

Aging mechanisms of perfluorocarbon emulsions using image analysis

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Abstract

The aging mechanisms of perfluorocarbon emulsions were investigated using image analysis. Oil-in-water emulsions of two perfluorocarbons, *n*-perfluorohexane and perfluorodecalin, were prepared with three emulsifiers, Lecithin, Span 20, and Pluronic F-68. The effect of the temperature and the replacement of water by an aqueous phase consisting of a microbial culture medium were also studied. The emulsions were prepared by sonication and their stability was followed through analysis of the evolution of mean droplet size. The results indicate that the stability of perfluorocarbon in water emulsions depends on all the parameters investigated and that two aging mechanisms, coalescence and molecular diffusion, may take place. Analysis of the evolution of the mean droplet size during long time periods indicate that coalescence is more common than previously reported for these systems and seems to be favored by a temperature increase.

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1. Introduction

The use of perfluorocarbons (PFCs) as oxygen carriers was first proposed in 1966, when Clark and Gollan at the University of Cincinnati demonstrated the capacity of these liquids to support animal life through liquid breathing [1]. PFCs are highly fluorinated, inert organic compounds that can dissolve large volumes of respiratory gases such as oxygen and carbon dioxide [2–5]. Liquid PFCs are colorless, odorless, and noncorrosive [6]. As they are immiscible in aqueous systems, including biological fluids such as plasma and cell culture medium, they have to be emulsified for intravascular administration and for use in biological reactors. The transport and delivery of oxygen *in vivo* by means other than red blood cells has become one of the most challenging research topics of the past 25 years and PFC emulsions have become one of the main candidates for a safe and reliable artificial blood substitute [6–8].

The application of PFC-based emulsions in the biomedical and biotechnological fields has experienced some developments and drawbacks. Apart from suitable thermophysical properties and inertness, emulsion stability plays a primary role in their use as injectable oxygen carriers, contrast agents, drug delivery systems, or cell culture medium supplements [7]. There has been a considerable effort to formulate stable PFC emulsions and to understand the driving forces and mechanisms responsible for their aging [9–11]. Emulsion stability can be studied through the evolution of the droplet size and size distribution. The increase in droplet diameter is an indicator of the loss of stability of the emulsion and the growth rate of the droplets reveals the mechanism responsible for their aging. Two main mechanisms have been proposed for the loss of stability of these emulsions: coalescence and molecular diffusion [12]. Previous works dealt with dilute emulsions and short times of analysis and contradictory results have been reported in the literature, with most researchers supporting molecular diffusion as the most frequent aging mechanism [13–18].

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2. Aging mechanisms

The physical degradation of emulsions is due to the spontaneous trend toward a reduction in the Gibbs free energy (ΔG), achieved by reducing the size of the oil/water interface, A , and/or of the interfacial tension between the continuous and the dispersed phases, γ :

$$\Delta G = \gamma \Delta A - T \Delta S. \quad (1)$$

The reduction of the interfacial area is achieved mainly by two mechanisms: coagulation, followed by coalescence, and molecular diffusion.

Coalescence is the formation of a larger droplet from the merging of smaller ones. This requires that the small droplets come into contact, with thinning and disruption of the film that covers them. Emulsion degradation by coalescence is characterized by a broadening in particle size distribution, with an exponential increase in the mean droplet size with time, according to the Van den Tempel Theory [19],

$$\bar{a}^3 = \bar{a}_0^3 \exp(Kt), \quad (2)$$

where \bar{a}_0 is the initial average particle radius, \bar{a} is the average particle radius at time t , and K is the coalescence constant.

The coarsening of emulsions through molecular diffusion, also known as Ostwald ripening, is due to the gradual growth of the larger droplets of the emulsion at the expenses of smaller ones. This is a direct consequence of the Kelvin effect, which states that the solubility of a particle is inversely proportional to its radius, meaning that individual molecules tend to leave smaller particles and to diffuse through the continuous phase to join larger ones. Thus, particle growth is achieved without physical contact of the particles. There is essentially one theoretical treatment known as the Lifshitz–Slyozov–Wagner (LSW) theory [20,21] and its modifications [22,23], where the molecular diffusion mechanism is characterized by linear growth of the droplet volume with time,

$$\frac{d}{dt}(\bar{a})^3 = \frac{8CD\gamma V_m^2}{9RT}, \quad (3)$$

where C and D are respectively the solubility and the diffusion coefficient of the dispersed phase in the continuous medium, V_m is the molar volume of the dispersed substance, R is the usual gas constant, and T is the absolute temperature. According to Eq. (3), an increase in the particle's volume is proportional to the solubility, the diffusion coefficient, and the interfacial tension of the dispersed phase (perfluorocarbon) in the continuous phase (water). Therefore, emulsions that undergo Ostwald ripening can be stabilized by decreasing at least one of these three factors.

It has been reported that molecular diffusion plays a decisive role in the coarsening of fluorocarbon-in-water microemulsions up to 50% (v/v) [8]. A higher percentage of oil in the emulsions results in a larger mean droplet diameter for the same homogenization conditions. As the organic phase

content increases the available surfactant decreases, limiting its stabilizing effects and acting in favor of oil droplet coalescence [24]. Since the effective transport of adequate amounts of oxygen requires the development of injectable emulsions with concentrations higher than 50% (w/v), it is important to verify whether molecular diffusion is still the primary degradation process in this PFC's concentration range and for a longer analysis period [11,25]. For biological reactors the composition of the aqueous phase, the cell culture medium, might also affect the aging mechanisms of the emulsion. The purpose of this study is to identify and understand the aging mechanisms of *n*-perfluorohexane and perfluorodecalin (50% (w/v)) in water emulsions and its dependence on the surfactant, the temperature, and the aqueous phase used.

3. Experimental

3.1. Materials and methods

The perfluorocarbons used were *n*-perfluorohexane (C₆F₁₄) and perfluorodecalin (C₁₀F₁₈), both 95% pure from Flutec (PP6 and PP1, respectively). Three surfactants were tested: lecithin (L- α -phosphatidylcholine) from egg yolk with an average purity of 88.6% (acid value < 25, iodine value < 80, peroxide value < 5) from Fluka, and Pluronic F-68, 10% aqueous solution, and Span 20, both from Sigma–Aldrich. Fluorocarbons and emulsifiers were used as received without any further purification. Deionized and double-distilled water was used. YPD medium containing yeast extract (1.0%) from Oxoid, casein peptone (0.64%) from Merck, and glucose (2.0%) from Vetec was prepared and autoclaved before being used in the formulation of the emulsions.

Emulsions of 50% (w/v) of each perfluorocarbon in aqueous phases using 5% (w/v) of each of the three surfactants were prepared by sonication, using a Branson Sonifier, model Cell Disruptor B15. The sonication was performed for two cycles of 1 min each, with 1 min of interval, in continuous phase and duty cycle at 40%, keeping the tube immersed in ice to avoid heating. The 10.0 ml of each emulsion was prepared with composition summarized in Table 1.

Table 1
Composition of the emulsions studied

Emulsion	Perfluorocarbon (50% (w/v))	Surfactant (5% (w/v))	Aqueous phase
1	C ₆ F ₁₄	Lecithin	Water
2	C ₆ F ₁₄	Span 20	Water
3	C ₆ F ₁₄	Pluronic F-68	Water
4	C ₁₀ F ₁₈	Lecithin	Water
5	C ₁₀ F ₁₈	Span 20	Water
6	C ₁₀ F ₁₈	Pluronic F-68	Water
7	C ₁₀ F ₁₈	Lecithin	YPD medium
8	C ₁₀ F ₁₈	Span 20	YPD medium
9	C ₁₀ F ₁₈	Pluronic F-68	YPD medium

The emulsions stability was studied through the evolution of the mean particle size at two different temperatures, 301.2 and 310.2 K. Two sets of emulsions 1–6 were prepared and each set was placed at a different temperature in a thermostatic oven (± 0.5 K) for 42 days. For emulsions 7–9 just one set was prepared and kept at 301.2 K. At the temperature of 310.2 K, emulsion 4 was followed up to 78 days and emulsions 5 and 6 were followed up to 106 days to confirm the observations. The former was kept for less time since after that period lecithin suffered degradation. The temperatures were chosen according to two common perfluorocarbon applications, as oxygen vectors in yeast cultures, for which 301 K is the optimum growth temperature of microorganisms such as *Yarrowia lipolytica* [26], and in artificial blood substitutes, 310 K.

The evolution of the particle size in the emulsions was followed with a Nikon optical microscope, model Eclipse 200, with a digital camera, Nikon Coolpix 990. The obtained images were processed with a program developed in Matlab 6.1 for this purpose. A micrometer and appropriate software, Image-Pro Plus 5.0, were used for calibration of the droplet size.

3.2. Validation of the image analysis

The image analysis performed followed a three-step sequence: image binarization, droplet quantification, and evaluation of statistical parameters.

The binarization consisted in the conversion of the captured image to black and white and in the removal of noise. The sequence of the operations performed can be divided into five primordial parts: (a) conversion of a RGB (red–green–blue) image to an intensity image with previous subtraction of the background; (b) creation of a binary image from an intensity image based on a luminance threshold; (c) use of a median filtering to remove salt and pepper noise; (d) suppression of the structures connected to image border; and (e) erosion of the binary image and morphological reconstruction using a mask previously created. This first step

is the vital part of the image analysis process, since all the measurable data are going to be taken from it.

The second step quantifies the droplets in the image, yielding parameters such as droplet diameter, droplet volume, droplet area, and particle sharpening.

During the last step a statistical analysis of the data acquired from several images was performed in order to evaluate the total number of analyzed objects, the average droplet diameter and its standard deviation. It also provides the particle roundness, useful in detecting if objects other than droplets are being analyzed, as well as the particle size distribution.

The use of this automatized procedure allowed the analysis of a larger number of objects with higher precision and accuracy compared to manual quantification.

4. Results and discussion

For each emulsion, the droplet diameter was measured periodically for 42 days, with the exception of emulsions 4–6 at 310.2 K. For each time an average of 100 droplets per image and about 12 different pictures from different samples of *n*-perfluorohexane emulsions and 200 droplets per image and 20 different pictures from different samples of perfluorodecalin emulsions were analyzed. The larger number of globules analyzed in perfluorodecalin emulsions is due to their smaller size. Since the precision of the program decreases with the decreasing size of objects a larger number were analyzed to reduce the systematic errors. An example of the microscopic images obtained for the two different PFC emulsions at the final state is presented in Fig. 1. The droplets in the perfluorodecalin emulsions are about 70% smaller than the droplets in the perfluorohexane emulsions, due to the differences in interfacial tension and viscosity between the two perfluorocarbons, as can be seen from Table 2. It has been shown that oils with high viscosity and low interfacial tension yield emulsions with smaller droplet size after homogenization [27].

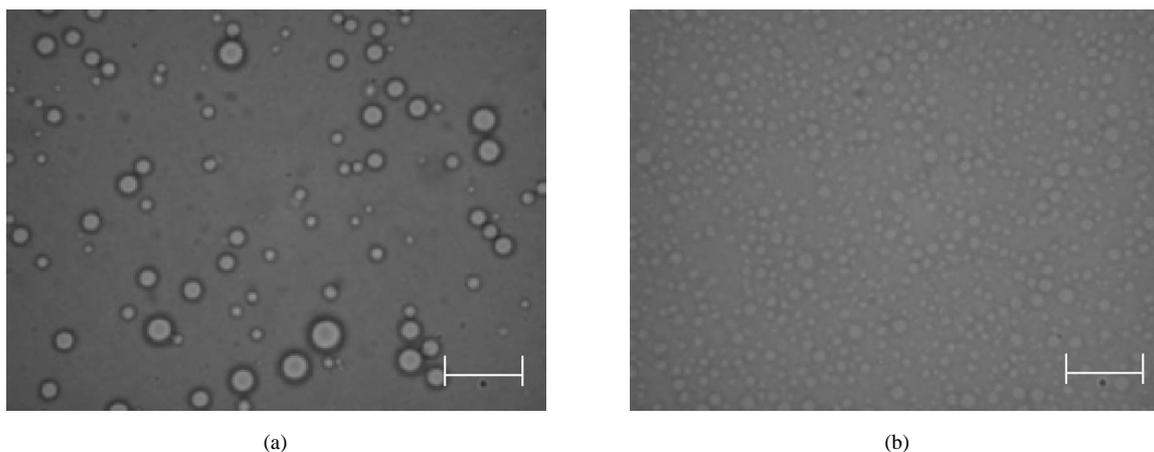


Fig. 1. Microscopic images for the final states (42 days) of Emulsion 1 (a) and Emulsion 4 (b). The bar corresponds to 10 μm .

Table 2

Comparison between the thermophysical properties of *n*-perfluorohexane, perfluorodecalin, and *n*-hexane at 298 K

Property	<i>n</i> -Perfluorohexane	Perfluorodecalin	<i>n</i> -Hexane
Molecular weight (g mol ⁻¹) [28]	337.9018	461.8983	86.1754
Density (kg m ⁻³) [29,30]	1.678	1.930	0.65521
Vapor pressure (kPa) [29,31]	29.41	1.02	19.98
Viscosity (N s m ⁻²) [32–34]	6.6×10^{-4}	5.14×10^{-3}	2.94×10^{-4}
Interfacial tension ^a (N m ⁻¹) [30,35]	0.05720	0.05661	0.0497
Water solubility (mol L ⁻¹) [36,37]	2.7×10^{-7}	9.9×10^{-9}	1.430×10^{-4}
Diffusion coefficient ^a (m ² s ⁻¹) [38]	6.79×10^{-10}	6.34×10^{-10}	9.97×10^{-10}
Ostwald ripening rate ^a (mol s ⁻¹)	3.9×10^{-22}	1.5×10^{-23}	1.8×10^{-19}

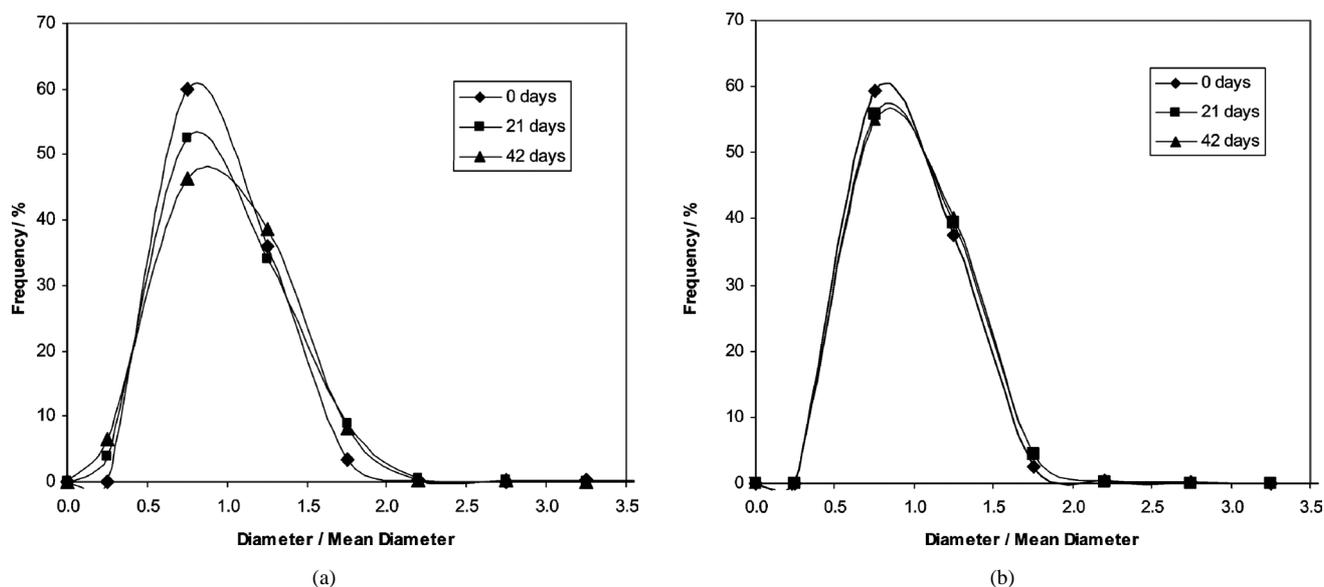
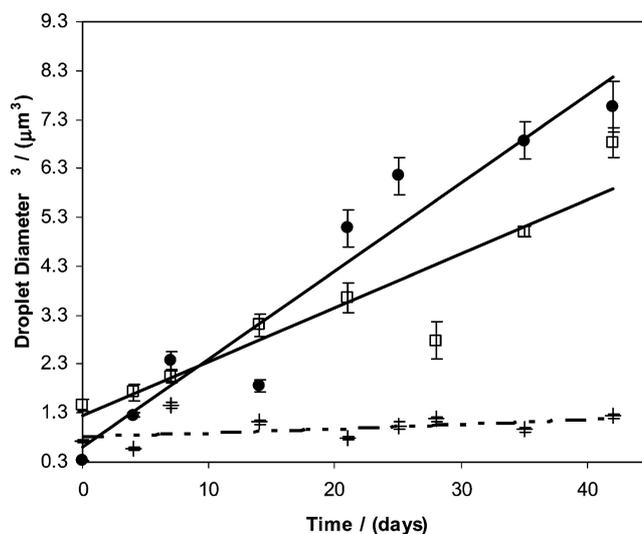
^a In water.

Fig. 2. Distribution of the droplet size populations for Emulsion 3 (a) and Emulsion 6 (b) at different periods of storage at 301.2 K.

A statistical analysis of the experimental data aiming at assuring that no systematic errors (or bias) were made during the particle diameter measurements was performed. Among other issues, it is important to ensure that the program is detecting the smallest droplets and that the samples are randomly analyzed with no preferential droplet size detection. When no systematic errors are present, the population follows a Gaussian distribution. This fact was observed in all the cases studied, as for example is depicted in Fig. 2 for Emulsions 3 and 6. Also note that these two examples show scaling of the particle size distribution functions as described by the LSW theory [20–23].

Figs. 3–8 show the evolution of the average droplet size with time and the respective error bars for Emulsions 1–9 at both temperatures. The uncertainty associated with \bar{a}^3 was calculated for the universe of objects analyzed in a 99% confidence interval [39]. Some emulsions presented very heterogeneous droplets size, thus leading to large error bars, which is a consequence of broadening in the population universe. Eqs. (2) and (3) were used to correlate the experimental data

Fig. 3. Cube of the droplet diameter of *n*-perfluorohexane emulsions as a function of time at 301.2 K for Emulsion 1 (+), Emulsion 2 (□), and Emulsion 3 (●). The dashed and the solid lines represent respectively the coalescence and the molecular diffusion mechanisms.

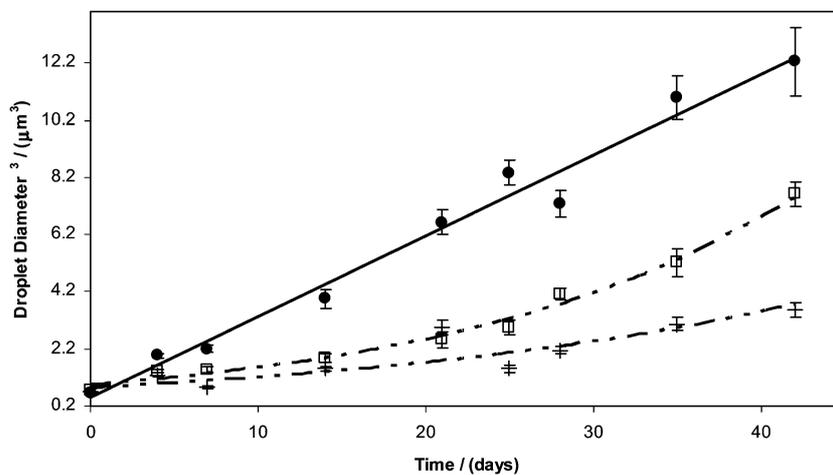


Fig. 4. Cube of the droplet diameter of *n*-perfluorohexane emulsions as function of time at 310.2 K for Emulsion 1 (+), Emulsion 2 (□), and Emulsion 3 (●). The dashed and solid lines represent respectively the coalescence and the molecular diffusion mechanisms.

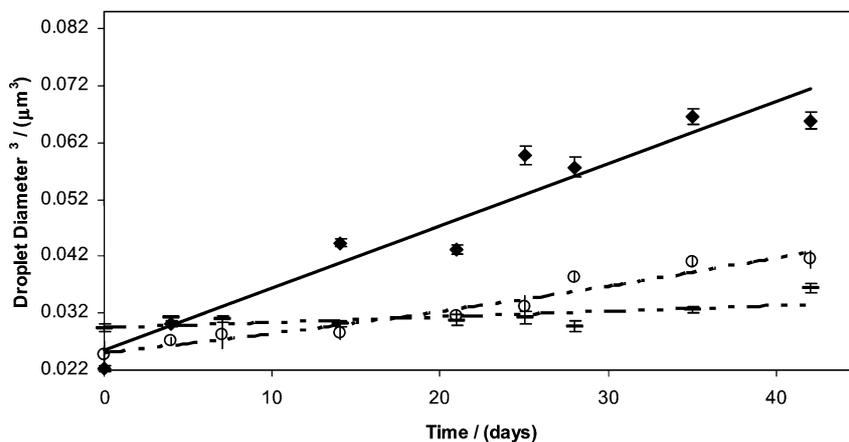


Fig. 5. Cubes of the droplet diameter of perfluorodecalin emulsions as function of time at 301.2 K for Emulsion 4 (—), Emulsion 5 (○), and Emulsion 6 (◆). The dashed and the solid lines represent respectively the coalescence and the molecular diffusion mechanisms.

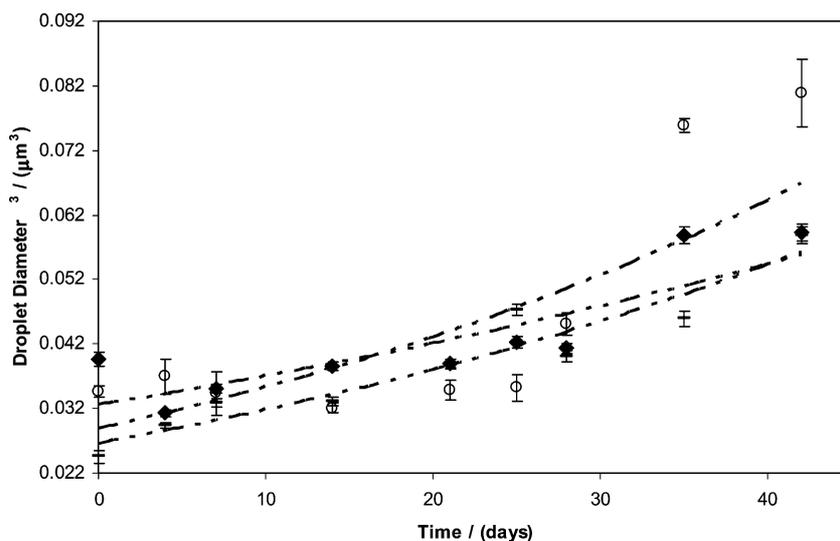


Fig. 6. Cube of the droplet diameter of perfluorodecalin emulsions as a function of time at 310.2 K for Emulsion 4 (—), Emulsion 5 (○), and Emulsion 6 (◆). The dashed lines represent the coalescence mechanism.

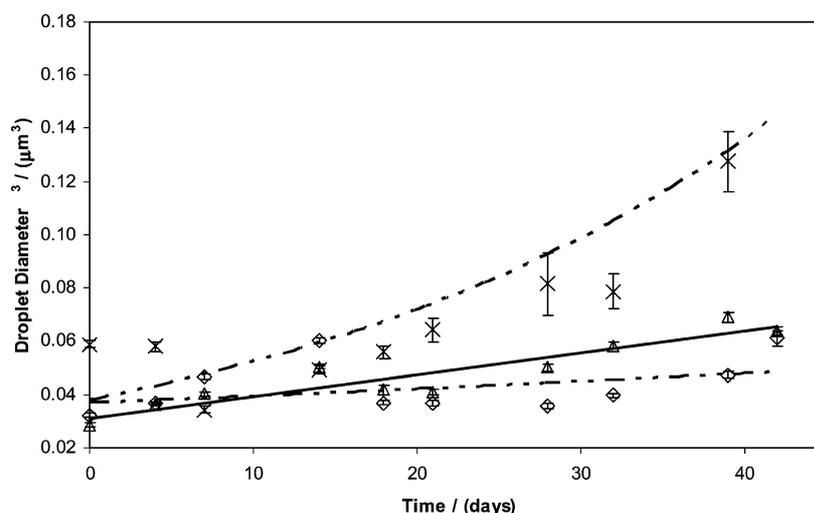


Fig. 7. Cube of the droplet diameter of perfluorodecalin in YPD medium emulsions as function of time at 301.2 K for Emulsion 7 (\times), Emulsion 8 (\diamond), and Emulsion 9 (\triangle). The dashed and solid lines represent respectively the coalescence and the molecular diffusion mechanisms.

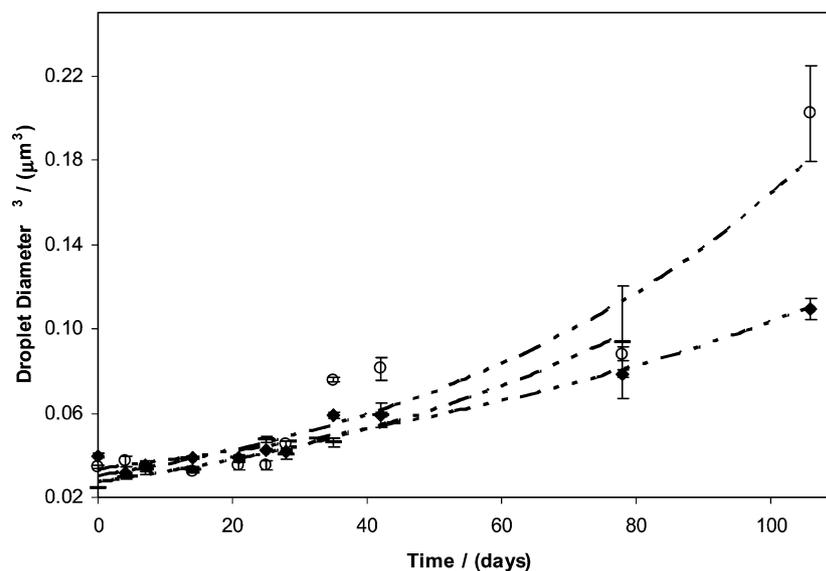


Fig. 8. Cube of the droplet diameter of perfluorodecalin emulsions as a function of time at 310.2 K for Emulsion 4 (—), Emulsion 5 (\circ), and Emulsion 6 (\blacklozenge) for 106 days of analysis. The dashed lines represent the coalescence mechanism.

and to identify the aging mechanisms. These correlations are presented in Table 3 and they were chosen according to the best correlation factor of the fitted equation. Both coalescence and molecular diffusion mechanisms were identified for different emulsions. The aging mechanism seems to be dependent on both the composition of the emulsion and the conditions of storage. It is also shown in Table 3 that some emulsions, although showing a clear exponential increase in the droplet size, present very low coalescence constants, K , which may lead to wrong conclusions about their aging mechanism if these are followed for periods of time shorter than 20 days, as done by several authors [14,15,17,18]. This is probably one of the reasons why so often the aging mechanism for these emulsions is described as molecular diffusion in contradiction to the more diversified picture displayed by this study.

Partial sedimentation or creaming was observed after about 15 days for emulsions with water and after about 20 days for cell culture media. This is due to the difference in densities between the continuous and the dispersed phases and favors droplet coagulation [40]. At the end of 42 days of storage, the loss of stability of the emulsions by phase separation at both temperatures is visible. The loss of stability is also detectable microscopically by the increase of the droplets size.

4.1. Temperature effect

It can be observed in Figs. 3–6 that there is an increase in the average droplet size with temperature indicating that it affects the stability of both PFC emulsions. There seems to be a generalized trend toward coalescence at higher tem-

Table 3

Optimal fitted equations for the mechanisms of loss of stability of the studied emulsions (y = droplet diameter³ (μm^3) and x = time (days))

Emulsion	Fitted equation ^a		Fitted equation ^b
	301.2 K	310.2 K	310.2 K
1	$y = 0.8188 \exp(0.0088x)$	$y = 0.8581 \exp(0.0353x)$	–
2	$y = 0.1104x + 1.2421$	$y = 0.9533 \exp(0.0491x)$	–
3	$y = 0.1801x + 0.6016$	$y = 0.2825x + 0.5082$	–
4	$y = 0.0295 \exp(0.0031x)$	$y = 0.0267 \exp(0.0178x)$	$y = 0.0273 \exp(0.0163x)$
5	$y = 0.0250 \exp(0.0128x)$	$y = 0.0290 \exp(0.0199x)$	$y = 0.0303 \exp(0.0169x)$
6	$y = 0.0011x + 0.0256$	$y = 0.0327 \exp(0.0128x)$	$y = 0.0335 \exp(0.0113x)$
7	$y = 0.0381 \exp(0.0317x)$	–	–
8	$y = 0.0370 \exp(0.0065x)$	–	–
9	$y = 0.0008x + 0.0312$	–	–

^a For a 42 days analysis.^b For a 120 days analysis.

peratures. Except for Emulsion 3, all the other Emulsions (1–6) show coalescence at higher temperatures. This is due to an increase of the thermal agitation of the droplets with the temperature, which leads to a higher frequency of contacts between them and to a higher probability of coalescence to occur. In some cases it is difficult to visualize the exponential growth of the average droplet volume with time due to the small size and small size change of the particles resulting also in low correlation coefficients of the fitted equations. For example, in Fig. 3, Emulsion 1 exhibits an exponential behavior with a correlation coefficient of 0.989 when the correlation coefficient for the Ostwald ripening is 0.966. This emulsion has lecithin in its formulation and it is known that the use of egg yolk phospholipids as emulsifiers lead to lower droplet size and lower polydispersity [41]. The same fact is observed in Fig. 7 for Emulsion 8, where the correlation factor for coalescence is 0.840 and for Ostwald ripening 0.781. The stability study of Emulsions 4–6 at 310.2 K was extended up to 106 days to confirm the obtained results. As can be observed in Fig. 8, the exponential volume growth with time becomes better defined, even for Emulsion 4, indicating that coalescence is the major process of stability loss for the studied perfluorocarbon emulsions under these conditions.

4.2. Perfluorocarbon effect

According to Kabalnov and Shchukin [42], the properties of the perfluorocarbon are the key factor in the degradation of this type of emulsions. This can be confirmed by the results presented in Table 3. They indicate again that there seems to be a generalized trend for the perfluorodecalin emulsions to suffer degradation by coalescence, except for the emulsions prepared with Pluronic F-68 at the lower temperature studied, where the molecular diffusion plays a dominant role. For the *n*-perfluorohexane emulsion both mechanisms can occur depending on the other variables studied, surfactant and temperature.

Perfluorodecalin emulsions present smaller droplets and lower growth rates than perfluorohexane emulsions. A similar behavior was observed in the hydrocarbon family by

Sakai et al. [43] and Weiss et al. [44], showing that the growth rate and droplet size of hydrocarbon-in-water emulsions decreases with increasing hydrocarbon size. This is a direct consequence of the interfacial tension decrease and viscosity increase in the same family.

The fact that molecular diffusion is more prone to occur in *n*-perfluorohexane than in perfluorodecalin emulsions can be associated with the larger solubility and diffusion coefficient of *n*-perfluorohexane than of perfluorodecalin in water. The solubility data found in the literature are reported in Table 2 and they agree with the obtained results. Since no experimental diffusion coefficients data for the perfluorocarbons in water were available, they were estimated using the Wilke–Chang Method [38] at 298 K and are reported in Table 2. The Wilke–Chang technique is an empirical modification of the Stokes–Einstein relation for diffusion coefficients at infinite dilution and can be applied to the studied systems. As it was expected, the diffusion coefficient for *n*-perfluorohexane in water is larger than that for perfluorodecalin in water, since it has a lower molar volume and density.

A comparison between the hydrocarbon-in-water emulsions and perfluorocarbon-in-water can be established. For example, considering the hydrocarbon-in-water emulsions, the diffusion coefficients of linear hydrocarbons in water reported in the literature are in the order of $10^{-9} \text{ m}^2 \text{ s}^{-1}$ [45], their solubilities in water vary from $10^{-1} \text{ mmol dm}^{-3}$ for *n*-hexane to $10^{-2} \text{ mmol dm}^{-3}$ for *n*-hexadecane [43], and their interfacial tensions with water are about 50 mN m^{-1} [46]. Due to their structure, perfluorocarbons are less soluble in water than hydrocarbons and have lower diffusion coefficients and higher interfacial tensions and molar volumes as shown in Table 2. These differences in the thermophysical properties between the hydrocarbon and the perfluorocarbon family lead to Ostwald ripening coefficients substantially lower for the perfluorocarbons than for the hydrocarbons. The Ostwald ripening coefficient for perfluorohexane emulsions, and thus the probability to suffer degradation by molecular diffusion is about 500 times smaller than in the analogous hydrocarbon at the same temperature. These Ostwald ripening coefficients are reported in Table 2 and were determined using Eq. (3).

Sakai et al. [43] concluded that oils with a higher vapor pressure have a higher molecular diffusion rate, due to the weaker interaction between their molecules. In fact, values in Table 2 show that perfluorodecalin has a larger molar volume and a lower vapor pressure than *n*-perfluorohexane and consequently has a lower molecular diffusing rate in the continuous medium, explaining the differences in the main mechanisms of both perfluorocarbons studied.

4.3. Surfactant effect

Unlike the results presented by Postel et al. [8], where Ostwald ripening is responsible for the aging of all perfluorocarbon emulsions studied by other authors, the surfactants used in this work seem to have a degradation mechanism associated to them. According to Table 3 and Figs. 3–7, lecithin emulsions (Emulsions 1 and 4) show stability loss by coalescence at both perfluorocarbons and temperatures. Emulsions prepared with Pluronic F-68 (Emulsions 3 and 6) usually degrade by molecular diffusion, with exception for Emulsion 6, where the temperature increase promotes the coalescence. Span 20 emulsions (Emulsions 2 and 5) seem to age by coalescence, with exception of Emulsion 2 where the aging mechanism changes with temperature.

The surfactant influence is usually explained based on the surfactant structure and on the hydrophilic–lipophilic balance (HLB). It has been showed that as HLB increases, coalescence decreases. Nonionic emulsifiers may stabilize oil-in-water emulsions through short-range steric forces, which are sufficiently strong to prevent droplets from getting close enough to aggregate. As HLB decreases, the number of hydrophilic head groups that protruded into the aqueous phase decreases and the prevention of the droplets to coming together also decrease [47,48]. According to the HLB values for the surfactants used (lecithin: 8.0, Span 20: 8.4, Pluronic F-68: 24) [47], emulsions prepared with Pluronic F-68 have a lower probability of stability loss by coalescence as verified in this work.

Kabalnov and Shchukin [42] concluded that phospholipid membranes possess very specific permeability for non-electrolytes and that the diffusion of highly hydrophobic substances through these membranes proceeds substantially slowly. This results in very low transmembrane permeability of perfluorocarbons, leading to very high stability due to the formation of an adsorption layer, and probably explains the fact that the lecithin emulsions always lose stability by coalescence in this work.

4.4. Aqueous phase effect

Comparing Figs. 5 and 7 and analyzing the results in Table 3, the solutes present in the aqueous phases of Emulsions 7–9 do not change the emulsions aging mechanism. However, a dramatic effect on the stability of the lecithin emulsions (Emulsions 4 and 7) was found. Lecithin, being a zwitterionic surfactant, is strongly affected by the ionic

compounds present in the YPD medium, leading to quicker degradation of the emulsion.

5. Conclusions

The stability of emulsions of two 50% (w/v) perfluorocarbons with three surfactants in two distinct aqueous phases and at two storage temperatures was studied by image analysis. The coupling of this automatized procedure with advanced digital microscopy allows the analysis of a larger number of objects with higher precision and accuracy.

The results indicate that, unlike previously reports in the literature for emulsions of lower perfluorocarbon content, coalescence often takes place in concentrated oil-in-water emulsions of perfluorocarbon compounds and seems to be favored by higher temperatures of storage. The introduction of charged species in the aqueous phase does not seem to affect the aging mechanism of the studied emulsions.

It should be noticed that some emulsions, due to their low coalescence constant, require analysis during long time periods for correct identification of the degradation mechanism.

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