Phase behavior and formulation of structured drops in triphasic systems

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***Abstract*—This work sets a first step towards the formulation of a two-domain drug carrier formed by an ATPS (Aqueous Two Phase System) of a solution of two biocompatible polymers, gelatin and poly(ethylene glycol) diacrylate (PEG-diAc), dispersed in an organic solvent. The main objectives of the work are the study of the phase behaviour and to achieve control over the morphology of the ATPS drops at micro and nanoscale by adjusting different parameters of the emulsification process, followed by optical microscopy.**

***Index Terms*—3. Nanoparmacotherapy: ATPS, emulsion, nanocarrier formulation, optical microscopy.**

I. INTRODUCTION

A. Aqueous Two-Phase Systems (ATPS)

Phase separation phenomena is expected for immiscible mixtures of oil and water. It is less known that this separation can also occur in mixtures that contain water as the only solvent, a kind of biphasic systems called Aqueous Two-Phase Systems (ATPS)[1]. Instead of incompatibility between solvents, what causes phase separation is the incompatibility between the substances dissolved in water.

Often, ATPS are formed in mixtures of two hydrophilic polymers, which form two liquid phases in equilibrium, each of them containing predominantly one of the two polymers. . The phase separation is due to thermodynamic incompatibility between the two polymers, which is mainly caused by either differences in hydration or molecular weight of the polymers. The different hydration capacities results in a positive enthalpy of mixing, associated with interactions between different molecular segments. Opposite to this enthalpic repulsion ($\Delta H_{Mixture}{}^{0}$) that promotes phase separation, intermolecular diffusion is described by differences in molecular weight produce a positive but smaller entropic contribution, $T\Delta S_{Mixture}{}^{0}$, which favors mixing. However, this term is rather small in the case of two molecules with large and different chain lengths, y of mixing, since shorter one polymers restricts the freedom of conformation of the other longer polymers. Altogether, enthalpy predominates and it produces a positive free energy of mixing ΔG_Mixture^0, as explained by equation (1), leading to unfavorable mixing.

$$\Delta G^{0}_{Mixture} = \Delta H^{0}_{Mixture} - T\Delta S^{0}_{Mixture} \quad (1)$$

At low concentrations the polymer molecules are separated enough in the aqueous solution, so the repulsive forces between them are almost non-existent, and therefore, the entropy of mixing predominates. Consequently, one single phase can be observed in the system at low concentration levels. However, upon rising the concentrations of the dissolved species, enthalpic repulsions predominate, resulting in the separation of the system in two aqueous phases.

In phase diagrams of ATPS, we can observe two different areas, corresponding to the monophasic and biphasic regions at different concentrations of the polymers, separated by a curve called binodal line. The tie lines link two points on the binodal line, representing the equilibrium concentrations of the two coexisting phases in the biphasic zone of the diagram.

Another factor to take into account in ATPS is that phase behavior of aqueous polymer solutions is highly influenced by the ionic strength of the solution. The presence of enough concentration of a salt, depending on the salting-out abilities of the salt species, can induce segregation in a salt-rich phase in the bottom and a polymer-rich phase in the top, due to the repulsive interactions between polymer chains and ions.

Most often, phase separation in non-ionic ATPS is very fast[2]. The repulsive forces between drops is almost non-existent, as the ζ-potential of the drops has often very low values, making w/w emulsions highly unstable. However, it is possible to have a certain degree of control over the rate of phase separation, by adding other components that temporarily stabilize the emulsion, which include particles or other polymers. Control over the phase separation will be very important not only to obtain more stable w/w emulsions but also for the formation of nanostructured emulsion drops, as explained in section I.B.

This kind of systems has many applications such as bioseparation[1], like a water-organic solvent system without the environmental risks that involves the use of organic solvent. It has also been reported[3] that ATPS can be used for separation of biological structures like cells, viruses, organels and proteins and that the polymers forming ATPS have stabilizing properties on particle structures and biological activities. Other kind of applications for ATPS are environmental remediation, biotechnological products recovery and other analytical applications such as separation of cells in subpopulations, surface charge and isoelectric point estimation via pH and ionic strength changes, quantify biomolecular interactions, etc[1].

The phase diagrams of many ATPS have been determined and published[4,5,6]. In this work the phase diagram of the aqueous mixture of gelatin and poly(ethylene glycol) diacrylate was studied and determined at two different temperatures.

B. Three-phase systems and structured drops

ATPS provide a simple route for obtaining three-phase systems as both aqueous phases are immiscible with hydrophobic solvents such as oils. In this way, we can obtain water-in-water-in-oil (w/w/o) multiple emulsions by simple processes, and tune the properties of these systems by optimizing the preparation method, in order to achieve different drop morphologies at micro and nanoscale, just by varying macroscopic parameters such as temperature, concentration of components, agitation conditions, etc., during preparation. The term structured drop refers to the morphology of w/w emulsion droplets inside the oil continuous phase. There is a great range of different drop morphologies that can be achieved, varying from typical multiple emulsions (multiple droplets in a random distribution inside a bigger drop) to more complex structures such as core-shell drops, ellipsoidal and spherical Janus drops or multi-core drop coverings. The main goal in this work was obtaining core-shell and Janus morphologies, by formulating three-liquid phase systems, combining an ATPS, consisting in two aqueous phases, with an oil.

In triphasic liquid systems, the morphology of droplets is controlled by interfacial tensions between various phases.According to Guzowski et al[7], it is possible to classify the morphology of the drops in three categories: (a) complete engulfing (core-shell), (b) partially engulfing (corresponding to various Janus morphologies) and (c) non-engulfing (individual drops of each of the two phases in the liquid host). The stability of the drop morphology is expressed in terms of the spreading coefficient of each phase over the others, S_i (eq. 2):

$$S_i = \gamma_{jk} - \gamma_{ij} - \gamma_{ik} \qquad (2)$$

Being *i*, *j* and *k* the different phases of the triphasic system, if this coefficient is negative (S_i < 0) for phase *i*, interface formation between the other two phases, *j* and *k*, is expected.

In a three phase system were all the phases are liquid there are three possible interfaces to be formed. From now on, we will refer to the interfacial tension of each aqueous phase against the oil continuous phase as γ_A and γ_B; while γ_{AB} refers to the interface between aqueous drops of different composition (Fig. 1a). The equilibrium shape is determined by the balance of forces acting on the interfaces, related to the contact angles θ_A and θ_B, that can also be expressed in the form of relations (Neumann triangle, Fig.1a) between them and the interfacial tensions:

$$\gamma_{AB}\cos\theta_B + \gamma_B + \gamma_A\cos(\theta_A + \theta_B) = 0 \qquad (3)$$

$$\gamma_{AB}\cos\theta_A + \gamma_A + \gamma_B\cos(\theta_A + \theta_B) = 0 \qquad (4)$$

Consequently, the values of the interfacial tensions play a major role in the final shape of the drops. The ratios between γ_A and γ_B with γ_{AB}, as schematized in Fig. 1b, determine the most stable morphology:

- *Core-shell* (complete engulfing): $\gamma_A > \gamma_B + \gamma_{AB}$, interface A (between aqueous solution A and oil) is not formed.
- *Separated drops* (non-engulfing): $\gamma_{AB} > \gamma_A + \gamma_B$, interface AB (between the two aqueous solutions) is not formed.
- *Janus* (partially engulfing): When neither of the inequalities above is fulfilled, all three interfaces coexist within the drops (A, B and AB).

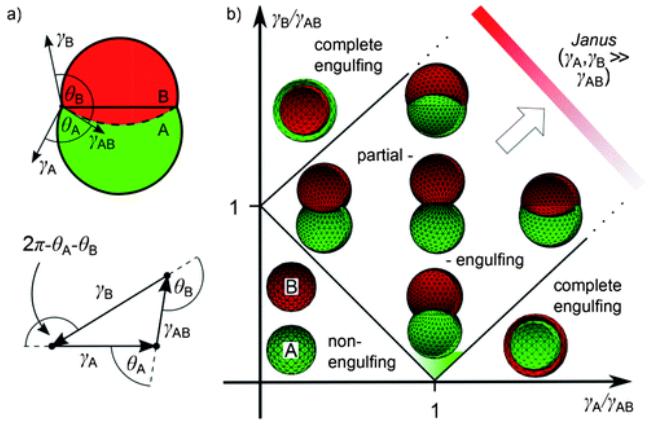

Fig. 1: *(a) Scheme of a Janus-shaped drop indicating the contact angles θ_A and θ_B and Neumann's triangle. (b) Diagram of the stable drop morphology for a biphasic drop as a function of the ratios between surface energy values. Extracted from Guzowski et al[7].*

There is a high interest in this complex drop morphologies, as different phases can become carriers for different materials (inorganic compounds, biostructures, particles, etc.). Specific loadings of materials will show preference for one of the three phases, resulting in an easy multifunctionalization of the drops, opening the door to many potential applications that can be developed in biopharmaceutics, food, cosmetics, separation sciences, etc. The search for controlled methods of production of complex drops, as many structured emulsions cannot be obtained by conventional procedures, is still ongoing. Combination of different emulsification methods, surfactant control of surface energy, use of microfluidic techniques and other new technologies are some of the new approaches and many of these methods are still a challenge in terms of parameter control. A very interesting approach is the phase-separation-induced technique for complex structured emulsions, based on the use of external stimulus (changes in salinity, pH, light or temperature) to achieve the segregation of the system by changing the solubility of its components. Applied to polymeric ATPS, phase separation is expected, and in order to control drop morphology, the mass ratio between the two solubilized components needs to be optimized.

The main drawback of the stimulus-induced phase separation method is the reduced number of systems in which it can be applied, as not many mixtures may show segregation induced by an external stimulus. Moreover, stimulus-induced phase separation cannot always be performed, for example, in

the case of emulsions containing labile bioactive ingredients, like delicate drugs, cells or proteins that may denaturalize.

The choice of the components must be strictly studied in order to achieve a viable product.

C. Biocompatible emulsions for future applications

As stated before, ATPS structures hold many potential applications in fields such as biopharmaceuticals, biotechnology and environmental sciences or food and cosmetics industries, among others. All the different uses of this kind of systems have one common issue: They set a series of conditions over the properties of the ATPS structure, requiring them to be biocompatible, biodegradable and stable.

The factors taken into account in order to fulfill all the requirements are the nature of the components, by using non-toxic polymers, and the physical properties they present, by studying ways of enhancing stability via different gelation processes.

Hydrogels, jelly-like materials formed by three dimensional networks obtained by crosslinking of polymeric chains in aqueous media are becoming a promising alternative route for drug encapsulation and controlled release[8]. According to the forces that sustain this crosslinked structure, gels are classified in physical gels, in the case of weak bonds formation due to Van der Waals and other non-covalent interactions, and chemical gels, when crosslinking comes from the formation of covalent bonds in the internal structure.

Gelatin is a natural polymer, product of the partial hydrolysis of collagen, that holds many interesting properties both as a polymer and a gel[9]. It forms a physical gel at room temperature with a reversible gel-sol transition around 35ºC, as long as the hydrolysis temperature, above 65ºC, is not reached. Gelatin has been vastly studied as a component for polymer-polymer as well as polymer-salt ATPSs, and its physicochemical properties make it a great choice for the kind of systems developed in this work.

Poly(ethylene glycol) (PEG) is a commonly used non-toxic polymer that can form ATPSs with gelatin[10]. For the sake of studying different gelation processes, a polymerizable derivate is chosen: Poly(ethylene glycol) diacrylate (PEG-diAc). It is expected of this compound that the main properties that make PEG an interesting component for this kind of systems are maintained, and the acrylate groups facilitate the chemical gellification as they provide polymerization capacity, induced by free radicals. This product is liquid at room temperature, but its melting point is between 12-17ºC, and below forms a ductile solid, so physical gellation by cooling is also possible.

Concerning the choice of organic solvent, any liquid hydrocarbon will be hydrophobic enough to be immiscible with both aqueous phases, and the optimal surfactant for the stability of complex drops will be studied in terms of biocompatibility, loading capacity of both aqueous phases in the organic matrix, stability of the emulsion formed and effect over the interfacial tensions, as discussed in section I.B. Decane was selected as a model hydrocarbon.

II. EXPERIMENTAL SECTION

A. Chemicals

Gelatin from bovine skin Type B (Sigma-Aldrich), Poly(ethylene glycol) diacrylate 700Da (Sigma-Aldrich), Fluorescein 5(6)-isothiocyanate (Sigma-Aldrich), Dimethyl sulfoxide (Sigma-Aldrich), sodium persulfate (Sigma-Aldrich), n-decane for synthesis (Sigma-Aldrich), Span-80 (SIGMA), Tween 80 (Sigma-Aldrich), Pluronic PE 10100 (BASF), KF-6104 (Shin Etsu), Hypermer 2296 (Croda), and deionized water were used.

B. Methods

- *Phase diagram construction*

For the determination of the phase diagram a series of sample compositions were selected, between 2-20 wt% content of gelatin and poly(ethylene glycol) diacrylate (PEG-diAc) in water. Each sample (3 g) is prepared following the same protocol: weighing the needed amount of gelatin, adding the correspondent quantity of water and letting it dissolve at 47ºC for about half an hour. Once it is dissolved, the weighted quantity of PEG-diAc is added and the system is mechanically stirred until complete mixing, and after that it is left for 24 h to rest at the desired temperature. Later on, visual inspection allows determining whether phase separation happens or not.

Two phase diagrams are constructed, one at a working temperature of 47ºC, using 32 samples and the other at 22ºC, with 12 samples.

- *Gelatin labelling*

In order to distinguish the gelatin-rich phase from the other aqueous phase when characterization by optical microscopy is performed, as both phases will have very similar refractive indexes, the labelling of the gelatin with fluorescein isothiocyanate (FITC) will be performed[11].

The process includes covalently bonding the fluorophore by adding into the water-gelatin solution 2 µL of a solution 2% (w/w) of fluorescein 5(6)-isothiocyanate in dimethyl sulfoxide (DMSO), after the gelatin has been completely solubilized, and left for 15 minutes at 47ºC so that the reaction between gelatin and the FITC takes place before adding the PEG diAc to the system.

- *Polymerization test*

Three samples containing a) pure PEG-diAc, b) 20% (w/w) PEG-diAc in water and c) 20% (w/w) PEG-diAc in water and a 1% of sodium persulfate were left for 2 months at a constant temperature of 47ºC. They were observed periodically in order to test: stability of the monomer at working temperature, behaviour of the sodium persulfate as initiator of PEG-diAc polymerization and time needed for the reaction to be completed in normal working conditions. Further characterization of the polymerized sample was carried out by SEM (TM-1000 Tabletop Microscope, HITACHI).

- *Identification of phases in biphasic mixtures*

Four emulsions were prepared by mechanical emulsification at 47ºC, by preparing both aqueous and organic phases separately and mixing them with an UltraTurrax homogenizer during 30 s at 13500 rpm (225 s^{-1}) and later observed under

optical microscopy. The compositions of these emulsions were:

a) w/o emulsion containing 10% (v/v) of a water solution 10 wt% of labelled gelatin in n-decane organic phase.

b) w/w emulsion containing 10 wt% of labelled gelatin and 20% of PEG-diAc in water.

c) w/o emulsion containing 5% (v/v) of a water solution 20 wt% of PEG-diAc in n-decane organic phase.

d) w/o emulsion containing 5% (v/v) of a water solution 20 wt% of PEG-diAc and 1 wt% in sodium persulfate in n-decane organic phase.

- *Preparation of double emulsions:*

In a typical experiment, the two phases are prepared separately and then mixed and emulsified. The organic phase is prepared by mixing 9 mL of n-decane and 0,5 mL of surfactant under magnetic stirring during 15 minutes. The aqueous phase (ATPS) consisted in an emulsion of labelled gelatin and PEG-diAc in water, prepared following the same steps as the samples for the phase diagram construction, and emulsified.

Finally, 500 µL of the ATPS are added to the prepared organic phase at 47ºC and emulsified either by:

A. *Mechanical emulsification*: Sample is mixed with UltraTurrax during 1 min at 13500 rpm (225 s^{-1}) and left under magnetic stirring at the desired temperature.

B. *Ultrasound emulsification*: First the sample is mixed with the UltraTurrax during 30s at 13500 rpm (225 s^{-1}) and then placed in the Bandelin SONOPLUS ultrasonic homogenizer at 10% power during 20s and left under magnetic stirring at the desired temperature.

Other parameters studied and optimized were:

-Emulsification of the ATPS, either by mechanical homogenization or temperature-induced phase separation.

- Surfactant species employed and optimal concentration in the organic phase

- Polymerization stage, by adding a 1% (w/v) of sodium persulfate at different points during the preparation.

- Storing temperature after the preparation; emulsion stability with time.

After the preparation, the samples are characterized by optical microscopy using an OLYMPUS BX51TRF-6 optical microscope in fluorescence mode at ambient conditions.

III. Results

A. *Gelatin/PEG-diAc Phase Diagram*

The phase diagram of the Gelatin/PEG-diAc ATPS has been obtained both at working temperature (47ºC) and storage temperature (22ºC), as shown in Fig. 2 and Fig. 2 (inset). We observed that the boundary between the region where phase separation occurs and the single phase region, the binodal line, is situated at higher concentrations for the PEG diacrylate compared to the Gelatin/PEG ATPS phase diagram[4], indicating that the PEG-diAc is more miscible with gelatin than PEG with higher molecular weight. This miscibility change might arise, probably, from the difference in molecular weight of the polymer, indicating that we could tune the position of the binodal line by using samples of different molecular weight of the same polymer. Lower molecular weight implies shorter chains within the molecules, increasing the entropy of mixing, and thus, facilitating the formation of a single phase that contains both polymers. It is known that reducing molecular weight enhances the mixing of polymers[12]. The average molecular mass of the PEG-diAc used in this work is 700Da, while the phase diagrams in Ref.4 were constructed using PEG with average molecular masses of 10.000-20.000Da. Also, comparing the same diagram at the two different temperatures (Fig. 1, inset) it is noted that at lower temperature the miscibility between the two polymers decreases, as expected, because higher temperatures also increase the entropy of mixing and thus, the single-phase region is bigger in the phase diagram. This opens the possibility of emulsification through a stimulus-induced phase separation method.

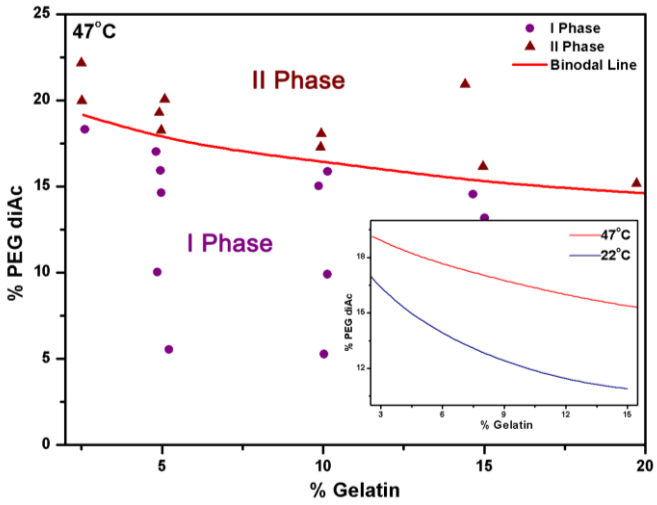

Fig. 2: *Phase diagram of the Gelatin/PEG-diAc ATPS at 47ºC). Inset: Comparison between the binodal line at 22ºC (blue) and at 47ºC.*

The position of the binodal line in ATPS phase diagrams is very important, as it establishes the conditions for emulsification. For mechanical emulsification of w/w and w/w/o samples, it is required to work above the red line, while in the case of stimulus-induced-phase-separation emulsification method of w/w/o samples the working concentration range would be between the blue and the red lines of the inset of Fig. 1.

It is also noticed that the binodal line of the phase diagram is not symmetrical. The gelatin-rich phase uptakes higher amounts of water than the PEG-diAc-rich phase, indicating that gelatin is the most hydrophilic compound of the two, requires larger amount of water for hydration and it produces a phase with larger volume.

B. *Polymerization Tests*

For the three samples stored ad 47ºC, polymerization occurred only in the one containing sodium persulfate. In this case, the reaction was completed in less than 1 h, obtaining a solid polymer engulfing the water in the system.

After 24 h, a syneresis effect was noted, with release of approximately 10% of the water content of the original

solution. The other two samples, containing no initiator, lasted over two months at 47ºC without showing any change, time enough to ensure the stability of the sample. No change in the polymer chemical state will betide unless we provoke it. Fig. 3.a and 3.b shows the changes observed in the samples.

The polymerization occurs through a free radical mechanism between the acrylate groups of the molecule, which is initiated by the persulfate. The SEM images (Fig. 3.c, d and e) showed the formation of hexagonal crystals. Probably, particles first nucleate, then grow into hexagonal particles (Fig. 3.d and 3.e), and finally the particles agglomerate, forming an amorphous mass, visible at low magnification (Fig. 3.c).

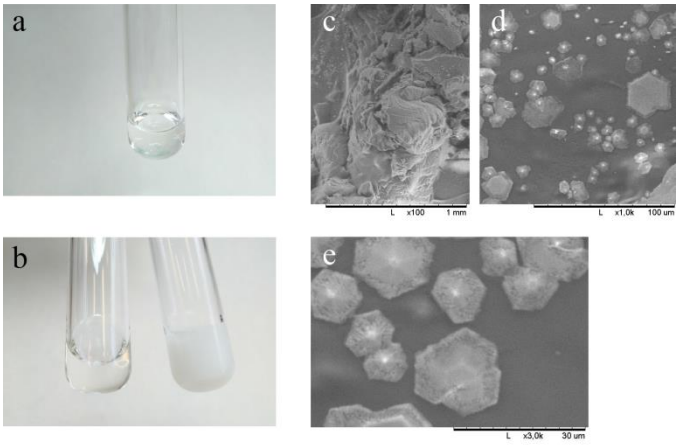

Fig. 3: *(a) Pure PEG-diAc after 2 months storage at 47ºC; (b) Comparison between before and 24h after adding sodium persulfate to a 20% (w/w) solution of PEG-diAc in water; (c), (d) and (e) SEM images of the sample at right in (b) at different magnification levels (scale bars 1mm, 100 µm and 30 µm respectively).*

C. Identification of phases in biphasic mixtures

It has been noted that both gelatin and PEG-diAc are immiscible in n-decane even at very small concentrations. Both emulsions of samples (a) and (c) (compositions indicated in section II.B) were stable for more than 12 h against phase separation, even without the use of any surfactant. However, the size distribution of the aqueous drops obtained in both cases is very polydisperse, as many populations of different sizes, ranging from droplets of 0.5-3 µm to drops around 20 µm, are present. For the case of emulsion (d), the polymerization caused the emulsion breaking into two phases, and emulsions could not be characterized since phase separation was too fast. Sodium persulfate used as initiator was added to the aqueous phase before mixing it with the organic one and emulsifying the sample. It has been proven that, in order to achieve a complete polymerization with no aggregation of polymerized drops, the reaction needs to be initiated after emulsification.

For the w/w emulsion containing labelled gelatin and PEG-diAc it is observed that the w/w emulsion consists of PEG-diAc drops dispersed in a gelatin continuous phase. This is consistent with the fact that PEG-diAc forms the phase with smaller volume. If we gradually increment the quantity of PEG-diAc, without changing the quantity of gelatin, we would reach the bicontinuous state, where both phases coexist both as continuous and discontinuous in different domains. Upon incrementing even more the proportion of PEG-diAc, phase inversion will take place. It is important to remark that, while the physical gelation of gelatin by temperature change is a reversible process, after the preparation of the gelatin/PEG-diAc ATPS it is noted that the gel formed by the gelatin rich phase cannot be disrupted once it has been cooled down, no matter how high the temperature is risen afterwards.

It is also noted that during the phase separation in the ATPS, the PEG-diAc rich phase acquires a certain degree of fluorescence under microscope observation, thanks to the residual content of fluorescein labelled gelatin within the PEG-diAc phase. Consequently, gelatin and PEG-diAc show different intensities of fluorescent emission, allowing identification of each phase in triphasic systems. In a single microscope observation using fluorescence filters, three different fluorescence levels will be distinguished, each corresponding to one of the three phases: the brightest drops correspond to the gelatin rich phase, the semi-bright ones to the PEG-diAc rich phase, and the dark regions correspond to the organic phase where the fluorescein dye is not soluble.

D. Drop morphology in triphasic systems

Samples in triphasic systems contain 90% in volume of the organic solvent, n-decane, and the remaining 10% includes the surfactant and the aqueous (inner) phase. This aqueous phase will contain 10 wt% of gelatin and 20 wt% of PEG-diAc. Several parameters are optimized in order to achieve control over the resultant drop shape:

• *Surfactant:* From the group of surfactants tested (Span 80, Tween 80, Pluronic PE 10100, KF-6104 and Hypermer 2296) in equal conditions of temperature and composition, the one that exhibited better solubilization and stability enhancement properties was Span 80, followed by KF-6104. Span 80 is the commercial name of the lipophilic surfactant sorbitant monooleate, insoluble in water and practically non-toxic[13] for human consumption, making it a good choice for this work.

To determine the optimal surfactant/inner phase volume ratio, three samples were prepared an characterized, in the same conditions, containing (1) 2.5% (v/v) of surfactant and 7.5% (v/v) of aqueous inner phase, (2) 5% (v/v) of surfactant and 5% (v/v) of aqueous inner phase and (3) 7.5% (v/v) of surfactant and 2.5% (v/v) of aqueous inner phase. The resultant drops can be seen in figure 4, where the dark background corresponds to the organic solvent, that contains no molecules labelled with fluorescein, the brighter drops correspond to the gelatin-rich phase of the ATPS and the semi-bright zones correspond to the PEG-diAc-rich phase.

In samples 1 and 3, the size distribution is quite polydisperse. Drops of both gelatin and PEG-diAc were found separated from each other (Fig. 4.1.a and 4.3.a), among PEG-diAc-coated gelatin drops, and aggregation between these gelatin drops was visible. Also, PEG-diAc drops lack the spherical shape expected, but it is found that it encapsulates gelatin drops in a multiple-core-like coating (Fig 4.1.b and 4.3.b). The size distribution in sample 2 (Fig 4.2) is far much monodisperse, the drops are smaller and the emulsion is more uniform. Smaller drops prove to be more kinetically stable than the bigger ones, so smaller the average size, longer time

before phase separation. One can also observe the formation of structured drops in this sample: small drops of gelatin depositing at the PEG-diAc/n-decane interface (Fig 4.2.a) and core-shell structures of one of the ATPS components encapsulating the other in a perfectly spherical shape (Fig 4.2.b). From these results, the optimal surfactant-to-inner phase volume ratio is found to be 1:1, corresponding to that of sample 2 (Fig. 4.2).

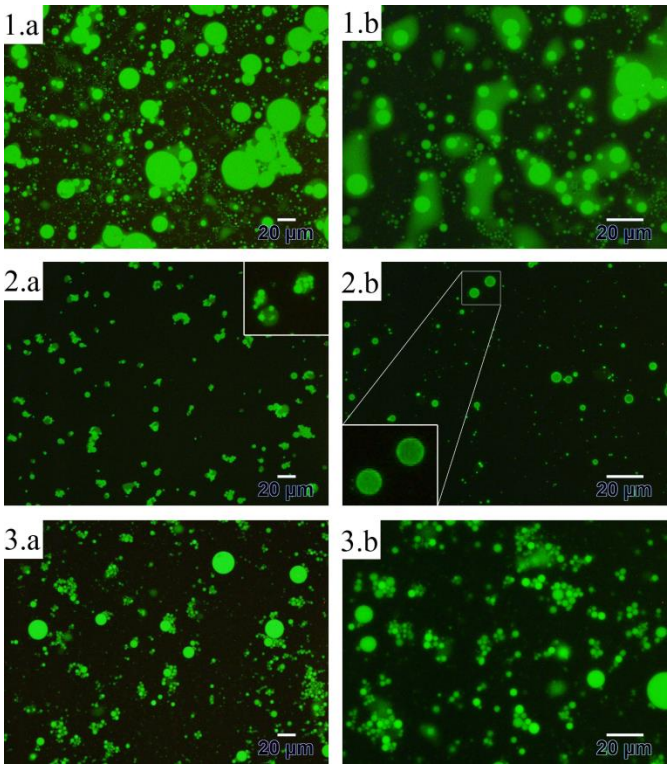

Fig. 4: *Fluorescence microscopy of samples 1, 2 and 3 at two different magnifications. Scale bar in each image: 20 µm. Insets: magnification of morphologies of interest.*

The surfactant might strongly affect the interfacial tensions between the ATPS drops and the organic solvent. Optimization was made in terms of stability of the inner emulsion within the organic solvent, without taking into account surface tension between the phases of the ATPS or other morphological parameters that are affected by the surfactant; that will be approached in following sections.

•*Gelation:* One of the drawbacks to avoid, or at least to reduce , is the aggregation that leads to phase separation. This could be achieved by gelification of the drops, which improves the stability of the dispersion. As it was previously stated in section I.C, the two polymers can be used to form hydrogels by different methods.

In the case of PEG-diAc, chemical gelation through radical polymerization reaction would produce an irreversible gelation of the PEG-diAc drops, obtaining a crosslinked microgel. The optimal concentration of the polymerization initiator (sodium persulfate) has been determined to be 1 wt%, so the main parameter to achieve control over the correct polymerization of the sample is the precise moment were the reaction should start. Several tests were conducted by adding the initiator to the mixture at different possible stages: before adding the PEG-diAc during the ATPS synthesis, after the ATPS emulsification, in the organic solvent before adding the aqueous phase to it and finally after the emulsification of the aqueous phase in the organic solvent. The observed results were that if the reaction starts too early the PEG-diAc coagulates, breaking the ATPS as the crosslinking between chains expels the gelatin from the drops, so the emulsification is not possible. In the case of adding the initiator to the organic phase alongside the surfactant, the dispersion is not possible because of the repulsive interactions between the solvent and the sodium persulfate, even in the presence of the surfactant, causing the initiator to precipitate. In this situation, when PEG-diAc-containing ATPS is added, the polymerization is not homogeneous and the obtained emulsions lasted just a few hours before experiencing phase separation. The best condition found for PEG-diAc polymerization within the emulsion was the polymerization after the whole w/w/o emulsion was produced, by adding the initiator and disperse it with the homogenizer. The drop morphology was not affected by the polymerization but failed to meet the expectations about stability. Further research over this aspect needs to be performed in order to explain the complete behavior.

In the case of gelatin, it presents the possibility of thermoreversible physical gelation, as under 30ºC it forms an hydrogel, so by cooling the sample after the emulsification and storing it at room temperature, elastic gels can be obtained, which do not affect the drop structure and efficiently improve the emulsion stability. As it has been previously stated, in this particular case, and due to the interaction with PEG-diAc, the reversibility of the gelation after cooling has been compromised, but far from being a disadvantage it has improved the emulsion performance as it can be heated again over 40ºC without breaking the emulsion. As the chemical gelation of PEG-diAc within the emulsion has not fulfilled the expectations, an alternative physical gelation by cooling is proposed. PEG-diAc below 12ºC forms an amorphous white solid with mechanical properties closer to those of the gelatin gel than the polymerized solid obtained in previous tests. Storage at 5ºC after the formation of the w/w/o emulsion was tested, but after 24h the emulsion starts to break because of coagulation.

Finally, the main pathway to slow down phase separation is by storing the samples at room temperature under continuous stirring and by reducing the drop size to nanometer size. The stability control needs to be evaluated in further works as the emulsions were not very stable.

•*Emulsification*: From the many existent methods to form emulsions[14, 15], we need to find the one that is more suitable to obtain stable, homogeneous emulsions with small and monodisperse drop size that allow the ATPS to segregate correctly into different morphologies upon parameter control.

For this procedure, mechanical emulsification using a high shear homogenizer (UltraTurrax) was performed at high speed, specified in section II.B, during 1 min, being the simplest and cheapest method, but the resultant emulsions lack homogeneity and stability. The size range of the drops is very broad and the drop structure control is lost due to the shear forces generated. In order to achieve less polydispersity, the emulsification needs to be homogeneous.

To overcome this problem, the available options are divided in two groups: External source providing energy enough in a

way that the whole sample receives the same quantity at the same time, or by a change in the internal energy of the system, coming from a phase inversion. As previously stated, ATPS are susceptible to changes in their miscibility by external stimulus such as changes in salinity, pH or temperature, so we can change the number of thermodynamically stable phases present without changing the polymer composition. This kind of changes affect the whole of sample so the emulsification occurs simultaneously, obtaining smaller drops and narrower size distributions[16]. The binodal line of the phase diagram changes its position when measuring it at room temperature, as the miscibility of the polymers increases with temperature. Adapting our starting composition for the ATPS to one between the binodal lines at the two temperatures (5 wt% content in gelatin and 15 wt% content in PEG-diAc) one can work with a w/o emulsion at high temperature, obtaining the desired dispersion of the aqueous phase in the organic solvent. Once all the parameters have been adjusted, the sample is cooled down, inducing the separation of aqueous phase into two phases, resulting a w/w/o emulsion with the desired drop structure.

Now, for the external w/o emulsion, induced phase separation is not achievable, since there are no conditions in which the aqueous and the organic phase can exist within a single phase, so there is need of an external energy source. As an improvement of the high shear homogenizer, the chosen approach uses ultrasonication, which can produce chemical or physical changes in a medium through acoustic cavitation. The expansion of acoustic waves generated by an ultrasonic device through the sample causes the formation of very small cavities with extreme temperature and pressure conditions over much reduced time windows, providing the energy to generate micro or nanodroplets with very narrow size distribution in a very stable emulsion[17, 18]. This energy contribution comes with a temperature rise during the process that helps maintaining the conditions for the aqueous phase not suffering segregation during the external emulsion formation.

•*Final drop morphology:* In Fig. 5 one can observe that, upon further refining of the technique, some Janus-like structures can be obtained. The composition is indicated in Table I.

The emulsion was produced by mechanical homogenization at 47ºC using UltraTurrax for 1 min at 13500 rpm (225 s^{-1}) and left under continuous magnetic stirring at 22ºC for two hours before observation. No polymerization of the PEG-diAc drops was performed on this sample. The result is an emulsion containing drops in a Janus-like structure, with PEG-diAc-rich ATPS drops around 10 µm and smaller (1-2 µm of diameter) gelatin-rich ATPS drops on the surface, forming a structured drop system of three interfaces as described in section I.B. However, the morphology of the drops is far from the ideal one. The size of both parts of the total ATPS drop should be similar, and the ideal contact angle between the phases should be around $\pi/2$. In contrast, the obtained contact angle is about $\pi/6$ (Fig. 6). The ATPS drop system should contain just one gelatin-rich drop in contact with one PEG-diAc-rich drop, but the difference in sizes causes that the smaller drops form more than one interface with the bigger drops of different composition.

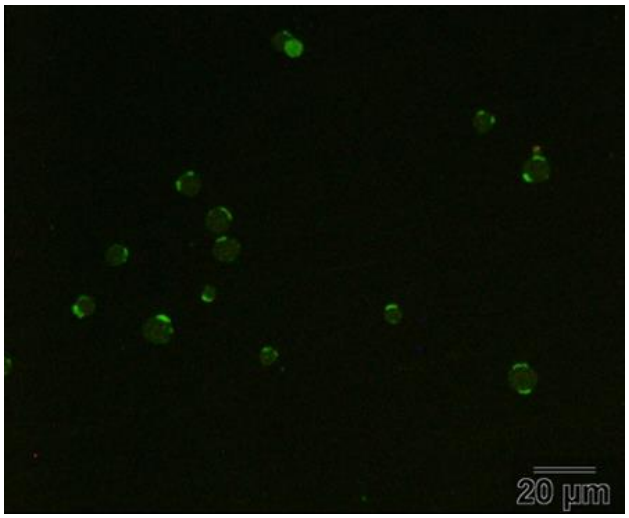

Fig. 5: *Optical microscope fluorescence image of sample containing Janus-like-shaped drops. Composition in Table I. Scale bar: 20 µm.*

TABLE I
COMPOSITION OF SAMPLES IN FIGURE 5

Volume % of each phase	Composition of each phase
95% Organic phase	n-decane
5% Surfactant	Span 80
5% Aqueous phase	10 wt% Gelatin 20 wt% PEG-diAc

In order to obtain single core-shell drops, instead of multicore ones, while attempting to solve the polydispersity problem, concentration of surfactant was changed, influencing the interfacial tensions. Samples were prepared by ultrasonic homogenization of the w/o emulsion and posterior w/w separation induced by temperature change, in order to obtain uniform size distribution and drop morphology, as well as increased stability. The composition of the samples was 5% volume of ATPS (containing 5 wt% of gelatin and 15 wt% of PEG-diAc), and a 95% in volume of n-decane and Span 80. The concentration of surfactant was varied from 1 wt% to 5 wt%. The obtained results can be seen in Fig. 6.

It is noted that the presence of multicore drops is decreased at higher surfactant concentration, while it is still present at low concentrations (Fig. 6.a), and polydispersity is also decreased in comparison with Fig. 5, so sample uniformity and monodispersity has been improved. For the obtained core-shell structures, it is noted that the core is always composed by gelatin and the shell by the PEG-diAc phase.

These results can be interpreted in terms of interfacial tensions, as indicated in Fig. 1 and Fig. 7. What we observe is that, with increasing surfactant concentration, there is a tendency of the drop morphology to evolve from single/multicore-shell morphology to a Janus morphology. Fig. 6.a shows that in the core-shell structure the nuclei are not perfectly centered, and from Fig. 6.b to Fig. 6.d we can see a displacement of such nuclei to the interface of the shell with the organic phase, that eventually creates the third interphase.

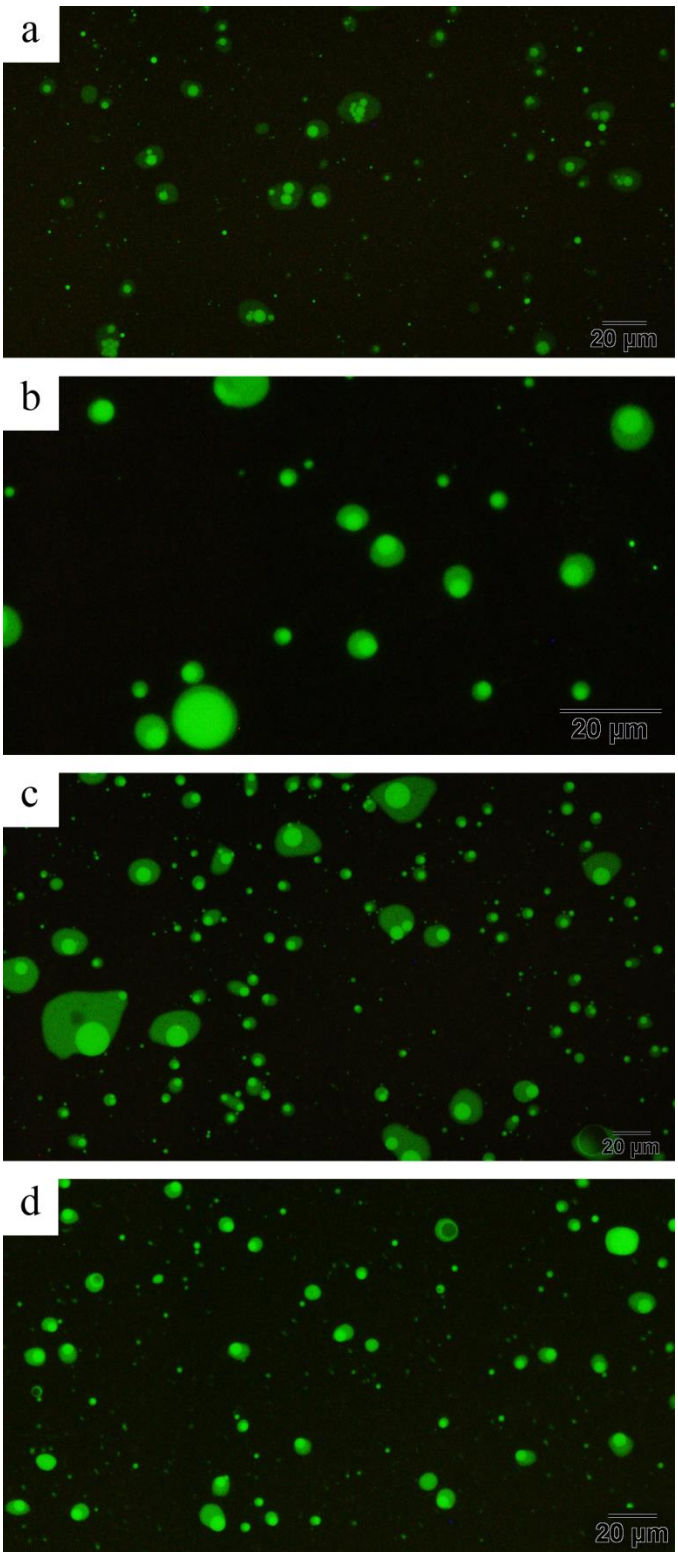

Fig. 6: *Optical microscope fluorescence image of a set of samples containing varying percentage in volume of surfactant: (a) 1%, (b) 2%, (c) 4% and (d) 5%. Scale bars: 20 µm.*

An interesting observation is that this mechanism of Janus drop formation leads to very low contact angles (Fig. 7). It may be that if we keep increasing the concentration of surfactant in the system we would get the desired Janus morphology at a π/2 contact angle between both phases and the organic solvent, but from previous test it is been concluded that too much surfactant can hinder the morphology control (Fig. 4.c). The fact that, in similar concentration conditions of both polymers, and similar sizes of the two ATPS phases, it is always the PEG-diAc the one engulfing the gelatin demonstrates that the interface of gelatin-rich phase with the organic solvent is the one with highest energy, and for that it tends to hide, provoking an expansion of the lower energy interfaces. It was stated in the first part of section III that PEG-diAc proved to be more hydrophobic than gelatin based on the difference of water uptake. This fact is corroborated by the difference in surface energies of both polymeric phases with the organic solvent and it can explain the lack of sphericity of the PEG-diAc drops observed in all the images of Fig. 4.

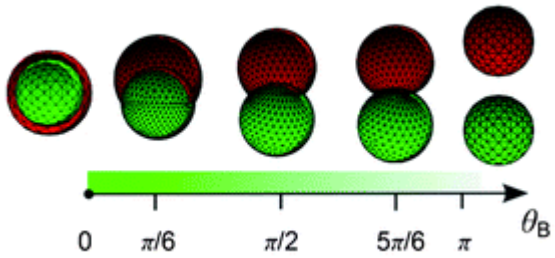

Fig. 7: *Scheme of the stable drop morphology as a function of the contact angle B, defined in Fig.1a, when γA ≈ γAB and γB << γAB (adapted from Guzowski et al[7]).*

In the studied systems, there is only one surfactant present, situated at the interfaces that involve the organic phase, but not at the interface between PEG-diAc and gelatin-rich aqueous phases, so only one kind of surface energy can be tuned. It is well known that interfacial tensions at w/w interfaces are very low, as small as 10^{-2} nN/m range[2], and that diffusion between both aqueous phases is high. For further work in this system, and to stabilize the w/w interface and make the three different surface energies equal, it would be possible to introduce different species between the PEG-diAc and gelatin-rich aqueous phases, acting as a stabilizing agent, such as a different kind of surfactant, because traditional amphiphilic ones would not be useful, or particles of different nature.

IV. Conclusion

The phase diagram of the gelatin/PEG-diAc ATPS has been determined at two different temperatures.

The results show that miscibility increases with temperature, expanding the region of one single phase. Surfactant Span 80 was selected for formation of drops in the w/w/o system. Multicore drops, consisting in various gelatin primary drops inside bigger PEG-diAc secondary drops, were formed at low surfactant concentration. Core-shell and Janus-like drops were achieved at a 1:1 surfactant-w/w mixture volume ratio. After studying the total surfactant concentration range between 1 wt% and 5 wt%, it wasdemonstrated that higher surfactant concentrations allow to obtain drops closer to Janus morphology.

However, results also showed that gelatin phase tends to hide and penetrate into PEG-diAc phase. This is probably due to a higher gelatin/decane interfacial tension, in comparison to PEG-diAc/decane interfacial tension. Therefore, more

balanced interfacial tensions would be required for obtaining Janus drops.

Another factor that needs to be further studied is the stability of the structured drops. Searching for alternative strategies to avoid aggregation or changes in the shape with time is needed as the attempts to turn the ATPS drops into microgels did not produce the desired results.

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