Food Hydrocolloids 45 (2015) 168–174

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd

Roughness analysis of single nanoparticles applied to atomic force microscopy images of hydrated casein micelles

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ARTICLE INFO

Article history:
Received 28 April 2014
Accepted 10 November 2014
Available online 20 November 2014

Keywords:
Atomic force microscopy
Casein micelle
Nanoparticle
Surface roughness
Image analysis

ABSTRACT

The topography of fully hydrated casein micelles was measured by Atomic Force Microscopy and quantitatively investigated by a custom-made roughness analysis procedure. Casein micelles in ultra filtrated permeate (UFP) were chosen as a typical example of food nanoparticles in order to validate the approach. Casein micelles were first covalently immobilized on solid substrates by carbodiimide chemistry and subsequently a number of individual particles were scanned at high resolution. Parameters for characterizing single particle surface roughness were defined and implemented in MATLAB. An essential feature of the analysis is the discrimination between overall particle shape and short-scale surface roughness. Parameters determined were: Root-mean-square roughness, ratio of area to projected area, particle compression and particle size. The method is generally applicable to particles with radii from ~15 nm–1 μm and will be useful for monitoring changes in hydrocolloid surface morphology in relation to environmental changes or industrial processing.

1. Introduction

The characterization of soft nanoparticles is of critical importance to a number of areas such as food science and drug formulation. Typical approaches are confined to the measurement of size distributions, which are readily available from a number of optical techniques such as light scattering. However, particle properties are not only determined by the hydrodynamic radius, but will also depend on the detailed shape and topography of single particles, in addition to their charge and chemical composition. An example is the exposed surface area, which is highly dependent to particle roughness and which will influence chemical reactivity.

Techniques such as Dynamic Light Scattering (DLS) and Static Light Scattering (SLS) measurements are used for obtaining the average size and shape of particles ranging from a few nanometers to micrometers in solution (Schärtl, 2007). Scanning Electron Microscopy (SEM) is often used for large matrices (Dudkiewicz et al., 2011) or powders (Wei, Dave, & Pfeffer, 2002) and provide insight at larger length scales depending on the circumstances and resolution limit (Danino, 2012). Fluorescence microscopy has been applied for more than a decade in food systems, for visualizing large structures in the micrometer range (Færgemand & Qvist, 1997; Hassan, Ipsen, Janzen, & Qvist, 2003).

Common for the above mentioned techniques concerning single particles, is that the quantitative information is mainly restricted to size, *e.g.* for DLS only the average size (Montasser, Fessi, & Coleman, 2002), and in some cases the average particle shape. For solid crystalline particles size might be sufficient, however there is a lack of analytical tools for particles with a nontrivial surface topography, porosity and complex internal structure. Typical examples of such particles are aggregated proteins and polymer clusters.

A quantitative approach to describing particle surface topography is by roughness analysis, which in surface physics is commonly used to analyze planar surfaces, but the same concepts can be used to describe surface structures of 3D objects, if the overall particle shape can be separated from the short-scale roughness. Such an analysis requires detailed data for the surface topography, and while a technique such as electron microscopy typically gives 3-dimensional information of a more qualitative character, the lack of accurate quantitative topographical information makes it difficult to perform roughness computations on *e.g.* SEM images.

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http://dx.doi.org/10.1016/j.foodhyd.2014.11.008
0268-005X/

A better technique to provide data for quantitative analysis is AFM, which gives highly resolved spatial information in the form of topographical maps. AFM supplies direct measurements without elaborate sample preparation as highlighted in a review by Sitterberg, Zcetin, Ehrhardt, and Bakowsky (2010) regarding nanoscale drug delivery systems. It has provided roughness measurements in various food systems for more than a decade (Baldwin, Adler, Davies, & Melia, 1998; Liu & Cheng, 2011), however mainly describing multiple particles on planar surfaces. By combining single particle AFM with surface topography analysis, particle surfaces in the nanometer size range, with various particle shapes both under dry and hydrated conditions can be analyzed.

AFM requires the immobilization of particles to a solid support which, for most particles, can be achieved via electrostatic interaction or an immobilization procedure (Martin, Douglas Goff, Smith, & Dalgleish, 2006). Soft particles can be scanned using low impact imaging modes such as magnetically alternating current mode (MAC-mode) (Han, Lindsay, & Jing, 1996).

Casein micelles are intrinsically disordered soft hydrocolloids, whose structure have been under scrutiny for more than a century (Fox & Brodkorb, 2008). In most mammals, casein micelles comprise four casein protein subtypes, α_{s1}, α_{s2}, β and κ, which are held together by calcium phosphate nanocrystals (Fennema, 2007; McSweeney & Fox, 2013). The spatial distribution of the proteins throughout the micelle along with the primary structure of the caseins has been studied intensively, but a definitive understanding of the casein micelle structure is still missing. Studies of the internal structure of casein micelles have been done using X-ray and neutron scattering (de Kruif, Huppertz, Urban, & Petukhov, 2012) and Nuclear Magnetic Resonance (NMR) (Gobet et al., 2013). However, the lack of a well-defined tertiary and quaternary protein structure, has made a full description of the casein micelle more difficult. Electron microscopy has previously been applied to study the internal casein micelle structure (Karlsson, Ipsen, & Ardö, 2007) and reports by Dalgleish, Spagnuolo, and Goff (2004) and McMahon and Gommen (2008), have used electron microscopy images to support particular models of the casein micelle. Dalgleish and Corredig have emphasized the importance of better data for the surface structure of casein micelles (Dalgleish & Corredig, 2012). Previous reports of individual casein micelles or casein particles visualized by AFM (Gebhardt, Doster, Friedrich, & Kulozik, 2006; Lin et al., 2014; Ouanezar, Guyomarc'h, & Bouchoux, 2012), have been concerned mainly with obtaining the particle height and volume.

In the present study we introduce a powerful method for analyzing surface roughness of multiple single particles under hydrated conditions, using single casein micelles bound to a chemically modified gold surface as a test case to validate the method. When introducing the quantitative analysis of particle topography we define roughness and shape parameters at the level of single particles. These are designed to be sensitive to changes in the surface structure and shape of particles resulting from different preparation conditions and/or environments.

2. Materials

2.1. Materials and apparature

All Chemicals were analytical grade: NH_4OH (28–30%), H_2O_2 (34.5–36.5%), EDC,[1] N-Hydroxysuccinimide, 11-MUA (11-Mercaptoundecanoic acid), DMSO (Dimethyl sulfoxide) (≥99.5%) (Sigma-Aldrith, Schnellendorf, Germany), EtOH (99.99%) (Kemetyl, Køge, Denmark), MilliQ H_2O (Millipore A/S, Hellerup, Denmark). Other materials: Silicon wafers (Si(100)) (Plano GmbH, Wetzlar, Germany).

Equipment used for substrate and sample preparation: Plasma Cleaner (Harrick Plasma, Ithaca New York, USA), Centrifuge Cr3i (ThermoFischer Scientific, Waltham Massachusetts, USA), E-beam evaporator Discovery 502 (Polyteknik AS, Østervrå, Denmark).

AFM imaging was done using a picoSPM operated in MAC-mode (Molecular Imaging, Phoenix Arizona, USA) and using a custom made fluid cell. Cantilevers were MAC levers type VII (Agilent Technologies, Arizona, USA) with a force constant of 0.14 N/m, a tip radius of lower than ~5 nm operated at 8–10 kHz. In the image analysis *SPIP*™ (Image Metrology, Hørsholm, Denmark) was used for single particle isolation and *MATLAB* (Mathworks, Boston, USA) was used for particle isolation from the substrate background, separation of overall spherical particle shape from the short-scale surface roughness variations and quantitative surface roughness analysis.

3. Methods

3.1. Substrate and sample preparation

Silicon wafers were prepared by chemical cleaning in a 4:1:1 volume solution of H_2O(l), NH_4OH (~29%) and H_2O_2 (~35.5%) (1 h, 100 °C), subsequently rinsed 3 times with MilliQ H_2O and dried under a stream of N_2. The clean slides were coated with 5 nm Ti followed by 35 nm Au using E-beam evaporation. For covalent immobilization of casein micelles, carbodiimide chemistry (Martin et al., 2006) was used to ensure a uniform lateral distribution of casein micelles on the slides. The slides were first suspended in a solution of 11-MUA in 100% EtOH. After 24 h they were rinsed 3 times with EtOH, dried under a stream of N_2 and subsequently exposed to a 2% solution of EDC in DMSO, followed by 3 times rinsing with DMSO and a 2% solution of N-Hydroxysuccinimide in DMSO, followed by 3 times rinsing by DMSO. After drying under a stream of N_2 the slides were immediately used in the next step.

Casein micelle solutions were made skimming fresh milk from a local farm (centrifugation at 1000 *g*, 20 °C, 1 h) less than 3 h before use.

Samples were prepared by immersing coated slides in the casein micelle solution (20 °C, 2 h) followed by an exchange of the supernatant 5 times with ultra filtrated permeate (UFP), ensuring removal of suspended casein micelles that may interfere with cantilever approach or adhere to the cantilever tip.

3.2. AFM imaging

Imaging was done by an AFM operated in MAC-mode, which employs magnetic excitation of the cantilever vibration. By using soft MAC-cantilevers, there is less sample perturbation than traditional mechanical excitation. The sample slides are placed in a custom made fluid cell filled with 0.8 mL UFP during the final preparation step.

During scanning the cantilever and piezo prism is immersed into the solution. To prevent broadening of the particles on the surface, sharp cantilevers were used and deformation of the casein micelle topography was avoided by using a low free cantilever amplitude. A low free amplitude minimizes the energy deposited by the cantilever onto the sample, ΔE, which is equal to the difference between the working amplitude, z, and the free amplitude, z_0 of the cantilever, where $\Delta E = k((z_0)^2 - (z)^2)/2$. During the scanning of the surface, both up- and downwards raster-scans were checked, to make sure no changes happened to the casein micelles during scanning.

[1] N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride.