Casein micelle as a natural nano-capsular vehicle for nutraceuticals

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Abstract

Casein micelles (CM) are in effect nano-capsules created by nature to deliver nutrients, such as calcium, phosphate and protein, to the neonate. A novel approach is herein presented, to harness CM, for nano-encapsulation and stabilization of hydrophobic nutraceutical substances for enrichment of non-fat or low-fat food products. Such nano-capsules may be incorporated in dairy products without modifying their sensory properties. This study introduces new possibilities for encapsulation and delivery of sensitive health-promoting substances using natural GRAS (generally regarded as safe) ingredients.

As a model hydrophobic nutraceutical compound we studied the fat-soluble vitamin D2, which is essential for calcium metabolism. A protocol for incorporation of vitamin D2 into CM was established. The encapsulation process was evaluated, and so was the protective effect of the micelles against photochemical degradation of the vitamin.

We have demonstrated, for the first time, the possibility to load a nutraceutical compound into CM, utilizing the natural self-assembly tendency of bovine caseins. The vitamin was about 5.5 times more concentrated within the micelles than in the serum where it was only present bound to residual soluble caseins. Moreover, the morphology and average diameter of the re-assembled micelles were similar to those of naturally occurring CM.

We have also demonstrated that the re-assembled CM can provide partial protection against UV-light-induced degradation to vitamin D2 contained in them.

This study suggests that CM may be useful as nano-vehicles for entrapment, protection, and delivery of sensitive hydrophobic nutraceuticals within food products.

Keywords: Casein micelles; Nano-encapsulation; Nutraceuticals delivery; Vitamin D; Milk proteins; Calcium

1. Introduction

Cow milk contains 30–35 g of protein per liter. Casein, which accounts for about 80% of milk protein, is organized in micelles. Casein micelles (CM) are designed by nature to concentrate, stabilize and transport essential nutrients, mainly calcium and protein, for the neonate (DeKruif & Holt, 2003). A CM is, in effect, a natural nano-delivery system.

The micelles are spherical colloids, 50–500 nm in diameter (150 nm in average) (Fox, 2003), composed of the main four caseins: \( \alpha_{s1}\)-casein (\( \alpha_{s1}\)-CN), \( \alpha_{s2}\)-CN, \( \beta\)-CN, and \( \kappa\)-CN (molar ratio \(~\approx 4:1:4:1\), respectively) (DeKruif & Holt, 2003; Fox, 2003; Swaisgood, 2003). The caseins are held together in the micelle by hydrophobic interactions and by bridging of calcium–phosphate nano-clusters bound to serine–phosphate residues of the caseins (DeKruif & Holt, 2003). The structure of CM is important for their biological activity in the mammary gland, for their stability in milk and during processing of milk into various products, as well as for the good digestibility of the nutrients comprising the micelles. The micelles are very stable to processing, and retain their basic structural identity through most of these processes (DeKruif & Holt, 2003; Fox, 2003; Swaisgood, 2003).

However, it has not yet been suggested in the literature, to harness these remarkable natural nano-capsules as vehicles for added nutraceutical substances. Caseinates have been used as microencapsulation wall materials (Hogan, McNamee, O’Riordan, & O’Sullivan, 2001). However, caseins forming such artificial capsules have lost

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the original micellar structure, as well as much of their natural functional behavior (e.g. during enzymatic coagulation of milk for cheese production). Moreover, the generally larger size of microcapsules is more likely to impair product smoothness.

CM can be re-assembled in vitro, by simulating their formation in the Golgi system of the mammary gland, according to a procedure developed by Knoop, Knoop, and Wiechen (1979). Re-assembled CM (rCM) with different casein and ion compositions were evaluated for micellar size, and for stability towards dialysis, pressure, ethanol and heat. It was shown that the size and stability of the micelles can be controlled by the relative proportion of kappa casein, which stabilizes the surface of the CM: the more κ-CN in the initial casein mixture, the smaller the micelles obtained, and the better their stability towards dialysis, pressure, ethanol and heat (Schmidt, 1979). This author demonstrated that for whole-casein-based preparations, micelle size may be somewhat reduced by reducing initial calcium and magnesium concentrations. Best results were obtained for CM re-assembled from whole-casein according to the conditions used by Knoop et al. (1979). These studies present a rather controllable process to obtain rCM which are very similar to naturally forming ones.

Vitamin D, which was chosen here as a model hydrophobic nutraceutical compound, is a fat-soluble vitamin of great importance in calcium and phosphate metabolism. It facilitates calcium absorption in the intestine, transport of calcium and phosphate to the bones, and re-absorption of calcium and phosphate in the kidneys. Vitamin D also takes part in the formation of osteoblasts, in fetal development and in the normal function of the nerve system, the pancreas and the immune system (Knoop et al., 1979).

The recommended daily intake of vitamin D is 5 μg per day for adults between 21 and 51 years of age, and 10 μg per day for children and pregnant women. Fortified cereals, eggs, butter and fish oil are all vitamin D sources (Eitenmiller & Landen Jr., 1999), but since it is fat-soluble it can hardly be found in skim milk and low-fat dairy products, which are important sources for calcium and phosphate.

Vitamin D has over 40 known metabolites, one of which is vitamin D2. Vitamin D2 originates from plants, and can be found in nature in limited amounts, but can be synthesized readily, and therefore, it is the main form of vitamin D used in the pharmaceutical industry. In the vitamin structure there are double bonds sensitive to oxidation. Light, air and high temperature induce vitamin isomerization and degradation into its inactive forms (Bell, 1978; Eitenmiller & Landen Jr., 1999).

The roles of vitamin D2 in calcium and phosphate utilization make it a prime candidate for enrichment of CM. Adsorption of vitamin D2, onto hydrophobic zones of the caseins, which tend to be found in the core of the micelle, would facilitate the enrichment of low-fat and fat-free dairy and other food products with vitamin D2, while minimizing the effect of its incorporation on the functional behavior of the system during processing.

The purpose of our research was to explore the possibility to encapsulate nutraceutical substances within CM and to develop the technology to achieve that with minimal changes to the functional properties of CM. Specific objectives included the establishment of a protocol for incorporation of the model hydrophobic nutraceutical, vitamin D2, into CM, and evaluation of the encapsulation process in terms of: (a) effectiveness and efficiency, (b) preservation of micelle properties: diameter, as determined by dynamic light scattering (DLS) and morphology, as determined by cryogenic transmission electron microscopy (cryo-TEM), stability to high shear, as determined by the relative change in average diameter following ultra-high pressure homogenization, (c) the protective effect which the micelles confer to vitamin D2 against photochemical degradation induced by UV exposure.

The main hypothesis of this study was that a hydrophobic nutraceutical compound may be entrapped within CM by association to soluble caseins, which are known to have hydrophobic domains, followed by re-assembly of the CM.

2. Materials and methods

2.1. Materials

Commercial sodium caseinate (Miprodan 30, 93.5% protein, MD Food Ingredients Amba (Videbæk, Denmark) was kindly donated by Strauss-Elite Ltd., Israel. Vitamin D2 was obtained from Sigma-Aldrich (Rehovot, Israel). Ethanol (absolute), hydrochloric acid (concentrated), petroleum ether and diethyl ether, were obtained from Bio-Lab (Jerusalem, Israel). Tripotassium citrate, sodium hydroxide, potassium hydroxide, and pyrogallol were obtained from Merck (Darmstadt, Germany). Calcium chloride was obtained from Carlo Erba (Rodano, Italy). Dipotassium hydrogen phosphate was obtained from Spectrum (CA, USA). Ethylene diamine tetraacetic acid (EDTA) was obtained from Acros (NJ, USA). Methanol and acetonitrile (both HPLC grade) were obtained from Lab Scan (Dublin, Ireland).

2.2. Methods

Non-covalent binding of vitamin D2 to sodium caseinate was achieved by dropwise addition 12.7 mM solution of the vitamin in absolute ethanol into a 5% sodium caseinate solution, while stirring, to a final concentration of 63.5 μM.

Re-assembly of CM: Preparation of rCM was done based on the method described by Knoop et al. (1979). However, unlike Knoop et al., we have started from a rehydrated commercial sodium caseinate powder, rather than a freshly prepared caseinate, in order to extend the commercial applicability of the method. The following
procedure was used: to 200 mL solutions of 5% sodium caseinate, with and without added vitamin D2, 4 mL 1 M tri-potassium citrate, 24 mL 0.2 M K2HPO4 and 20 mL 0.2 M CaCl2 were added. Eight consecutive additions of 2.5 mL 0.2 M K2HPO4 and 5 mL 0.2 M CaCl2 were performed, at 15 min intervals. During this process, samples were stirred in a thermostated bath at 37°C. The pH was maintained between 6.7 and 7, using 0.1 N HCl or 1 N NaOH. The volume was eventually brought up to 400 mL with water, the pH was adjusted to 6.7, and the final dispersions were stirred moderately for 1 h. Each experiment was performed in duplicate.

Analytical fractionation: Micelle preparations were centrifuged at 20°C and 25,000 × g for 1 h and the supernatant was collected. The supernatant was then ultra-filtered using Amicon 8050 stirred ultra-filtration cell with a 10,000 Da nominal molecular weight limit membrane (Millipore). Permeate was collected and analyzed for vitamin D2 content.

Ultra-high pressure homogenization was done using a Micro DeBee ultra-high pressure homogenizer (Bee Int’l Inc.). Samples of rCM and D2-rCM suspensions were homogenized, as well as a sample of skim milk reconstituted from powder, by 1 pass at the single-reversed-flow mode at 185 MPa, using a 0.1 mm orifice, and a back-pressure of 10 ± 3 MPa. Average diameter of rCM and D2-rCM was measured before and after the homogenization process by DLS (see method details below). Relative average diameter changes were then determined for each sample.

Evaluation of micelle protection against UV-light-induced degradation of Vitamin D2: Samples were placed in Petri dishes within a wooden light-proof cabinet, and exposed to a 254 nm UV light, at 200 µW/cm² intensity for 3, 6, 12, and 24 h. At each exposure time, three 20 mL samples were compared: a micelle dispersion preparation containing vitamin D2-enriched rCM (D2-rCM), a negative control (an identical sample covered with an aluminum foil to completely block the UV (control I)), and another control containing only serum from the D2-rCM preparation (control II). The serum samples were obtained by centrifuging D2-rCM dispersion as described above.

UV absorbance spectra determination for caseinate and vitamin D2: Samples of caseinate and vitamin D2 at concentrations similar to their concentrations in the rCM suspension (2.5% and 31.75 µM, respectively) were prepared. UV absorbance spectra of the samples were analyzed by absorbance scan at wavelengths between 220 and 360 nm, using a Pharmacia Biotech Ultrospec 3000 spectrophotometer.

Saponification and extraction: Ten mL samples were centrifuged as described above. The pellet, containing the micelles, was then separated from serum by decantation. Pellets were resuspended in a 100 mM EDTA solution (of same weight as the removed serum), and equilibrated for 6 h at 4°C to dissociate the micelles. Both pellet and serum from each sample underwent saponification and extraction procedures based on Renken and Warthesen (1993): five mL of each sample were placed into a 25 mL glass stoppered round flask wrapped with aluminum-foil. Three mL of 5% KOH and 1.5 mL of 1% pyrogallol in ethanol solutions were added. The samples were flushed with nitrogen, capped and then left to stir slowly in the dark for 12 h at room temperature. Each sample was then poured into a separatory funnel. The round bottomed flask was washed with 2 mL of water, then 0.75 mL of ethanol, and finally, 5 mL of petroleum ether: diethyl ether (90:10 v/v) mixture, adding each wash liquid into the separatory funnel. The mixture was blended and then allowed to phase-separate. The hydrophilic phase was poured into a second separatory funnel, into which, 0.75 mL ethanol and 5 mL of the ether mixture were added. After mixing the phases were allowed to separate. The hydrophobic phase was transferred into the first separatory funnel. A quantity of four and a half mL water was used to wash the hydrophobic phase, 4 times (Renken & Warthesen, 1993). The hydrophobic phase was collected and the solvents were evaporated using nitrogen. The dried sample was re-suspended in 1 mL solution of methanol:water (93:7 v/v). This solvent proportion was suggested by Mattila, Pironen, Uusi-Rauva, and Koivistoinen (1996) as appropriate for vitamin D2 analysis.

Determination of vitamin D2 content: Vitamin D2 content was analyzed using reversed phase HPLC (RP-HPLC). All samples were analyzed for vitamin D2 using a 4.6 × 100 mm C18-C2 Pharmacia RP-HPLC column and a UV detector at 265 nm. The gradient used was 0–75% acetonitrile as eluent B, while methanol:water (93:7 v/v) served as eluent A. A calibration curve was prepared using vitamin D2 standard dissolved in methanol:water (93:7 v/v) at seven concentrations ranging from 5 to 250 µg/mL. Vitamin D2 fractions were collected during RP-HPLC and analyzed for UV absorbance spectrum from 220 to 360 nm for further identity validation, using a Pharmacia Biotech Ultraspec 3000 spectrophotometer.

Size and morphology determination of rCM: For both rCM and D2-rCM, average diameter and size distribution were determined by DLS using a Malvern Zetasizer Nano-ZS. Morphology was determined using cryo-TEM: specimens were prepared in a controlled environment vitrification system (CEVS) at controlled temperature and humidity to avoid loss of volatiles. The samples were allowed to equilibrate in the CEVS for an hour (Bellare, Davis, Scriven, & Talmon, 1988). Then, a 7 µL drop of the examined dispersion was placed on a TEM copper grid covered with a perforated carbon film, and blotted with a filter paper to form a thin liquid film of the sample (100–200 nm thick). The thinned sample was immediately plunged into liquid ethane at its freezing temperature (−183°C) to form a vitrified specimen, and then transferred to liquid nitrogen (−196°C) for storage until examination. Some samples were examined in a Philips CM120 TEM operating at an accelerating voltage of 120 kV, using an Oxford CT3500 cryo-specimen holder.
that maintained the vitrified specimens below −175 °C during sample transfer and observation. Other samples were studied with a Tecnai 12 G2 TEM, at 120 kV, using a 626 Gatan cryo-holder. Images were recorded digitally on a cooled Gatan MultiScan 791 CCD camera or a high-resolution Gatan US1000 CCD camera using the Digital Micrograph 3.1 software, in the low-dose imaging mode to minimize beam exposure and electron-beam radiation damage (Danino, Bernheim-Groswasser, & Talmon, 2001). Brightness and contrast adjustments were done using Photoshop 7.0 ME.

3. Results and discussion

Fig. 1 presents the results of the analysis of vitamin D2 in preparations of re-assembled micelles, enriched with vitamin D2 (D2-rCM), and control rCM preparations without the vitamin. The analyses of both the micelle pellets obtained by centrifugation and their respective serum fractions are presented. In the chromatograms obtained for the control rCM preparation fractions (pellet — Fig. 1A, and serum — Fig. 1B) vitamin D2 peaks were absent, while in both D2-rCM fractions those peaks were observed. UV absorbance spectra of the peaks identified as vitamin D2 indicated good matching between vitamin D2 standard (see Fig. 4) and vitamin D2 eluted at the same position in the sample runs (not shown).

During the analysis, about 85% of total vitamin D2 added were recovered by the extraction procedure from the serum and the pellet together. Table 1 details the results of the analysis of the distribution of the vitamin between the micelles and the serum. Twenty seven percent of the analytically recovered vitamin D2 were found to be incorporated in the micelles. These micelle pellets accounted for 6.5% by weight of the total D2-rCM suspension prepared. The rest of the recovered vitamin was in the supernatant, presumably bound to soluble caseins which were not incorporated into the micelles. Ultra-filtered serum samples showed no traces of vitamin D2, supporting this presumption. Based on these results, we determined that vitamin D2 concentration in the rCM was about 5.5 times greater than its concentration in the serum surrounding these micelles. Milk fortified with such vitamin D2-enriched rCM accounting for only 0.6% of the total milk casein would contain about one third of the vitamin D2 recommended daily allowance (RDA) for adults in a single glass (200 mL) of milk.

Size and morphology determination of rCM: The rCM had average diameters of 147 and 156 nm without and with vitamin D2, respectively, and size distribution in both cases ranged from about 30 to 530 nm. CM of skim milk reconstituted from powder had an average diameter of 181 nm, which is slightly larger, but their size distribution was not much different as seen in Fig. 2. As mentioned above, the normal size range of CM in milk is ~50–500 nm, and the average is ~150 nm.

D2-rCM and rCM had similar morphology, which was also typical to naturally occurring CM, as may be judged from the TEM images presented in Fig. 3. These images suggest that the incorporation of vitamin D2 has a relatively small effect on the morphology of the CM.

Shear stability of rCM and D2-rCM: Following an ultra-high pressure homogenization process the average diameter of rCM was reduced to 122 nm (~26% reduction) and that of D2-rCM was reduced to 125 nm (~27% reduction). The reference micelles from reconstituted skim milk showed a 9% reduction in diameter during the homogenization. While this shows that the reformed micelles are expectedly somewhat weaker than the original micelles, their durability through such extreme shear suggests they could well withstand typical processing shear. The similar extent of reduction in size for rCM and D2-rCM suggests that the incorporation of vitamin D2 into rCM did not weaken their structure as reflected by shear stability.

Quantifying the protective effect of the micelles against UV-light-induced photochemical degradation of vitamin D2: To demonstrate one of the possible protective effects of the encapsulation of the vitamin within the micelles, that is protection against photochemical degradation, we exposed the micelle preparation to UV, and measured the residual
vitamin concentration with exposure time. The results are presented in Table 2.

The data show several interesting observations: first, the comparison of the UV exposed serum (control II) to the serum of control I (unexposed) shows how relatively quickly photochemical degradation of unprotected vitamin D2 occurs. As shown above, the vitamin in the serum is apparently bound to residual soluble casein molecules which did not aggregate into micelles, and provided hardly any protection. The main interesting observation emerges from the comparison of the rate of degradation of the vitamin within the micelles in the exposed preparation, to that of the UV exposed serum (control II). This comparison demonstrates the significant relative protection conferred by the micelles to the encapsulated vitamin. The micelles also confer some protection to vitamin in their surrounding serum, as the rate of degradation in the serum of the exposed micelle dispersion was lower than that in the exposed micelle-free serum (control II). This may be explained by a “shade” effect of the micelles which absorb and block much of the light from influencing their surroundings.

In order to better understand the protection mechanism of the micelles against photochemical-degradation of the
vitamin, the absorbance spectra for both caseinate and vitamin D2 were compared, as shown in Fig. 4. It is seen that at the concentrations each of the components (caseinate and vitamin D2) was present in the rCM suspension, caseinate, being a protein with aromatic side groups and double bonds, absorbs significantly more UV light than does vitamin D2. These data support the conclusion drawn for the protective effect that CM have over vitamin D2 in and around them.

4. Conclusion

CM were shown to serve as potential nano-vehicles for added nutraceuticals such as the fat-soluble vitamin D2 chosen here as a model for hydrophobic bioactive compounds. In terms of encapsulation efficiency, 27% of the vitamin recovered from the micelle suspension was found in the reformed micelles, which contained about 5.5 times higher concentration of the vitamin compared to the surrounding serum. Apparently, the vitamin D2 found in the serum was bound to soluble caseins which did not re-assemble into micelles. This conclusion is based on the result that the protein-free UF-permeate of the serum contained no trace of vitamin D, suggesting high affinity of the vitamin to caseins. We assume that the vitamin adhered to hydrophobic domains of the caseins; however, further experiments are needed to confirm this hypothesis, and to identify binding zones within the micelle structure. The morphology and size of the re-assembled micelles with and without the vitamin were similar to those of naturally occurring CM, in accord with our purpose to minimize modification of micelle properties. It was also shown that in addition to their effectiveness in stabilizing oil-soluble compounds in aqueous environment, the rCM have an additional protective affect against photochemical degradation of the entrapped hydrophobic nutraceutical compound. Other potential protective effects remain to be evaluated.

This study, therefore, demonstrated that CM can be used for nano-encapsulation of hydrophobic nutraceutical substances for potential enrichment of low- or non-fat food products.

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References


