rearrangement is initiated by the presence of a heteroatom and there must be other reactions similar to this one.

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[6] The photochemical reaction was carried out in degassed dichloromethane solution in a Pyrex tube using a Rayonet reactor (350 nm) at room temperature. The product **12** was isolated by column chromatography on silica gel, no other by-products were observed in the crude ^{1}H NMR spectra. **12**: ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 7.59$ (d, $J = 2.2$ Hz, 1H), 7.52 (d, $J = 0.8$ Hz, 1H), 7.40 (d, $J = 8.5$ Hz, 1H), 7.27 (dd, $J = 1.7$, 8.5 Hz, 1H), 6.72 (dd, $J = 0.8$, 2.2 Hz, 1H), 6.53 (d, $J = 12.2$ Hz, 1H), 6.17 (d, $J = 12.2$ Hz, 1H), 5.02 – 4.97 (m, 2H), 1.70 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 154.0$, 145.2, 142.1, 132.7, 132.1, 129.5, 127.1, 125.4, 121.3, 116.9, 110.6, 106.6, 22.1; MS (70 eV, EI): *m*/*z* (%): 184 (67, $[M^+]$), 169 (100, $[M^+ - CH_3]$), 155 (59), 141 (89), 115 (39), 105 (41), 91 (26), 77 (26); HR-MS: calcd for $C_{13}H_{12}O$: 184.0888; found: 184.0882.

[7] Spectral data for compound **13**: ^{1}H NMR (200 MHz, $CDCl_3$): $\delta =$ 7.58 – 7.57 (m, 2H), 7.39 (d, $J = 8.5$ Hz, 1H), 7.32 (dd, $J = 1.6$, 8.5 Hz, 1H), 6.71 (d, $J = 2.2$ Hz, 1H), 6.50 (d, $J = 12.3$ Hz, 1H), 6.10 (d, $J =$ 12.3 Hz, 1H), 4.98 (s, 2H), 2.10 (q, $J = 7.4$ Hz, 2H), 1.01 (t, $J = 7.4$ Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 154.0$, 147.7, 145.1, 132.6, 131.2, 129.7, 127.2, 125.4, 121.3, 113.5, 110.8, 106.6, 28.8, 12.8. **14**: ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 7.63$ (s, 1H), 7.58 (d, $J = 2.2$ Hz, 1H), 7.38 (s, 2H), 6.71 (d, $J = 2.2$, Hz, 1H), 6.50 (d, $J = 12.4$ Hz, 1H), 6.07 (d, $J =$ 12.4 Hz, 1H), 4.95 (s, 2H), 2.41 (sept, $J = 6.8$ Hz, 1H), 1.12 (d, $J =$ 6.8 Hz, 6H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 154.0$, 151.6, 145.1, 132.4, 130.4, 130.0, 127.3, 125.4, 121.3, 111.5, 110.8, 106.6, 34.0, 21.7. **15**: ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 7.59 – 7.56$ (m, 2H), 7.39 (d, $J =$ 8.5 Hz, 1H), 7.33 (dd, $J = 1.6$, 8.5 Hz, 1H), 6.71 (dd, $J = 0.7$, 2.2 Hz, 1H), 6.49 (d, $J = 12.3$ Hz, 1H), 6.07 (d, $J = 12.3$ Hz, 1H), 5.00 – 4.96 (m, 2H), 2.08 (t, $J = 7.6$ Hz, 2H), 1.54 – 1.35 (m, 2H), 0.83 (t, $J = 7.4$ Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 154.0$, 146.0, 145.1, 132.5, 131.1, 129.7, 129.5, 127.2, 125.4, 121.3, 114.7, 110.7, 106.6, 38.2, 21.5, 13.8. **16**: ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 7.66$ (m, 1H), 7.55 (d, $J = 2.2$ Hz, 1H), 7.45 (dd, $J = 1.8$, 8.6 Hz, 1H), 7.35 (d, $J = 8.6$ Hz, 1H), 6.64 (dd, $J = 0.8$, 2.2 Hz, 1H), 6.50 (d, $J = 12.4$ Hz, 1H), 6.17 (dd, $J = 1.3$, 12.4 Hz, 1H), 4.97 – 4.93 (m, 2H), 1.18 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$): $\delta = 153.9$, 153.7, 145.0, 132.3, 129.9, 129.8, 127.2, 125.5, 121.4, 111.3, 110.7, 106.6, 35.9, 29.3. **17**: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.57$ (d, $J = 2.2$ Hz, 1H), 7.50 (s, 1H), 7.38 (d, $J = 8.5$ Hz, 1H), 7.17 – 7.29 (m, 4H), 7.09 (d, $J = 8.3$ Hz, 2H), 6.69 (d, $J = 2.2$ Hz, 1H), 6.49 (d, $J =$ 12.3 Hz, 1H), 6.07 (d, $J = 12.3$ Hz, 1H), 5.09 (s, 1H), 4.96 (s, 1H), 3.40 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 154.1$, 145.2, 145.1, 139.4, 132.4, 130.5, 130.3, 129.0, 128.2, 127.3, 126.1, 125.3, 121.3, 116.6, 110.8, 106.6, 42.5. **18**: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.61 – 7.58$ (m, 2H), 7.42 (d, $J = 8.5$ Hz, 1H), 7.28 (td, $J = 2.1$, 8.5 Hz, 1H), 6.73 (dd, $J = 0.9$, 2.1 Hz, 1H), 6.64 (d, $J = 12.6$ Hz, 1H), 5.93 (dd, $J = 12.6$, 26.2 Hz, 1H), 4.75 (ddd, $J = 1.2$, 2.7, 16.4 Hz, 1H), 4.56 (dd, $J = 2.7$, 47.4 Hz, 1H). (*E*)-**19**: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.66$ (s, 1H), 7.61 (d, $J = 2.1$ Hz, 1H), 7.46 (d, $J = 8.7$ Hz, 1H), 7.41 (dd, $J = 1.5$, 8.7 Hz, 1H), 7.09 (d, $J =$ 15.3 Hz, 1H), 6.80 (d, $J = 15.3$ Hz, 1H), 6.75 (d, $J = 2.1$ Hz, 1H), 5.46 (s, 1H), 5.42 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 155.1$, 145.7, 138.8, 133.7, 131.1, 127.9, 124.5, 123.4, 120.0, 115.2, 111.7, 106.7.

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[12] **23**: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.39$ (d, $J = 1.9$ Hz, 1H), 7.02 (d, $J = 16.2$ Hz, 1H), 6.84 (d, $J = 16.2$ Hz, 1H), 6.41 (dd, $J = 1.9$, 3.1 Hz, 1H), 6.32 (d, $J = 3.1$ Hz, 1H).

[13] **24**: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.59$ (d, $J = 1.9$ Hz, 1H), 7.39 (d, $J = 8.6$ Hz, 1H), 7.26 (d, $J = 8.6$ Hz, 1H), 6.72 (d, $J = 1.9$ Hz, 1H), 5.01 (s, 1H).

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Cryo-TEM Snapshots of Ferritin Adsorbed on Small Zeolite Crystals**

Daniel Klint, Gunnel Karlsson, and Jan-Olov Bovin*

The development of cryo techniques in combination with transmission electron microscopy (TEM) has increased the number of biological systems that can be studied.[1] Cryo-TEM has become a common tool for the investigation of water/surfactant systems[2] and nucleation of inorganic crystals in solution.[3,4] We present for the first time direct imaging

[*] Prof. J.-O. Bovin, Dr. D. Klint
National Center for HREM, Inorganic Chemistry 2
Center for Chemistry and Chemical Engineering
Lund University
P.O. Box 124, S-221 00 Lund (Sweden)
Fax: (+46) 46-222-40-12
E-mail: jan-olov.bovin@oorg2.lth.se
G. Karlsson
The Biomicroscopy Unit, Chemical Center
Lund University (Sweden)

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of solutions containing proteins interacting with zeolite Y crystals. Pronounced adsorption of ferritin on ultrastable zeolite Y crystals is shown to be correlated to protein aggregation. Adsorption of ferritin molecules on low- and high-silica zeolite Y crystals results in different arrangements of the protein molecules. It is also shown that structural information, like unit cell parameters, can be obtained from inorganic materials present in vitrified solutions.

In recent years the use of zeolites, crystalline aluminum silicates, has been an alternative or complement to common biochemical methods in the purification of proteins.[5,6] Earlier studies of the adsorption of proteins on ultrastable zeolite Y (USY) have shown the protein adsorption to be dependent on pH value and ionic strength.[5] The process is believed to be predominantly mediated by protein aggregates interacting with the zeolite surface.

Ferritin is an iron storage protein present in animals and plants. The protein is spherical with a diameter of approximately 12 nm. The iron core can consist of up to 4500 Fe ions, thus giving a useful contrast when viewed with the microscope. In TEM images ferritin molecules are identified as black spots; the iron core is about 5 nm in diameter. The high contrast from the iron core makes it difficult to see the protein shell. Apoferritin—that is, ferritin without an iron core—is identified as a doughnut-shaped molecule with a diameter of 12 nm. Generally, a protein shows a minimum solubility around its isoelectric point (IEP), the pH value in solution where the sum of charges on the protein is zero. The IEP for ferritin is pH 4.5, and solutions with pH values at or close to the IEP will contain protein aggregates. The adsorbent matrix is USY, derived from the parent structure NaY by postsynthetic dealumination.[7] Both zeolites have the same FAU structure, but differ in Si/Al ratio and surface texture. The Si/Al ratio is 2.6 in NaY and 230 in USY, indicating a great difference in framework charge density and thus in surface charge density. The textural difference is due to mesopore formation during dealumination,[8] and results in less defined crystal shapes for USY.

Partial precipitation of ferritin was obtained in 20 mmol L^{-1} acetate solution at pH 3.6 (low ionic strength) and also in 20 mmol L^{-1} acetate at pH 5.2 containing 150 mmol L^{-1} NaCl (high ionic strength). No visible precipitation was observed in solutions of 20 mmol L^{-1} glycin at pH 3.0, either with or without the addition of 150 mmol L^{-1} NaCl, or in 20 mmol L^{-1} acetate at pH 5.2. Solutions of low ionic strength at pH 3.0 showed the presence of a wide distribution of intermediate-sized protein aggregates mainly consisting of less than 15–20 ferritin molecules. Cryo-TEM images of such solutions also containing USY showed that the aggregates were mainly adsorbed on the zeolite crystals (Figure 1a). Note that the solution surrounding the crystallite is depleted of ferritin molecules. There is no indication of monolayer adsorption, but rather a patchlike distribution of protein aggregates; only a fraction of the adsorbed molecules interacts directly with the adsorbent surface. Addition of 150 mmol L^{-1} salt at pH 3.0 increased the solubility of ferritin by reducing the average aggregate size. The increase in protein monomer concentration was followed by a decrease in the adsorbed amounts of ferritin (Figure 1b). The aggregate distribution at pH 5.2 (low ionic strength) resembled the distribution observed at pH 3.0 and at high ionic strength (Figure 1b). Also in this case, there was a high degree of monomers and small aggregates (3–10 molecules) and only a minor interaction with the zeolite crystals. The solution at pH 5.2 with 150 mmol L^{-1} NaCl was clarified by centrifugation prior to the incubation with USY in order to remove large precipitates. The majority of the wide distribution of protein aggregates remaining in solution consisted of less than 30 ferritin molecules. In the presence of these aggregates the adsorption on USY crystals was again more pronounced (Figure 1c). As in the case shown in Figure 1a aggregates of various sizes are adsorbed, with the solution surrounding the crystallite being depleted of ferritin. In Figures 1a–c the presence of mesopores in the USY crystals can be observed as lighter areas of various shapes, some indicated by white arrows.

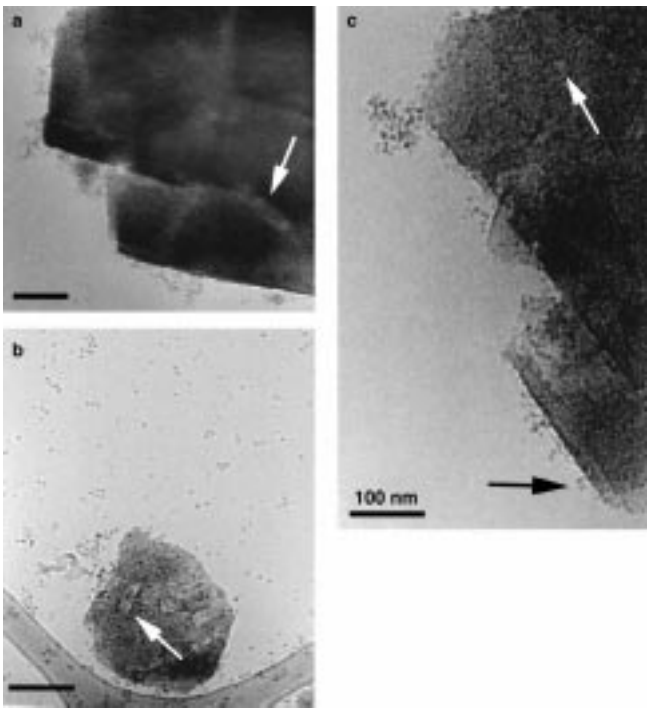

Figure 1. TEM images of frozen aqueous solutions containing horse spleen ferritin and USY crystals. The lighter areas of various shapes on the crystallites are interpreted as mesopores formed during dealumination; some are indicated by white arrows. Ferritin molecules are identified as black spots approximately 5 nm in diameter. a) Adsorption of ferritin on USY in a 20 mmol L^{-1} glycine solution at pH 3.0. Ferritin aggregates of various sizes are adsorbed in a patchlike manner on {111} surfaces of the twin crystal. The image has been subjected to background subtraction (script in DigitalMicrograph software) in order to increase the contrast. b) USY and ferritin in a 20 mmol L^{-1} glycine solution at pH 3.0 also containing 150 mmol L^{-1} NaCl. Ferritin molecules are mainly present as monomers and a large number of small aggregates consisting of five molecules or less. Only a few ferritin molecules are adsorbed on the zeolite. Poorly defined surfaces of the crystallite are probably also due to the dealumination. c) Ferritin aggregates of various sizes adsorbed on a USY crystal in 20 mmol L^{-1} buffer solution at pH 5.2 containing 150 mmol L^{-1} NaCl. Aggregates of various sizes were adsorbed on crystals. The black arrow indicates a dimer on the {100} surface of the crystal (see text for details).

Adsorption of ferritin molecules on NaY and USY crystals was performed in 20 mmol L^{-1} acetate solutions at pH 3.6. Again, the solution consisted of a wide distribution of mainly

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large protein aggregates, some containing 50 – 100 molecules. Nevertheless, the adsorption of ferritin resulted in a different protein arrangement on the NaY crystals than on the USY crystals. In Figure 2 a the NaY crystal is viewed approximately

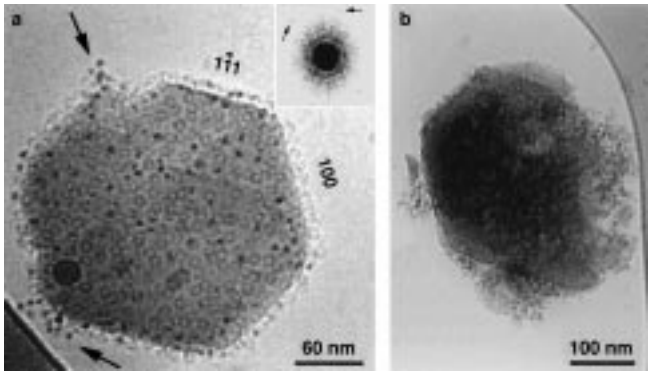

Figure 2. TEM images after the adsorption of ferritin molecules on NaY and USY crystals in a 20 mmol L^{-1} acetate buffer solution at pH 3.6. a) The NaY crystal is viewed approximately along [110], as indicated by the power spectrum in the inset; the arrows in the inset indicate the reciprocal distance to the {111} reflections. Due to partial precipitation of ferritin, the solution contained a wide distribution of mainly large aggregates (50 – 100 molecules). Nevertheless, ferritin molecules are almost uniformly distributed on the NaY crystal, except for a few aggregates indicated by arrows. The diameter of the doughnut shaped molecules is 12 nm, corresponding to the size of apoferritin. b) The adsorbed ferritin molecules on USY result in a different pattern. Large aggregates are adsorbed in a patchlike manner only covering parts of the crystallite. The crystallite in (b) has approximately the same orientation as that in (a).

along [110], displaying the {111} and {100} surfaces. The reciprocal distances to the {111} reflections correspond to 1.42 nm in real space, which agrees well with the calculated distance of 1.42 nm for NaY ($Fd\bar{3}m$, $a_0 = 2.47$ nm). There does not seem to be any preference for adsorption on any particular crystallographic surface. The ferritin molecules are uniformly distributed on the NaY crystal. The molecules adsorbed on the surfaces projected perpendicular to the viewing direction show traces of hexagonal close packing. A relatively large amount of molecules displays a doughnut appearance. Since many of these molecules are at the same focus as the dark spots, it can be ruled out that the doughnut shape is due to focal effects. Therefore, the NaY crystal is covered with both ferritin and apoferritin molecules. On USY ferritin is adsorbed patch-wise as aggregates, and only a fraction of the molecules interacts directly with the surface (Figure 2 b). The crystal in Figure 2 b also displays distinct crystallographic surfaces, although they are partly damaged due to the extensive dealumination.

Ferritin molecules carry a net positive charge at pH values below the IEP. As deprotonation of terminal hydroxyl groups occurs on the USY surfaces, attractive electrostatic forces arise between the zeolite surfaces and the ferritin molecules. These electrostatic interactions may play a role in the adsorption of the ferritin aggregates at ionic strength below pH 4.5. However, addition of salt breaks up protein – protein interactions and at the same time causes a shielding effect by decreasing the Debye length, thus reducing the effect of the electrostatic forces. The extent of adsorption of ferritin was consequently low in the pH 3.0 solution containing 150 mmol L^{-1} NaCl (Figure 1 b.) Increasing the pH value leads to an increased deprotonation of the USY crystal surfaces and hence an increase in negative charges. The reduced adsorption in 20 mmol L^{-1} buffer solution at pH 5.2 (low ionic strength) is reminiscent of the case of the solution at pH 3.0 containing 150 mmol L^{-1} NaCl (Figure 1 b) and is explained by a cooperative effect of two different events: 1) The net negative charge on the ferritin molecules is enough to maintain a low degree of aggregation, and 2) as the protein and the USY surfaces have charges of the same sign, interaction is reduced due to repulsive forces. The addition of salt causes a shielding effect of the repulsive forces between the USY surfaces and ferritin molecules, enabling protein aggregates to be formed and adsorbed (Figure 1 c).

The different adsorption behavior of ferritin on NaY and USY (Figure 2) could be explained by the great difference in chemical composition. On the NaY surface approximately every third tetrahedron contains Al, and it therefore displays a higher negative electric potential. As adsorption was performed in a solution of low ionic strength and at a pH value below the IEP for ferritin, the interactions are much more ionic in character at the NaY crystal surfaces than at USY surfaces.

Experimental Section

Suspensions of ultrastable zeolite Y particles (USY-HSZ-390HOA, Tosoh Co., Japan) in 20 mmol L^{-1} buffer solutions, either with or without the addition of 150 mmol L^{-1} NaCl, were degassed and ultrasonicated prior to sedimentation. Supernatants containing suitable particle sizes were obtained by centrifugation at 1000 – 1500 *g* for 5 min. Diameters of the zeolite crystals thus collected were in the range of 150 – 1000 nm, as estimated from low-magnification cryo-TEM images. Adsorption was performed by incubation of horse spleen ferritin with zeolite supernatant suspensions for 1 h on a rocking table at room temperature (ferritin concentration approximately 0.02 mg mL^{-1}). Sample were prepared by applying a drop of solution (8 μL) onto a lacey, carbon film covered TEM copper grid in a temperature and humidity controlled environment vitrification system (CEVS).[9] The excess solution was blotted off, and the remaining liquid film was plunged into a reservoir of liquid ethane at its freezing point (– 183 °C). The vitrified specimen was transferred by an Oxford CT3500 cryo-holder into a Philips CM120 BioTwin Cryo; the temperature was never allowed to raise above – 160 °C. The images were recorded under low-dose conditions using 117-kV energy filtered electrons on a Gatan 791 cooled multiscan CCD camera.

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