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ORIGINAL ARTICLE

Cold-set whey protein microgels containing immobilised lipid phases to modulate matrix digestion and release of a water-soluble bioactive

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Abstract

This study investigated the *in-vitro* digestibility of cold-set whey protein (WP) microgels prepared by two gelation methods (external and internal) containing lipids (0%, 10% or 20% w/w). The incorporation of lipids into these matrices achieved higher entrapment of the bioactive vitamin riboflavin, as well as significant reductions in rates of both the digestion of the protein matrix, and the subsequent diffusion of the water-soluble bioactive. A biexponential model accounted for the contribution of digestion- and diffusion-driven mechanisms in describing the release of riboflavin into enzyme containing simulated gastrointestinal fluids. In particular, for external gelation microgels, as the lipid load within the matrices increased, the contribution of a faster diffusion-driven release was almost completely negated by a slower digestion-assisted release. Lipid loads provided a composite matrix capable of alternating from a burst to a sustained release of bioactive.

Keywords

Biexponential model, encapsulation, food polymers, hydrogels, *in-vitro* digestion, sustained release

History

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Introduction

There has been an increasing trend in examining microcapsules as protective matrices for the controlled release of bioactive ingredients (Gouin, 2004; Champagne & Fustier, 2007). Clear advantages exist in the development of microencapsulation systems from proteins for use in the food industry; they are of a high nutritional value, readily available and are naturally occurring food components degradable by enzymes (Chen et al., 2006). In particular, whey proteins (WPs) have many excellent functional properties, namely emulsifying and gelling capabilities (Foegeding et al., 2002), which can permit the engineering of microcapsules and hydrogels that can be loaded with sensitive bioactive ingredients.

Whey proteins (from dairy) can be manipulated to form gels at room temperature (Barbut & Foegeding, 1993; Roff & Foegeding, 1996; Bryant & McClements, 1998). These ''cold-set'' gels provide the advantage of incorporating sensitive bioactive ingredients at a stage prior to gelling that does not require any further heat treatments. Recent results from our laboratory have shown that WP matrices, produced by two different cold-set gelation methods (an external and an internal method), had good stability in a variety of pH values and temperatures (Egan et al., 2013). The external method involves drop-wise addition of the denatured whey protein isolate (WPI) solution into a $CaCl_2$ solution to produce microgels. In the internal gelation method an insoluble calcium source is mixed into the denatured WPI, and these pre-gel droplets dispersed within an oil phase are gelled upon the addition of an oil soluble acid, which releases the calcium to initiate gelling from within the droplets. These WP microgels are very efficient immobilisers of lipid phases as previously reported by Egan et al. (2013), and by Beaulieu et al. (2002) for the external gelation method.

It has been reported that lipid loads (up to 20% v/v) within WP gels can function as ''active'' fillers (Dickinson & Chen, 1999). Hydrophobic residues of WPs, adsorbed at the interface of the oil droplets successfully bring those oil droplets into the network of the gel matrix, giving rise to 3D junctions interconnecting the protein and lipid, which can provide a physical restraint against gel swelling (Jost et al., 1986; Mor et al., 1999; Leung Sok Line et al., 2005). Additionally, Beaulieu et al. (2002) reported on the preparation of gastric resistant WP microgels containing a lipophilic bioactive by an extrusion based method. The researchers related this effect to pepsin-susceptible hydrophobic aromatic amino acids being masked from enzyme attack, because those groups were either interacting with the lipid phase or efficiently entrapped within the matrix after cross-linking with calcium. These studies indicate that WP microgels containing lipid phases can enhance the microgel structure through a combination of increased resilience to both polymer swelling and gastro-intestinal (GI) enzymatic degradation.

The incorporation of a lipid phase into a polymer matrix may modulate the rate at which a water-soluble bioactive (which could have little or no affinity for the matrix otherwise) is released. Lee and Rosenberg (2001) reported that the presence of an apolar filler material in WP-based matrices enhanced the retention of a water-soluble active ingredient during encapsulation, and provided a means to alter the rate of its release. Authors related this effect to the presence of lipid filler in increasing the overall hydrophobic nature of the matrix, thus decreasing the rate of diffusion of aqueous solutions into the matrix. Authors also suggested that droplets distributed throughout the matrix could provide a hindrance to the diffusion of water-soluble active

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material out of the matrix by virtue of tortuosity. The WP matrices applied in the study by Lee and Rosenberg (2001) required chemical cross-linking to make them water-insoluble. Additionally, the release of a water-soluble bioactive was contributed to a diffusion-driven process only, as the stability of the matrices in GI enzymes was not assessed.

The challenge, therefore, exists to prepare food-grade WP matrices (avoiding chemical cross-linking) that can incorporate a water-soluble bioactive in a stable manner. Ideally the premature release of bioactive and degradation of the matrix should be avoided. In this study, a water-soluble bioactive (riboflavin) was incorporated into WP emulsion gels, containing different lipid loads, formed by two cold set gelation techniques. The aim was to assess if incorporated lipid loads could provide a means to tailor; both encapsulation and release of the bioactive, as well as the digestibility of the matrices when subjected to simulated GI conditions.

Materials and methods

Materials

Whey protein isolate containing 95% protein was purchased from Davisco Food Ingredients Int. (Le Sueur, MN). Soybean oil was purchased from the Archer Daniels Midland Company (Decatur, IL). Micronized calcium carbonate (Microcarb 4; >99% $CaCO_3$) was donated by S.A. Reverte (Barcelona, Spain). Microcarb 4 had $d_{0.9}$, $d_{0.5}$ and $d_{0.1}$ of 9.5, 5 and 2.2 μm, respectively (manufacturer data). Calcium chloride, sodium chloride, hydrochloric acid, sodium hydroxide, potassium phosphate and glacial acetic acid were purchased from Fisher Scientific Co. (Pittsburgh, Pittsburgh, PA) and were all of analytical grade. Span 65 was obtained from Fluka Chemika (Sigma Aldrich, Saint Louis, MO) and Tween 80 from Acros Organics (Fisher Scientific, PA). Riboflavin (≥98% purity) was purchased from the Sigma Chemical Company (Sigma Aldrich, Saint Louis, MO), as were pepsin (from porcine stomach mucosa), and pancreatin 4X (from hog pancreas).

Emulsion preparation

The preparation of emulsions was as detailed in Egan et al. (2013). WPI solution (10% w/w of protein content and containing 0.02% sodium azide) with an intrinsic pH of 6.8 was prepared in deionised water at 40 °C. The solution was kept stirred gently overnight (at ambient temperature) to ensure complete hydration and swelling of the proteins. The following day the WPI solution was heated to 80 °C and held at that temperature for 30 min in a temperature-controlled water bath (Grant Instruments Ltd., Cambridge, UK). Temperature treatment of the WPI was conducted in a sealed, submerged 500 ml Duran® flask (Fisher Scientific, Pittsburgh, PA) to avoid evaporation. After heat treatment, the denatured WPI solution was cooled rapidly on ice to ambient temperature before further use.

Emulsions consisting of the denatured WPI solutions and 10% or 20% (w/w) soybean oil were prepared according to the two-stage emulsification procedure reported by Rosenberg and Lee (1993). First, a coarse emulsion was prepared using an Ultra-Turrax T-25 (IKA Works, Cincinnati, OH) homogeniser operated at 13 500 rpm for 30 s. The second stage consisted of four successive passes using a Niro Soavi VHP homogeniser system (GEA Niro Soavi, Bedford, NH) operated at 50 MPa. The emulsion constituents were heated to 40 °C prior to emulsification and this temperature was maintained throughout the process. Foaming during emulsification was prevented by adding Patcote 502 K (0.1% v/v) anti-foaming agent (Hydrite Chemical Company, Brookfield, WI) before passing through the Niro Soavi homogeniser.

Preparation of microgels

The denatured WPI solution and the emulsions were used for preparing protein-only and lipid containing microgels, respectively. Microgels were prepared using the calcium-mediated external and internal gelation methodologies.

In riboflavin-loaded microgels, 0.05 g/l equivalent riboflavin was added to the denatured WPI or denatured WPI-emulsion prior to the preparation of microgels. All equipment was covered in aluminium foil so as to prevent light degradation of riboflavin throughout the duration of the experiments.

External calcium-mediated gelling technique

Denatured WPI solution or denatured WPI-emulsions were dropped through a 200 μl pipette tip with an internal diameter of 0.35 mm (Bio-Rad Laboratories, Hercules, CA) into a stirred calcium chloride solution (100 mM) using a variable flow chemical transfer pump (Fisher Scientific, Pittsburgh, PA). An optimum distance of syringe tip to the hardening solution of 2.5 cm was maintained, as was an optimum dripping rate of 50 drops/min. Throughout the dropping process and for 15 min thereafter, the calcium chloride solution was gently agitated using a Nalgene floating magnetic stirring bar (Product code: DS6630, Fisher Scientific, Pittsburgh, PA). Microgels were recovered in a Buchner flask, before being stored at 4 °C in a 100 mM $CaCl_2$ solution containing 0.02% w/w sodium azide (Sigma Aldrich, Saint Louis, MO) pending analysis.

Internal calcium-mediated gelling technique

Microgels were prepared by a process consisting of a double emulsification based on Lee and Rosenberg (2000) and an internal calcium gelation method as adapted from Poncelet (2001). Briefly, micronized calcium carbonate was homogeneously dispersed at the level of 140 mM calcium equivalent to the denatured WPI solutions and to the denatured WPI-emulsions (primary emulsions). Soybean oil (100 g) containing 5 g Span 65 was placed in a 300 ml two-neck round-bottom flask. The contents of the round-bottomed flask were stirred by an over-head sheer mixer (Servodyne Inc., Chicago, IL) using a Teflon half-moon stirring blade at a speed of 400 rpm during both emulsification and gelling. The primary emulsion was added slowly to the round-bottomed flask as the mixing continued. A double emulsion was allowed to form for 15 min before a further solution of 20 g soybean oil containing 1 ml of glacial acetic acid was added to liberate calcium from the calcium carbonate and initiate gelling. Stirring continued at 400 rpm for 60 min.

Following gelation, the contents of the round-bottomed flask were transferred to a beaker containing a 100 mM calcium chloride solution with stirring facilitated by a Nalgene floating stirring bar (Fisher Scientific, Pittsburgh, PA).

Recovery of the microgels was permitted by decanting the oil phase. Microgels were washed repeatedly with a Tween 80 solution (1% v/v) until the external solution was clear of any oil residues. The final wash was in deionised water to remove residues of the surfactant.

The microgels were filtered using Whatman No. 1 filter paper. If particulates were evident in the filtrate, the filtrate was filtered once more through a 0.45 μm pore size filter. Samples were then stored in a 100 mM $CaCl_2$ solution containing 0.02% w/w sodium azide at 4 °C pending analysis.

Preparation of simulated gastric and intestinal fluids

Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as described by the US Pharmacopeia

(United States Pharmacopeial Convention Council of Experts, 2004), and as per the methods of Remondetto et al. (2004) and Maltais et al. (2009). SGF was prepared by an addition of 2 g sodium chloride and 7 ml of 37% hydrochloric acid diluted to 1000 ml with distilled, deionised water. The final pH was 1.2.

In the preparation of enzyme-containing SGF, pepsin was added (3.2 g/l) one hour before use of the fluid. The pepsin had an activity of 3200 units/mg protein. The addition of pepsin did not affect the pH of the solution.

SIF was prepared by the addition of 6.8 g monobasic potassium phosphate, 190 ml of 0.2 N NaOH, diluted to 1000 ml with distilled, deionised water. The final pH of the intestinal fluid was 7.5.

In the preparation of enzyme-containing SIF, pancreatin was added (10 g/l) one hour before starting the experiments. The pancreatin contained a mixture of enzymes including amylase, trypsin, lipase, ribonuclease and protease. The pancreatin had an activity that was 4X that required by the United States Pharmacopeia (USP) guidelines, meaning it should contain no less than 100 USP units of protease activity per mg of pancreatin (manufacturer data).

Examining protein digestion from microgels

Riboflavin-free microgels prepared from both the internal and external cold gelation method were tested for soluble protein (degradation of the protein matrix) over time in enzyme-free and enzyme-containing GI fluids. The total protein of the microgels had been previously determined by the macro-Kjeldahl method, with a conversion factor of 6.38 (IDF, 1993). Six double-walled glass beakers were connected by tubing to a circulating water bath (Haake Fisons DC3, Thermo Scientific Inc., Waltham, MA) at 37 °C. Aliquots of solution were removed at different time intervals, by pipetting through a specially prepared filter that allowed extraction of solution without extraction of microgels. After removal of each aliquot, an equivalent volume of fresh, warm medium was added to the solutions.

In the case of enzyme-free GI fluids, a 1 ml aliquot was centrifuged at 14 000 rpm for 20 min (Eppendorf Centrifuge S415C, Eppendorf, Westbury, NY) in 1.5 ml centrifuge tubes (Fisher Scientific, Pittsburgh, PA). Protein content was determined spectrophotometrically at 280 nm using a Bausch and Lomb Spectronic 1001 (Spectronic Instruments Inc., Rochester, NY) following sample filtration through a 0.45 μm Nalgene filter (Fisher Scientific, Pittsburgh, PA). Quantification was carried out using an appropriate standard curve.

In the case of enzyme-containing GI fluids, the presence of the enzymes gave rise to an absorption peak that disrupted an accurate reading of the protein concentration. For these samples, a one in five dilution was necessary prior to spectrophotometric examination. At this dilution, the interference from enzymes absorbing at 280 nm is negated, similar to the conditions applied by Tedeschi et al. (2009) in their work.

Release of riboflavin from microgels

Riboflavin-containing microgels prepared from both the internal and external cold gelation methods were tested for levels of riboflavin released over time into both the enzyme-free and enzyme-containing GI fluids. Samples were centrifuged and filtered as described above. The presence of enzymes did not affect the absorbance of riboflavin, which was determined spectrophotometrically at 445 nm using a Bausch and Lomb Spectronic 1001 (Spectronic Instruments Inc., Rochester, NY), similar to the work by Chen and Subirade (2006). Quantification was carried out using an appropriate standard curve.

The efficiency of entrapment of the riboflavin within the microgels was determined as

$$\%\,Entrapment = \frac{[ribo\,flavin]_{final}}{[ribo\,flavin]_{loaded}} \times 100\% \qquad (1)$$

where, $[riboflavin]_{loaded}$ is the concentration (g/l) of riboflavin that was initially loaded into the microgel forming solution and $[riboflavin]_{final}$ is the concentration (g/l) of riboflavin that was released from the completely degraded microgels after 6 h of incubation in SGF with pepsin.

Simulated GI conditions – data analysis

The digestion of protein, with time, from microgels (riboflavin-free), under *in-vitro* simulated GI conditions were fitted with a first-order model

$$\%\,Digestion = Max_{dig} \times \left(1 - e^{(-k_{dig}t)}\right) \qquad (2)$$

where % *Digestion* is the % matrix digestion at time t, Max_{dig} corresponds to maximum extent of matrix digestion for the duration of the experiment, and k_{dig} (h^{-1}) is the digestion apparent rate constant.

The diffusion of riboflavin with time from riboflavin-containing microgels in SGF and SIF (without enzymes) were also fitted with a first-order model

$$\%\,Digestion = Max_{diff} \times \left(1 - e^{(-k_{diff}t)}\right) \qquad (3)$$

where % *Diffusion* is the % riboflavin release at time t (into SGF and SIF without enzymes), Max_{diff} corresponds to the maximum extent of riboflavin release, from the microgels, for the duration of the experiment, and k_{diff} (h^{-1}) is the diffusion apparent rate constant.

The release of riboflavin with time from riboflavin-containing microgels in SGF and SIF (with enzymes) was fitted with a biexponential model, which considers the release profile as a two-phase process. This was in order to assess the contributions of both digestion and diffusion-assisted release of the riboflavin from the matrices.

$$\%\,Release = A_{dig} \times \left(1 - e^{-k_{dig}t}\right) + A_{diff} \times \left(1 - e^{-k_{diff}t}\right) \qquad (4)$$

where % *Release* is the % riboflavin release at a time t (into SGF and SIF with enzymes), A_{dig} and A_{diff} are parameters that indicate the contribution of digestion- and diffusion-assisted release of the riboflavin, and k_{dig} and k_{diff} are the apparent release rate constants (extracted from Equations (2) and (3), respectively) of the digestion and the diffusion release (Equation (4) was adapted from Heelan & Corrigan, 1997).

Statistical analysis

All experiments were performed at least in triplicate (independent samples). Mean results are presented, and error bars on graph represent standard deviations. Statistical analysis was conducted with SigmaStat software (Systat Software Inc., Chicago, IL) and model fits were analysed with the SigmaPlot software (Systat Software Inc.).

Results and discussion

Entrapment efficiency – implications of microgel manufacture and lipid load

The efficiency of riboflavin entrapment (Equation (1)), in microgels prepared by both the external and internal gelation methods, is shown in Figure 1. The entrapment of the riboflavin

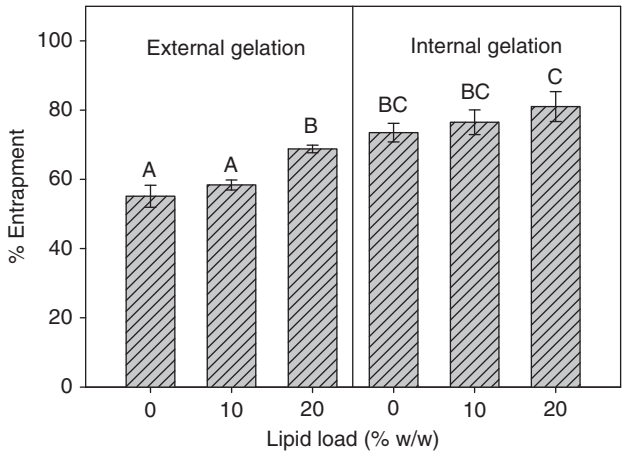

Figure 1. Entrapment efficiency of riboflavin in microgels of different lipid loads (0%, 10% or 20% w/w) prepared by either the external or internal gelation method. Means not sharing the same letter are significantly different ($p \leq 0.05$).

Table 1. First-order rates (k_{dig}) describing the digestion of protein matrices of microgels (external and internal gelation) with 0%, 10% or 20% w/w lipid loads in simulated GI fluids containing enzymes.

	External		Internal	
Lipid (% w/w)	SGF k_{dig} (h^{-1})	SIF k_{dig} (h^{-1})	SGF k_{dig} (h^{-1})	SIF k_{dig} (h^{-1})
0	1.7 ± 0.2^{a}	1.3 ± 0.1^{a}	19.6 ± 4.4^{a}	2.1 ± 0.3^{a}
10	1.7 ± 0.1^{a}	0.6 ± 0.1^{b}	5.5 ± 0.3^{b}	2.0 ± 0.2^{a}
20	1.3 ± 0.0^{b}	0.5 ± 0.1^{b}	2.9 ± 0.2^{c}	1.5 ± 0.1^{b}

[a,b,c]Means in a given column not sharing the same letter are significantly different ($p \leq 0.05$).
k_{dig} = digestion rate constant (h^{-1}) (Equation (2)).
SGF, simulated gastric fluid; SIF, simulated intestinal fluid.

was overall quite high, and consistently higher in microgels prepared by the internal gelation method (73%–81%) compared to the external gelation method (55%–68%). This could be explained by the methods of production for either set of microgels. In the external gelation method, the water-soluble riboflavin can diffuse into the external calcium solution used to induce gelation of the whey beads before gelation is complete, thereby explaining the loss in entrapment efficiency for these gels. In the case of the internal gelation method, where whey gelation takes place in an oil medium, there is little potential for the riboflavin to disperse in that oil until gelling is complete. The losses that did occur in this case were attributed to the washing steps necessary to remove the external oil from the surface of the microgels. Chen and Subirade (2007) reported improved bioactive retention with an acetone wash, however in order to avoid solvents, the microgels were washed with surfactant and water. Despite the losses of riboflavin incurred during washing, relatively high levels of riboflavin retention were still achieved similar to what has been reported by Chen and Subirade (2007).

There was a relationship between increasing the oil load within the gel matrix and an increase in the entrapment efficiency of the bioactive. As indicated in Figure 1, 20% lipids-containing externally gelled matrices had significantly ($p \leq 0.05$) higher entrapment of riboflavin than lipids-free microgels. Internally gelled matrices containing 20% lipids did not have significantly higher entrapment of riboflavin than lipids-free internally gelled matrices, but did have higher entrapment overall, and significantly ($p \leq 0.05$) higher entrapment than 20% lipids-containing externally gelled matrices.

This increase in entrapment observed with increasing lipids could be attributed to a limiting effect of lipids on the diffusion of aqueous solution (calcium chloride or washing solutions) into the microgels as previously reported by Lee and Rosenberg (2001). Among the factors discussed in that study was a possible increase in the hydrophobicity of the matrix, which could impede passage of aqueous solution, combined with tortuosity effects possibly exerted by lipid droplets distributed throughout the gel matrix (Lee & Rosenberg, 2001). It is likely a combination of these factors that had resulted in the enhanced entrapment of riboflavin observed upon increasing the lipids within the microgels.

Protein digestion from the microgels

The degradation of the protein matrices in microgels prepared by both gelation methods was examined in SGF or SIF in the presence or absence of enzymes. Without digestive enzymes present, there was practically no (0%–0.05%) degradation of the protein matrix over a 6-h period. Matrix degradation occurred by enzymatic digestion only. This suggests that the microgels prepared by both the external and internal gelation methods could be likely to remain stable within a food matrix until their ingestion, thereafter the action of GI enzymes could degrade these microgels. These results correlate with the reported results in Egan et al. (2013) showing excellent stability of these protein matrices over a range of pH values and temperatures. It is very promising from the perspective of feasibly adding these food-grade microgels into complex food products.

Digestion of the protein matrix from external and internal gelled microgels in SGF and SIF containing enzymes were fitted with a first-order model (Equation (2)). The rates of matrix digestion ($k_{dig}\,h^{-1}$) for the first-order model fits on microgels prepared by external and internal gelation in SGF or SIF are contained in Table 1. Coefficients of fits with this model were good with r^2 values of 0.96–0.98 for externally gelled matrices and r^2 values of 0.96–0.99 for internally gelled matrices. The degradation of the protein matrix in such an exponential manner is consistent with a pattern of peptide chain scission by the digestive enzymes (pepsin or pancreatin), which is typical in the bulk degradation of polymers (Burkersroda et al., 2002). Chen et al. (2008) reported a similar apparent first-order kinetic degradation/solubilisation of cross-linked soy protein matrices with time in SGF- and SIF-containing enzymes.

Effect of gelation method on protein digestion

The rates of matrix digestion ($k_{dig}\,h^{-1}$) were consistently faster in the microgels prepared by the internal gelation method when compared to the microgels prepared by the external gelation method (Table 1). In the microgels prepared by the external gelation method, the rates of matrix digestion ranged from 0.5 to $1.7\,h^{-1}$ (Table 1). In the microgels prepared by the internal gelation method the rates varied from 1.5 to $19.6\,h^{-1}$.

This difference in rates would be expected due to the difference in the size of the microgels prepared from both methods. Microgels prepared by the external gelation method tended to be about 1 mm in diameter, whilst those microgels prepared by the internal gelation method tended to be much smaller at typically 100 μm in diameter (Egan et al., 2013). The greater surface area of the smaller microgels (approximately 10 times smaller) facilitates enzyme activity, leading to faster protein matrix digestion.

Effect of digestion media on protein digestion

Rates of matrix digestion were overall much faster in the presence of enzyme-containing SGF rather than enzyme-containing

SIF (Table 1). Typically the pepsin activity (of SGF) was at least twice as fast at degrading the protein matrix compared to the pancreatin (SIF) on the same microgel. In some cases pepsin activity was quite considerably quicker, for example; the lipids-free internally gelled microgels, where the rate of degradation of the protein matrix with time was $19.6\,h^{-1}$ in SGF in comparison to a rate of $2.1\,h^{-1}$ in SIF (Table 1). This difference in rates between SGF and SIF was not as profound for externally gelled matrices, but was still significantly ($p \leq 0.05$) different with a rate of $1.7\,h^{-1}$ in SGF, dropping to a rate of $1.3\,h^{-1}$ in SIF, in lipids-free microgels.

The microgels produced from denatured WPI, should be very susceptible to enzymatic digestion, particularly the action of pepsin. Beta-lactoglobulin (β-Lg) is a major constituent of WP (~50% of total WP; Fox, 2003), and in its native form it is very resistant to gastric digestion, particularly the activity of pepsin (Reddy et al., 1988). This pepsin-resistant character of native β-Lg is believed to be because hydrophobic residues (very susceptible to digestion by pepsin) are orientated within the calyx-like core of the native structure. However, upon denaturation, the resistance of this protein to proteolysis is greatly reduced (Reddy et al., 1988), because denaturation involves unfolding of the protein structure. The pepsin-susceptible groups are then exposed to attack by the enzyme. Liang et al. (2010) reported a similar result, in which β-Lg gels were degraded much more quickly in the presence of enzyme-containing SGF than they were in SIF.

Although the matrix digestion was rapid in the presence of SGF, there was a marked effect on these rates of digestion in lipids-containing matrices.

Effect of lipid load on protein digestion

Lipid load within the matrices significantly ($p \leq 0.05$) slowed down the rates of digestion, regardless of whether digestion was in SGF or SIF from externally or internally gelled matrices. Results obtained with externally gelled microgels indicated that, the k value for digestion in SGF was $1.7\,h^{-1}$, for the lipid-free microgels and was significantly ($p \leq 0.05$) reduced to a k value of $1.3\,h^{-1}$ in microgels containing 20% w/w lipid (Table 1).

Matrix digestion was not as fast in the presence of SIF, but the presence of lipid within the microgels again significantly slowed down matrix digestion from a k_{dig} value of $1.3\,h^{-1}$ in the externally gelled microgels with no lipid, to $0.5\,h^{-1}$ in microgels loaded with 20% w/w lipid. The presence of a 20% w/w lipid load within these externally gelled microgels was particularly effective at hindering matrix digestion in SIF, in which only ~70% of the protein matrix had been digested after 6 h.

In the case of the internally gelled microgels the lipid load had a dramatic impact on reducing the rate of digestion from $19.6\,h^{-1}$ in lipids-free microgels, to a rate of $2.9\,h^{-1}$ in microgels containing 20% w/w lipid load (Table 1). This effect of lipid load in hindering enzymatic degradation of the protein matrix was very apparent in the internally gelled microgels, most likely resulting from the combination of the smaller size of these microgels and their susceptibility to rapid degradation by pepsin.

The effect of the lipid load within the matrix in delaying enzymatic digestion, and in particular impeding pepsin digestion is consistent with results reported by Beaulieu et al. (2002). Indeed, reports (Beaulieu et al., 2002) suggest that hydrophobic amino acid residues left exposed during the denaturation step interact with the lipid phase in the final microgels, thereby masking them against enzymatic digestion. It has also been suggested that the products of partial protein hydrolysis can again adsorb to the oil droplet surface and have an additional protective effect on impeding digestion of the matrices (Liang et al., 2010). So the incorporation of lipid into the matrix can provide a means by which to impede the enzymatic degradation of the WP microgels.

Release of riboflavin from microgels

Release without enzymes

The release of riboflavin from microgels prepared by both the external and internal gelation method was examined in SGF and SIF either in the presence or absence of enzymes. The release of riboflavin from these microgels in SGF or SIF without enzymes was best fitted to a first-order model (Equation (3)) to describe the diffusion-assisted release of the riboflavin. Table 2 shows the rate constants (k_{diff}) and the maximum extent of release (Max_{diff}) after 6 h for this data set.

The kinetics of release fitted to this first-order model showed good correlation of fit with r^2 values ranging from 0.90 to 0.99 in externally gelled microgels, and from 0.95 to 0.98 in internally gelled microgels.

Depending on the conditions, losses of between 27%–57% of the encapsulated riboflavin occurred during the 6-h duration of the experiments (Table 2). This diffusion of the riboflavin from microgels in simulated GI fluids, without enzymes, could be related to swelling of the matrices, consistent with the behaviour of the protein ionisable moieties, as has been observed in other studies (Beaulieu et al., 2002; Gunasekaren et al., 2006).

Geletion mode did not appear to have an impact on either the rate constant (k_{diff}) of release or the extent of release (Max_{diff}) over the 6-h duration of experiments. The one exception was the release of the riboflavin into SIF from externally gelled matrices. In these particular samples the maximum release was significantly lower ($p \leq 0.05$), from matrices containing lipid loads (27% release of the total encapsulated riboflavin in comparison to the typical 40%–50% (Max_{diff}) release observed in other samples, Table 2).

Increasing the lipid load within the matrices was consistently effective at reducing both the rate constants (k_{diff}) of release and the extent of release (Max_{diff}). The effect of lipid in slowing down the rate of diffusion of riboflavin was perhaps most noticeable

Table 2. First-order rates (k_{diff}) describing the diffusion and the maximum extent of release (Max_{diff}) for riboflavin from microgels (external and internal gelation) with 0%, 10% or 20% w/w lipid loads in simulated GI fluids (no enzymes).

	External				Internal			
	SGF		SIF		SGF		SIF	
Lipid (% w/w)	k_{diff} (h^{-1})	Max_{diff}	k_{diff} (h^{-1})	Max_{diff}	k_{diff} (h^{-1})	Max_{diff}	k_{diff} (h^{-1})	Max_{diff}
0	7.6 ± 2.3^{a}	57 ± 1^{a}	6.0 ± 0.3^{a}	40 ± 3^{a}	9.5 ± 3.6^{a}	52 ± 2^{a}	8.7 ± 0.7^{a}	49 ± 2^{b}
10	4.2 ± 1.4^{a}	51 ± 2^{b}	3.9 ± 1.3^{b}	30 ± 1^{b}	1.2 ± 0.1^{c}	55 ± 4^{a}	2.6 ± 0.3^{b}	55 ± 4^{a}
20	5.9 ± 0.6^{a}	50 ± 2^{b}	2.1 ± 0.5^{b}	27 ± 2^{c}	2.4 ± 0.1^{b}	41 ± 1^{b}	1.9 ± 0.1^{c}	44 ± 1^{c}

[a,b,c]Means in a given column not sharing the same letter are significantly different ($p \leq 0.05$).
k_{diff} = rate constant (h^{-1}); Max_{diff} = maximum extent of release (Equation (3)).

(and significant, $p \leq 0.05$) in the microgels prepared by internal gelation. The rate of release of riboflavin from these internally gelled microgels was initially 9.5 and 8.7 h^{-1} in SGF and SIF, respectively, in microgels containing no lipid phase (Table 2). These rates were then reduced to 2.4 and 1.9 h^{-1}, respectively, in SGF and SIF in microgels loaded with 20% w/w lipid.

The effect of the lipid loads in reducing both the rate constants (k_{diff}) of release and the extent of riboflavin release could be accounted for by two processes. First, due to the presence of ionisable fractions on WPs polypeptide structures, protein matrices are liable to a pH-related swelling mechanism (Beaulieu et al., 2002; Gunasekaren et al., 2006; Livney, 2010). Incorporating lipid loads into these matrices can provide anchor points for protein residues, leading to an interconnectivity between lipid droplets and protein residues that give rise to 3D junctions, which effectively strengthen matrices against swelling in aqueous media (Jost et al., 1986; Mor et al., 1999; Leung Sok Line et al., 2005). Second, the presence of lipid droplets can also limit the diffusion of aqueous solution into the matrices, impeding aqueous assisted swelling of the matrices but also hindering the diffusion of the water-soluble bioactive from the matrix (Lee & Rosenberg, 2001).

Release with enzymes

The release of riboflavin from externally gelled matrices into SGF and SIF containing enzymes are shown in Figure 2(a) and (b), respectively. There was 100% release of riboflavin into SGF containing enzymes (Figure 2a), regardless of lipid load, but incorporating 20% w/w lipid into the matrices had a visible impact in slowing down the release of the riboflavin.

There was 100% release of riboflavin from matrices containing 0 or 10% w/w lipid loads into SIF with enzymes (Figure 2b), but a significant reduction (only ~40%) in the release of the riboflavin after 6 h from the 20% w/w lipid-loaded matrices.

The release of the riboflavin from the externally gelled microgels with enzymes as shown in Figure 2(a) and (b) is consistent with the digestion of the protein matrix with enzymes. In which, the release of riboflavin is more rapid in the presence of pepsin (Figure 2a), rather than in the presence of pancreatin (Figure 2b). Also, it appears that microgels loaded with 20% lipid appear to exhibit a slower release of the riboflavin, particularly 20% lipid loaded microgels in the presence of pancreatin (Figure 2b), where after the 6-h duration of the experiment only ~40% of the total riboflavin had been released from the matrix, consistent with the protein degradation of these matrices, where only ~70% of the matrix had been digested in this time period.

The data sets describing the release of riboflavin from the internally gelled microgels in the presence of enzymes are shown in Figure 2(c) and (d). There was 100% release of riboflavin from the internally gelled matrices after 6 h in SGF (Figure 2c) and SIF (Figure 2d) in the presence of enzymes.

The release of the riboflavin into SGF and SIF with enzymes appeared consistent with the matrix digestion of the internally gelled microgels. In which, release appears fairly rapid in both the presence of pepsin and pancreatin, rather than the distinct difference that was apparent in the externally gelled matrices

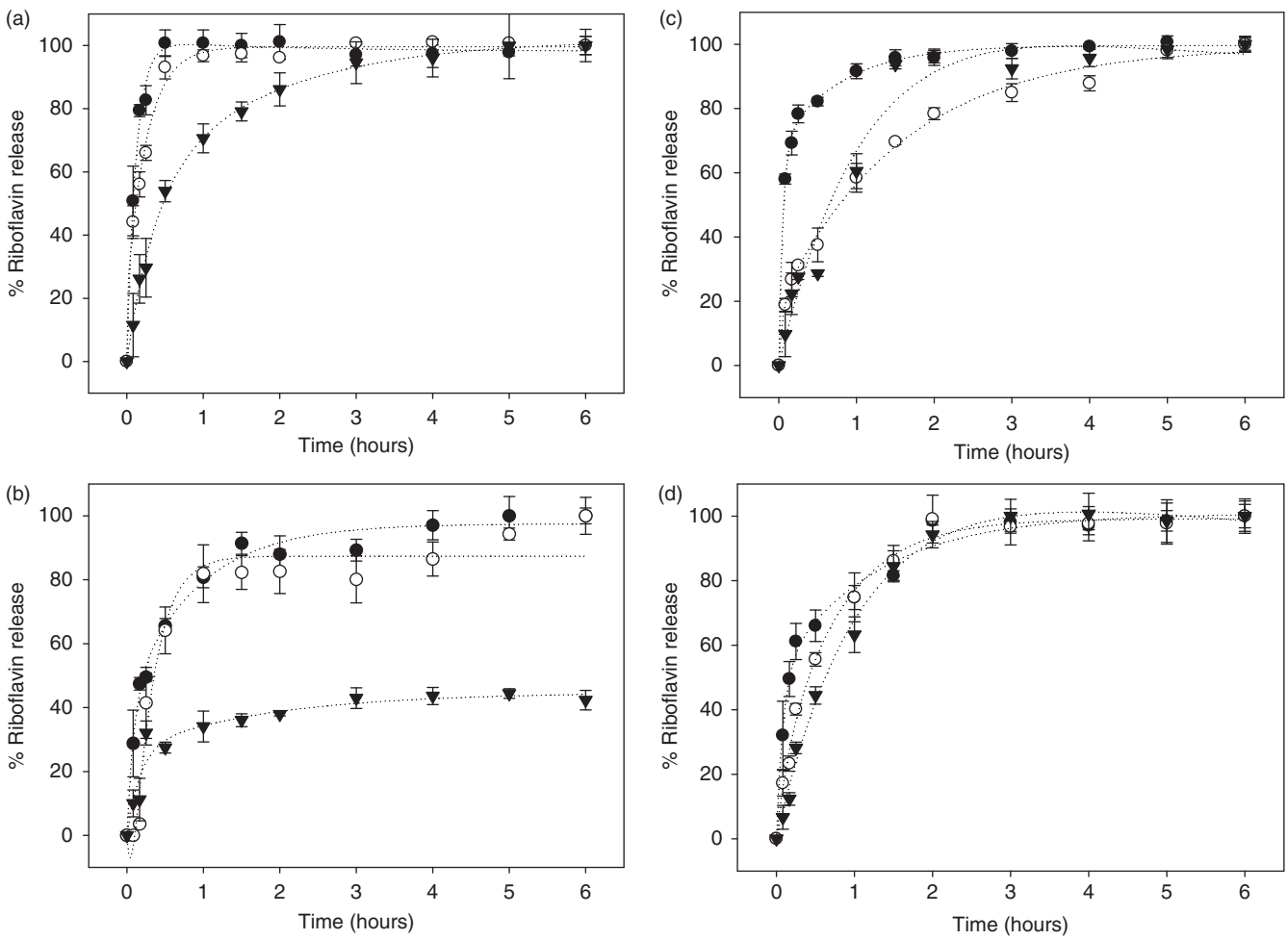

Figure 2. Effect of lipid load (●) 0%, (○) 10% or (▼) 20% w/w on the release of riboflavin with time from external gelation microgels in (a) SGF + enzymes, (b) SIF + enzymes, or internal gelation microgels in (c) SGF + enzymes, or (d) SIF + enzymes (data points represent averaged experimental data of three independent replicates, dotted line indicates biexponential fit).

where matrix digestion and riboflavin release was much slower in the presence of SIF over SGF.

The contribution of digestion and diffusion on the release of riboflavin

In order to correlate the contribution of diffusion-assisted release of riboflavin from matrices into GI fluids with no enzymes and the digestion-assisted release into GI fluids with enzymes, data from the release of riboflavin in the presence of enzymes were fitted with biexponential models (Equation (4)) as indicated by dotted lines in Figure 2 for externally and internally gelled matrices. Coefficients of fits (r^2 values) were in the range of 0.90–0.99 describing the release of riboflavin into SGF and SIF with enzymes from these externally gelled microgels. Such r^2 values were also observed for internally gelled matrices (0.95–0.99).

The biexponential model correlates the release as a two-phase process with one distinct phase of rapid release, and another distinct phase of a slower release (Heelan & Corrigan, 1997). Each of the phases has a specific parameter assigned to it, describing the contribution of both the fast phase and the slower phase. In general, in the data sets obtained in this study, diffusion (k_{diff}) was the faster process contributing to the release of riboflavin, and digestion (k_{dig}) was slower. The relevant k values were obtained from the first-order fits for (i) digestion of the protein matrix (Equation (2), Table 1), and (ii) diffusion of the riboflavin without enzymes (Equation (3), Table 2). These rate constants (k h^{-1}) for the first-order model fits for digestion and diffusion, were inserted into the biexponential model to aid interpretation of the contribution (A_{dig} and A_{diff}) of the digestion and diffusion mechanisms describing the release of riboflavin into GI fluids with enzymes.

An example of the models generated to describe diffusion- and digestion-assisted release of the riboflavin, and the corresponding experimental data are shown in Figure 3.

As shown in Figure 3, the biexponential model describing the contribution of both the diffusion- and digestion-driven processes of riboflavin release, works well with the data sets obtained for riboflavin release into simulated GI fluids with enzymes. This assisted the interpretation of the contribution of these mechanisms on the release of the riboflavin from both externally and internally gelled matrices. (All of the kinetic parameters for the biexponential model fits are contained in Appendix Tables A1 to A4.)

Contribution of digestion and diffusion processes on riboflavin release from externally gelled microgels

In the microgels produced by the external gelation method, diffusion-assisted release of the riboflavin was consistently found to be the faster process. The release rates for this diffusion-driven process ranged from 2.1 to 7.6 k h^{-1}, while the rates for digestion ranged from 0.5 to 1.7 k h^{-1} (Tables 2 and 1, respectively).

Although diffusion was consistently the faster mechanism of release of the riboflavin from these externally gelled matrices, as the lipid load within the microgels increased, there was a change in the contribution of either diffusion- or digestion-assisted release. The contribution of diffusion and digestion on the release of riboflavin from these externally gelled matrices with different lipid loads is shown in Figure 4.

As the lipid load within the microgels increased from 0% to 20% w/w, the relative contribution of the fast diffusion-driven phase to the release of riboflavin into SGF was significantly ($p \leq 0.05$) reduced. In microgels with no lipid, this fast diffusion-driven phase accounts for 95% of the release of the riboflavin as shown in Figure 4. In microgels loaded with 20% w/w lipid, this fast diffusion-driven phase only accounts for 13% of the total release of riboflavin. The majority (87%) of the riboflavin release from the microgels loaded with 20% lipid into SGF with enzymes occurs due to a slower (k value of 1.3 h^{-1}) digestion related process. Thus, a higher lipid content within the microgels changes the mechanism of riboflavin release into SGF from a diffusion-driven process to a digestion-assisted mechanism.

The release of riboflavin in the presence of SIF from both 0% and 10% lipid containing matrices was similar in the contribution of the diffusion or digestion phases (almost 50/50 diffusion and digestion, Figure 4). In microgels with the highest lipid load the contribution of diffusion-assisted release of riboflavin is significantly ($p \leq 0.05$) increased to 83% of the total release profile. It must be noted that the digestion of the protein matrix, and the riboflavin release was consistently slower in these externally gelled microgels in SIF in comparison to these processes in SGF. The 20% lipid-loaded microgels, in the presence of pancreatin, were particularly resistant to matrix digestion (only ~70% of total matrix digested after 6 h, and only ~40% of total riboflavin released after 6 h, Figure 2b). As the matrix digestion by pancreatin was shown to be impeded in these 20% lipid-loaded microgels, it follows that the major contributor to the release of the riboflavin from these matrices would be a diffusion process,

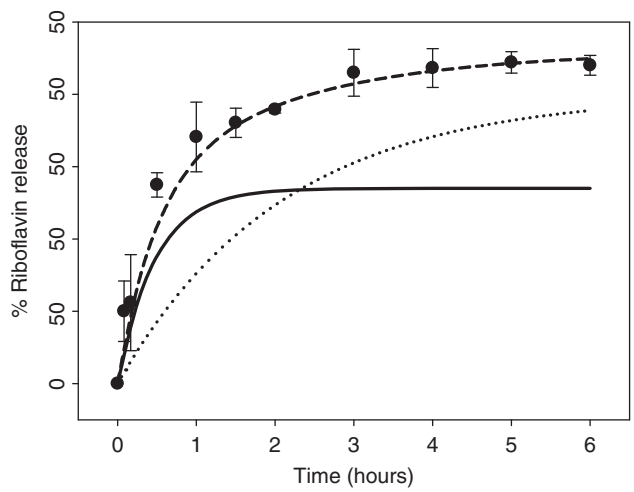

Figure 3. An example of the first-order model for diffusion (—), digestion (. . .), the biexponential model incorporating both contributions from diffusion and digestion (- - -) and the corresponding experimental data points (•) for the release of riboflavin.

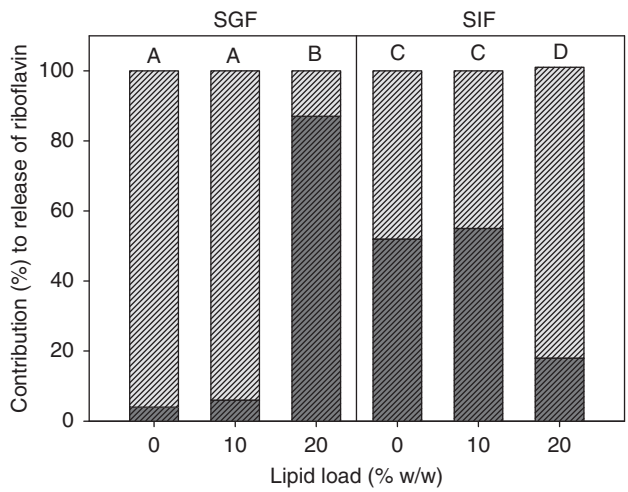

Figure 4. The contribution (%) of both diffusion (■) and digestion (■) on the release of riboflavin into SGF or SIF from externally gelled matrices containing 0%, 10% or 20% w/w lipid. Means not sharing the same letter are significantly different ($p \leq 0.05$).

rather than complete matrix digestion by GI enzymes as observed in other samples.

Contribution of digestion and diffusion processes on riboflavin release from internally gelled microgels

The contributions of digestion and diffusion processes to the release of riboflavin into SGF and SIF from internally gelled matrices were less clearly defined than in the case of externally gelled microgels. Digestion was the faster mechanism describing riboflavin release into SGF from lipid-free microgels where the rate constant (k_{dig}) was $19.7\,h^{-1}$ (Table 1). For the release of the riboflavin into SIF the opposite was true, where diffusion was the faster process with a rate constant of $8.7\,h^{-1}$ (Table 2, and additional data is contained in Appendix Tables A3 and A4).

Results obtained with lipids-containing microgels indicated similar release rate for the diffusion- and digestion-driven release mechanisms. For example, the release of riboflavin from 20% lipid-loaded matrices, into SGF, had a digestion-driven rate constant of $2.9\,h^{-1}$, whilst the diffusion driven process had a k value of $2.4\,h^{-1}$. Similarly, the release of riboflavin from 20% lipid-loaded matrices into SIF had a digestion-driven process rate constant of $1.5\,h^{-1}$, whilst the diffusion-driven process had a k value of $1.9\,h^{-1}$. This similarity in rates made the distinction of a faster or a slower driven process impossible. However, it is clear that there is significant ($p \leq 0.05$) reduction in both digestion and diffusion rates with an increase in the lipid load of these internally gelled matrices.

Conclusions

Modifying the lipid content of microgels (prepared by both an external and internal cold-gelation method) provided a means to obtain high (>60%) entrapment of a water-soluble food bioactive, and higher entrapment efficiency was obtained with the internal gelation method (>80%).

Microgels exhibited good physical stability in the absence of proteases yet underwent enzymatic degradation at simulated GI conditions. This suggests that microgels could be stable in food systems. Incorporating higher lipid loads (20% w/w) into the microgels delayed the proteolytic enzymatic digestion of the matrices. Considerably slower degradation rates were observed on the enzymatic digestion of 20% lipid-loaded microgels when compared to lipid-free microgels.

The release of riboflavin into enzyme containing GI fluids correlated well with the degradation of the protein matrix; microgels with higher lipid loads displaying a delayed or sustained release of this water-soluble bioactive. In particular for externally gelled matrices, at the highest lipid load (20% w/w), the contribution of a faster, diffusion-assisted release of bioactive was impeded, and the majority of bioactive release could then be accounted for by a slower, digestion-driven mechanism.

Modifying the lipid content within the microgels resulted in a composite matrix, capable of alternating from an initial burst release (lipid-free microgels) to a more sustained release (20% lipids-containing microgels).

Declaration of interest

The authors report no declarations of interest.

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Appendix A – Kinetic data for biexponential model fits

Table A1. Kinetic data for biexponential model fits describing the release of riboflavin with time from microgels (external gelation) with 0%, 10% or 20% w/w oil loads into SGF.

	Fast diffusion		Slow digestion	
Lipid (% w/w)	A_{diff}	k_{diff} (h^{-1})	A_{dig}	k_{dig} (h^{-1})
0	96 ± 1^{a}	7.6 ± 2.3^{a}	4 ± 1^{a}	1.7 ± 0.2^{a}
10	94 ± 2^{a}	4.2 ± 1.4^{a}	6 ± 2^{a}	1.7 ± 0.1^{a}
20	13 ± 6^{b}	5.9 ± 0.6^{a}	87 ± 7^{b}	1.3 ± 0.0^{b}

[a,b,c]Means in a given column not sharing the same letter are significantly different ($p \leq 0.05$).

A_{diff}/A_{dig} = Parameter indicating the contribution of diffusion/digestion-assisted release of riboflavin (Equation (4)); k_{diff}/k_{dig} = kinetic rate constant (h^{-1}) (diffusion/digestion) (Equation (2)/(3)).

Table A2. Kinetic data for biexponential model fits describing the release of riboflavin with time from microgels (external gelation) with 0%, 10% or 20% w/w oil loads into SIF.

	Fast diffusion		Slow digestion	
Lipid (% w/w)	A_{diff}	k_{diff} (h^{-1})	A_{dig}	k_{dig} (h^{-1})
0	48 ± 2^{a}	6.0 ± 0.3^{a}	52 ± 2^{a}	1.3 ± 0.1^{a}
10	45 ± 6^{a}	3.9 ± 1.3^{b}	55 ± 6^{a}	0.6 ± 0.1^{b}
20	83 ± 2^{b}	2.1 ± 0.5^{b}	18 ± 4^{b}	0.5 ± 0.1^{b}

[a,b,c]Means in a given column not sharing the same letter are significantly different ($p \leq 0.05$).

A_{diff}/A_{dig} = Parameter indicating the contribution of diffusion/digestion-assisted release of riboflavin (Equation (4)); k_{diff}/k_{dig} = kinetic rate constant (h^{-1}) (diffusion/digestion) (Equation (2)/(3)).

Table A3. Kinetic data for biexponential model fits describing the release of riboflavin with time from microgels (internal gelation) with 0%, 10% or 20% w/w oil loads into SGF.

	Slow diffusion		Fast digestion	
Lipid (% w/w)	A_{diff}	k_{diff} (h^{-1})	A_{dig}	k_{dig} (h^{-1})
0	95 ± 3^{a}	9.5 ± 3.6^{a}	3 ± 2^{a}	19.6 ± 4.4^{a}
10	82 ± 1^{b}	1.2 ± 0.2^{b}	17 ± 3^{b}	5.5 ± 0.3^{b}
20	100 ± 2^{c}	2.4 ± 0.1^{c}	–	2.9 ± 0.2^{c}

[a,b,c]Means in a given column not sharing the same letter are significantly different ($p \leq 0.05$).

A_{diff}/A_{dig} = Parameter indicating the contribution of diffusion/digestion-assisted release of riboflavin (Equation (4)); k_{diff}/k_{dig} = kinetic rate constant (h^{-1}) (diffusion/digestion) (Equation (2)/(3)).

Table A4. Kinetic data for biexponential model fits describing the release of riboflavin with time from microgels (internal gelation) with 0%, 10% or 20% w/w oil loads into SIF.

	Fast diffusion		Slow digestion	
Lipid s (% w/w)	A_{diff}	k_{diff} (h^{-1})	A_{dig}	k_{dig} (h^{-1})
0	40 ± 4	8.7 ± 0.7^{a}	55 ± 5^{a}	2.1 ± 0.3^{a}
10	–	2.6 ± 0.3^{b}	97 ± 5^{b}	2.0 ± 0.2^{a}
20	–	1.9 ± 0.1^{c}	101 ± 6^{b}	1.5 ± 0.1^{b}

[a,b,c]Means in a given column not sharing the same letter are significantly different ($p \leq 0.05$).

A_{diff}/A_{dig} = Parameter indicating the contribution of diffusion/digestion-assisted release of riboflavin (Equation (4)); k_{diff}/k_{dig} = kinetic rate constant (h^{-1}) (diffusion/digestion) (Equation (2)/(3)).