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Re-assembled casein micelles improve *in vitro* bioavailability of vitamin D in a Caco-2 cell model

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The pandemic of vitamin D (VD) deficiency, and the global rise in obesity stimulate a need for staple low-fat foods and beverages enriched with VD. In light of consumer demand for a clean label, the use of natural endogenous food ingredients as delivery vehicles is of great interest. To this end, reassembled casein micelles (rCM) have been shown to help retain VD during processing and shelf life as well as provide high bioavailability in low-fat milk and non-fat yoghurt. This follow up study focused on the performance of VD-loaded rCM after drying and reconstitution, considering VD retention during simulated digestion, and the subsequent *in-vitro* bioavailability of the vitamin. RCM conferred great protection to VD₃ during simulated digestion with significant ($p < 0.05$) increase in vitamin retention for 1 h under gastric conditions. This observation is believed to arise from the vitamin-casein binding and the system's natural gelation (curd formation) near the casein isoelectric point that seclude the vitamin from environmental stressors and couple its release with digestive proteolysis of the rCM matrix. Vitamin absorption by Caco-2 cells from digested rCM was not significantly different from absorption of digested free VD. However, thanks to the highly protective effect of the rCM, against VD gastric degradation, the overall effect of the rCM was a 4-fold higher bioavailability, compared to the free VD.

1. Introduction

Increasing evidence support the linkage between diet and human health¹. This has led to numerous attempts to develop functional foods that carry added nutritional value. Such attempts include the enrichment and fortification of food with bioactive ingredients such as vitamins, polyphenols, essential fatty acids and carotenoids². However, the incorporation of bioactive ingredients in food and drink systems poses several challenges. First, the enrichment of aqueous food or drink systems harbours a challenge of dosing accuracy and uniformity to avoid overdose, mainly in the case of oil-soluble bioactives, due to their poor solubility. Moreover, the incorporation of a bioactive ingredient within the food or drink system may result in its degradation and loss of activity during processing, product shelf life and digestion. Bioactive ingredients may cause unwanted effects on the sensory attributes of the enriched food system, and they can also react with other components present in the enriched system, which may decrease (or increase) their bioavailability¹⁻³. To overcome these challenges, various bioactive delivery systems are being developed². Delivery systems are commonly classified according to the main building blocks, which are necessarily GRAS (generally recognized as safe) food materials, preferably natural, and ideally- endogenous components of the product, thereby shortening the ingredient list and minimizing changes to the product sensory attributes, for higher consumer satisfaction. There are four main groups of delivery systems in food: protein-based, carbohydrate-based, lipid-based, and mixed systems². Due to the negative health implications of high fat consumption^{4, 5}, there is a need for enriching low-fat and non-fat food and drinks with health-promoting oil-soluble bioactives, preferably using non lipid, amphiphilic delivery systems.

Proteins are natural and diverse class of macronutrients, with various origins and techno-functionalities. Therefore, several studies have examined the development and use of protein-based delivery systems for lipophilic nutraceuticals⁶⁻¹³. Pertinent to vitamin D (VD), soybean β -conglycinin¹⁰, corn protein hydrolysate¹¹, hydrophobins⁹, potato-protein¹⁴ and milk proteins^{8, 12} have all shown good performances for the delivery of VD. Out of these, milk proteins seem particularly effective tools to deliver vitamins,

minerals and lipophilic compounds, due to their evolutionary role in milk, which provides total nutrition to new-borns¹⁵.

The recommended dietary allowance (RDA) of VD is 600 international units (IU)/day for children over 12 months of age, adolescents and adults according to Institute of medicine report¹⁶. The RDA for elderly people above 70 years is 800 IU/day¹⁶. Nowadays, the actual daily consumption of VD is low and does not exceed 400 international units (IU), in most populations^{17, 18}. Modern lifestyle and minimization of exposure to sun light render natural production of VD in the skin (by UVB radiation) to be generally insufficient, even in sunny countries¹⁹. VD is not only biologically important but also associated with prevention of various diseases^{20, 21}, therefore, its supplementation to foods is a sensible public health measure.

This study focuses on the potential use of caseins, and particularly of re-assembled casein micelles (rCM), as delivery vehicles for VD and extend a few recent studies. First, rCM were shown to be suitable for the enrichment of reduced-fat products with VD and other oil-soluble nutraceuticals^{8, 22, 23}. RCM were found to protect VD₃ during UV exposure²², thermal treatment (80°C, 1 min) and during 28 days of cold storage⁸. Then, a clinical human study concluded that the bioavailability of VD encapsulated in rCM within 1% fat milk was at least as good as that of VD in a commercial dietary supplement aqueous formulation in which VD₃ was emulsified using Tween-80⁸. In another clinical trial, the enrichment of fat-free yoghurt with VD₃ entrapped in rCM showed similarly high bioavailability as when using the synthetic surfactant Tween-80²⁴, though significantly better sensory properties were obtained with rCM. Another recent clinical study found that the bioavailability of VD in rCM in non-fat yoghurt was not significantly different from its bioavailability within the fat (in a 3% fat yoghurt), or in its presence (VD in rCM, in 3% fat yoghurt)²⁵.

The objectives of this follow up study were to evaluate the performance of the VD-loaded rCM after drying and reconstitution, and to gain deeper understanding of their behaviour during simulated digestion and of the consequent *in-vitro* bioavailability of the VD encapsulated in rCM.

2. Materials and Methods

2.1 Materials

Commercial sodium caseinate (Casinella-QN 94.2% protein Molkerei Meggle Wasserburg GMBH & Co. KG) was kindly donated by Kelta Ltd. Israel and Molkerei Meggle Wasserburg GMBH & Co. KG, Germany. VD_3 in powder (USP grade), potassium phosphate dibasic (USP), potassium citrate tribasic monohydrate (FCC) and calcium chloride dihydrate (FCC), pepsin (Sigma P7000, ≥ 250 units/mg protein), Trypsin (Sigma T0303, 13,000-20,000 BAEE units/mg protein), chymotrypsin (Sigma C4129, ≥ 40 units/mg protein), pancreatic lipase (Sigma L3126, 100-500 units/mg protein), sodium glycodeoxycholate (Sigma G9910) and taurocholic acid salt hydrate (Sigma T4009) were purchased from Sigma-Aldrich (Rehovot, Israel). Sodium chloride and Sodium bicarbonate were purchased from Frutarom Industries Ltd. (Herzliya, Israel) and Hydrochloric acid was purchased from Gadot group (Netanya, Israel). Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, penicillin/streptomycin, trypsin-EDTA (500 mg/L and 200 mg/L, respectively) and PBS were purchased from Life Technologies (Saint Aubin, France). Fetal bovine serum was obtained from PAA (Vélizy Villacourblay, France).

2.2 Methods

2.2.1. VD_3 -casein binding characterization, VD_3 -loaded rCM preparation, rCM freeze-drying and reconstitution

Casein solution and VD_3 loading: A solution of 6.11 mg/ml sodium caseinate was prepared with double distilled water and stirred overnight at room temperature for complete hydration prior to vitamin loading. Samples were prepared by adding ethanolic VD_3 solution (8 mg/ml) into a vial containing either double distilled water or casein solution while vortexing, followed by gently stirring overnight at 4°C. Final VD_3 and caseinate concentrations in the samples were 260 μ M each. A control of casein solution at

the same concentration without VD₃ was prepared by the same procedure except that an equivalent amount of pure ethanol was added to the pre-dissolved sodium caseinate. Final ethanol concentration in the samples was 1.2%.

Light microscopy: Light microscopy images of the pure VD₃ dispersions in water (containing 1.2% ethanol) were compared to those of VD₃: casein 1:1 molar ratio solutions. Images were taken at ×40 magnification using Olympus BX51 light microscope, operated in bright-field optical mode.

Binding studies by UV absorbance spectra measurements: The binding studies were performed according to the methods described by Israeli-Lev et al.⁹. UV-vis spectra were measured by UV spectrophotometer (Ultrospec 3000, GE Healthcare). All samples were diluted 10-fold before measurements. Measurements were made in duplicate at 23°C.

Preparation of VD₃ loaded rCM: VD₃ loaded rCM were prepared according to the protocol described by Haham et al.⁸ Briefly, to the solution of sodium caseinate loaded with VD₃, described above, K₂HPO₄ and tri potassium citrate solutions were added, followed by drop wise addition of CaCl₂ while stirring. Then, the samples were homogenized using a Micro-DeBee (Bee Int'l Inc., South Easton, MA, USA) ultra-high pressure homogenizer.

Freeze drying and reconstitution: 5% [wt/wt] Maltodextrin (MD, DE-18) was added to the rCM dispersion, as a cryoprotectant/drying aid, followed by stirring for 30 min at room temperature. Suspensions were then quench-frozen in liquid nitrogen and freeze-dried. Reconstitution was achieved by adding distilled water and stirring overnight at 4°C. Particle size distribution of the rCM before and after freeze-drying and reconstitution were determined by dynamic-light-scattering (DLS) using NICOMP™ 380, Particle size/zeta potential analyser (PSS, Santa Barbara, CA, USA). Measurements were made at 23°C. Particle size distributions were analysed by the NICOMP™ volume-weighted distribution analysis. VD₃ concentration before and after freeze-drying and reconstitution was evaluated by reversed phase (RP) HPLC.

2.2.2. Evaluation of VD₃ bioaccessibility using an *in vitro* simulated digestion model

Simulated digestion process: To assess casein digestibility and VD₃ retention a semi-dynamic *in vitro* digestion model was applied and comprised of two steps: first a 1h semi-dynamic gastric digestion followed by a two hour intestinal digestion step. *In vitro* digestion was based on a combination of the method described by C. Shani-Levi et al.²⁶ and D. Dupont et al.²⁷ with several modifications, with the most appreciable change done in the gastric pH profile adjusted to meet that of P. Marteau et al.²⁸ who worked on digestion of yoghurt. Simulated gastric fluid (SGF) was freshly prepared by dissolving 2 gr NaCl in 1000ml of double distilled water. Using 2M HCl, the pH was adjusted to 1.2 for pepsin dissolution, or to 5.0 for the initiation of the *in vitro* digestion model. In practice, the fresh VD₃-loaded rCM were mixed with SGF (mixing ratio of VD₃-loaded rCM :SGF 1:1 v/v) and were transferred to 100ml water jacketed reactor (6.1418.250, Metrohm, Switzerland), maintained at 37°C with continuous blending (~250rpm) to mimic the stomach conditions. The initial pH was adjusted to 5, and gastric pepsin (170 U/mg casein, pre-dissolved in SGF pH=1.2) was added to mimic the initial conditions in the stomach. The pH was gradually titrated to 1.8 during 1 hour by a dual auto titration unit (Titrand 902, Metrohm, Switzerland) using 0.5M HCl. The simulated gastric step consisted of five stages; pH was maintained as 5 for 7.5 min, then at pH 4.1, for additional 7.5 min, followed by 7.5 min at pH 3, 7.5 min at pH 2.1, and a final gastric step of 30 min at pH 1.8. The gradual titration was achieved using an auto-titration unit controlled by a dedicated software (Tiamo 2.3 software, Metrohm GA, Switzerland). Samples were then taken after 10, 30 and 60 min. Each sample was quench-frozen using liquid nitrogen to stop the enzyme activity. After 1 hour of simulated gastric step, the digesta underwent 2 hours of an upper intestinal step. Simulated duodenal fluid (SDF) solution was freshly prepared by dissolving 7.0 gr NaCl in 800ml distilled water and the pH was adjusted to 6.5 using 1 M NaOH. Trypsin (34.5 U/mg casein), chymotrypsin (0.4 U/mg casein), pancreatic lipase (58 U/ml), sodium glycodeoxycholate (4mM) and taurocholic acid salt hydrate (4mM) were pre-dissolved in SDF and were added to the digesta to mimic the intestinal conditions. The digesta pH was increased to 6.25 by titration with 1 M sodium bicarbonate solution and maintained constant for 2 hours.

Samples were then taken after 3, 10, 30 and 60 min. Each sample was quench-frozen using liquid nitrogen to stop enzymatic activity.

Protein degradation and VD₃ retention during simulated gastrointestinal digestion: The degradation of casein as a function of simulated digestion time was studied using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The bands of the protein were stained by coomassie brilliant blue. VD₃ retention as function of digestion time was assessed following the protocol of Kazmi et al.²⁹. Briefly, 0.5ml of each sample was transferred into a test tube. A 1.6 ml of mixture of methanol and chloroform (2:1 v/v) was added to each test tube, followed by mixing for 20 seconds. 1 ml of chloroform was then added to each tube, and mixed for 1 min. Samples were then centrifuged for 10 min at 1,500 g at 4°C. From each test tube, 1.2ml of the clear chloroform bottom layer was transferred to an evaporation vial using a glass syringe. The extract was dried from chloroform under a flow of nitrogen gas, reconstituted in 0.75 ml of the reversed phase HPLC (RP-HPLC) mobile phase [methanol: acetonitrile: water (49.5:49.5:1 v:v:v)], and equilibrated for 15 min. VD₃ was quantified by RP-HPLC using HP 1100 system with 4.6 × 250 mm Vydac 201TP C18 column, a diode array detector and an auto-sampler. Twenty microliter samples were run under isocratic flow of 1.3 ml/min. The area under the curve at 267nm (maximal VD absorbance wavelength) was calculated for VD₃ quantification. The ratio between the area under the curve at 228nm and 254 nm was calculated for VD₃ identification.

2.2.3. VD₃ *in-vitro* bioavailability analysis: uptake by Caco-2 cells

VD₃ uptake experiments: Caco-2 clone TC7 cells were cultured as previously described^{30,31}. Cells were seeded and grown in trans-wells for 21 days to obtain confluent and highly differentiated cell monolayers^{30, 31}. Twelve hours prior to each experiment, the media in apical and basolateral chambers were replaced by serum-free complete medium. At the beginning of the experiment, cell monolayers were washed with 0.5 mL PBS. The apical side of the cell monolayers received either the digested VD₃-loaded rCM or the digested free VD₃ diluted in DMEM (1/8), and the basolateral side received serum-free complete medium. After 1 hour of cell exposure with the samples, cells were washed with 0.5 mL ice-cold

PBS, then scraped and collected in 0.5 mL PBS. Absorbed VD₃ was estimated as VD₃ present in harvested cells or transported to the basolateral side. All the samples were sealed under nitrogen and stored at -80°C until analysed.

VD₃ extraction: VD₃ was extracted from 500 µL aqueous samples using the following method. Distilled water was added to sample volumes below 500 µL to reach a final volume of 500 µL. Ergocalciferol (VD₂), routinely used as internal standard, was added to the samples in 500 µL ethanol. The mixture was extracted once with two volumes of hexane. The hexane phase obtained after centrifugation (500 *g*, 10 min, 4°C) was evaporated to dryness under nitrogen, and the dried residue was dissolved in 200 µL of mobile phase (60% acetonitrile – 38% methanol – 2% water). A volume of 180 µL was used for HPLC analysis.

VD₃ analysis by HPLC: The HPLC system comprised a Shimadzu separation module (LC-20ADSP HPLC Pumps and SIL-20CHT autosampler, Shimadzu, Champs-sur-Marne, France), an SPD-M20A Shimadzu photodiode array detector (PDA, detection at 265 nm, spectral analysis between 190 and 300 nm). VD₃ and VD₂ were separated using a 250×4.6 nm RP C₁₈, 5 µm Zorbax column (Interchim, Montluçon, France) and a guard column, as previously described³². No VD₃ was detected in basal conditions. Quantification was performed using Chromeleon software (version 6.50 SP4 Build 1000, Dionex) comparing peak area with standard reference curves. All solvents used were HPLC grade from SDS (Peypin, France).

3. Results and discussion

Extending previous studies on rCM^{8, 24, 25, 33}, this study sought better understanding of the techno-functionality and digestive fate of this delivery system and particularly its implications to VD retention and bioavailability.

3.1 Light microscopy

Light microscopy images of pure VD₃ in water and VD₃ of the same concentration in the presence of casein (1:1 molar ratio), are presented in **Figure 1**. VD₃ concentration in the examined samples was 0.1 mg/ml (260 μM). The casein: VD₃ molar ratio was chosen to be similar to the molar ratio we use in rCM. At this concentration and after an overnight storage at 4°C, it can be seen from **Figure 1a** that VD₃ forms micron-sized aggregates. However, in the presence of casein, a clear solution is obtained without any visible particles under the light microscope (**Figure 1b**), indicating submicron co-assembly of the VD₃ and the casein. From this comparison, we can qualitatively conclude that caseins bind VD₃.

3.2 Binding studies by UV absorbance spectra measurements

The UV-absorbance spectrum of casein-only, VD₃ -only, and casein-VD₃ solutions were measured to qualitatively examine whether binding had occurred. The difference between the mathematical summation of UV-absorbance spectrum of the VD₃-only solution and that of the casein-only solution, and the UV-absorbance spectrum of their solution together is an indication for binding⁹. Observing **Figure 2**, it can be seen that the mathematical summation curve is lower than that of the mixed casein-VD₃ solution, indicating association between casein and VD₃. The results indicate that casein and VD₃ associate at this molar ratio (1:1), an assumption supported by the light microscopy images (**Figure 1**).

3.3 Freeze drying and reconstitution

Freeze-drying is widely used for improving chemical and physical stability and extending shelf-life of colloidal systems³⁴. During the freezing step there is phase separation into ice and freeze-concentrated solution. The high nanoparticle concentration in this phase may induce irreversible aggregation³⁴. Moreover, adsorption to ice may lead to protein denaturation³⁵. To minimize the harmful effect of the freeze-drying process, cryoprotectants are often added. Saccharides are the most popular cryoprotectants in food, as they protect proteins from denaturation, and promote matrix immobilization by raising the glass transition temperature, thereby preventing nanoparticle aggregation^{34, 36}. After their freeze-drying in the presence of MD, rCM were reconstituted by addition of water and stirring overnight. We have observed their reconstitution visually and by size distribution measurements by DLS, and the results are presented in **Figure 3**. The volume-weighted size distributions shows that rCM samples before

and after freeze-drying and reconstitution exhibited bimodal distributions (**Figure 3**). The first subpopulation was comprised of nanoparticles smaller than 100nm and the second was in the range of 100-350 nm. The volume-weighted-size distribution of the rCM before freeze-drying was in line with the previous work performed by our group⁸. It can be seen in **Figure 3** that freeze-drying and reconstitution slightly increased the volume weighted percentage of the larger subpopulation, while that of the smaller one decreased accordingly, but shifted to lower sizes. That resulted in a minor visual difference between the samples; it can be seen in the inset of **Figure 3** that the reconstituted sample (2) is slightly more turbid than the fresh sample (1). However, the process did not significantly affect the average size and it was even slightly lower after reconstitution (average size of 95 ± 2 nm and 89 ± 0.3 nm before and after freeze-drying and reconstitution respectively). About 90% of the VD was retained during the freeze-drying and reconstitution process (concentration of 167 ± 8 $\mu\text{g/ml}$ and 151 ± 0.3 $\mu\text{g/ml}$ before and after freeze-drying and reconstitution, respectively). These results indicate that freeze-drying aided by MD, and reconstitution, had only a minor influence on the rCM size distribution, the visual appearance and the concentration of VD.

3.4 Simulated gastric and intestinal digestion

In vitro digestion models are often used to screen and interrogate food delivery systems³⁷. Various simulated digestion models exist, mimicking the complex physicochemical and physiological conditions in the human gastrointestinal tract³⁷. Varying environmental conditions such as pH, ionic strength and enzyme activity induce structural and chemical changes in the delivery system which may trigger bioactive release³⁷ but might also cause its degradation. To assess casein digestibility and the retention of VD₃ remaining in the lumen of the human gastrointestinal tract, samples of VD₃ loaded rCM and free VD were challenged by a semi-dynamic human digestion model. Results of direct observations, assessment of digestive proteolysis and determination of VD retention are presented in **Figures 4-6**. As can be seen in **Figure 4**, under gastric conditions, VD₃ loaded rCM formed large curd clots, due to the proximity to the isoelectric point of casein ($\text{pH}=4.6^{15}$) and the activity of pepsin³⁸. The aggregate size significantly decreased in one hour of simulated gastric digestion, as can be seen in **Figure 4**. Pepsin

activity along with the gradual pH decrease below the isoelectric point, expectedly increased the solubility of the casein as well as its susceptibility to proteolysis. The observed casein clot degradation during the gastric phase was in-line with our SDS-PAGE results shown in **Figure 5**.

In lane 1 it is possible to see the initial protein composition in the VD₃ loaded rCM suspension. The four casein fractions, α_{s1} -, α_{s2} -, β - and κ - caseins were observed in molecular weight range of 19-25 KDa as naturally present in bovine milk¹⁵. The other bands in lane 1, whose sizes were lower than 19 KDa, were probably residual whey proteins such as β -lactoglobulin and α -lactalbumin (18.3 KDa and 14.2 KDa respectively¹⁵) from the sodium caseinate. A few weaker higher-MW bands above 40kDa are also seen, which may be serum albumin, lactoferrin and immunoglobulins, or some casein aggregates which weren't completely dissociated during sample preparation (less likely).

Lanes 2-4 represent the VD₃ loaded rCM under simulated gastric conditions after 10, 30 and 60 min respectively. In these lanes it is possible to witness the degradation of the caseins, as the bands representing the four casein fractions diminished and smaller peptides (2-15 KDa) appeared. The bands whose sizes were between 25 and 37 KDa, were apparently pepsin, but possibly also degradation products of the higher MW bands seen in lane 1. By the end of the simulated gastric phase, the large clot was enzymatically digested and only casein peptides remained (**Figure 4 and Figure 5**- lane 4). Our results regarding the gastric breakdown of the caseins are in accord with the literature^{15, 38, 39}. After 60 min of simulated gastric digestion, the digesta pH was raised to 6.25, and trypsin, chymotrypsin, lipase and bile salts were added to mimic duodenal conditions. Lanes 6-10 (**Figure 5**) represent 10, 30, 60, 90 and 120 min of simulated upper intestinal digestion. In these lanes it is possible to see bands in the size range of approximately 20-25 KDa which represent the intestinal chymotrypsin, trypsin and lipase that were added in this stage. The addition of duodenal enzymes expectedly caused further casein digestion. Several bands of casein peptides were observed in lane 6 representing a sample taken after 10 min of simulated duodenal digestion. These bands were not observed in samples taken after 30 min or more of simulated duodenal conditions (lanes 7-10). These SDS-PAGE results are in-line with the visual observations, as the digesta became more transparent with proceeding simulated digestion (**Figure 4**). In

the absence of protein no significant changes were observed over 60 min of simulated gastric digestion, as the vitamin remained dispersed in water and the system remained turbid (**Figure 4**). During the duodenal phase the system was clarified, most likely due to the addition of bile salts, natural surfactants known to solubilize lipid-soluble compounds and breakdown products, and to form mixed micelles⁴⁰. To study VD₃ retention during simulated gastric and duodenal digestion, extraction of VD₃ from the aqueous phase was performed, followed by its quantitative determination by RP-HPLC analysis. Results are presented in **Figure 6**. It can be seen from **Figure 6** that the presence of protein improved VD₃ stability during simulated gastric and intestinal digestion (retention of ~85% vs. ~15% at the end of simulated digestion process in presence and absence of protein respectively). VD₃ is sensitive to the presence of acid conditions and high temperatures, however, VD₃ has better stability under neutral and alkaline conditions⁴¹. As mentioned above, the initial pH of the simulated gastric phase was 5, which was gradually decreased to 1.8 during one hour. Expectedly, in the absence of protein only 37% of the vitamin survived under these acidic conditions. At the beginning of the simulated duodenal phase the pH was increased to 6.25 and maintained constant for two hours. Under these conditions VD₃ was less susceptible to degradation, and only additional 13% of the free vitamin were degraded in this phase, resulting in 15% of the initial VD₃ remaining after both simulated digestion stages. During simulated digestion of VD₃ loaded rCM, after 10 min of the gastric phase, there was an apparent decrease of the VD₃ residual amount to 40%, however, the apparent residual amount was then increased to 73% and 90% after 30 and 60 min of the gastric phase, respectively. Moreover, the measurement results of these samples were characterized by large error bars. As discussed above, at the beginning of the simulated gastric phase large casein aggregates formed, as seen in **Figure 4**. It is likely that most of the vitamin was entrapped in these aggregates, thus the samples taken from the bulk during the gastric phase contained less VD₃ and do not accurately represent the overall VD₃ retention status at this stage. The residual amount at the beginning of the duodenal phase, after breakdown and dissolution of the large aggregates, was 90% and it remained relatively constant during this phase. It can be seen in **Figure 4** that during the duodenal phase, the casein aggregates were digested and the digesta was clarified, making the sample

more homogenous. Thus, it is plausible that the VD_3 was no longer entrapped in casein aggregates but was extracted into mixed micelles and was more uniformly dispersed in the digesta. Therefore, the samples taken at this point better represent the actual VD_3 retention status. Hence, the high values of remaining VD_3 during the duodenal phase, demonstrate the protection conferred to the vitamin by the rCM mainly under simulated gastric digestion conditions. The use of protein nanovehicles to protect VD_3 during simulated digestion was previously examined. Levinson et al.¹⁰ found that 92% of the initial VD_3 concentration survived a two hours simulated gastric step digestion as the VD_3 was complexed within soybean β -conglycinin nanoparticles (relative to 50% retention for VD_3 in phosphate buffer pH 6.8). Lin et al.¹¹ found that nanocomplexes of corn protein hydrolysate (CPH) with VD_3 with CPH: VD_3 mass ratio of 15:1 and higher conferred protection to the vitamin during simulated digestion (1 hour simulated gastric step followed by 2 hours upper intestinal step), as the remaining vitamin concentration was approximately 95% of its initial concentration¹¹. Markman et al. also observed protection of VD by casein and casein-MD Maillard conjugates at pH 2.5⁴².

3.5 VD_3 in-vitro bioavailability analysis by Caco-2 cells

VD_3 *in vitro* bioavailability within rCM has been examined using Caco-2 cells. The results are presented in **Figure 7**. Caco-2 cells were exposed to VD_3 -loaded rCM and to free VD_3 samples taken after simulated digestion including one hour of a gastric step and two hours of upper-intestinal step. Cell exposure time with the digested samples was one hour. This exposure duration was chosen in view of the approximate exposure time of the digesta with a given intestinal epithelial cell, expected to occur in the small intestine considering atypical transit rate⁴³, and stomach content/chyme volume⁴⁴ (e.g. assuming a typical chyme volume of 1000 ml and intestinal flow rate of 15ml/min, exposure time is ~66 min). The uptake of VD_3 in both formulations is presented in **Figure 7A**. The VD_3 uptake rates were low for both formulations (approximately 10% and 15% uptake of VD for digested VD_3 -loaded rCM and for digested free VD_3 , respectively). The absorption rate for the digested free VD_3 was slightly, though not significantly higher, compared to that of digested VD_3 -loaded rCM. Taken together with the much higher retention, due to the protective effect of the rCM and the curd-aggregates they form mainly under the acidic gastric

conditions, the overall effect of the rCM was that a four-fold higher proportion of the ingested vitamin was bioavailable *in vitro*, compared to the unencapsulated VD (**Figure 7B**). These results regarding the high overall effect of rCM are in-line with the high oral bioavailability of VD observed in our preceding clinical studies. It is likely that VD was protected against the harmful acidic environment *in vivo* by its entrapment within the large protein clots formed at the beginning of the gastric step of digestion (as observed herein), and this can partly explain the high *in vivo* oral bioavailability obtained.

Conclusions

We studied the performance of VD-loaded rCM after drying and reconstitution, considering VD retention during simulated digestion, and the subsequent *in-vitro* bioavailability of the vitamin. Our results provided qualitative evidences for binding of VD₃ and caseins, using light microscopy and UV absorbance spectra measurements. Such VD₃-rCM co-assemblies aided by MD were well reconstituted after freeze-drying. The freeze-drying and reconstitution had marginal influence on the size distribution of the VD₃-loaded rCM, on their visual appearance and on the VD₃ concentration. Encapsulated VD₃ was also found to be significantly protected during simulated gastric and upper-intestinal conditions, compared with VD₃ dispersed in water. The VD₃ *in-vitro* bioavailability examined using Caco-2 cells indicated low uptake rates for both formulations, apparently due to limitations of this colon cell-based model (as our clinical studies^{8, 25, 45} showed high bioavailability). However, taken together with the much higher retention of the encapsulated VD₃ during digestion, the overall observed effect of rCM was an improved oral bioavailability of VD₃ compared to the unencapsulated VD₃. Thus, this work provides yet another support for the great potential of rCM to ensure efficient and more sustainable delivery of sensitive hydrophobic bioactive compounds in food.

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Figure captions:

Figure 1: VD₃ and casein form submicron co-assemblies. Light microscope images of (a) pure VD in water (containing 1.2% ethanol), (b) VD: casein 1:1 molar ratio in water (containing 1.2% ethanol). VD concentration was equal in both samples (0.1 mg/ml). Scale bar: 50 μm.

Figure 2: UV absorbance spectra measurements indicating casein-VD₃ binding. The UV-vis absorbance spectra of pure VD₃ in water (black, dashed), of casein solution in water (grey, dashed), of mixed VD₃ and casein (black, solid) and of the mathematical summation of the absorbance spectra of VD₃ alone and casein alone (grey, solid). [VD₃]=26 μM, [casein]=26 μM.

Figure 3: Freeze-drying aided by maltodextrin has only a minor influence on the size distribution and visual appearance of rCM suspensions. Volume-weighted size distributions of VD₃ loaded casein micelles before (solid line) and after (dashed line) freeze-drying and reconstitution with distilled water, determined by DLS; Inset: Vials containing: VD₃ loaded casein micelles before (1) and after freeze-drying and reconstitution with distilled water (2).

Figure 4: Visual observations of *in vitro* digestion of free VD₃ in water and VD₃ loaded rCM show differences in colloidal stability, and its changes during digestion.

Figure 5: RCM are digested mainly during the gastric step of the digestion process. SDS-PAGE breakdown analysis of VD₃-loaded rCM under two-step simulated digestion process consisting a one hour gastric step and a two hour upper-intestinal step, as a function of time. Lanes: (1) rCM-VD₃ suspension mixed with simulated gastric fluids (SGF), without pepsin (suspension: SGF ratio= 1:1 v/v) (2) gastric digestion- 10 min. (3) gastric digestion- 30 min (4) gastric digestion- 60 min, (5) size markers (top to bottom): 250, 150, 100, 75, 50, 37, 25, 20, 15, 10, 5, 2 kDa (6) 10 min duodenal digestion, (7) 30 min duodenal digestion, (8) 60 min duodenal digestion, (9) 90 min duodenal digestion, (10) 120 min duodenal digestion.

Figure 6: The entrapment in rCM improved VD₃ stability during simulated gastric and intestinal digestion. Retention of VD₃ during simulated gastric and intestinal digestion of free VD₃ (dashed) and VD₃ loaded rCM (solid) as a function of time. Notice that the apparent drop in VD during the gastric step was due to its entrapment in curd clots which were not very accessible upon sampling, and the real remaining concentration was revealed only upon complete curd breakdown in the intestinal stage.

Figure 7: The overall effect of the rCM is a higher proportion of the ingested vitamin being bioavailable *in vitro*, compared to the unencapsulated VD. Percentage of VD₃ absorbed (taken-up and transported) by Caco-2 cells after simulated gastric and intestinal digestion of VD₃-loaded rCM and of free VD₃; (A) VD uptake as percent of the amount found in the digesta after one hour of simulated gastric digestion and two hours of simulated upper-intestinal digestion; (B) VD absorbed as percent of initial VD amount at the beginning of simulated digestion; Different letters indicate significantly different results within each chart.

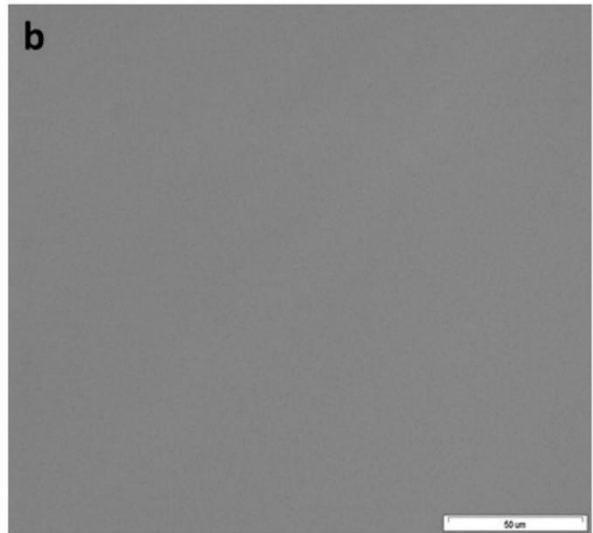
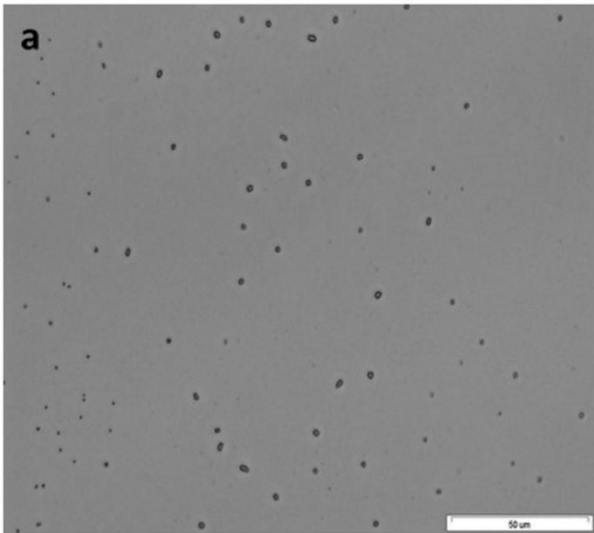
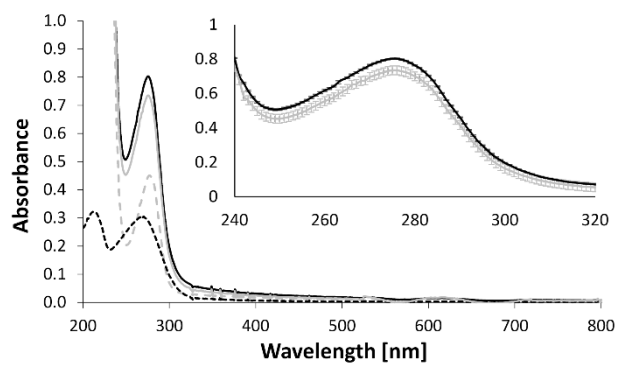
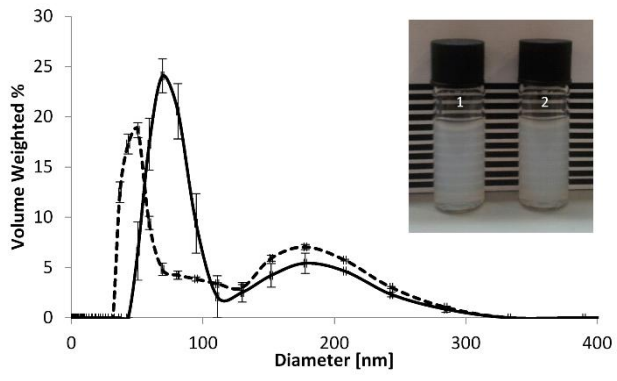


Figure 1:

Figure 2:





Figure

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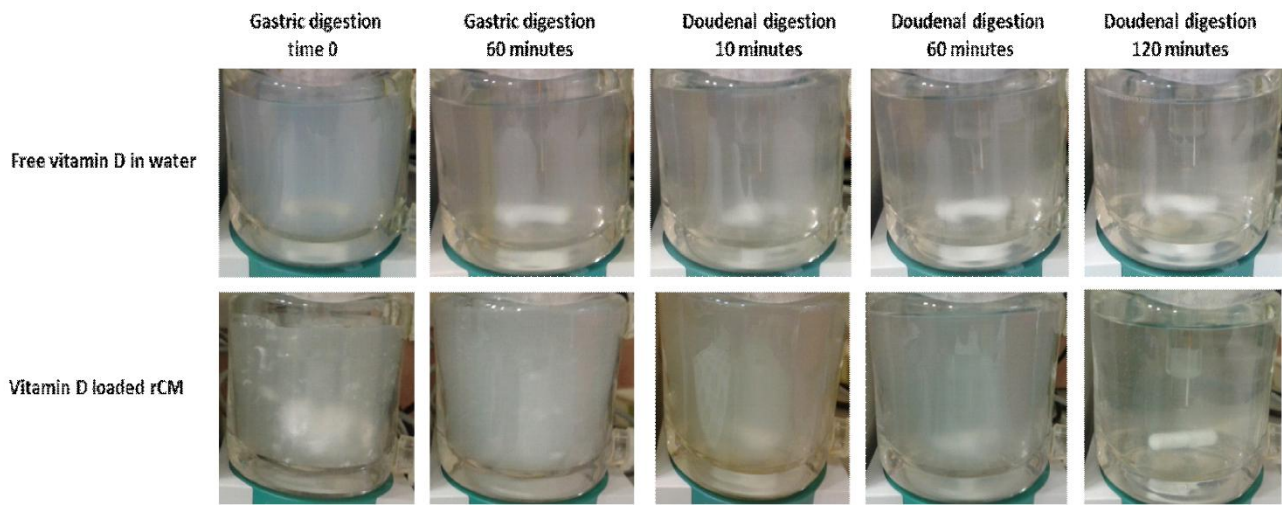
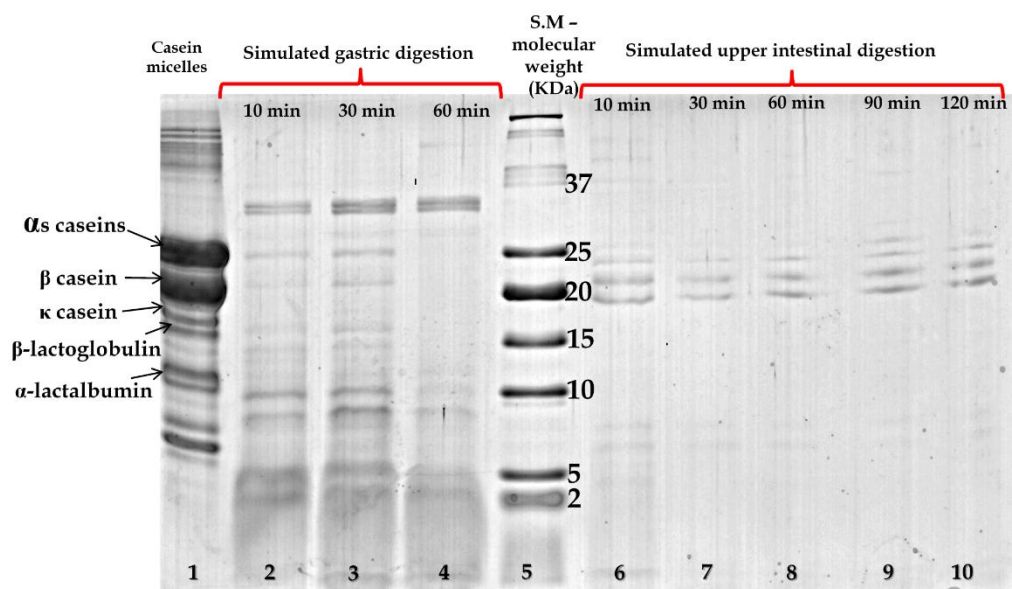


Figure 4:

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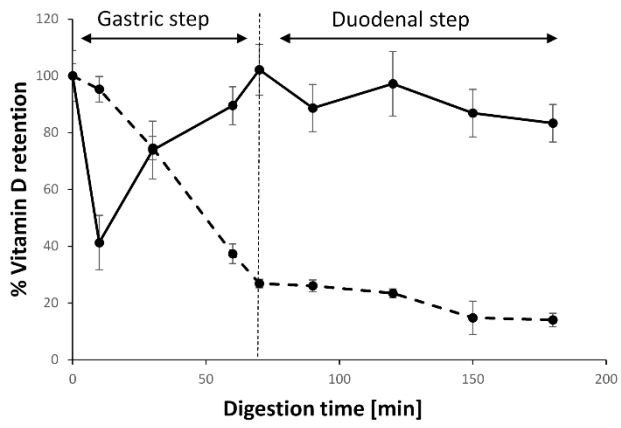


Figure 6:

Figure 7:

