Nanoencapsulation of Hydrophobic Nutraceutical Substances within Casein Micelles

Y. D. Livney*, E. Semo, D. Danino and E. Kesselman
Department of Biotechnology and Food Engineering, The Technion, Israel Institute of Technology, Haifa, Israel. (livney@technion.ac.il)

Introduction

Casein, which accounts for about 80% of milk protein, is organized in micelles. Casein micelles (CM) are designed by nature to stabilize and transport essential nutrients, mainly calcium and protein, for the neonate (DeKruif et al., 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) (Swaisgood, 2003; Fox, 2003; DeKruif et al., 2003). The caseins are held together in the micelle by hydrophobic interactions and by bridging of calcium-phosphate nanoclusters (DeKruif et al., 2003). The structure of CM is important for their biological activity, for their stability in milk and during processing of milk into products, as well as for the good digestibility of the nutrients comprising the micelles. (Swaisgood, 2003; Fox, 2003; DeKruif et al., 2003). However, it has not yet been suggested in the literature, to harness these remarkable natural nanocapsules as vehicles for added nutraceutical substances. Caseinates have been used as microencapsulation wall materials (Hogan et al., 2001). However, caseins forming such artificial capsules have lost 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 products smoothness. CM can be re-assembled in vitro, according to a procedure developed by Knoop et al. (1979). 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. (Eitenmiller et al., 1999). The recommended daily intake of vitamin D is 5 µg per day for adults between ages 21 and 51. Since vitamin D 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 D2 originates from plants, and can also be synthesized readily. It has double bonds sensitive to light, air and high temperature induced oxidation (Eitenmiller et al., 1999; Bell, 2005). The purpose of our research was to explore the possibility to encapsulate nutraceutical substances within CM and to develop a technology to achieve that with minimal changes to the 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 encapsulation process in terms of: (a) effectiveness and efficiency (b) preservation of micelle properties like size and morphology (c) the protective effect conferred by CM to vitamin D2 against UV induced degradation. The main hypothesis of this study was that a hydrophobic nutraceutical compound may be entrapped within CM by association to hydrophobic domains of soluble caseins, followed by reassembly of the casein micelles.

Materials and Methods

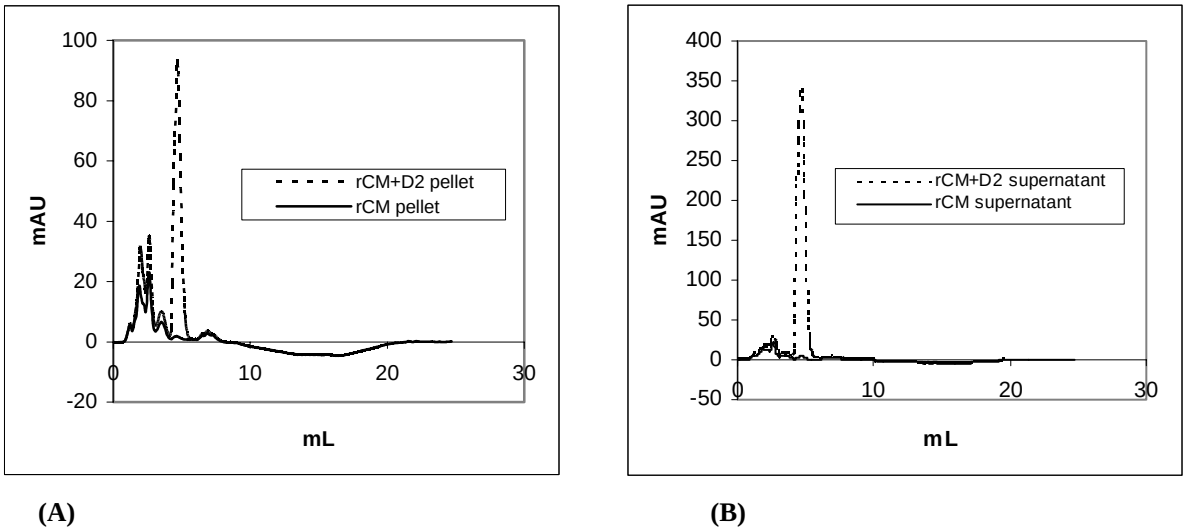
Materials: Sodium caseinate (Miprodan 30, MD Food Ingredients Amba Videbæk, Denmark). Vitamin D2 (Sigma-Aldrich, Rehovot, Israel). Absolute ethanol, conc. HCl, petroleum ether and diethyl ether (Bio-Lab, Jerusalem, Israel). Tripotassium citrate, NaOH, KOH, and pyrogallol (Merck, Darmstadt, Germany). $CaCl_2$ (Carlo Erba, Rodano, Italy). K_2HPO_4 (Spectrum, CA, USA). EDTA (Acros, NJ, USA). HPLC grade methanol and acetonitrile (Lab Scan, Dublin, Ireland).

Methods: Non-covalent binding of vitamin D2 to soluble caseins 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. ***Preparation of re-assembled CM*** (rCM) was

based on Knoop et al. (1979). 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 K_2HPO_4 and 20 mL 0.2 M $CaCl_2$ were added, while stirring at 37 °C. The pH was maintained between 6.7 and 7, using 0.1 N HCl or 1 N NaOH. The volume was adjusted to 400 mL with water, the pH was corrected to 6.7, and the final dispersions were stirred for one hour. Each experiment was performed in duplicate. ***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 $\mu W/cm^2$ intensity for 3, 6, 12 & 24 hours. 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 aluminum-foil covered identical sample (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 at 20 °C and 25,000xg one hour and collecting the supernatant. ***Determination of vitamin D2:*** Ten mL samples were centrifuged as above. The micelle-pellet was 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 hours at 4 °C. Both pellet and serum from each sample underwent saponification and extraction procedures based on Renken et al. (1993). Vitamin D2 content was analyzed using reversed phase HPLC (RP-HPLC) detecting at 265 nm. Vitamin D2 fractions were collected and scanned for absorbance (220-360 nm) for identity validation, using a Pharmacia-Ultrospec-3000 spectrophotometer. ***Size and morphology determination of rCM:*** For both rCM and D2-rCM, average size was measured by dynamic light scattering (DLS) (BIC 90Plus, Brookhaven Instruments). Morphology was studied using cryogenic-transmission electron microscopy (cryo-TEM). Specimens were prepared in a controlled environment vitrification system, at 25ºC and 100% R.H. (Bellare et al., 1988). Samples were examined in a Tecnai 12 G2 TEM, at 120 kV, using a cooled high-resolution Gatan US1000 CCD camera, under low-dose conditions (Danino et al., 2001).

Results and Discussion

Encapsulation efficiency: The results of the analysis of vitamin D2 in preparations of rCM, enriched with vitamin D2 (D2-rCM), and in control rCM preparations without the vitamin are presented in Figure 1. The analyses of both the micelle pellets obtained by centrifugation and their respective serum fractions are presented.



(A) **(B)**

Figure 1. RP-HPLC chromatograms of vitamin D2 in (A) pellets of rCM and of D2-rCM, and in (B) supernatants of rCM and of D2-rCM.

In the control rCM fractions (pellet-Fig1A, and serum-Fig 1B) vitamin D2 peaks were absent, while in both D2-rCM fractions those peaks were observed. The identity of the vitamin in these fractions was confirmed by matching of scanned UV absorbance spectra (not shown).

	% of the total weight of the sample	Vitamin D2 concentration (µg/mL)	% of the total amount of Vitamin D2 recovered by the analysis (85% recovery)
Micelles (pellet)	6.5% (±0.3)	43.8 (±0.7)	27.5% (±3.7)
Serum (supernatant)	93.5% (±0.3)	8.2 (±2.1)	72.5% (±3.7)

Table 1. Analysis of vitamin D2 distribution between the micelles and the serum.

About 85% of total vitamin D2 added were recovered by the analytical procedure (serum and pellet). Table 1 details the results of the vitamin distribution between the micelles and the serum. 27% of the analytically-recovered vitamin D2 were found to be incorporated in the micelles. These micelle pellets accounted for 6.5%w/w of the total D2-rCM suspension prepared. Based on these results, we determined that vitamin D2 concentration in the rCM was about 5.5 times greater than in the serum surrounding these micelles. Therefore, 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 (200mL) of milk. ***Size and morphology determination of rCM:*** The rCM had average diameters of 146 and 152 nm without and with vitamin D2 respectively (The average size of CM in milk is ~150 nm). rCM and D2-rCM had similar morphology, which was also typical to naturally occurring CM, as may be judged from the TEM images presented in Figure 2.

Exposure Time (Hrs)	UV exposed micelle suspension		Unexposed micelle suspension (control I)		UV exposed serum (control II)
	Micelle pellet	Serum	Micelle pellet	Serum	
0	100	100	100	100	100
3	20.9	7.2	107.8	43.1	0.11
6	2.7	1.7	41.0	74.9	UD
12	2.7	0.5	78.5	77.1	UD
24	1.8	0.9	135.1	62.0	UD

Table 2.
Vitamin D2 degradation, (Percent of the initial concentration remaining in each fraction with exposure time), in D2-rCM suspension exposed to UV light, D2-rCM suspension unexposed to UV light (control I) and in D2-rCM suspension serum exposed to UV light (control II). (UD=undetectable).

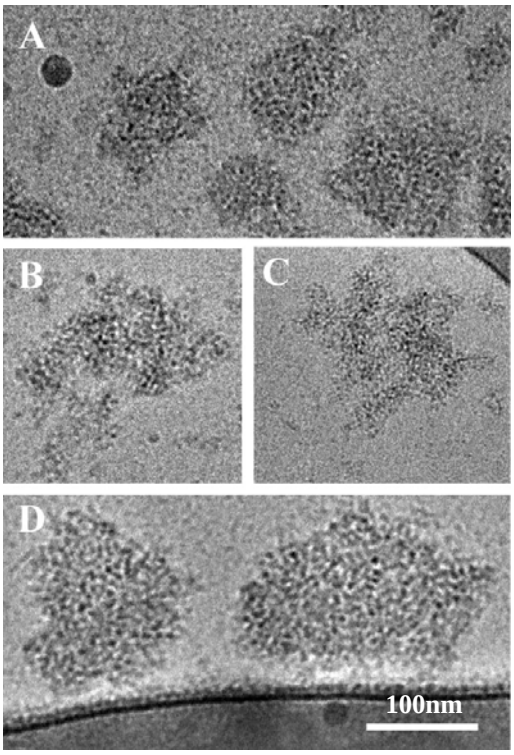


Figure 2. Cryo-TEM images of (A) naturally occurring CM in skim milk; (B) and (C) rCM; (D) D2-rCM .

Protective effect of the micelles against UV induced degradation of vitamin D2. We exposed the micelle preparation to UV, and measured the residual vitamin concentration with exposure time. The results are presented in Table 2. Comparison of control II and control I shows how relatively quickly photochemical degradation of unprotected vitamin D2 occurs. The vitamin in the serum is presumably bound to residual soluble casein molecules which did not aggregate into micelles, and provided hardly any protection. The main interesting observation emerges from comparing degradation rates of the vitamin within the CM in the UV exposed preparation, and that of control II. It demonstrates the significant protection conferred by the micelles to the encapsulated vitamin.

Conclusions

Casein micelles were shown for the first time to serve as potential nano-vehicles for added hydrophobic nutraceuticals such as the studied model-vitamin D2. The vitamin concentration was about 5.5 times higher in the reformed micelles compared to the surrounding serum, showing the good affinity of the vitamin to the CM. We assume that the vitamin adhered to hydrophobic domains of the caseins, however, this remains to be confirmed. The micelles morphology and size were very similar to those of naturally occurring CM. 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. This study, therefore, demonstrated that casein micelles can be used for nanoencapsulation of hydrophobic nutraceutical substances for enrichment of low- or non-fat foods.

Acknowledgements

This research was funded by the German-Israel Foundation, grant number I-2079-1309.5/2003.
We thank Prof. Douglas G. Dalgleish, Food Science Dept., University of Guelph, Guelph, Ontario, Canada, for helpful discussions.

References

Bell, A. B. (2005). The chemistry of the vitamins D. In D. E. M. Lawson, *Vitamin D.* (pp. 1-41). London: Academic Press.

Bellare, J. R. et al. (1988). *Controlled Environment Vitrification System - An Improved Sample Preparation Technique.* Journal of Electron Microscopy Technique, 10(1), 87-111.

Danino, D. et al. (2001). *Digital cryogenic transmission electron microscopy: an advanced tool for direct imaging of complex fluids.* Colloids and Surfaces A-Physicochemical and Engineering Aspects, 183113-122.

DeKruif, C. G. et al. (2003). Casein micelle structure, functions and interactions. In P. F. Fox and P. L. H. McSweeney, *Advanced Dairy Chemistry-1 Proteins Part A.* (pp. 233-276). New York: Kluwer Academic/ Plenum Publishers.

Eitenmiller, R. R. et al. (1999). Vitamin D. In , *Vitamin analysis for the health and food science.* (pp. 77-82). Boca Raton: CRC Press.

Fox, P. F. (2003). Milk proteins: general and historical aspects. In P. F. Fox and P. L. H. McSweeney, *Advanced Dairy Chemistry-1 Proteins Part A.* (pp. 1-48). New York: Kluwer Ackademic/ Plenum Publishers.

Hogan, S. A. et al. (2001). *Microencapsulating Properties of Sodium Caseinate.* Journal of Agricultural and Food Chemistry, 49(4), 1934-1938.

Knoop, A. M. et al. (1979). *Sub-structure of synthetic casein micelles.* Journal of Dairy Research, 46(2, Proc. IDF Symp. Phys. Chem. Milk Proteins), 347-350.

Renken, S. A. et al. (1993). *Vitamin-D Stability in Milk.* Journal of Food Science, 58(3), 552-&.

Swaisgood, H. E. (2003). Chemistry of the caseins. In P. F. Fox and P. L. H. McSweeney, *Advanced Dairy Chemistry-1 Proteins Part A.* (pp. 139-202). New York: Kluwer Academic/ Plenum Publishers.