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In situ study of skim milk structure changes under high hydrostatic pressure using synchrotron SAXS

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ABSTRACT

The structure evolution of casein micelles, as found in cow's milk, under high hydrostatic pressure (HHP) (up to ~1000 MPa) was investigated *in situ* using synchrotron small-angle X-ray scattering (SAXS) on samples held in a diamond anvil cell (DAC) at room temperature. During HHP, both scattering intensities at low q (~0.003 $Å^{-1}$) and at high q (~0.08 $Å^{-1}$) decreased, suggesting the expected disruption of the casein micelles and colloidal calcium phosphate (CCP) nanoclusters, respectively. As pressure increased, a scattering peak around 0.02 $Å^{-1}$ that corresponds to "sub-micelles" of casein micelles appeared and became marked with the increase in pressure. The pressure dependent SAXS profiles from 270 to 960 MPa showed two isosbestic points at $q = 0.013$ and 0.03 $Å^{-1}$ and the structural change can be well described using a two-state model. Upon release of pressure to atmospheric pressure, the pressure-induced change of casein micelles only partially reverted to its original scattering pattern.

1. Introduction

In milk, casein micelles are loose, highly hydrated, and dynamic complexes that consist of α-, β- and κ-caseins and minerals predominantly in the form of colloidal calcium phosphate (CCP) (de Kruif, Huppertz, Urban, & Petukhov, 2012). The size of the casein micelles in milk is polydisperse, with a diameter ranging from 50 to 600 nm (Horne & Dalgleish, 1985). The hydrophobic and electrostatic interactions are suggested to play predominant roles in holding together the casein monomers within the casein micelles (Holt, Carver, Ecroyd, & Thorn, 2013; Horne, 2006; Orlien, Boserup, & Olsen, 2010).

Although the structure of casein micelles has been studied extensively, the exact model for its fine structure assembly is still elusive (Day, Raynes, Leis, Liu, & Williams, 2017; Ingham et al., 2016). *In situ,* real time studies of casein micelles under external stressors, such as high hydrostatic pressure (HHP), and the reversibility of physical and chemical changes that result from the application of these stressors, may provide information on the casein micelle structure. In the last 20 years, small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) are increasingly employed to examine the internal structures of casein micelles (Loupiac, 2016; de Kruif, 2014). However, *in situ* investigations on the effect of HHP on milk systems using SAXS or SANS are very few (Jackson & McGillivray, 2011; Tromp, Huppertz, & Kohlbrecher, 2015). Jackson and McGillivray (2011) employed SANS and Ultra-small angle neutron scattering (USANS) to investigate *in situ* casein micelles structural changes up to 350 MPa at room temperature, and showed that HHP dissociated the casein micelles into smaller "sub-micelles". Tromp et al. (2015) used SANS to study *in situ* casein micelles disruption up to 300 MPa at room temperature (~23 °C), with each pressure applied for 45–60 min. Focusing on the size distribution changes, they demonstrated that the pressure-induced disruption of the casein micelles was partially reversed after releasing pressure back to ambient. It is worth noting that there are two limitations in these studies. Firstly, the HHP treatments were performed up to 300–350 MPa, which is lower than the maximum pressure achievable on most commercial HHP equipment (Yang, Chaib, Gu, & Hemar, 2017). Secondly, a characteristic scattering peak at q~ 0.08 $Å^{-1}$ corresponding to the CCP nanoclusters (Bouchoux, Gésan-Guiziou, Pérez, & Cabane, 2010; Gebhardt, Takeda, Kulozik, & Doster, 2011) was not resolved in

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previous *in situ* SANS studies. Either the SANS studies did not extend to sufficiently high-q to observe this feature (Tromp et al., 2015), or it is significantly broader and less predominant than in the SAXS spectra (de Kruif, 2014; Ingham et al., 2016).

In this study, synchrotron SAXS is used to probe *in situ* the effect of HHP on the internal structure of casein micelles up to ~1000 MPa. Synchrotron SAXS has the advantage over lab-bench SAXS, due to its higher intensity and collimation, thereby enabling the scattering patterns to be collected in real-time. SAXS has also an advantage over SANS as the measurements are very fast and require much smaller sample volume. This study is the first to report *in situ* synchrotron SAXS measurements on milk under HHP using a diamond anvil cell (DAC), in order to examine the internal structure changes of casein micelles under HHP. The reversible effects occurring upon releasing the pressure to ambient are also considered.

2. Materials and methods

2.1. Materials and sample preparation

Skim milk (Trim Anchor milk, Fonterra, New Zealand), a pasteurised skim milk with a content of 4 g/100 ml protein; 5 g/100 ml carbohydrate; and a total fat of 0.1 g/100 ml, was purchased from a local store. The milk was freeze dried into a powder for 4 days to allow long term storage. Prior to measurements, the milk powder was reconstituted in Milli-Q water by gentle mixing of the milk powder (10 wt%) under magnetic stirring for at least 2 h. The reconstituted milk was left overnight in the fridge at 4 °C to ensure full hydration. The milk (1 mL) was left to equilibrate at room temperature for at least 2 h before measurements.

2.2. Methods

Small amounts of milk samples were loaded along with micron size ruby balls, used as an internal pressure sensor, in a 0.5 mm diameter hole of a 0.2 mm thick gasket. A DAC (easylab, UK) was employed to apply the pressure. The four cap screws were tightened manually step by step to generate pressures of 160 MPa, 270 MPa, 380 MPa, 460 MPa, 570 MPa, and 960 MPa, incrementally. The pressure was measured from the shift of the ruby fluorescence using a LabRAM HR Evolution Raman system (Horiba scientific, France) with an accuracy of ±10 MPa (Piermarini, Block, Barnett, & Forman, 1975). Once the targeted pressure was reached, the DAC was loaded into the beamline to enable *in situ* collections of SAXS patterns. The typical data acquisition time for one SAXS pattern is about 4 min. At each pressure, three SAXS patterns were collected for a total time of about 12 min. After reaching the highest pressure (960 MPa), the pressure was immediately released to ambient pressure by unscrewing the DAC. The SAXS patterns were collected 20 min, 40 min, 60 min, and 80 min after the pressure was released back to ambient pressure.

In situ synchrotron SAXS experiments were performed on the BL19U2 BioSAXS beamline of National Centre for Protein Science Shanghai (NCPSS) at the Shanghai Synchrotron Radiation Facility (Shanghai, China). The sample-to-detector distance was set such that the detecting q range was between 0.003 and 0.17 $Å^{-1}$, $q = (4\pi\sin\theta)/\lambda$ (where θ is the scattering angle and $\lambda = 1$ Å is the X-ray wavelength). Scattered X-ray intensities were collected using a Pilatus 1M detector (DECTRIS Ltd.). The measured SAXS profiles of the milk samples at various pressures were reduced, normalized and averaged using the BioXTAS RAW software version 1.2.3. (Nielsen et al., 2009). All the measurements were performed at room temperature (~25 °C).

3. Results and discussion

In situ SAXS patterns of skim milk, under HHP, are reported in Fig. 1A, and the patterns are shifted in the y-axis direction for clarity. At atmospheric pressure, the SAXS spectra agrees well with previous SAXS studies of casein micelles reported in the literature (Bouchoux et al., 2010; Day et al., 2017; Ingham et al., 2016; Marchin, Putaux, Pignon, & Léonil, 2007; Shukla, Narayanan, & Zanchi, 2009). More specifically, the scattering in the low q region

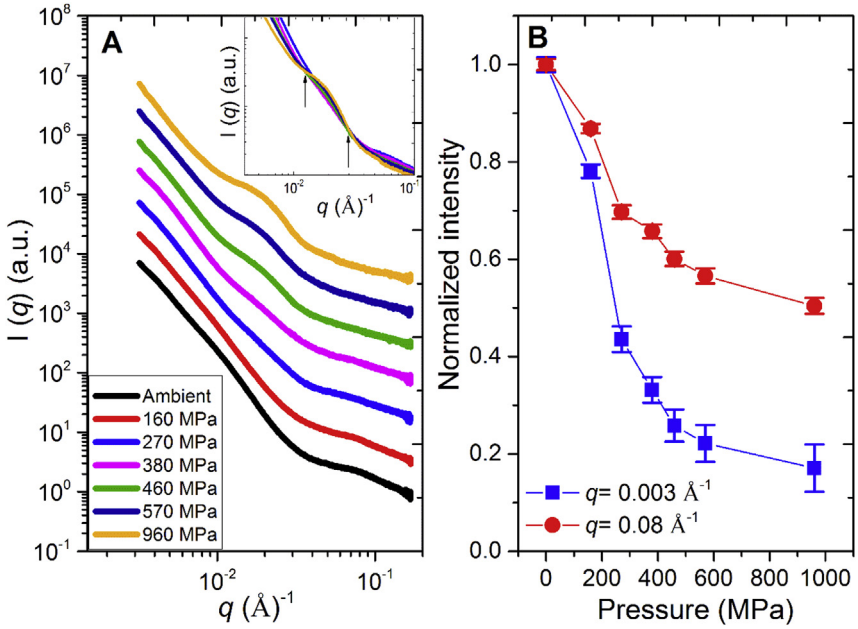

Fig. 1. (A) *In situ* synchrotron SAXS patterns of skim milk solution (10 wt%) under various pressures, the isosbectic point is indicated by an arrow as depicted in the insert. The scattering curves are vertically shifted for clarity. (B) Normalized scattering intensities at low- (0.003 $Å^{-1}$) and high-q (0.08 $Å^{-1}$) values with increasing pressures from atmospheric pressure to 960 MPa.