Gigantic Multilayered Vesicle-Like Hydrogel-Bilayer Composites

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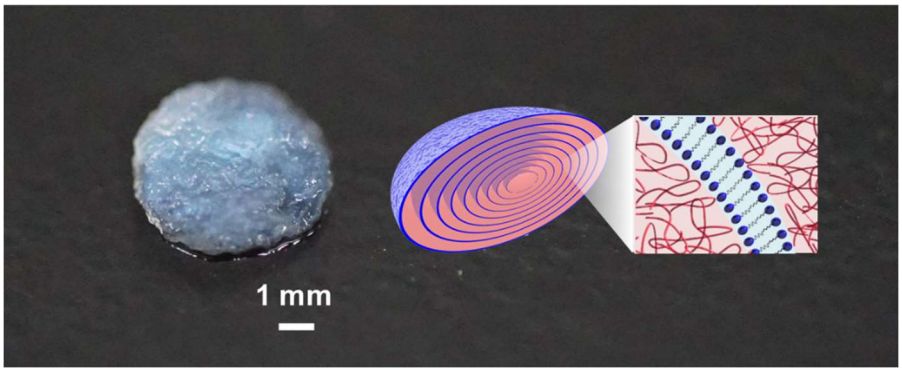
Abstract

Preparation of large vesicles with high stability is generally difficult. Here, we report the millimeter-scale multilayered vesicle-like hydrogel-bilayer composites with long-term stability through stabilization of the vesicular structure by polymerization of the lipid molecules and gelation of the inner solution. The well-oriented multilayered structure was examined by various methods. Reflecting the multilayered structure, the resulting vesicle-like composites exhibited extremely slow drug release behavior.

Keywords: vesicle, hydrogel, diffusion

Graphical abstract



Vesicles are capsule-like supramolecular assemblies formed by single or multilayered bilayers of amphiphilic molecules.[1,2] Vesicles have attracted much attention as substances for encapsulation, transportation, and selective release of molecules in liquid environments. Vesicles have also been applied to cell modeling and membrane protein anchoring.[3,4] The size of vesicles varies widely depending on the preparation methods and their chemical species.[5] While relatively small vesicles with a diameter of $<10^{-5}$ m have been widely prepared, the preparation of stable giant vesicles with larger diameters often faces technical issues because of their structural instability. Especially for millimeter-scale ones, the reported gigantic vesicles are unstable; they have short lifetimes and are extremely difficult to handle.[6,7] On the other hand, some methods have been reported to improve the structural stability of vesicles. One is the polymerization of lipids after the vesicle formation to stabilize the lipid bilayers.[8] Another is the gelation of the solution inside the vesicles to fix the shape of the vesicles.[9,10]

Here, we succeeded in preparing stable gigantic multilayered vesicle-like materials using the two stabilization methods mentioned above. The term *vesicle-like* instead of *vesicle* is because the lipid membranes of the materials would have many defects. We adopted the PDGI/PAAm gels to prepare such vesicle-like materials. PDGI/PAAm gels are gel-lipid bilayer composites consisting of multilayered poly(dodecyl glyceryl itaconate) (PDGI) lamellar lipid bilayers embedded in the polyacrylamide (PAAm) hydrogel matrix.[11–13] PDGI/PAAm gels are prepared from their precursor solution, which is the aqueous solution of DGI as polymerizable lipid with trace sodium dodecyl sulfate (SDS) as co-lipid, AAm as monomer, *N,N'*-methylene(bis)acrylamide as cross-linker, and 1-

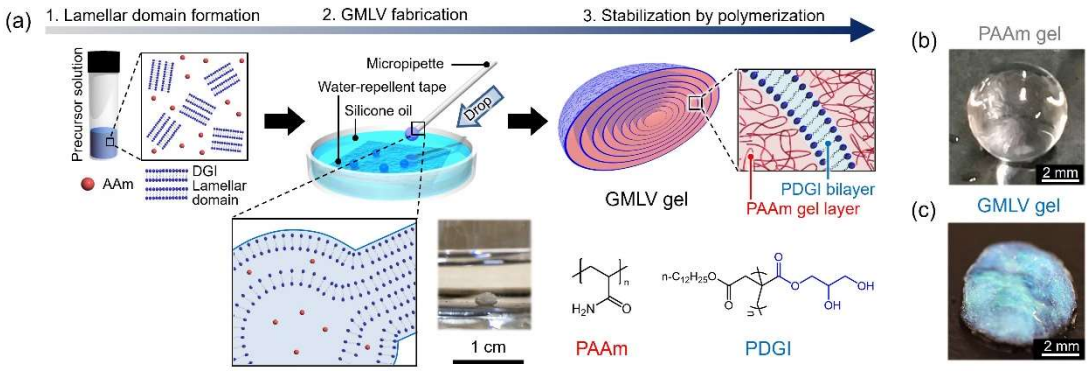


Fig. 1. (a) The preparation procedure of the gigantic multilayered vesicular PDGI/PAAm gel (GMLV gel). (b-c) The appearance of the PAAm and GMLV gels swollen in water

[4-(2-hydroxyethoxyl)-phenyl]-2-hydroxy-methylpropanone as photoinitiator. In the precursor solution, DGI (and SDS) assembles into the polydomain lamellar structure at a certain DGI concentration (1–2 wt%) above Kraft point of DGI (43 C°).[11,14] Electrostatic repulsion between the bilayers, originating from the ionic co-lipid, works to form the periodic lamellar structure. If strong mechanical shear is applied to the precursor solution, the random, polydomain lamellar structure of the DGI turns into the macroscopically well-ordered, monodomain lamellar structure.[15] By starting UV irradiation to the precursor solution within the lifetime of the monodomain structure (~10 min),[16] self-assembled DGI and the polymer network precursors (AAm and crosslinker) are independently polymerized to form the PDGI/PAAm gels.[17] The well-ordered orientation of the DGI in the precursor solution is stabilized in the PDGI/PAAm gels due to polymerization of the lipid itself for reduction of flowability of the lipid bilayer, chemical cross-linking of the PAAm network for immobilization of the whole system, and hydrogen bonding between the PAAm network and the PDGI bilayers for further stabilization.[18] We have prepared the PDGI/PAAm gels with sheet-like or cylindrical shapes with a well-ordered structure.[15,19] The obtained gels exhibit bright structural color,[13] high toughness,[20] controlled diffusions,[19] and anisotropic mechanical properties and deformations.[19,21]

In this work, we adopted this system to create gigantic multilamellar vesicle-like (GLMV) PDGI/PAAm gels. The preparation flow is shown in Figure 1(a), and the detailed experimental methods are shown in Supplementary Material. The water-repellent tape was put on the bottom of a glass petri dish filled with silicone oil. 30 μl of the precursor solution of the PDGI/PAAm gel was sucked by a micropipette and dispensed on the water-repellent tape in the silicone oil. During the dispensing process, the shear deformation of the solution against the tip wall generates a strong shear force on the solution. The precursor solution formed a spheroidal droplet on the water-repellent tape. The ratio of the minor to the major axes of the spheroid was about 3:10. By immediate 365 nm UV irradiation to the spheroidal droplets in the silicone oil, DGI and the gel precursors are independently polymerized in the droplets to form the spheroidal PDGI/PAAm gels (called GMLV gels). The GMLV gels were washed with isopropanol to remove the silicone oil. As a control, the spherical PAAm gel was prepared with the same synthesis procedure from the PAAm gel precursor solution without any lipids. After the synthesis, both gels were immersed in pure water. The obtained PAAm gel was almost spherical (Figure 1(b)), while the GMLV gel was spheroidal (Figure 1(c)). The spheroidal shape of the GMLV gel would be because of the slight wetting of the GMLV precursor solution on the water-repellent tape due to the presence of lipids. The swollen GMLV gel exhibited the bright blue structural color that originates from the periodic lamellar structure of PDGI in the gel. The structural color of the GMLV gel having the curved bilayers is angle-independent, while that of sheet-like PDGI/PAAm gels with the flat bilayers depends on the observation angle (Figure S1). The GMLV gel exhibited long-term stability, maintaining its structure color for at least 2 months.

For structural analyses, the swollen GMLV gel was sliced along the short axis at the center. Figure 2(a) exhibits the 2-dimensional small-angle X-ray diffraction (SAXD) pattern of the sliced GMLV gel near the edge, and Figure 2(b) shows the 1-dimensional profile extracted from the 2-D pattern. The strong diffraction spots with higher-order ones were observed in the 2-D pattern, corresponding to the radial orientation of the periodic PDGI lamellar bilayers. The ratios of the diffraction peaks are 1:2:3:4:5, suggesting the lamellar structure with the layer spacing of 183 nm. The appearance of the 5th-order peak suggests the well-ordered PDGI lamellar bilayers. Figure 2(c) shows the cross-sectional polarized optical microscope images of the GMLV gel with and without the 530 nm tint plate. A distorted Maltese cross pattern corresponds to the radial orientation of the PDGI bilayers in the gels. Figure 2(d) shows the cross-sectional scanning electron microscope image of the GMLV gel dried in air. The layered structure oriented along the gel surface was observed, corresponding to the large-scale orientation

of the PDGI bilayers. These results support the formation of the onion-like multilamellar vesicular structure. Under the assumption that the periodic PDGI bilayers homogeneously exist in the GMLV gels, the layer spacing of 183 nm corresponds to ~5,000 bilayers along the short axis of the GMLV gels.

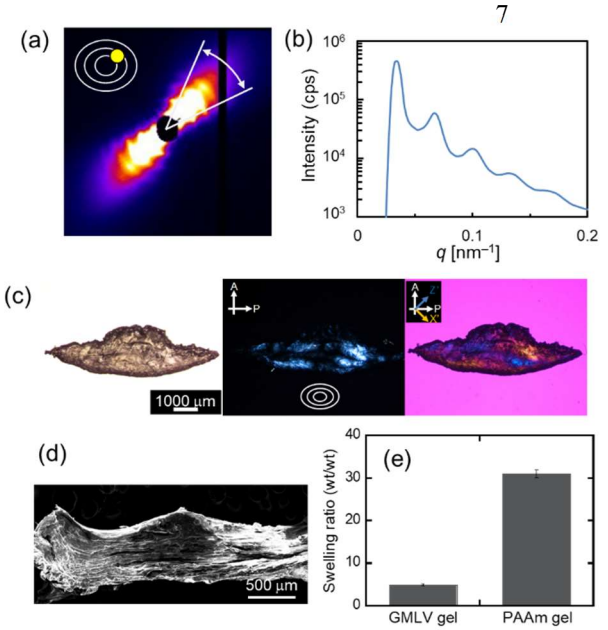


Fig. 2. (a) 2-D X-ray diffraction pattern of the cross-section of the GMLV gel. The region for integration into 1-D profile is shown by white lines. (b) The 1-D diffraction profile. (c) Polarized optical microscope images of the GMLV gel. From the left are the images of open Nicol, cross Nicol, and cross Nicol with the tint plate. (d) Scanning electron microscope image of the GMLV gel dried in air. (e) Equilibrium swelling ratio of the GLMV and PAAm gels in water.

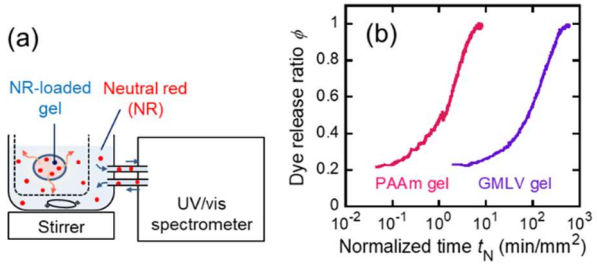


Fig. 3. (a) The setting of the dye diffusion test. NR concentration as a function of time was determined to obtain the diffusion ratio of NR. (b) The dye release ratio of the GMLV and PAAm gels as a function of normalized time, which is the time divided by the square of the short axis of the gel

Next, the GMLV and PAAm gels were dried and immersed in pure water to examine their swelling behavior. As shown in Figure 2(e), the equilibrium swelling ratio of the GMLV gel was smaller than that of the PAAm gel. The equilibrium swelling state of gels is generally determined by the balance of osmotic pressure and elasticity.[22] Since the GMLV and PAAm gels originally contained almost the same concentration of PAAm, their osmotic pressures at the same swelling ratio should be comparable. On the other hand, the elasticity of the PAAm gel is the rubber elasticity of the PAAm network, whereas that of the GMLV gel is the sum of the rubber elasticity of the PAAm network and the membrane + rubber elasticities of the PDGI bilayers.[23] The swelling (volume increase) of the GMLV gels must accompany the area expansion of the multilamellar bilayers. Such area expansion of the bilayers is energetically unfavorable and accompanies a remarkable increase in their elastic energy, which effectively suppresses the swelling of the GMLV gels. Regarding this, surface unevenness of the swollen GMLV gels was observed, while the as-prepared GMLV gels do not have such unevenness. As discussed above, the PDGI bilayers in the swollen GMLV gel always feel strong swelling pressure of the PAAm matrix. Thus, if there are some defects on the bilayers, the swelling pressure concentrates on the defects and expands them, which would lead the surface unevenness.

Finally, to investigate functions of the GMLV gels, their drug release properties are examined. The GMLV and PAAm gels were immersed in an aqueous solution of neutral red (NR), which is a common water-soluble dye, as a model drug for at least 2 days to fully load NR (Figure S2(a-b)). The drug-loaded gels were then immersed in a fixed volume of pure water, and the ratio of NR released from the gels was quantified as dye release ratio $\phi = c/c_\infty$, where c is the dye concentration of the external solution at the time of interest and c_∞ is c after reaching a plateau (Figure 3(a)). If the drug release is diffusion-dominant, the characteristic diffusion time is proportional to the square of the system size. Therefore, to normalize the differences in the size of GMLV and PAAm gels, the normalized time t_N, which is the time t divided by the square of the short axis of the sample l, was used as the effective time. The time-dependent dye release ratio is shown in Figure 3(b). The results show that the GMLV gel has an extremely longer normalized drug release time than the PAAm gel, suggesting the multiple PDGI bilayers effectively suppressed the diffusion of NR from the gel.

To investigate whether the diffusion rate of NR from the GMLV gel is controllable, we changed the cross-linking density of the PAAm network in the GMLV gels. The GMLV gels with varied cross-linker concentrations, 0.01, 0.05, 0.1, and 0.5 mol% to AAm (c_{link}), were prepared. The equilibrium swelling ratio of the gels decreased with increasing c_{link} (Figure 4(a)). A negative correlation was found between the dye diffusion rate and swelling ratio for the GMLV gels (Figure 4(b)). The results show that the diffusion rate of NR in the GMLV gels remarkably changes with very small changes in their swelling ratio. The PDGI bilayers in the swollen GMLV gels may have defects, acting as

channels for the diffusing substance (Figure 4(c)). The PDGI bilayers in the swollen GMLV gels are stretched, causing the formation and expansion of the defects. The diffusion rate of a substance increases with increasing number and size of the defects. Thus, the diffusion rate of NR from the GMLV gels increased with the swelling ratio (decreased with the cross-linker concentration). This suggests that the drug release rate of the GMLV gel can be controlled by the crosslinking density of the PAAm network; the denser crosslinking suppresses the defect formation and expansion, leading to prolonged drug release from the GMLV gels.

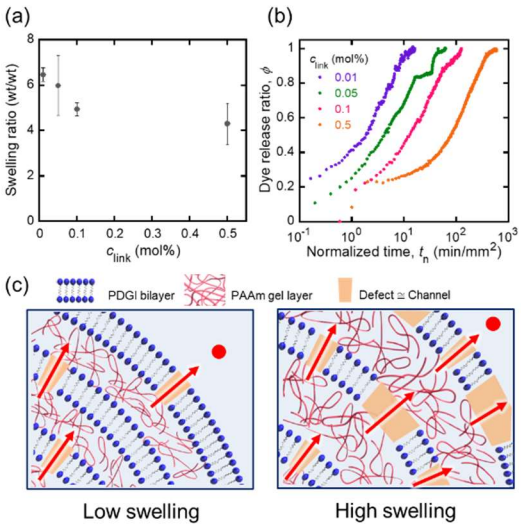


Fig. 4. (a) Swelling ratio of the GMLV gels with different molar ratios of the cross-linker to monomer, c_{link}. (b) Dye release ratio from the GMLV gels with a varied c_{link}. (c) Estimated pictures of the swelling ratio-dependent diffusion. The larger swelling ratio leads to larger and more defects as channels for diffusion.

In conclusion, the spheroidal multilayered vesicle-like hydrogel-bilayer composites with millimeter scale and long-term stability have been successfully prepared. The multilayered vesicular structure was stabilized by polymerization of the lipids and gelation of the inner solution after formation. Because of its multilayered structure, the composites show controlled release of the encapsulated substance. One of the drawbacks of this study is that the resulting vesicle is not spherical. The droplets of the precursor solution on a water-repellent tape were spheroidal due to the wetting. The spherical GMLV gels would be obtained by floating the precursor droplets in an oil with a density gradient. Another drawback is defects in the bilayer membrane. While the swelling-induced defects would contribute to the controlled release, such defects may impede the use of the GMLV gels as molecular containers. For defect-free composites, the swelling ratio needs to be suppressed by further increasing c_{link} or decreasing the PAAm concentration.

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Supplementary data

Supplementary material is available at *Chemistry Letters*

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Conflict of interest statement. None declared.

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Supplementary Material for

Gigantic Multilayered Vesicle-Like Hydrogel-Bilayer Composites

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Materials

Itaconic acid anhydride (Sigma Aldrich), dodecanol (Wako Pure Chemical Industries, Ltd. (Wako)), glycidol (Wako), pyridinium *p*–toluenesulfonate (Wako), *N,N'*-methylene(bis)acrylamide (Wako), 1-[4-(2-hydroxyethoxyl)-phenyl]-2-hydroxy-methylpropanone (Sigma Aldrich), sodium dodecyl sulfate (Wako), neutral red (NR) (Wako) were used as received. Acrylamide (AAm) (Wako) was used after recrystallization with chloroform.

Synthesis of DGI

DI (dodecyl itaconate) was synthesized as a precursor of the polymerizable surfactant DGI as reported in ref.11 of the main text. Itaconic acid anhydride (50.1 g, 0.44 mol) was mixed with dodecanol (80.0 g, 0.43 mol) and heated in an oil bath at 110-120°C for 50 minutes under an argon atmosphere to obtain DI. After the reaction, 100 ml of hexane was added to the solution, and the mixture was stirred for 5 minutes. The solution was then filtered to obtain crude DI crystals. The crude DI was purified twice by recrystallization with ethanol.

The obtained DI (20 g, 0.067 mol), toluene (20 ml), glycidol (15 g, 0.20 mol) and 40 mg of pyridinium *p*-toluenesulfonate as a catalyst were mixed, and the mixture was heated in an oil bath at 100 °C under an argon atmosphere for 5 hours to obtain the crude DGI (dodecyl glyceryl itaconate). After the reaction, toluene was removed from the solution by concentration under reduced pressure, and the DGI was then purified by silica gel column chromatography using hexane and ethyl acetate 1:1 mixture. The obtained DGI was recrystallized twice with acetone and hexane 1:1 mixture for further purification.

Synthesis of the GMLV gels and the PAAm gels

The gel precursor solutions were prepared by addition of DGI (0.1 M), AAm (2 M), *N,N'*-methylene(bis)acrylamide (0.2, 1, 2 or 10 mM, 2 mM was used unless specially mentioned), 1-[4-(2-hydroxyethoxyl)-phenyl]-2-hydroxymethylpropanone (2 mM) and SDS (0.025 mM) to pure water. The solutions were kept in a water bath at 55°C for 5 hours to form DGI lamellar structure. Subsequently, the water-repellent tape (Toyal Lotus aluminum tape, Toyo Aluminum) was put on the bottom of a glass petri dish. The dish was then filled with silicone oil (SRX310, Dow) and kept in a

glove box at 55°C for at least 2 hours to remove the dissolved oxygen from the oil. 30 μL of the gel precursor solution was dropped into the petri dish to form a spheroidal droplet in the silicone oil in a globe box. 365 nm UV light was then irradiated to the petri dish to induce the separate radical polymerization of the lipid precursor (DGI) and the polymer network precursor (AAm and the cross-linker) inside the droplet to obtain the spheroidal PDGI/PAAm gels (GMLV gels). As control samples, the spheroid PAAm gels without containing DGI were synthesized in the same way. The obtained GMLV and PAAm gels were washed three times with isopropanol and then three times with pure water to remove any unreacted reagents and the silicone oil. In addition, a sheet-like PDGI/PAAm gels were prepared according to ref.15 of the main text from the same gel precursor solutions.

Small angle X-ray diffraction measurements:

Periodic PDGI bilayers in the GMLV gels were evaluated by synchrotron small-angle X-ray diffraction test at BL05XU beamline in SPring-8 (Hyogo, Japan). X-ray wavelength and the camera length were 1 Å and 4 m, respectively. The GMLV gel swollen in pure water were sliced at the center to obtain the gel slices (thickness: ~1 mm). The sliced GMLV gel was irradiated with X-rays for 5 seconds to obtain a two-dimensional X-ray diffraction image. The 2-D images were analyzed using fit2d software to prepare the corresponding one-dimensional scattering profiles. The distance between the adjacent PDGI bilayers in the GMLV gels, d, was calculated from the first order peak of the scattering vector, q, extracted from the one-dimensional profiles by $d = 2\pi/q$.

Microscope observations

The GMLV and PAAm gels swollen in pure water were sliced at the center to obtain the gel slices (thickness: ~1 mm). For polarizing microscopic observation, the gel slice was placed on a glass plate and observed using a polarized light microscope (Nikon Eclipse LV100POL) under crossed Nicol at 25° C. Orientation direction of the PDGI bilayer was determined by the POM observation with a 530 nm tint plate. For scanning electron microscopic observation, the gel slices were dried in an ambient condition for more than 12 h. The dried gel slices were observed using a scanning electron microscope (JSM-6010LA, JEOL).

Swelling ratio measurement

The swelling ratio of the gels were determined based on the weight. The as-prepared gels were immersed into isopropanol, which is poor solvent for both GMLV and PAAm gels, for 24 h to remove solvent from the gels to prepare the dry gels. The dry gels are then immersed into pure water to obtain the wet gels for the desired time t. The swelling ratio of the wet gels were defined as $w(t)/w_0$, where w is weight of the wet gel at time t and w_0 is that of the dry gel.

Dye introduction/release measurements

For the dye introduction, the GMLV or PAAm gel swollen in pure water was immersed in a 0.1 mM neutral red (NR) aqueous solution with volume V_s. V_s was set to 30 times in comparison with the gel volume, V_g. Note that V_g did not change significantly during the immersion. The absorption density of NR in a gel, c_g, was estimated by the following equation;

$$c_g = \frac{V_s(c_b - c_a)}{V_g}$$

where c_b and c_a are NR concentration of the solution before and after gel immersion, respectively. c_b and c_a were determined from the peak height in the absorption spectra of the NR solution with the UV/vis spectrometer (Shimadzu UV-1800).

For the dye release measurement, the GMLV or PAAm gel was immersed in excess NR aqueous solution (0.1 mM) for a week to introduce the dye molecules in the gel. Then, the gel containing dye was covered by a Teflon net for protection from the rotating stirrer tip and immersed in a glass bottle filled with 100 mL of pure water. The water was continuously stirred and circulated with the measuring cell set in the UV/vis spectrometer (UV-1800, Shimadzu) using the peristaltic pump (AC-2110II-2, AS ONE), which enables the real-time UV/vis spectrum measurement of the solution. Ratio of the released NR from the gel to the solution, ϕ, is determined as

$$\phi = \frac{c(\mathrm{t})}{c_\infty}$$

where c is the NR concentration of the solution at time t, where t=0 is set when the gel was immersed in water.

For the GMLV gels with c_{link}=0.01 and 0.5 (mol%), the diffusion measurement was performed once. For those with c_{link} =0.05 and 0.1 (mol%), the two independent experiments were performed for each composition, and one representative data for each is shown in Figure 4(b) of the main text. All the data are shown in Figure S3.

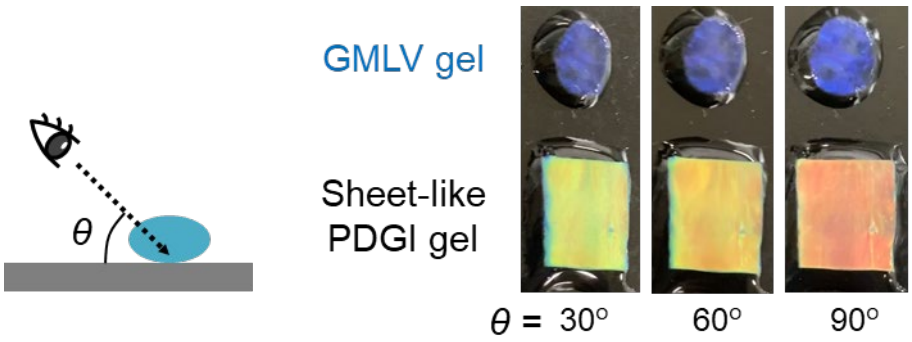

Figure S1. Appearance of the GMLV gel and the sheet-like PDGI/PAAm gel observed from various observation angle θ.

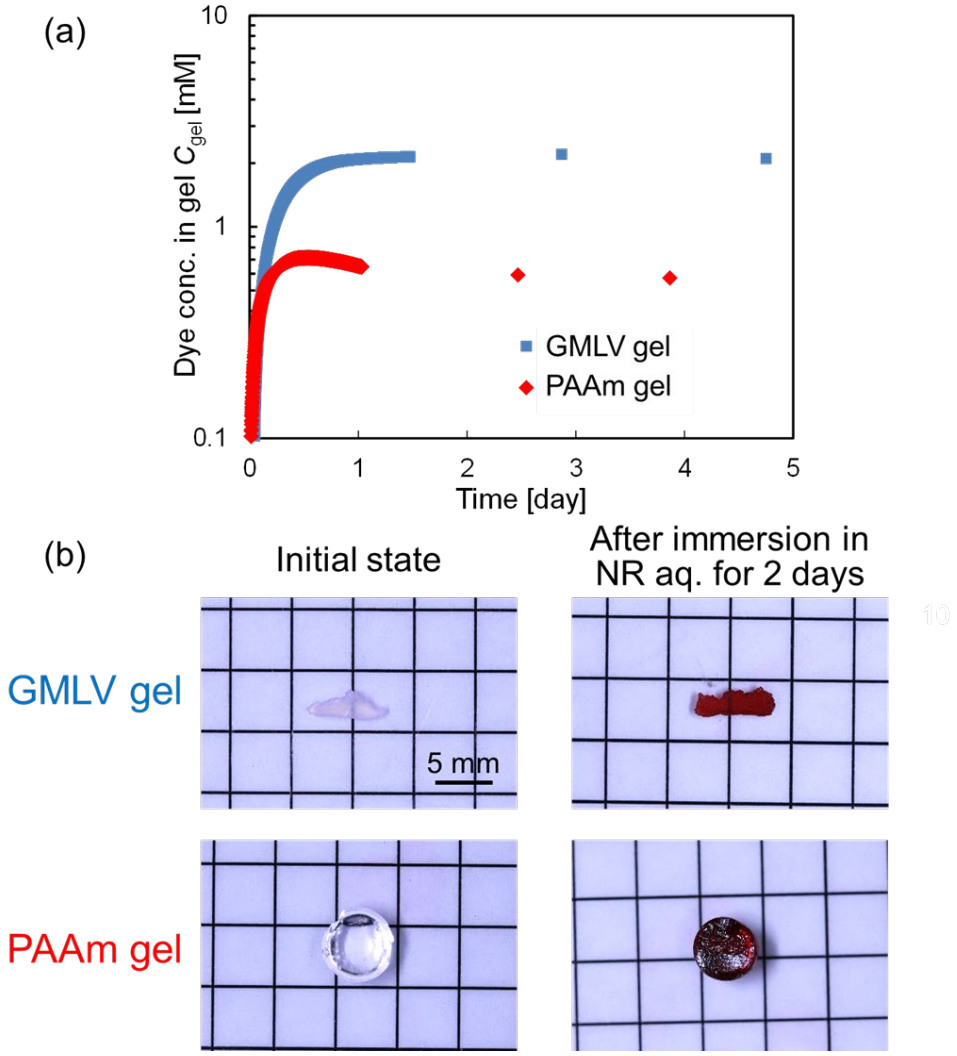

Figure S2. (a) Concentration of NR in the GMLV and PAAm gels as a function of the time after immersion into 0.1 mM NR aqueous solution. After 2 days, the NR concentration of each gel reached plateau. (b) Appearance of the GMLV and PAAm gels before and after immersion into the NR aqueous solution for 2 days.

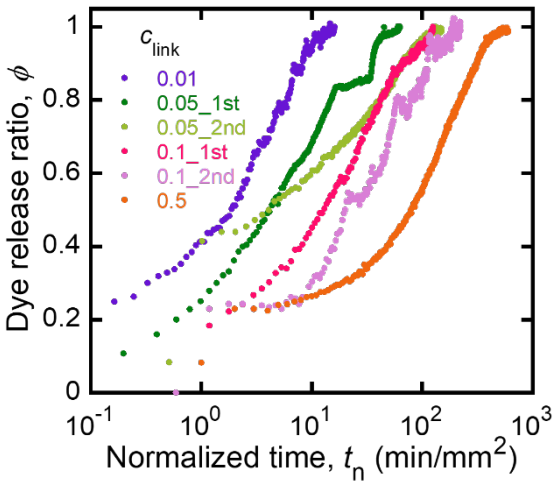

Figure S3. The dye release test results for all the samples.