Milk Protein Nanocapsules for Drug Delivery

R. Khalfin[*], M. Bachar[**] and D. Danino[**]

[*] Department of Chemical Engineering, rafail@tx.technion.ac.il
[**] Department of Biotechnology and Food Engineerin, mbachar@tx.technion.ac.il and dganitd@tx.technion.ac.il, Technion, Haifa 32000, Israel

ABSTRACT

In the presented work we investigated the possible use of β-casein micelles as a vehicle for delivery of water-insoluble molecules, particularly drugs. Our model drug, celecoxib, was encapsulated in micelles of β-casein. Freeze-dried and resuspended micelles retained their original pre-lyophilized structure, uniform size, and morphology. Small-angle X-ray scattering (SAXS) measurements show a transition from an oblate ellipsoid to a more spherical micelle shape and an increase in the micelle size upon drug loading, and confirm no influence of drying on the dimensions, as also indicated by cryo-TEM. SAXS points to a slight increase in the micelle density after drug loading, confirming mixed micelles formation.

Keywords: beta-casein, micelles, drug delivery, nanoencapsulation

1 INTRODUCTION

β-Casein, the main protein found in milk, is an amphiphilic molecule that forms micelles both above and below the pI. The micelles are of a nanometric scale, and are constructed of a hydrophobic core and a hydrophilic corona [1, 2]. Many drugs suffer from various drawbacks, such as poor stability in the digestive system, poor absorption, low solubility and more. Encapsulation by β-casein can improve drug delivery and therapeutic efficiency. As a natural food product β-casein is easily degradable in the body and does not provoke an immune system response, making it a great candidate as a drug carrier.

The purpose of this study was to develop β-casein nano-assemblies as a platform for oral delivery of drugs. As a model for a hydrophobic drug we used celecoxib, a poorly soluble drug with low bioavailability. Celecoxib is a nonsteroidal anti-inflammatory drug (NSAID), approved by the U.S Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) for the treatment of rheumatoid arthritis and osteoarthritis. It is an NSAID which inhibits cyclooxygenase (COX), the enzyme responsible for conversion of arachidonic acid to prostaglandins. Unlike most NSAIDs, celecoxib is selective for COX-2, one of the two COX isoforms, hence causing less ulceration of the stomach and intestines and other clinically important toxic effects [3,4]. This drug also shows distinct anticancer activities [5]. However the poor drug solubility and being a drug of variable absorption require to give high doses which therefore may lead to the severe toxicity [6]. Encapsulation of celecoxib in β-casein micelles may reduce the toxicity as it dramatically improves drug dispersibility. Additionally, β-casein carriers are expected to release the drug following natural digestion of the protein in the stomach and intestine, or by interaction of the micelles with the GI tract walls.

2 EXPERIMENTAL

2.1 Materials and solution preparation

Bovine β-casein (>90%; Sigma-Aldrich) was dissolved in pH 7.0 Hepes buffer containing 20 mM Hepes (MP Biomedicals), 1 mM $MgCl_2$ (Sigma-Aldrich), 2 mM EGTA (Sigma-Aldrich) and different amounts of NaCl (Loba Chemie) (10 to 150 mM), and was put over-night at 4 ºC under stirring. The protein solutions were prepared at a concentration 10 mg/mL, at least an order of magnitude above the CMC (0.5-2 mg/ml), where stable protein micelles exist. The solutions were filtered through a porous membrane of 0.45 μm to avoid large protein aggregates. Celecoxib ((4-[5-(4-methylphenyl)-3-(trifluoromethyl) -1*H*pyrazol-1-yl]-benzene-sulfonamide, MW of 381.373 g/mol) is weakly acidic (p*Ka* is 11.1), hydrophobic (log*P* = 3.5), and has low aqueous solubility (3–7 μg/ml). It was dissolved in 100% ethanol (Bio Lab) and a known amount of that solution was titrated to the protein micellar solution under stirring, to pre-determined protein:drug molar ratio. Ethanol concentration in the final solution did not exceed 5% v/v ethanol. Stirring continued for 30 minutes in room temperature. The obtained suspensions contained β-casein and celecoxib in protein:drug mole ratio of 1:8. All the solutions were transparent. As a control, a solution of celecoxib in 100% ethanol was titrated under stirring to the same Hepes buffer, and the stirring continued for 30 minutes in room temperature. All control drug solutions were cloudy, indicating the poor solubility of celecoxib in aqueous solution without β-casein.

Lyophilization. Protein and protein-drug solutions were lyophilized by freezing in liquid nitrogen followed by drying in a Christ Alpha 1-4 lyophilizer for 24 hours. The specimens were stored in 4 °C for over 6 months, then re-suspended in double distilled water, back to the original concentration. Re-suspension was performed by weighing

the powder, adding a measured amount of double distilled water and stirring for 30 minutes in room temperature. The suspensions obtained were transparent and stable.

2.2 Methods

Direct-imaging cryogenic-transmission *electron microscopy (cryo-TEM)*. Specimens were prepared either in an automated vitrification device (Vitrobot, FEI, The Netherlands) or in a homemade controlled environment vitrification system (CEVS) [27], at controlled temperature and humidity conditions (25 °C, 100% RH) to avoid loss of volatiles. A drop of the specimen was placed on a TEM copper grid covered with a perforated carbon film, and then blotted with a filter paper to form a thin liquid film of the specimen. The grid was immediately plunged into liquid ethane at its freezing temperature (-183 °C) to form a vitrified specimen then stored until examination in liquid nitrogen (-196 °C). Some of the specimens were examined in a Philips CM120 transmission electron microscope (Philips, The Netherlands) at an accelerating voltage of 120 kV. An Oxford CT3500 cryo-specimen holder was used (Oxford Instruments, UK) to maintain the vitrified specimens below -175 °C. Other specimens were examined in a Tecnai 12 G^2 TEM (FEI) using a Gatan 626 cryo-holder. Specimens were studied in a low-dose imaging mode to minimize beam exposure and electron beam radiation damage. Images were recorded digitally [28] in the CM120 on a cooled Gatan MultiScan 791 CCD camera (Gatan, UK), or in the Tecnai on an high-resolution 2k x2k Ultrascan 1000 cooled CCD camera (Gatan, UK), using the Digital Micrograph 3.6 software (Gatan, UK).

Wide-angle X-ray diffraction (*WAXS*). WAXS experiments with lyophilized protein samples, protein/celecoxib samples, or crystalline celecoxib were performed using a Philips PW 3020 powder diffractometer equipped with a graphite crystal monochromator (Philips). The operating conditions were CuKα radiation (0.154 nm), voltage 40 kV and current 40 mA, in 2θ recording range from 3° to 65°, at room temperature.

Small angle X-ray scattering (SAXS) was performed using a small-angle diffractometer (Molecular Metrology SAXS system with Cu K_α radiation from a sealed microfocus tube (MicroMax-002+S), two Göbel mirrors, and three-pinhole slits. Generator powered at 45 kV and 0.9 mA). The scattering patterns were recorded by a 20 x 20 cm two-dimensional position sensitive wire detector (gas filled proportional type of Gabriel design with 200 µm resolution) that is positioned 150 cm behind the sample. The resolution of the SAXS system is about 3^{-2} nm^{-1}.

The scattered intensity $I(h)$ was recorded in the interval $0.07 < h < 2.7$ nm^{-1}, where h is the scattering vector defined as $h = (4\pi / \lambda) \sin(\theta)$, 2θ is the scattering angle, and λ is the radiation wavelength (0.1542 nm).

The solutions under study were sealed in a thin-walled capillary (glass) of about 2 mm diameter and 0.01 mm wall thickness, and measured at ambient temperatures under vacuum. $I(h)$ was normalized to the following parameters: time, solid angle, primary beam intensity, capillary diameter, transmission, and the Thompson factor [7], $[I(h)] = nm^{-3}$. Scattering of the solvent (buffer), empty capillary and electronic noise were subtracted.

3 RESULTS

Celecoxib is a hydrophobic and highly permeable drug that can be absorbed throughout the GI tract, however its dissolution may be a rate-limiting factor for absorption from solid dosage forms [8]. Thus, keeping the drug in an amorphous state, as in our β-casein based formulation, may assist with its absorption and may, therefore, enable reducing the daily dose [9].

Celecoxib forms large crystals in buffer, as depicted visually and by light microscopy (data not shown). WAXS measurements show a loss of celecoxib crystallinity while present in the dried β-casein micelles (Fig. 1).

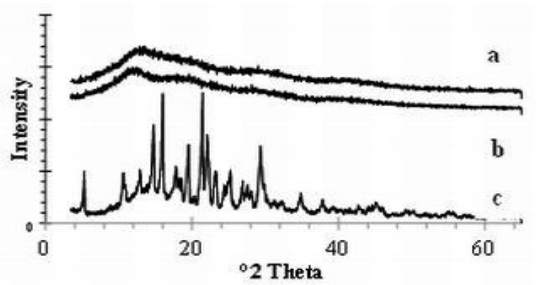

Figure 1: (a) Dried β-casein micelles, (b) dried β-casein/celecoxib micelles in 1:8 mole ratio, (c) celecoxib powder (c).

Cryo-TEM images show an increase in the micellar size upon increasing β-casein concentration and drug loading. In addition, loading the β-casein micelles with high amounts of drug results in a conformational change from an oblate ellipsoid to a spherical form (Fig. 2).

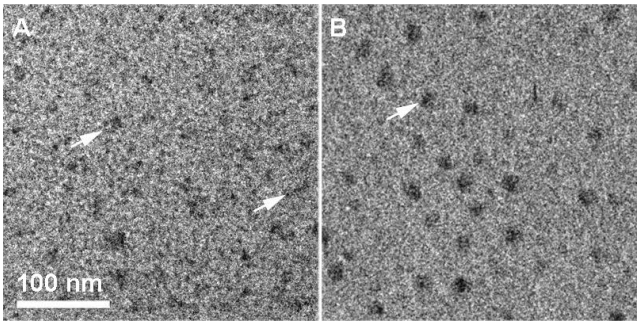

Figure 2: Cryo-TEM images: (A) 10 mg/mL empty β-casein micelles, (B) 10 mg/mL β-casein with 1:8 protein to celecoxib mole ratio.

In previous SAXS studieds we found that β-casin solutions (10 mg/ml) display an interparticle interference peak. Adding large enough NaCl concentrations (50 mM) to 10 mg/ml β-casein eliminates the interparticle interference peak (Fig. 3), allowing SAXS patterns to be analyzed more readily.

Table 1 summarizes the micelles parameters obtained from fitting procedures (Appendix 1) of the experimental SAXS profiles.

Table 1. Micelles structure parameters obtained from fitting of the experimental SAXS patterns.

Parameter	β-Casein	β-Casein +celecoxib
Rg (Guinier apr.), nm	7.8	9.0
Large axis (2a) from P(r), nm	24.0	27.5
a semiaxis, nm	12.0	13.5
b semiaxis, nm	4.0	5.7
Axis ratio	0.33	0.42
Rg (a,a,b), nm	7.8	8.9
V, nm^3, from a, b	2410	4336
Density, g / cm^3	1.23	1.27

When stored at 4 °C as an aqueous suspension, the stability of the drug-loaded complexes is about 3 weeks, and the assemblies remain stable when they are transferred to room temperature. However, this stability is still far from being satisfactory; therefore, we examined the ability to form a freeze-dried powder with a much better stability profile. By lyophilization we were able to keep the loaded micelles stable in a dry powder form for at least 6 months and probably for much longer. stability studies are ongoing.

Lyophilization did not have a significant effect on the SAXS profiles and the micelle parameters. The tails of the experimental SAXS curves (with and without celecoxib) coincide and go above the fitting profiles. The Kratky plots $I(h)*h^2$ *vs h* suggest this scattering might be connected with scattering of the β-casein molecule coils.

Gyration radii from the experimental SAXS profiles and from the fitting procedure coincide. Analysis with the p(r) function (eq. 1) confirms the selected micelles form (spheroid) and the values of spheroids axis obtained by the fitting procedure [2].

$$p(r) = \frac{r}{2\pi^2}\int_0^\infty I(h)[\sin hr]\,hdh \qquad (1)$$

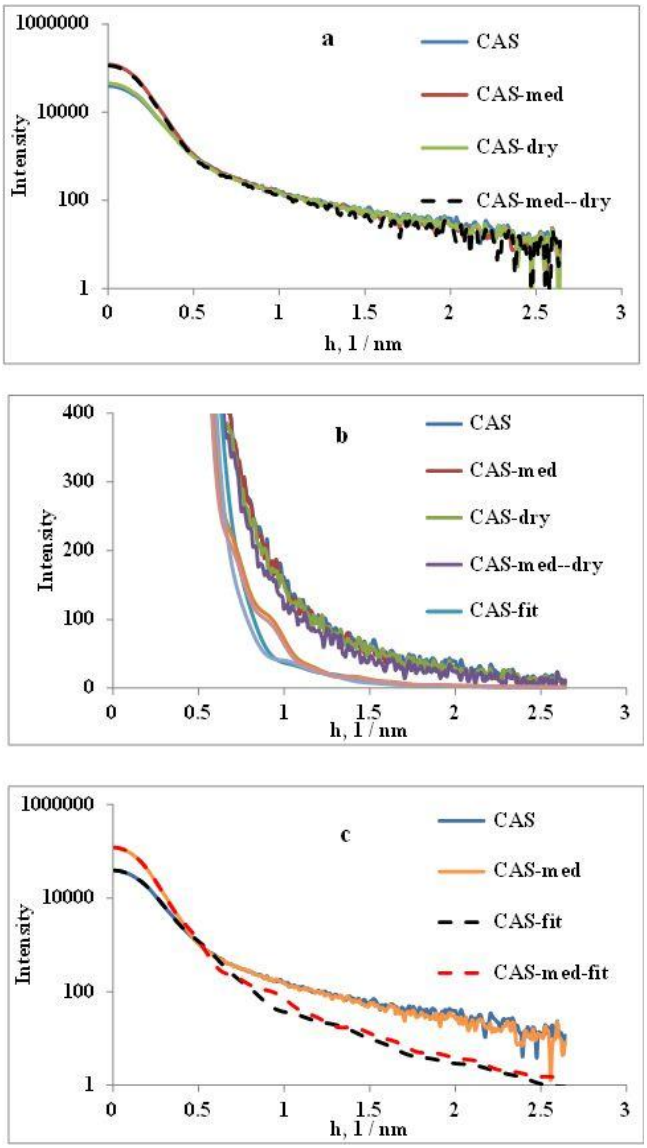

Figure 3. SAXS patterns without (a) and with (b, c) fitting. *Log-line* (a, c) and *line-line* scale (b). Specimens are of 10 mg/ml β-casein. Empty β-casein micelles marked as CAS, micelles loaded with 1:8 mole ratio celecoxib are marked as CAS-med. Lyophilized and resuspended specimens are marked as dry.

The absence of a peak at large *h* values indicates that the micelles do not have a core – shell structure with a constant shell thickness. The most probable structure is a mix of celecoxib – β-casein molecules that disturb the drug crystallization (Fig. 1). The density of the mixed micelles increases (Appendix 2) as their volume increases (Table 1).

4 CONCLUSIONS

- Celecoxib and β-casein form mixed micelles with a volume and density larger than those of pure casein micelles.
- The micelle shape changes from an oblate ellipsoid to a spherical micelle upon increasing casein concentration and drug loading.
- The stability of the drug-loaded complexes and especially the freeze-dried powder shows a good potential for practical application.

5 APPENDIX 1

SAXS of the oblate ellipsoid modeling was done using the formulas described in [10]:

$$\mathrm{I}(h) = N\rho^2 V^2 F_{ellip}{}^2(hr,\nu) = BVF_{ellip}^2(h,r,\nu) \quad \text{(A1.1)}$$

$$\mathrm{F}_{ellip}^2(h,r,\nu) = \int_0^1 \left\{ \mathrm{F}[hr\sqrt{1+\mathrm{x}^2(\nu^2\text{-}1)} \right\}^2 \mathrm{dx} \quad \text{(A1.2)}$$

$$\mathrm{F}(t) = 3\frac{\sin\ t - tR\cos\ t}{(t)^3} \quad \text{(A1.3)}$$

$$t = hr$$

Where $B=NV\rho^2=W\rho^2$, N is the number of micelles in one volume unit, W is the volume part (volume concentration) of the micelles, ρ is the electron density difference (micelle - solvent), and V is the average volume of one micelle $(V = 4\pi^3\nu/3)$.

6 APPENDIX 2

β-Casein micelle density, ρc, was calculated by the following formulas:

$$Q = 2\pi^2 W(\rho c - \rho s)^2 \quad \text{(A2.1)}$$

$$\rho ce = 6.0247\frac{ec}{mc}\pi^2 100\rho c \quad \text{(A2.2)}$$

$$\frac{\rho c \cdot W}{\rho c \cdot W + \rho s \cdot (1-W)} = C \quad \text{(A2.3)}$$

Where Q, [1 / nm^3], is the Porod integral value, W is the β-casein volume concentration, ρc and ρs are the mass density, g/cm^3, of β-casein and NaCl aqueous solution respectively, ρce and ρse are the electron density (el.number/nm^3) of β-casein and NaCl aqueous solution respectively, ec and mc are the electron number and mass (Dalton) of the β-casein molecule respectively. For the mixed micelles and the buffer the ec/em values were calculated as the average of the two components.

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