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Review

Electron Microscopy Studies of Soft Nanomaterials

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III Metrics & More

ABSTRACT: This review highlights recent efforts on applying electron microscopy (EM) to soft (including biological) nanomaterials. We will show how developments of both the hardware and software of EM have enabled new insights into the formation, assembly, and functioning (e.g., energy conversion and storage, phonon/photon modulation) of these materials by providing shape, size, phase, structural, and chemical information at the nanometer or higher spatial resolution. Specifically, we first discuss standard real-space two-dimensional imaging and analytical techniques which are offered conveniently by microscopes without special holders or advanced beam technology. The discussion is then extended to recent advancements, including visualizing three-dimensional morphology



Article Recommendations

of soft nanomaterials using electron tomography and its variations, identifying local structure and strain by electron diffraction, and recording motions and transformation by in situ EM. On these advancements, we cover state-of-the-art technologies designed for overcoming the technical barriers for EM to characterize soft materials as well as representative application examples. The even more recent integration of machine learning and its impacts on EM are also discussed in detail. With our perspectives of future opportunities offered at the end, we expect this review to inspire and stimulate more efforts in developing and utilizing EM-based characterization methods for soft nanomaterials at the atomic to nanometer length scales in academic research and industrial applications.

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1. INTRODUCTION

This review highlights recent progresses at the intersection of electron microscopy (EM) and soft materials. Electron microscopy utilizes electrons, which are charged particles that can be focused and positioned with magnetic lenses. When accelerated to high energies (keV -10^2 keV), they become ultrafine probes with large scattering cross sections, ideal for nanoscale resolution characterization. New detectors, beam technologies, and data science promise to capture images and movies on-demand with unprecedented speed, precision, and attenuation of beam effects. A diversity of systems that were traditionally considered as the wild west for EM imaging are now made possible to study, with nanoscale structure and dynamics unveiled and understood. Soft materials (different from metallurgy and ceramics) are among those systems-a huge and ever-growing class of materials-first defined by de Gennes in his 1991 Nobel lecture. Soft materials describe broadly soft and "squishy" systems that are complex and flexible, constructed often from tiny constituents that can be agitated by thermal fluctuations in a suspended liquid media during synthesis, processing, and functioning.¹ For these systems, questions of interest can range from the structural, phase, and functional changes of the system as a whole (such as in a colloidal suspension or a thin film assembled from conjugated polymers) to the crystallinity, strain, defects, transformation of a single constitute such as a colloidal nanoparticle (NP), coordinated organic frameworks, polymer molecules, and proteins. Those questions belong to different fields of research as diverse as colloid and surface science, polymer physics, structural biology, and biochemistry, with designated tools such as dynamic light scattering (DLS), X-ray diffraction (XRD), small-angle X-ray/neutron scattering (SAXS/SANS), differential scanning calorimetry (DSC), and crystallography that address samples at discrete length scales and often on an ensemble level. Thus, it is exciting to see an emergent, unified theme of developing and utilizing softmaterials friendly EM technologies as a general route to investigate and image the spatial and temporal structures and behaviors at the atomic to nanoscale.

This review thus focuses on these exciting advancements and user applications, and points toward the future of this exciting and newly formed frontier, especially to invite crosstalk between the communities of EM (M&M meetings in the

US) and soft materials (scattered in APS, ACS, MRS meetings in the US) to stimulate more active research. As shown in Figure 1, we will focus on colloidal NPs and their synthesis, assembly, reaction, and application, polymers and their assembly forms, various types of carbonaceous materials, coordinated organic frameworks, and biostructures. We will review the technological developments to address key issues in characterizing those materials using the commonly accessible techniques of scanning electron microscopy (SEM), transmission electron microscopy (TEM), and scanning transmission electron microscopy (STEM), together with a suite of functional modules to enable imaging in three-dimension (3D), alongside the temporal, composition, and structural order dimensions. These technological developments are covered to give the soft materials readers a jump start on the history and possible techniques to use for their own purpose, with the types of measurable properties introduced by extensive user examples. Based on this survey of state-of-theart EM technologies and what have been applied to soft materials, it is clear for us to spot the remaining uncharted territories that we see as promising directions in the next five to ten years.

Given the rich reconfigurability, structural complexity, and spatiotemporal heterogeneity of soft materials, massive datasets are generated as one investigates them using EM.² As a result, this review also contains a huge section with forward-looking views on how data science has been used and will be used further in analyzing EM data, and even in acquiring and controlling data in how we understand interfaces, how we discover new materials with new functionality, and even how we do EM.

We would like to also point out that EM has drawn various initiatives and attention worldwide and it is a timely contribution now to review the current status and future of EM on soft materials, one of the areas that has traditionally escaped EM's investigation. This goes beyond the Nobel prize recognition of cryogenic EM in 2017. In the US, the Department of Energy recently published a roundtable discussion on "Research Opportunities in the Physical Sciences Enabled by Cryogenic Electron Microscopy".³ In Europe, it has been decades of years to build and maintain the "European Network for Electron Microscopy" to consolidate the EM resources and provide access to leading European state-of-theart EM research infrastructures for translating research. We hope to stimulate a wider range of academic and industrial research communities to use EM for the analysis and engineering of new materials in physical, chemical, and biological science in the long run.

2. STANDARD REAL-SPACE, TWO-DIMENSIONAL (2D) IMAGING BY EM

Among the EM techniques, SEM, TEM, and STEM are the most commonly used in soft material characterization. In this section, we will describe each of them with a focus on standard, so-called "ex situ" imaging of 2D features of the samples that is accessible on all common machines without complicated adaptation. The applications and challenges in characterizing different materials, ranging from colloidal inorganic NPs to polymers and biomaterials, will be introduced, together with the utilization of versatile analytical techniques (energy dispersive X-ray spectroscopy (EDX), electron energy loss spectroscopy (EELS), and energy-filtered TEM (EFTEM)) to unveil their chemical and plasmonic properties.



Figure 1. Illustration showing the application of EM, with assistance of ML, in the characterization of soft nanomaterials. The EM images and illustrations are adapted with permission from refs 4–9. Copyrights 2020 Springer Nature under CC-BY 4.0 [https://creativecommons.org/licenses/by/4.0/], 2022 The Royal Society of Chemistry, 2022 AAAS under CC BY-NC 4.0 [https://creativecommons.org/licenses/by-nc/4.0/], 2017 American Chemical Society, 2019 American Chemical Society.

2.1. Introduction to SEM, (S)TEM, and Cryo-EM

By scanning samples with a focused electron beam of an accelerating voltage <30 keV and collecting the signals from scattered electrons and generated X-rays, one can obtain information about surface topography and composition of the sample using SEM with 0.5-4 nm resolution. Compared to TEM and STEM, SEM brings weaker damage to the sample and, instead of penetrating through the sample, maps the interaction of electron with the surface. It does not have a limitation on the sample thickness and provides topology and elemental characterization of surface structures. The contrast due to more secondary electrons emitted at a sharp edge of a feature can also bear immediate information on the 3D shape of the sample-while one might not differentiate between triangular plates and tetrahedron based on their projected view in TEM, the corner and faceted shapes of tetrahedron would be clearly captured by SEM with its great depth of field (a few micrometers to several millimeters).¹⁰ While such a surfacedominant interaction could lead to the accumulations of charge on insulating materials, strategies such as conformal conducting coating (e.g., a few nanometers of carbon or gold) and low voltage ($\sim 0.3-5$ keV) at which the emitted electron current can balance the beam current are proved convenient and efficient.

In TEM and STEM, an electron beam of a high accelerating voltage (60-400 keV) is transmitted through the sample, focused by a series of electromagnetic lenses, and projected onto a screen or camera to produce images, enabling a higher resolution of 0.5-2 Å in imaging. In the obtained TEM or

STEM images, information such as (i) the size and shape (based on bright-field (BF) TEM images), (ii) atomic arrangement and crystal structure, orientation, and strain (high-angle annular dark field (HAADF) images based on STEM or diffraction patterns), and (iii) composition (EDX and EELS based on STEM) can be obtained at high resolution. Thin samples (for optimized resolution, typically less than 200 nm), such as a thin film or a suspension on a grid, are usually required for TEM imaging to allow the penetration of electrons. Due to the high voltage applied, samples such as organic materials that may be easily deteriorated by electron beam need to be treated carefully (see Section 2.2.2 for more details).¹¹ Specifically, high-resolution TEM (HRTEM), also referred to as phase-contrast TEM, utilizes transmitted and scattered electrons to create an interference image of highenough resolution to detect arrays of atoms in crystalline structures. In comparison, in STEM, the electron beam is focused into a fine spot (with a typical size of 0.05-0.2 nm) and raster scans across the sample to enable various imaging and analytical modes, such as annular dark-field (ADF) imaging, EDX, and EELS, the signal of which can be obtained at the same location or even simultaneously. Combined with aberration corrector, the electron probes can be further focused to sub-Angstrom diameter in size, allowing location of individual atoms in the HAADF mode, in which the contrast in the HAADF image is highly sensitive to variations in the atomic number (Z) and the sample thickness.^{12,13} For low-Z samples, the difficulty in STEM imaging is increased due to their small scattering cross section and sensitivity to radiation



Figure 2. (a) High- and (b) low-magnification SEM images of TOH Au@Pd NPs. (c) Individual NPs in different orientations with corresponding 3D models (right of each SEM image). (d, e) TEM images showing (d) the overall morphology of TOH Au@Pd NPs and (e) a single NP viewed from the $\langle 110 \rangle$ direction with the measured projection angles marked. Inset in panel e: the corresponding electron diffraction pattern. (f) HRTEM image showing the edge of a TOH Au@Pd NP, revealing the atomic steps made of $\{221\}$ and $\{331\}$ subfacets in the surface. Inset: the corresponding FFT pattern. (g, h) TEM images and schematics with angle notation showing the self-assembly of patchy prisms into (g) twisted star and (h) slanting diamond structures. (i) HAADF-STEM image of amorphous Pd NPs, and (j) FFT pattern of the selected area marked in panel i. (k) SAED pattern of amorphous Pd NPs. (l) HAADF-STEM image of 2H–Pd NPs. (h) Magnified HAADF-STEM image and (i) FFT pattern of the selected areas marked in panel g. (o–q) Characteristic projections of binary superlattices self-assembled from different NPs: (k) 13.4 m γ -Fe₂O₃ and 5.0 nm Au; (l) 7.6 nm PbSe and 5.0 nm Au; (m) 6.2 nm PbSe and 3.0 nm Pd. The lattice projection is labeled above the scale bar in each panel. Scale bars: (c) 50 nm, (g, h) 50 nm, and (o–q) 20 nm. (a–f) Reprinted with permission from ref 18. Copyright 2010 American Chemical Society. (g, h) Reprinted with permission from ref 21. Copyright 2019 American Chemical Society. (i–n) Adapted with permission from ref 25. Copyright 2020 American Chemical Society. (o–q) Reprinted with permission from ref 33. Copyright 2006 Nature Springer.

damage. Note that both TEM and STEM usually provide 2D projection of a material. For example, it is hard for STEM to characterize the vertical structure of a material, such as distinguishing the atoms distributed above or beneath a certain (x,y) plane. To obtain 3D structures, it is necessary to utilize tomography methods by taking a tilt-series of a sample (Section 3).

Cryo-EM is a subclass of EM that allows the visualization of samples in a solvated environment by rapidly cooling a solution sample to ultralow temperatures (85-100 K). The recent achievement of near atomic resolution cryo-EM imaging through improved detector technology and reconstruction algorithms has attracted more attention and applications beyond imaging biomolecules (Section 2.2.2).¹⁴ The potency of flash-freezing samples in their solvated environments and reducing damage from the electron beam makes cryo-EM a powerful tool for imaging polymer specimens¹⁵ and air/oxygen sensitive materials in batteries.¹⁶ It is also an emerging trend to incorporate cryo-EM (Section 2.2.1) and liquid-phase TEM (Section 5) to obtain both high resolution and temporal changes in the presence of solvents to understand the dynamics of nanomaterials during formation, processing, and function.¹²

2.2. 2D Real-Space EM Imaging Techniques

2.2.1. Inorganic Colloidal NPs. Colloidal NPs have attracted a lot of attention in various research fields, including chemistry, materials science, biology, and physics, as they exhibit optical, electrical, and chemical properties that are different from their bulk counterparts.¹⁷ Among various

research topics, one important task is to characterize the structure of NPs, as well as their assemblies, and understand the structure—performance relationship. Most inorganic NPs are made of metals or metal oxides that are stable against an electron beam, allowing the leverage of different modes of electron microscopes and beam energies for comprehensive characterization. Electron microscopy methods provide convenient and efficient high-resolution imaging of individual NPs, which is of particular significance to heterogeneous nanomaterials if one is eager to know the precise shape, structure, and elemental distribution of the material.

NP Size, Shape, Crystal Structure, Strain, and Assembly. Use trisoctahedral (TOH) Au@Pd NPs, which are a promising candidate for catalytic reactions due to the high-density atomic steps and kinks on surface, as an example on shape and surface structure analysis.¹⁸ By conformally growing Pd layers on concave TOH Au seeds, Au@Pd core-shell NPs featuring a similar shape were obtained and their exact size, shape, facets, as well as surface structures can be resolved by EM. As shown in Figure 2a-c, the uniform size distribution and concavity of the products were confirmed by SEM, and images from different orientations further helped construct the particle's 3D shape. From TEM images (Figure 2d,e), the projection angles in selected crystallographic directions were measured, which were later used to determine the high-index facets exposed on the surface. For instance, viewing from the $\langle 110 \rangle$ direction, the four edge-on facets of the TOH Au@Pd NP were identified as {552} facets as the projection angles were measured to be around 140°, 95°, and 150°, consistent with theoretical values



Figure 3. (a-d) Ex situ SEM images of Ag and AgCl NPs obtained at different stages during the synthesis of Ag nanocubes: (a) 5, (b) 30, (c) 90, and (d) 120 min. The octahedral NPs are made of AgCl, while the small, bright dots are Ag NPs generated from the decomposition of AgCl upon exposure to electron beam, suggesting the potential existence of Ag nuclei in AgCl octahedra before SEM imaging. Scale bar: 500 nm. (e) Electrochemical cell with (f) carbon-coated gold grid as working electrode. (g, h) IL-TEM showing the same location of Pt NPs (g) before and (h) after 1000 potential cycles between 0.05 and +1.3 V. Effects of coalescence and migration are marked in panel h. (a-d) Reprinted with permission from ref 36. Copyright 2016 American Chemical Society. (e–h) Reprinted with permission from ref 45. Copyright 2015 American Chemical Society.

(Figure 2e). Besides, the Miller indices of high-index facets can be also examined by HRTEM, where the arrangement of atoms in the edge-on facets was characterized and analyzed (Figure 2f). On the surface of the NP, a series of alternating {221} and {331} subfacets were captured, resulting in an overall profile close to the {552} facet, consistent with the conclusion from projection angles. Interestingly, the Au/Pd interface showed no disruption in the lattice pattern from HRTEM, which indicated epitaxial growth of Pd on the Au seed, as well as the capability of ex situ TEM in understanding the reaction mechanism.

In BF-TEM imaging, elements with large differences in Z will give rise to different contrasts. Such a capability of distinguishing different components can be useful for hybrid materials. Representative examples include Janus and patchy particles.¹⁹ For example, in Au-organosilica Janus NPs, the metallic and silica domains can be simply resolved in TEM images.²⁰ Similarly, one can also easily tell the polymers and quantum dots from noble metals, and their positions in patchy particles can be identified such as coatings at vertices, edges, or faces of metallic cores (Figure 2g,h).^{21–23} For elements with close Z numbers, HAADF-STEM with higher sensitivity to Zcontrast and EDX or EELS with high energy resolution in detection of elements can provide more reliable results than BF-TEM (more discussions on analytical techniques in Section 2.3).²⁴ Besides, aside from the Z of elements, the contrast shown in TEM images can arise from many other factors, such as thickness, crystal orientation, and strain, which should be noted for interpretation.

The capability of EM in resolving atomic scale structures of NPs also shows great significance in unveiling unconventional crystal structures of metals and amorphous materials. For instance, it was shown that amorphous Pd NPs can be synthesized by reducing palladium(II) acetate in oleylamine with 1-dodecanethiol serving as a surfactant.²⁵ With HAADF-

STEM, the arrangement of atoms inside Pd NPs can be clearly observed, the randomness of which indicates the unusual amorphous structure of the sample. The conclusion was further verified through fast Fourier transform (FFT) pattern, selected-area electron diffraction (SAED), and XRD, as shown in Figure 2i-k. Interestingly, by heating the amorphous Pd NPs dispersed in oleylamine in air and under vacuum, a thermodynamically stable face-centered cubic (fcc) phase and an unconventional 2H phase were obtained, respectively. The HAADF-STEM images clearly present the typical "ABCABC" atomic stacking along the [111]_f direction of *fcc*-Pd and the "ABAB" stacking along the [001]_h direction of 2H–Pd (Figure 2l-n), the latter of which was reported for the first time and are expected to find wide applications in the synthesis of heterophase nanostructures and catalysis. Similar cases can also be found in many other metal NPs synthesized from welldesigned one-pot or epitaxial growth methods, where atomicresolution STEM images serve as a powerful means for confirming their powel crustal structures 2^{26-28} confirming their novel crystal structures.²

In addition to the overall external size, shape, structure, and rough elemental distributions in single NPs, EM can also resolve structure-alteration-induced strain in NPs. As an example, the lattice spacings of Pd in Pd nanosheets with different atomic layer thicknesses can be measured from aberration-corrected HRTEM images, from which the strain inside the nanosheets was calculated.²⁹ Due to attraction between surface atoms, the nanosheets experienced larger compressive strain when their thickness got smaller. This intrinsic strain was later proved to be a key factor affecting the catalytic performance of catalysts, revealing the significance of characterization methods in assisting the discovery of reaction mechanisms and catalyst design. Similar strain control can also be realized by tuning lattice parameters of supports. The resulted expansion or contraction in catalysts can be visualized and quantified from atomic-resolution STEM images, which was further correlated to their catalytic performance and used for understanding the mechanisms.^{30,31}

Besides imaging individual particles at the atomic scale, an electron microscope can also be used to characterize the ensemble of NPs, such as self-assemblies, to visualize their structure.³² For example, from TEM images of samples at different tilt angles without performing extensive tomography, the twisted dimeric structures of patchy particle assemblies were observed and misaligned angles between patches were measured, assisting further explanation of their formation mechanism involving electrostatic repulsion and steric hindrance (Figure 2g,h).²¹ Under a lower magnification, a larger scale of ordered NPs can be characterized, with their positions, orientations, and components clearly identified under electron microscope. Figure 2k-m shows some characteristic projections of binary superlattices self-assembled from different materials and in a variety of structures.³³ The tight placement of NPs also suggested the maximization of packing density as a driving force for the formation of binary superlattices. By tuning the size, shape, component, surface ligand, and charge of building blocks, one could expect more diversified superlattices to be realized with programmable physical and chemical properties.

Pseudodynamics of Colloidal NPs. Before the advent of in situ EM, standard TEM utilizes the strategy of taking static images of aliquots of samples quenched in a time sequence (e.g., during a synthesis or assembly process) to understand the formation mechanism. Such quenching can be done on a regular TEM where the products obtained at different time steps can be centrifuged out of the reaction solution and thus maintain a "paused" state of growth, processing, or assembly. One prominent type of examples is in morphology-controlled synthesis of NPs.³⁴⁻³⁶ For instance, during the synthesis of sharp-cornered Ag nanocubes with CF₃COOAg serving as the precursor and cetyltrimethylammonium chloride (CTAC) as the surfactant, TEM images at different stages provided important evidence of the growth mechanism. The generation of AgCl nanoscale octahedra was observed at the initial stage (5 min into reaction, Figure 3a), followed by nucleation and growth of Ag nanocrystals both on the surface and in the interior of the AgCl octahedra (Figure 3b-d), which finally evolved into cubic shapes in the presence of CTAC and FeCl₃.³⁶ Aside from the nucleation and growth pathways of NPs, the change in crystal structure of NPs during various processing conditions, such as heating and ligand exchange, can also be characterized using this quenching method.³⁷ For example, the transformation of Pd NPs from fcc to an amorphous phase after ligand exchange was faithfully recorded by ex situ HRTEM image series and corresponding SAED patterns.³⁸ The crystalline Pd NP transformed into a coreshell structure containing a crystalline core and an amorphous shell after replacing oleylamine with bismuthiol I as surface ligands for 1 h, and finally into an amorphous phase after 24 h with Pd atoms arranged in a non-close-packed manner. In this case, HRTEM serves as a powerful tool for resolving the crystal structures and thus unveiling the reaction process.

Aside from regular TEM, cryo-EM is also widely used for characterizing the growth of inorganic NPs where NPs in a solution could be flash-frozen and quenched. The advantages over the "quenching" for a regular TEM experiment are that (i) beam-sensitive NPs can be protected better with the vitrified ice and the optimized low dose conditions in cryo-EM,

enabling studies such as the transformation of amorphous aggregates to crystals for zeolite NPs³⁹ and the nucleation and growth of polystyrene (PS) nodules on silica NPs,⁴⁰ (ii) species other than NPs in solution, such as shape-directing molecular assemblies and ligand complex, can also be maintained and imaged under TEM.41 A representative example is the morphology change of cetyltrimethylammonium bromide (CTAB) micelles in the synthesis of Au nanorods (NRs).⁴² It was observed that the addition of Hofmeister salts (e.g., NaNO₃, NaCl, NaHSO₄) increased the proportion of ellipsoidal (1.5 < aspect ratio <3) and rod-like (aspect ratio >3) micelles, affecting the aspect ratio of final products. Complex NP assemblies with beam-sensitive materials can also be characterized by cryo-EM, such as binary NP superlattices whose formation was guided by protein cages.4

By comparing the particle position before and after reactions, the motion of NPs can be inferred, such as their aggregation after electrochemical activation characterized using identical location (IL) TEM.44,45 In a typical experiment, a TEM grid is loaded with catalyst particles and used as a working electrode for electrochemical reactions such as oxygen reduction reaction (ORR) and water splitting (Figure 3e,f). The morphology and location of NPs are recorded before and after experiment, providing a wealth of information about the degradation mechanism of catalysts, including element leaching, aggregation, and detachment from carbon support, among others (Figure 3g,h). It should be noted that compared to ex situ characterizations, recording the shape evolution or movement of nanomaterials in real time can be more direct, which is now possible by the development of in situ TEM including environmental EM (ETEM), gas-heating EM, and liquid-phase EM. More details will be discussed in Section 5.5.

2.2.2. Organic and Biological Nanomaterials. Compared to the imaging strategies developed for inorganic NPs, imaging organic and biological nanomaterials presents additional challenges in low contrast due to low-Z elements⁴⁶ and surface charge build-up in SEM.^{47–50} Besides, they also show high sensitivity to beam effects (e.g., charging, ionization, heating⁵¹), which usually result in structural deformation and chemical damage. When it comes to biological materials with high water content, the challenge is exacerbated as EM is performed under high vacuum. Thus, optimization in sample preparation, special instrumentation of EM, and advanced postimage and data processing methods are often implemented for imaging these samples.

Surface Morphology and Internal Structures of Nanomaterials Using SEM Techniques. Surface and cross-sectional structures are often imaged in SEM. In doing SEM, sputter coating samples with a thin film (<5 nm) of noble metals, such as Au, Pd, or their alloys, or lower acceleration voltages between 5 keV and as low as 0.3 keV are used to reduce beam damage caused by accumulated charges built on a semiconducting surface.^{47,52} The former solution may introduce damages to the sample substrate and may even result in a nonuniform coating, thereby damaging the sample integrity.⁴⁷ For the latter method, a decrease of the signal-to-noise ratio (SNR) could occur, which ultimately affects the final image quality⁵³ and can be compensated with advanced image processing methods. For example, a machine learning (ML)based method was reported to super-resolve low-resolution SEM images, where a convolutional neural network (CNN) was trained using a set of low-resolution SEM images and the



Figure 4. (a) Cryo-FIB SEM image of the solid–liquid interface of strontium titanate substrate, layer of alternating lanthanum ferrite/bismuth ferrite and frozen water droplet. (b) Magnified cryo-SEM image of the solid–liquid interface shown in panel b. (c) SEM micrograph of Liesegang band patterns seen in silver nitrate–chromium(VI) system in gelatin medium. (d) FIB-SEM image of the cross section of the band pattern showing the particle distribution along the thickness of the gel film. Scale bars: (a) 50 μ m, (b) 500 nm, and (c, d) 10 μ m. (a, b) Adapted with permission from ref 58. Copyright 2020 American Chemical Society. (c, d) Adapted with permission from ref 59. Copyright 2016 The Royal Society of Chemistry.

high-resolution counterparts.⁴⁹ In a similar study, SEM-based secondary electron spectroscopy and secondary electron hyperspectral imaging was used to examine the structure of semicrystalline poly(3-hexylthiophene).⁵⁴ By coupling SEM with simulation and ML, this study presented the fraction of amorphous and crystalline regions in poly(3-hexylthiophene).⁵⁴

Since its development in the 1970s, focused ion beam (FIB)milling has been widely used as a sample preparation method for soft materials to obtain electron-transparent samples suitable for TEM and, more prominently, coupled with SEM to view the cross-sectional morphology.⁵⁵ The technique, known as FIB-SEM, can be coupled with cryogenic sample preparation methods and used for a myriad of applications in organic and biological soft material characterization, such as differentiating domains of block copolymers⁵⁶ and cell envelope thickness measurements during bacterial biofilm formation.⁵⁷ For example, a study demonstrated using cryogenic sample preparation methods to characterize solidliquid interfaces using the FIB-SEM technique.⁵⁸Figure 4a,b shows the FIB-SEM images of the interfacial layer of lanthanum ferrite and bismuth ferrite on a strontium titanate substrate, where the sample was prepared by freezing a drop of water on the surface, followed by depositing a layer of organometallic platinum to facilitate a high-resolution cryo-SEM micrograph and cryo-EDX elemental mapping of a solidliquid interface.⁵⁸ FIB-SEM was also used to image the spatial distribution of micro- and nanoparticles in Liesegang band patterns seen in the periodic precipitation processes of silver nitrate-chromium(VI) in a gelatin gel medium.⁵⁹ It showed that the micro- and nanoparticle bands were not only confined to the surface, but also spread across the thickness of the gel film (Figure 4c,d).59

Size and Shape Characterization Using TEM Techniques. Comprehensive understanding of the size and shape of nanoscale organic and biological samples has been achieved in TEM imaging. To increase the TEM contrast, one of the commonly used methods is staining. Stains also distribute the energy from the electron beam over a larger area of sample

compared to unstained samples, effectively reducing the beam damage.⁴⁶ A myriad of staining chemicals exist, which mostly consist of a heavy metal element interacting with the sample to generate contrast by scattering electrons at high angles.⁶⁰ Positive stains, such as iodine, ruthenium, and ruthenium tetroxide, preferentially react with certain chemical bonds (e.g., heavy metals react with anionic carboxyl, phosphoric acid, and sulfhydryl groups in biological tissue⁶¹) in the soft material sample, hence localized in certain regions. In comparison, uranyl acetate, ammonium molybdate, phosphotungstic acid, and other electron-opaque negative stains cover the sample exterior, acting to enhance the outline. Characterization of deoxyribonucleic acid (DNA) origami structures under TEM commonly uses negative staining as a means of enhancing the inherently low contrast of DNA.62-65 DNA origami involves folding the nucleic acid into well-defined 2D and 3D shapes by utilizing hydrogen bonds between complementary base pairs.^{63,64,66,67} In one example, by staining with uranyl formate, DNA origami designs of a wireframe beach ball, concave and convex triangles were clearly resolved, which was achieved by targeted insertion and deletion of base pairs in bundled DNA strands (Figure 5a).⁶³ Interestingly, coating DNA origami structures in a silica shell was recently reported, leading to a higher image contrast from the electron-dense Si atoms when compared to the uranyl negative stain (Figure 5b).⁶⁷ While the latter stains the background, the former directly coats silica onto DNA origami and thus more efficiently increases its contrast.

Due to the diversities of accessible designs, nowadays DNA origami assemblies have been widely used as templating materials for NPs, $^{68-72}$ nanowires, 73 lipids, 66 peptides, 74 carbon nanotubes (CNTs), $^{75-77}$ and graphene. 78 It was demonstrated that DNA-lipid complexes can be produced by assembling multilamellar lipid structures around a DNA origami template through electrostatic interactions between the positively charged lipid and negatively charged DNA groups (Figure 5c). 66 This DNA-lipid assembly was clearly visible in negatively stained HRTEM images (Figure 5d). 66



Figure 5. (a) DNA origami designs of a beach ball (left), concave triangle (middle), and convex triangle (right). (b) TEM images of uranyl formate-stained DNA origami rings (left) and DNA origami rings coated in silica (right) with high magnification insets. (c) Structure of the DNA-lipid complex with DNA origami template covered with multilamellar lipid sheets. The interactions between the positively charged lipid head and negatively charged DNA backbone is shown. (d) HRTEM images of negatively stained DNA-lipid complexes, with the schematic representation of the areas in dotted lines. (e) Schematic of DNA origami nanoplate imaged in panels f and g. (f, g) STEM images of (f) uranyl acetate-stained and (g) unstained nanoplates on graphene substrate showing the contrast difference. (h) STEM image of stained DNA origami nanoplates on amorphous carbon and (i) a zoomed-in view of the dashed area. Scale bars: (a) 20 nm, (b) 300 nm, 30 nm (inset), and (c) 200 nm. (a) Adapted with permission from ref 63. Copyright 2009 The AAAS. (b) Adapted with permission from ref 79. Copyright 2017 Wiley-VCH.

tions into DNA origami structures (Figure 5e) using stained and unstained samples on free-standing graphene or amorphous carbon films, giving interesting insight on how the sample preparation methods affect image contrast and resolution.⁷⁹ The work showed that the negatively stained DNA origami nanoplates mounted on free-standing graphene has the highest contrast (Figure 5f), whereas the unstained counterparts showed both lower contrast and resolution (Figure 5g).⁷⁹ Interestingly, the negatively stained DNA origami nanoplates on an amorphous carbon substrate depicted the best image quality (Figure 5h,i), which was attributed to the heavy-metal stain and the lack of interactions between the carbon substrate and the DNA origami sample.⁷⁹ Despite these examples, it should be noted that 2D imaging is far from enough to illustrate the complex 3D morphology of DNA origami. To this end, 3D characterization methods such as electron tomography will be more suitable in capturing such complex 3D morphologies (see Section 3.2 for more examples).

Beam-induced radiation damage is a common challenge in soft material characterization using EM. At the atomic level, the electron beam interacts with the specimen to break



Figure 6. (a, b) Grayscale intensity-based plots of a TEM micrograph of a PA membrane, imaged under low-dose conditions: (a) Colored plot based on intensity and (b) a line-plot showing the thickness as 20.4 nm. (c-e) TEM images of (c) negatively stained and (d) unstained amyloid fibrils, and (e) unstained amyloid fibrils on graphene coated grids which showed high contrast in regions where the fibrils overlap. Scale bars: 100 nm. (f) Intensity profile of the fibrils along the lines marked in panel e. The overlapping region (green) shows twice as much the grayscale intensity as those of individual fibrils (blue and red). (a, b) Adapted with permission from ref 80. Copyright 2019 The Royal Society of Chemistry. (c-f) Adapted with permission from ref 85. Copyright 2013 Elsevier.

chemical bonds by displacing atoms.⁵⁰ This chemical damage is manifested as structural deformations such as thinning, shrinking, and warping of the sample under the electron beam.⁵⁰ Radiation damage of an electron beam can be effectively reduced by decreasing the electron dose rate and total doses.⁵³ TEM imaging of polyamide (PA) membranes by An et al. used a low dose rate of $4.0-6.9 \text{ e}^{-} \text{Å}^{-2} \text{ s}^{-1}$ to preserve the heterogeneous membrane morphology.⁸⁰ Membrane protrusions, i.e., crumples, were imaged with minimized electron beam damage, and elucidated a relationship between the monomer concentrations used in the synthesis and topological parameter of thickness (Figure 6a,b).⁸⁰ Another example used a low accelerating voltage (\leq 15 keV) to analyze the surface of plasma-modified and unmodified poly(ε caprolactone) scaffolds to show that both surfaces are morphologically similar.⁴⁷

Recently, graphene has emerged as a support substrate in EM, particularly in TEM techniques, because it reduces damage from the electron beam⁸¹ and its low thickness reduces both elastic and inelastic scattering,^{82,83} resulting in highcontrast⁷⁹—and sometimes high-resolution^{48,84}—images. A study which used graphene-coated TEM grids to image amyloid fibrils compared unstained and stained specimens.⁸ Although the stained amyloid samples showed an overall higher contrast due to the strong scattering of electrons by heavy metal ions in the staining agent (Figure 6c,d), with the use of graphene-coated grids, overlapping fibers of unstained amyloid fibrils were better resolved as darker regions compared to individual fibers, attributed to the mass-thickness contrast⁸⁵ (Figure 6e,f). Such detail was unobservable in the stained images, emphasizing the benefits of graphene in soft material EM.

Concluding the challenges and strategies described above, electron microscopic imaging has found wide applications in the study of organic and biological materials, and this is also evident from a myriad of reviews and papers on different aspects of the topic,^{86–89} such as polymers,⁵³ biological and biobased soft matter,⁸⁸ and battery materials.^{16,89}

2.3. 2D Real-Space EM-Based Analytical Techniques

2.3.1. Composition in Hybrid NPs and Polymer Membranes. Both EDX and EELS are commonly used techniques for chemical characterization and elemental analysis of heterogeneous soft nanomaterials, providing high-resolution elemental mapping even down to the atomic scale. For EDX measurements, as the sample is excited by the electron beam, the characteristic X-ray generated from different elements are separated into an energy spectrum, enabling the mapping and quantification of material composition. EDX is generally better suited for the detection of high-Z elements, as the detection of low-Z elements is limited by their low fluorescence yield, difficulty in absorption correction, and the potential absorption from the detector window.⁹⁰ Also, EDX identifies elements but does not provide more detailed chemical information such as electronic structure and chemical bonding of the sample.⁹¹ As a comparison, EELS shows higher detection efficiency for low-Z elements, for which the excitation edges tend to be sharp and well-defined, but the signal gets weaker beyond around 3 keV energy loss, making its detection on high-Z elements not comparable to EDX. EELS is a technique measuring the energy loss of electrons that pass through the sample and undergo inelastic scattering,92 which can give more information about the chemical and dielectric properties of a sample. EELS's unique ability to differentiate different valences of the same element, originating from the higher energy resolution of



Figure 7. (a) HAADF-STEM image, (b) secondary-electron (SE)-STEM image, and (c) EDX mapping of CoFe@LSCFM. (d) Atomic-resolution elemental mapping of LSCFM marked by a green box in panel a. (e) Normalized EELS spectra of Mn from the core (green) and shell (red) of a $MnO@Mn_3O_4$ NP. (f) The valence states distribution of Mn in a typical $MnO@Mn_3O_4$ NP, in which Mn_3O_4 and MnO are colored in red and green, respectively. (g) ADF-STEM image of SiC specimen with a stacking fault located at the center. (h) Line profile of the angle-resolved vibrational spectra across the stacking fault in the direction denoted by the black arrow in panel g. Color scale shows the signal intensity normalized by its maximum. TA, LA, TO, and LO represent the transverse acoustic, longitudinal acoustic, transverse optical, and longitudinal optical modes, respectively. (a–d) Reprinted with permission from ref 96. Copyright 2020 Wiley-VCH. (e, f) Reprinted with permission from ref 101. Copyright 2018 Wiley-VCH. (g, h) Reprinted with permission from ref 102. Copyright 2021 Nature Springer.

EELS, is a strong advantage over EDX. However, more complex data processing is usually required for EELS.

Generally, EDX is much easier in operation and data analysis, enabling the identification of different atomic compositions in a heterogeneous nanomaterial. For example, the presence of elements can be easily revealed by EDX in bimetallic NPs, showing their side-by-side, tip-coated, coreshell, or mixed distribution.^{93,94} Aside from colloidal NPs, the position of heavy-metal ions on polymers can also be resolved by EDX, such as the widespread and site-localized distribution of Zn²⁺ and Pb²⁺ ions, respectively, after adsorption to PA membrane.95 With the development of modern technology, the attainable signal is largely improved and EDX can now be performed even at atomic resolution. For instance, the atomicresolution EDX mapping clearly showed the distribution of different atoms in La_{0.4}Sr_{0.6}Co_{0.2}Fe_{0.7}Mo_{0.1}O_{3-δ} (LSCFM) substrate and confirmed the exsolution of CoFe alloy NPs, where all the elements were evenly distributed in the substrate whereas only Co and Fe were detected in the NPs outside the substrate (Figure 7a-d).⁹⁶ The as-generated metal-oxide interface was later demonstrated as the active site for CO₂ adsorption and activation, contributing to a higher CO₂ electrolysis performance than the LSCFM counterparts. The atomic-scale EDX provides a viable method to understand the atomic structures and their functionalities in nanomaterials.

EFTEM is another technique that can be used for elemental mapping of hybrid NPs.⁹⁷ In EFTEM, a beam of high-energy electrons interacts with the specimen and results in elastic or inelastic scattering. After the electron energy loss spectrum forms, an adjustable energy slit can be used to allow only electrons with certain energy loss to pass through, forming compositionally sensitive images. If one chooses electrons that have no loss to pass, named zero-loss filtering, the inelastically scattered electrons will be blocked. In this way, the image contrast and resolution can be improved.^{98,99} By imaging with the electrons that have an energy loss corresponding to core losses of particular elements, one can obtain elemental information with high spatial resolution using EFTEM. For example, a BF-TEM image, alongside EFTEM Co L_{2,3} and Fe $L_{2,3}$ maps, clearly showed the high concentration of Co in the cores and the tendency for iron and oxygen accretion to form a shell in Co@Fe₃O₄ core-shell NPs, demonstrating the capability of EFTEM in elemental mapping.¹⁰⁰

2.3.2. Valence State, Plasmonic Properties, and Vibrational Spectroscopy of Colloidal NPs. Compared to EDX, EELS can not only reveal the elemental distribution in a nanomaterial, but also its physical and chemical information, such as valence state of elements and interactions of atoms with their neighbors. For example, EELS mapping showed the atomic-scale elemental distributions of Pt and Co in hundreds of Pt-Co NPs across different stages of catalyst aging, relating Pt-shell thickness to heat or acid treatment, particle size, surface orientation, and ordering.¹⁰³ In another report, the valence-state distribution of Mn in a MnO@Mn₃O₄ NP was detected by EELS mapping, verifying its core-shell structure that is hard to be distinguished by EDX (Figure 7e,f).¹⁰¹ By comparing the measured EELS spectra with standard ones, the chemical environment around atoms can be indicated. Using Al-doped TiO₂ NPs as an example, EELS spectra confirmed the entering of Al atoms into TiO2 lattice, as well as the transition of local structure around Al atoms from a TiO2-like environment to an Al₂O₃-like environment as the dopant concentration increases.¹⁰⁴ The observation of the transition

was realized through the comparison of EELS data of samples to the $\mathrm{Al}_2\mathrm{O}_3$ standard one.

EELS is also a powerful tool for studying localized surface plasmon resonance (LSPR) in metallic NPs, allowing the visualization of LSPR modes in NPs with high spatial resolution. For example, with STEM-EELS, the LSPRs and bulk plasmons can be probed on arrays of AuAg and AuPd nanodisks with varying Au contents, with the electron beam positioned 5-10 nm away from the edge of the nanodisks and passing through the center of disks, respectively.¹⁰⁵ The complex permittivity function can be derived as a function of Au content. The results highlighted the wide tunability of LSPRs with composition and charted different trends in optical properties (e.g., LSPR peak position and width) of AuAg and AuPd with varying compositions. EELS can also be used to investigate the various coupling interactions of metalsemiconductor nanocomposites, such as the strong coupling between the excitons of ZnO nanowires and the LSPRs of Ag NPs.¹⁰⁶ Besides, it can also be employed to measure the position and density of interband impurity states in nonstoichiometric oxides, to investigate the optical and electronic properties of atomic and nanoscale defects in electrically conducting and optically active oxides.¹⁰⁷

Recent developments in aberration correction technology have improved the attainable energy resolution in STEM-EELS to a record of ~ 10 meV, allowing vibrational spectroscopy to be carried out in the STEM.^{108,109} Such a technique promises the analysis of vibrational modes at fine structural features such as surfaces, interfaces, and grain boundaries with high spatial resolution (0.1-1 nm), and enables the direct detection of even hydrogen in organics and hydrogen storage materials. Taking advantage of this technique, mapping of the vibrational spectra of individual crystal defects was reported recently, unveiling how the imperfections in solids influence thermal conductivity and diffusivity.¹⁰² It has been demonstrated that crystal defects can affect the heat-transport properties of materials by scattering phonons and modifying phonon spectra. With SiC as a model material, a red shift of several millielectronvolts were detected in the energy of acoustic vibration modes near a single stacking fault, as well as an intensity change and the confinement of the changes to within a few nanometers of the stacking fault (Figure 7g,h), indicating the impediment of thermal transport and reduction in local phonon propagation caused by the defects.¹⁰² Aside from structural defects and thermal properties, nanoscale vibrational spectroscopy collected by STEM-EELS has shown great impact in investigating different properties of nanomaterials, though still an emerging field. Typical examples include the measurement of surface and bulk vibrational excitations in MgO nanocubes,¹¹⁰ phonon dispersion in hexagonal and cubic boron nitride nanoflakes,¹¹¹ and measuring the temperature of a nanomaterial with precision down to 1 K.^{112,113}

3. ELECTRON TOMOGRAPHY OF THE 3D STRUCTURE, MORPHOLOGY, aND COMPOSITION OF NANOMATERIALS

Building on the efforts and procedures in Section 2 for standard 2D real-space imaging, there are ever-growing needs and advancements in imaging nanomaterials' 3D structure, morphology, and composition. In particular, with advanced knowledge on the manipulation of biomaterials and biomimetic soft materials, synthetic soft nanomaterials such as polymeric membranes, nanocomposites, as well as colloidal



Figure 8. (a) Schematic of electron tomography showing the tilting of a cryogenically fixed sample. Tomography involves (b) collecting projections of the object and then (c) combining the projections to reconstruct the object in 3D. (d) Continuous rotation and recording approach applied to $Pd@SiO_2$ aggregates with different acquisition times of 6, 8, and 7 s (left to right). (e) Corresponding reconstructed volumes of $Pd@SiO_2$ aggregates. (a–c) Adapted with permission from ref 134. Copyright 2018 Elsevier. (d, e) Adapted with permission from ref 135. Copyright 2019 Elsevier.

and patchy NPs can reach the complexity of their natural counterparts, such as cellular organelles, viruses, proteins, and lipid assemblies. Imaging merely 2D projections is insufficient and 3D characterization is necessitated. 3D characterization carries different challenges for colloidal inorganic NPs and organic, biological materials. The former is robust against imaging electrons and can be reconstructed conventionally at a few nm resolution. The challenge lies in pushing for resolving single atom positions and corresponding strains, which are found relevant to their applications in catalysis, charge transport, and mechanical devices. For organic and biological materials, the radiation damage in the TEM mode is more serious when it comes to 3D imaging which requires constant showering of electrons onto the same sample area to collect a tilt series. Moreover, they exhibit complex and irregular 3D morphologies that are hard to quantify using simple shape parameters. In this section, the principles and applications of electron tomography to heterogeneous soft nanomaterials including the recent development of cryo-electron tomography (cryo-ET) will be discussed, ended with an introduction to emerging techniques including single particle analysis, atom electron tomography (AET), and Brownian one particle reconstruction.

3.1. Introduction to Electron Tomography

3.1.1. Brief History of Electron Tomography and Other 3D Imaging Techniques. Historically, laser-scanning confocal microscopy and X-ray computerized tomography are two imaging techniques commonly used in characterizing 3D structures of soft materials. The former was first introduced by Jinnai et al. in the mid-1990s, to resolve the morphology of bicontinuous polymer mixtures, ^{114–116} and the latter has been reported in imaging fiber-reinforced or porous polymers.^{117,118} While both imaging techniques allow 3D visualization, their spatial resolutions are traditionally limited to micrometer-scale with the recent innovation of pushing it to 7 nm in resolving NP lattices and multimaterial frameworks,¹¹⁹ still far below that of electron beam-based methods (0.1–0.3 nm for TEM and 0.5-1 nm for SEM).¹²⁰ On the other hand, field ion microscopy was introduced in 1951, which resolved individual atoms in 1955, evolved into a 3D imaging technique called atom probe tomography (APT) in 1980s.¹²¹⁻¹²³ While APT offers a high resolution below 0.15 nm on composition,¹²⁴ it has a few drawbacks; the most prominent one is that APT is a destructive technique.^{125–127} Apart from consuming the specimen during analysis, APT also has limited sample size and requires special sample preparation methods such as FIB-



Figure 9. (a, b) Schematics of (a) sample tilting in electron tomography with the tilt angle α labeled, and (b) the missing wedge of information. (*c*, d) Reconstruction of (c) sample with missing wedge showing elongation along the beam direction, and (d) sample reconstructed without missing wedge. (e, f) Schematics showing the missing wedge (f) replaced by a missing pyramid (e) in dual axis tomography. (g, h) Missing domains of poly(styrene-*block*-isoprene) in the (g) single axis electron tomographic reconstruction, as opposed to the (h) dual axis reconstruction. (a) Adapted with permission from ref 95. Copyright 2019 American Chemical Society. (b–d) Adapted with permission from ref 149. Copyright 2019 Springer Nature. (e–h) Adapted with permission from ref 156. Copyright 2005 American Chemical Society.

milling and chemical fixation, which may interact with samples and introduce unwanted artifacts.¹²⁸

Thus, electron tomography remains a versatile means of high-resolution (up to Å) 3D imaging by utilizing 2D projections to construct 3D structures, enabling the visualization and quantification of the complex structures of various samples. The possibility of using a series of 2D images to reconstruct a 3D object was mathematically postulated in 1917 by Randon.¹²⁹ Since its introduction, the technique was first applied in the field of medicine in 1963,¹³⁰ for the development of the computer-aided tomography (CAT) scanner, which has been even extended today as a diagnostic tool. In 1968, the first studies on the theory and application of electron tomography were proposed by De Rosier and Klug,¹³¹ Hart,¹³² and Hoppe et al.¹³³ At that time, the computational power required for practical application of electron tomography was lacking. With the developments in computation, microscopy (especially on automation) and spectroscopy, electron tomography is now widely used as a tool to elucidate complex 3D structures at nanoscale resolutions. In summary, electron tomography enables the access to the 3D morphology of an object via a three-step process (Figure 8a-c): (i) image series acquisition by tilting the sample typically at a range of $\pm 70^{\circ}$, at a certain fixed or varied angle increment, (*ii*) tilt series alignment to correct for axis and angle positions, and (iii) tomogram generation by computer-aided reconstruction methods (Sections 3.1.2-3.1.4).

3.1.2. Tilt Series Acquisition. Electron tomography regardless of TEM, STEM, or EDX modes commonly involves sequentially tilting the sample about an axis, i.e., the tilt axis, to acquire 2D image projections of a 3D object. The projections around the tilt axis are taken over a negative and positive angle range primarily via two methods. The first method involves tilting the object at equal angle intervals. In this case, the

resolution of the tomogram (d) depends on the number of projections (N) and the thickness of the sample (D), as proposed by Crowther et al.¹³⁶

$$d = \frac{\pi D}{N} \tag{1}$$

Thus, increasing the number of projections with equal angle intervals within a given tilt range improves the resolution of the final tomogram.¹³⁷ To this end, angle increments between 1° and 3° are usually employed in electron tomographic reconstruction.^{137,138} It should be noted that eq 1 only shows the theoretically achievable resolution, while the real resolution of the tomogram will be lower due to specimen positioning, tilt angle accuracy, and image quality. Using a fixed angle of increment assumes the sample to be spherical, with its thickness constant across the tilt range. However, this assumption does not hold true for most soft nanomaterials, such as polymer membranes, nanofibril assemblies, and porous hydrogels, where the thickness of the sample increases at higher tilt angles. For instance, for a cuboid-like sample, the thickness increases with the tilt angle α by $1/\cos \alpha$.^{139,140} To address this issue, a Saxton scheme was developed, where the tilt increments become smaller at higher tilt angles compensating for the increase in sample thickness due to tilting.¹⁴¹ A Saxton scheme requires a larger number of image projections when compared to the equal angle increment method, but it inputs data evenly in the Fourier space, which renders higher resolution tomograms.

Compared to hard materials, electron tomography of soft nanomaterials faces a unique challenge due to their beam sensitivity given the needed large number of tilt images and imaging time. This challenge can be overcome by using lowdose imaging and cryogenic sample preparation.^{95,142} As an example, the tomographic reconstruction of PA membranes used a constant 2° angle increment across a tilt range of -60° to $+60^{\circ}$ to acquire 61 projections with a low electron dose rate of 7.4 e⁻ Å⁻² s^{-1.95} Moreover, it has been recognized that manual tilting to acquire images is time-consuming and leads to high accumulative dose. To overcome this issue, semi-manual and automated image acquisition methods have been introduced.¹⁴³ For example, an automated, continuous rotation and recording approach was reported in 2019.¹³⁵ Even with limitations such as image blurring, Pd@SiO₂ aggregates were successfully reconstructed by acquiring tilt recordings in the range of $+72^{\circ}$ to -71° in 6-8 s,¹³⁵ as opposed to manual tilting of the samples which can take 2–5 h¹⁴⁴ (Figure 8d,e). Additionally, soft materials have a low TEM contrast which can

be remedied by sample staining or improving the amplitude

contrast⁴⁶ (see more details in Section 2.2.2). Both manual and automated image acquisition may introduce misalignments in the x-tilt axis and tilt angle. Hence, the projected images need to be aligned onto a commonly defined x-tilt axis at the tilt angle intervals, using fiducial tracking,¹⁴⁵ patch tracking⁹⁵ and cross-correlation,¹⁴ to ensure the quality of the final tomographic reconstruction. As an example, the tomographic reconstruction of the active layer of a polymer solar cell containing an alternating copolymer of fluorene and fullerene derivative used 10 nm Au NPs as fiducial markers to align the projected images.¹⁴⁷ The tomographic reconstruction of PA membranes instead used patch tracking for alignment.95 In recent years, ML has attracted increasing interest in improving the image quality through minimizing the tilting alignment and missing wedge problems, and more details will be discussed in Section 6.4.

3.1.3. Missing Wedge Effect. As shown in eq 1, the tomogram resolution can be increased by tilting the sample at a larger range. However, eq 1 does not account for experimental constraints, such as mechanical limitations of the sample holder, limited space between the objective lenses in electron microscopes, and sample thickness which blocks the electron beam and reduces image contrast at high tilt angles due to inelastic scattering.¹³⁷ These tilt range limitations gives rise to a wedge of missing data in the Fourier space of the tomogram, which translates to a low resolution tomogram in real space (Figure 9a,b).¹³⁸ Theoretically, this so-called missing-wedge effect causes undesirable artifacts such as elongation along the z-axis (beam direction)^{137,148,149} and structure distortion,¹⁵⁰ thereby affecting the integrity of the final tomographic reconstruction (Figure 9c,d). Note that in practical applications of tomography, the common maximum tilt range is -70° to +70°, which can already result in successful reconstructions, even without any corrections to the missing wedge effect. $^{153-152}$

The missing-wedge effect can be reduced by employing dual axis tomography, where the sample projected image series around two tilt axes are combined, replacing the missing wedge by a missing pyramid (Figure 9e,f).^{154,155} Dual axis electron tomography increases the tomogram resolution along the plane and depth of the object.¹⁵⁵ Furthermore, it is capable of reconstructing morphological features in soft materials with fiber-like,¹⁵⁴ cylindrical, and lamellar nanostructures,¹⁵⁶ which fail to appear in single tilt tomography due to elongation artifacts. Reconstruction of the anisotropic nanodomains of poly(styrene-*block*-isoprene) block copolymer demonstrated the ability of dual axis electron tomography to capture cylindrical morphologies.¹⁵⁶ While single-axis electron tomographic reconstruction of the block copolymer showed missing

domains, a dual axis tomography was able to reconstruct the complete 3D nanomorphology (Figure 9g,h).¹⁵⁶ Interestingly, the same group also introduced a special sample preparation method and a special holder to achieve a tilt range of -90° to $+90^{\circ}$.¹⁵⁷ A rod-shaped sample of a metal–polymer nanocomposite, fabricated by FIB, was attached to a modified molybdenum grid to acquire 181 images in the range of $\pm 90^{\circ}$, thereby eliminating the missing-wedge effect.¹⁵⁷

To completely rule out the missing-wedging effect, one method is to use the concept of "Brownian one particle reconstruction" (also known as "Brownian tomography") coined by Jungwon Park and his co-workers,¹⁵⁸ which utilizes the free rotation of a sample when dispersed in liquid to collect the 2D projections in all orientations, thereby reducing and nearly eliminating the missing-wedge effect. This approach was proposed and implemented by Park et al. in reconstructing the 3D morphology of colloidal Pt NPs in solution, sandwiched in a graphene liquid cell.¹⁴⁴ The random orientations of the tilts were aligned using a probabilistic initial 3D model generation procedure,¹⁵⁹ followed by 3D reconstructions using an image series containing 1,561 and 1,171 projections, to reveal the asymmetric structure of the Pt nanocrystals.¹⁴⁴ In a recent study,¹⁶⁰ the heterogeneity of 3D atomic arrangement of Pt nanocrystals in solution was studied, through acquiring a large range of projections from the Brownian motion of crystals within a short time span at 400 frames/s and using 1,000-3,000 projections for the 3D reconstruction. A resolution higher than 0.72 Å was reported, allowing the mapping of atomic positions within individual nanocrystals and quantitative studies of strain and lattice distortion.¹⁶⁰ As evident from the above examples, single particle tomography uses a higher number of projection images when compared to tilt seriesbased tomography to eliminate the missing-wedge effect, and to match the orientation of the random projections to the 3D structure of the sample. More discussion on this method can be found in Section 3.4.3.

3.1.4. Tomogram Reconstruction. Early tomography reconstruction work used a Fourier-space approach which is currently replaced by back-projection and algebraic reconstruction techniques. Back-projection involves superimposing and aligning each of the projected 2D images to yield the 3D reconstruction of the object. Depending on the sample filter used to correct an imbalance caused by nonuniform frequency sampling, two back-projection methods have been proposed: weighted back-projection (WBP) using a weighting filter to correct for the sampling error, and filtered back-projection (FBP) applying a high-pass filter (Figure 10a).¹⁶¹ An algebraic reconstruction technique was presented by Gordon et al. in 1970,¹⁶² which was later improved by Andersen et al. through



Figure 10. Central *xz*-slices showing missing wedge corrections of tomograms generated by (a) WBP, (b) SIRT, (c) Iterative compressed-sensing optimized nonuniform fast Fourier transform reconstruction (ICON) and (d) MBIR. Among them, only MBIR shows data in the missing wedge region. Adapted with permission from ref 167. Copyright 2019 Elsevier.



Figure 11. (a) STEM-HAADF image of the Au-TiO₂ thin film. (b) Orthogonal cross sections through the matrix of the reconstructions showing the distribution of larger and smaller NPs in the TiO_2 matrix. (c) Watershed analysis of silica NPs with the reconstructed volume (bottom right). (d) Voronoi cell tessellation (top) and closest neighbor identification (bottom) of silica spheres. (e) Representative tilt projections of the DNA-Au NP handcuff assembly showing the raw images with a signal-to-noise ratio of ~0.31 for the DNA strands in the first column, followed by intermediate and final projection views during iterative refinement. The last column shows the 3D reconstruction views at the given tilt angles. (f) 3D density map of the DNA strands at a SNR ratio of ~2.44. (g) Final reconstruction of the DNA-Au handcuff assembly showing the AuNPs and DNA strands. Scale bar: 10 nm. (a, b) Adapted with permission from ref 174. Copyright 2018 American Chemical Society. (c, d) Adapted with permission from ref 175. Copyright 2014 American Chemical Society. (e–g) Adapted with permission from ref 176. Copyright 2016 Springer Nature.

the introduction of the simultaneous algebraic reconstruction technique (SART).¹⁶³ The simultaneous iterative reconstruction technique (SIRT), presented by Gilbert in 1971,¹⁶⁴ is an algebraic reconstruction method which uses back-projection with iterative cycles (Figure 10b).¹³⁸ Even with the wide use of FBP and SIRT approaches, as well as improvements to these traditional techniques (e.g., discrete algebraic reconstruction technique¹⁶⁵ and weighted SIRT¹³⁸), these methods show limitations in correcting artifacts caused by the missing wedge, tilt axis, angle alignment variations,^{137,138} and low SNR caused by low-dose conditions used in soft nanomaterial tomography. To address these issues, Venkatakrishnan et al. introduced a model-based iterative reconstruction (MBIR) technique and highlighted that this approach improved the resolution and contrast of the final tomogram while reducing artifacts due to missing wedge,¹⁶⁶ when compared to WBP and SIRT methods (Figure 10d).¹⁶⁷ Recently, there is an increasing interest in ML-based methods to further improve the quality of reconstruction, the details of which will be discussed in Section 6.4.

3.2. Visualizing the 3D Structure and Morphology of Nanomaterials

3.2.1. Inorganic Colloidal NPs. Various properties of inorganic NPs, such as size, 3D shapes, and spatial distribution in a matrix, can be imaged by electron tomography. One notable example is the 3D reconstruction of Pd–Ru bimetallic NPs supported on mesoporous silica by Midgley et al.,¹⁶⁸ where HAADF-STEM tomography was first introduced. Since then, this technique has been applied to imaging the 3D structure of a variety of inorganic systems.^{169–172} For example,

the distribution of Pt NPs within a porous ceria nanocrystallite aggregate was characterized.¹⁷³ The 3D reconstruction revealed two types of pores existing in the ceria crystallites: micron-sized mesopores and nanosized micropores, contributing to their imperfect assembly. Furthermore, nearly half the 3-4 nm Pt NPs were found to be embedded within the ceria aggregate, revealing that the fraction of the Pt NPs exposed on the surface was lower than expected and a significant number of active sites for catalysis were lost.¹⁷³ Another example involves the leverage of HAADF-STEM tomography to reveal the size-dependent distribution of Au NPs on a TiO₂ dielectric matrix.¹⁷⁴ Smaller-sized Au NPs were found to embed into a dielectric TiO₂ matrix, whereas larger NPs are positioned on the surface of the matrix, showing a bimodal distribution of Au NP sizes after annealing (Figure 11a,b).¹⁷⁴ TEM tomography can also be applied to analyze the spatial distribution of inorganic NPs in polymers, where the quality of grafted silica NP dispersions in polymer matrices was quantified. The silica NPs were subjected to watershed treatment for segmentation, followed by Voronoi tessellation to map the interparticle distance and number of neighboring NPs (Figure 11c,d).¹⁷⁵

3.2.2. Hybrid Structures with Organic or Biological Polymers Coated on Inorganic NPs. Nanoparticles patterned with synthetic or biopolymer patches have gained research interest due to their capability to act as molecular analogous building blocks. The polymer patches on the NPs are often of nm–10s of nm in size and arranged in 3D over the NP surface. The patch arrangement is important in such systems, which determines the NPs' packing structure through directed assembly and contributes to applications such as drug



Figure 12. (a) Schematic for the microdomain structure of star terpolymer and (b) cross-sectional view showing PI (black), PS (white) and PDMS (gray) phases. (c, d) Two viewing angles of the reconstructed terpolymer. (e) Tomographic reconstruction of double gyroid and hexagonally packed cylinder phases with (f) the sample region used for reconstruction shown in the TEM image. (g) TEM and 3D structures of PS-*b*-PI copolymer NPs showing multipod structures with PS phase in blue and PI phase in green. Scale bar: 100 nm. (a–d) Adapted with permission from ref 182. Copyright 2003 American Chemical Society. (e and f) Adapted with permission from ref 185. Copyright 2009 American Chemical Society. (g) Adapted with permission from ref 183. Copyright 2016 Wiley VCH.

delivery, nanorobotics, and mechanical metamaterials.²¹ Many studies employ electron tomography as a means to determine patch arrangement in 3D. For example, in reconstruction of PS patches on spherical Au NPs, a tilt range of $\pm 70^{\circ}$ and tilt angle intervals of 2° was used, with the WBP approach for tomogram generation.¹⁵² This study used an interesting trick to increase the contrast of the low-intensity polymer patches against the Au NP core, where the projection images were defocused to create a Freshnel fringe, which was used as a boundary to identify the polymer from the background.¹⁵²

The first reconstruction of an individual double-stranded DNA (dsDNA) was reported by Zhang et al. with the TEM tomographic imaging of a dsDNA-Au nanosphere conjugate, where two nanospheres are bound together by dsDNA in a handcuff-like assembly (Figure 11e,f).¹⁷⁶ This work used a reconstruction method called individual particle electron tomography, which addresses tilt errors and image distortions by applying automatically generated masks and filters to small regions of the projections, giving rise to high resolution reconstructions.^{176–178} The negatively stained dsDNA had an average SNR of 0.56 in the acquired tilt images, which was improved to \sim 3.26 after image alignment to achieve a final reconstruction resolution of ~17.1 Å. In a more recent example, electron tomographic reconstruction was applied to visualize the distribution of particles in assemblies of DNAmodified Au NPs.¹⁵³ The work presented a method of selectively blocking Au NP surface with a polystyrene-bpoly(acrylic acid) copolymer and modifying the polymer free surfaces with single-stranded DNA, thereby facilitating the assembly of NPs via complementary strand interactions.¹⁵³ The reconstruction of the assembly between the polymercoated, DNA-modified nanocubes and nanospheres resolved not only the Au NPs orientations but also the polymer coating using FBP reconstruction.¹⁵³ Both studies used TEM for image acquisition, with tilt ranges of $\pm 60^{\circ}$ and $\pm 70^{\circ}$, along with tilt angle increments of 1.5° and 2°, respectively.^{153,176}

A common limitation acknowledged by such studies is the effect of substrate on the reconstruction of patchy NPs. In the case of the PS-patched NPs, the TEM grid caused the patches to form into a meniscus, which was attributed to partial wetting of the grid by PS.¹⁵² In the DNA-modified nanocube and nanosphere assembly, the face of the cube resting on the substrate cannot be reconstructed.¹⁵³ Similar to the patchy NP examples presented above, TEM tomography was also used to map the spatial arrangement of NPs in nanoclusters formed from Au NPs stamped with DNA patches¹⁷⁹ and the anisotropic growth of benzene-1,4-dithiol patches on Au NPs.¹⁸⁰

3.2.3. Polymeric Nanomaterials and Assemblies. Polymeric materials are often structurally complex due to synthesis and processing conditions such as reactant gradients in polymerization processes, stratification, and phase separation of multicomponent polymer blends.

Electron tomography was first applied to polymers in 1988 by Spontak et al. to reconstruct the microphase separated domains in a styrene-butadiene-styrene copolymer.¹⁸¹ This study was followed by a similar study in 2003, which imaged a block copolymer self-assembling into a nanoscale microphaseseparated morphology.¹⁸² As a popular type of systems employing TEM tomography, block copolymers with bicontinuous morphologies have the challenge that they are hard to be reconstructed using TEM tomography due to insufficient contrast difference between different polymer phases. In TEM tomography of such systems, selective staining of one phase is commonly employed. An impressive example is the use of a combination of staining and EFTEM to identify the microdomains of a PS, poly(dimethylsiloxane) (PDMS), and polyisoprene (PI) star terpolymer (Figure 12a-d).¹⁸² At zero energy-loss ($\Delta E = 0$), the unstained star terpolymer showed PDMS domains as high contrast regions due to the presence of Si atoms. Since the ionization energy for the Kshell electrons of Si atom is 99 eV, the inelastically scattered electrons from Si-rich PDMS decreased when $\Delta E > 99$ eV,



Figure 13. (a) Grayscale intensity-based tomographic reconstructions of PA membranes belonging to six different synthesis conditions. The concentrations of m-phenylenediamine (MPD) and trimethosoyl chloride (TMC) monomers used to synthesize each membrane are given within parentheses. (b) PA crumple reconstruction and the shape parameters extracted. (c, d) Perspective views (left) and cross sections (right) of water diffusion pathways of two PA films. (e) Tomographic reconstruction of the polymer particle and (f) the clusters (left) and matrix (right) segmented to show internal clusters. (a, b) Adapted with permission from ref 6. Copyright 2022 AAAS. (c, d) Adapted with permission from ref 190. Copyright 2021 AAAS. (e, f) Adapted with permission from ref 192. Copyright 2019 American Chemical Society.



Figure 14. (a, b) Cryo-ET reconstruction of the (a) outer surface and (b) cross section of the herpes simplex virus showing the membrane (blue), glycoproteins (yellow), capsid (light blue), and the tegument (orange). (c-e) Cryo-EM images (top) and reconstructions (bottom) of budding events of SARS-CoV-2 virus infected cells showing (c) early virion budding stage, (d) assembled viron in close proximity to the infected cell membrane, and (e) further away from the cell membrane. Scale bars: 100 nm. (a, b) Adapted with permission from ref 209. Copyright 2003 AAAS. (c-e) Adapted with permission from ref 212. Copyright 2020 Springer Nature.

resulting in brighter PDMS microdomains at high energy-loss $(\Delta E = 230 \text{ eV})$ and darker PI and PS domains. Taking advantage of the contrast change at different energy-loss levels, selective staining was used to further differentiate between the PI and PS domains of similar contrast. Osmium tetroxide was used to selectively stain PI, giving it the highest contrast at ΔE = 0 with PDMS and PS having comparatively lower contrast. When switching to $\Delta E = 230$ eV, PDMS microdomains emerged as the brightest regions, whereas PI and PS regions were dark, allowing for the successful identification of the three different domains. In similar studies, Higuchi et al., Jinnai et al., and Park et al. stained the PI phase of PS-b-PI bicontinuous systems on multiple occasions and successfully reconstructed the corresponding microdomain morphologies (Figure 12e,f).¹⁸³⁻¹⁸⁵ Three-dimensional confinement of the PS-b-PI copolymer yielded tennis-ball, mushroom, and multipod structures, which were resolved using selective staining and TEM tomography (Figure 12g).¹⁸³ However, staining might cause structural changes to the sample, which is worth attention.¹⁸⁶ Furthermore, in most cases, selective staining cannot be used for in situ imaging as it fixates the structure and hinders any dynamics,¹⁸⁷ except for special instances such as beam-induced staining on liposomes in liquid-phase TEM.¹⁸⁸ In comparison, STEM-based tomography methods, where the contrast depends on the chemical differences of the phases, can eliminate the need for staining and are sometimes more suited for tomography of dynamic systems.¹⁸⁷

Beyond recognizing the 3D shape and microstructures of polymeric materials, recent study on PA membrane reconstruction demonstrated the utilization of TEM tomography for quantitative morphometric studies (Figure 13a). Shape parameters such as the surface area-to-volume and mass-perarea were measured and local thickness and surface curvatures of the PA membrane were mapped.95 Groups of distinct morphologies showing the protruding structures of the PA membranes, named crumples, were presented. In a follow-up study, unsupervised ML was used to group the crumples into three categories, named dome, dimple, and pancake, based on a collection of more than 50 shape parameters derived from electron tomograms (Figure 13b, details on ML in Section 6.5.1).⁶ Importantly, unlike previous examples in Section 3.2, MBIR method was used in this study for tomograph reconstruction to minimize missing-wedge effect.⁶ In both studies, tomographic reconstruction was applied to visualize internal void and pores. Tilt ranges of $\pm 60^{\circ}$ or $\pm 70^{\circ}$, with angle increments of 2°, for collection and back-projection methods for tomogram reconstructions were used, as common settings for polymer tomographic reconstructions.¹⁸⁵

In addition to TEM-based tomography, the HAADF-STEM mode is widely used to generate contrast through Zcontrast,¹⁶⁸ mass, and thickness.¹⁹⁰ For example, a recent study used HAADF-STEM tomography to analyze a PA membrane.¹⁹¹ After reconstruction, the local polymer density variation was mapped to show a higher water permeance at regions of the PA membrane with a lower average density.¹⁹¹ In combination with EFTEM, the average density, free volume, and the water diffusion coefficient were calculated, followed by modeling the water diffusion pathways through the films (Figure 13c,d).¹⁹⁰ The 3D intensity distributions from the tomograms were converted to density, followed by calculating the fractional free volume, to show that the water diffusivity is low in regions of high PA density and low fractional free volume and vice versa. Similarly, Rajabalinia et al. demonstrated the cluster size characterization for structured polymerpolymer latex particles using the 3D reconstruction of STEM tomography, achieved by removing the polymer matrix to visualize internal and external clusters (Figure 13e,f).¹⁹²

Composite systems commonly used in photovoltaic devices have also been characterized using electron tomography. Polymer solar cells, for example, where the active layer constitutes blends of polymer and derivatives of fullerene,¹⁹³ are morphologically complex because the two phases separate to form a vast interface area between the polymer donor and the fullerene-derivative acceptor, which affects the performance of the solar cell.^{194,195} Atomic force microscopy (AFM) is conventionally used to characterize such interfaces,^{196,197} along with X-ray scattering,¹⁹⁸ SEM,¹⁹⁹ and TEM.²⁰⁰ However, AFM is a surface characterization technique and is unable to provide a 3D representation of the different phases.¹⁴⁷ In the late 2000s, Andersson et al. reported the use of electron tomography to visualize the active layer of polymer solar cells.¹⁴⁷ This electron tomography-based 3D reconstruction was adopted in subsequent studies not only to visualize the morphology of the active layer, but also for quantitative morphological analysis.^{201,202} In another example, HAADF-STEM was used to tomographically reconstruct a blend of poly[2-methoxy-5-(3',7'-dimethyloctyloxy)-1,4-phenylenevinylene] polymer and CdSe NPs, discovering regions with highly connected networks of CdSe NPs and those surrounding areas with low connectivity and density.²⁰²

3.2.4. Cryo-ET of Biological Soft Materials. When subjected to high vacuum environments, a biological sample dehydrates, thereby destroying its structural integrity. To overcome this challenge, in 1974, Taylor and Glaeser proposed a frozen hydration method to preserve the structure of protein crystals in electron diffraction studies.²⁰³ This work was



Figure 15. (a) Zero-loss BF image and (c) EF image of bulk-heterojunction thin film with poly(3-hexylthiophene) and butyric acid methyl ester phases. (b, d) The phase distribution at different depths obtained from tomograms reconstructed from (b) zero-loss BF images and (d) EFTEM images with field views of ~900 nm. (e) Reconstructions of different ratios of polyisoprene rubber (light blue) and styrene-butadiene (dark blue) rubber blends with silica distributed between the two phases (purple and yellow). Scale bars: 200 nm. (f) Plot of silica distribution as a function of blend ratio. (a–d) Adapted with permission from ref 151. Copyright 2010 American Chemical Society. (e, f) Adapted with permission from ref 220. Copyright 2021 Wiley-VCH.

followed by McDowall et al., who introduced the plunge freezing method in the 1980s,^{204,205} where the sample in a thin liquid film is plunged into liquid nitrogen-cooled ethane to capture the sample within a layer of thin amorphous ice.²⁰⁶ Over the years, this method has developed into a powerful tool to characterize fragile soft material nanomorphologies, by capturing them in their native hydrated states.

Cryo-ET is widely used in visualizing heterogeneous morphologies of biological materials with resolutions of 3-4 nm.^{207,208} For example, it was applied to reconstruct the structure of the Herpes simplex virus, with the viral envelope, glycoprotein spike protrusions, interior tegument, and intraviral nucleocapsid clearly segmented (Figure 14a,b).²⁰⁹ Unlike viruses, using cryo-ET to reconstruct cells is quite challenging due to their larger size. Like most tomographic methods, cryo-ET uses dose-limiting conditions to preserve the chemical and structural integrity of the biological material by minimizing beam damage. Most cells are too thick to be used in these dose-limited conditions as the size exceeds the range of the mean-free path of inelastically scattered electrons. Therefore, samples are sliced to obtain thin sections which are transparent to the electron beam. There are two widely used approaches for sample slicing: mechanical sectioning of the cryogenic

sample using microtome knife, and FIB milling. As the former introduces more artifacts, such as blade indentations and crevasses,²¹⁰ FIB-milling is widely used in cryo-ET of interior structures in large cells.²¹¹ A recent study of SARS-CoV-2 virus used FIB-SEM to obtain sections with a thickness of 150 nm coupled with cryo-ET to reconstruct the budding and reassembly of the SARS-CoV-2 virion in entering a host cell (Figure 14c-e).²¹² To facilitate cryo-ET of larger samples, such as biological tissues or small organisms, a special cryo-FIB lifting technique was recently introduced.²¹³ This method uses special TEM grids and gripper-tips to cut-out sample sections for cryo-ET. They demonstrated the viability of this method by reconstructing the ribosomes within a Caenorhabditis elegans worm.²¹³ In comparison with the single-particle analysis (see Section 3.4.1), though they both use similar cryogenic sample preparation methods, cryo-ET analyses tilt projections of a given sample unit, while single-particle analysis examines different orientations of a collection of sample units to determine the 3D structure.

3.3. Spectroscopic Electron Tomography of Soft Nanomaterials

Recent development in EDX and EELS also allows them to be combined with electron tomography, enabling the character-

ization of 3D elemental distribution in NPs.²¹⁴ Though there are lots of difficulties in realizing tomography using EELS or EDX signals, including the relatively low SNR, change in projected specimen thickness during a tilt-series acquisition, large datasets, and complication in interpretation, a number of different strategies were proposed for addressing these challenges, such as compressed sensing and total variation minimization (TVM) approaches.²¹⁵ These strategies have enabled EDX and EELS tomography for nanomaterials, such as mapping the elemental distribution in an Al-Si alloy, galvanic replacement of a Ag nanocube by Au, and the oxidation state in iron oxide NPs. Compared to EDX, STEM-EELS tomography can be more powerful in resolving not only the elemental distribution, but also oxidation state and plasmonic properties of a nanomaterial. For example, the 3D images showing the spatial distribution of LSPRs of an individual Ag nanocube can be reconstructed through STEM-EELS and electron tomography, mapping the excitation across a range of orientations.

EFTEM and STEM-EELS tomography have also been used to visualize different components in organic materials. For example, poly(3-hexylthiophene) and butyric acid methyl ester, which show low differential contrast under BF-TEM, are widely used in organic solar cells as bulk-heterojunction thin films. Though their 3D morphology can be analyzed by TEM tomography,^{199,216,217} most studies use phase contrast as a mechanism to differentiate the two phases, which is low in spatial resolution and suffers from image blurring. Furthermore, unlike amplitude contrast that can be achieved by staining methods, phase contrast is not related to the mass thickness of the sample, so the bright and dark contrast in tomogram reconstructions cannot be simply considered as low and high mass thicknesses, respectively. To avoid potential misinterpretation, EFTEM tomography was introduced to differentiate the phases in bulk-heterojunction thin films (Figure 15a,c).¹⁵¹ The researchers used the Saxton scheme (see Section 3.1.2) to acquire 141 EFTEM images in a tilt range of $\pm 70^{\circ}$, with an energy slit of $\Delta E = 5$ eV centered at E =19 eV on the low-energy shoulder of the bulk plasmon peak for the thin film, to provide the optimal contrast between the poly(3-hexylthiophene) and butyric acid methyl ester phases (Figure 15b,d).¹⁵¹ The image contrast in EFTEM is generated by the differences in chemical structures between the components of the system,²¹⁸ and elemental mapping can be further obtained from EELS.²¹⁹

A recently developed multidetector EDX system contains four silicon-drift detectors placed symmetrically around the optical axis near the specimen, which allows for the acquisition of elemental maps with equal quality and high-detection efficiency over the entire range of tilt angles. One example is the utilization of STEM-EDX and HAADF-STEM tomography on beam-sensitive composites consisting of silica distributed between two phases of PI rubber and styrene-butadiene rubber (Figure 15e).²²⁰ The reconstructions showed that silica was preferentially distributed in the styrene-butadiene rubber phase, but it could also be found in the PI rubber phase when the PI rubber fraction reached higher than 40 wt % (Figure 15f).²²⁰

Incorporating into different types of electron microscopes, EDX and EELS can provide chemical information on nanomaterials in a wider range of situations. For example, with cryo-EM, EDX and EELS permit the characterization of native state or in situ analysis of NPs in aqueous suspensions. In one study, cryo-TEM and cryo-STEM were compared. It was found that relative to TEM mode, far higher total electron fluences were needed to induce damage to the vitreous ice in STEM mode, indicating the opportunity for individual NP identification using EDX and EELS with cryo-STEM. Besides, 2D EDX and EELS mapping have also been used with liquid-phase TEM, enabling in situ characterization of elemental and valence distribution inside NPs during chemical reactions (Section 5). Considering the developments in EM, we are expecting more future research on combining in situ techniques with spectroscopic electron tomography, uncovering the 3D distribution of elements/components in NPs in their native states or during reactions.

3.4. Recent Developments in Electron Tomography

3.4.1. Single Particle Analysis of Synthetic Soft Materials. Electron crystallography is used to determine the structure of proteins at the atomic resolution.^{221,222} This method, however, requires ordered 2D crystals, which is challenging to grow. In 1975, Joachim Frank proposed that, instead of using 2D protein crystals, the structure of the protein can be reconstructed by combining and aligning images of different orientations of similar protein particles.²² In combination with cryo-EM, a method called single particle analysis was developed, for which the 2017 Nobel prize was awarded. Since its introduction, single particle analysis has been widely used to determine the structures of biological macromolecules, and is now introduced to reconstruct the structure of synthetic materials by imaging different orientations within a cryogenically fixed sample.^{224–226} The major advantage of single particle analysis as a biomaterial imaging technique is that it requires neither crystallization (as needed in crystallographic techniques) nor large amounts of sample (as needed in nuclear magnetic resonance, NMR) and has been widely used to analyze biosamples such as small ribonucleic acid (RNA) molecules, membrane proteins,²²⁷ and nanoassemblies with multiple conformations. It should be noted that single particle analysis usually requires identical samples, both morphologically and structurally, which may set barriers to synthetic materials.

A low electron dose of $\sim 20-40 \text{ e}^- \text{ Å}^{-2}$ is usually leveraged in single particle analysis to avoid beam-related damage to the samples. This significantly reduces the SNR of the acquired images.²²⁸ When compared to conventional EM tomography, a larger number of images need to be collected for a successful reconstruction. The number of images may range from 10⁴ to 10⁶, depending on the sample and reconstruction algorithm used.²²⁸ The major challenge in single particle analysis is identification of relative orientations of the samples. One method is to use projection matching, where the acquired images are assigned an orientation based on a reference model of the structure.²²⁹ Later in the early 2000s, software incorporating iterative methods of assigning orientations was developed, which improves resolution and corrects for the contrast transfer function of the microscope.²³⁰⁻²³² Another challenge is to analyze conformationally heterogeneous samples. To address this issue, computational tools, such as RELION introduced by Scheres in 2012,²³³ have been developed with the ability to classify different conformations into different structural subsets, ^{233,234} even using noisy images.

With the development of programmable self-assembly of soft materials to create conformationally identical nanosystems, single particle analysis becomes also accessible to those systems. For example, it was applied to reconstruct silica cages of sizes smaller than 10 nm, with dodecahedral symmetry.²³⁵ This study used a ML algorithm to assign the orientation of the collected images, while simultaneously identifying and reconstructing the particles.²³⁵ RELION software was used in this work for reconstruction. Single particle analysis was also used to determine the structure of Au₆₈ clusters at the atomic resolution (Figure 16a).²³⁶ The reconstructions of Au atom



Figure 16. (a) TEM images (right) of the Au_{68} NPs, back projection reconstructions (middle) and class average images (left). (b) Electron density map (blue mesh), (c) atomic coordinates (red stars) of Au atoms with Au–Au distances, and (d) arrangement of atoms in the Au_{68} NP as determined by single particle analysis. Reprinted with permission from ref 236. Copyright 2014 AAAS.

positions revealed a truncated *fcc*-like packing, with interatomic distances of 2.72–3.1 Å, where 12 atoms were arranged in a cuboctahedron surrounding a central atom, followed by 24 atoms assembled in the *fcc*-like packing (Figure 16b,c). The rest of the atoms were observed to deviate from the *fcc*-framework due to the small cluster size (Figure 16d).²³⁶

3.4.2. Atom Electron Tomography. In 2012, AET was first demonstrated, which reached a 2.4 Å resolution without assuming crystallinity.²³⁷ With the Au NP as a model system, individual atoms were observed in some regions, and several grains were identified inside the NP. Since then, the 3D atomic structures of a variety of nanomaterials have been studied by AET, either crystalline or amorphous, unveiling the 3D structures of grain boundaries, stacking faults, screw dislocations, and point defects that are hidden in conventional 2D projections.^{144,238-240} With the coordinates of individual atoms determined, strain inside the NP could also be derived. The structural information can be further correlated to material properties, such as conductivity, mechanical property, and catalytic activity, enhancing our understanding of materials at the single atom level and guiding the engineering of materials to achieve better performance.

A notable example is the measurement of the atomic structure of an FePt NP, where the 3D coordinates (\pm 22 pm precision) and chemical species (99% accuracy) were precisely determined by AET.²⁴¹ A rich variety of structures inside the NP with 3D details were identified, including atomic composition, grain boundaries, antiphase boundaries, antisite point defects, and swap defects (Figure 17a,b). The atomic coordinates and chemical species experimentally measured were further input to density functional theory (DFT) calculations, from which the atomic spin, orbital magnetic moments, and local magneto-crystalline anisotropy energy were obtained and their direct correlation with local chemical ordering were identified. Similar work was presented using AET to visualize atom positions in a multiply twinned Pt NP, where dislocations and atomic steps at twin boundaries were resolved.²⁴² From the relative distance between atoms, the strain inside NPs can be measured. For example, a novel model-based method of measuring lattice coordinates was presented to investigate the lattice strain in Au decahedra,

revealing a systematic outward expansion of the lattice in both the *x* and *z* directions.²⁴³ Aside from internal defects and strain distributions in crystalline structures, atom arrangements in amorphous NPs can also be captured by AET.^{244,245} In a recent report, pentagonal bipyramids were identified as the most abundant atomic motifs in amorphous Pd NPs, which were found to arrange into pentagonal bipyramid networks with medium-range order.²⁴⁵ Such discoveries expand our understanding of nanomaterials to a wider range of structures at the 3D level.

Besides 3D imaging, AET can be further applied to capture atom motion in 4D (one dimension for reaction time), revealing the structural change and dynamics of atoms in nanomaterials during reactions (Figure 17c). The 3D atomic structure of FePt NPs with an accumulated annealing time of 9, 16, and 26 min were imaged and reconstructed.²⁴⁶ It was found that the Pt-rich core stayed nearly the same, while the atoms on and near the surface rearranged to form the L10 phase. The early stage nuclei were also tracked, each containing a core of one to a few atoms, and their behaviors of growth, fluctuation, dissolution, merging, and division were captured, which showed great dependence on the order parameter gradient and its distribution, as well as thermodynamics and kinetics.

With such an advanced technique of AET, deep exploration into the structure-property relationship and 3D atomic dynamics of nanomaterials is allowed and more fundamental research in materials science, nanoscience, and condensed matter physics are expected. So far the limitation of this method is that it works well for thin and small samples (usually NPs < 10 nm in size); otherwise, the electron beam cannot penetrate to obtain an atomic resolution. Besides, to our knowledge, the characterization of biological or organic materials using AET is still in absence, potentially due to their beam sensitivity and low contrast of light elements. With developments in low-dose imaging and contrast enhancement methods, atomic resolution tomography on these materials can be expected. Data acquisition speed can also be improved, enabling the tracking of dynamics of individual atoms and defects.

3.4.3. Brownian One Particle Reconstruction. As briefly mentioned in Section 3.1.3, from the Brownian rotational motion of NPs in liquid-phase TEM, their 3D structures down to the atomic resolution can be reconstructed, which is named Brownian one particle reconstruction (Figure 18a). Consider other 3D structure analysis methods: conventional electron tomography has a missing wedge problem where the tilting angle is usually limited to $\pm 70^{\circ}$, while single particle analysis (Section 3.4.1) requires a large quantity of particles identical in structure. Neither is needed for Brownian one particle reconstruction. In liquid-phase TEM, theoretically one can observe the full range of orientations of an individual particle during its random rotation, avoiding the missing wedge problem. Such orientations are obtained from one particle without the need for obtaining many identical copies of the same particle. Typically, a NP suspension is sandwiched between two sheets of graphene, forming a liquid cell with a thin liquid thickness. A fast detector is required, such as direct electron detector, to reduce rotation averaged blurring of rotating NPs and improve the spatial resolution.¹⁵⁸ The successful atomic-resolution 3D reconstruction also relies on the correct 2D/3D alignment and averaging distinct from



Figure 17. (a) Complex grain structure of an FePt NP experimentally determined by AET. The NP consists of two large $L1_2$ grains, three small $L1_2$ grains, three small $L1_0$ grains, and a Pt-rich A1 grain. (b) 3D atomic positions overlaid on the 3D reconstructed intensity illustrating antisite point defects (highlighted by white arrows): an Fe atom site occupied by a Pt atom (top left), a Pt atom site occupied by an Fe atom (top right), swapping between a pair of nearest-neighbor Fe and Pt atoms (swap defect) (bottom left), and an ideal $L1_2$ FePt₃ phase for reference (bottom right). (c) Experimental observation of nuclei undergoing growth, fluctuation, dissolution, merging and/or division at 4D atomic resolution: (a'-c') a representative growing nucleus at an annealing time of 9, 16, and 26 min, respectively; (d'-l') three representative fluctuating nuclei at three annealing times, showing merging and division of nuclei; (m'-o') a representative dissolving nucleus at three annealing times, which dissolved at 26 min (o'). The atomic models show Fe (red) and Pt (blue) atoms with an order parameter ≥ 0.3 , and the 3D contour maps show the distribution of an order parameter of 0.7 (red), 0.5 (purple) and 0.3 (light blue). (a, b) Reprinted with permission from ref 241. Copyright 2017 Springer Nature. (c) Reprinted with permission from ref 246. Copyright 2019 Springer Nature.

those used in biological cryo-EM, where more information can be found in a recent paper by Reboul et al. 247

The first report on Brownian one particle reconstruction is based on Pt nanocrystals with sizes smaller than 2 nm.¹⁴⁴ The as-obtained near-atomic structure indicates the presence of multiple domains in a single particle, with twisted grain boundaries existing at their interface (Figure 18b–e). These domains merge along the low-index crystal planes, such as

(100) and (110), which can be attributed to the coalescence between small particles during particle growth as a route to minimize excess surface energy. This finding also provides important evidence supporting the nucleation and growth model of nanocrystals that proceeds via merging of smaller NPs. Further analysis on the Pt nanocrystals synthesized from one batch manifests the structural heterogeneity of the products owing to the complexity of their growth trajecto-

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Figure 18. (a) Schematic illustration of in situ TEM imaging of Pt nanocrystals freely rotating in a graphene liquid cell. (b) EM density map obtained from the 3D reconstruction of two particles. (c) Cross-sectional view of the EM density map of particle 1 and 2 along the vertical plane. (d) 3D density map of particle 1 with color coding to highlight the three sections. Cross sections 1 and 2 are in arbitrary positions near crystal domain interfaces. (e) Slab through the 3D reconstruction of particle 1 along the vertical plane, with tentative atomic positions indicated. ABC repeats of $\{111\}$ planes are visible. (f) Sliced maps of the six components of the strain tensors for a single-crystalline particle. (g) Corresponding histograms of the strain tensors of all atoms (top), core atoms (middle), and surface atoms (bottom). Scale bars in panels b and d: 0.5 nm. (a–e) Reprinted with permission from ref 144. Copyright 2015 AAAS. (f, g) Reprinted with permission from ref 160. Copyright 2020 AAAS.

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ries.^{248,249} Shear distortions and dislocations were analyzed in the reconstructed 3D density maps, as shown in Figure 18f,g. Surprisingly, an overall expansion of the *fcc* lattice, varying from 0.95 to 2.56%, is observed in the poly(vinylpyrrolidone) (PVP)-protected Pt nanocrystals in solution, with the domain boundaries, dislocation edges, and surfaces experiencing an even larger strain. This finding contrasts with the expectation for vacuum-exposed NPs, where the lattice parameters are generally compressed due to effect of surface stress and low

coordination numbers. Further DFT calculations reveal the bonding between Pt atoms near the surface can be weakened from the binding of PVP ligands, resulting in the lattice expansion in the colloidal Pt nanocrystals.¹⁶⁰

From reconstructed 3D density and atomic position maps, the coordination number and generalized coordination number of surface atoms in individual Pt nanocrystals can also be measured.²⁵⁰ The high ratio of low-coordination surface atoms, small domain size of low-index facets, and rich types of high-



Figure 19. An overview of electron diffraction techniques. (a) SAED can select the sample area contributing to the electron diffraction via the aperture in the image plane of the objective lens. (b) NBD using parallel electron beam in a TEM. It achieves a small electron probe via imaging the condenser aperture on the sample with a third condenser lens. The convergence angle remains small for the parallel electron beam. (c) CBED in a TEM. A focused probe with large convergence angle is used. (d) Schematic showing electron diffraction patterns obtained by scanning electron nanobeam across the sample. Based on the electron diffraction patterns, the strain, phase, and phase orientation can be deduced. (a-c) Reprinted with permission from ref 264. Copyright 2005 Springer-Verlag. (d) Reprinted with permission from ref 265. Copyright 2021 American Chemical Society.

index facets are all important features explaining the outstanding activities of Pt nanocrystals in a variety of catalytic reactions. Aside from colloidal metallic NPs, a similar technique is also recently reported on ferritin.²⁵¹ When imaging proteins in Brownian motion using liquid-phase TEM, the acquisition time can be reduced from hours required for sample preparation for cryo-EM to seconds. The dynamics of the protein, such as conformational changes, hydration layer dynamics, and thermal fluctuations, can be potentially tracked, so-called the 4D (three dimensions plus time) characterization of biological nanomaterials.

Though powerful in high-resolution 3D reconstruction of single particles, the technique is limited by the potential shape degradation and structural transformation of NPs under an electron beam, which can be potentially solved by carefully selecting the solvent and analyzing the effect of dose rate. Besides, the fast movement of NPs also poses restrictions on its potential integration with other techniques such as EDX and EELS which require a relatively long time for signal collection. Currently, the NPs analyzed by Brownian one particle reconstruction are all smaller than 5 nm, and further research on larger particles are expected, while the restricted rotation in the confined space of a graphene liquid pocket might be an obstacle.¹⁵⁸

4. LOCAL STRUCTURAL ORDER AND DEFECTS CHARACTERIZED BY ELECTRON DIFFRACTION

As discussed in Sections 2 and 3, shape, size, morphology, and elemental composition can be measured from the real-space direct imaging at nanometer or even subnanometer resolution. Yet details such as the local structural order or defects, including the distortions of colloidal NPs due to their multiple components,²⁵² chemical reactions (e.g., during synthesis or catalysis),²⁵³ or phase transformations (e.g., stimulated by temperature or electrochemical biasing),^{254,255} are hard to see from direct imaging of EM. X-ray-based diffraction techniques^{256,257} have been the major methods for such structural characterization, but they are generally ensemble methods and cannot distinguish the local structural variations. Electron diffraction, on the other hand, is a powerful and versatile technique capable of identifying the structure of nanomaterials in a spatially resolved manner. Traditionally, this method is mostly applied to metals and ceramics, namely "hard materials", and has been the foundation of the theme of "microstructural engineering" that has led to recent advances in high-entropy alloys (HEAs),²⁵⁸ ultrastrong ceramics under extreme conditions,²⁵⁹ magnetic compounds,²⁶⁰ plasmonic materials and fibers,²⁶¹ and various solid electrolytes.²⁶² The extension of the electron diffraction-based method to soft materials has been limited due to highly focused beam during diffraction and the lack of universally robust crystalline order (i.e., nanocrystalline or even amorphous properties). Yet with recent development of new detector technology and data

processing algorithms, electron diffraction has been adapted increasingly to probe the structure (the arrangement of atoms and molecules) of soft materials with high spatial resolution (down to atomic scale) and with a large field of view (up to micrometers).

In this section, we will start with an introduction to the development and concepts of different electron diffraction techniques (Section 4.1). Their applications in various nanomaterials will be discussed next, from colloidal NPs, polymers, carbonaceous materials to metal–organic frameworks (MOFs), to unveil their structural details such as crystallinity, grain orientation, and atomic arrangement, among others.

4.1. Introduction to Electron Diffraction Techniques

Traditionally, transmission electron diffraction is conducted on a selected area of the sample using a broad beam illumination in TEM, known as SAED. SAED is conducted by inserting an aperture in the imaging plane of the objective lens (Figure 19a), and only the electron beam passing through the aperture contributes to the diffraction pattern in the far field. A regionof-interest (ROI) on the sample (typically from hundreds of nanometers to micrometers) is therefore selected by this aperture. To increase the spatial resolution of electron diffraction microscopy, the electron beam can be focused into a nanometer-sized probe in electron nanodiffraction modes. The application, named nanobeam electron diffraction (NBD), can be dated back to the 1980s.²⁶³ As shown in Figure 19b, the electron beam is focused to the focal plane of the upper objective and then forms a parallel beam incident on the sample. The beam size can vary from a few to tens of nanometers, which is smaller than that of SAED to detect local structural variations. In contrast to the parallel beam in NBD, convergent beam electron diffraction (CBED) focuses the beam at a large convergence angle at the sample (Figure 19c) down to a size of sub-Å. One unique feature of CBED is that the recorded diffraction intensities are composed of diffraction disks. Each pixel in each disk approximately corresponds to one incident beam direction. The information contained in the diffraction disks is particularly helpful for quantitative analysis of the local structure of materials. In these methods, the spatial resolution is approximately determined by the size of the primary peak (r_0) of the electron probe:

$$r_0 = 0.61\lambda/\theta_c \tag{2}$$

where θ_c is the beam semiconvergence angle and λ is the electron wavelength. As r_0 increases (or θ_c decreases), the electron diffraction patterns change from Ronchigram to CBED patterns and then NBD patterns. These three diffraction patterns provide local structural information on complementary length scales, varying from the atomic scale to the nanoscale, and are important as the foundation for electron diffraction imaging. More information can be found in the book chapter written by Zuo.²⁶⁴

Based on the nanodiffraction modes, the electron probe can be further controlled to scan across the sample automatedly with the diffraction patterns $(k_x \text{ and } k_y)$ recorded at various sample locations (x and y). This technique is known as scanning electron nanodiffraction (SEND) or four-dimensional STEM (4D-STEM), where the 4D space consists of the 2D real-space electron probe positions and 2D diffraction space electron wave intensity (Figure 19d). 4D-STEM is a high throughput method to determine and map the local structural properties of the samples, such as phase, orientation, strain, and short- to medium-range ordering. The development of 4D-STEM draws on the advancement of electron detector in TEM/STEM. The electron detector would ideally have readout speeds comparable to the scan rate of the electron probe $(10^{-3} \text{ s to } 10^{-6} \text{ s time scales})$, a large dynamic range to measure both high intensity in the bright field disk and low intensity from the high-angle scattered electrons, and high electron sensitivity.

Dynamical diffraction occurs if the electrons are scattered multiple times before they exit the sample, mostly found in thick samples. It can result in loss of the structure factor details in the diffraction patterns, generation of fine structures in each Bragg disk, and false reflections. All these effects will increase the difficulties in indexing, fitting, and other quantitative measurements of the Bragg spots. One method to reduce dynamic diffraction is to use the illumination mode of precession electron diffraction (PED), to collect multiple diffraction patterns from various incident beam tilt angles and then average them,²⁶⁶ which has been integrated with NBD and 4D-STEM.²⁶⁷ An alternative approach of multibeam electron diffraction (MBED) was proposed and tested.²⁶⁸ Multibeam experiments were conducted previously in SEM^{269,270} and electron beam lithography^{271,272} as it increases the throughput of the experiments. In TEM, an amplitude grating was placed in the TEM condenser optics to allow one on-axis beam and several tilted beams to pass through and illuminate on the sample simultaneously. Since multiple electron beams were employed, it covered a larger reciprocal space compared to a typical NBD pattern and therefore could provide more precision to determine the crystal orientations.

When it comes to weakly scattering samples of low-Z elements, dose-efficient techniques based on phase contrast imaging, including differential phase contrast (DPC) imaging and electron ptychography, have been used.^{273,274} DPC imaging is a STEM technique to reveal the electromagnetic field in a sample by measuring the deflection of the electron probe due to the field at each probe position in the sample. The method simplifies the diffraction pattern collected at each probe position to a two-element vector that describes the mean shift of the electron beam influenced by the electromagnetic field from the electron cloud and nucleus in the weakly scattering samples including various soft materials. Electron ptychography, on the other hand, is a phase retrieval method related to the coherent diffraction imaging. By performing overlapping measurements of the 4D-STEM and recording the full coherent diffraction pattern at each probe position, computational methods can be used to reconstruct the complex sample potential with superior spatial resolution. Recently, atomic resolution imaging of bilayer graphene was demonstrated based on electron ptychography.²⁷⁵ The highest spatial resolution has been reported on bilayer MoS₂, which estimates a spatial resolution of 0.39 Å at an acceleration voltage of 80 keV in comparison to the conventional ADF-STEM imaging resolution of 0.98 Å.²⁷⁶

The capability of 4D-STEM can be extended to image the sample in real space in the confocal configuration of STEM, which is known as scanning confocal electron microscopy (SCEM). The confocal imaging mode has been used for long in optical microscopy,²⁷⁷ and later introduced to EM in 2002.²⁷⁸ One of the potentials of SCEM is to reveal the 3D information on the sample without the need for sample tilting, as SCEM improves the depth resolution. The depth-sectioning



Figure 20. Electron nanodiffraction studies to determine crystal structure and facets of NPs. (a, c) SAED patterns collected from Pt THH NPs with the NPs tilted along the [001] and [011] zone axes, respectively. Inset: A dark-field TEM image of the THH NP. (b, d) Zoom-in experimental diffraction spots (left) and the simulated results (right). (e) NBD pattern of a 101 nm Au TOH NP oriented on the [110] zone axis. (f) Magnified view of (220) reflection spot showing streaks from this and other reflections, indicating additional facets growth. (g) A TEM image of the TOH NP in NBD mode. (h) Schematic of a TOH NP generated by computer. (i, j) Comparison between (i) experimental and (j) theoretical NBD patterns of Au₁₄₄(SCH₃)₆₀ cluster. A projection with 16 reflections is observed, which are indexed in the patterns. Inset: Au₁₄₄(SCH₃)₆₀ structure viewed along a 3-fold symmetry axis optimized by first-principles DFT calculations. (k) The SAED pattern of an individual grain in the YAG:Er polycrystalline sample oriented along the $\langle 113 \rangle$ zone axis. (l) SAED/PED pattern collected at 1° in the same zone axis as that in panel d. (m) PDF fit analysis of cryo-electron diffraction results obtained from ~4.5 nm Au NPs with precession, in comparison with structure model of decahedron (Dh) (Rw = 22%). (a–d) Adapted with permission from ref 289. Copyright 2007 American Institute of Physics. (e–h) Reprinted with permission from ref 296. Copyright 2021 The Royal Society of Chemistry. (m) Reprinted with permission from ref 297. Copyright 2019 American Chemical Society.

measurements can be performed by placing a pinhole aperture at a conjugate plane that can block electrons outside the focal plane, akin to the working mechanism of confocal optical microscopy.²⁷⁹ Comparing to electron tomography, SCEM also faces challenges such as achieving a high spatial resolution, penetration depth, and a large field of view.

Note that all these electron diffraction techniques can further be integrated with the atomic pair distribution function (PDF) analysis to quantify structurally disordered materials in terms of short- and medium-range ordering. For example, the local atomic environment and degree of the internal disorder in a nanomaterial can be unveiled by PDF method, such as strain in semiconductor alloys.²⁸⁰ PDF analysis is traditionally performed for XRD data obtained from synchrotron beamline. When combining PDF analysis with electron diffraction (ePDF), it enables the analysis on the single NP level.

4.2. Applications of Electron Diffraction Methods in Inorganic Colloidal NPs

Inorganic NPs and their assemblies in both the liquid state and dry state exhibit novel physical and chemical properties, playing a pivotal role in the emerging technologies such as catalysis, plasmonics, and semiconductors.^{281–283} The crystal



Figure 21. Crystallographic orientation of NPs revealed by scanning electron nanodiffraction. (a) The model of the decahedral NP and the reference frame for the crystal orientation mapping. Inset: TEM image of the decahedral NP close to the 5-fold $\langle 110 \rangle$ zone axis. (b) The color code figure from the inverse pole diagram along the [001] direction. (c) Crystal orientation maps of the decahedral NP corresponding to *x*, *y*, and *z* directions. The *x*, *y*, and *z* directions are also noted in (c). Scale bar: 50 nm. (d) Schematic of 4D-STEM mapping of the local lattice structure in cathode NPs that undergo the cubic spinel to tetragonal spinel phase transformation. (e) Virtual ADF image of a spinel cathode NP at the end of the first galvanostatic discharge. Scale bar: 20 nm. (f–h) Mn oxidation state map (f), crystallographic orientation map (g), and strain maps (h) for the same spinel cathode NP shown in panel i. (i, j) Left panels: The cubic spinel structure viewed at $[110]_c$ can transform to either tetragonal spinel structure of $[100]_t$ orientation when *c* axis is strained (i) or of $[111]_t$ orientation when *a* or *b* axis is strained (f). Right panels: experimental HAADF images show the atomic structures of tetragonal spinel $[100]_t$ and $[111]_t$ orientation in the cathode NPs at the end of discharge. Scale bars: 0.5 nm. (a–c) Reprinted with permission ref 291. Copyright 2016 Elsevier. (d–j) Reprinted with permission ref 306. Copyright 2022 Springer Nature.

structures of NPs, depending on factors such as the chemical composition, number of atoms, and the interatom interactions, can significantly influence the material behaviors, thus emphasizing the importance of identifying the microscopic structures of NPs. In this Section, we are going to introduce recent high-resolution electron diffraction methods employed to determine the atomic structures, crystallographic orientations, and even 3D structures of inorganic NPs.

4.2.1. Surface Facets and Crystallinity of NPs. Traditionally, the crystalline structure of NPs can be identified by SAED, including plasmonic metallic NPs such as Au and Ag,²⁸⁴ catalytic NPs such as Pt,²⁸⁵ Pd,²⁸⁶ Fe and Fe oxides,²⁸⁷ and more.²⁸⁸

Recently, the capability of SAED was extended to determine the high-index facets of NPs that could not be easily attained previously, with Pt tetrahexahedral (THH) NPs serving as a typical example.²⁸⁹Figure 20a shows the SAED pattern from a THH NP tilting along the [001] zone axis of the *fcc* structure. Fine features such as streaks can be identified around each diffraction spots, especially for those with high indices (e.g., (400) in Figure 20b). By comparing the experimental results with simulations, the fine features around the diffraction spots can be used to refine the shape of the NP. To this end, the researchers calculated the diffraction patterns of THH NPs tilted along the [001] and [011] zone axes with surface facets of {110}, {210}, {310}, {520}, and {730}. The results showed that the best matched result for the fine features was achieved when the NP shape was enclosed by {730} facets (Figure 20a–d), revealing the capability of electron diffraction in determining the complex structures that are hard to distinguish in conventional TEM.²⁸⁹

The 3D structural change of NPs can also be revealed by a similar NBD method, such as the change of surface facets on Au TOH NPs as their size increases.²⁹⁰ Nanorods, small (44 nm) and large (101 nm) TOH NPs were studied (Figure 20e-h). The direction and angles of the diffraction streaks were first measured for the 44 nm TOH NP. The streaks observed in the diffraction pattern were found to be normal to a series of high-index facets that were identified in the TEM image, confirming that the fine features could convey the facet information on the NP. As the NP size grew from 44 to 101 nm, more streaks were found around the diffraction spots, suggesting that more facets were formed during the growth of the TOH NP. Such NBD analysis of NP facets has also been applied to Au decahedral NPs to understand their surface structures.²⁹¹

Compared to NPs, metallic clusters consisted of a dozen or more atoms coordinated by ligand molecules have a much smaller size (usually <1 nm). They are scientifically interesting as they no longer follow classical physical laws, but could be interpreted by quantum mechanics. $^{292}\,$ The structure of the metallic cluster remains challenging to explore, except for a few cases studied by single-crystal XRD.^{293,294} Recently, the structure of the individual metallic cluster was studied by SAED by comparing electron diffraction with simulated ones.²⁹⁵ SAED was performed at a low acceleration voltage (80 keV) and fast dwell times to avoid beam damage. Figure 20i-j shows a set of NBD patterns used to illustrate the structure of $Au_{144}(SCH_3)_{60}$ clusters. The electron diffraction patterns collected in NBD-STEM mode showed well-defined diffraction spots, suggesting that the $Au_{144}(SCH_3)_{60}$ cluster likely retained its structural integrity during the collection of the diffraction pattern. The experimental diffraction pattern was further compared with the simulated ones calculated using a theoretical model. While most parameters matched with each other, the distances between Bragg spots in the experiments were slightly smaller than those of the first reflections in refined simulations. The mismatch was attributed to the diffraction from Au atoms attached to S atoms, which likely exhibits stretched bond lengths.

In addition, the precision in determining NP facets depends on the quality of electron diffraction patterns, which can be further improved by the integration of PED. Figure 20k shows the electron diffraction patterns taken from a single grain area on the YAG:Er sample.²⁹⁶ Diffuse scattering is clearly observed between the Bragg spots likely due to the sample thickness and multiple scattering of electrons in the sample. This dynamical diffraction effect (see Section 4.1) can result in false reflections and nonproportional intensities in the diffraction patterns, making the analysis under the kinematical framework difficult. In this case, PED can be used to reduce the dynamic effects by rotating the incident electron beam and averaging the diffraction patterns. With precession activated, the quality of electron diffraction patterns is clearly enhanced by reducing diffuse scattering (Figure 201).

By integrating SAED with PDF, ePDF analysis has been conducted for colloidal NPs of different sizes to study their crystalline structures.^{297–299}Figure 20m shows the ePDF analysis performed for aqueous-phase Au NPs covered by lipoic acid ligands (~4.5 nm) using PED at cryo-temperature. By fitting and comparing the results to different Au structural models (icosahedron, octahedron, and decahedron), the authors indicated that the PED-ePDF data was sensitive to different crystalline structures such as monocrystalline (octahedron) and multiply twinned (decahedron, icosahedron) structures.^{300,301} Compared to local atomic resolution imaging of HAADF-STEM, PDF can probe a larger sample area, obtaining lattice information statistically and enabling the detection of a large number of samples.

4.2.2. Inner Microstructure of NPs: Domains, Their Orientations, and Grain Boundaries. While Section 4.2.1 discusses the overall crystallinity and surface facets of single-crystalline NPs and disordered materials, this section discusses the complicated cases of crystals consisting of multiple domains of different orientations, such as twinned crystals and polycrystals. In those systems, the local variations of crystallographic orientations in the NP structures may pose great influence on the optical and catalytic properties of the NPs, such as the damping of light^{302,303} and enhancement of catalytic activity.³⁰⁴ One of the well-known twinned NPs with heterogeneity in crystallographic orientation is the decahedral NP of 5-fold symmetry. By applying 4D-STEM to the Au

decahedral NPs at 1 nm resolution,³⁰⁵ inner structural features of each domain and the grain boundaries in the NPs were clearly resolved through a correlation analysis of the spatially resolved diffraction patterns. Alternative to the correlation analysis of the experimental data alone, theoretical calculation can be integrated to elucidate more complicated structures.²⁷⁴ One example is the multiply twinned, decahedral Au NPs with a barrel-like shape studied by scanning PED with the same step size and probe size of 1.5 nm (Figure 21a-c).²⁹¹ The electron beam was parallel to the 5-fold symmetry of the (110) zone axis of the barrel-like NP (Figure 21a). When viewing from different directions, the five crystal domains in a Au decahedron can be clearly recognized, confirming the twinned structure and equivalent pentagonal symmetry of Au decahedron similar to known decahedral NPs. Although NBD and 4D-STEM are widely used in the determination of the crystal structure and orientation, the recorded diffraction patterns could be impacted by the dynamic diffraction effects and the mistilt of sample from the zone axis. To this end, MBED can be used to improve the accuracy of crystal orientation determination. Similarly, MBED was conducted on the polycrystalline Au NPs as a proof-of-concept.²⁶⁸ The authors compared the MBED and single beam diffraction for orientation mapping of the polycrystalline Au NPs. The MBED method gave more contrast in the virtual dark-field (VDF) image, and it clearly revealed that the overall trend of the NP orientation was biased toward an (011) orientation. MBED could also reveal the height information of the Au NPs, despite that the tilt angles of the electron beam were relatively small (60 mrad $\approx 3.5^{\circ}$).

Microstructural domains are present not only in as-prepared NPs, but can be formed in otherwise single-crystalline NPs during ion insertion in energy storage and separation technologies. Electrochemical phase transformation induced by ion insertion is usually accompanied by both compositional and structural changes, including the microstructural development of domains. In the studies of insertion compounds, extensive efforts have been devoted to identifying the chemical composition heterogeneities associated with the diffusion- and reaction-limited mechanisms during charge and discharge. In contrast, the transformation-induced microstructure resulted from the loss of symmetry elements remain underexplored, despite the general importance of domains and microstructures in alloys and ceramics. Recently, the formation of oriented phase domains in the cathode NPs during the electrochemical phase transformation was mapped and elucidated quantita-tively for the first time.³⁰⁶ A collocated 4D-STEM and EELS imaging approach was employed for the study (Figure 21d). The model cathode materials of λ -MnO₂ NPs transformed from the high-symmetry cubic spinel structure (space group: $Fd\overline{3}m$) to the low-symmetry tetragonal spinel structure (space group: $I4_1/amd$) during Mg²⁺ insertion, with one of the principal axes strained up to 15%. The monochromated EELS mapping showed that the NPs underwent a solid-solution type transition during ion insertion, maintaining a relatively uniform intraparticle distribution of electronic structures (Figure 21e,f). On the other hand, the collocated 4D-STEM mapping showed that the newly formed tetragonal spinel phase in the cathode NPs exhibited different crystallographic orientations at the nanometric length scale (Figure 21g). Nonuniform strain distribution was present in the cathode NPs and correlated with the distribution of domains in space (Figure 21h). The results clearly showed the spatial decoupling between chemical



Figure 22. (a–c) Wide-field ptychographic reconstruction of Au NPs and graphitized carbon on a holey carbon support film. (a) Modulus and (b) phase of the ptychographic reconstruction are compared with the conventional (c) TEM image of the same area. The strong contrast in (a) and (b) corresponds to thick areas on the sample where the phase passes from π to $-\pi$. (d) Ptychographic reconstruction of Au NPs showing the atomic fringes at 0.236 nm. Inset: the full field-of-view is shown in the inset image. The modulus and phase of the reconstructions are shown together in the figure. The phase is represented by color and modulus by brightness as indicated by the color wheel scale. (e) Transverse profile of the modulus of the ptychographic reconstruction along the optic axis of the microscope. Insets: The x-y profiles of the probe at two different z positions indicated by the arrows. The left-hand inset plots the probe at the sample plane and the right-hand inset is at the focus of the beam. (f) HAADF image of the cerium dioxide NP used in the ptychographic experiment. The green box indicates the region where the ptychographic reconstruction process was applied and the transmission function was obtained. (g) Reconstructed real-space illumination intensity. (h) The phase of transmission function of the reconstructed sample using the reconstructed illumination. Scale bars: (a–c) 50 nm, (d) 5 nm (inset: 15 nm), and (g, h) 1 nm. (a– e) Reprinted with permission from ref 314. Copyright 2016 Springer Nature. (f–h) Reprinted with permission from ref 316. Copyright 2014 American Physical Society.

phases and crystallographic orientation in the electrochemical phase transformation. The principal axes (a, b, and c) of cubic spinel structure are equally likely to be stretched in the phase transformation due to the *fcc* symmetry. Therefore, the cubic spinel structure at the $[110]_c$ zone axis can transform into either $[100]_t$ or $[111]_t$ as the *c* axis or a/b axis is stretched (Figure 21i,j). The large strain gradients built up from the development of oriented phase domains were also found to have a large impact on the chemical diffusion coefficient by a factor of more than 10. In another study, electrochemical ion intercalation was used to extract lithium from diluted water

sources.³⁰⁷ During extraction and separation, lithium and sodium ions could potentially cointercalate into the cathode particles, such as olivine iron phosphaste (FePO₄), which is not favorable for lithium extraction. The cointercalation behavior could be manipulated through intercalation kinetics. 4D-STEM was applied to map the local lattice parameters of the ion-inserted FePO₄. By comparing the local lattice parameters with the theoretical ones, the study identified the lithium and sodium phases within the cathode particles at specific kinetic conditions.

Powder electron diffraction patterns, which were reported recently, are also shown to be helpful in analyzing the NP crystal structure in selected, nanoscale regions. During this process, a stack of diffraction patterns recorded in an SEM equipped with the pixelated detector (4D-STEM performed in the SEM) are combined into one composite diffraction pattern, showing diffraction rings that resemble the powder electron diffraction pattern. In a recent study, powder electron diffraction was performed on a Au nanoisland (~ 20 nm), small TbF₃ NPs (<5 nm), and large NaYF₄ NPs (>100 nm).³⁰⁸ The scanning area was chosen to be about a few hundreds of nanometers. The composite diffraction patterns and the radially averaged intensity profiles revealed the diffraction peaks characteristic to the samples, and the diffraction results in SEM were found to be comparable to the SAED patterns collected in TEM.

4.2.3. Atomic Structures Resolved by Electron **Ptychography.** The direct imaging of atomic structures of NPs holds significance in understanding the material structure, phase, and defects. Electron ptychography can overcome the aberration limitations in electron imaging since it records diffraction patterns without the objective lens. If the correct phase structure can be assigned to the diffraction data, the diffraction patterns could be used to calculate an aberration-free image. The theoretical concept of the ptychography was first suggested decades ago.^{309,310} More recently, practical iterative solution methods were developed for ptychography.^{311,312} The wave function can be calculated at the object plane via back-propagation to construct an image with a resolution mostly determined by the effective numerical aperture of the detector.

Small NPs with negligible dynamic effects provide outstanding model systems for electron ptychography. Since 2010, research efforts have emerged for using electron ptychography to solve the phase problem at the nanoscale. For example, the ptychographical iterative engine (PIE) was used to retrieve the phase change at the object plane in TEM.³¹² Based on it, an extended-PIE (e-PIE) was developed and showed improvement over PIE for its ability to reconstruct both the object and illumination functions simultaneously, with Fe_{0.3}Ni_{0.7} NPs as an example.³¹³ It also provides robustness to noise and fast speed of convergence. More recently, the electron ptychography was demonstrated in the TEM mode within an SEM, achieving a spatial resolution of 0.24 nm that surpassed the intrinsic resolution of SEM of 1.2 nm.³¹⁴ The experiment was performed by recording the diffraction patterns of a mixture of Au NPs and graphitized carbon. The images of sample (modulus and phase of ptychographic reconstruction) were obtained, together with the conventional TEM image (Figure 22a-c). The ptychographic reconstruction was found to be more sensitive to the thickness of the graphitized carbon layer at the top of the Au NPs than the conventional TEM image. It also indicates that the thickness of the sample was approaching the limit of validity of the projection approximation that the reconstruction algorithm relies on. In the sample area where Au NPs were not covered by thick graphitized carbon, the (111) atomic planes of Au NPs could be observed (Figure 22d). The lattice spacing measured from the image was close to 0.236 nm, largely improved over the conventional spatial resolution of SEM at 1.2 nm. By propagating the wavefront, the profile of the illumination along the optical axis (z) of the SEM was also reconstructed (Figure 22e). The results indicate that the NP thickness and inelastic scattering do not

significantly affect the ptychographic reconstruction, even though a low acceleration voltage of 30 keV was used where multiple scattering could occur.

More approaches for the ptychographic reconstructions have been developed at the nanoscale for robustness and being computationally efficient, including methods of iterative linear retrieval using Fourier transforms (ILRUFT) and global ptychographic ILRUFT (GPILRUFT).^{315,316} For example, the fast deterministic approach of GPILRUFT was demonstrated on a CeO₂ NP characterized in an aberration-corrected STEM, as indicated in Figure 22f.³¹⁶ The reconstructed realspace illumination (Figure 22g) and the reconstructed specimen transmission function phase (Figure 22h) revealed the atomic structure of the CeO₂ NP which was comparable to the results obtained from the established ePIE method.

4.3. Structural Ordering and Molecular Packing in Polymers and Biomaterials

A significant task in polymer science is to design nanoarchitectures of polymer assemblies with precisely controlled structures and properties. The chain conformation and structure are therefore needed to be characterized from the atomic to mesoscale. Electron diffraction provides the needed high spatial resolution as the wavelength of electron beams (typically <0.004 nm for 100 keV or higher) is much shorter than that of X-rays (typically 0.15 nm), although X-rays usually have a much larger interaction volume. In 1938, Storks applied electron diffraction to characterize some linear high polymers of polyethylenes (PEs).³¹⁷ Later, Keller and coauthors applied both EM and electron diffraction to study the submicroscopic morphology and related crystal orientations in crystalline high polymers such as PA.³¹⁸ Since then, electron diffraction techniques, such as SAED, have been applied in complementary to X-ray methods to study the structures of a variety of polymers such as poly(ethylene oxide), polypropylene, block copolymers like poly(ethylene oxide)-b-polystyrene, and semiconducting polymers like poly(3-hexyl-thiophene-2,5-diyl) (P3HT).^{319–323} The radiation damage of the electron beam is an important factor to consider in the characterization of polymers, which usually requires a case-by-case study considering the temperature, sample thickness, and the different types of polymers.^{324,50}

4.3.1. Local Crystallinity and Orientation in Synthetic Nanocrystalline Polymers or Stacking of Conjugated Polymers. Nanocrystalline polymers can form various types of complicated hierarchical structures, varying from the angstrom to micrometer length scales, such as row structure, spherulites, and shish kebab formed during crystallization.^{265,325} For example, planar-zigzag or helical chains can be repeatedly packed to form unit cells of the polymer. Flexible polymeric chains can fold and form thin lamellar crystal of 10-20 nm thickness.³²⁶ Due to the chain folding mechanism, crystalline domains in a polymer are usually bounded by amorphous regions. These complex structures of the polymeric chains and their assembly are, on the other hand, closely linked with the mechanical, electronic, and optical properties of synthetic polymers.³²⁷ For example, the nanoscale orientation of stacked crystalline domains in semiconductive polymers³²⁸ is an important factor influencing their electronic properties, such as the ability to generate excitons.³²⁹ The preferential crystallization of P3HT along the $\pi-\pi$ bond facilitates the movement of electrons and holes within the bulk crystal.³³⁰ Therefore, a fundamental understanding of polymeric



Figure 23. (a, b) ADF-STEM images and (c, d) orientation maps of (a, c) nonoriented and (b, d) orientated polyethylene obtained from 4D-STEM. The arrow in (b) indicates the stretching direction. The azimuth difference between the stretching and chain directions is denoted by colors in the color wheel with a range of $\pm 90^{\circ}$. (e) 4D-STEM mapping of P3HT/PS sample. The Bragg diffraction angle map of the scanned region with the angle map overlaid onto the ADF image of P3HT/PS sample. The legend is shown above as a colorwheel with the beamstop. (f-i) 4D-STEM mapping of the sample of p-DTS(FBTTh₂)₂ (T1) dropcast (f, h) without the cosolvent of 1,8-diiodooctane (DIO) and (g, i) with DIO. (f, g) The orientation maps show the direction of the brightest reflection spot in the diffraction pattern collected at the probe location. (h, i) The flow line maps show the molecular backbone structure: the T1 sample shows gradual lattice rotations while the T1/DIO sample shows rigid crystalline domains with overlap. (a–d) Reprinted with permission from ref 265. Copyright 2021 American Chemical Society. (e) Reprinted with permission from ref 332. Copyright 2016 Elsevier. (f–i) Reprinted with permission from ref 335. Copyright 2019 Springer Nature.

structures is important for designing high-order hierarchical structures of nanocrystalline polymers for devices. While electron backscattered scanning diffraction (EBSD) can effectively map the local grains in metals and ceramics at the nanoscale,³³¹ the sensitivity of polymers to EBSD is low, which would require an extended exposure time. Furthermore, polymers are usually composed of light elements such as C, O, N, and H, which lead to weak contrast between different nanostructures.

4D-STEM provides an outstanding platform for mapping the local crystallinity and lattice orientation from the atomic to the nanometer length scales. Figure 23a-d shows a 4D-STEM study of the nanoscale spatial distribution and orientation of lamellar crystals of PE.²⁶³ PE is a representative example of a beam-sensitive semicrystalline polymer. Two PE samples with significantly different crystalline properties were prepared: one was fabricated using thermal annealing (nonoriented) while the other was fabricated using stretching followed by thermal annealing (oriented). In the nonoriented sample, the orientation of the lamellae was observed to vary significantly (Figure 23c), forming orientation domains of ~ 100 nm in the sample. No clear correlation between lamellae orientations was found between the adjacent domains. In contrast to the lamellar crystals in the nonoriented sample, those in the oriented sample were uniformly aligned in the stretching direction forming the row structure (Figure 23d). The lamellae orientation was highly aligned in a homogeneous manner. The results demonstrated that hierarchical structures in PE can be captured by 4D-STEM, which did not require pretreatment of staining to enhance the image contrast. It is worth noting that electron irradiation damage was observed in the repeated scanning of the same sample area, although the samples were carbon-coated for reducing irradiation damage. As the scans were repeated, the diffraction intensity decreased, and the peaks became broadened and shifted.

Studies have been conducted to map the local lattice orientations of semiconductive polymers using 4D-STEM. Figure 23e shows an example of a P3HT/PS sample.³³² It is

worth noting that the electron diffraction patterns in soft materials usually have weak Bragg reflection intensities due to the low electron dose and light elements. In addition, there could be a decaying background (amorphous halo), resulting from the amorphous regions of the polymers, overlapping with the Bragg reflections, which could increase the difficulty in determining the Bragg reflections. In this work,³³² the authors took advantage of the amorphous halo to first align the diffraction patterns and then subtract the amorphous halo from each diffraction pattern based on a virtual annular aperture and developed algorithm to facilitate the identification of Bragg reflections. The crystallites tended to be distributed within the oval clusters of the polymer in a relatively uniform way (Figure 23e), and the crystallite orientations were likely to be randomly distributed with respect to one another. The measured periodicity at ~3.8 Å could match the 3.9 Å stacking period of successive polythiophene backbones along the *b*-axis of the monoclinic structure,³³³ which is the $\pi - \pi$ bond spacing, suggesting that the crystallite is oriented edge-on. Alternatively, the measured periodicity could also match the (002) reflection characteristic to the monomer spacing (3.88 Å) along the backbone, which could be resulted from face-on oriented crystals. It was difficult to distinguish these two configurations due to the fewer Bragg reflections for soft materials.

In addition to P3HT, the structures of a variety of semiconductive polymers, including p-DTS(FBTTh₂)₂ (denoted as T1 in the article), poly[2,5-bis(3-tetradecylthiophen-2-yl)thieno[3,2-b]thiophene], and poly(ethylene oxide), have been studied and revealed by 4D-STEM.^{334,335}Figure 23f-i shows two samples of the T1 molecule: one with cosolvent of 1,8-diiodooctane (DIO) and the other one without DIO.³³⁵ To control the electron beam damage, the samples were cooled with liquid nitrogen. In the collected diffraction patterns, the brightest π - π diffraction spot pair was used to determine the crystal orientation for each probe location. The T1 molecules without DIO exhibited ordered domains smoothly twisting over 180° range, reminiscent of the structure of liquid crystals (Figure 23f). In contrast, the T1 molecules with DIO showed a



Figure 24. (a) 4D-STEM mapping data obtained from the sample of a twisting tunicate cellulose nanofiber. The different zone axes of the cellulose $I\beta$ structure (bottom panel) can be identified from the electron diffraction patterns. (b) The representative diffraction pattern indexed as the [010] direction of the cellulose $I\beta$ structure. (c) HAADF image of a peptide nanocrystal (QYNNQNNFV). (d) Clustering of the diffraction patterns recorded by 4D-STEM scanning across the sample in panel c. Inset: the color wheel stands for the relative orientation from the mean in *x* and *y* tilts. The maximum deviation denoted by the color wheel is about 4°. (e) The average diffraction patterns from the clustering in panel c. (f) Low-dose cryo-ptychographic reconstruction of rotavirus double-layered particles at 22.8 e⁻ Å⁻². Scale bar: 100 nm. (g) Scheme showing the experimental setup for the 3D electron ptychography. (h) The 3D phase reconstruction of a DNA origami framework computed from the ptychographic data set. (i) Frequency histogram showing the background (green), DNA strands (orange), and Au NPs (light blue) that are indicated by the colored boxes in panel h. (a, b) Reprinted with permission from ref 341. Copyright 2021 American Chemical Society. (c–e) Reprinted with permission from ref 345. Copyright 2022 Springer Nature. (g–i) Reprinted with permission from ref 345. Copyright 2022 Springer Nature.

very different structure with segmented grains and domain boundaries (Figure 23g). The lattice orientation at each probe position is further represented by a colored and oriented line (Figure 23h,i). It is obvious that the nanostructure of the polymers, by adding DIO, transited from a liquid-crystal-like, continuous film to a partially segmented grain structure. Such high-resolution mapping of the polymer structures at different conditions can uncover important information about the crystallization pathways of polymer materials.

4.3.2. Local Crystallinity, Orientation, and Phase Information in Natural Polymers and Proteins. The understanding of the local structures in the natural polymers, for example, cellulose nanofibers, peptides, and proteins, is fundamentally important to explain their physical properties and biological functions. For example, cellulose is ubiquitous in our daily life and has a range of applications from paperboards

to biological and optical materials.³³⁶⁻³³⁹ Nanocrystalline cellulose can consist of various nanostructures, including nanofibers and nanocrystals. The mechanical properties of cellulose nanofibers are dependent on the formation of helical superstructures and the ordering of polysaccharide chains, which is conventionally characterized by ensemble methods such as X-ray and neutron diffraction.³⁴⁰ Recently, 4D-STEM was used to characterize the local ordering of polysaccharide chains in cellulose nanofibers (Figure 24a).³⁴¹ The recorded diffraction patterns (Figure 24b) showed strong Bragg reflections, which indicated that 4D-STEM can serve as an appropriate technique for the characterization where the structure of cellulose nanofiber was not obviously damaged by the electron beam. The recorded diffraction patterns along a single cellulose nanofiber were indexed to different crystal orientations, such as [010], [110], and $[1\overline{10}]$, corresponding to

the monoclinic I β cellulose structure. These orientations were sequentially arranged along the cellulose nanofiber, suggesting a twist of the nanofiber along the *c*-axis of the structure.

The local crystallinity in protein crystals can be probed and revealed by 4D-STEM. Figure 24c-e shows the 4D-STEM mapping of the lattice structure across micrometer-sized areas of cryogenically preserved 3D peptide crystal. The number and intensity of Bragg reflections varied across the different regions on the peptide crystal, with weaker reflections generally observed in the thicker regions. Using an unsupervised classification method, nanodomains within the peptide crystal were distinguished from the diffraction pattern clusters. The changes in diffraction patterns across the peptide crystal were found to be originated from about $\pm 1^{\circ}$ tilt of the lattice away from the mean orientation of the crystal (Figure 24d).

Aside from local crystallinity, the phase image of biological specimens can also be recovered by electron diffraction techniques, such as the utilization of cryo-electron ptychog-raphy in the scanning electron diffraction (SED) mode.^{342,343} The conventional cryo-EM technique usually takes advantage of the phase contrast imaging at high defocus to enhance information transfer at low spatial frequencies, but can suffer from the loss of information transfer at high spatial frequencies.³⁴⁴ Electron ptychography can enable continuous information transfer across a larger bandwidth of the spatial frequencies. In a recent study, cryo-electron ptychography was used to retrieve phase images of rotavirus double-layered particles and HIV-1 virus-like particles.³⁴³ An electron probe of 1.03 mrad convergence semiangle, 26.9 nm diameter, and -13 μ m defocus was used to scan across the specimens to achieve a low-dose condition (22.8 to 5.7 e^- Å⁻²). The reconstructed phase images of the virus particles clearly resolved the capsid trimmers of viral protein 6 and the channels (Figure 24f). The power spectra were further calculated to compare information transfer between the two methods: ptychography and conventional phase contrast imaging at high defocus. The power spectrum from the ptychographic reconstruction was continuous over a wide range of spatial frequencies and did not show thon rings which were obvious in that of TEM images at high defocus. The results suggested that ptychography could retrieve phase information over a wider range of spatial frequencies without contrast reversal. This method can be used to image the heterogeneous and low-concentration biological specimens which are traditionally challenging to resolve by single particle analysis.³⁴³

The phase information on biological specimens can also be resolved in 3D by integrating electron ptychography with tomography. A recent study demonstrated the 3D phase reconstruction for the unstained DNA origami with Au NPs.³⁴⁵ A tilt series of ptychography-based tomography was performed in the tilting range of $\pm 70^{\circ}$ with a defocused electron probe of approximately 120 nm diameter. At each tilt angle, ptychographic data were acquired in a 10×10 scanning area (Figure 24g). The 3D rendering of the phase of the DNA origami framework computed from the ptychographic data resolved both the DNA strain and the Au NPs (Figure 24h). This was supported by the histogram of the intensity, which showed that the peaks of the DNA strand and Au NPs were separated from the background (Figure 24i). In contrast, the electron tomography based on the tilt series of defocused contrast TEM images found great difficulty in resolving the lowcontrast DNA strand from the Au NPs, where the DNA strand could be hardly distinguished from the background.

4.3.3. Electron Crystallography on Small-Sized Protein Crystals and Synthetic Molecules to Resolve 3D Structures. Protein structures have sizes ranging from tens to several thousands of amino acids. By physical size, the dimension of proteins varies between 1 and 100 nm.³⁴⁷ To perform biological functions, proteins fold into one or more spatial conformations driven by a number of noncovalent interactions such as hydrogen bonding, van der Waals forces, ionic interactions, and hydrophobic packing. It is often necessary to resolve the 3D structure of proteins in order to understand their functions at the molecular level.

Electron diffraction has been used to study thin protein crystals for several decades. $^{\rm 348-351}$ Early studies showed that 3D protein crystals could produce well-defined electron diffraction data.³⁴⁸⁻³⁵⁰ More recently, with the development of microcrystal electron diffraction (MicroED) in cryo-EM, the structures of beam-sensitive protein microcrystals can be efficiently determined with high precision from the diffraction patterns based on 3D crystallography.^{352,353} The MicroED method was developed for small nanocrystals of proteins (from a few hundreds of nanometers to a few micrometers) that are not suitable for X-ray crystallography which would typically require a crystal size larger than 20 μ m.³⁵⁴ The samples are frozen in a hydrated state, similar to other cryo-EM techniques, and the diffraction data is taken as a movie when the sample is continuously rotated in a STEM/TEM.³⁵² For biological samples, the electron dose rate is controlled to be very low (usually <0.01 e⁻ Å⁻² s⁻¹).³⁵⁵ MicroED was first demonstrated in 2013 to determine the 3D structure of protein crystals,³⁵⁶ where diffraction data at an atomic resolution (2.9 Å) was collected from lysozyme crystals that were about 6 orders of magnitude smaller in volume than those conventionally used for X-ray crystallography. Since then, several research groups have been successfully solving the protein structures based on submicrometer-sized protein crystals via 3D rotation electron diffraction (RED).³⁵⁷⁻³⁵⁹ More than 40 different proteins, oligopeptides, and organic molecules have been studied for structural determination.³⁵⁶ By increasing the sampling in the reciprocal space and improving the data processing, the structure resolution has been steadily improved. For example, the resolution of the lysosome structure has been improved from 2.9 Å in 2013³⁵⁶ to 1.8 Å in 2017.³⁶⁰ Several novel structures that were not solved previously have been studied and characterized by MicroED, including the structures of fragments derived from amyloidogenic proteins³⁶¹ and ligandcapped Au NPs (Au₁₄₆(p-MBA)₅₇).³⁶² Furthermore, automated procedures are being developed for identifying protein crystals, acquiring diffraction series while rotating and positioning the crystal, and characterizing many crystals sequentially.^{363,364} A more detailed description of the MicroED method and the structures determined by MicroED can be found in the recent review paper from Nannenga and Gonen.355

It is also worth mentioning that MicroED is different from other electron diffraction techniques with rotational illumination, such as automated diffraction tomography (ADT)³⁶⁵ and RED,³⁶⁶ in the experimental setup. Special software is required to process ADT and RED data.³⁶⁶ Whether ADT and RED can be applied to beam-sensitive biological samples also remains elusive, since both techniques were mostly demonstrated on inorganic and organic samples (e.g., zeolites)^{367–370} that are generally less sensitive to electron beam damage.



Figure 25. (a) STEM image of randomly distributed granuloviruses on TEM grid. Inset: a zoom-in view of a representative virus. The red circle corresponds to the electron beam probe of ~110 nm diameter. Colored lines indicate the lattice directions after indexing the diffraction pattern. Scale bar: 5 μ m. (b) A representative diffraction pattern acquired from panel a. (c) 2Fo–Fc map of the entire structure of granulin and zoom-in view of a randomly chosen region of the structure. Reprinted with permission from ref 374. Copyright 2020 Springer Nature.



Figure 26. (a) Schematic showing the simultaneous collection of ADF image signals and the coherent diffraction patterns on pixelated detector as the electron probe was scanned across the carbon nanostructure. (b) The ADF image of carbon nanostructure. (c) The phase image of the same carbon nanostructure reconstructed from the simultaneously collected coherent diffraction patterns (4D data set) via ptychography. (d, e) An example of the reconstructed modulus and phase map at a spatial frequency $+Q_P$. The two diffracted beams $(+Q_P \text{ and } -Q_P)$ overlap with the undiffracted beam. By analyzing the phase information in all spatial frequencies in the image, phase image c was reconstructed. (f) An ADF image of CNT conjugates. The iodine atoms were indicated. (g) The phase image reconstructed from ptychography shows the fullerenes in the CNT. The gray scale bar is in the unit of radians. (h) The same phase image as that in panel g with annotated fullerenes (dotted circles) and iodine atoms (cross marks). For comparison, conventional phase-contrast images including (i) BF, (j) annular BF, (k, 1) DPC, and (m) DPC using the center of mass approach were reconstructed from the same 4D data set. Inset: the area of the detector used in each imaging technique. Scale bars: 1 nm. Reprinted with permission from ref 376. Copyright 2016 Springer Nature.



Figure 27. (a) Schematic illustrating the BF-SCEM (left) and ADF-SCEM (right). The setup contains the annular and pinhole apertures and a stage-scanning system in TEM/STEM. The stage enables the movement of the sample in *x*, *y*, and *z* directions. (b) The HAADF images of carbon nanocoils that are composed of fibers with helical structures collected at various *z* positions (-400, 0, and +400 nm). (c) ADF-SCEM images of the same sample at corresponding *z* positions. (d) Animated image of the 3D reconstruction of the carbon nanocoil based on collected ADF-SCEM images at various *z* positions. Reprinted with permission from ref 387. Copyright 2009 American Institute of Physics.

Although MicroED has a high dose efficiency of electrons, the accumulation of electron damage during the rotation of the crystal potentially remains a limiting factor. Recently, a serial ED (SerialED) method has been developed and demonstrated³⁷¹ by combining MicroED and serial crystallography.^{372,373} In SerialED, snapshots of diffraction patterns were obtained from a single orientation from each crystal. The completeness of dataset was achieved by taking thousands of snapshots of different crystals. The electron beam damage is therefore intrinsically reduced. More recently, SerialED has been applied to study protein crystals of granulovirus occlusion bodies and lysozyme.³⁷⁴ In the experiment, the protein crystals randomly dispersed on a TEM grid were first mapped by lowdose STEM (low-magnification mode) over a large area to identify the crystal features (Figure 25a). Then the electron beam (~100 nm in diameter) was sequentially scanned over each identified crystal position to record the diffraction data (Figure 25b). The structures of the protein crystals were solved to resolutions of 1.55 and 1.80 Å (Figure 25c), respectively, which are comparable to the previous MicroED method of the same type of lysozyme sample. This work shows that the 3D structures of proteins can be identified with high resolution, low beam damage, and automation by using SerialED, during which process no sample rotation is required.

4.4. Atomic Structures of Carbonaceous Materials

Carbonaceous materials, such as fullerene peapod materials and CNTs,^{375,376} have found important applications in quantum processing,³⁷⁷ magnetic resonance imaging (MRI),³⁷⁸ and organic photovoltaics.³⁷⁹ However, their characterization suffers a lot from beam damage, through either knock-on or ionization mechanism.³⁷⁵ They are also weakly scattering materials. Therefore, the compositionsensitive HAADF-STEM technique described in Section 2 that collects electrons at a high scattering angle is less effective. HRTEM, with its phase contrast imaging capability, has been frequently used to characterize the shape and size of fullerene peapods.³⁸⁰⁻³⁸⁴ EELS spectroscopy, on the other hand, can show the fine structure of the chemical environment of the heavy atoms in an endofullerene peapod structure, which allows the identification of the chemical species and location.³⁷⁵ The heavy dopant atoms in the carbon nanostructure can also be revealed via STEM imaging,^{385,3}

where the isolation of single atoms and groups of atoms attracts lots of research interest.

It remains challenging to resolve the atomic structures of carbonaceous materials. Recently, considering its capability of providing phase imaging of weakly scattering samples with high SNR, electron ptychography was employed to study carbonaceous materials,³⁷⁶ combining with the ADF imaging that is sensitive to the atomic weight (Figure 26a,b). The peptidic covalently attached "tether" between a single-walled CNT and a few carbon fullerene molecules was chosen as a model system. The coherent diffraction patterns contained the phase information on carbon structures (Figure 26c-e), while the ADF image was more sensitive to the heavy iodine atoms in the CNT conjugates (Figure 26f). The reconstructed phase image from 4D-STEM dataset had a low sensitivity to the Z, but it clearly revealed the engulfed fullerene molecules in the CNT (Figure 26g,h). The distance of the fullerene molecules traveled into the CNT was not consistent with the length of the peptide tether, suggesting that not all the fullerene and CNT were successfully attached by the peptide tether. Other existing phase-sensitive imaging techniques, such as the BF, annular BF (ABF), DPC, and the DPC using the center of mass approach, were also reconstructed from the 4D dataset of CNT conjugates (Figure 26i-m). These images, however, were not able to resolve the "peapod" structure of the CNT conjugate as clearly as the electron ptychography, which was attributed to the worse SNR under the near-zero aberration conditions. In addition, the contrast of the phase image obtained from ptychography could be further improved by the postacquisition correction of lens aberrations.

Carbon-based materials can have complex 3D morphologies, which can be resolved by SCEM (Figure 27a).³⁸⁷ Carbon nanocoils were employed as a model system,³⁸⁷ which consists of carbon fibers with helical structures, inner and outer diameters of about 500 and 1000 nm, respectively, and the pitch of 600–1000 nm. Compared with electron tomography introduced in Section 3, SCEM works efficiently to reveal the morphology of relatively large samples when the spatial resolution is not a stringent requirement. Figure 27b,c shows a series of HAADF-STEM and ADF-SCEM images of carbon nanocoils at various *z* positions of the sample. In the HAADF mode, the images of the carbon nanocoils did not change when


Figure 28. (a) Schematic showing SED performed on a defect-engineered UiO-66(Hf) particle. (b) Calculated diffraction patterns for a defective UiO-66(Hf) particle (blue) along [011] zone axis. (c) An ADF-STEM image of a 6(Hf):5(BDC) UiO-66(Hf) particle with high defect density. (d) Diffraction pattern collected in the magenta region as indicated in panel c is indexed to **fcu** phase. (e) Diffraction pattern collected in the green region as indicated in panel a contains both parent and superlattice reflections marked in panel c, respectively. (h) ADF image of a MOF-CGC particle from the (MIL-53)_{0.6}(a_g ZIF-62)_{0.4} sample. (i) Crystallinity map showing the number of Bragg peaks at each electron probe location from SED data. The color scale shows the number of Bragg peaks. (j) SED mapping of (MIL-53)_{0.25}(a_g ZIF-62)_{0.75} CGC showing the number of Bragg peaks found in the diffraction pattern recorded at each probe position and (k) the corresponding STEM-EDX mapping of the sample. (a–g) Reprinted with permission from ref 390. Copyright 2020 American Chemical Society. (h, i) Reprinted with permission from ref 399. Copyright 2019 American Chemical Society. (j, k) Reprinted with permission from ref 401. Copyright 2019 Springer Nature.

the sample was moved along the *z* direction for a few hundred nanometers (Figure 27b), since HAADF images collect the projection of the sample. In the ADF-SCEM mode, different parts of the nanocoils were resolved when the sample was moved along the *z* direction (Figure 27c), demonstrating the capability of optical depth sectioning. The depth resolution for ADF-SCEM was about 100 nm. 3D reconstruction of the carbon nanocoils could be further achieved based on the optical depth sectioning (Figure 27d). It is worth noting that the ADF-SCEM images were obtained from the average of several measurements in order to enhance the detected signals (60–90 s for one image).

4.5. Crystallinity and Local Distortion in MOF from Synthesis or upon Guest-Host Interactions

MOFs are hybrid framework materials composed of metal nodes (ions or clusters) that are connected into an extended network by organic molecules.³⁸⁸ MOFs are well-known for their record-breaking surface area and adjustable pore size,³⁸⁹ and have been studied for a variety of applications including gas storage, separation, catalysis, and sensing.³⁸⁸ The structure of MOFs, however, can be complicated as they may contain lattice defects at the nanoscale,³⁹⁰ which can originate from the generic heterogeneity during synthesis or structural distortion during usage. Synthetic control of distribution of defects in the crystalline structures enables the engineering of MOF functions. For example, the performance of heterogeneous catalysis is closely associated with the number of active sites and the diffusion limitation. By incorporation of missing linkers or nodes in MOFs, the catalytic performance can be improved.³⁹¹ Such defects can be introduced to form nanoscale defect domains in MOFs under appropriate synthesis conditions.³⁹¹ Measurements of the bulk defect concentration has been implemented based on a variety of ensemble

techniques, such as NMR spectroscopy, thermogravimetric analysis (TGA), Fourier transform infrared spectroscopy (FTIR), and XRD.³⁹⁰ However, they do not spatially resolve the defect distribution in individual MOFs. To this end, EM-based diffraction techniques can be applied to study MOFs and zeolites at a single-particle level with high resolution.^{392–394} It should be noted that MOFs can be significantly damaged by an accumulated electron fluence of $10-20 \text{ e}^- \text{ Å}^{-2}$, so low-dose EM is required.³⁹⁵

The distribution of defects in MOF materials can be resolved via SED, a 4D-STEM technique (Figure 28a), as demonstrated in a recent study.³⁹⁰ A focused electron probe was used to scan across the defective UiO-66(Hf) particle at an estimated electron dose of 5 e⁻ Å⁻². UiO-66 has a nominal formula of $[M_6O_4(OH)_4][C_6H_4(COO)_2]_6$, where M represents Zr, Hf, or other tetravalent metals. In the defect-free region, the structure consists of metal oxyhydroxide clusters coordinated by terephthalate ligands, forming a fcu structure of cubic-F lattice. In the defective region, the face-center symmetry was broken, and the structure consisted of cubic-P phase with reo topology (Figure 28b). A defective UiO-66 particle (Figure 28c) was found to contain both a defect-free region (fcu structure, Figure 28d) and a defect region (reo structure, Figure 28e), which was confirmed based on the identification of the superlattice reflections in diffraction patterns. The authors performed diffraction contrast imaging by plotting the summed intensity within the selected disk integration windows in each diffraction pattern for all the probe positions to form VDF images (Figure 28f,g). The parent phase of defect-free region was observed in the whole particle (Figure 28f), while the superlattice reflections corresponding to defect region were observed only in certain regions (Figure 28g), forming a lamellar domain morphology.



Figure 29. (a) Schematic showing the configuration of iDPC technique. (b) Two representative images of MOF MIL-101 nanocrystal sample detected by the A and C segments of DPC detector, respectively. (c) DPC image from the two images in panel b. (d) iDPC-STEM image obtained from a 2D integration of the DPC image in panel c. (e) Magnified iDPC-STEM image showing the MOF structure. (f) iDPC image (left) of the lattice structure of 29 Å cage in the MOF MIL-101. Schematic of the corresponding MOF MIL-101 structure is on the right. Scale bar: (b) 20 nm, (d) 10 nm, (e) 5 nm, and (f) 1 nm. Reprinted with permission from ref 408. Copyright 2020 Springer Nature.

The **reo** structure formed rectangular domains and the domain boundary appeared to be aligned with the particle facets, which could be associated with the minimization of strain energy within the lattice structure.³⁹⁶ Such defect domain structures were consistently observed in UiO-66 particles with varied defect densities.

Typically, MOFs are synthesized as microcrystalline powders composed of particles with nanometer to millimeter sizes. Their physical form is not well suited to industrial applications without prior processing.³⁹⁷ One potential solution is to use a secondary material to aggregate the MOF particles to form composites, for example, MOF-insilica,³⁹⁸ MOF crystal-glass composites (MOF-CGCs),³⁹⁹ and MOF-based mixed-matrix membranes.⁴⁰⁰ In these composites, the nanoscale structures of MOFs and their distribution can impact the functionalities. Recently, SED was employed to study MOF composites to distinguish the crystalline materials from the amorphous matrix. The sample was room-temperature stabilized open-pore form of MIL-53(Al) within a ZIF-62(Zn) MOF glass matrix.^{399,401} The crystalline and noncrystalline regions within the MOF-CGC sample were distinguished based on the Bragg peaks in the recorded diffraction patterns at various probe positions (Figure 28h-k), where the crystalline map of the MOF-CGC samples indicated the number of Bragg peaks in the diffraction pattern (Figure 28i,j). Comparing the crystalline map with the compositional map obtained from STEM-EDX (Figure 28j,k), the crystalline regions were found to correspond to the metal centers (aluminum), which was expected for MIL-53(Al) samples. The quantitative determination of the distribution of crystalline components in the composites can be important, for example, to identify the optimal loading necessary for maximum gas absorption capacity.

The direct imaging of the atomic structures of beamsensitive MOF materials is important to understand the framework lattice structures and their related functions. STEM imaging is powerful in resolving atomic structures, but suffers from a few limitations such as the relatively high energy

electrons and therefore severe electron beam damage.⁴⁰² Recently, the employment of the direct-detection electroncounting camera enables the low-dose HRTEM imaging of MOF subunit lattice structures.^{403,404} The introduction of the cryogenic condition can further reduce the electron beam damage to the lattice structure.⁴⁰⁵ In addition to HRTEM, another solution to image beam-sensitive materials is the utilization of the integrated DPC (iDPC) technique based on electron diffraction. The iDPC technique is available in the new generation of Cs-STEM (corrections of spherical aberration) with a DPC detector, and has been applied to image zeolite frameworks^{406,407} and the MOF MIL-101 framework⁴⁰⁸ with a low dose (~40 e⁻ Å⁻²) at the atomic scale. In the iDPC-STEM setup, four images were collected from the four segments of the DPC detector in the diffraction mode (Figure 29a,b). The DPC images were calculated as $DPC_x = A - C$ and $DPC_y = B - D$, respectively (an example of DPC_r of MIL-101 nanocrystal is shown in Figure 29c). In this way, the iDPC technique enables linear imaging of the projected electrostatic potential in the lattice structure, different from the HAADF imaging which collects electrons at a high scattering angle. The resulting contrast in the iDPC image was proportional to Z and was expected to distinguish the light elements. The obtained iDPC images showed the atomic structures of MIL-101 nanocrystals (Figure 29d,e). The corresponding FFT pattern of the iDPC image suggested an information transfer of ~ 1.8 Å, which was higher than that of 2.5 Å in HRTEM (equipped with the direct-detection electroncounting camera) in a previous study.⁴⁰⁴ The averaged iDPC image of 29 Å cage in the MOF MIL-101 (Figure 29f) was consistent with the modeled structure. The atomic-scale imaging with iDPC-STEM is expected to be applicable to various beam-sensitive soft nanomaterials.409

The 3D structure determination of MOFs and covalent organic frameworks (COFs) is an important characterization step to understand their functional properties. In recent years, 3D electron diffraction (3DED) methods emerge and are increasingly being used for the structure determination of



Figure 30. Schematic illustrations of (a) standard graphene liquid cell made by sandwiching the solution between two graphene-coated grids; (b) graphene liquid cell consisting of a thin lithographically patterned hBN crystal encapsulated by two graphene windows; (c) MOS_2 liquid cell with one grid coated with MOS_2 and another with graphene; (d) SiN_x cell with two silicon chips placed together and separated by a spacer (yellow); (e) side view of a SiN_x cell with patterned nanochannels and hydrophobic Au nanoparticles encapsulated; (f) side view of a microwell made of SiN_x ; (g) side view of a bypass flow cell consisting of two SiN_x chips with liquid flow between them (left) and top view showing liquid flowing around the microchips (right); (h) side view of a direct flow cell containing a flow path through the microchips and avoiding liquid flow around the windows; (i) the unassembled nanochannel chip and top plate (left) and cross-sectional view (right) of a graphene flow cell. (a–f) Circled by the blue dashed line are representative examples of closed cells, and (g–i) in the orange dashed line are flow cells. (a) Reprinted with permission from ref 439. Copyright 2012 AAAS. (b) Reprinted with permission from ref 419. Copyright 2018 American Chemical Society. (c) Reprinted with permission from ref 426. Copyright 2019 American Chemical Society. (e) Reprinted with permission from ref 429. Copyright 2017 Wiley-VCH. (f) Reprinted with permission from ref 440. Copyright 2014 Microscopy Society of America. (g) Reprinted with permission from ref 433. Copyright 2010 Microscopy Society of America. (h) Reprinted with permission from ref 434. Copyright 2013 American Chemical Society. (i) Reprinted with permission from ref 435. Copyright 2020 American Chemical Society.

nanosized and submicrometer-sized crystals. 3DED data collection techniques advance from stepwise rotation to continuous rotation of the MOF crystals, which enables fast data collection for beam-sensitive materials. For detailed information, we recommend a recent review paper from Huang et al.⁴¹⁰

5. IN SITU CHARACTERIZATION OF SOFT NANOMATERIALS' FULL LIFE CYCLE: SYNTHESIS, RECONFIGURATION, AND FUNCTIONING

Soft nanomaterials, ranging from colloidal NPs to biomolecules, are mostly grown from, react, and function in a dynamic process, which can entail a solvent to maintain diffussion and thermal fluctuation, can be heated to overcome a reaction barrier, and can be pressurized with a feed gas to catalyze reactions. Such dynamic processes necessitate in situ observations where the sample environment can be modulated during imaging with the help of specialized holders (liquid flow, gas flow, heating, biasing, etc.). Compared to X-ray or light-based spectroscopy, such as XRD, X-ray photoelectron spectroscopy (XPS), X-ray absorption spectroscopy (XAS), FTIR, and surface enhanced Raman spectroscopy (SERS), which deliver ensemble information on the samples, EM provides nanometer or even atomic resolutions and real-space imaging. In this section, in situ characterizations of various soft nanomaterials based on EM will be discussed, ranging from shape and structural transformations during chemical reactions to self-assembly in liquid environments. Liquid-phase EM will be highlighted, detailing different types of liquid cells for specific applications in inorganic, organic, and biological samples, followed by a brief introduction of other in situ techniques applied at elevated temperatures and varied gas environments. Both real-space direct imaging and diffractionbased methods will be covered.

5.1. Introduction to EM-Based In Situ Characterization Techniques

5.1.1. Liquid-Phase EM. Conventional practices of SEM, TEM, and STEM require samples to be casted and dried on a thin substrate, during which its native structures might be altered such as solvated polymers and proteins or the real dynamics since solution-based thermal motions is lost. Although strategies such as sequential drying or vitrification of materials at different stages may be separated and imaged (Section 2.2.1), the gaps between these discrete images are unresolved, let alone tracking the real-time continuous dynamics.⁴¹¹ To this end, liquid-phase EM, including TEM, STEM, and SEM, serves a powerful platform enabling time-resolved nanoscale imaging of solvated samples or samples immersed in liquids.

The concept of liquid-phase TEM and a vacuum-sealed liquid chamber design can be traced back to 1944, and the first experiment on in situ liquid EM was conducted in 1973.⁴¹²

However, the early studies suffered from poor spatial resolution and challenges to seal the liquid sufficiently in the chamber under high vacuum.⁴¹³ Owing to the advances in EM resolution and liquid cell fabrication, a wide range of materials can be resolved nowadays, with their size ranging from a few nm to several μ m, composition from metallic NPs to carbonaceous polymers and biomaterials, and properties from structures to tracking of shape evolution and assembly processes.⁴¹⁴ Recently, the integration of liquid-phase EM with advanced, analytical techniques, including EELS,^{48,415} EDX,⁴¹⁶ tomography,¹⁴⁴ and SAED⁴¹⁷ allows more informative detection of morphological and compositional changes of materials and inspires deeper exploration into related material properties and reaction mechanisms.

Design of Liquid Cells to Account for Different Dynamics Studies. The achievement in liquid-phase (S)TEM is closely associated with different generations of designs of liquid cells. Liquid cells currently used can be divided into two categories: closed cells and flow cells.

The graphene liquid cell is one of the commonly used closed cells, prepared usually by encapsulating pockets of solutions between two graphene-coated TEM grids (Figure 30a). The thin (tens to 500 nm) liquid pocket and thinnest possible window material enable atomic-resolution imaging of NPs and compatibility with other techniques such as diffraction,⁴¹⁸ EDX,^{419,420} and EELS⁴⁸ due to reduced scattering and thus small background noise.⁴¹⁴ Besides, the high thermal and electric conductivities of graphene and its capability of scavenging radicals contribute to fast transfer of accumulated charges and heat, as well as minimization of radical damage, making graphene liquid cells exceptionally suitable for beamsensitive materials such as polymers, biomolecules, and hybrid materials.⁴²¹ When the size of the samples demands for larger pockets (e.g., some organic compounds, DNA-Au superlattices⁴²²), the cell fabrication protocol can be modified by sealing the solution between a graphene-coated TEM grid and free-standing graphene, named graphene channel cell, to allow more flexible coverage to wrap up the samples. In these cases, the thickness of the liquid pockets could exceed micrometers.⁴²³ In more recent efforts where delicate control of liquid pocket thickness is desired, liquid samples can be trapped in nano- or micrometer cavities made of $\hat{Si}_{,v}^{424} \hat{SiN}_{,v}^{424}$ aluminum oxide,⁴²⁵ or hexagonal boron nitride (hBN)⁴¹⁹ and sealed with graphene layers. When hBN is used as the spacer, its height can be reduced to 30 nm, where the minimized background absorption allows elemental mapping of NPs with nanometer resolution (Figure 30b).⁴¹⁹ Aside from graphene, other 2D materials like MoS₂ can also be coated onto grids and serve as a functional and sealing substrate (Figure 30c).⁴²⁶ Moreover, it has been recently reported that commercial Cu grids covered by thin amorphous carbon can be directly used to sandwich liquid samples. Studies are still ongoing to understand the pocket stability in these Cu carbon liquid cells, and demonstration on high resolution imaging to atomic scale has been achieved.⁴¹³

Owing to the mature silicon microfabrication industry, SiN_x cells, where a SiN_x membrane serves as the window, can be reliably mass-produced and are the most commercialized type of liquid cells, both as closed and open cells. The thickness of the SiN_x membrane can be reduced to 10-50 nm, greatly limiting the background scattering, while its mechanical robustness can still be maintained, allowing the integration with multiline-flow, heating, and electrochemical biasing. A

liquid sample can be sandwiched between a pair of SiN_x chips, and the liquid thickness can be controlled by adjusting the height of the spacer (Figure 30d) (plus bulging effect of the membrane under high vacuum). Different patterns such as nanochannels can be fabricated on the bottom SiN_x membrane (Figure 30e), enabling the investigation of dynamics of chemical or physical processes under confinement.⁴²⁹ Such a design is quite similar to microwells, where an array of wells are etched into the surface of the SiN_x window and function like individual compartments that greatly suppress the longdistance motion of samples (Figure 30f). The separation between individual microwells also allows mitigation of beam damage if the samples within microwells are imaged sequentially.^{430,431}

For all different types of closed cells, however, once they are sealed, no liquid exchange is allowed, let alone the observation of materials' response to external stimuli, such as pH and concentration change of solvents. The limited volume of liquid is also hard to be compatible with electrochemistry owing to the lack of enough reactant and imposed restriction on mass transfer. To address these issues, flow cells and the associated liquid flow holder are designed to enable replenishing or changing solvents during imaging. SiN, cells with better robustness than graphene cells are mostly employed, with defined flow paths guiding the liquid flow fabricated with the spacer.^{432,433} Flow cells can be further divided into two types: bypass flow cell and direct flow cell, with the former more commonly used. In the bypass flow cell, the injected liquid flows around the chips as well as between them, enabling a rapid liquid exchange in the whole flow system (Figure 30g). However, clogging and uneven diffusion speed may occur when the spacer gets smaller. In contrast, the liquid exchange can be forced even in a direct flow cell due to the high pressure in the flow channels between windows, but the flushing of the flow system takes a longer time (Figure 30h).^{432,434} Graphene can also be incorporated into a flow cell to reduce charging effect and radiation damage. Figure 30i shows a typical example of graphene flow cell with a series of circular viewing apertures covered by graphene, while the bottom is windowed with ultrathin SiN_x .⁴³⁵ The compatibility of the flow cell with electrochemistry, heating, and light illumination also significantly broadens its applications, enabling the imaging of materials' temporal change upon elevating the temperature,436 cycling between a certain potential range,437 or exposing to light of a certain wavelength.⁴³⁸ Such capabilities are realized by patterning the electrodes or heaters onto SiN_x chips or introducing a light/optical fiber inside the (S)TEM.

Beam Effects and Liquid-Phase SEM. Electron beam induced artifacts and damage are one of the key aspects of concern in liquid-phase TEM, including but not limited to alteration of the electrostatic and redox environment in samples, etching and regrowth of NPs, breakage of chemical bonds and structural disintegration of polymers and biomaterials, charging of the membranes, and generation of bubbles. Beam effects can be complex, with various ions, gases, and radicals generated in different solvents. To minimize the beam effect, low dose imaging can be the simplest and most effective way, which is easier to be realized nowadays while maintaining the high resolution due to the development of sensitive detectors. Besides, coating with graphene or introducing radical scavengers such as alcohols may also help. Note that beam effect can also be beneficial depending on one's purpose, such as initiating polymerization⁴⁴¹ and

enabling the investigation into the reactions at the liquid–gas interface. 442 More details can be found in the review articles by Woehl et al. 443,444

Aside from TEM, liquid-phase SEM is another powerful tool to image soft materials in the liquid state at a high temporal resolution, with a slightly compromised spatial resolution (10 nm for high-Z materials and several tens of nanometers for low-Z materials) but the capability to image extended-sized samples.^{432,445} By integrating with STEM detector, the resolution can be further improved to 2-4 nm.⁴⁴⁶ The large sample chamber of SEM provides significant design freedom, such as the incorporation of high numerical aperture epifluorescence microscopy.⁴⁴⁷ More examples with liquid-phase SEM will be discussed in Section 5.4.

5.1.2. Other In Situ Studies of Dry Samples (Heating, Gas Environment, Diffraction). Most industrial reactions are conducted at high temperatures and under various gas environments, such as the Fischer–Tropsch process (150–300 $^{\circ}$ C, H₂ and CO) and ammonia synthesis (400–600 $^{\circ}$ C, H₂ and N_2), which deviates from conventional EM imaging conditions (room temperature, vacuum). To investigate the nanomaterials under such harsh environments, different functional modules have been integrated with EM. With the development of microelectromechanical system (MEMS), microheaters, such as those with spiral heating unit surrounding the SiN_r observation window, can now achieve fast heating rate, accurate and stable temperature control, tiny sample drift, as well as compatibility with spectroscopic techniques, enabling in-depth research of shape and structural transformation of nanomaterials at elevated temperatures.448,449 Compared to heating in a vacuum, the introduction of gas into EM is more challenging due to the potential interaction between the gas molecules and the electron beam. Two techniques are commonly used: ETEM that allows the existence of gases with a low pressure around the sample area, and microfabricated gas cells with samples sealed between two MEMS chips and gases flowing between the chips.⁴⁴⁸ The pressure inside the gas cell can even reach ambient pressure, which is much higher than that in ETEM, and thus the reaction condition in the gas cell can be closer to that in practical applications. Both techniques can be combined with heating functions, and even connected to a mass spectrometer⁴⁵⁰ to enable the simultaneous detection of gas products during chemical reactions, correlating nanomaterials' structures with their catalytic performance. Aside from real-space imaging, electron diffraction patterns can also be recorded in situ to track the fast-speed structural and compositional change of NPs during reactions. More detailed examples of in situ heating experiments and gas-solid reactions can be found in Section 5.5.

5.2. Liquid-Phase (S)TEM Studies of the Shape and Composition Evolution of Colloidal NPs during Chemical Reactions

One of the hallmark applications of liquid-phase (S)TEM is to characterize the shape evolution and compositional change of nanomaterials during chemical reactions, such as nucleation, growth, oxidative etching, and catalysis. This section will focus on inorganic colloidal NPs, while organic and biological materials are discussed in Section 5.3. We will also focus on NPs during reactions, which has not been reviewed so far. Note that a big area of application of liquid-phase (S)TEM is to understand the motion and assembly of NPs in a liquid

environment, which is of great significance in biomedicine, catalysis, and chemical or biosensors. Combined with simulations, statistical analysis of NPs' motion and interactions with environment can be realized. Such studies start from fundamental research on the particle–substrate interactions,⁴⁵¹ electron beam effects,⁴⁵² and confinement in a liquid cell environment,^{424,453,454} which enhance our understanding and allow better control of the technique, and then extend to particle-particle interactions,⁴⁵⁵⁻⁴⁵⁷ where van der Waals interactions, electrostatic repulsion, hydration effects, and steric repulsion were analyzed and parameters such as ion concentration, pH, surface ligand, temperature, and electric field were investigated.⁴⁵⁸ Aside from random movements, by carefully tuning the aforementioned parameters, NPs can be guided to assemble into a library of superlattices. The investigation into driving force for crystallization,^{429,459–462} transformation from random distributions to ordered struc-tures,⁴⁶² effects of anisotropic shapes,⁴⁶³ from 1D, 2D to 3D superlattice⁴⁶⁴ are all trending in the liquid-phase TEM community. A deeper investigation into the assembly processes and mechanisms can be found in a recent review paper by Chen et al.465

5.2.1. Growth Kinetics. Most liquid-phase (S)TEM studies on NP growth mechanisms have been focused on metallic NPs. It is generally considered that the reduction of precursors in a solution will reach a saturation of atoms and their self-nucleation into nuclei, which further grow larger from continuous deposition of atoms, named classical nucleation.⁴⁶⁶ Such a monomer addition process has been captured by liquidphase TEM in Ag and Pt NPs.^{425,467–469} By using electron beam as a reducing agent, the nucleation process of Ag nanocrystals was recorded, and the nuclei were observed to form directly from the solution after an initial induction.⁴⁶ Aside from classical nucleation, direct imaging also uncovered other mechanisms, such as amorphous-phase-mediated crystallization,⁴⁷⁰ oriented attachment,^{428,469,471-473} and multistep pathways where the precursor solution separates into soluterich and solute-poor phases, followed by the nucleation of amorphous nanoclusters from the metal-rich phase and their further crystallization into a nanocrystal.⁴⁷⁰ Classical and nonclassical nucleation pathways may also coexist, as shown in a recent study tracking the mineralization of hydroxyapatite in an artificial saliva solution. Ion-rich and ion-poor solutions were formed at the beginning, followed by coexistence of an amorphous calcium phosphate-mediated nonclassical nucleation path and a classical, direct nucleation path.⁴⁷

In addition to self-nucleation, seed- and template-mediated growth can also be characterized by liquid-phase TEM, unveiling the different growth pathways (monomer addition or coalescence),⁴⁷⁵ mechanisms of epitaxial growth and site-selective growth,^{426,431,476} influence of temperature,⁴⁷⁶ and existence of phase transition in seeds.⁴⁷⁷ Aside from the nucleation stage, liquid-phase TEM also provides direct visualization of 3D NPs to 2D nanosheets⁴⁷⁸ and oscillatory growth of Bi NPs where pairwise Ostwald and anti-Ostwald ripening were observed.⁴⁷⁹ A more thorough discussion on the application of liquid-phase TEM to uncover the growth mechanism of nanomaterials can be found in recent review papers and book chapters.^{465,480}

5.2.2. Oxidative Etching, Galvanic Corrosion, and Galvanic Replacement. Studies of etching kinetics of NPs on one hand provide mechanistic understanding of the



Figure 31. (a) Boundaries of Au nanorods colored to their local surface curvatures during etching. Scale bar: 50 nm. (b) Three stages of etching based on the distinctive relationships between the etching rate and curvature at a local site. (c-h) Transition of (c-e) a cube and (f-h) a RDD to tetrahexahedron during nonequilibrium etching: (c, f) Model of (c) a cube and (f) a RDD (gray) with intermediate THH NPs shown internally (blue). (d, g) THH intermediates for (d) cube and (g) RDD with labeled zone axis and calculated {*hk0*} facet angles. (e, h) Time-lapse TEM images and corresponding snapshots from Monte Carlo simulations showing the etching process of (e) a cube and (h) a RDD. (a, b) Reprinted with permission from ref 485. Copyright 2020 American Chemical Society. (c–h) Reprinted with permission from ref 484. Copyright 2016 AAAS.



Figure 32. (a, b) Time-lapse TEM images and corresponding schematics showing the shape evolution of Pd nanocubes during the etching process by (a) 0.1 mM and (b) 1.0 mM Fe(acac)₃. (c) TEM image showing the Fe(acac)₃ molecular layers grown on the Pd (100) facet in the liquid cell at 1.0 mM Fe(acac)₃. (d–i) Etching trajectory and corresponding projected area as a function of reaction time for Au NPs in an aqueous encapsulating solution of (d, e) 40 mM Fe(III) and 190 mM Cl⁻; (f, g) 40 mM Fe(III), 190 mM Cl⁻, and 0.38 mM Br⁻; (h, (i) 40 mM Fe(III), 190 mM Cl⁻, and 0.38 mM I⁻. (j) Standard reduction potentials of Au(I) complexes with different halides. (a–c) Reprinted with permission from ref 488. Copyright 2021 American Chemical Society. (d–i) Reprinted with permission from ref 490. Copyright 2021 American Chemical Society.



Figure 33. (a) Time-lapse TEM images showing the etching process of a Pd@Pt octahedron with a terrace defect. Scale bar: 20 nm. (b) Illustration of atomic structures demonstrating the etching process with measured distance at three stages. TS, CS, and HC represent tensile strain, compressive strain, and high curvature, respectively. (c) Strain map of the octahedron before corrosion. The color-coded bar represents a range from -3% CS to +3% TS. (d) Time-lapse TEM images and corresponding simulation snapshots showing a faster etching of Pd core than the Au shell, and the as-generated hexapod-like Pd core capped with Au pyramids. (e) Time-lapse TEM images and corresponding cut-section snapshots showing rapid dissolution of the Cu core upon exposure to the oxidative environment while the dissolution of the Au shell was negligible. (a-c) Reprinted with permission from ref 495. Copyright 2020 Elsevier. (d, e) Reprinted with permission from ref 496. Copyright 2020 Springer Nature.

reactivity of different facets of NPs which can be relevant to understanding of corrosion and catalytic stability of NPs, and on the other hand can serve as a means of harvesting nonequilibrium shapes of NPs (e.g., concave, cages, and branched) with desired properties. Different etching mechanisms have been reported so far, including the conventional oxidative etching where metal atoms are oxidized back to ions by oxidative species,481 the galvanic corrosion where two metals are in contact and one is preferentially etched due to its higher oxidation potential to protect another,⁴⁸² and the galvanic replacement where one metal is replaced by another with a higher reduction potential.⁴⁸³ Different from the role of a reducing agent when imaging the growth of NPs, an electron beam can also generate oxidative species from radiolysis of solvents, which either are involved in redox reaction networks of the system or serve as the real etchant.

Etching of Single Component NPs: Effects of Surface Facets, Ligands, and Temperature. For monometallic NPs, the etching rates are dependent on surface curvature and facets. Localized etching preferentially initiates at the sites of high surface energy, such as corners and defects, where lowcoordinated atoms accumulate and are easier to be oxidized. A model system is Au nanocrystals with well-defined shapes, the etching of which in FeCl₃ solution has been monitored in several systematic studies in graphene liquid cells. Faster etching was observed at the tips of Au NRs (Figure 31a), owing to a higher curvature and lower areal ligand density at 4,485 The quantitative analysis of etching rates shows tips.4 three stages involved in this process (Figure 31b): (*i*) isotropic etching dominates in the first stage when an excess of oxidative species overwhelms the curvature dependence, (ii) directional etching in the intermediate stage with the higher-curvature sites etched faster, and (iii) a sudden drop of etching rate at the

end of the etching process due to local accumulation of gold ions or depletion of oxidative species in a sealed environment.⁴⁸⁵ Interestingly, when a cube or a rhombic dodecahedral (RDD) Au NP was studied, a THH nanocrystal was consistently observed as an intermediate, owing to a steprecession mechanism where peripheral edge atoms with coordination numbers smaller than 6 were preferentially etched (Figure 31c-h).484 By tuning the concentration of FeCl₃ and thus the chemical potential of the oxidative environment, cubes enclosed by $\{100\}$ facets were found to adopt an $\{hk0\}$ -faceted THH intermediate with h/k dependent on chemical potential, while {110}-faceted RDD adopted a {210}-faceted THH intermediate regardless of the driving force. Monte Carlo simulations indicate that 6- and 7coordinate atoms coexist on cubes during etching and their sensitivity to the chemical potential leads to different THH facets. In contrast, only 6-coordinate atoms exist on RDD, either at the edge or in the interior after removal of edge atoms, resulting in negligible effect of chemical potential on the intermediate shape.486 The facet dependency in etching was also observed in ceria nanocrystals, where specific facets $({100})$ for reduction and ${111}$ for oxidation) were identified as governing the overall etching under different chemical conditions.4

In addition to the intrinsic NP surface, the local environment also plays an important role in the etching of nanocrystals, which includes but is not limited to the type and concentration of surface ligands, halides, and temperatures.

Through selective adsorption onto certain NP sites, ligands can effectively suppress the etching on these sites. Using Pd nanocrystals as an example, in the presence of iron acetylacetonate (Fe(acac)₃, 0.1 mM), etching originated from {100} facets due to the protection of {110} facets by acac molecules generated from Fe(acac)₃ dissociation (Figure 32a).⁴⁸⁸ In contrast, when increasing the concentration of Fe(acac)₃ to 1.0 mM, the protection of the Fe(acac)₃ film on the Pd {100} facets greatly mitigated the etching on them, leading to preferential initiation of etching occurring on the Pd {110} facets (Figure 32b,c).

As another commonly used ligand, halide ions also impose influence on the etching of metallic NPs, usually leading to accelerated etching. For Pd nanocubes, no noticeable dissolution was observed in the absence of Br⁻ ions even after increasing the electron dose rate (1140 e⁻ Å⁻² s⁻¹ in SiN_x cell) or replacing the Br⁻ with Cl⁻ ions.⁴⁸⁹ This can be attributed to the impressive stability of $[PdBr_4]^{2-}$, 10³ times higher than that of $[PdCl_4]^{2-}$, which greatly promotes the oxidative etching of Pd nanocrystals. A similar phenomenon was also observed in Au nanocrystals, where the etching rate increased when the halide ions were changed from Cl⁻ to Br⁻ and then to I⁻, consistent with the mechanism that halides serve as complexation agents for oxidized Au species (Figure 32d-j).⁴⁹⁰

Temperature is another factor that affects the rate of chemical reactions. A recent work introduced the design of "stream liquid heating holder", which consists of an on-chip flow channel combined with a microheater and allows the imaging of samples at elevated temperatures with the solution rapidly replenished.⁴⁹¹ An acceleration in the etching of silica NPs was observed when the temperature increased from 20 to 60 °C, consistent with in-flask experiments. Interestingly, the structural change of nanocrystals can, in return, enhance our understanding on the electron beam-induced heating effect.

Through parallel-beam electron diffraction (PBED), the lattice expansion of Au nanocrystals and the change in scattering angles can be tracked and correlated to the exact temperatures, suggesting the leverage of metal nanocrystals as a reference for calibrating the beam-induced heating.^{492,493}

Etching and Galvanic Exchange of Bicomponent or Multicomponent NPs. With the introduction of a second component, bimetallic nanomaterials also attract lots of interest considering their wide applications and, in most cases, improved performance relative to their monometallic counterparts. Considering NP structures, in addition to surface curvature and facet effect in monometallic nanocrystals, defects and strains, especially those at the interfaces of components, play an important role in the etching kinetics. One representative example is the etching of Pd@Pt core-shell nanocubes into Pt nanocages, wherein both components serve as effective catalysts for electrochemical reactions such as ORR and water splitting.⁴⁹⁴ Through liquid-phase TEM, two types of etching pathways were identified: the indirect-contacting galvanic etching on nondefected site with slow kinetics, and the direct-contacting halogen-induced etching at defected site with faster rates. Corners were preferred by both pathways as the initial and faster etching site, indicating the importance of protecting corners and engineering surface defects to improve the stability of electrocatalysts. Combining ex situ strain mapping with in situ liquid-phase TEM, a faster etching was observed in a Pd@Pt octahedron at locations with tensile strain and high local curvature (Figure 33a-c), which was supported by DFT calculations that a lower reduction potential of Pd associated with increased strain and tensile strain tends to facilitate corrosion more effectively than compressive strain.49

Etching rates can also diverge for different components of bior multimetallic nanocrystals due to the variation in oxidation potentials, and those with a higher oxidation potential are more vulnerable to etchants. For example, for Pd@Au coreshell nanocubes imaged in graphene liquid cells, when the cubic Au shell was etched to a THH shape, the corners of the Pd core would be exposed.⁴⁹⁶ The higher oxidation potential of Pd/Pd²⁺ than Au/Au³⁺ (-0.92 and -1.52 V vs standard hydrogen electrode, SHE, respectively) contributed to a faster etching of Pd core than the Au shell, leading to a hexapod-like Pd core capped with Au pyramids (Figure 33d). By plotting the particle volume V with reaction time t, the etching process can be monitored using the Lifshitz, Slyozov, and Wagner (LSW) theory. The plot of $V^{2/3}$ vs t gave a more satisfactory fit to the data points prior to exposure of the Pd core, whereas $V^{1/3}$ vs t fitted better to the data when Au and Pd were simultaneously exposed to the etching solution. Such results indicate a transition of etching mechanism from edge-selective to layer-by-layer removal of atoms after the exposure of Pd. Besides, it was also observed that etching of the Au shell slowed down once the Pd core contacted the etchant, which can be attributed to the galvanic corrosion where the more active metal (i.e., the anode) is preferentially etched and the less active metal (i.e., the cathode) is protected. Further increasing the difference in reduction potentials by replacing Pd with Cu (-0.34 V vs SHE for Cu/Cu²⁺ pair) led to the formation of Au nanobox as an intermediate due to the rapid dissolution of Cu once it was exposed to the etching solution (Figure 33e).

Compared with core-shell structures, galvanic corrosion can be more obvious in Janus NPs, where both components are



Figure 34. (a, b) HAADF frames showing the corrosion of Ag–Cu Janus NPs (a) without and (b) with Ar plasma treatment. (c) Time-lapse TEM images and corresponding schematics showing the morphological evolution of a Ag nanocube during galvanic replacement reaction with HAuCl₄ at 23 °C. Green arrows indicate pores that form in the deposited Au shell, and a cyan arrow points to a second galvanic replacement reaction on the residual Ag core after it is again exposed to HAuCl₄ through pores on the outer shell. (a, b) Reprinted with permission from ref 482. Copyright 2021 Wiley-VCH. (c) Reprinted with permission from ref 436. Copyright 2017 Springer Nature.

exposed to the etchant. In a recent paper, a set of Janus NPs were synthesized and their etching under the electron beam was observed.⁴⁸² For a Ag-Cu nanocrystal, due to the higher oxidation potential of Cu, the breakdown of Cu occurred first, followed by the dissolution of Ag (Figure 34a). Instead of dissolving gradually, the Cu portion was broken into pieces and flowed away from the particle, which could be attributed to the presence of CuO that has a crystal symmetry distinct from Cu and forms a nonconformal protective layer on metal surface. Besides, no groove was observed along the Ag-Cu interface at the initial stage, indicating that etching was not preferred at the interface while the electrochemical potential gradient across the interface drove the corrosion process. With a mild Ar plasma treatment, the amorphous oxide on the Cu surface can be converted into a dense, crystalline CuO shell, effectively suppressing the current flow required for galvanic corrosion and preventing the dissolving of Cu. Surprisingly, when the vapor-phase corrosion was examined by in situ STEM, a faster corrosion of Ag was observed albeit its significantly lower oxidation potential (Figure 34b), strongly suggesting the formation of a dense oxide shell as a powerful approach to enhance the stability of NPs. The influence of surface oxides is also reflected in PtNi NPs with a segregated bimetallic structure.497 Localized pits were observed in the initial stage of chemical etching (mixed with 5% (v/v) aqueous acetic acid solution at 90 °C), while a uniform dissolution of Ni was observed in electrochemical etching (applied with a positive potential of +0.5 V, vs Pt reference electrode, with 0.1

M H_2SO_4 serving as the electrolyte) due to the destabilization of the surface oxide layer.

While etching of compartmentalized bimetallic NPs usually results in the complete removal of one component while leaving another almost intact, etching of alloy NPs can be more complex and voids are commonly generated inside the particles. With AuAg alloy NPs as an example, encapsulated in a SiN_x cell with 25 nm-thick windows, their shape evolution during corrosion was tracked.⁴⁹⁸ In the initial stage of etching, the removal of Ag led to the formation of a Au-rich passivation layer on the NP surface, helping retain the particle's shape when the underneath Ag was further etched away. By using 0.3 M HNO₃ (chemical etching) as the etchant, a great variation in the pore size was observed, while etching through applying positive potentials (electrochemical etching, with 0.1% v/v acetic acid as the electrolyte) can better control the pore size, with larger pores preferred at lower potentials.

As a unique form of oxidative etching, galvanic replacement is widely used to generate alloy NPs, where a metal with a higher oxidation potential serves as a sacrificial template and is replaced by another metal. By leveraging liquid-phase TEM, such complex reaction process can be tracked, and one example is the galvanic replacement on Ag nanocubes with Au(III) ions where nucleation, growth, and coalescence of voids inside the nanocube were observed (Figure 34c).⁴³⁶ The higher surface energy at corners led to the favored nucleation of voids at these sites. The Kirkendall effect combined with galvanic replacement facilitated the expanding of voids and



Figure 35. (a) Schematic of polymer solution sandwiched between graphene sheets. (b) Low-mag TEM image showing a representative liquid channel. (c) Representative images for PEO (top) and PSS (bottom, $M_w = 2200 \text{ kDa}$) in deionized water. (d) Probability distributions compared for PSS ($M_w = 2200 \text{ kDa}$) in 0.2 M NaCl (top), deionized water (middle), and PSS ($M_w = 1030 \text{ kDa}$) in deionized water (bottom). The dotted vertical line shows that relative to the peak of the distribution in the middle panel, the peak is smaller in salt solution and also for a sample with lower molecular weight. (e–g) Direct observation of micelles within the liquid cell. The micelle solution was continuously irradiated at 0.25 e⁻/Å² s for 2 min. Insets are zoomed-in images of the particles boxed with the corresponding color. Scale bars: 20 nm. (a–d) Reprinted with permission from ref 422. Copyright 2017 Wiley-VCH. (e–g) Reprinted with permission from ref 441. Copyright 2018 American Chemical Society.

finally the production of hollow cubes mainly composed of Au. Similar products with a hollow structure but different surface roughnesses were observed at varied reaction conditions (e.g., elevated temperatures approaching 90 °C or replacing Au(III) with Au(I)), indicating the prevailing of the two mechanisms.

5.2.3. Shape Degradation of NPs and Their Relevance to Cycling Performance in Electrochemical and Photocatalytic Reactions. Characterizing the shape deformation of nanomaterials in battery cycling, electro- and photocatalytic reactions is of great significance in understanding their active sites and degradation mechanisms, providing insightful guidance in improving their activity and stability. For example, the investigation into the volume expansion of Si during lithiation is essential for designing robust electrodes. By encapsulating Si NPs in the liquid electrolyte (LiPF₆ in a solvent mixture of ethylene carbonate, dimethyl carbonates, and diethyl carbonates) and initiating lithiation by electronbeam irradiation, it was found that facet-dependency existed at the beginning of the lithiation.⁴²⁰ An anisotropic volume expansion was observed with the $\langle 110 \rangle$ direction preferred due to the smaller Li diffusion energy barrier at the Si-electrolyte interface along this direction. Once passing the initial barrier, Li diffusion progressed isotropically. Such an observation indicates that the rate-limiting diffusion barrier is at the Si-electrolyte interface and is different based on the lattice orientations.

Liquid-phase TEM is also a powerful tool to study NP degradation, namely the shape deformation and aggregation commonly seen as issues of NP in catalysis applications. By patterning electrodes onto SiN_x chips and integrating optical fibers in TEM, both electro- and photocatalytic reactions can be characterized. A notable example is resolving the degradation mechanisms of metal nanocrystals during electrochemical reactions such as ORR and CO₂ reduction reaction

 (CO_2RR) . A series of behaviors were observed, including the dissolution, aggregation, and Ostwald ripening, among others, which can benefit the fundamental understanding and rational design of more stable catalysts.^{437,499,500} Such observations can be expanded to surface amorphization of Co_3O_4 NPs in oxygen evolution reaction (OER),⁵⁰¹ reduction of Cu_2O to metallic Cu in CO_2RR ,⁴¹⁷ formation of self-hydrogenated shell on TiO₂ surface in photocatalytic hydrogen evolution reaction (HER),⁴³⁸ determination of active facets on TiO₂ from bubble generation in photocatalytic water splitting,⁵⁰² and so on.

5.3. Liquid-Phase TEM Characterization of the Morphology, Transformation, and Self-Assembly of Organic and Biological Nanomaterials

Regarding organic compounds made of small molecules and polymers, and biological materials such as hybrids, proteins, vesicles, and even living cells,⁴³² these materials have complex solvent-mediated intra- and intermolecular interactions, where the interplay of enthalpic and entropic effects causes many interesting phenomena such as assembly, structural reorganization upon stimulus, and so on. To understand and maneuver the interactions, in situ imaging of the structures and behaviors plays a pivotal role. Currently, the majority of experiments involve the use of bulk-averaging techniques including light, neutron, and X-ray scattering, as well as the static imaging with cryo-EM. Considering the fact that most of these soft materials are synthesized or functioning in liquid environment, the direct imaging enabled by liquid-phase TEM and integration with molecular simulations are expected to provide more significant insights into the dynamic nature of soft materials.

Generally, there are two main impediments for imaging organic and biomaterials: the sample damage from incident electrons, mostly from heating, charging, and ionization, and weak electron scattering due to atoms with low Z values. Different techniques have been applied to retard the damage and enhance the contrast, such as incorporation of graphene,⁴²¹ staining or labeling with heavy metal ions,¹⁸⁸ and leverage of radiolysis-resistant solvents, as discussed in Section 5.1.1.⁵⁰³ In this section, we will focus on how these challenges are met for liquid-phase TEM imaging, starting from organic compounds, mostly on polymers and organic reticular crystals, including MOF and COF. These will be followed by discussions on biomaterials, an important category of soft materials. A review paper from Smith et al. has summarized studies on biomaterials prior to 2020,⁵⁰⁴ and we will focus on more recent publications in this section, from fibers, proteins, to hybrid structures.

5.3.1. Morphology and Transformation of Polymeric Nanomaterials and Their Assemblies. With the development of liquid cells and detectors, polymers can now be imaged at molecular level. By trapping the polymer solution in a graphene channel cell (Figure 35a,b), single polymers including polystyrenesulfonate (PSS) and poly(ethylene oxide) (PEO) were resolved (Figure 35c).⁴²² In addition to the size distribution of polymers measured from liquid-phase TEM images, other features of the polymers were also confirmed, such as conformational fluctuations, adsorption and desorption on substrates, and radiation damage involving chain scission and recombination after long exposure to electrons. It should be noted that subdiffusive motion is mostly observed for particulate samples in liquid-phase TEM, no matter inorganic or organic.⁴⁶⁰ While efforts are ongoing to understand how to alleviate such effects to observe the generic

motions of NPs, which have been shown successful in the studies of NP superlattices,^{461,462,505} the slowed motion of polymers could suppress motion blurring to allow recognition of a molecular structure.

Beyond the single molecule configuration where intermolecular forces are negligible, self-assembly of amphiphilic molecules and polymers is a typical approach to forming macromolecular compartments and bilayers. Their formation, phase change, and stimuli-response behaviors play a pivotal role in the basic functions of life and have inspired a vast diversity of industrial products, from biomedicines to detergents, lubricants, and coatings.⁵⁰⁶ Controlling the pathway can offer modulation of the morphology of polymer assemblies, such as packing, crystallinity, domain size, orientation, and connectivity, enabling property control.⁵⁰⁷

The polymerization of an amphiphilic diblock copolymer was first observed by Touve et al., who used beam-induced radiolysis of the aqueous solution to create a chemical environment that mimics the condition of bulk polymerization.⁴⁴¹ The radicals generated from electron beam drove the monomer polymerization in solution via reversible addition-fragmentation chain transfer (RAFT). The polymerization then triggered the formation of micelles through the growth of a hydrophobic block on a hydrophilic polymer, named polymerization-induced self-assembly (PISA). To avoid uncontrolled chain growth and morphology evolution induced by high concentrations of radicals, a low electron flux, pulsed imaging, and a sufficiently high macrochain transfer agent (macroCTA) concentration were used, promoting the initiation of polymerization from carbon-centered radicals and the degenerative chain transfer over uncontrolled conventional radical polymerization. The growth of micelles and their equilibration into similar sizes were observed under liquidphase TEM (Figure 35e-g). Recently, with the help of variable-temperature liquid-phase TEM (VT-LPTEM), a thermally initiated RAFT polymerization process was observed, leading to the formation of an amphiphilic block copolymer that assemble upon dispersion PISA.⁵⁰⁸ Due to the thermoresponsive property of the polymers, a substantial reduction in particle dimensions was observed at elevated temperatures, followed by a hydrophobic-to-hydrophilic phase transition upon cooling. With the capability of temperature control, we expect more exploration into the thermal phasetransition behavior of polymers.

Micelles and vesicles are among the most commonly formed assembly structures from amphiphilic molecules such as block copolymers studied in liquid-phase TEM. The first paper reporting the imaging of individual micellar NPs made of amphiphilic block copolymers is by Proetto et al.⁵⁰⁹ Pt was coordinated to monomers to enhance the contrast of the micelles, and their motions in an aqueous solution was recorded and analyzed. Interestingly, when three particles adjacent to each other were tracked, a concerted motion was observed, which could be due to the beam induced charging effects between particles, charging effects induced from solution, or from SiN_x substrate. Later studies investigated the self-assembly of amphiphiles via liquid-liquid phase separation. Unimers of diblock copolymer, poly(ethylene oxide)-block-poly(caprolactone) (PEO-b-PCL), was dispersed in acetone, followed by solvent exchange with water to prompt the formation of polymer-rich liquid droplets.⁵¹⁰ The assemblies were observed to form at the interface between the droplet and the bulk solution with the size of the liquid



Figure 36. (a) Time-lapse TEM images showing the formation of PEO-*b*-PCL block copolymer vesicles. Scale bars: 400 nm. (b) Micelle–micelle fusion process captured by liquid-phase TEM. The video frame where collision between the red and blue micelles first occurred is referred to as t = 0 s. Frames labeled as t = 55 and 110 s show a single assembly (micelle labeled by a black arrow) after morphological relaxation. (c) Magnified time-lapse images of the fusion-relaxation process between the red and blue micelles. (d) Schematic showing the encapsulation of a Au NP with the triblock copolymers. (e) Time-lapse TEM images showing the encapsulation process. In addition to the NP encapsulation, other micelles also form in the solution and cluster around the NP. (a) Reprinted with permission from ref 510. Copyright 2019 Springer Nature. (b, c) Reprinted with permission from ref 506. Copyright 2017 American Chemical Society. (d, e) Reprinted with permission from ref 513. Copyright 2019 Royal Society of Chemistry.

droplet determining the final size of the vesicles (Figure 36a). The onset of kinetic traps was found to play a determinant role in the membrane thickness and affected the organization or composition of the assemblies. During the growth process, the assemblies were initially spherical, then transferred to cylinders or lamella before forming the vesicle membrane, confirming the existence of different vesicle formation pathways.

Aside from the growth of micelles, their fusion, fragmentation, and morphology switching also attract lots of interest. In an early study, the growth and fusion of block copolymer micelles made of (phenyl-b-peptide-co-hydroxyl) were tracked, where both unimer addition and particle-particle fusion were observed (Figure 36b,c). Combining with in silico modeling, fusion was found to be associated with external solvent entrapment, and a variety of complex vesicles and biocontinuous micelles were generated from initially spherical micelles.⁵⁰⁶ In another work, fragmentation of 1,2-polybutadiene-block-poly(ethylene oxide) (PB-b-PEO) micelles in ionic liquid was observed.⁵¹¹ Upon heating to 170 °C, a series of transition of micelle shapes were resolved, from a spherical shape, a prolate ellipsoid, further elongating to a peanut shape, followed by necking, neck thinning, and ultimate separation into two micelles. Aside from shape transition in aqueous solutions, the solvents can also be expanded to organics, such as dimethylformamide (DMF) and methanol. For example, a

worm-to-micelle transformation in poly(styrene)-*b*-poly(4vinylpyridine) (PS-*b*-P4VP) was observed, which was triggered by a solvent switch from methanol to DMF.⁵¹² Interestingly, it was suggested that water is more radiolytically sensitive than organic solvents, indicating the great potential of organic solvents to be utilized for the characterization of polymers.

Micelles formed by amphiphilic block copolymers can also be used to encapsulate metal NPs and drugs to enhance their solubility, stability, and reduce toxicity, as a useful strategy in biomedicine and bioimaging. The encapsulation process can be analyzed by liquid-phase TEM, which was found to be a selflimiting process. ⁵¹³ The block copolymers, (ethylene oxide)₁₀₀*block*-(propylene oxide)₆₅-*block*-(ethylene oxide)₁₀₀, (EO₁₀₀-PO₆₅-EO₁₀₀), gradually adsorbed onto the hydrophobic, PScoated Au NP, grew around the particle to form a shell, and ceased when particle was fully covered by the polymers (Figure 36d,e). The strong hydrophobic interaction between PS and EO₆₅-block was regarded as the main contributor to the adsorption of copolymers to Au NPs. When switching PS to hydrophilic citrate, as a comparison, no encapsulation was observed in either in situ or ex situ experiments. The incorporation of heavy metals can also label the polymer assemblies and make their positions easier to be recognized, a strategy widely used in the characterization of proteins and



Figure 37. (a) Time-lapse TEM images showing the formation of ZIF-8 nanocubes. The solution (t = 1 s) phase separates into solute-rich (dark gray contrast) and solute-poor (lighter gray contrast) regions (t = 15 s). Next, these solute-rich regions condense into aggregates (t = 31 s), which then crystallize into ZIF-8 nanocubes (t = 62 s). (b) Schematic illustration of the nucleation process. (c) (top) Time-lapse liquid-phase TEM images showing the room-temperature conversion of ZIF-8 nanocubes into LDH nanocages. t_0 represents the time point corresponding to the onset of the etching. (bottom) Schematic illustration depicting the conversion process of a ZIF-8 nanocube into an LDH nanocage. (a, b) Reprinted with permission from ref 519. Copyright 2021 National Academy of Science. (c) Reprinted with permission from ref 523. Copyright 2021 American Chemical Society.

cells and tracking their motions in SEM (more examples can be found in Section 5.4).

5.3.2. Nucleation, Growth, and Transformation Mechanisms of Organic Reticular Crystals (MOF and **COF**). Organic reticular crystals, including MOFs and COFs, are highly porous and tunable materials, which have attracted great attention due to their impressive performance in a vast variety of applications, ranging from gas storage, separation, to catalysis, drug delivery.⁵¹⁴ A deep mechanistic understanding of the growth, phase transition, assembly, and other dynamic processes of these organic reticular materials would benefit the rational design and precise control of their morphologies. In applying liquid-phase TEM to observe such processes, one needs to be aware of the beam sensitivity of MOFs and COFs.¹¹ Low dose and careful selection of solvents are necessary. Post-mortem analysis using ex situ techniques, such as dry TEM and XRD, can help evaluate the composition of the in situ products and potential beam artifacts.^{514,515}

In one example, methanol was found to generate fewer reactive radiolysis products than water, enabling the observa-

tion of the growth process of the zeolitic imidazolate framework-8 (ZIF-8).⁵¹⁶ Small MOF seeds were observed at the initial stage, which grew larger without coalescence, indicating a monomer addition mechanism. The nucleation of ZIF-8 was also found to be limited under local depletion of monomers in solution, with particle diameter $\propto t^{1/2}$ to $t^{2/3}$ based on the LSW model,^{517,518} suggesting that the growth is a surface reaction-limited monomer addition process. However, the process examined above is more of postnucleation. A recent paper looked deeper into the challenging nucleation process of ZIF-8.⁵¹⁹ With ultralow-electron-flux ($\leq 0.05 \text{ e}^- \text{ Å}^{-2}$ s^{-1}) liquid-phase and cryo-TEM, three steps were observed throughout the nucleation: liquid-liquid phase separation into solute-rich and solute-poor regions, condensation of the soluterich region into an amorphous aggregate, and finally the crystallization of the aggregate into a MOF (Figure 37a,b). Though the generality of the nonclassical nucleation pathway still needs further proving on other MOF particles, the integration of different characterization techniques and the



Figure 38. (a) Liquid-phase TEM snapshots of radial growth of FF nanotube with integrated line profiles illustrating the increase in diameter and hollowness of the nanotube. (b) Outer diameter plotted as a function of time shows the linear trend in increasing diameter. (c) Schematic of FF nanotube cross section illustrating the increase in tube diameter with time owing to the association and dissociation of monomers. (d, e) Liquid-phase TEM frames of POPC-sLPS vesicles in the (d) absence and (e) presence of 1 mM NiCl₂. (f) Snapshots of POPC-sLPS vesicles in 1 mM NiCl₂ as a function of time and cumulative dose. Red strip represents a time period of 120 s when the electron beam is turned off. (g) A snapshot showing the decrease in contrast after a 2 min beam blank. (h) Schematic illustration of partial reversible interaction of Ni²⁺ ions with vesicles. (a– c) Reprinted with permission from ref 503. Copyright 2021 American Chemical Society. (d–h) Reprinted with permission from ref 188. Copyright 2020 American Chemical Society.

improved resolution even under low dose set the foundation for more studies of synthetic mechanisms.

In another report, the growth of Cu₂Br₂-based metalorganic nanotubes (MONTs) at room temperature and 85 °C was captured.⁵²⁰ It was found that tubular crystal growth was reaction limited at both temperatures, with particle size $\propto t^{1/2}$, suggesting the thermodynamically driven surface-specific monomer attachment mechanism. Besides, instead of reacting instantly, precursor ions would instead seek the lowest energy face of the growing crystal, inducing the anisotropic growth and the formation of tubular shape. In a more recent paper, the formation of Ag-based MONTs was characterized using liquidphase TEM, and multiple pathways were observed by tuning the metal to ligand ratios (AgNO₃:L1).⁵²¹ At low concentrations of AgNO₃, aggregation and short-range clustering of precursor ions occurred to form primary particles. As the supersaturation increased, MONT bundles were formed by heterogeneous nucleation from the primary particles. In contrast, when an excess amount of AgNO₃ was used, coalescence and oriented attachment of ensemble particles were observed, generating anisotropic MONT crystals.

Aside from nucleation and growth, the postnucleation morphology transformation of MOF NPs has also been investigated, upon external stimulus such as heating and etching. Phase transition from a microporous MOF, NU-906, to a mesoporous MOF, NU-1008, was discovered and monitored with VT-LPTEM.⁵²² By heating for around 2 h in formic acid at 80 °C, spherical NU-906 particles were transformed into NU-1008 flakes and rods. Liquid-phase TEM clearly captured the transformation process, where the NU-906 particles first broke into units whose sizes were too

small and contrast were too low to be resolved. These small units then reassembled into new seeds, which further grew into NU-1008 flakes and then rods. The shape transformation can also be induced by etching. It is recently reported that hollow layered double hydroxide (LDH) nanocages could be converted from ZIF-8 NPs with their shapes of a cube or a RDD largely retained (Figure 37c).⁵²³ With liquid-phase TEM, the nucleation and growth of LDH nanosheets on the surface of ZIF-8 particles were observed as the MOF particles gradually got etched. The in situ observations highlighted the importance of maintaining comparable etching and growth rates if one wants to obtain hollow LDH cages with welldefined, consistent shapes with the MOF template.

Similar to MOFs, COFs are crystalline organic polymers with high porosity and tunable molecular structures. One of the key challenges in the synthesis of COFs is to understand their nucleation and growth processes.⁵²⁴ With VT-LPTEM, direct imaging of hundreds of individual COF-5 (a prototypical 2D boronate-ester linked COF) NPs was realized. The uniform, radial growth of seed particles at 80 °C and the absence of particle coalescence and ripening indicated the monomer or oligomer addition mechanism involved in the growth of COF-5 particles. It is noted that both the COF crystals and monomer solutions are highly susceptible to beaminduced artifacts at 80 °C, thus snapshots were acquired periodically with beam off during intervals, instead of continuous videotaping, providing an alternative and effective method for imaging beam-sensitive materials.

5.3.3. Structure and Dynamics of Biomaterials and Their Assemblies. Biomaterials, including cells, fibers, lipids, proteins, and nucleic acids, are another type of soft materials

that are largely investigated by liquid-phase EM. Life processes are tightly connected with water, either occurring within or reacting with this liquid solvent, such as the folding of proteins, ion transition through membranes, and catalysis with enzymes.⁵⁰⁴ For real-time imaging of biomaterials, optical microscopy is mostly used, such as phase contrast microscopy and confocal fluorescence microscopy. Though dynamics can be directly imaged, the limited spatial resolution (usually 200 nm, can also be improved to approximately 10 nm in superresolution optical microscopy) restricted the characterization of biomaterials at the nanometer or atomic resolution of EM. Liquid-phase TEM applied to solvated biological nanostructures was first demonstrated by Mirsaidov et al. in 2012.⁵²⁵ By using a low electron flux, around 100 nm wide crystalline acrosomal bundles were imaged at a sub-3 nm resolution, giving similar results to those imaged in vitrified ice. The capability of imaging unstained and unlabeled macromolecular assemblies in an aqueous solution encouraged more studies using liquid-phase TEM. Besides publications introduced in our previous review paper in 2020,⁵⁰⁴ in this section, we will give an updated review of recent studies from single molecules and proteins to peptide assemblies and protein crystallites.

Fibers. Cytoskeletal fibers including the acrosomal actin bundles and microtubules were among the first protein structures to be imaged in liquid-phase TEM, with their periodic features and sensitivity to electron beam deeply investigated.^{525,526} In a recent work, diphenylalanine (FF) nanotubes, a key motif forming amyloid fibers that are associated with Alzheimer's disease and type II diabetes, were analyzed.⁵⁰³ By sealing the FF monomers inside SiN_x chips, the growth of FF nanotubes was recorded, with a pulsed imaging protocol leveraged to reduce the beam damage. The initial nucleation stage of the nanotubes was observed, where the oligomers formed immediately upon cooling from 80 °C and aggregated into ring-like motifs. These motifs could further elongate into nanotubes, proceeding through a monomer addition mechanism. Aside from the elongation, for the first time, the radial growth of nanotubes was resolved, which was attributed to the association and dissociation of FF monomers that occurred radially (Figure 38a-c). The investigation into the influence of electron beam was also covered, where the nanotube assemblies remained intact under a low electron flux (~0.5 e $^{-}$ Å $^{-2}$ s $^{-1})$ and aggregated to form anisotropic chains under a high electron flux (~100 e⁻ Å⁻² s^{-1}). Post-mortem analysis via time-of-flight secondary ion mass spectroscopy (ToF-SIMS) showed the unavoidable fragmentation of FF over time even under low electron flux, which can be effectively reduced when compared to high flux. Such molecular level damage still existed when switching the solvents from water to alcohols, which are regarded as ·OH radical scavengers, indicating that the damage was directly posed on FF nanostructures instead of through indirect solvent radiolysis. Interestingly, the edge sharpness of FF nanotubes was improved in alcohols when compared to water, owing to the lessening of scattering effects and beam broadening as the mean free path of electrons decreased from isopropyl alcohol to ethanol and then to water. Such a phenomenon points out the possibility of using alcoholic solvents to improve the resolution of low contrast materials.

Lipid Assemblies. The structure and dynamics of lipid assemblies can also be resolved in liquid-phase TEM. To enhance the contrast of carbonaceous species with low-Z elements, similar to ex situ TEM, staining with metal ions is proposed.⁵²⁷ In a recent work, reversible staining of Ni²⁺ ions on vesicles formed from 1-palmitoyl-2-oleoyl-*sn*-glycero-3phosphocholine (POPC) and smooth lipopolysaccharides (sLPS) was introduced.¹⁸⁸ The idea was inspired by a recent finding that Ni²⁺ ions bind reversibly to the O-antigen of sLPS. While no species were visible in the absence of Ni salt until ~20 min, the vesicles were immediately observed at the same dose rate when 1 mM NiCl₂ was present, with size comparable to that in cryo-TEM (Figure 38d,e). Flowing with buffer solutions or blank the beam can both lead to the partial desorption of Ni²⁺ ions (Figure 38f–h). Considering that prolonged staining may perturb the dynamics of the system, partial reversible staining may allow the observation of dynamic processes in a more natural way along with a decent contrast.

Viruses, Ferritins, and Other Large Protein Structures. Large protein structures such as viruses and ferritins (and many other metalloproteins) are widely explored by liquidphase TEM as they exhibit higher contrast against the liquid background, especially for those containing high Z elements. Previous studies include resolving the structure of H3N2 influenza viruses,⁵²⁸ movement of rotavirus double-layered particles due to Brownian motion or biological activity, 430,440 atomic and electronic structure of hydrated ferritin,48 rotational and translational diffusion of Au-labeled DNA strands,⁵²⁹ and diffusion, interaction, and assembly of unhybridized DNA oligomers.⁵³⁰ In a recent report, a liquidflowing graphene chip (LFGC) was introduced, allowing imaging of beam-sensitive materials with high resolution and rapid liquid circulation.⁵³¹ The chip was fabricated by transferring few-layered graphene onto the holey-patterned SiN_x membrane, followed by assembly of top and bottom chips on a liquid-flowing holder equipped with a liquid injection system. The thin liquid layer (with a 50 nm spacer for monodisperse NPs and 200 nm spacer for aggregates), capability of flushing radicals, and graphene combined are capable of tolerating high dose and thus achieving highresolution imaging. Typical wet samples, including PS beads, liposomes, E. coli, and colloidal Au NPs were characterized. The spherical shape of PS beads, 5 nm size of liposome lipid bilayer, and the rod-shaped morphology of *E. coli* and their pili with a width of 5 nm were clearly resolved in the LFGC. By sealing particles in 150 nm thick microwells, the structural details of adeno-associated virus (AAV) dispersed in liquid can be visualized with the help of a high-frame-rate direct detector and parallel computing processes.⁵³² A spatial resolution comparable to cryo-EM was achieved, as well as the tracking of dynamic conformational change of viruses, suggesting the major benefit and great potential of the liquid imaging method.

Combining with tomography, the 3D structure of biomaterials can be reconstructed, allowing the recognition of material morphology and properties in a higher dimension than simple 2D projection. To achieve different viewing angles, a hybrid chamber was introduced by sealing the aqueous samples between a SiN_x microchip and a collodion/carboncoated TEM grid.⁸ A flagellotropic phage was imaged at tilting angles ($\pm 35^{\circ}$), and the capsid head and tail can be clearly resolved (Figure 39a,b). The interactions between the phage and its bacterium host, *Agrobacterium sp.* H13-3, were also analyzed, with the phage attachment to the host observed in the liquid cell and their contact further confirmed by tilt images. The tomographic images also showed the direct contact between the bacterial surface and the heads of phage



Figure 39. (a) Density map (magenta) of a reconstructed bacteriophage displayed at different angles. (b) An image stack showing differences in phage dimensions and features from $\pm 35^{\circ}$ in the tilt series. Scale bar: 50 nm. (c) TEM image of DNA-Au NP superlattices taken using graphene liquid cell TEM. Scale bar: 200 nm. The inset shows the corresponding FFT pattern which matches the diffraction pattern of a *bcc* crystal along the [320] zone axis. Scale bar: 0.1 nm⁻¹. (d) High-magnification TEM image showing the ordered arrangement of Au NPs. The lattice spacing corresponds to (002) planes in a *bcc* crystal structure. Scale bar: 50 nm. (a, b) Reprinted with permission from ref 8. Copyright 2019 American Chemical Society. (c, d) Reprinted with permission from ref 421. Copyright 2017 American Chemical Society.

particles instead of their tail fibers during infection. As previously mentioned in Section 3.4.3, besides tilting deliberately, by imaging NPs rotating with Brownian motion, one could also obtain images of NPs from different orientations and reconstruct their 3D structures.²⁵¹

Peptide/Protein Assembly and Aggregation, Hybrids. The assembly of peptides and proteins creates important structural motifs, such as fibrils, matrices, and gels, with functions and applications ranging from scaffolding to biocatalysis and biomedicine. Real-time imaging of the self-assembly process can aid our understanding of the underlying science and enable rational manipulation of this process. Aside from ordered assembly structures, the aggregation of proteins into disordered structures is of direct relevance to the stability of pharmaceuticals, partitioning of biomolecules into cellular droplets, and pathogenic protein aggregation.⁵⁰⁴ Lysozyme was mostly used as a model system for fundamental studies using liquid-phase TEM due to their different polymorphic structures from different crystallization pathways, including the formation of ordered crystals and amorphous clusters⁵ and the high mobility of defects and defect annealing due to rapid rearrangement of crystal bonds.⁵³⁴ Other than lysozyme, a variety of peptides and proteins and their behaviors were also investigated, such as formation of peptide assemblies in response to chemical reduction and enzymatic cleavage,⁵ the aggregation of PEGylated Interferon α_{2a} (Pegasys) under different temperatures and pH,536 and the phase separation of histidine-rich beak protein 2 (HBP-2) in solutions with varied ionic strengths and initial protein concentrations.⁵³⁷

Coating graphene onto SiN_x membrane is a strategy to image beam-sensitive biomaterials like DNA-Au NP superlattice while still allowing for liquid flow and other functional modules of a flow holder. Graphene can effectively scavenge reactive radical species, hydroxyl radicals in particular, which mainly contribute to breaking of DNA strands. With the protection of graphene, the long-range periodic ordering of Au NPs in the superlattice was resolved with clearly visible lattice spacings (Figure 39c,d).⁴²¹ The FFT image measured a bodycentered cubic (*bcc*) structure of the superlattice with a (002)plane lattice spacing of 16.3 nm, consistent with SAXS results (16.6 nm). No significant rearrangement was visible, while at the same imaging conditions, dissociation and aggregation of Au NPs were immediately observed upon electron beam illumination in bare SiN_x cells without graphene coating. Raman spectroscopy suggested that graphene served as a radical scavenger, with radicals reacting with graphene-based nanomaterials via electrophilic addition to conjugated C=C π bonds or further oxidation of existing oxygen-containing functionalities. Switching graphene to graphene oxide or graphene quantum dots also enabled the stabilization of DNA-Au NP assemblies.

5.4. Liquid-Phase SEM (and STEM-in-SEM) Characterization of the Motion and Configuration of Soft Nanomaterials

Liquid-phase SEM is a more recently emerged tool compared with liquid-phase TEM, with lower spatial resolution but compatibility with large samples. The shape evolution and motion of NPs can be observed by liquid-phase SEM. In one example, growth of Au NPs in an ionic liquid droplet was recorded, where the reduction of Au(III) precursor was realized by hydrated electrons. Small particles appeared in the first 30 s after exposure to the electron beam, followed by their formation into heterogeneous aggregates on the surface of the ionic liquid.⁵³⁸ Taking advantage of the nonvolatility of ionic liquid, its droplet was placed on a glass slide and was not required to be fully sealed. Similarly, one can also create a freestanding film by placing a drop of ionic liquid dispersion on a lacey carbon-coated grid and blotting with filter paper to remove excess liquid. To this end, NPs can be trapped inside the ionic liquid film and their motions can be tracked. Using silica spheres and rods as model systems, their motions in the ionic liquid films with different thickness were analyzed.539 While the diffusion coefficients of dilute nanospheres agree well with theoretic predictions in thick films, a tendency toward pairing was observed when changing to thin films, unveiling a balance of capillary and hydrodynamic interactions. Au-coated silica NPs also work for this system and in fact appeared brighter, enabling their motions amidst neighboring NPs clearly resolved (Figure 40a). With a high concentration, these nanospheres displayed a subdiffusive caged motion and irreversible adherence to each other. In contrast, concentrated NRs preferred assembling into finite stacks. The use of an ionic liquid as a solvent and a large field of view in SEM benefit the in situ characterization of particles with fast diffusion and interaction with macroscopic interfaces. However, the use of ionic liquids to avoid evaporation was a limitation.

To extend to other solvents, liquid-phase SEM utilizes SiN_x microchips to achieve sealing against a high vacuum and benefits from a configuration of STEM-in-SEM or low-voltage



Figure 40. (a) (Left) Bare (dark) and Au-coated (bright) silica NPs mixed at the periphery of an ionic liquid film. (Right) Image sequences showing the diffusion of single (top row) and dimer (bottom row) Au-coated silica NPs at ~57% areal coverage of bare NPs. Scale bar: 2 μ m. (b) Overview dark-field environmental STEM-in-SEM image showing the flat regions of the cells (gray) and the thicker cellular areas (white). (c) Image recorded at the location of rectangle in panel b. (d) Image showing individual Au NPs as white spots in the region marked by a rectangle in panel c. (e–h) Magnified regions from panel d showing individual Au NPs, dimers, and larger clusters indicated with numbers 1–4, respectively, in panel d. (a) Reprinted with permission from ref 539. Copyright 2016 American Chemical Society. (b–h) Reprinted with permission from ref 541. Copyright 2013 Springer Nature.

STEM. By sandwiching Au-labeled epidermal growth factor receptors (EGFRs) (on triple-negative breast cancer (TNBC) cells) between two SiN_x microchips, the localization, distribution, and configuration of labeled membrane proteins can be precisely recognized.⁵⁴⁰ One can also directly grow live cells on Si microchips.⁵⁴¹ Maintained in a saturated water vapor atmosphere, the hydrated cell is covered with a thin layer of water, mimicking the liquid environment. In combination with specific labeling with Au NPs, the activated EGFRs in the plasma membranes of COS7 and A549 cells can be localized, and their further dimerization and clustering can be determined and quantified (Figure 40b-h). In both studies, an integration of STEM with SEM, so-called STEM-in-SEM, was used, which can improve the resolution of SEM to subnanometer in a dry cell and 2-4 nm in a liquid cell. The high contrast and low sample damage from low accelerating voltage provide more opportunities for the characterization of beam-sensitive materials.^{446,542–545} By coating graphene, the beam damage in liquid-phase SEM can be further reduced. In a recent report, a single-layer graphene veil casted on a silicon substrate with bacteria and buffer solution confined within was

introduced.⁵⁴⁶ The shape, size, and morphology of cells can be discriminated with their functions well maintained after imaging, attributing to the effective dissipation of charged electrons via conductive graphene. The graphene veil provides a new possibility of close-to-non-invasive imaging of live cells and functional biomolecules.

5.5. In Situ Characterization of Nanomaterials at Dry State

5.5.1. Shape Evolution and Compositional Change of Nanomaterials at Elevated Temperatures. The properties of colloidal NPs can be easily tuned by maneuvering their size, shape, structure, and composition. Meanwhile, the shape deformation, structural, and compositional changes of NPs in harsh environments, such as elevated temperatures and various gas environments, can also lead to degradation in their performance. In this case, direct observation of NPs' behaviors under external stimuli is essential to the fundamental understanding of their stability and provides guidance for the design of nanomaterials. In situ TEM/STEM has been a powerful tool to provide imaging and quantification of the morphological and structural changes of nanomaterials in

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harsh environments.^{547–550} For example, using in situ HRTEM, the stability of decahedral Au NPs was investigated at elevating temperatures from 300 to 1000 K (Figure 41a).



Figure 41. (a) Representative HRTEM images taken from a decahedral Au NP at different temperatures. The average NP diameter is about 10.3 nm. The initial Au NP close to a $\langle 110 \rangle$ projection at (A) 300 K (room temperature), (B) 600 K, (C) 750, (D) 800 K, (E, F) 900 K (the structure was distorted with significant surface roughening), (G) 950, (H) 1000 K (the surface became almost fluid while the crystalline areas are still present). (b, c) In situ HAADF-STEM images recorded from Pd@Pt_{4L} (b) octahedron and (c) cube under different heating conditions. (a) Adapted with permission from ref 553. Copyright 2009 American Chemical Society. (b, c) Adapted with permission from ref 552. Copyright 2017 American Chemical Society.

The onset of surface melting was observed at about 800 K, suggesting the threshold temperature below which the shape of decahedra is maintained.⁵⁴⁷ In another example, Pd NPs with a concave, icosahedral shape were heated to 600 °C.⁵⁵¹ A dramatic change occurred with the multitwinned structure transformed to single crystal due to the elimination of twin defects and thus the alleviation of strain energy.

Compared to monocomponent NPs, investigating the stability of bicomponent NPs could be more complicated considering possible simultaneous occurrence of shape deformation and element mixing or segregation. For example, the response of Pd@Pt_{4L} core–shell (4L means four layers of Pt) cubes and octahedra to high temperatures was resolved by in situ STEM.⁵⁵² After heating to 600 °C, alloying between Pd and Pt was observed in the octahedron, revealed by the loss of Z-contrast between shell and core in the HAADF-STEM image (Figure 41b), while the two elements were clearly distinguished until 800 °C in the cube (Figure 41c). Converse to the structural stability, cubic shape started to deform and became spherical at 500 °C, which was about 400 °C lower

than that of the octahedral shape. Such comparison shows the role of facets in maintaining the shape and structure of NPs.

High-entropy alloys are emerging alloy compounds composed of five or more principal elements existing as disordered solid solutions. HEAs are well-known for their high mechanical strength, thermal stability, and corrosion resistance.²⁵⁸ Similar to conventional alloy NPs, HEAs are also studied under elevated temperatures and various gas environment to understand their physical properties. In situ TEM combined with the gas-cell holder was used for such studies, as shown in Figure 42a, where the oxidation of the HEA NPs (Fe_{0.28}Co_{0.21}Ni_{0.20}Cu_{0.08}Pt_{0.23}) was performed in atmospheric ⁵⁴ The in situ heating and imaging of the NPs showed that air. the oxidation layer gradually grew and covered the NP surface, which was indicated by the lighter contrast of the oxides (Figure 42b). The oxidation process was found to be guided by Kirkendall effects, where the different diffusion rates of metals led to a composition variation in the oxide layer and HEA core. In comparison to the oxidation of monometallic and bimetallic alloy NPs (e.g., Co, NiFe, NiCr) with similar sizes, 555,556 HEA NPs in this experiment were more stable and showed slower overall oxidation kinetics. The thickness of the oxide layer on HEA NPs was found to follow logarithmic law other than a parabolic one predicted by Wagner's theory,557 which was attributed to the spherical shape of the NPs and the resulting stronger polarization across the oxide layer. In a following study by the same group, the HEA NPs were first oxidized (red box) and then reduced in H₂ gas environment by in situ heating (Figure 42c).⁵⁵⁸ In the reduction, the external surface of the oxide layer transformed from dense to porous structures with cracks, and the Cu atoms reduced from oxides segregated into Cu NPs, while Fe, Co, and Ni remained in the oxide phase (Figure 42d,e). It is worth noting that the in situ TEM imaging can be combined with chemical analysis, such as EDX, to analyze the variations of the principal elements in the NPs at elevated temperatures, further enhancing our understanding of the reaction process. 554,558

5.5.2. Tracking Motions and Reactions of Atoms and Single Molecules. The use of HRTEM or aberrationcorrected (S)TEM can further push in situ imaging to the atomic resolution. A notable example is the individual Fe atoms residing at graphene edges examined by low-voltage aberration-corrected TEM.559 The individual Fe atom diffused along an edge of the graphene with the neighboring carbon atoms removed or added. The serial TEM images further showed the pentagon-to-hexagon transition during this catalytic process: the Fe atom was initially connected to four carbon atoms in a pentagonal shape, followed by absorbing nearby carbon atoms into a distorted hexagonal structure, moving to the right, and finally stopping in a pentagonal structure again (Figure 43a-d). Recently, the formation of 5fold twins through repeated oriented attachment of 3 nm Au, Pt, and Pd NPs was observed, in which HRTEM played an indispensable role.⁵⁶⁰ The time-lapse atomic resolution images, combining with molecular dynamics (MD) simulations, clearly suggested two formation mechanisms: forming and decomposing of high-energy grain boundaries, and partial dislocation slipping. Such findings provide essential insights into the interpretation and controlling of twinned crystal structures, guiding the design and synthesis of nanomaterials with diverse morphologies and applications.

Compared to metallic NPs, resolving the conformation and motion of organic molecules is much more challenging due to



Figure 42. (a) Schematic showing the in situ TEM imaging of HEA NPs in a gas cell. (b) In situ TEM image sequences of HEA NPs during annealing in air to show the growth of oxide layer. (c) Time-dependent TEM images of a HEA NP oxidized in air (red box) and then reduced in H_2 (gray box). (d) STEM (HAADF and low-angle ADF) images of HEA NP shown in panel c, as well as the corresponding EDX mapping. (e) Atomic percentage distribution of metal elements and O. The red arrow points to the directions in the same manner as marked in the HAADF image in panel d. (a, b) Reprinted with permission from ref 554. Copyright 2020 American Chemical Society. (c–e) Reprinted with permission from ref 558. Copyright 2021 American Chemical Society.

their low contrast and high sensitivity to beam damage. In 2007, Koshino et al. proposed a single-molecule atomicresolution real-time TEM (SMART-TEM) method, where the organic molecule was captured on a single-walled CNT, resembling a fish captured by a fishing rod. In this way, the stepped conformation change and translational motion of hydrocarbon molecules confined in CNTs were clearly tracked.⁵⁶¹ The molecule changed from one conformation to another twice in the first 20 s and varied to more afterwards (33.6 s) (Figure 43e-f). The same technique was applied to study the structure of a hydrocarbon chain as it passed through the nanoscale pore in the wall of a single-wall CNT. Figure 43g shows the time-lapse TEM images of this process. The hydrocarbon chain underwent rotation in the tube and then the chain terminus entered into the nanopore with a diameter of about 0.5 nm located at the top of the CNT. The chain was retarded by the nanopore for a few seconds, potentially due to the pore/chain interactions, and later drawn back into the CNT. The SMART-TEM technique elucidated the motions and interactions of organic molecules on a single-molecule basis, providing opportunities for more fundamental research

and engineering applications such as gas adsorption and storage in carbonaceous materials.

5.5.3. In Situ Electron Diffraction Study of Phase Transformation of NPs. Nanoparticles can undergo fast and subtle microstructural changes, such as domain formation, phase transition, and coalescence, at the atomic scale during reactions, which is hard to probe using methods such as TGA, XRD, and even real-space imaging of EM due to the limited size or morphology change involved. In situ electron diffraction techniques provide an alternative method.⁵⁶³⁻⁵⁶⁵ In one study, time-resolved SAED patterns were collected from Ni/NiO NPs during the oxidation reaction. The experiment combines the atmospheric gas cell system and fast in situ electron diffraction camera with millisecond time resolution, which are essential for capturing the fast oxidation process of Ni NPs. As the NPs are small (<10 nm), the intensities of the diffraction peaks of Ni and NiO can be regarded to be proportional to their volumes,⁵⁶⁶ and therefore can be used to determine the ratio of Ni and NiO phases. It is worth noting that this proportional relationship cannot be applied to thick samples where dynamic scattering occurs. The whole reaction process occurred within a few seconds and could be divided into two



Figure 43. (a-d) In situ HRTEM imaging of one cycle of catalytic growth of graphene edge that lasted for 4 s. The Fe atom and C atoms are marked as red and black dots, respectively. Bottom panel: the corresponding atomic structures showing the track of the Fe atom during the translocation. (e) In situ TEM images resolving intertwined hydrocarbon chains confined in a CNT. Scale bar: 1 nm. (f) Models of the structure of hydrocarbon chains confined in CNT at 4.2 and 6.3 s, respectively. (g) Time-lapse TEM images of alkenyl fullerene for consecutive seconds at room temperature. Bottom panel: the corresponding molecular models. The red arrow indicates the hole on the CNT side wall. Scale bar: 1 nm. (h) SAED intensity profile as a function of reaction time during the lithiation of Co₃O₄ NPs. (i) Integrated intensity profiles at 0, 200, 760, and 2000 s, together with references of Co₃O₄, LiCo₃O₄, Co, and Li₂O. (a-d) Reprinted with permission from ref 559. Copyright 2014 National Academy of Sciences. (e-f) Reprinted with permission from ref 561. Copyright 2007 AAAS. (g) Reprinted with permission from ref 562. Copyright 2008 Nature Springer. (h, i) Adapted with permission from ref 563. Copyright 2016 American Chemical Society.

stages: nucleation and growth.⁵⁶⁷ The oxidation rate of Ni NPs remained constant at the initial stage (<0.5 s), while later accelerated oxidation and growth of oxide layer fit better to the Avrami-Erofeev solid-state reaction model, instead of the Wagner and Mott-Cabrera models where ion diffusion was regarded as the control step. Figure 43h,i shows another example of in situ SAED performed throughout the lithiation process of Co₃O₄, which confirmed the phase transformation

of Co₃O₄ (spinel) to a composite of metallic Co (hexagonal close-packed, hcp, phase) and Li₂O (cubic phase).⁵⁶³ The example highlights the capability of in situ diffraction in collecting millisecond kinetics information on NP variations, both compositionally and structurally.

6. APPLICATION OF ML IN EM DATA ANALYSIS

Unlike most conventional spectroscopy methods where the collected dataset is one-dimensional (1D), the real-space imaging advantage of EM characterization by default leads to 2D data. With additional data collection from sample tilting (tomography), reciprocal space (electron diffraction), and temporal series (video), the dimensionality of EM data can increase to 3D and 4D. Consequently, analysis of EM data can be benefited from a sophisticated and high-throughput algorithm. The analysis starts from detecting the contours of features (i.e., segmentation) to obtaining the features' size, shape, and position. Manual annotation assisted by software (e.g., ImageJ/FIJI) can sometimes locate the desired feature, but is inefficient for large-volume data processing and can be biased in detecting blurry features against noisy background. Automated, non-ML-based EM image processing algorithms are limited to frequency filtering and thresholding.

Machine learning-based EM data analysis thus has emerged for different tasks. As an elementary and traditional imagerelated ML task, supervised image classification classifies experimental EM images into categories representing different materials, compositions, or structures—much like labeling dog and cat photos-and thus enables qualitative descriptions of mixture or heterogeneous samples. The classification task is usually achieved by CNNs, which can take thousands of images as input and output predefined categorical labels for each of them. Meanwhile, image classification treats and labels images as whole without extracting the spatial information from them. As a result, complicated tasks including image segmentation and object detection have been identified and achieved by advanced CNN and other ML algorithms. Image segmentation dedicates to labeling every pixel in an image to identify the shape, location, and species of features in EM images. The pixelwise classification nature of image segmentation makes it necessary to be combined with other algorithms to single out the feature of interest (e.g., NPs or atoms) for statistical analysis. Different from image segmentation, the object detection focuses on recognizing and locating every individual object in an image, but without giving the pixelwise information. Both image segmentation and object detection are useful in measuring the species abundance (e.g., yield of valuable products or fractions of different components in a mixture), spatial arrangement, and self-assembly structures, while image segmentation is also proficient on morphology characterization.

The ML techniques mentioned above belong to supervised learning, where training process and definition of the tasks are required. For example, the labels one wishes to predict in image classification have to be predefined. For underexplored material systems with less prior knowledge, where the predefined labels are not available, unsupervised learning such as dimension reduction and clustering algorithms have been used. The relevant task of EM analysis is to group samples, such as differentiating desirable products from impurities as well as recognizing and qualifying spatial heterogeneity in samples. Such heterogeneity can include polydisperse morphologies, multiple structural domains, and grain boundaries.

Other than the supervised and unsupervised learning for prediction-driven tasks, ML can also be used for data generation and decision making, which are known as generative learning and reinforcement learning, respectively. Generative learning models such as the variational autoencoder (VAE)⁵⁶⁸ and the generative adversarial network (GAN)⁵⁶⁹ capture the important features of existing dataset to synthesize nonexisting data. This learning method proves advantageous in the design of novel material structures to achieve desired functions,^{570,571} prediction of material morphologies to facilitate investigation into their physical properties,⁵⁷² fabrication of training datasets for supervised learning,⁵⁷³ and refinement of tomography reconstruction for improved quality.⁵⁷⁴ Lastly, reinforcement learning has seen applications in guiding microscopy-based experiments by real-time decision making, which can be a promising future direction in EM and will be covered in the outlook.

In the following sections, we will review the challenges and application examples of ML. First, we will start from the analysis of real-space 2D EM images, highlighting the morphology and structural characterizations enabled by image segmentation and object detection. We will next push to real-space 3D tomography, focusing on ML-assisted high quality and artifact-free tomography reconstruction. Lastly, we will cover material morphology grouping via unsupervised learning techniques based on real-space EM images and diffraction patterns.

6.1. Introduction and General Challenges of ML for EM Data Analysis

6.1.1. Overview of ML Methods Used in EM Data Analysis. ML has been increasingly applied to accelerated discovery and synthesis optimization of nanomaterials.^{575–580} In those applications, ML model predicts or regresses material property from a relatively small and fixed material descriptor space. In comparison, the inputs of EM data are huge image matrices which contain thousands to millions of pixel values with strong spatial correlation. This poses a challenge also recognized for ML-based analysis of optical microscopy images. The solution is to use CNNs that are designed for image matrices. In CNNs, small matrices, usually 3 \times 3 or 5 \times 5 (also known as kernels), replace the densely connected neurons in the conventional deep neural network, which greatly decreases the model size and makes the computation feasible. As an example, 128⁴ weights are needed to connect two hidden layers with sizes as small as 128×128 pixels in the densely connected neural networks. With convolution, the information can be passed through hidden layers simply by sliding a few tens of kernels containing only a few hundred weights across the input image matrix to improve efficiency. Additionally, the convolution operation introduces translational equivariance, which enables CNN to capture objects regardless of their position in one image. Lastly, in most CNNs, pooling layers are employed to down sample the input images, enabling feature detection at multiple length scales at fixed kernel sizes. All those advantages resulting from the architectures of CNNs make them the first choice in initial processing of EM images.

Most CNNs models belong to supervised learning. In supervised learning, the model optimizes its weights by learning from the exampled input and output pairs (i.e., training dataset), which can be divided to the forward propagation and the back-propagation steps. In the forward propagation, the input image goes through a series of convolution, pooling, and densely connected layers to compute the output, which can be a scalar, a vector, or even another image matrix. During the back-propagation, the output is compared with the exampled output (i.e., ground truth), and a "loss" representing the difference between the current output and the ground truth is calculated. Then the weights in the supervised learning model will be updated to decrease the loss value through gradient descend. In the training process, the training dataset will be iterated through several times until the loss converges to an acceptable value. By tailoring the output and the architectures, CNNs can be applied to image analysis tasks as diverse as image denoising, classification, segmentation, object detection, and even helping tomography reconstruction.

Meanwhile, the real-space imaging advantage of EM allows for comprehensive description of the heterogeneity by collecting a statistically large number of morphologies in samples. Several ML-based unsupervised algorithms, including k-means, Gaussian mixture model (GMM), and density-based spatial clustering of applications with noise (DBSCAN), have been used to cluster samples into groups. Those algorithms recognize and group morphologies with high similarity while maintaining heterogeneity across the whole sample.581-583 Among them, k-means and GMM assign each data point (i.e., each sample) to a cluster center in the feature space, where each center corresponds to one group and the number of cluster centers, namely the number of feature groups, is predetermined by users. The k-means minimizes the squared Euclidean distances between all samples and its cluster center in the feature space, while GMM maximizes the likelihood of the samples belonging to its cluster center by assuming the data point distribution is a mixture of several multivariant Gaussian distributions. k-means tends to find clusters of comparable spatial extent with a shorter computation time, while GMM allows clusters to have different shapes but has a slow speed. DBSCAN is a data point distribution density-based clustering algorithm, where the closely packed data points are grouped together, and the outliers in low-density regions are assigned as noises. Compared with k-means and GMM, DBSCAN does not require the specification of the number of clusters and is more applicable to samples with arbitrarily shaped distributions in the feature space (e.g., do not need to follow Gaussian distributions). For these morphology clustering strategies based on unsupervised learning, they do not require training, and can directly use certain feature descriptors (e.g., NP size and shape) that can be generated from the segmentation of EM images as the inputs.

Unsupervised learning algorithms can also be applied to classify features using complicated input datatypes, such as XRD⁵⁸⁴ and electron diffraction pattern, and shape contours of features as a whole. One prominent example is to associate different diffraction patterns with lattice structures and orientations in 4D-STEM. In these applications, one needs to consider that different from the simple feature vectors made up of a few scalar shape descriptors, those complicated datatypes have high dimensionality (>10) and are hard to be classified due to the well-known "curse of dimensionality". 585,586 To address this issue, dimension reduction techniques (which are also considered as unsupervised learning methods) are applied prior to classification. Commonly, the top two or three dimensions after reduction are either directly visualized to show the variations and similarities among different samples or serving as the input of unsupervised classifications. Such dimension reduction algorithms include principal component analysis (PCA),⁵⁸⁷ independent component analysis (ICA), t-distributed stochastic neighbor embedding (t-SNE),⁵⁸⁸ and uniform manifold approximation and projection (UMAP).⁵⁸⁹

6.1.2. Challenges and Solutions in ML Model Training: Generation of Training Dataset, the Issue of Unbalanced Data, and Model Validation. Among the ML-based EM data analysis methods, supervised learning requires training of the model before usage, the common challenges and solutions of which we detail below.

Generation of Training Dataset. Similar to humans, most CNNs require a training process to learn how to use and analyze the input images to predict the output. The common and open-source ML platforms (e.g., TensorFlow and PyTorch) provide user-friendly libraries for model constructing, training, and predicting. However, users still have to provide the training dataset for their own customized prediction tasks. For example, to predict NP shape (e.g., rod vs sphere) from an EM image, the training dataset should contain pairs of exampled input images (experimental EM images of rod or sphere) and output classes (e.g., categorical variables such as 0 for rod and 1 for sphere). The training dataset also needs to be large enough (at least hundreds of input and output pairs) to cover the sample variations and to optimize the kernel and neuron weights in the model.

Two methods are commonly used in EM image training dataset construction: manual annotation and image synthesis. Manual annotation means that researchers manually determine and label the outputs as ground truth on their EM data, which can be difficult and time-consuming, compromising the efficiency of CNN-based image analysis. Image augmentation can be used to expand these manually annotated ground truth—usually a small portion of the full experimental data—to a sufficient training dataset through random combinations of image transformations, such as translation, rotation, shearing, zooming, flipping, and brightness change. Augmentation captures random sample shape and orientation, image magnification, and brightness/contrast fluctuations that can occur during EM imaging, thereby making the trained ML model robust against them.

On the other hand, regarding image synthesis as a means to generate synthetic experimental data as the training dataset, the advantage is that the ground truth is well-defined and no experimental data is needed. It has been successfully applied to train neural networks that identify atoms and NPs from various microscopies including STEM, TEM, and helium ion microscopy (HIM). Taking the example of TEM images of NPs, one can build a NP geometric model (with composition, size, spatial shape profile defined), which is also the ground truth, and "synthesize" the corresponding TEM image, i.e., the input image for training following the contrast formation mechanism of TEM and computer graphics. This method can in principle provide training datasets with unlimited sizes. It also bypasses the uncertainty and inconsistency of manual annotation of images of relatively low imaging resolution and SNR.

Note that the key to generate a good synthetic training dataset is to resemble and cover the features of the experimental dataset as much as possible, such as pixel size, background noise, and graininess for TEM images.^{485,590} Otherwise, the trained model can perform poorly because of the difference between the synthesized datasets used in training and the real experimental data. Such a difference can be qualitatively spotted by human observers. It also can be quantitatively evaluated. For example, Mill et al.⁵⁹⁰ applied t-

SNE dimension reduction on the patches cropped from their synthetic and experimental HIM images of metal oxide NPs. In the reduced dimension, a high similarity is revealed by good mixing of data points from the synthetic and experimental image patches, while data point separation suggested the opposite. Similarly, Ma et al.⁵⁷³ applied the sliced Wasserstein distance (SWD) to measure the similarity at various length scales between their artificial SEM images of uraniummolybdenum alloy microstructures synthesized by GAN models and experimental images. In the same work, features describing the area, spatial distribution, and texture were extracted from the synthetic and experimental images, serving as the input of Gaussian process classifiers (GPCs) to learn the difference between two types of images. The successful image synthesis was then validated by the low cross-validation accuracy of GPC models. Meanwhile, there are also studies suggesting that some details of the experimental data can be discarded in training dataset synthesis. For example, it was reported that by using the synthetic STEM images of graphene with vacancies and Si dopants simply generated by removing and replacing the atoms without any structural relaxation as the training dataset, the resulted CNN models can be powerful enough to identify more complex defects in the experimental data that were not explicitly included in the training dataset.¹⁵ This robustness of trained CNNs suggests that ML models are able to learn and extrapolate beyond the training dataset provided to more complicated samples.

Unbalanced Data. For the task of image classification, most naturally sampled datasets are unbalanced, which means the number of observations (or samples) from each class are not the same. As a result, a highly unbalanced training dataset might result in low prediction accuracy on the minority classes. As an example, for a binary classification task with a training dataset containing 99% positive samples and 1% negative samples, a "naive" model that outputs 100% "positive" can reach an accuracy of 99% on the training dataset while missing all the negative samples. Resampling the training dataset and collecting more negative samples may help solve this issue. However, oversampling (replicating the minority classes) and undersampling (skipping the data from the majority groups) may also become an issue consequently. The former could cause overfitting because of duplicating the same data points, while the latter is based on arbitrary decisions and does not fully make use of the training data.⁵⁹¹ One solution is to use data augmentation. For example, Luo et al.⁵⁹² created augmented artificial images to supplement the minority image classes during their classification of CNT images. Their prediction accuracies in all classes were improved and the accuracy variations among different classes were also reduced. Tuning the class weights during the training process is another way to avoid the waste of training data while solving the data unbalance. Decreasing the class weights of the majority groups asks the model to "learn less" from the majorities and consequently rebalances the training dataset, improving the overall prediction accuracies. Data balancing is also a task sensitive practice. If the unbalanced training datasets truly resemble the feature of experimental data, directly using the unbalanced datasets could give the highest overall accuracy and should be accepted as what it is. It also depends on the specific metric that one cares about: overall accuracy (defined as the percentage of predictions that match with the ground truth), precision (defined as the percentage of positive predictions that match with the ground truth), or recall

(defined as the percentage of positive samples that are correctly predicted)—to achieve a higher percentage of correct predictions, to only give the absolutely positive data, or to collect all the possibly positive data with tolerance on the negative data that are mixed in.

Model Validation. The trained model needs evaluation. The loss and evaluation metrics (e.g., accuracy) are embedded in the ML algorithms and can be monitored throughout the training process. Depending on the tasks of ML-based EM analysis, different evaluation metrics are used. For the numerical regression tasks (e.g., EM image denoising^{593,594} and tomography reconstruction^{149,595,596} where the numerical output is required instead of classes), the mean absolute error (MAE), mean squared error (MSE), and root mean squared error (RMSE) are usually used. For classification tasks, in addition to the accuracy, precision, and recall mentioned above, the F1-score and the area under the receiver operating characteristic curve (AUROC) are also common choices. Specifically, for image segmentation, the object of interest (e.g., NPs) can only account for a small area in the whole TEM image. As a result, a high accuracy cannot represent a good prediction performance.⁵⁹⁷ Instead, the intersection-overunion (IoU, or Jaccard Index) can be used to calculate the overlapped area of the predicted object segmentation and the ground truth object segmentation, divided by the area of their union to reflect the performance of feature segmentation itself. Segmentation performance can also be evaluated by an even more rigorous metric such as boundary F1 that only considers the segmentation border difference between the prediction and the ground truth.598

These metrices alone, however, do not reflect the generalizability of the model to real experimental data after training. Overfitting describes this situation where a trained ML model has high accuracy on the training dataset but has poor performance on the new experimental data. The overfitting problem can be solved by decreasing the model complexity, adding regularization and dropout layers, stopping the training early, or expanding the training dataset. In order to identify overfitting, in a practical training process, the generated training data can be split into two portions: one for model training, other for model validation-it does not participate in the back-propagation and is only used to calculate the loss and evaluation metrics. This hold-out validation split enables the model to be tested on the untrained data. During the training, if the accuracy on the validation data starts to drop after certain training epochs, overfitting might start. Note that although convenient, in this data validation process, the randomness of splitting of the training datasets into two pools can lead to deceiving validation. For example, most samples in the validation pool can happen to be easy to predict. In this case, the k-fold cross-validation can be used instead. The k-fold cross-validation is an ensemble approach to evaluate the performance on multiple models trained with different validation splits, during which the total training data will be split into k batches and k different models will be trained on k-1 batches while using the rest one for validation. While the k-fold cross-validation demands more computation capability, it can provide the averaged model performance and variance without wasting any experimental data. After an acceptable cross-validation, all k batches are jointed to train the final model.⁵⁹¹



Figure 44. Studies of using CNNs for image classification and segmentation. (a) A schematic showing the carbon nanostructures image classification workflow. (b) Experimental STEM image of graphene doped with silicon, overlaid with the FCN output of atoms, dopants, and vacancies classes. (c, d) Schematics showing the U-Net-based segmentation of HIM images of TiO_2 NPs. (c) The U-Net model trained on synthetic data used to segment the experimental images. (d) The analysis of TiO_2 NP images. The U-Net prediction is further processed to give individual particles labeled by different colors, which provides particle size distribution and other information. (e) TEM image of tip-coated patchy Au nanoprisms and the corresponding U-Net prediction with three output channels: nanoprism core (black), polymer patch (white), and background (gray). (f) Workflow showing the overlaying tetrahedral Au NP contour reconstruction using a multiple channel U-Net. The nonoverlaying region of NPs (gray, channel 1) and the overlaying region of NPs (white, channel 2) are combined to reconstruct individual NP contours. (a) Adapted with permission from ref 592. Copyright 2021 The Royal Society of Chemistry. (b) Adapted with permission from ref 603. Copyright 2017 American Chemical Society. (c, d) Adapted with permission from ref 590. Copyright 2021 John Wiley & Sons. (e, f) Adapted with permission from ref 5. Copyright 2022 The Royal Society of Chemistry.

6.2. Applications of ML in Analysis of 2D EM Image for Feature Recognition

6.2.1. Supervised EM Image Classification of Nanomaterials. CNN-based models such as LeNet,⁵⁹⁹ AlexNet,⁶⁰⁰ VGGNet,⁶⁰¹ and ResNet⁶⁰² are designed for the supervised image classification task, namely outputting one (multiclass classification) or multiple (multilabel classification) categorical labels representing the predefined classes given an image input. They have shown excellent performance on classifying daily life photographs and hand-written numbers. For EM images, supervised CNNs were reported to classify the TEM images of CNTs and carbon nanofibers (CNFs).⁵⁹² The authors innovatively extracted outputs from intermediate layers in the VGG-16 model representing the information at various length scales, and clustered them via k-means and vector of locally aggregated descriptors to serve as the input of a softmax classifier (Figure 44a). The trained model is able to classify thousands of CNT/CNF TEM images into several classes including clusters formed by loosely packed fibers, single fibers, condensed matrix structures with embedded CNT/CNFs, oversized mixed structures with CNT/CNFs on surfaces, and non-CNT structures, with an accuracy over 90%. Given the potential health risks associated with occupational exposure to these CNT/CNFs, such automated structural classification from TEM images can efficiently recognize their different structures and thus accelerate their exposure assessments for health risk-related studies.

6.2.2. Segmentation of EM Images with CNN. Image segmentation, also referred to as semantic segmentation in ML, serves as a basic step in quantitative analysis of nanomaterial features, such as yields in NP synthesis, NP size histogram,

atomic lattice structure, and NP self-assembly structure. Image segmentation algorithms separate the input image of grayscale pixel values into several domains assigned with different physical meanings (e.g., NPs, atoms, lattice structural domains, background). Mathematically, this is done by translating the EM image matrix into another image matrix with the corresponding pixels assigned with predicted categorical values of the domains. These segmented domains can be then processed by other algorithms to identify properties such as their size, shape, and position.

CNNs with image-sized outputs, such as the fully convolution network $(FCN)^{604}$ and U-Net,⁶⁰⁵ have been used for image segmentation. Those CNN models can make pixelwise predictions on the probability of each pixel in the original image belonging to different species (e.g., NPs, background), by using their special encoder-decoder architectures. The encoder part is connected to the input layer, and the pooling layers inside can allow the detection of features on multiple length scales. The decoder part is responsible for reconstructing the output image back in the original spatial domain with full spatial resolution through its up-pooling layers. Unlike conventional non-ML thresholdbased segmentation algorithms, where only the absolute pixel intensity value is used as the criteria to assign pixels, FCN and U-Net can judge the semantic meaning by considering the neighboring pixels' intensities through the kernel convolution with prelearned weights. This way of making the prediction mimics the decision-making process by human experts and thus provides the "smartness" to ML models to give precise and robust prediction results.



Figure 45. Studies of using ML models other than CNN for image segmentation and instance segmentation. (a) Training setup of the genetic algorithm for Au NR TEM image segmentation. Genes containing the parameters of various image analysis methods are evaluated to select the best genes by crossing and mutating genes with high scores over generations. Images of nanoparticles obtained from individual high-scoring genes are combined in the end to collect more individual Au NRs. (b) Schematic of the few-shot learning model. The raw STEM image is broken into smaller chips and a few user-defined chips are used to represent desired segmentation classes in the support set. Each chip then acts as a query and is compared against the support set and get categorized, yielding the segmented image. Scale bar: 5 nm. (c) Mask R-CNN-based nanoparticle shape characterization workflow from TEM images. (d) Comparison of NP TEM image segmentation results obtained by semantic segmentation (U-Net and SegNet) and instance segmentation (Mask R-CNN and improved Mask R-CNN). The improved mask R-CNN can have better performance on weak-edge nanoparticles and adhesion NPs. (a) Adapted with permission from ref 581. Copyright 2020 American Chemical Society. (b) Adapted with permission from ref 612. Copyright 2021 Springer Nature. (c, d) Adapted with permission from ref 628. Copyright 2020 Springer Nature.

At the atomic scale, CNNs are used to extract the accurate atom positions from TEM⁶⁰⁶ and STEM^{603,607,608} images to study the lattice structures in 2D materials, which is otherwise hard for traditional image analysis due to high noise. In one example,⁶⁰³ FCN was trained to segment the lattice atoms, dopants, and vacancies from the STEM images of 2D lattices of graphene and MoSe₂ (Figure 44b). Synthetic STEM images of 2D lattices with the pixel intensity proportional to $Z^{1.5-1.8}$ were generated as the training dataset with known ground truth. The arrangement of atoms in these images were generated from ab initio or MD simulations. The trained FCN model was able to extract the atomic coordinates in experimental STEM images to identify various defect structures associated with the vacancies and dopants, such as single or multiple atom vacancies, 5-7 reconstructions (Stone-Wales defects), Si dopants bonded with different numbers of carbon atoms in graphene, and dimer or trimer dopant atoms. Combining such an automated and high throughput defect extraction workflow with time-series STEM data could further allow the analysis on defect diffusion and structural transformation behavior over time.^{603,607} The defect structures can also be used to construct libraries of experimentally stable defect configurations. When serving as the input to DFT calculations, such defect configurations yield theorical scanning tunneling microscopy (STM) patterns which match well the experimental STM images, indicating

such ML-assisted atomic structure characterization is able to build links between the material structure (STEM) and functionality (STM). 608

For colloidal NPs, CNN segmentation aids the quantification of NP shape and size distributions by its precise binarization of an EM image. One example is training U-Net to segment SiO₂ and TiO₂ NPs in HIM images by Mill et al.⁵⁹⁰ In this study, a rendering software (Blender) was used to generate synthetic and photorealistic microscopic NP images comprising NPs of randomized sizes, shapes, and positions. The trained-Net model can segment isolated NPs as well as the partially obscured NPs in aggregations (Figure 44c), with accuracies comparable to human experts. Enabled by the segmentation of individual NPs, the particle size distribution was obtained (Figure 44d). Such automated and highthroughput particle segmentation and characterization can benefit studies related to nanotoxicology and other fields such as nano- and biomedicine, consumer product efficacy testing, and anticounterfeiting. Moreover, the multiple output channels of CNNs can facilitate the segmentation of different components in the hybrid nanomaterials. For example, the U-Net with three output channels is capable of segmenting both the high-contrast Au NP core and weak-contrast polymer patches in the patchy Au nanoprism (Figure 44e).⁵ The careful design of output channels can also achieve the segmentation of overlaying NPs. As shown in Figure 44f, when assigned to

predict the overlaying region and nonoverlaying region respectively, the two output channels in U-Net can be combined to reconstruct the contour of partially overlaying tetrahedral Au NPs in TEM images.⁵ The ability to separate the overlaying NPs benefits ML-based automatic nanomorphology characterization, making it more robust for aggregated samples.

CNN also facilitates the analysis of assembled nanostructures.^{609,610} For example, FCN has been used to segment the low-contrast protein nanofibers from Au NR-protein nanofiber assemblies in SEM images.⁶¹⁰ A few initial images were manually labeled and augmented as the training dataset. Combined with the conventional thresholding-based image segmentation of high-contrast Au NRs, the identification of Au NR and protein fiber orientations showed a preference of Au NRs to parallelly align to the nanofibers, the extent of which decreased with increasing salt concentration and was weakly sensitive to the rod aspect ratio. Another example exists in the segmentation of liquid-phase TEM movies. Due to the existence of thick liquid layer and the leverage of low electron dose rate for minimizing beam damage, the SNR is usually low and accompanied by blurred features. Training U-Net models on synthetic TEM images of randomly positioned NPs superimposed with artificial noises is proven to segment individual NPs, as well as to separate overlaying NPs, in the layered self-assembly structures. 485,611 Such segmentation can allow the detection of NP positions, and thus facilitate studies on NP diffusion and the dynamic self-assembly behavior in their native, liquid environment. More details will be discussed in Section 6.3.

6.2.3. Segmentation of Nanomaterial Images with Models Other Than CNNs. Though CNNs have excellent performance in image segmentation, their training process requires a large number of either manually annotated or simulated image data as the input, which are not always available. Efforts in other types of ML algorithms have also been devoted to simplifying training data acquisition for segmentation of EM images. For example, Lee et al.⁵⁸¹ trained a genetic algorithm to optimize the sequence and parameter selection in a stepwise TEM image segmentation approach with pretreatments and filtering. Conventionally, such parameters (e.g., filter kernel size, edge detection methods, etc.) are selected by humans empirically. In this study, a genetic algorithm resembles the natural selection process in species evolution was trained to find the best combination of the parameters that gives good segmentation results (Figure 45a). Benefited from the efficient and accurate segmentation, more than 15,000 Au NRs were extracted from hundreds of TEM images and their sizes and shapes were used for the computer simulation of UV-visible extinction spectra via the boundary element method. By matching the simulated spectrum with the experimentally obtained spectrum, it was shown that at least 1,500 individual Au NRs are necessary to converge to the experimentally measured extinction, which in return emphasizes the importance of the automated and high throughput analysis of NP morphology.

To achieve the adaptability to new and underexplored materials, the few-shot learning model is another ML model that does not require a thorough training dataset. Akers et al.⁶¹² developed a few-shot learning model to segment and differentiate the phases, defects, and microstructural features in the STEM images of SrTiO₃ (STO)/Ge heterostructures, $La_{0.8}Sr_{0.2}FeO_3$ (LSFO) thin films, and MoO₃ NPs (Figure

45b). A high-resolution STEM image was first divided into image "chips" to be classified individually. Then a few chips from each class representing different phases or microstructures were manually picked and annotated as the support set while the majority of the chips were free of annotation. Next, all chips were transformed by a prototypical network (ResNet101 in this work) to give the prototype representations of the chips. The distances between the unlabeled prototypes and the center of each support set of labeled prototypes were calculated and compared to predict the labels for unlabeled chips. Finally, all classified chips were stitched together to give the semantic segmentation of the original high-resolution image. As shown in Figure 45b, the few-shot learning can deliver satisfying segmentation results, except that the segmentation resolution is limited by the size of image chips. The major advantage of few-shot learning over CNN models is that few-shot learning only requires one to a few images per class for training and can be applied more rapidly and flexibly to new materials and new classification tasks.

6.2.4. Object Detection of NPs and Proteins in EM Images and Its Combination with Image Segmentation. CNN-based image segmentation relies on pixelwise assignment, which makes it challenging to recognize individual features when they are partially overlapped. For example, NPs assembled in a lattice or aggregating randomly can leave a few or no pixels between the boundaries of the neighboring NPs; 3D stacking can lead to partially obscured NPs in SEM images or overlaid NPs in projected TEM images. In these scenarios, mere image segmentation leads to the detection of the assembled structures or aggregates as a whole, without recognizing the NP components inside. Postsegmentation algorithms such as watershed transformation,⁶¹ ³ ultimate erosion for convex sets,⁶¹⁴ and bounded erosion-fast radial symmetry⁶¹⁵ have been applied after the segmentation to cut the aggregated or overlaying particles apart. However, such algorithms only exploit the information in the segmented binarized image without referring to the intensity profile in the original image. They generally work better for features of simple rounded shapes with minor overlaying.

For some applications such as yield analysis, self-assembly, and trajectory analysis of NPs, locating and counting the individual components without knowing the exact shape contours are sufficient. To this end, several ML models were developed to perform only the object detection task, to directly output the identity and position of nanomaterial instances from EM images without segmentation. In object detection, bounding boxes are generated by the ML model to locate and annotate each individual object of interest without specifying their contour shapes. Region-based CNNs (e.g., R-CNN,⁶¹⁷ Fast R-CNN,⁶¹⁷ Faster R-CNN,⁶¹⁸ YOLO,⁶¹⁹ and Retina-Net⁶²⁰) are the most common models for object detection, which were originally designed for processing real-life photographs and later applied to EM images. Using Faster R-CNN as an example to discuss the architecture of region-based CNNs: In Faster R-CNN, a region proposal network (RPN) proposes ROIs representing highly possible regions for finding the objects and generating proposed bounding boxes around them. Another half of the model consists of a classifier and a regressor network, which takes the proposed bounding boxes as inputs to give the object an identity and regress the location of the bounding box to inscribe the object, respectively. When applying the Faster R-CNN to STEM images of FeCrAl alloys,⁶²¹ the model simultaneously recognized and located

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dislocation loops with four different types of morphologies in irradiated steels at a speed of around 0.1 s per image, which is hundreds of times faster than manual analysis.

CNN-based object detection has also been applied to the analysis of biological samples such as automated single particle picking in cryo-EM reconstruction. The particle picking from 2D EM images in cryo-EM remains a challenge due to the low SNR, particle overlap, ice contamination, and amorphous carbon. Although several computational methods have been proposed such as template matching and edge detection, they can either be sensitive to noise and low contrast or still require human supervision.⁶²² Several studies employ CNNs to develop single particle picking platforms including Deep-Picker,⁶²³ DeepEM,⁶²⁴ and DeepCryoPicker.⁶²² Such studies rely on the image classification CNNs to judge whether a cropped region from the EM image contains a good protein particle or background for automated particle picking. Through demonstrating on published protein particle EM datasets including Keyhole Limpet Hemocyanin and EMPIAR, the platforms are proven to be efficient, automatic, and accurate approaches to pick particles in cryo-EM images. More recently, Xiao et al.⁶²⁵ implemented Fast R-CNN to pick protein particles in cryo-EM micrographs. Tested on the recent published cryo-EM data of three proteins including human gammas-secretase, yeast spliceosome, and TRPV1, their Fast R-CNN model can achieve a fast protein particle detection speed at 2 s per image, which is much faster than the conventional manual picking process that can take a few days or weeks. This work also innovatively distinguished between the ice contamination and protein particles to significantly reduce the false detection rate.

In more recent studies, the robust capability of object detection to recognize individual materials instances even in crowded or aggregated samples and the capability of image segmentation to elicit the full contour of the instances are combined for thorough analysis of samples. Such tasks are also known as instance segmentation. Starting from the regionbased R-CNN, another segmentation network offers the ability to segment each detected object, giving rise to the mask R-CNN model (Figure 45c). The mask R-CNN model and its variant have been used in detecting NP size and orientation from TEM and SEM images,⁶²⁶⁻⁶³⁰ showing superior segmentation results on aggregated NPs (Figure 45d) to pixelwise classification models. However, the addition of the segmentation network in mask R-CNN burdens the computation efficiency, leading to slow model training and image prediction. Further study also suggests that mask R-CNN can still suffer from missing detections during its nonmaximum suppression (NMS) step when the overlaying area between adjacent bounding-boxes is large.⁶³¹ To address these problems, more lightweight and bounding-box-free algorithms such as StarDist⁶³¹ and MultiStar⁶³² have been purposed. Similar to the image-to-image CNN models, the StarDist directly regresses the pixel-to-boundary distance for every pixel inside the object at different radial directions, which is realized through a U-Net architecture with multiple output channels. Meanwhile, another parallel regression task will be conducted to find the pixels near individual objects' center. Finally, the object contour is reconstructed from the radial distances measured at pixels near each object's center. Although the original designs of such proposal-free instance segmentation algorithms are demonstrated with optical microscopy images of aggregated cells as examples, 631,632

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several recent studies have employed them to analyze SEM and TEM images. For example, the StarDist has been demonstrated to give an accurate measurement of the particle size distribution of the Pt catalysts supported on carbon for polymer electrolyte fuel cells,⁶³³ by training on datasets as small as 30 images. Such imaging-based characterization of a catalyst enables the high-throughput screening in the design and fabrication of novel materials. Similarly, a Bayesian particle instance segmentation algorithm was employed to measure the particle-size distributions, radial-distribution functions, average sizes, and aspect ratios from the TEM and SEM images of various nanomaterials ranging from ZnO microrods, steel powders, bacterial cells, Pd NPs, AuNRs, dendritic-like mesoporous silica, to PS spheres and Pt₃Co NPs,⁶³⁴ indicating the versatility of instance segmentation algorithms in EM image analysis.

6.3. Machine Learning Applications in Temporal Microscopy Data Obtained from In Situ EM

Other than conventional imaging of static nanomaterial samples, EM can also be combined with different in situ modules (see more details in Section 5) where a video—stacks of time-lapse EM images—is collected. In addition to the 2D positional information in a single TEM image, time is now an additional dimension to consider in analysis. Currently, ML has been applied to the following in situ EM studies, all of which are real-space images (no diffraction or tomography involved).

In Situ Liquid-Phase TEM of NP Dynamics. For liquid-phase TEM studies, ML methods aid the analysis of NP morphology change, diffusional dynamics, and self-assembly kinetics. Owing to the existences of a liquid layer and sealing windows, as well as low dose rates to have the electron beam intervention of samples under control, SNR is commonly lower in liquid-phase EM videos than in the EM images of dry samples, raising challenges in analyzing individual video frames. Meanwhile, the high-volume nature of video data also necessitates automated and high throughput analysis methods where minimal human involvement is needed. Recently, Yao et al. used U-Net for accelerated and highfidelity segmentation to analyze liquid-phase TEM videos.⁴⁸⁵ Specifically, these liquid-phase TEM videos have high noise and blurriness. After trained on synthesized TEM images containing randomly distributed NPs with different shapes and sizes, the U-Net model precisely delineated the shape contours of individual NPs, providing not only their positions but also their orientations, both as a function of time. The contours were then used to calculate the etching kinetics, diffusion coefficient, interaction potential, and self-assembly kinetics of NPs. Another example of applying the object detection models is to achieve real-time detection of early nucleation events during liquid-phase TEM experiments.^{635'} The study focused on observing the NaCl crystallization in liquid cell starting from NaClO₃ dissolved in acetone. Upon beam illumination, the Cl⁻ produced by ClO₃⁻ radiolysis crystallizes with Na⁺. However, due to the uncertainty of where the nucleation event happens, early detection of the nucleation events is important for guiding the high-magnification observation. The YOLOV5 network was trained to detect crystalline objects with a detection time as short as 15-35 ms, which made real-time observation possible through integrating the detection program with the TEM hardware. With the crystal volume estimated by the bounding box size and recorded as a function of time, a

higher growth rate was observed on small particles comparing to large particles, which contradicted with the Gibbs-Thomson effect in the classical nucleation theory. This phenomenon was explained by a nonclassical, two-step nucleation process, where an unknown, amorphous, or dense-liquid phase of NaCl with smaller interfacial energy compared to NaCl crystal was formed at the initial stage of nucleation.

Machine learning can also help sort the trajectory of NPs recorded by liquid-phase TEM, where the particle-substrate interaction, heterogeneity of the liquid cell substrate, and the liquid structure in the liquid cell environment may result in anomalous diffusion of NPs. For such trajectory data arranged as a time series with uncertain duration, one type of neural network models, such as long short-term memory (LSTM), was designed to exploit the correlation from the whole time sequence instead of individual time points, which have been widely demonstrated in the trajectory analysis of col-loids⁶³⁶⁻⁶³⁹ and biomedical molecules⁶⁴⁰⁻⁶⁴⁴ imaged by optical microscopy. Jamali et al.⁶⁴⁵ used MoNet, a neural network, to classify the trajectories of Au NRs into different types of diffusion behaviors. MoNet comprises six parallel 1D convolutional sublayers with different filter sizes and step sizes to capture local dynamics of NPs with different spatial resolutions. It was followed by concatenation and fully connected layers to give the final classification and regression outputs. After being trained on the simulated trajectories with predefined physical models including Brownian motion, fractional Brownian motion, and continuous-time random walk (CTRW), MoNet took the trajectory data extracted from experimental liquid-phase TEM videos as input and revealed a diffusion transition of NPs from fractional Brownian motion to CTRW as the electron beam dose rate increases. This transition was explained by electron-beam-radiolysis induced passivation of the functional groups on the liquid cell substrate, which weakened the attraction between NPs and the substrate and caused detrapping and long-distance jumping of NPs as in the CTRW model.

In Situ STEM of Atomic Structure Dynamics. Similar to NP dynamic observations in liquid-phase TEM, analysis of atomic structural evolutions in in situ STEM has also been challenging due to the lack of automated analysis methods for extracting dynamic information from the high dimensional, enormous amount of data. To address the low efficiency in manual analysis, low-speed, and frequently needed human supervision in existing analysis algorithms, Maksov et al.⁶⁰⁷ trained an FCN model to segment and identify the atoms in in situ STEM movies of the defect structure transformation in a 2D Modoped WS₂ monolayer. Only trained on one annotated frame combined with the image augmentation, their FCN model was able to segment the atomic positions in the rest of 99 frames in a movie at an accuracy of 99% in model evaluation. Benefited from the high throughput and automated segmentation, $\sim 10^4$ defect structures were extracted from the movie and consequently grouped into five classes by a GMM, which were associated with Mo dopant atom, vacancy, and carbon contamination. The trajectories of different types of defects were tracked and compared. It was found that the vacancy defects typically generated shorter trajectories compared with the dopant defects, which might be due to the filling of vacancies by W and S atoms. The diffusion coefficient of vacancy defects was estimated to be in between 3×10^{-4} nm²/ s and 6×10^{-4} nm²/s. On the other hand, the dopant defects

were further grouped into four subclasses determined by different couplings with their neighboring vacancies, and the transition probability of those individual defects over time was studied via the Markov process model. The observation that the Mo dopant defects may couple to a S vacancy in the dynamic STEM experiment can be explained by the lower diffusion barrier of a S vacancy, and the higher probability of S atoms being knocked-out by an electron beam, which led to the occupation of the as-formed S vacancy by the Mo dopant. This work highlights the capability of ML to extract pointdefect dynamics and map chemical transformation pathways in real time at the atomic level.

6.4. Machine Learning Applications in 3D Tomography Reconstruction

6.4.1. Introduction of the Applications of ML to Solve Challenges in Electron Tomography of Soft Materials. As elaborated in Section 3, electron tomography is a powerful method to image directly the 3D structure, morphology, and composition of soft nanomaterials. However, complications in the tilting series acquisition process can result in poor reconstruction quality. Such complications include the tilting angle limitations, SNR, and tilting image alignment.

Tilting Angle Challenge. During the tilting image acquisition, due to the mechanical limitation of the holder and the fact the holder can block the electron beam, the accessible tilting angles usually range from $\pm 60^{\circ 6,95}$ to $\pm 70^{\circ 153}$ which is lower than the ideal tilting range of $\pm 90^{\circ}$ to collect all the information necessary for reconstruction. This missing-wedge effect reduces the resolution and reliability of the reconstruction and can potentially lead to serious misinter-pretation (see Section 3.1.3).⁶⁴⁶ On the other hand, to avoid the electron beam damage to beam-sensitive samples such as polymer membranes⁹⁵ or to samples under EDX tomography that requires high electron dose rates, the number of sinograms in tilting series can be limited, which leads to sparse tilting angle intervals and limits the spatial resolution in reconstruction.⁶⁴⁷

SNR Challenge. Another way of preventing the sample damage is to lower the dose rate or reduce the exposure time during the image acquisition,⁶⁴⁷ but the resulting low dose can lead to sinograms with a low SNR and consequently noisy reconstruction. This problem is more severe for EDX tomography, where the low probability of X-ray generation and low efficiency of detection⁵⁹⁵ makes the SNR lower than other EM tomography techniques.

Alignment Challenge. Moreover, the inevitable projection translations and rotations can be caused by the specimen movement, mechanical instabilities, and optical imprecisions of the imaging system during the tilting series acquisition. Such issues call for accurate tilting series alignment before the reconstruction, which is critical for reconstructing high-quality tomograms.⁶⁴⁸

While experimental solutions to such complications rely on the development of advanced cameras, tilting holders, and imaging protocols, the efforts in designing reconstruction algorithms also show success in improving reconstruction from sinograms with limited SNRs and tilting angles. Iterative reconstruction algorithms without ML such as SIRT,¹⁶⁴ MBIR, and DART⁶⁴⁹ provide better performance compared with the simplest analytic algorithms such as WBP, through comparing the reconstructions with the experimental observations iteratively until the error vanishes and the solution



Figure 46. Application of ML in tomography reconstruction. (a) The training pipeline of the IRDM model to predict information recovered and deartifacted tomogram from WBP reconstructions. (b) IRDM in comparison with conventional methods by "phantom face" reconstruction with 40° missing wedges. The lower panels show enlarged images of the boxed regions in upper panels. (c) The unsupervised tomography reconstruction method. A low-resolution 3D reconstruction is generated first using MBIR. Then a sinogram generation CNN produces EDX projection images across all angles, which are later used by FBP to give the final high-resolution 3D reconstruction. (d) A schematic showing using U-Net for elemental map denoising to decrease the acquisition time and electron dose in EDX tomography of NPs. (e) Comparison of sinogram denoising performance of different methods on simulated 2D EDX elemental maps with noise. The references are noiseless ground truth maps used in the simulation. (f) Comparison of 3D tomography from experimental data denoised using different methods. The references are experimental data taken with long acquisition times and high electron doses. The left column are 3D models of the used core–shell Au/Ag NPs, with the orientation of the depicted orthoslices. Scale bars: 30 nm. (a, b) Adapted with permission from ref 574. Copyright 2020 John Wiley & Sons. (c) Adapted with permission from ref 595. Copyright 2021 The Royal Society of Chemistry.

converges.⁶⁵⁰ However, iterative algorithms may potentially suffer from over smoothing of feature and high computational costs.⁶⁵¹ In recent years, an increasing number of ML reconstruction studies demonstrate their application in transmission X-ray microscopy (TXM), HAADF-STEM, and STEM-EDX tomography of nanomaterials.^{574,595,596,647} During the reconstruction of EM tomography, ML models such as image-to-image CNNs and GAN have been reported to be helpful in (i) detecting the fiducial markers for sinogram alignment,⁶⁴⁸ (ii) preprocessing the sinograms to enhance the SNR⁵⁹⁵ or to generate sinograms at unmeasured angles, ^{149,647} and (iii) postprocessing the tomograms reconstructed via simple algorithms to recover the missing wedge information.⁵⁷⁴ Moreover, although they have not been applied to EM tomography, neural networks with fully connected layers are proven to be capable of the direct end-to-end reconstruction with reduced noises and artifacts in MRI and positron emission tomography (PET) imaging.⁶⁵² In addition to the reconstruction process, the 3D segmentation of tomograms in the presence of missing-wedge effect and noises can also be achieved by image-to-image CNNs.653-655

6.4.2. Machine Learning in Solving the Tilting Angle Challenge. To recover the lost information and remove artifacts in the tomograms reconstructed from limited tilting

angles, a series of ML methods have been developed. Wang et al.⁵⁷⁴ developed the information recovery and deartifact model (IRDM), which is a GAN consisting of U-Net++ as the generator and an image classification CNN as the discriminator (Figure 46a). During training, the deartifacted tomograms generated by U-Net++ from the simple reconstruction (e. g., WBP) were compared to the ground truth tomograms without missing-wedge effect by the discriminator network, and the loss was differentiated and backpropagated through the discriminator to help the generator better deceive the discriminator. This adversarial evolution between the generator and the discriminator finally led to better information recovered and deartifacted tomograms that resemble the ground truth tomographs. The trained IRDM was then tested on tomograms with missing wedge angles from 40° to 80° . Compared with other reconstruction algorithms including WBP, SART, and TVM, where either blurriness or elongation in the vertical direction can occur in the final reconstruction, IRDM can consistently make predictions nearly identical to the ground truth at all missing wedge angles (Figure 46b). The trained IRDM was applied to the HAADF-STEM atom tomography of a Au nanocrystal with a tilting angle range of $\pm 70^{\circ}$, showing the reduced missing wedge artifacts and noise in the reconstruction compared to WBP results. As a result, the

higher order Bragg spots in the power spectrum of the reconstruction were resolved by the IRDM algorithm, achieving a 3D atomic resolution as high as 0.71 Å. This work highlighted the power of ML in solving the missing-wedge challenge in 3D atomic tomography of nanomaterials. However, because the ML model used in the work was implemented in 2D, each 2D slice along the tilting axis in the 3D reconstruction has to be processed separately, and the information in the third dimension was not fully exploited. Meanwhile, due to the nature of supervised learning, the model performance also depends on the selection of features in the training data set.

Other than refining the simple reconstructions with generative learning, GAN models can also directly recover the missed angles in the sinogram, which avoids the additional artifacts introduced through the reconstruction of tilting series with missed angles. Ding et al.¹⁴⁹ developed a two-step ML algorithm to solve the missing-wedge problem in electron tomography. In this work, an image inpainting GAN model first filled the sinogram at missed angles, followed by another deartifact GAN model to further remove the residual artifacts in the tomogram reconstructed from the filled sinogram. With being trained on hybrid datasets comprised of simulated images of random polygons and images acquired from open databases, their model showed a superior peak SNR, structural similarity index, and perceptual index compared to existing reconstruction algorithms including WBP, SART, and TVM. The method was also tested on the experimental EM data of Au NRs and layered cathode materials that are not presented in the training dataset,¹⁴⁹ both showing greater performance comparing with conventional methods. Such a sinogram inpainting method offers a new angle to solve the tilting angle limitations in electron tomography. More recently, Han et al.⁶⁴⁷ used an unsupervised ML

workflow to analyze the tomography tilt series of NPs with a limited tilting angle range and sparse tilting interval. In this workflow, MBIR was first used to build a low-resolution reconstruction from the noisy and sparse sinograms experimentally obtained (Figure 46c). This noisy reconstruction was then reprojected to generate sinograms, which were later processed by a CNN with the U-Net type architecture to fill the missed and under-sampled tilting angles (Figure 46c). The optimization of CNNs was done by imposing the consistency between the original and enhanced sinograms, thus only the experimental sinogram but no matching ground truth was needed. In the final step, the sinograms recovered by the CNN were reconstructed by a simple algorithm (WBP) to give the tomogram with a significantly higher resolution than the original one. The workflow was successfully applied to reconstruct the STEM-HAADF and EDX elemental map tilting series of Au NPs and InP/ZnSe/ZnS quantum dots at a tilt range of $\pm 60^{\circ}$ and an angular interval of 10° . High-quality 3D reconstructions of the geometry and elemental distribution of quantum dots were obtained, which revealed the absence of S in specific areas in quantum dots that was not identified from the conventional 2D image analysis. The absence of S indicates the nongrowth of ZnS shell on the quantum dots, which was later identified as the cause of lower photoluminescent quantum yield and is correlated with different synthesis conditions. This unsupervised ML, combined with STEM-EDX tomography, shows its great potential as a comprehensive tool for physicochemical analysis of soft nanomaterials.

6.4.3. Machine Learning in Solving the SNR Challenge. The low SNR in sinograms of soft materials can be preprocessed to ensure a clean reconstruction process and reduce noise presented in the tomography reconstruction. To this end, a recent work from Skorikov et al.⁵⁹⁵ reported a U-Net model for elemental map denoising before the tomography reconstruction of STEM-EDX tilt series of NPs. The U-Net model was trained by a series of simulated STEM-EDX tilting projections of various-shaped NPs with randomizable geometric parameters and artificial noises (Figure 46d). Compared with conventional, non-ML-based denoising algorithms, the trained model behaved better in recovery of sharp NP boundaries and low intensity variations within the particle from the noisy sinograms (Figure 46e). Inputting the U-Net denoised sinograms of core-shell Au/Ag NPs with different shapes for reconstruction gave clear particle shape and elemental distribution, in contrast to the tomograms with fracturing and blurring reconstructed from the sinograms denoised by non-ML-based methods (Figure 46f). The utilization of U-Net to increase the sinogram SNR not only produces more pronounced 3D elemental map of NPs but also potentially allows a reduction in EDX acquisition time and electron dose, which can benefit the studies of beam-sensitive nanomaterials.

6.4.4. Machine Learning for Tilting Series Alignment **Challenge.** As mentioned in Section 3.1.2, the fiducial marker is one of the most frequently used methods for tilting image alignment in electron tomography. During the detection of fiducial markers, the common strategies rely on the template matching between an averaged or synthetic fiducial marker image and the patches in the image to be detected. However, the dark regions, size variation of the marker particle, and low SNR at high tilting angles could all contribute to the challenge in fiducial marker detection under various imaging conditions. To solve these problems, Hao et al.⁶⁴⁸ introduced a CNNbased image classification model to help the marker detection process in electron tomography. In the workflow, the candidate marker regions were first generated by a conventional method, followed by a CNN performing the binary classification to keep the successful detections of the markers and discard the failed ones. Trained on the experimental tilting images consisting of positive fiducial marker image patches and negative background patches, the model can overcome the marker overlaying, blurriness, and low contrast issues commonly occurred at high tilting angles, identifying more fiducial markers with longer trajectories comparing with the conventional method. Such advantages were demonstrated with the EM tilting series of protein samples. This work shows the contribution of ML to the image alignment, which is a critical step before the reconstruction in electron tomography.

6.4.5. Machine Learning for Post-Reconstruction Tomogram Segmentation. While the previous sections focus on the application of ML to facilitate reconstruction of tomographs, in this section, we discuss postreconstruction tomogram segmentation. Like 2D EM images (Section 6.2), quantitative analysis of electron tomograms for nanomorphology characterization (e.g., the measurement of volume, surface curvature, roughness) requires image segmentation to binarize the materials out of the background based on grayscale tomography reconstructions. Such segmentation is often conducted by thresholding the reconstructed voxels, which suffers from the unavoidable reconstruction artifacts including the noises, imperfect alignment, and missing-wedge effect.

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Figure 47. Application of unsupervised ML in nanomaterials shape classification. (a) AutoDetect-mNP unsupervised classification result of a Au NR mixture with different aspect ratios. The optimal class number is first determined by the maximum entropy. The exampled particle shapes from each class produced by AutoDetect-mNP are shown together with the original TEM images. The histogram of four features used for classification are shown and denoted by colors corresponding to their shape classes. (b) Eight shape descriptors describing the 3D morphology of crumples on the PA membrane are dimension reduced and projected onto the space of the first two principal components. (c) Distribution of the crumples in the PC space and the GMM clustering results. Color represents the grouping given by the GMM. The colored regions are confidence ellipsoids of the fractions of the surface element descriptors. (e) Measurement of the fingerprint function $d(\theta)$ to describe the shape of Au tetrahedral NPs. (f) Full set of $d(\theta)$ collected from all Au tetrahedral NPs from TEM images. Different sizes, and the gray $d(\theta)$ stands for impurities. (g) Full set of $d(\theta)$ projected onto their first two principal components. Insets show the average NP shape of the classes identified by the GMM. (h) Average edge length (triangular symbols) and truncation (circular symbols) as functions of seed amount. (a) Adapted with permission from ref 583. Copyright 2021 American Chemical Society. (b–d) Adapted with permission from ref 6. Copyright 2022 AAAS. (e–h) Adapted with permission from ref 5. Copyright 2022 The Royal Society of Chemistry.

Alternatively, segmentation can be done manually to better accommodate those artifacts. However, the manual segmentation of 3D images is extremely time-consuming and inconsistent for different operators. For robustness against the artifacts while keeping the high efficiency, the Trainable Weka Segmentation plugin in ImageJ was utilized to optimize the conventional segmentation parameters in a ML approach to achieve the multiclass segmentation of electron tomogram slice-by-slice.⁶⁵⁵ The approach was applied to the reconstructed electron tomography of a composite of silica NPs embedded in a rubber matrix. Compared to the thresholdingbased segmentation with or without additional filtering, the silica NPs were distinguished more clearly with their positions inside the rubber matrix better resolved. This work highlighted the capability of ML segmentation in the presence of reconstruction artifacts through ImageJ in a coding-free manner. More recently, the development of image-to-image CNNs with 3D architectures paves the way to ML-based segmentation of 3D images,⁶⁵⁶ which resembles the 2D imageto-image CNNs (such as U-Net) but with input, output, and convolution kernels shaped in 3D. Such methods have been applied to electron tomography data, showing their excellent

performance in segmenting the cryo-electron tomograms with high noise and a missing-wedge effect.^{653,654} For example, a 3D CNN-based platform, named DeepFinder, was reported for multiclass macromolecule identification from cryo-electron tomograms.⁶⁵⁴ DeepFinder predicted the macromolecule categories voxel-wisely and then utilized them to determine the location of macromolecular particles in the tomogram, which outperformed the conventional template matching and other deep learning methods on synthetic datasets. When applied to the experimental datasets, DeepFinder successfully distinguished and located ribosomes with two different states including the membrane-bound 80S ribosome and the cytosolic ribosome, at a 3D structural resolution of 24 Å, which is comparable to that of expert annotations at 23 Å. DeepFinder also showed similar performance on macromolecules with smaller sizes such as Rubisco and photosystem II at resolutions of 15 Å. Moreover, in this work, the voxel-wise annotation labels used for training were converted from the expert labeled 3D coordinates of the macromolecules, in order to avoid the time-consuming step of directly generating the voxel-wise ground truth by manual selection. This makes it a

promising algorithm for the efficient semiautomated analysis of the cryo-ET data.

6.5. Unsupervised Learning for Grouping of Features (e.g., Morphology, Size, Composition) in Analyzing EM Images and Electron Diffractions

While classification, segmentation, and object detection are mostly used for extracting information from TEM, SEM, and STEM images, unsupervised learning has been applied to analyzing the features extracted from the real space images and electron diffraction patterns. For real-space images, such analysis focuses on the clustering (or grouping) of morphology (e.g., size, shape, structure). For electron diffraction, such analysis is about clustering the electron diffraction data into different phases (crystallinity, phase orientations, etc.).

6.5.1. Morphology Grouping of NPs and Polymers in Real Space. To gain a comprehensive understanding of nanomaterial morphology and build the linkage between synthesis conditions and macroscopic material properties (e.g., optical, electronic, and catalytic properties) by EM characterization, morphology grouping and quantitative analysis are needed. Such quantitative analysis usually requires a big dataset of samples to be statistically significant. For example, a recent study on Au NR shows that at least 1,500 individual particle contours collected from TEM images are necessary to predict the UV-vis extinction spectra that match with the experimental measurement.⁵⁸¹ Although supervised learning algorithms introduced in Section 6.2 are capable of accelerating and automating the morphology extraction from EM images, postcharacterization of these morphological parameters often relies on direct averaging, which smears out heterogeneity, if any, in the samples.

To address this issue, morphology classification by unsupervised clustering algorithms prior to any averaging has been recently employed for heterogeneous nanomaterials. Such a clustering algorithm usually starts from converting the nanoscopic features (e.g., shape contours) to appropriate descriptors, based on the distribution of those nanoscopic features in the descriptor space, the features can be divided into relatively homogeneous groups by the clustering algorithm. One example is the AutoDetect-mNP algorithm developed by Wang et al. to group Au NP mixtures (including Au NR with different aspect ratio and Au triangular prism) into different shapes from TEM images.⁵⁸³ In this work, four shape descriptors including area, eccentricity, aspect ratio, and circularity of segmented NP projections were first calculated, followed by a k-means algorithm for clustering (Figure 47a). The results showed that AutoDetect-mNP reliably distinguished Au NRs or triangular prisms from impurities and further identified subgroups of Au NRs with different aspect ratios or triangular prisms of corner truncations without human intervention. In another work by Lee et al.,⁵⁸¹ a twostep method was used to group a Au NP shape mixture. A center-to-perimeter distance first distinguished the particles with obvious geometries such as square, sphere, and rod. Then in the second step, three descriptors (major axis length, minor axis length, and solidity) served as the input of a GMM classifier to further divide the spheres and rods into subgroups with varying shape details, which was later used for estimating their optical properties as stated in Section 6.2.3. A similar report by Slater et al.582 utilized several different clustering algorithms including the k-means, DBSCAN, and ordering points to identify the clustering structure (OPTICS) to group

the TEM projections of Au, Pd, and PtNi NPs for reconstruction to reveal their 3D geometries. In this work, the solidity, maximum pixel intensity, and the area of individual particles were extracted from the TEM images as the clustering input. Finally, the projections identified as the same groups showed sufficient homogeneity in size and shape, providing the high-fidelity single-particle reconstruction.

Unsupervised morphology clustering also applies to 3D morphologies of polymeric materials. For example, An et al. reported the utilization of GMM to classify the 3D crumple structures on the PA filtration membrane,⁶ which are formed following Turing's pattern during interfacial polymerization. The individual volumetric crumple images were first binarized and cropped from the reconstructed electron tomogram. Eight surface curvature-related descriptors were measured and collected from the 3D geometries for each crumple instance. The descriptors were then dimension reduced via PCA and classified into three groups by GMM (Figure 47b,c), including shapes resembling a dome, a dimple, and a pancake (Figure 47d). The pervasiveness of the grouping as well as the high similarity within each group can be consistently confirmed across the PA synthesized in different conditions. On the other hand, the fractions of each group in different synthesis conditions are found to be changing with the monomer concentrations. For example, the dome-like crumples with smaller size occur more frequently with increasing monomer ratio, highlighting the synthetic control over morphology. Moreover, the distinctive mechanical response of different morphological groups was confirmed by liquid-phase AFM, showing the one-to-one relationship between the material propriety and morphology for each group.

In general, unsupervised nanomorphology grouping demands no training dataset but might require the input of the number of clusters (groups), especially in k-means and GMM. Different numbers of clusters can lead to different clustering results. Research has shown that the optimal number of clusters, which gives the best data point separation, can be evaluated and determined by metrics such as maximum entropy⁵⁸³ and Bayesian information criterion⁵⁸¹ to further decrease the level of human intervention. Moreover, the input morphological parameters tend to be a system-specific choice. For example, the descriptors that researchers have chosen to representing NP shapes in TEM images include solidity, convexity, area, eccentricity, aspect ratio, circularity, major/ minor axis length, and even the maximum pixel intensity in the projection area. While those descriptors, each emphasizing a certain aspect of the nanomorphology, lead to different classification results, they can sometimes be correlated, which causes problems in clustering. Some studies utilize more universal descriptors such as the NP contour data with dimension reduction as input to group algorithms,⁶⁵⁷ which avoids the manual descriptor selection and can be widely applied to different shapes. For example, the center-toperimeter distance as a function of angle can be used as a fingerprint describing the nanomorphology and serve as inputs of dimension reduction and clustering algorithms (Figure 47e,f).⁵ With the ability to prevent clustering discrepancies introduced by the choice of descriptors, this method has been proven to be compatible with nanomaterials with various shapes, in both 2D and 3D. Demonstrated on Au tetrahedral NPs, Au nanoprisms decorated with polymer patches, and crumples on the PA filtration membrane, it is shown that the dimension-reduced fingerprint functions can directly visualize



Figure 48. Application of unsupervised ML in SPED diffraction pattern classification of a GaAs nanowire with a twin boundary at an oblique angle to the beam. (a) Visualization of the first three SVD component, first three ICA component, and two NMF component patterns corresponding to the crystal orientations. The slim images on the right parts visualize the loading of the corresponding components in the real space. In SVD and ICA, intensities in red and blue indicate positive and negative values, respectively. Pattern and loading scale bars are common to all subfigures and measure 1 Å⁻¹ and 150 nm, respectively. (b) 2D projection of three component SVD loadings onto the plane of the second and third loading with cluster membership predicted by the fuzzy *c*-means as contours. (c) The quantitative diffraction pattern unmixing in the real space by VDF images formed from manually selected diffraction spots, NMF, and fuzzy *c*-means clustering. The real space images and the line scan profiles show the spatial distribution and fraction of structures corresponding to diffraction patterns highlighted by the same colors. The blue color represents the background component in NMF and fuzzy *c*-means. Pattern and loading scale bars are common to all subfigures and measure 1 Å⁻¹ and 70 nm, respectively. Adapted with permission from ref 660. Copyright 2019 Springer Nature.

the morphology diversity in the principal component space (Figure 47g). When combined with GMM, this method could further distinguish the major components from the heterogeneous nanomaterial sample with different sizes and shapes (Figure 47g). Via clustering, shape details of interest such as the edge length and the corner truncation of Au tetrahedral NPs were measured, where the decreasing edge length was correlated with the increasing seed concentration used in the synthesis (Figure 47h). Aside from metal NPs, the method was also extended to characterize the polymer patches on Au nanoprisms and 3D PA membrane crumples. Asymmetric polymer patch expansion was observed with increasing ligand concentration in the Au nanoprism system.

6.5.2. Diffraction Pattern Classification for 4D-STEM. As elaborated on in Section 4, electron diffraction is a powerful tool to resolve the local orientation, crystallography, and strain in heterogeneous nanomaterials. In such techniques, diffraction patterns in the 2D reciprocal space can be recorded at different locations in the 2D real space, thus producing data in four dimensions named as 4D-STEM (Section 4.1).⁶⁵⁸ 4D-STEM data are large in volume and thus demand high throughput algorithms to analyze, visualize, and extract useful information from them.⁶⁵⁹ On the other hand, in heterogeneous materials, the number of grains with distinct crystalline structures or orientations in the scanned area are much smaller than the number of diffraction patterns scanned by the 4D-STEM, resulting in data redundancy. 660 Such data redundancy can be exploited by unsupervised learning such as matrix decomposition and clustering algorithms to automate and accelerate the crystal structure mapping in 4D-STEM data analysis.

The Matrix Decomposition and Dimension Reduction of Diffraction Patterns. In 4D-STEM analysis, matrix decomposition methods can be used to group diffraction patterns corresponding to different spatial structural domains and to unmix the signals in the same diffraction pattern coming from multiple grains along the beam path.⁶⁶⁰ Taking PCA decomposition as an example, the 4D dataset containing the 2D diffraction patterns at different locations in the real space are first reshaped to a $M \times N$ matrix, where M corresponds to each observation in the real space and N corresponds to each

location in the diffraction pattern. Then through PCA, each point in the diffraction pattern will be transformed to N linearly uncorrelated principal components to maximize the data variance. Usually, the first few principal components contain the most variance among all diffraction patterns and thus capture the most information from the original data.⁶⁵⁹ While PCA allows the identification of structures/phases/ orientations with different diffraction patterns in the material, the physical interpretation of each principal component with respect to the original diffraction pattern is still challenging due to the positive and negative values resulting from the orthogonal factorization constraint.^{658–661} To this end, other alternative decomposition/dimension reduction algorithms such as ICA and nonnegative matrix factorization (NMF) can be used. Compared to PCA, which maximizes the data variance in the reduced dimension, ICA maximizes the independence of resulting components. Similarly, in the NMF decomposition, both the components and the resulting decomposed coefficients are restricted to be positive, which can allow easier physical interpretation.⁶⁶¹ A comparison between singular value decomposition (SVD, similar to PCA), ICA, and NMF decomposition can be found in a recent study by Martineau et al.,660 where the 4D-scanning PED data of GaAs nanowires were analyzed by those unsupervised learning algorithms. The top components (i.e., the more important components) identified by three methods as well as their distribution in the real space are plotted in Figure 48a. Both the SVD and ICA were able to tell the difference between the diffraction patterns from the two twined crystal orientations in the nanowire as well as the vacuum background. However, due to the existence of negative values in the components of SVD and ICA, none of their component patterns could be directly associated with the crystal structures. In contrast, benefited from the nonnegative constrains, the top three components obtained from NMF well corresponded to the two twinned crystal orientations and the background (Figure 48a). Similar phenomenon was also observed in the grain classification of Au-Pd NPs⁶⁵⁸ and crystalline structure studies of TiO₂,⁶⁶² where the classifications of 4D-STEM diffraction patterns produced by PCA and NMF were compared. Unlike the PCA,

the NMF results represented real diffraction patterns containing positive peaks only, highlighting its capability of identifying discrete components with less tendency to capture mixed phases. It should be noted that NMF also requires the more expensive computation and higher sensitivity to variations in the input data.⁶⁵⁸

Structure Classification with Unsupervised Clustering Algorithms. Although the diffraction decomposition methods could potentially produce patterns regraded to be associated with the physical crystalline structures/orientations, they still simply decompose the patterns into statistically significant functions and associated weightings.⁶⁶¹ Other than directly yielding the structure classification results, matrix decomposition methods such as PCA and NMF can be used as a reliable dimension reduction tool to provide inputs to unsupervised clustering algorithms. To cluster the diffraction patterns with unsupervised classification, the patterns can be regarded as a high-dimensional feature vector with each element corresponding to a point in the reciprocal space. However, due to the curse of dimensionality, the direct clustering of such high-dimensional (usually larger than 10) data is unreliable.⁶⁶⁰ To address this issue, the patterns can first be transformed into the top components produced by the matrix decomposition methods to greatly decrease the dimensionality while maintaining the most information.⁶⁵⁹ In one example,⁶⁶⁰ the diffraction data dimension reduced by the SVD were used as the input to a fuzzy c-means algorithm to differentiate different crystalline orientations in GaAs nanowires. Scattering plots representing diffraction patterns in the reduced 2D space of SVD showed three naturally formed clusters, corresponding to the three different types of diffraction patterns coming from the two crystalline domains and the background (Figure 48b). The membership of each diffraction pattern can then be assigned by the fuzzy c-means and mapped in the real space to reveal the spatial distribution of each crystalline domain. In this work, the quantitative unmixing of the diffraction patterns corresponding to two crystalline orientations was also studied. In the region of mixed diffraction patterns, the loading of the NMF and the membership predicted by fuzzy c-means were found to be quantitatively correlated with the VDF image reconstructed from the diffraction spots uniquely coming from two crystalline domains, where the loading, membership, or the VDF intensity of one domain decreased as a line profile was scanned from the domain to another (Figure 48c). This indicated both unsupervised learning algorithms were reliable and more automated for the diffraction pattern unmixing. The similar classification approach has also been applied to the ptychography data analysis.⁶⁵⁹ In one example, PCA followed by k-means was leveraged to classify CBED patterns of strained polymorph bismuth ferrite thin film. More than 30,000 diffraction patterns with size of 96 \times 96 taken at different locations were dimension-reduced by PCA to keep the top 300 principal components suggested by the inflection point in the scree plot. The k-means was then used to classify the dimension-reduced patterns into 36 clusters, which visualized the variability of the ptychographic dataset on the atomic level, and effectively sorts the scattering information at an atomic resolution.

7. OUTLOOK

We see great opportunities at the intersection of the rapidly growing fields of EM and soft materials and below we outline the directions we see as particularly promising from two aspects: what are the systems of soft materials that EM techniques have not yet been extensively used, and how to integrate various EM modes to generate comprehensive structure, morphology, and chemical information.

7.1. Opportunities in Real-Space 2D and 3D Imaging by EM

7.1.1. Extending EELS and EDX to Chemical Imaging of the Compositional and Chemical Heterogeneity of Polymeric, Carbonaceous, and Biological Samples. As nanoscale analytical probes, EDX and EELS have been extensively used to characterize the composition of hybrid NPs (Section 2.3.1) as well as the chemical valency transformation and vibrational spectroscopy (Section 2.3.2) of colloidal NPs. Instruments of high energy resolutions can now be used to map the spatial distributions of adsorbates (such as metal ions filtered by PA membranes),⁹⁵ and to track (electro)chemical reaction pathways on the surface and within a NP, which is associated with not only composition but also valency change.⁶⁶³ If this capability is extended to polymeric, carbonaceous, and biological samples, more molecular insights will be obtained at a spatial resolution not accessible to the traditional analytical methods (NMR, ICP, etc.). For example, in understanding complicated assembly structures of block copolymers, EDX and EELS can circumvent the use of selective staining to distinguish blocks and instead directly detect the chemical identity of the dominant species in a spatially heterogeneous sample. Similarly, for conjugated polymers and composite carbonaceous materials widely used for optoelectronics, solar cells, transistors, and flexible electronics, mapping valency changes during electrochemical reactions or locations of "dopants" or "fillers" (often added to enhance electrical and mechanical properties) using EDX and EELS can revolutionize the modeling and understanding of such processes.

To realize these experiments, several aspects of EELS and EDX need improvements. The foremost is the dosage, which is harder to control than in the conventional direct imaging mode of (S)TEM. Dosage is coupled with the sensitivity of the detector, i.e., how many molecules need to be present to generate enough signal in EELS and EDX. Highly sensitive detectors are being used, enabling detection of small molecules and other beam-sensitive materials. In a recent work, through averaging and using an aberration-corrected STEM, the subtle difference of CTAB packing density on Au NR tips and sides was revealed.⁶⁶⁴ It should be noted that under beam illumination, some elements may easily be kicked out of the sample, such as N and S, the sensitivity of which can be characterized by a dose threshold.⁶⁶⁵ Collecting enough signals of these elements without damage is still challenging. Further developments in EDX and EELS are expected to push the limits of signal collection at low dose levels and enhanced speed, as well as higher energy resolution and lower detection limit (enabling trace element detection at a concentration even <0.02 wt %).

7.1.2. Fast Tomography for Lowering Beam Damage and In Situ Imaging. Fast data acquisition of tomography is another highly sought-after frontier of research, both for the purpose of lowering total accumulated dose for beam sensitive samples (e.g., polymeric, carbonaceous, biological samples) and for incorporation with in situ imaging to record dynamic processes. For polymeric materials, empirically, highly cross-



Figure 49. (a) 4D-STEM tomography of Ni-based superalloy (*fcc* matrix γ -phase, orange) containing the faceted metal carbide (blue) and the hexagonal η -phase precipitate (green). (b–d) 4D-STEM tomography of the TiN sample containing grains of different crystallographic orientations. The reconstructed grains are shown for the (b) side view, (c) front view, and (d) top view. (e) illustration of the orientations of the seven grains shown in panels b–d. Each cube is labeled with the color corresponding to the grain in panels b–d. (a) Reprinted with permission from ref 666. Copyright 2015 Springer Nature. (b–d) Reprinted with permission from ref 666. Copyright 2016 International Union of Crystallography.

linked materials (e.g., PA membranes) have been shown to be decently beam-stable to obtain tomography tilt series. We expect conjugated polymers which are good to conduct charges to have similar robustness. Cryogenic temperatures have also been shown to keep biological samples stable for cryo tomography. As noted in Section 3.2.3, for low contrast samples, staining could be used for tomography, but might cause structural changes to the sample and fixate the structure to hinder dynamics,^{186,187} except for special instances such as beam-induced staining on liposomes in liquid-phase TEM.¹⁸⁸ In comparison, STEM-based tomography methods, where the contrast depends on the chemical differences of the phases, can potentially eliminate the need for staining and are sometimes more suited for tomography of dynamic systems.¹⁸⁷

For in situ imaging, besides the Brownian one particle method, tomography studies are very limited since most dynamic processes of nanoscale systems occur on the time scale of seconds and minutes, much shorter than the typical time needed to acquire tilt series. Moreover, for liquid-phase TEM studies using customized holders, the SiN_x membrane design currently allows only for a maximum of $\pm 30^{\circ}$ tilt angle range.⁸ Thus, even for liquid samples that are largely stationary and hydrated such as the virus imaging by Kelly and coworkers, only a coarse sinogram set was obtained to qualitatively identify some structural features but not the full 3D reconstruction. For these issues, liquid cells with large-sized windows such as graphene liquid wraps can be superior to allowing for large tilt angle ranges.

Fast tomography is being pushed both with advanced hardware for automated data acquisition and ML-aided reconstruction algorithms. For example, as discussed in Section 3.1.2, a continuous rotation and recording method was introduced with the ability to capture a typical tilt range ($\sim \pm 70^{\circ}$) in less than 10 s.¹³⁵ Therefore, mechanical precalibration and image analysis methods are required, such as U-Net-based sinogram denoising that can lower the acquisition time⁵⁹⁵ and GAN-based missing sinogram generation that can tolerate a narrow tilting range and sparse tilting intervals.^{149,647}

7.1.3. Tomography Integrated with EDX, EELS, and 4D-STEM. Coupling electron tomography with the advanced and fast-developing modules of EDX, EELS, and 4D-STEM will be an exciting direction to pursue. We envision that EDX tomography progresses to realize quantitative 3D element mapping, while EELS tomography can provide 3D maps of properties from optical to valence states of NPs and organic materials, with nanometer resolution. For EDX and EELS

tomography which requires high beam intensity, reducing the time for tomography data acquisition will be beneficial in minimizing the radiation damage. It requires not only the fast imaging during tilting but also fast signal collection from EDX and EELS, which largely relies on the development of more sensitive detectors. Besides, ML is expected to play a significant role in EDX and EELS tomography, which may realize accelerated processing of large datasets, denoising of elemental maps, and analysis of 3D plasmon maps.²¹⁵

4D-STEM tomography, on the other hand, can potentially map the morphology and orientations of internal structures in 3D. 4D-STEM tomography has been demonstrated for mapping 3D grain boundaries and orientations of precipitates in the superalloy (Figure 49a)⁶⁶¹ and the nanostructures in the TiN sample (Figure 49b-d).⁶⁶⁶ The results provide the detailed knowledge of the microstructures in the polycrystal-line materials. We envision that the extension of 4D-STEM tomography to soft materials (where lowered beam damage and shorter acquisiton time are desired) offers a potential means to study strains, defects, molecular packing, and various types of short- and long-range structural order (and disorders) during the processing and functioning of the materials.

7.2. Opportunities in In Situ (S)TEM

7.2.1. Toward Higher Temporal and Spatial Resolution Imaging. Achieving a high spatial resolution by usage of aberration correction and a high temporal resolution in in situ EM imaging is always the goal. A high temporal resolution is important to match with the time scale of the dynamic process of interest and to minimize motion blurring (thus also improve the spatial resolution). Current state-of-the-art direct electron detector pushes the capturing speed to 1,500 full frames per second (fps)-3.75 times the speed of the K2IS camera.¹⁵⁸ Yet it should be noted that even with this detector, it does not match with the time scales of most of molecular reactions (e.g., stretching and breakage of bonds), biomolecular transformations, and even the Brownian motions of NPs of a few nanometers in size (about 10⁸ nm²/s diffusion coefficient per the Stokes-Einstein relation). To resolve those ultrafast reaction dynamics, one possibility is to integrate ultrafast electron microscopy, a technique that uses pulse laser to bring subpicosecond time scale into reach, with in situ EM imaging.⁶⁶⁷ Meanwhile, MD simulation is complementary with the experimental in situ movies in terms of time scales. The former resolves nanosecond dynamics and is limited to an observation window up to μ s, while the in situ movies resolve



Figure 50. (a-f) Time-lapse SEM images showing (a-c) lithium plating and (d-f) stripping on the Li/Cu electrode. The artificial colors from purple to green, yellow, and red represent the contrasts from bright to dark in grayscale. The purple (bright contrasts) indicates Li/Cu electrode and the charged area, and the red and yellow (dark contrasts) indicate lithium dendrites. Scale bar: 20 μ m. (g) Schematic diagram presenting the high-voltage STEM connected to a quadrupole mass spectrometer. OL PP, TMP, and Scroll P represent the objective lens pole piece, turbo-molecular pump, and scroll pump, respectively. (a-f) Reprinted with permission from ref 676. Copyright 2017 Wiley-VCH. (g) Reprinted with permission from ref 450. Copyright 2019 Oxford University Press.

the millisecond dynamics and are only limited in the observation window by accumulated dose.

7.2.2. Understanding and Manipulating the Electron Beam Effects in Liquid-Phase EM. Regarding liquid-phase TEM, the influence and elimination of the beam effect is an unavoidable topic, where pursuit of observing samples and dynamics in a "native" environment and avoiding misinterpretation of artifactual data always exists. In most liquid-phase TEM experiments on organic and biological samples, a low electron dose rate (generally, $0.1-5 \text{ e}^- \text{Å}^{-2} \text{ s}^{-1}$) or single snapshot imaging with the beam turned off between acquisitions was used to minimize the influence of electron beam. Radiolysis-resistant solvents may also be applied to reduce the damage on biomaterials, such as D_2O and alcohols.⁵⁰⁴

The bottleneck, however, is that the understanding of beam effects in liquid-phase TEM remains largely empirical. The theoretical calculations based on radiation reaction networks in water published in 2014 are still the only quantitative model of the radiolysis process.⁶⁶⁸ It does not necessarily apply to systems that, for example, have more redox species than water or contain organic solvents which have become increasingly
relevant as electrolytes for electrochemical systems (e.g., batteries, fuel cells, catalysis) and as dispersant for polymer systems in painting and self-assembly. It is also not clear how to account for the limited volume of the liquid cells (especially in graphene wraps) which might lead to exhaustion of reaction species and pose mass transport limits, or how to consider the effects of a scanning and dwelling beam (used in various modes of STEM, such as HAADF, EELS, EDX, and 4D-STEM). Thus, we foresee a fundamental leap in understanding radiation effects in liquid-phase EM will occur if one could measure experimentally the concentrations of the molecular, radical, and ionic species under beam illumination. Such measurements are currently challenging due to the ultrasmall volume of liquid cells ($<\mu$ L) and the fact that the products are only present under an electron beam. One potential experiment would be to do the beam illumination for bulk liquid samples that are compatible with analytical methods such as NMR or mass spectrometry outside EM and perform the measurement in situ. These steady-state radiolysis product concentrations can be inputted into theories to establish a comprehensive understanding of radiolysis.

We see such experiment—theory understanding of beam effects to be crucial in pushing the next-level of liquid-phase EM. For example, reconciling ex situ electrochemical performance (e.g., cyclability, CV curves, capacity) with in situ TEM characterizations has been the focus of research attention. Low-dose conditions and control experiments (e.g., comparison between areas exposed or away from the beam) are usually required to minimize or clarify potential beam damage. More discussions and perspectives on the application of liquid-phase TEM in electrocatalysis can be found in a recent review by Yang et al.⁶⁶⁹

7.2.3. Incorporation with Analytical Methods. We expect more efforts devoted to the integration of in situ EM with EDX,⁶⁷⁰ EFTEM,⁶⁷¹ EELS, 4D-STEM, and electron tomography,⁶⁷² which are still in their infancy. The integration with tomography has been discussed in Section 7.1.2, and here we focus on the analytic methods. High electron energy and a slow scanning speed are usually required for these analytical methods which can lead to conformational, structural, and chemical alteration in nanomaterials. Controlled beam damage and fast scanning will be the key issue to tackle.^{48,188} Moreover, the existence of liquid can contribute greatly to background noises in EDX, EELS, and 4D-STEM measurements. Tricks to thin the liquid thickness, such as generating a bubble,^{499,673,674} inducing partial solvent evaporation,⁶⁷⁵ or utilizing new liquid cells that are ultrathin,⁴¹⁹ can all be possible to lower the background noise from the liquid and allow measurement of the sample. These tricks are still limited though in the sense that a thin liquid (thus a confined space or limited solvent supply) is needed to make the measurement possible. Alternative strategies are called for.

Obtaining chemical information can also be achieved at a decently high resolution using SEM and ETEM. For example, given SEM's compatibility with surface characterization and large/thick samples, wider application of liquid-phase SEM is expected, such as tracking the 3D assembly process of NPs, characterizing reactions at the electrode–electrolyte interface (Figure 50a–f),⁶⁷⁶ and resolving the dynamic structures of proteins and living cells,⁵⁴⁰ among others. In particular, considering the large size of protein assemblies and eukaryotic cells, liquid-phase SEM equipped with analytical capability might serve as a complementary approach with TEM. By

labeling with Au NPs or fluorescent quantum dots and binding them to features of interest, the spatial position, density, and arrangement of these electron-dense labels can provide significant insights into the corresponding positions and functions of the targeted intracellular structures^{676,677} and resolving the dynamic structures of proteins and living cells.⁵⁴⁰ Similarly, a recent study reported the connection of mass spectrometry to ETEM (Figure 50g), realizing the real-time detection of gas species from oxidation of CNTs.⁴⁵⁰ Such design may open more opportunities to the deeper investigation into reactions such as alcohol oxidation and the Fischer–Tropsch process, and the combination of mass spectrometry (and even NMR, high-performance liquid chromatography) with liquid-phase EM can be expected.

7.3. Opportunities in Electron Diffraction and Soft Materials

There have been amazing breakthroughs of electron diffraction techniques on how the beam is generated, how patterns are collected, and how data are analyzed over the past decade, as we discussed in the long list of MicroED, 4D-STEM, ptychography, and so on. Here we discuss two particularly inspirational directions for future research of utilizing electron diffraction for soft materials.

7.3.1. ML-Guided Dose Efficient Acquisition and Ptychography for Soft Macromolecular Systems. The use of high-resolution STEM imaging, ptychography, and tomography is limited for beam-sensitive soft materials due to radiation damage. Recently, compressive sensing provides a promising method to tackle the radiation challenge with reduced number of scan pixels in STEM. Compressive sensing has been successfully demonstrated on a variety of beamsensitive samples, including zeolites and organic/inorganic core-shell nanowires.^{678,679} It was shown that by reducing the sample pixels down to 5% of the original image, the original image could still be recovered from the reduced data set via computational algorithms.^{678,680} With the assistance of ML, the electron beam coverage could be further reduced by 87 times.⁶⁸¹ When integrating compressive sensing with ptychography, the acquisition time was reduced by 100 times,⁶¹ increasing the competency of STEM-ptychography for imaging dose-fragile materials. In tomography, compressive sensing and reconstruction codes were shown to retrieve reconstruction based on reduced datasets that were limited on image pixels and angular tilts.⁶⁷⁹ Dose-efficient image acquisition methods are actively developed and will play a significant role in imaging soft macromolecular materials.

7.3.2. Beyond Elucidating Structural Ordering: Diffuse Scattering. Traditional electron diffraction analysis focuses on Bragg diffraction spots which measures long-range structural order. We see enormous opportunities in mapping local structural disorder, and to understand their effects on a material's property via different forms of fundamental interactions such as defect-strain coupling, charge-lattice coupling, and intermolecular stacking. For example, intrinsically disordered regions in proteins were recently discovered,⁶⁸³ which are polypeptide segments that do not contain sufficient hydrophobic amino acids to mediate co-operative folding. However, these local disorders can escape Bragg diffraction-based analysis and get buried within a material. Diffuse scattering promises to be the solution, which is often manifested as a mixture of streaks, diffuse plane, satellite reflections, or cloudy patterns. Its relevance in electron



Figure 51. (a) Schematic showing AUTOMAP replaces the conventional image reconstruction stages with a unified image reconstruction framework that learns the reconstruction relationship between sensor and image domain without expert knowledge. (b) A schematic showing AUTOMAP model for sensor data to reconstruction image domain transformation. The model is implemented with a deep neural network architecture composed of fully connected layers, followed by convolutional layers. Adapted with permission from ref 652. Copyright 2018 Springer Nature.

diffraction has been discussed as early as 1968,684 but the practical use of it only becomes possible until recently. There are two hurdles. First, sensitive detectors are needed to enable collection of high-quality electron diffraction patterns with lower noise, higher dynamic range, and not suffering from blooming overloads that obscures diffuse signals.⁶⁸⁵ Second, interpretation of the diffuse scattering data has been difficult. Right now there are two directions to interpret a diffuse scattering dataset. One is to derive the atomic PDF from the total scattering (including Bragg peaks and diffuse scattering), which has a spectrum shape and depicts complete structures.²⁹⁷ PDF needs to be coupled with computational modeling and simulation to understand the atomic structures of nanoscale materials and disorders in ordered systems. The other is to directly compare the diffraction pattern with Monte Carlo simulations of model crystals.⁶⁸⁶ In both models, it is critical to make educated guesses on the cause of the 3D diffuse data from either 3D concerted motions of atoms or local disorders. This is a burgeoning direction of interdisciplinary research that we see potentials in.

7.4. Opportunities in Integrating ML with EM

Beyond the postcollection data analysis (Section 6 and outlooks we already discussed) and dose efficient acquisition (Section 7.3.1), the development of ML for EM can be inspired by the ML methods used in other microscopy platforms (e.g., the Faster R-CNN used for enhanced materials discovery in STM⁶⁸⁷) and in live image capturing, such as the advanced ML algorithms used in tracking multiple object motion trajectories in videos of pedestrian and automobiles,^{688–691} which could be used to facilitate a fully automated NP diffusion and self-assembly study workflow in EM movies. In addition, we also would like to note two directions we see as particularly relevant.

7.4.1. ML for End-to-End Tomography Reconstruction. Different from the ML applications in individual steps in tomography reconstruction mentioned in Section 6, studies in medical computed tomography $(CT)^{692-694}$ and PET⁶⁹⁵ imaging systems have shown that ML can achieve direct volumetric reconstruction from the sensor domain. If applied to electron tomography, such end-to-end reconstruction models can replace conventional reconstruction algorithms such as WBP, SIRT, and MBIR, potentially providing more

accurate and faster reconstruction with fewer artifacts and noises (Figure 51a). One relevent example is an end-to-end reconstruction model study versatile to various imaging systems. Zhu et al.⁶⁵² developed automated transform by manifold approximation (AUTOMAP), which is a supervised learning framework that allows the mapping between the sensor data and the image domain (Figure 51b). In this work, the model was trained and tested on several datasets from experimental MRI and PET sensor data and simulated Radon transform sensor data (similar to the tilting series in electron tomography) and showed improved artifact reduction and reconstruction accuracy on noisy and under-sampled data. It was suggested that AUTOMAP could learn reconstruction functions for arbitrary acquisition strategies with a broad range of applications including X-ray CT, large-baseline radio astronomy, and rapid volumetric optical coherence tomography.

7.4.2. ML Algorithms for Automated Imaging. ML image classification also contributes to the automation of EM operations. For example, Rashidi et al.⁶⁹⁶ utilized a CNN to help the sharp tip restoration in scanning probe microscopy (SPM)-based nanofabrication, which would otherwise need to be repeatedly done by human operators at low efficiency. The CNN was trained on a manually labeled library of SPM images of isolated silicon dangling bonds to make binary classification on whether the SPM tip was sharp or doubled. When integrated into the in situ double tip restoration routine, the CNN model can assess the probe quality by evaluating images of known atomic defects and automatically repeat the restoration until a sharp tip was detected. This method can serve as a module and automate the atomic-scale nanofabrication as well as be applied to other material systems and nanoscale imaging techniques. Various other ML techniques such as Gaussian processes, Bayesian optimization, reinforcement learning, and active learning have been applied to achieve the automated experiments in SPM and STEM, allowing applications in microscope tuning, spectroscopic material fabrication, and controlling the external stimuli for observation of dynamic phenomena. For more detailed examples of MLbased automated EM experiments, we refer the readers to a recent review paper by Kalinin et al.⁶⁹⁷ on this topic.

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Author Contributions

Z.L. led the writing of this review and wrote part of Section 2 and Section 5. L.Y. designed and made original schematic and wrote Section 6. W.C. contributed to Section 4 and provided critical reading of Section 2. F.K. wrote part of Section 2 and Section 3. Q.C. defined the scope of the review, supervised the writing process, and wrote Sections 1 and 7. CRediT: **Zhiheng Lyu** conceptualization, writing-original draft, writing-review & editing; **Lehan Yao** writing-original draft, writing-review & editing; **Falon Kalutantirige** writing-original draft, writingreview & editing; **Qian Chen** conceptualization, funding acquisition, project administration, supervision, writing-original draft, writing-original draft, writing-original draft, writing-original draft, writing-original

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

1/2/3/4D	one/two/three/four-dimensional		
3DED	three-dimensional electron diffraction		
4D-STEM	four-dimensional scanning transmission electron		
	microscopy		
ABF	annular bright-field		
ADF	annular dark-field		
ADT	automated diffraction tomography		
AET	atom electron tomography		
AFM	atomic force microscopy		
APT	atom probe tomography		
AUTOMAP	automated transform by manifold approximation		
AUROC	area under the receiver operating characteristic		
	1 0		
	curve		
bcc	curve body-centered cubic		
bcc BF	curve body-centered cubic bright-field		
bcc BF CAT	curve body-centered cubic bright-field computer-aided tomography		
bcc BF CAT CBED	curve body-centered cubic bright-field computer-aided tomography convergent beam electron diffraction		
bcc BF CAT CBED CNF	curve body-centered cubic bright-field computer-aided tomography convergent beam electron diffraction carbon nanofiber		
bcc BF CAT CBED CNF CNN	curve body-centered cubic bright-field computer-aided tomography convergent beam electron diffraction carbon nanofiber convolutional neural network		
bcc BF CAT CBED CNF CNN CNT	curve body-centered cubic bright-field computer-aided tomography convergent beam electron diffraction carbon nanofiber convolutional neural network carbon nanotube		
bcc BF CAT CBED CNF CNN CNT CO ₂ RR	curve body-centered cubic bright-field computer-aided tomography convergent beam electron diffraction carbon nanofiber convolutional neural network carbon nanotube CO ₂ reduction reaction		
bcc BF CAT CBED CNF CNN CNT CO ₂ RR COF	curve body-centered cubic bright-field computer-aided tomography convergent beam electron diffraction carbon nanofiber convolutional neural network carbon nanotube CO ₂ reduction reaction covalent organic framework		
bcc BF CAT CBED CNF CNN CNT CO ₂ RR COF cryo-EM	curve body-centered cubic bright-field computer-aided tomography convergent beam electron diffraction carbon nanofiber convolutional neural network carbon nanotube CO ₂ reduction reaction covalent organic framework cryo-electron microscopy		

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СТ	computed tomography	NMS	nonmaximum suppression
CTAC	cetyltrimethylammonium chloride	NP	nanoparticle
СТАВ	cetyltrimethylammonium bromide	NR	nanorod
CTRW	continuous-time random walk	OER	oxygen evolution reaction
DBSCAN	density-based spatial clustering of applications	OPTICS	ordering points to identify the clustering
DET	With holse	ODD	structure
DFI	density functional theory	OKK	oxygen reduction reaction
DIO	1,8-dilodoociane		poly(3-nexyl-thiophene-2,3-diyl)
DMF	dimetryitormamide		polyamide
DNA	deoxyribonucieic acid	PDED	paranei-beam electron diffraction
	double strended DNA	PCA	principal component analysis
	alactron hadvacattered scenning diffraction	PDF	pair distribution function
EDSD	energy dispersive X ray spectroscopy	P DNIS DE	poly(diffetily(shoxafie)
FEIS	electron energy loss spectroscopy	DED	projectively electron diffraction
FETEM	energy filtered transmission electron microscopy	DET	positron emission tomography
ECER	endermal growth factor recentor	DI	polyisoprepa
EM	electron microscony	DIE	ntychographical iterative engine
PDF	electron microscopy	DISA	polymerization_induced self-assembly
FTFM	environmental TFM	PS	polymenzation-induced sen-assembly
FRP	filtered back-projection	PVP	polystyrene poly(vinylpyrrolidone)
fcc	face-centered cubic	RAFT	reversible addition-fragmentation chain transfer
FCN	fully convolution network	RED	rotation electron diffraction
FF	diphenylalanine	RDD	rhombic dodecahedral
FFT	fast Fourier transform	RMSE	root mean squared error
FIB	focused ion beam	RNA	ribonucleic acid
FTIR	Fourier transform infrared spectroscopy	ROI	region-of-interest
GAN	generative adversarial network	RPN	region proposal network
GMM	Gaussian mixture model	SAED	selected-area electron diffraction
GPC	Gaussian process classifiers	SART	simultaneous algebraic reconstruction technique
HAADF	high-angle annular dark field	SAXS	small-angle X-ray scattering
hBN	hexagonal boron nitride	SCEM	scanning confocal electron microscopy
HEA	high-entropy alloy	SED	scanning electron diffraction
HER	hydrogen evolution reaction	SEM	scanning electron microscopy
HIM	helium ion microscopy	SEND	scanning electron nanodiffraction
HRTEM	high-resolution transmission electron microsco-	SerialED	serial electron diffraction
	ру	SERS	surface enhanced Raman spectroscopy
ICA	independent component analysis	SHE	standard hydrogen electrode
iDPC	integrated differential phase contrast	SIRT	simultaneous iterative reconstruction technique
IL-TEM	identical location transmission electron micros-	SNR	signal-to-noise ratio
	сору	SPM	scanning probe microscopy
IoU	intersection-overunion	STEM	scanning transmission electron microscopy
IRDM	information recovery and deartifact model	STM	scanning tunneling microscope
LDH	layered double hydroxide	SWD	sliced Wasserstein distance
LFGC	liquid-flowing graphene chip	SVD	singular value decomposition
LSPR	localized surface plasmon resonance	TEM	transmission electron microscopy
macroCTA	macrochain transfer agent	TGA	thermogravimetric analysis
MAE	mean absolute error	THH	tetrahexahedral
MEMS	microelectromechanical system	TOH	trisoctahedral
MBIR	model-based iterative reconstruction	t-SNE	t-distributed stochastic neighbor embedding
MBED	multibeam electron diffraction	TVM	total variation minimization
MD	molecular dynamics	TXM	transmission X-ray microscopy
MicroED	microcrystal electron diffraction	UMAP	uniform manifold approximation and projection
ML	machine learning	VAE	variational autoencoder
MOF	metal-organic framework	VDF VT I DTEM	virtual dark field
MOF-CGC	inetai-organic framework crystal-glass compo-	VI-LPIEM	variable-temperature liquid-phase transmission
MONT	site		electron microscopy
MDI	metal—organic hanotube	VVDP VAC	Y ray absorption spectroscopy
MCE	magnetic resonance imaging	AAO VDC	X-ray absorption spectroscopy
NBD	nean squared error	Aro VDD	X ray diffraction
NME	nonnegative matrix factorization	7	atomic number
NMR	nuclear magnetic resonance		

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