Preparation and Characterization of Crosslinked Gelatin Microspheres

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SYNOPSIS

Gelatin, a natural macromolecule, is widely used in biomedical and biotechnological applications and is a good candidate for preparation of microspheres and microcapsules for the purpose of controlled release applications of drugs. In this study, gelatin microspheres crosslinked with glutaraldehyde with different crosslink densities were prepared by the phase separation technique induced by temperature change. The chemical structures of the microspheres were examined with FTIR and formation of crosslinks were observed. Topography, size, and shape of the microspheres were examined with scanning electron microscopy (SEM) and a narrow size distribution (approximately 1 μm) was observed. Thermal properties of microspheres were analyzed by differential scanning calorimetry and an increase in glass transition temperature values with an increase in crosslinking was observed. For each sample, dry and swollen densities of microspheres were determined by pycnometric methods and the average molecular weights between the crosslinks, M_C, were calculated from the equilibrium swelling experiments using the modified Flory–Rehner thermodynamic theory. A decrease in percent solvent content values with decreasing average molecular weight between the crosslinks was observed. © 1995 John Wiley & Sons, Inc.

INTRODUCTION

Many classes of polymers have been studied for controlled release applications.[1-4] Among different types of polymeric delivery systems, degradable systems have the advantage of obviating the need to surgically remove the drug-depleted device.[5-7] Potentially, degradable matrix systems also have a number of advantages such as simplicity of design and predictability of release if the release is controlled solely by matrix degradation.[8,9] After considering properties desirable for a degradable polymer matrix, gelatin was selected especially because it is natural and nontoxic. It is used in matrix type devices, and also in microsphere form. The microsphere sample geometry offers distinct advantages relative to the more conventional film and fiber geometries. The extremely small characteristic dimensions obtainable with spherical samples makes convenient experimental times for exceedingly slow processes possible.[10]

Drug delivery systems based on polymeric microspheres are becoming very popular and colloidal carriers in the form of microspheres and nanoparticles are being investigated as potential advanced drug delivery systems.[11] These systems involve microspheres in diameters ranging from below 1 μm to over 100 μm. Considerable interest has been shown in the formation of biodegradable nanoparticles.[12]

A great number of microencapsulation techniques are available for the preparation of microspheres. Solvent evaporation and organic phase separation are two processes by which microencapsulation can readily be performed in the laboratory without specialized equipment. The choice of one particular method is primarily determined by the solubility characteristics of the drug and the polymer. If the core material is water insoluble, either of the methods can be considered. For water-soluble core materials, only the organic phase separation procedure is suitable.[13] Organic phase separation of the wall-forming polymer can be induced by one of the following methods: addition of nonsolvent to the polymer, addition of a second polymer incompatible with

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Journal of Applied Polymer Science, Vol. 58, 95–100 (1995)
 CCC 0021-8995/95/010095-06

the wall-forming polymer, and change in temperature.

A number of workers have prepared microspheres and nanospheres from natural polymers like gelatin, serum albumin, and starch. In the case of gelatin, the insolubilization process may simply involve chilling an aqueous gelatin solution to 4°C, thereby forming a gel structure. The gel structure is labile and readily destroyed by moderate temperature increases. Hashida and coworkers described the preparation of drug-loaded gelatin microspheres that are not chemically crosslinked.[14,15] If a chemical crosslinking agent is added to the system, a stable structure is formed. Aldehydes are commonly used to crosslink or fix the protein structures. Thus it is not surprising that reports of aldehyde crosslinked protein microspheres have appeared. Lee et al.[16] describe the preparation of glutaraldehyde treated serum albumin microspheres. Yoshioka et al.[17] prepared drug-loaded gelatin microspheres by ultrasonically emulsifying an aqueous drug/gelatin solution in excess sesame seed oil. In these systems environmentally undesirable solvents such as diethyl ether or chloroform are required to remove the oil phase of the emulsion and the resulting microspheres often contain residual surfactants used as emulsifiers.

The rate of permeation of chemicals through the polymer network structure depends on the network structure swelling ability, that in return is highly influenced by factors such as crosslink density, crystallinity, porosity, and hydrophilicity as well as by the chemical structure of the polymer. The degree of crosslinking affects the diffusion coefficient due to the change of the number of junctions and the added restrictions of the macromolecular chains. The average molecular weight between crosslinks, M_C, is a good parameter characterizing the crosslinking density, but an accurate calculation of it may be impossible because of experimental difficulties.[12]

In this study, phase separation induced by temperature change was used for the preparation of gelatin microspheres. This method takes advantage of the fact that gelatin is soluble in a hot aqueous solution but insoluble on cooling to room temperature. Temperature can therefore be used as another means to induce phase separation. Crosslinking of microspheres by glutaraldehyde was achieved and the number-average molecular weights between the crosslinks were calculated.

EXPERIMENTAL

Materials

Gelatin (Oxoid Co., bacteriological grade) and glutaraldehyde (50%, w/w, BDH, UK) were used as the matrix material and the crosslinking agent, respectively. *n*-Decane (Merck, F.R.G.) was used in the pycnometric density determination as the inert liquid that is immiscible with water.

Microsphere Preparation

Aqueous gelatin solution (20% w/v) was heated to 65°C and was sprayed through a capillary with a 0.5 kg/cm^2 pressure of nitrogen into cold water containing glutaraldehyde. Microspheres were reacted with glutaraldehyde for 3 h. For this crosslinking process, solutions containing 4, 8, 16, and 24% glutaraldehyde were used and the resultant microspheres were named as gel-1x, gel-2x, gel-3x, and gel-4x, respectively. After the completion of reaction, hardened gelatin microspheres were filtered and washed with cold water. To maintain spherical geometry and to avoid fissure or crack formation, the microspheres were dried slowly at 4°C in a sealed chamber at 40% humidity maintained by a saturated solution of $CaCl_2 \cdot 6H_2O$.

DSC and SEM Examinations

Thermal properties of crosslinked gelatin microspheres were analyzed by using a DuPont 1090 Differential Scanning Calorimeter attached to a DuPont 9900 thermal analyzer. The glass transition temperatures (T_g) of the microspheres were deter-

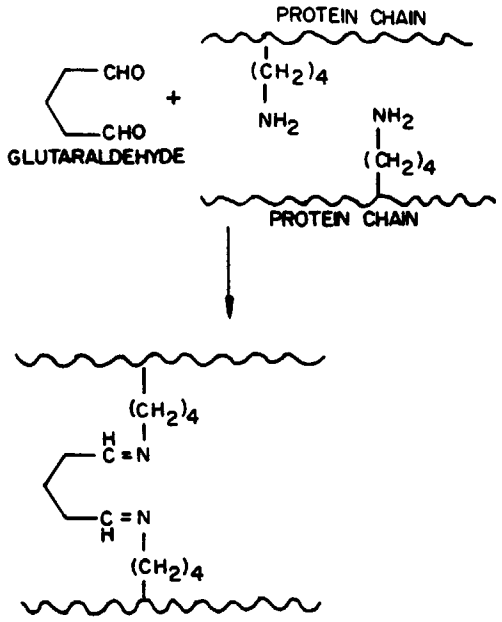

Figure 1 Scheme of reaction between glutaraldehyde and the ε-amino group of the lysine residue of the protein chain.

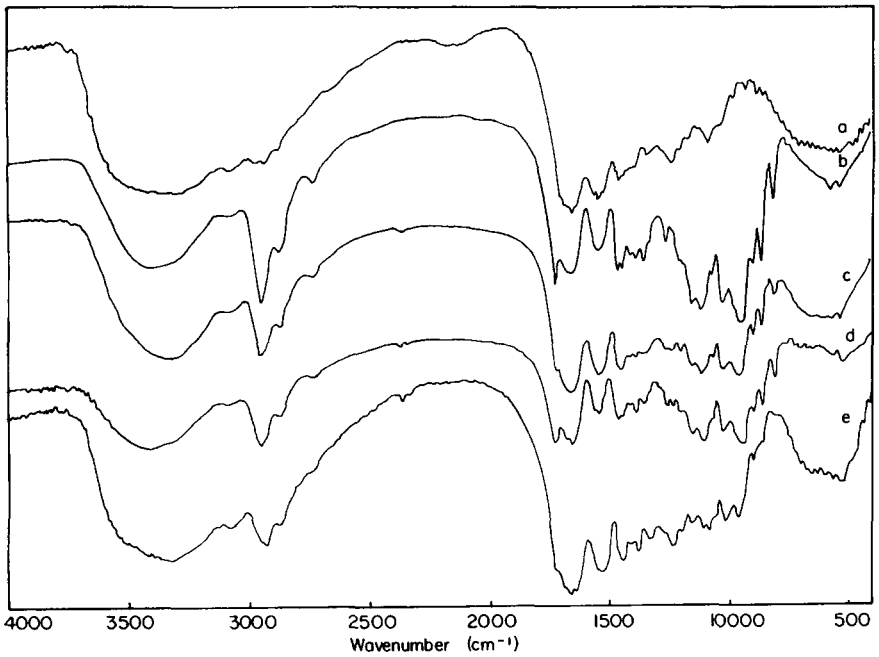

Figure 2 IR spectra: (a) gelatin, (b) gel-1x, (c) gel-2x, (d) gel-3x, (e) gel-4x.

mined under nitrogen atmosphere by using the Interactive DSC.V2.0 program.

The microsphere structure and the surface morphology of the samples were studied under vacuum by using a Jeol (Model 6400) scanning electron microscope. The particle size was determined by measuring the diameters of approximately 50 particles.

Measurement of Water Contents and Densities

Gelatin microspheres were placed into water at 25°C for 24 h to swell. Degradation of gelatin was assumed to be very small in this period and was not considered in the calculations. Dry and swollen densities of microspheres were determined by using a modified pycnometric method.[18]

Calculation of M_C and Some Swelling Parameters

The microspheres were characterized by equilibrium swelling experiments and their average molecular weights between crosslinks, M_C, were calculated from the modified Flory–Rehner equation given below [eq. (1)] in which $\nu_{2,s}$ is the volume fraction of polymer in the swollen state, X is the polymer–solvent interaction parameter, F is the functionality of crosslinking agent, d_p is the density of dry polymer, and V_1 is the molar volume of the solvent.[19] X was taken as 0.77 for collagen and water.[20]

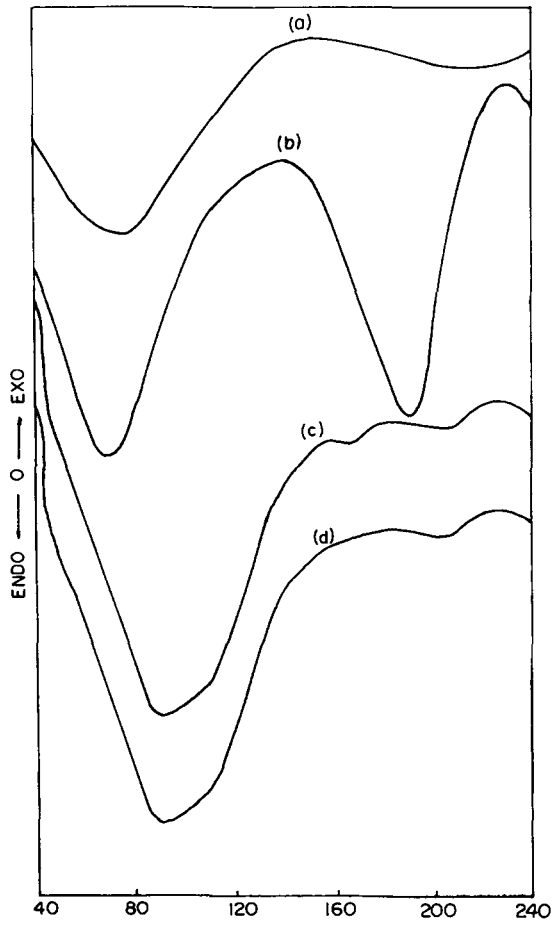

Figure 3 DSC curves: (a) gel-1x, (b) gel-2x, (c) gel-3x, (d) gel-4x.

Table I Swelling Properties of Crosslinked Microspheres

Sample	% H_2O (w/w)	VAS (mL/g)	Q (g, s/g, d)	Dry Density (g/cm^3)	Swollen Density (g/cm^3)
Gel-1x	30.4631	0.4380	1.4380	1.0121	1.0070
Gel-2x	25.4566	0.3374	1.3376	1.0309	1.0204
Gel-3x	21.5318	0.2744	1.2744	1.0975	1.0973
Gel-4x	19.4234	0.2410	1.2410	1.1362	1.1360

$$1/M_C = -[\ln(1 - \nu_{2,s}) + \nu_{2,s} + X\nu_{2,s}^2]/ d_p V_1[(\nu_{2,s})^{1/3} - (2\nu_{2,s}/F)]. \quad (1)$$

The polymer volume fraction, $\nu_{2,s}$, and weight fraction, $\omega_{2,s}$, of the swollen crosslinked microspheres were calculated using the following equations[21]:

$$\nu_{2,s} = V_{p,d}/V_{p,s} = (W_a - W_n)/(W_{a,s} - W_{n,s}) \quad (2)$$

$$\omega_{2,s} = W_a - W_{a,s}. \quad (3)$$

Here $V_{p,d}$ and $V_{p,s}$ are the volume of dry and swollen microspheres, W_a and $W_{a,s}$ are the weight of the dry and swollen microspheres in air, and W_n and $W_{n,s}$ are the weight of the dry and swollen microspheres in n-decane at 25°C, respectively.

Solvent content (wt %), weight swelling ratio (Q), and the volume of adsorbed solvent (VAS) were calculated according to the following equations.[18]

$$\text{wt}(\%) = (W_{a,s} - W_a)/W_{a,s} \times 100 \quad (4)$$

$$Q = W_{a,s}/W_a \quad (5)$$

$$\text{VAS} = (W_{a,s} - W_a)/d_{so}W_a \quad (6)$$

where d_{so} is the density of solvent.

Using the swelling data, change in volume (q_ν) or change in weight (q_ω), can be presented as;

$$q_\nu = V_s/V_p = 1/\nu_{2,s} \quad (7)$$

$$q_\omega = (W_{a,s} - W_a)d_m/W_a d_{so}. \quad (8)$$

RESULTS AND DISCUSSION

Glutaraldehyde is one of the most popular crosslinking reagents, especially for proteins. Because it reacts easily at room temperature with obvious color change characteristic of Schiff base linkages, it is well established that the aldehyde group of the reagent reacts with the amino group of the lysine residues of the protein chain to form a Schiff base.[22] Figure 1 shows the reaction between glutaraldehyde and gelatin. In this study, crosslinking was observed as the color changed from the pale yellow to deep orange within a few minutes on treatment with glutaraldehyde. The color change is due to the establishment of aldimine linkages (CH=N), between the free amino groups of protein and glutaraldehyde.

Crosslinking with glutaraldehyde is an instantaneous reaction and leads to immediate formation of microspheres from the dispersed gelatin droplets.

Further proof for crosslinking came from IR spectra. FTIR spectrum of microspheres and native gelatin is shown in Figure 2. The IR spectra of polypeptides and proteins have two distinct absorption bands, the C=O stretching at 1650 cm^{-1} and the NH stretching at 3300 cm^{-1}. The characteristic absorptions of the backbone occurring at 1540 and 1650 cm^{-1} are the only distinguishing features of the gelatin. The crosslinked gelatin shows, in addition to the previously mentioned peaks, the aldimine absorption peak at 1450 cm^{-1}. Similar spectra are given for native and crosslinked gelatin in the literature.[21]

The DSC curves of microspheres are given in Figure 3. Generally, an endotherm located at a T_g indicates time-dependent thermal relaxations in the

Table II Structural Characteristics of Crosslinked Gelatin Microspheres

Sample	$\nu_{2,s}$	$\omega_{2,s}$	q_ν	q_ω	M_c (g/mol)
Gel-1x	0.5302	0.6954	1.8861	0.4433	578.5
Gel-2x	0.5488	0.7476	1.8222	0.3478	330.9
Gel-3x	0.5632	0.7847	1.7756	0.3044	249.0
Gel-4x	0.5678	0.8059	1.7612	0.2738	233.2

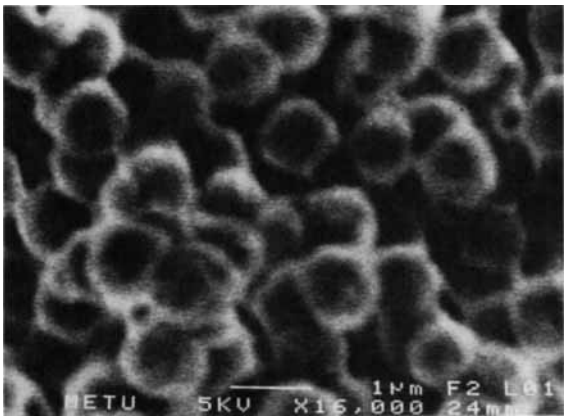

Figure 4 SE micrograph of gel-1x microspheres.

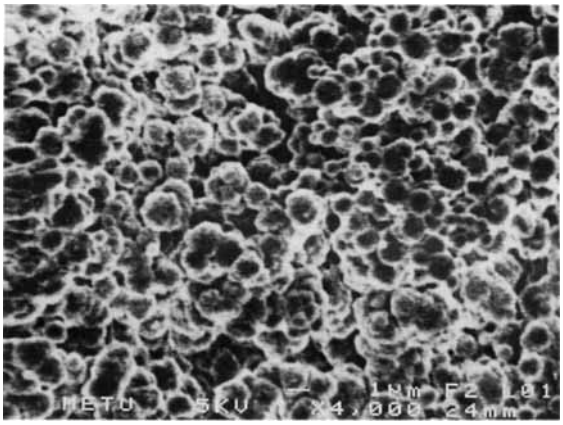

Figure 6 SE micrograph of gel-3x microspheres.

polymer. Two glass transition temperatures for gelatin were observed in each thermogram. This occurrence has been explained by the block copolymer model for the amino acid content of gelatin.[23] The first glass transition temperature is a minor one, located around 80–100°C and associated with the glass transition of α-amino acid blocks in the peptide chain. Detection of this is not easy with a highly hydrophilic material like collagen because evaporation of water takes place, in the same temperature range, with a strong endothermic peak. The second, more intense glass transition temperature is located around 180–200°C, depending on the method of evaluation, and represents the blocks of imino acids, proline, hydroxyproline, and glycine. The second T_g is apparently responsible for the overall physical behavior of gelatin and is one of the most cited and studied.[23,24] The first T_g value of around 95°C agrees well with glass transition temperatures reported.[25–27] The first and second glass transition temperatures shift from around 80 to 95°C and from around 180 to 210°C, respectively, with increasing degree of crosslinking.

Analytical study of the swelling properties of gelatin can be more difficult than that for other synthetic hydrophilic polymers due to the susceptibility of the protein chains to hydrolysis. For all other crosslinked microspheres, except the native gelatin, the percent solvent content (% H_2O), weight swelling ratio (Q), and VAS values were found and are listed in Table I. A decrease from 30.5 to 19.4% in % H_2O, from 1.44 to 1.24 in Q, and from 0.44 to 0.24 in VAS was observed with an increase in crosslinking density obtained by increasing glutaraldehyde concentration from 4 to 24% in the crosslinking medium. An increase in crosslink density also caused an increase in dry densities of microspheres from 1.0121 to 1.1362 g/cm^3. The values of $\nu_{2,s}$, $w_{2,s}$, q_ν, q_ω, and M_C are listed in Table II. The values of these parameters varies between 0.5302 and 0.5678 for $\nu_{2,s}$, 0.695 and 0.806 for $w_{2,s}$, 1.886 and 1.761 for q_ν, and 0.443 and 0.273 for q_ω as the crosslink density increases (from gel-1x to gel-4x). The M_C values were found to be in between 578.5 and 233.2 and are quite low. This might be due to calculating the M_C from

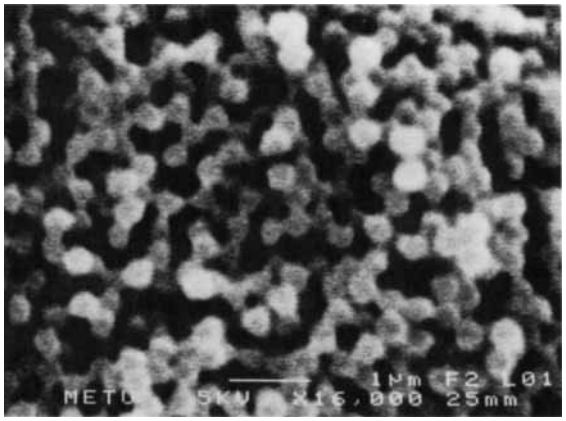

Figure 5 SE micrograph of gel-2x microspheres.

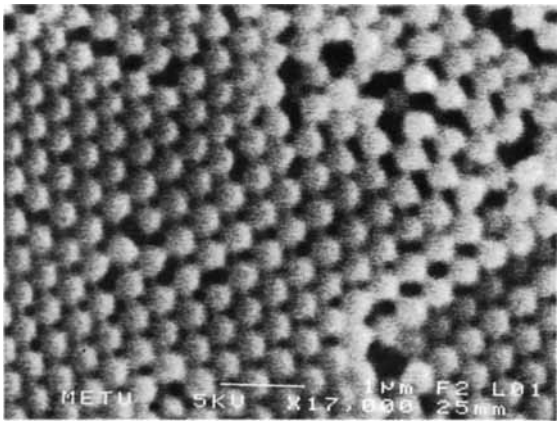

Figure 7 SE micrograph of gel-4x microspheres.

swelling experiments without differentiating between physical and chemical crosslinks. Some of the gelatin chains have a collagenlike helical conformation and because of denser packing, they swell more slowly than the domains of randomly coiled chains.[28]

The microspheres prepared had good spherical geometry and narrow particle size distribution as evidenced by SEM (Figs. 4–7). The average particle size was about 1.0 μm for gel-1x and gel-2x, 0.5 μm for gel-3x, and 0.25 μm for gel-4x microspheres. The extent of crosslinking is an effective parameter in the control of the shape and the size of the beads. When the amount of crosslinker was increased, the size of the microspheres decreased and this occurrence may be explained by increased crosslinking density resulting in the formation of densely packed structurelike helical formation causing a shrinkage of the particles. Increasing crosslinking results in slower rate of water evaporation and leads to the formation of microspheres with a smooth surface. Aggregation observed for the microspheres also decreased with increased amount of glutaraldehyde.

In conclusion, gelatin microspheres approximately 1.0 μm in diameter can be reproducibly prepared by controlling the concentration of the crosslinker, the pressure of the nitrogen gas (atomizing gas), and the temperature of the medium. Water is a true solvent for gelatin, but the reaction between gelatin and glutaraldehyde is an instantaneous reaction and this leads to formation of crosslinked microspheres from the gelatin droplets. This is a promising technique for the large-scale preparation of gelatin microparticles as drug delivery systems without using undesirable solvents and other ingredients.

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Received October 4, 1994
Accepted April 1, 1995