Swelling Behavior and Controlled Release of Theophylline and Sulfamethoxazole Drugs in β-Lactoglobulin Protein Gels Obtained by Phase Separation in Water/Ethanol Mixture

Thimma T. Reddy, Laurence Lavenant, Jacques Lefebvre, and Denis Renard*

Institut National de la Recherche Agronomique, Centre de Nantes, BP 71627-44316 Nantes Cedex 03, France

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Physically cross-linked β-lactoglobulin (BLG) protein gels containing theophylline and sulfamethoxazole low molecular weight drugs were prepared in 50% ethanol solution at pH 8 and two protein concentrations (6 and 7% (w/v)). Swelling behavior of cylindrical gels showed that, irrespective of the hydrated or dehydrated state of the gel, the rate of swelling was the highest in water. When the gels were exposed to water, they first showed a swelling phase in which their weight increased 3 and 30 times for hydrated and dehydrated gels, respectively, due to absorption of water, followed by a dissolution phase. The absorption of solvent was however considerably reduced when the gels were exposed to aqueous buffer solutions. The release behavior of both theophylline and sulfamethoxazole drugs from BLG gels was achieved in a time window ranging from 6 to 24 h. The drug release depended mainly on the solubility of the drugs and the physical state of the gel (hydrated or dry form). Analysis of drug release profiles using the model of Peppas showed that diffusion through hydrated gels was governed by a Fickian process whereas diffusion through dehydrated gels was governed partly by the swelling capacities of the gel but also by the structural rearrangements inside the network occurring during dehydration step. By a judicious selection of protein concentration, hydrated or dehydrated gel state, drug release may be modulated to be engineered suitable for pharmaceutical as well as cosmetics and food applications.

Introduction

Chemical and physical cross-linked hydrogels for controlled and/or sustained core release have been developed for pharmaceutical and other applications using both synthetic and natural polymers. Natural polymers suitable for developing hydrogels for such applications include mainly proteins and polysaccharides. Therefore, there is an increasing interest in physically cross-linked hydrogels because the use of cross-linking agents to prepare such hydrogels is avoided. Several physical interactions were exploited to design hydrogels such as ionic interactions, e.g., alginate gels,[1−3] hydrophobic interhelical interfaces (coiled-coil interactions) taking place in self-assembled artificial proteins,[4,5] or antigen antibody interactions by making an antigen-sensitive hydrogel prepared by the application of the antigen−antibody binding at cross-linking points in the hydrogel.[6]

Proteins have been shown to present a significant potential as wall materials for controlled and sustained release of different drugs, and most of the reported research has been focused on different albumins.[7−11] Both chemical and physical cross-linking methods have been used to create protein hydrogels. In chemically cross-linked gels, covalent bonds are present between different protein aggregates. In physically cross-linked protein gels, dissolution is prevented by physical interactions existing between protein aggregates.

Among proteins, whey proteins (WP) or β-lactoglobulin (BLG) are widely used as food ingredients, because of their high nutritional value and their remarkable functional properties.[12,13] Among these is their gel forming ability, which is particularly of great importance to the food industry. In recent years, the concept of using whey proteins as microencapsulating agents has been established.[14,15] Emulsification, heat gelation and chemically cross-linked whey protein based microcapsules have been developed for controlled release applications.[15−17] Wall matrixes consisting of whey proteins isolate (WPI) have been reported to provide effective protection against oxidation of encapsulated lipids in storage conditions that are known to promote lipid oxidation.[18] A series of studies have demonstrated that whey proteins exhibit excellent microencapsulating properties and are suitable for microencapsulation of volatile and nonvolatile core materials.[19−25] However, thermal treatment or chemical cross-linking[26] needed to induce gelation of BLG limits their application in formulations containing heat sensitive ingredients and toxicity problems of chemical agents may damage incorporated active agents. Furthermore, for whey proteins, an alternate gelation method, involving low temperature (i.e., cold gelation) have been exploited to overcome this limitation.[26] This method requires preheating of protein solution followed by addition of chilled Ca^{2+} salt solution. The amount of salt used to form a cold-set gel is likely to be the major determinant of the structure and spatial organisation of protein aggregates.[27]

To create physically cross-linked gels, another alternative and original method based on gelation/phase separation at room temperature using cosolvent such as water/ethanol solution has been investigated.[28−30] The protein hydrogel formed by this way can be used directly in virtually any biological system, as the ingredients used to prepare gels are biocompatible and biodegradable provided part or total ethanol amount is removed. It has significant advantages for purification and drug loading in comparison to previously methods reported,[8] since high temperature and small toxic cross-linkers can be avoided. The effect

* To whom correspondence should be addressed. Tel: 33 2 40 67 50 52. Fax: 33 2 40 67 50 25. E-mail: drenard@nantes.inra.fr.

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of ethanol on structural changes of BLG in dilute solutions has been extensively studied using spectroscopic methods and revealed that BLG shifted from a predominantly β-sheet structure to an α-helical structure when ethanol percentage increased.[28] This conformational change has been demonstrated to be reversible, i.e., the protein fully recovered its native conformation after removal of ethanol.[31] The gelation kinetics of BLG in 50% (v/v) water/ethanol solution were studied at 25 °C by using rheological measurements and microscopic methods.[29] The phase state diagrams as a function of pH and ethanol concentration showed that a gel structure appeared after a period ranging from 1 min to 1 week, depending on the physicochemical conditions.[30] Viscoelasticity and infrared measurements indicated that alcohol-induced gelation would proceed via a two-step mechanism: small aggregates loosely connected between them were first built up, and a real network took place in a second step. The coarse and irregular structures formed in aqueous ethanol gels revealed by confocal laser scanning microscopy could be analyzed in terms of a phase separation.[30] BLG in water−ethanol solution would undergo either an inhibition of the demixing by gelation or a binary phase separation accompanied by an irreversible gelation transition.

In this paper, results from the encapsulation of two different model drugs, theophylline (TP) and sulfamethoxazole (SF), in BLG networks formed by the gelation/phase separation method at room temperature and pH 8 were presented. The objectives were to demonstrate the combined swelling and controlled release capacities of these protein hydrogels in order to find potential applications in the microencapsulation field.

Materials and Methods

β-lactoglobulin (BLG) was obtained from Davisco International, Inc. (Lot number JE 001-1-922, Le Sueur, MN). Protein content was determined with the semi-micro Kjeldahl method (AOAC, 1984) using N-factor of 6.38 and was found to be 89.8%. Hydrophobic C18 chromatography using a water-TFA (0.1%) gradient (solvent A) and a TFA (0.1%)-acetonitrile gradient (solvent B) gave two major peaks corresponding to BLG variants A and B (98.2%) and a minor peak (1.6%) corresponding to α-lactalbumin. The mineral composition was (g per 100 g) 0.013 Mg^{2+}, 0.079 Ca^{2+}, 0.576 Na^{+}, 0.097 K^{+}, and 0.050 Cl^{-}. BLG was extensively dialyzed against water (until conductivity close to water was reached) before use and freeze-dried. SF (lot no. 101K1461) and TP (anhydrous, minimum 99% purity, lot no. 093K0122) compounds were purchased from Sigma-Aldrich. TP has a moderate aqueous solubility of 8.3 g/L at 25 °C, whereas its solubility in ethanol is 12.5 g/L at 25 °C. SF has a very low aqueous solubility of 0.2 g/L at 40 °C, whereas it is sparingly soluble in alcohol. Buffer solutions were prepared using analytical grade chemicals.

Protein Solutions Preparation. The protein was dissolved in water (stock solution 20%, w/w, dry-matter basis) and the pH adjusted to 8 with NaOH solutions (0.1 or 1 M). The solutions were stirred overnight at 4 °C to ensure both complete hydration and maximum solubility of the material. The undissolved material was, therefore, removed by centrifugation at 11 000*g* for 40 min. The concentration of the protein stock solution was determined from the optical density at 278 nm, corrected for turbidity, using the value $A_{1cm}^{1\%} = 9.6$ for the specific absorption coefficient.

Hydrated Gels Preparation. Appropriate volumes of water and ethanol were added to BLG stock solution to give the required concentrations of BLG (6 or 7%, w/v) in aqueous ethanol solutions, the final ethanol concentration in the mixture being 50% (w/v). These two protein concentrations were chosen for two reasons: first, critical gelation time t_g was appropriate for swelling and drug release studies, i.e., $t_g = 4000$ and 500 min for 6 and 7% (w/v) protein concentration, respectively) and second, gel strength was in agreement with further gels manipulation. 25 mL of protein solutions at the two different concentrations and two different pHs (2 and 8) were homogenized by vortexing the tubes for a few seconds followed by degassing to remove air bubbles. The solutions were then poured into circular Teflon moulds, which were previously fixed onto Teflon plate with the help of silicon grease. The upper part of the moulds were covered with glass plates to avoid samples dehydration. Depending on the pH and concentration used, the equilibrium gel state was reached in a time period ranging from 1 to 7 days. Constant equilibrium time of one week was chosen whatever the conditions used to obtain BLG gels in order to achieve complete gelation and to get stiff gels. After one week, gels were removed from the Teflon moulds and punched them into cylindrical shapes except those formed at pH 2 which were too brittle and finally unabled to be punched into cylindrical shapes. As it was impossible to handle gels without breaking the structure, BLG gels formed at pH 2 were discarded from our study.

Dehydrated Gel Preparation and Characterization. BLG hydrated gels were dried at room temperature until a constant weight was reached. The solvent content after complete dehydration of protein gels was evaluated by the Karl Fisher method and was found to be 4% for all of the gels. Wide-angle X-ray diffraction was used to characterize the amorphous or crystalline phases present in the solid transparent protein matrixes.

Encapsulation. 10% (w/w) of two different drugs, SF and TP, with respect to protein dry mass, were dispersed into the protein solutions at pH 8 separately in 30 mL plastic tubes. An appropriate volume of ethanol was then added giving a 50% (w/v) ethanol concentration and a total protein concentration of 6 or 7% (w/v). It was checked that dispersion of model drugs in ethanol before mixing with protein solutions did not affect drug release kinetics. Immediately after adding ethanol, the tubes were subjected to homogenization for a few seconds followed by degassing to remove air bubbles. The drug containing solutions were placed into circular Teflon moulds to form gels. The Teflon moulds were covered with glass plates to avoid samples dehydration. After one week, circular gel disks were removed from Teflon moulds and punched into cylindrical shape gels. Some pieces of gels were dried at room temperature while other pieces of gels were stored in closed plastic bottles before use.

Swelling of Hydrated and Dehydrated Gels in Different Solvents. A known weight of both hydrated and dehydrated protein gels was put in different conical flasks, and 50 mL of different solvents such as water, water/ethanol, phosphate buffer solution (PBS) pH 7.8, PBS + dithiothreitol (DTT), PBS + 1 M urea, PBS + 3 M urea, sodium chloride (NaCl) solution, and NaCl−HCl buffer pH 1.2 were added separately. The flasks were kept in a shaking water bath (100 rpm) at 37 °C, the gels being removed at different times, blotted with a tissue paper to remove surface water, and weighed. The swelling ratio was defined as the weight of absorbed solvent (W_2) per weight of initial gel weight (W_1). The swelling ratio Q of the gels was then calculated using the equation:

$$Q = \frac{W_2 - W_1}{W_1} \tag{1}$$

Each swelling experiment was repeated two times ($n = 2$).

In Vitro Release. Two different concentrations (6 and 7%, w/v) of hydrated and dehydrated protein gels containing two different drugs of 10% w/w (with respect to protein weight) were used for in vitro release studies in three different solvents: water, PBS pH 7.8, and NaCl−HCl buffer pH 1.2. Briefly, known weights of drug containing hydrated and dehydrated gel pieces were taken in 200 mL conical flasks, and 100 mL of water and buffer solutions were added separately to each flask. The flasks were placed in a constant shaking water bath at 37 °C. Aliquots (0.5 mL) were withdrawn periodically to determine drug concentration and, in all cases, equal volumes of dissolution medium were immediately added to maintain a constant volume. TP and SF concentrations were determined spectrophotometrically at 274

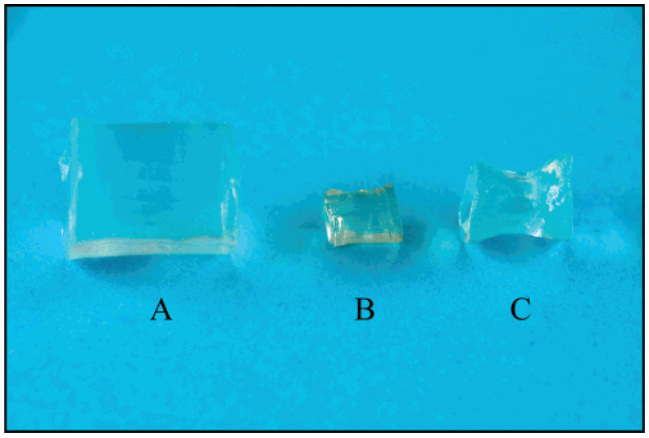

Figure 1. Morphologies of β-lactoglobulin (BLG) gels ($C = 7\%$, w/v) formed in 50/50 (w/v) water/ethanol solvent (A) fresh hydrated gel (B) dehydrated gel (C) dehydrated gel after equilibrium swelling in PBS.

and 264 nm, respectively. Absorbance from blank (gels without drug) as a function of time was systematically measured and subtracted from the drug loaded gels absorbance value. This measurement ensured to take into account the partial solubilization of gels in the external solvent that might occur during the time of release experiments. Samples were withdrawn until two successive aliquots showed no increase in absorbance. The amount of TP and SF released from the hydrated and dehydrated gels in a dissolution medium, at a given time, was calculated using standard curves of TP and sulfamethazole in corresponding buffers and expressed as percentage of total drug content of the investigated gels. Each drug release experiment was repeated three times ($n = 3$).

Drug Release Mechanism. The controlled-swelling characteristic of the hydrogel allows its release kinetics to be analyzed with Fickian and non-Fickian diffusional behavior.[32] Equation 2 displays the model which the TP and SF release data can be fit to

$$\frac{M_t}{M_\infty} = kt^n \qquad (2)$$

where M_t and M_∞ represent drug release at time, t, and at equilibrium, respectively, k is the rate constant characteristic of the system, and n is the diffusional exponent.[33] Equation 2 can only be applied to the first 60% of drug release. The diffusional exponent (n) is calculated as the slope, and the rate constant (k) is calculated as the intercept of linear regression lines fitted to the $\log(M_t/M_\infty)$ versus log time plots.[34] Ultimately, the value of n determines if the hydrogel release represents Fickian ($n = 0.5$) or non-Fickian diffusion ($n > 0.5$).

Results and Discussion

The morphologies of BLG gels produced in water/ethanol solvent in various physical states, swelled in water after 24 h, totally dehydrated and freshly hydrated, were depicted in Figure 1. Totally transparent and homogeneous samples were observed whatever the physical state. Using wide-angle X-ray diffraction to identify crystalline or amorphous phases, no changes in both intensity and peak position were observed on the dehydrated and the annealed sample. As in native protein powder, two peaks at 9.9 and 4.5 Å were observed (results not shown). The absence of new peaks in the dehydrated gels would mean that no increase of crystalline phases, therefore, appeared in the solid transparent matrix (dehydrated state).

The samples corresponding to the equilibrium states of swollen gels in water were liquid after 30 days. This observation would lead to the conclusion that gels formed in water/ethanol solutions are reversible. However, some protein fibrous structures were identified in the flasks leading to the hypothesis that the reversibility would not be total.

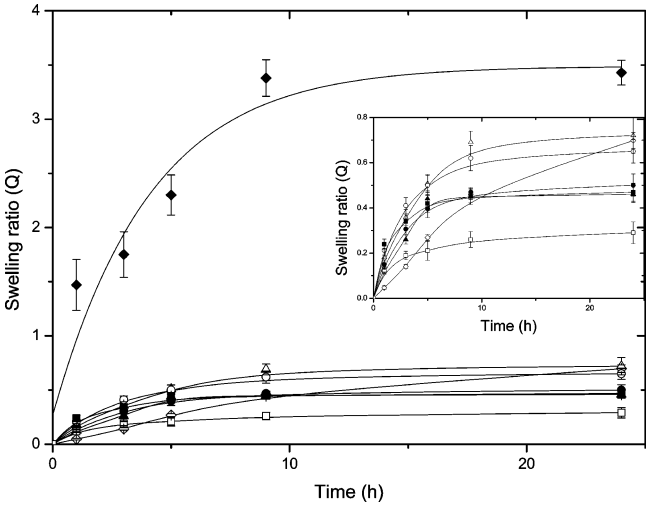

Figure 2. Swelling kinetics of β-lactoglobulin hydrated gels ($C = 7\%$ (w/v), 50/50 (w/v) water/ethanol) in different solvents ($n = 2$). Inset: same figure without data collected in water, (◆) water, (◇) 50/50 (w/v) water/ethanol, (▲) PBS pH 7.8, (△) PBS + DTT, (●) PBS + 1 M urea, (○) PBS + 3 M urea, (■) NaCl, and (□) NaCl−HCl buffer pH 1.2.

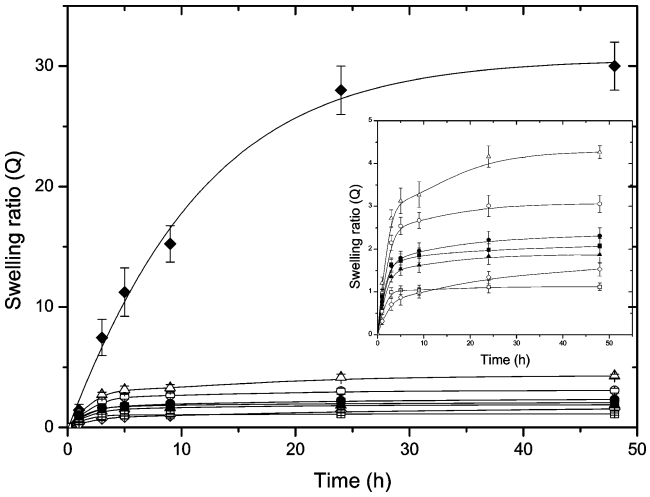

Figure 3. Swelling kinetics of β-lactoglobulin dehydrated gels (resulting from a hydrated gel prepared at $C = 7\%$ (w/v) and 50/50 (w/v) water/ethanol) in different solvents ($n = 2$). Inset: same figure without data collected in water, (◆) water, (◇) 50/50 (w/v) water/ethanol, (▲) PBS pH 7.8, (△) PBS + DTT, (●) PBS + 1 M urea, (○) PBS + 3 M urea, (■) NaCl, and (□) NaCl−HCl buffer pH 1.2

Swelling Kinetics. BLG gels produced in aqueous ethanol solutions were essentially formed by intermolecular hydrogen bonding and/or hydrophobic interactions. Since intramolecular cross-links do not contribute to the effective elasticity of the network, the swelling of the network is mainly governed by the intermolecular cross-links. Intramolecular cross-links are predominantly formed when networks are prepared by cross-linking diluted low molecular weight polymer solutions, whereas intermolecular cross-links are formed by cross-linking concentrated polymer solutions.[35,36] The ratio inter/intramolecular cross-linking will then depend on the extent the polymer chains are entangled. The polymer chains will start to entangle when the total volume occupied by the hydrated BLG molecules equals the total volume of the solution.

Figures 2 and 3 displayed the swelling capacities of hydrated and dehydrated gels in different solvents, respectively. The

solvents used to study the swelling of the gels were water, 50/50 (w/v) water/ethanol, PBS pH 7.8, PBS + DTT, PBS + 1 M urea, PBS + 3 M urea, NaCl solution, and NaCl−HCl buffer pH 1.2.

In the case of BLG gels swelling in different solvents except water, results in Figure 2 (inset) showed that the rate of swelling of hydrated gels decreased in the order PBS + DTT, 50/50 (w/v) water/ethanol, PBS + urea 3 M, PBS + urea 1 M, NaCl solution, PBS pH 7.8, and NaCl−HCl pH 1.2. The results in Figure 3 (inset) differed only for dehydrated gels swelled in water/ethanol where the swelling ratio (Q) was quite similar to those obtained in NaCl−HCl pH 1.2. The peculiar swelling profile of the hydrated gel in the presence of water/ethanol (Figure 2) was unexplained. The highest swelling ratio obtained in the case of PBS + DTT, whatever the physical state of the gels, would mean that disulfide bonds between protein aggregates would exist in these gels and would be disrupted through the action of DTT. In addition, the difference in swelling behavior between PBS and PBS + DTT was obvious on dehydrated gels (Figure 3). This result would mean that disulfide bonds were very likely to be formed in the dehydrated state. As a consequence, solvent molecules would enter the protein network and disrupt protein aggregates allowing the solvent−solute interactions to occur through hydrogen bonding. The mesh size of the network would thus increase more easily than in other solvents and would cause the gel to swell. The use of urea alone provoked the disruption of hydrogen bonding between protein aggregates and allowed the gel to swell at a rate however lower than in the case of DTT.

In the case of the swelling media such as PBS alone, NaCl solution, or NaCl−HCl buffer, the rate of swelling of both hydrated and dehydrated gels was the lowest. The presence of these ions would cause a reduction of the interactions between protein and solvent molecules decreasing thus drastically the swelling ratio (Q). The possible consequences of salt ions in the swelling medium was to vary the osmotic pressure due to differences in the ionic concentration of the interior of the gel and the external solution. This would be due to decrement in the expansion of the gel network because of repulsive forces of counterions acting on the polymeric chain shielded by the bound ionic charges. Therefore, the difference in the osmotic pressure between the gel network and the external solution decreased with an increase in the ionic strength of the salt concentration. This osmotic pressure effect could compete with chemical effects in the particular case of PBS + DTT external solvent. Structural changes inside protein network would be the driving force instead of ionic strength differences between the interior of the gel and the external solution to explain the higher swelling ratio Q obtained in the particular case of PBS + DTT. The results obtained in the case of PBS and NaCl−HCl buffer were in good agreement with those previously reported in the case of protein based microcapsules.[14]

The results displayed in Figures 2 and 3 showed also that, irrespective of the state of the gel, whether hydrated or dehydrated, the rate of swelling was the highest in water. The increase of swelling ratio (Q) was however 10 fold higher in the case of the dehydrated gels. The lower Q values obtained with hydrated gels could be due to the presence of ethanol, which may interrupt further penetration of water molecules by forming hydrogen bonds with protein molecules thereby reducing the swelling capacity of the hydrated state gel. When hydrated and dehydrated protein gels swelled in water, the interactions solvent−solute were predominant other solute−solute interactions leading to a rate of swelling much important in water than in other solvents. From a thermodynamic point of view, three forces which interact to either expand or shrink polymer networks were identified.[37] The forces are the rubber elasticity, the polymer−polymer affinity and the hydrogen-ion pressure. The sum of these three forces was called the osmotic pressure of the gel. The hydrogen-ion pressure gives rise to pressure in the gel. At equilibrium, the total free energy variation is zero, which means that for swollen hydrogels, a decrease in free energy, due to mixing ions and solvent molecules with the network chains, is balanced by an increase in free energy due to stretching of the network chains. It can be expected that, upon increasing the number of anionic residues in the hydrogel, the driving force for swelling increases. This thermodynamic consideration could explain the results obtained in the case of BLG swollen gels where the ionization of amino and carboxyl groups increases noticeably in aqueous solvent. This hypothesis was given to explain the higher degree of swelling when heat-set albumin or gelatine gels swelled in solutions prepared at pH's far from the isoelectric point.[38,39]

Matrix Mesh Sizes. To fully characterize BLG hydrogels, the mesh sizes of the protein matrixes were calculated based on a variation of the Flory−Rehner method. The underlying physics is that tighter cross-linked structures will swell (or shrink) less than opened cross-linked structures. The method relates the volumetric swelling ratio Q to the hydrogel mesh size, ξ, according to the following equation:

$$\xi = Q^{1/3}\sqrt{r_0^{\,2}} \tag{3}$$

where r_0^2 is the root-mean square distance between the cross-links and for BLG, it is assumed that this distance is the hydrodynamic diameter ($D_h = 5.5$ nm for BLG in its dimeric form). This assumption was confirmed and measured in the case of BSA networks.[40] The root-mean square distance is related to this diameter as follows:

$$\left(\frac{r_0^{\,2}}{2n}\right)^{1/2} \cong 5.5 \text{ nm} \tag{4}$$

where n is the number of repeat units or residues in the protein, and $n = 162$ for BLG. The mesh sizes values based on swelling ratios determined for both hydrated and dehydrated BLG gels were listed in Table 1. Mesh sizes values ranged from 65 to 149 nm for the hydrated state and from 103 to 308 nm for the dehydrated state depending on the solvent conditions used to swell protein gels. These findings would mean that some rearrangements inside the networks occurred during dehydration and leaded to more open structures during swelling, i.e., higher ξ values. In addition, ξ values obtained in our case would be in agreement with a pure Fickian diffusion mechanism of molecules with sizes lower than the mesh size. This hypothesis will be checked in the next part dealing with in vitro release kinetics of model drugs and data fitting according to eq 2.

In Vitro Drug Release Kinetics. The release of the drugs from gels can be controlled by increasing the physical or chemical cross-linking density.[41] Physically entangled polymer systems slowly dissolve and release the drug simultaneously with polymer dissolution. Chemically cross-linked polymer gels degrade due to hydrolysis or enzymatic digestion and drug in these delivery systems is released at a rate that is dependent on the rate of polymer degradation.[42] Hydrogels that swell after contact with water permit diffusion of macromolecules throughout the entire matrix, drugs being released through a porous structure that expands during swelling.[43] The size of the pores

Table 1. Swelling Ratios (Q), Mesh Sizes (ξ, nm) and Diffusion Exponents (n) for β-Lactoglobulin (BLG) Hydrogels ($C = 7\%$ (w/v), 50/50 (w/v) Water/Ethanol) in Both Hydrated and Dehydrated States

solvent conditions	hydrated $Q^{1/3}$	hydrated ξ (nm)	drug	diffusion exponent n
water	1.51	149	SF	0.43
			TP	0.51
water/ethanol	0.89	88		
PBS pH 7.8	0.77	76	SF	0.45
			TP	0.46
PBS+DTT	0.90	89		
PBS+urea 1M	0.79	78		
PBS+urea 3M	0.87	86		
NaCl	0.78	77		
NaCl+HCl pH 1.2	0.66	65	SF	
			TP	0.36

solvent conditions	dehydrated $Q^{1/3}$	dehydrated ξ (nm)	drug	diffusion exponent n
water	3.11	308	SF	0.67
			TP	0.66
water/ethanol	1.15	114		
PBS pH 7.8	1.23	122	SF	0.63
			TP	0.65
PBS+DTT	1.62	160		
PBS+urea 1M	1.32	131		
PBS+urea 3M	1.45	144		
NaCl	1.27	126		
NaCl+HCl pH 1.2	1.04	103	SF	
			TP	0.74

(i.e., mesh sizes) located within the network, which is related to the extent of cross-linking and the degree of swelling, determines the drug release rate. The rate of drug release from hydrogel networks can be modified by varying the degree of physical entanglements within the gel, i.e., by varying concentration of the matrix or altering the number of chemical (or physical) cross-links between the polymer matrix and the molecule of interest.[44] For example, polymer−drug interaction can be modified by the use of ionisable groups on the polymer network, so that oppositely charged molecules are stabilized by the matrix and like charged molecules are excluded from the polymer matrix.[45]

The 10% SF and TP loaded hydrated (Figure 4A,C) and dehydrated (Figure 4B,D) 7% (w/v) BLG hydrogels were used to conduct release studies in three different external solvents such as water, PBS pH 7.8, and NaCl−HCl buffer pH 1.2.

It was first demonstrated that the drug release was not dependent on the drug concentration loaded in the gels nor the protein concentration used (6 or 7% w/v) to prepare gels (data not shown). It was however noticed that slight differences existed in the case of loaded TP dehydrated gels where the release rate was always slightly higher for the 6% (w/v) prepared protein gels. The independence of drug release rate on protein concentration used to prepare gels would mean that the network structure, and in particular the matrix mesh size, was unaffected by the difference of protein concentration used to prepare gels.

The release of the drugs depended mainly on the solubility of the drug in the medium, the physical state of the gel and the nature of the external solvent.

Effect of Drug Solubility on Release Kinetics. TP is known to have a moderate solubility in water and ethanol while SF has a very low solubility in both solvents (see Materials and Methods). However, it was recently demonstrated that the solubility of drug-like organic compounds could be enhanced in cosolvents.[46] SF solubility increased by a factor of 100 when drug was solubilized in PEG/water (50/50 v/v) mixed solvent. TP solubility was rather unchanged in pure aqueous and PEG/water solvent. These findings, demonstrated on 122 drug-like organic compounds, would mean that cosolvents such as water/ethanol may also enhance drugs solubility. In addition, complicated solubility behavior of TP and SF could arise from the fact that the nature of external solvent (water, PBS, or NaCl−HCl) was in all cases different from the solvent used to prepare gels (water/ethanol).

Panels A−C on hydrated gels and panels B−D on dehydrated gels in Figure 4 showed that the percentage of drug release of drugs was not directly related to the drug solubility. Panels A and C of Figure 4 revealed that the release of drugs from hydrated BLG gels in water and PBS as external solvent were identical. It was thus demonstrated that total release of SF and TP occurred after ~6 h, whereas the solubility of these two drugs are known to be very different in aqueous solvent. Another hypothesis would be that SF has a higher solubility in the water/ethanol mixed solvent as suggested below, allowing a diffusion similar to TP outside the protein matrix. Drug release differences were more pronounced in the dehydrated gels case (Figure 4B,D). It was thus demonstrated for instance that 95% of TP was released in water as external solvent after 6 h, whereas only 80% of SF was released during the same time. The difference of TP and SF drug release percentage was however less important in the case of PBS as external solvent (95% vs 87%, respectively, after 9 h). These results would mean that TP and SF release are in accordance with their different solubility behavior only in the case where protein gels were dehydrated.

The percentage of drug release from hydrated gels in NaCl−HCl pH 1.2 as external solvent was however very different between TP and SF. 24% of SF was released after 9 h (26% only after 24 h), whereas 93% of TP was released during the same time. The slow release of SF could be related to its poor solubility in acidic buffer. The consequence would be that the percentage of SF released after 24 h would correspond in fact to the total soluble fraction of the drug. The slow release of SF with time was also noticed when the drug was loaded in the dehydrated gels (Figure 4B,D).

It appeared from the above results that the drug release of both TP and SF may be independent of their solubility behavior except when NaCl−HCl pH 1.2 was used as external solvent where the low pH would play a crucial role on SF diffusion. These results were partly in agreement with previously data reported on release rate of drugs by using hydrophilic polysaccharide matrixes where it was concluded that TP release was always faster than those of SF.[47] The faster release of TP compare to SF was therefore evident in our study in the dehydrated protein gels case. The release of drug is generally controlled, from hydrophilic matrixes, by both diffusion and erosion phenomena, erosion phenomenon dominating the release rate of low aqueous solubility drugs.[48] About 70% of SF and TP drugs were released in 4 h in water and PBS buffer from both hydrated and dehydrated gels, whereas only 20% of SF was released in NaCl−HCl buffer even after 24 h. These huge differences between water and PBS on one hand and NaCl−HCl buffer on the other hand could be related to both an abnormal solubility of drugs and a very low swelling behavior of protein gels in acidic medium.

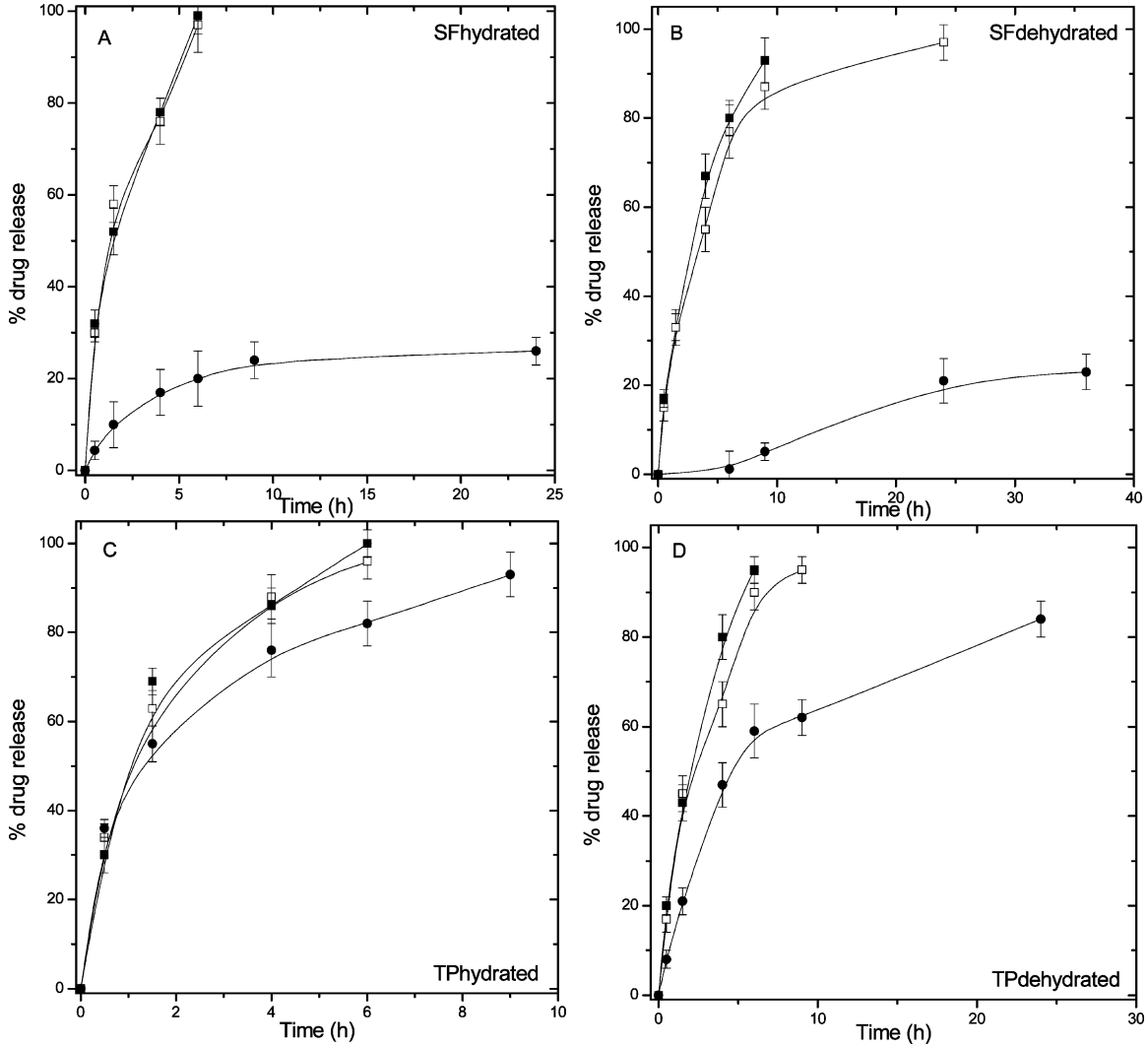

Figure 4. Sulfamethoxazole (SF) and theophylline (TP) release from hydrated (A, C) and dehydrated (B, D) 7% (w/v) BLG gels in different external solvents (n=3): (■) water, (□) PBS pH 7.8, and (●) NaCl−HCl pH 1.2

Effect of Gel State on Release Kinetics. Comparison in Figure 4 of panels A and B on one hand and panels C and D on the other hand revealed that the drugs release depended on the state of the gel (hydrated or dehydrated forms). The percentage of drug release was always higher in the case of hydrated gels. This higher drug release percentage was noticed for both water and PBS external solvents and particularly pronounced in the case of NaCl−HCl buffer external solvent. However, drug release percentage plateau values, for both hydrated and dehydrated gels, were reached at approximately the same time except those of SF release in NaCl−HCl buffer. In addition, the drug release percentage would be partly independent of the swelling capacities of hydrated and dehydrated gels. In the case of hydrated gels, the rate of release of both drugs in water and PBS was similar whereas the swelling ratio Q was 7-fold higher in water compare to PBS. This behavior could be attributed to the fact that the release of drugs is governed by a purely diffusion process through the hydrated protein matrix independently of its swelling capacities. Small molecules like drugs are thus released immediately through a porous structure that expands during swelling after the hydrogels are in contact with either water or buffer. Swelling tends thus to expand the mesh size of the network and allows the drug to be totally released into the medium.

In the case of dehydrated gels, a reasonable agreement was found between the rate of release of drugs and the swelling capacity of gel in water vs PBS buffer. The rate of release of both drugs in dehydrated gels was thus always faster while the swelling ratio Q was the highest in water. The release of drugs through the protein matrix would be partly governed by the onset of swelling allowing a diffusion of molecules in the external solvent. The process of release would be however considerably slowed in agreement with the swelling kinetics occurring in the dry matrixes.

These hypotheses dealing with the release of drugs in the case of both hydrated and dehydrated protein gels will be checked in the analysis of drug release through the calculation of the diffusion exponent n in the different external solvents (see the next section).

However, the higher percentage drug release measured in hydrated gels compared to dehydrated gels would not only depend partly on swelling of the matrix but also on its glass-transition temperature T_g. In the hydrated state, the protein aggregates (or polymer) are wet, soft and in the rubbery state and would have lower T_g, favoring molecules diffusion both inside and outside polymeric matrix.

Effect of External Solvent on Release Kinetics. It appeared clearly from Figure 4 that external solvent played an important role in the drug release kinetics particularly in the SF release case whatever the gel state. It was therefore demonstrated that SF release percentage was much higher in PBS buffer pH 7.8 than in NaCl−HCl pH 1.2. These differences could not however

be directly related-to-swelling ratio differences, the Q value differences being only 14 and 15% for PBS buffer and NaCl−HCl buffer (see Table 1) for hydrated and dehydrated gels, respectively. The drug release differences could be much more related to the SF solubility behavior as a function of pH. These different external solvents used were chosen in agreement with the gastro-intestinal tract pH conditions and the results obtained could have importance for pharmaceutical applications of these biocompatible hydrogels. The drug release kinetics were therefore much less affected by the nature of external solvent in the TP case, results in agreement with the higher solubility of the drug whatever the pH used.

Analysis of Drug Release. The diffusion exponents (n) were calculated according to eq 2. According to the model of Peppas for swellable cylindrical devices,[49] Fickian diffusion corresponds to a diffusion exponent $n = 0.45$, whereas polymer relaxation occurs at higher n values ($n = 0.89$). Data fitting results from eq 2 (Table 1) showed that n for TP and SF ranged from 0.43 to 0.74 irrespective of the solubility of the drug. The exponent n was not determined in the case of SF drug release in NaCl−HCl buffer as only ∼20% of drug was released whatever the physical state of the gel. The diffusion exponent values were quite similar whatever the type of drug and solvent conditions. However, it appeared clearly from Table 1 that the diffusion exponents n for hydrated gels were very close to the value given for a Fickian diffusion ($n = 0.45$) while those calculated for dehydrated gels were intermediate between Fickian diffusion and polymer relaxation according to Peppas's model.

These findings would indicate that the most important mechanism of drug release is by Fickian diffusion irrespective of the swelling ratios for the hydrated protein matrixes. This would confirm the fact that the diffusion of drugs through the mesh size of the network was independent of the swelling capacities of BLG hydrated gels, the diffusion rate being however in all cases faster in water.

The mechanism of drug release for the dehydrated gel matrixes would be more complicated and governed partly by the swelling capacities of the gel but also by the structural rearrangements inside the network occurring during dehydration step. These structural rearrangements would provoke some polymer relaxation, i.e., dissociation of the cross-links through protein aggregates disruption, during the dehydration step and further contact with external solvent. These combined processes would explain the intermediate values found for the diffusion exponent n in the framework of the model of Peppas developed for cylindrical swellable matrixes.

Conclusion

This study showed that β-lactoglobulin hydrogels could be used to encapsulate active compounds, gelation proceeded through a phase separation method using 50% ethanol solution at room temperature. This technique was thus conducted in mild conditions without using any harsh chemicals. Entrapment of TP and SF drugs inside these biodegradable matrixes, provided part or total ethanol was removed, revealed that drug release depended mainly on the solubility of the drugs and the physical state of the gel (hydrated or dry form). Analysis of drug release profiles using the model of Peppas showed that diffusion through hydrated gels was governed by a Fickian process, whereas diffusion through dehydrated gels was governed partly by the swelling capacities of the gel but also by the structural rearrangements inside the network occurring during dehydration step. By a judicious selection of hydrophilic or hydrophobic drugs, hydrated or dehydrated gel state, drug release may be modulated to be engineered suitable for pharmaceutical as well as food applications. Strategy applying mechanical treatments (i.e., shearing) during or after protein gelation would lead to fluid loaded gels that could find relevant cosmetics applications.

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