Available online at www.sciencedirect.com

ScienceDirect

Mendeleev Commun., 2021, **31**, 88–90

Mendeleev Communications

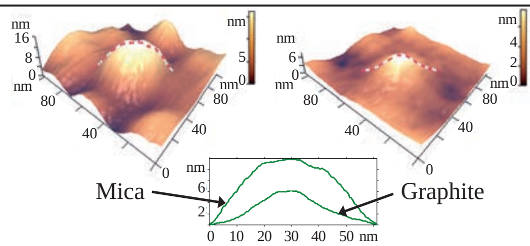
Atomic force microscopy characterization of β-casein nanoparticles on mica and graphite

Olga V. Sinitsyna* and Mikhail M. Vorob'ev

A. N. Nesmeyanov Institute of Organoelement Compounds, Russian Academy of Sciences, 119991 Moscow, Russian Federation. Fax: +7 499 135 5085; e-mail: sinitsyna@gmail.com

DOI: 10.1016/j.mencom.2021.01.027

The morphology and size distribution of β-casein nanoparticles adsorbed on mica and graphite were determined using atomic force microscopy. The height of the dried nanoparticles on graphite was about twice as smaller as that on mica, and the cross-sectional profiles of particles were different for these substrates. It is due to the hydrophobic interaction of nanoparticles with the graphite surface.



Keywords: β-casein, AFM, micelles, enzymatic hydrolysis, mica, graphite, hydrophobic interaction.

Interest in β-casein micelles stems from their potential as a delivery vehicle for water-insoluble drugs.[1–4] As a result of enzymatic hydrolysis of β-casein, new polypeptide nanoparticles with different sizes and different ability to interact with hydrophobic low-molecular-weight substances can be obtained.[5] In experiments on static light scattering,[6] the formation and subsequent degradation of β-casein particles with a size of 10–200 nm were observed during partial hydrolysis of β-casein by trypsin. In the course of hydrolysis, the maximum concentration of nanoparticles was achieved at a low degree of hydrolysis of peptide bonds (0.5–2%).[4,6,7] Since modified β-casein particles are polydisperse and the mechanism of their formation and further hydrolysis may depend on their size, it is important to study their size distribution in detail. Atomic force microscopy (AFM) was chosen for this purpose,[7,8] since it gave the highest spatial resolution without additional staining, shading or freezing,[9,10] and proved to be informative when studying the size distribution and morphology of food proteins and their molecular aggregates.[11] However, to obtain an image, the particles under examination must be attached to a smooth surface. The most common substrates are mica and graphite. Their important advantages are their ultra-low roughness and easy preparation of a clean surface by cleavage. Mica is hydrophilic;[12] in water, it acquires a negative charge due to the leaching of potassium ions.[13] Graphite is considered a hydrophobic substrate since its contact angle with water is about 90°.[14] The aim of this work was to study the effect of the substrate on the shape and size of native β-casein micelles and nanoparticles formed after their partial hydrolysis.

β-Casein micelles were adsorbed on mica and graphite before and after their modification and then studied by AFM.[†] The medium fraction of particles ranging in size from 30 to 100 nm was analyzed. Too large and too small particles were excluded from consideration. An increase in the dilution factor was required to study nanoparticles less than 30 nm in size. A detailed analysis of small nanoparticles was performed in a previous study.[8]

The representative images of β-casein particles on mica and graphite surfaces are shown in Figure 1. Axially symmetric particles are visible on both surfaces. AFM images show no

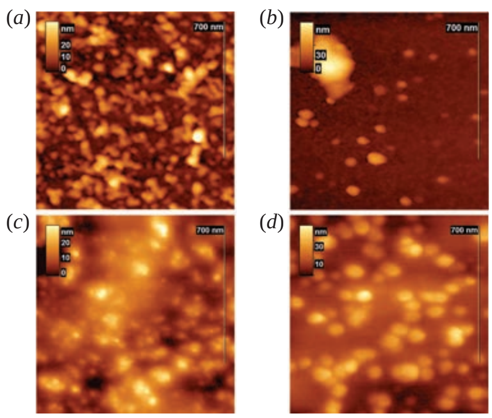


Figure 1 AFM images of β-casein particles on the surface of (*a*,*b*) mica and (*c*,*d*) graphite (*a*,*c*) before and (*b*,*d*) after hydrolysis.

[†] β-Casein (C6905, Sigma–Aldrich) was solubilized in 50 mM phosphate buffer to reconstitute micelles. Hydrolysis of β-casein micelles (10 ml, 3.0 g dm^{-3}) was initiated by adding a trypsin solution (10 μl, 1 g dm^{-3}). Soybean trypsin inhibitor was used with a mass ratio of trypsin to the inhibitor of 1 : 3 to terminate hydrolysis. In our experiment, the concentration of β-casein significantly exceeds the critical micelle concentration (0.5 g dm^{-3}),[15] which can contribute to the formation of micelles in a wide range of sizes. Details of the preparation and hydrolysis of micelles have been published recently.[8] Portions of the solution, taken before and after hydrolysis, were diluted 10 times with Milli-Q water, and 2.5 μl of samples were immediately applied to the freshly cleaved surface of mica and graphite (HOPG with a mosaic angle of 0.8 ± 0.2º). The samples were left to dry under ambient conditions for one day. AFM measurements were carried out in the air using a FemtoScan scanning probe microscope (Advanced Technologies Center) in the tapping mode. We used ScanSens cantilevers (ETALON series) with a resonant frequency of 230 and 380 kHz and a tip radius of about 10 nm. To minimize particle deformation, scanning was performed under the mildest conditions making it possible to obtain a stable image. FemtoScan Online software[16] was used for image processing and analysis. Surface cuts were made through the peaks of the nanoparticles at the angle of 45° to the fast scanning axis. They were used to measure the height (h) and width (d) of particles. The apparent contact angle (θ) was calculated by the height–width method using the following equation: $\theta = 2\mathrm{arctg}(2h/d)$.

Table 1 Height (h), width (d), density (h/d) and apparent contact angle (θ) of the β-casein particles adsorbed on different substrates.[a]

Substrate	h/nm	d/nm	h/d	θ/°
Before hydrolysis				
mica	11 ± 5	49 ± 13	0.21 ± 0.07	45 ± 13
graphite	6 ± 3	53 ± 19	0.10 ± 0.04	23 ± 9
After hydrolysis				
mica	25 ± 12	54 ± 14	0.45 ± 0.14	82 ± 18
graphite	12 ± 5	71 ± 14	0.16 ± 0.06	35 ± 12

[a] 50 particles were measured in each group.

influence of the graphite cleavage steps on the micelle arrangement. Therefore, the adsorption of the β-casein micelle was related to the interaction with the basal plane of graphite. The same applies to mica since the scan areas were selected without defects. The results of particle size measurements are summarized in Table 1. Particle size distribution before and after partial enzymatic hydrolysis is shown in Figure 2. The average diameter of native micelles on both substrates is about 50 nm. The height of micelles on graphite is approximately half that on mica. The height-to-diameter (h/d) ratio used to estimate the density of dried particles is also smaller for micelles adsorbed on graphite.

For the particles on mica, it was previously shown that enzymatic hydrolysis leads to an increase in the particle density (h/d), probably due to the shrinkage of micelles in solution.[8] In the case of graphite, we observed changes both in the density and in the shape of the particles. If the shape of the native micelles is close to a spherical cap on mica, then on graphite, the particles have a high center and flattened edges. It can be assumed that the disruption of micelles occurs due to the dominant hydrophobic interaction of β-casein with graphite as a hydrophobic surface.[17] β-Casein micelles have a detergent-like micellar structure with a hydrophobic core and a charged hydrophilic exterior.[15] The absence of covalent bonds between casein molecules in the micelle core can lead to the exposure of its hydrophobic interior to the substrate.[18] An additional driving force for the binding of β-casein to graphite may be π–π stacking interaction between the top graphite layer and aromatic amino acids in the protein molecule.[19,20] For hydrolytically modified micelles, their shape on graphite becomes closer to the spherical cap, as on mica, which may indicate their greater stability.

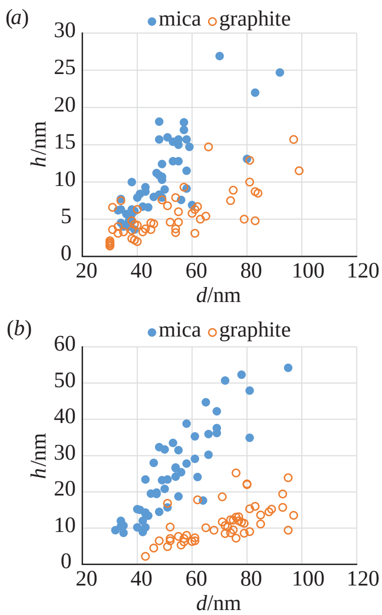


Figure 2 Particle size distribution of β-casein particles adsorbed on mica and graphite (*a*) before and (*b*) after partial hydrolysis.

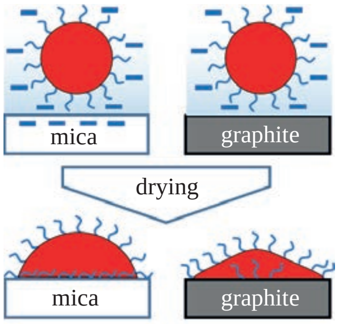


Figure 3 Schematic diagram of adsorption of a native micelle on mica and graphite. 'Hairs' denote the negatively charged hydrophilic N-terminal parts of β-casein molecules.

The contact angle, which in our approximation is a function of h/d, is also smaller for graphite. This can be explained as follows. In an aqueous solution, both the surface of mica and micelles have negative charges, the latter due to the N-terminus of the β-casein molecule. Electrostatic repulsion prevents adsorption and 'spreading' of micelles over the surface. It can be assumed that the contact of a micelle with mica is formed at the final stage of drying the solution. Unlike mica, probably a positive charge can be induced on the surface of conductive graphite by an approaching micelle, which leads to an electrostatic attraction between the micelle and graphite. The micelle is anchored to the surface, and its rearrangement begins, driven by hydrophobic interaction between graphite and the highly hydrophobic C-region of β-casein. The processes described above are illustrated in Figure 3.

According to published data,[17] the height of the β-casein monolayer on a hydrophobic surface is about 3 nm. Taking into account the shape and height of native micelles on graphite (about 6 nm), it can be assumed that a bilayer film is formed in the central part, and a monolayer film is formed at the edges after the disruption of the native β-casein micelle.

In conclusion, it should be noted that the choice of substrate is critical for AFM studies of structures such as micelles, in which macromolecules are not covalently bound, since interaction with the surface can cause significant distortion and even destruction of the adsorbed particles. Although the particles under investigation can vary in shape, AFM can be effectively used to track the relative changes in particles during their modification.

This work was supported by the Russian Foundation for Basic Research (project no. 20-53-46006) and the Ministry of Science and Higher Education of the Russian Federation.

References

1 A. O. Elzoghby, W. M. Samy and N. A. Elgindy, *J. Controlled Release*, 2012, **161**, 38.
2 S. A. Forrest, R. Y. Yada and D. Rousseau, *J. Agric. Food Chem.*, 2005, **53**, 8003.
3 A. Shapira, Y. G. Assaraf and Y. D. Livney, *Nanomedicine*, 2010, **6**, 119.
4 M. Bar-Zeev, Y. G. Assaraf and Y. D. Livney, *Oncotarget*, 2016, **7**, 23322.
5 M. M. Vorob'ev, V. S. Khomenkova, O. V. Sinitsyna, O. A. Levinskaya, D. K. Kitaeva, A. V. Kalistratova, M. S. Oshchepkov, L. V. Kovalenko and K. A. Kochetkov, *Russ. Chem. Bull., Int. Ed.*, 2018, **67**, 1508 (*Izv. Akad. Nauk, Ser. Khim.*, 2018, 1508).
6 M. M. Vorob'ev, K. Strauss, V. Vogel and W. Mäntele, *Food Biophysics*, 2015, **10**, 309.
7 O. V. Sinitsyna and M. M. Vorob'ev, *INEOS Open*, 2019, **2**, 50.
8 M. M. Vorob'ev and O. V. Sinitsyna, *Int. Dairy J.*, 2020, **104**, 104652.
9 G. Binnig, C. F. Quate and C. Gerber, *Phys. Rev. Lett.*, 1986, **56**, 930.
10 A. P. Gunning and V. J. Morris, *Food Hydrocolloids*, 2018, **78**, 62.
11 C. Shi, Y. He, M. Ding, Y. Wang and J. Zhong, *Trends Food Sci. Technol.*, 2019, **87**, 14.

12 S. Nishimura, S. Biggs, P. J. Scales, T. W. Healy, K. Tsunematsu and T. Tateyama, *Langmuir*, 1994, **10**, 4554.
13 M. V. Maslova, L. G. Gerasimova and W. Forsling, *Colloid J.*, 2004, **66**, 322 (*Kolloidn. Zh.*, 2004, **66**, 364).
14 S. Wang, Y. Zhang, N. Abidi and L. Cabrales, *Langmuir*, 2009, **25**, 11078.
15 J. E. O'Connell, V. Y. Grinberg and C. G. de Kruif, *J. Colloid Interface Sci.*, 2003, **258**, 33.
16 I. Yaminsky, A. Akhmetova and G. Meshkov, *Nanoindustriya*, 2018, **11**, 414 (in Russian).
17 L. Pérez-Fuentes, C. Drummond, J. Faraudo and D. Bastos-González, *Materials*, 2017, **10**, 893.
18 K. Helstad, M. Rayner, T. van Vliet, M. Paulsson and P. Dejmek, *Food Hydrocolloids*, 2007, **21**, 726.
19 S. Kumar and S. H. Parekh, *Commun. Chem.*, 2020, **3**, 8.
20 J. Tian and C. Ge, in *Biomedical Applications and Toxicology of Carbon Nanomaterials*, eds. C. Chen and H. Wang, Wiley-VCH, Weinheim, 2016, pp. 97–130.

Received: 2nd September 2020; Com. 20/6301