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ORIGINAL PAPER

Effect of heating process on the formation of nanoparticles of elastin model polypeptide, $(GVGVP)_{251}$, by gamma-ray crosslinking

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Abstract Nanoparticles were prepared by the thermosensitive aggregation of the elastin model polypeptide, $(GVGVP)_{251}$, and gamma-ray crosslinking. Three different heating processes, "slow heating," "fast heating," and "heat shock," were used for the aggregation of the peptide, followed by gamma-ray crosslinking. Only the "heat shock" process successfully yielded stable nanoparticles with diameters of less than ca. 150 nm and a narrow size distribution. Circular dichroism (CD) spectrometry showed that this polypeptide formed a type-II β-turn structure when the temperature was increased to above the cloudy point in the case of the "heat shock" process; suggesting that this structure might contribute to stable nanoparticle formation by gamma-rays. CD spectrometry also suggested that this structure would be affected during the formation of stable crosslinked particles.

Keywords Polypeptide · Elastin · Nanoparticle · Gamma-rays · Structure

Introduction

In recent years, nanoparticles have become an important device for drug delivery because they have advantages as compared to larger micro-particles in that nanoparticles are better suited for intravenous delivery, reduce nonselective uptake by macrophages of the reticuloendothelial system (RES), and exhibit enhanced

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permeability and retention effects (EPR effect) at solid tumor tissue sites. Choosing biocompatible and biodegradable materials is also important in the fabrication of nanoparticles [1–5].

Elastin as well as collagen is an essential component of animal tissues. Elastin molecules forms covalently crosslinked network, and a network of these molecules can stretch and recoil like a rubber band. These molecules provide elasticity to skin, blood vessels, lung etc. [6]. Elastin molecules are composed largely of hydrophobic amino acid residues such as glycine (G), alanine (A), proline (P), and valine (V), and amino acid repeats, such as $(GVGVP)_n$, of these elastin molecules exist in the animals [7]. On increasing the temperature to a value higher than a specific temperature called cloudy point (CP), these molecules folded and assembled to form a helical structure of a type-II β-turn [8]. Using these hydrophobic amino acid residues, Urry et al. [9] synthesized unique thermoresponsive polypeptides: $(GVGVP)_{251}$ using *Escherichia coli* recombinant DNA technology; these polypeptides exhibit a remarkable biocompatibility. On the basis of this thermosensitive feature, an elastic crosslinked polypeptide sheet has already been developed using gamma irradiation [10]. Urry et al. [11] have also obtained nanoparticles by gamma irradiation. Irradiation-induced crosslink causes no residual chemical reagent, such as toxic crosslinkers, in the irradiated materials, which are effectively sterilized as well.

In this article, we focus on the heating process for optimizing nanoparticle formation. We changed the heating rate and monitored the aggregation process of the polypeptides through a circular dichroism (CD) spectrometry and dynamic light scattering as well as TEM analysis to yield the nanoparticles more efficiently.

Experimental

Materials

Bioelastic polypeptides, $(GVGVP)_{251}$, were synthesized by recombinant DNA technology using *E. coli*-carrying plasmids containing the gene encoding (GVGVP) repeat [12]. These polypeptides were supplied from BRL (Bioelastic Research Ltd.) through Bioelastic Japan Co.

Preparation of $(GVGVP)_{251}$ aggregates in aqueous solutions by heating

Aqueous solutions of the polypeptide were prepared at 4 °C and heated to 42 °C using three different heating processes, "slow heating," "fast heating," and "heat shock." The sizes of the nanoparticles obtained were analyzed after the heating (Fig. 1) [13].

Slow heating

Twenty-five milligram of the polymer was dissolved in 5 mL of deionized water (5 mg/mL according to Urry et al. [11]) in a stoppered test tube made of Pyrex glass and cooled on ice. The polymer solution was slowly heated from 4 to 42 °C at a rate

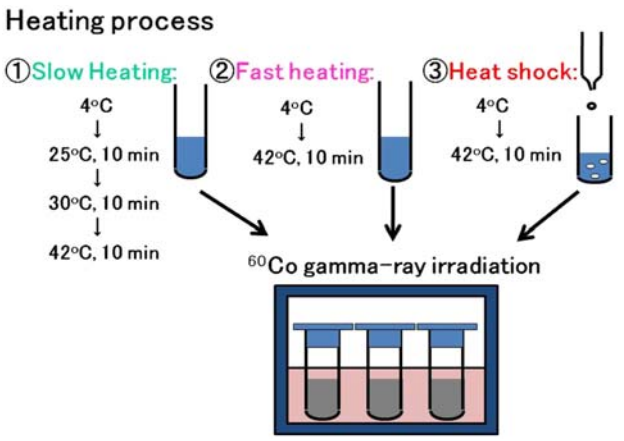

Fig. 1 Process for heating and irradiation of the $(GVGVP)_{251}$ solution

of 1.3 °C/min while stirring in a water bath (EYELA SB-350) with a magnetic stirrer (EYELA RCN-3D) at a speed of 600 rpm.

Fast heating

25 mg of the polymer was dissolved in 5 mL of deionized water at room temperature (5 mg/mL) in a stoppered test tube made of Pyrex glass and cooled to 4 °C. The polymer solution was quickly heated from 4 to 42 °C for 10 min while stirring in a water bath (EYELA SB-350) with a magnetic stirrer (EYELA RCN-3D) at a speed of 600 rpm.

Heat shock

25 mg of the polymer was dissolved in 1 mL of deionized water at room temperature and cooled to 4 °C. Drops of the solution at 4 °C were added to a 4-fold volume of water at 42 °C in a stoppered test tube made of Pyrex glass using a 1-mL tuberculin syringe with a 27-G needle (0.4 × 19 mm) (TERUMO) while stirring in a water bath (EYELA SB-350) with a magnetic stirrer (EYELA RCN-3D) at a speed of 600 rpm.

Characterization of nanoparticles before gamma irradiation

Transmittances were measured with a Hitachi U-3210 UV–Vis spectrophotometer under various temperatures using a water bath (EYELA SB-350) with a magnetic stirrer/hot plate (CORNING). Turbidity was assessed using the change in absorbance at 300 nm for a 1-mg/mL solution according to Urry et al. [14]. At a given temperature, the disposable plastic cuvette containing the sample was allowed to sit until a constant turbidity value was obtained. This steady value is considered to be the actual turbidity for the sample at the given temperature.

Particles sizes were measured by dynamic light scattering (DLS) (Particle Sizing Systems, Nicomp 370, Santa Barbara, CA) at a 90° scattering angle. The polymer solution described above was diluted to 1 mg/mL with deionized water. The solution was placed into a disposable plastic cuvette (Kartell ART.01961-00) and incubated at 42 °C in the cuvette holder of Nicomp 370.

This polypeptide structure was analyzed using circular dichroism (CD). CD spectra were measured by a J-720 spectropolarimeter (Jasco Co., Tokyo, Japan) with a standard analysis program. The temperature was controlled using a recirculating water bath, and the spectra were recorded with a cell having a path length of 0.1 cm, at a scanning speed of 50 nm/min, spectral bandwidth of 1.0 nm, integration time of 5 s, over the wavelength range from 190 to 250 nm. The samples were prepared by dissolving polypeptides in deionized water. The concentration of all the samples was 0.5 mg/mL. Absorbance was within the measurable range (<1.0) for CD spectrometry. Data are represented as molar ellipticities ([θ] deg cm^2/d mol).

Further, we checked the size of the particles and the circular dichroism (CD) spectra of "fast heating" and "heat shock." We increased the volume of the droplet during the preparation of the nanoparticles by the "heat shock" process using a different needle gauge to increase the droplet volume and modulate the "heat shock" such that this condition is closer to the "fast heating" condition. In the case of the 27-G needle, the droplet volume was 5 μL, whereas in the case of the 18-G needle (1.2 × 38 mm) (TERUMO), the droplet volume was 20 μL. In the latter case, the sample was prepared in the same manner as that of the above "heat shock" process.

Gamma irradiation

The heated polymer samples were placed in thermos bottles (TIGER MWE-C350) and irradiated with ^{60}Co gamma-rays at doses of 8.5–30 kGy using ^{60}Co gamma irradiation pool at Osaka Prefecture University (dose rate: 10.5 kGy/h). The temperature of the sample was maintained at 42 °C during irradiation.

The size distributions of the polymers before and after irradiation were measured by DLS at the temperatures in the range of 15–42 °C for $(GVGVP)_{251}$ (three measurements were carried out at intervals of 2 min).

Characterizations of nanoparticles after gamma irradiation

Particle size, structure, and transmittance were measured in the same manner as described in the case before gamma irradiation.

The morphology and particle size distribution of the aggregated polypeptide particles were examined using a HITACHI, EF2000 Transmission Electron Microscope (TEM) operated at 200 keV. Samples for the TEM were dissolved in deionized water to a final concentration of 10 mg/mL. The sample solution was added to carbon-coated 400-mesh copper grids (VECO) on ice and excess sample was absorbed in filter paper and the grid was dried at room temperature before observing under TEM.

Results and discussion

We examined the effect of the heating process on the particle formation from the polypeptides in aqueous solution. The polypeptides in the solution aggregated at a temperature higher than the CP (30 °C) irrespective of the heating process—"slow heating," "fast heating," or "heat shock"—used. The average size of the aggregated particles was within the range of 380–440 nm in the case of all the heating processes before irradiation according to the DLS measurement (Table 1).

We also investigated the effect of gamma irradiation on the aggregated polypeptides prepared by each heating process at a temperature higher than the CP. After irradiation, "slow heating" yielded a gel of the aggregated polypeptides, and "fast heating" yielded a sediment of the aggregated polypeptides (aggregated polypeptide could not form nanoparticles). In contrast, "heat shock" yielded a clouded suspension of nanoparticles (ca. 150 nm). The results of the DLS measurements are shown in Table 1 and Fig. 2. The nanoparticles were not soluble at temperatures lower than the CP, as shown by turbidity measurement (Fig. 3). These results strongly suggested that the nanoparticles were crosslinked by gamma-rays. We obtained the crosslinked nanoparticle image from TEM, as shown in Fig. 4a. The figure shows that almost all the crosslinked nanoparticles were spherical. We measured the size of the 120 particles appeared in the TEM image based on the scale bar. The obtained size distribution histogram (average size and standard deviation: 150 ± 60 nm) (Fig. 4b) was consistent with the DLS result

Table 1 Effect of the heating process on $(GVGVP)_{251}$ particle size

Mean size of the $(GVGVP)_{251}$ measured by DLS analysis at 42 °C

Heating process	Irradiation	Size (nm)
Slow heating	Before	440 ± 100
	After	No nanoparticles
Fast heating	Before	380 ± 70
	After	No nanoparticles
Heat shock	Before	370 ± 80
	After	150 ± 30

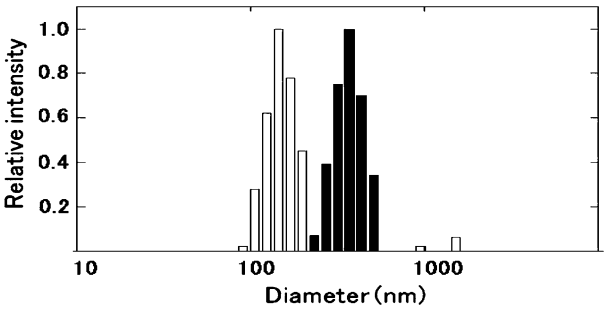

Fig. 2 Mean size of the $(GVGVP)_{251}$ particles measured by DLS analysis. The sample is prepared using the heat shock process at 42 °C in deionized water. The *close square* in the figure is before irradiation. The *open square* in the figure is after irradiation

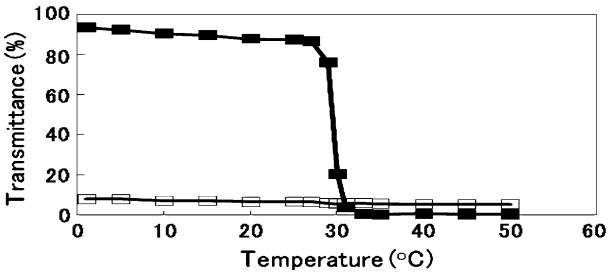

Fig. 3 Temperature dependence of light transmittance at 300 nm for the 1-mg/mL aqueous polypeptide solution before and after irradiation. The CP is considered as the temperature at which the transmittance reaches a value of 50. The *close square* in the figure is before irradiation. The *open square* in the figure is after irradiation

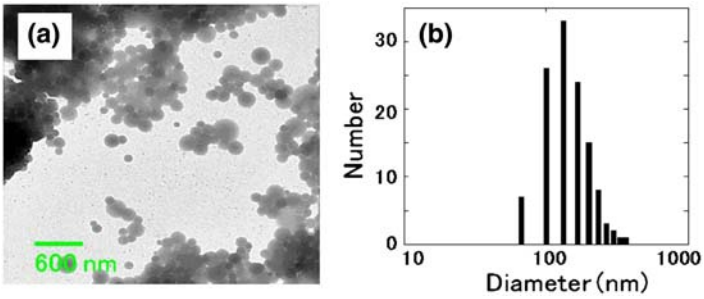

Fig. 4 TEM image of the $(GVGVP)_{251}$ particles crosslinked by gamma irradiation (**a**) and the size distribution histogram obtain from the TEM image (**b**). The scale bar in the micrograph represents the length of 600 nm. The size distribution histogram was obtained by the diameter measurement of 120 particles in the TEM image based on the scale

Table 2 Radiation dose response of the yield of the particles

Radiation doses (kGy)	Yield of particles (%)
8.5	36
17	45
34	62

Deposit of the particles obtained by centrifugation of the particles solution for 1 h at 19,000 rpm at 4 °C and it dissolved in deionized water. Yield is determined by measuring its dry weight

(average size and standard deviation: 150 ± 30 nm) (Fig. 2). The yield of the crosslinked nanoparticles increased with an increase in doses, as shown in Table 2.

During the "heat shock" process, because the polypeptide concentration was 25 mg/mL, the initial polypeptide concentration might be higher than that during any other heating process at the moment of the particle formation of the polypeptides when the polypeptide solutions were dropped into hot deionized water have a temperature higher than the CP; although, the final concentration after the

particle formation was 5 mg/mL. In contrast, the polypeptide concentration was maintained at 5 mg/mL all the time during "slow heating" and "fast heating." Therefore, we compared particle formation by "slow heating" and "fast heating" in aqueous solutions with different concentrations of polypeptides (5 and 25 mg/mL). Both the concentrations of the polypeptide solutions yielded clouded suspensions after "slow heating" and "fast heating," and any significant difference could not be observed before irradiation. After irradiation, nanoparticles were not formed in the aggregated polypeptide solutions in the case of the 25 mg/mL solution as well as the 5 mg/mL solution, as described above. These results suggested that the "heat shock" process was the most important with respect to the formation of nanoparticles.

We also tried to increase the sample volume of the droplet of the polypeptide solution during the "heat shock" process using needles with a different gauge size. The cooled $(GVGVP)_{251}$ solution (25 mg/mL cooled at 4 °C) dropped into hot deionized water at 42 °C with a tuberculin syringe using 18-G (droplet volume was 20 μL) and 27-G (droplet volume was 5 μL) needles, and the final concentration after the aggregation of $(GVGVP)_{251}$ was 5 mg/mL. The stable particle was not obtained in the case of using the 18-G needle; however, the stable particle was obtained in the case of using the 27-G needle. The average size of the particles was 150 nm in the case of using the 27-G needle. Thus, the 27-G needle was more effective than the 18-G needle in forming stable crosslinked nanoparticles distributed within narrow size range.

In order to obtain further information on the advantage of using the "heat shock" process to yield the stable nanoparticles, we performed CD spectrometry for the polypeptide solutions (0.5 mg/mL in deionized water) at 42 °C (above CP) prepared by the three heating processes "slow heating," "fast heating," and "heat shock." The CD spectra of the samples taken at 42 °C achieved by "slow heating," "fast heating," and "heat shock" are shown in Fig. 5. Absorbance of the aggregated polypeptides in an aqueous solution was under OD 1.0, and the intensity of the CD spectra was within the measurable range. As shown in Fig. 5, the CD spectrum of the sample prepared using "heat shock" showed a negative band near 225 nm which is reported as a characteristic band of type-II β-turn by Urry et al. [8], and that spectrum is more remarkable than those of the other cases. We also conducted

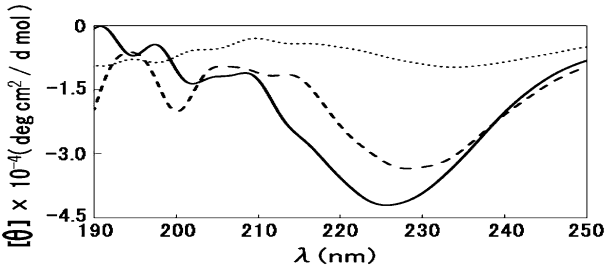

Fig. 5 Circular dichroism (CD) spectra of $(GVGVP)_{251}$ before irradiation. The concentration of all the samples is 0.5 mg/mL. The *dot line* in the figure is slow heating. The *broken line* in the figure is fast heating. The *normal line* in the figure is heat shock

the CD spectrometry on the gamma-ray crosslinked samples prepared by the best heating process (i.e., the "heat shock" process) the 27-G needle. The intensity of the negative band near 225 nm reduced after gamma irradiation when compared with that of before irradiation, as shown in Fig. 6, suggesting that the type-II β-turn structure was partially distorted by gamma-ray irradiation. The type-II β-turn is formed by hydrogen-bonded ring at the Val-Pro-Gly-Val portion. These Val residues are closer to each other [14–19]. Molecular dynamic simulation of $(VPGVG)_{18}$ in water showed that side-chain contact at 42 °C is 1.5 times that at 20 °C [20], which is caused by an increase in hydrophobic interactions between aliphatic side-chain group. From these evidences, it is suggested that crosslinking might be caused between Val and Val. Therefore, as shown in Fig. 5, the formation of more type-II β-turns within the $(GVGVP)_{251}$ chains by the "heat shock" process would be effective to form the organized structure stimulating crosslinking between the polypeptides to yield stable nanoparticles by gamma irradiation [21]. In case of "slow heating" and "fast heating", the hydrophobic side-chain interaction of the polypeptides might be poor and it might not be effective to form the crosslinked structure. As a result, "slow heating" and "fast heating" would yield gels and

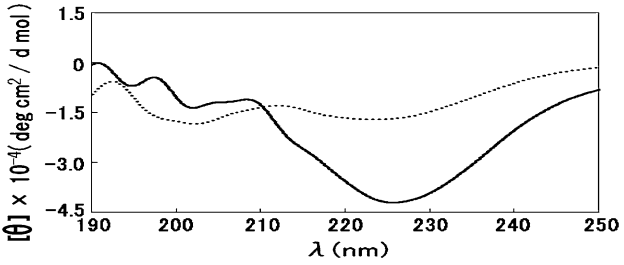

Fig. 6 Circular dichroism (CD) spectra of $(GVGVP)_{251}$ prepared using heat shock, before and after irradiation. The concentration of all the samples is 0.5 mg/mL. The *normal line* in the figure is before irradiation. The *dot line* in the figure is after irradiation

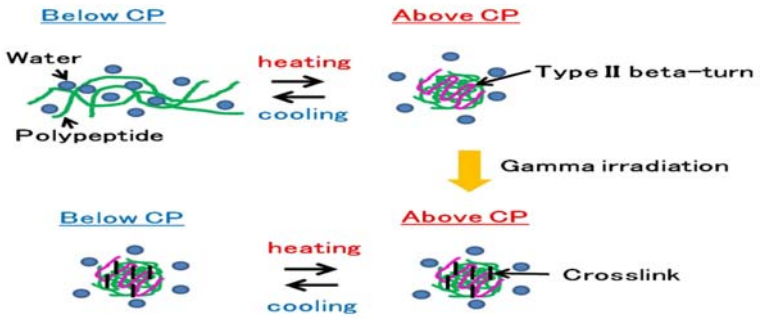

Fig. 7 Postulated reaction scheme for the formation of the crosslinked nanoparticles. $(GVGVP)_{251}$ dissolves in water at 4 °C (below CP). Increasing the temperature to 42 °C (above CP) by heat shock makes the polypeptides form type-II beta-turn structure during the course of aggregation. The aggregated polypeptides are crosslinked by gamma irradiation under 42 °C (above CP) resulting in stable nanoparticle formation even below CP

sediments, respectively. It was supposed that the inter-molecular contact between hydrophobic side-chains was essentially important to form crosslinked structures. Therefore, if polypeptide side-chain does not form organized structure within the aggregated polypeptides before irradiation, the crosslinked nanoparticle formation may be become difficult, even though all average sizes of the aggregated particles of "heat shock", "slow heating," and "fast heating" are similar before irradiation. Schematic model of the efficient nanoparticle formation process was illustrated in Fig. 7.

Conclusions

Stable nanoparticles of $(GVGVP)_{251}$ were successfully obtained by gamma-ray crosslinking on increasing the temperature to a value higher than the CP by the "heat shock" process using a needle having a small droplet volume before irradiation and maintaining the temperature during the gamma irradiation. It was suggested that specific formation of the aggregated polypeptides and thermal efficiency on the polypeptide solutions under high temperature (above CP) before irradiation would be crucial to yield stable crosslinked nanoparticles by gamma irradiation. The size of the nanoparticles was within the range of appropriate size (ca. 150 nm) expected for a drug delivery carrier to deliver hydrophobic drugs to solid tumor tissue sites by the enhanced permeability and retention effect.

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