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27	microscopy, Double Network, Mineral staining, Inhomogeneity, Dangling chain
28	
29	In this work, we succeeded in direct visualization of the network structure of synthetic
30	hydrogels with TEM by developing a novel staining and network fixation method. Such a
31	direct visualization had not been carried out because sample preparation and obtaining
32	sufficient contrast are challenging for these soft materials. TEM images revealed robust
33	heterogeneous network architectures at mesh size scale and defects at micro-scale. TEM
34	images also revealed the presence of abundant dangling chains on the surface of the hydrogel
35	network. The real space structural information provides a comprehensive perspective that
36	links bulk properties with a nanoscale network structure, including fracture, adhesion, sliding
37	friction, and lubrication. The presented method has the potential to advance the field.
38	

## 39 1. Introduction

40	Hydrogels are important soft materials with broad applications in society, especially
41	as biomaterials, stretchable electronics, etc <sup>[1–4]</sup> . A typical hydrogel is a crosslinked hydrophilic
42	polymer that shows rubber elasticity and does not dissolve in water. As a feature of soft
43	matter, the structure of the hydrogel network does not have long-range order and is
44	heterogeneous at various scale levels. Typically, at the nanoscale, the polymer chains between
45	crosslinking points have distribution in molecular weight along with loops and dangling
46	chains <sup>[5]</sup> ; at the microscale, large voids and defects induced during coagulation of microgels
47	to bulk gels often exist <sup>[6–8]</sup> . The physical properties of a hydrogel strongly depend on the
48	architecture of the polymer at different scales. For example, loops and dangling chains
49	contribute to solvation and thereby swelling ability as like network chains but do not
50	contribute to the elasticity of a hydrogel; the network mesh size regulates the molecular
51	diffusion and transportation; the largest defect in the hydrogel formed during preparation
52	process governs the fracture strength of the material <sup>[9]</sup> . Observing the structure of network
53	materials from the nanoscale network level to the microscale level is of paramount importance
54	for understanding and predicting the physical properties and rational design of this class of
55	materials.

As the state-of-the-art approach, the X-ray/neutron/light scattering are powerful
methods to characterize the physical structure of hydrogels at different length scales from

58	nano to micro <sup>[7,10,11]</sup> . However, the scattering methods give average structural information
59	over a scattering volume several orders of magnitude larger than the mesh size of the network
60	(typically 10 $\sim$ 100 nm). Moreover, explaining the scattering results in reciprocal space is
61	usually difficult without complementary real space structure information.
62	Transmission electron microscopy (TEM) is one of the most promising methods to
63	directly image material structures with nanoscale resolution in real space. Recent progress in
64	tomography TEM technology clarifies the three-dimensional structure of polymer materials at
65	the nanoscale <sup>[12]</sup> . However, applying TEM to observe the network structure of hydrogels that
66	consist of thin, flexible polymer chains swollen in water is highly challenging. Existing TEM
67	observation methods, such as electron staining, phase contrast, and scanning TEM, are
68	difficult to give sufficient electron density contrast for individual polymer chains of synthetic
69	hydrogels <sup>[13–16]</sup> . Resin substitution or cryogenic environment (Cryo-TEM), required to
70	suppress the thermal fluctuation of the soft materials <sup>[17,18]</sup> , will provide a similar electron
71	density between the thin polymer strands of a hydrogel network and the surrounding matrix,
72	resulting in no contrast. The difficulty is to develop a method that can selectively stain the
73	thin target polymer strand to produce a sufficient electron density contrast and spatial
74	resolution while maintaining the polymer network architecture. The extremely soft and thin
75	synthetic polymers undergo large deformation during staining or dehydration compared to
76	relatively rigid and thick polymers like DNA <sup>[19,20]</sup> .

77	Here, we developed a method to prepare hydrogel specimens for TEM observation.
78	This method allows for performing selective staining, dehydration, and resin substitution
79	without causing fatal changes in network architecture. With this method, for the first time, we
80	succeeded in observing the network architecture of chemically crosslinked, vinyl-type
81	polyelectrolyte hydrogels in real space over a wide length scale, from the network architecture
82	in tens nm to meso-scale heterogeneity in $\mu$ m. For the first time, we also succeeded in
83	visualizing the existence of graft chains on the surface of the network. These observations
84	provide important insights for understanding the kinetics of polymer network formation,
85	fracture behavior, and the surface properties of hydrogels. This TEM observation method has
86	the potential to be applied to other rubbery network materials with various chemical structures
87	and has the potential to advance the field.





**Figure 1. (a)** Schematic illustration of the double-network method to stain a polyelectrolyte network at isovolumic condition for TEM observation.  $\xi$  is cut-off length for structure preservation. Crosslinking positions and strand conformation of the polymer network at large scale (> $\xi$ ) are preserved, while interaction between polyelectrolyte and mineral generates shrinking force, and the network strands collapse, loosing conformational information at small scale (< $\xi$ ). Conformational information of relatively short dangling chains is also not

96	preserved. (b) Appearance of the PAMPS-4 network gel with and without skeleton PDMAAm
97	network before and after staining. (c) Thickness swelling ratio of the PAMPS networks with
98	different crosslinker ratio during skeleton network inducing, staining, and resin substitution.
99	Resin substitution is a process to replace the water in the hydrogels with solidified resin for
100	TEM observation in vacuum (see details in Experimental section). Samples are coded as
101	PAMPS- $C_x$ , where $C_x$ stands for the crosslinker ratio (mol% in relative to monomer). T <sub>0</sub> is the
102	thickness of the PAMPS single network swelled in water. The black dashed line in the graph
103	indicates the iso-volume line (T/T <sub>0</sub> =1). By inducing the PDMAAm skeleton, the samples
104	swell slightly (T/T <sub>0</sub> =1.1-1.2). The samples without a skeleton network significantly shrink in
105	staining solution and in resin precursor solution (T/T <sub>0</sub> =0.1-0.4). With the skeleton network,
106	the change in the final thickness of the resin-cured specimen is within 10% (T/T <sub>0</sub> =0.9-1.1).
107	The error bars indicate the standard deviation for five samples.
108	
109	2. Results and Discussion
110	2.1. Strategy to staining target networks while reserving structure
111	The strategy is illustrated in Figure 1a. We chose a polyelectrolyte network carrying
112	negative charges as our target network for two purposes. One reason for such selection is to
113	pre-stretch the network strands into a highly extended state in favouring the network
114	architecture observation. The counter-ions of a polyelectrolyte network exert a large osmotic

115	pressure to significantly swell the network in low ionic strength aqueous solution,
116	spontaneously achieving the highly pre-stretched state of the polymer strands. The other
117	reason is to selectively stain the target network by mineralization using metal ions. To hold
118	the polyelectrolyte network in the pre-stretched state during staining and the afterward resin
119	substitution process for TEM observation under vacuum condition, we introduce a neutral
120	polymer network of high concentration as a skeleton matrix before staining <sup>[21–23]</sup> . The neutral
121	polymer network, topologically interpenetrated with the target polyelectrolyte network,
122	generates a high osmotic pressure to prevent the shrinkage of the polyelectrolyte network in
123	the ionic staining solution <sup>[24]</sup> . The isovolumic staining preserves the crosslinking position and
124	mesh size of the target network and therefore allows us to clarify the architectural feature of
125	the target network. The conformation of the polyelectrolyte strands below a certain small
126	length scale is not preserved and changes upon staining. This cut-off length scale $\xi c$ below
127	which the strand conformation is not preserved should depend on the balance between the
128	energy gain by mineral staining and the elasticity penalty of both the target network and the
129	skeleton network. On the other hand, loops and undulating structures of chains larger than $\xi c$
130	can be preserved because their deformation is inhibited by entanglement with the neutral
131	network.
132	Specifically, we adopted poly(2-acrylamido-2-methyl propanesulfonic acid)

133 (PAMPS) hydrogels synthesized by free radical polymerization as the target network. For the

134	skeleton network, we adopted poly(dimethylacrylamide) (PDMAAm) since we discovered
135	that among several neutral polymers tested, PDMAAm could maintain the isovolumic
136	condition of the target network not only during the mineral staining but also during the later
137	resin substituting process (see details in experimental section). We introduced the skeleton
138	network by synthesizing the PDMAAm network in the presence of the target PAMPS
139	network, using the double network technique <sup>[21–23]</sup> . To selectively stain the target
140	polyelectrolyte network with enough electron density contrast, we adopted Fe <sup>3+</sup> that forms
141	amorphous ferric oxide (AFO) nanoparticles through heterogeneous nucleation on the
142	sulfonic acid groups of PAMPS chains <sup>[25]</sup> . In this study, we synthesized all hydrogels with a
143	commonly used crosslinker: N, N'-methylenebisacrylamide (MBAA) and UV initiator: 2-
144	oxoglutaric acid (α-keto).
145	The polymer volume fraction of the water-swollen PAMPS gels studied in this work
146	was $0.2 - 3.8$ vol%, depending on the chemical crosslinker ratio relative to monomer in the
147	precursor solutions of PAMPS gels (see Experimental section). Direct staining of AFO on
148	water swollen PAMPS network caused substantial shrinkage of the gel (Figure 1b) and large
149	mineral formation (Figure S1), indicating the collapse of the network strands. The volume
150	shrinkage of the PAMPS network during AFO staining is suppressed in the presence of the
151	skeleton network PDMAAm, as confirmed by the small change in sample size (Figure 1b). In
152	these PAMPS hydrogels, the volume ratio of the skeleton network to the target PAMPS

153	network was 5 to 100, depending on the crosslinker ratio of PAMPS. We quantitatively
154	studied the volume changes of PAMPS hydrogels during the specimen preparation process by
155	characterizing the specimen thickness relative to that in pure water (Figure 1c). PDMAAm
156	can maintain approximately the same thickness of the specimen not only in staining solution
157	but also in ethanol and in the precursor solution of resin for substitution. As a result, the final
158	thickness change of PAMPS network relative to their water swollen state is less than 10% for
159	all PAMPS gel samples prepared with different formulations. This isovolumic condition
160	means the positions of the crosslinking points, and therefore the distribution of end-to-end
161	distances of the PAMPS network strands between crosslinking points (i.e., the mesh size), are
162	maintained in the TEM specimen.
163	

## 164 2.2. Nanoscale TEM observation of the networks

165To confirm that AFO is selectively mineralized on the PAMPS network but not on the166PDMAAm network, we first performed the TEM observation on the PAMPS microgels167embedded in the bulk PDMAAm gel<sup>[26]</sup>. Figure S2 shows the optical microscopic image of168the PAMPS microgels, TEM images of PAMPS microgels in the PDMAAm gel, and their169corresponding schematic illustrations. The PAMPS microgels before mineralization (not170shown in the figure) are unseen by TEM, while spherical microgels with several micrometer171diameters are clearly identified after mineralization. This result indicates that AFO is

selectively stained on the PAMPS network but not on the PDMAAm network since thesulfonic group of PAMPS can catalyze the AFO deposition.

174	To observe the nanoscale PAMPS network structure, first, we adopted a porous
175	PAMPS hydrogel containing large interconnected pores with ~10 $\mu$ m gel walls <sup>[27]</sup> . The large
176	pores provide a fast iron ions supply, thereby favoring sufficient mineral staining. The TEM
177	micrograph shows the fine network architecture of the PAMPS (Figure 2a). Under high
178	magnification, it is clearly observed that AFO nanoparticles with a diameter of several
179	nanometers are stained on the network with a mesh size of several tens of nanometers. The
180	two-dimensional Fourier transform image of a single nanoparticle has no obvious peaks
181	(lower right in Figure 2a), indicating its amorphous nature. Element mapping by using a
182	scanning TEM further shows that the positions of sulphur of PAMPS and iron of AFO overlap
183	on the nanoscale (Figure 2b), confirming that only the PAMPS network was selectively
184	stained with ferric oxide. Furthermore, TEM tomography was performed to observe the 3D
185	network structure (Figure 2c and Supplementary Video). 3D connection of the network
186	consisting of four-arm crosslinking points is clearly observed. To the authors' knowledge, this
187	is the first 3D nanoscale direct observation of network structure for soft synthetic polymers.







100 nm

189	Figure 2. TEM observation of PAMPS network. (a) Low and high magnification TEM
190	images and 2D FFT from a single mineral nanoparticle. (b) STEM elemental mapping. Fe and
191	S shows the iron in the ferric oxide nanoparticles and sulphur in the PAMPS gel network,
192	respectively. (c) TEM tomography image. A porous PAMPS gel with a wall thickness of
193	approximately 10 $\mu$ m was used for the observation. TEM sample thickness was about 100 nm
194	
195	To understand the applicable range of this method, PAMPS hydrogels prepared with
196	different crosslinker ratios were observed (Figure 3 and S3). There are two possible causes to
197	make a large difference between the actual network structure and the TEM image: under-
198	staining and inter-chain aggregation. When under-staining occurs, the mineral particles fail to
199	adhere to the entire chain, resulting in a dots-like appearance in TEM images. This
200	phenomenon occurred with too fine mesh gels (more than 3 mol% crosslinker, Figure S3),
201	while the staining progressed sufficiently for large mesh gels (1 to 3 mol% crosslinker, Figure
202	3), and continuous network structures were observed. The under-staining is considered due to
203	insufficient mineral supply. Higher concentration mineral source or longer reaction time may
204	be the solution. When inter-chain aggregation occurs, multiple chains combined with a large
205	mineral are formed, as shown in Figure S1, and the individual polymer structure collapses. In
206	the PAMPS gels containing the skeletal network (Figure 3b), the formation of such huge
207	minerals was inhibited, and there were only small minerals of uniform size, so it is considered

208	that inter-chain aggregation hardly occurred, and individual polymer structure was observed.
209	Rarely, in areas where the mesh size is locally small, relatively large minerals of about 10 nm
210	formed by interchain aggregation can be seen (Figure 3b, PAMPS-2 and PAMPS-3) because
211	the distance between chains is close and the skeleton mesh cannot sufficiently inhibit
212	aggregation. Such mineral particles may act as artificial cross-linking points, creating fake
213	network structure, so caution is necessary. As a result, we concluded that in gels with a
214	crosslinker concentration of 1 to 3 mol %, under-staining and inter-chain aggregation are
215	negligible, and the network structure above the cut-off length remains almost unchanged in
216	the TEM image.





Figure 3. TEM images of the PAMPS networks prepared with different crosslinker ratio. (a) Low magnification images. (b) High magnification images. Samples are coded as PAMPS- $C_x$ , where  $C_x$  stands for the crosslinker ratio (mol%). The mesh sizes calculated from PAMPS gel bulk modulus (**Table S1**) are shown by bars in the bottom. Since the specimen thickness is about 100 nm, the mesh with a size larger than 100 nm appears as unconnected.

218

Next, we discuss polymer conformation change on a small scale. TEM images reveal that the network strands have a persistent length of about 40 nm (**Figure S4**). This persistent length could be understood as the cut-off length  $\xi_c$  below which the initial conformation

228	information of the polymer strands is lost by mineralization. TEM images should represent
229	unperturbed polymer strands conformation at a scale larger than this value. This is revealed by
230	the winding structures (tens nm) between the cross-linking points in the PAMPS-1 sample that
231	has a relatively long strand length among the three PAMPS networks (Figure 3). We notice
232	that the cut-off length $\xi_c$ is larger than the mesh size of the skeleton (8 ~ 10 nm) ( <b>Table S2</b> ).
233	This could be understood by the low crosslinking ratio (0.1 mol%) of the skeleton network,
234	which gives a relatively small elastic penalty for the collapse of the target strands by staining.
235	During staining, partial collapse of the strands at a scale below $\xi_c$ should exert additional
236	tension on the target network. Since each crosslinking point is stretched from four different
237	directions, the tension induced by shrinkage should be cancelled. As a result, the positions of
238	crosslinking points should be preserved at a scale smaller than the cut-off length $\xi_c$ . In fact,
239	we can observe a network structure (about 20 nm mesh size) smaller than $\xi_c$ in PAMPS-3
240	(Fig.3).
241	Based on the above discussion, we quantitatively confirmed whether the network
242	structure was accurately observed by TEM. For that, we estimate the accumulative length of
243	the polymer strands observed in the TEM image, using the PAMPS-1 sample as an example
244	(Figure S5a). The high magnification TEM images of the PAMPS-1 are shown in Figure
245	S5b. A fine PAMPS network morphology with a mesh size of a hundred nano-meter is
246	observed. The discontinuity of the network can be attributed to the mesh structure that is

247	frequently larger than the thickness of the TEM specimen (100 nm). The accumulative strand
248	length in the volume of the TEM view (896×896×100 nm <sup>3</sup> ) is measured as $L_{2D}$ =17700±2700
249	nm (see experimental part and Figure S5a). Since this length in the 2D TEM image is the
250	projection of length in 3D space, we correct it into 3D length by multiplying a factor of $\pi/2$ as
251	$L_{3D} = 2.8 \times 10^4$ nm (see experimental part). This is 21% of the total length of polymer $1.3 \times 10^5$
252	nm calculated from the amount of AMPS monomer unit in the viewing volume. Since the
253	polymer strands at a scale smaller than the cut-off length $\xi_c$ are collapsed by staining, this
254	accumulative strand length should be corrected to get the true contour length of the single
255	polymer chain. A simple model derived a corrected factor of 1.4 for this effect (see
256	experimental part), which gives a value of approximately 30% of the total length of the
257	polymer. Accordingly, the TEM observation reproduced almost the 1/3 length of the network.
258	The remaining 70% monomers can be assigned for dangling chains and small loops which
259	cannot entangle with the skeleton, and they should take collapsed conformation and appear as
260	small dots in the TEM image. The large aggregations in Figure S5b indicated by black arrows
261	are ascribed to dangling chains on the network.
262	Furthermore, we qualitatively compare the mesh size in TEM images with that
263	calculated from Young's modulus of the bulk samples based on rubber elasticity theory <sup>[28]</sup>
264	(Table S1, Figure 3b). The increase of the crosslinker ratio results in the decrease in mesh
265	size in TEM images. The average mesh sizes calculated from the mechanical measurement at

266	the corresponding swelling state of TEM observation were shown by length bars under
267	corresponding TEM images in Figure 3b. These mechanically determined mesh sizes are in
268	the same order of magnitude with TEM images. Since the high-magnification TEM images
269	(Figure 3b) captured relatively sparse network density area for easy to observe, the network
270	sizes look larger than the calculated values, but as low-magnification images (Figure 3a)
271	indicate, hydrogel network is extremely inhomogeneous. Areas of high mesh density push
272	down the average mesh size and may carry more load. Considering that the accumulated
273	contour length of the polymer network in TEM is about 30% of the theoretical length, these
274	network structures observed in TEM are almost consistent with the prediction from
275	mechanical property and not a result of under-staining or inter-chain aggregation.
276	
277	2.3. Surface structure of network
278	The surface feature, such as friction, adhesions, and permeability for small molecules
279	and ions, is an essential characteristics of network materials. Especially, hydrogels show
280	attractive surface nature, for example, in nature, hyaline cartilage exhibits both incredibly low
281	friction property and semi-permeability that allows nutrient diffusion <sup>[29,30]</sup> . Our TEM
282	observation for the first time revealed the precise structure of the polymer strands at the
283	chemically synthesized hydrogel network surface. When observing the bulk network
284	structure, we fixed the target network by physical entanglement with the skeleton network,

- but dangling chains inside the network or on the surface cannot be fixed by this mechanism.
- 286 Therefore, we adapt another strategy based on two previously established pieces of
- 287 knowledge.
- 288





Figure 4. Surface network structure of the PAMPS gel. (a) Schematic of the surface dangling
chain extension by the skeleton network. (b, c) Virgin PAMPS-4 network surface with (b) and
without (c) inter-network crosslinking to the skeleton network. Before skeleton network
polymerization, the residual crosslinkers in PAMPS network (c) were inactivated with the
excess amount of initiators<sup>[31]</sup>. (d) Cut PAMPS-4 network surface with inter-network

295	crosslinking to the skeleton network. The PAMPS hydrogel was cut with a microtome knife
296	and then was immersed in the precursor solution of the skeleton network to form PAMPS
297	network embedded in skeleton matrix. The virgin surfaces in $(b)$ and $(c)$ were synthesized on
298	a flat glass mould. The black arrows in the TEM images indicate surface dangling chains. The
299	plain regions without network structure on the right part of the images (b-d) correspond to the
300	skeleton network.

302	One is fixing the dangling chains to the skeleton network through the inter-network
303	chemical crosslink <sup>[31]</sup> . As illustrated in <b>Figure 4a</b> , the PAMPS network usually contains a few
304	unreacted vinyl groups after polymerization due to partial reaction of the crosslinkers. During
305	the polymerization of the second skeleton network, some PAMPS dangling chains with
306	residual vinyl groups are incorporated into the PDMAAm network. The other is inducing a
307	layer of skeleton network on PAMPS surface during the skeleton network formation in
308	PAMPS. When inducing the neutral skeleton network, a PAMPS network is sandwiched
309	between glass plates. Counter-ion osmotic repulsion between the PAMPS and glass that are
310	both negatively charged in water induces a layer of PDMAAm precursor solution at the
311	interface. As a result, the PAMPS sample surface is covered by a layer of the neutral network
312	of several micrometers-thick after polymerization of the skeleton network <sup>[32]</sup> . In fact, the
313	neutral PDMAAm layer is clearly seen on the topmost surface of the specimen in a TEM

314	image at low magnification (Fig. S6). Therefore, the immersion of the PAMPS gel with the
315	PDMAAm network in water induces the unidirectional swelling of the PDMAAm network
316	surface layer towards the thickness direction since the already highly stretched PAMPS
317	network confines the lateral direction. As a result, the PAMPS strands connected to the
318	PDMAAm network are stretched in the direction vertical to the PAMPS network surface, and
319	we can observe the approximate length of the PAMPS dangling chains.
320	Figure 4b shows the outermost as-prepared surface of a PAMPS-4 hydrogel
321	synthesized on a flat glass mould. Dangling chains of several hundred nano-meter lengths
322	(indicated by the black arrows) in a fully elongated state from the PAMPS network surface are
323	observed. These dangling chain lengths are in the same order as network mesh size in the bulk
324	region. Therefore, dangling chains can be assumed to be strands that could not find a
325	crosslinking partner at one end. In addition, this result indicates that the length of the surface
326	dangling chain of the chemically crosslinked network is comparable to the bulk network mesh
327	size; thus, the length of the dangling chains of the gel surface can be estimated based on the
328	mesh size of the bulk network. It should be noted that only dangling chains that have inter-
329	network crosslinking with the neutral skeleton network are observed as elongated strands.
330	Dangling chains not covalently connected to the skeleton network collapse into a globule
331	conformation by staining. This is clearly seen by the dark rough line on the gel surface,
332	similar to the dangling chains inside the gel (Figure S5b). In addition, no elongated dangling

333	chains were observed on the surface of the PAMPS network that had been treated with the
334	excess amount of initiators to inactivate the residual crosslinkers by radicals (Figure 4c) <sup>[31]</sup> .
335	We also observed the surface of the PAMPS gel cut using a fine microtome knife
336	with an edge thickness of 76 $\mu$ m. We synthesized the skeleton network after cutting the
337	PAMPS gel so that some dangling chains formed by cutting were also connected to the
338	skeleton by residual crosslinkers and elongated by uniaxial swelling of the skeleton network.
339	The cut surface is significantly rougher than the as-prepared surface (Figure 4d). In addition,
340	it has a disordered network structure approximately 1 $\mu$ m deep from the outermost surface,
341	while the mesh structure is maintained in the inner region. The length of the created dangling
342	chains at the cut surface is nearly equal to the bulk mesh size since these dangling chains
343	originated from the mesh. Considering that cutting creates two fracture surfaces, the damage
344	zone has a total depth of approximately 2 $\mu$ m, suggesting that brittle hydrogel is fractured
345	near the surface, despite the cut edge thickness of 76 $\mu$ m. This observation provides molecular
346	information about the relation between the microscale damage zone and macroscopic fracture.
347	
348	2.4. Observation of large size heterogeneity

Characterization of large size heterogeneity and defects is vital in understanding the fracture of the material, as the latter is governed by local defects, not by the average structure<sup>[9,33]</sup>. Particularly, the fracture is always initiated from the largest defect in a material.

352 Here, we applied the developed method to directly observe the large-size defects in the



354



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Figure 5. Micro-scale defects in the bulk PAMPS-4 gel. (a)–(c) Low magnification TEM images at different locations. The white areas reveal the presence of micro-voids with no PAMPS network. (d) High magnification TEM image around a micro-void and the polymer strands are aligned around the void as shown by the illustration.

360

**Figure 5** shows the TEM images of a bulk PAMPS-4 hydrogel with the PDMAAm

362	skeleton network and its schematic illustrations. The PAMPS hydrogel contains many voids
363	of approximately 10 $\mu$ m in diameter (Figure 5a–c). These micro-scale defects are not
364	observed in small microgels with a diameter of several micro-meters (Figure S2). TEM
365	observation allows simultaneous observation of the hydrogel structure at multiple scales.
366	Figure 5d shows a high magnification image of a void and its schematic illustrations.
367	Polymer strands around the void are oriented along the defect circumference, indicating that
368	these strands are highly elongated compared to those 2 $\mu$ m away from the void surface. This
369	result denotes that microscale heterogeneity, such as void, induces nanoscale stress
370	concentration. Note that these micro voids are not always generated, depending on
371	polymerization kinetics and gel composition. The void formation has been predicted for the
372	hydrogels formed by free radical polymerization from monomer and crosslinker of the current
373	study <sup>[34,35]</sup> . During the polymerization, microgels are first formed, then these microgels grow
374	and coalesce to form bulk gels, resulting in voids in the unfilled space of microgels. Hence,
375	the TEM observation confirms that a microgel (Figure S2) is relatively homogeneous and
376	free of large defects, while bulk gels inevitably contain defects, thereby affecting their
377	mechanical properties.
378	
379	3. Conclusion

380 We develop for the first time a method to image the hydrogel networks with nanoscale

381	resolution. This method allows us to directly observe the structure of the polyelectrolyte
382	network from the nanoscale level to the microscale level. In this method, we selectively stain
383	the target network at isovolumic conditions by mineralizing nanoparticles to achieve
384	sufficient electron contrast and high spatial resolution. Although we only reported TEM
385	imaging of hydrogels carrying negative charges, this method could be expanded to the
386	hydrogels carrying positive charges using proper staining conditions. Moreover, it also could
387	be applied to neutral polymers by appropriate chemical modification of side chains. For
388	example, polyacrylamide hydrogel, one of the most common synthetic hydrogels, could be
389	visualized by converting the amide side chain to carboxylic acid by hydrolysis.
390	The molecular structure of the network in bulk and on the surface was unveiled for the
391	first time in real space. We clarified the presence of dangling chains on the surface of the as-
392	prepared gels with a length scale the same as the mesh size. These results are essential in
393	understanding the surface properties of hydrogels, including surface wetting-dewetting,
394	sliding friction, adhesion, and bonding. Furthermore, numerous micro-scale defects are
395	observed, around which polymer strands are aligned. These results are essential in
396	understanding the origin of the brittleness of hydrogels. This direct imaging method provides
397	rich information for understanding the underlying mechanism for many physical behaviors of

### 400 4. Experimental Methods

Materials: The 2-acrylamido-2-methyl propanesulfonic acid (AMPS) monomer was 401 provided by Toagosei Co. Ltd, Japan. The dimethylacrylamide (DMAAm) monomer, N, N'-402 403 methylenebisacrylamide (MBAA) crosslinker, 2-oxoglutaric acid ( $\alpha$ -keto) initiator, iron (III) 404 chloride hexahydrate and 2-morpholinoethanesulfonic acid monohydrate (MES) buffer were 405 purchased from Wako Pure Chemical Ind., Ltd., Japan. Osmium tetroxide (OsO4) was 406 acquired from TAAB Laboratories Equipment Ltd., England. DMAAm monomer was 407 purified by reduced pressure distillation before usage. The other chemicals were used as 408 received.

409 Network sample synthesis: The PAMPS network was synthesized from precursor 410 solution comprised of 1 M AMPS as the monomer, 1-8 mol% MBAA as the crosslinker, and 411  $0.1 \text{ mol}\% \alpha$ -keto as the initiator in water. Each mol% is relative to the corresponding 412 monomer. The precursor solution was poured into a mould made of two glass plates separated 413 with a 2 mm thick silicone spacer, and then was irradiated by UV for 8 h in a chamber filled 414 with inert Ar gas. Then, the plate-shaped PAMPS gel was removed from the mould and 415 immersed in water. The polyelectrolyte gel significantly swelled in water and the sample 416 thickness swelling ratio  $\lambda_s$  in relative to its state at synthesis was 4.5 - 1.7 (Supplementary 417 **Table S1**) for crosslinker ratio Cx of 1 - 8 mol%. The corresponding PAMPS polymer volume 418 fraction was 0.2 - 3.8 vol.%. The samples were coded according to C<sub>x</sub> as PAMPS-C<sub>x</sub>. 419 To induce the skeleton network, the PAMPS gels removed from the mould were 420 directly immersed in PDMAAm network precursor solution for 1 day until reaching 421 equilibrium. The precursor solution of PDMAAm network was comprised of 2 M DMAAm 422 monomer, 0.1 mol% MBAA, and 0.1 mol% α-keto in water. The PAMPS gel, substantially swelled in the PDMAAm network precursor solution, was sandwiched between two glass 423

424	plates and irradiated with UV for 6 h in Ar atmosphere to form PDMAAm skeleton network
425	inside the target PAMPS network <sup>[21]</sup> . The obtained samples were immersed in distilled water
426	for one week to completely remove residual chemicals. All glass plates used in the moulds
427	were heated in a 500 °C oven for 1 h to remove any residual organics before use.
428	PAMPS-4 microgels and supermacroporous PAMPS-0.5 gel were also prepared by
429	suspension polymerization and cryogelation, respectively, using the protocols reported. <sup>[26,27]</sup>
430	The mesh size of the supermacroporous PAMPS-0.5 gel is less than that of conventional
431	PAMPS-0.5 gel since the concentration of the precursor solution were increased by ice
432	formation during freezing in the cryo-gelation. The PDMAAm network in these gels was
433	introduced using the protocol described above.
434	Average mesh size estimation from bulk modulus: The average mesh sizes of the PAMPS
435	network and the PDMAAm network was estimated from their bulk Young's modulus according
436	to rubber elasticity theory. If we assume affine model, the Young's modulus $E_0$ of the network
437	samples at the as-prepared state is related to the density of elastically effective strands $v_0$ as
438	follows <sup>[28]</sup> .

439 
$$E_0 = 3v_0 k_B T = 3k_B T / \xi_0^3$$
 (1)

440 where  $k_B$  and T are Boltzmann constant and temperature, respectively, and  $\xi_0$  is the mesh size 441 at the as-prepared state. Here, it is assumed that each elastically active strand has energy of  $k_B T$ 442 in the as-synthesized state. The average mesh sizes  $\xi$  of the PAMPS network and PDMAAm 443 network in TEM observation state were estimated from the thickness ratio  $\lambda_s$  of the resin-cured 444 sample relative to their corresponding as-prepared states. Assuming the affine deformation, the 445 polymer network mesh size was increased by a factor of  $\lambda_s$  in comparing with that in their as-446 prepared state.

447 
$$\xi = \lambda_s \times \xi_0$$
 (2)

The compressive stress-strain curves of the PAMPS single network gel and PDMAAm single network gel in their as-prepared state were measured 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<sup>-1</sup>.  $E_0$  was estimated from the initial slopes of the stress-strain curves. Each test was performed on five samples.

Mineral staining: For mineral staining, the PAMPS hydrogels with PDMAAm skeleton 453 were immersed in a staining aqueous solution of 2.5 M FeCl<sub>3</sub> for 1 day at 25 °C. Subsequently, 454 the gels were immersed in pure water or 0.1 M MES buffer solution at 25 °C to increase pH 455 and amorphous ferric oxide (AFO) nanoparticles were mineralized on the PAMPS network. For 456 TEM observation under vacuum condition, the water in the hydrogels must be removed without 457 causing structure change of the target network. For this purpose, we adopted the established 458 resin substitution process for preparing TEM specimen of biological tissues.<sup>[17]</sup> The mineralized 459 460 hydrogels were immersed in ethanol to replace water with ethanol. The ethanol was then step wisely replaced by resin precursor of acrylic monomer liquid (London Resin white, medium), 461

462	and finally the samples were heated at 55 °C to obtain solidified resin in the chamber of an
463	automatic freeze substitution system (EM AFS2, Leica Microsystems, Germany). For all
464	PAMPS samples with different formulations, the final thickness changes of the resin-cured
465	specimen are within 10% relative to that of the PAMPS samples in water (Figure 1c, Table S1).
466	Then, 100 nm thick resin-cured specimens were cut using an ultra-microtome knife (EM UC7i,
467	Leica Microsystems, Germany) and then placed on a carbon-supported copper mesh grid for
468	TEM observation. We confirmed that the conventional electron stain (Osmium (VIII) oxide)
469	that labels one carbon-carbon double bond with one heavy atom could not provide enough
470	contrast to the sparse and extremely thin network of PAMPS hydrogels in TEM observation.
471	Transmission electron microscopy (TEM) observation: 2D TEM observations (H-7650,
472	Hitachi, Japan) were performed at an acceleration voltage of 100 kV of the electron gun. 3D
473	TEM <sup>[12,14]</sup> and energy dispersive X-ray spectrometry (EDS) were performed using a TEM
474	tomographic system (JEM-1400 and EM-05500TGP, JEOL, Japan) and a scanning transmission
475	electron microscope (STEM, JEM-F200, JEOL, Japan). The acceleration voltage of the electron
476	guns was 120 and 200 kV, respectively.
477	TEM image analysis: The total apparent polymer length in the 2D TEM images of PAMPS-

binarized and then skeletonized and the total pixels were measured in the images of fivedifferent places of the sample. Because TEM image shows projection of three-dimensional

478

1 samples was estimated using ImageJ software<sup>[36]</sup> (Figure S5). The original TEM images were

object into two-dimensional plane, we corrected the 2D apparent polymer length to 3D length. 481 When a three-dimensional straight line is projected in 2D, the 2D apparent length is the original 482 length multiplied by  $\cos\theta$ , where  $\theta$  is the angle of the line with respect to the projection plane. 483 In the view field, there are polymers with various directions, 0° to 180°. The integral value of 484  $\cos\theta$  in the range of 0° to 180° is  $2/\pi$ . Thus, 2D apparent polymer contour length in the viewed 485 field is related to the 3D length as, 486  $L_{3D} = (\pi/2)L_{2D}$ 487 (3) 488 Estimation of theoretical contour length in TEM image: The theoretical network strand contour length L was calculated from the AMPS concentration  $C = C_0 / \lambda_s^3$  in the specimen after 489 490 resin exchange, where  $C_0$  is the PAMPS monomer concentration at synthesis (The conversion

491 ratio of monomers in synthesis was close to 100%). The total contour length of PAMPS chain

492 in a TEM view volume V is

493 
$$L = b C N_A V = b C_0 N_A V / \lambda_s^3 \qquad (4)$$

Here *b* is the monomeric length of PAMPS, and  $N_A$  is the Avogadro number. For PAMPS-1 sample, the swelling ratio from its as-prepared state  $\lambda_s$ =4.5 and *b*=0.25 nm. In this experiment, the view field has a volume of *V*=896×896×100 nm<sup>3</sup> = 8.0×10<sup>-20</sup> m<sup>3</sup>. Thus, the theoretical network strand contour length, including the loops and dangling chains, is estimated as *L*=1.3 ×10<sup>5</sup> nm.

499 *A simple model to estimate contour length from cut-off length of stained polymer:* Since

the polymer strands could collapse at a length scale smaller than the cut-off length  $\xi_c$  by mineral 500 501 staining, we need make a correction of this effect to estimate the total true contour length from the total apparent chain length obtained from TEM images. Denoting  $N_{sub}$  as the Kuhn monomer 502 number of a subsection of the strand with the end-to-end distance  $\xi_{sub}$  equal to cut-off length  $\xi_c$ 503  $(\xi_{sub} = \xi_c)$  before staining, the contour length of such subsection of strand  $L_{sub} = b_K N_{sub}$ , where  $b_K$ 504 is Kuhn length of the polymer at the as-prepared state. On the other hand, the corresponding 505 end-to-end distance of the subsection strand at the as-prepared state  $\xi_{sub0} = \xi_{sub}/\lambda s$  assuming 506 affine deformation by swelling. Since water is good solvent for PAMPS ( $\chi = 0.30 - 0.35$ )<sup>[37]</sup>, 507  $\xi_{sub0} = b_K N_{sub}^{3/5}$ . Hence, the fraction of shrinkage 508  $L_{sub}/\xi_{sub} = (\xi_c/\lambda sb_K)^{2/3}/\lambda s \quad (5)$ 509 Using  $\lambda s=4.5$ ,  $\xi_{sub}=\xi_c=40$  nm, and  $b_K=0.5$  nm<sup>[38]</sup>, we have  $L_{sub}/\xi_{sub}=1.4$  for PAMPS-1 sample. 510 Therefore, a correct factor of 1.4 should be considered to get the true contour length from the 511 stained contour length of TEM. 512 513 514 **Supporting Information** Supporting Information is available from the Wiley Online Library or from the author. 515 516 Acknowledgements 517



519	results. R.K. performed most experiments and analysed the data. M.Y. performed TEM			
520	observation for the gels with different crosslinker ratios. T.S. prepared the porous gel. H.J.			
521	performed STEM measurements. All the authors participated the discussion. R.K., T.N. and			
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530				
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#### 590 **Table of Content**

- Direct observation of the nanoscale polymer network of hydrogels is essential in 591
- understanding its properties. Herein, for the first time, we developed a novel staining and 592
- structure fixation method for TEM observation to visualize the hydrogel network architecture 593
- in its unperturbed conformation with nanometer resolution. The revealed molecular structures 594
- 595 on surface and bulk hydrogels provide important insights for the friction, adhesion, and fracture of hydrogels.
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- 597
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- 600

#### Nanoscale TEM Imaging of Hydrogel Network Architecture 601

