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Application of confocal Raman microscopy to investigate casein micro-particles in blend casein/pectin films

Yu Zhuang[a], Julia Sterr[b], Ulrich Kulozik[c], Ronald Gebhardt[a,*]

[a] Chair of Food Process Engineering and Dairy Technology, Technische Universität München, 85354 Freising-Weihenstephan, Germany
[b] Chair of Food Packaging Technology, Technische Universität München, 85354 Freising, Germany
[c] Research Centre for Nutrition and Food Science (ZIEL) – Section Technology, Technische Universität München, 85354 Freising-Weihenstephan, Germany

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ABSTRACT

Pectin triggers formation of casein micro-particles during solution casting. Confocal Raman microscopy revealed their composition and spatial dimension in resulting films. Peaks in the Raman spectra corresponded to those found in films prepared by either casein or pectin. This suggested that no conformational changes occurred after mixing. Raman images revealed incompatibility of both polymers because particles consisted of casein only and the surrounding matrix of pectin. Deformation of micro-particles into an oblate shape took place during film formation. In dried films, an empty space between casein and pectin was found in lateral dimension. In contrast, casein micro-particles overlapped with the pectin matrix in the vertical dimension.

1. Introduction

Micro-particles from protein have been of interest in a number of applications involving carrying or releasing of bioactive compounds [1–3]. Caseins (αs1-, αs2-, β-, and κ-caseins), the abundant protein in milk, are receiving increasing attention from the food, cosmetics and pharmaceutical industries. They are organized in micelles (CM) together with colloidal calcium phosphate [4]. Integrity of CM is ensured by electrostatic and hydrophobic interactions [5] while steric stabilization is provided by an outer surface layer [6].

The addition of polysaccharides have been used to introduce new functionality in foodstuffs [7,8]. Pectin is an anionic polysaccharide, which consists of a partially methyl-esterified galacturonic acid backbone with branches of arabinose, galactose and xylose. It is commonly utilized as a stabilizer, gel former and/or viscosity enhancer in protein rich dairy products [9]. High-methoxyl pectin (HMP) has a high degree of methyl-esterification (degree of methyl esters >50%) and is efficient in textural modification and stabilization of acidified milk dairy products [10,11].

The mechanism of complex formation between CM and HMP at various pHs has been widely discussed. At neutral pH, individual molecules are mostly negatively charged and normally separated from each other due to electrostatic repulsion [12]. Under these conditions pectin molecules are expelled from CM. This phenomenon is generally related to volume exclusion behavior in two component systems. Excluded volume of large polymers, i.e. CM, restricts the accessibility of small polymers, i.e. pectin. This may lead subsequently to depletion flocculation of large molecules [13].

When pH decreases to pH 4.6 (isoelectric point of CM), the micelles become unstable and start to form flocculated aggregates. This is because of collapse of the 'hairy' κ-casein layer [14]. At low pH, pectin is able to bind on the surface of CM. Bridging between neighboring CM occurs at low pectin concentrations which may lead to subsequent aggregation [12]. In contrast, a surface layer of pectin is formed at high carbohydrate concentrations preventing interconnection between CM.

Films can be used to study phase transformation [15], crystal structure formation [16] and deformation [17] of biopolymers. It has been shown that particles rearrange and concentrate by capillary force on a substrate at the early stage of film drying [18]. Later, drying forces compress the particles in vertical direction leading to a deformed shape [18]. Thereafter, biopolymers are embedded within the dried film matrix [15].

Raman microscopy is suitable to visualize the structural homogeneity or composition of biopolymer films [19]. Raman scattering provides information on the vibrational modes which are characteristic of chemical groups within the sample [20,21]. By combining confocal Raman scattering with high resolution scans, it is possible to analyze the distribution of components in spatial dimension.

* Corresponding author. Tel.: +49 8161 71 3536; fax: +49 8161 71 4384.
E-mail address: ronald.gebhardt@tum.de (R. Gebhardt).

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Previous researchers successfully used confocal Raman microscopy to localize micro-components in cells or tissues [22–24]. Another advantage of confocal Raman spectroscopy is that it offers a possibility to look inside the samples in a non-destructive way [25,26].

In this study we investigated casein micro-particles in films after addition of pectin to a solution of CM prior to film formation. We used confocal Raman microscopy to explore the spatial distribution of the polymers in the film matrix. This allowed us to gain molecular information about the micro-particles concerning their structure, composition and flexibility, which is not easily accessible in solution.

2. Materials and methods

2.1. Materials

CM were extracted from raw skim milk (Molkerei Weihenstephan GmbH & Co, Germany) by centrifugation for 65 min at 31,000 rpm and 25 °C [27]. A Bis-Tris base buffer (50 mM Bis-Tris with 10 mM $CaCl_2$) was used to dissolve the pellets. After stirring for 5 h at 37 °C, dust and large casein aggregates were separated by a third centrifugation step (6000 rpm at 25 °C for 10 min). Thereafter, the solution was again centrifuged for 15 min at 12,000 rpm. The supernatant containing the size-fractionated CM was used for the structural study in a final concentration of w/w = 3%, similar to the casein concentration in conventional milk.

HM pectin (Herbstreith & Fox KG, Germany) was dissolved in the same Bis-Tris buffer at 80 °C and adjusted to pH 6.8 for further use.

After mixing at room temperature, the protein concentration in solution was 3% (w/w). The final ratio between casein and HM-pectin was 1:0.1 (w/w). Solutions of 0.1 M HCL and 0.1 M NaOH were used for pH adjustment. After stirring for 15 min at 20 °C, the CM/HMP mixture was stored in a cold room (4 °C) for 24 h.

2.2. Film preparation

After pH adjustment, 2 mL of the CM/HMP matrix was carefully deposited on microscope slides (VWR international, Germany). All slides were placed in an isolated box of 28.8 cm × 40.5 cm × 15.7 cm (length × width × height) at a constant temperature of 20 °C and relative humidity of 30% for drying. Temperature and relative humidity were monitored by Almemo 2490-1sensor for more than 36 h. Thereafter, resulting films were removed from the drying chamber and stored at room temperature for Raman measurements.

2.3. Confocal Raman measurements

Raman spectra were obtained at room temperature using a confocal Raman microscope (Alpha 500, WITec GmbH, Germany) equipped with a Nd:Yag Laser (λ = 532 nm). The polarized laser passes through a polarization-preserving single-mode optical fiber to the microscope. The beam is deflected by a dichroic beam splitter to a microscope objective (here: 100× Nikon objective, NA = 1.0) and focused on the sample. The laser polarization is set in the *x*-direction. The scattered Raman radiation passes the same objective and is focused by a multi-mode fiber (here 50 μm core diameter), which is connected to a UHTS 300 spectrometer with a black-illuminated charged coupled device (CCD) camera (Newton DU970 N-BV, Andor, Inc., cooled to −59 °C) and a grating with groove density of 600 g/mm and 500 nm blaze wavelength. A typically Raman image was recorded collecting single spectra at every image pixel (80 × 80 pixel, total 6400 spectra) with a lateral size of 60 μm × 60 μm (horizontal area scan) and 60 μm × 20 μm (vertical depth scan). The integration time was 37.02 ms per spectrum.

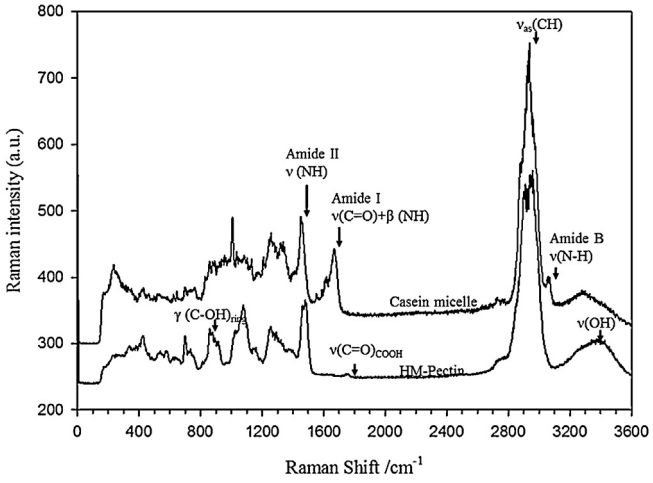

Fig. 1. Confocal Raman spectra of films consisting of either CM or HM-pectin.

For Raman data analysis the program WITec Project (version 2.10, WITec GmbH, Germany) was used. The Raman images showing the distribution of certain constituents were calculated by integrating the corresponding Raman band region of each spectrum in the image. The integrated value was then displayed with a special color profile, where bright colors represent high and dark colors represent low integrated values.

3. Results and discussion

We used confocal Raman microscopy to identify the spatial distribution of casein and HM-pectin in dried films. At first we measured reference spectra by applying Raman spectroscopy to films consisting of either casein or pectin. The peaks in the spectra are assigned to characteristic vibrational modes as shown in Fig. 1. The Raman spectrum of CM showed strong intensities at 1669 cm^{-1}, 1454 cm^{-1} and 3065 cm^{-1} corresponding to the Amide I, II and B bands, respectively. They are typical assignments for protein backbone vibrations, as stated in literature [28]. For HM-pectin we assigned the peaks at 699 cm^{-1} and 1748 cm^{-1} to out-of-plane vibration of hydroxyls (ring configuration of HM-pectin) and to the C=O stretching of COOH groups, respectively [29]. Intense bands between 2852 cm^{-1} and 2988 cm^{-1} are visible in both HM-pectin and casein spectra. These bands belonged to the stretching of C—H bonds and their different shapes suggested the varying contributions from CH_2 and CH_3 modes [30]. The band between 3200 cm^{-1} and 3500 cm^{-1} corresponded to the inter-molecular hydrogen bonding of the polymers [29].

We mixed 3% casein with HM-pectin (mixing weight ratio 1:0.1) and prepared films using solution casting. Fig. 2 shows the resulting film morphology at different magnification. Contrary to films consisting of casein or pectin only (see S1 – supplementary notes), films of casein/pectin mixtures had a turbid appearance as the digital photograph in Fig. 2A shows. Optical micrographs at 10× (Fig. 2B) and 100× (Fig. 2C) magnifications provide a more detailed view. Spherical structures of different size are distributed throughout the film surface. We applied scanning Raman microscopy to access information about the lateral and vertical structure of three spherical objects as shown in Fig. 2C. A lateral scan was applied over the whole area of Fig. 2C. The black line in the same figure indicates where the vertical depth scan (*X*–*Z* direction) was performed.

We grouped the spectra of the scans into three classes: spherical structure, surrounding and border region. Fig. 3 shows averaged spectra of the three groups. While spectra sampled inside the spherical structures belonged to casein, those in the surrounding