Lipid Membrane Effect on the Elasticity of Gelatin Microgel Prepared inside Lipid Microdroplets

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In a previous study (Sakai A., et al., ACS Cent. Sci., 4, 477 (2018)), a spherical microgel of gelatin prepared inside a lipid droplet was reported to have a higher surface elasticity than the bulk gel. In this study, we investigate the role of contact or lack of contact between gelatin and the lipid membrane as well as the micrometric confinement to isolate the dominant cause of this higher elasticity of microgels. For our experiment, we prepared a concave microgel of gelatin with two surfaces, with one surface in contact with the lipid membrane and the other without being in contact with the membrane. Next, we measured the elasticities of both the surfaces by using micropipette aspiration. Although the elasticity of the surface not in contact with the lipid membrane was slightly lower than that of the surface in contact with the membrane, the elasticity value was much higher than that for the bulk gel. Further, it was found that the droplet confinement without lipids did not decrease the elasticity of gelatin microgels. These results demonstrate that the dominant factor responsible for the higher elasticity of gelatin microgels is micrometric confinement and not their contact with the lipid membrane.

Key Words: Janus microgel / Emulsion polymerization / Biopolymer hydrogel / Micropipette aspiration / Phase separation

1. INTRODUCTION

Micrometer-sized gels (microgels) of biopolymers such as gelatin have extensive applications as food, cosmetics, and biomedical materials¹,² because of their biocompatibility, biodegradability, and fast response to external environments³-⁵. Basically, functional microgels have been developed based on physicochemical properties of bulk gels. However, recent reports have revealed that “single” microgels have different properties from the bulk gels⁶-¹⁰. Thus, the elucidation of single microgel properties is indispensable for further functionalization of microgels.

In a previous study, we have reported that a spherical microgel of gelatin prepared inside a lipid droplet was reported to have a higher surface elasticity than the bulk gel prepared by using micropipette aspiration⁶. That finding raised the question of whether this higher surface elasticity was derived from the contact between the lipid membrane and gelatin or micrometric confinement inside the small droplet. To answer this question, we need to prepare a gelatin microgel with one surface in contact and the other not in contact with the lipid membrane and then measure the elasticity of each surface. If the interaction between gelatin and the membrane is the dominant factor, the difference between the elasticities of the two surfaces is expected to be large. Conversely, if the confinement is the dominant factor, the difference between the two elasticities is expected to be small.

In this study, we prepared microgels with two surfaces, with one surface in contact and the other not in contact with the lipid membrane, by using a binary polymer solution of gelatin and poly(ethylene glycol) (PEG) confined inside di-oleoylphosphatidylcholine (PC) droplets as reported previously¹¹. With a decrease in the temperature, the gelatin/PEG solution inside the PC droplet proceeds with phase separation, and the partially wet gelatin-rich phase on the PC membrane turns into a concave microgel. By using micropipette aspiration⁸,¹⁰,¹²,¹³, we measured the local elasticities for the convex and concave sides, where the surfaces were in contact and not in contact with the membrane, respectively. Experimental results show that the elasticities of both surfaces are much higher than that of the bulk gels, which strongly suggests that micrometric confinement of gelatin is more dominant than microgels contact with the lipid membrane. These findings indicate that the confinement space can be a
factor responsible for change in the elasticity of biopolymer microgels.

2. MATERIALS AND METHODS

2.1 Materials

1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (PC) and PEG (nominal molecular weight of 20,000) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Mineral oil was purchased from Nacalai Tesque (Kyoto, Japan). Alkali-treated gelatin was supplied by Nitta Gelatin Co. (Osaka, Japan). The average molecular weight of the gelatin determined by gel permeation chromatography was 145 kDa. Thioflavin T (ThT; Wako Pure Chemical Industries) was used to detect β sheet rich structures (like amyloid structure) inside the gelatin microgels. All of the materials were used without further purification.

2.2 Preparation of concave microgels and collection in aqueous phase

Concave microgels of gelatin were prepared as reported previously11). Shortly, a binary polymer solution of 5.0 wt% gelatin and 1.7 wt% PEG was emulsificated with lipid-in-oil via pipetting and confined inside droplets covered with a lipid layer. With a decrease in the temperature, the gelatin/PEG system underwent a phase separation into two liquid phases before gelation took place. In the case of PC droplets, the partially wet gelatin-rich phase on the PC membrane formed concave microgels inside the droplets as reported previously11). We placed an aliquot containing gelatin/PEG droplets on a silicone-coated cover glass to prevent the droplets from sticking to the glass plate. The samples were gradually cooled to 27 °C, a temperature lower than the gelation temperature, at a rate of approximately 0.2 °C/min and gelated by leaving them to stand for 24 h. We confirmed that the volume changes of the droplets after 24 h were negligible.

To measure the elasticity of the exposed microgels, we removed the lipid membrane covering the microgels by adding a 3.0 wt% PEG solution to the microgel droplets in oil, as previously reported (Fig. 1). This procedure ruptured the lipid membrane and exposed the microgel in the PEG aqueous phase.

2.3 Fluorescence observation of microgels

To visualize the spatial distribution of self-assembled β sheet structures in gelatin microgels, we added a 5 μM ThT to the 5.0 wt% gelatin solution before droplet encapsulation. The cross-sectional images of the microgels are taken by using a confocal laser scanning fluorescence microscope (CLSM; Olympus IX83 with FV1200).

2.4 Measurement of local elasticity of microgel using micropipette aspiration

We selected concave microgels with a radius \( R \) from 15 μm to 50 μm (\( R = 35 ± 18 \mu m \), average (Ave.) ± standard error (SE)) and measured the local Young’s modulus \( E \) for the two different surfaces by using micropipette aspiration (Fig. 2). We derived \( E \) from the following expression for a homogeneous half-space model:14, 15)

\[
E = \frac{3\Delta P R_p \Phi/2\pi}{\Delta L}. \tag{1}
\]

Here \( \Delta P \) is the aspirate pressure, \( \Delta L \) is the protrusion length of microgel into the micropipette, \( R_p \) is the inner radius of the mouth of the micropipette, and \( \Phi \) is a constant determined by the geometry of the micropipette and fixed at 2.0. We aspirated the concave microgels by using a micropipette manipulator system (MMO-202ND and MN-4; Narishige, Tokyo, Japan) and a microinjector (IM-11-2; Narishige) with a differential pressure transducer (DP15; Validyne, Northridge, CA), which was equipped with a microscope (Axiovert 40CFL; Carl Zeiss, Göttingen, Germany). We prepared a glass micropipette having a tip with inner diameter \( R_p \) of 20 ± 10 μm (Ave. ± SE; \( R_p/R < 0.4 \)) by pulling glass capillaries (GC-1; inner diameter 0.5 mm, Narishige) using a puller (PC-10; Narishige), a microforge (MF-900; Narishige), and a polishing machine (EG-401; Narishige).

3. RESULTS AND DISCUSSION

3.1 Elasticity measurement of concave microgels

We prepared concave microgels of gelatin by using gelatin/PEG droplets covered with a PC layer (Fig. 1). The gelatin/PEG solution in one phase was confined inside the PC droplet at a high temperature prior to phase separation and gelation. When the temperature was gradually lowered, the gelatin/PEG solution initially separated into two liquid phases, and the partially wet gelatin-rich phase on the PC membrane turned into a gel phase to form concave microgels. Under the experimental condition of 5.0 wt% gelatin and 1.7 wt% PEG, the gelatin concentration in the concave microgels was approximately double after phase separation, i.e., 10 wt%. Given that the volume of droplets was almost constant before and after the transitions, the change in the gelatin concentration because of evaporation was found to be negligible. For elasticity measurement, microgel was collected...
from the droplet after adding 3.0 wt% PEG solution to it.

As illustrated in Fig. 1 (right edge), the obtained concave microgels prepared inside lipid droplets have two surfaces: a concave surface with no contact with the lipid membrane and a convex surface in contact with the membrane.

To obtain the elasticity of each surface by using micropipette aspiration, we needed to confirm the linear relationship between the aspirate pressure \( \Delta P \) and the protrusion length of the microgel into the micropipette \( \Delta L \).

In the case of the convex side, the linear relationship was satisfied for a small deformation region (Fig. 2a, \( \Delta L < 7 \mu m \)), similar to spherical microgels in previous reports.\(^8,10,12,13\). However, the negative curvature of the concave surface probably derived a deviation from the linear relationship.

To account for this concern, we plotted the aspirate pressure and the protrusion length of the microgel for the concave surface (Fig. 2b). Although the deviation from the linear response was observed in a minute deformation region (<2 \( \mu m \)), it was confirmed that the linearity is satisfied well in the subsequent deformation region (2 \( \mu m < \Delta L < 8 \mu m \)) similar to the convex surface. Therefore, we used these small elongation ranges with and without the minute deformation region to obtain gel elasticities for convex and concave surfaces, respectively.

### 3.2 Local elasticity of concave microgels

By using micropipette aspiration, we derived Young’s moduli \( E \) for the concave side with no contact with the PC lipid membrane \( (E_0, \text{Fig. 2b}) \) and the convex side in contact with the membrane before gelation \( (E_m, \text{Fig. 2a}) \). It should be mentioned that we selected microgels with \( R = 35 \pm 18 \mu m \) for the \( E \) measurement because smaller microgels with \( R < 50 \mu m \) are found to have a similar \( E \) value irrespective of \( R \).\(^8\). The obtained \( E \) values of collected microgels for each side are plotted as a histogram in Fig. 3(a, b). The average values for the convex side in contact with the lipid membrane and for the concave side with no contact with the membrane are \( E_m = 43.6 \pm 4 \text{ kPa (N = 86) and } E_0 = 28.0 \pm 4 \text{ kPa (N = 55)} \), respectively. This means that the convex side in contact with the lipid membrane was stiffer than the concave part with no contact before gelation, \( i.e., E_m > E_0 \). The difference between the two cases in the same microgel \( \Delta E \) \( (= E_m – E_0) \) is 19.3 \pm 4 \text{ kPa (N = 50; Fig. 3c)}.\(^5\)

Owing to phase separation, the gelatin concentration of the collected microgels is estimated to be at least twice the charged concentration, \( i.e., 10.0 \text{ wt\%} \). Given the \( E \) value for

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Fig. 1 Schematic illustration of concave microgel preparation from gelatin/PEG droplets covered with a lipid layer of PC.

Fig. 2 (left) Microscopic view showing elasticity measurements of the microgel on the convex side and concave side. (right) Relationship between the aspiration pressure and elongation length of the microgel into the micropipette. The solid and dashed lines indicate the linear fitting area and the deviating area, respectively.

Fig. 3 Histograms of Young’s moduli \( E \) of (a) the concave microgels of 5.0 wt% gelatin for the convex side in contact with the membrane \( E_m \) and (b) the concave side not in contact with the membrane \( E_0 \). (c) The elasticity difference \( \Delta E \) between them. (d) Comparison of the values of \( E_m \) and \( E_0 \) with the corresponding bulk gels (10 wt% gelatin) and the spherical microgels without PEG and lipid (5 wt% gelatin).
bulk gels of 10 wt% gelatin is 6.4 ± 4 kPa, both the convex side \((E_m = 44 \text{ kPa})\) and the concave side \((E_0 = 28 \text{ kPa})\) have much higher elasticities than the corresponding bulk gels (Fig. 3d). This tendency of a higher elasticity of concave microgels than that of bulk gels is confirmed by using atomic force microscopy (AFM) nanoindentation \((E_m = 19.2 \text{ kPa})\). This \(E_m\) value was smaller than the value obtained by using micropipette aspiration \((E_m = 43.6 \text{ kPa})\). It might be due to chemically fixing the microgel on a glass substrate pre-coated with a tissue section adhesive (Biobond; Electron Microscopy Science, PA, USA) that was required for the AFM measurement.

These results strongly suggest that the dominant factor for the higher elasticity of microgels prepared inside lipid droplets is not the interaction between gelatin and lipid but their confinement inside the droplets. This conclusion is also supported by the fact that the absence of lipids does not change the elasticity of gelatin microgels so much. Young’s modulus of spherical microgels of 5.0 wt% gelatin prepared from gelatin droplets in hexadecane without lipids is \(E = 26 ± 2 \text{ kPa} \ (R = 167 ± 19 \mu \text{m}, \ N = 21)\) (Fig. 3d, right edge).

### 3.3 Spatial uniformity in the structure of concave microgels

The elasticity difference \(\Delta E\) between the concave and convex sides (Fig. 3c), suggests a spatial difference in the internal structure of concave microgels. To verify the existence of such spatial difference in the structure, Thioflavin T (ThT) — a fluorescent dye binding to the \(\beta\) sheet rich structure like the amyloid structure (considered to be the source for the higher elasticity of gelatin microgels\(^8\, 16\)) — was added to the PEG/gelatin solution in droplets before phase separation and gelation.

Figure 4 shows the confocal fluorescence image of a concave microgel with ThT and the intensity profile along the dashed line. In addition, in the melting process of the concave microgels upon temperature increase, the concave microgels maintained their shapes (Fig. 5).

Together with the previous report that the fluorescent (FITC) labeled gelatin is uniformly distributed in the concave microgel\(^13\), the inhomogeneity of the \(\beta\) sheet distribution in the concave microgel is found to be negligibly small. However, the gelatin concentration should be small at the phase boundary (concave side). Therefore, the heterogeneous distribution of the triple helix structure, which is the cross-linking point of the gelatin gel, might result in the elasticity difference between both sides.

### 3.4 Elasticity change of concave microgels upon volume phase transition

With a change in the external osmotic pressure by adding PEG and ethanol solutions, the concave microgels show a volume phase transition. Figure 6a shows examples of swelling and shrinking microgels as differential interference contrast (DIC) images. The concave microgel retained its shape even at this volume transition, as in the melting process shown in Fig. 5. The volume of the concave microgel \(V\) normalized by the initial volume in the droplet \(V_0\) before the collection in 3.0 wt% PEG was plotted against the solution concentration added to the outside (Fig. 6b). Under the experimental condition of 1–20 wt% PEG and 0–80 vol% ethanol, the collected microgels swelled, \(V/V_0 > 1\). The convex side \(E_m\) of the microgels swollen more than two times \((V/V_0 > 2)\) had similar elasticity as 43 kPa (dash line in Fig. 6c), as in the case of Fig. 3a.

By adding much higher concentrations of PEG above 10 wt%, the microgels shrink with \(V/V_0 < 2\) and their \(E_m\) values approach 100 kPa. The \(E_m\) value of the concave microgels with a small swelling ratio \((< 2)\) of 100 kPa corresponds to the maximum elasticity of gelatin bulk gels having a concentration of 20 wt% or more. Although the shrinkage \((V/V_0 < 2)\) increases the elasticity of the microgels up to the maximum elasticity of the bulk gels (~100 kPa), the swelling of the microgel does not decrease the elasticity below 43 kPa, which is unlike the bulk gels.

The higher elasticity of swelling microgels than that of bulk gels might be also originating from the difference in the structures, \(i.e., \) unlike bulk gels, gelatin microgels prepared...
inside droplets contain β sheet rich structure. Both the increasing gelatin concentration and droplet confinement of gelatin increase the gel elasticity. However, the microgels prepared inside droplets might have different rheological properties from bulk gels because of the unique β sheet rich structure.

4. CONCLUSION

We investigated the local elasticity of microgels of gelatin with two different surface conditions — convex surface in contact with the lipid membrane and concave surface not in contact with the lipid membrane — by using the phase separating gelatin/PEG system inside lipid droplets. The elasticities for the concave and convex surfaces with and without contacting the lipid membrane are 28 kPa and 43 kPa, respectively. The values are much higher than that of the corresponding bulk gel (6 kPa). In addition, the gelatin microgels prepared inside droplets without lipid membrane have similar elasticities. These results demonstrate that the dominant factor for the higher elasticity of gelatin microgels prepared inside lipid droplets is micrometric confinement rather than their contact with the lipid membrane.

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