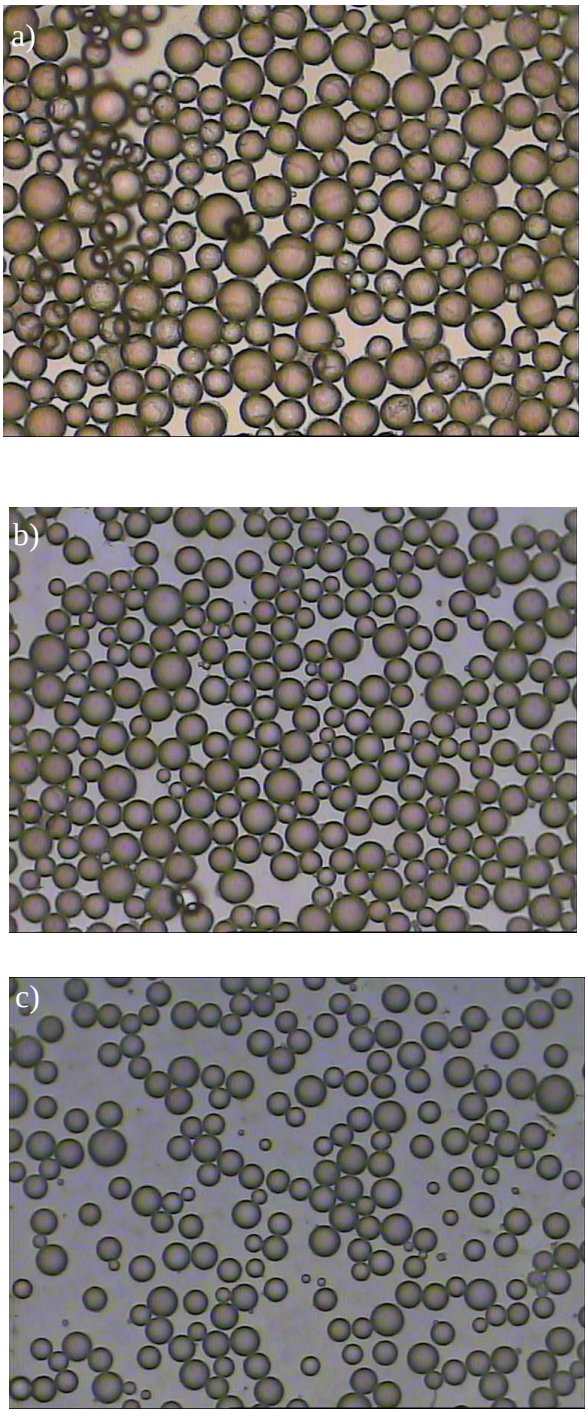


Fig. 3.7. Optical Microscopy images (10x) of emulsions produced at shear stress value of (a) 44.51 Dyne/cm^2, (b) 66.12 Dyne/cm^2, (c) 74.68 Dyne/cm^2. Limonene was used as continuous phase

The Fig. 3.8 combines the D(3,2) and span obtained using different continuous phases and at different shear stress conditions.

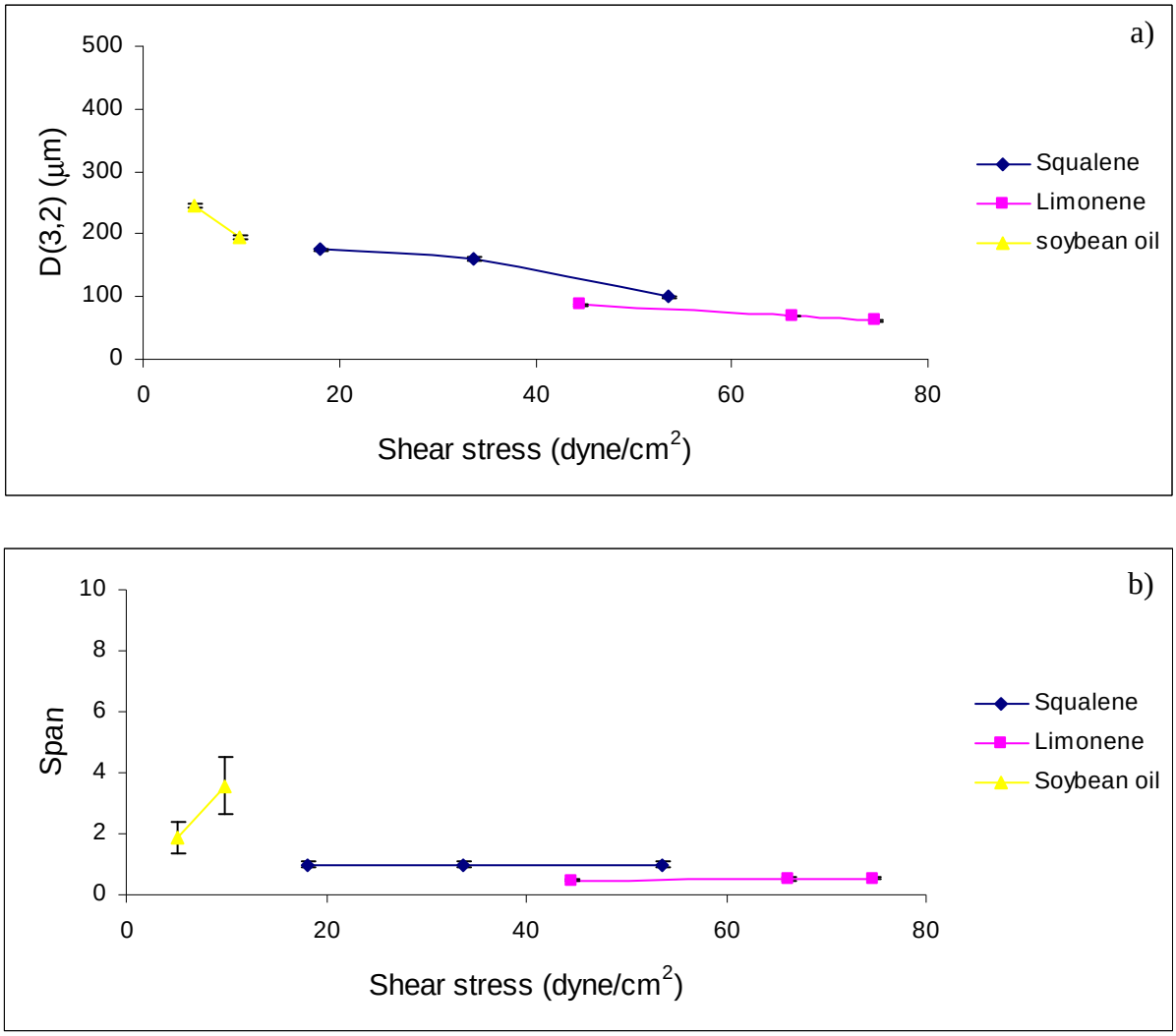


Fig. 3.8. The suitable range of shear stress conditions when using viscous fluids. Effects on: a) mean particles diameter and b) span when continuous phases with different viscosity were used

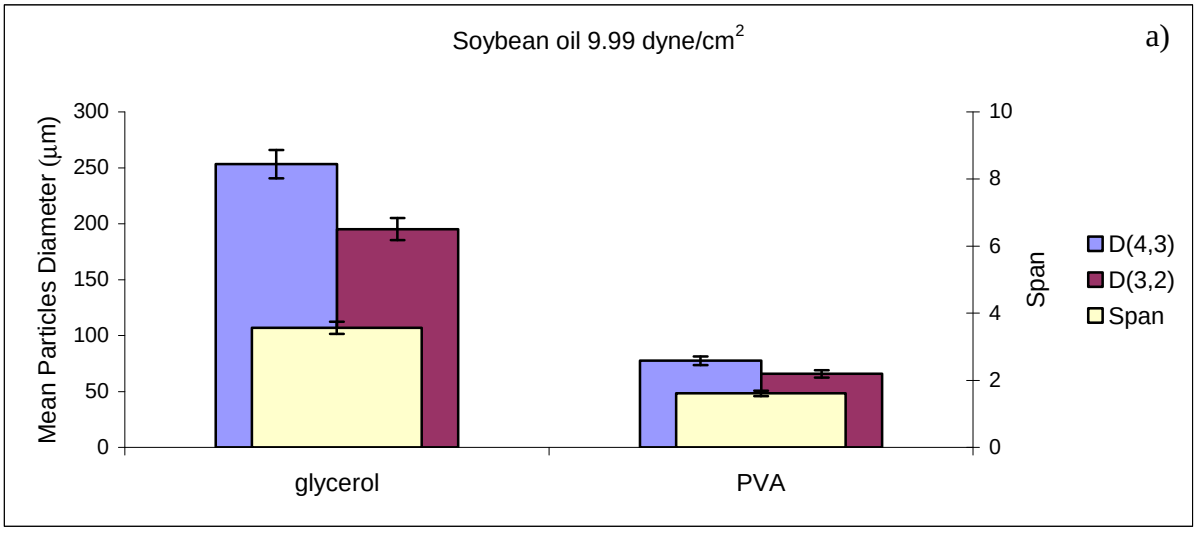
The effect of continuous phase on the W/O emulsion preparation using dispersion cell system depends on the combination of two factors: the shear stress (or angular velocity) and the viscosity of the continuous phase. The data shows that with soybean oil already at an angular velocity of 24.14 s^{-1} (5.18 D/cm^2) droplets breaking was observed and span value was high (drawn in yellow in Fig. 3.8 b). A further increase of angular velocity could cause loss of control of the emulsification process through the membrane pores. For limonene, higher values of angular velocity (up to 94.14 s^{-1}) do not cause emulsion droplets breaking and monosized droplets are obtained (span = 0.53). The low viscosity of limonene allows to use high values of angular velocity and shear stress without affect the

membrane process. Squalene has viscosity properties intermediate between soybean oil and limonene. Up to values of angular velocity of about 46.42 s-1 (33.75 D/cm^2) the effect of breaking does not occur but the further increase of angular velocity of up to 58.03 s-1 (53.54 D/cm^2) involves a loss of process control through the membrane pore size and got several droplets of smaller size.

3.3.2. Effect of dispersed phase composition

The study evaluated the effect of the dispersed phase composition using two different stabilizers added to the aqueous phase: glycerol and polivinilalcol (PVA) (13.000-23.000 daltons). Glycerol is used as plasticizer because it confers plasticity to the droplets while the PVA works as a thickening by increasing dispersed phase viscosity and thus reducing the kinetic energy of the droplets and hence their mobility and association by preventing the destabilization reversible phenomena such as flocculation. PVA is known to associate with anionic surfactant such as SDS in solution and form polyelectrolyte complex at the interface, leading to the decrease of interfacial tension [21, 22]. Experiments carried out in the same operative conditions with different continuous phases in the presence of PVA or glycerol in the dispersed phase are compared (Fig. 3.9).

In all experiments, droplets with lower size and lower size distribution are obtained when PVA is used.



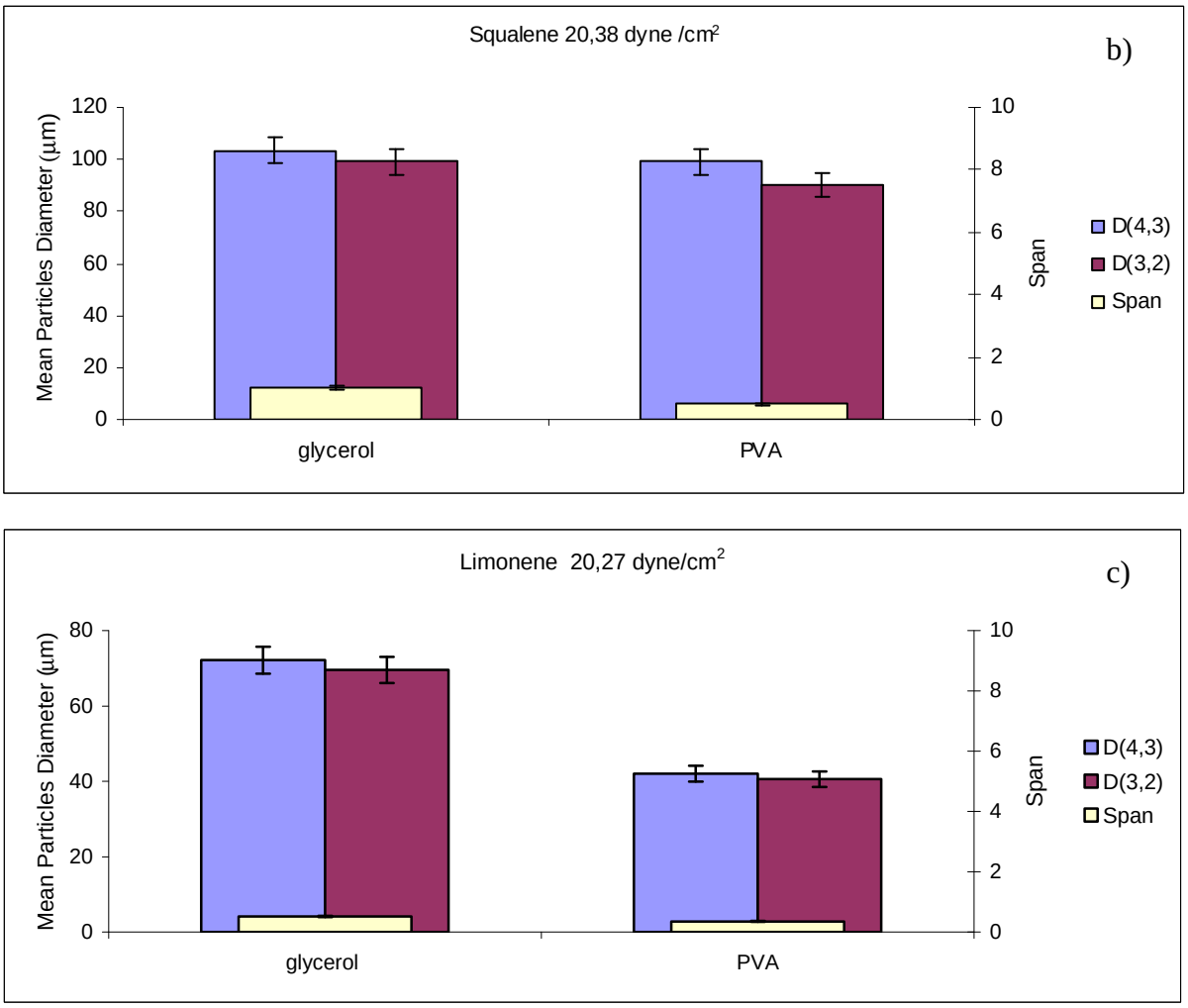


Fig. 3.9. Effect of PVA or Glycerol added in the dispersed phase on mean particles diameter and span when a) soybean oil, b) squalene or c) limonene was used as continuous phase

The physical stability of dispersed systems is expected to be lower when droplets with lower size are obtained. According to Stokes law:

$$v = d^2(\rho_s - \rho_0) - \frac{g}{18\eta_0}$$

where v is the particles' settling velocity (m/s) (vertically downwards if $\rho_s > \rho_o$, upwards if $\rho_p < \rho_f$), d is the men partciles diameter, g is the gravitational acceleration (m/s^2), ρ_p is the mass density of the particles (kg/m^3), and ρ_o is the mass density of the continuous phase (kg/m^3).

The difference between W/O emulsions obatined using PVA or glycerol are also evident in the Fig. 3.10 for all continuous phase used.

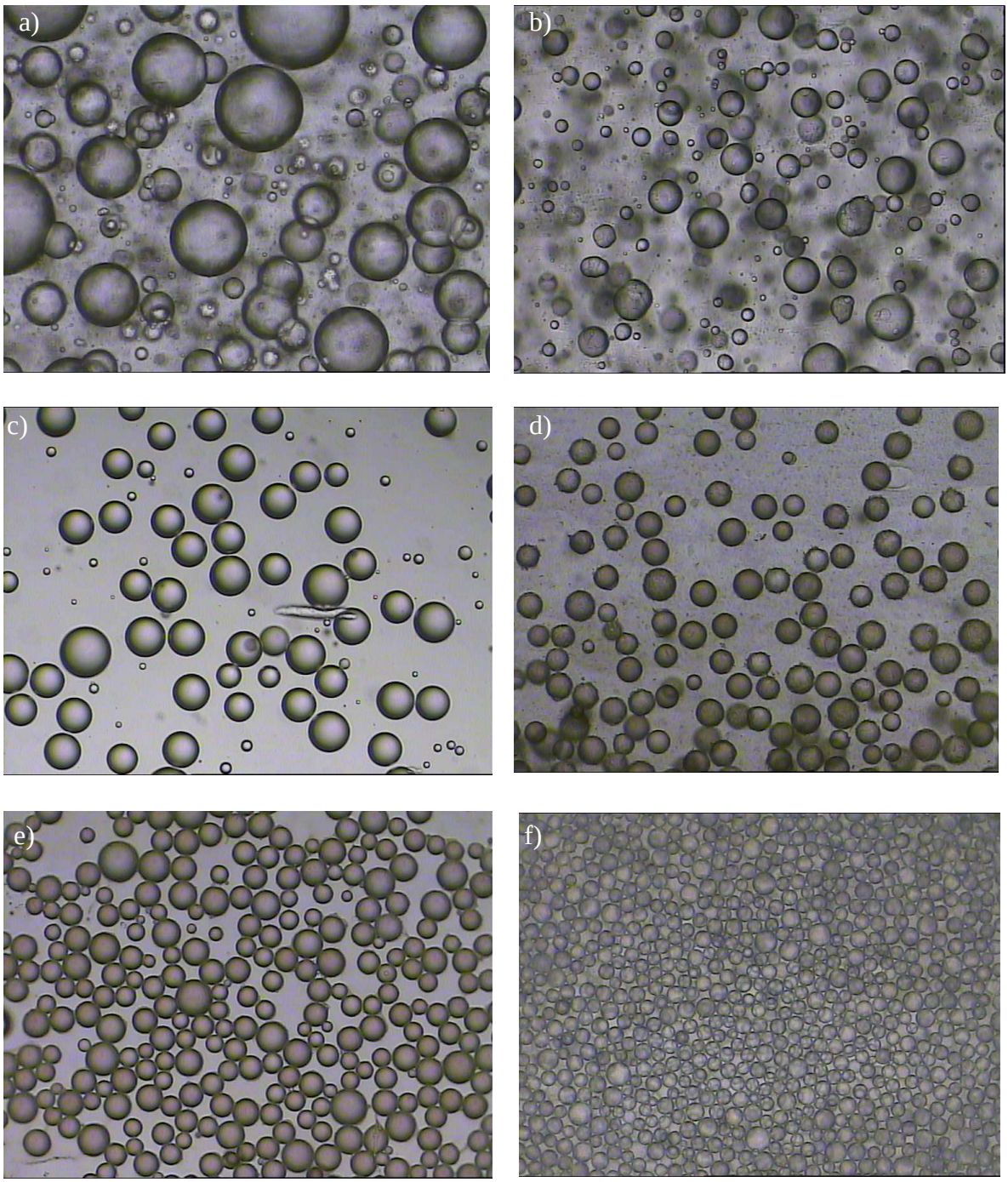


Fig. 3.10. Optical microscopy images of a) Soybean Oil + glycerol, b) Soybean Oil + PVA; c) Squalene + glycerol, d) Squalene + PVA; e) Limonene + glycerol, f) Limonene + PVA.

3.3.3. Comparison between membrane emulsification and mechanical emulsification.

Membrane emulsification process was compared with the mechanical process. The dispersion cell was used but the dispersed phase was added along with the continuous

phase and not been made to permeate through the membrane pores. The mixture was stirred using the same angular velocity used with the membrane emulsification process. In particular, some experimental data obtained using squalene as a continuous phase were compared. The aim is to verify whether the emulsification is achieved through the dispersion of the aqueous phase in oil phase (continuous phase) through the membrane pores or by mechanical agitation. The data confirm that emulsion droplets are obtained by membrane emulsification process. More uniform and monodisperse emulsions were obtained using the membrane compared to tests in with only mechanical agitation was used. In particular, this is clear if span values are compared (Fig. 3.11). In addition, the results obtained from mechanical tests shows very low reproducibility as indicated from the error bar in Fig. 3.11.

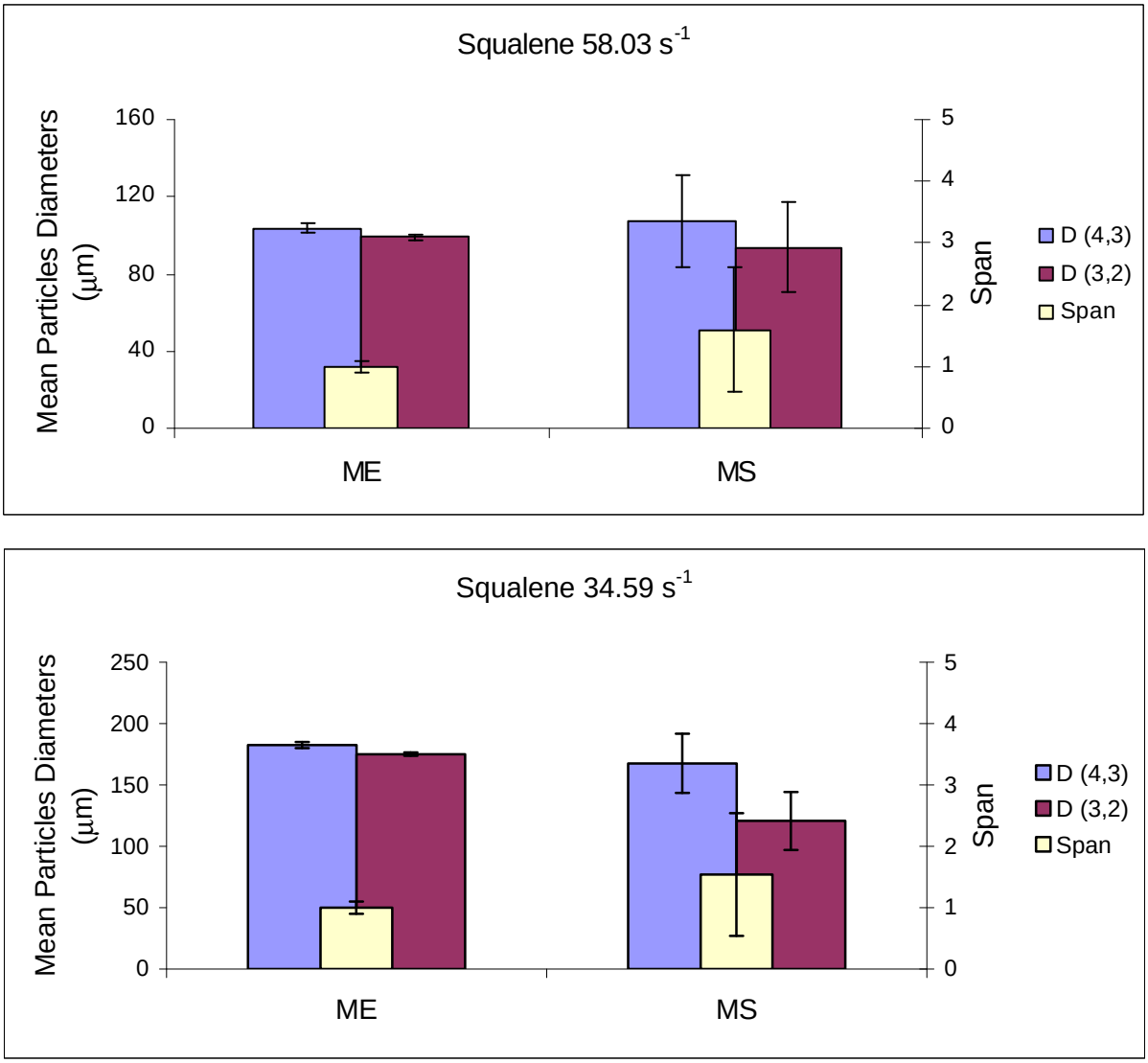


Fig. 3.11. Comparison between Membrane emulsification (ME) and mechanical stirring (MS) when squalene was used at two different angular velocity (ω) values a) 34.59 s^{-1}, b) 58.03 s^{-1}.

3.4. Conclusions

Stirred membrane emulsification can be used with successful to produce W/O emulsion using Limonene (+ Span 80 2% wt) as continuous phase and PVA as stabilizer in the dispersed phase. The continuous phase viscosity influence the process of droplets emulsions production controlled by membrane pore.
These results shows the combined effect between phase properties and operative conditions in the preparation of w/o and o/w emulsion by membrane emulsification using continuous phases with high viscosity.

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Chapter 4

Evaluation of protein behaviour in membrane emulsification process

4.1. Introduction

Proteins are the single most commonly used class of foaming and emulsifying agent used in the food industry. They are natural, non-toxic, cheap and widely available, thus making them ideal ingredients. Their interfacial properties have made them the subject of much study over the years [1,2]. They stabilise emulsions by forming a viscoelastic adsorbed layer [3], the mechanical properties of which are thought to influence the stability of emulsions and foams [4]. Proteins have a complex structural morphology. It is known that the extent of protein adsorption is influenced by surface hydrophobicity [5,6] and charge [2,7]. Once adsorbed, they unfold and rearrange their secondary and tertiary structure [8-10] to expose hydrophobic residues to the hydrophobic phase. Proteins (enzyme or functional proteins) are also used as high value components in emulsion formulation in food and pharmaceutical field. Commonly used emulsification technique due to the high shear forces can be cause lose of bioactivity of shear stress or thermosensitive ingredients like proteins [11]. Membrane emulsification technology is a suitable process to produce particles with precisely controlled size and size distribution with low energy and low shear. However, when proteins are used in membrane emulsifications some aspects must be considered.

Fouling can occur as accumulation of proteins on the top surface of the membrane (external fouling), or as deposition and adsorption of proteins within the internal pore structure of the membrane (internal fouling). Fouling phenomena can determine large dispersed phase flux decline, modification of pore size during emulsification process, modification of membrane wettability and loss of protein amount needed as emulsifier or as functional ingredients in the specific formulation.

The aim of this work to identify the conditions influencing the process of membrane proteins adsorption. Proteins with different properties and potential use as emulsifier were selected to study membrane adsorption. Bovine serum albumin (BSA), β-lactoglobilin, Concanavalin A (Conc A) and Lipase from *candida rugosa* was used as proteins models. Hydrophilic ceramic membrane with pore size of 50 nm, hydrophilic SPG membrane with pore size of 100 nm and hydrophobic SPG membrane with pore size of 400 nm was use to carried out the adsorption membrane experiments.

Two different conditions were investigated: a) protein solution recirculation in tangential flow and b) protein solution permeation through the membrane.

In this work, the membrane wettability modification by protein adsorption was used to obtain a hydrophilic membrane with asymmetric properties used to prepare a water-in-oil emulsion (w/o). The w/o emulsion prepared following membrane modification was compared with the w/o emulsion prepared without membrane modification.

4.2. Materials and Methods

4.2.1. Materials

The proteins and buffer solutions used are summarized in table 1.

Table 4.1.

Proteins	Buffer solution
BSA (Sigma)	NaH_2PO_4/ Na_2HPO_4 10 mM pH 7
β-lactoglobilin (Sigma)	NaH_2PO_4/ Na_2HPO_4 10 mM pH 7
Conc A (Sigma)	TrisHCl 20 mM, $MnCl_2$ 1 mM, NaCl 1 M, $CaCl_2$ 1 mM pH 5.3.
Lipase from *candida rugosa* (Sigma)	NaH_2PO_4/ Na_2HPO_4 50 mM pH 7

Isooctane (Carlo Erba) and Span 80 (Sigma) was used as organic phase and emulsifier in the preparation of o/w emulsion.

4.2.2. Membranes and membrane plant

Membranes used in this work are ceramic membrane (Membraflow, Germany) and shirasu porous glass membrane (SPG).

Tubular microporous ceramic zirconia oxide (ZrO_2) membrane with mean pore sizes of 50 nm, inner diameter of 7 mm, outer diameter of 10 mm and length of 100 mm were employed. The membrane is constructed from multiple ceramic layers:

- first layer with alfa-allumina, pore size about 1 μm, layer thickness about 50 μm;
- second layer with alfa-allumina, pore size about 200 μm, layer thickness about 50 μm;
- active layer with zirconia , with layer thickness1-2 μm.

Tubular hydrophilic and hydrophobic SPG membrane with mean pore sizes respectively of 100 nm and 400 nm, inner diameter of 9 mm, outer diameter of 10 mm and length of 100 mm were also employed. The membrane plants used to carried out the experiments of protein adsorption are showed in Fig. 4.1 and 4.2. Fig. 4.3 shows the cross-flow membrane emulsification plant used to prepare w/o emulsions.

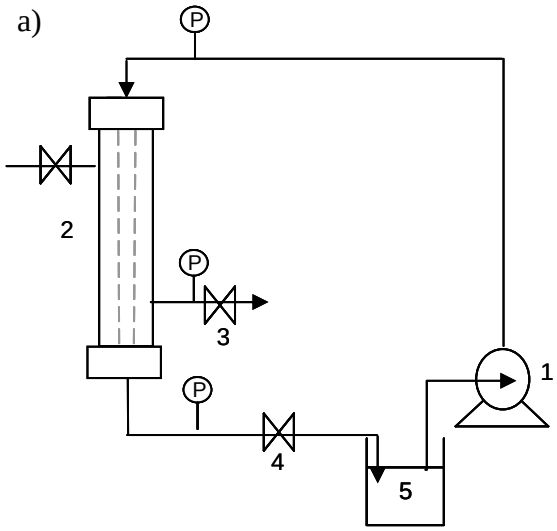

Fig. 4.1. Equipment scheme of protein adsorption experiments when protein solution was recirculated along the lumen membrane
1: peristaltic pump; 2: membrane module; 3: purge valve; 4: backpressure valve; 5: continuous phase container

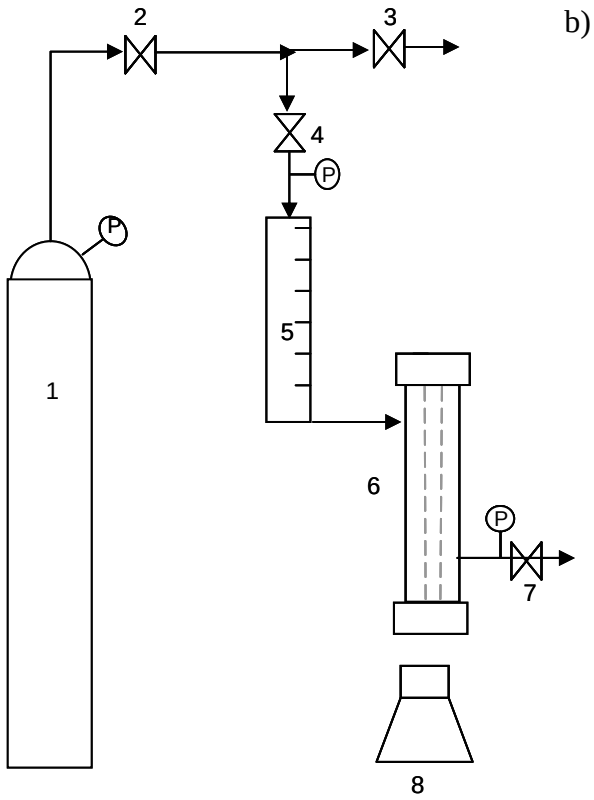

Fig. 4.2. Equipment scheme of protein adsorption experiments when protein solution was pressurized trough the membrane pores
1: nitrogen gas cylinder; 2: backpressure valve; 3: purge valve; 4: backpressure valve; 5: initial Conc A solution graduated vessel; 6: membrane module; 7: purge valve, 8: permeated solution vessel

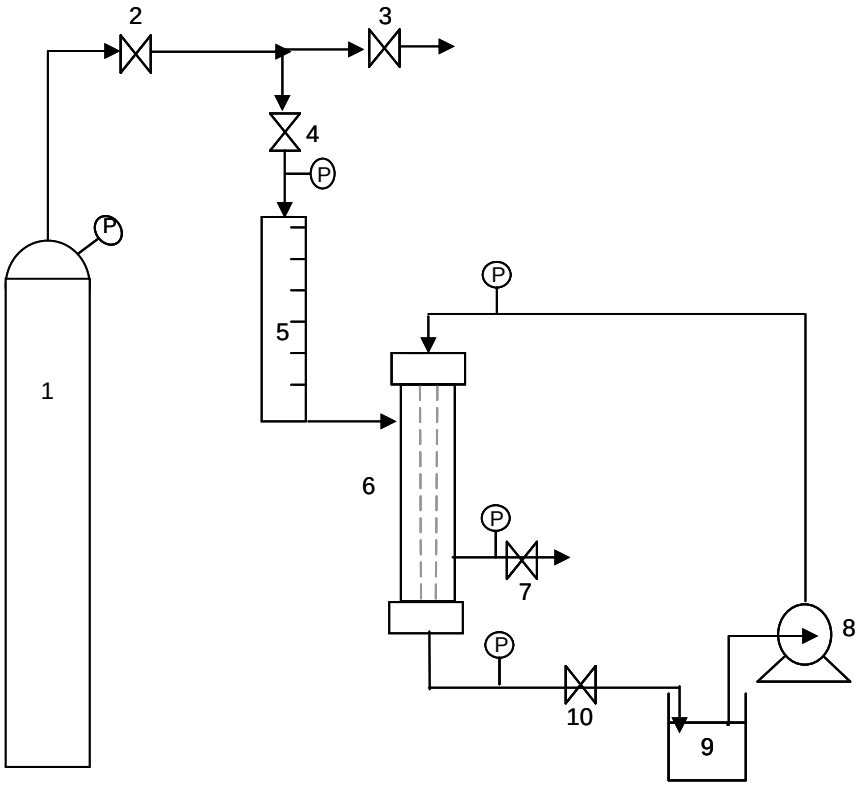

Fig. 4.3 . Crossflow membrane emulsification equipment scheme.
1: nitrogen gas cylinder; 2: backpressure valve; 3: purge valve; 4: backpressure valve; 5: dispersed phase graduated vessel; 6: membrane module; 7: purge valve; 8: gear pump; 9: continuous phase container; 10: backpressure valve

4.2.3. Experimental set-up

In this work two main experimental parts: a) Protein adsorption and b) emulsion preparation will be discussed.

4.2.3.1 Protein adsorption in condition of tangential flux

The membrane plant showed in figure A was used. The protein solution was ricirculated in the lumen side using a peristaltic pump (Amicon 958622) for 45 min. During the time, a samples was taken from the solution vessel after 5, 15, 30, 45 min. The protein amount was quantified by BCA Test. The adsorbed protein amount (m_{ads}) was calculated as following:

$$m_{ads} = m_i - m_s \quad (1)$$

in which m_i and m_s represents, respectively, the initial mass and the mass measured in the samples taken form solution vessel at different time (t_n) of protein.

The membrane permeability to ultra pure water was measured at beginning and at the end of each experiments. The permeability reduction was measured according to following equation:

$$P_{red} = \frac{P_i - P_f}{P_i} * 100 \quad (2)$$

in which Pi and P_f represents respectively the initial water permeability and the water permeability at the end of protein adsorption experiment.

In some case, the protein adsorption % was reported and calculated according to following equation:

$$m_{ads}\% = \frac{m_{ads}}{m_i} * 100 \quad (3)$$

In addition, the surface that the protein would occupy as mono strait (S_{prot}) was calculated considering proteins crystallographic data.

After each experiment the membrane and membrane plant were cleaned according the procedure described in the section 2.3.3. The experimental conditions are showed in Table 4.2. The effect of protein concentration, axial velocity and type of membrane was evaluated.

Table 4.2. Experimental conditions

Protein	Protein concentration (g/l)	Axial velocity (m/s)	Type of membrane
BSA	0.5	0.09	Hydrophilic Ceramic d_p 50 nm
		0.28	
		0.43	
		0.58	
	1	0.09	
		0.58	
	2	0.09	
		0.28	
Lipase	2	0.09	Hydrophilic Ceramic d_p 50 nm
		0.28	
		0.43	
		0.58	
	4	0.09	
		0.28	
	6	0.09	
		0.28	
	2	0.28	Hydrophilic SPG d_p 0.1µm
Concanavalin A	1	0.09	Hydrophilic Ceramic d_p 50 nm
β-Lattoglobulin	1	0.09	Hydrophilic Ceramic d_p 50 nm

4.2.3.2 Protein adsorption in condition of cross flux

The membrane plant showed in figure A was used. To evaluate membrane protein adsorption, protein solution was pressurized with N_2 gas at 30 psi and filtered from shell-to-lumen in dead-end mode. The flux and protein concentration in permeate solution was measured during the protein filtration process. In this case, the adsorbtion of Conc A, PhAla was evaluated. The conditions applied during the experiments were showed in the table 3.

Protein concentration was evaluated using at spectrophotometry, at 280nm. The Conc A adsorption was evaluated from the C_p/C_f ratio (the ratio of Conc A concentration in permeate, C_p, to that in initial feed, C_f). A value of C_p/C_f equal to 1 indicate no protein retention occurred. When C_p/C_f <1 indicates protein retention. The mass adsorbed into the membrane pores (m_a) and the concentration of protein eventually adsorbed (C_a) were calculated as follows, respectively:

$$m_a = m_f - \sum_i m_{p_i} \tag{4}$$

$$C_a = \frac{m_f - \sum_i m_{p_i}}{V_v} \tag{5}$$

where m_f and m_p are, the mass of protein in the initial feed and in the permeated collected samples, respectively, and V_v is the void volume of the membrane.

$$\text{flux reduction \%} = \frac{J_0 - J}{J_0} * 100 \tag{6}$$

where J_0 and J are the buffer solution flux without and with Conc A or PhAla or Conc A/PhAla, respectively.

Table 4.3. Experimental conditions applied when protein adsorption experiment was carried out applying a transmembrane pressure

Protein	Concentration (g/l)	Pression (Psi)	Type of membrane
PhAla	4	30	Idrophobic SPG membrane d_p 0.4 μm
Conc A	1	30	Idrophobic SPG membrane d_p 0.4 μm
Conc A + PhAla	1 8	30	Idrophobic SPG membrane d_p 0.4 μm

4.2.3.3 Membrane and membrane plant cleaning

The cleaning procedure was carried out trough different step using detergent solution of 10-30 wt% of NaOH and 1-5 wt% di NaOCl:

1. 1% v detergent solution was recirculated along the lumen side for 30min at 50°C;
2. 3% v detergent solution was a) recirculated along the lumen side for 30min at 70°C and b) permeated through the membrane at low flux after 30 min
3. ultrapure water was recirculated 2 or 3 times along the lumen side

After this procedure, the water permeability was completely restored.

Alla fine di queste operazioni, la permeabilità della membrana è ritornata al valore di partenza.

4.2.3.4. W/O emulsion preparation

W/O emulsions were prepared using the plant showed in Fig. 4.3 and the hydrophilic ceramic membrane with 50 nm of pores diameter. The emulsion was prepared after membrane modification after lipase adsorption. 6 g/l lipase solution was ricirculetd along the lumen membrane side using a peristaltic pump as previously described (4.2.3.1 section) at 0.58 m/s as axial velocity for 45 min. The modified membrane was used to prepare a w/o emulsion. The continuous phase was a 2% wt Span 80 solution in Isooctane and the dispersed phase was ultrapure water. The water phase was pressurized trough the membrane at the transmembrane

pressure of 0.05 bar and dispersed phase flux was 30 l/hm^2. The continuous phase was recirculated using an axial velocity of 0.05 m/s.

A w/o emulsion using the same conditions, previously described, was prepared without membrane surface modification. In this case, a transmembrane pressure of around 0.025 bar was required.

The emulsions was analyzed by optical microscopy.

4.2.4. Protein quantification

The overall protein amount present in the solution was quantified by BCA Protein Assay (Sigma Aldrich) based on the aromatic amino acid absorbance at 562 nm. BSA solutions were used as standard proteins.

4.2.5. HPLC analysis

The concentration of PhAla was measured by using a CROWNPAK CR (+) column (150mm×4 mm) (Daicel Chemical Industries, ltd.), a mobile phase made of $HClO_4$ pH 7 flowed at 0.8 ml/min, 200 nm, 25 °C. Calibration curve of chromatographic peak area versus PhAla concentration was obtained from (D,L) PhAla standard solutions.

4.2.6. Droplets size analysis

W/o emulsion droplet size distribution and droplet size were measured using optical microscope (Zeiss, model Axiovert 25) equipped with a camera (JVC, model TK-C1481BEG) to capture the images of the emulsions was used. The droplets size was measure using scion image software.

The mean particle size was expressed as the surface weighted mean diameter (or Sauter diameter), D[3,2]and as the volume weighted mean diameter, (or De Brouckere diameter), D[4,3]. D[3,2] and D[4,3] were determined, respectively, as follows:

$$D[3,2] = \frac{\sum D_i^3 n_i}{\sum D_i^2 n_i} \tag{7}$$

$$D[4,3] = \frac{\sum D_i^4 n_i}{\sum D_i^3 n_i} \tag{8}$$

where D_i = particle diameter of class i and n_i = number of particle in class i.

The width of droplet size distribution was expressed as a Span number, calculated by the following expression:

$$Span = \frac{D[0.9] - D[0.1]}{D[0.5]} \quad (9)$$

where D[x0] is the diameter corresponding to x0 vol.% on a relative cumulative droplet size curve.

4.3. Results and discussion

4.3.1. Protein adsorption in condition of tangential flux as a function of protein concentration and axial velocity

The conditions applied during the protein adsorption experiments was summarized in table and the results were discussed separately for each protein studied.

4.3.1.1 BSA

The protein amount adsorbed during the time and the membrane permeability, measured before and after protein adsorption experiments, at different axial velocity, are showed in figure 4 for 0.5 g/l BSA solution. The results showed that BSA rapidly adsorb at membrane surface in 5-15 min and after this time the amount of protein adsorbed remained constant. In the pH conditions used the protein show very high water solubility for the presence of negative charge groups and present the expanded structure. The protein adsorption determine water permeability reduction. Same results were observed when 1 and 2 g/l BSA solution were used (Fig. 4.5 and Fig. 4.6).

The data suggest that protein adsorption depended on protein concentration and axial velocity. In particular when high protein solution was used, the amount of protein adsorbed on membrane surface increased. This phenomenon determined a major reduction of membrane permeability. The protein adsorbed amount and permeability reduction as a function of initial protein solution concentration are showed in the Fig. 4.6 a) and b), respectively, at different axial velocity used.

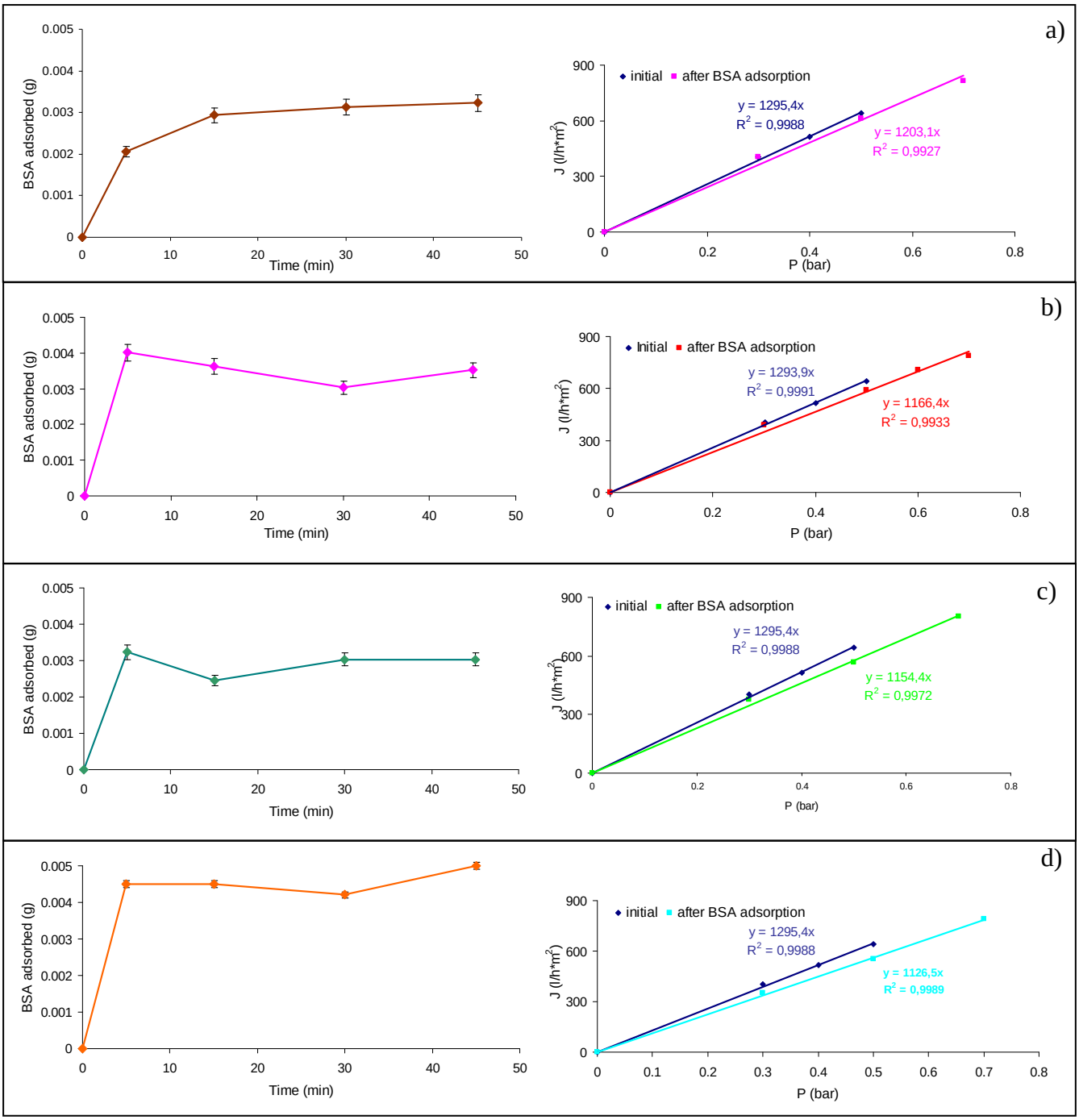

Fig. 4.4. BSA adsorbed amount and membrane permeability when a) 0.09 m/s, b) 0.28 m/s, c) 0.43 m/s and d) 0.58 m/s as axial velocity was applied. The initial protein solution concentration was 0.5 g/l.

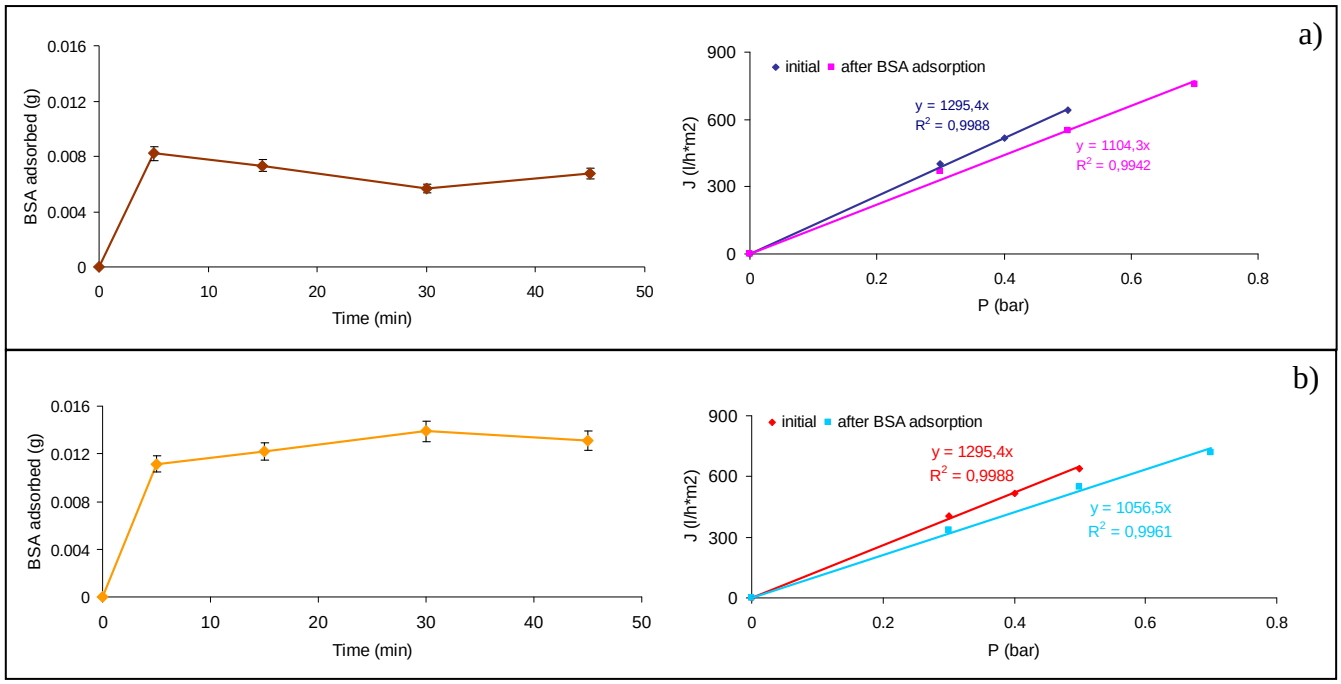

Fig. 4.5. BSA adsorbed amount and membrane permeability when a) 0.09 m/s, b) 0.58 m/s as axial velocity was applied. The initial protein solution concentration was 1 g/l.

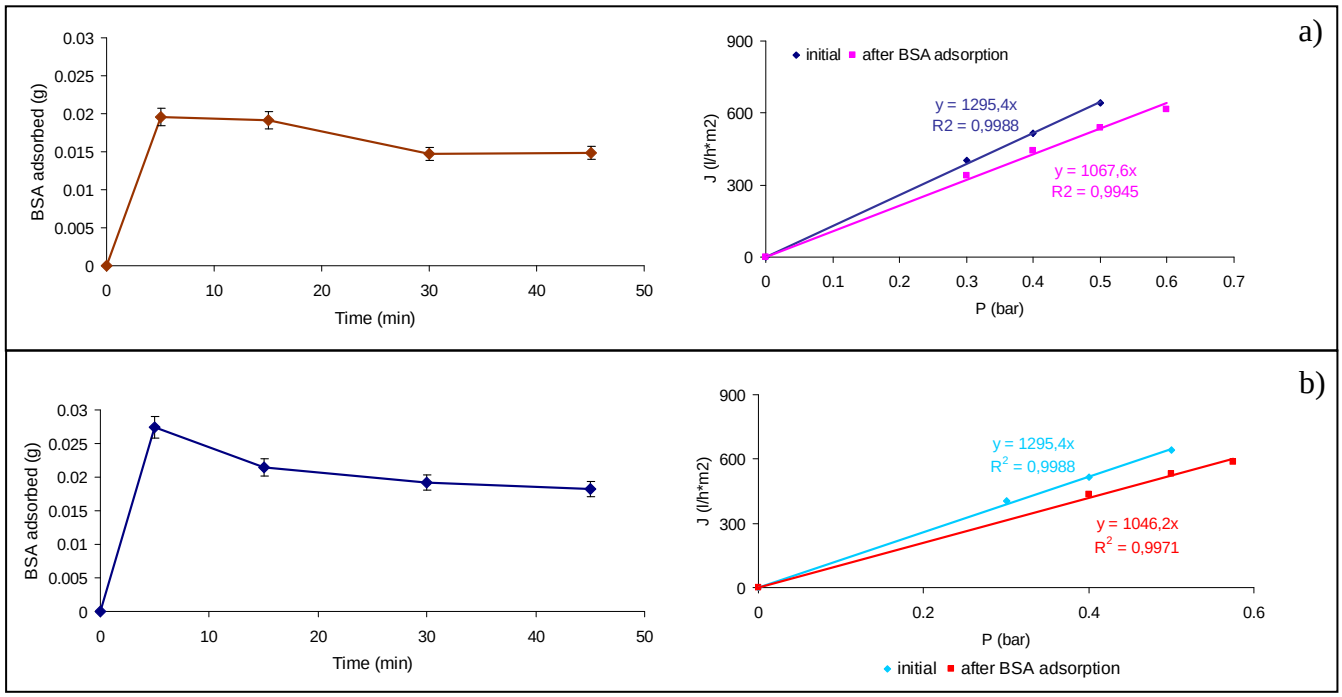

Fig. 4.6. BSA adsorbed amount and membrane permeability when a) 0.09 m/s, b) 0.28 m/s as axial velocity was applied. The initial protein solution concentration was 2 g/l.

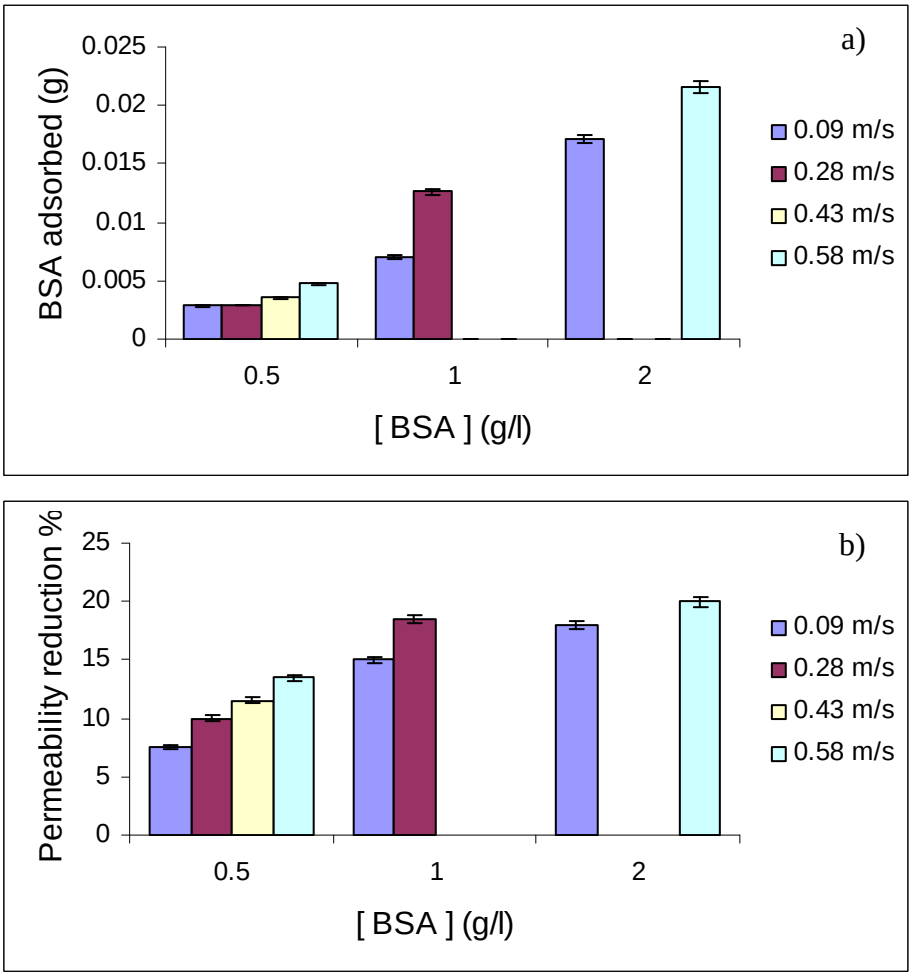

Fig. 4.6. a) BSA adsorption amount and b) permeability reduction as a function of initial BSA solution concentration

The same relationship was observed when axial velocity was increased as showed in the Fig. 4.7 and 4.8, respectively.

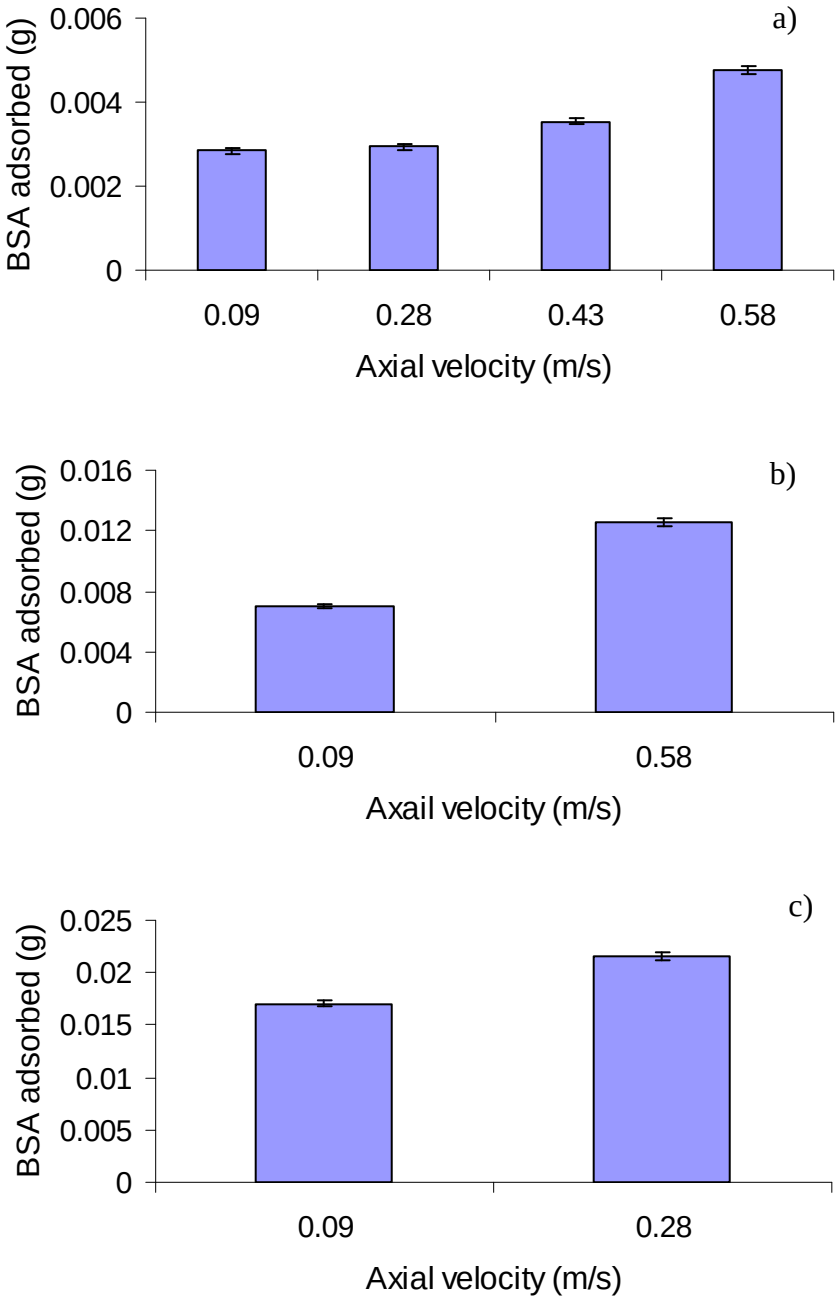

Fig. 4.7. BSA amount adsorbed as a function of axial velocity when a) 0.5 g/l, b) 1g/l and c) 2 g/l BSA solution was used

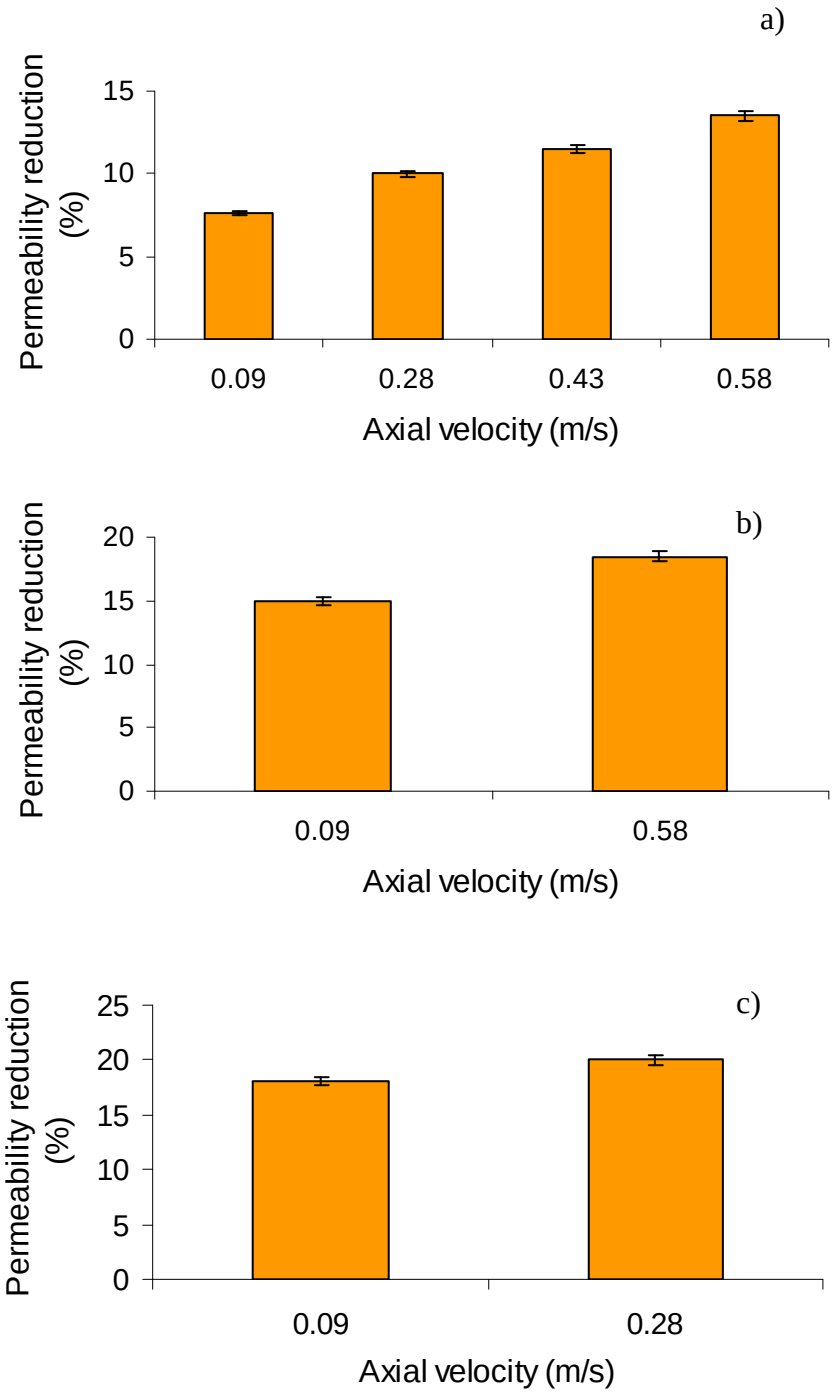

Fig. 4.8. Permeability reduction after BSA adsorption as a function of axial velocity when a) 0.5 g/l, b) 1g/l and c) 2 g/l BSA solution was used

All results obtained using BSA are summarized in Table 4

Table 4.4 Protein adsorption and Permeability reduction with BSA

BSA concentration (g/l)	Axial velocity (m/s)	Protein adsorption amount (mg)	Permeability reduction (%)
0.5	0.09	2.8	7.6
	0.28	2.9	10
	0.43	3.5	11.5
	0.58	4.8	13.5
1	0.09	6.9	15
	0.58	1.26	18.5
2	0.09	1.7	18
	0.28	2.16	20

4.3.1.2 Lipase

Lipases are well-known biocatalysts able to interact with interfaces where they undergo a conformational activation. The lipase active site is covered by a lyophilises surface loop, the so-called lid (or flip), which upon binding to the interface moves away, turning the "closed" form of the enzyme into an "open" form.

The protein amount adsorbed during the time and permeability measurement at different axial velocity are showed in Fig. 4.9 when 2 g/l lipase solution was used. The results showed that the lipase rapidly adsorb at membrane surface in 5-20 min and after this time the amount of protein adsorbed remained constant. Same results were observed when 4 and 6 g/l lipase solution was used (Fig. 4.10 and 4.11).

The data showed very high protein amount adsorption and permeability reduction. In addition, as showed to the BSA, also when the lipase was used, the protein adsorption and permeability reduction increase when initial protein concentration and axial velocity was increased as showed in Fig. 4.12 and 4.13.

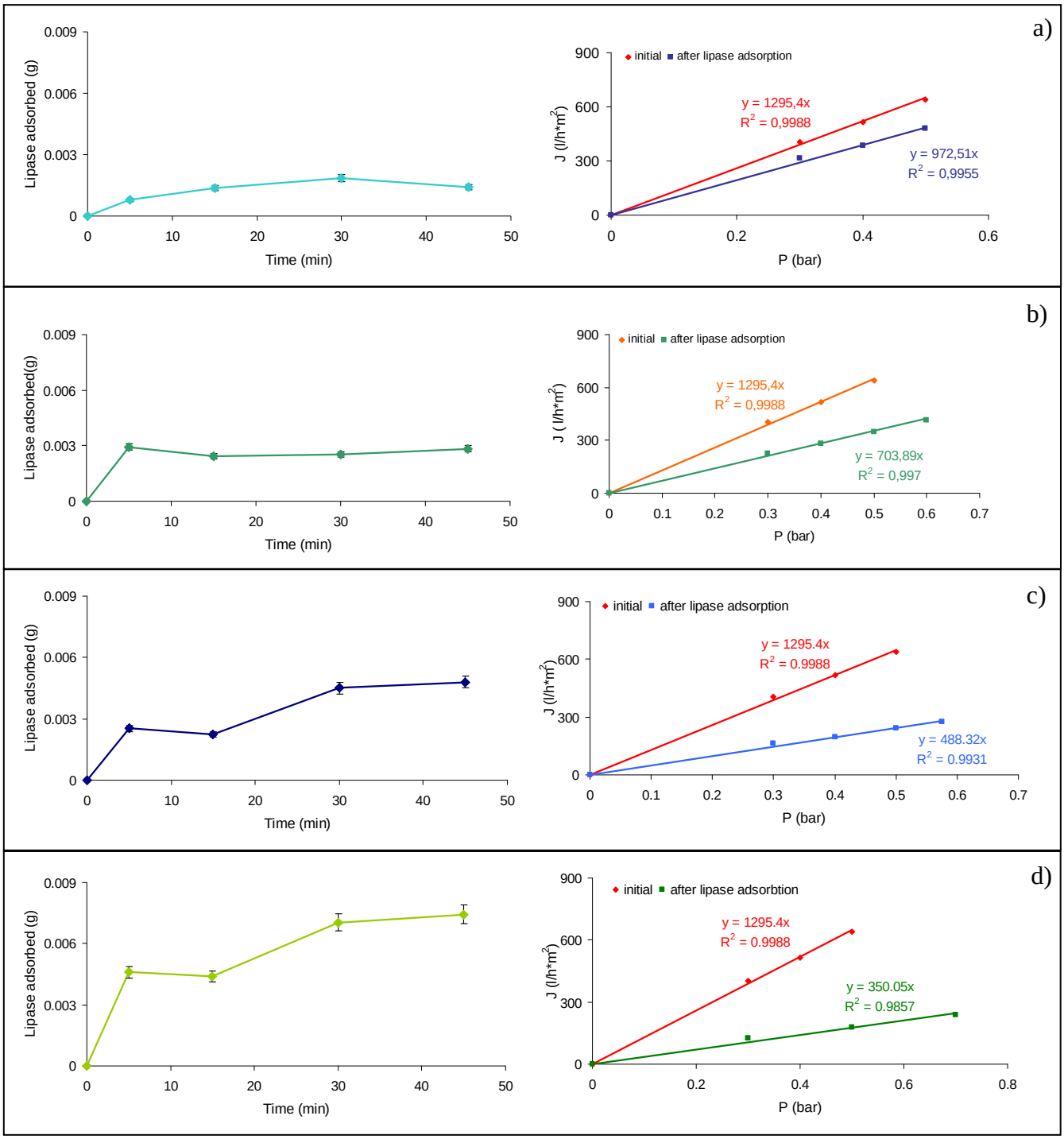

Fig. 4.9. Lipase adsorbed amount and membrane permeability when a) 0.09 m/s, b) 0.28 m/s, c) 0.43 m/s and d) 0.58 m/s as axial velocity was applied. The initial protein solution concentration was 2 g/l.

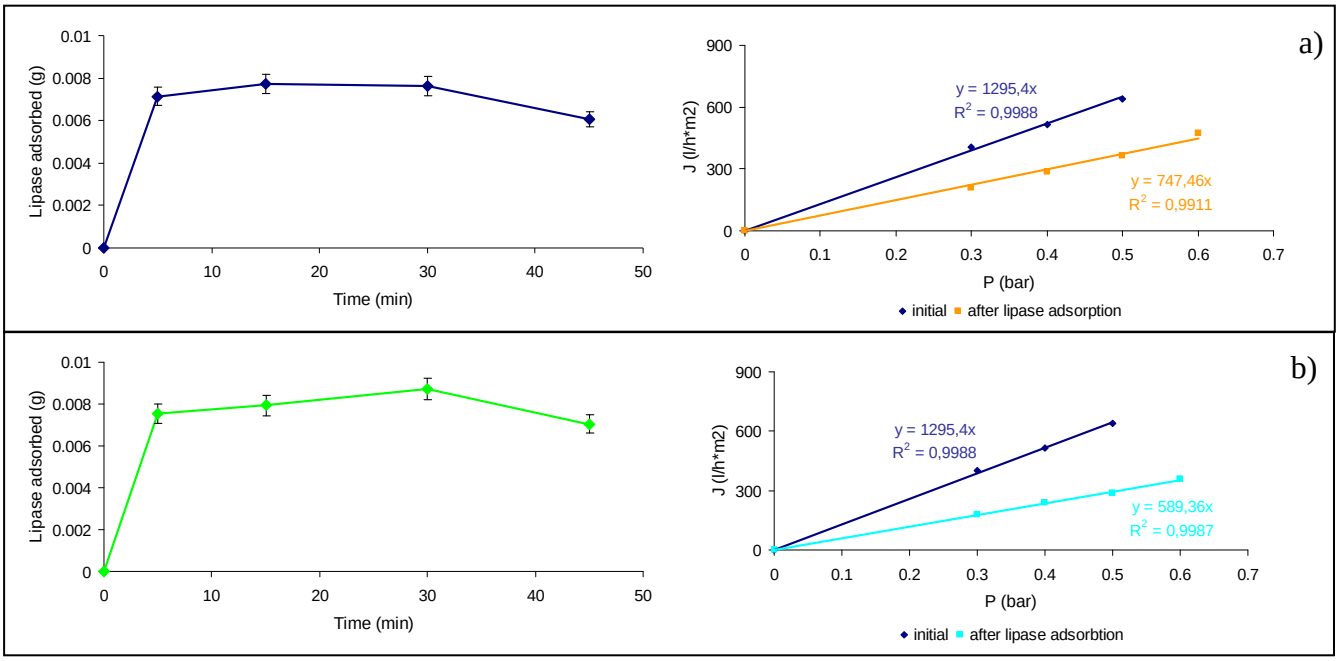

Fig.4.10. Lipase adsorbed amount and membrane permeability when a) 0.09 m/s, b) 0.28 m/s as axial velocity was applied. The initial protein solution concentration was 4 g/l.

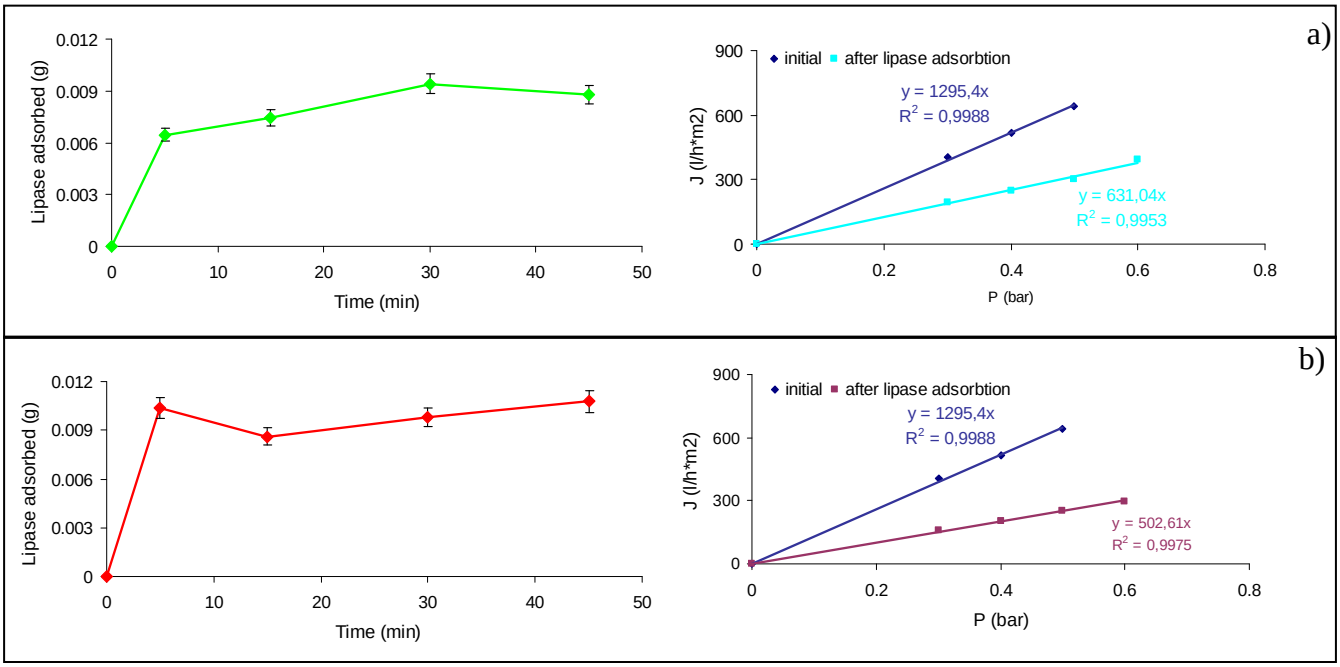

Fig. 4.11. Lipase adsorbed amount and membrane permeability when a) 0.09 m/s, b) 0.28 m/s as axial velocity was applied. The initial protein solution concentration was 6 g/l.

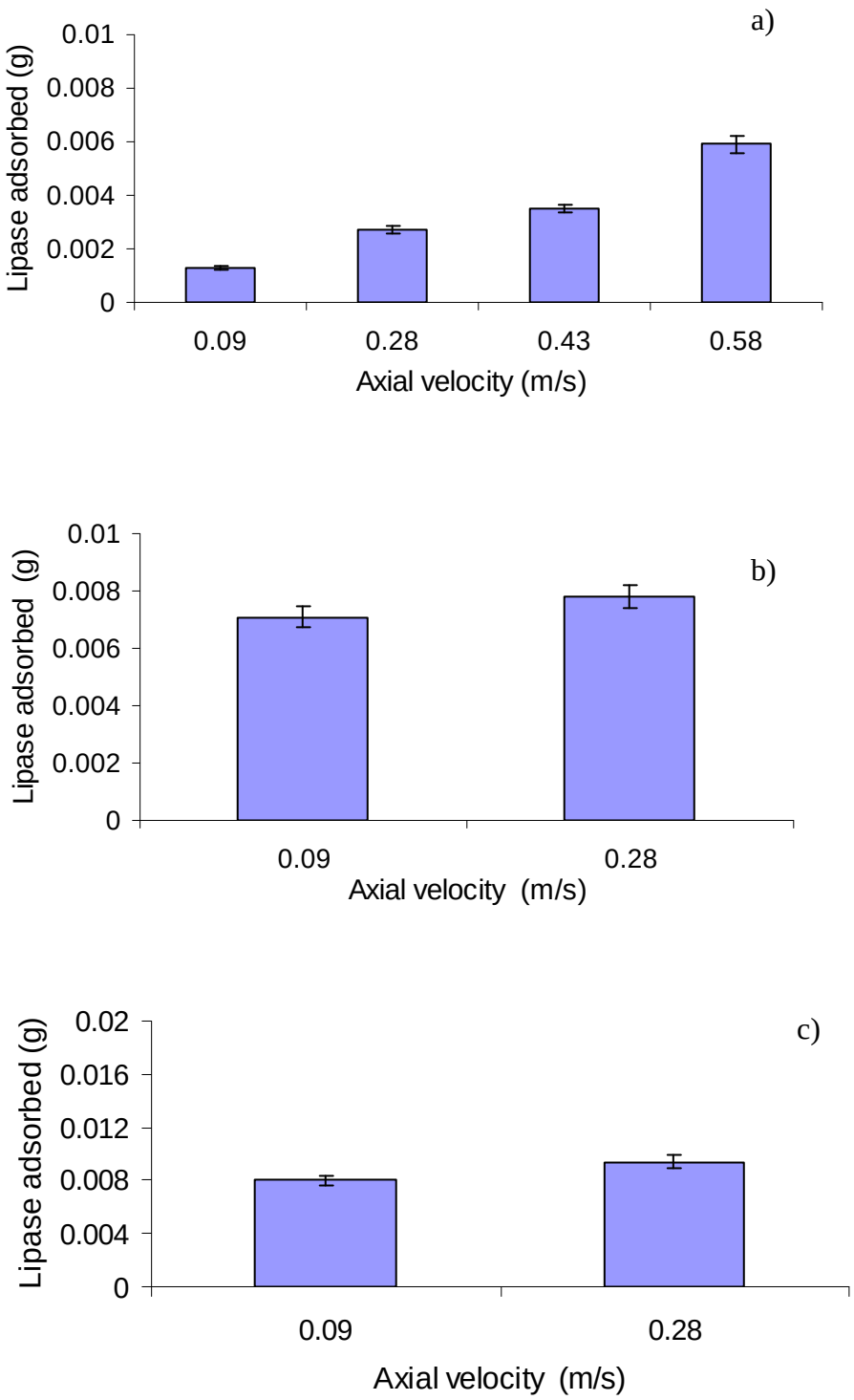

Fig. 4.12. Lipase amount adsorbed as a function of axial velocity when a) 2 g/l, b) 4 g/l and c) 6 g/l initial lipase solution was used

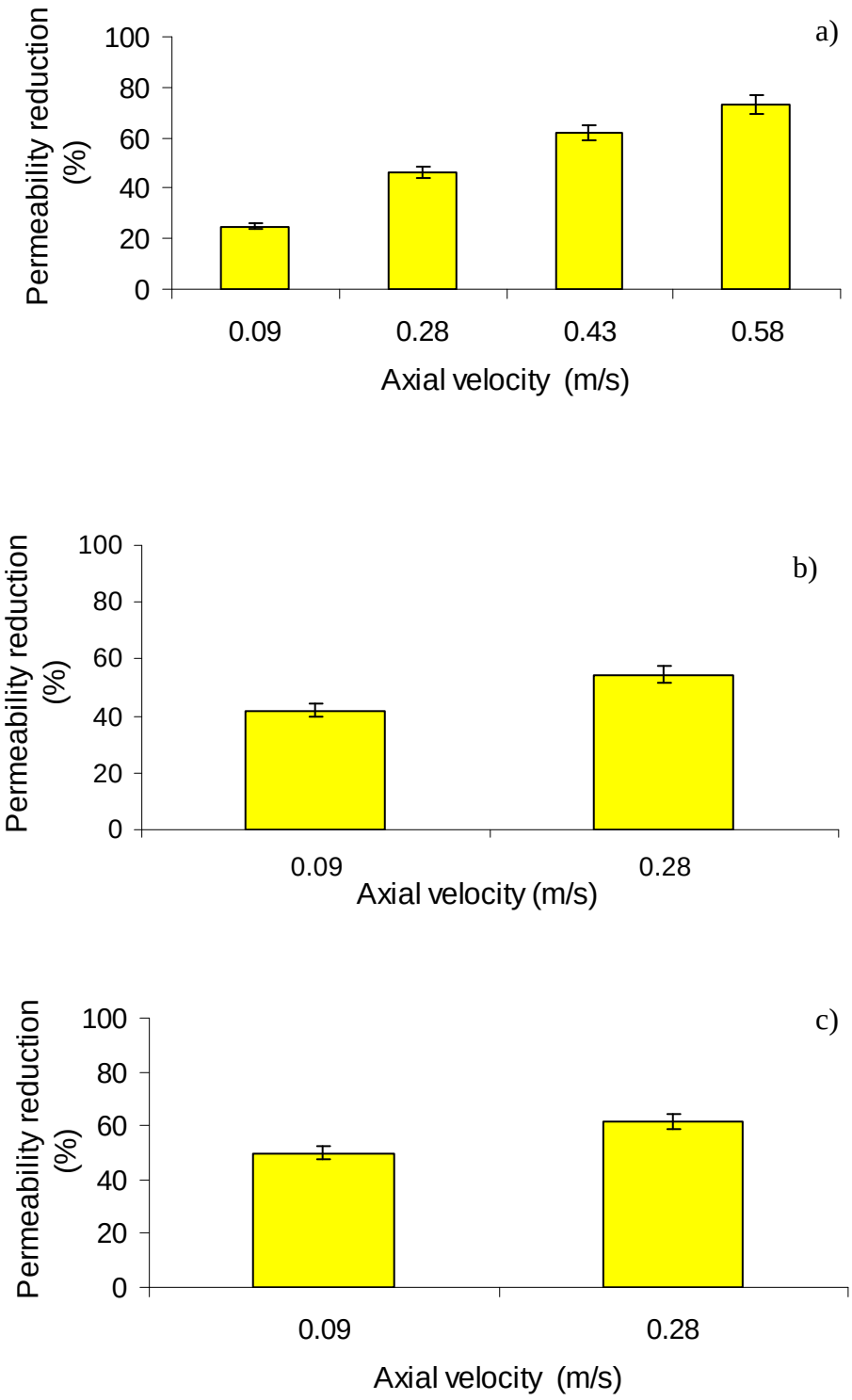

Fig. 4.13. Permeability reduction after lipase adsorption as a function of axial velocity when a) 2 g/l, b) 4 g/l and c) 6 g/l lipase initial solution was used

4.3.2. Protein adsorption in condition of tangential flux as a function of membrane type

2 g/l lipase solution was used to investigate the effect of protein adsorption as a function of membrane type. SPG membrane with pore size of 0.1 mm and ceramic membrane with pore size of 50 nm was compared. Axial velocity 0f 0.09 m/s was used. The protein adsorption profile and reduction permeability for SPG membrane are showed in the figure 4.14.

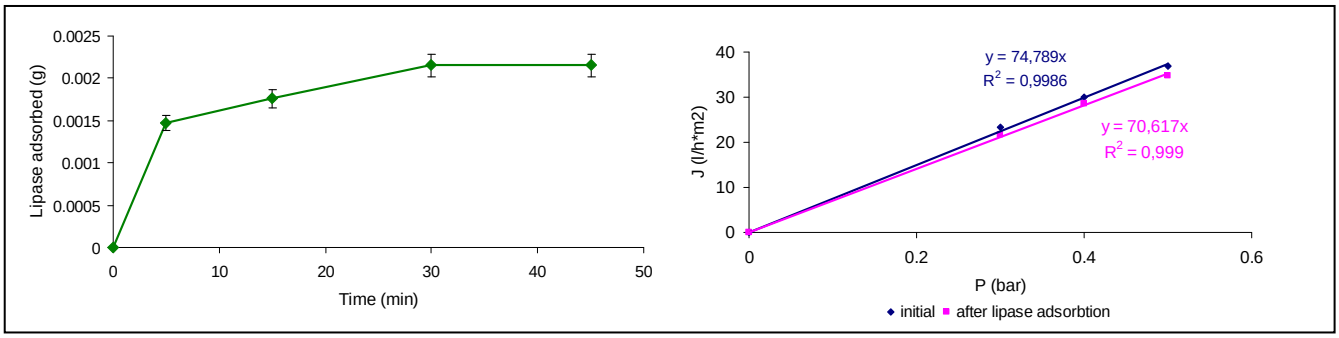

Fig. 4.14. Lipase 2 g/l adsorption and permeability reduction when SPG membrane was used

In Fig. 4.15 the experimental data obtained using the two type of membranes are compared.

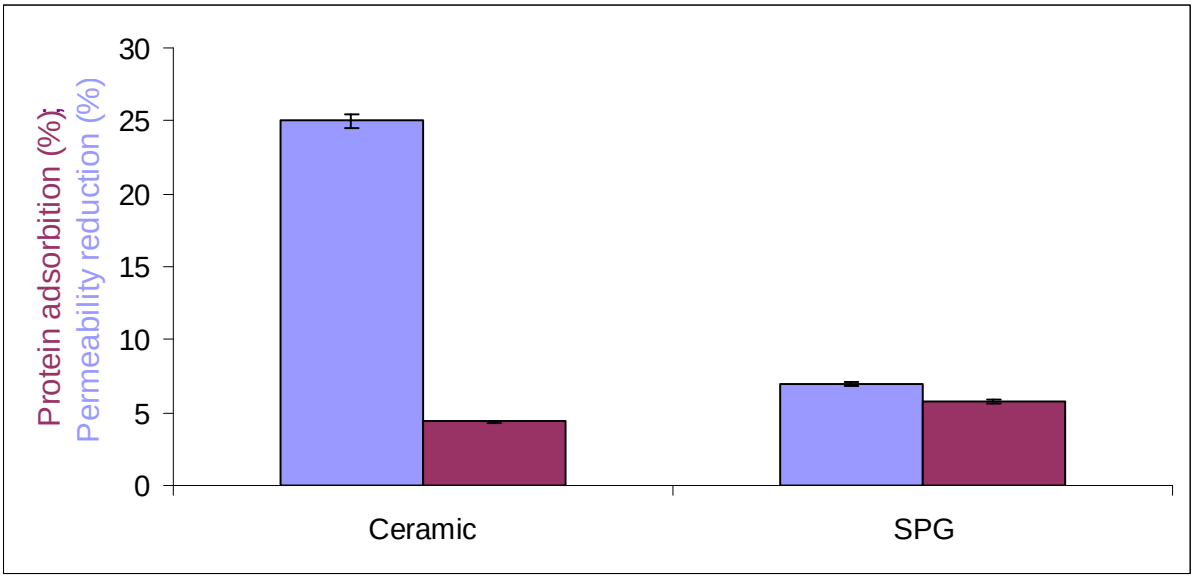

Fig. 4.15. Lipase adsorption and permeability reduction when SPG membrane and ceramic membrane were used

The data show that the protein amount adsorbed on membrane surface was the same for the two type of the membranes. However, the permeability reduction was major for the ceramic membrane. This can be do to the fact that the membranes used have different pores size. In particular, when ceramic membrane with pore size of 50 nm was used the protein adsorption can be determine a major decrease of membrane permeability.

4.3.3 Protein adsorption in condition of tangential flux as a function of proteins used

The protein adsorption profile and permeability measurement for the 1 g/l β-lactoglobulin and Conc A solutions are showed in Fig. 4.16. The axial velocity used was 0.09m/s. The adsorption experiments were carried out using 50 nm ceramic membrane.

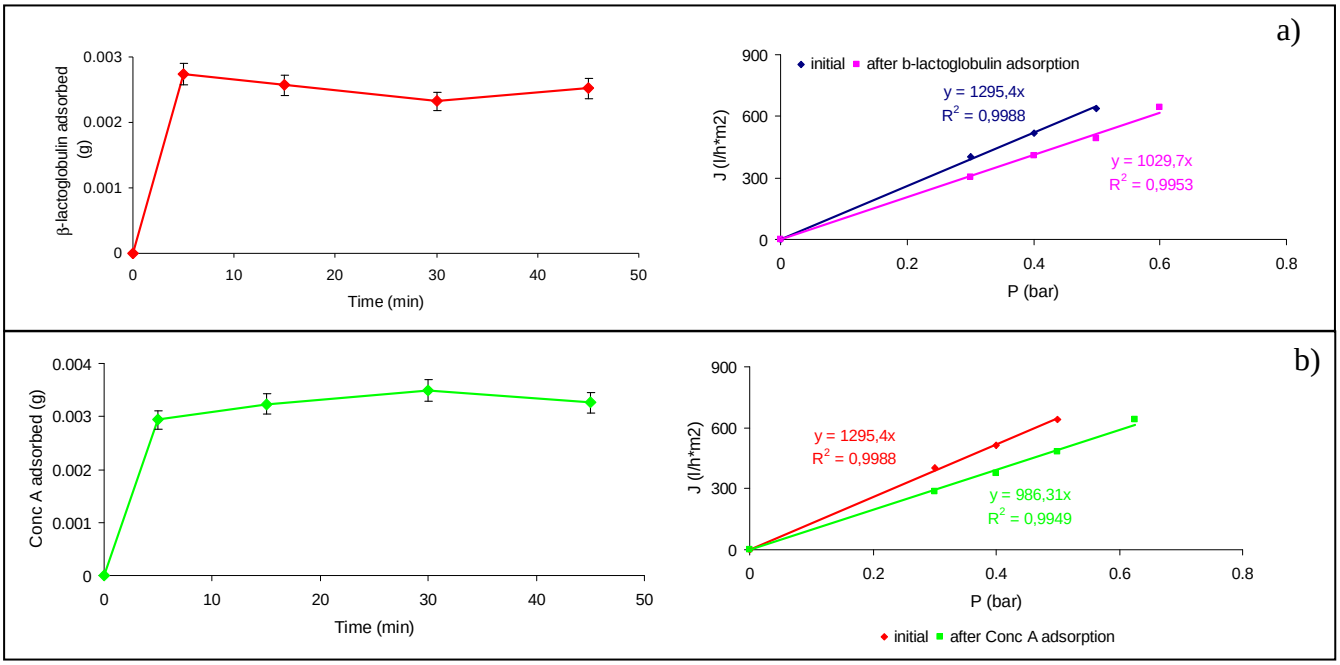

Fig. 4.16. a) 1 g/l β-lactoglobulin and b) 1g/l Conc A adsorption and permeability reduction.

Also when β-lactoglobulin and Conc A were used, the protein adsorption occurred in the fist 5-10 min and after this time the amount of protein adsorbed did not change over time.

In Fig. 4.17, a comparison between different proteins used in this work, at the same condition of protein concentration, axial velocity and membrane type, are showed.

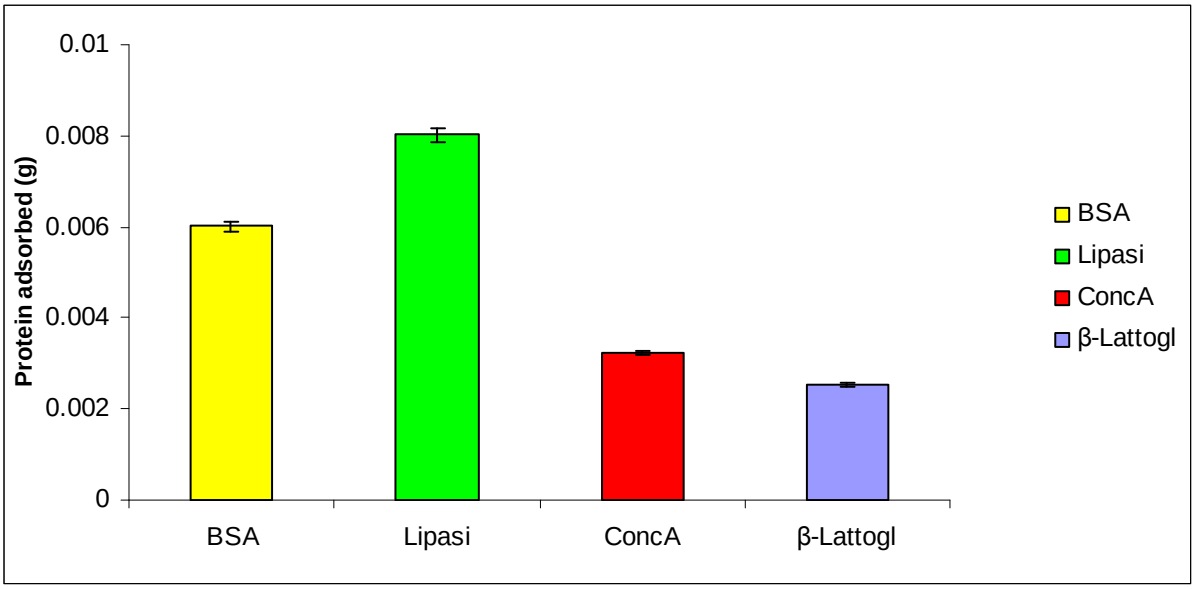

Fig. 4.17. Amount of protein adsorbed as a function of protein type

The results showed that proteins structural properties influence the adsorption process. Under the conditions studied, ceramic membrane is free of charge, since its isoelectric point falls into a rather wide range, between 5 and 7. The results showed that proteins structural properties influence the adsorption process. Under the conditions studied, ceramic membrane is free of charge, since its isoelectric point falls into a rather wide range, between 5 and 7. For this reason, the protein-membrane interactions are of hydrophilic-hydrophilic type and the electrostatic attractions effect is absent. The different protein behaviour was explain by the differences between the protein structural properties at the chemical conditions investigated.

Fig. 4.18 shows the permeability reduction and total surface ideally occupied for a monostrait of each protein. The data show that the lipase was the protein having higher adsorption properties compared to the others because the permeability reduction was greater after lipase adsorption experiment. This can be do to the hydrophobic sites present and to protein-protein aggregation phenomena.

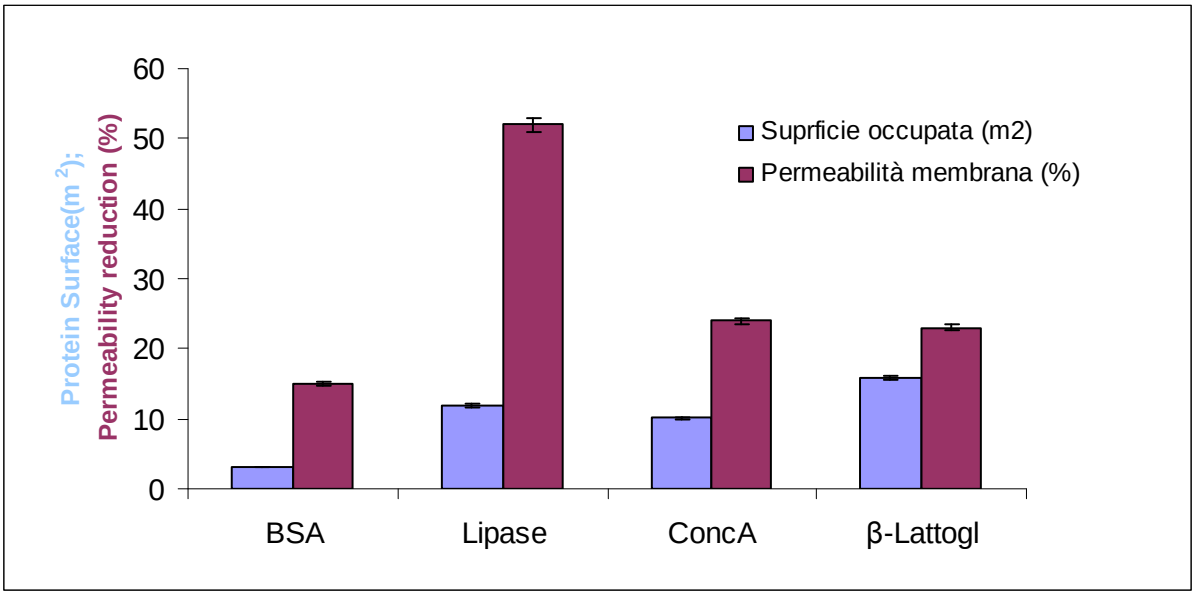

Fig. 4.18. Permeability reduction and area occupied on the membrane for each protein used

4.4.4. Protein adsorption in flow-through condition by applying transmembrane pressure

Hydrophobic SPG membrane was used in this experiments. The membrane pores were much larger (0.4 μm) than Conc A size. Conc A in solution largely consists of dimmers. The monomeric molecular weight of Con-A is 25,500 [12]. At pH values less than 6 (such as that used in the present work) the maximum dimer extension is slightly less than 8 nm as it was deduced from crystallographic structures (for the dimmer form, 1APN from the Protein Data Bank) [13]. Therefore, based on the size, the protein should easily pass through the 0.4 μm membrane pores. However, due to electrostatic interactions it could be adsorbed to the membrane pore wall. Also the PhAla is a small aminoacid of 165.19 but the electrostatic interactions could cause membrane adsorption.

In order to verify the steady-state permeation properties of the Conc A and PhAla solution through the membrane, pre-screening experiments were carried out during which both volumetric flux and C_p/C_f ratio as a function of time were measured.

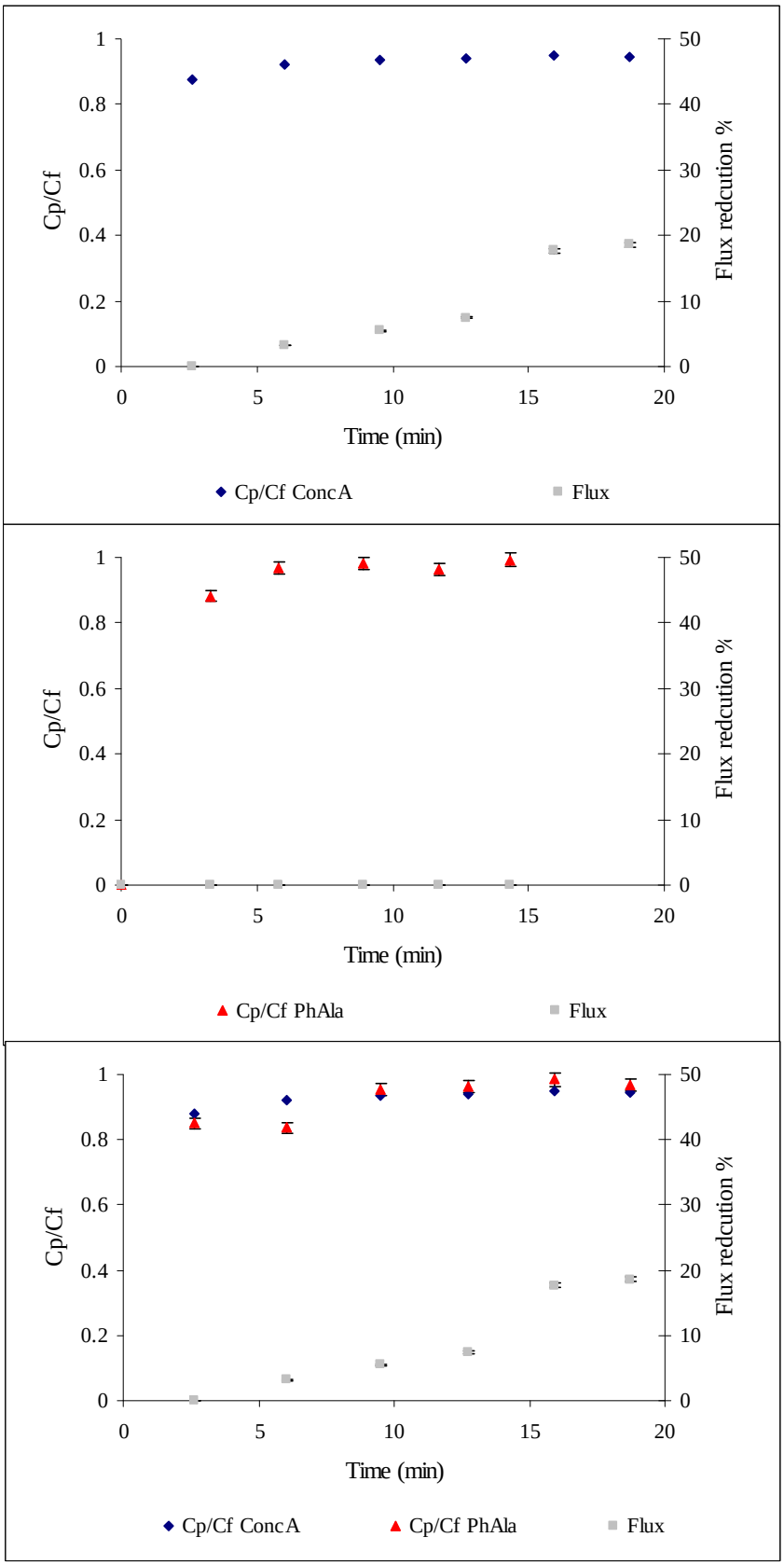

Fig. 4.19. C_p/C_f ratio and flux reduction % for a) Conc A, b) PhAla and c) Conc A/PhAla solution during the experimental time

As shown in Fig. 4.19, the C_p/C_f ratio increase during the time until 0.94 and 0.98 to Conc A and PhAla respectively. If no protein was adsorbed by the membrane, C_p/C_f was 1. In the present experiment, the C_p/C_f value close to 1 indicates that only the small amount of Conc A or PhAla was adsorbed during the solution permeation. However, the mass adsorbed into the membrane pores (m_a) and the protein concentration adsorbed calculated according to (1) and (2), respectively, was 0.43 mg and 0.49 mg/ml. Although the membrane pore was much larger than protein size the electrostatic interaction between protein and membrane occurred. At start of the solution permeation, the flux is constant but the protein adsorbed into the membrane determine membrane fouling that causes flux decline of 20%.

4.3.5 W/O emulsion preparation

The hydrophilic ceramic membrane after lipase adsorption was used to prepare a w/o emulsion. The emulsion prepared with the modified membrane was compared with the emulsion prepared with the hydrophilic membrane without modification. The pictures of two emulsions are showed in the Fig. 4.20.

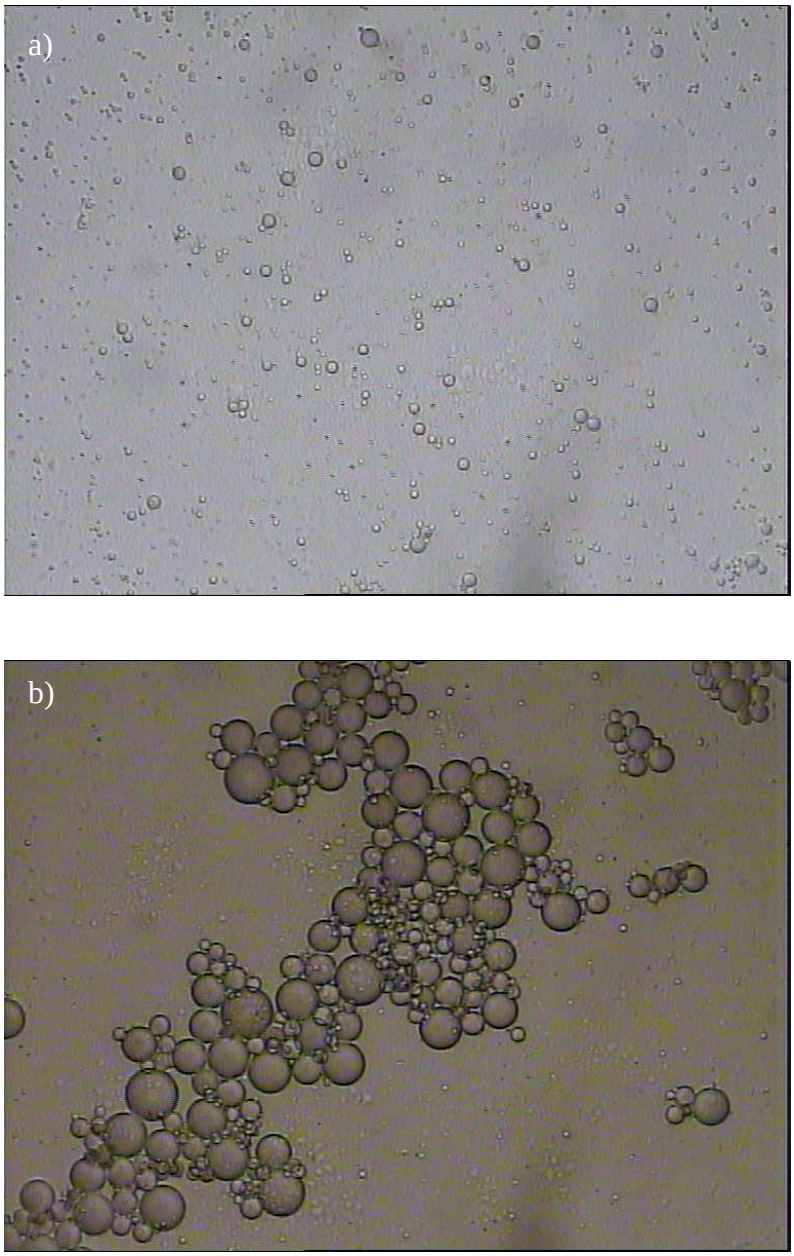

Fig. 20. Droplets emulsions prepared using a) modified membrane and b) non modified membrane

When modified membrane was used, mean 8 μm as droplets size and 1 as span was obtained. In the other case, 24 μm and 1.6 were obtained respectively as droplets size and span e lo span (Fig 4.21).

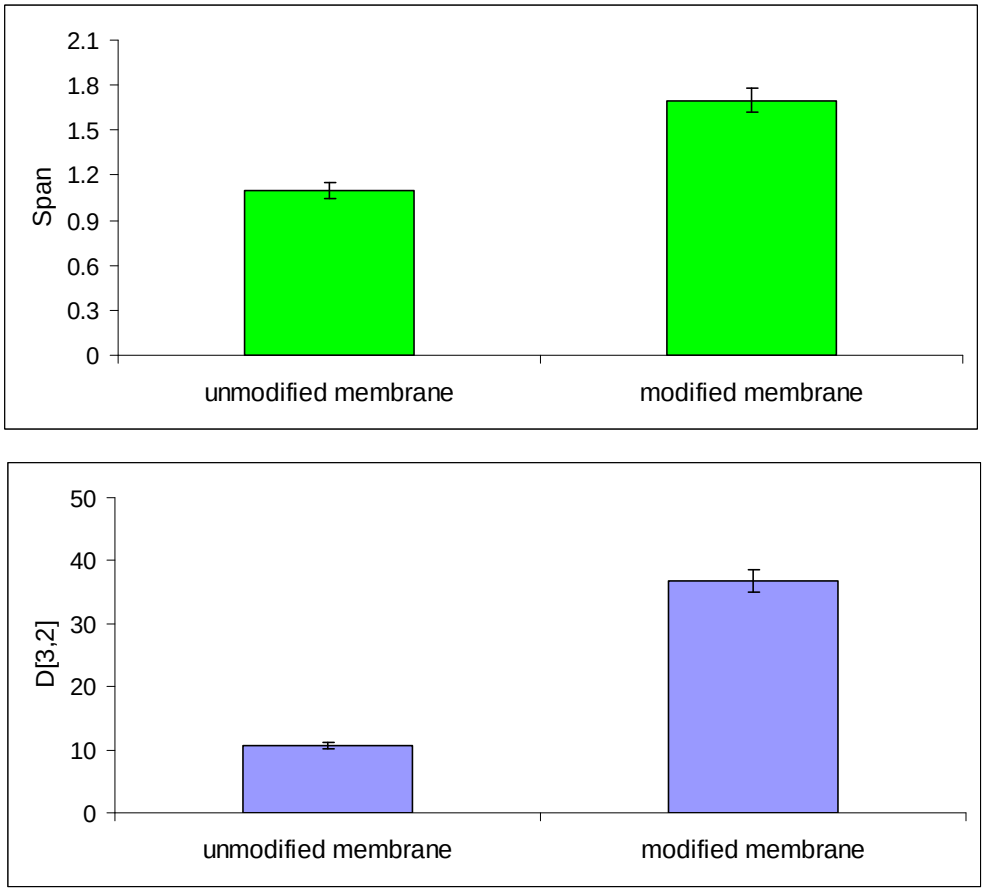

Fig. 4.21. Mean particles size and span of emulsion prepared using modified and not modified membrane

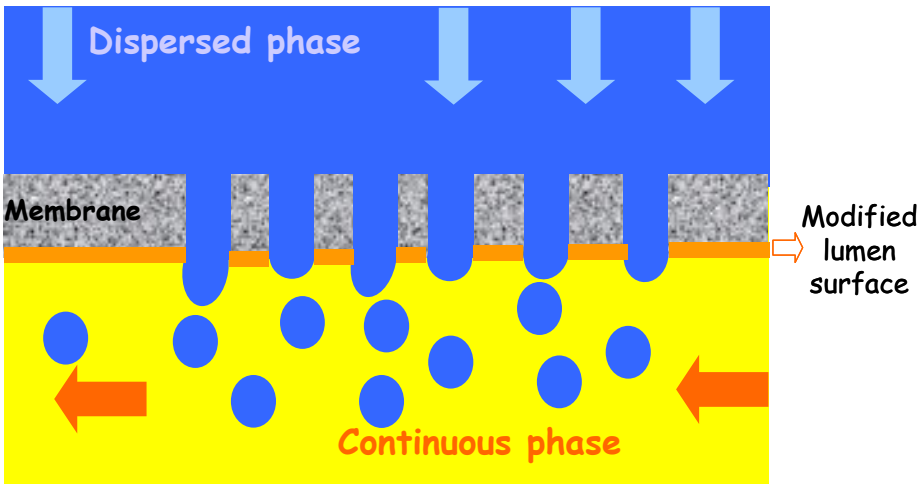

Fig. 4.22. Schematic representation of modified membrane used to prepare w/o emulsions

The lipase adsorption modified membrane wettability improving the hydriphobicity properties as suggested from the permeability reduction data obtained in the section 3.1.2. The water flux decrease a higher value of transmembrane pressure was required to carried out the experiment at the same dispersed phase flux used when the hydrophilic membrane was used. The hydrophobic properties of membrane lumen side permit to increase the interfacial tension between dispersed phase and membrane surface so that the water is detached as a forms of droplets into the oil phase ricirculeted in the lumen side. The schematic representation of modified membrane was shown in Fig. 4.22.

This method permits to use hydrophilic asymmetric membrane to prepare w/o emulsion with the significant advantages for the emulsification process time because the dispersed phase flux was higher compared to the hydrophobic symmetric membrane.

4.4. Conclusions

Fouling phenomenon was influenced by: protein parameters (concentration, chemical-physical properties), membrane parameters (pore size, wettability), operating parameters (flow rate, flux). Membrane-protein interaction influence the real amount of protein able to work as emulsifier or as functional ingredients during membrane emulsification process and the emulsification process efficiency (dispersed phase flux, membrane wettability). These results show that the use of protein as emulsifier or functional ingredients during membrane emulsification process has limitations but also some advantages if properly managed. Understanding all the phenomena involved in the membrane-protein interaction permit to control membrane emulsification process performance and the quality of dispersed material produced.

Membrane-protein interaction phenomenon was used with successful to produce a membrane with different wettability properties between shell and lumen side.This membrane was used to produce W/O emulsion with smaller droplets size and size distribution compared with the non modified hydrophilic membrane. These results shows that the membrane-protein interaction can be used to functionalize opportunely membranes to be used in membrane emulsification process reducing emulsification time and increasing dispersed phase flux.

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Chapter 5

New operational strategies to promote droplets detachment from the membrane pore at low shear conditions

5.1. Introduction

Membrane emulsification is an alternative emulsification methodology which allows particles preparation by forcing a dispersed phase through membrane pores into a continuous phase [1-3]. Generally, in order to ensure a regular droplet detachment from the pore outlet, the continuous phase is recirculated along the membrane surface. This technique is called “cross-flow membrane emulsification” [4-7] . The flow rate of the continuous phase should be high enough to provide the required tangential shear on the membrane surface. However, during droplet formation process with continuous-phase flow, droplets can be liable to further break up due to the shear force caused by the cross-flow pump [8,9].

In this work, O/W emulsions were prepared by cross-flow membrane emulsification process using a hydrophilic SPG membrane with pore size of 3 μm. The aim of the present work is the study of new operational strategies to promote droplets detachment from the pore outlet reducing shear stress conditions during the continuous phase recirculation. The main strategies suggested were:

- Reduction of membrane section in order to reduce continuous phase flow rate
- Reversal of continuous phase flow at appropriate intervals of time as innovative detachment of droplets at the pores membranes in a continuous and controlled operation mode

5.2. Materials and Methods

5.2.1. Materials

2% wt Tween 80 (Polyoxyethylene (20) sorbitan monooleate, Sigma) was used as a water soluble emulsifier. The dispersed oil phase was soybean oil (Sigma). The dispersed phase % in emulsions prepared was 7% wt.

5.2.2. Membranes and membrane emulsification equipment

The o/w emulsion was prepared using an hydrophilic tubular SPG membrane with pore size of 3 μm. Homemade lab-scale cross flow emulsification plant is schematised in Figure 5.1. The phase to be dispersed was contained in a graduated vessel and circulated in the shell side using a peristaltic micropump (Ismatec, model C.P. 78016-30). The transmembrane pressure was fixed by regulating two backpressure valves located between the pump and the membrane module. In all the experiments the dispersed phase flux was 1 l/hm^2. The continuous phase was re-cycled inside the lumen of the membrane using pump to work at the desired continuous phase tangential velocity by selecting the adequate flow rate in the pump regulator.

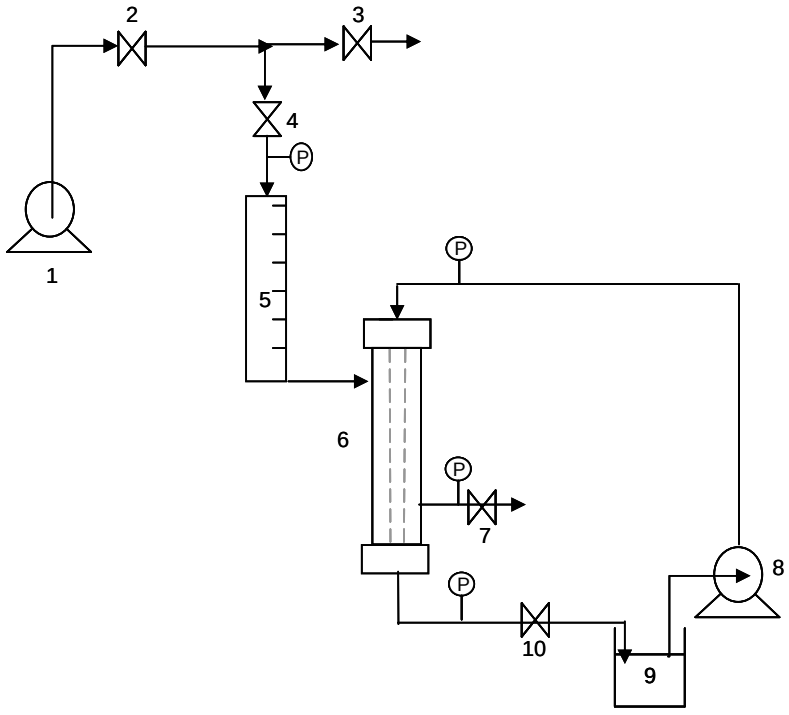

Fig. 5.1. Crossflow membrane emulsification equipment scheme. 1: peristaltic pump; 2: backpressure valve; 3: purge valve; 4: backpressure valve; 5: dispersed phase graduated vessel; 6: membrane module; 7: purge valve; 8: peristaltic pump; 9: continuous phase container; 10: backpressure valve

Some preliminary experiments were carried out in order to evaluate the break up phenomena eventually occurred during the continuous phase recirculation along the membrane surface. Three different pump were used: a gear pump (model 130-000), a conventional peristaltic pump (Amicon, model 54113#1728) and a low shear peristaltic pump (Digi-Staltic double-Y Masterflex® pump Micropump, model GJ-N23.JF1SAB1). Minimal pulsation ensures accuracy in peristaltic dispensing while pulsation causes variations in flow rate. Combining the split-channel tubing configuration with the offset rollers of two stacked Easy-Load® II pump heads merges a pulse from one channel with a trough from the other. In this way, the flow of the continuous phase doubles while the pulses are reduced by 90% allowing that there are not variations of the continuous phase velocity during membrane emulsification process.

When the effects of the reduction of membrane section and the reversal of continuous phase flow at appropriate intervals of time were studied the low shear peristaltic pump (Digi-Staltic double-Y Masterflex® pump Micropump, model GJ-N23.JF1SAB1) was used.

All the experiments were carried out at room temperature (21°C).

5.2.3. Measurement and analytical methods

Emulsions droplet size distribution and droplet size were measured using a laser light scattering system (Malvern Mastersizer 2000, Malvern Instruments). Microscopic analysis was also carried out. Optical microscope (Zeiss, model Axiovert 25) equipped with a camera (JVC, model TK-C1481BEG) to capture the images of the emulsions was used.

The mean particle size was expressed as the surface weighted mean diameter (or Sauter diameter), D[3,2] and as the volume weighted mean diameter, (or De Brouckere diameter), D[4,3]. D[3,2] and D[4,3] were determined, respectively, as follows:

$$D[3,2] = \frac{\sum D_i^3 n_i}{\sum D_i^2 n_i} \tag{3}$$

$$D[4,3] = \frac{\sum D_i^4 n_i}{\sum D_i^3 n_i} \tag{4}$$

where D_i = particle diameter of class i and n_i = number of particle in class i.

The width of droplet size distribution was expressed as a Span number, calculated by the following expression:

$$Span = \frac{D[0.9] - D[0.1]}{D[0.5]} \quad (5)$$

where D[x0] is the diameter corresponding to x0 vol.% on a relative cumulative droplet size curve.

The mass of oil-water emulsion samples were measured by means of a moisture analyser (Ohaus, model MB45) composed by a precision balance and a dryer unit. The oil percentage was determined from the weight of sample dried by heating at 100°C because only water phase was evaporated at this temperature. The percentage of water-in-oil (%W/O) can be determined as follows:

$$\%W/O = \frac{m_f}{m_i} * 100 \quad (6)$$

where m_f was the mass of sample after evaporation (corresponding to the mass of oil not evaporated at 100°C) and m_i was the mass of initial sample (at the moment in which it was taken from emulsion).

5.3. Results and Discussion

5.3.1. Effect of continuous phase recirculation by low shear pump

To evaluate the break up phenomena eventually occurred during the continuous phase recirculation along the membrane surface, three pumps with different operation mechanism were used: ear pump, a peristaltic pump and a low shear peristaltic pump. A flow rate of 1700 ml/min (0.55 m/s) was applied. The figure 5.2 shows the mean particle diameter and span of emulsions produced using the three different pumps.

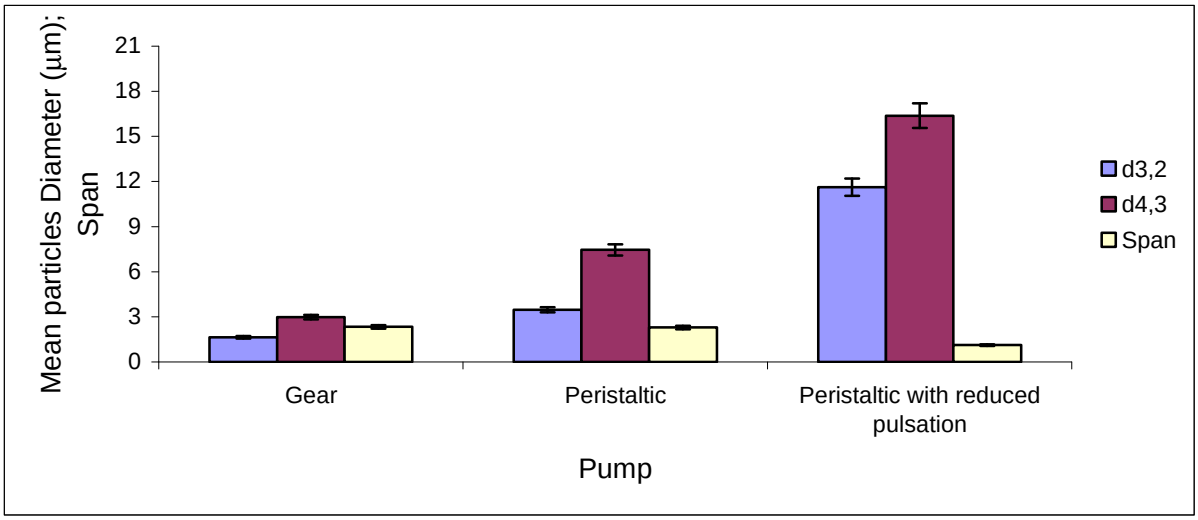

Fig. 5.2. Mean particles diameters of O/W emulsions prepared recirculating the continuous phase at 0.55 m/s using a gear pump, a peristaltic pump and a peristaltic pump with reduced pulsation

When gear and peristaltic pumps were used, emulsions droplet size were very close to membrane pores size. In particular, when gear pump was used, a D[3,2] of 1.6 µm and D[4,3] of 2.9 µm were obtained. In addition, emulsion temperature during the recirculation was 30°C. The small droplets diameter obtained can be explained due to the recirculation of the continuous phase inside the gear of the pump that determines droplets break up. The high shear stress hypothesized is confirmed by temperature increasing during emulsification process. In the case in which peristaltic pump was used, due to the absence of gear, break up phenomena doe to the mechanical stress are not expected. This hypothesis was confirmed by D[4,3] value of 7.5 µm that is more sensitive to the presence of large particles. Despite of some break-up phenomena also occurred as showed by D[3,2] value of 3.5 µm. This suggested that, also when peristaltic pump was used, not controlled shear conditions were obtained as confirmed by emulsion temperature increase (35°C). In addition to droplets size and emulsion temperature, also the droplets size distribution is an important parameters to evaluate the control of emulsion production by the membrane pores. In fact, a special property of membrane emulsification process is the capacity to control droplet size and size distribution using a membrane with a well-defined pore structure. The emulsions produced with both mentioned pumps showed a span of 2.3 that indicate emulsion polidispersed. When the pump with reduced pulsation and low shear was used, the operating conditions selected allow an efficient control of particle size to obtain droplets with a diameter about 4 times the diameter of the pore, according to data in the

literature [2]. No solution temperature increase was observed during the recirculation time and also a span value of 1.2 was obtained.
The low shear peristaltic pump was used in the other experiments in order to avoid droplets break up caused by not controlled shear conditions.

5.3.2. Effect on membrane section reduction

The operational strategy used to improve the efficiency of the cross-flow emulsification process was to reduce the membrane section so that obtain high values of axial velocity at low flow rate values. Through this method, it is possible to obtain high value of axial velocity only in the membrane compartment where high shear force are necessary to detach faster emulsion droplets at the membrane pores.
To reduce SPG membrane section, a plastic tube of 6 mm in diameter and 150 mm in length was placed inside the membrane lumen. The length of the tube is equal to the distance between the two ends of the membrane module, so that the pipe rests on the form but not in contact with the surface of the membrane as shown in Fig. 5.3.

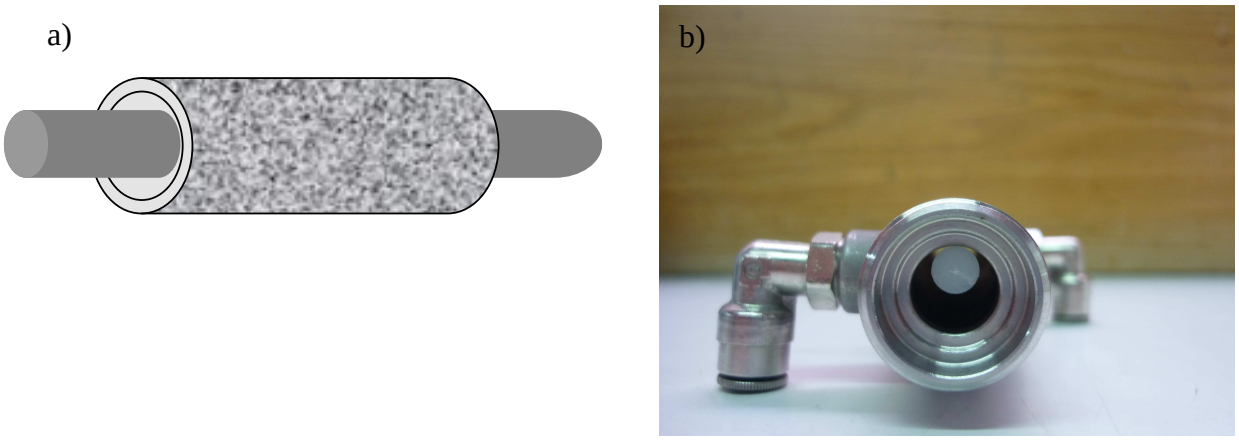

Fig. 5.3. SPG membrane module with reduction section a) schematic representation and b) picture

In order to evaluate the effect of membrane reduction section the continuous phase was ricirculated along the membrane surface at the flow rate of 850 ml and the data was compared to those obtained in the same conditions but using the membrane with the unmodified section. Table 5.1 and Fi. 5.4 summarize conditions used and data obtained.

Table 1. Experimental conditions and main results obtained when membrane section strategy was evaluated to produce O/W emulsions

Flow rate (ml/min)	Axial velocity along the membrane surface (m/s)	Section (mm^2)	D[4,3] (μm)	D[3,2] (μm)	Span
850	0.28	50.2	38.4	24.21	1.49
850	0.64	22	32	15.41	1.9

Data show that the reduction section of 56% permit to obtain droplets size reduction of 36% when D[3,2], more sensitive to the presence of small particles, was considered.

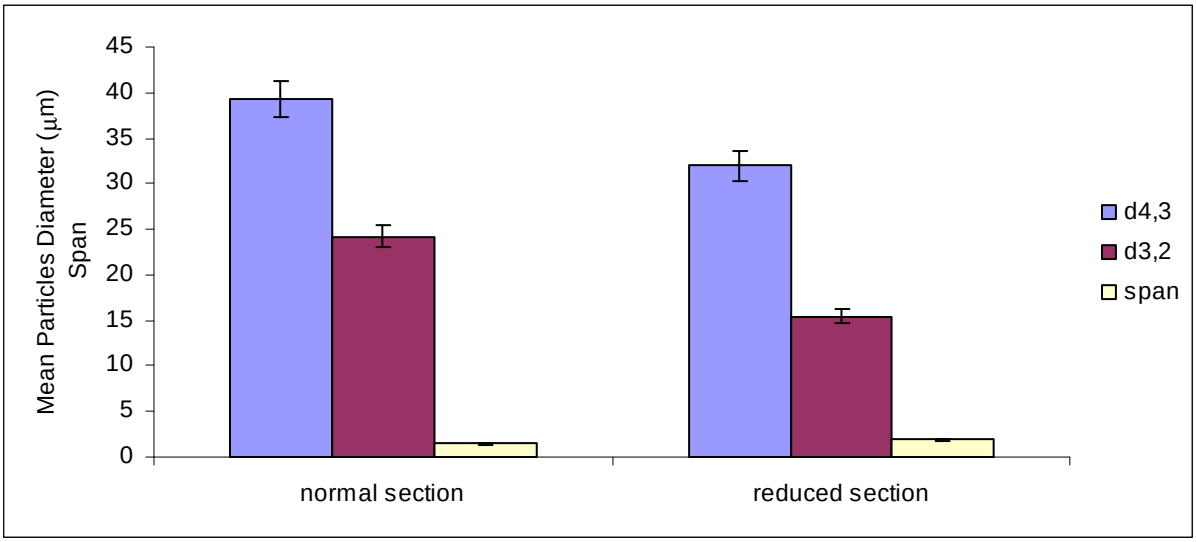

Fig. 5.4. Mean particles diameter and span of O/W emulsions obtained using SPG membrane with normal section (50.2 mm^2) and reduced section (22 mm^2) when continuous phase was recirculated at 850 ml/min as flow rate.

5.3.3 Effect of continuous phase "vibrating" along the lumen membrane surface

An alternative methodology to cross-flow membrane emulsification was carried out reversing the continuous phase flow, alternately, at appropriate intervals of time, during the emulsification process. The peristaltic pump with reduced pulsation used can be configured so as to alternate the flow direction of the continuous phase in one direction and the opposite, consecutively. A new emulsification method has been developed using rotating membrane [10-12]. Compared with cross membrane emulsification methods, the rotating membrane emulsification can be particularly advantageous to the production of coarse emulsions and fragile structured products in which the droplets and/or particles are subject to breakage during the pump recirculation. The dispersed phase passes radially through the porous membrane wall and forms droplets moving into the continuous phase. In the

method investigated in this work, the shear stress is developed by reverting the continuous phase flow direction rather than by circulating the continuous phase in cross-flow direction. In the table 5.2 are showed the operative conditions studied. The parameters evaluated are the flow rate, the number of clockwise ($REV_{\rightarrow}$) and counterclockwise ($REV_{\leftarrow}$) revolution, the time of clockwise ($T_{REV\rightarrow}$) and counterclockwise ($T_{REV\leftarrow}$) revolution.

Table 5.2. Operative conditions studied when effect of continuous phase "vibrating" along the lumen membrane surface was evaluated

Flow rate (ml/min)	Axial velocity (m/s)	$REV_{\rightarrow}$ Number	$REV_{\rightarrow}$ Time ($T_{REV\rightarrow}$) (s)	$REV_{\leftarrow}$ Number	$REV_{\leftarrow}$ Time ($T_{REV\leftarrow}$) (s)	$\frac{T_{REV_{\rightarrow}}}{T_{REV_{\leftarrow}}}$
1700	0.55	2	0.2	1	0.1	2
1700	0.55	1	0.1	1	0.1	1
1700	0.55	2	0.2	1/10	0.01	20

The experiments were carried out at the same flow rate (1700 ml/min). The only difference between them was in the ratio of clockwise ($REV_{\rightarrow}$) and counterclockwise ($REV_{\leftarrow}$) time ($\frac{T_{REV_{\rightarrow}}}{T_{REV_{\leftarrow}}}$) that corresponds to a range of time in which the continuous phase flow direction was reverted. The small $\frac{T_{REV_{\rightarrow}}}{T_{REV_{\leftarrow}}}$ value corresponds to a fast change of continuous phase flow direction.

The mean particles diameter and span as a function of $\frac{T_{REV_{\rightarrow}}}{T_{REV_{\leftarrow}}}$ at flow rate constant of 1700 ml/min are shown in the figure 5.5. The effect in the droplets size are observed only when the $\frac{T_{REV_{\rightarrow}}}{T_{REV_{\leftarrow}}}$ value was 20. In this case, the time of counterclockwise revolution is much smaller than the time of clockwise revolution and the droplets size increase. Could be that this time is not sufficient to determine droplets detachment as a result of reversal flow rate as the continuous phase was shacked along the membrane surface.

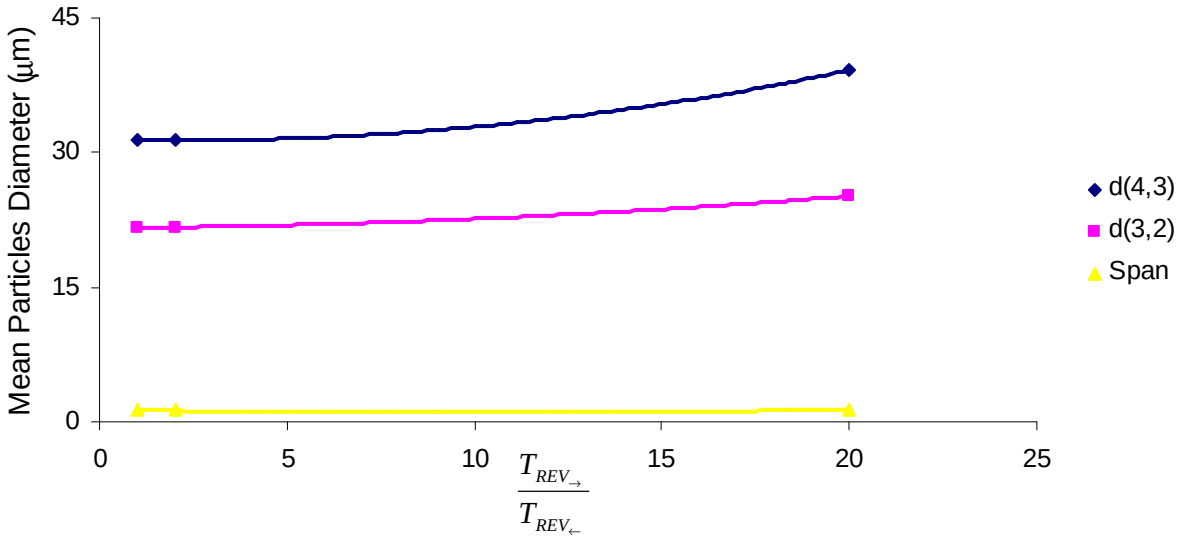

Fig. 5.5. Mean particles diameter and span of emulsion droplets as a function of $\frac{T_{REV_{\rightarrow}}}{T_{REV_{\leftarrow}}}$

5.4. Conclusions

In this work, new operational strategies to promote droplets detachment from the pore outlet at low shear stress conditions have been reported.

O/W emulsions were produced in condition of low flow rate of continuous phase but at high wall shear stress along the membrane, reducing membrane section. The reduction section of 56% permitted to obtain droplets size reduction of 36% compared with the droplets size obtained at the same flow rate.

O/W emulsions were produced by “shaking” the continuous phase along the membrane surface by inverting the axial flow versus. This strategy is useful to reduce recirculation of formed emulsion and increase dispersed phase concentration for single passage along the module.

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Innovative Microstructured Formulations

Chapter 6

Proofs of concept on use of Conc A as glucose sensor in multiple emulsions prepared using membrane emulsification

6.1. Introduction

Effort have been made, in recent years, to develop alternative novel drug delivery systems that would mimic and/or replace important physiological functions. It would be highly beneficial if the active agents were delivered by a system that sensed the signal and acted to release. The majority of stimuli responsive biomaterials are hydrogels, polymeric materials having great interest to their hydrophilic character and potential to be biocompatible. Stimuli responsive hydrogels undergo volume changes in response to environmental changes such as pH [1], temperature [2] and electric field [3]. Such stimuli-responsive have been extensively investigated as smart materials in the biomedical fields since they can sense environmental changes and induce structural changes by themselves. For example, as pH and temperature are the most widely utilized triggering signals for modulated systems, pH- and temperature-responsive hydrogels have many potential applications in self regulated drug delivery [4, 5]. In addition, biomolecule-responsive hydrogels, which recognize specific biomolecule and undergo volume changes in response to them, are promising materials as the next generation of biomaterials and drug-delivery devices [6]. A few biomolecule-responsive hydrogels, have been reported since such biomolecule-responsive hydrogels became increasingly important in the biomedical field.

Liquid membrane systems known as water-in-oil-in-water emulsions could be one class of possible candidates to obtain stimuli responsive biomaterials. Multiple emulsions are dispersed systems where both water in oil and oil in water emulsion exists simultaneously in a single system. The two major types of multiple emulsions are the water-in-oil-in-water emulsion ($W_1/O/W_2$) and oil-in-water-in-oil emulsion ($O_1/W/O_2$) double emulsions. They can be used for entrapment of both hydrophilic and hydrophobic solutes that have to transverse through the middle immiscible phase (liquid membrane) in order to come from inner miscible

phase to outer miscible phase. The potential applications for this technology are enormous, mainly in the areas of food, cosmetics, pharmacology. Usually multiple emulsions are used to provide high capacity of entrapment, protection of fragile substances, combination of incompatible substance in one product and controlled release. Few models of stimuli responsive multiple emulsions are reported in literature. Temperature-induced release from $W_1/O/W_2$ emulsion [7] and pH-responsive pickering emulsion [8] have been investigated. Biomolecule-responsive emulsions are not yet reported.

In the present work, the manufacturing of multiple emulsions as self-regulated drug delivery systems has been investigated. Glucose-sensitive multiple emulsion has been selected as study-case. Concanavalin A (Conc A), a glucose-binding protein obtained from the jack bean plant, *Canavalia ensiformis*, was selected as biomolecule-sensor. Conc A has been frequently used in glucose sensitive delivery systems. In one approach, insulin was chemically modified to introduce glucose using a glycosylated insulin derivative complexed with Conc A [9]. Insulin release occurred in presence of glucose because the free glucose molecules compete with glucose-insulin conjugates bound to Conc A and thus, the glycosylated insulin is desorbed from the Conc A host in the presence of free glucose. In another approach, a highly specific interaction between glucose and Conc A was used to form crosslinks between glucose-containing polymer chains [10]. Because of the non-covalent interaction between glucose and Conc A, the formed crosslinks are reversible. As the external glucose molecules diffuse into the hydrogel, individual free glucose molecules can compete with the polymer-attached glucose molecules and exchange with the polymer-attached glucose molecules exchange with them.

In the present work, Conc A will be also usend as emulsifier in the present work. Proteins form an interfacial films when adsorb at the liquid interface. Amphiphilic proteins such as BSA and casein along with monomeric emulsifiers were used to improve stability and release properties in double emulsions [11] but the emulsifier properties of Conc A have not been studied yet. However, the presence of hydrophobic and hydrophilic sites suggest the possibility to use Conc A as macromolecular emulsifier. In fact, the study of Conc A structure evidences that Conc A hydrophobically bind carbohydrates [12, 13].

In the present work Conc A will be used as emulsifier, dissolved in the aqueous dispersed phase, in the preparation of water-in-oil emulsion (W_1/O). In addition, Conc A will be used, also together with other emulsifiers, in the preparation of water-in-oil-in-water ($W_1/O/W_2$) emulsions. The aim is to functionalize emulsion interface with Conc A so that to recognize specifically the glucose molecules free. Previous works showed that same substance are able

to act as demulsifier agents modifying the interfacial films properties and determining phase separation when added in emulsions systems [8, 14].

We have hypothesized that the Conc A-glucose interaction determine changes in the properties of Conc A interfacial film so that to promote the release of a molecule of interest added in the W_1 aqueous dispersed phase when glucose is added in the W_2 aqueous phase.

Membrane emulsification technology has been selected to prepare W_1/O and $W_1/O/W_2$ emulsions. In fact, conventional methods to prepare emulsions utilize strong shearing stress which may affects physicochemical properties of proteins used as emulsion ingredients and drops rupture during secondary $W_1/O/W_2$ emulsion preparation. The membrane emulsification process is highly alternative attractive technique [15-17]. This method uses the microporous membrane to disperse one of two immiscible liquids into another by applying a low pressure. This technique permit a fine control of droplets size using low shear stress and shear-sensitive ingredients. The distinguishing feature is that the resulting droplet size is controlled primarily by the choice of the membrane pore. In order to ensure a regular droplet detachment from the pore outlets, shear stress is generated at the membrane/continuous phase interface by recirculating the continuous phase tangentially along membrane surface (cross-flow mode) or by stirring vessel.

In order to provide a proofs of concept on use of Conc A as glucose sensor in multiple emulsions prepared using membrane emulsification, this work first attempts to:

1. formulate a W/O emulsion in which Conc A is distributed at the interface using cross-flow membrane emulsification. W/O emulsion properties will be evaluated.
2. formulate $W_1/O/W_2$ emulsion using stirred cell membrane emulsification. $W_1/O/W_2$ emulsion properties will be evaluated.
3. measure of release in presence of glucose-stimulus and in absence of stimulus.

6.2. Materials and methods

6.2.1. Materials

Concanavalin A (Conc A) from *Canavalia ensiformis* (Jack bean) Type IV, lyophilized powder, (Sigma,) was used as glucose-sensor and as emulsifier for the preparation of both water-in-oil (W_1/O) and water-in-oil-in-water ($W_1/O/W_2$) emulsions. The protein was dissolved in a 20 mM Tris HCl (>99%, Sigma) buffer (pH 5.4) with 1 mM $CaCl_2$ (96%,Sigma), 1 mM $MnCl_2$ (>99%, Sigma) and 1 M NaCl (Sigma). The inner aqueous phase (W_1) contained 0.1 wt.% Conc A and an easy molecule, such as (D, L)-Phenylalanine (PhAla, 99%, Sigma), used as an indicator to study and verify the controlled release. The outer aqueous phase (W_2) contained 2 wt.% Polyethylene glycol sorbitan monooleate (Tween 80, Sigma) was used as emulsifier also with 0.2 wt.% Conc A. Soybean oil (Sigma,) was used as oil phase in the ($W_1/O/W_2$) emulsion preparation. In same experiments, soybean oil containing 2 wt.% sorbitan monooleate (Span 80, Sigma) was used. All the aqueous solutions ware prepared using ultrapure water (USF Elsa, model Purelab Classic PL5221) with a resistivity of 18.2 MΩ·cm. Perchloric acid (70% redistilled, 99.999%, Sigma) was used to prepare the mobile phase for HPLC analysis.

6.2.2. Membranes and membrane emulsification equipment

Shirasu Porous Glass (SPG) hydrophobic membrane tube, having mean pore size (d_p) of 0.4 μm, supplied from SPG Technology (Japan), was used to prepare (W_1/O) emulsions. Homemade lab-scale cross flow emulsification plant is schematised in Fig. 6.1 A.

The phase to be dispersed was contained in a graduated vessel pressurised by nitrogen vessel. The transmembrane pressure was fixed by regulating two backpressure valves located between the gas cylinder and the stainless steel membrane module. The permeate flux of dispersed phase was perfectly controlled and kept constant during the experiments. The continuous phase was re-cycled inside the lumen of the membrane using a gear pump (Micropump, model GJ-N23.JF1SAB1) to work at the desired continuous phase tangential velocity by selecting the adequate flow rate in the pump regulator.

An hydrophilic metallic membranes, having 10 μm d_p, was used to prepare $W_1/O/W_2$ emulsion in the stirred emulsification cell (Micropore Technologies Ltd) [18, 19]. The system consists in a PTFE base (named injection chamber) that hosts a flat membrane coupled with a dismountable threaded glass cylinder. A stainless steal stirrer is placed over the glass cylinder

and regulated by a voltage regulator connected to the electricity. The feeding system of the cell was modified from the original version, where instead of a gravimeter cylinder a peristaltic micropump (Ismatec, model C.P. 78016-30) was connected to the injection chamber to permeate the dispersed phase at finely controlled flow rate. A schematic representation of the stirred emulsification plant is reported in Fig. 6.1 B.

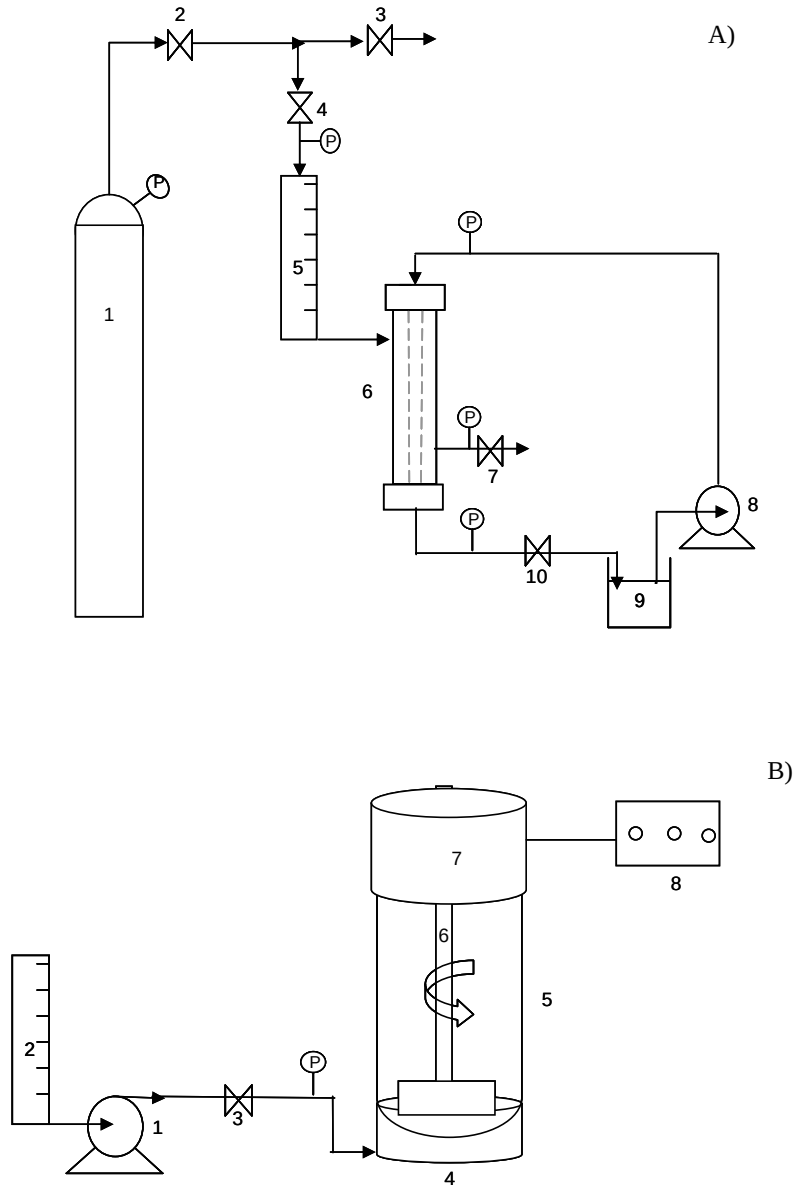

Fig. 6.1. Membrane emulsification equipment scheme.

A) Crossflow membrane emulsification. 1: nitrogen gas cylinder; 2: backpressure valve; 3: purge valve; 4: backpressure valve; 5: dispersed phase graduated vessel; 6: membrane module; 7: purge valve; 8: gear pump; 9: continuous phase container; 10: backpressure valve

B) Stirred membrane emulsification. 1: peristaltic pump nitrogen gas cylinder; 2: dispersed phase graduated vessel; 3: backpressure valve; 4: injection chamber; 5: glass cylinder; 6: stainless steal stirrer, with a blade at the bottom ending; 7: motor; 8:voltage regulator

6.2.3. Experimental procedure

6.2.3.1. W_1/O emulsion preparation with Conc A in the dispersed phase

W_1/O emulsion containing 0.1wt % Conc A as emulsifier was first prepared using cross-flow emulsification mode using plant shown in the Figure 6.1 A. A volume of 45 ml was re-cycled inside the lumen of the SPG hydrophobic membrane (0.4 μm d_p). The dispersed phase flux was 1.5 ±0.5 l/hm^2 in all experiments. The axial velocity ranging from 0.003 to 0.05 m/s (shear stress 0.17 to 2.37 Pa) was used. Soybean oil (also with Span 80 2%wt, where specified) was used as continuous phase.

2.3.1.1. Property of Conc A adsorption onto SPG hydrophobic membrane

To evaluate membrane protein adsorption, a 0.1wt % Conc A solution was pressurized with N_2 gas at 30 psi and filtered from shell-to-lumen in dead-end mode. The set-up in Figure 6.1 A was used with valves 7 and 10 closed and collecting permeate from lumen (valve 11 open). The membrane used was the same in the emulsification process (SPG hydrophobic membrane, 0.4 μm d_p). The experiment was carried out at ambient temperature (23 ± 1°C). The flux and protein concentration in permeate solution was measured during the protein filtration process. In this case, the setting of a three way valve (11 in Figure 6.1 A) was changed to allowed to collected permeated protein solution. Protein concentration was evaluated using at spectrophotometry, at 280 nm. The Conc A adsorption was evaluated from the C_p/C_f ratio (the ratio of Conc A concentration in permeate, C_p, to that in initial feed, C_f). A value of C_p/C_f equal to 1 indicate no protein retention occurred. When C_p/C_f <1 indicates protein retention. The mass adsorbed into the membrane pores (m_a) and the concentration of protein eventually adsorbed (C_a) were calculated as follows, respectively:

$$m_a = m_f - \sum_i m_{p_i} \quad (1)$$

$$C_a = \frac{m_f - \sum_i m_{p_i}}{V_v} \quad (2)$$

where m_f and m_p are, the mass of protein in the initial feed and in the permeated collected samples, respectively, and V_v is the void volume of the membrane.

$$\text{flux reduction \%} = \frac{J_0 - J}{J_0} * 100 \quad (3)$$

where J_0 and J are the buffer solution flux without and with Conc A and PhAla, respectively

6.2.3.2. $W_1/O/W_2$ emulsion preparation

W_1/O emulsion containing 0.1wt % Conc A as emulsifier was used as dispersed phase. Different compositions of W_2 phase were used to tested the effect of different emulsifiers on droplets size, droplet size distribution and controlled release. The following W_2 phases were tested:

0.2wt % Conc A

2wt % Tween 80

0.2wt % Conc A + 2wt % Tween 80

$W_1/O/W_2$ emulsion was prepared using the stirred emulsification cell. The W_2 volume used in all experiments was 90 ml. The dispersed phase flux was 8 l/hm^2 in all experiments. The speed velocity used was 116.05 s^{-1} (56.51 dine/cm^2). The final % O/W in $W_1/O/W_2$ emulsion was 10%.

6.2.4. Emulsion characterisation techniques

$W_1/O/W_2$ emulsion droplet size distribution and droplet size were measured using a laser light scattering system (Malvern Mastersizer 2000, Malvern Instruments). Microscopic analysis of both $W_1/O/W_2$ and W_1/O emulsion characterisation was also carried out. Optical microscope (Zeiss, model Axiovert 25) equipped with a camera (JVC, model TK-C1481BEG) to capture the images of the emulsions was used. The droplets size was measure using scion image software.

The mean particle size was expressed as the surface weighted mean diameter (or Sauter diameter), D[3,2]and as the volume weighted mean diameter, (or De Brouckere diameter), D[4,3]. D[3,2] and D[4,3] were determined, respectively, as follows:

$$D[3,2] = \frac{\sum D_i^3 n_i}{\sum D_i^2 n_i} \quad (4)$$

$$D[4,3] = \frac{\sum D_i^4 n_i}{\sum D_i^3 n_i} \quad (5)$$

where D_i = particle diameter of class i and n_i = number of particle in class i.

The width of droplet size distribution was expressed as a Span number, calculated by the following expression:

$$Span = \frac{D[0.9] - D[0.1]}{D[0.5]} \quad (6)$$

where D[x0] is the diameter corresponding to x0 vol.% on a relative cumulative droplet size curve.

The mass of water-oil emulsion samples and of water present in the samples were measured by means of a moisture analyser (Ohaus, model MB45) composed by a precision balance and a dryer unit. The oil percentage was determined from the weight of sample dried by heating at 100°C because only water phase was evaporated at this temperature. The percentage of water-in-oil (%W/O) can be determined as follows:

$$\%W/O = \frac{m_f}{m_i} * 100 \quad (7)$$

where m_f was the mass of sample after evaporation (corresponding to the mass of oil not evaporated at 100°C) and m_i was the mass of initial sample (at the moment in which it was taken from emulsion). The analysis was carried out immediately after the emulsification and the % W/O evaluated with this procedure was compared with the one calculated from the dispersed phase permeated during the emulsification experiment. In such experiment, these measurements were repeated during the time to monitor the eventual changes of the %W/O caused by phase separation from the emulsion. The phase eventually separated from the emulsion, was collected using a separating funnels and the resulting %W/O only refers to the stable %W/O emulsified.

6.2.5. Measurement of controlled release as a function of glucose stimulus

PhAla was used as a marker substance contained in the W_1 droplets to monitor its release in the W_2 phase after glucose addition. Therefore, in order to determine the PhAla release from the $W_1/O/W_2$ emulsion under glucose stimulus, a glucose amount was added to $W_1/O/W_2$ emulsion (+Glu) to have in the aqueous phase a final concentration of 5g/l. The effect of different composition of $W_1/O/W_2$ emulsion prepared with different emulsifier was tested using the devices shown in Fig. 6.2. In all experiments, $W_1/O/W_2$ emulsion without glucose stimulus (No Glu) was also monitored and used as control/blanc reference system. At

appropriate intervals, samples were taken from the glucose testing vessel and from control system and the concentrations of (D)- and (L)-isomer were measured by chiral HPLC. Samples from the aqueous phase of the emulsion were obtained by filtering a small amount of emulsion thorough a 0.2 μm cellulose acetate filter attached to a syringe. The concentration of PhAla released was measured by using a CROWNPAK CR (+) column (150mm×4 mm) (Daicel Chemical Industries, ltd.), a mobile phase made of $HClO_4$ pH 7 flowed at 0.8 ml/min, 200 nm, 25 °C. Calibration curve of chromatographic peak area versus PhAla concentration was obtained from (D,L) PhAla standard solutions.

The amount of released PhAla by the glucose stimulus effect (PhAla*) was calculated as follows:

$$PhAla^* = PhAla_{+Glu} - PhAla_{NoGlu} \qquad (8)$$

where $PhAla_{+Glu}$ and $PhAla_{+NoGlu}$ were the PhAla concentration released in presence and in absence of glucose stimulus, respectively.

In some experiments, the same glucose amount used as initial stimulus was added into the water phase (to 10 g/l and 15 g/l), at appropriate time intervals, to observe the effect of progressive stimulus on the release properties.

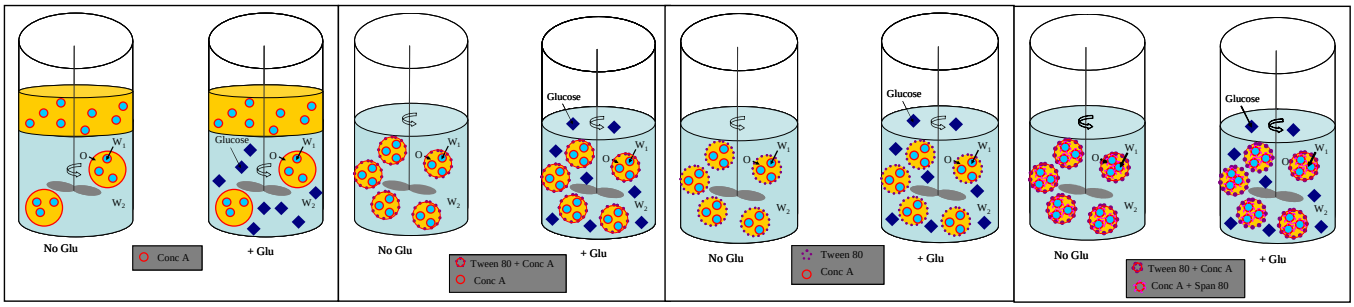

Fig. 6.2. Scheme describing the $W_1/O/W_2$ emulsions tested to the controlled release as a function of glucose stimulus. For all emulsions a device without glucose stimulus was represented. The emulsions differ for the emulsifiers composition as specified in each scheme.

6.3. Result and discussion

6.3.1.Property of Conc A adsorption onto SPG hydrophobic membrane

The first W/O emulsion was prepared by using Conc A as emulsifier and as glucose sensor to be dispersed at the interface. Conc A was solubilized into the water phase (dispersed phase) that had to be permeated through the porous hydrophobic SPG membrane. The membrane pores were much larger (0.4 μm) than Conc A size. Conc A in solution largely consists of dimmers. The monomeric molecular weight of Con-A is 25,500 [20]. At pH values less than 6 (such as that used in the present work) the maximum dimer extension is slightly less than 8 nm as it was deduced from crystallographic structures (for the dimmer form, 1APN from the Protein Data Bank) [21]. Therefore, based on the size, the protein should easily pass through the 0.4 μm membrane pores. However, due to electrostatic interactions it could be adsorbed to the membrane pore wall. Also the PhAla is a small aminoacid of 165.19 but the electrostatic interactions could cause membrane adsorbtion.

In order to verify the steady-state permeation properties of the Conc A and PhAla solution through the membrane, pre-screening experiments were carried out during which both volumetric flux and C_p/C_f ratio as a function of time were measured. Experiments were carried out at pH 5.4, ionic strength 1.026 M and room temperature (23±1°C).

As shown in Figure 6.3, the C_p/C_f ratio increase during the time until 0.94 and 0.98 to Conc A and PhAla respectively. If no protein was adsorbed by the membrane, C_p/C_f was 1. In the present experiment, the C_p/C_f value close to 1 indicates that only the small amount of Conc A or PhAla was adsorbed during the solution permeation. However, the mass adsorbed into the membrane pores (m_a) and the protein concentration adsorbed calculated according to (1) and (2), respectively, was 0.43 mg and 0.49 mg/ml. Although the membrane pore was much larger than protein size the electrostatic interaction between protein and membrane occurred. At start of the solution permeation the flux is constant but the protein adsorbed into the membrane determine membrane fouling that causes flux decline of 20%.

This pre-screening tests are important because permit to confirm that Conc A and PhAla was available to arrange at the water/oil interface and to its transport through droplets emulsions, respectively.

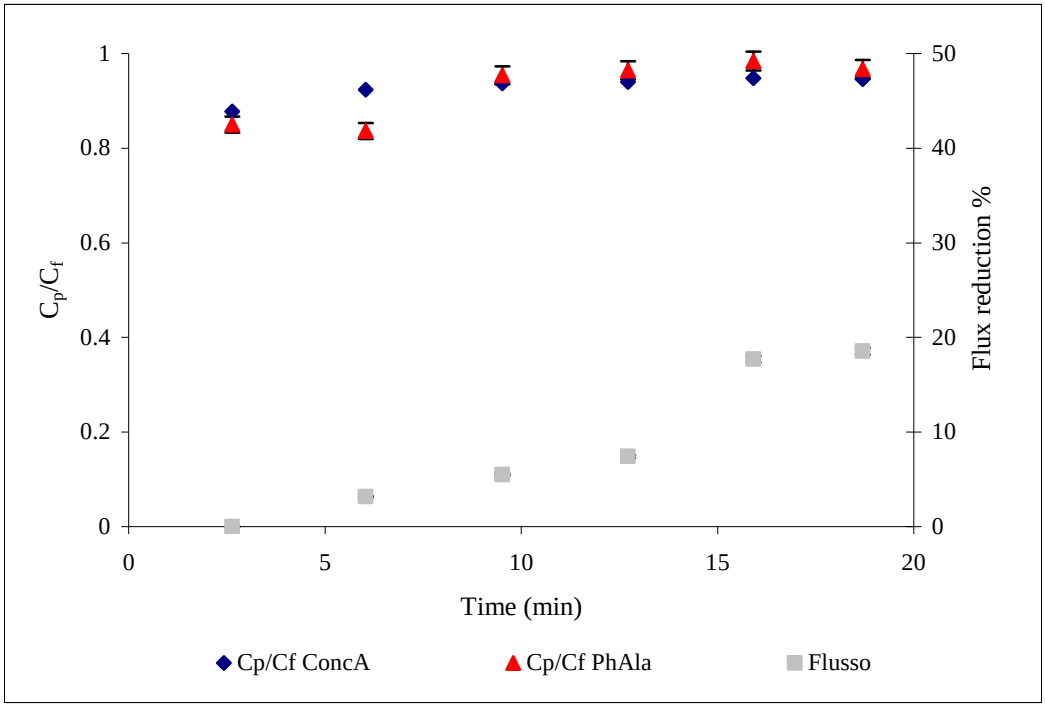

Fig. 6.3. C_p/C_f ratio (for Conc A and PhAla) and flux reduction during adsorption pre-screening text onto SPG hydrophobic membrane.

6.3.2. W_1/O emulsion preparation with Conc A as emulsifier present in the dispersed phase

The preparation of W_1/O emulsion was carried out using Conc A in the dispersed phase that would have been displaced at the interface so that to stabilize emulsion and function as glucose sensor. W/O emulsion preparation was carried out using low shear stress. The dispersed phase flux of 1.5 l/hm^2 was used in all experiments. Effect of axial velocity on mean particle size and Span is presented in Fig. 6.4, while A, B, C illustrate the relative emulsion droplets pictures.

Continuous phase axial velocity is a fundamental process parameter to determine membrane emulsification characteristics, because wall shear stress caused by the continuous phase is a major force to drive the droplets that are detached from the membrane pore.

Figure 6.4 shows droplet size decrease with axial velocity and shear stress increasing from 0.003 to 0.05 m/s and from 0.17 to 2.37 Pa, respectively. Also span shows the same behaviour.

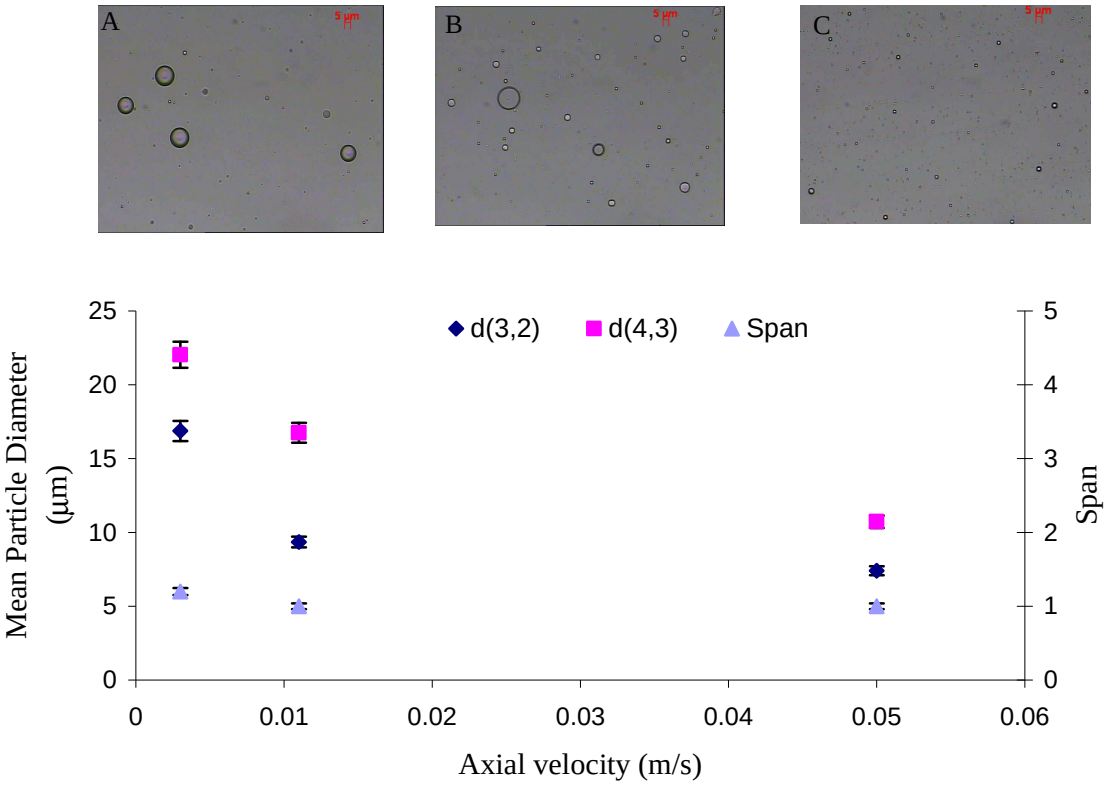

Fig. 6.4. Effect of axial velocity on mean particle size and Span of W/O emulsion. Emulsion droplets pictures prepared at A) 0.003 m/s, B) 0.011 m/s, C) 0.05 m/s as axial velocity.

Water separation from the prepared emulsions occurred and only the stable % W/O was measured. Fig. 6.5 shows the stable %W/O present in W/O emulsion prepared using different value of axial velocity. The stable %W/O was more when higher axial velocity (and shear stress) was used (see Fig. 6.5). In fact, larger droplets are formed at low shear stress and the emulsion results less stable.

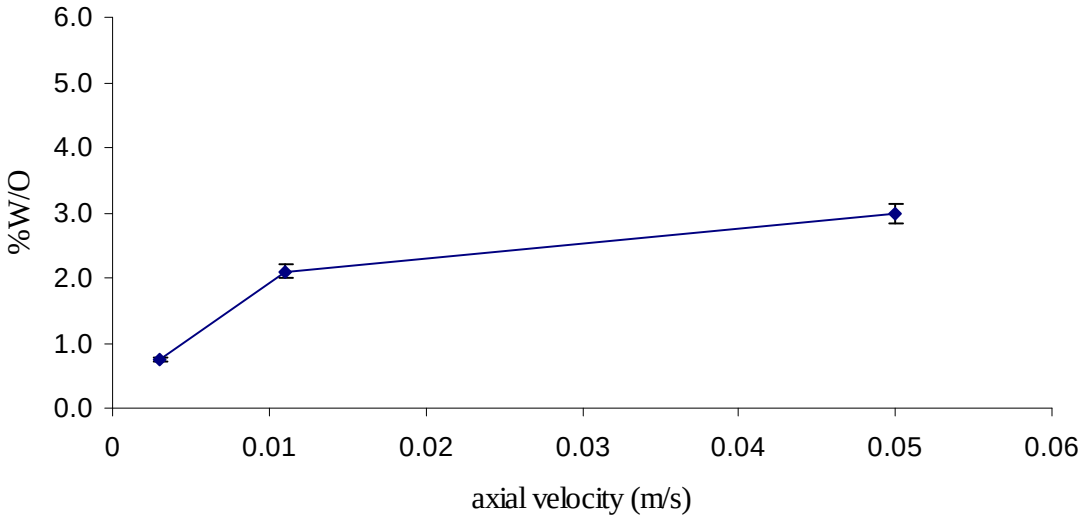

Fig. 6.5. The stable %W/O present in W/O emulsion prepared using different values of axial velocity.

Results showed that 0.1wt % Conc A in the dispersed phase could stably emulsify about 3% of W_1 phase in oil at 0.05 m/s as axial velocity. Using 1.5 l/hm^2 as dispersed phase flux and 0.05 m/s as axial velocity we prepare a W_1/O emulsion in the presence of 2% wt Span 80. In Fig. 6.6 mean particle diameter and span of emulsion prepared using only Conc A or Conc A with Span 80 were compared. The volume-surface mean particle diameter (d(3,2)), which is more sensitive to the presence of small particles, decreases slightly in the presence of Span 80. The more significant effect there was in the volume-weighted mean particle diameter (d(4,3)) which is more sensitive to the presence of any large particles.

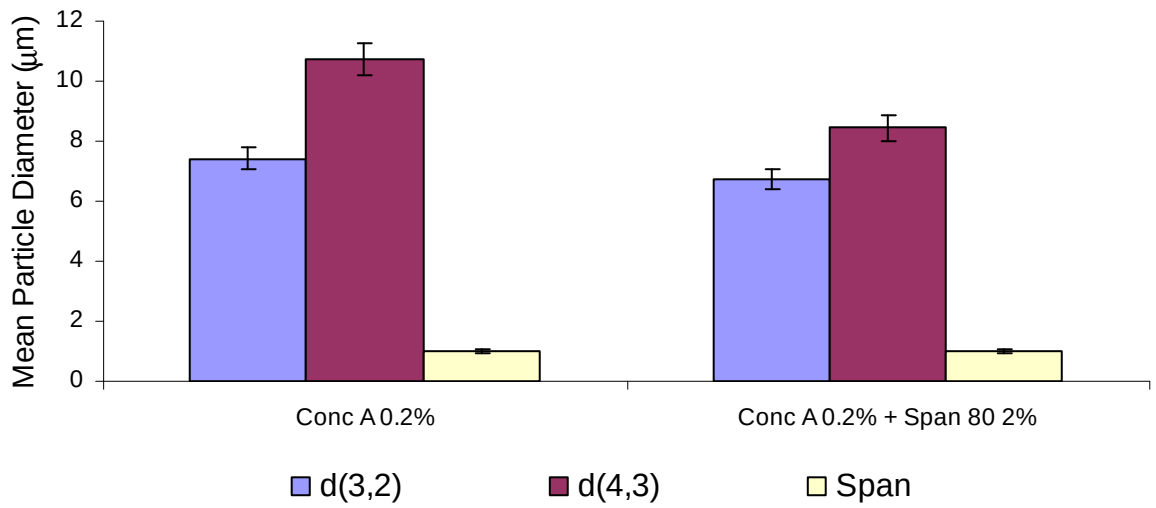

Fig. 6.6. Mean particle diameter and span of W/O emulsion prepared using only Conc A 0.2% wt or Conc A 0.2 % wt and Span 80 2% wt.

The stable %W/O present in the stored W/O emulsion also was compared in Fig. 6.7. The water amount stably emulsified in the oil phase decrease in the time more slowly in the presence of Span 80. Results shows that the combination of Conc A and monomeric emulsifier added in the oil phase (Span 80) influence emulsion stability. Other authors [22, 23] found significant stability enhancement in the presence of proteins (such as BSA or β-lactoglobulin) and oil soluble surfactant.

The w/o emulsions prepared using Conc A and Conc A with Span 80 as above described were used as dispersed phase for the subsequent W_1/O/W_2 emulsion preparation.

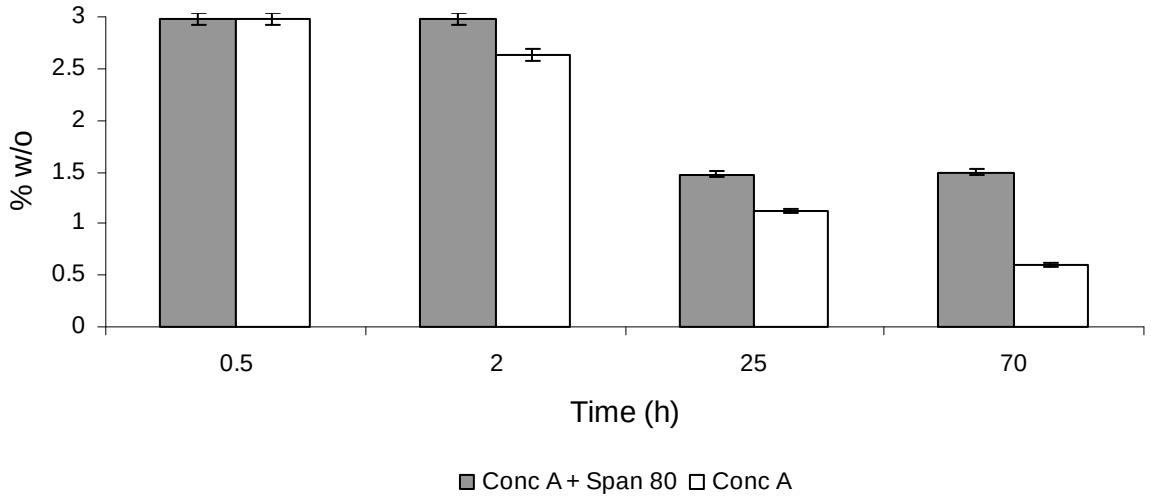

Fig. 6.7. The stable %W/O present in the stored W/O emulsion prepared using only Conc A 0.2% wt or Conc A 0.2 % wt and Span 80 2% wt as a function of time.

3.3. $W_1/O/W_2$ emulsion preparation

Hydrophilic flat sheet metallic membrane with 10 μm d_p was used to prepare $W_1/O/W_2$ emulsion. The aim was to prepare a stable interface containing Conc A able to interact with glucose and activate the release. Fig. 6.8 shows the effect of different emulsifier on the emulsion properties. When Conc A was used, droplets having a larger mean particle diameter (D[3,2] and D[4,3]) was obtained. This is a typical behaviour of most proteins. Because of their higher molecular weight and lower flexibility they can not adsorb and unfold at the interface as quick as surfactants can [24]. In fact, as shown in Fig. 6.8, emulsions prepared using Tween 80 as emulsifier have a more narrow droplet size and droplet size distribution compared with other prepared emulsions. They have d(3,2) and d(4,3) of 55.7 μm and 59.6 μm, respectively and span of 0.6. In Fig. 6.8 one sees that the addition of Tween 80 to Conc A aqueous solution decreases the mean particle diameter, 92.4 versus 62.6 μm and 134.2 versus 73 μm for D[3,2] and D[4,3], respectively, compared with the emulsion prepared using Conc A as emulsifier. However, mean particle diameter and span of emulsion prepared using Conc A with Tween 80 are more similar to emulsion prepared only with Tween 80. We could hypothesize that the emulsifier having low molecular weight such as Tween 80 adsorbs at vacant holes in the interfacial protein network but emulsion droplet size and size distribution is influenced mostly by the adsorption of Tween 80 at emulsion interface.

The presence of Span 80 in the oil showed an additive effect with Tween 80 and Conc A in the W2 continuous phase (Fig. 6.9) and both mean particle diameter and Span decrease.

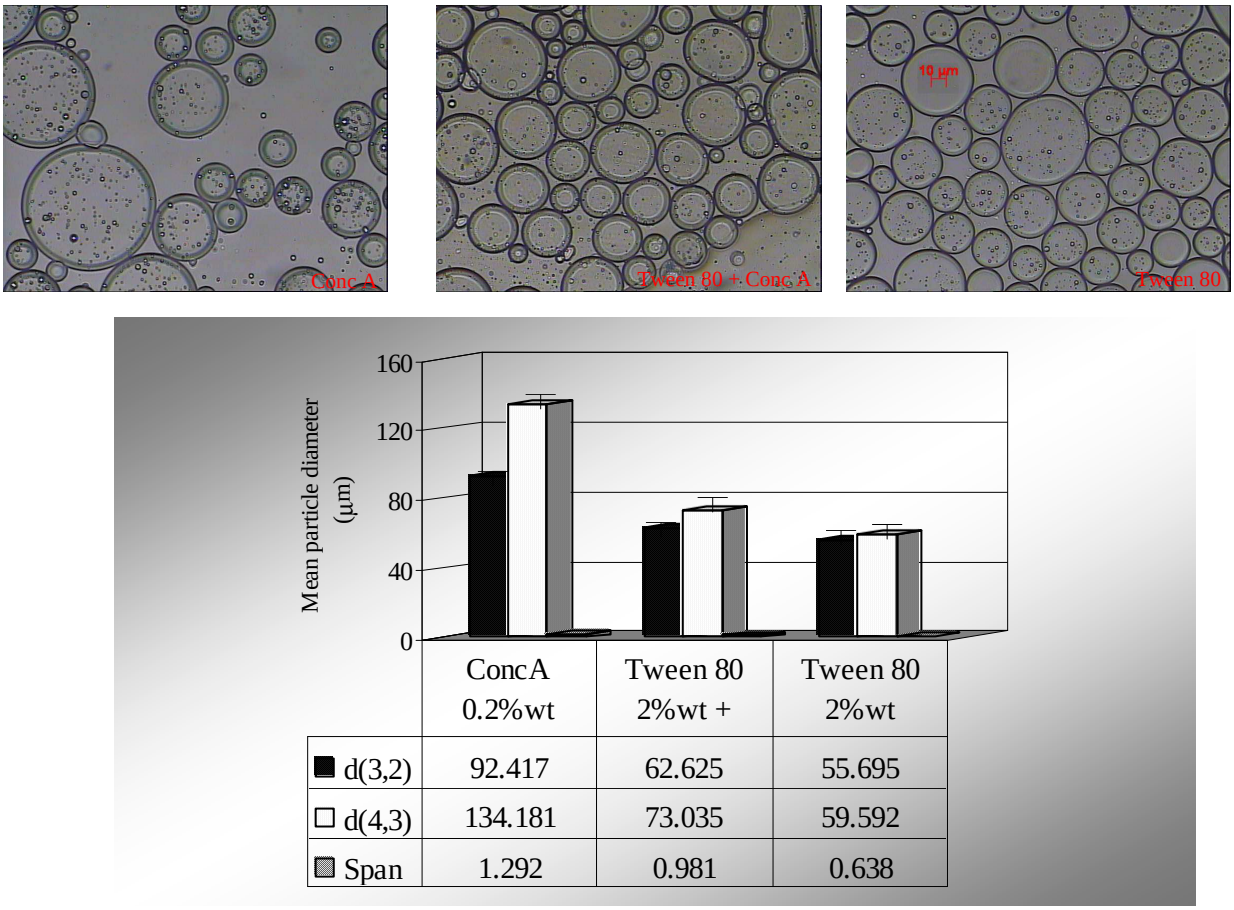

Fig. 6.8. Mean particle diameter and span of $W_1/O/W_2$ emulsion prepared using different emulsifier on. Emulsion droplets pictures prepared using A) Conc A 0.2% wt, B) Conc A 0.2% wt and Tween 80 2%wt, C) Tween 80 2%wt.

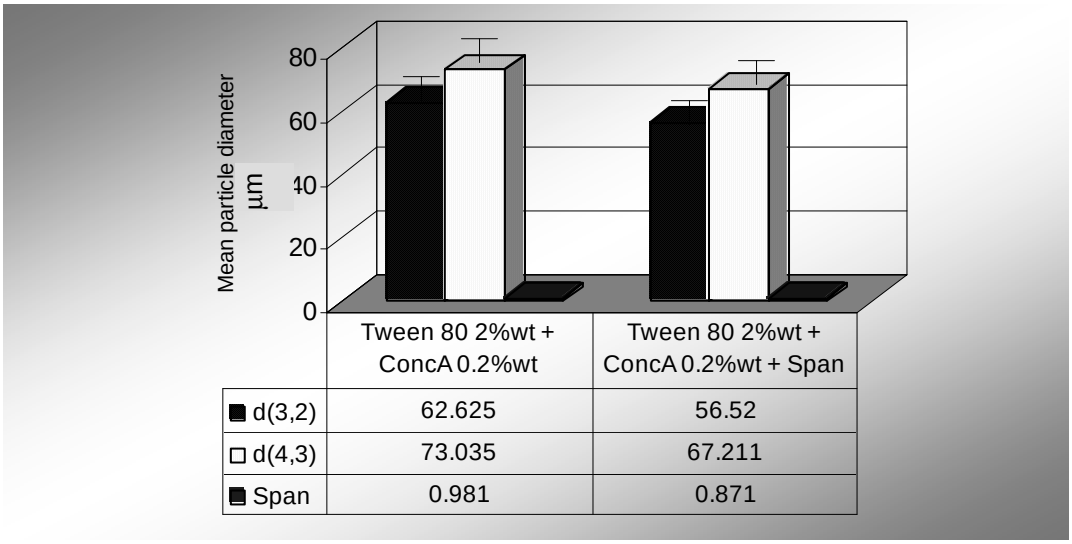

Fig. 6.9. Mean particle diameter and span of $W_1/O/W_2$ emulsion prepared using as dispersed phase W/O emulsion prepared using only Conc A 0.2 % wt or Conc A 0.2% wt and Span 80 2%wt.

6.3.4. Measurement of controlled release as a function of glucose stimulus

In order to evaluate Conc A glucose sensor property in $W_1/O/W_2$ emulsion the release of a marker substance, i.e. PhAla, dissolved in W_1 aqueous phase was followed during the time. Fig. 6.10 A, B, and C shows the %PhAla release from emulsions prepared using different emulsifier as a function of time. For all experiment, the amount of PhAla released with and without glucose stimulus was compared.

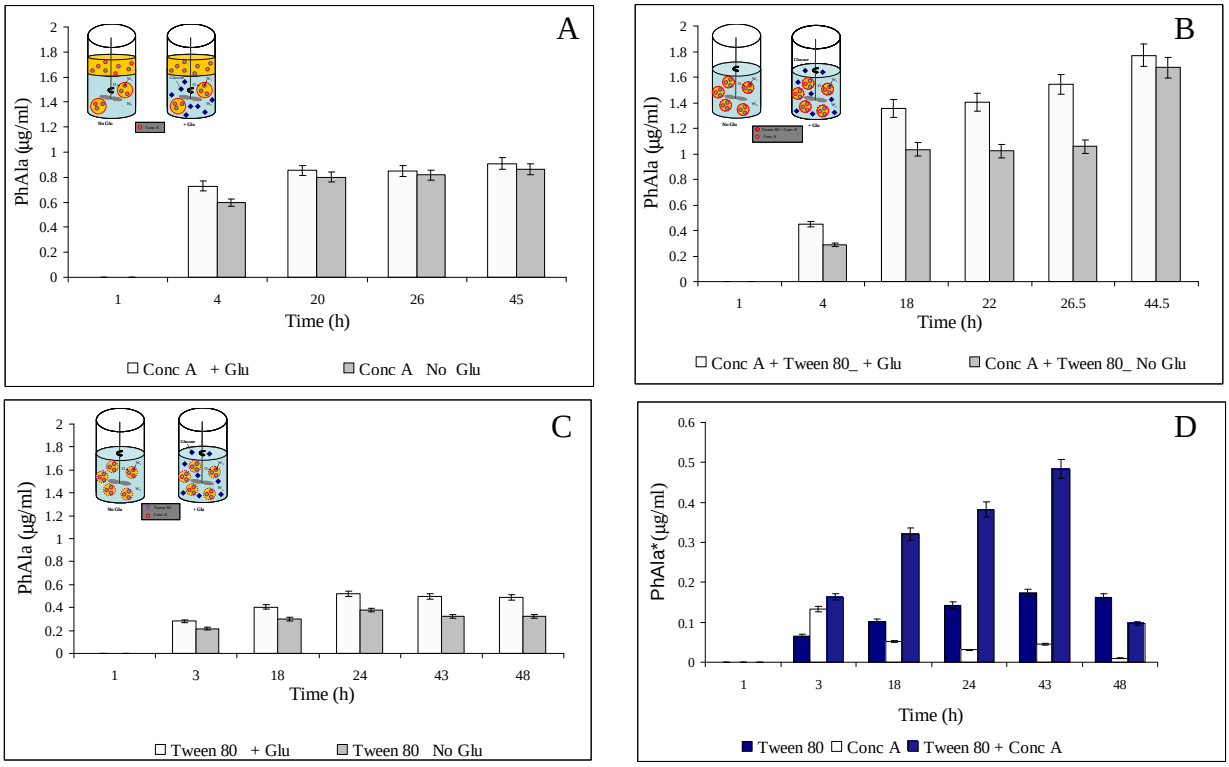

Fig. 6.10. PhAla amount released as a function of time from $W_1/O/W_2$ emulsions prepared using different emulsifier at W_2/O interface: A) Conc A 0.2% wt, B) Conc A 0.2% wt and Tween 80 2% wt, C) Twenn 80 2% wt. D) The effective release of PhAla due to response to stimulus for the $W_1/O/W_2$ emulsions tested.

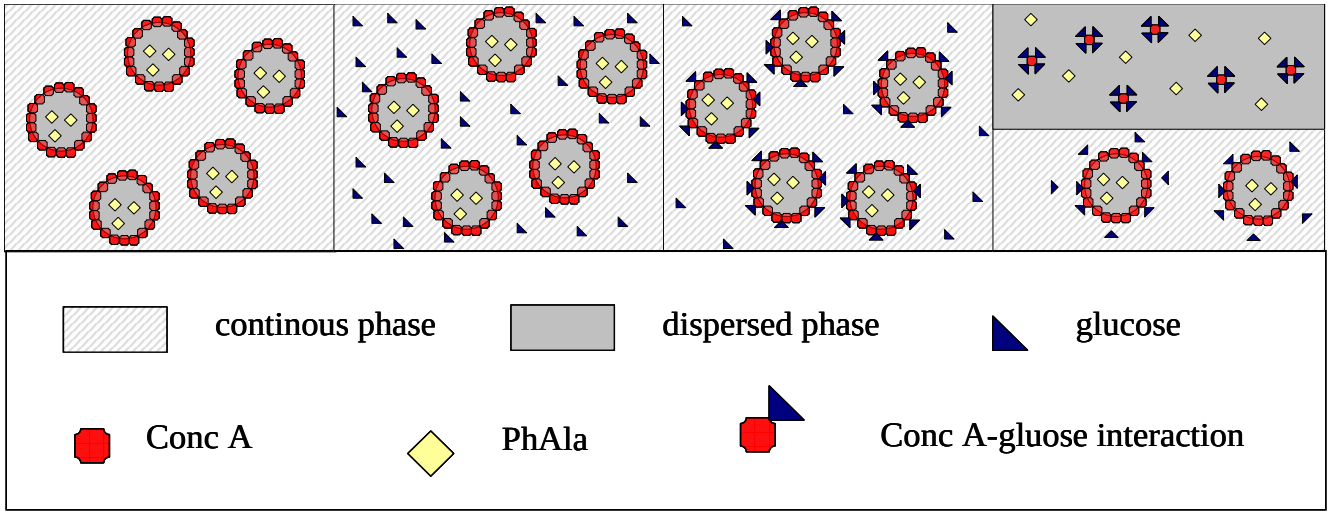

Fig. 6.11. The hypothesized mechanism to promote the controlled release in the presence of glucose stimulus when Conc A was distributed at the interface.

Fig. 6.10 shows that, in all considered systems, Conc A was able to control the marker substance release as function of glucose stimulus. In fact, the amount of PhAla release from $W_1/O/W_2$ emulsion, in all cases considered, was more in the presence of glucose. The high affinity between Conc A and glucose determined a preferential interaction between them, causing the protein displacement from emulsion interface with phase separation and marker substance release. This is the hypothesized mechanism to promote the controlled release (Fig. 6.11).

PhAla amount released in absence of glucose stimulus was due by the two possible mechanism for permeation through oil phase suggested for release from the double emulsion [25]: the first being via the "reverse micellar transport" and the second by "diffusion across a very thin lamellae" of surfactant phase formed in areas where the oil layer is very thin. The different PhAla amount release profile, in considered $W_1/O/W_2$ emulsions, depending by the different properties of interfacial film. We could hypothesize the following mechanism:

1. Conc A is distributed at W_1/O emulsion interface and at $W_1/O/W_2$ emulsion interface with Tween 80 (1° system)

   Conc A functions as glucose sensor at W_1/O and $W_1/O/W_2$ emulsion interface. The presence of Tween 80 stabilize the multiple emulsion. Conc A at O/W_2 interface bonds the glucose dissolved in the aqueous phase promoting its displacement from the interface with consequent rupture of double droplets and than the interaction between Conc A at W_1/O interface and glucose. The release of PhAla marker substance increased progressively during the time (Fig 6.10 A).

2. Conc A is distributed at W_1/O emulsion interface and at $W_1/O/W_2$ emulsion interface (2° system)

   Conc A functions as glucose sensor at W_1/O and $W_1/O/W_2$ emulsion interface. The $W_1/O/W_2$ emulsion was not stable and phase separation was observed. The intrinsic emulsion instability determines no remarkable difference between the system with and without glucose stimulus, respectively. The amount of PhAla marker substance release in this emulsion system is lower than the system previously discussed (Fig. 6.10 B). This was probably determined to the phase separation phenomena. In fact, the interaction between the glucose dissolved in the aqueous phase and Conc A at W_1/O interface can be delayed by the glucose diffusion through the separated oil phase. In addition, although the same amount of

glucose is used, the free glucose amount is less because of Conc A- glucose interaction in the aqueous phase.

3. Conc A is distributed only at W_1/O emulsion interface (3° system)

Conc A functions as glucose sensor only at W_1/O. The glucose diffusion through the O/W_2 droplets delayed the ConcA- glucose interaction. For this reason, PhAla marker substance release resulted lower and slower compared the first system discussed (Fig. 6.10 C).

The effect of glucose stimulus on PhAla marker substance release from multiple emulsion systems considered is better shown in the Figure 6.10 D which reports the effective release due to response to stimulus. PhAla release really determine to the effect of glucose stimulus (PhAla*), calculated according to equation 4, was considered. When only Tween 80 was used as emulsifier, PhAla* release slowly increased during the time. Instead, when Conc A was used together with Tween 80, PhAla* release significantly and continuously increased in the time. When only Conc A was used, PhAla* release decreased because of emulsion instability.
In order to develop a controlled release system it is of great important to consider factorial design such as emulsion interface stability and glucose concentration effect. The presence of Span 80 in the oil phase showed an improve in the w/o stability evaluated during the time. For this reason, the multiple emulsion prepared using Conc A, Span 80 and Tween 80 has been selected to measure PhAla release as a function of progressive progressive addition of glucose stimulus (Fig. 6.12).
In this case, Conc A functions as glucose sensor at W_1/O and W_1/O/W_2 emulsion interface. The PhAla marker substance release in the presence of glucose stimulus is more significant compared to the others systems studied when 10 g/l glucose concentration is reached. When 5 g/l glucose concentration is used no difference between the system with and without glucose stimulus was observed. It is possible that in this case the amount of free Conc A is major compared with the amount of Conc A distributed at the interface because of Span 80 and Tween 80 contemporary presence. A major concentration of glucose is required to promote PhAla release. In fact, the progressive addition of glucose in the water phase (to 10g/l and 15 g/l) determine the reactivation of PhAla marker substance release. This results proof the concept that Conc A functions as glucose sensor in the multiple emulsion considered controlling the release as a function of glucose stimulus. The glucose concentration able to promote PhAla release depend on Conc A amount distributed to the interface or free in the

aqueous phase. The presence of Span 80 at improving W_1/O interface stability seems to permit a best interaction between Conc A and glucose.

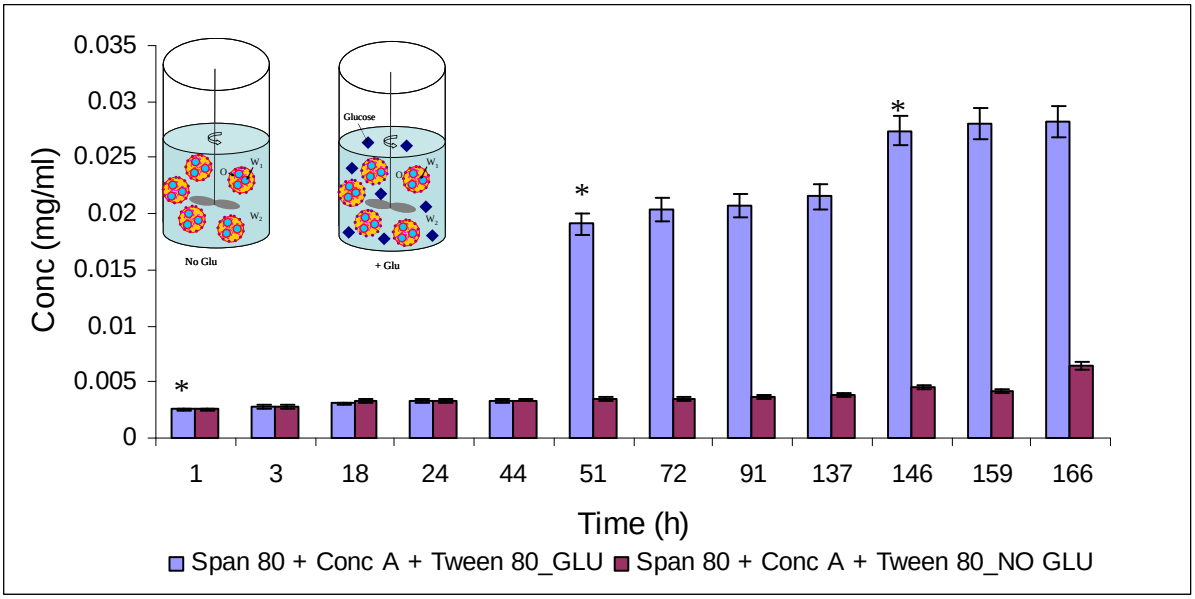

Fig. 6.12. The PhAla amount released as a function of progressive addition of glucose stimulus in the emulsion prepared using using Conc A 0.02 % wt (at W_1/O and W_2/O interfaces), Span 80 2% wt and Tween 80 2% wt.

6.4. Conclusions

Preparation of stimulus responsive multiple emulsions has been investigated in this work. A glucose-sensitive multiple emulsion was used as model system. A protein that specifically bind glucose such as Conc A was tested as emulsifier and as glucose-sensor in the multiple emulsions preparation. The membrane emulsification technique was selected as emulsification technique both in the W/O and W/O/W emulsion preparation. Conc A showed emulsifier property in the W_1/O emulsion prepared in the controlled shear stress conditions using crossflow membrane emulsification. W_1/O emulsion prepared using cross-flow membrane emulsification containing a marker substance (PhAla) to testing controlled release, was used as dispersed phase in $W_1/O/W_2$ preparation. The multiple emulsion having the best properties in terms of stability and controlled release was prepared investigating the effect of O/W_2 interface composition. Results showed that it is possible to modulate the drug release velocity modifying interface properties with different emulsifier molecule. The combination

of Tween 80and Span 80 as emulsifier and Conc A as glucose-sensor showed the better controlled release property in the presence of glucose stimulus. The progressive addition of glucose in the water phase determine the reactivation of marker substance release confirming the effect of self-regulating drug delivery property of multiple emulsion functionalized with Conc A at the interface.

These results show the flexibility and suitability of membrane emulsification process for controlled production of self-regulating drug delivery W/O/W emulsions that would mimic and/or replace important physiological functions.

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Chapter 7

Membrane emulsification as a novel method to distribute phase-transfer biocatalysts at the oil/water interface in bioorganic reactions

7.1. Introduction

Emulsions are a unique microheterogeneous medium for enzymatic reactions [1]. They are usually prepared using high-pressure homogenizers, ultrasound homogenizers and rotor/stator systems, such as stirred vessels, colloid mills or toothed disc dispersing machines [2]. In the dispersing zone of these machines high shear stresses are applied to deform and disrupt large droplets. Therefore, high-energy imputes are required and shear-sensitive ingredients such as proteins or starches may lose their functional properties [3].

In membrane emulsification process, a dispersed liquid phase is pressed through the membrane pores to form droplets on the membrane permeate side; droplets are then removed from the pore mouth by a continuous phase flowing along the membrane surface [4, 5]. In this kind of process, shear stress is much lower than the above-mentioned emulsification systems and therefore it is possible to use shear-sensitive ingredients such as proteins. Furthermore, monodispersed emulsions with narrow droplet size can be produced [6, 7].

These properties suggested that the membrane emulsification process could be a very suitable method to prepare multiphase microstructured reaction systems containing phase transfer biocatalysts.

Lipases are well-known biocatalysts able to interact with interfaces where they undergo a conformational activation. The lipase active site is covered by a lypophilic surface loop, the so-called lid (or flip), which upon binding to the interface moves away, turning the “closed” form of the enzyme into an “open” form [8]. This makes the active site accessible to the organic solvent and also exposes a large hydrophobic surface, which is thought to facilitate binding of lipase to the interface. This phenomenon, known as interfacial activation [9],

enhances lipase hydrolytic activity and also favours the lipid-water interface with high regio- and/or enantioselectivity [10].

Studies on lipase in multiphase systems have reported on the use of surfactant molecules to stabilize the interface. Usually lipase activity in emulsion depends on the type of surfactant [11].

In this work, the distribution of lipase at the interface of the oil droplets was assisted by membrane emulsification. The process allowed avoiding the use of other surfactants and lipase itself functioned both as phase transfer biocatalyst and surfactant. The interface stability provided a constant reaction interface at steady-state.

The efficiency of the multiphase emulsion reaction system prepared in this way was tested using the kinetic resolution of racemic naproxen methyl ester mixture as a reaction model to produce (S)-naproxen acid, a member of the arylacetic acid group of nonsteroidal anti-inflammatory drugs.

7.2. Materials and methods

7.2.1. Chemicals

Lipase from *Candida rugosa*, type VII, containing 1 $g_{protein}$/5.88 $g_{raw\ powder}$ and 1.180 units/mg of solid was purchased from Sigma Aldrich. Racemic naproxen methyl ester was used as substrate after it was dissolved in isooctane reagent grade. Ultrapure water was made by bi-distilled water filtered through a 0.2 μm membrane; sodium dihydrogen phosphate anhydrous (NaH_2PO_4) and disodium hydrogen phosphate anhydrous (Na_2HPO_4) were used to prepare phosphate buffer solution (pH 7.0). Pure (S)-naproxen isomer was used as standard. THF (tetrahydrofuran) and TEA (triethylamine) HPLC grade, were obtained from Sigma Aldrich. Methanol and ethanol HPLC grade were obtained from Carlo Erba.

7.2.2. Equipment and Procedure

The experimental apparatus is illustrated in Fig. 7.1. The main parts of the system consisted of i) a membrane module where the emulsion was formed; ii) a circuit feeding the organic phase containing the substrate; and iii) a reaction circuit where emulsion was collected into an aqueous phase.

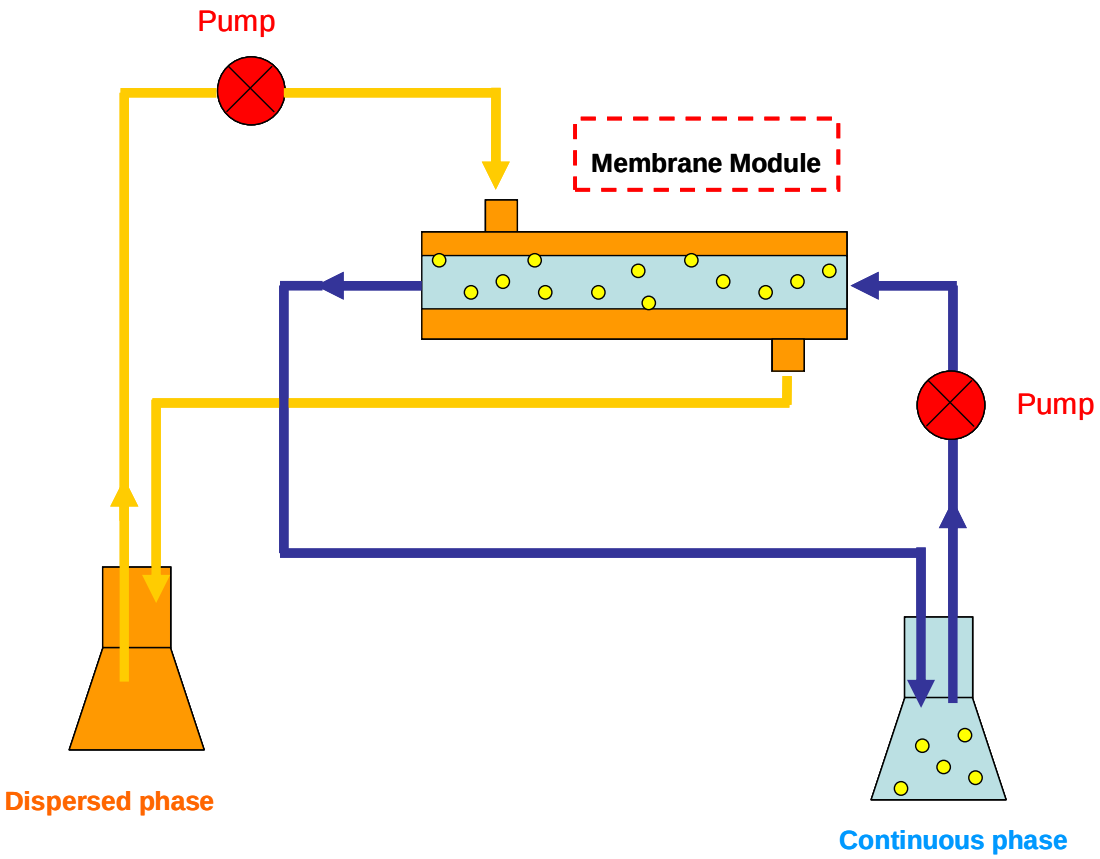

Fig. 7.1. Schematic representation of the experimental apparatus used to distribute the lipase at the interface of the oil/water emulsion.

SPG tubular membranes (from SPG Technology Company, Japan) having 0.1 µm pore diameter, 10 mm outside diameter, 1 mm thickness and 100 mm length were used. An SPG tube was assembled in a Pyrex vessel. A 50 mM phosphate buffer solution (pH 7.0) containing lipase was circulated by a gear pump, along the lumen side of the SPG membrane. The aqueous phase axial velocity was 0.16 m/s, which corresponded to the maximum axial velocity value of the used system, causing no pressure increase at the lumen inlet. The volume of the initial aqueous phase was 100 ml. Emulsification was carried out at room temperature (20 ± 2 °C).

The effect of protein concentration on emulsification and reaction systems was studied using four different lipase concentrations (1, 2, 4 and 8 g/l).

The oil phase, isooctane containing 5 mM racemic naproxen methyl ester mixture, was circulated along the shell side of the SPG membrane and forced to permeate through the

membrane by applying a transmembrane pressure of about 0.1 bar. The initial volume of the organic phase was 100 ml, the volume permeated through the membrane to form an emulsion dependent on the emulsifier (lipase) concentration and will be reported case by case. The emulsion preparation time ranged from 30 to 60 min.

When the volume of organic phase in the emulsion was equivalent to the maximum volume of organic phase that the lipase was able to steadily disperse in the aqueous phase, the circulation of the continuous phase between the batch and the emulsification module was stopped and the reaction was continued in the stirred tank reactor, thermostated 30 °C. At appropriate intervals, samples were taken from the reaction vessel and the concentrations of (S)- and (R)-isomer were measured by chiral HPLC.

Samples from the aqueous phase of the emulsion were obtained by filtering a small amount of emulsion thorough 0.2 μm cellulose acetate membranes. The protein not complexed at the interface and remaining in the aqueous phase was measured both by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and by catalytic activity test. Enzyme activity in the filtered aqueous solution was measured using triglycerides present in olive oil as a substrate. This substrate was also used to study the effect of axial velocity during emulsion preparation on lipase functional properties.

7.2.3. Droplet measurement

Droplet size was measured by Zeiss Axiovert 25 optical microscopy with a 20x lens. The images were obtained with an AxioCam (Carl Zeiss) using AxioVision 3.1 software (Carl Zeiss). The images were processed by the "Scion Image" program. This software automatically counts identified particles and measures their diameter. From these measurements, the mean droplet size and size distribution were evaluated. For each series of experiments, standard deviation was calculated from at least three different experiments. For each experiment, three samples were taken at the same sampling time and for each one at least five photos were analysed.

In addition to optical microscopy, light scattering analyses were also carried out, using Brookhaven 90 Plus, which has an analytical range between 2 nm and 3 μm. The two analytical methods gave similar droplet size values, with an overestimation of about 0.2 μm for the optical microscopy analyses.

7.2.4. HPLC analysis

(S)- and (R)-naproxen acid were measured by HPLC (Merk Hitachi) coupled with a UV detector (Merk Hitachi L-4000) at 254 nm. Chirobiotic V (Astec) column was used. The mobile phase was a 10/90 mixture of THF/aqueous solution at pH 7.0 containing 0.1% TEA. The analysis was made at a flow rate of 1.0 ml/min. (S)-naproxen was eluted faster than (R)-naproxen. Calibration curve of chromatographic peak area versus naproxen concentration was obtained from standards solutions.

Mass, produced at time t, was calculated by considering the concentration of naproxen acid in the aqueous phase volume and the concentrations of previous sampling volumes, according to:

$$Mass_{produced} = V_t \cdot C_t + \sum V_n \cdot C_n \qquad (1)$$

Where V_t is the total volume of aqueous phase in emulsion at a certain sampling time, C_t is the naproxen concentration measured by HPLC, V_n is the volume of a previous sample, and C_n is the naproxen acid concentration of a previous sample.

Enantiomeric excess of (S)-isomer in the product, ee_p, was calculated according to:

$$ee_p = (S_p - R_p) / (S_p + R_p) \qquad (2)$$

where S_p and R_p are the mass of product of (S)- and (R)-naproxen acid, respectively.

The conversion of the substrate was calculated according to:

Conversion = $(S_p + R_p) / M_s$ (3)

where M_s is the mass of substrate.

7.2.5. Electrophoresis

Proteins present in the collected samples, e.g. lipase in the initial solution and in the aqueous phase after filtration of emulsion samples (hereinafter also called unadsorbed lipase), were analysed by one-dimensional SDS-PAGE on a 10-15% PhastGel^{TM} gradient using buffer strips. An 8/1 μl sample applicator was used (Amersham Biosciences, UK). The gel has a continuous 10 to 15% gradient gel zone with 2% crosslinking. The buffer system in PhastGel SDS Strip is composed of 0.20 M Tris-glicine 0.20 M Tris and 0.55% SDS at pH 8.1.

Sample treatment: to the final volume, 2.5% SDS (Sigma-Aldrich), 5% β-mercaptoethanol (Sigma-Aldrich), were added and heated at 100°C and then 0.01% of bromophenol blue (Sigma-Aldrich) was added. Each sample was loaded onto a separate lane of the gel containing 1 μL of sample. The gels were stained with silver and then distained with 3.7% Tris-HCl, and 1.6% sodium tiosulphate. The solution for preserving the gels contained 10% glycerol.

The gel images captured by scanner were analysed by Image Quant TL Software (Amersham Biosciences, UK), which permitted the identification of the band molecular weights (MW) and concentration. The estimation of protein MW was calculated by using the molecular size calibration mode in a gel image containing standard MWs. The MWs of the proteins of unknown samples were calculated from the logarithm curve fitting, which relate the standard MWs with the relative mobility as a pixel position by using calibration Kit proteins (LMW, low molecular weight). The amounts of proteins identified in the gels were calculated, using standard proteins, from the quantitative calibration curve, which relates the band volume and intensity to protein amount.

7.2.6. Enzyme activity test with olive oil

A 19 ml phosphate buffer solution (pH 7.0) and 1 ml of olive oil were placed in a 50 ml volume tank and stirred at 500 rpm to form a uniform mixture and then 1 ml of lipase solution was added. The mixture was stirred with a magnetic stirrer and the temperature was maintained at a constant value of 30 (±1) °C. The reaction was monitored by automatic titration using a Mettler DL21 titrator and 20 mM NaOH solution.

7.3. Results and Discussion

In this section, results on the use of membrane emulsification to distribute lipase at the interface of oil/water multiphase reaction systems are presented. Initially, it was verified that the operating conditions during emulsion preparation did not have a negative effect on enzyme activity. Then, the influence of the emulsifier (lipase) concentration on the emulsion properties, as well as on the catalytic activity and enantioselectivity of lipase distributed at the interface, were investigated.

7.3.1. Effect of axial velocity on lipase performance

The effect of shear stress caused by axial velocity on the lipase functional properties was investigated. A 2 g/l lipase solution was recirculated along the membrane emulsifier lumen at the same axial velocity used during the emulsion preparation (0.16 m/s). The solution was recirculated for more than 60 min and samples were taken every 30 min to test the enzyme activity. Catalytic activity was measured using olive oil as substrate and the results were compared with native lipase activity. Fig. 7.2 shows the concentration of free fatty acids as a function of time produced by a native lipase solution and a lipase solution after recirculation along the membrane emulsifier circuit. The initial reaction rate for the native lipase solution and the lipase solution after recirculation (at 30 and 60 min) resulted 0.012 mM/min and 0.013 mM/min, respectively. Results confirmed that no negative effect on the lipase activity was caused by operating conditions.

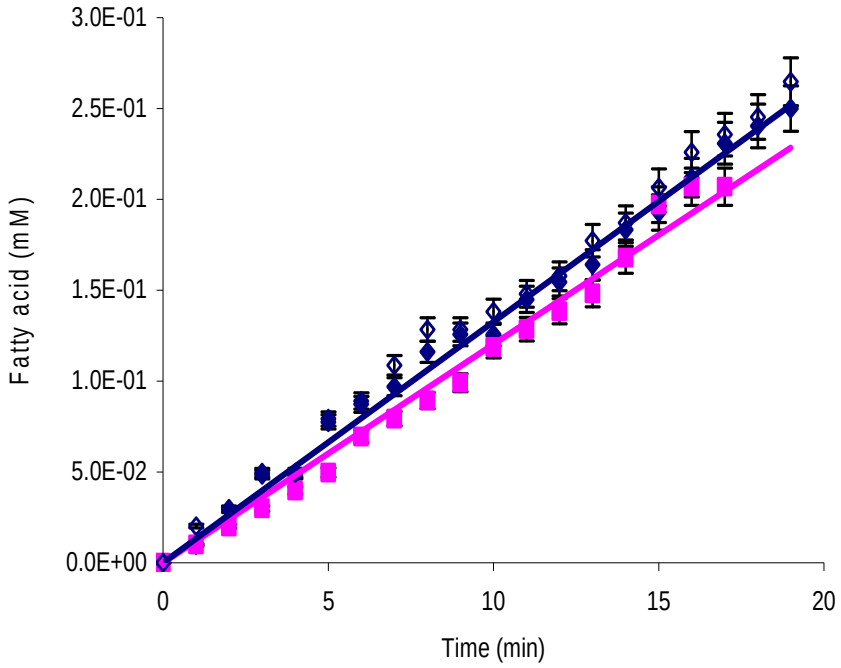

Fig. 7.2. Reaction rate of lipase native solution and lipase solution recirculated along the lumen of SPG membrane at 0.16 m/s. (lipase solution concentration = 2g/l).

7.3.2. Emulsion properties

7.3.2.1. Influence of lipase concentration on emulsion droplets size distribution

Emulsions were prepared using isooctane containing 5 mM naproxen methyl ester as dispersed phase and phosphate buffer containing lipase as continuous phase. Four lipase concentrations were used: 1, 2, 4 and 8g/l.

Droplet size distributions for different lipase concentrations, measured by optical microscopy, are shown in Fig. 7.3.

There was no difference between the droplet size distribution of the different emulsions. In all cases, droplet sizes ranged from 0.7 (±0.01) to 3.1 (±0.2) µm with a maximum at 1.6 (±0.4) µm, representing more than 90% of the droplet emulsions. Analyses carried out by light scattering resulted in a maximum droplet size distribution of 1.4 (±0.3) µm. Although the two methods resulted in a 0.2 µm difference, the overall range value can be considered trustworthy.

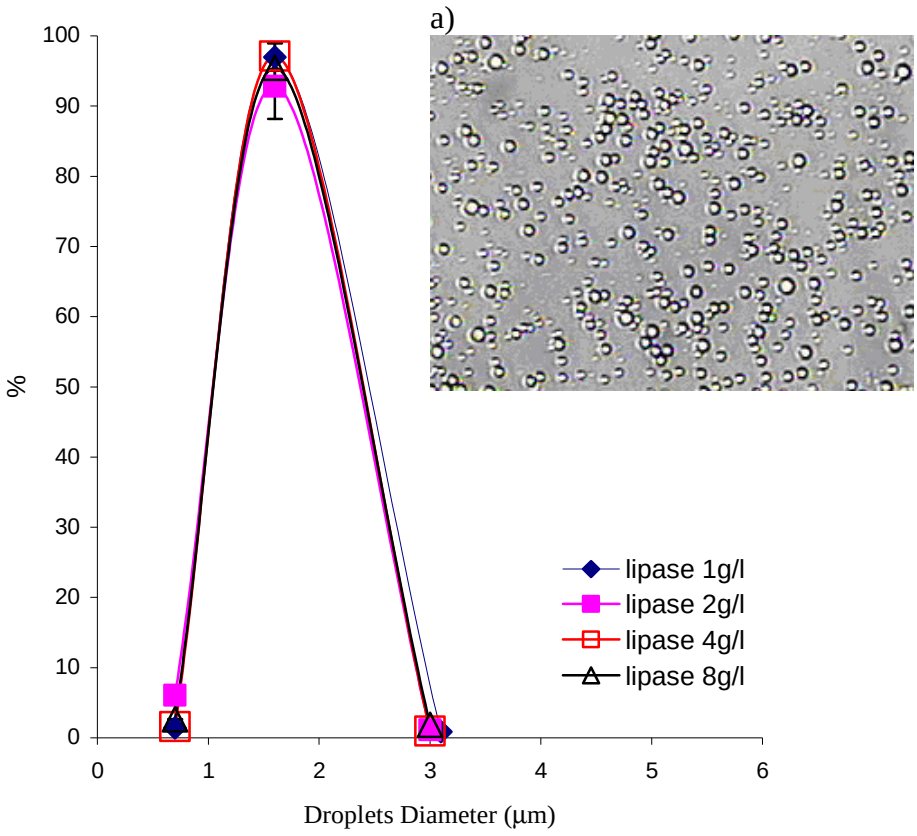

Fig. 7.3. Droplet size distribution of emulsions obtained using 1, 2, 4 and 8 g/l of lipase initial concentration as emulsifier. a) Oil in water emulsion picture captured at the optical microscope (20x). (Oil phase = isooctane containing 50 mM racemic naproxen methyl ester mixture; water phase = phosphate buffer containing lipase solution; transmembrane pressure (TMP) = 0.1 bar; axial velocity = 0.16 m/s).

The fact that droplet size did not depend on the emulsifier concentration implied that hydrodynamic conditions played a decisive role in the used emulsifier concentration. Similar results were reported by Tcholakova [12]. The experimental data from those studies showed that there was a considerable decrease of droplet size at low surfactant concentrations (< 0,1 wt%) on the contrary there was a plateau region for high surfactant concentration values (> 0,1 wt%).

Fig. 7.4 shows the percentage of organic phase in the water phase as a function of lipase concentration solution used as emulsifier. At low emulsifier concentration (1 to 4 g/l) the percentage of organic phase increased linearly with increasing lipase concentration, reaching a plateau after 4 g/l. This behaviour is probably due to the fact that lipase forms stable aggregates at a concentration of 8 g/l, which prevents the lipase from being available to stabilize additional emulsion droplets. This point will be further discussed in the next section.

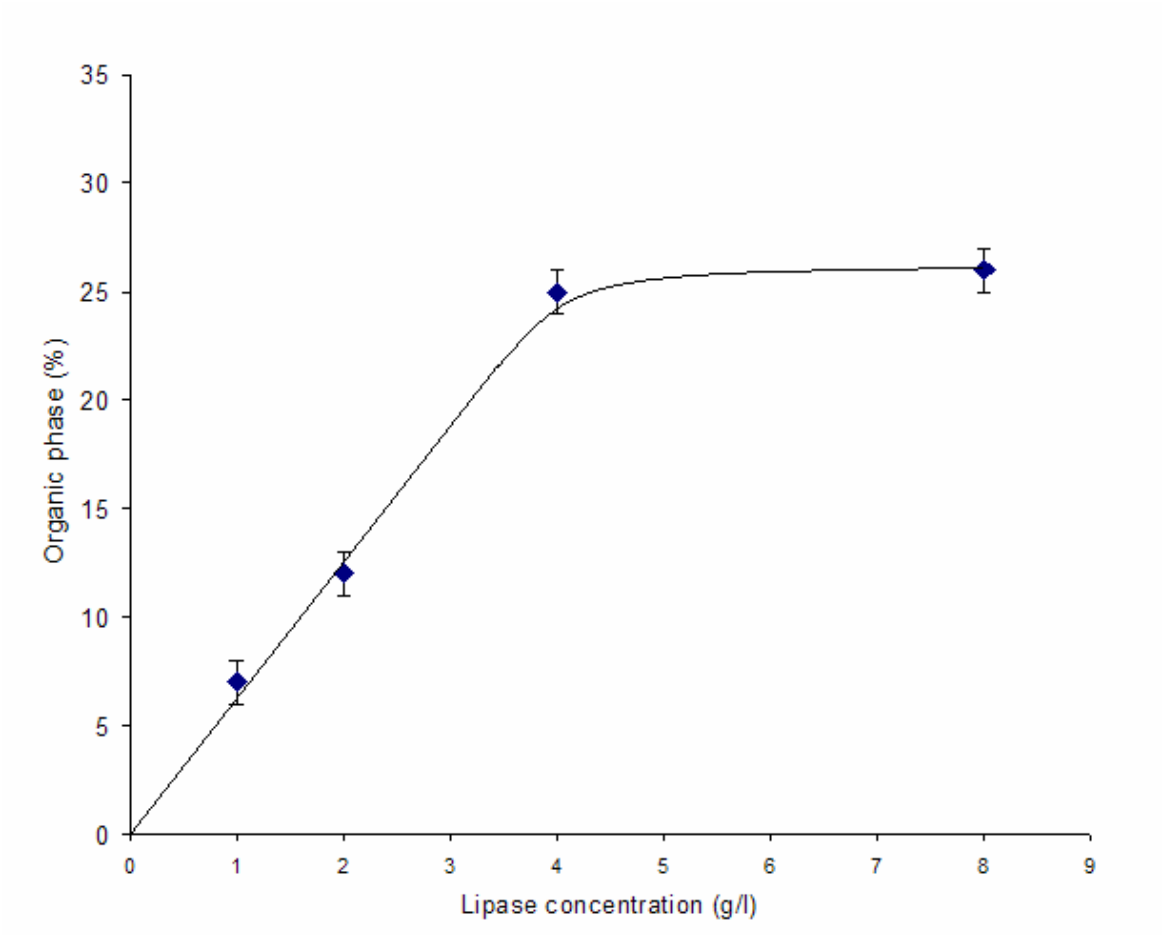

Fig. 7.4. Behaviour of organic phase percentage in water phase as a function of lipase solution concentration.

Thanks to the highly droplet uniformity and stability the membrane emulsification methodology offered a possibility to accurately evaluate catalyst basic parameters such as the hydrodynamic diameter. This information is useful in the bioreactor optimization design. In this work, macromolecule diameter at the interface was evaluated and compared to the molecular diameter calculated from crystallographic data.

In fact, each emulsion droplet is a sphere for which it was calculated the volume of each sphere (V_g) knowing the radius of each drop (r_g):

$$V_g = \frac{4\mu r_g^3}{3} \tag{4}$$

Knowing the total volume (V_{tot}) of organic phase permeated in the aqueous phase is possible to calculate the total number of droplets (N_{tot}):

$$N_{tot} = \frac{V_{tot}}{V_g} \tag{5}$$

The total droplets number (N_{tot}) multiplied by the area of each drop (A_g) gives the total area occupied by droplets (A_{tot}):

$$A_{tot} = N_{tot} \times A_g \tag{6}$$

The area of each droplet (A_g) will be:

$$A_g = 4\,\pi r_g^2 \tag{7}$$

In which r_g is the drop ray.

The area of each droplet (A_g) can also be expressed as the product of the number of lipase molecules that covers every single droplet ($N_{molecoleLipasi}$) and the area of lipase to the interface (A_{Lipase}).

$$A_g = N_{lipasemolecules} \times A_{lipase} \tag{8}$$

If we approximate the lipase as spherical molecule ($A_{Lipasi} = \pi r_{Lipasi}^2$,dove r_{lipasi} è il raggio della lipasi) the equation 6 can be rewrite as following:

$$A_{tot} = N_{tot} \times N_{molecoleLipase} \times \mu r_{Lipase}^{2} \quad (9)$$

$N \times N_{molecoleLipase}$ is the lipase total number ($N_{totLipase}$):

$$N_{totLipase} = N_A \times \frac{g_{Lipase}}{MV_{Lipase}} \quad (10)$$

in which N_A is Avogadro number, MV_{Lipase} and g_{Lipase} are respectively, the lipase molecular weight and lipase amount distributed at emulsion interface.

Combining equation 9) and 6), we obtain the following relationship:

$$\frac{V_{tot}}{\frac{4\mu r_g^3}{3}} \times 4\mu r_g^2 = N_A \times \frac{g_{Lipase}}{MV_{Lipase}} \times \mu r_{Lipase}^{2} \quad (11)$$

that permit to obtain lipase molecular ray knowing the dispersed phase volume (V_{tot}) stably distributed in the water phase and the lipase amount distributed at the interface (g_{Lipase}).

The molecular ray of lipase from *candida rugosa* obtained using equation 11) is 112.28 (± 6 Å).

Usually this information is obtained from crystallographic data. However, when protein are absorbed at the emulsion interface they undergo conformational changes from which molecular ray depends. Membrane emulsification methodology permit to obtain the hydrodynamic diameter of the protein distributed at the interface.

7.3.2.2. Measure of lipase not present at the interface

The emulsification process was stopped after the organic phase was separated on the top surface of the aqueous phase. This was considered as the signal that no more free lipase was available for emulsion stabilisation. The organic volume excess was collected and measured in order not to consider it in the percentage of organic phase in emulsion.

After emulsion preparation, 5 ml emulsion was filtered to separate 2 ml of aqueous phase and it was analysed to determine whether any uncomplexed lipase was present. Analyses were carried out by electrophoresis and activity tests with olive oil. The results are summarized in

Fig. 7.5. The graphic shows fatty acids production as a function of time obtained with the solutions filtered from emulsions prepared with different lipase concentrations. Enzyme activity was detected only when 8 g/l lipase concentration was used. The electrophoretic gel (Fig. 7.5 a) shows the bands of initial and filtered lipase solution for the used concentrations (1-8 g/l). Also in this case, only when the 8 g/l lipase solution was used uncomplexed enzyme was present in the aqueous phase (lane 9).

In the used operating conditions of transmembrane pressure, axial flow rate, temperature, etc., lipase 8 g/l was able to stabilize a certain volume of organic phase, over this value, additional organic phase that permeated through the membrane separated as a continuous phase on the top of the emulsion mixture, even though the uncomplexed lipase was still present in the aqueous phase. Since the organic phase separates, there is no lack of organic phase to be emulsioned, but on the contrary lack of emulsifier available to stabilize the droplets. Therefore, in the conditions used, lipase concentration higher than 4 g/l does not increase the oil/water ratio.

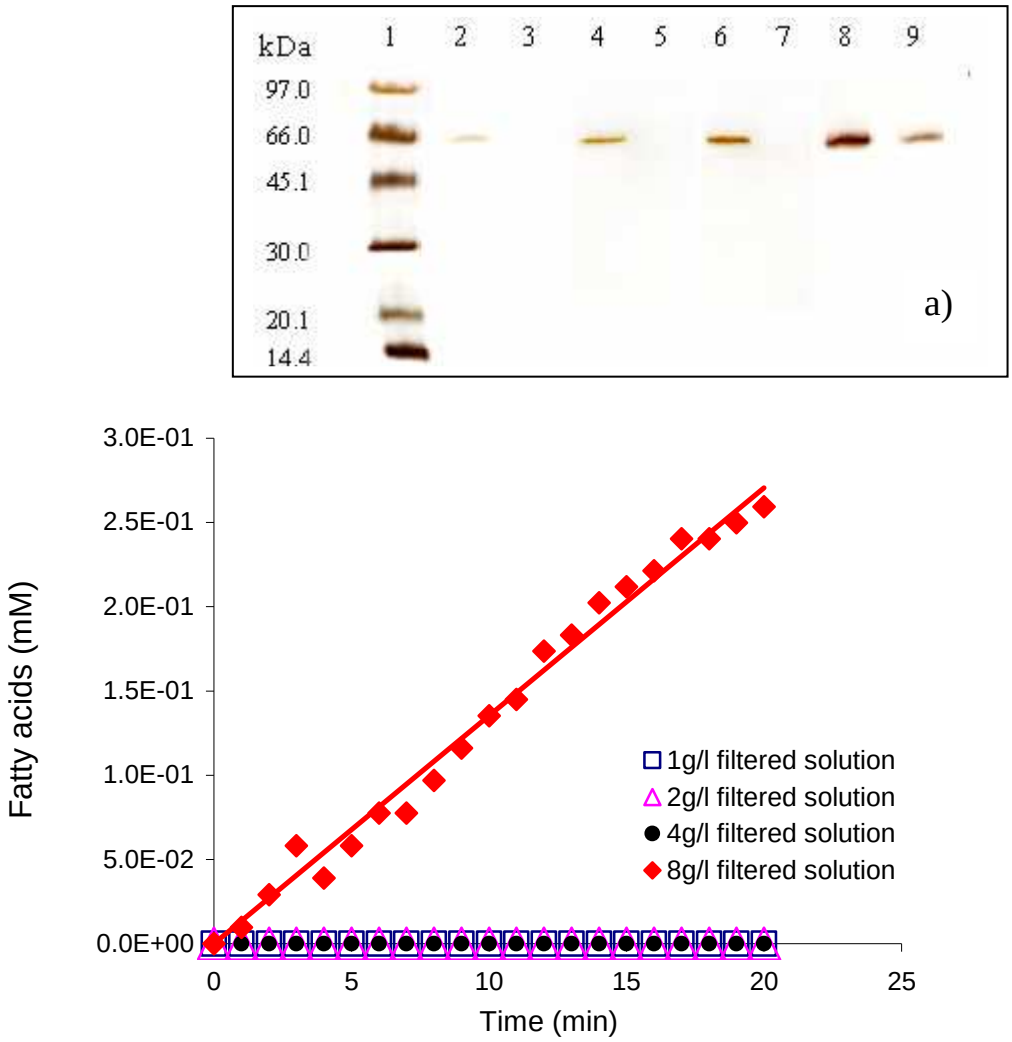

Fig. 7.5. Activity tests of aqueous solutions filtered from the oil/water emulsion to detect uncomplexed lipase. a) SDS-PAGE of lipase initial solution and filtered aqueous phase after emulsion preparation. Line 1: Standard solution; Lines 2, 4, 6 and 8 represent the initial lipase solution at 1, 2, 4 and 8 g/l, respectively; Lines 3, 5, 7 and 9 represent the uncomplexed lipase present in the filtered aqueous phase of emulsion prepared using the lipase solution of 1, 2, 4 and 8g/l respectively.

The results are in agreement with the previous observation that at 8 g/l lipase formed stable aggregates that made it unavailable for stabilizing the oil/water interface during the emulsion preparation.

It is worth to mention that, the protein aggregated also at lower concentrations (as light scattering analyses indicated – data not shown). However, while at lower concentrations all the enzyme was available to be distributed at the oil/water interface (in fact, no protein nor activity was found in the water phase filtered from the emulsion) at 8 g/l, after a certain value, no additional oil phase was emulsioned even though enzyme was found in the filtered

aqueous phase. This led to the hypothesis that such aggregates were more stable than in the other cases, and therefore the protein was not available for stabilizing additional interface.

Previous studies on the behaviour of *Candida rugosa* lipase aggregation showed that aggregation is a function of protein concentration [13]. Although there are no clear indications about the reversibility/irreversibility of aggregates formation, it is likely that aggregates formed at higher lipase concentration are more stable.

7.3.3. Performance of lipase at oil/water interface in bioorganic reactions

Activity and enantioselectivity of lipase distributed at the oil/water interface by membrane emulsification was evaluated by means of enantioselective hydrolysis of 5 mM racemic naproxen methyl ester present in organic droplets. For each lipase concentration (1, 2, 4 and 8 g/l) the production of naproxen acid, the enantiomeric excess and the conversion degree were evaluated. The time course of (S)- and (R)-naproxen acid produced as a function of time is shown in the Fig. 7.6, whilst conversion and enantiomeric excess are reported in Fig. 7.7.

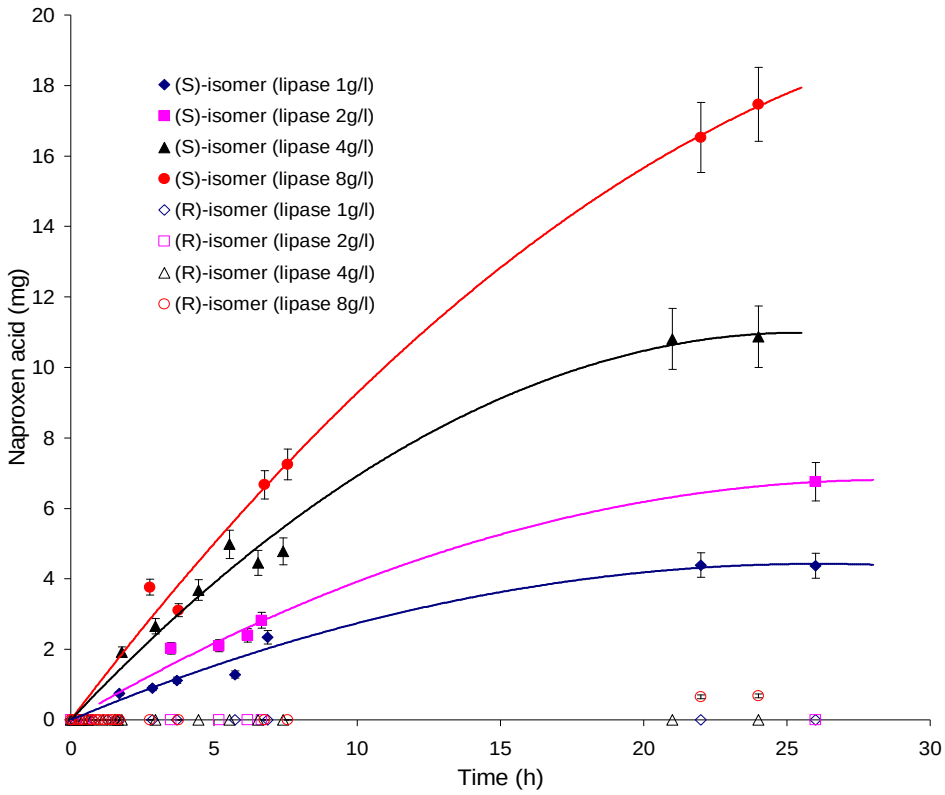

Fig. 7.6. Behaviour of naproxen acid mass production as a function of time in bioorganic reactions carried out using the lipase distributed at the oil/water interface by membrane emulsifier. (Oil phase = isooctane containing 5 mM racemic naproxen methyl ester mixture; water phase = phosphate buffer containing lipase at 1, 2, 4 and 8 g/l).

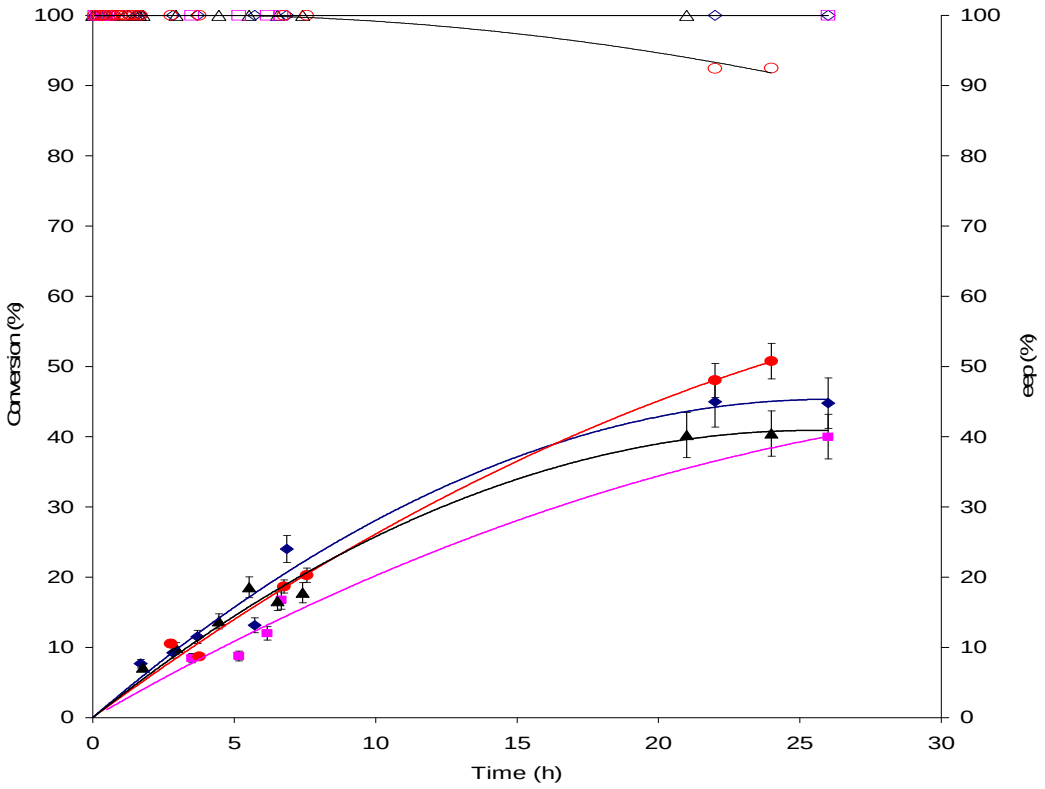

Fig. 7.7. Time course of conversion and enantiomeric excess of the bioorganic reactions carried out using the lipase distributed at the oil/water interface by membrane emulsion.

For each series of experiments, the production of (S)-naproxen acid increased as a function of time. The productivity, calculated as the slope between the mass of naproxen acid produced and the reaction time, increased with increasing the lipase concentration approaching to a plateau at the highest lipase concentration (8 g/l). The results are congruent with the data obtained in previous experiments. In fact, the productivity increased with the lipase at the oil/water interface and the substrate availability (percentage of oil phase) increased with increasing lipase concentration, up to a critical concentration that most probably caused the formation of stable lipase aggregates which externally exhibited the polar part of the macromolecules, thus maintaining them in the aqueous phase and preventing their adsorption at the oil/water interface.

In all cases, the enantiomeric excess of the (S)-naproxen acid was 100% and no decrease was observed as long as the conversion of the naproxen methyl ester did not exceed 45%. After this conversion value, the enantiomeric excess decreased to about 93%. This can be explained by the fact that the reaction is a kinetic resolution, therefore lipase catalyzes the enantioselective hydrolysis of the racemic naproxen methyl ester mixture until (S)-naproxen

methyl ester is available. When (S)-naproxen methyl ester concentration is remarkably reduced, competition of (R)-naproxen methyl ester becomes significant and (R)-naproxen acid is also produced.

The results indicated that lipase did not undergo deactivation, in fact production of (S)-naproxen acid increased with time and enantiomeric excess was 100% as long as the proper isomer substrate was available. It has been reported that reaction rate and productivity of hydrolysis reaction catalyzed by lipase in microemulsions depends on the nature of the surfactant [14-18]. In this work, lipase itself was used as a surfactant and therefore the enzyme activity was not influenced by additives.

7.5. Conclusions

The suitability of membrane emulsification to optimise the distribution of the lipase at the oil/water interface of heterogeneous bioorganic reaction systems has been demonstrated.

The mild operative conditions in terms of shear stress of this technique allowed emulsions to be obtained with droplets stabilised by the lipase itself without the need of additional emulsifier.

Stable emulsions with droplet size distribution having a maximum at 1.4-1.6 □m representing more than 90% of the droplets, were obtained. Thanks to low shear stress and enzyme optimal spatial arrangement at the stable and constant oil/water interface, lipase showed very high enantioselectivity (100%) at high conversion degree (up to 90% of the(S)-naproxen ester).

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Chapter 8

Ferrous ion effects on the stability and properties of O/W emulsions formulated by membrane emulsification

8.1. Introduction

Emulsions are formed by two immiscible phases in which one (dispersed phase) is dispersed in the other (continuous phase). Emulsions are generally unstable and, therefore, they tend to separate. The addition of surfactants is frequently employed to avoid phase separation and to stabilise the emulsion droplets because they possess amphiphilic nature. This is, they have a hydrophilic head and a hydrophobic tail in their molecular structure. Surfactants not only reduce the interfacial tension of the media, thus facilitating the formation of emulsions, but also are supposed to induce repulsive forces between emulsion droplets, stabilising the emulsion. Due to the importance of the interfacial charge in emulsion systems, surfactants have been typically classified according to the charge that they have in the molecule. Classically, surfactants have been classified as anionic, cationic, non-ionic and amphoteric. Thus, it is logical to assume that emulsions obtained with charged surfactants are highly stable due to the charge repulsions between the head groups of the surfactant molecules located in the interface of the emulsion droplets. Even though emulsions can benefit from the charge repulsions occurring between emulsion droplets, it has been previously demonstrated that non-ionic surfactants are also capable of stabilising emulsions although they are non-charged surfactants [1]. In general, depending on the emulsion ingredients and emulsification conditions, the emulsions can suffer from coalescence and even breakage. The so-called stabilisers or the presence of solid particles may increase the stability of emulsions [2]. When dealing with charged surfactants, the presence of counterions in the media can decrease the stability of the emulsions due to their electrostatic attraction with the charged emulsion droplets [3]. This may cause a decrease on the repulsions occurring between droplets and, in consequence, their coalescence and, at the end, phase separation phenomena. Thus, a

definitive formula for obtaining stable emulsions does not exist and an accurate study of each emulsion to be prepared has to be carried out to ensure both its stability and its final properties.

Emulsions can be prepared in several devices such as high-pressure homogenisers [4-6], ultrasonication devices [7, 8], rotor-stator systems [9, 10]. However, these methods both consume notable amounts of energy and the size distributions of the emulsion droplets are generally wide. Recently, several new methods that utilize various microfabricated structures have been developed for preparing monodisperse emulsions with low energy requirements. Microchannel [11], microfluidic devices [12, 13] and membrane emulsification [14], offer an alternative and versatile routes to produce emulsions.

Membrane emulsification has been shown to be very attractive for the production of high value compounds. One example is the formulation of capsules containing drugs for its controlled release [15-18]. The technique is based on the formation of emulsions by forcing the dispersed phase to permeate through a membrane and being collected into the contiuous phase drop-by-drop. There exist several membrane emulsification configurations depending on how the dispersed phase droplets detach from the membrane surface [19]. For instance, previous works have shown that stable emulsions can be successfully produced by crossflow membrane emulsification [15, 20-22] and dead-end stirred membrane emulsification [23-25]. In such equipments, membrane material, pore size, shear stress, and dispersed phase flux are some of the operating variables having a strong influence on the properties of the prepared emulsions. Obviously, the previous variables have to be added to the others influencing variables such as the surfactant percentage, the O/W percentage in the final emulsion, the density and viscosity of both phases, the composition of the continuous phase, etc.

This work aimed at the preparation of oil-in-water emulsions containing Fe(II) at their interface. SDS was used as anionic emulsifier. In this system, Fe(II) functioned as a counter ion of SDS and it could be adsorbed at the oil/water droplet interface. On the other hand, as previously mentioned, this may cause destabilization of the emulsion.

One of major interest of producing stable Fe(II) –containing emulsions could be to test them as catalyst for the partial oxidation of organic contaminants. The Fenton process has been typically used for this purpose. However, this process uses Fe(II) ions as homogeneous catalyst [26]. Thus, the catalyst continuously leaves the oxidation reactor together with the treated effluent often causing environmental problems [27] and increasing the treatment costs. The use of Fe(II) ions confined in emulsion droplets could avoid those problems. However, this work is not focused on the use of oil/water droplets as catalyst but to identify the

appropriate operating conditions for the preparation of stable oil/water droplets containing Fe(II) ions. This is considered to be the most important point for ensuring the adequate development of this new application. The effect of Fe(II) presence in the continuous phase or added after droplet formation, Fe(II) concentration, emulsifier concentration, disperse phase percentage and droplets size on emulsion stability were investigated. Emulsions were prepared in a stirred cell, by using flat metallic membranes, and by cross flow emulsification using tubular ceramic membranes. Membrane pore size from 20 down to 0.5 μm have been used. A relationship between droplets size and Fe(II) on emulsion stability was identified.

8.2. Materials and methods

8.2.1. Chemicals

Commercially available corn oil was used as dispersed phase of the prepared emulsions. SDS for molecular biology (Sigma, purity 99%) was selected as model anionic surfactant. Iron (II) sulphate heptahydrate (Sigma-Aldrich, purity 99%) was used as source of Fe(II) ions. The continuous phases was an aqueous solution made of ultrapure water (USF Elsa, model Purelab Classic PL5221) with a resistivity of 18.2 MΩ·cm, containing SDS, Fe(II) or both.

8.2.2. Membrane emulsification devices, membranes and operation

8.2.2.1. Stirred membrane emulsification equipment and experiments

The stirred emulsification cell was manufactured by Micropore Technologies Ltd. (Leicestershire, United Kingdom). The system consists in a PTFE base (named injection chamber) that hosts a flat membrane coupled with a dismountable threaded glass cylinder. Between the glass cylinder and the membrane, a PTFE joint is placed in order to prevent leaks during the emulsification. A stainless steal stirrer, with a blade at the bottom ending, a motor at the top and a PTFE vortex breaker fixed at the stirrer, is placed over the glass cylinder and regulated by a voltage regulator connected to the electricity. A detailed scheme of the stirred emulsification cell was published by Stillwell et al. [24]. The feeding system of the cell was modified from the original version, where instead of a gravimeter cylinder a peristaltic micropump (Ismatec, model C.P. 78016-30) was connected to the injection chamber to permeate the dispersed phase at finely controlled flow rate.

A typical emulsification experiment started preparing the continuous phase by dissolving the appropriate amount of surfactant into ultrapure water and, depending on the experiment, also iron sulphate. A graduated glass cylinder was subsequently filled with the liquid to be dispersed. Then, both pump impulsion and aspiration tubes, as well as the injection chamber, were filled with the liquid to be dispersed. Then, the membrane was placed inside the injection chamber, below the joint, and the glass cylinder was threaded. At that moment, 90 mL of continuous phase were added to the cylinder and the stirrer was placed at its correct position. The voltage regulator was set at the desired voltage position and the pump was subsequently switched on. The pump was always operated so that the oil permeate flow rate through the membrane was 0.15 mL/min. The decrease of the level in the graduated cylinder of the liquid to be dispersed was controlled throughout the experiment to assure that the permeate flux was constant. When the desired volume of the liquid to be dispersed had permeated, the pump and the stirrer were switched off and the emulsion was poured out into a glass beaker. After this, the system was dismounted and submitted to cleaning with soap and water.

Two hydrophilic metallic membranes were used in the stirred emulsification experiments, both purchased at Micropore Technologies Ltd. The pore sizes of the membranes were 10 and 20 μm. The membranes were also cleaned with soap and water after being used and were maintained submerged in ultrapure water until their next use.

8.2.2.2. Crossflow membrane emulsification equipment and experiments

Emulsification experiments in crossflow mode were carried out in a homemade lab-scale emulsification plant, schematised in Fig. 8.1. The phase to be dispersed was contained in a pressurised nitrogen vessel. The transmembrane pressure was fixed by regulating two backpressure valves located between the gas cylinder and the stainless steel membrane module. This way, the permeate flux of oil was perfectly controlled and kept constant during the experiments. Two valves that allowed the depressurisation of the system were installed before the oil vessel and at the shell of the module. An Amicon (model 54113#1728, Beverly, USA) peristaltic pump was used to work at the desired continuous phase tangential velocity by selecting the adequate flow rate in the pump regulator. A 100-mL glass beaker was used as continuous phase container during the experiments. Finally, several manometers were installed to control and monitor the pressure during the emulsification tests.

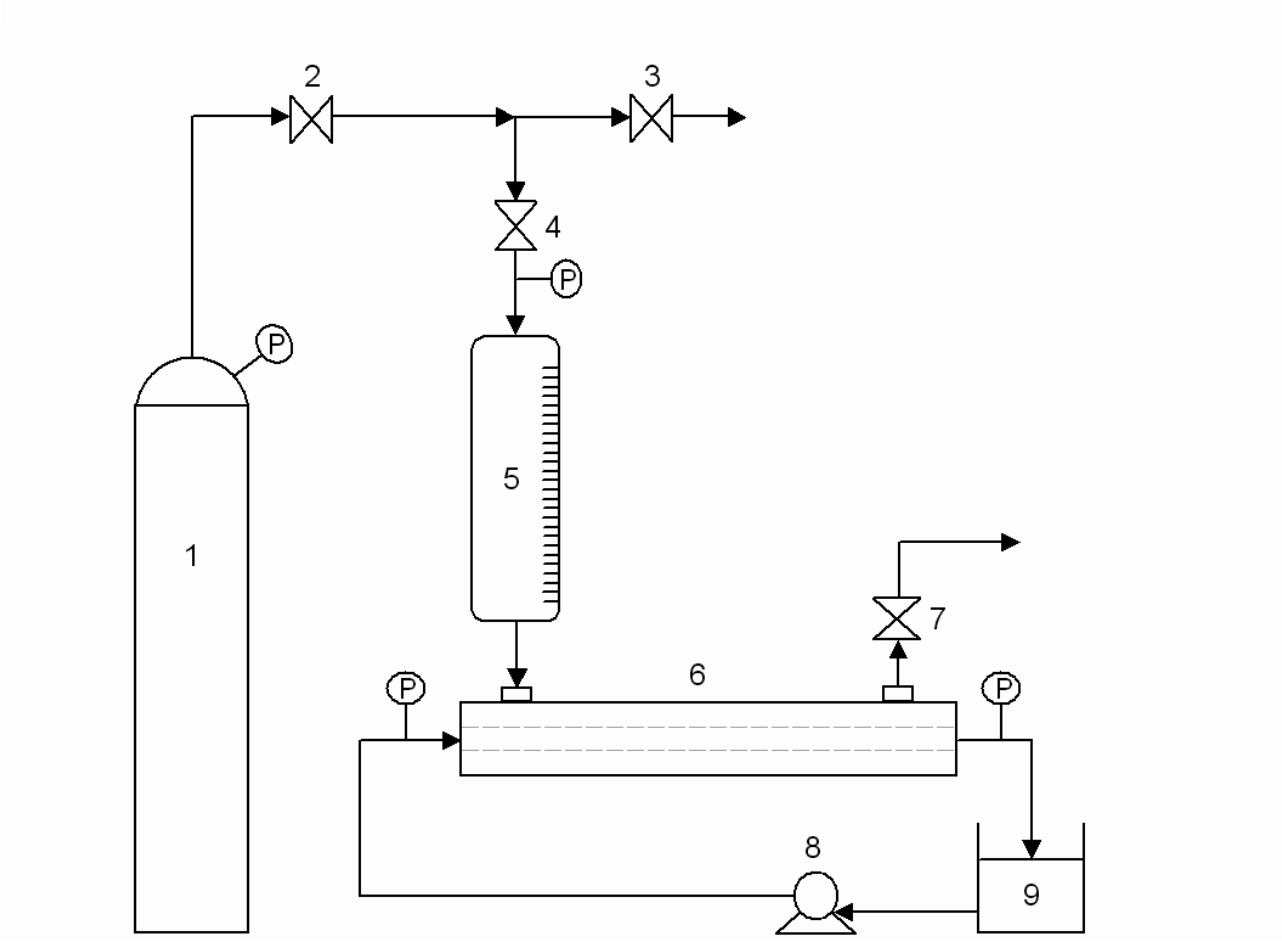

Fig. 8.1. Crossflow membrane emulsification equipment scheme.
1: nitrogen gas cylinder; 2: backpressure valve; 3: purge valve; 4: backpressure valve; 5: oil vessel; 6: membrane module; 7: purge valve; 8: peristaltic pump; 9: continuous phase container.

The membrane tested with this system was a tubular hydrophilic ceramic membrane with a pore size of 0.5 μm (manufactured by Membraflow, Germany). The membrane active layer was made of zirconium oxide and the total length of the membrane was 100 mm. The internal and external diameter of the membrane was 7 and 10 mm, respectively.

A crossflow membrane emulsification experiment started by filling with oil the dispersed phase vessel, the tubes connecting the vessel and the membrane module and also the shell of the module. The vessel containing the continuous phase was filled with 90 mL of ultrapure water and the pump was switched at the desired flow rate for performing the emulsification experiment. The continuous phase was always re-cycled inside the lumen of the membrane. Once all the air was purged out from the module, a nitrogen pressure of 5 bar was fixed and the dispersed phase was allowed to permeate during one hour. After this, the dispersed phase circuit was depressurised, the water that had been circulating was purged out and a solution containing the ingredients desired for the emulsification experiment was circulated for five minutes in order to remove some oil that could have remained in the continuous phase circuit. 90 mL of continuous phase, prepared as in the stirred cell experiments, was then replaced to the continuous phase vessel and kept circulating at the same flow rate than when water was circulated. Then, the dispersed phase circuit was pressurised at 1 bar and, at that moment, the emulsification process started. Once the desired volume of oil had permeated, the dispersed phase circuit was depressurised to stop the oil permeation and the obtained emulsion was collected from the circuit and continuous phase vessel and kept in a glass beaker for further characterisation and/or iron addition experiments.

8.2.2.3. Preparation of Fe(II)-containing emulsions

The presence of Fe(II) in the continuous phase before the emulsification experiments started was done by dissolving a known amount of Fe(II) and SDS in ultrapure water, as abovementioned. Then, this solution was used as continuous phase and the experiment operation was as when no Fe(II) was present.

When the effect of the Fe(II) presence and concentration on the emulsion properties of previously-prepared emulsions wanted to be explored, emulsions without Fe(II) in the initial continuous phase were obtained, following the protocols described in the previous sections. Then, the obtained emulsions were left to settle overnight in separating funnels and the separated continuous phase was replaced by an Fe(II)-containing aqueous solution of known concentration. The new emulsion system was gently mixed at 50 rpm at room temperature (20

±1ºC). At this point, the new emulsion was subjected to the same analysis than those prepared with an Fe(II)-containing continuous phase. Control experiments were also carried out in which the separated continuous phase was replaced by pure water.

8.2.3. Emulsion characterisation techniques

Emulsions obtained in the stirred and crossflow membrane emulsification devices were characterised with the same techniques and following the same sampling and analysis protocol. After the emulsification experiments, emulsions were preserved unstirred in closed glass beakers at room temperature (20±1 ºC). Given the size of the droplets, the emulsions suffered from creaming. To obtain an homogenous sample from emulsions that were analysed, they were gently stirred (50 rpm) before the characterisation analyses were performed. The emulsion sample was withdrawn from the centre of the beaker. A pipette was immersed, the liquid entered in the tip during immersion was purged off and then the sample was saked. Any eventually separated oil was carefully eliminated from the sample and its mass and volume were measured. Five different samples were collected for each measure to evaluate the analysis reproducibility. After the sample withdrawal, the emulsions were left again without stirring.

The mass of oil-water emulsion samples and of oil present in the samples were measured by means of a moisture analyser (Ohaus, model MB45) composed by a precision balance and a dryer unit. The instrument operates on the thermo gravimetric principle: the oil percentage is determined from the weight of sample dried by heating at 100°C. The percentage of oil-in-water (%O/W) can be determined from the initial and final (after evaporation) sample weight measurements. The analysis was carried out immediately after the emulsification and the oil in water percentage evaluated with this procedure was compared with the one calculated from the the oil permeated during the emulsification experiment. For certain emulsification experiments, these measurements were repeated along one week to monitor the eventual changes of the %O/W as a function of time, caused by droplet breakage and oil separation from the emulsion. As explained before, as separated oil was not collected during the sampling, the resulting %O/W only refers to the emulsified oil-in-water percentage. Therefore, a decrease of %O/W was an indicator of the emulsion breakage.

The droplet size distribution of the emulsion was obtained in a Malvern Mastersizer analyser (Malvern Instruments, model 2000), a laser light scattering analyser, equipped with a liquid sampling unit (Malvern Instruments, model Hydro 2000 MU). Reported data are averages of ten readings of at least three samples from at least two separate experiments. The analyser

was used to obtain the droplet size distribution curve, the surface weighted mean diameter (or Sauter diameter), represented by D[3,2], the volume weighted mean diameter, represented by D[4,3] (or De Brouckere diameter), and the value of the Span (indicator of the width of the distribution curve). D[3,2] and D[4,3] were determined, respectively, as follows:

$$D[3,2] = \frac{\sum D_i^3 n_i}{\sum D_i^2 n_i} \quad (1)$$

$$D[4,3] = \frac{\sum D_i^4 n_i}{\sum D_i^3 n_i} \quad (2)$$

where D_i = particle diameter of class i and n_i = number of particle in class i

Span number is calculated by the following expression:

$$Span = \frac{D[0.9] - D[0.1]}{D[0.5]} \quad (3)$$

Where D[0.5] is the diameter in microns at which 50% of sample is smaller and 50% is larger; D[0.1] and D[0.9] are the diameters below which 10% and 90% of the sample lie, respectively.

The droplet size distribution analysis was performed immediately after preparing the emulsions and followed during one week. The changes of the D[3,2], D[4,3] and Span are therefore an indicator of the changes on the properties of the prepared emulsions. D[4,3] value is more sensitive to the presence of larger droplets than D[3,2] value, and therefore it gives an indication of droplet coalescence. Emulsion stability index (ESI) was expressed as the ratio between the emulsion stored data (including D[3,2], D[4,3] and Span) and the initial emulsion data. Combining ESI value with %O/W measurements for stored emulsions it is possible to understand the occurred destabilization process. In particular, ESI = 1 and constant %O/W indicate that emulsion is stable. ESI>1 and decrease in the %O/W indicate emulsion coalescence and droplets breakage. ESI<1 and decrease in the %O/W indicate emulsion breakage, i.e. larger droplets coalesce and then breaks causing oil separation. In this last case, as the larger droplets disappear the analysis catches mainly the smaller stable droplets.

Optical microscopy characterisation of the prepared emulsions was also carried out in some of the experiments to compare the visual observations of the droplet size distribution of the emulsions with those obtained using the light scattering analyser. The optical microscope (Zeiss, model Axiovert 25) was equipped with a camera (JVC, model TK-C1481BEG) to capture the images of the emulsions, which were then processed by scion image software.

The Fe(II) content was obtained in an atomic absorption spectrometer (Hitachi, model Z-8200). Emulsions were filtered through Nylon membrane disks of 0.2 μm pore size and 47 mm diameter (Whatman International) prior to Fe(II) analysis. A vacuum pump (Edwards, model D1) was used to filter the emulsion samples. The difference between the initial iron concentration in the continuous phase (original or exchanged) and the final iron concentration allowed the determination of the Fe(II) linked to the emulsion droplets.

8.3. Results

This section is structured in two main parts. The first deals with the exploration of the effect of emulsification parameters to obtain emulsions having a high degree of uniformity and stability. These emulsions were prepared only with corn oil as dispersed phase and SDS solution as continuous phase in the stirred emulsification cell. These emulsions were then used as a base reference to identify the effect of the presence of Fe(II).

The second part includes the effect of the presence (and concentration) of Fe(II) dissolved in the continuous phase used for preparing emulsions (W_o) at the same conditions than when prepared without Fe(II). In this section, it is also presented the study of the effect of the Fe(II) addition after the emulsification process, therefore, after exchanging the continuous phase of a formulated emulsion by a new Fe(II)-containing aqueous phase (W_n). The emulsions used in this part were prepared by the two membrane emulsification devices employed in this work.

8.3.1. Formulation of O/W emulsions

8.3.1.1. Effect of the stirring rate and membrane pore size on the emulsion properties

The study of the effect of the stirring rate on the properties of the prepared emulsions has been carried out with 2% SDS aqueous solution as W_o. The %O/W was 10% in all experiments and the tested stirring rates varied from 583 to 2333 rpm. The two metallic membranes (10 and 20 μm) were used to test the effect of the membrane pore size on the final characteristics and stability of the obtained emulsions.

Figure 8.2 shows the effect of the stirring rate on the mean particle diameter.

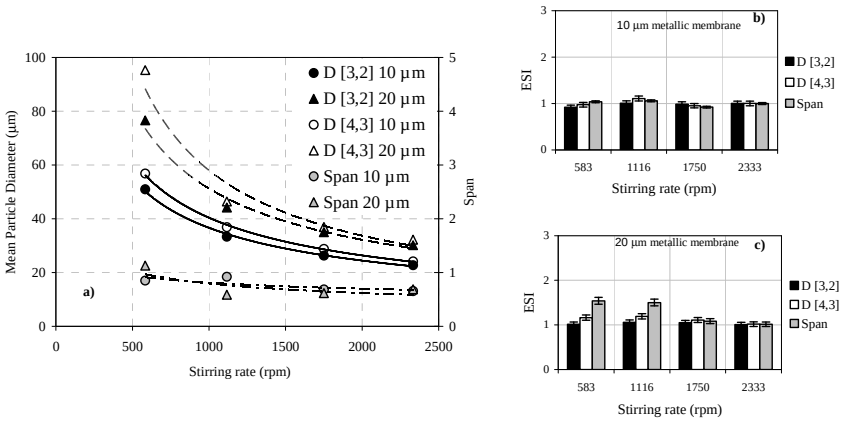

Fig. 8.2. a) Effect of the stirring rate and membrane pore size on the D[3,2], D[4,3] and Span. Influence of stirring rate on the emulsion stability index (ESI) of stored emulsion prepared using b) 10 µm metallic membrane and c) 20 µm metallic membrane. (10% O/W; 2%SDS in W_o).

In particular, Fig. 8.2a illustrates the D[3,2], D[4,3] and Span of the just prepared emulsions obtained with the stirred cell using the two metallic membranes. As it can be seen, for both membrane pore size, the higher the stirring rate, the lower the mean particle diameters. This can be explained by a higher shear stress at the vicinities of the membrane, causing a higher degree of detachment of the oil droplets per time unit. The span is slightly higher at lower stirring rate. The emulsion stability was evaluated by comparing these data with the ones obtained after 7 days. Fig. 8.2b and 8.2c show the ESI for 10 µm and 20 µm metallic membranes, respectively. Results show that emulsions prepared at stirring rate higher than 1750 rpm are very stable regardless to the type of membrane. For lower stirring rate, the value of ESI>1 indicates that emulsions coalesce. The effect is more severe for 20 µm membrane pore size. However, no phase separation occurred in the observed time. Finally, 1750 rpm of stirring rate and the 10 µm metallic membrane were selected for carrying out all the experiments presented in the following sections.

8.3.1.2. Effect of the SDS concentration on the emulsion properties

This section shows the emulsion features as a function of the SDS weight percentage (%SDS) in the W_o. The %SDS varied from 0.2 to 2%. 10 %O/W emulsions were prepared using the 10 µm metallic membrane in the stirred cell at 1750 rpm of stirring rate. Fig. 8.3 shows the effect of the %SDS on the D[3,2], D[4,3] and Span of the formulated emulsions.

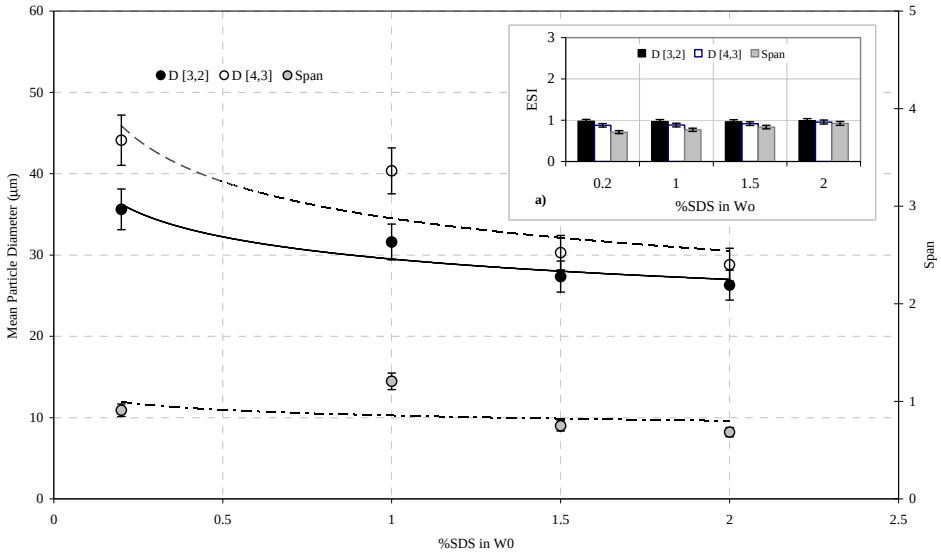

Fig. 8.3. Effect of the %SDS in W_0 on the D[3,2], D[4,3] and Span; a) Influence of %SDS on the emulsion stability index (ESI) of stored emulsion (10% O/W; 10 µm metallic membrane; 1750 rpm)

As it can be seen, the mean particle diameters decrease with increasing of emulsifier concentration. In particular, the major change is obtained between 0.2 and 1.5 % of SDS while between 1.5 and 2% the particle properties are similar. This behaviour is due to the decrease of interfacial tension with increasing of emulsifier concentration [14]. In fact, the emulsion droplet formation and detachment is governed by the balance between the interfacial tension force and the shear force exerted by the continuous phase. Since in these experiments, shear force was constant, droplet size distribution changed due to the interfacial tension decrease. This effect is more significant at 0.2%. In this case, separation of oil phase after emulsion preparation was also observed. The behaviour is mainly due to the fact that a SDS concentration lower than its critical micellar concentration (0.24% w/w) was used [28, 14]. As shown in Fig. 8.3a the emulsion prepared by using 2% SDS was the most stable compared the others.

8.3.1.3. Effect of the %O/W on the emulsion properties

2% SDS in W_o was employed to prepare O/W emulsions at different %O/W in the stirred cell at 1750 rpm. Fig. 8.4 shows the D[3,2], D[4,3] and Span of the prepared emulsions as a function of the %O/W.

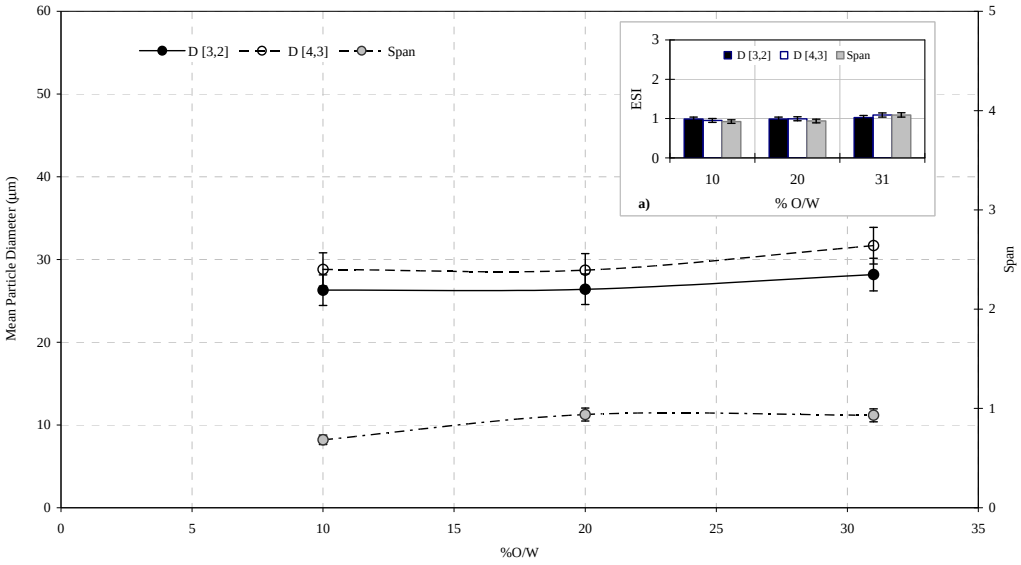

Fig. 8.4. Effect of the %O/W on the D[3,2], D[4,3] and Span; a) Influence of %O/W on the emulsion stability index (ESI) of stored emulsion (2% SDS in W_o; 10 µm metallic membrane; 1750 rpm)

As it can be seen, an increase on the %O/W from 10 to 20% does not significantly affect the D[3,2] and D[4,3] of the obtained emulsions while they slightly increase for the 31% O/W emulsion. Contrarily, the Span increases when passing from 10 to 20% remaining constant afterwards. Less SDS is available per oil volume unit at higher %O/W and, consequently, larger droplets are produced, which agrees with literature data [29]. Even though higher Span values are observed at high %O/W, none of the emulsions were broken during one week of observation.

8.3.2. Formulation of ferrous O/W emulsions

Two operating modes have been explored to investigate the effect of the presence of Fe(II) in the final properties of O/W emulsions. The first methodology contemplates the addition of Fe(II) in the W_o together with 2% SDS. Besides, the second one consists on exchanging the separated aqueous phase of a previously prepared emulsion for a new Fe(II)-containing aqueous solution (W_n).

8.3.2.1. Formulation of emulsions with Fe(II)-containing W_o

The emulsions presented in this section have been prepared with the stirred cell emulsification set-up at a constant stirring rate of 1750 rpm and employing the 10 μm metallic membrane. The %SDS was 2% and the %O/W 10%, unless otherwise noted. Thus, the only variable changed during the experiments was the Fe(II) concentration ([Fe(II)]) in W_o.

Figure 8.5 shows the behaviour of D[3,2], D[4,3] and Span as a function of time for emulsion prepared with different [Fe(II)]. Figure 5 also includes the properties of the emulsion prepared without Fe(II) in the W_o.

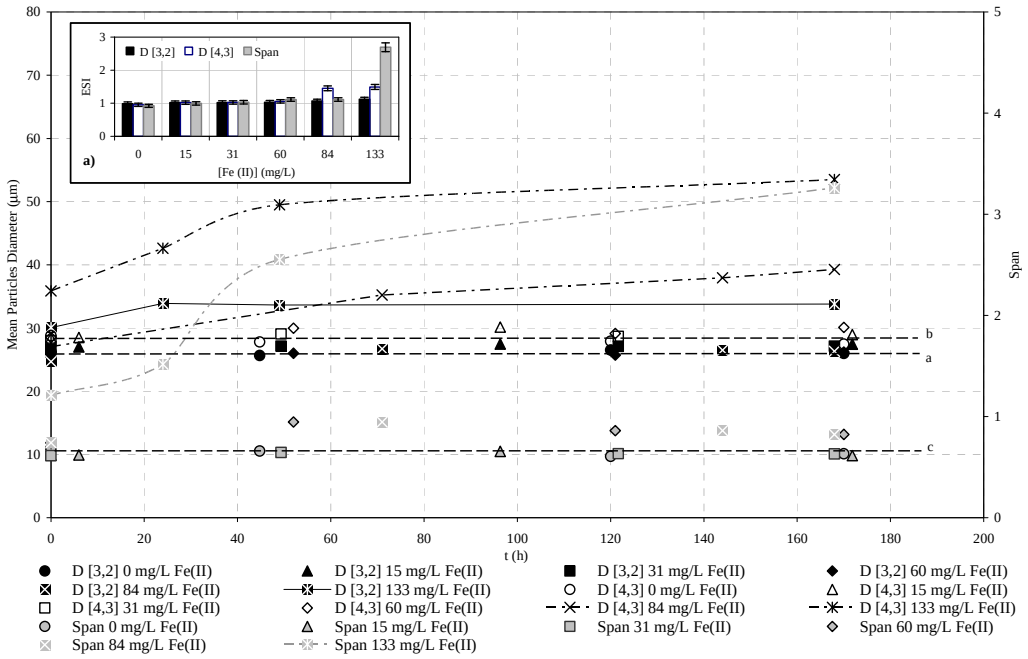

Fig. 8.5. Effect of the [Fe(II)] in W_o on the D[3,2], D[4,3] and Span. Dashed lines represent: *a* D[3,2], *b* D[4,3] and *c* Span of the virgin emulsion; a) Influence of the [Fe(II)] in W_o on the emulsion stability index (ESI) of stored emulsion (10% O/W; 2% SDS in W_o; 10 μm metallic membrane; 1750 rpm)

Fig. 8.5a illustrates the ESI values and Table 1 summarises the actual %O/W for one-week-aged emulsions as well as the final [Fe(II)] in the continuous phase of the prepared emulsions.

Table 8.1. Remaining %O/W and final [Fe(II)] in W after emulsification. 10% O/W ; 2% SDS; 10 μm metallic membrane; 1750 rpm.

[Fe(II)] in W_o (mg/L)	Emulsion breakage	%O/W after seven days	Remaining [Fe(II)] in W (mg/L)
0	No	10	0
15	No	10	12
31	No	10	28
60	7^{th} day	7.1	NA
84	6^{th} day	3.2	NA
133	1^{st} day	1.4	NA

NA: not analysed due to emulsion destabilisation.

The emulsion prepared with a W_o containing the highest [Fe(II)], this is 133 mg/L, broke just in few hours after its formulation. After one week, the real %O/W of the remaining emulsion was only 1.4%, as shown in Table 8.1. Fig. 8.5 shows that, at this [Fe(II)] in W_o, D[3,2] increased from 30.1 to 33.8 μm and D[4,3] increased from 35.87 to 53.5 in one week. Similarly, Span increased from 1.2 to 3.3. These parameters were 26.1 μm and 0.65, respectively, when no Fe(II) was present in the W_o. This can be explained by the charge interactions between Fe(II) cations and the negatively-charged emulsion droplets, made of SDS, which is an anionic surfactant. As shown by Mei et al. using zeta potential measurements, Fe(II) and Fe(III) ions are associated with emulsion droplets prepared with SDS as emulsifier due to electrostatic attractions between iron ions and the charged interfaces of the emulsion droplets and this phenomena determine a decrease of zeta potential [30]. Similarly, other works have dealt with the association of calcium ions with emulsion droplets, causing destabilisation phenomena [3, 31].

When 84 mg/L Fe(II) and 60 mg/L Fe(II) were present in W_o, the resultant emulsions were also broken six and seven days after the emulsion formulation, respectively. The remaining %O/W were 3.2 and 7.1% when 84 and 60 mg/L Fe(II) were present in Wo, as shown in Table 8.1. In both emulsions, D[3,2] remained unchanged along the time and very close to the D[3,2] of the emulsion without Fe(II) in the W_o. Nonetheless, Span and D[4,3] slightly

increased along the time, being the indicator of the emulsion destabilisation few days after the emulsion formulation. ESI value are in agreement with this behaviour.

For 31 and 15 mg/L Fe(II), the emulsions remained stable and their droplet size distributions unchanged along one week. As Table 8.1 shows, the %O/W of both emulsions was constant and equal to 10% and the [Fe(II)] in the prepared emulsions after one week were 28 and 12 mg/L, respectively. Thus, even though Fe(II) destabilises the emulsions, it is possible to obtain stable emulsions in presence of Fe(II) below 31 mg/L. Taking into account the differences in the [Fe(II)] before and after the emulsification, summarised in Table 8.1, around 27 mg Fe(II) per litre of emulsified oil was linked to the emulsion droplets or located at their interfaces.

The effect of the presence of Fe(II) in W_o was also studied at several %O/W. The [Fe(II)] in W_o was 15 mg/L and the rest of the emulsification variables remained unchanged and equal to those used for performing the previous experiments. Thus, 2% SDS was also in the W_o together with Fe(II), the stirred emulsification cell was selected for preparing the emulsions at 1750 rpm of stirring rate and the 10 µm metallic membrane was used. The D[3,2], D[4,3] and Span of the prepared emulsions are shown in Fig. 8.6.

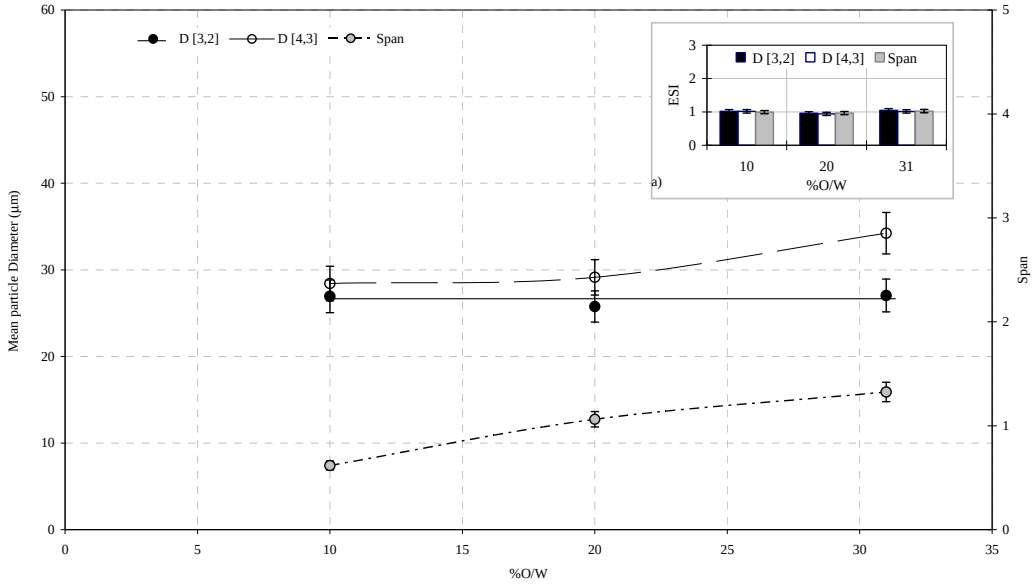

Fig. 8.6. Effect of the %O/W on the D[3,2], D[4,3] and Span of emulsions prepared with Fe(II). (10% 0/W ; 2% SDS and 15 mg/L in W_o; 10 µm metallic membrane; 1750 rpm).

The behaviour is similar to the one related to experiments carried out without iron in the continuous phase (see Fig. 8.4). However, in the present case the change of D[4,3] and Span is more evident. Fig. 8.6a also reports the ESI after one week. Neither D[3,2], D[4,3] nor Span suffered significant changes along one-week observation but when 31% emulsion was produced, three days after the emulsion formulation, free oil was visually observed. The remaining %O/W one week after the emulsion production can be seen in Table 8.2.

Table 8.2. Remaining %O/W and final [Fe(II)] in W after emulsification.
2% SDS; 15 mg/L Fe(II); 10 μm metallic membrane; 1750 rpm.

%O/W	[Fe(II)] in W_o (mg/L)	Emulsion breakage?	%O/W after seven days	Remaining [Fe(II)] in W (mg/L)
10	15	No	10	12
20	15	No	20	9.3
31	15	3rd day	18	NA

NA: not analysed due to emulsion destabilisation.

As the droplets of the 31% O/W emulsion were originally larger, they were more sensitive to be destabilised by coalescence and, finally, breakage. As also shown in this table, the remaining %O/W one week after the emulsion preparation was 18%. Therefore, even though ESI = 1 the emulsion was not stable. On the other hand, as it can be seen, neither 10% nor 20% O/W emulsions containing 15 mg/L Fe(II) in the W_o were destabilised and their properties remained unaltered (ESI = 1 and %O/W constant) at least during the observation period. Regarding the remaining [Fe(II)] in the emulsion continuous phase, Table 8.2 shows that, for the 10% and 20% O/W emulsions, 12 and 9.3 mg/L Fe(II) appear in the continuous phase. As shown before, for 10% O/W emulsion, this represents a presence of 27 mg Fe(II) per litre of emulsified oil. For 20% O/W emulsion, this ratio decreased to 26 mg Fe(II) per litre of emulsified oil. Thus, the decrease on the mass of Fe(II) linked to the emulsion per volume unit is not significantly affected by the %O/W and can be assumed to be between 26 and 27 mg/L.

8.3.2.2. Addition of Fe(II) to previously-prepared O/W emulsions

The effect of the presence of Fe(II) and its concentration on the stability changes and properties of previously-produced O/W emulsions is discussed in this section. In the

experiments, the emulsions were prepared without Fe(II) in the W_o. After this, the emulsions were left to separate and the continuous phase was replaced by an Fe(II)-containing (W_n) aqueous solution. The difference between this type of experiments and those carried out with Fe(II) in W_o was that, replacing the continuous phase by W_n, practically no SDS was free in the continuous phase and the Fe(II) could not be attracted by free SDS molecules, which are also negatively charged. Hence, the effect of the presence of free SDS molecules (or micelles) is also considered in this section. In addition, in this part of the study emulsions were obtained by crossflow membrane emulsification with a 0.5 μm ceramic membrane. This way, the effect of the [Fe(II)] in W_n is also examined with emulsions having smaller mean droplet diameter, obtained using a membrane with smaller pores in crossflow mode. In addition, the replacement of the emulsions' continuous phases was also examined when no Fe(II) was present in the new continuous phase (W_n). For this, the original continuous phases of the prepared emulsions were removed and replaced by ultrapure water. This way, the effect of the intrinsic instability, which might arise from the elimination of the emulsifier from the continuous phases, was also studied. The blank experiment allowed the discussion of the destabilisation phenomena occurring when W_o are replaced by Fe(II)-containing W_n. The emulsions prepared had a %O/W equal to 10% and the %SDS was 2% in W_o. The emulsions prepared in the stirred cell were done at 1750 rpm of stirring rate and those obtained with the crossflow membrane emulsification set-up were done at 760 mL/min of continuous phase circulation flowrate. This flowrate corresponds to a tangential velocity of the continuous phase in the membrane lumen of 0.33 m/s.

First, the discussion of the emulsions prepared in the stirred cell is presented. Fig. 8.7 shows the D[3,2], D[4,3] and Span evolution along the time at several [Fe(II)] in W_n and of the virgin emulsion, therefore, when no continuous phase replacement was performed.

As it can also be seen, when the W_n did not contain Fe(II) (blank experiment) or contained 0.44 mg/L Fe(II), neither D[3,2] and D[4,3] nor Span differed from the values of the virgin emulsion. However, when 5.1 and 15 mg/L Fe(II) were present in W_n, D[3,2] and D[4.3] decreased during the first hours after the continuous phase exchange. Span values remained practically unchanged after the substitution of the continuous phase by an Fe(II)-containing W_n, regardless the [Fe(II)] in W_n.

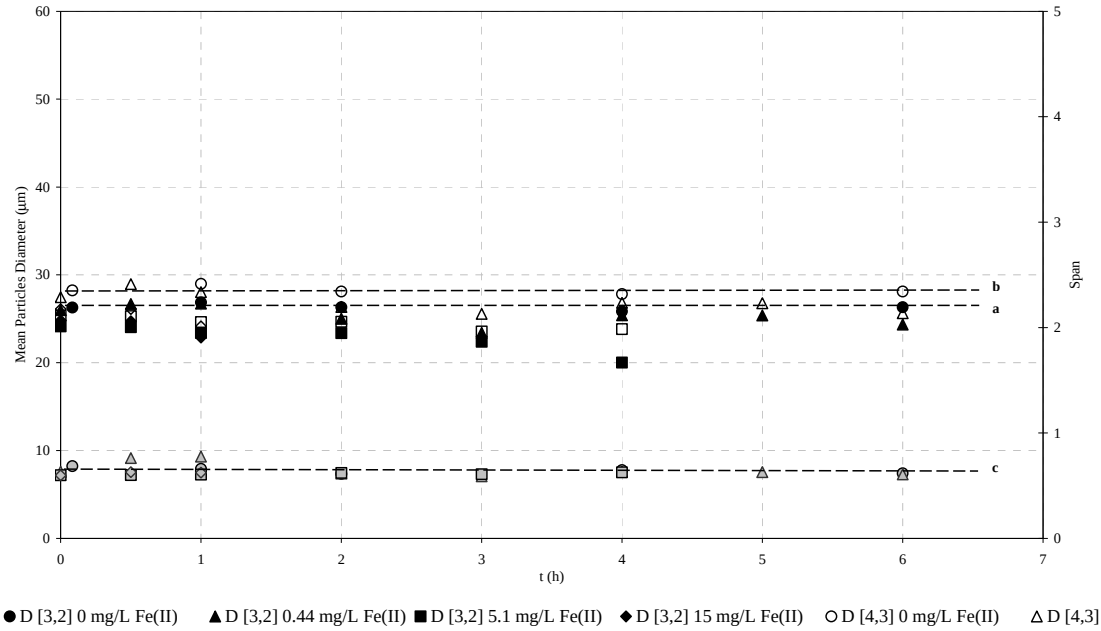

Fig. 8.7. Effect of the [Fe(II)] in W_n on the D[3,2], D[4,3] and Span. (10% 0/W ; 2% SDS in W_o; 10 µm metallic membrane; 1750 rpm). Dashed lines represent *a* D[3,2], *b* D[4,3] and *c* Span of the virgin emulsion.

Besides, Fig. 8.8 shows the evolution of %O/W along the time as an indicator of the emulsion breakage due to the presence of Fe(II) in W_n.

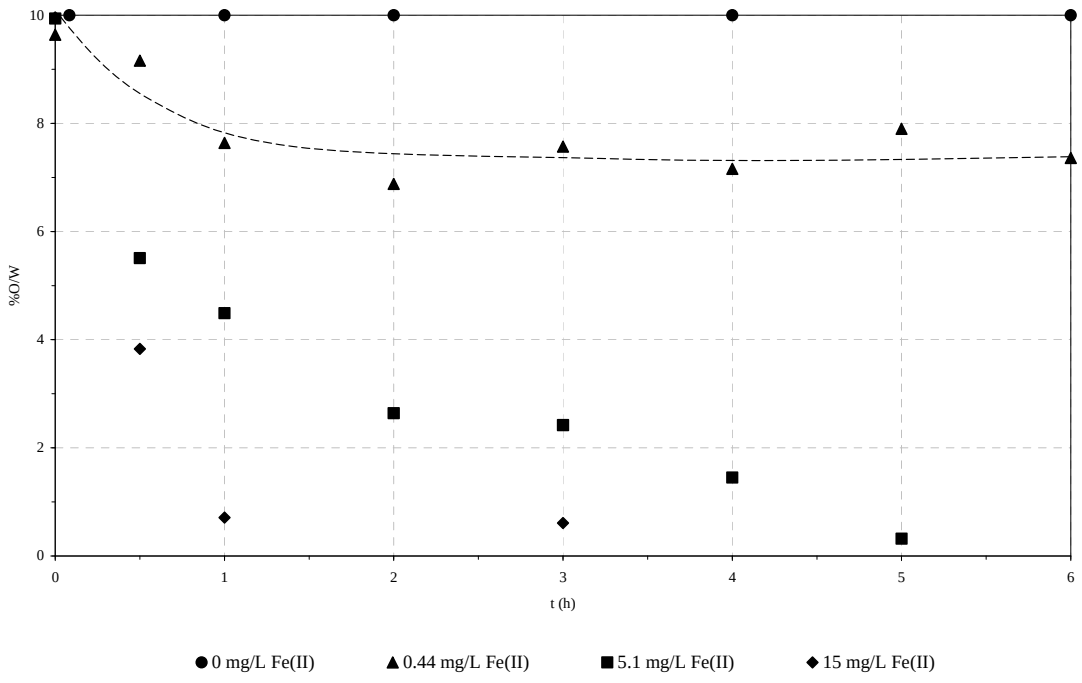

Fig. 8.8. Effect of the [Fe(II)] in W_n on the remaining % O/W (10% O/W ; 2% SDS in W_o 10 µm metallic membrane; 1750 rpm).

It can be noticed that, when no Fe(II) was present in the W_n (blank experiment), no emulsion destabilisation occurred and the emulsion behaved as the virgin one. In addition, regardless the [Fe(II)] in W_n, %O/W reduction occurred and, therefore, emulsion breakage was observed during the first hours after the continuous phase exchange. These results are in agreement with the fact that Fe(II) cations are linked to the emulsion droplets causing an increase on the instability of the emulsions due to the decrease on the repulsion forces between the emulsion droplets, as explained in the previous section. Fig. 8.8 demonstrated that, as the [Fe(II)] in W_n increases, emulsion breakage occurs earlier. For instance, at 0.44 mg/L Fe(II) in W_n, the %O/W decreased down to 7.5% during the first six hours after the continuous phase exchange. After one week, the %O/W of this emulsion was 5.9%. Nevertheless, at 5.1 and 15 mg/L Fe(II) in W_n, total breakage of the emulsion occurred five and one hour after the continuous phase exchange, respectively. Thus, [Fe(II)] in W_n strongly affects the stability of the previously-formulated O/W emulsions. Comparing the previous results with those of the stability of O/W emulsions produced with Fe(II)-containing W_o, at the same [Fe(II)], the latter were more stable than the former. This could be explained by the fact that SDS molecules (or micelles) were present in the continuous phase when Fe(II) was present before the emulsification process in W_o and could also attract Fe(II) cations. However, when Fe(II) was present in W_n, where no free SDS molecules were present, Fe(II) cations were more available to be attracted by the emulsion droplets and, in consequence, higher emulsion instability can be expected in this case.

Emulsions obtained with the crossflow membrane emulsification device, equipped with a 0.5 μm ceramic membrane were also prepared (without Fe(II) in W_o) and subjected to exchange of their continuous phases by Fe(II)-containing aqueous solutions. This way, the [Fe(II)] in W_n effect on the stability of the emulsions could also be studied in emulsions having smaller mean droplet diameters. The original emulsions obtained in crossflow mode had a D[3,2] of 5.8 μm, D[4.3] of 7.9 μm and a Span of 1.6. The obtained emulsions remained stable during one week and the previous parameters unchanged during this period. The same [Fe(II)] in W_n than with the emulsions obtained in the stirred emulsification cell, already presented before, were tested, this is, 0.44, 5.1 and 15 mg/L Fe(II). The evolution of D[3,2], D[4.3], Span and %O/W were followed along the time to characterise the changes in the emulsion characteristics and stability. Fig. 8.9 shows that the replacement of W_o by a W_n without Fe(II) (blank experiment) did not cause any change on the mean particle diameter and Span compared to the virgin emulsion.

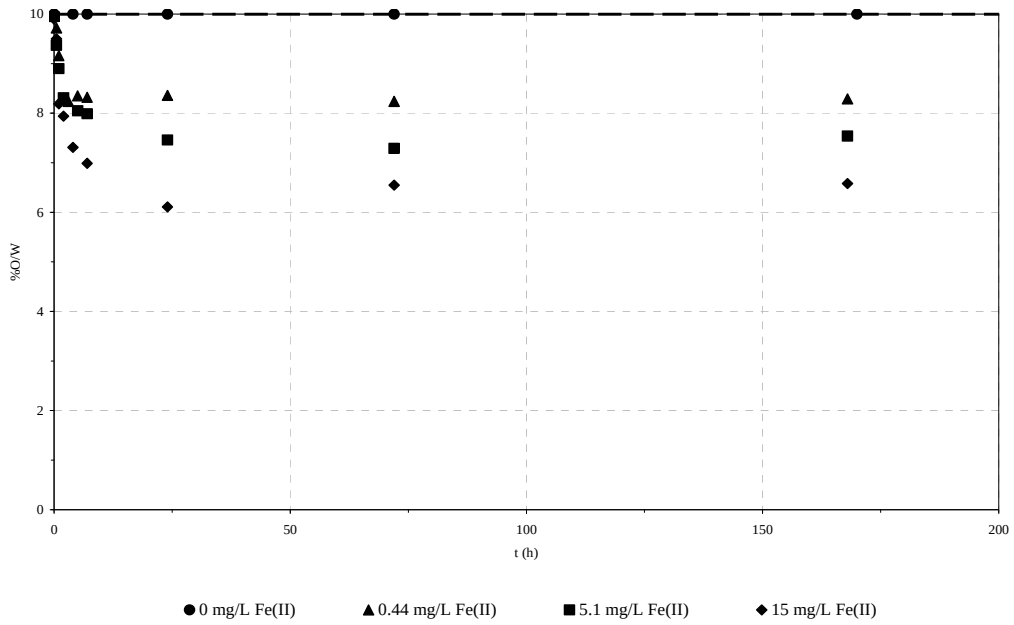

Fig. 8.9. Effect of the [Fe(II)] in W_n on the D[3,2], D[4,3] and Span (10% 0/W; 2% SDS in W_o, 0.5 μm ceramic membrane). Dashed lines represent *a* D[3,2], *b* D[4,3] and *c* Span of the virgin emulsion.

Thus, the removal of SDS from the W_o (and its replacement by ultrapure water) did not cause destabilisation on the emulsions prepared. This was not observed in the other emulsions after the replacement of their W_o by Fe(II)-containing W_n, which indicates that destabilisation phenomena occur when Fe(II) is present. Fig. 8.10 shows the %O/W evolution at the three tested [Fe(II)] in W_n together with the virgin (represented by a dashed line) and blank emulsion.

Fig. 8.10 demonstrates that the emulsions, after the exchange of their original continuous phases by Fe(II)-containing ones suffered from destabilisation, regardless the [Fe(II)]. The remaining %O/W one week after the continuous phase replacement was 6.6, 7.5 and 8.3% when 15, 5.1 and 0.44 mg/L Fe(II) aqueous solutions were employed as W_n. The possible mechanism causing destabilisation may be, as above mentioned, the lower repulsion forces due to the decrease on the net charge of the interfaces of the droplets when Fe(II) is present.

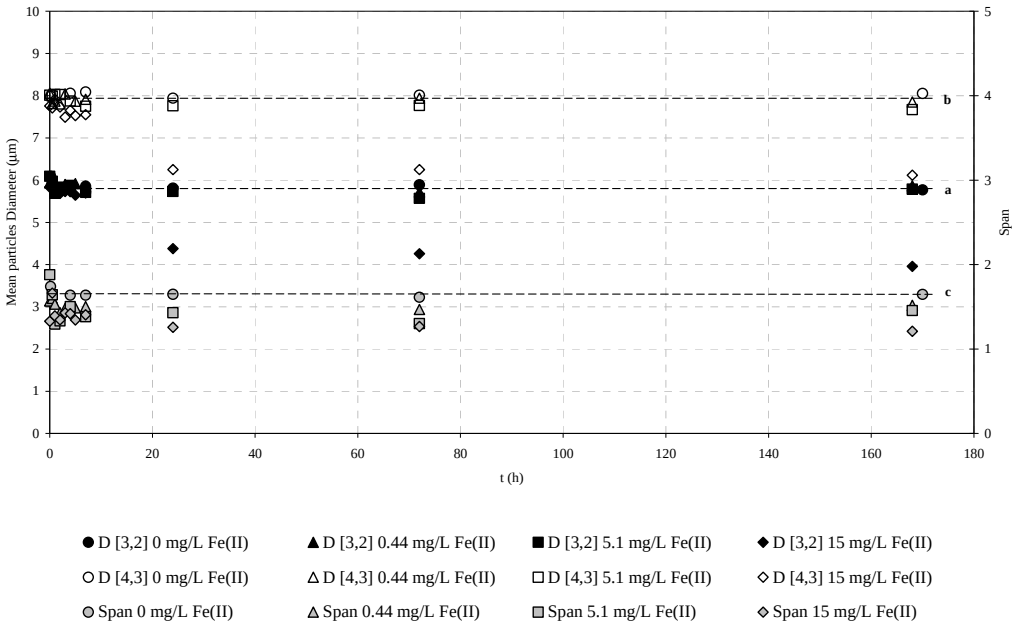

Figure 8.10. Effect of the [Fe(II)] in W_n on the remaining % O/W. (10% O/W ; 2% SDS in W_o 0.5 µm ceramic membrane).

Fig. 8.11 shows the optical micrographs of both the virgin emulsion obtained in crossflow mode before exchanging the continuous phase (Fig. 8.11A) by a 15 mg/L W_n and after the exchange (Fig. 8.11B).

It is clearly shown that, when Fe(II) is present in the emulsified system, droplets are agglomerated due to the lower charge repulsion between droplets, which can be the starting point for coalescence and result, at the end, in emulsion rupture. Instead, when no Fe(II) was present, agglomeration between droplets is not visually observed. Thus, the observations by optical microscopy agree with the decrease on the %O/W along the time due to emulsion breakage. This also supports the proposed destabilisation mechanism based on the decrease on the droplets repulsions when Fe(II) is present due to its electrostatic attraction towards the emulsion droplets.

Comparing the response of the larger-droplet-size emulsions with that of the smaller ones, it can be deducted that, the former are more negatively-affected by Fe(II) ions and are easily broken at the same [Fe(II)] in W_n that the latter. Thus, not only the [Fe(II)] in W_n is an important parameter affecting the emulsion droplet stability but also the original droplet size of the emulsions plays a crucial role on it.

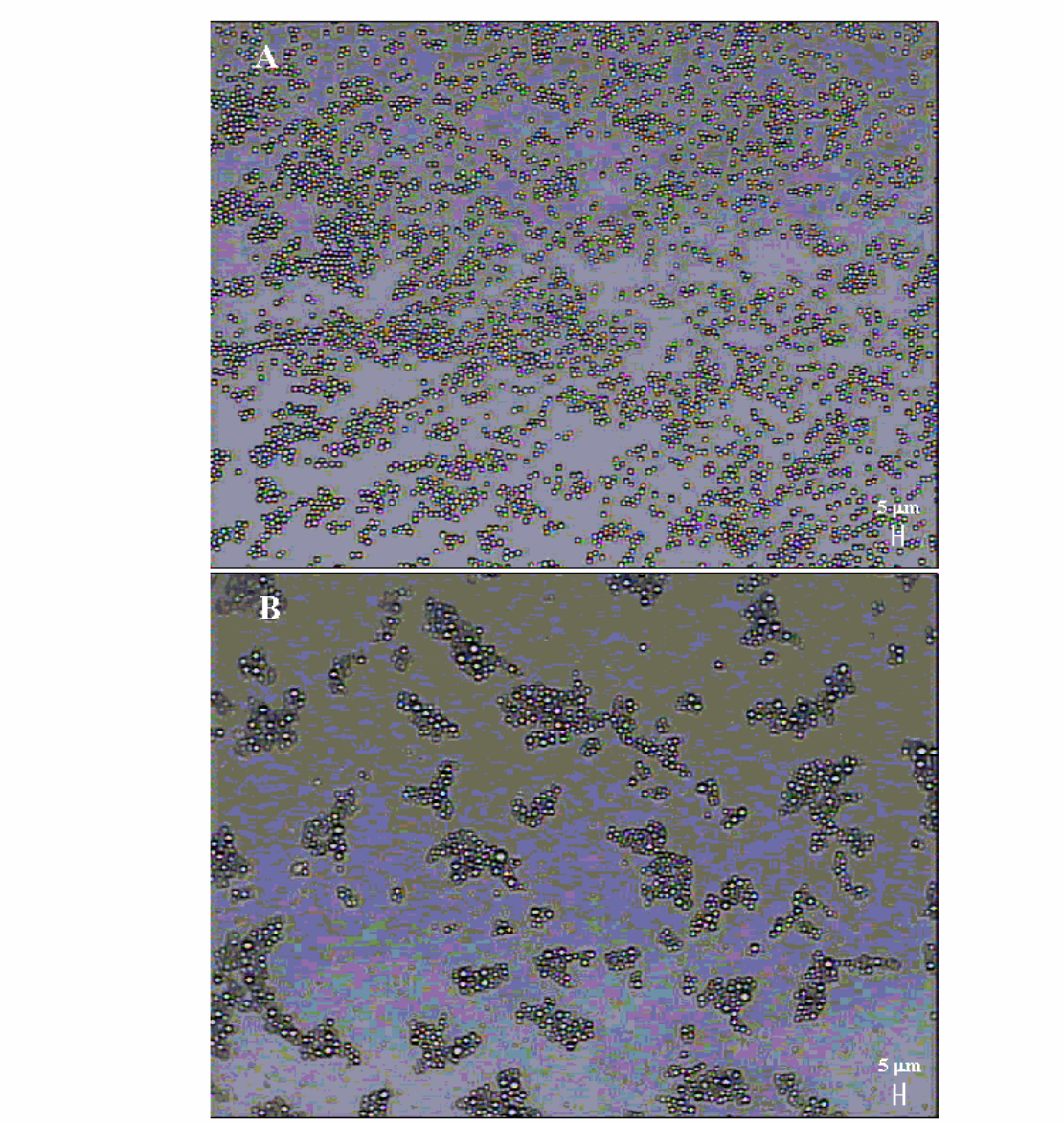

Fig. 8.11. Optical micrographies at 20x of virgin emulsion (A) and after exchanging the original continuous phase by a 15 mg/L Fe(II) solution (B). (10% 01W; 2% SDS; 0.5 µm ceramic membrane).

8.4. Conclusions

Membrane emulsification, operated in stirred or crossflow mode, has demonstrated to be an efficient technology to prepare stable oil/water droplets containing Fe(II) linked to the surfactant molecules present in their interfaces.

The stability of O/W emulsions is strongly affected by the presence of Fe(II) dissolved in the continuous phase. It was found that the maximal tested Fe(II) concentration that did not cause emulsion breakage along one week was 31 mg/L with a 10% O/W emulsion. The %O/W was also found to be crucial for the stability of the emulsions prepared in presence of Fe(II). Destabilisation phenomena occurring in presence of Fe(II) cations are due to the attraction of Fe(II) to the negatively-charged emulsion interfaces. As a consequence, the interfacial charge of emulsion droplets is decreased and charge repulsions between emulsion droplets are also diminished, causing coalescence and even rupture. Iron analysis in the continuous phase of the stable emulsions confirms that Fe(II) is bound to the SDS present in the droplets' interfaces.

Free SDS negatively-charged molecules and/or micelles in the emulsion continuous phases have also been found to be possible Fe(II) attractors due to charge interactions. Experiments based on the removal of the free SDS from the continuous phase and subsequent addition of Fe(II)-containing solutions demonstrated that the absence of free SDS in solution caused an increase on the instability of the emulsions at the same Fe(II) concentrations than when SDS was present. In addition the absence of free SDS in emulsion with continuous phase containing no Fe(II) did not destabilize the emulsion. Thus, it is evident that free SDS can act as breakage retardant (or even evader) when Fe(II) is present in the continuous phase. The droplet size distribution of the emulsions also plays a crucial role when Fe(II) is present in the continuous phase. Emulsions with larger droplet diameters are more sensitive to be broken by Fe(II) dissolved in the continuous phase. Thus, not only the Fe(II) concentration and presence of free SDS molecules (or micelles) in the continuous phase are important variables affecting the stability of O/W emulsions but also the droplet size is a key variable in the destabilisation phenomena.

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Chapter 9

Use of Membrane Emulsification Process to produce solid lipid microstructured systems at high temperature

9.1. Introduction

Solid lipid particles (SLP) were realised by exchanging the liquid lipid (oil) of the emulsions by a solid lipid, which means lipids being solid at room temperature. At the beginning of the 1990ssolid lipid nanapartciles was introduced as an alternative to solid nanoparticles, emulsions and liposomes in cosmetic and pharmaceutical preparations [1–3].

The use of solid lipids instead of liquid oils is a very attractive idea to achieve controlled drug release, because drug mobility in a solid is considerably lower compared with liquid oil. Other advantages of SLN are reported to be increased on biotoxicity, avoidance of organic solvents, drug stability, high drug payload, and incorporation of liophilic and hydrophilic drugs [1].

The high pressure homogenization technique has been demonstrated to be the more effective method for the production of SLP [4–5]. Recently, the membrane emulsification process was applied to prepare SLP [6–8].

In this work, membrane emulsification process is investigated to prepare paraffin-in-water emulsions. Paraffin is wax solid, with a typical melting point between about 47 °C to 64 °C. In this work, paraffin wax is used as dispersed phase at 70°C to obtain highly concentrated emulsions. The aim is to investigate the suitable operation conditions to obtain stable paraffin wax droplets using membrane emulsification process carried out at high temperature. In particular, the effect of continuous phase axial velocity and dispersed phase flux on droplets size and droplets size distribution was investigated. Emulsion stability is also evaluated during the storage time of up to 60 days.

9.2. Materials and methods

O/W emulsions were prepared using paraffin wax (Sigma-Aldrich, 6.5 cP viscosity at 70 °C,melting point is 58/62 °C), 9 wt. % Polyoxyethylene 20 stearyl ether (Brij78, HLB=15, Sigma-Aldrich) in ultrapure water as continuous phase. Paraffin wax. Emulsions were prepared with tubular hydrophilic microporous ceramic membrane from Membraflow, Germany with pore size of 0.1 (surface of $1.87*10^{-3}$ m^2).

Droplet size distribution was determined using a light-scattering Malvern Mastersizer 2000 apparatus, which operates based on laser diffraction of particles. The droplet size was expressed as the d(3,2) or Sauter mean diameter. The droplet uniformity was expressed as the span, this value is a measure of the size distribution width. The narrower the distribution, the smaller the span becomes.

The emulsions paraffin content has been determined through a humidity analyser (thermo-balance). The humidity is determined through the loss of weight of the sample dried by heating. This analysis is possible for fluid witch different boiling points. The sample desiccation has been carried out at 100 °C. The amount of water is calculated by the difference between initial mass of the sample (containing paraffin wax, emulsifier and water) and the residual weight (paraffin wax and emulsifier) after total evaporation of the water.

9.2.1 Membrane emulsification apparatus

The experimental apparatus used for membrane emulsification is shown in Fig 9.1. The tank containing paraffin wax and the membrane module are thermostated at 70 (±2)°C. Continuous phase is not thermostatically controlled, but it reaches a temperature of about 65 (±2)°C when it is recirculated.

The dispersion phase was circulated with piston pump in the shell side of membrane module and under certain pressure permeates through membrane into continuous phase. This phase circulated with gear pump in lumen side of module membrane where the emulsion was formed.

The effect of continuous phase axial velocity is investigated carrying out the experiments at 0.15, 0.3 and 0.58 m/sec. Also the effect of dispersed phase flux (221 and 600 $g/h*m^2$) is evaluated at two different value of transmembrane pressure 80 and 140 kPa, while the axial velocity was maintained constant at 0.58 m/s. In all experiments, 40% (*v/v*) of dispersed phase in emulsion is obtained.

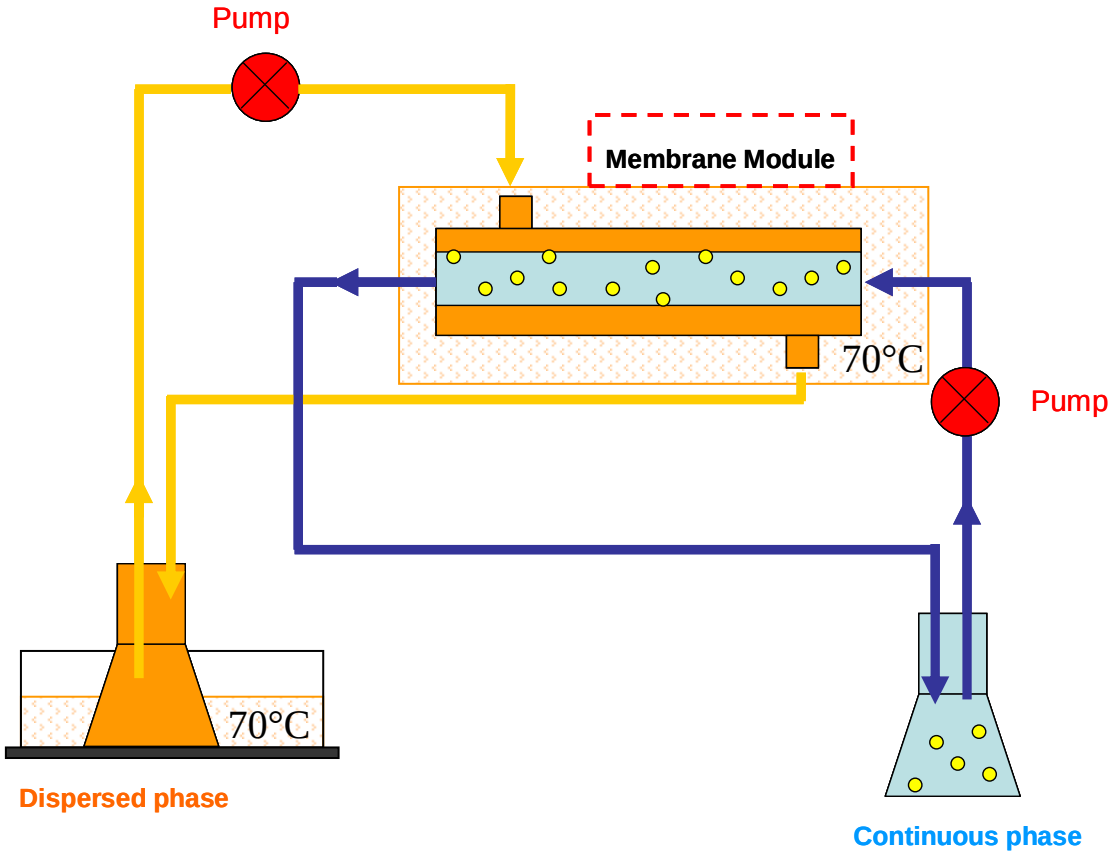

Fig. 9.1. Emulsification plant

9.3. Results and discussion

9.3.1 Influence of continuous phase flow velocity on droplets size and droplet size distribution

The o/w emulsions consisting of paraffin wax as dispersed phase and 9 wt. % brij 78 dissolved in ultrapure water as the continuous phase were prepared. The influence of continuous phase velocity on the mean droplet size was investigated. Furthermore, an optimal condition for monodispersed emulsion production was also found.

The continuous phase flow is one of fundamental process parameters that determine the membrane emulsification characteristics; this parameter influences the droplet size produced as it generates drag force on the droplet. The continuous phase velocity was varied from 0.15 to 0.58 m/sec. Dispersed phase flux was kept constant at 600 g/h*m^2 (transmembrane pressure140 kPa). In Fig. 9.2, it can be seen that mean droplets diameter decreased when cross flow velocity increased. Whit increasing axial velocity from 0.15 to 0.58 m/s, droplet size

distribution become narrower and span shift from 3.2 to 0.9. At high axial velocity (0.58 m/s) o/w emulsions with a droplets size of 2.3 micron and span of 0.9 were obtained.

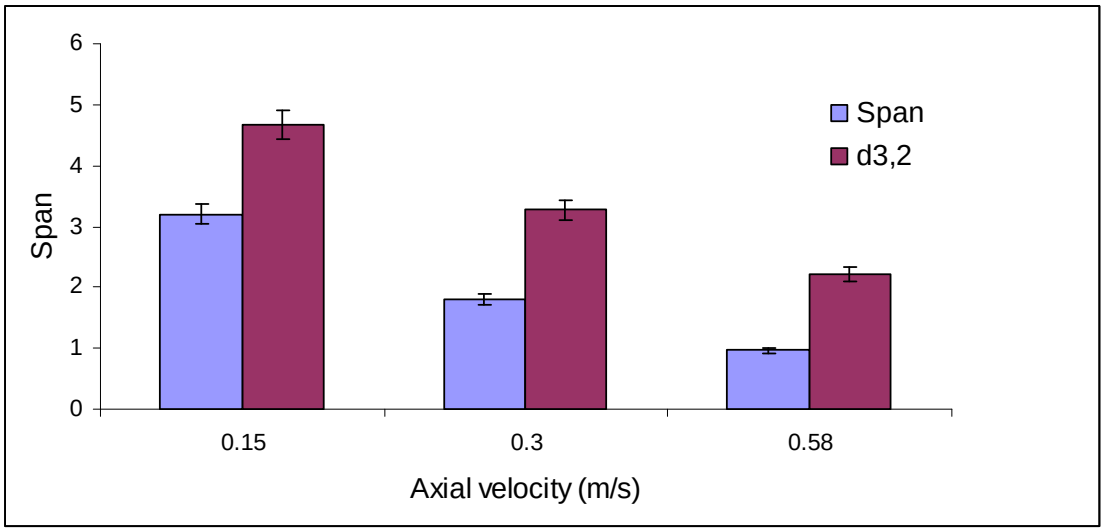

Fig. 9.2. Effect of continuous phase velocity on droplet size and droplets size distribution for paraffin wax-in-water emulsions

9.3.2 Influence of transmembrane pressure on droplets size and droplet size distribution

Transmembrane pressure also influences droplets size and high pressure leads to jets of the dispersed phase and large droplet. However, low pressure leads to low flux of dispersed phase, which means a long emulsification time. In Fig.9.3 the influence of transmembrane pressure on droplets size and dispersed phase flux was evaluated.

When transmembrane pressure increase linearly increase the dispersed phase flux. This determine the formation of larger droplets emulsions.

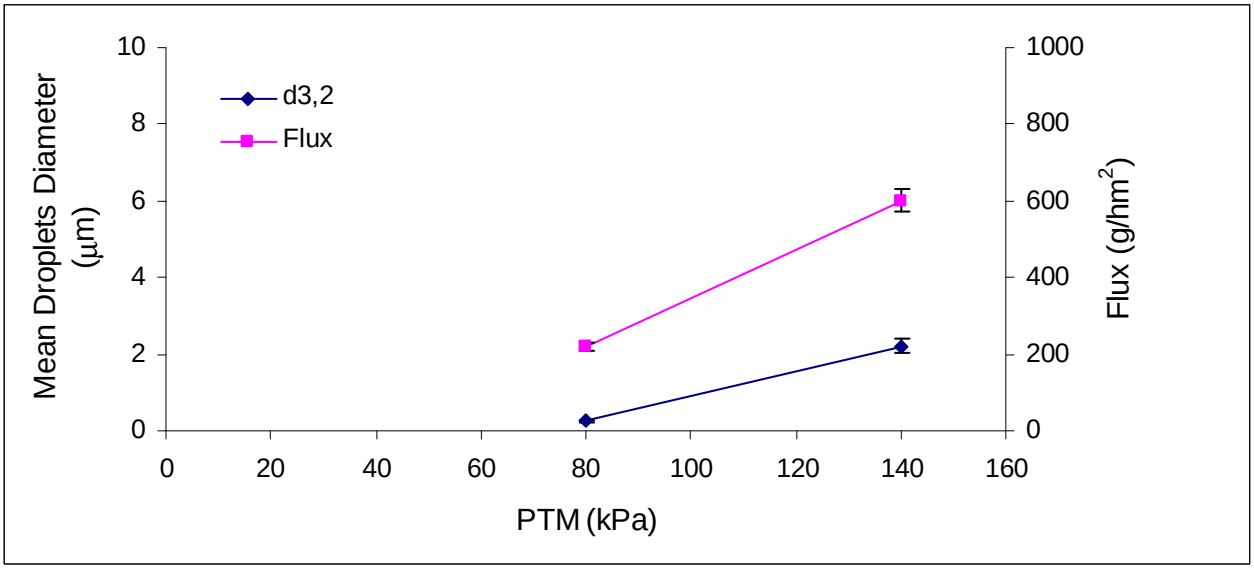

Fig. 9.3. Effect of continuous phase velocity on droplet size for paraffin wax-in-water emulsions

However when droplets size distribution is evaluated as a function of dispersed phase flux, an increase in span value was observed (Fig. 9.4). In fact when 221 g/hm2 was used as dispersed phase flux, a bimodal distribution was obtained (Fig. 9.4 a) and also a certain amount of larger droplets were formed.

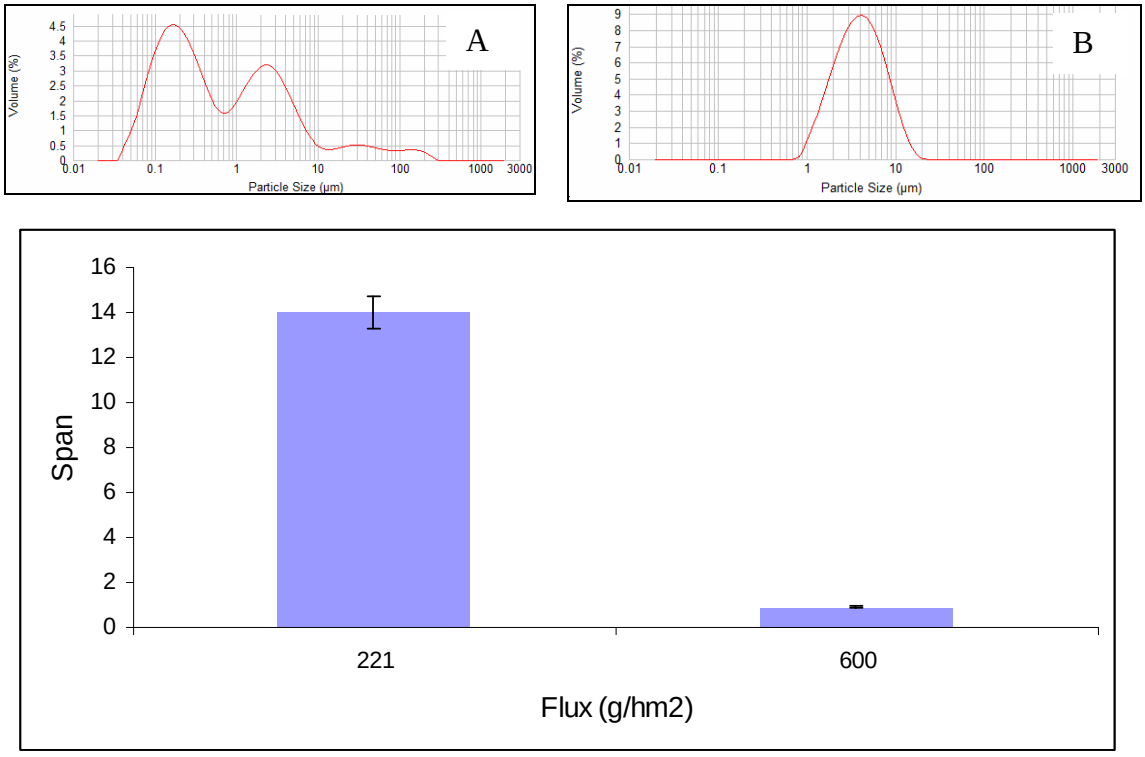

Fig. 9.4. Effect of dispersed phase flux on droplet size distribution for paraffin wax-in-water emulsions. Droplets size distribution obtained using A) 221 g/hm^2 and B) 600 g/hm^2 as dispersed phase

This broader droplet size distribution can be explain by lower proportion of active pore a lower trans membrane pressure. The fact that small and big pores are activated determine the formation of droplets with different size.

An additional important evidence is the behaviour of dispersed phase flux during the time showed in Fig. 9.5. The instantaneous dispersed phase flux decreases versus time due to fouling which is higher for membrane with smaller pore size.

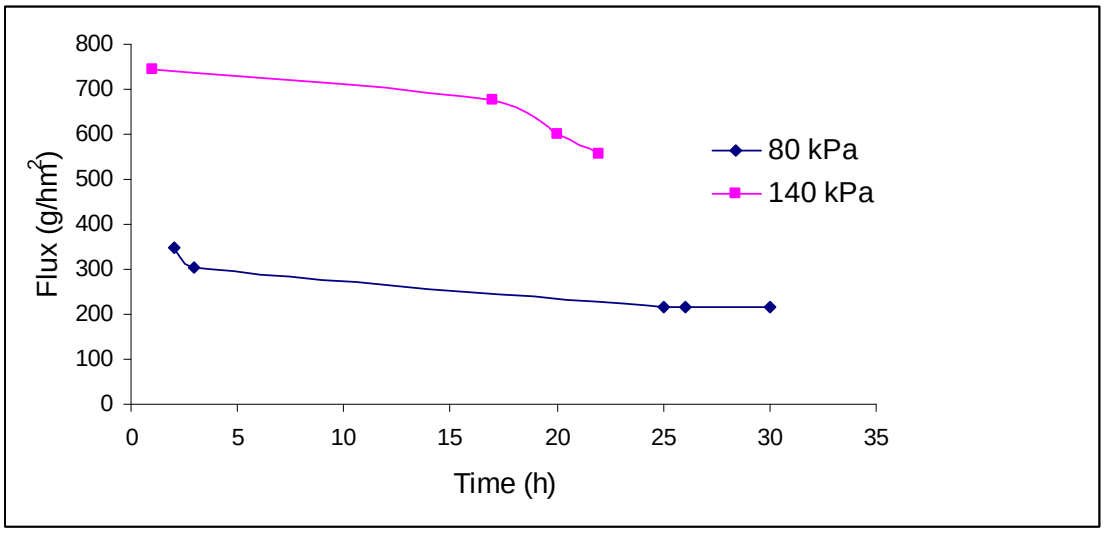

Fig. 9.5. The variation of dispersed phase flux with time

9.3.3. Studies on stability of o/w emulsions

The stability of emulsions was carried by analyzing a sample of emulsion over time a light-scattering. This study showed that size of the droplets is not changed during the storage time. In Fig. 9.6 are reported result the analysis of emulsions prepared with continuous phase velocity of 0.15, 0.3 and 0.58 m/sec. The stability of emulsions is illustrated in terms of average size as a function of time.

Any significant change in droplets size distribution of prepared O/W emulsions was observed during the storage time of up to 60 days.

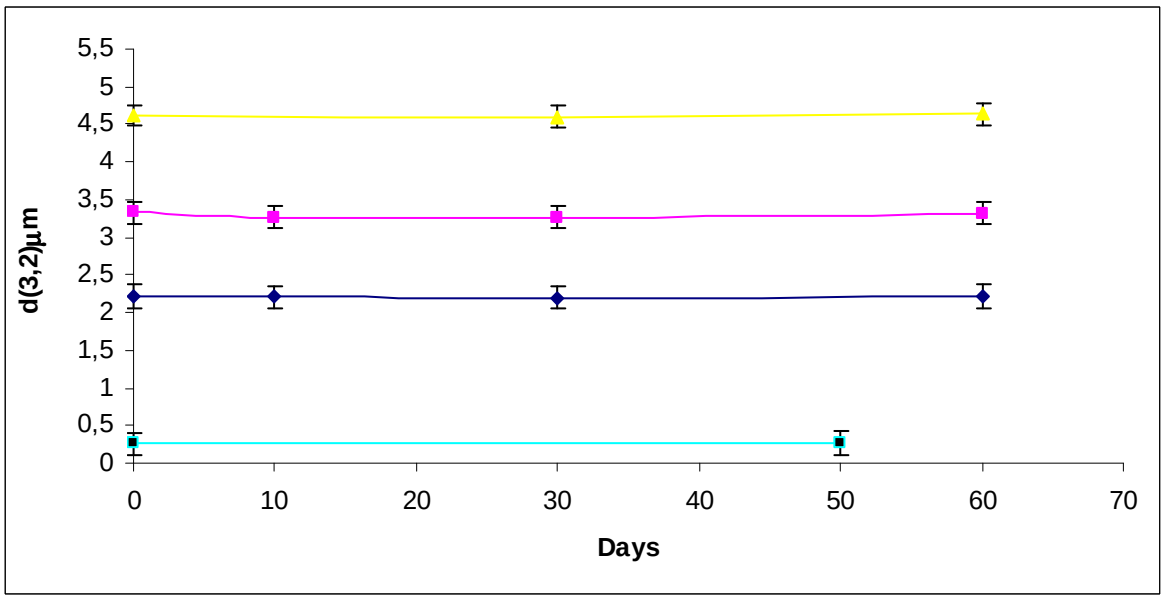

Fig. 9.6. Average droplet size as a function of time

9.4. Conclusions

The o/w emulsions with a narrow droplets size distribution and dispersed phase content up to 38 % were successful prepared using ceramic membrane with a nominal pore size of 0.1 μm at high temperature. The mean size and the span value of the emulsion droplet decreased with increasing the continuous phase flow velocity. The pore activation influence droplets size distribution when low transmembrane pressure was applied. The prepared o/w emulsions were stable and no significant change in mean droplet size was observed during the storage time of two months.

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Overall Conclusions

In this work the development and application of membrane emulsification process to prepare biohybrid microstructured and multifunctional systems were investigated.

Starting from the critical analysis of literature data and patents developed in the membrane emulsification field, some problems regarding the process have been identified and several technological advances have been proposed. Knowledge gained and advances proposed have been applied to the preparation of new formulations with potential applications in various fields such as pharmaceuticals, chemicals, biotechnology and food showing the flexibility of membrane technology.

In particular, the correlation between emulsions phase compositions and shear stress conditions have been identified for the preparation of water-in-oil emulsion by stirred membrane emulsification. Few studies have been conducted on the preparation of water-in-oil emulsions. The results obtained can be applied at the production of dispersed systems by membrane emulsifications processes in which continuous phase with high viscosity are required to reduce emulsions destabilization phenomena caused by particles' settling velocity (Stokes law).

The interaction between proteins with different properties and membranes has been also investigated in order to understand and prevent fouling phenomena during membrane emulsification process. The work showed the possibility to identified the best conditions in which proteins can be used in membrane emulsification without negatively influence the emulsification process. In addition, the positively use of membrane-proteins interaction was shown in the development of an asymmetric membrane, modifying wettability membrane surface only in the lumen side. This strategy permitted to achieve high aqueous dispersed phase flux trough an hydrophilic membrane as well as to control droplets size distribution. In fact, when membrane emulsification process is used, low dispersed phase flux is usually required in order to obtain small droplets size but this determine low process productivity. The opportune modification of membrane properties permits to control dispersed phase flux during membrane emulsification.

A problem connected with the application of cross-flow membrane emulsification was also investigated and some solution has been investigated. The shear stress generated by the recirculation of the continuous phase determines droplets breaking due to the shear force caused by the flow along the circuit. The technological advances proposed in this work to

prevent droplets breaking were: i) applying high axial velocity at the membrane level but low continuous phase flow along the circuit and ii) “shaking” the continuous phase along the membrane surface by inverting the axial flow direction. This strategy permit to reduce recirculation of formed emulsion and increase disperse phase concentration for single passage along the module

From a point of view of the exploration of new and advanced applications of membrane emulsification process, multiple emulsions as self-regulated drug delivery systems were investigated. Few examples of liquid membrane systems (such as water-in-oil-in- water emulsions) are used as stimuli responsive biomaterials. In this work, the membrane emulsification permitted tightly controlled production of particulate multifunctional materials at low shear conditions.

Using membrane emulsification, also a biocatalyst was distributed at emulsion interface and used at the same time as catalyst and as surfactant. Phase transfer biocatalysts are usually distributed at the interface by stirred tank reactor (STR) but some disadvantages are observed such as enzyme deactivation and non constant interfacial area. In this work, the membrane process was proposed with success as suitable alternative methodology.

The application of membrane emulsification was also investigated to organic catalysis preparing droplets emulsions complexing Fe(II) ions. The use of Fe(II) ions confined in emulsion droplets could avoid the problems related to the homogeneous catalysis. The appropriate operating conditions for the preparation of stable oil/water droplets containing Fe(II) ions are evaluated for ensuring the adequate development of one of major interest of producing stable Fe(II) –containing emulsions as catalyst .

In this work, the preparation of Solid Lipid Particles at high temperature by membrane emulsification process was also investigated. Few study has been developed in the preparation of SLP by membrane emulsification. The study carried out in this work permitted to evaluate the influence of process parameters on SLP size and size distribution at high temperature and high dispersed phase percentage. Sub-micron sized droplets have been produced.

Overall, improvements in technological aspects, operation mode, phases composition, innovative formulation capacities and application versatility of the membrane emulsification process have been promoted.

Appendix

Relevant Publications from 2006 to 2009

Chapters in Books

L. Giorno, G. De Luca, A. Figoli, **E. Piacentini**, E. Drioli, Membrane Emulsification: Principles and Applications, Membrane Operations. Innovative separations and transformations, Cap 21 (2009) 463-494, Wiley

Articles in International Journals

E. Piacentini, L. Giorno, E. Drioli, Proofs of concept on use of Conc A as glucose sensor in multiple emulsions prepared using membrane emulsification, Journal of Controlled Release, Submitted

X. Bernat, L. Giorno, **E. Piacentini**, F. Bazzarelli, C. Bengoa, A. Fabregat, E. Drioli, J. Font. Ferrous ion effects on the stability and properties of O/W emulsions formulated by membrane emulsification, Ind. Eng Chem Res., Submitted

E. Piacentini, L. Giorno, R. Mazzei, E. Drioli, New development for the controlled fabrication of microstructured multiphase bioreactor using membrane emulsification technology, *New Biotechnology*, (2009) 25S, pp S168.

L. Giorno, **E. Piacentini**, R. Mazzei, E. Drioli. Membrane emulsification as a novel method to distribute phase-transfer biocatalysts at the oil/water interface in bioorganic reactions, *Journal of Membrane Science* 317 (2008) 19-25.

L. Giorno, E. D'Amore, R. Mazzei, **E. Piacentini**, J. Zhang, E. Drioli, R. Cassano, N. Picci, An innovative approach to improve the performance of a two separate phase enzyme membrane reactor by immobilizing lipase in presence of emulsion, Journal of Membrane Science, 295 (2007) 95-101.

L. Giorno, E. Piacentini, R. Mazzei, E. Drioli, Distribution of phase transfer biocatalyst at the oil/water interface by membrane emulsifier and evaluation of enantiocatalytic performance, Desalination 199 (2006) 182-184.

Proceedings

E. Piacentini, L.Giorno, E. Drioli, Preparation of stimulus-responsive multiple emulsion by membrane emulsification, *IMeTI* Workshop on "Membrane Applications in Agrofood" 2009, Cetraro (CS), Italy

E. Piacentini, L.Giorno, E. Drioli, "Glucose-sensitive delivery systems" prepartion using membrane emulsification technology, *Euromembrane 2009*, Montpellier, France

L. Giorno, **E. Piacentini,** R. Mazzei, F. Bazzarelli, E. Drioli, Membrane Emulsification Technology to Enhance Phase Transfer Biocatalyst Properties and Multiphase Membrane Reactor Performance, ICOM 2008, Honolulu/Kahuku Hawaii

L. Giorno, **E. Piacentini**, R. Mazzei, F. Bazzarelli, E. Drioli, Membrane emulsification and its application in production system optimization, China-EU Seminar on the Application of Membrane Technology & Cooperation Fair 2007, Weihai, China

E. Piacentini, L. Giorno, E. Drioli, Membrane emulsification in the preparation of microstructured functionalized systems, *NanoMemCourse EF1: "Nanostructured materials and membranes, synthesis and characterization" 2007*, University of Zaragoza, Spain

L. Giorno, **E. Piacentini**, S. Piluso, E. Drioli, Preparation of stable O/W emulsion containing water insoluble drugs by membrane emulsification, *ECI Conference 2006*, Cetraro (CS) Italy

Other Publications

Articles in International Journals

R. Mazzei, L. Giorno, E. Piacentini, S. Mazzuca, E. Drioli, Kinetic study of a biocatalytic membrane reactor containing immobilized -glucosidase for the hydrolysis of oleuropein, *Journal of Membrane Science*, 339 (2009) 215-223.

R. Mazzei, L. Giorno, E. Piacentini, E. Drioli, Advances in Biocatalytic Membrane Reactors for the production of non commercially available pharmacologically active compounds from vegetal material, *New Biotechnology*, (2009) 25S, p. S276.

Proceedings

L. Giorno, R. Mazzei, **E. Piacentini**, S. Chakraborty, E. Drioli, Membrane Bioreactors for production of nutraceuticals, *IMeTI* Workshop on "Membrane Applications in Agrofood" 2009, Cetraro (CS), Italy

R. Mazzei, L. Giorno, **E. Piacentini**, E. Drioli, Advances in Biocatalytic Membrane Reactors for the production of non commercially available pharmacologically active compounds from vegetal material, *Symbiosis 2009*, Barcelona, Spain

L. Giorno, **E. Piacentini**, R. Mazzei, E. Drioli, R. Cassano, N. Picci, Multiphase enzyme membrane reactors for kinetic resolution of racemic esters: influence of OR-ester group lenght and oil/water interface, Permea 2007, Siófok, Hungary (2-6 September 2007)

L. Giorno, R. Mazzei, **E. Piacentini**, E. Drioli, Membranes in food industrial applications and research perspectives, Permea 2007, Siófok, Hungary (2-6 September 2007)

L. Giorno, **E. Piacentini**, R. Mazzei, F. Bazzarelli, E. Drioli, Biocatalytic membrane reactors: Advances in biotechnology and pharmaceutical applications, ICCMR8 2007, Kolkata (India)

R. Mazzei, L. Giorno, S. Mazzuca, A. Spadafora, **E. Piacentini**, E. Drioli, β-glucosidase separation from Olea europaea fruit and its use in membrane bioreactors for hydrolysis of oleuropein, Euromembrane 2006, Taormina (Messina) Italy.

Education and training from 2006 to 2009

18-20 October, 2009
Partecipation to IMeTi Workshop on “Membrane Apllications in Agrofood”, Cetraro, Italy.

12-16 September, 2009
Partecipation to Symbiosis 2009, Barcelona, Spain

6-10 Septemebr, 2009
Partecipation to Euromembrane 2009, Montpellier, France

15 June-3 July, 2009
Course on “Fundamentals of mass trasport in membrane processes” and “ Preparation of membranes for mass separation and energy conversion processes”, Institute on Membrane Technology, ITM-CNR, Prof. Heiner Strathmann

November 2008 -Maggio 2009
Inglease Course, Centro linguistico d’ateneo (CLA), Unical , Rende (CS)

24-29 May, 2008
Partecipation to Chemical Engineering Conference for Collaborative Research in Eastern Mediterranean Countries EMCC5 5th, Cetraro (CS), Italy

7-16 November, 2007
NanoMemCourse EF1: “Nanostructured materials and membranes, synthesis and characterization”. University of Zaragoza, Spain

16-19 September, 2007
Partecipation to Symbiosis 2007, Barcelona, Spain

29 May- 6 June, 2007

Course on “Preparation, Characterization and Application of Phase Inversion Hollow Fiber Membranes” Istitute on Membrane Technology (ITM-CNR), Rende CS, prof. Heiner Strathmann.

24-28 September, 2006

Partecipation to Euromembrane 2006, Giardini Naxos, Taormina (Messina), Italy

11-15 June, 2006

Partecipation to ECI 2006, Cetraro-Cosenza, Italy

Awarded Presentations

EMS Prize for the best oral presentation at Euromembrane 2006 “Distribution of phase transfer biocatalyst at the oil/water interface by membrane emulsifier and evaluation of enantiocatalytic performance”, L. Giorno, **E. Piacentini,** R. Mazzei, E. Drioli (Presenter: Emma Piacentini)

Teaching Activities

Tutoring activity of Course of “*Laboratorio di Ingegneria Chimica I*”, Faculty of Ingegneria Chimica A.A. 2007/08, Unical