European Journal of Pharmaceutics and Biopharmaceutics 73 (2009) 66–73

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Interactions between poly(ethylene glycol) and protein in dichloromethane/water emulsions. 2. Conditions required to obtain spontaneous emulsification allowing the formation of bioresorbable poly(D,L lactic acid) microparticles

Aurélie Malzert-Fréon [a,*], Karin Schönhammer [b], Jean-Pierre Benoît [b], Frank Boury [b,*]

[a] Centre d'Etudes et de Recherche sur le Médicament de Normandie, UPRES EA 4258, UFR des Sciences Pharmaceutiques, Université de Caen Basse-Normandie, Bd Becquerel, 14032 Caen, France
[b] Inserm U646 Ingénierie de la Vectorisation Particulaire, Université d'Angers, 10 rue André Boquel, 49100 Angers, France

ARTICLE INFO

Article history:
Received 31 October 2008
Accepted in revised form 30 April 2009
Available online 7 May 2009

Keywords:
PEG
Lysozyme
BSA
Spontaneous emulsification
Microparticles
Pendant drop tensiometer

ABSTRACT

From microscopic observations, it was established that an oil-in-water emulsion with droplets of a size in the micrometer range can spontaneously form at room temperature without additional external stirring as soon as a solvent that is only partly miscible to water-like dichloromethane (DCM) is put in contact with an aqueous mixture of polyethylene glycol (PEG) and a protein. Experimental results show that emulsification only occurs if the system simultaneously includes PEG with middle chain, an organic solvent partly miscible to water and for which PEG affinity is sufficiently high, and a protein. From adsorption kinetics, it appears that this spontaneous emulsification process is related to the rapid diffusion of DCM towards water through the formation of interfacial turbulences, once the accumulation of PEG close to the DCM/water interface occurs. The oil droplets formed would be then stabilized by adsorbed protein molecules. Since the presence of polylactic acid in the organic phase did not prevent the emulsion formation, we studied the feasibility of formulating microparticles using this polymer. From results, it appears that microcapsules with a polymeric shell, with a homogeneous size of about 50 μm and able to encapsulate a model hydrophobic drug, such as amiodarone, can be obtained by using this spontaneous emulsification method.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

The emulsification solvent evaporation technique is commonly used to encapsulate a wide range of drugs, such as proteins, at high concentration levels in polymer matrices [1,2]. To optimize protein stability within the formulation, polymers, and in particular poly(ethylene glycol) (PEG), can be used to minimize protein adsorption at interfaces created during the emulsification process [3–5]. PEG is a peculiar polymer which is soluble both in water and in organic phases such as dichloromethane (DCM) [6]. From the results of adsorption kinetics and interfacial rheological studies performed on mixed films of PEG and hen egg-white lysozyme (HEWL), a model protein, it appears that the introduction of PEG in water or in DCM influences protein adsorption prevention [7]. Thus, exposure of HEWL at the water/DCM interface is prevented for a longer time if PEG is dissolved in DCM instead of in water. Moreover, it was observed that the simultaneous dissolution of HEWL and PEG in water induces the apparition of a spontaneous emulsion process [7].

Generally speaking, spontaneous emulsification refers to the formation of small droplets having diameters on the order of 1 μm when two immiscible liquids are placed in contact with each other and when high shear rates are not required. Typically, the droplets form spontaneously, without requiring any supply of external energy of agitation, the entire energy required for the emulsification coming from the redistribution of material within the system [8,9].

In 1878, Johannes Gad first observed that a solution of lauric acid in oil would spontaneously form emulsions when placed on top of aqueous alkali [10]. In the past, the self-emulsification process was reported to occur in oil–water–alcohol systems and in various systems containing surfactants [11]. There is considerable literature on the formation of microemulsions using alcohols such as butanol, hexanol, and octanol which can help solubilize large

Abbreviations: BSA, bovine serum albumin; DCM, dichloromethane; HEWL, hen egg-white lysozyme; PEG, poly(ethylene glycol); PLA, poly(lactic acid); SEM, scanning electron microscopy; γ_{eq}, surface tension at equilibrium.

* Corresponding authors. Tel.: +33 231 566819; fax: +33 231 566020 (A. Malzert-Fréon), tel.: +33 241 735848; fax: +33 241 735853 (F. Boury).
E-mail addresses: aurelie.malzert-freon@unicaen.fr (A. Malzert-Fréon), frank.boury@univ-angers.fr (F. Boury).

0939-6411/$ - see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2009.04.011

quantities of oil and water, but these agents are not appropriate pharmaceutical ingredients [12].

In the present paper, we have developed innovative conditions required to obtain a spontaneous oil-in-water emulsion using PEG and a protein. We have tried to explain the mechanism leading to spontaneous emulsification. In order to further analyze this mechanism and to link it with the formulation area, preliminary studies of formulation were carried out by using the spontaneous emulsification method to produce microparticles and to encapsulate a model drug, amiodarone.

2. Materials and methods

2.1. Materials

PEG 2000 was an α-methoxy, ω-hydroxy-PEG 2000. The number-average molecular weight ($\overline{M}n$) of the polymer determined by nuclear magnetic resonance at 360 MHz was 2200. PEG 400, PEG 2000, PEG 5000, PEG 8000, PEG 17,500, HEWL (no. L6876, dialyzed and lyophilized, containing the buffer salts sodium acetate and sodium chloride, 3 × crystallized, protein at approx. 95%), human serum albumin and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (L'Isle d'Abeau, France), and were used without further purification. Amiodarone was obtained from Sanofi AG (Montpellier, France). Poly(lactid acid) (PLA50) was supplied by Phusis (Saint-Ismier, France). According to the Mauduit and Vert classification [13], the gross composition was 50% D-repeating units and 50% L-repeating units. The weight-average molecular weight ($\overline{M}_w$) determined by size exclusion chromatography was 52,000 g/mol. The polydispersity index was 1.7. All the organic solvents were purchased from Prolabo (Paris, France). NaCl (for analysis, ACS, ISO) was supplied by Merck (Nogent-sur-Marne, France). Ultrapure water was obtained from a Millipore® system (Milli-Q Plus 185, Molsheim, France).

2.2. Drop images and interfacial tension measurements

Adsorption kinetics and images of drops were recorded by means of a pendant-drop tensiometer (Tracker®, Teclis, Longessaigne, France) [14]. A drop was formed with an Exmire microsyringe (Prolabo, Paris, France) into an optical glass bowl (Hellma, France) containing the other phase. From the analysis of the numerical image of the drop with the Laplace equation integrating the points of the drop profile, the interfacial tension was recorded in real time (up to 20 measurements per second). The drop surface area was maintained constant during the experiments thanks to a drop volume control system, in such way that the surface tension variation was only related to the adsorption of molecules at the interface.

Images (real size of 5 mm × 5 mm) of the studied drops were recorded in real time. The size of droplets appearing at the surface of the drop in some experiments (see results) was determined by using the software ImageJ on the recorded images.

2.3. Microscopy observations

Spontaneous emulsification was also followed using optical microscopy (Axioskop 2, Zeiss, Le Pecq, France), and images were recorded using a Sony digital still camera supplied by Zeiss (Le Pecq, France). A droplet of the oil was injected using an Exmire microsyringe (Prolabo, Paris, France) into a mixed solution of HEWL (1 mg/mL) and PEG 2000 or PEG 17,500 (10 mg/mL) contained in a rectangular 30 mm × 5 mm glass cell of 0.5 mm thick (VitroCom, New Jersey, USA) directly under the microscope.

2.4. Microparticle preparation

The aqueous phase contained PEG 2000 (10 or 20 mg/mL) and protein (HEWL or BSA at 1 mg/mL), and the organic solution was made of DCM and PLA50 (5 mg/mL). In case of drug-loaded microparticles, amiodarone was solubilized in the organic phase prior to experiments. Using a syringe, 2.5 mL of the organic phase was injected into the aqueous solution (50 mL), and then the mixture was left at room temperature for 90 min, without any external agitation, to allow the spontaneous emulsification to form. The resulting emulsion was poured into deionized water (500 mL) and magnetically stirred for 45 min to extract the DCM. Finally, the formed microparticles were filtered through a 0.45 μm filter (HVLP type, Millipore SA, Saint-Quentin en Yvelines, France), washed with deionized water, freeze-dried (Virtis, France), and stored at 4 °C.

2.5. Microparticle characterization

2.5.1. Morphology and size

The obtained microparticles were observed by optical microscopy (BH2, Olympus, Tokyo, Japan). The surface and the internal morphology of the microparticles were investigated by using scanning electron microscopy (SEM; JSM 6310F, JEOL, Paris, France). Freeze-dried microparticles were mounted onto metal stubs using double-sided adhesive tape, sputter-coated with a fine coat of gold and carbon (JEOL JFC 1100, Paris, France) and examined under SEM. To characterize the internal morphology, the adhesive tape with stuck particles was first folded on itself and secondly roughly unfolded to fracture the microparticles according to Pean et al. [15]. The coating was carried out as previously described. The average particle size and distribution were determined using a Coulter® counter Multisizer (Coultronics, Margency, France).

2.5.2. Drug encapsulation efficiency

Encapsulation tests were performed with amiodarone as a model drug. Amiodarone-loaded microparticles were prepared according to the previously described protocol. Entrapment efficiency was determined by HPLC (Waters 996, Waters, Saint-Quentin en Yvelines, France) based on the method described by Weir and Ueda [16]. Microparticles were dissolved with dimethylsulfoxide (DMSO) prior to injection in the column (RP-18 column, LiChrospher® 100 Merck, Darmstadt, Germany). Samples of 50 μL were injected into the column. Analysis was performed using a mobile phase consisting of methanol, water and ammonium hydroxide (94:5:1 v/v) delivered at a flow rate of 1.5 ml/min. Amiodarone was detected by UV absorbance at 244 nm.

3. Results and discussion

Fig. 1a shows a pendant drop of DCM freshly formed in an aqueous solution of PEG 2000 ($C_{PEG2000}$ = 10 mg/mL) and HEWL (C_{HEWL} = 1 mg/mL). A few seconds after the formation of the drop, a flow of material is observed from the organic phase towards the aqueous phase, and dark spots appear on the surface of the DCM drop (Fig. 1b). With time, more and more of these spots can be distinguished (Fig. 1c). After about 60 min, well-individualized droplets (50 ± 20 μm in diameter) are visible (Fig. 1d). Fig. 2a shows that the formation of a rising drop of the mixed aqueous phase in DCM is not followed by any material flow but by the apparition of the spontaneous formation of droplets in the aqueous drop.

From these results, once a DCM drop is brought into contact with an aqueous mixture of a PEG and a protein, an oil-in-water (o/w)

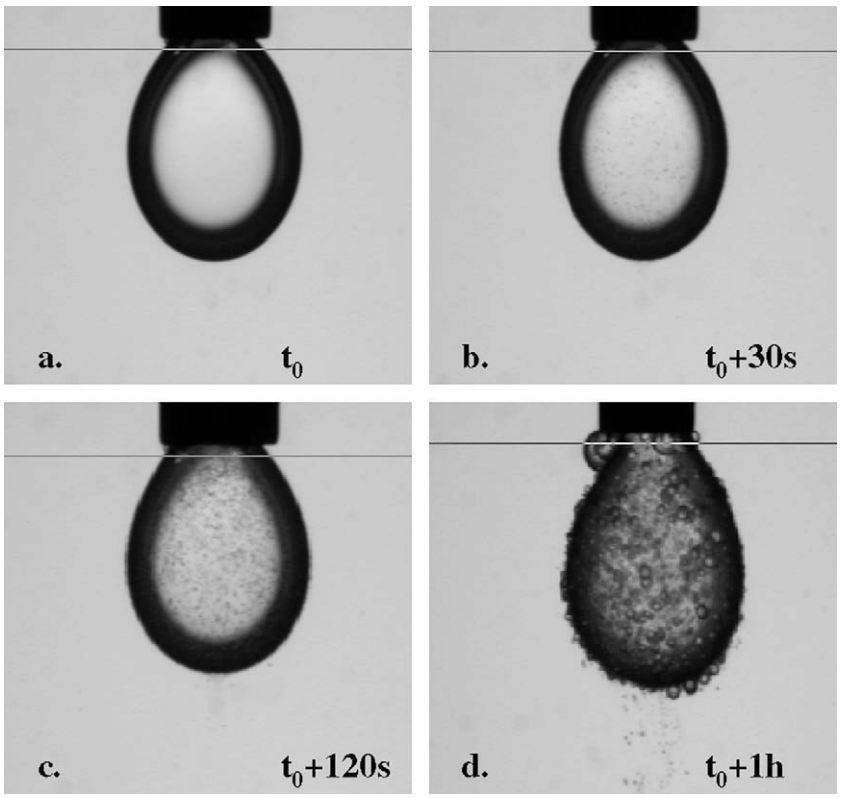

Fig. 1. Processing of a spontaneous emulsion at the surface of a DCM drop immersed in an aqueous phase of HEWL (1 mg/mL) and PEG 2000 (10 mg/mL). As observed in the images taken at (a) t0, (b) t0 + 30 s, (c) t0 + 2 min and (d) t0 + 1 h, a spontaneous emulsification process occurs with the formation of microdroplets leaving the interfacial regions.

emulsion spontaneously appears, and oil droplets of 50 ± 20 μm diameter are formed after keeping the phases in contact for 1 h.

From observations made by Malzert-Fréon et al. [7], such spontaneous emulsification depends on experimental conditions. Thus, the influence of various experimental parameters on the formation of the spontaneous emulsification was assessed. The results are summarized in Table 1.

PEG is a peculiar polymer which is soluble both in water and in various organic solvents, one of them being DCM [6]. As previously observed [7], if HEWL is dissolved in water (C_{HEWL} = 1 mg/mL) and PEG 2000 in DCM ($C_{PEG2000}$ = 10 mg/mL), no spontaneous emulsification occurs. Thus, the polymer and protein must be simultaneously present in the aqueous phase to make the spontaneous formation of the o/w emulsion possible. This result is different from other self-emulsification processes using ethyleneglycol derivatives as described in the literature. For example, Rang et al. [17,18] report that an emulsion spontaneously forms when a mixture of ethyleneglycol derivates (15 wt%)/n-octanol/n-hexadecane is injected into water. In this case, emulsification would be explained by the diffusion of water into the initial oil drop, first converting it to a microemulsion, and then causing this phase to become supersaturated in oil, so that many oil droplets nucleate in the aqueous phase [18].

In the absence of PEG, i.e. in the case of a drop of DCM immersed in a protein aqueous solution (C_{HEWL} = 1 mg/mL), no spontaneous emulsification occurs. At fixed C_{HEWL} in water (1 mg/mL), results show that a PEG 2000 threshold concentration in water of 0.1 mg/mL must be reached to enable the spontaneous emulsification process to occur.

If PEG 400 (C_{PEG400} = 10 mg/mL) is used instead of PEG 2000, no emulsification is observed. With PEG 5000, similar spontaneous emulsification to that obtained with PEG 2000 is observed. The use of PEGs with higher molecular weights not only enables the formation of a spontaneous emulsification outside the drop but also induces a significant intensification of the material flow from the organic phase towards the aqueous phase (marked on Fig. 2b with a black arrow). This amplification of the emulsification was also observed under the microscope after the injection of a DCM drop with a microsyringe in a horizontal glass cell containing the aqueous phase (Fig. 3). In these conditions, if PEG 2000 or PEG 17,500 is used, immediately after the formation of the DCM drop, vesicles of about 1 μm in diameter appear all around the drop; the layer of droplets becomes thicker and thicker over the course of the experiment, and the progression of this layer towards the aqueous phase is observed. The use of higher molecular weight PEGs induces the formation of many more droplets (Fig. 3b). In both cases, the droplets coalesce to form stable drops of about 50 μm in diameter, obtained 1 h after the beginning of the experiment.

In the case of a system composed of a mixed polymer ($C_{PEG2000}$ = 10 mg/mL) and protein (C_{HEWL} = 1 mg/mL) aqueous phase, and chloroform instead of DCM, the emulsification process occurs (Table 1). However, if DCM is substituted by ethyl acetate or ethyl ether, no solvent flow and no spontaneous emulsion are observed. This is also the case if decane is used. If ethanol is added to decane in low proportions (ethanol/decane, 10/90), no emulsification process is observed. The fivefold increase in the alcohol proportion (ethanol/decane, 50/50) leads to the apparition of a small solvent flow from the drop towards the bulk, but it does not induce droplet formation.

These different observations show that the formation of spontaneous emulsification requires the use of an organic solvent partly

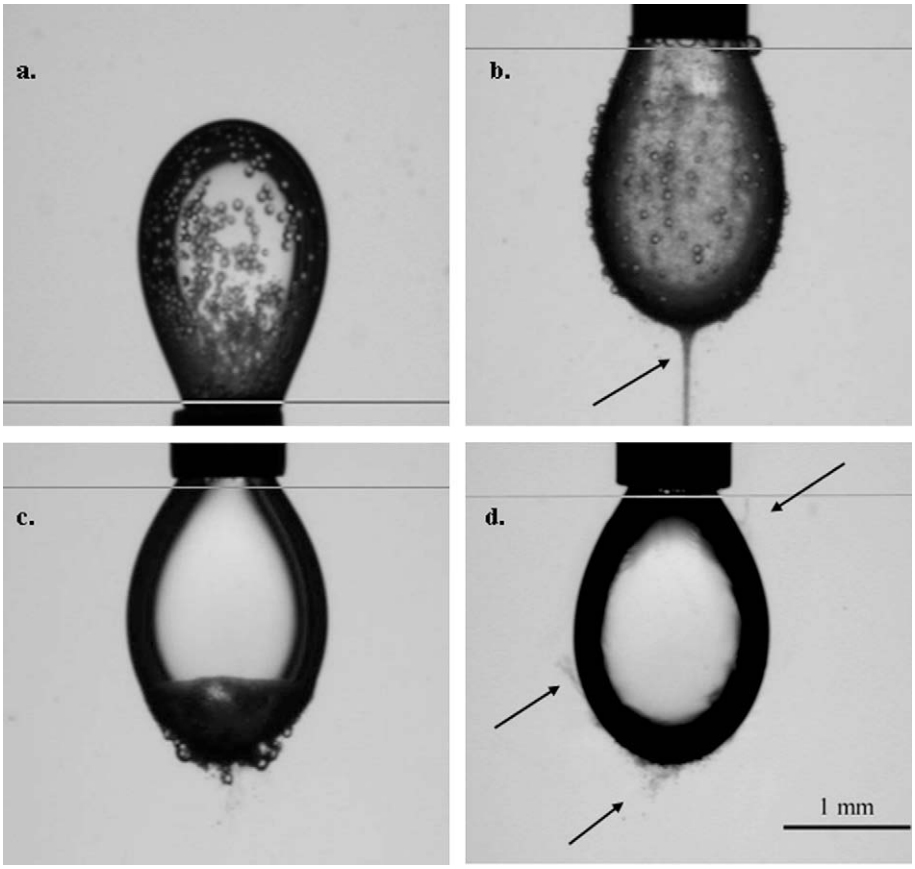

Fig. 2. Observation of (a) a rising aqueous drop of HEWL (1 mg/mL) and PEG 2000 (10 mg/mL) immersed in DCM, (b) a pendant drop of DCM immersed in an aqueous phase of HEWL (1 mg/mL) and PEG 8000 (10 mg/mL), (c) a pendant drop of DCM immersed in an aqueous phase of PEG 2000 (10 mg/mL), and (d) a pendant drop of DCM immersed in an aqueous phase of PEG 2000 (50 mg/mL).

Table 1
The influence of various experimental parameters on the formation of a spontaneous emulsification process (SEP). "yes" indicates that a well-defined SEP occurs; "no", that no SEP is observed; and ∼, that some emulsification occurs but no formation of organized droplets is observed.

PEG	Dissolution phase of PEG	Organic phase	C_{peg} (mg/mL)	C_{hewl} (mg/mL)	C_{Hsa} (mg/mL)	Ionic strength (M)	SEP
2000	DCM	DCM	10	1	–	0	No
2000	Water	DCM	10	1	–	0	Yes
2000	Water	Chloroform	10	1	–	0	Yes
2000	Water	Ethyl acetate	10	1	–	0	No
2000	Water	Ethyl ether	10	1	–	0	No
2000	Water	Decane	10	1	–	0	No
2000	Water	Ethanol/ decane	10	1	–	0	No
–	–	DCM	–	1	–	0	No
2000	Water	DCM	0.01	1	–	0	No
2000	Water	DCM	0.1	1	–	0	Yes
2000	Water	DCM	1	1	–	0	Yes
2000	Water	DCM	10	–	–	0	∼
2000	Water	DCM	50	–	–	0	∼
2000	Water	DCM	10	0.1	–	0	Yes
2000	Water	DCM	10	0.5	–	0	Yes
2000	Water	DCM	10	–	1	0	Yes
2000	Water	DCM	10	1	–	0.1	Yes
400	Water	DCM	10	1	–	0	No
5000	Water	DCM	10	1	–	0	Yes
8000	Water	DCM	10	1	–	0	Yes
17,500	Water	DCM	10	1	–	0	Yes

miscible to water (Table 2), and the presence of PEG in the aqueous phase above a threshold PEG concentration value. Furthermore, the higher the PEG molecular weight, the greater its affinity for the organic phase [6]. The fact that no emulsion occurs with organic solvents in which PEG is insoluble (Table 2), and the intensification of the process with higher polymers, indicates that the affinity of PEG for the solvents used must be sufficient to allow emulsification to occur.

In the absence of protein, i.e. in the case of a drop of DCM immersed in a polymer aqueous solution ($C_{PEG2000}$ = 10 mg/mL), some material flow is observed from the organic phase towards the aqueous phase. Nevertheless, after 90 min, only a few droplets outside the drop and a slight precipitate inside the drop are visible (Fig. 2c). A fivefold increase in the PEG 2000 concentration in water results in the formation of large eddies of DCM at the interface all around the drop (indicated on Fig. 2d with black arrows) but does not induce the formation of organized droplets.

At fixed $C_{PEG2000}$ in water (10 mg/mL), the spontaneous emulsification process occurs independently of the HEWL concentration (Table 1). Furthermore, the use of human serum albumin instead of HEWL induces a similar spontaneous emulsification.

Thus, spontaneous emulsification occurs only if the system includes a protein, PEG, and an organic solvent partly miscible to water and for which PEG affinity is sufficiently high.

Fig. 4 shows adsorption kinetics of PEG and HEWL at the water/DCM interface. Although a decrease in surface tension is observed, only high, positive interfacial tension values are monitored. Negative interfacial tensions have been proposed as the cause of spontaneous emulsification for some experiments using surfactants [11,19]; they spontaneously cause an increase in the surface area and lead to the formation of droplets that leave the macroscopic interface and disperse [19]. However, from the results obtained

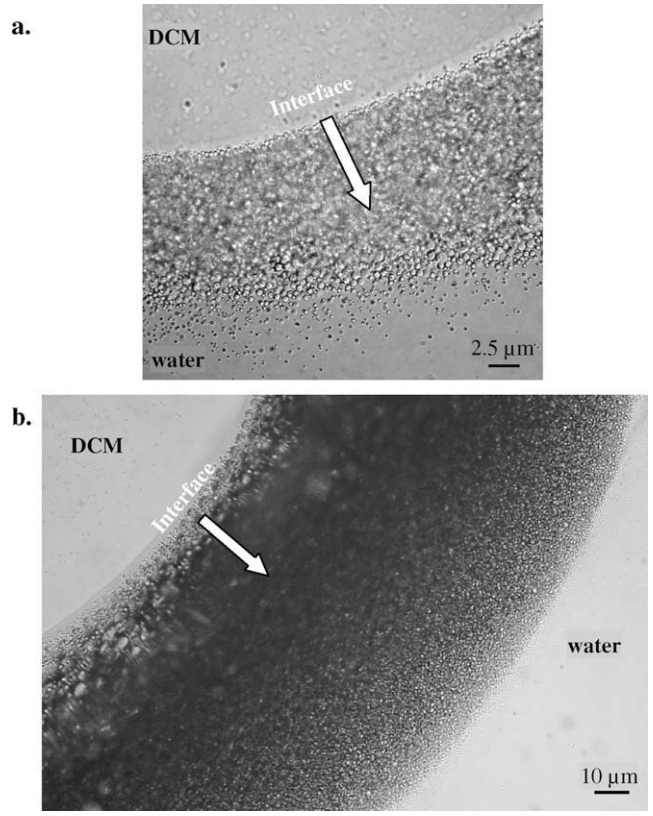

Fig. 3. Microscopic observations of spontaneous emulsification processes occurring when a DCM drop is immersed in an aqueous solution of HEWL (1 mg/mL) and PEG 2000 (10 mg/mL) (a) or PEG 17,500 (10 mg/mL) (b).

for our system, emulsification would not be due to such locally negative interfacial tensions.

From adsorption kinetics, it appears that immediately after the formation of a fresh drop of DCM, PEG segments adsorb at the interface (Fig. 4). Furthermore, it is known that PEG presents an important exclusion volume [20,21]. Because of the correlated steric exclusion, and as the osmotic stress induced by PEG decreases the water activity, the presence of PEG in any protein solution would provide the driving force to generate phase separation [22,23]. In these conditions, the presence of protein in the aqueous phase can indirectly lead to local accumulations of PEG, in particular close to the DCM/water interface, as indicated by the polymer adsorption kinetics, and even more so when the polymer bulk concentration is high.

Local supersaturation of surfactants can lead to the formation of intermediate phases such as micellar solutions, microemulsions, and lyotropic liquid crystals. In some cases, intermediate phase formation is accompanied by spontaneous emulsification [11]. From the examination of our systems in polarizing microscopy, no anisotropic structures were observed (results not shown). However, intermediate phase formation is not always present in systems where spontaneous emulsification occurs [9,18].

From that, local accumulation of PEG close to the DCM/water interface, favoured by the mutual presence of the protein in the aqueous phase, can enhance interfacial tension gradients, and thereabouts even enhance interfacial turbulences and diffusion-stranding processes [11,24,25]. In these conditions, emulsification can be spontaneously produced without supplying energy by mechanical agitation.

Fig. 4 shows that adsorption equilibrium is reached after 1000 s, γ variation remaining lower than 0.1 mN/m. The value of interfacial tension at equilibrium (γ_{eq}) for the PEG 2000 film is 14.6 mN/m, 7.3 mN/m for that of HEWL, and 6.5 mN/m in the case of the mixed HEWL/PEG 2000 adsorption film. By comparing γ_{eq} values obtained for the HEWL film and the mixed film, it appears that either both PEG segments and HEWL molecules are present at the interface at equilibrium or only HEWL molecules are present, the difference between γ_{eq} values being regarded as negligible, and correlated with artifacts of measurement because of the emulsification. From surface rheology studies performed on the mixed film at equilibrium (detailed results not shown), it appears that the elastic behavior of the protein is recovered, and the same value of elasticity (12 ± 1 mN/m) is found. Thus, during the late stage of adsorption, HEWL molecules entirely push away the PEG 2000 segments from the interface, and at equilibrium, protein molecules are present at the interface and predominantly influence the interfacial properties of the mixed polymer/protein film.

An increase in the ionic strength of the aqueous phase does not prevent emulsification and even leads to the formation of larger droplets outside the DCM drop (110 ± 40 µm). The conditions of the experiment included a pH value of the aqueous phase of 6, HEWL with a pI of 11.2, and positively charged protein molecules [26]. An increase in the ionic strength makes the system less hydrophilic [27] and leads to higher hydrophobic interactions, lower electrostatic repulsions within HEWL molecules, and a further adsorption of the protein molecules at the interfaces [13,28]. A decrease in electrostatic repulsions between HEWL molecules which are adsorbed at the surface of droplets improves coalescence of droplets and provides larger droplets. This corroborates the fact that HEWL molecules are present at the surface of spontaneously formed droplets and help in their stabilization.

From the results obtained, the organization of droplets could be schematized as follows (Fig. 5): DCM-swollen PEG droplets stabilized by HEWL molecules and turned towards water.

Poly(lactid acid)s (PLAs) are polymers belonging to the family of the poly(α-hydroxy acid)s, i.e. they are biocompatible and bioresorbable polymers, which are used in the formulation of polymer microparticles [13]. The addition of PLA50 in DCM (C_{PLA50} = 5 mg/mL) induces no modification of spontaneous emulsification. Thus, the process could be interestingly applied to the formulation of drug delivery particles of micrometric size, without requiring any agitation.

Considering this last result, and to confirm the hypothesis formulated above about the observed spontaneous emulsification, formulation assays of microparticles were performed.

The organic phase (composed of PLA50 and DCM) was added by a syringe into the aqueous solution (composed of water, PEG 2000 and protein). The concentrations of the compounds used were based on the results presented above. The mixture was left at room temperature for 90 min, without additional external agitation, to let the oil/water emulsion form spontaneously. Then, in a second stage, the resulting emulsion was poured into deionized water and magnetically stirred for 45 min in order to extract DCM and

Table 2
The solubility of studied organic solvents in water at 25 °C according to [39,40]; PEG solubility in studied organic solvents according to [6].

Solvent	Solubility in water from [39,40]	Solubility of PEG 2000 from [6]
DCM	1.30 wt%: sparingly soluble in water	Yes
Chloroform	0.815 wt%: very slightly soluble in water	Yes
Ethyl ether	6.04 wt%: soluble in water	No
Ethyl acetate	8.08 wt%: soluble in water	No
n-Decane	$5.2 \cdot 10^{-8}$ wt% : practically insoluble in water	No

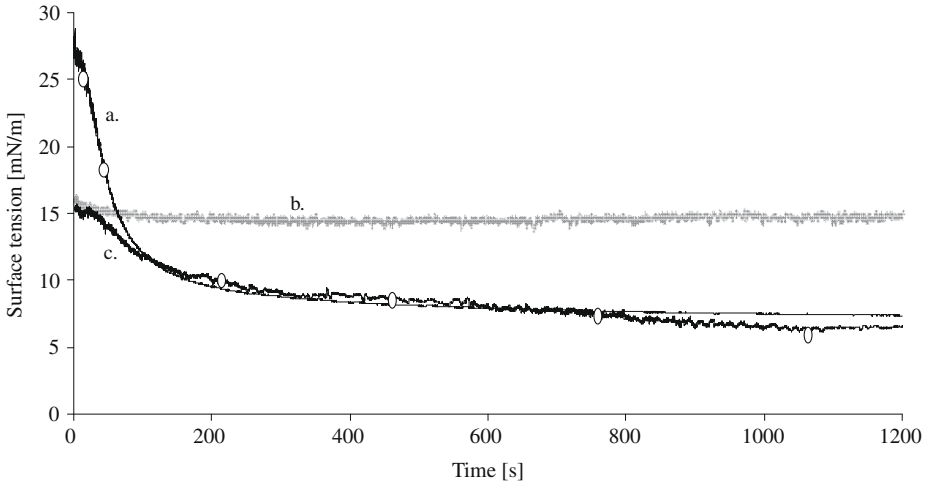

Fig. 4. Adsorption kinetics of (a) HEWL (1 mg/mL), (b) PEG 2000 (10 mg/mL), and (c) HEWL (1 mg/mL)/PEG 2000 (10 mg/mL) mixture from water at the DCM–water interface.

to promote polymer desolvation. Finally, at the end of the process, the objects formed were recovered *via* filtration and washed with water.

The formation of microparticles was assessed under optical microscopy. Two proteins, i.e. HEWL and BSA were tested. When HEWL is used, both spherical and non-spherical objects such as fragments or fibers are obtained (results not shown). In contrast, with BSA, microparticles appear as well-individualized and spherical objects (Fig. 6). This result suggests a better stabilization effect of BSA in comparison with HEWL, probably due to the higher surface activity of BSA [29,30]. As reported in the literature in another context, the use of BSA instead of HEWL is more beneficial to the stabilization of w/o emulsions [31].

Blank microparticles obtained with BSA as a protein have a mean size of 50 μm. Under SEM, they exhibit a smooth skin without any pores (Fig. 7A). Particles are formed by a dense and homogeneous structure. According to the internal morphology of microparticles observed after section (Fig. 7C), the particles are microcapsules with a well-defined shell.

Amiodarone was used as a model substance to be entrapped in microparticles. This drug, which is widely used in heart therapy because of its antianginal and antiarrythmic properties [32,33], is not very soluble in water at room temperature. By applying the spontaneous emulsification process, particles with a size of 50 ± 27 μm were obtained. This diameter is of the same order of magnitude as that of the droplets observed outside the DCM drop. From SEM observations (Fig. 7B and D), drug-loaded microparticles are spherical with a smooth external surface and a rough internal structure. From HPLC measurements, the encapsulation yield reaches 33%. These results indicate the presence of amiodarone in the core of the capsules. The rough internal surface tends to confirm the diffusion toward the aqueous phase of droplets of the DCM phase, draining with them amiodarone, and stabilized by PEG and protein.

Results of the experiments show that the new spontaneous emulsification method allows the efficient entrapment of a model drug. Thus, we can suppose that it could also be successfully applied to the encapsulation of other lipophilic drugs. The traditional formulation process based on solvent-displacement implies a water-miscible organic solvent such as acetone towards water [12,26,34–38]. In our case, the process could involve a partly water-miscible solvent. It could be convenient for encapsulation of oil-soluble molecules which exhibit a partition coefficient highly favorable for the organic solvent used [24]. Only depending on the presence of PEG and a protein, the polymer acting partly as the initiator of the process, and the protein partly as a stabilizing agent, this method could permit the formulation of microparticles using a continuous process and avoiding the use of a high-energy-input step (like sonication). This would be simple to use and relatively easy to scale up.

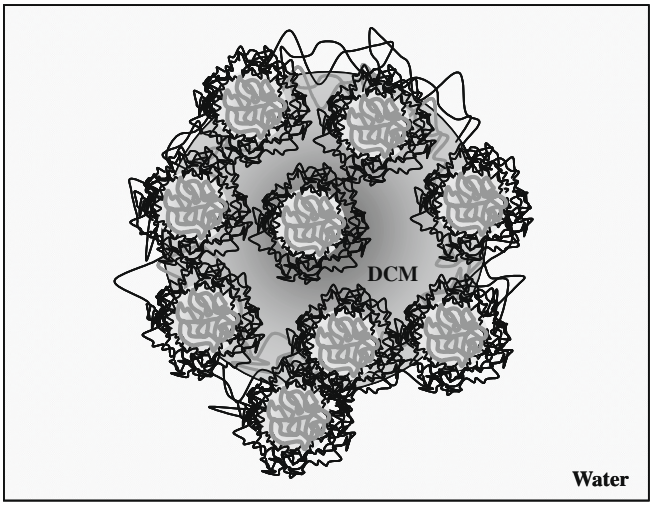

Fig. 5. Schematic view of the organization of oil droplets around the DCM drop immersed in an aqueous phase of a protein and a PEG.

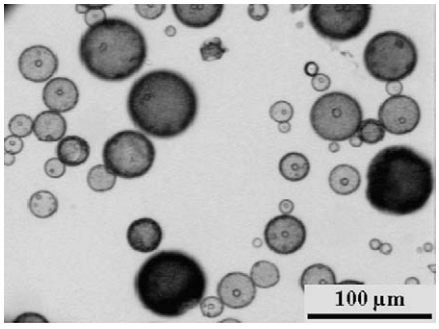

Fig. 6. Optical micrograph showing blank microparticles obtained if the protein used is BSA.

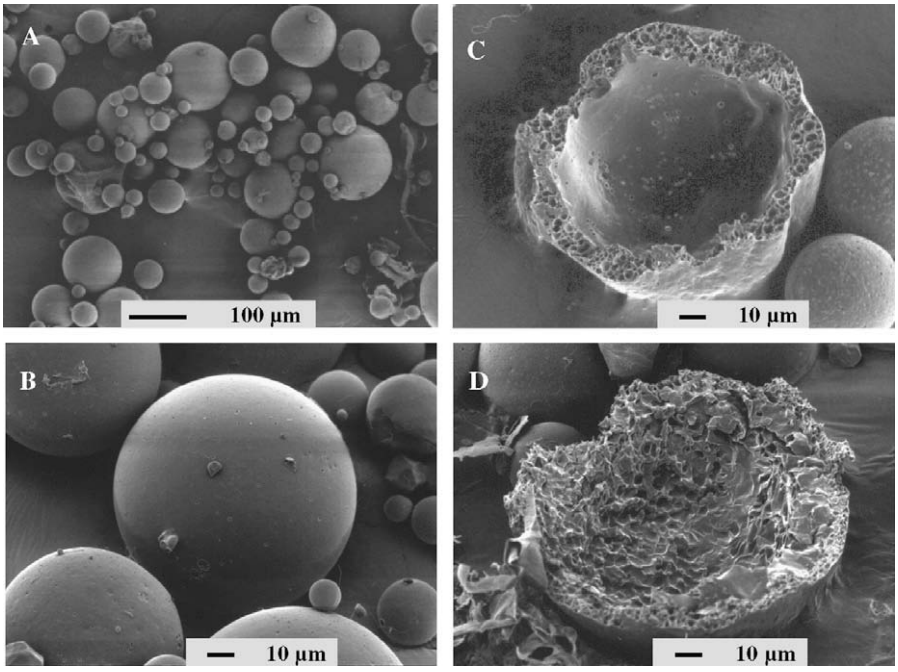

Fig. 7. Scanning electron microscopy (SEM) images showing the surface morphology of blank (A) or amiodarone-loaded microparticles (B), and the internal morphology of the fractured corresponding blank (C) or amiodarone-loaded microparticles (D). The protein used in both cases is BSA.

4. Conclusions

It was established that an oil-in-water emulsion is spontaneously formed at room temperature, without additional external stirring, as soon as an organic solvent partly miscible to water is put into contact with an aqueous phase containing protein and PEG with middle chain length, soluble in the oil phase, and used above a relatively low concentration threshold. From the results of adsorption kinetics, it appears that PEG molecules adsorb at the interface as soon as a drop of DCM is put in contact with the aqueous phase. These molecules would initiate rapid diffusion of DCM towards the aqueous phase through the formation of interfacial turbulences. Then, the protein molecules would act as a stabilizing agent of the DCM droplets newly created. After some recombination for about 1 h, probably by coalescence, oil droplets appear as stable structures of about 50 µm in diameter.

Furthermore, it was shown that the presence of polylactid acid in the organic phase does not prevent spontaneous emulsification to occur, and formulation of microparticles could be achieved. Thus, by applying this new spontaneous emulsification self-diffusion method, spherical microcapsules of about 50 µm were produced, and preliminary tests allowed a model drug, amiodarone, to be encapsulated. Optimization and applications of this novel and very simple formulation process will be the object of further studies.

Acknowledgments

The authors thank Dr. R. Filmon (Service Commun d'Imageries et d'Analyses Microscopiques (SCIAM), University of Angers) for the SEM photographs, and J. Colleau-Haffner for her technical assistance. K. Schönhammer was financially supported by the Socrates Program.

References

[1] K.F. Pistel, B. Bittner, H. Koll, G. Winter, T. Kissel, Biodegradable recombinant human erythropoietin loaded microspheres prepared from linear and star-branched block copolymers: influence of encapsulation technique and polymer composition on particle characteristics, J. Control. Release 59 (1999) 309–325.
[2] I.D. Rosca, F. Watari, M. Uo, Microparticle formation and its mechanism in single and double emulsion solvent evaporation, J. Control. Release 99 (2004) 271–280.
[3] U. Bilati, E. Allemann, E. Doelker, Strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microparticles, Eur. J. Pharm. Biopharm. 59 (2005) 375–388.
[4] I.J. Castellanos, R. Crespo, K. Griebenow, Poly(ethylene glycol) as stabilizer and emulsifying agent: a novel stabilization approach preventing aggregation and inactivation of proteins upon encapsulation in bioerodible polyester microspheres, J. Control. Release 88 (2003) 135–145.
[5] J.M. Pean, F. Boury, M.C. Venier-Julienne, P. Menei, J.E. Proust, J.P. Benoit, Why does PEG 400 co-encapsulation improve NGF stability and release from PLGA biodegradable microspheres?, Pharm Res. 16 (1999) 1294–1299.
[6] J.M. Harris, Introduction to biotechnical and biomedical applications of poly(ethylene glycol), in: J.M. Harris (Ed.), Poly(ethylene glycol) Chemistry, Plenum Press, New York, 1992, pp. 1–14.
[7] A. Malzert-Freon, F. Boury, J.P. Benoit, Interactions between poly(ethylene glycol) and protein in dichloromethane/water emulsions: a study of interfacial properties, Eur. J. Pharm. Biopharm. 69 (2008) 835–843.
[8] J.T. Davies, E.K. Rideal, Interfacial Phenomena, Academic Press, New York, London, 1963.
[9] N. Shahidzadeh, D. Bonn, J. Meunier, Dynamics of spontaneous emulsification for fabrication of oil in water emulsions, Langmuir 16 (2000) 9703–9708.
[10] J. Gad, Archives of anatomy and physiology 181 (1878).
[11] C.A. Miller, Spontaneous emulsification produced by diffusion – a review, Colloid. Surface 29 (1988) 89–102.
[12] C.W. Pouton, Formulation of self-emulsifying drug delivery systems, Adv. Drug Deliver. Rev. 25 (1997) 47–58.
[13] J. Mauduit, M. Vert, Les polymères à base d'acides lactiques et glycolique et la délivrance contrôlée des principes actifs, S.T.P. Pharma Sci. 3 (1993) 197–212.
[14] A. Malzert, F. Boury, D. Renard, P. Robert, J.E. Proust, J.P. Benoit, Influence of some formulation parameters on lysozyme adsorption and on its stability in solution, Int. J. Pharm. 242 (2002) 405–409.
[15] J.M. Pean, M.C. Venier-Julienne, R. Filmon, M. Sergent, R. Phan-Tan-Luu, J.P. Benoit, Optimization of HSA and NGF encapsulation yields in PLGA microparticles, Int. J. Pharm. 166 (1998) 105–115.
[16] S.J. Weir, C.T. Ueda, Rapid liquid chromatographic assay for the determination of amiodarone and its N-diethyl metabolite in plasma, urine, and bile, J. Pharm. Sci. 74 (1985) 460–465.
[17] M.J. Rang, C.A. Miller, H.H. Hoffmann, C. Thunig, Behavior of hydrocarbon/alcohol drops injected into dilute solutions of an amine oxide surfactant, Ind. Eng. Chem. Res. 35 (1996) 3233–3240.
[18] M.J. Rang, C.A. Miller, Spontaneous emulsification of oils containing hydrocarbon, nonionic surfactant, and oleyl alcohol, J. Colloid Interf. Sci. 209 (1999) 179–192.

[19] N. Shahidzadeh, D. Bonn, O. Aguerre-Chabriol, J. Meunier, Spontaneous emulsification: relation to microemulsion phase behaviour, Colloid. Surface A 147 (1999) 375–380.
[20] M. Ide, D. Yoshikawa, Y. Maeda, H. Kitano, State of water inside and at the surface of poly(ethylene glycol) films examined by FT–IR, Langmuir 15 (1999) 926–929.
[21] Z. Xu, N.B. Holland, R.E. Marchant, Conformations of short-chain poly(ethylene oxide) lipopolymers at the air–water interface: a combined film balance and surface tension study, Langmuir 17 (2001) 377–383.
[22] J.C. Lee, L.L. Lee, Preferential solvent interactions between proteins and polyethylene glycols, J. Biol. Chem. 256 (1981) 625–631.
[23] T. Arakawa, S.N. Timasheff, Mechanism of poly(ethylene glycol) interaction with proteins, Biochemistry 24 (1985) 6756–6762.
[24] D. Quintanar-Guerrero, E. Alléman, H. Fessi, E. Doelker, Preparation techniques and mechanisms of formation of biodegradable nanoparticles from preformed polymers, Drug Dev. Ind. Pharm. 24 (12) (1998) 1113–1128.
[25] S. Galindo-Rodriguez, E. Alléman, H. Fessi, E. Doelker, Physicochemical parameters associated with nanoparticle formation in the salting-out, emulsification–diffusion, and nanoprecipitation methods, Pharm. Res. 21 (8) (2004) 1428–1439.
[26] H.S. Yoo, H.K. Choi, T.G. Park, Protein–fatty acid complex for enhanced loading and stability within biodegradable nanoparticles, J. Pharm. Sci. 90 (2001) 194–201.
[27] T. Nishimi, C.A. Miller, Spontaneous emulsification of oil in aerosol–OT/water/hydrocarbon systems, Langmuir 16 (2000) 9233–9241.
[28] S. Damodaran, K. Anand, L. Razumovsky, Competitive adsorption of egg white proteins at the air–water interface: direct evidence for electrostatic complex formation between lysozyme and other egg proteins at the interface, J. Agr. Food Chem. 46 (1998) 872–876.
[29] K. Anand, S. Damodaran, Kinetics of adsorption of lysozyme and bovine serum albumin at the air–water interface from a binary mixture, J. Colloid Interf. Sci. 176 (1995) 63–73.
[30] C.J. Beverung, C.J. Radke, H.W. Blanch, Protein adsorption at the oil/water interface: characterization of adsorption kinetics by dynamic interfacial measurements, Biophys. Chem. 81 (1999) 59–80.
[31] M. Saito, L.J. Yin, I. Kobayashi, M. Nakajima, Preparation characteristics of monodispersed oil-in-water emulsions with large particles stabilized by proteins in straight-through microchannel emulsification, Food Hydrocolloid. 19 (2005) 745–751.
[32] F.I. Marcus, G.H. Fontaine, R. Frank, Y. Grosgogeat, Clinical pharmacology and therapeutic applications of the antiarrhythmic agent amiodarone, Am. Heart J. 101 (1981) 480–493.
[33] J. Gill, R.C. Heel, A. Fitton, Amiodarone: an overview of its pharmacological properties and a review of its therapeutic use in cardiac arrhythmias, Drugs 43 (1992) 69–110.
[34] T. Niwa, H. Takeuchi, T. Hino, N. Kunou, Y. Kawashima, Preparations of biodegradable nanospheres of water-soluble and insoluble drugs with D,L-lactide/glycolide copolymer by a novel spontaneous emulsification solvent diffusion method, and the drug release behavior, J. Control. Release 25 (1993) 89–98.
[35] A. Schade, T. Niwa, H. Takeuchi, T. Hino, Y. Kawashima, Aqueous colloidal polymer dispersions of biodegradable DL-lactide/glycolide copolymer as basis for latex films: a new approach for the development of biodegradable depot systems, Int. J. Pharm. 117 (1995) 209–217.
[36] H. Murakami, M. Kobayashi, H. Takeuchi, Y. Kawashima, Preparation of poly(DL-lactide-co-glycolide) nanoparticles by modified spontaneous emulsification solvent diffusion method, Int. J. Pharm. 187 (1999) 143–152.
[37] K.M. Park, M.K. Lee, K.J. Hwang, C.K. Kim, Phospholipid-based microemulsions of flurbiprofen by the spontaneous emulsification process, Int. J. Pharm. 183 (1999) 145–154.
[38] N.S. Santos-Magalhaes, A. Pontes, V.M.W. Pereira, M.N.P. Caetano, Colloidal carriers for benzathine penicillin G: nanoemulsions and nanocapsules, Int. J. Pharm. 208 (2000) 71–80.
[39] J.A. Reddick, W.B. Bunger, Techniques of chemistry, Organic Solvents: Physical Properties and Methods of Purification, third ed, vol II, Wiley-Interscience, 1970, ISBN 0 471 92726 0.
[40] European Pharmacopoeia, sixth ed., 2008.