Ultrastructure and Composition of Asteroid Bodies

Jörg Winkler\textsuperscript{1,2} and Heinrich Lünsdorf\textsuperscript{2}

Purpose. Asteroid hyalosis is a disease of the vitreous, characterized by brilliant reflecting particles, termed asteroid bodies, which are surrounded by a tightly adhering network of fibrils. The composition and mode of formation of asteroid bodies is not yet understood in detail. The purpose of this study was to investigate the ultrastructure of asteroid bodies and to identify the intrinsic inorganic and organic components that contribute to the nature and development of asteroid bodies.

Methods. Electron energy loss spectroscopy and energy-filtered transmission electron microscopy were used for the elemental analysis of asteroid bodies. The ultrastructural localization of glycosaminoglycans was investigated, using lectin and antibody conjugates in conjunction with transmission electron microscopy and epifluorescence microscopy. Anionic sites of glycosaminoglycans were detected with 15 nm cationic colloidal gold at low pH, applied as a postembedding technique. Ultrastructural details of asteroid bodies were documented using fast Fourier transform analysis of zero-loss filtered images.

Results. Element mapping of asteroid bodies by electron spectroscopic imaging revealed a homogeneous distribution of calcium, phosphorus, and oxygen. The electron energy loss spectra of these elements showed details similar to those found for hydroxyapatite. Additionally, high contrast and sensitivity against a calcium-specific chelator highlighted the crystalline, apatite-like nature of asteroid bodies. Immunofluorescence microscopy revealed the presence of chondroitin-6-sulfate at the periphery of asteroid bodies, which is in agreement with the ultrastructural colocalization of anionic sites. Fast Fourier transform analysis revealed that each 7-nm periodicity of asteroid lamellar stacks is divided by a fine, parallel-oriented line, separating each 7-nm layer into two halves of 3.5-nm thickness. Carbohydrates specific for hyaluronic acid were observed by lectin-gold labeling to be part of the inner matrix of asteroid bodies.

Conclusions. The results of this study demonstrate the structural and elemental similarity of asteroid bodies with hydroxyapatite. Proteoglycans and their glycosaminoglycan side chains are implicated in playing a role in regulating the biomineralization process.


Asteroid hyalosis is a vitreous disease occurring, predominantly, unilaterally without any recognizable predisposition to gender or race but commonly is diagnosed in elderly patients >60 years old. The disease is characterized by a deposition of glistening particles, termed asteroid bodies (ABs), in the vitreous of the eye, which have been reported to consist of lipid-containing calcium compounds, often surrounded by an adhering network of fibrils. Using high resolution transmission electron microscopy (TEM), the ultrastructure of ABs was described as a myelin-like, multilamellar construct with a lamellar periodicity of 6 to 8 nm, or 4.6 nm. The positive-staining of ABs with Alcian Blue, colloidal iron, and osmium tetroxide has led to the suggestion that the asteroids are predominately composed of acidic lipids. Elemental analysis of ABs, using energy-dispersive x-ray spectroscopy (EDX), demonstrated calcium and phosphorus as the main detectable elements and, in some cases, potassium and sulfur. Electron diffraction analysis performed by Streeter suggested that electron-dense areas lying between the lamellar stacks could represent calcium apatite crystals.

Although ABs have been extensively investigated, their composition, origin, and mode of formation is still incompletely elucidated. In an attempt to further unravel the conflicting data reported in the literature, we have reinvestigated the structure, elemental composition, and distribution of anionic sites of ABs.

Materials and Methods

Fixation and Embedding Procedures

The study included four individual vitreous aspirates obtained from two female and two male patients ranging in age from 68 to 83 years, who underwent pars plana vitrectomy. Informed consent was obtained from every patient, and the tenets of the Declaration of Helsinki were followed. Asteroid hyalosis occurred in all patients unilaterally and coincided with other pathologic conditions. Preoperative diagnoses included two eyes with proliferative diabetic retinopathy with posterior retinal detachment, one eye with a pseudophakic retinal detachment and one eye with a macular hole. A posterior vitreous detachment occurred in two eyes. The aspirates were centrifuged (10,000g, 5 minutes) and transferred into Sörensen’s or sodium cacodylate buffer (0.1 M, pH 7.2). Fixation of the samples was performed with 2% paraformaldehyde (PFA), left overnight at 4°C. The samples were embedded in 2% (w/v) noble agar (Difco, Detroit, MI), cut into small cubes, washed, and postfixixed in 1% (w/v) osmium tetroxide. Samples that were used for postembedding labeling were washed three times with 40 mM glycine. Subsequent dehydration of samples was performed in a graded series of ethanol (30%–100%). Infiltration of the cubes with LR-White resin (Plano, Marburg, Germany) and curing was performed according to the instructions of the manufacturers. Ultrathin sections were counterstained with 2% (w/v) uranyl acetate and 0.5% (w/v) lead citrate. Examination of the sections was performed with a Zeiss EM9 transmission electron microscope (Oberkochen, Germany) using an accelerating voltage of 60 kV.

Postembedding Labeling with Cationic Gold Conjugates

Ultrathin sections were mounted on Formvar-coated nickel grids and floated mount-side down on drops of reaction buffer. One-step incubation of sections was carried out using poly-l-lysine-coated cationic colloidal gold (CCG, mean diameter: 15 nm; British BioCell, Cardiff, United Kingdom) diluted 1:50 in 0.1 M phosphate buffer (pH 7.0). After incubating for 12 hours at 4°C, unbound gold particles were washed off by rinsing the grids with buffer and distilled water. Controls were carried out with colloidal gold instead of cationic gold conjugate or with poly-l-lysine, to block anionic sites before incubation in CCG. Sections of gold labeling were air-dried and examined by TEM.

From the 1Department of Ophthalmology, Medical University of Lübeck; and 2Department of Microbiology, GFB, German Research Center for Biotechnology, Braunschweig, Germany.

Submitted for publication May 11, 2000; revised November 13, 2000; accepted November 29, 2000.

Commercial relationships policy: N.

Corresponding author: Jörg Winkler, Medizinische Universität zu Lübeck, Labor für experimentelle Ophthalmologie, Ratzeburger Allee 160, D-23538 Lübeck, Germany. jorgw@yahoo.com
Ultrastructure and Composition of Asteroid Bodies

Electron Spectroscopic Imaging

Suitable aspects of asteroid bodies were sampled at the element-specific energy settings for calcium, phosphorus, and oxygen, according to the EELS reference atlas. Images were captured with a cooled 14-bit CCD camera (Proscans, Scheuring, Germany). Image processing and background correction were carried out using the ESI Vision Pro Software 3.0 (Soft Imaging System Ltd, Münster, Germany).

RESULTS

Ultrastructural Characteristics of Asteroid Bodies

Figure 1A shows a low-resolution micrograph of an AB. The particle appeared to be roughly spherical, with electron-dense angular material that tended to crack out of the section. ABS were surrounded, predominantly, by fibrillar collagen-like material. High-resolution ultrastructural analysis of ABS revealed an irregular orientation of their protrusions when observed from ultrathin sections with the EFTEM (Fig. 1B). This is recognized, on the one hand, as a distinct alignment of many layers in stacks, reflecting a perpendicular orientation relative to the plane of sectioning (Fig. 1B, square box) and, on the other hand, as a diffuse dark-gray region, adjacent to the stack border, changing progressively to lighter gray tones (Fig. 1B, asterisk). These changes of gray levels indicate different degrees of tilted orientations of the lamellar stack, relative to the electron beam. Higher magnifications of ABS revealed additional linear substructures located in a parallel orientation between the main lamellae (Fig. 1B, inset). The spacing of the lamellae (i.e., the lamellar thickness) was determined by FFT analysis of different multilamellar stacks, by the first-order diffraction spot, which has a frequency in reciprocal space of 0.14 nm⁻¹, or a lattice constant of 7.1 nm (Fig. 1C). A second-order diffraction spot possessed a spatial frequency of 0.28 nm⁻¹ or a spacing of 3.6 nm, followed by a highest diffraction spot at 0.42 nm⁻¹ spatial frequency according to a spacing of 2.4 nm (Fig. 1C, crossed circle). Finally, structural details with a resolution of 0.46 nm⁻¹ or 2.2 nm could be observed (Fig. 1C, circle). Main diffraction spots were used to construct a mask for noise-filtering of the IFFT. The IFFT displays the unit lamella of a regular crystalline array, which is composed of two layer halves of 3.6 nm thickness each (Fig. 1D, arrowheads). These layer halves, showing a grainy particulate substructure (Fig. 1D, circle), are separated by a fine dark line (Fig. 1D, arrow) that corresponds with the linear substructures shown in Figure 1B (inset). At the periphery of ABS, individual asteroid lamellae often appeared, interspersed with collagen-like fibrils, frequently accompanied by a surrounding greyish halo (Fig. 2A). Because ABS are assumed to be, at least in part, composed of apatite-like material, microbeads of hydroxyapatite were prepared for comparison. Most microcrystals were clustered in aggregates and possessed a crystal width of 4.6 to 8.6 nm (Fig. 2B, inset).

High-Resolution Labeling of Anionic Constituents and Lectin (WGA) Binding Sites

The distribution of anionic components was analyzed using a postembedding procedure performed at low pH to increase the specificity toward sulfated and sialylated glycoconjugates, which are known to be the only matrix constituents dissociated under such acidic conditions. Figure 3A shows an AB surrounded by vitreous fibrils. The matrix of the AB revealed only a low label intensity, whereas its periphery displayed several aggregations of gold particles (Fig. 3A, encircled). The surrounding vitreous showed the strongest gold labeling. Clusters of gold particles were frequently associated with collagen-like fibrils, indicating accumulations of anionic sites (Fig. 3A, inset). Additional areas of
strong focal gold labeling were scattered irregularly within the vitreous (Fig. 3B, arrows). The distribution of lectin binding sites within the inner matrix of an AB is shown in Figure 3C. Aggregates of gold conjugates were frequently associated with lamellar stacks. Preincubations of WGA–gold conjugates in 0.25 M $\text{N}-\text{acetyl glucosamine}$ reduced labeling to low amounts, indicating the specificity of binding (data not shown).

Fluorescence Staining of Glycosaminoglycans

The nature of anionic sites found at the periphery of ABs and in the vitreous was further studied, using FITC-conjugated antibodies and lectins specific for carbohydrate moieties of glycosaminoglycans (GAGs; Figs. 4A, 4B). Figure 4A shows the immunocytochemical localization of chondroitin-6-sulfate at the periphery of ABs (arrowheads). Additionally, granules of small size scattered irregularly in the vitreous displayed a positive staining (Fig. 4A, arrows). Both reactions correspond with the distribution of anionic sites shown in Figures 3A and 3B. Control experiments performed with a secondary antibody alone or with unspecific IgMs revealed a low background fluorescence, probably caused by immunoglobulin–mineral interactions (data not shown). WGA-specific binding of $\text{N}-\text{acetyl glucosamine}$, typically found in hyaluronic acid, was found to be rather homogeneous, being distributed throughout the vitreous and in ABs (Fig. 4B). Individual ABs revealed strong peripheral staining (Fig. 4B, arrowheads). Control experiments in which the lectin had been preincubated with the inhibitory sugar (0.3 M) revealed a less intense fluorescence (data not shown).

EELS Analysis and Electron Spectroscopic Imaging of ABs

Serial EELS of distinct multilamellar stacks of ABs revealed the presence of characteristic elements, i.e., phosphorus, calcium, and oxygen (Fig. 5). The chemical composition of ABs was compared with myelinated axons of the optical nerve, as a homologous ultrastructural feature (data not shown), and with hydroxyapatite microcrystals. These materials were treated under the same conditions and analyzed with similar probe volumes under identical measuring conditions. Serial EELS of ABs was in accordance with the spectroscopic data of hydroxyapatite microcrystals with respect to signal intensities and spec-
trum details (Fig. 5). In contrast, the myelinated axon did not reveal the similar signal intensities of phosphorus by EELS and, thus, contained lower amounts of this element (data not shown). Interestingly, the energy loss near-edge fine structures (ELNES) at the PL2,3 (Fig. 5, boxed area) indicate that the phosphorus of ABs and hydroxyapatite shows the same chemical environment (i.e., they are chemically identical). The higher amount of carbon (C\text{K}) relative to calcium (CaL\text{2,3}) in ABs versus hydroxyapatite indicates a lower amount of calcium in the resin matrix of ABs, relative to the hydroxyapatite reference. The distribution of calcium, phosphorus, and oxygen within ABs was further analyzed in the electron spectroscopic imaging (ESI) mode. Electron spectroscopic images showed a homogeneous distribution of these elements with a mapping resolution of approximately 7 nm, reflecting the size of a single asteroid lamella (Fig. 6, arrowheads). Particularly, the calcium distribution map demonstrated that the dark-greyish halos at the stack borders (Fig. 6C, asterisks) are composed of asteroid matter, representing the lamellar stack continuum at an inclined angle relative to the electron beam, which is characteristic for the highly irregular shape of ABs.

**Ca-Chelator Sensitivity of ABs**

The sensitivity of the lamellar structure of ABs against a Ca-specific chelator (EGTA) was examined. Figure 7 shows the successive structural degradation of the ordered lamellar array of an AB over time. After 20 minutes, the lamellar stacks revealed early disarrangements (Fig. 7A). Lamellar sheets that detached from the particle surface were superimposed with underlying structures, resulting in a projection of a crosswise pattern (Fig. 7A, arrowheads). After 3 hours, increasing amounts of lamellar stacks started to dissolve (Fig. 7B). An incubation of 12 hours led to a complete dissolution of the particle (Fig. 7C). Residual lamellar structures were distributed randomly within electron-dense regions of undefined morphology (Fig. 7D, arrows). The subsequent labeling of decalcified structures with CCG revealed only low amounts of anionic sites (Fig. 7D).

**DISCUSSION**

Multiple studies concerning the composition and structure of ABs, using biochemical methods,2,3 light3 or electron microscopy4–6,8, have been reported. The elemental detection of calcium and phosphorus, using EDX analysis,4,8 and the intense reaction of ABs with lipid stains,3 together with the membrane-like appearance of lamellar stacks4–6, supported the hypothesis that Ca-associated phospholipids are the major...
structural component of asteroids. To further investigate the elemental composition and distribution within ABs we used EELS/ESI analysis, which are of high sensitivity and resolution, especially for the biological important elements of low atomic number, and thus are superior to previously used detection methods such as x-ray or electron diffraction techniques. The application of ESI demonstrated a homogeneous distribution of calcium, phosphorus, and oxygen in ABs (Fig. 6), and data from EELS analysis of ABs and hydroxyapatite crystals revealed identical electron energy loss details with similar ELNES features (Fig. 5). The absence of an osmiophilic reaction of asteroid lamellae, formerly characterized as osmiophilic, is a further argument against the suggested lipid character of ABs. Although composed of elements with low atomic mass (Fig. 5), ABs exhibited a strong contrast in TEM without heavy metal staining, which indicates a high material density. Thus, the dark osmium staining of ABs, which occurred occasionally during postfixation, is the result of a cortical reaction with vitreous material surrounding the asteroid particles. Moreover, the relatively acellular vitreous is not expected to contain sufficient amounts of phospholipids for the formation of hundreds or thousands of ABs often observed in asteroid hyalosis. Vesicles or cells associated with ABs, which could serve as a source for lipids, were rarely seen in our preparations. These data, together with the sensitivity of the asteroid lamellar structure against a Ca-specific chelator (Fig. 7), provide strong evidence that the whole AB, including the multilamellar stacks, is uniquely composed of Ca-apatite-like material.

Miller et al. compared the parallel lamellae of asteroid particles with the regular pattern observed in the liquid crystalline phase of phospholipids in water, which, at room temperature only, forms after fixation in OsO. In our experiments, the ultrastructure of ABs was proven to be stable at a wide range of temperatures (−80°C, 4°C, and 20°C) and even in the absence of fixative, contradicting the earlier reports. Rodman et al. described a positive staining of ABs with lipid stains, such as Sudan black B and Oil red O, and a marked stability of ABs in lipid solvents. Both reactions can be explained, on the one hand, by an inclusion of organic macromolecules (e.g., lipids) commonly found in biological minerals causing defects in the ordered crystal lattice and, on the other hand, by the observation that acidic phospholipids found in

FIGURE 5. EELS of an asteroid body and a cluster of hydroxyapatite microcrystals. The spectra show identical edges from oxygen (O_K = 532 eV), calcium (Ca_L2,3 = 346 eV), carbon (C_K = 284 eV), and phosphorus (P_L1 = 189 eV, P_L2,3 = 132 eV). The boxed area outlines the P_L2,3 ELNES of the phosphorus atom. The three intensity peaks of both spectra are highly similar in morphology but different in intensity. This indicates an identical chemical environment of the phosphorus in relation to the coordination sphere of its surrounding nearest atom neighbors (i.e., phosphate-oxygen).

FIGURE 6. ESI analysis of an AB revealing the elemental distribution of phosphorus, calcium, and oxygen. (A) High-contrast image (250 eV), printed in reversed contrast, used for orientation. Elemental mapping revealed a homogeneous distribution of net phosphorus (B), net calcium (C), and net oxygen (D). Arrowheads: an individual lamella; asterisks: (A, C) a lamellar stack, obliquely oriented relative to the electron beam, shown as a distinct intensity in the calcium elemental map.

FIGURE 7. Na-EGTA–induced degradation of an AB over time. (A) After 20 minutes, lamellar stacks started to detach from the asteroid surface, resulting in a crosswise pattern (arrowheads). (B) After 3 hours, lamellar stacks started to dissolve. (C) After 12 hours, the AB dissolved completely, leaving a hole in the ultrathin section. (D) Subsequent labeling of residual structures with CCG revealed a low binding intensity. Small fragments of lamellar stacks are rarely seen (opposing arrows).
dentin, enamel, calcified cartilage and bone could also not be extracted by lipid solvents before demineralization.\textsuperscript{12-15}

It is unknown which elements form the nucleation center to initiate apatite crystallization. However, an intense association of vitreous fibrils with single crystalline lamellae and stacks of ABs (Fig. 2) and a low prevalence of vitreous liquefaction associated with asteroid hyalosis\textsuperscript{10} provides evidence that a relatively intact vitreous may serve as an organic matrix in the formation process of ABs. The aggregation of collagen fibers into bundles of parallel fibrils with aging\textsuperscript{17} and the association of vitreous fibrils with anionic groups (Fig. 3) results in a strong anionic field, to which calcium ions are attracted and in which apatite crystals could form, aligned parallel to the collagen macromolecules. The interaction of ABs with GAGs (Fig. 4) and, possibly, other acidic matrix molecules is in accordance with the general view that polyanions may interact electrostatically with calcium sites present in the crystal lattice of hydroxyapatite.\textsuperscript{18,19} In bone and dentin, such interactions greatly influence the precipitation and growth process of calcium phosphates in vivo and may also do so in AB development.\textsuperscript{20} The reaction of lectin (WGA) with matrix components of ABs (Figs. 3 and 4) indicates that hyaluronic acid, which contains N-acetyl glucosamine as the WGA binding motif, may be included in the calcification process. This hypothesis is further supported by the development of particles similar to ABs in rabbit eyes after intravitreous injection of hyaluronidase\textsuperscript{41} or hyaluronic acid.\textsuperscript{22}

There has been considerable controversy regarding the relation between asteroid hyalosis and systemic diseases.\textsuperscript{1,16,23,24} However, a convincing statistically significant association between asteroid hyalosis and diabetes was described by Bergren et al.\textsuperscript{23} The increased permeability of basal membranes found in diabetic eyes\textsuperscript{25} might explain a positive correlation with asteroid hyalosis. Because the inner limiting membrane (ILM) revealed structural alterations in diabetic\textsuperscript{26,27} and aged\textsuperscript{28,29} eyes, it is conceivable that the ILM becomes more permeable for AB-relevant ions and macromolecules released by vascular changes of the retina, which could initiate the long-term development and growth of ABs. Because biological macromolecules appear to be an integral part of, if not all, biomimlar structures and are often assumed to play a pivotal role for the initial formation of calcium hydroxyapatite crystals, our future studies will include biochemical analysis of organic macromolecules obtained after complete demineralization of ABs.

**Acknowledgments**

The authors are grateful to Horst Laqua (MUL-Lübeck) for his continuing support, Viktoria Frank (MUL-Lübeck) and Elke Haase (GBF-Braunschweig) for their excellent technical assistance, and Edward R. B. Moore (GBF-Braunschweig) for his helpful comments on this manuscript.

**References**


**Ultrastucture and Composition of Asteroid Bodies**