Gelatin Microspheres Crosslinked with γ-ray: Preparation, Sorption of Proteins, and Biodegradability

Ken Terao,[1] Tomotake Karino,[1] Naotsugu Nagasawa,[2] Fumio Yoshii,[2] Miyoko Kubo,[3] Toshiaki Dobashi[1]

[1]*Department of Biological and Chemical Engineering, Faculty of Engineering, Gunma University, 1-5-1, Tenjin-cho, Kiryu, Gunma 376-8515, Japan*
[2]*Takasaki Radiation Chemistry Research Establishment, Japan Atomic Energy Research Institute, 1233 Watanuki-machi, Takasaki, Gunma 370-1292, Japan*
[3]*Department of Plastic and Reconstructive Surgery, Kawasaki Medical School, 577, Matsushima, Kurashiki, Okayama 701-0192, Japan*

Received 5 June 2003; accepted 11 August 2003

ABSTRACT: A new type of gelatin microspheres was manufactured with the crosslinking method by using γ-ray irradiation. Microspheres thus obtained were characterized by microscopic observation and enzymatic degradability. The mean diameter of the microspheres was in the range from 2.4 to 3.6 μm and the size distribution was quite small. The size decreased with increasing the shear rate of the emulsifier used in the preparation and was not affected by the radiation dose. The enzymatic degradability decreased with increasing the radiation dose and decreasing the gelatin concentration of the microsphere. In other words, the rate of the enzymatic decomposition of microspheres can be controlled by these two parameters. These trends were consistent with our previous results for the gel sheet made of gelatin. Their sorption of proteins was also investigated with the use of three types of proteins labeled by fluorescent pigment. From the fluorescence micrographs, it was observed that the gelatin microspheres adsorbed only proteins having the opposite charge. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 91: 3083–3087, 2004

Key words: gels; irradiation; crosslinking; biodegradable; proteins

INTRODUCTION

Microspheres prepared by crosslinking of polyelectrolytes are a group of useful carriers of the drug delivery system because they can be injected into the body and it is expected to adsorb many kinds of proteins by the electrostatic force of attraction. Indeed, Tabata and Ikada prepared microspheres consisting of gelatin crosslinked by glutaraldehyde and demonstrated the applicability as a carrier of the drug delivery system.[1] The crosslinking reagent, glutaraldehyde, has, however, unneglectable toxicity and oncogenicity.[2,3]

Recently, we found that concentrated gelatin gels (physical gel) can be crosslinked by γ-ray or electron beam.[4] We prepared gel sheets (chemical gel) by the irradiation of electron beam and concluded that the enzymatic degradability of the sheet could be controlled over a wide range of degradation times by varying the gelatin concentration and the radiation dose. The hydrogel thus obtained is useful for some clinical medicine and/or food processing because no crosslinking reagents and organic solvents are required in the preparation process and it is expected that a release of polyelectrolytes from the gel could be controlled by the biodegradability of the hydrogel.[5,6]

In this work, we manufactured hydrogel microspheres (physical gel) made of two kinds of gelatin samples originated from porcine skin (type A) and bovine skin (type B) and irradiated γ-ray to them to crosslink the gelatin molecules. Microscopic observation was carried out for the microspheres to determine the average size and the size distribution. Enzymatic degradability was estimated from the time course of optical absorbance from the suspension of the microspheres containing proteinase. The sorption of polypeptides was also investigated with acidic or basic proteins labeled with fluorescein-4-isothiocyanate (FITC-I). Selective sorption obtained from the experiment could be one of the most useful functions of these microspheres.

EXPERIMENTAL

Materials, preparation of microspheres, and irradiation

Commercially obtained gelatin samples from porcine skin (type A) were purchased from Sigma Co. (St. Loius, Mo) and used for this study without further

Correspondence to: K. Terao (terao@bce.gunma-u.ac.jp).

Journal of Applied Polymer Science, Vol. 91, 3083–3087 (2004)

purification. Three grams of gelatin solution (5 or 10 wt % in pure water) was mixed with cyclohexane containing a small amount of SY-glyster CR-310 (tetraglycerine polyrisinolate), a gift from Sakamoto Yakuhin Kogyo Co. (Osaka, Japan), as a surfactant. This surfactant has been approved as a nontoxic food additive. Each mixture was homogenized at 6000 or 16,000 rpm at 50°C for 15 min by an emulsifier (Excel Auto, Nihon Seiki Co. (Nagaoka, Japan)). The resultant suspension was cooled to room temperature and stored at low temperature (about 5°C) until irradiation of the γ-ray. The suspensions were irradiated with γ-ray generated from a ^{60}Co source (Takasaki Radiation Chemistry Research Establishment) at a dose rate of 10 kGy/h. Then, the suspensions were frozen with liquid nitrogen and the gel spheres were separated by the conventional freeze-dry method.

To compare the biodegradability of the newly prepared gelatin microspheres with those crosslinked by other methods, we prepared two other microspheres as follows. Unirradiated microspheres prepared with the same method as described above were freeze-dried and irradiated by ultraviolet light in air at a distance of 20 cm from a UV lamp (254 nm, 4 W) for 3 days or soaked into 25% glutaraldehyde aqueous solution. The latter microspheres were separated by washing with ethanol several times and dried in vacuum.

Size distribution of microspheres

Size distribution measurements were carried out for gelatin microspheres dispersed in water by using a Nikon Microphot-FXA microscope (Tokyo, Japan) combined with ×10 objective lens (Nikon). Micrographs were taken with a Nikon CCD camera unit. The diameter d and the distribution of the microspheres were estimated from the micrographs and about 1000 spheres (20–30 micrographs) were counted for each run.

Enzymatic degradation

Each enzymatic degradability measurement was carried out for the gelatin microspheres in 0.02*M* sodium bicarbonate/0.001*M* calcium chloride buffer solutions with proteinase (Tokyo Kasei, Tokyo, Japan, from *bacillus subtilis*) of 0.01 g cm^{-3} as an enzyme at 40°C. The activity of the enzyme was stated to be 89,000 units g^{-1} with the use of casein as a substrate according to the data sheet issued by the Corp. A small amount of microsphere dispersing liquid was poured into a 1-cm quartz cell filled with the proteinase solution. UV absorbance at 275 nm wavelength resulting from the aromatic amino acids consisting of the dissolved gelatin was measured at appropriate time intervals. We note that the solution was stirred with a magnetic stirrer while conducting the measurement.

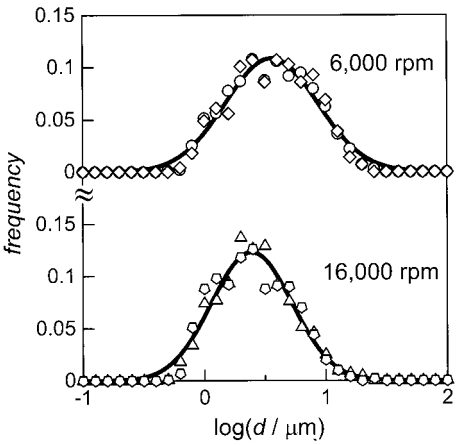

Figure 1 Droplet size distributions of the porcine gelatin microspheres in water. Circles: microspheres irradiated with 30 kGy and homogenized at 6000 rpm; squares: 60 kGy and 6000 rpm; triangles: 30 kGy and 16,000 rpm; pentagon: 60 kGy and 16,000 rpm. Curves are normal logarithmic ones with the mean value and the deviation listed in the text.

Sorption of proteins to microspheres

To examine if the microspheres adsorb proteins, microscopic fluorescence observations were performed under a Nikon Microphot-FXA microscope for the microspheres-mixed proteins labeled with fluorescein-4-isothiocyanate (FITC-I, Dojindo, Kumamoto, Japan). We chose an acidic protein, bovine serum albumin (BSA, Sigma Co.), and two basic proteins, protamine sulfate (Wako Co. (Osaka, Japan), and polylysine (Lot No. 2000306SF) gifted from Chisso Co. (Tokyo, Japan). These proteins were reacted with FITC-I in the following manner: An aliquot of FITC-I aqueous solution was poured into protamine sulfate, or BSA, or polylysine aqueous solution and the mixture was stirred at 20°C for 24 h. The isothiocyanate group of FITC-I was bonded to the amino groups of each protein in this process. To test the dependence of the type of gelatin, acidic or basic, the fluorescence observation was also carried out for the microspheres made of bovine skin gelatin (type B), purchased from Sigma Co.

RESULTS AND DISCUSSION

Size distribution of microspheres

Figure 1 shows the size distribution of the microspheres made of 5 wt % porcine gelatin solution. The size distribution curve is almost independent of the radiation dose, in contrast with considerable effects of the radiation dose on the gel fraction and the swelling shown in our previous study.[4] This apparent incon-

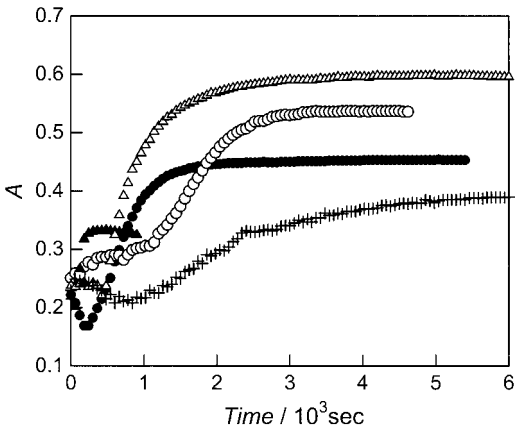

Figure 2 Time courses of the absorbance A at 275 nm of the buffer solution including proteinase and gelatin microspheres irradiated with γ-ray. Filled circles: microspheres made of 5 wt % solution, irradiated with 30 kGy, and homogenized with 16,000 rpm; unfilled circles: 5 wt %, 60 kGy, 16,000 rpm; filled triangles: 10 wt %, 30 kGy, 16,000 rpm; unfilled triangles: 10 wt %, 60 kGy, 16,000 rpm; crosses: 5 wt %, 60 kGy, 6000 rpm.

sistency results from the difference in the range of radiation dose; the radiation dose used in this work is quite large and the dose dependence of swelling behavior is negligibly small in this range. The size distribution of the microspheres made of 10 wt % gelatin solution is essentially the same as that of 5 wt % solution. Each size distribution of microspheres obeys the logarithmic normal distribution curve, and the mean value is determined to be 2.4 and 3.6 μm for those prepared by the emulsification at 16,000 and 6000 rpm, respectively. The standard deviation is estimated to be 0.33 and 0.38 for 16,000 and 6000 rpm, respectively. Thus, the mean size increases appreciably with decreasing emulsification rate. This trend is natural in the process of formation of emulsion. We note that the mean size of microspheres in water is not as large as that in cyclohexane, but the difference is quite small.

Enzymatic biodegradation behavior

Figure 2 illustrates the time course of the optical absorbance from the mixture of the proteinase and the microspheres made of gelatin from porcine skin. Data points near $t = 0$ are complicated, but the entire curve is S-shaped and approaches each asymptotic value when t is large. It is expected from these data that two effects, turbidity from the microspheres and absorbance from degraded gelatin molecules, affect the total absorbance. To explain this complicated behavior of absorbance, we examined these effects by evaluating the absorbance A of the solution under degradation (see Appendix). The resultant expression for A is expressed as the sum of absorbance A_g from degraded gelatin molecules and turbidity τ_m of the microspheres, for instance,

$$A = A_g + \tau_m \quad t \le \frac{r_0}{v} \tag{1}$$

$$= A_\infty \qquad t > \frac{r_0}{v}$$

where

$$A_g = A_\infty\left[3\left(\frac{vt}{r_0}\right) - 3\left(\frac{vt}{r_0}\right)^2 + \left(\frac{vt}{r_0}\right)^3\right] \tag{2}$$

$$\tau_m = 6\pi K' \int_0^\pi \frac{[\sin k(r_0 - vt) - k(r_0 - vt)\cos k(r_0 - vt)]^2}{k^6} \times (1 + \cos^2\theta)\sin\theta d\theta \tag{3}$$

Here r_0 is the initial radius of the microsphere, v, is the rate of reducing radius, K' is the constant shown in eq. (A6), A_∞ is the absorbance at infinitely long time, and k is the absolute value of the scattering vector defined as

$$k = \frac{4\pi n_0}{\lambda_0}\sin\frac{\theta}{2} \tag{4}$$

where n_0 and λ_0 are the refractive index of the solvent and the wavelength of the incident light in vacuum, respectively.

An example of theoretical calculations of eq. (1) for $A_\infty = 1$, $K' = 0.35$ g^{-1} cm^4, $n_0 = 1.33$, and $\lambda_0 = 0.275$ μm are illustrated in Figure 3. Figure 3(a) shows A plotted against time t for the same v and a little different r_0. These theoretical calculations reproduce the complicated curve illustrated in Figure 2, at least qualitatively. However, a small difference in r_0 results in considerable change of A in the small t range. It is expected from this result that the polydispersity of microspheres affects serious transformation of the time course in this range. On the other hand, the time courses become independent of r_0 at large t. Figure 3(b) illustrates the plot of A versus t for the same r_0 and different v. It is seen that the difference of v can be distinguished significantly from the time when A approaches each asymptotic value. In other words, the difference of v can be estimated from such data. In the light of this conclusion, the degradation rate increases with decreasing dose and increases with increasing concentration of gelatin. The same trend is observed for the microspheres made at low shear rate of 6000 rpm. These results are consistent with our enzymatic degradation data[4] for gelatin sheets irradiated by electron beam. On the other hand, the degradation rate

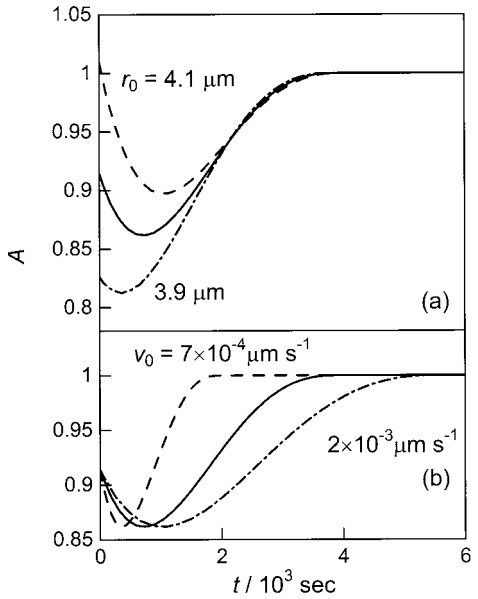

Figure 3 Theoretical time courses of absorbance A. Curves are calculated from eqs. (1) to (3) with $A_\infty = 1$, $K' = 0.35$ g^{-1} cm^4, $n_0 = 1.33$, and $\lambda_0 = 0.275$ μm. Other parameters are indicated in the figure: (A) radius dependence, (B) degradation velocity dependence.

decreases with decreasing the rotation speed of the emulsifier at the preparation of microsphere (not shown). This is because the mean diameter of the microsphere increases with decreasing the rotation speed.

Figure 4 illustrates the comparison of the time courses thus obtained with those for the other microspheres crosslinked with glutaraldehyde or ultraviolet light. Compared with Figure 3, it is shown that the enzymatic degradability of the microspheres crosslinked with γ-ray is as changeable (or controllable) as those crosslinked with glutaraldehyde, and the degradability of those irradiated with ultraviolet light is much higher.

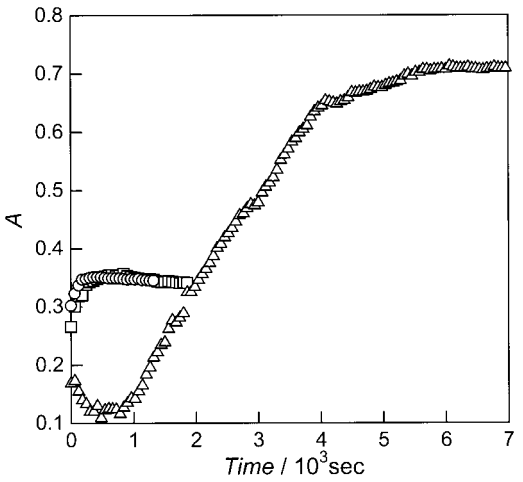

Figure 4 Time courses of the absorbance A at 275 nm of the buffer solution including proteinase and gelatin microspheres. Circles: microspheres crosslinked with UV for 3 days; triangles: crosslinked with glutaraldehyde for 15 h; squares: crosslinked with glutaraldehyde for 20 min.

Sorption of proteins to microspheres

An example of a pair of micrographs is shown in Figure 5. These pictures were taken for the mixture of protamine sulfate labeled with FITC-I and the microspheres made of bovine skin gelatin. Figure 5(A) is a (normal) micrograph and many microspheres are observed. A lot of luminous points in Figure 5(B), fluorescent picture, are observed at the same positions of the microspheres in Figure 5(A). This observation indicates that protamine adhered to the microspheres in this case. In fact, the fluorescence did not disappear even after colored microspheres were washed in buffer solution several times. We judged if each pro-

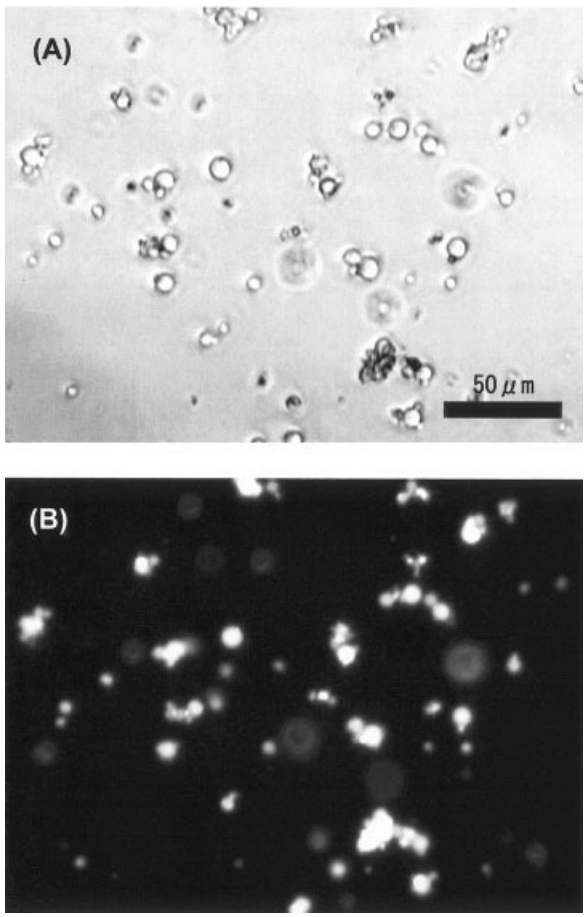

Figure 5 An example of the optical photomicrograph of the mixture of microspheres and the protein labeled with fluorescence in water. The microspheres in these pictures were made of 5 wt % solution of bovine gelatin, irradiated with 30 kGy, and homogenized at 16,000 rpm. (A) normal micrograph, (B) fluorescent micrograph.

TABLE I
Adsorptive Behavior of Proteins to Gelatin Microspheres

Protein	Origin of gelatin (type)	
	Porcine skin (A)	Bovine skin (B)
BSA	O	X
Protamine	X	O
Polylysine	X	O

O, adsorptive; X, not adsorptive.

tein adhered to microspheres from such pictures and summarized the results in Table I. Here, BSA and type B gelatin (from bovine skin) are acidic proteins and have minus charges. On the contrary, protamine sulfate, polylysine, and type A gelatin (from porcine skin) are basic proteins and have plus charges. Therefore, it is concluded from this table that microspheres made of each type of gelatin adsorbed only proteins having opposite charge. Thus, the newly prepared gelatin microspheres have the same sorption properties as microspheres crosslinked by glutaraldehyde.

In conclusion, gelatin microspheres crosslinked by γ-ray without any chemical reagent have been manufactured. The average-size microspheres can be controlled by emulsification speed in the preparation. Enzymatic degradability and polypeptide sorption properties are controllable as much as microspheres crosslinked by glutaraldehyde.

APPENDIX

Derivation of A_g

When the degradation of the gel microsphere occurs on the surface, the absorbance increment dA_g from a sphere dissolved in buffer is proportional to the surface area S

$$dA_g = -\gamma l_c V_c^{-1} n C_g S(r) dr = -4\pi\gamma l_c V_c^{-1} n C_g r^2 dr \qquad 0 \le r \le r_0 \quad \text{(A1)}$$

where r is the radius of the sphere at t, r_0 is the original radius (the radius at $t = 0$), l_c is the light path length of the cell, V_c is the volume of the suspension in the cell, n is the number of spheres in the cell, C_g is the gelatin concentration of the sphere, and γ is a constant. The differential equation can be solved by using a boundary condition $A_g(r_0) = 0$ as

$$A_g = \frac{4}{3}\pi\gamma l_c V_c^{-1} n C_g (r_0^3 - r^3) \quad 0 \le r \le r_0 \quad \text{(A2)}$$

If the gel is degraded with a uniform velocity v, $r(t)$ is expressed as

$$r(t) = r_0 - vt \quad 0 \le t \le r_0/v, r(t) = 0 \qquad t > r_0/v \quad \text{(A3)}$$

Substitution of eq. (A3) into eq. (A2) leads eq. (2)

Derivation of τ_m

The turbidity τ_m from the microspheres can be expressed as the total sum of the intensity of the scattered light as

$$\tau_m = 2\pi \int_0^\pi R_\theta (1 + \cos^2\theta) \sin\theta d\theta \quad \text{(A4)}$$

where θ and R_θ represent the scattering angle and the Rayleigh ratio of the suspension, respectively. When the light scattering intensity can be assumed to represent with the particle scattering function $P(\theta)$ for rigid spheres, R_θ is expressed as

$$R_\theta = KcMP(\theta) = K' r^6 P(\theta) = \frac{3K'(\sin kr - kr\cos kr)^2}{k^6} \quad \text{(A5)}$$

$$K' = 16\pi^2 K n C_g^2 N_A / 9 V_c \quad \text{(A6)}$$

where K is the optical constant, c is the mass concentration of gelatin in the suspension and equal to $4\pi r^3 n C_g / 3V_c$, M is the molecular weight of a microsphere without water, and N_A is the Avogadro number. Substitution of eqs. (A3) and (A5) into eq. (A4) leads to eq. (3).

References

1. Tabata, Y.; Ikada, Y. Adv Drug Delivery Rev 1998, 31, 287.
2. Ballantyne, B.; Jordan, S. L. J Appl Toxicol 2001, 21, 131.
3. Van Miller, J. P.; Hermansky, S. J.; Losco, P. E.; Ballantyne, B. Toxicology 2002, 175, 177.
4. Terao, K.; Nagasawa, N.; Nishida, H.; Furusawa, K.; Mori, Y.; Dobashi, T.; Yoshii, F. J Biomater Sci Polym Ed 2003, 14, 1197.
5. Tabata, Y.; Nagano, A.; Muniruzzaman, M.; Ikada, Y. Biomaterials 1998, 19, 1781.
6. Yamamoto, M.; Ikada, Y.; Tabata, Y. J Biomater Sci Polym Ed 2001, 12, 77.