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European Journal of Pharmaceutics and Biopharmaceutics 69 (2008) 835–843
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Interactions between poly(ethylene glycol) and protein in dichloromethane/water emulsions: A study of interfacial properties

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Received 6 June 2007; accepted in revised form 25 January 2008
Available online 31 January 2008

Abstract

From adsorption kinetics and interfacial rheological studies performed by using a pendant-drop method, i.e. in conditions close to those of the primary emulsion of the water-in-oil-in-water emulsion-encapsulation technique, it was shown that adsorption of the hen egg-white lysozyme (HEWL) at the water/dichloromethane (DCM) interface can be efficiently slowed down by modulating some parameters. It was shown that a decrease of the ionic strength of the aqueous phase, and the optimization of the density of the poly(ethylene glycol) (PEG) adsorbed film by increasing the PEG concentration or by modulating the polymer chain length, can significantly decrease the rate of adsorption of HEWL at the water/DCM interface. Moreover, it was shown that the choice of the dissolution phase of PEG (DCM or water) clearly influences the results.

Keywords: Poly(ethylene glycol); Hen egg-white lysozyme; Emulsion; Pendant-drop tensiometer; Adsorption

1. Introduction

Rapid growth in the field of biotechnology has led to the development of numerous recombinant protein drugs such as hormones and vaccines [1]. The use of protein delivery systems is a promising way to prevent and cure diseases [2]; in fact microspheres can provide various advantages such as sustained drug delivery, localized release, enhanced drug safety and efficacy, and improvement of patient compliance [3]. Extensive studies are ongoing using biodegradable microspheres for sustained protein delivery [4].

The water-in-oil-in-water (w/o/w) emulsion technique, followed by solvent extraction and freeze-drying steps, is the method commonly used to load peptides or proteins into microparticles based on biocompatible polymers such as poly(D,L-lactide) (PLA) or poly(D,L-lactide-co-glycolide) (PLAGA). With this technique, concentrated proteins can be encapsulated in polymer matrices [5]. However, the first step of the encapsulation process can be detrimental to the integrity of proteins. The exposure of proteins to the water/organic solvent (frequently dichloromethane (DCM)) interface induces structural changes, modifications of protein stability, and results in a loss of biological activity and an increase of an unwanted immune response [6–8]. Moreover, the anchoring of protein in the polymer matrix can result in an incomplete release of the protein [9].

Reviews dealing with the different strategies commonly used to prevent protein adsorption, report that tensioactive compounds competing with proteins at interfaces can minimize protein adsorption [4,10]. Among these compounds,

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Abbreviations: C_{bPEG}, concentration of PEG in the bulk; DCM, dichloromethane; HEWL, hen egg-white lysozyme; PEG, poly(ethylene glycol); γ, interfacial tension; γ_{eq}, interfacial tension at the equilibrium.

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doi:10.1016/j.ejpb.2008.01.021

poly(ethylene glycol) (PEG) is an interesting polymer with various potential applications in the formulation area [11]. PEG, being a flexible, hydrophilic, uncharged, and highly hydrated polymer, presents all the basic requirements for efficient protein rejection [12]. Studies report a potential increase of protein stability in the presence of PEG in the aqueous phase [9,13,14]. Nevertheless, PEG is a peculiar polymer, soluble both in water and in DCM [11]. From bulk FTIR spectroscopy measurements and enzymatic activity assays to assess the structural changes of proteins induced by its exposition to interfaces created during the emulsification process, it was concluded that the addition of PEG in the organic phase could optimize the reduction of the amount of non-covalent protein aggregates and a subsequent loss of activity [2,15]. In such studies, the protecting effect of PEG is not directly estimated at the interface but in the bulk. However, the prevention of protein adsorption at the water/DCM interface directly influences the success of the formulation.

Even if PEG has already been the focus of many interfacial characterization studies, alone [16–19] or in combination with proteins [12,20], the influence of the dissolution phase on the ability of PEG chains to prevent protein adsorption has been poorly investigated.

The pendant-drop method is a technique which makes it possible to experimentally model the conditions of the primary emulsion. Thus, in the present study, by using the pendant-drop method, adsorption kinetics and interfacial rheological properties of PEG films adsorbed from water or from DCM, and of a model protein, the hen egg-white lysozyme (HEWL), were characterized. Moreover, as some parameters like PEG molecular weight and interfacial PEG chain density may influence protein-rejecting properties of PEG-containing coatings [12,21], the impact of PEG bulk concentration and PEG molecular weight on the adsorption of the protein at the water/DCM interface was studied. Finally, we also checked the influence of the ionic strength of the aqueous phase on the adsorption of HEWL at the water/DCM interface.

2. Materials and methods

2.1. Materials

PEG 2000 was a α-methoxy, ω-hydroxy-PEG 2000. The number-average molecular weight ($\bar{M}n$) of the polymer determined by nuclear magnetic resonance at 360 MHz was 2200. PEG 2000 and α-methoxy, ω-hydroxy-PEG 5000, α-methoxy, ω-hydroxy-PEG 17500 HEWL (n° L6876, dialyzed and lyophilized, containing the buffer salts sodium acetate and sodium chloride, 3× crystallized protein at approx. 95%) were purchased from Sigma Chemical Co. (L'Isle d'Abeau, France), and were used without further purification. Dichloromethane (DCM) was purchased from Prolabo (Paris, France). NaCl, for analysis, ACS, and ISO was supplied from Merck (Nogent-sur-Marne, France). Ultrapure water was obtained from a Millipore® system (Milli-Q Plus 185, Molsheim, France).

2.2. Interfacial tension measurements

Adsorption kinetics of HEWL or/and PEG were obtained at the water/DCM interface by means of a pendant-drop method (TRACKER®, TECLIS, Longessaigne, France) [22]. A DCM drop was formed with an Exmire microsyringe (Prolabo, Paris, France) into an optical glass bowl (Hellma, France) containing the aqueous phase (eventually dissolving HEWL). PEG was dissolved in one or the other phases depending upon the experiment. The axial symmetric shape (Laplacian profile) of the drop was analyzed by using a video camera connected to a microcomputer. The computer calculated up to twenty times per second the characteristic parameters of the drop (surface area, volume, and surface tension) according to the Laplacian equation applied to the profile of the drop. Movements of the syringe piston were controlled by a stepping motor. It was thus possible to compress or expand the surface of the drop within a few percent, and to maintain the drop surface area constant during the experiments, so that the surface tension variation was only related to the adsorption of the molecules at the interface.

2.3. Rheological measurements

By using a theoretical approach based on two-dimensional rheology, the dynamic response of interfacial films to a controlled dilatational perturbation (drop compression) was investigated. Figs. 1A and B, corresponding, respectively, to the relative surface area and surface pressure variations against time, present theoretical results that could be related to the compression of a surface film.

If a dilatational mechanical stress of the film is followed by a relaxation of the surface pressure over time, the rheological behavior of the interfacial film is called viscoelastic. In this case, the analysis of the dynamic response of the film allows us to estimate, according to a modified Maxwell model (Fig. 1C), the viscoelastic parameters of the interfacial film: E_e, the conservative part of the dilatational elasticity, significant for the lateral interactions between segments of macromolecules on the interface plane, and relevant to the rigidity of the interfacial film; E_{ne}, the dissipative part of the dilatational elasticity, related to molecular reorganization like the expulsion of molecular chains upon compression, and interactions of molecules with the adjacent liquid phase molecules; and τ, the characteristic time of relaxation, i.e. the necessary time for the interface to reach a new equilibrium energy state after the perturbation. This rheological approach has been discussed in previous papers [23–26].

If a dilatational mechanical stress of the film is not followed by any relaxation, the rheological behavior of the interfacial film is called purely elastic. In that case,

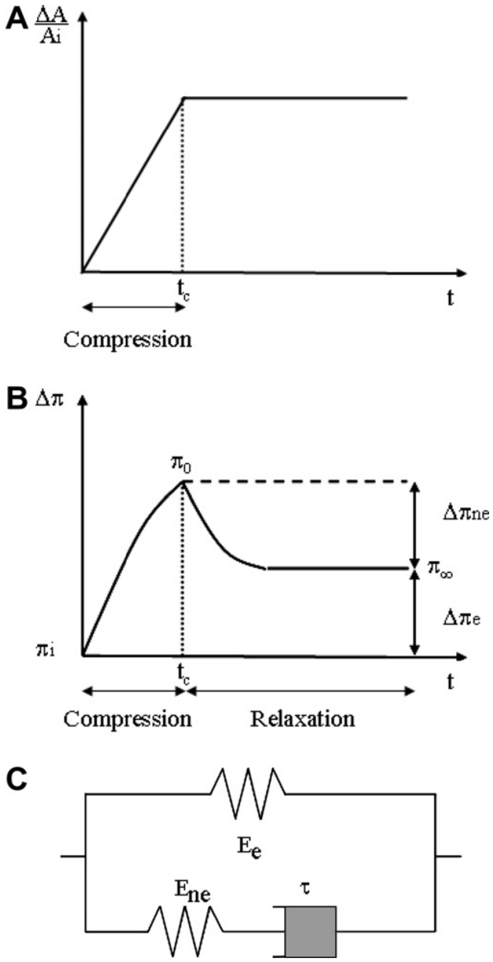

Fig. 1. (A) Schematic surface area variation during the compression ($0 < t < t_c$) of a surface film. For $t > t_c$, the surface area is maintained constant. (B) Schematic related surface pressure change $\Delta\pi$ during the time of compression ($0 < t < t_c$), followed by a relaxation (———) or not (– – – –). (C) Rheological model used to study the dilatational properties of monolayers is based on a generalized Maxwell model. The viscoelastic parameters of the interfacial film: E_e, the conservative part of the dilatational elasticity, E_{ne}, the dissipative part of the dilatational elasticity, and τ, the characteristic time of relaxation.

$E_{ne} = 0$, and the elasticity of the film (E_e) can be directly deduced from Eq. (1)

$$E_e = -A\frac{d\pi}{dA} \tag{1}$$

3. Results and discussion

Fig. 2 shows the adsorption kinetics of HEWL (0.05 mg/mL) obtained at the water/DCM interface in the absence or presence of PEG 2000 (0.1 mg/mL) in water or in DCM.

The adsorption kinetics of HEWL obtained in the absence of PEG in any phase (Fig. 2a) can be divided into three parts:

(1) A "lag time" also called "induction time" [27] corresponding to the first 350 s during which the initial surface tension remains close to that of the pure water/DCM interface, i.e. 28 mN/m. During this period, protein segments would be present at the interface but would not appreciably reduce the interfacial tension [27,28].

(2) A rapid decrease of γ in the interval comprised between 350 s and 6000 s indicating an accelerated adsorption of HEWL. Adsorption can be related to the affinity of HEWL for amphiphilic interfaces since HEWL is a globular protein exhibiting high effective surface hydrophobicity [29]. Acceleration can be also due to attraction interactions being established between adsorbed protein segments and bulk molecules [28]. Indeed, adsorbed protein molecules adopt a more energetically favorable conformation for both hydrophobic and hydrophilic side-chains. Order/disorder transitions take place leading to a significant unfolding of the protein and changes in its secondary structure, i.e. turning α-helixes into β-sheets [28,30,31]. Such conformational changes may provide attraction interactions between adsorbed protein segments and bulk molecules [28].

(3) A very slow decrease in γ is observed after 6000 s. After 7500 s, γ variation remains lower than 0.1 mN/m, indicating that adsorption equilibrium has been reached, and the equilibrium surface tension value (γ_{eq}) is 7.9 mN/m. This third regime would correspond to a slow unfolding and molecular rearrangement, i.e. two-dimensional precipitation, intermolecular association, or the aggregation of the individual unfolded protein molecules, resulting in the formation of additional layers [28–30,32]. Protein self-aggregation upon adsorption would lead to the formation of intermolecular antiparallel β-sheets [33]. It is reported that such aggregation can be objectified in emulsions by the apparition of a continuous membrane coating the oil–water interface [33]. Interestingly, the formation of a quite visible skin around the DCM drop can be directly observed upon compression of the drop ($\Delta\gamma \approx 4$ mN/m) (Fig. 3). This skin is typical of concentrated protein systems but also of surface aggregation [33,34].

To better understand and characterize the interfacial molecular organization, the interactions within the interfacial layer, and the interactions between the interfacial film and adjacent phases, the dilatational, rheological properties of the HEWL interfacial film were studied after adsorption kinetics reached an equilibrium state, typically after 8000 s.

Compression ($\Delta\gamma < 1$ mN/m) of the interfacial film was followed by an immediate decrease of γ; no relaxation of γ was observed, indicating purely elastic behavior. E_e was calculated from Eq. (1), and a value of 13 ± 1 mN/m was found. Such pure elastic rheological behavior indicates that compression of the film induces only protein segment compression in the plane of the interface, but no major reorganization such as the expulsion of protein segments toward adjacent phases [24,25]. This result corroborates the formation by protein segments of a coagulum at the interface at equilibrium.

From Fig. 2, curves b and c, we can see that whether the phase of dissolution of PEG 2000 (0.1 mg/mL) is water

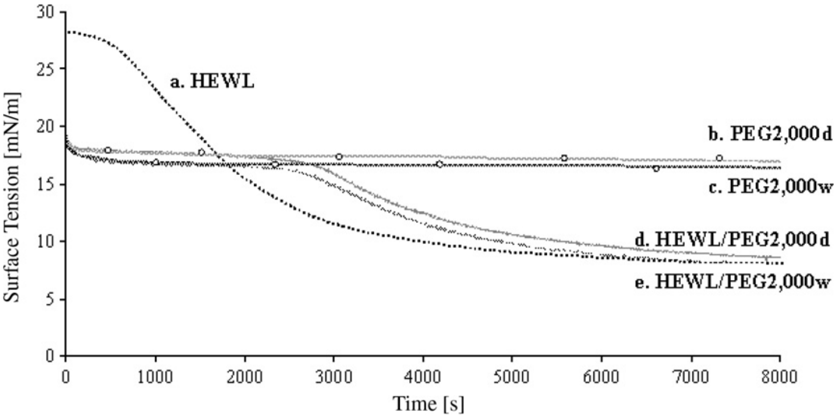

Fig. 2. Adsorption kinetics obtained at the water/DCM interface for, (a) HEWL (0.05 mg/mL ··········), (b) PEG 2000 from DCM (PEG 2000d, 0.1 mg/mL —○—), (c) PEG 2000 from water (PEG 2000w, 0.1 mg/mL —○—), (d) mixture of HEWL and PEG 2000d (——), (e) mixture of HEWL and PEG 2000w (——).

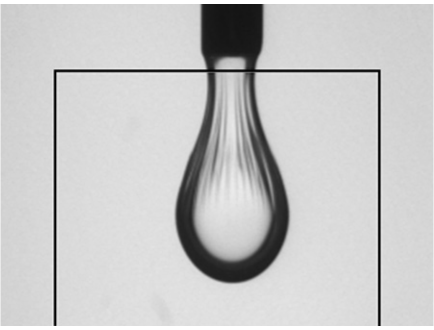

Fig. 3. Presence of a skin upon compression ($\Delta\gamma \approx 4$mN/m) of a DCM drop immersed in an aqueous phase of HEWL (1 mg/mL).

(PEG 2000w) or DCM (PEG 2000 d), the initial surface tension value (γ_0) is 19 mN/m. In both cases, a decrease in γ is observed, and adsorption equilibrium is reached after about 300 s. From γ_{eq} values, 17.5 mN/m for PEG 2000 d, and 16.6mN/m for PEG 2000w, PEG 2000 is more surface-active at the water/DCM when it is dissolved in water rather than in DCM. This is in accordance with the higher affinity of PEG 2000 for DCM than for water, and corroborates results previously obtained for PEG 2000 (0.001 mg/mL) [19].

The study of the rheological properties of the polymer films shows for PEG 2000w purely elastic behavior, and gives an E_e value of 4.5 mN/m. For PEG 2000d, compression ($\Delta\gamma < 1$ mN/m) of the interfacial film is not followed by any rheological response. As already suggested for other polymer interfacial films, reorganization of the interfacial films could take place upon compression, but too fast to be quantified by our method which is sensitive to phenomena having characteristic times typically greater than 1 s [19,35].

Adsorption kinetics obtained for the mixed protein/polymer films (Fig. 2, curves c and d) show that during the initial 2500 s, the γ values are identical to those measured for the corresponding pure PEG 2000 films. γ then decreases, and curves obtained for mixed films tend to become superimposed with those of HEWL. At equilibrium, γ_{eq} is 7.9 mN/m, i.e. the same γ_{eq} value obtained for the pure HEWL film. Moreover, although the presence of PEG 2000 in water or in DCM, compression of the mixed interfacial film ($\Delta\gamma \approx 4$ mN/m) shows the existence of a skin coating the drop (results not shown). Dilatational, rheological studies carried out at equilibrium (≈after 8000 s) on mixed HEWL/PEG 2000 films show purely elastic behavior of the films in both cases, and a value of 13 ± 1mN/m is obtained for E_e.

These results indicate that whatever the dissolution phase of PEG 2000, PEG 2000 segments first adsorb when a fresh water/DCM interface is created. Nevertheless, protection promoted by PEG 2000 segments is only partial since protein segments progressively adsorb and replace PEG 2000 segments at the water/DCM interface. At equilibrium, as attested by the γ_{eq} value, the rheological properties of the mixed interfacial films, and the presence of a skin around the DCM drop, only HEWL segments are present at the water/DCM interface in an unfolded and aggregated state.

However, one can note with interest that the replacement of the PEG segments by those of HEWL is slower when PEG 2000 is initially dissolved in DCM rather than in water. From this, it is reasonable to believe that the dissolution of PEG 2000 in DCM rather than in water should be preferred in order to slow down the adsorption of HEWL at the water/DCM interface.

Since in the emulsion formulation processes, physicochemical parameters such as ionic strength may alter the stability of proteins and modify their conformation [36], the ionic strength (I) of the aqueous phase was adjusted to 0.1 M with NaCl, and the influence of PEG 2000 on the adsorption kinetics of HEWL at the water/DCM interface was determined. Fig. 4 shows the adsorption kinetics obtained. In these conditions, for HEWL (Fig. 4a), the initial γ value is 22.1 mN/m. No induction time is seen. A drop of γ is observed, and a γ_{eq} value of 7.9 mN/m, i.e. identical to that obtained in pure water, is reached after

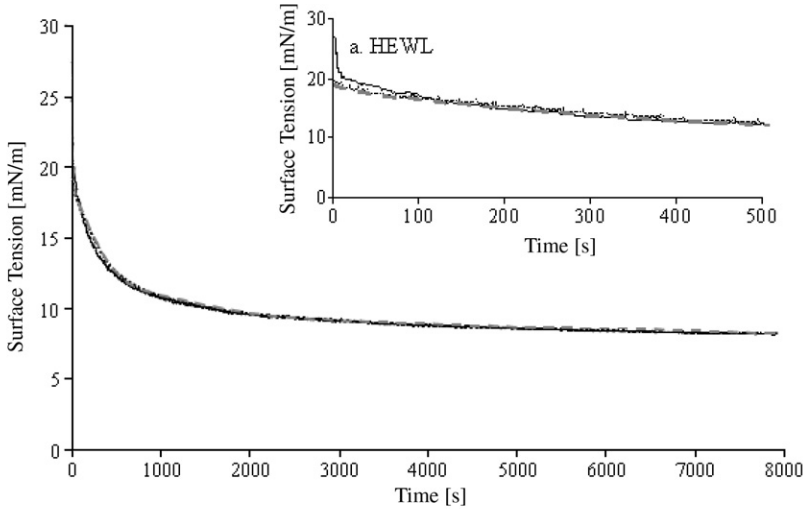

Fig. 4. Adsorption kinetics obtained at the DCM/NaCl 0.1 M interface (and closer insight during the first 500 s) for (a) HEWL (0.05 mg/mL ············), or for the mixed films of HEWL (0.05 mg/mL) and PEG 2000w (0.1 mg/mL ———) or PEG 2000d (0.1 mg/mL — — —).

about 4000 s. As for the DCM-pure water interface, compression of the protein film at equilibrium shows purely elastic behavior, and E_e is 13 ± 1 mN/m.

Thus, the HEWL molecules adsorption rate at the water/DCM interface increases when ionic strength increases. As previously shown, this accelerated adsorption can be related to lower electrostatic interactions within protein molecules when $I = 0.1$ M but also to the absence of protein aggregates in the bulk – contrary to in pure water – facilitating the diffusion and the adsorption of protein molecules toward the interface [37,38]. At equilibrium, as indicated by identical E_e values, the rigidity of the HEWL films is not influenced by the ionic strength.

The addition of PEG 2000 (0.1 mg/mL) to the aqueous or the organic phase does not induce any change in the adsorption kinetics of HEWL (Fig. 4), or in the interfacial rheological data. The increase of ionic strength of the aqueous phase leads to a salting-out effect and PEG chains become less soluble [39]. This could thus lead to a decrease in steric effects, explaining the facilitated adsorption of HEWL. From these results, it appears that PEG 2000 segments slow down protein adsorption more efficiently when the protein is dissolved in a bad solvent like pure water. This result could be interesting for the formulation of protein microparticles by the emulsion technique since it was previously determined that no irreversible conformational change of HEWL occurred in pure water [38].

By taking these different results into consideration, various parameters likely to influence the efficiency of adsorption of HEWL at the DCM–NaCl 0.1 M interface were studied, i.e. PEG bulk concentration and PEG molecular weight.

When the dissolution phase of PEG (10 mg/mL) was water, an unexpected event happened: immediately after the formation of a new interface, tiny droplets spontaneously appeared at the surface of the DCM drop, then detached, and moved into the aqueous phase (Fig. 5). Progressively, this spontaneous emulsification process intensified.

The majority of transport phenomena in emulsions described in the literature deal with the diffusion of water through the oil phase [40,41]: they are correlated to the presence of an osmotic pressure gradient explained by an excess of hydrophobic emulsifiers in the organic phase. In the present case, such an osmotic pressure gradient due to an excess of emulsifiers, i.e. PEG and HEWL, can take place, leading to a diffusion of DCM toward the aqueous phase. Additional studies are required to precisely characterize the appearance of this phenomenon, and its precise explanation. This will be the object of further work. Nevertheless, it is reasonable to think that such a diffusion of DCM in the aqueous phase probably also occurs during the emulsion-encapsulation process. It may induce a destabilizing effect because of the likelihood of rupture of the inner and multiple droplets [42], but also influence the organization and the porosity of the polymer matrix, and in time, the protein-release kinetics profile.

When the dissolution phase of PEG (10 mg/mL) was DCM, no spontaneous emulsion appeared. DCM could be chosen as a PEG dissolution phase for this reason. In this case, by increasing the PEG 2000 bulk concentration (10 mg/mL instead of 0.1 mg/mL), γ values obtained for the mixed HEWL/polymer film remain identical to those of PEG 2000 during the first 150 s (Fig. 6A), i.e. during a time sufficiently long to permit the formation of the primary emulsion [9]. From that point, when the protein is dissolved in a better solvent, protein adsorption can be more efficiently prevented by increasing PEG 2000 bulk concentration.

Protein-rejecting properties of PEG-containing interfacial films are attributed to a steric repulsion effect between tethered polymer layers and protein molecules, and the simultaneous absence of strong attraction interactions between proteins and PEG [20,43]. As theoretically pro-

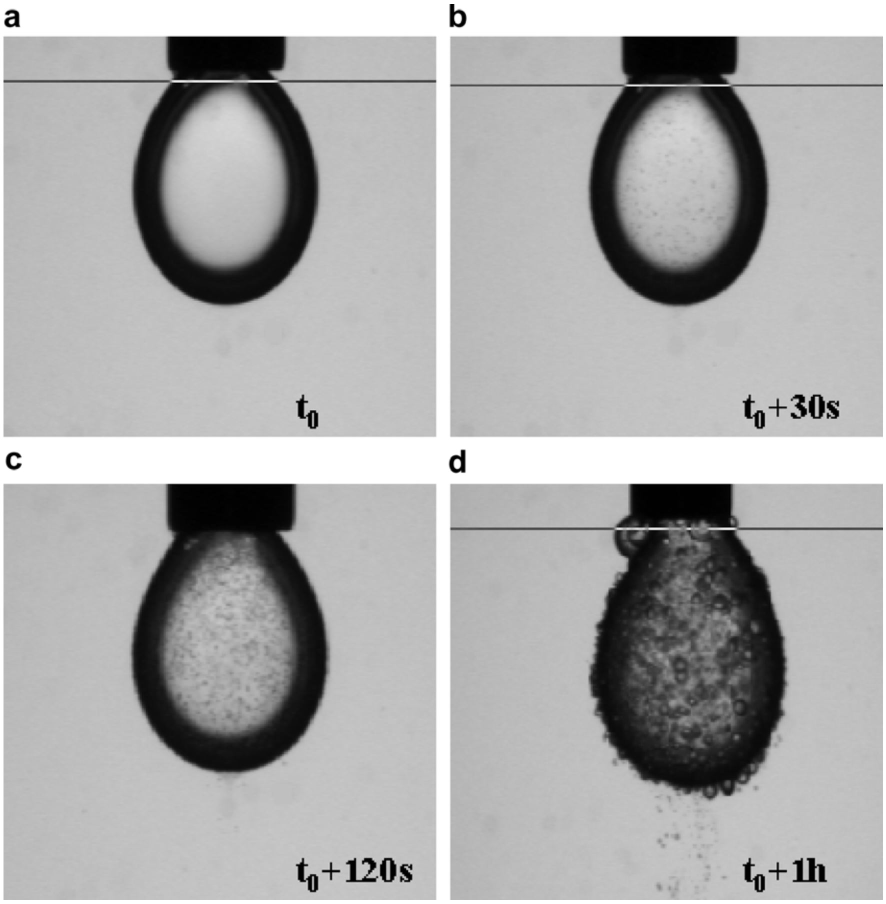

Fig. 5. Processing of a spontaneous emulsion at the surface of a DCM drop immersed in an aqueous phase of HEWL (0.05 mg/mL) and PEG 2000 (10 mg/mL). As observed in the images taken at (a) t_0, (b) $t_0 + 30$ s, (c) $t_0 + 2$ min, (c) $t_0 + 1$ h, a spontaneous emulsification process occurs with the formation of microdroplets leaving the interfacial regions.

posed [44,45], an increase in the PEG concentration enables the formation of a denser and more rigid film. For PEG moieties grafted at an interface, it is reported that the PEG chain conformation is dependent on the available interfacial surface area [16,46,47]. If the PEG surface concentration increases, the PEG surface layers pass from a "mushroom" organization to a "brush" conformation, allowing for more efficient protein steric rejection. Such brush conformation designs systems where water-insoluble moieties anchor at the interface while PEG blocks are stretched and solubilized into the aqueous phase. Although at liquid/liquid interfaces, polymer chains can penetrate into both phases [27,48], and being organized in both sides of the interface making such a PEG brush conformation less conceivable, results of the present study show that the steric contribution to the protein–surface interaction becomes more repulsive with a higher PEG 2000 bulk concentration.

Fig. 6B shows that when PEG 5000 (0.1 mg/mL) is used instead of PEG 2000 (10 mg/mL), during the first 150 s, PEG segments are present at the interface. At $C_{\mathrm{bPEG}} = 10$ mg/mL in DCM, whatever the PEG chain length, no obvious difference is observed regarding the HEWL rejection time. Then, in all cases, after 150 s, as indicated by the superimposition of curves, HEWL segments progressively adsorb and replace PEG moieties at the interface. At equilibrium, the different mixed films exhibit purely elastic behavior, and E_e is 13 ± 1 mN/m, i.e. a value identical to that determined for the pure HEWL film. Thus, at equilibrium, only HEWL segments are present in the interfacial film and govern the properties of the interfacial film.

From the Single Chain Mean-Field theory considering the adsorption of HEWL molecules on surfaces on which PEG moieties are grafted, above the PEG molecular weight threshold of 1540 g/mol, adsorption isotherms of HEWL become polymer chain-length independent, and HEWL adsorption depends only on the polymer chain density [43,49]. Our experimental results clearly corroborate this theory and show that even at a liquid/liquid interface, and even with PEG moieties adsorbed instead being grafted at the interface, when PEG bulk concentration is high enough, the prevention of HEWL molecule adsorption becomes PEG molecular weight independent. The critical parameter becomes only the PEG surface density.

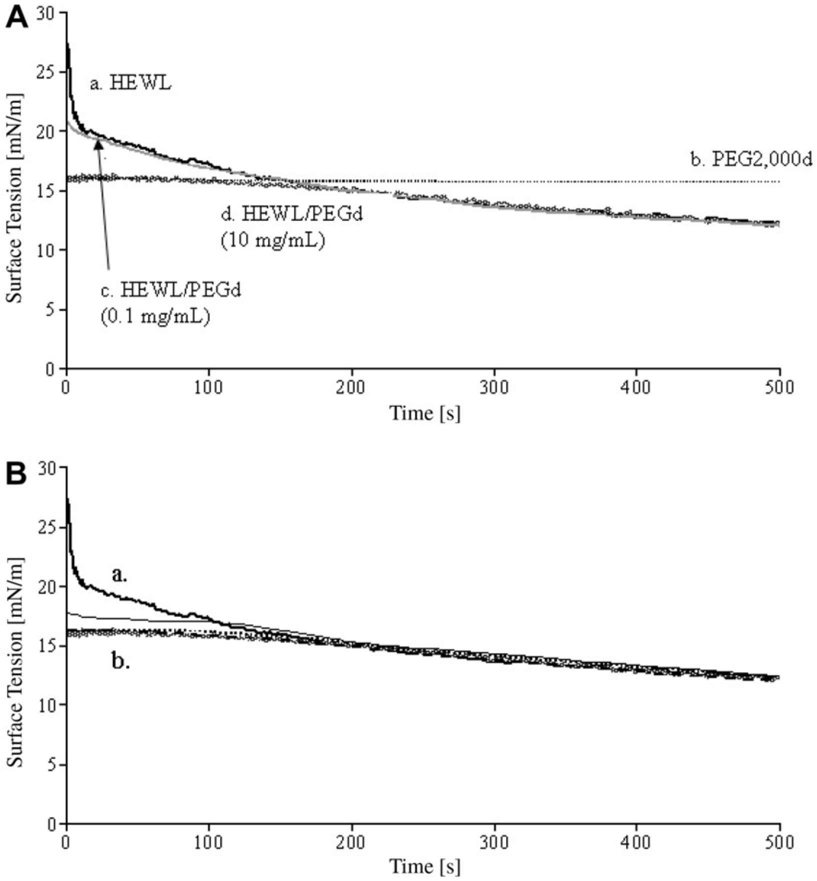

Fig. 6. (A) Adsorption kinetics obtained at the DCM–NaCl 0.1 M interface for (a) HEWL (0.05 mg/mL ▬▬), (b) PEG 2000 (10 mg/mL ·········), or for the mixed films with HEWL (0.05 mg/mL) and PEG 2000d ((c) 0.1 mg/mL ——; (d) 10 mg/mL ▬▬). (B) Adsorption kinetics obtained at the DCM–NaCl 0.1 M interface for a. HEWL (0.05 mg/mL ▬▬), (b) the mixed films with HEWL (0.05 mg/mL) and PEG 2000 d (10 mg/mL ▬▬), or PEG 5000d (0.1 mg/mL ——), PEG 5000d (10 mg/mL – – –), or PEG 17500d (10 mg/mL ·········).

From this, by modulating the PEG concentration in DCM and/or the polymer chain length, a sufficient PEG surface density could be obtained to allow efficient protein rejection, avoid the exposure of proteins at the water/organic phase interface during a time sufficiently long to permit the formation of the primary emulsion, and positively influence the success of the formulation.

4. Conclusion

From results obtained in the present study by using the pendant-drop method, a technique which makes it possible to experimentally model the conditions of the primary emulsion of the w/o/w emulsion-encapsulation technique, different strategies to prevent rapid adsorption of a model protein, the HEWL, at the water/DCM interface were drawn up. Amongst possible methods, the use of an aqueous phase with a very low ionic strength would allow the efficient decrease of the rate of HEWL adsorption at the liquid/liquid interface, and that without inducing irreversible conformational changes of the protein.

Moreover, the use of PEG would prevent exposure of the protein to the water/DCM interface during a sufficiently long time to permit the formation of the primary emulsion as soon as critical polymer chain surface density is reached at the interface. This critical PEG chain density adsorbed at the interface can be obtained by modulating the PEG bulk concentration or the PEG molecular weight.

From the adsorption kinetics performed, it appears that the adsorption of HEWL at the water/DCM interface is longer prevented by dissolving PEG in DCM rather than in water. Thus, by optimizing the choice of the PEG introduction phase in the formulation process, exposure of HEWL to interfaces could be further reduced, and thus, protein stability could be improved. Moreover, it was observed that the dissolution of PEG in water rather than in DCM induces an unwanted spontaneous emulsion process at the surface of the DCM drop. It is reasonable to think that such a phenomenon also occurs during the emulsion-encapsulation process if PEG and HEWL are present in water. To determine if this event negatively influences the efficiency of the encapsulation of proteins in microparticles, characterization studies of this spontaneous emulsion process should be carried out, and translation to a formulation work should be undertaken. This will be the object of further work.

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