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# Synthesis and characterization of alkylated caseinate, and its structure-curcumin loading property relationship in water

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ABSTRACT

Alkylated caseinates (Cn-caseinates) containing selected C8–C16 alkyl groups were successfully synthesized through a two-step reaction between the protein and fatty acids. The substitution degree (SD) of the alkyl groups was 5.2–72.9%, which depended on the feed molar ratio of reagents and fatty acid chain length. The SD value was positively associated with the surface hydrophobicity index ($S_0$) of the Cn-caseinate. Among the tested Cn-caseinates (n = 0, 8, 12, 14 and 16), C16-caseinate showed the best self-assembly and curcumin-loading properties in water. With increased palmitoyl group SD, the critical micelle concentration (*CMC*) of C16-caseinate was decreased from 5.15 to 3.77 mg/L and the encapsulation efficiency of curcumin-loaded C16-caseinate self-assemblies was increased from 31.16% to 69.87%. Transmission electron microscopy analysis demonstrated a desirable sphere assembly. In addition, the C16-caseinate self-assemblies had good re-dispersibility and storage stability at 4 °C for 6 weeks.

## 1. Introduction

Sodium caseinate, the sodium salt of casein, is widely used in food industry for its nutritional and functional properties (Damin, Alcântara, Nunes, & Oliveira, 2009; Goff, Kinsella, & Jordan, 1989; Schou et al., 2005). Recently, it has also been developed as a natural carrier for encapsulation and delivery of hydrophobic bioactive food components, since it may form self-assemblies in the aqueous phase (Livney, 2010; Tavares, Croguennec, Carvalho, & Bouhallab, 2014). For instance, Semo, Kesselman, Danino, and Livney (2007) have demonstrated that encapsulation of fat-soluble vitamin $D_2$ within the self-assemblies of bovine caseinate was able to improve its water solubility and UV-light stability. These caseinate self-assemblies were also effective in protecting thermolabile β-carotene against thermal degradation during industrial processes such as pasteurization, sterilization and baking (Sáiz-Abajo, González-Ferrero, Moreno-Ruiz, Romo-Hualde, & González-Navarro, 2013). Delivery systems based on caseinate self-assemblies possess many advantages, including but not being limited to non-toxicity, desirable biocompatibility and biodegradability, which facilitate their food applications (Livney, 2010; Sahu, Kasoju, & Bora, 2008).

Despite these advantages, the food application of delivery systems based on caseinate self-assemblies is limited by several drawbacks. First, the encapsulation efficiency (EE) for hydrophobic compounds of caseinate self-assemblies is relatively undesirable, compared to those formed from synthetic polymers with an amphiphilic nature (Sanna et al., 2015) or other encapsulation systems (Luo, Teng, Wang, & Wang, 2013). Semo et al. (2007) showed that the encapsulation efficiency for vitamin $D_2$-loaded caseinate self-assemblies was only 27% (Semo et al., 2007). Besides, ions adding (e.g., $Ca^{2+}$, $PO_4^{3-}$) (Sáiz-Abajo et al., 2013) and high energy processing, such as high pressure homogenization (Haham et al., 2012; Roach, Dunlap, & Harte, 2009), were also required to form self-assemblies with desirable characteristic properties, which complicated the preparation procedure for caseinate self-assemblies.

To overcome these challenges, there have been efforts to modify the structure of caseinate including covalently conjugating selected hydrophobic moieties into its molecular structure. In 2014, a novel biphasic enzyme-catalyzed protocol was developed to incorporate non-polar octyl groups into caseinate molecules (Zhang, Niu, Yao, et al., 2014). However, the intra- and intermolecular cross-linking occurred simultaneously in the octylated caseinates. Therefore, additional approaches to obtain alkylated caseinates with only alkyl conjugation and investigate their potential use in encapsulating and delivering hydrophobic food components are needed.

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This study first synthesized and characterized 24 alkylated caseinates with the selected alkyl chain lengths and different substitution degrees. These alkylated caseinates were also evaluated for their physicochemical properties. In addition, the effects of alkyl chain length and substitution degree of alkyl groups on the curcumin-loading property of the caseinate self-assemblies were investigated using curcumin as a probe component. Curcumin was selected because of its hydrophobicity, low bioavailability and health benefits. Moreover, the best curcumin-loaded alkylated caseinate self-assemblies were characterized for their size, size distribution, encapsulation efficiency, morphology, re-dispersibility and storage stability. To the best of our knowledge, few studies have investigated the self-assembly behavior of alkylated caseinate, and little is known about the effect of structural difference (chain length and substitution degree of the alkyl group) of alkylated caseinate on the encapsulation properties for hydrophobic components of the formed self-assemblies.

## 2. Materials and methods

### 2.1. Chemicals

Sodium caseinate (NaCas, 87% purity), 8-anilino-1-naphthalenesulfonic acid (ANS, 97% purity) and curcumin (≥99.5% purity) were purchased from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, MO, USA). Octanoic, lauric, myristic and palmitic acids (≥99% purity), *N*-hydroxysuccinimide (NHS, 98% purity) and dicyclohexylcarbodiimide (DCC, 99% purity) were obtained from Shanghai Jingchun Biochemical Technology Co., Ltd. (Shanghai, China). The Bradford reagent was purchased from Bio-Rad Co. (Richmond, CA, USA). Dialysis membranes were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA). All other reagents were of analytical grade and used without further purification. Water purified with a Milli-Q system was used for all experiments.

### 2.2. Synthesis of alkylated caseinates (Cn-caseinates)

Palmitoyl caseinates with different substitution degrees were synthesized and purified according to a previously reported method with modifications (Haque & Kito, 1983). Briefly, the esterification reaction was carried out under $N_2$ using pre-dried glasswares. Palmitic acid (6.4 g, 25 mmol) and NHS (2.9 g, 26 mmol) were dissolved in 75 mL of 1,4-dioxane. To this solution, dicyclohexylcarbodiimide (DCC) (7.6 g, 37 mmol) was added and the resultant mixture was stirred overnight at 25 °C. At the end of the reaction, the formed dicyclohexylurea (DCU) was removed by filtration, and the filtrate was concentrated under a reduced pressure to obtain the crude ester as colorless oil. Purification of the crude ester was done by column chromatography with silica gel (100–200 mesh) as the solid phase and 12% ethyl acetate/hexane as the elution agent. After desiccation, the product was obtained as white flaky solid. Then, the covalent attachment of palmitoyl group to the ε-$NH_2$ in lysyl residue of caseinate by a base-catalyzed ester exchange reaction was carried out. Dimethylformamide (DMF) or dimethyl sulfoxide (DMSO) containing the appropriate amount of C16-NHS (0.6, 1.2, 2.4, 4.8 and 6 mM final concentration in the reaction mixture) was gradually added to 100 mL of bicarbonate buffer (50 mM, pH 9.0), containing a single concentration of NaCas (0.2 mM). The calculated feeding molar ratio of the ε-$NH_2$ in lysyl residue of NaCas to C16-NHS was 1:0.25, 1:0.5, 1:1, 1:2, 1:4 and 1:5, respectively. The reaction mixture was incubated for 1 h under continuous stirring at 25 °C. After the reaction, the mixture was centrifuged at 9390*g* for 10 min (5415R Centrifuge, Eppendorf AG, Hamburg, Germany), and the resultant supernatant was subsequently dialyzed against distilled water for 48 h. The palmitoyl caseinates were then lyophilized and stored at −20 °C for the other tests. Alkylated caseinates with other chain lengths were also obtained following the same protocol. The nomenclature for alkylated caseinates was designated as Cn-caseinates, where n stands for the carbon number in the conjugated alkyl groups (n = 8, 12, 14 and 16).

### 2.3. Characterization of alkylated caseinates (Cn-caseinates)

#### 2.3.1. Substitution degree (SD)

The substitution degree (SD) of the alkyl groups conjugated into caseinate molecules were determined using a $^1$H NMR method following a laboratory procedure (Zhang, Niu, Yao, et al., 2014). Cn-caseinate was dissolved in $D_2O$ at a concentration of 0.5% (w/v), using TMS as an internal reference (0 ppm). $D_2O$ signal was suppressed by the presaturation method. The parameters for the $^1$H NMR spectra of protein samples were: number of data points, 32 K; spectral width, 8012.82 Hz; acquisition time, 2.04 s; relaxation delay time, 5.0 s; and number of scans, 256. An exponential line broadening (0.30 Hz), automatic phase correction, and a baseline correction (degree of polynomial equal to 5) were applied to each spectrum.

#### 2.3.2. Surface hydrophobicity

ANS fluorescence assay (Teng, Li, Luo, Zhang, & Wang, 2013) was carried out to determine the surface hydrophobicity of NaCas and Cn-caseinates with different substitution degrees of alkyl groups. Protein samples (10 mg) were dissolved in 1 mL of 0.1 M phosphate buffer (pH 7.0) and stirred overnight at ambient temperature. The sample dispersions were then centrifuged at 20000*g* for 20 min to sediment insoluble proteins (5415R Centrifuge, Eppendorf AG, Hamburg, Germany). The resultant supernatants were subjected to protein content determination using the Bradford method (Jahaniaval, Kakuda, Abraham, & Marcone, 2000). Then, the protein solutions were diluted 10, 20, 50, 80 and 100 times to make a series of diluted protein solutions. The ANS (100 μg/mL) was prepared in the same buffer and filtered through a 0.22 μm Acrodisc syringe filter membrane. The diluted protein solutions were mixed with the same volume of ANS solution. After 1 h of incubation at 25 °C, the mixture was observed for its fluorescence intensity (FI) on a TECAN Infinite M200 PRO multilabel plate reader (Tecan Group Ltd., Mannedorf, Switzerland). The excitation and emission wavelengths were 355 and 460 nm, respectively. For each protein sample, a linear regression equation was established for FI versus protein concentration ($R^2 > 0.99$), and the slope ($S_0$) was used as an index for surface hydrophobicity.

#### 2.3.3. Critical micelle concentration (CMC)

The *CMC* of NaCas and Cn-caseinates were determined by measuring the surface tension of each sample solution at the selected concentrations at 25 °C. The surface tension was measured with a Krüss Tensiometer (K100, KRÜSS GmbH, Hamburg, Germany) using a Wilhelmy small platinum plate of ca. 4 cm perimeter. Prior to the measurement, the plate was first rinsed with doubly distilled water and burned to red to make sure that there were no surfactants left on the plate. The equipment was tested by measuring the surface tension of double distilled water (72 ± 0.2 mN/m) at 25.0 °C. The *CMC* values were obtained by plotting surface tension versus logarithm of the concentration of samples. The surface tension value decreased continuously and then remained constant with the increase of the sample concentration. The point of break, when the constancy of surface tension begins, was taken as the *CMC* of each sample (Alam, Ragupathy, & Mandal, 2016).

### 2.4. Preparation of the curcumin-loaded Cn-caseinates self-assemblies

Curcumin-loaded Cn-caseinate self-assemblies were prepared using the dialysis method (Gong et al., 2009). Briefly, 1 mL of curcumin ethanol solution (1 mg/mL) was added dropwise to 10 mL of Cn-caseinate aqueous solutions (2 mg/mL) with continuous stirring using a magnetic stirrer (IKA_C-MAG HS 7, IKA WORKS Inc., Wilmington, NC, USA) for 30 min. The obtained dispersion of caseinate self-assemblies was dialyzed against distilled water for 24 h, followed by centrifuging