

# Preparation of stimuli-responsive protein nanogel by quantum-ray irradiation

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**Abstract** To prepare the functional nanoparticle with biological affinity, we tried to control the particle diameter and volume phase transition point of protein nanogel by quantum-ray irradiation. We succeeded in controlling the particle diameter of gelatin nanogel in the range of 20–70 nm by gamma-ray irradiation. It was also found that the prepared gelatin nanogel reversibly swelled and shrunk by pH and temperature change. Volume phase transition point and swelling ratio were found to change, depending on the absorbed dose and gelatin concentration.

**Keywords** Protein nanogel · Gelatin · Gamma-ray · Cross-linking · Volume phase transition

## Introduction

Recently, the artificial nanoparticles are attracting great interest and are applied to cosmetics and medicines because of their high absorption efficiency into the biological tissues. They have various functions such as controlled release of drugs [1, 2], molecular chaperons [3], and contrast medias [4], although they may have side effects such as cell toxicity and accumulation in the human body. Protein nanogels with lower cell toxicity, higher biological affinity, and biodegradability compared with synthetic polymer nanogels can reduce these side effects.

In this study, we tried to control the particle diameter and volume phase transition point of gelatin nanogel by quantum-ray irradiation. The preparation of polymer gel using quantum-ray has advantages, such that the reaction proceeds without initiator or cross-linker and that the degree of polymerization or cross-linking can be controlled by the absorbed dose. In many studies on control over the volume phase transition point in synthetic polymers, introduction of a functional group by copolymerization [5] or cross-linking by agents [6] was performed. In the case of quantum-ray irradiation, cross-linking density can be controlled by the absorbed dose, and sterilization is also possible. These advantages are suitable for application to the human body. Preparation of nanogel using quantum-ray by intramolecular cross-linking of synthetic polymers was reported by Ulanski et al. [7, 8], and they also reported that the nanogel can respond to temperature or pH change [9, 10]. On the other hand, for biopolymers, preparation of gelatin nanogel using gamma-ray irradiation is reported by Furusawa et al. [11], but the response of irradiated gelatin nanogel has not been reported.

To apply the protein nanogel for the drug carriers and nanoactuators in bio microelectromechanical systems (bio-MEMS), we tried to control the particle diameter and the response to external stimuli of gelatin nanogel using  $^{60}\text{Co}$  gamma-ray, which has been widely used in the food industry.

## Materials and methods

### Nanogel processing

Gelatin powder with average molecular weight of about 100,000 (Merck KGaA, Darmstadt, Germany) was used as

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a starting material. Distilled water was added to the gelatin powder and adjusted to the concentration of 0.1–5.0 mass%. After the gelatin powder was swollen in distilled water for 10 min at room temperature, samples were heated to 313 K for 30 min with gentle stirring. After cooling to room temperature, the gelatin aqueous solutions were irradiated with gamma-ray from a  $^{60}\text{Co}$  source at room temperature in the Institute of Scientific and Industrial Research, Osaka University. In the present study, the absorbed dose was changed from 0 to 20 kGy.

#### pH and Temperature Adjustment

The solution pH involving gelatin nanogel was adjusted by adding 0.1 N sodium hydroxide aqueous solution (Nakalai Tesque, Kyoto, Japan) and 0.1 N hydrochloric acid (Nakalai Tesque). The pH levels of the sample solutions were measured with pH meter (HM-60, TOA-DKK, Tokyo, Japan) at room temperature.

#### Characterization of nanogels

Particle size distribution of gelatin nanogel was measured by means of dynamic light scattering spectroscopy (FOQELS, Brookhaven Instruments, NY, USA). The scattering angle was set at 153 degrees, and the used laser source was 10 mW and 671 nm in wavelength. Measurements of particle size distribution were performed after filtration through a membrane (0.2- $\mu\text{m}$  pore size).

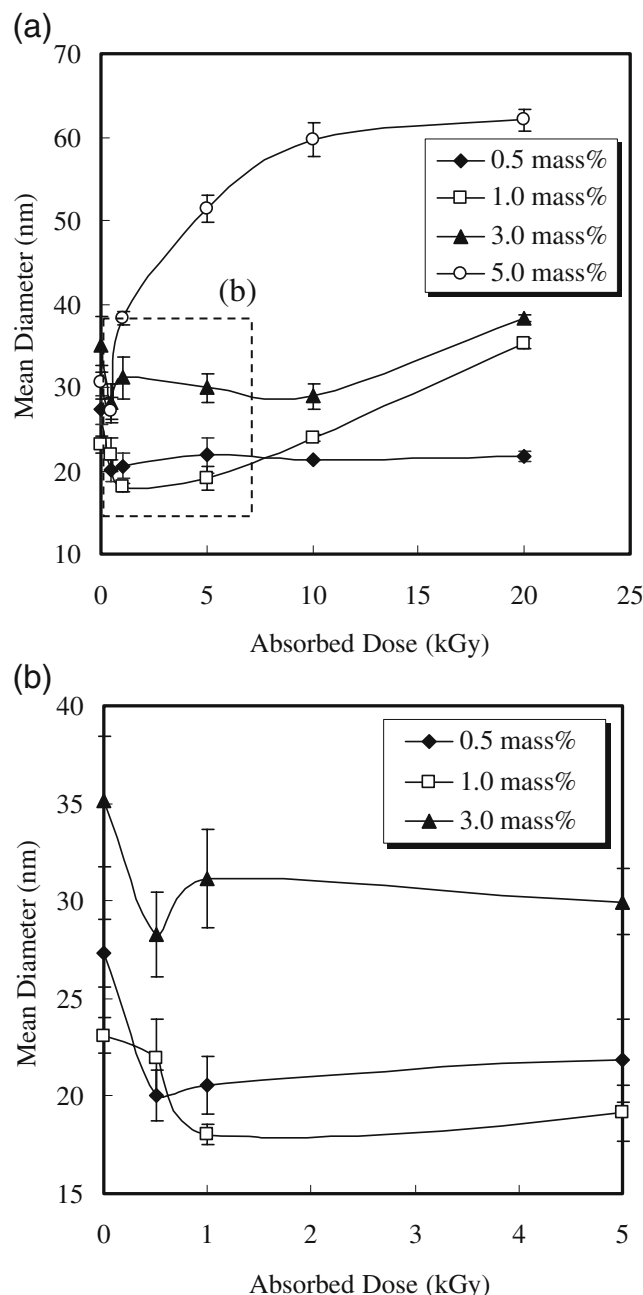
### Results and discussion

#### Control of the particle size diameter of gelatin nanogel

Figure 1a shows the change in median diameters of the nanogel obtained from the 0.5–5.0 mass% gelatin solutions as a function of the absorbed dose. These samples were macroscopically fluids and in sol states, and the median diameters of the gelatin nanogels changed from 20–35 nm (before irradiation) to 15–65 nm, depending on the absorbed dose. Diluted nanogel solutions after irradiation were also measured, but significant difference by concentration was not observed. As shown in Fig. 1a, the median particle size increased with an increase in the absorbed dose at 5.0 mass%. On the other hand, at 0.5–3.0 mass%, the median particle size decreased with an increase in the absorbed dose in the range of 0–5.0 kGy, as shown in Fig. 1b (enlarged view). However, in the range of 10–20 kGy, the median size of the particle tends to slightly increase with an increase in the absorbed dose, even in the range of 0.5–3.0 mass%. From these results, the median diameter of the nanogel increased by irradiation at higher

gelatin concentration and larger absorbed dose, whereas this decreased by irradiation at lower concentration and smaller absorbed dose.

We previously made a measurement of particle sizes of nanogel prepared by gamma ray irradiation using acrylic acid monomer solution and found that the median diameter of particles linearly increased with the increase in the absorbed dose. In the case of gelatin solutions, the manner of cross-linking caused by gamma ray irradiation seems to be different, depending on the concentration.



**Fig. 1** Relation between the absorbed dose and the median diameter of gelatin nanogel (a) and that at lower dose (b)

When the aqueous solution involving the polymer was irradiated with gamma-ray, reactive oxygen species such as hydroxyl radicals were formed by radiolysis of water, and they eliminate the hydrogen atom from the carboxyl group or the hydroxyl group to form polymeric radicals. The three kinds of reactions such as cross-linking, main chain scission, and side chain scission occurred by these polymeric radicals. In the case of protein, only cross-linking will be a main reaction.

Increase in particle size by gamma-ray irradiation may be due to the interparticle cross-linking, whereas decrease in particle size may be due to the intraparticle cross-linking. The cross-linking manner depends on the concentration of the gelatin and the absorbed dose. At lower concentration of the gelatin and at lower absorbed dose, intraparticle cross-linking was dominant, and smaller particles were mainly formed. On the other hand, at higher gelatin concentration and higher absorbed dose, interparticle cross-linking proceeded to form larger particles.

Particle size distribution (volume based distribution) of the sample before gamma-ray irradiation at 0.5 mass% is shown in Fig. 2a, and that at 5.0 mass% is shown in Fig. 3a. In these figures, two peaks around 10–20 nm (peak 1) and around 50–300 nm (peak 2) were observed for both samples. The values of these two peaks as a function of the absorbed dose are shown in Figs. 2b and 3b. Peak 1 did not change by the absorbed dose, whereas peak 2 decreased at 0.5 mass% (Fig. 2b) and increased at 5.0 mass% (Fig. 3b) along with increase in the absorbed dose. Because peak 1 did not change by the absorbed dose, it is considered that peak 1 corresponds to the primary particle size and peak 2 corresponds to the flocculated particle size consisting of several primary particles. These results show that the diameters of comparatively large particles changed by gamma-ray irradiation at both low and high concentrations. Figures 1, 2, and 3 also indicate that the extent of intraparticle cross-linking was saturated at the absorbed dose of 1 kGy and that interparticle cross-linking was saturated at the absorbed dose of 10 kGy.

As it is expected that intraparticle or interparticle cross-linking is formed depending on the interparticle distance, we tried to estimate the average interparticle distance at each concentration. We considered that the gelatin nanogel was a monodispersed spherical particle of 10 nm in diameter, which consists of one molecule from peak 1 shown in Figs. 2 and 3. The volume fraction occupied by gelatin nanogel particles was calculated using the following equation [12]:

$$\phi = \frac{\pi D^3}{6} \cdot \frac{N_A}{M_w} \cdot C \times 10^{-26} \quad (1)$$

where  $D$  is the particle diameter and  $C$  is the particle concentration. The volume occupied by one particle was

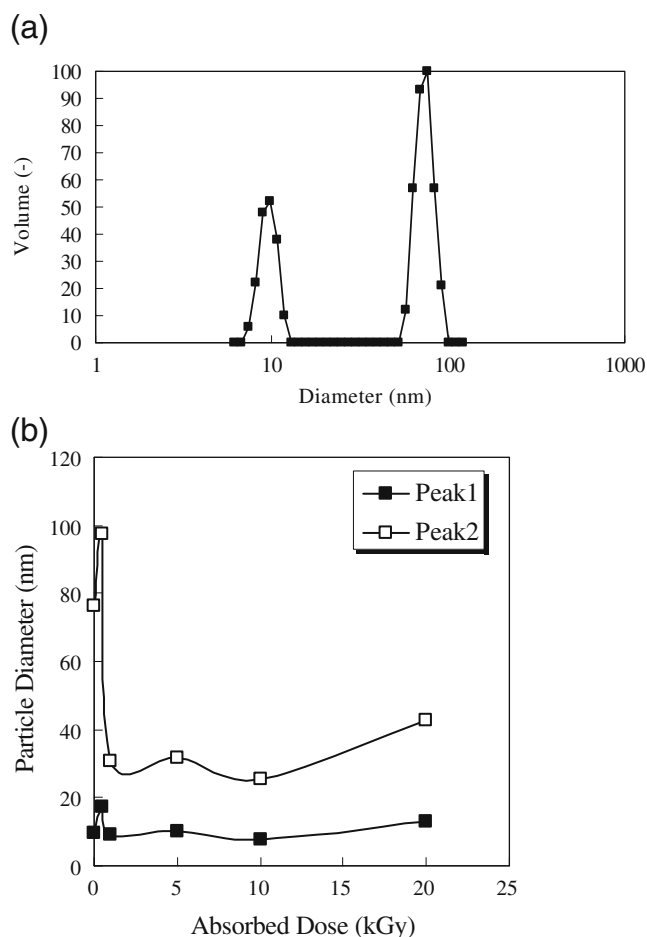
approximated as a sphere, and its diameter  $b$  is calculated from volume fraction by the following equation.

$$b = \frac{D}{\sqrt[3]{\phi}} \quad (2)$$

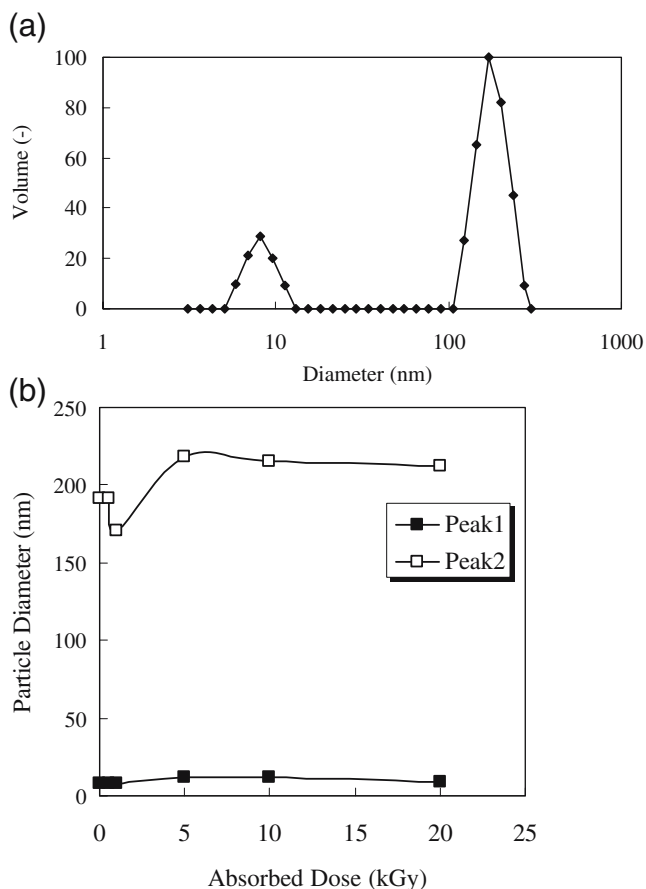
Interparticle distance is obtained from  $(b-D)$ . The calculated interparticle distance is 29.0 nm at 0.5 mass% and 8.5 nm at 5.0 mass%. Because particle diameter was presumed to be 10 nm, interparticle distance is larger than the particle diameter of nanogel at 0.5 mass% and is smaller at 5.0 mass%.

#### Control of the extent of response of nanogels

We tried to examine the response upon pH and temperature change with irradiated gelatin nanogel and to control the volume phase transition point by gamma-ray irradiation. To examine the response to stimuli of gelatin nanogel, particle size distribution was measured by means of dynamic light



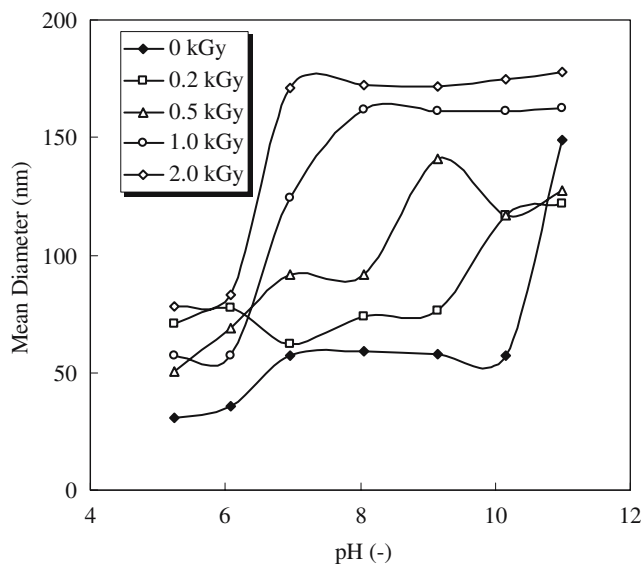
**Fig. 2** Particle size distribution of 0.5 mass% gelatin nanogel before irradiation (a) and shift of peak 1 and peak 2 with the absorbed dose (b)



**Fig. 3** Particle size distribution of 5.0 mass% gelatin nanogel before irradiation (a) and shift of peak 1 and peak 2 with the absorbed dose (b)

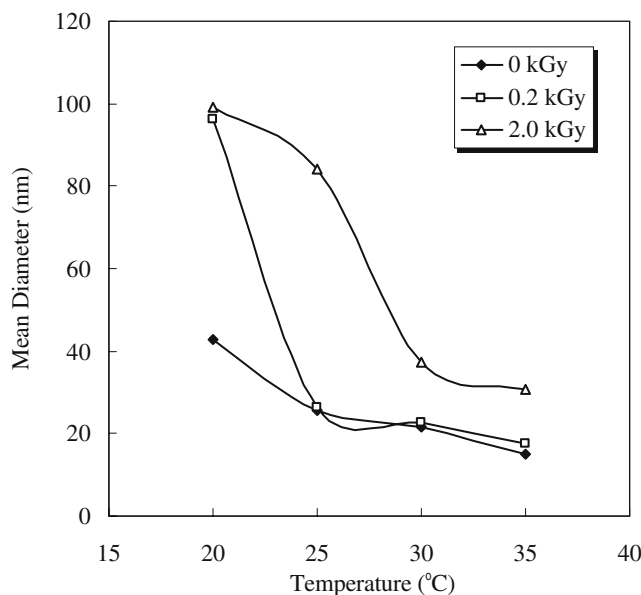
scattering when the solution pH and temperature changed. Accompanied with pH and temperature change, a peak of several hundreds of nanometers (peak 3) sometimes appeared in addition to the two peaks shown in Figs. 2 and 3, particularly in the swelled state, whereas peak 1 was not changed by pH and temperature change. Thus, we adopted the median diameter of whole nanoparticles to evaluate the pH and temperature response that reflects the change in both peaks 2 and 3.

The pH-dependent response of 5.0 mass% nanogel before and after irradiation with various absorbed doses are shown in Fig. 4. The equilibrium swelling ratio of the nanogel particle was about 5 in median diameter at the maximum, and the volume phase transition point was shifted to lower pH with an increase in the absorbed dose. As it is reported that the swelling ratios of *N*-isopropylacrylamide (NIPAAm) gel was about 2.4 [13] and that of poly(methacrylic acid) (PMAA) gel was about 6 [14] in particle diameter, protein nanogel also shows swelling–shrinking behavior as well as synthetic polymers. Temperature-dependent responses of 5.0 mass% nanogel before and after irradiation with various absorbed doses are shown



**Fig. 4** Relation between pH and median diameters of 5.0 mass% gelatin nanogels during pH-increasing process

in Fig. 5. Non-irradiated sample was not responsive to temperature. The slight decrease in diameter by heating was considered to be by dissolution of gelatin by gel–sol transition, whereas irradiated samples markedly were swelled by cooling compared with initial particle diameter at room temperature (298 K), which means that the irradiated samples were responsive to temperature. We previously elucidated that irradiated macro- and nano-sized gelatin gels are more stable at higher temperatures compared with non-irradiated samples. The irradiated gel was hardly dissolved in water at 310 K. It shows that the

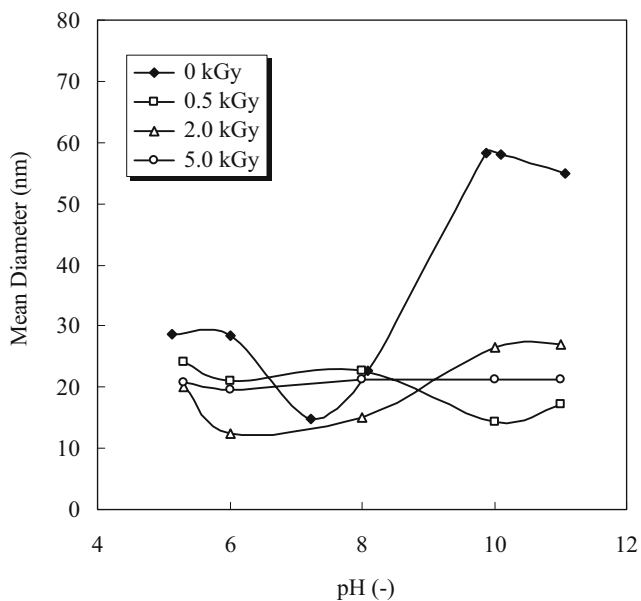


**Fig. 5** Relation between temperature and median diameters of 5.0 mass% gelatin nanogels during heating process

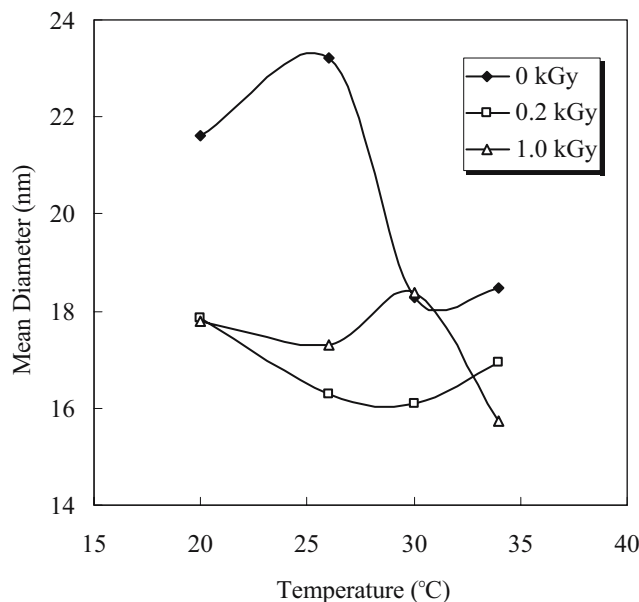
gel–sol transition temperature was elevated by cross-linking by gamma-ray irradiation. Dhara et al. [15] reported that gelatin hydrogels did not show the temperature-induced volume phase transition, but we succeeded in synthesizing temperature-responsive gelatin nanogel by gamma-ray irradiation. Decrease in water solubility of gelatin by cross-linking with glutaraldehyde at temperatures above 310 K was observed in the work for drug delivery system [16, 17]. It is known that part of the gelatin consists of a helical structure similar to that of collagen constructed with a polypeptide chain with about 1,000 amino acid residues, and the structure of one helical coil is in cylindrical form of 1.5 nm in inner diameter and 300 nm in length. There is glycine, NH<sub>2</sub>CH<sub>2</sub>COOH, at the inner surface, and another hydrophobic residue exists on the outside of the helix structure by steric hindrance [18–20]. A random part of the gelatin is considered to be hydrophilic. Decrease in water solubility of the gelatin by cross-linking may be caused by structural change of the gelatin to collagen-like structure with more ordered helix structure, which makes gelatin more hydrophobic. It is considered that appearance of temperature response is related to hydrophobicity, but the reason why the temperature response of gelatin arose by cross-linking is not certain. More detailed conformation analysis is necessary to clarify this point.

The maximum equilibrium swelling ratio was about 4 in median diameter. The volume phase transition point shifted to higher temperature with increase in the absorbed dose.

As described above, irradiated gelatin nanogel showed pH- and temperature-dependent response. The volume phase transition behavior of the polymer is generally



**Fig. 6** Relation between pH and median diameter of 0.5 mass% gelatin nanogels during pH-increasing process

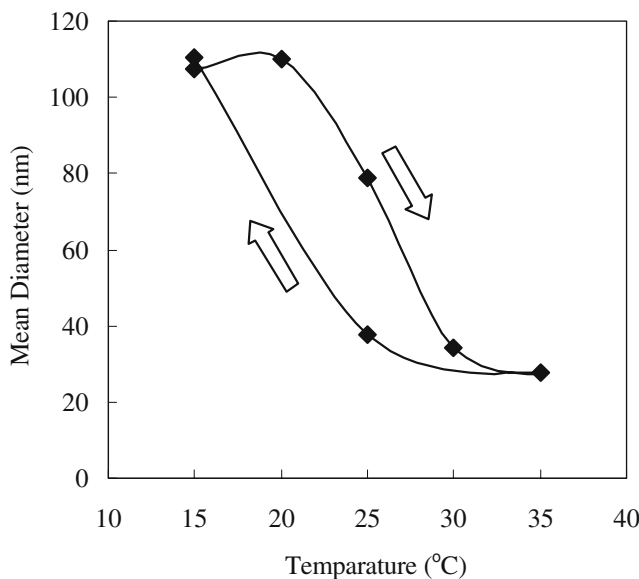


**Fig. 7** Relation between temperature and median diameter of 0.5 mass% gelatin nanogels during heating process

described by Flory–Huggins equation, and the osmotic pressure of gel  $\pi$  is given by the following equation [21]:

$$\pi = \nu kT \left\{ \frac{\phi}{2\phi_0} - \left( \frac{\phi}{\phi_0} \right)^{1/3} \right\} - \frac{\Delta F}{V} \phi^2 - \frac{kT}{V} \{ \ln(1 - \phi) + \phi \} + f \nu kT \frac{\phi}{\phi_0} \quad (3)$$

where  $k$  is Boltzmann constant,  $\nu$  is the number of polymer molecules per unit volume,  $V$  is the volume of a polymer



**Fig. 8** Relation between temperature and median diameters of 5.0 mass% gelatin nanogels after irradiation (1 kGy of the absorbed dose) during heating–cooling process

molecule,  $T$  is absolute temperature,  $\phi$  is the volume fraction of the polymer,  $\phi_0$  is the volume fraction of polymer in random conformation state,  $f$  is the number of counterions per one polymer chain between the cross-linking points, and  $\Delta F$  is free energy of interaction between polymer network and solvent.  $\phi/\phi_0$  corresponds to the reciprocal of the swelling ratio.

At higher pH, the number of dissociated counterions per chain between cross-linking points  $f$  increases fourth terms, and it makes osmotic pressure positive and makes the gel swell. In the case of the gelatin gel, the carboxyl group is mainly dissociated. Ogawa et al. [22] pointed out that Coulomb repulsion between  $\text{COO}^-$  is also contributing to shrinking of NIPAAm gels, and it may be also applied to gelatin gel. On the other hand, increase in temperature affects first, third, and fourth terms in Eq. 3. As the negative effects of first and third terms are larger than the positive effect of fourth term, osmotic pressure becomes positive, and the gel shrinks. It was shown that the state equation generally applied to bulk gel could also be applied to the protein nanogel.

As shown in Fig. 4, the volume phase transition point was changed by the absorbed dose. When the hydrogel in aqueous solution is in its equilibrium state, the osmotic pressure of gel  $\pi$  can be regarded as zero in Flory–Huggins equation, and the following equation is obtained [21]:

$$\begin{aligned} \tau &\equiv 1 - \frac{\Delta F}{kT} \\ &= -\frac{V_v}{N\phi^2} \left[ (2f+1) \left( \frac{\phi}{\phi_0} \right) - 2 \left( \frac{\phi}{\phi_0} \right)^{1/3} \right] + 1 + \frac{2}{\phi} \\ &\quad + \frac{2 \ln(1-\phi)}{\phi^2} \end{aligned} \quad (4)$$

where  $\tau$  is the reduced temperature and is a function of  $\Delta F$  and  $T$ . When the gelatin nanogel was cross-linked by gamma-ray irradiation, decrease in  $f$  leads to increase in the reduced temperature  $\tau$ . It means that the transition temperature shifts to a higher temperature. In the case of pH response, increase in the reduced temperature  $\tau$  corresponds to decrease in pH because  $\Delta F$  increases with increase in pH by dissociation of the carboxyl group. Therefore, it means that the transition pH shifts to a lower pH by cross-linking of the gelatin.

However, as shown in Figs. 6 and 7, at a concentration of 0.5 mass%, particle size of the nanogel did not change obviously by pH and temperature change after gamma-ray irradiation. At lower gelatin concentration, the gelatin nanogel after irradiation did not show the sensitive response for changing pH and temperature after gamma-ray irradiation. This fact shows that the swelling ratio of the nanogel can be also controllable by adjustment of gelatin concentration. Difference in response by gelatin concentration will

be due to interparticle and intraparticle cross-linking. It is supposed that the flexibility of the polymer chain was decreased by intraparticle cross-linking, but it was not decreased because the conformation of one polymer chain is maintained by interparticle cross-linking.

To check the reversibility of the swelling–shrinking behavior, the change in particle diameter during the heating and cooling process was investigated. As shown in Fig. 8, the median diameter of the gelatin nanogel changed reversibly by temperature change, and it means that the gelatin nanogel reversibly swelled and shrunk by external stimuli. Reversible response was also obtained for pH-induced response.

From these facts, gelatin nanogel showed reversible response to pH and temperature, and the volume phase transition point could be controlled by the absorbed dose. In addition, it is effective to control interparticle distance by gelatin concentration and form interparticle cross-linking to prepare nanogel with a large swelling rate.

## Summary

The particle size of protein nanogels could be controlled by  $^{60}\text{Co}$  gamma-ray irradiation processing, and irradiated protein nanogel shrunk and swelled reversibly by stimuli of pH and temperature changes. The volume phase transition point and swelling ratio were controlled by cross-linking density and structure. It will be possible to design protein nanogels with volume phase transition points and swelling ratios to accommodate various applications. Protein nanogels can be expected to be of nature-derived functional nanobiomaterials with high biological affinity.

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