Calcium phosphate mineralization in bone tissues directly observed in aqueous liquid by atmospheric SEM (ASEM) without staining: microfluidics crystallization chamber and immuno-EM

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The malformation and disordered remodeling of bones induce various diseases, including osteoporosis. We have developed atmospheric SEM (ASEM) to directly observe aldehyde-fixed bone tissue immersed in radical scavenger buffer without thin sectioning. The short procedure realized the observation of bone mineralization surrounded by many cells and matrices in natural aqueous buffer, decreasing the risk of changes. In osteoblast primary cultures, mineralization was visible without staining. Correlative energy dispersive X-ray spectrometry indicated the formation of calcium phosphate mineral. Fixed bone was sectioned, and the section surface was inspected by ASEM. Mineralized trabeculae of talus spongy bone were directly visible. Associated large and small cells were revealed by phoshphotungstic acid staining, suggesting remodeling by bone-absorbing osteoclasts and bone-rebuilding osteoblasts. In tibia, cortical bone layer including dense grains, was bordered by many cells with protrusions. Tissue immuno-EM performed in solution for the first time and anti-cathepsin-K antibody, successfully identified osteoclasts in femur spongy bone. A microfluidics chamber fabricated on the silicon nitride film window of an ASEM dish allowed mineralization to be monitored in vitro; calcium phosphate crystals as small as 50 nm were imaged. ASEM is expected to be widely applied to study bio-mineralization and bone-remodeling, and to help diagnose bone-related diseases.

Mineralization plays a crucial role in life. As animals evolved, the formation of extracellular collagen conferred mechanical strength to multicellular organisms, allowing the size of their bodies to increase. Later, calcium phosphate (CaP) precipitation followed by oxidation formed hydroxylapatite (HA) on crosslinked collagen fibers, leading to bone formation. This resulted in further body enlargement, especially during the evolution of air-breathing vertebrates. Mineralization also serves as a calcium stock and plays significant roles in calcium metabolism. In human bodies, bones are constantly remodeled to maintain their structural matrix and strength; bone is partially absorbed by osteoclasts and rebuilt by osteoblasts in micro-remodeling pits1. The malformation and disordered remodeling of bones caused, in part, by the unbalanced activity of these cells, are related to diseases such as osteoporosis, osteopetrosis and osteoarthritis2.

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The mineralization that takes place during bone development has been intensively studied by optical microscopy (OM) and electron microscopy (EM)\(^1,2\), but the methodological limitations of high-resolution microscopy for bone tissues make it difficult to study bone remodeling and mineralization. Transmission EM (TEM) of Epon-embedded specimens is widely employed\(^3\). While lightly mineralized tissues can be embedded and thin-sectioned without decalcification, densely mineralized tissues generally need to be demineralized (decalcified) beforehand\(^4,5\). This involves the use of EDTA for a relatively long period of time (typically 3 weeks). Further, Epon-embedding and thin-sectioning include hydrophobic treatments and take additional days, which together might affect mineralization and attached hydrophilic tissues. A new fixation method to preserve tiny precipitates under anhydrous conditions developed by Boonrengsiman et al., makes it possible to image the mineral granules less than 50 nm in mitochondria in osteoblasts by TEM\(^6\).

Quantitative backscattered electron imaging (qBSE/qBEI) scanning EM (SEM) techniques are generally used to examine non-decalcified, dehydrated, resin-embedded and polished thick sections of bone\(^7,8\). The measurement can differentiate between changes in bone volume or in the degree of mineralization of the bone matrix. Methods that allow bone to be observed without prior hydrophobic treatment are lacking.

Environmental capsules were developed to image wet samples for TEM\(^9,10\) and SEM\(^11\). Such systems have been successfully employed to image hydrophilic molecular complexes\(^12\), cells\(^13,14\) and tissues\(^15,16\). This development led to the first visualization of biological mineralization in wet conditions. Vidavsky et al. observed calcium carbonate mineralization in sea urchin embryos in wet conditions\(^17\) using a B-nano microscope (a field emission SEM (FE-SEM) column sealed by a SiN film at the bottom end)\(^18\). Until now, CaP mineralization has not been observed in liquid by EM.

In this report, we report the use of our developed atmospheric SEM (ASEM)\(^17,19,20\) to investigate CaP mineralization in bone and in vitro. Based on protocols developed for other samples including neurons\(^21,22\), bacteria biofilm\(^23\), Langerhans islet in pancreas\(^24\) and cardiac- and skeletal- muscles\(^25\) bearing end plates\(^26\), the cells and tissues were aldehyde fixed, immersed in aqueous solution containing a radical scavenger (glucose) - to minimize the effect of radicals formed by electron radiation at low-accelerating voltage (30 kV) suppressing knock-on damage - and imaged by ASEM (Fig. 1). We successfully observed mineralization using osteoblast culture. We also observed fine and large mineralization in cortical and spongy bone tissues and the attached non mineralized cells and structures using staining with metal solution. Further, to identify osteoclasts, immuno-EM of tissue in solution was realized for the first time using anti-cathepsin K protease antibody.

Results

Mineralization process in primary cultured osteoblasts. To follow mineralization, mouse osteoblast primary cultures were imaged over time without staining using ASEM. Since CaP precipitates and the HA subsequently formed both have a high electron density (atomic numbers: Ca = 20, P = 15), we expected the process to be visible without staining, even in aqueous solution (atomic numbers: H = 1, O = 8). Osteoblasts from neonatal mouse calvaria were cultured on 8-window ASEM dishes\(^27\) in a CO\(_2\) incubator\(^28\), and at a given time point the cells in some dishes were fixed with glutaraldehyde (GA) and observed in radical scavenger glucose solution (Fig. 1a, left path). On culture day 5 (DID5) from induction of differentiation, slight signals started to emerge in some of the dishes (Fig. 1b, inset). Mineralization onset varied from area to area on the same dish and from dish to dish: almost half of the ASEM dishes were not mineralized at this time-point. On DID8, bright patches indicating mineral were detected in the region where the CaCl\(_2\) and PB (pH 7.4) solutions started to mix. At higher magnification, the dots were imaged as ambiguous densities and crystals as small as 50 nm in width (Fig. 3b, arrows). The patches were surrounded by small bright spots (arrowheads). The number and intensity of the signals, which we assign to mineralization, generally increased as culture proceeded. The degree of mineralization varied from location to location (Fig. 1d and Supplementary Fig. 1c,d).

Elemental composition analysis confirmed CaP mineralization. To confirm that the electron-dense signals were CaP mineralization, cells cultured on the ASEM dish were GA-fixed, observed, then dehydrated using an alcohol series, dried and placed in the vacuum chamber of a standard FE-SEM equipped with an energy dispersive X-ray spectrometer (EDS). EDS spectra were recorded from various areas of the sample. Typical results are shown in Fig. 2. The two-dimensional localization of calcium and phosphate determined (Fig. 2b, c) correlates well with the bright signals observed by ASEM (Fig. 2a); bright areas in the ASEM image usually gave EDS spectra with large Ca and P peaks and darker areas EDS spectra with smaller Ca and P peaks, i.e., the Ca and P content increased as ASEM image brightness increased (Fig. 2a1 – a6). The slight difference between the Ca and P localization maps (Fig. 2b, c) and the ASEM image (Fig. 2a) is attributable to deformation due to drying. The oxygen content also increased as image brightness increased. However, it was not possible to determine the phase of the mineral. The relatively high C and N content measured in the mineralized areas might reflect the integration of proteins, including collagens. The large Si peak is from the silicon nitride (SiN) film-window substrate (Fig. 1a bottom). The results confirm that CaP mineralization can be imaged in aqueous solution by ASEM.

CaP crystallization imaged in vitro by ASEM. To follow the formation of simple calcium phosphate crystals in aqueous solution by ASEM, we designed and fabricated a small volume-microfluidics chamber with two inlets on the ASEM dish; the chip with SiN windows formed the base of the chamber (Fig. 3a). Approximately 2µl of CaCl\(_2\) solution and 2µl of sodium phosphate buffer (PB, pH 7.4) were added to the inlet lines, and parts of the volumes were simultaneously introduced to the chamber with glow-discharged SiN windows, so that the interface formed above the windows. This was aided by OM observation from the top (Fig. 3a). After 30 seconds, many fine dots were detected in the region where the CaCl\(_2\) and PB (pH 7.4) solutions started to mix. At higher magnification, the dots were imaged as ambiguous densities and crystals as small as 50 nm in width (Fig. 3b, c). The ambiguous density was interpreted as amorphous calcium precipitates (ACP) that can be transformed to a crystalline phase.
To visualize crystal growth in a bulk mixture, 0.75 ml of 2.5 mM CaCl₂ solution and 0.75 ml of 1 mM PB (pH 7.4) were mixed and centrifuged at 18000 × g at room temperature (RT). The resulting precipitate was resuspended in a small volume of the supernatant solution, and a small aliquot of the suspension was placed on the SiN windows of the ASEM dish and imaged by ASEM (Fig. 3d,e). Elongated CaP crystals (100 nm–1 μm) were observed amongst ambiguous density that was presumably ACP. Together, the results suggest that the technique can image crystallization in real-time for smaller and larger precipitates.

NCMIR and PTA staining visualized cell organelle surrounding CaP mineralization in osteoblast cultures. On DID10, osteoblast primary cultures were fixed with GA, and examined by ASEM before (Fig. 4a) and after staining (Fig. 4b) by metal solution. Imaging precisely the same region twice, revealed mineralization alone and together with cellular structures in the proximity. The National Center for Microscopy and Imaging...
Research (NCMIR) staining method moderately stained membraneous structures, nuclei (※) and fine intracellular structures as well as filopodia, and strongly stained round structures (arrowheads) in the cytoplasm (Fig. 4b). In a more cell dense area, the NCMIR method again strongly stained round structures (arrowheads) around the nucleus sometimes including clear nucleoli (open arrowheads) (Fig. 4d–f). From their shape and distribution, these (arrowheads) could be oil droplets, that are reported to be present in COS7 cells \(^{21}\) and fat liver tissue \(^{31}\).

In contrast, phosphotungstic acid (PTA) staining methods known to stain proteins and nucleic acids, stained nuclei, nucleoli and intracellular structures (Fig. 4g–i). The membranous structures around nucleus (※) might be mitochondria (Fig. 4i), that are reported for cultured cells using ASEM \(^{31}\). The brightly imaged extracellular filaments (Fig. 4h, open arrow) might be collagen fibers. These results indicate that PTA or NCMIR staining can be employed to specifically stain subcellular structures surrounding mineralization, but have different staining preferences. For the following tissue observation, we adopted PTA staining which preferably stains proteins.
Since mouse bones begin to mineralize around embryonic day 15 (E15), mineralization was expected to be observed at later timepoints without staining. After confirming that the mineralization-negative control, mouse liver, exhibited very faint signals under the imaging conditions used, bones were directly imaged in solution by ASEM.

**ASEM observation of bone tissues before and after staining.** Since mouse bones begin to mineralize around embryonic day 15 (E15), mineralization was expected to be observed at later timepoints without staining. After confirming that the mineralization-negative control, mouse liver, exhibited very faint signals under the imaging conditions used, bones were directly imaged in solution by ASEM.

**Spongy bone of Talus.** A new born mouse (P0) talus was fixed using GA in cacodylate buffer, cut along the longitudinal axis using a vibratome, and the section face was placed on the SiN film of an ASEM dish (Fig. 1a, right path). The unstained tissue slab was immersed in glucose buffer and inspected to find a well mineralized area. This was achieved using the inverted SEM, by sliding the tissue on the SiN film window aided by OM observation of the unstained tissue from the top. Well-calciﬁed areas were examined at a range of magniﬁcations. An example is shown in Fig. 5. In the images, dark chambers are separated by bright walls (Fig. 5a bottom right, white arrow) that are interpreted as mineralized trabeculae of spongy bone. Afterwards, the sample was stained with PTA and the same area was observed using ASEM (Fig. 5a top left). Higher magniﬁcation images reveal differently sized cells attached to the mineralized trabeculae (Fig. 5b). Round cells that exhibited only a faint signal before staining are prominent around the trabeculae (Fig. 5b black arrows) and might be haematopoietic cells in the bone marrow. Large cells hardly visible before staining observed in some small clefts of the trabeculae, either attached to them or separated from them by a slight gap, might be osteoclasts (Fig. 5b white arrowheads).
Smaller cells only faintly visible before staining, are attached to the bright bone and might be osteoblasts (black arrowheads). Fine filaments close to some walls might be collagen fibers (Fig. 5b, open arrow). Another spongy bone region of the same talus slab was similarly observed (Fig. 6). Cells larger than 10 μm were attached to bright trabeculae with many holes, and are considered to be osteoclasts (Fig. 6b, white arrowheads).

Cortical bone of tibia. Cortical bone, i.e., compact bone, forms the hard outer shell of most bones in our body and bears much of the hard structure of the skeleton. Mouse tibia was sectioned into 200 μm slabs, stained with PTA, and observed using ASEM. To observe developing cortical bone, we first found spongy bone of the tibia and then shifted the observation area towards the proximal articular joint between the tibia and the femur. At the outer edge of the cartilage tissue, a highly dense continuous thick envelop was observed, i.e., cortical bone (Fig. 7). On the inner side, the bone was lined by many chamber-like structures isolated by homogeneous bright partitions (Fig. 7, black arrowhead). The chambers closest to the cortical bone were more elongated (flatter), suggesting that they are hypertrophic chondrocytes. The outsides of the cortical bone was mainly surrounded by connective tissue. At higher magnification, the layered structure of cortical bone included many fine grains (Fig. 7b,c, white arrowhead). Cortical bone was sometimes attached to cells via thin protrusions (Fig. 7c arrow) extending from the cell body, suggesting an interaction between cells and bone.

Distribution of cathepsin K in spongy bone using immuno-gold labeling. To identify the large cells attached to bone, spongy bone tissue was immuno-labelled for the osteoclast marker cathepsin K and imaged by ASEM. Cathepsin K protease secreted by osteoclasts degrades bone collagen and thus plays a critical role in bone absorption. Femur slabs, 200 μm thick, were labeled with anti-cathepsin K antibody and further with a secondary antibody conjugated to FluoroNanogold, and gold-enhanced. Afterwards, the slabs were counter-stained with PTA. ASEM images showed that trabeculae in the spongy bone were surrounded by and attached to many cells of various shape (Fig. 8) as in Figs 5 and 6. Higher magnification revealed that large cells attached to the bone might be osteoclasts.
thin trabecula walls had many small bright dots, i.e., gold particles, indicating that they are cathepsin K positive (Fig. 8c,e,f arrowheads); such signals were hardly detected in the corresponding areas of a negative control made without primary antibody (Supplementary Fig. 2). Moreover, because the cathepsin K-positive cells sometimes extended filopodia (open arrowheads) and attached to trabeculae displaying light or heavy mineralization, they are interpreted as osteoclasts. Some of the cathepsin K-positive cells covered a pit of thin mouse trabeculae, with the result that many probably secreted and/or digested substances were observed between the cell and the trabeculae (Fig. 8c,e). The trabeculae themselves were also sometimes partly immuno-labelled (arrow), in good agreement with the observation that cathepsin K secreted from osteoclasts associates with bone.

Discussion

Here, we demonstrate that CaP mineralization can be observed in aqueous solution at atmospheric pressure by ASEM. Using bone tissue from newborn mice (Fig. 5), we were able to monitor the early stages of mineralization by ASEM. EDS showed that the bright signals in the ASEM images arose from structures containing Ca, P and O (Fig. 2). Since the sample pretreatment required for ASEM is quick, consisting of medium changes of aldehyde solution followed by washing at one stage, fine mineralization is only minimally affected.

We also successfully observed mineralization and the surrounding soft cells and matrices in cortical- and spongy- bone tissues immersed in aqueous solution using ASEM (Figs 5–8), avoiding the time-consuming decalcification-Epon embedding required for TEM. The ASEM method has three main advantages for tissues.
First, hard tissue, such as bone, can simply be cut using a sharp scalpel. The two new surfaces resulting from this single cut can then be imaged by ASEM. For hard tissues and some skins, this is much easier than cutting 2–8 μm-thick slices for OM or 70–200 nm-thick Epon slices for TEM. Second, sample pretreatment is short, simple and performed in an aqueous environment. The time required can be limited to the time it takes the reagents, e.g., fixatives, to penetrate the sample to a depth of a few micrometers and interact, because the specimen thickness observable using the inverted SEM is just 2–3 μm at an acceleration voltage of 30 kV. Third, the high-resolution SEM imaging (ca. 8 nm) and single-section preparation, allow bone to be assessed for microcracks with minimum perturbation.

Sudden fracture of bone in spite of sufficient bone mass is suspected to be caused by the accumulation of microcracks, altered collagen crosslinking and spatial variance of mineral and other components as well as other factors. Microcracks are known to have various causes. One is the physical stress bone is exposed to when osteoporosis patients are treated with the osteoclast inhibitor bisphosphonate (BP). In this case, microcrack accumulation is attributable to reduced remodeling activity and/or to the abnormal HA alignment caused by direct BP binding.

qBSE/qBEI techniques using SEM also do not require thin sections and can quantify the mineralization in the surface of an embedded thick tissue. In ASEM, if part of the tissue is not attached to the SiN film window due to surface roughness, the quantification is not guaranteed, because the electron signal from this region is decreased by the buffer layer between the tissue and the SiN film. The advantage of ASEM is the ability to observe fully wet soft tissues without hydrophobic treatment and embedding.

The observations of inorganic CaP crystallization in aqueous solution using the cell-free microfluidic crystallization system were performed without any pretreatment. The result was consistent with previous reports on calcium phosphate crystallization. The mixed 0.2 M CaCl₂ and sodium phosphate buffer solution (pH 7.4) is supersaturated with respect to amorphous calcium phosphate (ACP), octacalcium phosphate and HA. In supersaturated calcium phosphate solutions, mineralization includes three steps: formation of prenucleation clusters, the aggregation of clusters to form ACP, and transformation from ACP to a crystalline phase. The second and third steps are associated with densification previously revealed by dynamic light scattering and cryo-TEM techniques. The observations shown in Fig. 3b,c can be attributed to the formation of ACP and subsequent densification.

The cell-free microfluidic CaP crystallization system developed here (Fig. 3a) could be also used as a simplified in vitro model to investigate abnormal HA alignment, possibly via time-lapse imaging, because live-imaging of CuSO₄ crystal growth in the crystallization solution has already been demonstrated at 17 frames/sec. For this purpose, additional inlets for possible crystallization modulators, including BP, should be developed to realize real time monitoring of abnormal HA alignments. The new dual inlet chamber developed here would also allow various kinds of inorganic mineralization and crystal formation to be studied.

While mineralization can be observed directly in the ASEM without staining, heavy metal stains or other labeling procedures are required to visualize less electron dense material such as protein. PTA generally stains proteins and chromatins, while NCMIR stains lipids, membranes and proteins. Importantly, the hydrophilic environment in the ASEM dish preserves the antigenicity of proteins allowing them to be identified and localized by immuno-gold labeling; the immuno-gold labeling of various proteins has been demonstrated.
for different cell cultures\textsuperscript{22–26,41}. Here, immuno-EM of tissue in solution was realized for the first time (Fig. 8). It determined the localization of cathepsin K and identified osteoclasts in mouse femur. The open configuration of the ASEM dish and the OM above, is advantageous. It facilitates targeting the immuno-labeled area by ASEM and allows operations to be carried out on the tissues during observation, e.g., fine position adjustment by sliding the tissue on SiN film window. Repeated shifting-imaging cycles allow the observation area to be increased as demonstrated\textsuperscript{28}. Immuno-EM in solution should now be extended to other molecules important for the bone-metabolism and –remodeling, particularly to investigate protein-mineralization relationships, including the bridging of aged collagen by advanced glycation end products (AGEs) that leads to abnormal HA alignment\textsuperscript{34}.

Figure 7. Cortical bone of tibia stained with PTA and imaged with ASEM. Tibia was stained with PTA and the cortical bone near the joint connecting the tibia to the femur was imaged using ASEM. Bone runs diagonally in the micrographs; the region closest to the joint is top left and the region furthest from the joint is bottom right. (a) Low magnification. (b,c) Higher magnification of the annotated squares in panel (a). The images reveal cortical bone layer (white arrowhead) lined on the inner side by many chamber-like structures isolated by homogeneous bright partitions (black arrowhead). (c) Some cells close to the outer side of the cortical bone have thin connections to the bone (arrow).
For the elementary analysis of CaP minerals by EDS, we did not coat the sample because a metallic conductive coating (e.g. platinum) would make interpreting the P signal challenging. In the analysis, CaP minerals were observed, but their phase was not determined. To overcome the limitations of the method, the combination of ASEM with a different technique, such as Raman spectroscopy in aqueous solution, should be investigated in future studies.

Figure 8. Distribution of cathepsin K in femur spongy bone in solution using immuno-gold labeling and ASEM. Femur thick slabs were labeled with anti-cathepsin K antibody and further with secondary antibody conjugated with FluoroNanogold, gold-enhanced, and then counter-stained with PTA. (a) ASEM image of spongy bone. Trabeculae are clearly observed. (b–f) Higher magnification of the annotated white rectangles indicated in the preceding panels. (c) A cathepsin K positive cell attached to a thin trabecula. Gold signals are observed as strong bright dots. (d) Another area in (a) was observed at higher magnification. Trabeculae are surrounded by many cells of various shape. (e,f) Higher magnification images of the annotated rectangles in (d). (c,e) A cathepsin K positive cell (arrowheads) that covers a pit of thin trabeculae. In both cases, many substances that were probably digested or secreted by the cell were observed between the cell and the trabecula (c,e). Gold signals also observed on the trabeculae (arrow) might correspond to cathepsin K secreted from osteoclast cell. (f) Cathepsin K positive cells attached to trabeculae.
Many potential applications of ASEM have a high clinical relevance. For example, it could be employed to study the osseointegration of bone implants. Artificial bone, hip-joint implants and dental implants have been intensively developed using metal, surface-roughened metals and various kinds of non-metallic materials. High-throughput ASEM could be used to image the interface between such materials and tissue, allowing the degree of osseointegration to be directly assessed. For this, the osteoblast culture could be combined with the fabrication of micro metal platforms on the SiN film window of an ASEM dish. The latter technique was developed and employed to make electrodes and study electrochemical processes at the molecular level by ASEM40.

As already demonstrated, ASEM can be used to precisely image bacterial biofilms26,41. This is important, because decalcification is known to be highly related to biofilm formation42. Biofilm formation is also an important factor in periodontitis, a major cause of tooth loss42. Based on the results presented here, ASEM could be applied to study the formation of such biofilms and their relationship to the osteogenesis and gum inflammation that are symptoms of this widespread dental condition. Biofilm formation by calcite-precipitating bacteria (e.g., Sporosarcina pasteurii) and its mineralization43 is also a fascinating target for ASEM.

The rapid, high-throughput ASEM method that uses 'single-cut' preparations rather than thin sections, is also a potential research tool for mineralization-related diseases including osteoporosis and chondrocalcinosis. Since ASEM successfully visualized haematoepoietic cells in spongy bone (Figs 5 and 8), it could be applied to study haematoepoiesis and the immune system in bone marrow. In an earlier study, ASEM also quickly identified breast cancer cells metastasized to the lung and spinal cord, using their larger nucleus as a cancer marker44. Since definitive diagnosis of bone cancer is carried out using bone biopsy and OM, the single cut method made possible by ASEM in combination with or without immuno-labeling could be applied to investigate osteosarcoma and leukemia, and for the intraoperative diagnosis of primary bone cancers and various cancers metastasized to bone.

Conclusions
The rapid ASEM method outlined and demonstrated here, allows osteoblasts and bone tissues to be imaged in aqueous solution at atmospheric pressure. The single section protocol is useful to observe hard cortical bone and delicate spongy bone. Additional staining using different metal solutions visualized bone metabolism-related cells and large protein complexes as well as haematoepoietic cells immersed in natural aqueous buffer. The new dual inlet chamber developed here allowed formation and visualization of CaP crystals as small as 50 nm using ASEM. It would also allow the effect of additional reagents on CaP mineralization to be investigated, and the study of CaP and other inorganic mineralization and crystal formation processes to reveal the dynamics of crystal growth. Quick immuno-labelling method of tissues should be applied together with in-solution ASEM to study bone-metabolism and -remodeling, to study teeth and related diseases and to other fields, including the diagnosis of various bone-related diseases and other diseases, such as cancers.

Methods
Animals. For osteoblast primary culture and tissue preparation, C57BL/6J mice were sacrificed. The methods and the experiments were conducted with the approval of the Animal Research Committee according to the regulations on animal experimentation at National Institute of Advanced Industrial Science and Technology (AIST), Nagasaki University and Kyoto University.

Primary culture of osteoblasts. Primary cultured osteoblasts were prepared30 and analyzed30,31 as described previously. In each case, 5 Culture dishes were fixed at 3, 5, 8 and 10 days after the induction of osteoblast differentiation. Detailed information of the culture is given in the Supplementary Methods.

Tissue sample preparation. Post-natal day 0 C57BL/6 mice (Riken Bioresource Center, Tsukuba, Japan) were euthanized and fixed in 4% PFA in 0.1 M cacodylate buffer (CB, pH7.4) at RT overnight. Tissues were excised from the organ, further fixed with 4% PFA in CB at RT for 15 min and sliced with a PRO7 linear slicer (Dosaka, Kyoto, Japan) to obtain 200 μm thick slabs27, or cut manually using a scalpel for immuno-labeling. For PTA staining, the excised tissue was further fixed with 2.5% GA in CB at RT for 2 h25 and sliced similarly.

Staining. Fixed tissues were stained with 2% PTA (TAAB Laboratories Equipment Ltd., Aldermaston, UK) for 3 h at RT for ASEM as described previously27. Cells observed by inverted SEM, were counter-stained using PTA or by the NCMIR staining method28,44 developed by the Ellisman group44. Detailed information of the NCMIR staining is given in the Supplementary Methods.

Immuno-gold labeling. Immuno-gold labeling of cathepsin K of bone tissue was performed as described27. Detailed information of the labeling is given in the Supplementary Methods.

ASEM imaging. ASEM images were recorded using the ClairScope ASEM system (JASM-6200; JEOL Ltd., Tokyo, Japan) as described previously27. Briefly, the standard 35 mm bio-ASEM dish with eight 100-nm thick (250 × 250-μm) SiN film windows was employed to observe samples in aqueous liquid28. A fixed tissue slab was laid cut face down on the SiN film of the ASEM dish, immersed in a buffer containing 10 mg/ml (w/v) glucose as radical scavenger and 1 mM CB (pH 7.4) and 60 mM KCl, and observed using the inverted SEM as described31. The fixed osteoblast cultures were imaged in 10 mg/ml (w/v) glucose solution using the ASEM. The acceleration voltage of the inverted SEM was 20 kV for osteoblast cultures and 30 kV for bone tissues and inorganic mineralizations. Backscattered electrons were recorded by a backscattered electron imaging (BEI) detector (Fig. 1a) for visualization. The electron dose applied for biological samples was less than 0.43 e⁻/Å², which is less than 1% of the dose of 47 e⁻/Å² permitted in low-dose cryo-EM aiming at atomic-resolution single-particle reconstructions. The penetration depth was 2–3 μm at an accelerating voltage of 30 kV, and slightly less (around 2 μm) at an accelerating voltage 20 kV27.
EDS-SEM. EDS of cultured osteoblasts was carried out as described previously. Briefly, after observation in aqueous solution using the inverted SEM, osteoblast cells on the ASEM dish were dehydrated using an ethanol series and dried. The specimen on the chip was mounted on the aluminum holder with adhesive carbon tape, and the uncoated specimen was examined in an FE-SEM (JSM 7400F; JEOL) equipped with an EDS (EDAX Genesis, Ametek) at an acceleration voltage of 20 kV.

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**Author Contributions**

C.S., H.T., D.Y., Y.H. and E.S. designed and performed experiments. C.S., H.T. and E.S. analyzed data and wrote the paper. T.T., N.M. and M.S. performed experiments.

**Additional Information**

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