Beta- casein micelles as nano-delivery vehicles for chemotherapeutic drugs

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Introduction

Beta-casein (β-CN), one of the four main caseins in bovine milk, has a hydrophilic N-terminal domain, and a hydrophobic C- terminal domain (Kumosinski et al., 1993; Livney et al., 2004), an amphiphilic structure resembling low molecular weight surfactants. Similarly, it tends to self-associate under appropriate conditions forming stable micelle-like structures in aqueous solution (Mikheeva et al., 2003). The monomers (single β-CN molecules) have a radius of gyration (R_g) of 4.6 nm, and the micelles, containing 15-60 molecules, have R_g values ranging between 7.3 and 13.5 nm. The critical micellization concentration (CMC) ranges between 0.05 and 0.2% w/v, depending on temperature, pH, solvent composition and ionic strength (Portnaya et al., 2006).
Few studies have investigated the binding of lipophilic molecules to β-CN, including vitamin D3, vitamin A, sucrose esters and sodium dodecyl sulfate. These studies suggested that hydrophobic interactions are largely responsible for the binding (Forrest et al., 2005).
Recently, in a study conducted in our group (Semo et al., 2007), it was suggested that casein micelles are in fact natural nano-delivery vehicles, and it was shown that they could be used to encapsulate, stabilize and protect hydrophobic bioactive compounds (e.g. vitamin D). It is expectable that a similar technology may be used for encapsulating hydrophobic drugs, including anti-cancer chemotherapeutics, for oral delivery.
Currently available cancer treatments include surgery, chemotherapy, radiation therapy, and immunotherapy (Nautiyal et al., 2006). Current chemotherapy approach has a few problems: first, the drugs are usually administered intravenously (IV), a major source of discomfort, stress, complications (infections, bleeding), and cost to patients as well as require multiple hospitalizations to complete the long chemotherapeutic combination regimen. The availability of oral agents would make a significant contribution to patients' quality of life and may significantly reduce cost (Liu et al., 1997). Secondly, many chemotherapeutic drugs are hydrophobic and are thus hardly soluble in aqueous solutions. Furthermore, conventional chemotherapy often results in toxic side effects. Therefore it is highly desirable to develop novel treatment strategies that target malignant tissues, in order to reduce side effects and enhance the therapeutic efficacy (Nautiyal et al., 2006). The major goal of the current study is to develop a rationally designed drug delivery system comprising hydrophobic anticancer drugs encapsulated within β-CN based nanoparticles. This drug delivery system will allow the lipid-soluble drug to be thermodynamically stable in aqueous solutions and to be readily delivered to the gastrointestinal tract (GIT). Additional coatings may be added to direct these antitumor agents harboring nanoparticles to the required target regions along the GIT. Mitoxantrone (MX), a hydrophobic chemotherapeutic drug with an endogenous blue color, was chosen as a model for chemotherapeutic drugs to characterize encapsulation in β-CN micelles.

Materials and Methods

Mitoxantrone (M6545, purity >97%), and β-CN from bovine milk (C6905, purity 90%) were purchased from Sigma-Aldrich Israel Ltd. A stock solution of 10 mM mitoxantrone (MX) in dimethyl sulfoxide (DMSO) was prepared. Beta-CN was dissolved in sodium phosphate buffer

solution (PBS) pH 7.0, ionic strength 0.1. The encapsulation of MX in β-CN at different MX: β-CN molar ratios, was performed by titration of different volumes of MX in DMSO solution to β-CN solution in PBS while stirring. The volume percentage of DMSO in PBS did not exceed 5%. The samples were equilibrated overnight at room temperature.

Tryptophan (Trp) Fluorescence: Trp143 is located in the main hydrophobic domain of β-CN. Quenching of protein fluorescence due to energy transfer from this Trp residue to a bound ligand serves to determine the binding affinity (Cogan et al., 1976). Beta-CN-MX interaction was studied by monitoring the changes in the Trp fluorescence emission of β-CN upon addition of MX. Trp fluorescence was determined by excitation at 287 nm and emission at 332 nm (Forrest et al., 2005), using the Fluorolog 3-22 spectrofluoremeter (Jobin Yvon, Horiba, Longjumeau cedex, France). The apparent dissociation constant and the number of MX molecules involved in binding per β-CN molecule were calculated from plots of the fluorescence intensity, expressed as the percentage of the initial fluorescence of MX-free β-CN vs. added MX concentration. The data was analyzed using Matlab (MathWorks), by means of the derivation of equation (1) for high affinity associations

$$F = \left\{F_0 + F_1 \left(1/K_\mathrm{d}\right)\left[L_\mathrm{tot}\right]\right\} / \left\{1 + \left(1/K_\mathrm{d}\right)\left[L_\mathrm{tot}\right]\right\} \qquad (1)$$

where F is the fluorescence intensity at a given ligand (MX) concentration, F_0 the fluorescence intensity at the beginning of the titration, F_1 the fluorescence at the end of the titration, K_d the dissociation constant, and L_{tot} the total ligand concentration (Christiaens et al., 2002).

MX Fluorescence: MX is a fluorescent molecule with emission at 675 nm following excitation at 609 nm according to a 3-D fluorescence spectra analysis we performed using the Fluorolog 3-22. We further studied β-CN-MX interaction by monitoring the changes in the MX fluorescence emission upon addition of β-CN. Data were analyzed as detailed above for the Trp quenching.

Light scattering: Particle diameter was analyzed by dynamic light scattering (DLS) (NICOMP 380, Particle Sizing Systems, Santa Barbara, CA, USA). The effects of MX: β-CN molar ratio on the Gaussian mean particle diameter, and on the particle size distribution were determined.

Scattered light intensity measurement of MX encapsulated in β-CN at different MX: β-CN molar ratios, was performed as a measure of turbidity, using the Fluorolog 3-22 at a front-face mode. To study elastic light-scattering the excitation and emission wavelengths were both set at 480 nm, (a wavelength at which both MX and β-CN have minimal absorbance) and using slit widths of 1 nm.

Results and Discussion

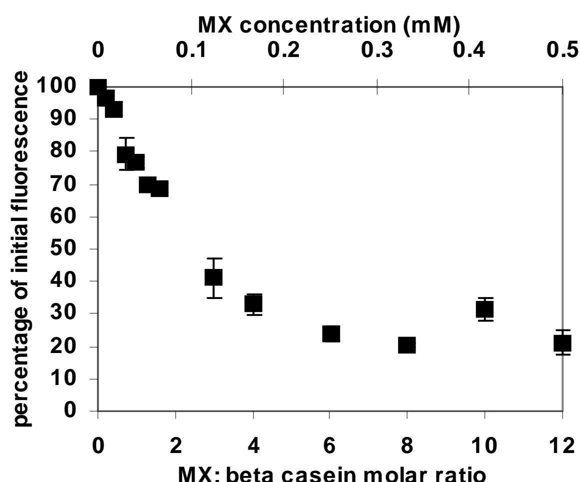

Figure 1 – Fluorescence quenching of Trp 143 (excitation 287 nm, emission 332 nm), vs. MX: β-CN molar ratio at 1mg/ml β-CN, and vs. MX concentration (upper axis).

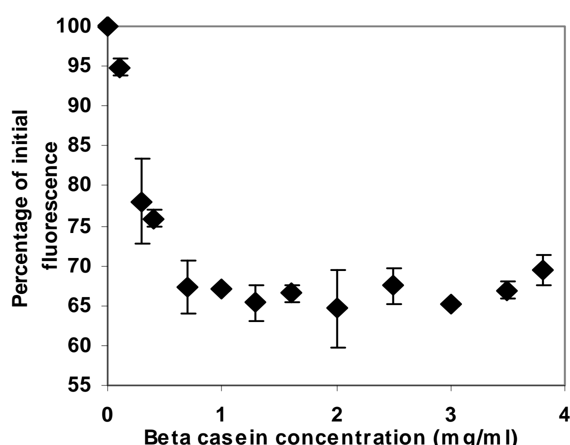

Figure 2 – Fluorescence quenching of MX (excitation 609 nm, emission 675 nm) vs. β-CN concentration at 42 μM MX.

The binding of MX to β-CN was studied using both the fluorescence of the protein and of the drug. Figure 1 shows a decrease in Trp emission intensity (as percentage of its initial value) as a function of MX: β-CN molar ratio, at 1 mg/ml protein, which is above the CMC of β-CN at these conditions (~0.1 mg/ml). Trp emission reached a plateau at MX: β-CN molar ratio of 6:1, a ratio at which all of the accessible Trp 143 residues were apparently binding MX; Evidently, MX quenched 80% of the initial Trp emission intensity, as saturation was reached. While the Trp 143 is the reporting site, there are apparently more binding sites of higher or similar affinity for MX on β-CN that were saturated prior to- or with Trp 143, to account for the molar binding ratio obtained.

Figure 2 presents the MX emission intensity (as percentage of its initial value) vs. β-CN concentration. Beta-CN quenched the over-all MX emission to 65% of its initial intensity. MX emission reached a plateau at 0.7mg/ml β-CN, a concentration at which practically all of the MX molecules were bound to β-CN. The dissociation constant, K_d, and the stoichiometric binding ratio were calculated from both the quenching of Trp and MX fluorescence, and the results are summarized in Table 1. Both methods resulted in K_d values in the micro molar range, suggesting a high affinity binding of MX to β-CN.

Observed fluorophore	K_d (μM)	MX: β-CN molar binding ratio
β-CN (Trp 143)	8.43±6.06	3.28±0.03
MX	0.73±0.29	2.22±0.39

Table 1 – Apparent dissociation constant (K_d) and the "stoichiometric" molar ratio

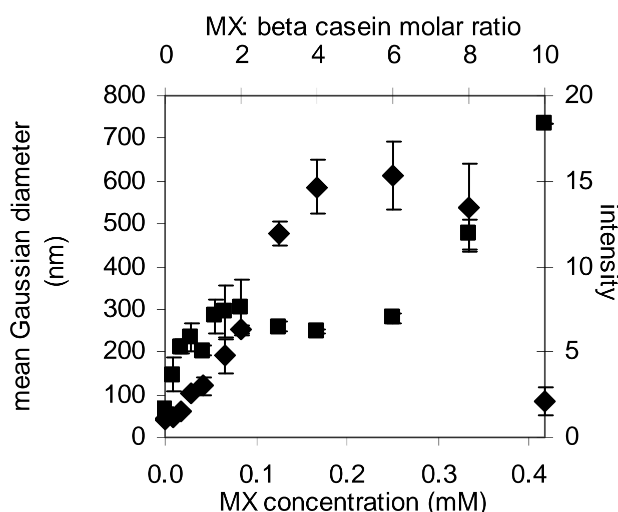

Figure 3 -Mean Gaussian diameter (■) and scattered light intensity (♦) of β-CN - MX particles vs. MX concentration, or vs. MX: β-CN molar ratio (upper axis).

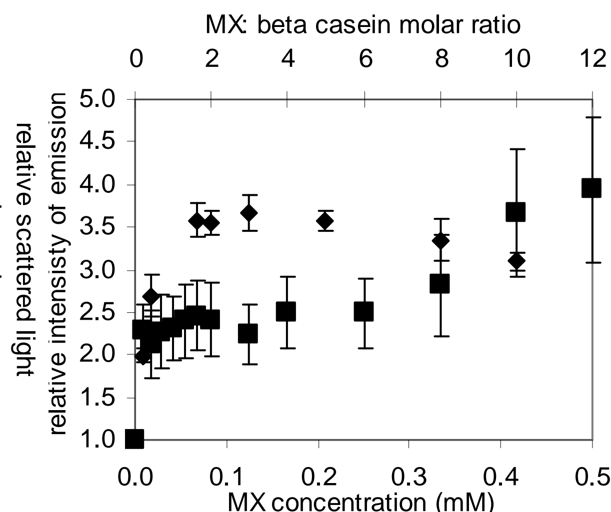

Figure 4–MX emission intensity vs. MX concentration, or vs. MX : β-CN molar ratio (for the solutions containing both components; upper axis). MX only (♦) vs. 1mg/ml β-CN+MX (■) (ex.: 609 nm em.: 675 nm).

Further to studying the binding of MX to β-CN, we characterized the formation of the drug-loaded nanoparticles by light scattering. Mean Gaussian diameter results and scattered light intensity as a function of MX: β-CN molar ratio are presented in Figure 3. One can observe an increase of β-CN+MX particle size with MX:β-CN molar ratio up to 2:1. Between molar ratios 2:1 and 6:1, the average particle diameter remained constant but the number of particles increased, as may be deduced from the increased turbidity. At MX: β-CN molar ratio above 6:1, particles apparently aggregated and sedimented due to the excess MX. Figure 4 shows relative emission intensity of MX vs. MX concentration, and to MX: β-CN molar ratio at 1mg/ml β-CN, compared to the

emission intensity of MX without the protein. Encapsulated MX emits less than MX alone at same concentrations because β-CN quenches MX emission up to a molar ratio of 6:1. Above this ratio, MX emission increases, hence we deduce that at these MX concentrations MX is not all covered by β-CN micelles anymore. It could either exist in particles without β-CN, or its excess appears as uncoated patches on the surfaces of overloaded β-CN micelles.

Conclusions

We have shown for the first time that MX may be encapsulated within β-CN micelles. The maximal MX loading of a β-CN micelle system containing 1mg/ml β-CN was 6:1 mole MX: mole β-CN. Assuming that a single β-CN micelle contains ~15-60 β-CN molecules (as reported for pure β-CN micelles (Portnaya et al., 2006)), then the maximal MX loading per β-CN micelle is in the range of ~90-360 MX molecules per β-CN micelle. This assumption requires further verification as it is highly likely that the binding of a hydrophobic drug cargo raises the aggregation number of the protein. We conclude that β-CN displays a very good binding and encapsulation capacity for this model of hydrophobic anticancer drug, and thus may serve as a useful nanoscopic vehicle for the solubilization and delivery in aqueous drug preparations for oral delivery of MX and possibly other hydrophobic therapeutic drugs, and drug combinations aimed at treating malignant and non-malignant disorders.

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