Single-Macromolecular Level Imaging of a Hydrogel Structure

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Hydrogels are promising materials for several applications, including cell scaffolds and artificial load-bearing substitutes (cartilages, ligaments, tendons, etc.). Direct observation of the nanoscale polymer network of hydrogels is essential in understanding its properties. However, imaging of individual network strands at the molecular level is not achieved yet due to the lack of suitable methods. Herein, for the first time, we developed a novel mineral-staining method and network fixation method for transmission electron microscopy observation to visualize the hydrogel network in its unperturbed conformation with nanometer
Surface network observation indicates that the length of surface dangling chains, which play a major role in friction and wetting, can be estimated from the gel mesh size. Moreover, bulk observations reveals a hierarchical formation mechanism of gel heterogeneity. These observations have the great potential to advance gel science by providing comprehensive perspective that link bulk gel properties with nanoscale.

1. Introduction

Hydrogel is a water-abundant three-dimensional polymer network that is a promising soft material for various applications, including bioengineering, soft robotics, and stretchable electronics, owing to its high water content, low friction, permeability of small molecules and ions, flexibility, and biocompatibility\textsuperscript{[1–4]}. The surface and bulk properties of hydrogels strongly depend on their molecular structure. However, state-of-the-art approaches to characterise the hydrogel network structure, especially synthetic hydrogel, are still limited at the average structure level derived from bulk measurements, such as the mesh size of a network is determined from the bulk modulus or X-ray/light scattering\textsuperscript{[5–7]}. Direct observation of individual network strands at the molecular level is yet to be achieved due to insufficient suitable methods. Transmission electron microscopy (TEM) and atomic force microscopy (AFM) are the most commonly used imaging techniques to directly observe the nanostructures of materials. Compared to AFM, TEM is more suitable for gels as...
it can eliminate the influence of the thermal motion of the network using resin substitution or water freezing at cryogenic temperatures\[8,9\]. However, existing TEM observation methods, such as electron staining, phase contrast, and scanning TEM\[10–13\], cannot produce a sufficient contrast for chemical gels consisting of thin polymer chains; therefore, a contrast enhancement method is desired. We considered the development of an improved staining method to be the most promising approach in terms of simplicity and contrast enhancement efficiency.

Here, we developed a novel staining method for the TEM imaging of a network structure of synthetic polyelectrolyte hydrogels in the unperturbed state with approximately 10 nm resolution. We introduced a skeleton neutral polymer network in the polyelectrolyte hydrogel to fix the conformation of the polyelectrolyte strands during specimen preparation. We adopted the mineralization of amorphous ferric oxide (AFO) nanoparticles to stain the polyelectrolyte strands in the unperturbed state. This method allowed the imaging of a network structure in a wide mesh size range (tens to hundreds of nanometers) for the first time and surface molecular structure of hydrogels. These observations provide important insights for the friction and fracture of hydrogels.

2. Results and Discussion

2.1. Mineral nanoparticle staining and double-network fixation method
A conventional electron stain (Osmium (VIII) oxide) that labels one carbon–carbon double bond with one heavy atom does not provide enough contrast to the sparse and extremely thin network of hydrogels. Thus, we used inorganic nanoparticles as a bigger marker.

To accurately attach the nanoparticles to hydrogel network, we used the heterogeneous nucleation of ferric oxide\textsuperscript{[14]}. Poly(2-acrylamido-2-methyl propanesulfonic acid) (PAMPS) hydrogel was adopted since sulfonic acid functional groups are expected to function as nucleation points for the growth of ferric oxide particles. PAMPS gel was synthesized by radical Fizgation, resulting in its significant swelling in water due to its polyelectrolyte nature, thereby reaching a polymer weight fraction as low as 0.3–5 wt.%. The heterogeneous nucleation of amorphous ferric oxide was gently performed in the gel by iron (III) hydrolysis and nano-sized AFO particles grew along the polymer network (Figure 1a). However, this AFO deposition caused significant irreversible shrinking of the PAMPS gel due to the high ionic osmotic pressure of the stain solution and intermolecular cross-linking (Figure 1b and Figure S1). Therefore, direct AFO deposition on a single PAMPS gel network can cause the collapse and aggregation of the polymer strands in the gel.
Figure 1. (a) Schematic illustration of the double-network method to stain a polyelectrolyte hydrogel without causing a collapse of the polymer network strands. (b) Appearance of the PAMPS-4 single network gel (SN) and double-network gel (DN) with poly(dimethylacrylamide) (PDMAAm) before and after mineral staining (upper), and chemical structure of PAMPS and PDMAAm, and the mineralization reaction during staining (lower).

To prevent network structure changes during staining, we applied the double-network (DN) method. DN method was originally developed to toughen the brittle polyelectrolyte gels by inducing excess amount of neutral polymer network into the polyelectrolyte gel[15–17]. In this study, we applied this method to fix the target structure of the polyelectrolyte hydrogel.
As the skeleton neutral polymer network, poly(dimethylacrylamide) (PDMAAm), which is non-reactive to the staining solution, was polymerized in excess in the PAMPS hydrogel prior to AFO staining. By creating this interpenetrating DN structure with strong topological entanglement, the shrinking of the PAMPS network during staining is suppressed, as demonstrated by the small change in its gel size (Figure 1a and 1b). This is attributed to the neutral PDMAAm network that generates a large osmotic pressure to maintain gel swelling\(^{[18]}\). DN method is applicable to any polymer network. For this study, PDMAAm can maintain approximately the same volume of the specimen even in the resin solution for substitution. Figure S1 shows the detailed change in thickness of the PAMPS gels in each experimental step. The final thickness change is less than 10% for all PAMPS gel samples prepared with different formulations, indicating that the strands of the PAMPS gel was nearly unperturbed in the TEM specimen.

2.2. Nanoscale TEM observation of the hydrogel network

To confirm that AFO selectively mineralized only the PAMPS network, the PAMPS micro-gels embedded in the PDMAAm gel matrix were stained\(^{[19]}\). Figure S2 shows the optical microscopic image of the PAMPS micro-gels, TEM images of PAMPS micro-gels in the PDMAAm gel, and their corresponding schematic illustrations. The PAMPS micro-gels before mineralization (not shown in the figure) are unseen by TEM, while spherical micro-
gels with several micrometer diameters are clearly identified after mineralization. This confirms that AFO selectively stained the PAMPS network, but not the PDMAAm network since the sulfonic group of PAMPS can catalyse the AFO deposition.

During staining, insufficient mineral binding to the PAMPS network results in understaining. To obtain the best TEM image, we prepared a supermacroporous sponge-like PAMPS/PDMAAm hydrogel comprising an interconnected porous structure with thin gel walls, thereby supplying iron ions faster than that for bulk gel. In this sample, sufficient minerals were attached to the gel mesh. The TEM micrograph shows the finest morphology of the PAMPS in this study (Figure 2a). Under high magnification, it is clearly observed that AFO nanoparticles with a diameter of several nanometers form network structure with a mesh size of several tens of nanometers.

Figure 2. (a) Low and high magnification TEM images of the PAMPS gel and 2D FFT from a single mineral nanoparticle. (b) STEM elemental mapping of the PAMPS gel. Fe and S shows the iron in the ferric oxide nanoparticles and sulphur in the PAMPS gel network, respectively.
A supermacroporous PAMPS gel with a wall thickness of approximately 10 µm was used for the observation.

The two-dimensional Fourier transform image of a single nanoparticle has no obvious peaks (lower right in Figure 2a), indicating its amorphous nature. Furthermore, 3D TEM was performed to obtain a 3D network structure (Supplementary Video)[21], clearly presenting the 3D connections of the network. Notably, this is the first 3D nanoscale direct observation of a hydrogel network structure. To prove that only the PAMPS network was mineral stained at the nanoscale, element mapping (STEM–EDS) was carried out (Figure 2b). The positions of sulphur of PAMPS and iron of AFO overlap with each other on the nanoscale, confirming that only the PAMPS network was selectively stained with ferric oxide.

The high magnification TEM observation of the bulk non-porous PAMPS/PDMAAm gel is shown in Figure S3. A fine PAMPS network morphology is observed with the mesh sized in tens of nanometers, which roughly agrees with the previously reported average mesh size from small-angle X-ray scattering and mechanical estimation[22]. Considering the polymer mesh size and thickness of the specimen slice (100 nm), the discontinuity of the network can be attributed to the mesh structure that is frequently larger than thickness of the specimen. The accumulative strand length in the volume of the measured view (1200×1200×100 nm^3) is 9100±2100 nm, which is approximately 1/10 of the contour length of 1.3×10^5 nm calculated from the total amount of PAMPS in the viewing volume (Figure
S3a). Even considering that TEM measured a 2D projected length of approximately 0.64 the true length, there is still a large difference from the calculated value. These indicate that the observed accumulative strand length does not represent the full contour length of the polymer network and the fine structures of the polymer strands, such as blobs, were not included. Moreover, the dangling chains that should be in a collapsed conformation (Figure S3b) are also not included in the observed accumulative strand length. On the other hand, this measurement provides a rough answer to the big question in the gel field, what percentage of polymer chains are effective network (not dangling chains). For example, in this gel, at least one-sixth or more of the polymer chains (which probably be more than one-third because the real chains are loose and not fully extended) function as effective network. The TEM images of the PAMPS hydrogel prepared with different crosslinker densities are shown in Figure S4. The polymer density increases with increasing crosslinker density. In addition, the structure becomes finer, while the network morphology is unclear owing to the insufficient amount of mineral precursor to stain all polymer strands. These demonstrate that we achieved the direct observation of an actual hydrogel polymer-network structure for the first time. This method was then applied to characterise the surface and bulk structure of hydrogels.

2.3. Surface structure of hydrogel

The surface feature, such as low friction, adhesions, and permeability for small
molecules and ions, is an important characteristic of hydrogel materials. In nature, hyaline cartilage exhibits both incredibly low friction property and semipermeability that allows nutrient diffusion\cite{23,24}. The TEM observation of the specimen prepared by our method for the first time revealed the precise structure of the polymer strands at the hydrogel surface. Figure 3A(i) shows the outermost as-prepared surface of a PAMPS hydrogel synthesized on a flat glass mold. PDMAAm was induced by second polymerization with the method described in the previous section. Several hundred nanometer-sized dangling chains (indicated by the black arrows) in the fully elongated state from the bulk region are observed. These surface dangling chains do not collapse as they are covalently connected to the PDMAAm network and are stretched by its swelling (Figure 3a(i)). As illustrated in Figure 3b, the PAMPS network usually has a few unreacted vinyl groups after PAMPS polymerization\cite{25}. During the polymerization of the second skeleton network, some dangling chains with remaining vinyl groups on the PAMPS gel surface are incorporated into the PDMAAm network. Therefore, the surface of the formed PAMPS/PDMAAm DN hydrogel is covered by the second PDMAAm network partially connected with the PAMPS dangling chains\cite{26}. The immersion of the PAMPS/PDMAAm gel in water induces the swelling of the PDMAAm network surface layer and stretching of the connected PAMPS strands. In Figure 3a(i), the approximate length of the dangling chains (several hundred nanometers) is approximately equal as that of the network mesh size in the bulk region. Therefore, these dangling strands can be assumed to be
strands that could not find a cross-linking partner at one end. In addition, this result indicates that the length of the surface dangling chain of the chemically crosslinked gel is comparable to the bulk network mesh size; thus, the length of the dangling chains of the gel surface can be estimated based on the mesh size of the bulk network. It should be noted that only dangling chains that are covalently connected to the second network are observed as elongated strands. As most dangling chains are not covalently connected to the second network, they collapsed into a globule conformation, as seen by the dark rough line on the gel surface, similar with the dangling chains inside the gel (Figure S3b).

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\[\text{Figure 3. Surface network structure of the PAMPS-4 gel. (a) (i) As-prepared gel surface synthesized on a flat glass mold; (ii) Surface cut with a microtome knife. The black arrows in the TEM images indicate surface dangling chains. (b) Schematic of the surface dangling chain}\]
extension by the skeleton network. The PDMAAm network in the PAMPS gel was omitted for schematic visibility.

We also observed the surface of the PAMPS gel cut using a fine microtome knife with an edge thickness of 76 µm. The cut surface is significantly rougher than the as-prepared surface (Figure 3a(ii)). In addition, it has a disordered network structure approximately 1 µm deep from the outermost surface, while the mesh structure is maintained in the inner region. The length of the created dangling chains at the surface is nearly equal to the bulk mesh size since these dangling chains are originated from the mesh. Considering that cutting creates two fracture surfaces, the damage zone has a total depth of approximately 2 µm, suggesting that brittle hydrogel is fractured near the surface, despite the cutting edge thickness of 76 µm. This observation can be used to estimate the relation between the microscale damage zone and macroscopic fracture.

2.4. Heterogeneity of the hydrogel

The characterisation of the defects or heterogeneity is vital in understanding the fracture of the material as it is governed by local structure, not by the average structure\(^{[27,28]}\). Particularly, fracture is always originated in the largest defect in a material. Here, we applied the developed method to directly observe the defects in hydrogels.

Figure 4 shows the TEM images of a bulk PAMPS hydrogel and its schematic
illustrations. The PAMPS hydrogel contains several local voids that are approximately 10 µm (Figure 4a–c). These voids do not have a PAMPS network. The origin of defects is the nonhomogeneous formation of the micro-gels[29,30]. In the initial stage of hydrogel polymerization from the monomer and crosslinker, several micro-gels are instantaneously formed with non-uniform spacing. When these microgels grow and coalesce together to form the bulk gel, a void structure is formed where there are no micro-gels. These micro-scale defects are not observed in small particle gels with a diameter of several micrometers (Figure S2). Hence, a small gel is relatively homogeneous and free of large defects, while defect formation is inevitable for a bulk gel, thereby affecting its mechanical properties. This observation allows a simultaneous observation of the hydrogel structure at multiple scales. Figure 4d-f shows a high magnification image of a void and its schematic illustrations. Polymer strands around the void are oriented along the defect circumference, indicating that these strands are highly elongated than those farther from the voids. This result denotes that microscale heterogeneity, such as voids, induces nanoscale heterogeneity.
**Figure 4.** Micro-scale network defects in the bulk PAMPS-4 gel. (a)–(c) Low magnification TEM images at different locations. The white areas are microvoids without a PAMPS network. (d)–(f) High magnification TEM image around a microvoid and its schematic illustration.

### 3. Conclusion

We demonstrated the combination of a novel staining method with DN technique to directly observe a hydrogel network at the nanoscale level. The molecular structure of polymer hydrogel in bulk and on the surface was unveiled for the first time in real space. We clarified the presence of dangling chains on the surface of the as-prepared gels with a length
on the same scale as the mesh size. These results are important in understanding the surface properties of hydrogels, including surface wetting–dewetting, sliding friction, adhesion, and bonding. Furthermore, numerous micro-scale defects noted in these gels can induce stress concentration of the nanoscale polymer strands. These results are essential in understanding the origin of the brittleness of hydrogels. This method has the potential to be widely used not only for hydrogels but also for single macromolecular level imaging of polymer materials.

4. Experimental Methods

Materials: The 2-acrylamido-2-methyl propanesulfonic acid (AMPS) monomer was provided by Toagosei Co. Ltd, Japan. The dimethylacrylamide (DMAAm) monomer, N, N’-methylenebisacrylamide (MBAA) crosslinker, and 2-oxoglutaric acid (α-keto) initiator, iron (III) chloride hexahydrate, and iron (II) chloride tetrahydrate were purchased from Wako Pure Chemical Ind., Ltd., Japan. Osmium tetroxide (OsO₄) was acquired from TAAB Laboratories Equipment Ltd., England. DMAAm monomer was purified by reduced pressure distillation before usage. The other chemicals were used as received. The precursor solution for the PAMPS gel comprised 1 M AMPS as the monomer, 1–8 mol% MBAA as the crosslinker, and 0.1 mol% α-keto as the initiator. Meanwhile, the formulation of the precursor solution for the PDMAAm network was 2 M DMAAm monomer, 0.1 mol% MBAA, and 0.1 mol% α-keto. Each mol% is relative to the corresponding monomer.

Gel synthesis: The plate-shaped PAMPS network was synthesized from the precursor solution in a mold, which was made of a 2 mm thick silicone spacer sandwiched between two glass plates, by UV polymerization for 8 h in a chamber filled with inert Ar gas. Then, the PAMPS gel was removed from the mold and soaked in a PDMAAm network precursor solution
for 1 day until reaching equilibrium. The PAMPS gel containing the PDMAAm network precursor solution was sandwiched between two glass plates and irradiated with UV for its 2nd polymerization for 6 h in Ar atmosphere\[15\]. The obtained samples were immersed in distilled water for one week to completely remove residual chemicals. All glass plates used in the molds were heated in a 500 °C oven for 1 h to remove any residual organics before use. The samples were coded according to the PAMPS network crosslinker ratio $C_{MBAA}$ (mol%), as PAMPS-$C_{MBAA}$. The thickness of these swelled PAMPS gel samples were 8.99, 5.37, and 3.36 mm for PAMPS-1, PAMPS-3, and PAMPS-8, respectively. PAMPS-4 micro-gels and supermacroporous PAMPS-0.5 gel were prepared by suspension polymerization and cryogelation using the protocols reported.\[19,20\] The mesh size of the supermacroporous PAMPS-0.5 gel is less than that of conventional bulk PAMPS gel since the concentration of the precursor solution increases with freezing in the cryogelation. The PDMAAm network in these gels was introduced using the protocol described above.

**Compression test:** The compressive mechanical properties of the PAMPS gel were examined using a mechanical tester (Tensilon RTC-1310A, Orientic Co., Japan). The samples were cut into discs with 15 mm diameter using a cutting machine. The test was performed at a strain rate of 1/600 s\(^{-1}\). Each test was performed on five samples.

**Mineral staining and transmission electron microscopy (TEM) observation:** For mineral staining, the hydrogels were immersed in a staining solution of 2.5 M FeCl\(_3\) and 1.5 M FeCl\(_2\) mixture for 1 day at 25 °C. Subsequently, the gels were immersed in pure water to increase pH and amorphous ferric oxide nanoparticles were mineralised on the PAMPS network. TEM observations (H-7650, Hitachi, Japan) were performed to observe the polymer network morphology of the stained hydrogels. The specimens for TEM observations were prepared by freezing the hydrogels in liquid nitrogen and the water of the hydrogels was substituted with ethanol and then acrylic resin (London Resin white, medium) in the chamber of an automatic freeze substitution system (EM AFS2, Leica Microsystems, Germany). The gel thickness in
these processes is shown in Figure S1. For all PAMPS gels with different formulations, the final thickness changes of the resin-cured specimen are within 10% relative to that of the PAMPS gels in water. Then, 100 nm thick resin-cured specimens were cut using an ultramicrotome knife (EM UC7i, Leica Microsystems, Germany) and then placed on a carbon-supported copper mesh grid. The electron gun has an acceleration voltage of 100 kV. Subsequently, 3D TEM and energy dispersive X-ray spectrometry were performed using a TEM tomographic system (JEM-1400 and EM-05500TGP, JEOL, Japan) and scanning electron microscope (JEM-F200, JEOL, Japan). The acceleration voltage of the electron guns was 120 and 200 kV, respectively. ImageJ software\textsuperscript{[31]} was used to calculate the total chain length in the TEM image of PAMPS-1.5 (Figure S3). The original TEM images were skeletonised and the total pixels were measured in the image of five samples. This total chain length is considered as the apparent length in 2D. When a three-dimensional straight line is projected in 2D, the 2D apparent length can be written by the original length multiplied by cosθ, where θ is the angle of the line with respect to the projection plane. The average value of cosθ in the range of 0° to 180° is 2/π, which is approximately 0.64.

Data analysis: The theoretical network strand contour length was calculated from the PAMPS concentration in the specimen after resin exchange. The gel composition was PAMPS-1.5. The PAMPS monomer unit charged concentration at synthesis was 1 M. After equilibrium swelling, staining, and resin exchange, the PAMPS-1.5 hydrogel has a volume swelling ratio of 166 relative to its as-prepared state. Thus, the TEM sample has a PAMPS monomer unit concentration of 1/166 M. By considering the Avogadro constant of $6.02\times10^{23}$ (mol$^{-1}$):

$$\text{Number of monomers per volume} = \frac{1}{166} \left( \frac{\text{mol}}{\text{L}} \right) \times 6.02 \times 10^{23} \ (\text{mol}^{-1})$$

$$= 3.6 \times 10^{24} \ (\text{monomer/m}^3).$$

In this experiment, the view field has a volume of $1200\times1200\times100 \ \text{nm}^3 = 1.44 \times 10^{-19} \ \text{m}^3$. Thus,

$$\text{Monomer in the view field} = 3.6 \times 10^{24} \left( \frac{\text{monomer}}{\text{m}^3} \right) \times 1.44 \times 10^{-19} \ (\text{m}^3)$$
One monomer unit has a contour length of approximately 0.25 nm. Thus, the theoretical network strand contour length in the fully stretched state, excluding the blob and dangling chains, is:

Total polymer length in the view field = 518400 (monomer) \times 0.25 (\text{nm/monomer}) = 1.3 \times 10^5 (\text{nm}).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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R.K., T.N. and J.P.G. designed the experiments and interpreted the results. R.K. performed the almost experiments and analysed the data. T.S. prepared the supermacroporous gel. H.J. performed STEM measurements. R.K., T.N. and J.P.G. wrote the paper. This research was financially supported by a Grant-in-Aid for Scientific Research (No. JP17H06144, JP17H06376), a Grant-in-Aid for JSPS Research Fellow (No. JP16J05057), and the Ambitious Leaders Program. J. P. Gong thanks the Institute for Chemical Reaction Design and Discovery (ICReDD) established by the World Premier International Research Initiative (WPI), MEXT, Japan. H. J. is grateful to the JSPS Kakenhi for the partial support of this research through Grant No. 19H00905. R. K. thanks Kazuki Fukao, Takahiro Matsuda, and Taiki Fukuda (Hokkaido University) for their useful comments. The authors also thank
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References


Supporting Information

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**Figure S1.** Thickness swelling ratio of the PAMPS gels with different crosslinker density during staining and substitution. $T_0$ is the thickness of the SN gels swelled in water. The black dashed line in the graph indicates the constant size ($T/T_0=1$). By introducing the PDMAAm skeleton, the gel swells slightly ($T/T_0=1.1$-$1.2$). The samples without a skeleton network significantly shrink ($T/T_0=0.1$-$0.4$). With the skeleton network, the change in the final size of the resin-cured specimen is within 10% ($T/T_0=0.9$-$1.1$). The error bars indicate the standard deviation for five samples.
Figure S2. TEM image of the particle PAMPS-4 micro-gels in the bulk PDMAAm gel. (a) Optical microscopy image of the pristine PAMPS micro-gel particles. (b) Low magnification (×300) TEM image. (c) High magnification (×5000) TEM image. (d) Schematic illustration of (b).
Figure S3. (a) Calculation process of the polymer length in the field of view for the PAMPS-1.5 gel. The volume swelling ratio at the measured state was 166 times that of the as-prepared state for this sample. (b) High magnification TEM images of the PAMPS network at different locations. The mesh structures sized in tens of nanometres were clearly observed. The black arrows indicate the apparent aggregated dangling chains in the structure.
Figure S4. Effect of crosslinker ratio of the PAMPS gels. (a) TEM images. (b) (i) Stress–strain curves of the PAMPS gels in their water swollen state; (ii) Gel properties. (c) Illustrations of the network structure with low (upper) and high (lower) crosslinker density of the PAMPS gels before and after mineral staining.
Supplementary Video 3D TEM movie of the supermacroporous PAMPS gel.