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Coacervates of whey proteins to protect and improve the oral delivery of a bioactive molecule

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ABSTRACT

The potentiality of heteroprotein complex coacervates as biocarrier for a bioactive was investigated. Vitamin B9 (B9), also known as folic acid, was encapsulated by complex coacervation of two whey proteins (WP), β-lactoglobulin and lactoferrin. The stability and bioavailability of formed B9-WP coacervates was then characterized. Under degradative conditions *in vitro* (UV light irradiation, and oxidation by H_2O_2), WP coacervates protected the vitamin against chemical degradation. B9-WP coacervates also showed considerable physical stability over time when incorporated in real food matrices. Compared to unencapsulated B9, oral administration of B9-WP coacervates to healthy rats enhanced the plasma level of the vitamin. This improved bioavailability can be ascribed to the improvement in the solubility of B9 throughout the gastro-intestinal tract. We thus demonstrate the efficiency of WP coacervates as biocarrier for the protection and delivery of small bioactive molecules.

1. Introduction

In recent years, the interest in functional foods and nutraceuticals has been motivated by their potential to contribute to healthy lifestyles. Their ability to enhance the daily intakes of specific nutrients and prevent deficiencies can possibly reduce the risks of certain diseases. (Annunziata & Vecchio, 2011). To formulate functional foods, bioactive molecules like minerals, antioxidants, omega-3 fatty acids or vitamins can be incorporated into food matrices (de Boer, Urlings, & Bast, 2016). Yet, in industrial settings where processes and storage can affect stability, solubility and bioavailability, the incorporation of these molecules and the preservation of their biological properties remain challenging. In this context, encapsulating agents can act as carriers or delivery systems to overcome these limitations (Shimoni, 2009). To ensure industrial sustainability, such systems should be developed from natural food-grade ingredients while using economical and reliable raw materials and processes (McClements, Decker, Park, & Weiss, 2009). The design of natural food-grade carriers, thereafter called "biocarriers" represents an important source of innovation for current food technologies. By evaluating how coacervates prepared from whey proteins can increase the stability and bioavailability of a model bioactive molecule, the present study highlights important parameters for a promising technology.

Among standard food components, edible proteins are good candidates for the design of natural biocarriers. Proteins are generally recognized as safe (GRAS) by the food industry and display unique functional properties such as their ability to form gels, to spontaneously self-assemble or to bind specific ligands. These characteristics make them ideal materials for the encapsulation of bioactive molecules (Chen, Remondetto, & Subirade, 2006; Christophe Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). While supplying essential amino-acids and providing nutritional benefits, some food proteins exhibit additional and specific bioactive properties. For instance, lactoferrin an iron-binding glycoprotein is described as a natural modulator of the immune system (Farnaud & Evans, 2003) and beta-lactoglobulin has demonstrated interesting binding abilities for bioactives such as curcumin

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(Sneharani, Karakkat, Singh, & Rao, 2010), essential fatty acids (Loch et al., 2013) or polyphenols (Zorilla, Liang, Remondetto, & Subirade, 2011).

Several encapsulation techniques exist that use food proteins as building blocks for the preparation of biocarriers (Matalanis, Jones, & McClements, 2011). Among them, the complex coacervation of biopolymers has demonstrated interesting properties; the process offers high loading efficiency (up to 99%) and good control over the release of bioactives, through mechanical stress, temperature or passive diffusion (Gouin, 2004). Most systems currently studied rely on the complex coacervation between proteins and polysaccharides (Garti & McClements, 2012; Turgeon, Schmitt, & Sanchez, 2007). The use of complex coacervates involving only proteins is less described and might be an attractive alternative in the context of clean label foods.

The formation of biocarriers throughout complex coacervation of a protein-protein system, named heteroprotein complex coacervation, is a two-step process. First, the bioactive is incorporated in a solution of protein A. Next, a solution of protein B, carrying opposite electric charges, is added to the system. Driven by electrostatic interactions between the two proteins, complex coacervation occurs, resulting in the formation of supramolecular co-assemblies of the two proteins called coacervates, entrapping the bioactive. As coarcervation is mainly driven by electrostatic interactions, the nature of the proteins, their stoichiometric ratios, as well as the pH and ionic strength of the solutions all strongly affect the coacervation process (Croguennec, Tavares, & Bouhallab, 2016; Schmitt & Turgeon, 2011). Thus, the optimal choice of proteins and the methods for heteroprotein complex coacervation are still under investigation to maximize the efficiency and benefits of the encapsulation process.

Recently the coacervation of two whey proteins, beta-lactoglobulin (BLG) and lactoferrin (LF), have been investigated in details (Anema & de Kruif, 2014; Flanagan et al., 2015; Tavares, Croguennec, Hamon, Carvalho, & Bouhallab, 2015; Yan et al., 2013). Beyond their ability to form coacervates, whey proteins display other features that are interesting from industrial and physicochemical standpoints. First, they are common by-products of the dairy industry: finding differentiated uses and functionalities for them could contribute to their industrial valorization. Second, whey proteins demonstrate interesting binding properties with various bioactive molecules. For instance, BLG can bind many small bioactives as indicated above and LF is able to bind several molecules of vitamin B9 (Tavares et al., 2015). Such proteins-ligands interactions can be exploited to encapsulate a variety of bioactives. Finally, consumers seem to favor foods with minimal amounts of additives, closer to natural products. In this regard, the preparation of biocarriers using milk components could pave the way for "clean-label" or "green product" functional foods and dairy products (Diaz, 2013). Altogether, efficiently exploiting whey protein's coacervates for encapsulation could elegantly complement alginates, gums, kappa-carrageenans and other biopolymers used in the food industry (Tavares, Croguennec, Carvalho, & Bouhallab, 2014).

We recently described the optimal conditions for optimal encapsulation of a model bioactive, vitamin B9 (B9) by BLG-LF coacervates (Chapeau et al., 2016). B9 is involved in several important biochemical processes, notably DNA synthesis and repair (Adank, Green, Skeaff, & Briars, 2003; Lucock, 2000). Since it is not synthetized by the human metabolism, B9 must be found in sufficient quantity in the daily diet (Basset, Quinlivan, Gregory, & Hanson, 2005). Certain populations, like pregnant women and elderly people, are more vulnerable to deficiencies (Mills & Signore, 2004) and could benefit from food enriched with B9 (Gregory, 2001). This represents a technological challenge since B9, as many other bioactives, is poorly soluble in acidic aqueous solutions. Moreover the relative instability and precipitation of B9 in some food matrices and in the gastro-intestinal tract might comprise its overall oral bioavailability (McNulty & Pentieva, 2004). The encapsulation of B9 in biocarriers might therefore provide an interesting alternative to enrich acidic foods while preserving the bioavailability of the vitamin.

Building on previous work which optimized the coacervation of whey proteins at the bench scale level (Chapeau et al., 2017), the present study validates how the encapsulation of B9 can contribute to its physicochemical stability and increase its bioavailability. To progress toward the complete formulation of an efficient biocarrier, we investigated here (i) the protective properties of the coacervates under different stresses occurring during storage or transformation processes (UV-light radiations and oxidation), (ii) the stability of B9-loaded coacervates in food matrices, and (iii) the ability of the coacervates to promote the gastrointestinal absorption of B9 in rats.

2. Material and methods

2.1. Stock solutions

B9 stock solutions at 1 g/L were prepared by solubilizing folic acid powder (purity > 97%, Sigma Aldrich, St. Louis, MO, USA) in aqueous NaOH solution (pH > 8). The pH was adjusted to pH 5.5 using 1 M HCl solution. This pH value was found to be optimal for complex coacervation between the two whey proteins (Chapeau et al., 2016). The prepared B9 solution was centrifuged at 28,000*g* for 30 min at room temperature (centrifuge Heraeus Biofuge Primo, Thermo Scientific, Waltham, MA, USA) to remove the insoluble fraction of B9. The exact vitamin B9 concentration was determined by absorbance at 283 nm (spectrometer UVmc2, Safas, Monaco) using 25.1 $L{\cdot}g^{-1}{\cdot}cm^{-1}$ as the extinction coefficient.

BLG (confidential industrial source) was further purified before use. BLG powder was dispersed in deionized water (45 g/L), adjusted to pH 4.6 with 1 M HCl, and kept at 30 °C for 5 min to precipitate non-native forms of BLG. The dispersion was centrifuged at 20,000*g* for 10 min at room temperature. The BLG suspension was then freeze-dried and stored at -20 °C until use. LF from bovine milk (purity 90% and iron saturation level of 10–20%, Fonterra Cooperative Group, New Zealand) was used without further purification. LF and BLG stock solutions were prepared by solubilizing protein powders in ultrapure water adjusted to pH 5.5 using 1 M HCl. The exact protein concentrations of LF and BLG (16.6 and 18.3 g/L, respectively) were determined by absorbance at 280 nm (spectrometer $UVmc^2$, Safas, Monaco) using 0.96 $L{\cdot}g^{-1}{\cdot}cm^{-1}$ and 1.47 $L{\cdot}g^{-1}{\cdot}cm^{-1}$ as extinction coefficients, respectively.

2.2. Preparation of B9-whey proteins coacervates

Coacervates of B9 and whey proteins (B9-WP) were prepared according to the previous study that defined the B9 and WP concentration ranges and B9:WP optimal ratios for complex coacervation (Chapeau et al. (2017). Briefly, a solution containing 0.11 g/L of B9 and 4.15 g/L of LF was prepared, and equilibrated for 10 min, at room temperature. Then, an equivalent volume of the BLG solution was added (final BLG concentration of 9.15 g/L). Spontaneous coacervation occurred at pH 5.5 without significant pH change after mixing. The B9-WP coacervate phase was recovered by centrifugation at 38,000*g* for 45 min at 20 °C (Centrifuge Avanti J-26S XP, Beckman Coulter Inc., Brea, CA 92821 USA). The recovered pellet had a final protein concentration of 26% w/w, with a 2:1 ratio of BLG to LF, and a final B9 loading of 0.04% w/w and at pH 5.5. Coacervates of the two whey proteins without B9 were also prepared and used as control when required.

2.3. Photodegradation

2.3.1. Method

Photodegradation was induced according to a method adapted from (Akhtar, Khan, & Ahmad, 1999). A Philips 30 W TW tube was used to expose experimental solutions to UV-light radiation, simulating a daylight exposure. The UV tube emits 88.7% of its radiation energy at 254 nm, corresponding to one of the absorption maxima (256 nm) of B9.

The experimental solutions contained B9 at 0.4 g/L alone, in combination with LF (4.15 g/L) or as B9-WP coacervates (as described above). Aliquots of 500 μL in 1.5 mL microcentrifuge tubes were irradiated with UV-light, while controls were kept in the dark, at 20 °C. After 0, 8, 24 or 48 h, two aliquots of each solutions were withdrawn and analyzed by reverse-phase high performance liquid chromatography (RP-HPLC), and mass spectrometry analysis.

2.3.2. RP-HPLC analysis

B9 and derived products were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC). Briefly, FA was separated on a PLRPS column (S/N 1006329-5, Varian Inc., Shropshire, UK) connected to a Waters 2695 HPLC system. Elution was performed using a gradient of solvent (acetonitrile 80%, water 20%, trifluoroacetic acid 0.1%) in water containing 0.1% trifluoroacetic acid. Detection was performed by absorbance at 283 nm. B9 degradation products, P-aminobenzoylglutamic acid (Pa) and a Pteridine residue (Pr), were identified by mass spectrometry as described below.

2.3.3. Mass spectrometry analysis

Electrospray mass spectrometer operating in positive ion mode with a voltage of 4 kV one-line with RP-HPLC was used for the identification of B9 degradation products. Spectra were recorded in full MS mode and selected in a mass range 130–2000 *m*/*z* for MS spectra with a resolution of 70,000 at *m*/*z* 200. The instrument was externally calibrated according to the supplier's instructions. Ions recovered in the sample were analyzed from the MS spectra using Xcalibur 2.2 Software (Thermo Scientific, San Jose, USA). The specific ions of B9 and its photoproducts, Pr and Pa, were identified by their respective molecular weights: B9 (Mw = 442 Da), Pr (Mw = 267 Da) and Pa (Mw = 192 Da) (Patring & Jastrebova, 2007; Santos, Scurachio, & Cardoso, 2014). The relative abundance of various species after treatments was estimated from the intensity of their corresponding ions before and after treatment. Experiments were conducted in triplicates.

2.4. Oxidative degradation

Oxidative degradation was induced by exposure to hydrogen peroxide (H_2O_2). Experimental solutions containing 1 g/L of B9 alone or as or as B9-WP coacervates were prepared. Solutions of B9 (1 g/L) with either LF (27.8 g/L) or BLG (55.2 g/L) were also used. In each solution, H_2O_2 (30% v/v, Sigma Aldrich, St. Louis, MO, USA) was added to reach final concentrations of 0.33 g/L B9 and 20% (v/v) H_2O_2. Samples were incubated at 37 °C in the dark. At 0, 2, 4, 6, and 18 h after the addition of H_2O_2, the remaining residual concentration of B9 in the solution were quantified by the enzyme linked immune assay (ELISA) method. Experiments were conducted in triplicates.

2.5. B9 quantification by enzyme-linked immunosorbent assay

Concentrations of B9 were determined using a competitive ELISA (Vitamin B9 Elisa Kit 96T, Abbexa, Cambridge, UK). Each sample was analyzed in technical duplicates (with no dilution or a 0.5 dilution in Abbexa kit diluent buffer). Appropriately diluted samples (50 μL) were added into each well of the microplate precoated with B9. Then, 50 μL of biotinylated anti-B9 antibodies were added into each well. The microplate was incubated for 45 min à 37 °C, then washed three times with the Abbexa washing solution. Then, 100 μL of horseradish peroxidase (HRP) streptavidin solution was added into each well, followed by incubation for 30 min à 37 °C. The microplate was washed five times with the washing solution. For quantification, 90 μL of tetramethylbenzidine substrate was added into each well and the microplate was incubated for 15 min in the dark at 37 °C. The reaction was stopped by the addition of 50 μL of Abbexa stop solution. Finally, the absorbance of each well was measured at 450 nm using a spectrophotometer microplate reader (NanoQuant infinite M200 Pro, Tecan, Switzerland). For each analysis, a calibration curve was performed over the range of 1000 to 12.5 ng/mL ($R^2 > 0.98$). Under these experimental conditions, the limit of quantification of this method was calculated to be 7.5 ng/mL.

2.6. Stability of B9-WP coacervates as assessed by dialysis method

Dialysis method was set up to explore the stability and release behavior of B9 from the B9-WP coacervates when incorporated into a dairy product such as milk. Two processed milk samples were used: Ultra High Temperature (UHT) semi skimmed milk (Sodiaal, France) and pasteurized whole milk (Québon, Canada). Each of the two types of milk was enriched by the addition of free B9, B9-WP coacervates or free B9 and additional WP coacervates. Suspension was made using three-repeated ultrasonication cycles of 1 min (ultrasonication bath Bransonic 220, Branson Ultrasonics, St Louis, USA) followed by 2 min of vortex (Topmix-94223, Fisher Scientific Bioblock, Whaltam, USA).

To ascertain stability in enriched milks, 5 mL of milk samples with added B9 (50 mg/L of B9) were placed in a 5-cm long cellulose-ester dialysis tubing with a molecular weight cut-off of 100 kDa (Spectra/Por product, Spectrum Laboratories, Inc., USA). Dialysis experiments were performed under constant slow agitation in the dark and at 4 °C against two liters of corresponding milk. B9 concentration inside the dialysis membrane was monitored by ELISA method at 0, 1, 2, 4, 8 and 24 h. Each experiment was carried in triplicate.

The B9 released from the dialysis bag was calculated using the following equation:

$$B9\ release(\%) = \left(1 - \frac{[B9]_t}{[B9]_0}\right) \times 100 \quad (1)$$

where $[B9]_t$ represents the concentration inside the dialysis bag at time t, and $[B9]_0$, the initial concentration. The absence of interference from the dairy matrices on the ELISA quantification was checked prior to the experiments.

2.7. Phase contrast microscopy

B9-WP coacervates suspended in ultrafiltered milk permeate were observed by a phase contrast optical microscope (Olympus BX51TF, Olympus, Hamburg, Germany) set at the magnification of ×40 or ×100. A 10 μL sample of the B9-WP coacervates dairy solution was deposed on standard microscope glass slide, covered by a glass coverslip (22 mm × 22 mm) and immediately observed. The samples and observations were performed three times to ensure the representability of the results.

2.8. In vivo pharmacokinetic studies in male rats

2.8.1. Ethics

All experiments were performed in accordance with the Canadian Council on Animal Care and were approved by the Institutional Committee of the CHU de Quebec Research Center (CHUL) (protocol number 2015074-2).

2.8.2. Tested solutions

The four solutions used in this experiment were prepared by dissolving B9, BLG and LF or B9-WP coacervates in PBS. The concentration of B9 in all solutions was set at 1 g/L, while the concentrations of BLG and LF were 55.2 and 27.8 g/L, respectively. Coacervates contained both proteins at these concentrations.

2.8.3. Pharmacokinetic studies

Pharmacokinetic studies were performed in male Sprague Dawley rats (350–450 g) obtained from Charles River (St-Constant, QC, Canada) and housed in 12 h light/dark cycles at 20 ± 2 °C and 55–60% relative humidity. Prior to the experiment, animals were fed *ad libitum* for a minimum of 10 days with a folic acid deficient diet (TD 95247, Envigo Teklad Diets, Madison Wisconsin, USA) and free access to drinking water. For the experiment, rats were randomly divided into 4 groups of 5 animals each. Each group was administrated a specific PBS solution: B9, B9-WP coacervates, B9 and BLG or B9 and LF. Each animal received a dose of 500 μg of B9. Solutions were administered using a blunt needle via the esophagus into the stomach. Just before the oral administration (0 min) and at different times post administration (0.5, 1, 2, 4, 6 and 10 h), 200 μL blood samples were collected via the saphenous vein using capillary tubes (Microvette CB300K2E, Sarstedt, Germany). Blood samples were centrifuged at 2 000 *rcf* for 15 min at 4 °C, and the isolated plasma was kept in microcentrifuge tubes at −20 °C until further analysis (5–7 days). Concentrations of B9 recovered in rat plasma were determined by ELISA.

2.8.4. Pharmacokinetic parameters

The maximal B9 concentration in rat plasma (C_{max}) and the time taken to reach C_{max} (T_{max}) were obtained from experimental data. The area under the plasma concentration *vs.* time curve from time 0–10 h (AUC) was calculated using the trapezoidal method.

2.8.5. Statistical analysis

A series of ANOVA followed by *post hoc* Tuckey tests were performed to investigate potential statistical differences between the pharmacokinetic parameters. In all cases, $p < 0.05$ was considered to be statistically significant.

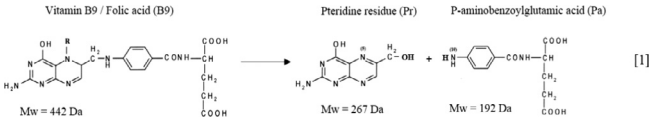
3. Results

3.1. Protective effect of B9-WP coacervates

To investigate the ability of WP coacervates to limit the degradation of B9, unencapsulated B9, mixtures of B9 with LF and BLG and B9-WP coacervates were exposed to either UV-light (photodegradation) or H_2O_2 (oxidative degradation). These accelerated degradation conditions provided surrogates for processes occurring during food transformation or long time storage. B9 and LF mixture was studied because we recently showed that B9 forms nanocomplexes with LF (Tavares et al., 2015).

3.1.1. Photodegradation

In aqueous environments, UV-light converts B9 into two main photoproducts, a pteridine residue (Pr) and a P-aminobenzoylglutamic acid (Pa), with the capture of a water molecule according to the following chemical scheme [1] (Jamil Akhtar, Ataullah Khan, & Ahmad, 2003; Off et al., 2005):



[1]

Fig. 1 shows the elution profiles of B9 of the three treated samples after exposure to UV light for 0, 8 or 24 h. On shown, native B9 had a retention time of 3.3 min (Fig. 1A–C). When B9 was not encapsulated (*i.e.*, solutions of B9 alone or in complex with LF), degradation products were evidenced after 8 h of irradiation with the apparition of a peak with a retention time of 2.7 min. The rel-

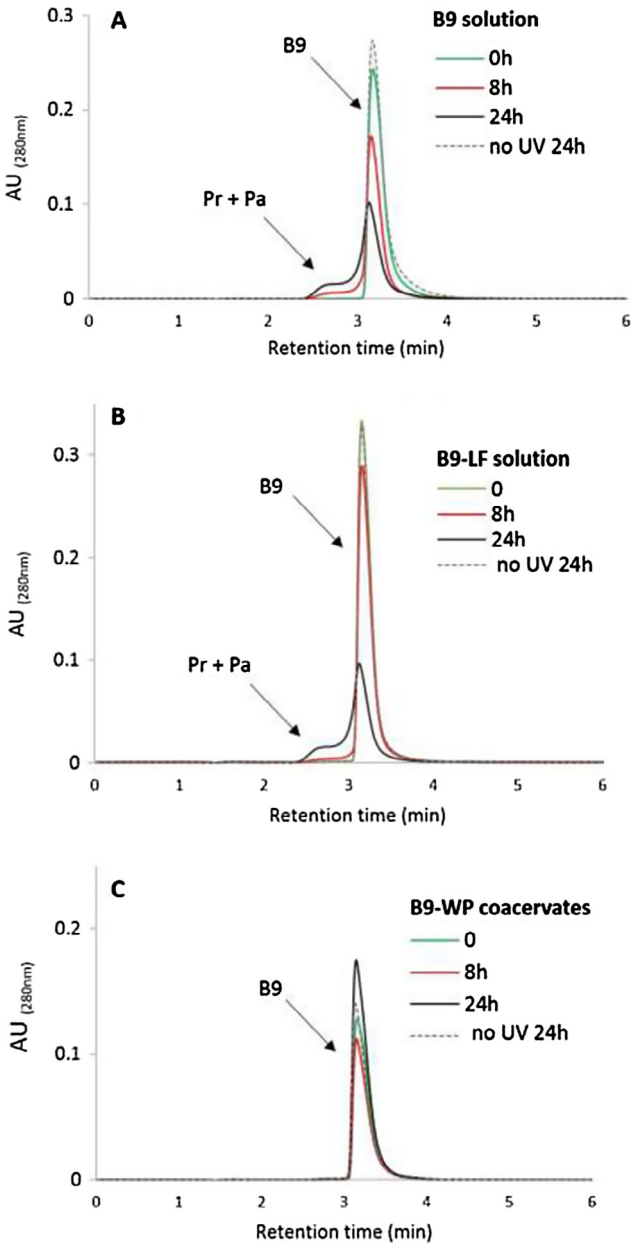


Fig. 1. Evolution of RP-HPLC profiles of vitamin B9 after exposure to UV light during 24 h. Vitamin B9 was treated with UV either alone in solution (A), pre-complexed with lactoferrin (B) or encapsulated in whey protein coacervates (C).

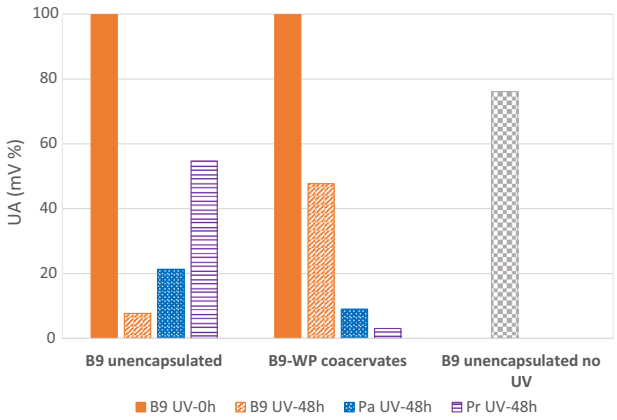


Fig. 2. Protective effect of WP coacervates against UV light induced degradation of vitamin B9 as assessed by mass spectrometry. Ion intensities of B9 and derived products from electrospray mass spectrometry analysis before and after 48 h exposure to UV light. The intensity of untreated B9 after 48 h is also shown. Under used conditions, experimental variations were ≤5%.

ative abundance of this peak increased after 24 h of irradiation for treated B9 alone or in complex with LF with concomitant decrease of B9 peak (Fig. 1A, B). Mass spectrometry indicated the presence of ions with molecular masses, *m*/*z* of 442, 267, and 192 corresponding to B9 and its two main degradation products Pr and Pa, respectively. In the absence of UV light, these two ions were not detected suggesting that they origin from the photodegradation of B9. The formation of these products from B9 has been already reported by Santos et al. (2014). Interestingly, when B9 was encapsulated in WP coacervates, the two degradation products were not detected (Fig. 1C), suggesting the WP coacervates can mitigate the degradation of B9 induced by UV light.

An estimation of the relative degradation of B9 alone or protected in WP coacervates with and without UV irradiation was assessed by mass spectrometry analysis ((Fig. 2). Based on the initial ion intensity, about 90% of free B9 disappeared after 48 h under UV light with simultaneous appearance of degradation products Pr and Pa (see the scheme above). Meanwhile, despite a slight decrease in B9 ion intensity, the untreated B9 solution stored in the dark was relatively stable (Fig. 2). Encapsulation into WP coacervates limited the observed degradation of B9. More than 50% of initial B9 (initial ion intensity) were still detected after 48 h of UV light treatment. This was also confirmed by a lower detection of the ions corresponding to the degradation products Pr and Pa.

3.1.2. Oxidative degradation

Hydrogen peroxide was used to accelerate oxidative degradation, an efficient method to study drug decomposition in pharmaceutical sciences (Blessy, Patel, Prajapati, & Agrawal, 2014). Fig. 3 presents the remaining quantities of B9 in solutions (B9 alone or in complex with LF) and encapsulated into WP coacervates after exposure to 20 % H_2O_2, at 37 °C, for 18 h. In these experiments, the ELISA assay allowed quantification of only the native form of B9 (without interference from H_2O_2). For all the samples, native B9 decreased over time, yet the kinetics were different depending on whether B9 was free, in complex with LF, or encapsulated in WP coacervates. High degradation rate was found for B9 either in free form or in complex with LF. In these samples, degradation of about 75 % on the initial vitamin was found after 18 h exposure to H_2O_2. Hence, complexation of B9 with LF does not seems to be enough for efficient protection. In contrast, the encapsulated B9 exhibited much slower degradation rate underlying a protective effect.

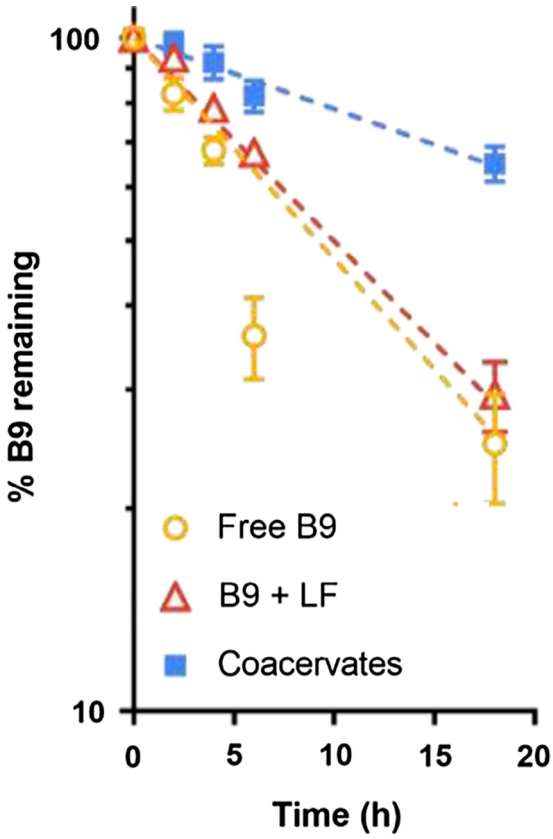


Fig. 3. Comparative degradation kinetics of vitamin B9 either in free form, complexed to Lf or encapsulated into WP coacervates after 18 h exposure to 20% hydrogen peroxide at 37 °C (n = 3, values represent means ± SD).

Approximately, 80 and 70 % of the initial B9 into WP coacervates were quantified after 6 and 18 h of treatment, respectively.

3.2. Stability of coacervates prepared from WP in dairy solutions

The ability of the coacervates to maintain interactions with B9 in conditions where other components of food matrices can compete and alter the nature of the protein complexes was checked. Two dairy matrices commonly consumed in North America and Europe were studied: whole full-fat pasteurized milk and UHT semi-skimmed milk. The release of B9 from the coacervates was checked by dialysis approach. Free B9 or B9-WP coacervates were added to milk and submitted to dialysis against the same milk. The free B9 solution is assumed to represent a control sample where the diffusion is only limited by passage through the dialysis membrane (Bertrand, Leclair, & Hildgen, 2007). In the absence of interactions between B9 and coacervates, for example if the protein complexes are totally disrupted, B9 should be released at rates comparable with the free vitamin solution. The same dialysis results were obtained for the two studied milks.

Encapsulated B9 showed a completely different dialysis behavior as compared to free B9 (Fig. 4). The release of free B9 was nearly complete, i.e. 90–95% after 24 h of dialysis experiment. Comparatively, the encapsulated B9 seemed to be more retained in the dialysis bag. The release of B9 was then lower and plateaued around 45% of the initial B9 added to milk indicating a partial stability of B9-WP coacervates. Of note, the effect of the coacervates on the diffusion of B9 was not solely due to the presence of additional WP. In a control experiment, unencapsulated B9 incubated with coacervates prepared in the absence of vitamin, showed a release kinetic similar to that found for free B9.

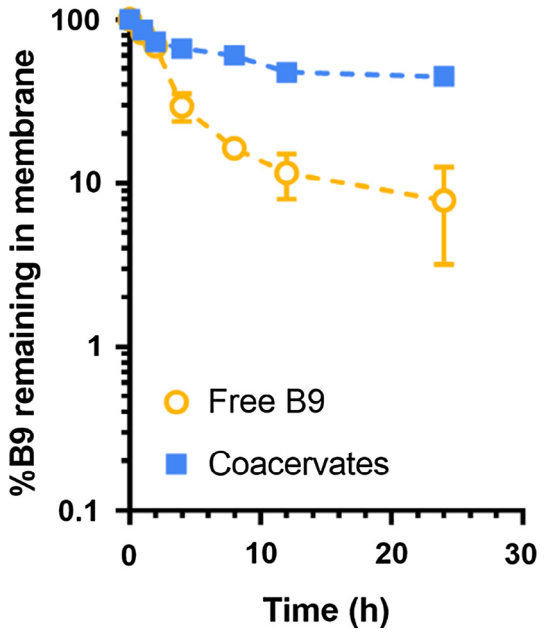


Fig. 4. Kinetic release of B9 from enriched whole pasteurized milk as assessed by dialysis approach. Five ml of B9 enriched milk were placed into dialysis tubing and submitted to dialysis against 2 l of unenriched milk for 24 h at 4 °C (n = 3, values represent means ± SD).

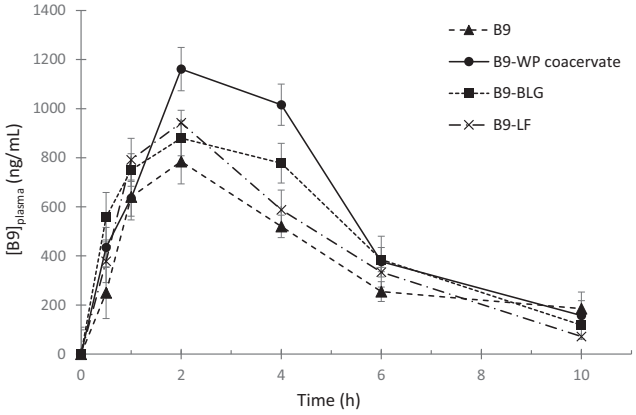


Fig. 5. Pharmacokinetics of vitamin B9 concentrations in rat plasma *vs* time after a single oral administration at T0 of B9, B9-WP coacervate, B9-BLG, or B9-LF solutions corresponding to a dose of 500 μg B9/oral administration. Values represent mean ±SD, n = 5.

3.3. In vivo pharmacokinetics of B9-whey proteins coacervates

Fig. 5 shows the pharmacokinetics of B9 concentrations recovered in plasma after a single oral gavage of rats with either B9 alone, B9 complexed with lactoferrin, B9 in the presence of BLG or encapsulated in WP coacervates. For free B9 group, the B9 level in the plasma of animals increased rapidly during the two first hours post-administration followed by a phase where the detected vitamin decreased slowly until the end of the experiment. The others groups displayed similar profiles. Nevertheless, the plasma level of B9 from B9-WP coacervates was significantly higher than that found for groups receiving free B9. The plasma level of B9 in the other groups (B9 + LF and B9 + BLG) exhibited intermediate levels. Table 1 summarizes the pharmacokinetic parameters (C_{max}, T_{max}, and AUC) obtained for each group. Maximum concentrations were obtained within 1.8–3 h of gavage. The group receiving B9-WP coacervates showed the highest C_{max} value and the largest total blood exposure (*i.e.*, AUC). The B9-WP coacervates group provided a blood exposure which was 1.5-fold higher than the free B9 solution (5868 *vs.* 3804 ng·h/mL). Theses parameters were also slightly higher with WP coacervates compared to proteins taken individually at the same concentration.

4. Discussion

In this study, B9 was investigated as a model bioactive molecule to illustrate the potential of heteroprotein coacervates for the preparation of biocarriers. Vitamin B9 plays a crucial role in various metabolic and biochemical processes such as the replication of DNA and cell division, notably during the early stages of embryology (Herrmann & Obeid, 2011; Lucock, 2000). This bioactivity is mostly due to the native form of B9 (Gazzali et al., 2016; Hirakawa, Suzuki, Oikawa, & Kawanishi, 2003; Patro, Adhikari, Mukherjee, & Chattopadhyay, 2005). To prepare biocarriers that maintain the bioactivity of the vitamin, it is therefore critical to preserve its chemical integrity.

Under accelerated degradation conditions, unencapsulated free B9 was efficiently converted into various degradation products. B9 can be photo-chemically cleaved, yielding 6-formylpterin and p-aminobenzoylglutamic acid (Araújo et al., 2015; Thomas, Suárez, Cabrerizo, Martino, & Capparelli, 2000). This photodegradation preferentially occurs in acidic solutions and in the presence of oxygen (Thomas et al., 2000). Although B9 is known to remain mostly stable in aqueous solution at 37 °C (Tripet & Kesselring, 1975; Vora, Riga, Dollimore, & Alexander, 2002), addition of H_2O_2 significantly increases oxidative degradation via the production of singlet oxygen (Gazzali et al., 2016; Thomas et al., 2000). These oxidative conditions allowed degradation of native B9 forming peteridin product that was identified by mass spectrometry. In our conditions, unencapsulated B9 degraded within a few hours.

Under both UV light irradiation and oxidative conditions, WP coacervates significantly hindered the degradation of B9. After up to 48 h of exposure to UV light, few degradation products were detected for the encapsulated vitamin, in stark contrast with the degradation observed for free B9. Although small fluctuations in concentrations were observed over time, these differences were similar in samples that were kept in the dark; therefore, these changes can hardly be ascribed to photodegradation. The observed protective effect of the coacervates could be attributed to decreased contact between the vitamin, entrapped in a hydrophobic environment within the proteins, and water or oxygen mole-

Table 1
Characteristic parameters of pharmacokinetics of vitamin B9 concentrations in rat plasma *vs* time after a single oral administration at T0 of B9, B9-WP coacervates, B9-BLG, or B9-LF solutions corresponding to a dose of 500 μg B9/oral administration. Statistical analysis by a, b, c, statistically different at a significance level of $p < 0.05$.

	C_{max} (ng/mL)	*SD* (ng/mL)	T_{max} (h)	*SD* (h)	AUC (ng·h/mL)	*SD* (ng·h/mL)
B9	872[a]	*±49*	1.8	*±0.3*	3804[a]	*±190*
B9-BLG	942[ab]	*±95*	2.8	*±0.3*	5108[ab]	*±602*
B9-LF	1054[bc]	*±77*	1.8	*±0.3*	4516[ab]	*±496*
B9-WP coacervates	1162[c]	*94*	1.8	*±0.3*	5868[b]	*±574*

cules. About 0.4 g B9 is protected in a highly concentrated environment, with nearly 260 g proteins per kg of coacervates. Similar protective effects have been reported for B9 bound to BLG at neutral pH, essentially by decreasing the effective concentration of all reagents involved in the photodecomposition reaction (Liang, Zhang, Zhou, & Subirade, 2013).

The encapsulation of B9 in WP coacervates also strongly limits its H_2O_2 induced oxidation. After 6 h of oxidative stress, more than twice as much native B9 was still detectable in the sample containing B9-coacervates compared to free B9. After 18 h, more than 60% of the initial vitamin remains detectable in the coacervates (compared to less than 30% for treated free B9). B9-LF complex showed intermediate protective effect toward oxidative degradation, underlying that the WP coacervates present the more efficient protective effect. Once again, the encapsulation of the vitamin within a dehydrated protein complex would limit its reactivity toward the solution containing singlet oxygen. It is also conceivable that the protein could capture singlet oxygen and act as a buffering antioxidant to preserve the vitamin. As such, additional benefit of coacervates could result from the higher concentrations of proteins present in these samples.

Because interactions governing the architecture of protein complexes in the coacervates are non-covalent and could be displaced by proteins, pH or the higher ionic strength of food matrices, the ability of B9 to remain encapsulated within the coacervates was investigated in milk. Model dialysis experiments where B9-coacervates were strongly diluted in milk samples showed that WP complexes retained large part of the vitamin for up to 24 h. The release kinetics of B9 from the coacervates were found to be similar in whole and semi-skimmed milks. This suggests that B9-WP coacervates are stable in food matrices such as milk with complex chemical composition. However, such partial stability requires encapsulation of B9 into WP coacervates before addition to milk. These results support the benefits and potentiality of WP coacervates as biocarrier of bioactive in complex food products.

Both the protective effect of WP coacervates and their stability in a dairy matrix would be of little scientific merit if the molecule is not bioavailable after oral administration. Pharmacokinetic experiments in rats demonstrated that the encapsulation of B9 in coacervates increased the overall bioavailability of the vitamin, notably by increasing the maximum concentrations measured in blood after a single oral dose. This could be explained by the ability of coacervates to increase the apparent solubility of B9, especially at the acidic pH values found in the stomach. It has already been reported that the binding between B9 and a whey protein (BLG) can increase the water solubility of the vitamin (Liang & Subirade, 2010). Since molecules require dissolving to be absorbed through the gastro-intestinal wall, any interactions preventing the precipitation of B9 could be beneficial to increase bioavailability. Similar results were obtained with egg white proteins and B9 (Arzeni, Pérez, LeBlanc, & Pilosof, 2015), as well as with curcumin and BLG (Teng, Li, & Wang, 2014), and more generally with many pharmaceutical formulations (Saha, 2010). The intrinsic resistance of BLG to the gastric enzyme pepsin (Mandalari et al., 2009; Reddy, Kella, & Kinsella, 1988), combined with a rapid proteolysis during duodenal digestion, can increase the bioavailability of B9 during the digestion of B9-BLG complexes (Pérez, David-Birman, Kesselman, Levi-Tal, & Lesmes, 2014). These observations agree with the present pharmacokinetic data indicating longer times taken to reach maximal concentrations (T_{max}) and potentially delayed adsorption when B9 is administered in presence of BLG alone.

Considering the results obtained *in vivo*, it is conceivable that proteins, including BLG, LF and coacervates, could protect B9 from gastric degradations. Penalva et al. (2015) suggested that the delayed release of B9 from casein nanoparticles in acidic environment could prevent degradation of the vitamin and improve its oral bioavailability. Herein, the improved protection observed *in vitro*, added to the differences in pharmacokinetic measured between WP coacervates and simple protein solutions, suggests the WP coacervates architecture provides benefits over plain interactions between vitamin and proteins.

5. Conclusion

Herein, coacervates prepared from whey proteins demonstrated their capacity to protect a model bioactive molecule from chemical degradation *in vitro* and potentiate its bioavailability *in vivo*. Furthermore, by showing that the B9-coacevates maintain their structural integrity upon addition in a food matrix containing proteins, ions and fat, the potential of the platform for the preparation of enriched food product was evidenced. Because the biocarrier uses whey proteins (lactoferrin and beta-lactoglobulin) which are currently undervalued in the milk transformation industry, the encapsulation approach might have additional merit, from an economic standpoint. Further work, notably incorporating other bioactive molecules, will further position the importance of this encapsulation approach for the preparation of differentiated, natural and additive-free food products.

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