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Gelatin Microspheres and Sponges for Delivery of Macromolecules

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ABSTRACT: Gelatin microspheres and gelatin sponges were prepared by coacervation and freeze drying techniques, respectively. Both systems were crosslinked with glutaraldehyde. The mean diameter of the microspheres were in the range of 40–80 μm and the mean pore size of the sponges was 130–220 μm depending on the preparation conditions. Bovine serum albumin (BSA) was added into the preparation solutions and entrapped in the microspheres and sponges. BSA addition to sponges was also achieved by addition of BSA-containing microspheres into the sponges. The release kinetics of BSA from the prepared systems were examined. Studies demonstrated that release is dependent on the amount of BSA present in the system and crosslinking densities of microspheres. It was concluded that gelatin microspheres and gelatin sponges are promising carrier matrices for macromolecules.

KEY WORDS: gelatin, microsphere, sponge, protein, controlled release.

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INTRODUCTION

Use of gelatin in pharmaceutical and biomedical field is very attractive since it is a nontoxic, biodegradable, inexpensive, nonimmunogenic material and has a very high potential for use with a variety of medicinal agents. It has also been used as wound dressings [1–5], as hemostatic and wound healing agents [6,7], as sealant for vascular prosthesis [8,9], and in drug delivery systems such as hard and soft capsules [10,11], hydrogels [12], or microspheres [13–15].

Many biodegradable materials are used in the preparation of microspheres as carriers for macromolecules and proteins. But in the preparation processes of microspheres there are some limitations, such as need for high temperatures, surfactants, solvents or other ingredients. All these factors may affect the protein structure and cause denaturation. Because of their delicate tertiary structures, proteins are very sensitive and often denature either during the incorporation process or in the body for prolonged periods. Efficient protein delivery requires not only controlling the release rate but also solving the problems of inactivation and protein aggregation. For polymeric matrices, biocompatibility and the degradation products of the polymer are of extreme importance. Gelatin is successfully used in medicine especially for drug delivery applications because of its biological origin and inherent biocompatibility. Preparation of gelatin microspheres and their use in the controlled release of macromolecules, proteins and anticancer drugs has received wide attention especially in the last decades [16–19]. It was reported that gelatin-based microspheres offer excellent potential as carrier systems for *in-vivo* administration of both single and double-stranded DNA molecules [20]. For controlled release of growth hormones monolithic gelatin microspheres were successfully developed [21]. It was also shown that gelatin microspheres are promising carriers for bFGF to enhance the vascularization effect [22]. More recently gelatin scaffolds are proposed for tissue engineering applications [23].

The purpose of the present study was to prepare gelatin systems in microsphere, sponge or combined forms that are capable of delivering macromolecules and proteins in the applied region and at the target area.

MATERIALS AND METHODS

Materials

Fluorescein isothiocyanate labelled bovine serum albumin (FITC-BSA, FITC content 11 mol/mol, Mw ~ 66,000 g/mol) was purchased from

Sigma, USA. Gelatin (bacterial grade) was obtained from Difco (USA) and glutaraldehyde was a product of BDH (UK). All other reagents were of analytical grade and used as received.

Preparation of Gelatin Microspheres

General Procedure

Gelatin microspheres were prepared by coacervation technique [24]. Briefly, the procedure was as follows: Aqueous gelatin solution (15%, w/v) was added to paraffin oil and stirred at 1000 rpm to form a water–oil emulsion. Then the solution was rapidly cooled and filtered. The removal of residual oil was performed by washing the formed microspheres with acetone. These gelatin microspheres were dried at room temperature and labelled as GM.

Preparation of Gelatin Microspheres with FITC-BSA

Gelatin microspheres containing FITC-BSA were prepared in the same manner except that the bioactive agent (FITC-BSA, 50 mg in 1 mL phosphate buffer, 0.01 M, pH = 7.4) was added to the gelatin solution at the beginning. These microspheres were labelled as GM-BSA. The microspheres were hardened with glutaraldehyde treatment. For this purpose, aqueous glutaraldehyde solutions (1 mL, at various concentrations of 0.1%, 1% or 5%) were added to the water–oil emulsion of microspheres. The unreacted glutaraldehyde and paraffin oil were removed by filtering and washing with water and acetone. The obtained microspheres were dried at room temperature. Depending on the concentrations of added glutaraldehyde, these microspheres were labelled as; GMH0.1-BSA, GMH1-BSA and GMH5-BSA. The microspheres prepared in this research are tabulated in Table 1.

Preparation of Gelatin Sponges

General Procedure

Aqueous gelatin solutions (2% or 5%) were stirred at about 2000 rpm for 30 min and then glutaraldehyde solution (1 mL of 0.1%) were added continuing stirring. The foaming solutions were poured into the molds, frozen and freeze-dried. These sponges were labelled as GS and GS5 depending on the gelatin concentration.

Preparation of Gelatin Sponges with FITC-BSA

FITC-BSA solution (50 mg FITC-BSA dissolved in 1 mL phosphate buffer 0.01 M, pH = 7.4) was added into the foaming gelatin solution

Table 1. Prepared gelatin microspheres

Samples	Glutaraldehyde (%)	Bioactive agent
GM	–	–
GM-BSA	–	FITC-BSA (50 mg)
GMH0.1-BSA	0.1	FITC-BSA (50 mg)
GMH1-BSA	1.0	FITC-BSA (50 mg)
GMH5-BSA	5.0	FITC-BSA (50 mg)

Table 2. Prepared gelatin sponges

Samples*	Gelatin solution (w/v) (%)	Bioactive agents
GS	2	–
GS5	5	–
GS-BSA	2	FITC-BSA (free form)
GS-GM-BSA	2	FITC-BSA (in microspheres)

*all sponges contain 0.1% glutaraldehyde solution

(2% w/v), poured into molds, frozen and freeze-dried. These sponges were labelled as GS-BSA.

Preparation of Gelatin Sponges with FITC-BSA Loaded Microspheres

FITC-BSA containing microspheres (GM-BSA) prepared previously were added into the foaming gelatin solution (2% w/v), poured into molds, frozen and freeze-dried. These sponges were labelled as GS-GM-BSA. Various types of sponges prepared in this research are tabulated in Table 2.

Morphological Analysis

The morphologies of microspheres and sponges were examined by a scanning electron microscope (SEM, Jeol Model 6400). For this purpose, the samples were coated with gold under vacuum and their scanning electron micrographs were obtained.

IR Analysis

IR spectra of microspheres were obtained from KBr discs of the samples by using Nicolet 510 FTIR spectrophotometer at a resolution of $2\,cm^{-1}$.

Thermal Analysis

Thermal properties of gelatin microspheres were studied by using a DuPont 2000 Differential Scanning Calorimeter. Measurements were carried out under nitrogen atmosphere by using about 4–6 mg samples with heating and cooling rates of $10^{\circ}C\,min^{-1}$. The range of heating cycles was varied from 10 to 200°C for the first heating and 10 to 300°C for the second heating runs.

Particle Size Analysis

The average size and size distribution curves of microspheres were obtained in acetone by using a particle size analyzer (Malvern Instruments Ltd., Model Mastersizer S, Malvern, UK).

Mechanical Testing

Tensile properties of the prepared sponges were investigated by using Lloyd® Mechanical Testing Machine (Lloyd Instruments, LRX 5K). For this purpose rectangular sponge samples (2 cm × 5 cm × 0.8 cm) were placed on the fixed and movable member of the mechanical testing machine by special clamps. The applied test speed was 2 mm/min. Results were obtained from a computer which was connected to the mechanical testing machine. Six samples were subjected to tensile tests and average values for tensile strength and elastic modulus were obtained.

In Vitro FITC-BSA Release

The release of FITC-BSA from microspheres was studied in a constant temperature shaking bath by incubating the samples in phosphate buffer solutions (0.01 M, pH = 7.4) at 37°C. For this purpose, microspheres (30 mg) or sponge samples (1 cm^2) were put into buffer solutions (5 mL). Sodium azide was added into the release medium to prevent microbial growth. At certain time intervals liquid part was withdrawn, filtered through 0.45 μm millipore filters, analyzed spectrophotometrically at $\lambda = 495$ nm and released FITC-BSA amounts were calculated from the calibration curves [25].

RESULTS AND DISCUSSION

Use of gelatin in pharmaceutical and biomedical applications is particularly attractive by virtue of its biocompatibility and biodegradability

Table 3. Microspheres recovery

Samples	Microspheres recovery (%)*
GM	90.2 ± 2.3
GM-BSA	88.5 ± 4.1

*Data represent the average of 5 independent experiments

together with the total absence of toxicity or allergic problems generally associated with the use of synthetic polymers.

In this study gelatin microspheres and sponges were prepared with rather simple methods by avoiding the use of surfactants, organic solvents or high temperature.

For the prepared microspheres, the yields (the ratio of microsphere weight to initial weight) were found to be over 88% (Table 3). This point is particularly appealing since it allows minimal loss of material during the preparation. This could profoundly influence the total cost of the procedure, especially in the case of addition of expensive bioactive agents such as growth factors, DNA, etc. On the other hand, the prepared sponges were very soft, white, porous and highly elastic structures (Figure 1). Mechanical properties of these sponges were tested by Lloyd® LRX5K mechanical test machine. Because of the very porous structure of the sponges, it was very difficult to determine the tensile strength or modulus of elasticity values. The measurements were very carefully carried out for 6 samples and the average values were determined. The values for tensile strength and elastic modulus were found as 9.701 ± 1.974 KPa and 0.6912 ± 0.1021 MPa, respectively.

Morphological Analysis Results

SEM micrographs indicated that no aggregation between the microspheres had occurred (Figure 2). The surfaces of some of the microspheres were quite smooth and no pores were observed. SEM micrographs of BSA containing microspheres demonstrated very similar shapes indicating almost no effect of addition of BSA or hardening procedure (Figure 2B through E).

SEM micrographs of the gelatin sponges are shown in Figure 3. All the prepared sponges demonstrated very similar lattice structures. It was observed that pore sizes were dependent on the concentration of the initial gelatin solutions. Sponges which were prepared by using 2% gelatin solution (GS) had pores with a diameter of approximately 220 μm

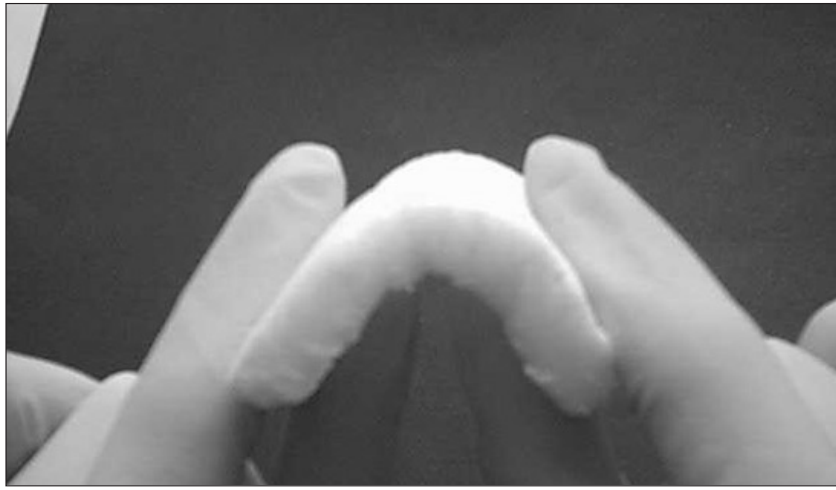

Figure 1. Photographs of gelatin sponges.

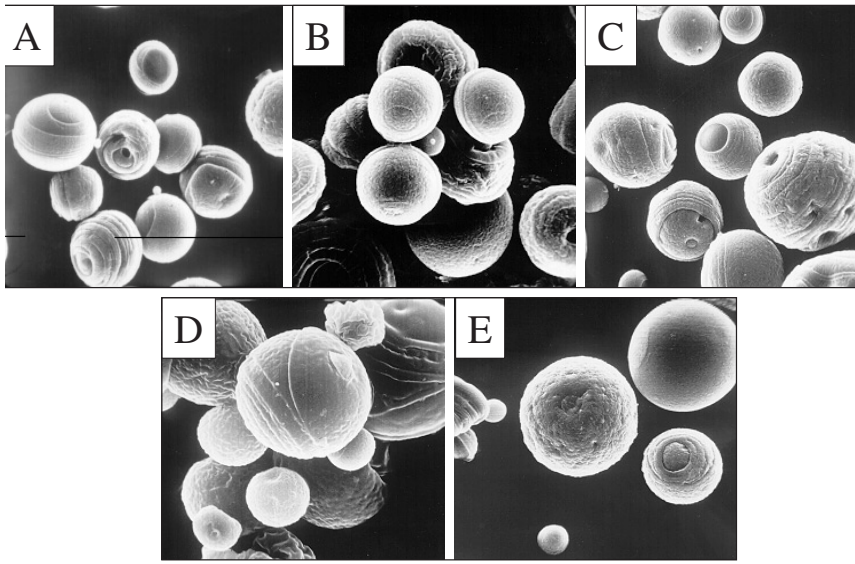

Figure 2. SEM of gelatin microspheres (×1600) (A) GM (B) GM-BSA (C) GMH0.1-BSA (D) GMH1-BSA (E) GMH5-BSA.

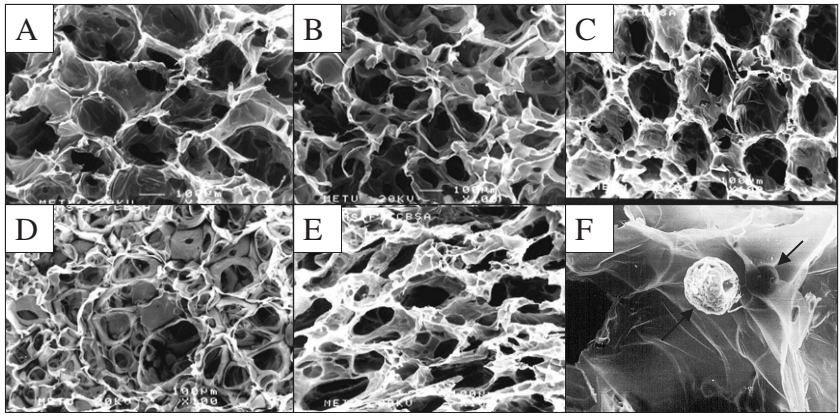

Figure 3. SEM of gelatin sponges (A) GS, cross-section × 100 (B) GS5, cross-section × 100 (C) GS-BSA, cross-section × 100 (D) GS-GM-BSA, surface × 100 (E) GS-GM-BSA, cross-section × 100 (F) GS-GM-BDA cross-section × 1600.

while the ones prepared by using 5% gelatin solution (GS5) had approximately 130 μm pore diameter (Figures 3A and B). There was no significant difference between the structures of gelatin sponges loaded with BSA (GS-BSA) and the ones without BSA (GS) (Figures 3A and C). Gelatin sponges containing BSA loaded microspheres (GS-GM-BSA) are shown in Figures 3D and E. At higher magnifications the microspheres inside the sponge structure are clearly evident (Figure 3F).

FTIR Analysis

FTIR spectra of gelatin microspheres are given in Figure 4. The infrared spectra of polypeptides and proteins have two characteristic absorption bands, C=O stretching at 1650 cm^{-1} and NH stretching at 3000 cm^{-1}. The characteristic absorptions of the backbone peptide bond (amide) occurring at 1540 and 1650 cm^{-1} are the only distinguishing features of the gelatin. For the samples which were crosslinked by glutaraldehyde, the characteristic absorption of aldimine groups occurs at 1450 cm^{-1} [26].

For the examined samples these characteristic bands of gelatin were observed for all samples. Similar spectra were obtained with BSA loaded samples implying that very low amounts of BSA did not cause any alterations in gelatin spectra.

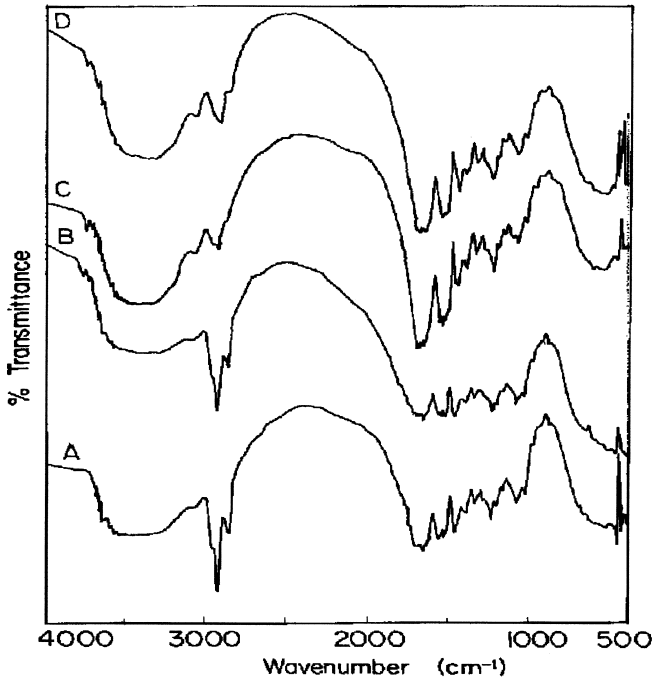

Figure 4. FTIR Spectra of gelatin microspheres (A) GM (B) GMH5 (C) GM-BSA (D) GMH5-BSA.

Thermal Analysis

The DSC curves of the prepared microspheres and sponges are given in Figure 5. The peaks observed at around 70°C for the microspheres might be the removal of acetone and the peaks observed around 100°C are most probably caused by dehydration and water removal. The second heating cycle (Figure 5B) demonstrated intense glass transition at almost 220°C, characterized by energy absorption overlapped with the change in the specific heat in each thermogram. This glass transition is responsible for the overall behavior of the material (glass or rubbery). Fraga and Williams reported two glass transition temperatures, at 120°C and 180–190°C. The low temperature transition was assigned to the devitrification of blocks rich in α-amino acids (soft blocks), while the high temperature transition was attributed to the devitrification of blocks rich in imino acids (rigid blocks) [27]. In Figure 5, a large peak was observed at the temperature range of

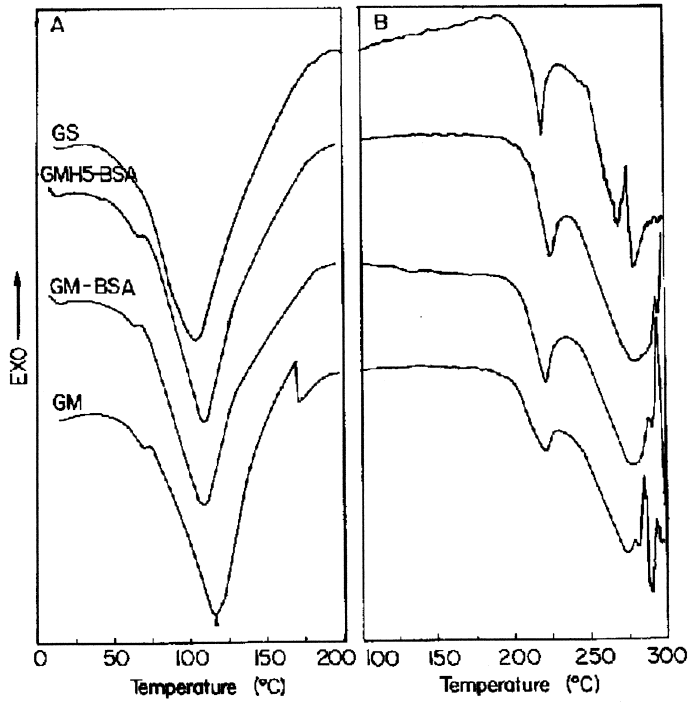

Figure 5. DSC thermograms of gelatin samples (A) first heating (B) second heating.

95–118°C probably due to the removal of water. This endotherm was not observed in the second heating run. It was observed that addition of crosslinker shifts the T_g from 220 to 225°C (Figure 5B). In addition, endothermal peaks associated with thermal degradation were observed at temperatures in the range of 235–300°C.

Particle Size Analysis of Microspheres

Experimental parameters employed for microsphere preparation (speed of mixing, aqueous to organic phase ratio, etc.) affect the particle size of the microspheres. By using a stirring speed of 1000 rpm, microspheres with mean diameters of 39.84 and 81.18 μm were obtained (Table 4). The percentages of the cumulative sizes of the microspheres are as follows:

For GM, GM-BSA, GMH0.1-BSA, GMH1-BSA and GMH5-BSA, 10% of the particles were found to be under 16.50, 15.87, 26.70, 26.40 and

Table 4. Particle size analysis results

Microsphere type	$D(v, 0.1)$ (μm)	$D(v, 0.5)$ (μm)	$D(v, 0.9)$ (μm)	VMD (μm)	SMD (μm)
GM	16.50	38.11	76.16	42.80	17.85
GM-BSA	15.87	35.18	70.27	39.84	16.52
GMH0.1-BSA	26.70	58.19	107.12	62.62	27.02
GMH1-BSA	26.40	58.73	117.87	81.18	27.25
GMH5-BSA	25.22	53.79	92.94	54.83	23.92

$D(v, 0.1)$ is the size of particle which 10% of the sample is below this size.
$D(v, 0.5)$ is the size of particle at which 50% of the sample is smaller and 50% is larger than this size. This value also known as the mass median diameter (MMD).
$D(v, 0.9)$ gives a size of particle which 90% of the sample is below this size.
VMD is the volume mean diameter.
SMD is the surface area mean diameter. Also known as the Sauter mean.

25.22 μm in diameter, respectively and for the same samples 50% of the samples were found to be under 38.11, 35.18, 58.19, 58.73 and 53.79 μm.

The effect of BSA loading and addition of crosslinker on the average particle size and size distribution curves of the microspheres were not very significant. But it could still be concluded that increasing the amount of crosslinker caused a slight increase in the mean diameter of the microspheres. However, it would be possible to obtain smaller microspheres by changing the experimental conditions such as stirring speed, concentration of the gelatin solution or addition of surface active materials to the reaction medium during the preparation step [24]. It should be noted that addition of surfactants might decrease biocompatibility and cause some adverse effects. Therefore, in this study, no surfactants were used to take precautions for the mentioned problems as well as to prevent their possible effect on the denaturation of BSA.

In Vitro FITC-BSA Release

For release studies, a high molecular weight protein, FITC-BSA was chosen as a model compound and release was studied for three systems described below,

1. FITC-BSA containing microspheres with varying crosslink densities
2. Gelatin sponges containing FITC-BSA in free form
3. Gelatin sponges containing FITC-BSA loaded microspheres

For each system, release experiments were repeated at least three times. The deviations of the obtained values were less than 3% and the average values were plotted on the graphs.

Figures 6 and 7 show the release profiles of FITC-BSA in 0.01 M phosphate buffer (pH 7.4) at 37°C from the prepared systems. The

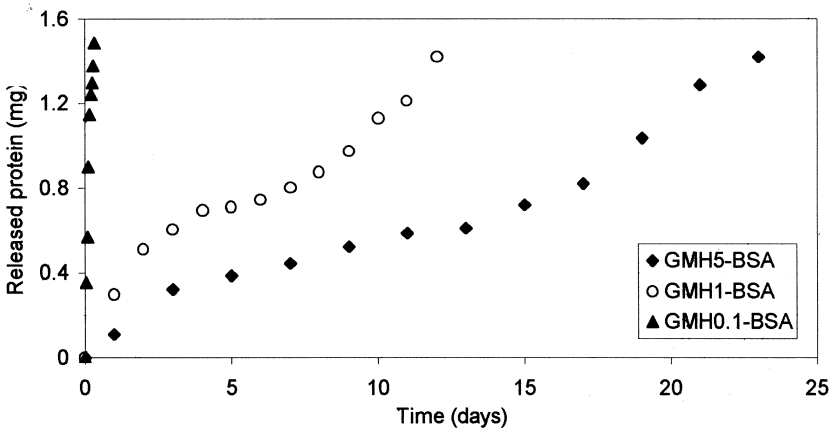

Figure 6. Release of BSA from gelatin microspheres.

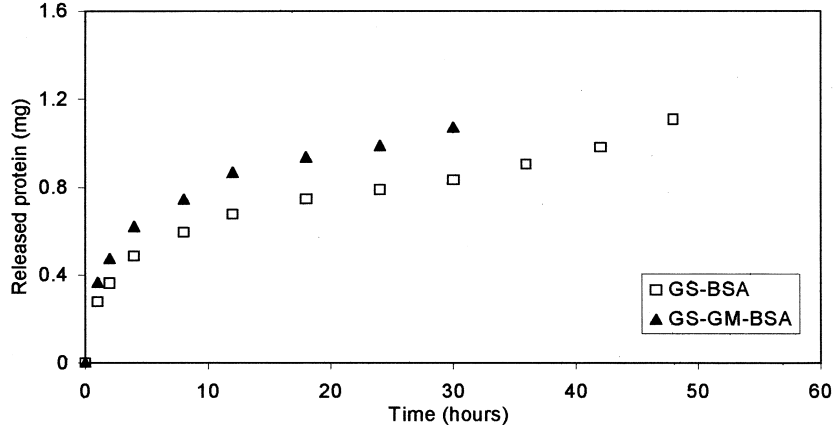

Figure 7. Release of BSA from gelatin sponges.

release duration of BSA varied from hours to days, depending on the structures of the systems and crosslinking densities of microspheres. For microspheres, at higher crosslinking densities, swellability, rate of degradation and rate of release are slower (Figure 6). For GMH0.1-BSA system, the release lasted for about 8 h and approximately 1.4 mg of BSA was released from these microspheres during this period. For GMH1-BSA and GMH5-BSA systems, characteristic release profiles were observed. Initial fast release reached equilibrium in about 3 days and

then a second increase in rate, which was caused by the degradation of microspheres, was observed and continued until complete erosion. For GMH1-BSA and GMH5-BSA systems, approximately 1.4 mg FITC-BSA was released in 12 and in 24 days, respectively. By increasing crosslinking density of microspheres, it became possible to extend the release duration from a few hours to certain weeks.

BSA release profiles from gelatin sponges are shown in Figure 7. From GS-BSA, approximately 1 mg BSA was released in 48 h while from GS-GM-BSA system, 1 mg BSA was released in 30 h. Both curves are quite similar to each other. The microspheres in GS-GM-BSA systems were not initially crosslinked. Therefore a faster release, which would complete in few hours, was expected. But the release was completed in few days. This, most probably, is the result of presence of glutaraldehyde (1 mL of 0.1%) in the sponge solution. Glutaraldehyde caused some crosslinking reactions in the microspheres or binding reactions of proteins to gelatin microspheres. In both cases the result is a slower and delayed protein release. For both systems, typical two-step release was observed, where the initial rapid release was due to the diffusion while the second step was due to erosion of the matrix.

Gelatin has a hydrophilic structure; therefore both bulk and surface erosion take place. The release of high molecular weight bioactive materials occurs in a combined process of diffusion and erosion. These processes either follow each other or occur simultaneously. In these studies, initial fast rates were obtained for the diffusion of protein close to surface. The second step gives the results of degradation. Time duration for this second step and the slope of it depends on crosslinking densities of microspheres.

CONCLUSIONS

In conclusion, high molecular weight proteins can be efficiently incorporated with gelatin microspheres and sponges and the release rates of these macromolecules can be controlled and can be extended from hours to weeks by adjusting some parameters of the structures. This type of systems can be used for delivering of proteins, macromolecules, growth factors, hormones etc. In fact, the matrices prepared by the addition of EGF were tested *in vivo* on the wounds created on rabbits and significantly good results in wound healing were observed [2].

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REFERENCES

1. Choi, Y.S., Hong, S.R., Lee, Y.M., Song, K.W., Park, M.H. and Nam, Y.S. (1999). Study on gelatin-containing artificial skin – I – Preparation and characteristics of novel gelatin-alginate sponge. *Biomaterials*, **20**: 409–417.
2. Ulubayram, K., Çakar, A.N., Korkusuz, P., Ertan, C. and Hasirci, N. (2001). EGF containing gelatin based wound dressing. *Biomaterials*, **22**(11): 1345–1356.
3. Drave, J.P., Delaey, B., Van de Voorde, A., Van Den, Bulcke, A., De Reu, B. and Schacht, E. (1998). *In vitro* and *in vivo* biocompatibility of dextran dialdehyde cross-linked gelatin hydrogel films. *Biomaterials*, **19**: 1677–1687.
4. Takahaski, H., Miyoshi, T. and Boki, K. (1993). Study on hydrophilic properties of gelatin as clinical wound dressing. II. Water-absorbing property and hemostatic effect of gelatin. *Tokushima J. Exp. Med*., **40**: 169–175.
5. Neumann, P.M., Zur, B. and Ehrenreich, Y. (1981). Gelatin-based sprayable foams as skin substitute. *J. Biomed. Mater. Res*., **15**: 9–18.
6. Petersen, J.K., Krogsgaard, J., Nielson, K.M. and Norgaard, E.B. (1984). A comparison between 2 absorbable hemostatic agents: gelatin sponge (spongostan) and oxidized regenerated cellulose (surgical). *Int. J. Oral Surg*., **13**: 406–410.
7. Sung, H.W., Huang, D.M., Chang, W.H., Huang, L.L., Tsai, C.C. and Liang, I.L. (1999). Gelatin-derived bioadhesives for closing skin wounds: an *in vivo* study. *J. Biomater. Sci. Polym. Ed*., **10**: 751–771.
8. Jonas, R.A., Ziemer, G., Schoen, F.J., Britton, L. and Castaneda, A.R. (1988). A new sealant for knitted Dacron prostheses minimally cross-linked gelatin. *J. Vasc. Surg*., **7**: 414–419.
9. Chakfe, N., Marois, Y., Guidoin, R., Deng, X., Marois, M., Roy, R., King, M. and Douville, Y. (1993). Biocompatibility and biofunctionality of a gelatin-impregnated polyester arterial prosthesis. *Polym. Composites*, **1**: 229–252.
10. Digenis, G.A., Gold, T.B. and Shah, V.P. (1994). Cross-linking of gelatin capsules and its relevance to their *in vitro–in vivo* performance. *J. Pharmaceu. Sci*., **83**(7): 915–921.
11. Li, J.K., Wang, N. and Wu, X.S. (1998). Gelatin nanoencapsulation of protein/peptide drug using an emulsifier-free emulsion method. *J. Microencapsulation*, **15**(2): 163–172.
12. Tabata, Y., Nagano, A., Md, M. and Ikada, Y. (1998). *In vitro* sorption and desorption of basic fibroblast growth factor from biodegradable hydrogels. *Biomaterials*, **19**: 1781–1789.
13. Cortesi, R., Nastruzzi, C. and Davis, S.S. (1998). Sugar cross-linked gelatin for controlled release: microspheres and disks. *Biomaterials*, **19**: 1641–1649.
14. Narayani, R. and Rao, K.P. (1994). Controlled release of anticancer drug methotrexate from biodegradable gelatin microspheres. *J. Microencapsulation*, **11**: 69–77.
15. Lou, Y. and Groves, M.J. (1995). The use of gelatin microparticles to delay the release of readily water-soluble materials. *Pharm. Pharmacol*., **47**: 97–102.

16. Nastruzzi, C., Pastecini, C., Cortesi, R., Esposito, E., Gambari, R. and Menegatti, E. (1994). Production and *in-vitro* evaluation of gelatin microspheres containing an antitumor tetra-amidine. *J. Microencapsulation*, **3**: 249–260.
17. Jeanthi, R. and Panduranga, R. (1987). Preparation of gelatin microspheres of bleomycin. *Int. J. Pharma.*, **35**: 177–179.
18. Yan, C., Li, X., Chan, X., Wang, D., Zhong, D., Tan, T. and Tano, H. (1991). Anticancer gelatin microspheres with multiple functions. *Biomaterials*, **12**: 640–644.
19. Narayani, K. and Panduranga, R. (1993). Preparation, characterization and *in vitro* stability of hydrophilic gelatin microspheres using a gelatin-methotrexate conjugate. *Int. J. Pharmaceutics*, **95**: 85–91.
20. Cortesi, R., Esposito, E., Menegatti, E., Gambariç, R. and Nastruzzi, C. (1994). Gelatin microspheres as a new approach for the controlled delivery of synthetic oligonucleotides and PCR-generated DNA fragments. *Int. J. Pharmaceutics*, **105**: 181–186.
21. Di Silvio, L., Gurav, L., Kayser, M.V., Braden, M. and Downes, S. (1994). Biodegradable microspheres: a new delivery system for growth hormone. *Biomaterials*, **15**: 11.
22. Ikada, Y., Munıruzzaman, Md., Hıjıkata, S. and Tabata, Y. (1999). Neovascularization effect of biodegradation gelatin microspheres incorporating basic fibroblast growth factor. *J. Biomater. Sci. Polymer Edn.*, **10**(1): 79–94.
23. Ikada, Y., Tabata, Y. and Kang, H.Y. (1999). Fabrication of porous gelatin scaffolds for tissue engineering. *Biomaterials*, **20**: 1339–1344.
24. Esposito, E., Cortesi, R. and Nastruzzi, C. (1996). Gelatin microspheres: influence of preparation parameters and thermal treatment on chemico-physical and biopharmaceutical properties. *Biomaterials*, **17**(20): 2009–2020.
25. Cohen, S., Yoshioka, T., Lucarelli, M., Hwang, L.H. and Langer, R. (1991). Controlled delivery systems for protein based on poly(lactic/glycolic acid) microspheres. *Pharmaceutical Research*, **8**(6): 713–720.
26. Chatterji, P.R. (1989). Gelatin with hydrophilic/hydrophobic grafts and glutaraldehyde crosslinkings. *J. Applied Polym. Science*, **37**: 2203–2212.
27. Fraga, A.N. and Williams, R.J.J. (1985). Thermal properties of gelatin films. *Polymer*, **26**: 113–114.