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ARTICLE

Influence of Ionic Complexation on Release Rate Profiles from Multiple Water-in-Oil-in-Water (W/O/W) Emulsions

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Water-in-oil-in-water (W/O/W) double emulsions were prepared, and the kinetics of release of magnesium ions from the internal to the external water phase was followed. Different chelating agents (phosvitin and gluconate) were used to bind magnesium within the prospect of improving the ion retention in the internal aqueous droplets. Magnesium release was monitored for 1 month of storage, for each formulation, with and without chelation, at two storage temperatures (4 and 25 °C). Leakage occurred without film rupturing (coalescence) and was mainly due to entropically driven diffusion/permeation phenomena. The experimental results revealed a clear correlation between the effectiveness of chelating agents to delay the delivery and their binding capacity characterized by the equilibrium affinity constant. The kinetic data (percent released versus time curves) were interpreted within the framework of a kinetic model based on diffusion and taking into account magnesium chelation.

KEYWORDS: W/O/W emulsions; sustained release; chelating agent; release mechanisms; diffusion/permeation

INTRODUCTION

Recently, there has been increasing interest in the development of multiple or double emulsions, for example, water-in-oil-in-water (W/O/W) and oil-in-water-in-oil (O/W/O). W/O/W emulsions consist of small water droplets dispersed in larger oil globules, which are themselves dispersed in an aqueous continuous phase. Numerous studies have shown that W/O/W emulsions provide a high capacity of entrapment and protection of the encapsulated species toward degradation. The compartmented structure allows the introduction of incompatible substances into the same system and subsequent sustained release (*1*−*8*). Because of their encapsulation and protection efficiency, W/O/W emulsions are potentially suitable materials for applications in various domains such as cosmetics, pharmaceutics, and foods. In this latter domain, double emulsions provide a number of potential benefits over conventional emulsions, such as reduction of fat content (*9*), taste masking, and protection of labile ingredients or sensitive probiotics (*8*). Double emulsions of the W/O/W type are generally stabilized by a couple of surface-active species following the empirical Bancroft rule: one is water-soluble to stabilize the oil globules in water and the other is oil-soluble to stabilize the internal aqueous droplets dispersed in oil. Nevertheless, there have been many difficulties associated with preparing this type of multiple emulsion with sufficient stability for commercial utilization, due to coalescence or due to diffusion of water molecules and of the whole set of hydrophilic solutes from the internal aqueous phase to the bulk aqueous phase or vice versa (*10*). During years of investigations to improve stability and to control sustained release of active species, short surfactant molecules have been progressively replaced by polymeric emulsifiers. Polymeric amphiphilic molecules, synthetic or naturally occurring ones, are known to impart better emulsion stability, to ensure better encapsulation and controlled release of the encapsulated species than low molecular weight surfactants (*11*−*15*).

In this paper, we addressed a common issue that arises with potential uses of double emulsions: achieving sufficient stability for commercial applications. This issue is relevant in all of the sectors that may potentially use double emulsions (food industry, pharmacy, and cosmetics). The main objective of the present study was to develop a novel strategy based on the complexation (chelation) of ionic encapsulated species to improve retention in W/O/W emulsions. Magnesium (Mg^{2+}) was used not only as a model species to probe the release mechanism but also as a valuable compound for food supplementation. Indeed, magnesium plays a role as a physiologic modulator affecting muscular contraction, cardiovascular function, and nerve impulse transmission. Due to a change in nutrition habits, the daily intake in magnesium is lower than the recommended value. Thus, magnesium supplementation of food could be an alternative to prevent magnesium deficiency and its clinical disorders, for example, hypertension, cardiovascular diseases, muscular weakness, and diarrhea. However, magnesium addition in foods can induce chemical degradations and protein aggregation and generate an unpleasant taste. These drawbacks could be avoided or at least reduced by encapsulation. In recent studies, we have studied the

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kinetics of release of magnesium ions in W/O/W double emulsions based on triglyceride oils, using sodium caseinate (water-soluble) and a polyglycerol ester (oil-soluble) as the surface-active species. Magnesium leakage occurred without film rupturing through diffusion/permeation mechanisms. The characteristic retention time varied between weeks and months depending on the oil chemical nature (*16*). When the emulsions were placed in the presence of pancreatic lipase, the triglycerides composing the oil phase were rapidly hydrolyzed (within minutes) by the enzyme, suggesting that magnesium could be available at the intestinal site. These results indicated a possible use of W/O/W emulsions loaded with magnesium ions in food.

In this study, we investigated the possibility of improving magnesium retention in W/O/W through ion binding by introducing metal chelatants in the internal droplets at sufficiently large concentrations: gluconate ions or phosvitin. By forming water-soluble complexes with magnesium, such chelatants were expected to decrease the concentration of free ions and, thus, to lower the rate of leakage. Gluconic acid is a polyhydroxycarboxylic acid derived from glucose. It possesses a carboxylic group and five hydroxy groups that are responsible for its chelating properties. Phosvitin is a hen egg yolk protein consisting of a chain of 217 amino acid residues and containing 123 serine residues, 98% of which are phosphorylated (*17*). The high proportion of phosphate groups provides to the protein a strong chelating ability for cations such as Ca^{2+}, Fe^{2+}, and Mg^{2+} (*18*−*20*). In particular, at neutral pH, the phosphoserine residues being doubly negatively charged, the binding is optimal, and approximately 100 Mg^{2+} ions can be chelated by one phosvitin molecule (*18*). Magnesium complexation in the internal aqueous phase of double emulsions based on olive oil was performed in the presence of one of the two above-mentioned chelatants, and its effect on the release in the external aqueous phase was investigated. Ion leakage was studied over a month, at two storage temperatures, 4 and 25 °C. The experimental results were interpreted within the framework of a mean-field model based on Fick's law, taking into account the binding of magnesium by gluconate or phosvitin molecules in the inner droplets and by sodium caseinate in the external phase. On the whole, chelation of magnesium improved its retention in W/O/W emulsions. This appeared to be a novel and simple strategy to formulate materials based on edible components, with long retention times and compatible with technological food applications.

MATERIALS AND METHODS

Materials. Olive oil (Lesieur, extra virgin) was used as the oil phase of the W/O/W emulsions. It was essentially composed of triglyceride molecules with the following fatty acid chain composition: 77% oleic acid (18:1), 11% palmitic acid (16:0), 7% linoleic acid (18:2), and 3% stearic acid (18:0). The refractive index of the oil at 20 °C under white light was 1.466, and the viscosity was equal to 228 and 80 mPa·s at 4 and 25 °C, respectively. Olive oil was chosen because of its biological origin, its wide use in the food industry, and its healthy properties due to the high amount of oleic acid. Moreover, this oil was selected because it provides fast ion release when present in W/O/W emulsions compared with other triglyceride-based oils (*16*). Olive oil was thus one of the best candidates to probe novel strategies to improve ion retention. Polyglycerol polyricinoleate (PGPR) (Grinsted PGPR 90, esters of polyglycerol and polyricinoleate fatty acids, $M_w \approx 1766$ g mol^{-1}) was purchased from Palsgraad (France), and sodium caseinate ($M_w \approx 20000$ g mol^{-1}) from Lactoprot (Germany). Magnesium chloride (hexahydrate 99%) was from Acros Organics (Geel, Belgium) and sodium azide from Merck (Darmstadt, Germany). Lactose, gluconic acid hemimagnesium salt, and sorbitan monooleate (Span 80) were purchased from Sigma-Aldrich (Steinheim, Germany). All of the species were used as received. Phosvitin ($M_w \approx 35000$ g mol^{-1}) was isolated from egg yolk according to the method described in ref *21*. The water used in the experiments was deionized with a resistivity close to 15 MΩ·cm at 20 °C.

Table 1. Composition of the W/O/W Emulsions

globules (70 wt %)		
oil phase (42 wt %)	internal aqueous droplets (28 wt %)	external aqueous phase (30 wt %)
	WI	
	$MgCl_2$ (0.014 M) lactose (0.2 M)	
	WII	
olive oil (95 wt %)	gluconic acid hemimagnesium salt[a] (0.028 M)	sodium caseinate (12 wt %)
PGPR (5 wt %)	lactose (0.2 M)	sodium azide (0.08 wt %) lactose (0.2 M)
	WIII	
	phosvitin[b] (5 g/L) $MgCl_2$ (0.005 M) lactose (0.2 M)	

[a] Gluconic acid hemimagnesium salt was used so that Mg^{2+} concentration was 0.014 M. [b] Phosvitin contained 4 wt % magnesium.

W/O/W Emulsion Formulation and Preparation. W/O/W emulsions were prepared at room temperature using a two-step emulsification process, under laminar flow conditions (low Reynold's number). In dilute emulsions, for droplet deformation to occur, the applied shear stress, $\eta_c G$, where η_c is the viscosity of the continuous phase and G is the applied shear rate, must overcome the characteristic Laplace pressure of the droplets, $2\gamma/d$, where γ is the oil/water interfacial tension and d is the droplet diameter (see ref *22* and references therein). For rupturing to occur, the capillary number, $Ca = \eta_c G d/2\gamma$, must exceed a critical value Ca_c. This implies that the droplet has been elongated by the viscous shear before rupturing. The average droplet diameter of the ruptured droplets is thus given by

$$d = \frac{2Ca_c\gamma}{\eta_c G} \quad (1)$$

The parameter Ca_c depends on the type and history of the shear flow and on the continuous-to-dispersed phase viscosity ratio (*22*). In concentrated emulsions (i.e., with droplet fractions exceeding 64%), it has been demonstrated that eq 1 remains valid but the viscosity of the continuous phase has to be replaced by the overall average viscosity η_e of the emulsion (*22*). Following eq 1, to fragment the drops at relatively low shear rates, the viscosity had to be large enough. This was achieved by increasing the droplet fraction and by dissolving a large amount of surface-active species in the continuous phases.

Three different aqueous phases were prepared, one without any chelatant (WI), another one containing gluconate ions (WII), and a third one containing phosvitin (WIII). In all cases, the magnesium concentration was set to 14 mM and lactose was dissolved at a concentration of 0.2 M (**Table 1**). Phosvitin used in this study initially contained magnesium. Using flame atomic absorption spectroscopy (see below), we evaluated that magnesium represented 4 wt % of the dried product, corresponding to 60% of the available sites for chelation (*20*). A solution of $MgCl_2$ was added to achieve the targeted Mg^{2+} concentration of 14 mM, exactly equal to the concentration of available sites for chelation ("stoichiometric" conditions).

The different aqueous phases were manually dispersed at 80 wt % into the oil phase (olive oil) containing PGPR (30 wt %). The obtained crude W/O emulsions were fragmented in laminar flow conditions at a shear rate of 1580 s^{-1} using a Couette's cell (concentric cylinders geometry, Ademtech SA, Pessac, France), with a gap of 200 μm. Once fragmented, the emulsions were diluted with olive oil, at a droplet content of 40 wt %. In the second step, the W/O emulsions were incorporated into an aqueous phase, up to 70 wt %. The external aqueous phase contained 0.2 M lactose,

12 wt % sodium caseinate, and 0.08 wt % sodium azide (bactericide agent). Lactose was introduced in the internal and external aqueous phases at the same concentration (0.2 M) as an osmotic pressure regulator to limit water transfer (*23*−*25*). The W/O/W emulsions were fragmented in the Couette cell at a shear rate close to 5000 s^{-1}. The final compositions of all formulations are reported in **Table 1**. The pH of both internal and external aqueous phases was always close to 6.5. The emulsions were stored at 4 and 25 °C for 1 month. The globules tended to cream after a few hours of settling. To maintain homogeneity, instead of applying a continuous mechanical stirring that could accelerate the release process because of convective effects, the samples were turned upside down at regular time intervals (5 h).

W/O/W Emulsion Characterization. The structural evolution of the double emulsions was followed by means of an Olympus BX51 microscope equipped with a phase-contrast device, an oil immersion ×100/1.3 objective (Zeiss, Oberkochen, Germany) and a video camera. The size distribution of the water droplets in the primary W/O emulsions and of the oil globules in the W/O/W emulsion were measured by light scattering using a Coulter LS 230, as previously described (*16*). For each type of formulation, three samples of the W/O and W/O/W emulsions were analyzed right after preparation and after 1 month of storage at 4 and 25 °C.

Quantification of Magnesium Release from W/O/W Emulsions. Magnesium release was measured right after preparation and during 1 month of storage. A small amount of the double emulsion was collected from the stock volume at regular time intervals for further titration of the continuous phase. To facilitate the separation of the aqueous external phase and the oil globules, a dilution of the W/O/W emulsions with an isoosmotic lactose solution (0.2 M) was performed prior to centrifugation. The diluted emulsions were centrifuged at 1100*g* (*g* being the earth gravitational constant) for 30 min (Jouan CR 1000), which resulted in the separation of the globules from the external aqueous phase. We checked that the applied centrifugation did not lead to coalescence phenomena that could produce further release of Mg^{2+}. The cream was redispersed again after centrifugation, and the globules were observed under the microscope. Both the internal droplet size and the droplet concentration within the globules remained apparently invariant. Moreover, no significant variation in the average globule diameter before and after centrifugation was measured by static light scattering. Magnesium in the subnatant aqueous phase was titrated by flame atomic absorption spectroscopy (Perkin-Elmer AAnalyst 100) as described in ref *16*. Magnesium release was measured for 1 month for emulsions stored at 4 and 25 °C. Three samples from each type of emulsion were analyzed. Magnesium was always hardly detectable right after emulsion fabrication ($t = 0$). This indicates that the encapsulation yield was almost equal to 100%. Moreover, we can again conclude from this very first measurement that the experimental procedure used for the quantification of magnesium release did not generate any artifact (e.g., droplet/globule coalescence that would produce further release of Mg^{2+}).

The percentage of magnesium released from the internal (subscript 1) to the external (subscript 2) aqueous phase was calculated according to the following formula:

$$\%_{Mg^{2+}}(t) = 100 \times \frac{N_{2,Mg^{2+}}(t)}{N^0_{1,Mg^{2+}}} \quad (2)$$

In eq 2, $N_{1,Mg^{2+}}{}^0$ is the initial encapsulated magnesium in the internal droplets (in moles) and $N_{2,Mg^{2+}}$ is the total magnesium present in the external aqueous phase at time *t*:

$$N_{2,Mg^{2+}}(t) = C_{2,Mg^{2+}}(t) \times V_2 \quad (3)$$

Here $C_{2,Mg^{2+}}(t)$ is the molar concentration measured by flame spectroscopy at time *t* and V_2 the volume of the external aqueous phase.

The initial internal and external osmotic pressures, Π_1 and Π_2, respectively, were mainly determined by lactose, the concentration of which was larger than that of the other solutes. However, they were not perfectly matched, and the volumes of the aqueous compartments had to be corrected. Assuming ideal behavior and constant temperature, the osmotic pressure is simply proportional to the total concentration of the different dissolved species

$$\Pi = RT \sum_i C_i$$

where *R* is the ideal gas constant and *T* is the absolute temperature. In the internal droplets, magnesium ions were supposed to be fully chelated in the case of WII and WIII phases and, consequently, they contributed as a single species together with the ligand molecules (phosvitin or gluconate). In the particular case of WI, magnesium ions were initially free at a concentration of 0.014 M. Regardless of the internal aqueous phase compositions, chloride ions emanating from $MgCl_2$ had a maximum contribution of 0.028 M (2 × 0.014 M). In the external aqueous phase, caseinate molecules had a small influence because of their very large molar mass. However, each protein yielded on average 25 counterions Na^+ (the sodium caseinate used contained 3 wt % of sodium; for the sake of simplicity, we assumed that sodium caseinate was fully dissociated). This contribution was significant, because the Na^+ concentration was equal to 0.15 M, representing an osmotic pressure increment of 75% with respect to the contribution of lactose. Thus, for all of the formulations, the osmotic pressure in the external aqueous phase was initially greater than the internal one, and osmotic equilibration by water transfer was supposed to occur. The internal and external volumes, V_1 and V_2, respectively, were deduced from the equilibrium criterion, assuming ideal behavior of the solutes for the calculation of the osmotic pressures (van't Hoff equation)

$$\Pi_1 = \Pi_2 = RT\frac{(C^0_{1,lact} + C^0_{1,chel} + C^{0f}_{1,Mg^{2+}} + C^0_{1,Cl^-})V^0_1}{V_1} = RT\frac{(C^0_{2,lact} + C^0_{2,chel} + C^0_{2,Na^+})V^0_2}{V_2} \quad (4)$$

with

$$V_1 + V_2 = V^0_1 + V^0_2 \quad (5)$$

V^0_1 and V^0_2 being the volumes of the internal droplets and of the external aqueous phase, respectively, before osmotic equilibration (volumes of the aqueous phases initially incorporated). In eq 4, the different terms are relative to the initial conditions and are defined as follows: $C^0_{1,lact}$ = concentration of lactose in the internal droplets (0.2 M); $C^0_{2,lact}$ = concentration of lactose in the external aqueous phase (0.2 M); $C^0_{1,chel}$ = concentration of the chelated species in the internal droplets (0 for WI, 0.014 M for WII, and 1.4 × 10^{-4} M for WIII); $C^0_{2,chel}$ = concentration of caseinate in the external aqueous phase (6 × 10^{-3} M); $C_{1,Mg^{2+}}{}^{0f}$ = concentration of free magnesium ions in the internal droplets (0.014 M for WI, 0 for WII and WIII); $C_{1,Cl^-}{}^0$ = concentration of chloride ions in the internal droplets (0.028 M for WI, 0 for WII, and 0.01 M for WIII); and $C_{2,Na^+}{}^0$ = concentration of sodium ions in the external aqueous phase (0.15 M).

The osmotic pressure was always dominated by lactose and Na^+ concentrations, and the corrected volumes deduced from eq 4 were such that $V_1 \approx 0.8V^0_1$ and $V_2 \approx 1.2V^0_2$ for all of the systems. It should be noted that a *k*-fold variation in the volume corresponds to a $k^{1/3}$-fold variation in the droplet size. Thus, the variation of the internal droplet volume, which was almost equal to within 20%, induced only a variation of the internal droplet radius within 6%.

Osmotic equilibration was supposed to occur instantaneously compared with the characteristic time scale of magnesium leakage (*24*, *26*). We assumed that volumes V_1 and V_2 given by eq 4 remained constant after the initial osmotic equilibration. This assumption will be justified under Characterization of W/O/W Emulsions and Structural Evolution on the basis of observations under the microscope.

Isothermal Titration Calorimetry. The isothermal titration calorimetric experiments were carried out on a VIP-ITC (MicroCal Inc., Northampton, MA) to determine the binding constant *K* of gluconic acid and phosvitin with magnesium. The measurements were performed on fresh solutions (< 24 h after preparation), which were thoroughly degassed before loading. Titrations were carried out using a 300 μL syringe and a sample cell of 1.4 mL. The solutions were titrated by 30 successive injections of 5 μL of magnesium. Each injection lasted 5 s, with an interval of 200 s in two consecutive injections. The heat produced, δQ, for each injection was measured as the change in power required for maintaining the same temperature between the sample and the reference cell. This energy was calculated by integrating the area under the peaks of the recorded time course of change of power and then subtracted with control

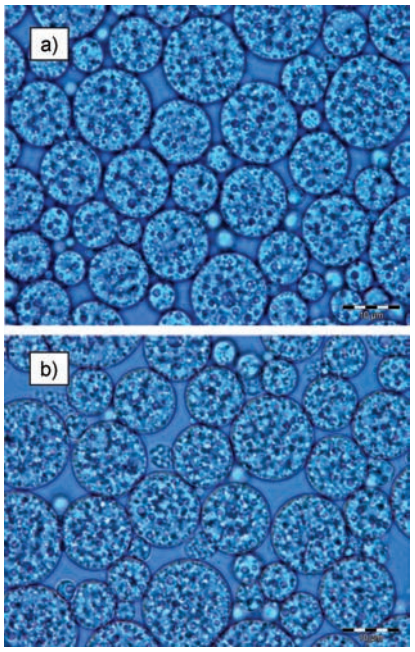

Figure 1. Microscope images of W/O/W emulsions encapsulating $MgCl_2$ (WI solution) immediately after preparation (**a**) and after a 30 day storage period at 4 °C (**b**).

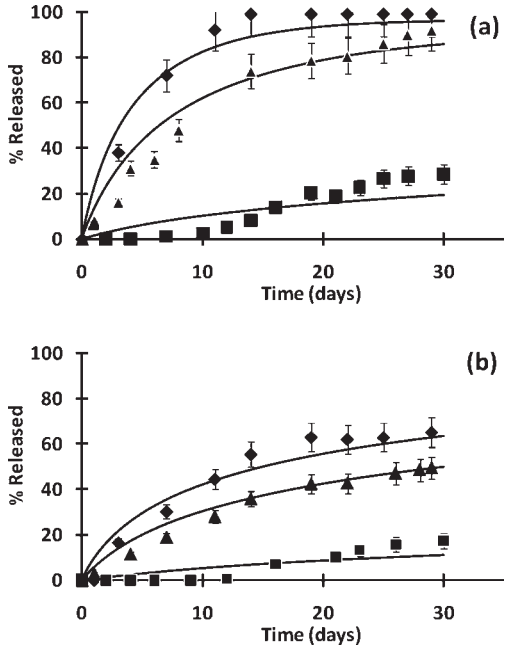

Figure 2. Magnesium release for W/O/W emulsions based on WI (no chelatant, ◆), WII (gluconic acid, ▲), and WIII (phosvitin, ■) internal aqueous solutions for 30 days and stored at 25 °C (**a**) or at 4 °C (**b**). Magnesium chloride at 0.014 M was initially encapsulated into the internal droplets. The solid lines are theoretical calculations using the model developed under Theoretical Approach (see text for details; the following parameters were adopted: $K_{2\ (T=25°C)}$= 1200 M^{-1}; $K_{2(T=4°C)}$= 860 M^{-1}; s_2 = 5; $C_{1,Mg^{2+}}{}^0$ = 0.014 M; $C^0_{2,chel}$ = 0.006 M; $C^0_{1,lact}$ = $C^0_{2,lact}$ = 0.2 M; $C_{2,Na^+}{}^0$= 0.15 M; V_1^0 = 0.270 L; V_2^0 = 0.280 L; V_{oil}= 0.450 L; $d_g{}^0$ = 1 μm; the values of K_1, s_1, R_m, V_1, $P_{Mg^{2+}}{}^0$, and k are provided in **Table 2**).

titrations. The heat produced during each injection was proportional to the amount of complex formed. Each experiment was reproduced in duplicate. The equilibrium binding constant K, the binding enthalpy, Δh^0, and the total number of available binding sites per chelatant molecule, s, can be deduced from the experimental curve $Q = f(C_{Mg^{2+}})$, where $C_{Mg^{2+}}$ is the total concentration of magnesium introduced into the measuring cell (*27*). For a 1:1 binding stoichiometry, that is, one ligand site per Mg^{2+} (case of phosvitin)

$$\frac{dQ}{dC_{Mg^{2+}}} = \frac{\Delta h^0 V_0}{2}\left(1 + \frac{1 - \frac{C_{Mg^{2+}}}{sC_{chel}} - \frac{1}{KsC_{chel}}}{\sqrt{\left(1 - \frac{C_{Mg^{2+}}}{sC_{chel}} + \frac{1}{KsC_{chel}}\right)^2 + \frac{4C_{Mg^{2+}}}{Ks^2C^2_{chel}}}}\right) \quad (6)$$

In this equation, V_0 is the sample volume (assumed to remain constant) and C_{chel} is the concentration of chelatant molecules. For a 1:2 stoichiometry (case of gluconate) $dQ/dC_{Mg^{2+}}$ cannot be expressed analytically and a third-degree equation has to be solved numerically.

RESULTS

Characterization of W/O/W Emulsions and Structural Evolution. Because of the diffusive transfer at different rates of the various solutes from one compartment to the other, water should permanently migrate to match the osmotic pressures. Moreover, coalescence of the internal droplets on the globule surface, if any, should modify the volume ratio V_2/V_1. However, microscopic observations did not allow visualizing any significant evolution of the internal droplet size and concentration over the time scale of the experiments (30 days). **Figure 1** shows typical microscope images taken right after fabrication and after 30 days, showing that the compartmented structure remained apparently unchanged during storage. The size distributions measured by static light scattering were almost identical irrespective of the formulations. The mean diameters of the internal droplets, d_d, in the primary W/O emulsion and of the oil globules, d_g, in W/O/W emulsions were in the same range for all of the prepared emulsions: d_d = (1.2 ± 0.4) μm and d_g = (10.2 ± 2.5) μm. Within experimental uncertainty (±0.2 μm), both diameters remained invariant over the whole storage period for each type of emulsion. This is why, for the calculation of the amount of magnesium release, volumes V_1 and V_2 were assumed to remain constant. Moreover, because the structural parameters of the double emulsions were almost identical irrespective of the composition of the water compartments, the rates of magnesium release could be straightforwardly compared for all of the W/O/W emulsions.

Magnesium Release. The release kinetics of magnesium from multiple emulsions stored at 25 °C are presented in **Figure 2a**. The leakage was achieved (100%) after 15 days in the presence of $MgCl_2$ alone (no chelatant in the internal droplets). Because coalescence was marginal, the progressive transfer of magnesium from the internal to the external aqueous phase occurred principally by diffusion and/or permeation across the oil globule (*16*, *25*). Equilibrium (asymptotic regime) was reached when free magnesium concentrations in the internal and in the external aqueous phases become equal (Laplace pressure contribution is negligible as demonstrated in ref *25*). Assuming that magnesium remained free (no chelation) in both compartments, the final percentage of release should be equal to

$$R_m = \%_{Mg^{2+}}(t_\infty) = 100\frac{V_2}{V_2 + V_1} \approx 62\%$$

Because coalescence was not significant, the measured R_m value close to 100% could account for the chelation capacity of caseinate in the external aqueous phase. Indeed, it is well-known that such protein can strongly bind divalent cations (Ca^{2+}, Mg^{2+}, ...), mostly because of the presence of phosphoryl groups (*28*−*30*). Moreover, its large molecular mass and hydrophilicity precluded

diffusion of the protein across the oil phase. In the experimental conditions probed here, each caseinate molecule could bind on average five magnesium ions (*29*). With 12 wt % of sodium caseinate in the external aqueous phase, the amount of available protein sites for chelation was about twice larger than the total number of Mg^{2+} ions. Thus, caseinate proteins acted as a chemical "pump", which trapped almost all of the magnesium ions initially encapsulated into the internal droplets. The presence of phosvitin in the internal aqueous phase significantly decreased the amount released at any time t compared with the system obtained without internal chelatant (**Figure 2a**). It was unlikely that phosvitin crossed the oil barrier because of its high molecular weight and hydrophilicity. According to Fick's law, the diffusion rate is proportional to the concentration difference between the internal and external aqueous phases. Chelation of magnesium by phosvitin decreased the internal concentration of free ions, a fact that obviously contributed to slow the release process. Measurements were interrupted after 1 month, before the asymptotic regime was reached. From empirical considerations, it could be stated that the percent released at equilibrium, R_m, was significantly smaller than 100% due to the strong binding capacity of phosvitin (*20*). This will be confirmed by the theoretical calculations performed under Application of the Model to Experimental Data. The amount of available sites for binding in the internal droplets was exactly equal to the total amount of magnesium ions initially encapsulated. Interestingly, the release was negligible during approximately 10 days. After this induction period, the release occurred at a faster rate. The amount released at any time in the presence of gluconate ions was greater than with phosvitin but smaller than in the absence of chelatant (**Figure 2a**). This could be due to the higher magnesium binding constant of phosvitin compared with that of gluconate (see below): the greater the constant, the smaller the concentration of free (unbound) magnesium ions in the internal droplets and the smaller the amount released, as observed experimentally. Also, the asymptotic value, R_m, was expected to be a decreasing function of the binding constant in the internal droplets. Another explanation could be the possible transfer of the chelated species across the oil phase. Indeed, the molecular weight of hemimagnesium gluconate (196 g mol^{-1}) was rather small, and the release of magnesium in its bound form could not be ruled out.

The same qualitative evolution was observed when the double emulsions were stored at 4 °C (**Figure 2b**). However, decreasing the storage temperature from 25 to 4 °C prolonged magnesium retention. This latter result could not be explained by the evolution of the binding constants with temperature. Indeed, it is well-known that the hydration layer around ions tends to become thicker as the temperature decreases. Hydration has the effect to create an effective barrier (spacer) between ions of opposite sign. As a consequence, the binding constant is generally an increasing function of temperature (*29*). Such temperature dependence was experimentally confirmed for the binding constants of both gluconate and phosvitin (see below). The evolution of the rate of release with temperature actually reflected the thermally activated nature of the permeation process. As discussed in ref *11*, the rate of diffusion/permeation is controlled by an activation barrier, which can be overcome only by thermal fluctuations.

THEORETICAL APPROACH

Basic Principles. We propose to interpret the curves reported in **Figure 2** within the framework of an extended version of the mean-field model initially developed by Pays et al. (*11*). The extended model accounts for the transfer of ions by diffusion, considering their possible chelation by ligand molecules. Ligands are hydrophilic molecules (gluconate, phosvitin, caseinate), each one bearing several sites for magnesium binding. Magnesium is initially encapsulated in the internal droplets at concentration $C_{1,Mg^{2+}}{}^0$. The evolutions of magnesium concentrations in the internal and external aqueous compartments are fixed by diffusion and by a binding equilibrium involving free (subscript f) and bound (subscript b) ions according to the equation

$$Mg_f^{2+} + nL \Leftrightarrow Mg_b^{2+} \tag{7}$$

where L represents an available (free) ligand site and n defines the "stoichiometry" of the binding process. Sodium caseinate and phosvitin are supposed to carry independent binding sites, each characterized by a 1:1 stoichiometry. Thus, we adopt $n = 1$ for these two molecules. However, the binding of magnesium with gluconate obeys a 1:2 stoichiometry and, consequently, we adopt $n = 2$ in this case. Because ligand molecules located in the internal and external compartments are of different chemical natures, we define two equilibrium binding constants, K_1 and K_2, as

$$K_1 = \frac{C^b_{1,Mg^{2+}}}{C^f_{1,Mg^{2+}}(C^0_{1,L} - n_1 C^b_{1,Mg^{2+}})^{n_1}} \tag{8}$$

$$K_2 = \frac{C^b_{2,Mg^{2+}}}{C^f_{2,Mg^{2+}}(C^0_{2,L} - n_2 C^b_{2,Mg^{2+}})^{n_2}} \tag{9}$$

where $C^0_{1,L}$ and $C^0_{2,L}$ are the concentration of ligand sites in the internal and external phases, respectively. For sodium caseinate and phosvitin, such concentrations are linked to the ligand (chelatant) concentrations by

$$C^0_{i,L} = s_i C^0_{i,chel} \quad (i = 1,2) \tag{10}$$

where s_i ($i = 1, 2$) is the total number of ligand sites per molecule. For gluconate,

$$C^0_{1,L} = C^0_{1,chel} \tag{11}$$

It is worth noting that the binding constants have different units depending on the stoichiometry. In the case of gluconate, the binding constant is expressed in M^{-2}, whereas it is expressed in M^{-1} for sodium caseinate and phosvitin.

The total magnesium concentrations $C_{i,Mg^{2+}}$ ($i = 1, 2$) can be expressed as

$$C_{i,Mg^{2+}} = C^f_{i,Mg^{2+}} + C^b_{i,Mg^{2+}} \tag{12}$$

Diffusion from the internal droplets to the external water phase is entropic in origin and occurs mainly because of the difference in free ion concentrations. In the asymptotic regime ($t \rightarrow \infty$), the concentrations $C_{i,Mg^{2+}}{}^f$ in both compartments tend to equilibrate. Before reaching the asymptotic regime, the molar flow $J_{Mg^{2+}}$ of magnesium ions across the oil phase is provided by Fick's law

$$J_{Mg^{2+}} = -\frac{dN_{1,Mg^{2+}}}{dt} = P_{Mg^{2+}} A (C^f_{1,Mg^{2+}} - C^f_{2,Mg^{2+}}) \tag{13}$$

where A is the surface area involved in the permeation process and $P_{Mg^{2+}}$ is the permeability coefficient. We assume that A is equal to the total globule surface area

$$A = \frac{V_g}{6d_g} = \frac{(V_1 + V_{oil})^{2/3}(V_1^0 + V_{oil})^{1/3}}{6d_g^0} \tag{14}$$

Table 2. Permeation Coefficients of Magnesium ($\times 10^{-11}$ m s^{-1}) Deduced from the Best Fits of the Experimental Curves (**Figure 2**) Using the Model Developed under Theoretical Approach[a]

solution[b]	temperature (°C)	K_1	$C^0_{1,\text{chel}}$(M)	s_1	V_1(L)	R_m (%)	$P_{Mg^{2+}}$($\times 10^{-11}$ m s^{-1})	k
WI	4		0		0.218	95.5	2.5	8
	25					96.5	6	2
WII	4	2400 M^{-2}	0.028		0.215	90.3	2.5	8
	25	2500 M^{-2}				92.2	6	2
WIII	4	10600 M^{-1}	1.4×10^{-4}	100	0.222	31.7	2.5	8
	25	11250 M^{-1}				35.1	6	2

[a] The parameters used were the following: $K_{2(T=25°C)}$ = 1200 M^{-1}; $K_{2(T=4°C)}$ = 860 M^{-1}; s_2 = 5; $C_{1,Mg^{2+}}{}^0$ = 0.014 M; $C^0_{2,\text{chel}}$ = 0.006 M; $C^0_{1,\text{lact}} = C^0_{2,\text{lact}}$ = 0.2 M; $C_{2,Na^+}{}^0$ = 0.15 M; V^0_1 = 0.270 L; V^0_2 = 0.280 L; V_{oil} = 0.450 L; d^0_g = 1 μm. [b] The compositions of WI, WII, and WIII phases are reported in **Table 1**.

where V_g is the volume of the oil globules ($V_g = V_1 + V_{oil}$). The right-hand term of eq 14 accounts for the conservation of the total number of inner droplets after osmotic equilibration, $d_g{}^0$ being the initial globule diameter.

Several possible mechanisms have been proposed in the literature to account for the diffusive transport or permeation of the encapsulated compounds: (i) direct solubilization of the entrapped species in the oil phase (for neutral molecules), (ii) transport via the hydrophilic surfactant polar headgroup in the case of water (*26*, *31*), (iii) transport through the oil phase into reverse micelles (*1*, *4*−*6*, *31*), and (iv) formation of thermally activated transient holes in the thin liquid films separating the internal droplets and the globule surface (*11*). In the case of magnesium ions, mechanisms iii and iv are the most likely. The permeability coefficient $P_{Mg^{2+}}$ in eq 13 characterizes the rate of release across the oil phase. It can be considered as a phenomenological constant that reflects the influence of all the parameters involved in the transfer. Of course, it is expected that $P_{Mg^{2+}}$ depends on, among other things, the chemical nature of the encapsulated substance, the chemical nature of the oil, the interfacial composition, the droplet and globule sizes, and the aqueous phase composition. For the sake of simplicity, our model considers only the diffusion of magnesium ions, but it is clear that other solutes (Cl^-, lactose, Na^+) also cross the oil barrier. Because of the electroneutrality requirement and osmotic pressure equilibration, magnesium release is influenced by the other solutes and consequently $P_{Mg^{2+}}$ must be regarded as an effective (system-dependent) permeation coefficient. Equation 13 assumes a quasi-stationary process in which the diffusion/permeation across the oil phase is the rate-determining factor (the binding equilibrium is supposed to be a much faster process than diffusion/permeation).

The total number of moles $N_{i,Mg^{2+}}$ (i = 1, 2) in each compartment at time t can be calculated by integration of eq 13, considering eqs 2−14 and the conservation equation

$$N^0_{1,Mg^{2+}} = N_{1,Mg^{2+}} + N_{2,Mg^{2+}} \quad (15)$$

Volumes V_i are deduced from V^0_i (i = 1, 2) (volumes of the aqueous phases initially incorporated) using eqs 4 and 5 and are assumed to remain constant over time. This assumption implies that after the initial (instantaneous) osmotic equilibration, the total net flow of the different solutes k which may potentially cross the oil membrane remains permanently balanced:

$$J_{\text{total}} = \sum_k J_k \approx 0 \quad (16)$$

The whole set of equations was solved numerically. The percentage of magnesium released at time t, defined by eq 1, as well as its asymptotic value, R_m, were then straightforwardly obtained.

Application of the Model to Experimental Data. The model was used to fit the experimental data, considering the permeation coefficient $P_{Mg^{2+}}$ as the unique free parameter. The binding constants and the number of binding sites per chelatant molecule were obtained through isothermal titration calorimetric experiments: (i) for gluconate, K_1 (T = 25 °C) = 2500 ± 250 M^{-2}, K_1 (T = 4 °C) = 2400 ± 240 M^{-2}, (ii) K_1 (T = 25 °C) = (11250 ± 830) M^{-1}, K_1 (T = 4 °C) = 10600 ± 380 M^{-1}, s_1 = 100 for phosvitin. Data relative to sodium caseinate were K_2 (T = 25 °C) ≈ 1200 M^{-1}, K_2 (T = 4 °C) ≈ 860 M^{-1}, and s_2 = 5 (*25*). The asymptotic value provided by the model, R_m, was fixed by "thermodynamic" (K_i, s_i, $C_{1,Mg^{2+}}{}^0$, $C^0_{i,\text{chel}}$, $C^0_{i,\text{lact}}$ (i = 1, 2)) and "geometrical" parameters (V^0_1 = 0.27 L; V^0_2 = 0.28 L) and was independent of the permeation coefficient $P_{Mg^{2+}}$ ("kinetic" parameter). Thus, R_m could be obtained from the model considering any arbitrary value of $P_{Mg^{2+}}$. The numerical values of R_m and of the parameters used to obtain them are reported in **Table 2**.

In a previous work, it was demonstrated that the experimental curves (% released = $f(t)$) could not be properly fitted using a constant value of the permeation coefficient (*16*, *25*). It was argued that the interfacial properties and, thus, ion permeability are influenced by the aggregation state of the proteins, which depends on the relative proportion of ligand sites occupied by magnesium ions in the external phase. Caseinate molecules form a dense adsorbed layer at the globule/water interface with a significant capacity to concentrate (released) magnesium ions because of the relatively strong affinity constant. This layer acts as a barrier to further ion release. To account for the impact of magnesium released, the following empirical expression for the permeation coefficient was proposed:

$$P_{Mg^{2+}} = P^0_{Mg^{2+}} \exp\left(-k \frac{C^b_{2,Mg^{2+}}}{C^0_{2,L}}\right) \quad (17)$$

$P_{Mg^{2+}}{}^0$ is the value in the absence of Mg^{2+} ions in the external phase, and k is a dimensionless constant. Equation 17 reflects the fact that the permeation coefficient decreases with the extent of protein binding, represented by the fraction of sites occupied by magnesium ions

$$\frac{C^b_{2,Mg^{2+}}}{C^0_{2,L}}$$

First, the data of **Figure 2** in the absence of ligand molecules (W1 solution) were fitted using $P_{Mg^{2+}}{}^0$ and k as adjustable parameters. The numerical values deduced from the best fits (using least-squares method) are reported in **Table 2**. The same values were then used to calculate the theoretical curves in the presence of chelatant molecules in the inner droplets. In **Figure 2**, theoretical calculations (solid lines) were in reasonable agreement

with the experimental data regardless of the chelatant (at a given temperature), thus proving the global consistency of our approach.

The double emulsion formulated without chelatant in the internal droplets (WI phase) was taken as a "reference" system for the sake of comparison. At 25 °C, the improved magnesium retention in the presence of phosvitin (WIII solution) was due to the regime occurring at short times ($t < 10$ days), when the release was negligible, as well as to the comparatively lower R_m value (35.1% compared with 96.5% in the reference system, see **Table 2**). The latter was imposed by the binding constant K_1 and by the ligand concentration $C^0_{1,chel}$, both of them being elevated, thus favoring magnesium retention in the internal droplets. Phosvitin is surface-active and as such is adsorbed at the internal water/oil interface. Moreover, phosvitin is known to adopt a dense and multilayered interfacial organization in the presence of divalent cations such as Ca^{2+} (*32*). It is likely that, when adsorbed at the interface, the protein reduced the permeation coefficient compared with the reference system and consequently, the percent released was overestimated at short times. The transition from a slow permeation regime to a relatively fast one for $t > 10$ days was surprising and suggested an evolution of the permeation properties. One explanation could be some aging of the interfacial layer and/or a change in the interfacial conformation of phosvitin resulting from the variation of the ionic strength. Indeed, at short times, most of the magnesium ions were bound and the only significant contribution to the ionic strength came from chloride ions at a concentration of 0.01 M. As time passed, Na^+ from the outer phase migrated into the droplets, thus increasing the ionic strength. At equilibrium and considering the volume ratio V_1/V_2, the expected concentration of Na^+ in the droplets should be close to 0.08 M. Polyelectrolyte molecules such as phosvitin adopt a more compact conformation as the ionic strength increases because of screening effects. In addition, the binding constant and the number of ligand sites per protein are generally decreasing functions of the ionic strength (*28*, *29*). Obviously in the case of phosvitin a more elaborate model should be required to perfectly fit the experimental data. Equation 17 reflects the impact on the permeation coefficient of the adsorbed casein layer at the oil/external water interface. The impact of the phosvitin layer adsorbed at the internal water/oil interface should be identically considered, as well as the influence of the ionic strength on the equilibrium binding constant K_1. This will be within the reach of future work.

In the case of gluconate (W2 solution), the agreement between the theoretical and experimental data was satisfactory. This could account for the fact that, unlike phosvitin, gluconate is not surface-active and is therefore not supposed to modify the permeation properties. The better retention in the presence of gluconate compared with the reference system (**Figure 2**) resulted from the lower concentration of free ions in the internal droplets because of magnesium complexation and was also reflected in the slightly lower R_m value (92.2 vs 96.5% for the reference). However, because of its much weaker affinity with respect to magnesium ions, gluconate influenced to a much lesser extent the release kinetics than phosvitin. For instance, from the values of the binding constants K_1 given above, we could estimate that when the total Mg^{2+} concentration was equal to 0.014 M (initial conditions), 42.3% of the ions were bound to gluconate, whereas 92.3% were bound to phosvitin. The model implicitly assumed that the ligand molecules could not cross the oil barrier either in the free or in the bound state. As discussed above, the transfer of gluconate could not be ruled out because of its relatively small molecular size. Nevertheless, the agreement of the experimental data with the theoretical curves suggested that the transfer of gluconate could be considered as negligible.

The permeation coefficients obtained at $T = 4$ °C followed the same qualitative tendencies as those obtained at $T = 25$ °C. However, because of the thermally activated nature of the permeation process (*11*), release at 4 °C occurred at significantly lower rates as evidenced by the smaller $P_{Mg^{2+}}{}^0$ value and by the larger attenuation coefficient k (see **Table 2**).

In this paper, we have examined the effect of ion chelation on the release kinetics from multiple W/O/W emulsions based on triglyceride oil and polymeric (food grade) surface-active species. Magnesium was initially encapsulated in the inner water droplets. The release was mainly due to diffusion/permeation phenomena (coalescence was marginal) originating from the concentration mismatch between the internal and external water compartments. We demonstrated that the use of a strong chelating agent such as phosvitin is a simple and efficient method to appreciably prolong the retention of encapsulated species in the internal aqueous droplets. The data were interpreted using a mean-field model combining permeation/diffusion of magnesium ions across the oil phase and chelation induced by the presence of ligand molecules. The permeation coefficients were obtained from the best fit to the experimental data. Finally, magnesium retention could be further improved by lowering the storage temperature. From a practical point of view, our results should provide some guidance in encapsulation design and colloidal sustained release.

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