Chitosan/Polyethylene Glycol–Alginate Microcapsules for Oral Delivery of Hirudin

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Received 12 January 1998; accepted 14 March 1998

ABSTRACT: A mild chitosan/calcium alginate microencapsulation process, as applied to encapsulation of biological macromolecules such as albumin and hirudin, was investigated. The polysaccharide chitosan was reacted with sodium alginate in the presence of calcium chloride to form microcapsules with a polyelectrolyte complex membrane. Hirudin-entrapped alginate beads were further surface coated with polyethylene glycol (PEG) via glutaraldehyde functionalities. It was observed that approximately 70% of the content is being released into Tris-HCl buffer, pH 7.4 within the initial 6 h and about 35% release of hirudin was also observed during treatment with 0.1 *M* HCl, pH 1.2 for 4 h. But acid-treated capsules had released almost all the entrapped hirudin into Tris-HCl, pH 7.4 media within 6 h. From scanning electron microscopic and swelling studies, it appears that the chitosan and PEG have modified the alginate microcapsules and subsequently the protein release. The microcapsules were also prepared by adding dropwise albumin-containing sodium alginate mixture into a PEG– $CaCl_2$ system. Increasing the PEG concentration resulted in a decrease rate of albumin release. The results indicate the possibility of modifying the formulation to obtain the desired controlled release of bioactive peptides (hirudin), for a convenient gastrointestinal tract delivery system. © 1998 John Wiley & Sons, Inc. J Appl Polym Sci 70: 2143–2153, 1998

Key words: chitosan; polyethylene glycol; alginate; microencapsulation; hirudin delivery; surface modification

INTRODUCTION

Proteins and enzymes represent a growing and promising field of therapeutics and are currently administered by injection. Many of these are stable only under physiological conditions, and their therapeutic application is limited by their short half-lives *in vivo*. Peptide drug delivery by routes other than the parenteral one has gained much attention in recent years. Routes include the nasal, pulmonal, buccal, transdermal, rectal and peroral routes.[1,2] Although peptide drug absorption is strongly hampered by the high metabolic activity and the low permeability of the absorbing tissues, several nasal peptide drug formulations such as buserelin and desmopressin have already been successfully introduced to the market. Many studies are currently being conducted to address other absorption sides for peptide delivery through different targeting mechanisms such as entrance via the Pyers patch or mucoadhesion.[3,4] Oral delivery is the easiest method of administration, and allows for a more varied load to be released; however, proteins are quickly denatured and degraded in the hostile environment of the stomach. A potential solution to these problems is the use of microencapsulation process for oral release of therapeutic agents.[5] The protein is en-

Correspondence to: G. H. R. Rao.

Contract grant sponsor: NIH; contract grant number: HL 1880.

Contract grant sponsor: Biomedical Engineering Institute.

Journal of Applied Polymer Science, Vol. 70, 2143–2153 (1998)

 CCC 0021-8995/98/112143-11

capsulated in a core material that is covered by a biocompatible, semipermeable membrane. The membrane controls the diffuse release rate of the protein from the capsule to the surrounding medium while protecting the remaining encapsulated protein from biodegradation. Alginates, a naturally occuring copolymer of guluronic and manuronic acid, are widely used in biomedical applications and are capable of being processed under mild conditions. The calcium alginate microcapsules have been utilized to encapsulate cells,[6] for hybrid artificial organs,[7] hemoglobin carriers, macromolecular delivery,[8,9] and recently, for oral delivery of transforming growth factor beta (GF-β_1).[10] Alginate, being polyanionic, polycationic polymer coatings of polylysine, polyvinyl amine,[9] chitosan,[8,11,12] etc., are employed to increase the stability of alginate capsules or to minimize the loss of encapsulated material.

Chitosan, a natural polysaccaride, having structural characteristics similar to glycosaminoglycans, seems to be nontoxic and bioabsorbable,[13] and has been explored for the release of several drugs.[14,15] Inouye et al.[16] developed a method for controlling the release of agents with a chitosan matrix. At low pH, the matrix remained gel form, whereas at a pH of 6.8, the matrix completely disintegrated. The diffusion of the bioactive agent is generally retarded when chitosan is in gel form. The gel-forming property of chitosan at low pH may be useful because a constant release rate is preferred in the gastrointestinal fluid.

Polyethylene glycol (PEG) is a water-soluble polymer that exhibits properties such as protein resistance, low toxicity, and immunogenicity.[17] Further, it has been observed that the PEGs can abrogate the immunogenicity of proteins and are capable of preserving their biological properties.[18,19] PEGs are used for improving the biocompatibility of polymers.

A major concern with the use of artificial organs and biomedical devices is the untoward interactions of blood upon contacting a foreign surface, resulting in surface thrombosis. Controlled release of appropriate drugs alone and in combinations is one of the approaches for treating coronary obstructions, balloon angioplasty, restenosis associated with thrombosis, and calcification.[20] Hirudin, naturally occuring specific, and highly potent direct inhibitor of thrombin has been shown in experimental studies to inhibit platelet deposition and thrombus formation after deep arterial injury to a greater extent than heparin[21,22] and to inhibit neointimal thickening after balloon injury in a rat hypercholesterolemic rabbit model of restenosis. Inhibition of neointimal thickening by systemic hirudin therapy has also been demonstrated in atherogenic rabbit iliac artery model. The convenient absorption route for patients, however, remains the peroral application. Such administration of drugs will lead to an improved compliance and, consequently, to a higher therapeutic benefit.

The intestinal absorption of peptide drugs is generally very poor. However, different drug carriers to transport the peptide to the most preferable site of absorption, e.g., colon targeting or through the absorbing tissue of the gut have been studied.[23,24] It is observed that a number of mucoadhesive polymers have promising effects on the modulation of the physiological barriers and subsequently enhancing the peptide drug absorption. Recent studies of Lueben et al.[25] have shown that chitosan can enhance the intestinal transport of peptide drugs, by increasing the paracellular permeability of the intestinal epithelium.

Presently, our aim is to utilize calcium alginate as a matrix to deliver hirudin (a polypeptide having M_w of 10,800 and a potent antithrombin agent[22]) orally in the gastrointestinal tract. A chitosan and PEG coatings are employed to increase the capsule strength and flexibility. Further, the effect of PEG in the formation of alginate microbeads and their role in protein delivery is also investigated. Bovine serum albumin (BSA) is chosen as a model drug for encapsulation studies in an alginate–PEG system. It seems that bioactive peptides can be conveniently released in the gastrointestinal tract via alginate–PEG/chitosan systems.

MATERIALS AND METHODS

Sodium alginate of medium viscosity ($\sim$ 3500 cps for a 2% solution at 25°C), polyethylene glycol, M_w 20,000 (PEG) and chitosan derived from crab shell chitin by greater than 85% deacetylation, having a particle size of 1–3 mm and M_w of approximately 1.0×10^6 were obtained from Sigma Co., St. Louis, MO. Hirudin (a polypeptide having M_w of 10,800), a bioactive protein having antithrombin activity, 1360 units/mg protein activity, bovine serum albumin (BSA), and protein assay kit (Lowry method) were also from Sigma Chemical Co. All other chemicals were of analytical reagent grade.

Microcapsule Formation

Sodium alginate (2% w/v) was dissolved in distilled water containing hirudin (160 μg%) or BSA (0.3 g%) and 0.9% NaCl. Approximately 20 mL of this solution was dropped through a needle (0.15-mm diameter), from a plastic syringe in a beaker containing 100 mL of calcium chloride solution (1.5 g%) under gentle stirring. The capsules formed were allowed to harden for 15 min in $CaCl_2$ solution, and then were filtered and rinsed with distilled water. A chitosan solution in 0.01 *M* HCl was prepared and the pH was adjusted to 5.7 using 0.1 *N* NaOH solution, and filtered to get a clear solution. A 100 mL chitosan solution containing 1.5 g% $CaCl_2$ was used for gellation of alginate.

In another method, a polyethylene glycol solution of various concentrations (100 mL) containing 1.5 g% $CaCl_2$ was used for bead formation. A series of PEG solutions in 0.1 *M* Tris-HCl buffer, pH 5.0 (0.05, 0.1, 0.2, and 0.3%) were prepared and filtered to get a clear solution. The capsules were prepared from these PEG solutions and the alginate, as mentioned above. The rinsed capsules were allowed to dry in air at room temperature until constant weight was achieved.

Hirudin-encapsulated alginate beads were also surface coated with PEG-20,000 for improving their surface and the relese characteristics. For that, 450 mg of hirudin-encapsulated alginate beads were exposed overnight to 2.5% glutaraldehyde vapors. They were further incubated in 5% solution of polyethylene glycol (PEG-20,000) in 0.1 *M* Tris-HCl buffer, pH 5.0 for 5 h.[26] The PEG-grafted samples were then washed with distilled water to remove the unattached PEGs, then were air dried.

Effect of pH on Swelling and Water of Hydration of Beads

Alginate beads were placed in 20 mL of Tris-HCl buffer at different pHs, (pH 1.0, 3.0, 5.0, 7.4, and 9.0) at 37°C. Beads were removed at selected time intervals, and the average diameter of 10 beads were determined with the use of a micrometer, as reported elsewhere.[27] The water of hydration to these beads were also determined by dipping them in Tris-HCl buffer at varying pHs overnight. The beads were removed, the excess water blotted using a filter paper, and their wet weight was determined. They were dried well in an oven at 60°C, and the dry weights were noted and the percentage water of hydration determined from the following equation:

$$\% \text{ Water of hydration} = \frac{W1 - W2}{W1} \times 100$$

where W1 is the wet weight of beads, and W2 is the dry weight of beads.

In Vitro Hirudin/BSA Release

The release of encapsulated protein was carried out into Tris-HCl buffer, pH 7.4 at 37°C and samples at appropriate intervals were withdrawn and assayed using Lowry's method[28] for protein estimation. An equal volume of the same dissolution medium was added to maintain a constant volume. The release profile into Tris-HCl, pH 7.4 after 4-h acid treatment (0.1 *M* HCl) was also evaluated. The amount loaded (100% loading level) was quantitated by disruption of a representative sample of beads and subsequent dissolution of the drugs in Tris-HCl, pH 7.4. Each determination was carried out in triplicate, and the release results are plotted as the cumulative amount and percentage of the content into dissolution medium versus time.

Evaluation of Recalcification Time

Plasma recalcification time (PRT) was determined with released hirudin to certain specific time intervals, according to the standard techniques of Austen and Rhymes.[28] The test system consisted of a known amount of released hirudin (30–100 ng) solution, incubating 0.1 mL citrated human plasma (1 mL of 3.8% sodium citrate for 9 mL of blood), and 0.1 mL $CaCl_2$ (0.025 *M*) at pH 7.4 and 37°C. The time required to form a firm clot was registered. A known amount of soluble hirudin (150 ng) was also used as standard, for comparing the biological activity of released hirudin. The test was repeated at least five times, and the recalcification time expressed in seconds, with standard deviation.

Scanning Electron Microscopy

The beads were initially screened under a light microscope to distinguish the features, such as wrinkling, that were artifacts of the drying and coating steps for the SEM. The selected beads were observed under SEM. The surface morphol-

Table I Plasma Recalcification Time (PRT) as a Function of Released Hirudin

Time (h)	Amount of Hirudin (ngs)[a]	Mean Plasma Recalcification Time (s ± SD)		
		Alginate	Alg–Chitosan	PEG-Coated Alginate
Zero (Bare Glass)—115.4 ± 10.1				
Bare glass	150 ng soluble hirudin 206.4 ± 9.0			
1	30–40	152.7 ± 6.4[c]	143.5 ± 13.2[b]	164.0 ± 6.8[c]
2	40–45	150.5 ± 11.6[c]	155.7 ± 4.3[c]	161.6 ± 11.0[c]
4	41–47	161.0 ± 8.7[c]	154.5 ± 12.7[c]	180.7 ± 6.5[c]
6	58–67	162.5 ± 9.9[c]	161.7 ± 4.8[c]	171.6 ± 9.0[c]
24	75–95	164.2 ± 6.4[c]	160.0 ± 4.3[c]	183.0 ± 5.6[c]
48	80–100	153.2 ± 4.8[c]	146.2 ± 5.7[b]	179.0 ± 12.4[c]

[a] 0.2 mL of released hirudin used for PRT assay.
[b] $p < 0.05$, [c] $p < 0.001$, where the PRT values of all hirudin released cases were compared with the bare glass.

ogies of bare and chitosan/PEG-modified alginate beads were examined using scanning electron microscopy (Hitachi Model S-800). Samples were mounted on metal grids, using double-sided adhesive tape, gold coated under vacuum, and were then observed.

Statistical Analysis

Statistical analysis of important observations were also done and probability values (p) for significance were calculated using the Student's t-test. The mean, standard deviation and p-values are provided in Table I, for comparing the significance of different observations. p-Values of < 0.05 are considered as being statistically significant.

RESULTS

Scanning electron micrographs of hirudin-loaded alginate and PEG surface-grafted alginate beads and their surface morphology are shown in Figure 1. The beads were about 900–1000 μm in size, and not spherical in shape, and had a relatively rough surface with wrinkles [Fig. 1(A)]. The surface morphology [Fig. 1(B)] revealed this to be highly porous with open channels. Grafting of PEG on these beads had dramatically modified the bead and its surface, as is evident from Figure 1(C,D). The open porous structures on alginate beads were filled with polyethylene glycols.

Figure 2 shows the SEM photo micrographs of hirudin-loaded alginate–chitosan (0.3%) beads and their surface morphology. The incorporation of chitosan had changed the overall shape and size of the beads with a smoother outer structure. The surface morphology had been modified with this membranous structure with intermitent micropores (0.5 to 1.5 μm), as indicated in Figure 2(B,C). In other words, the incorporation of chitosan modifies the surface of the alginate beads.

Scanning electron photomicrographs of albumin-loaded alginate–PEG beads and their surface morphology are depicted in Figure 3. Very low levels of PEG (0.05 to 0.2%) in the bead formation helped to have relatively spherical beads with smooth surface [Fig. 3(A)]. The surface morphology had been modified with incorporation of very low levels of PEG (0.1%) to alginate beads [Fig. 3(B,C)]. It seems that the hydrophilic polyethylene glycol makes the alginate surface smoother and microporous. However, increasing the PEG concentration to 0.5% could result in poor bead formation, as indicated in Figure 3(D). The encapsulated molecules (hirudin or albumin) did not reveal any effect on surface morphology of the beads. However, SEM images of the interior of the beads may reveal more informations on the changes due to the encapsulated species. These studies lack such informations and are being planned in the future to come.

Figure 4 shows the dissolution rates of hirudin from various alginate beads as a function of time, in Tris-HCl buffer, pH 7.4. Here, an initial burst release (within 2 h) followed by a constant release (from a 2- to 24-h period) of hirudin from the microbeads had been observed over the 48-h period. It is also evident that the percent of hirudin

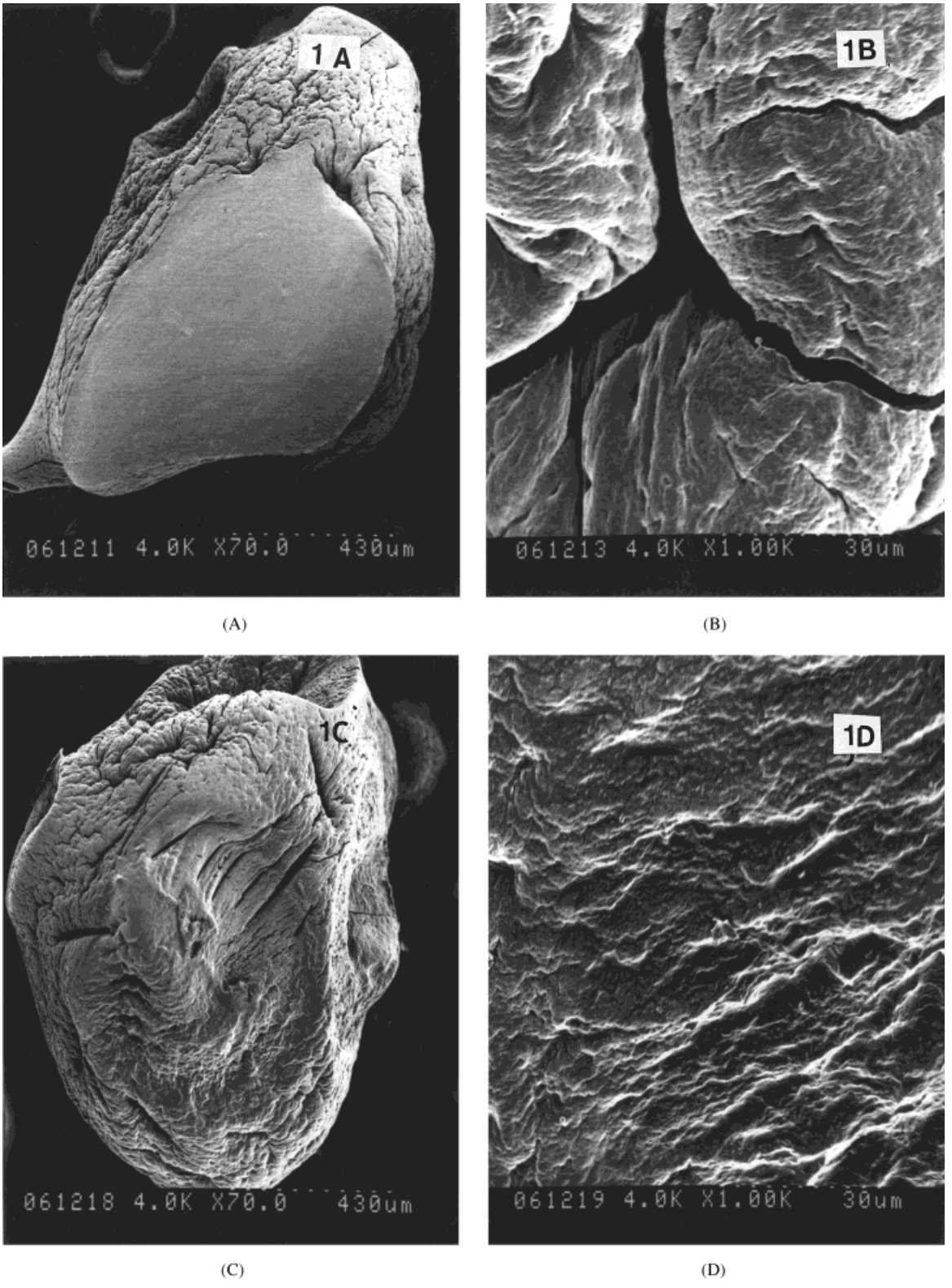
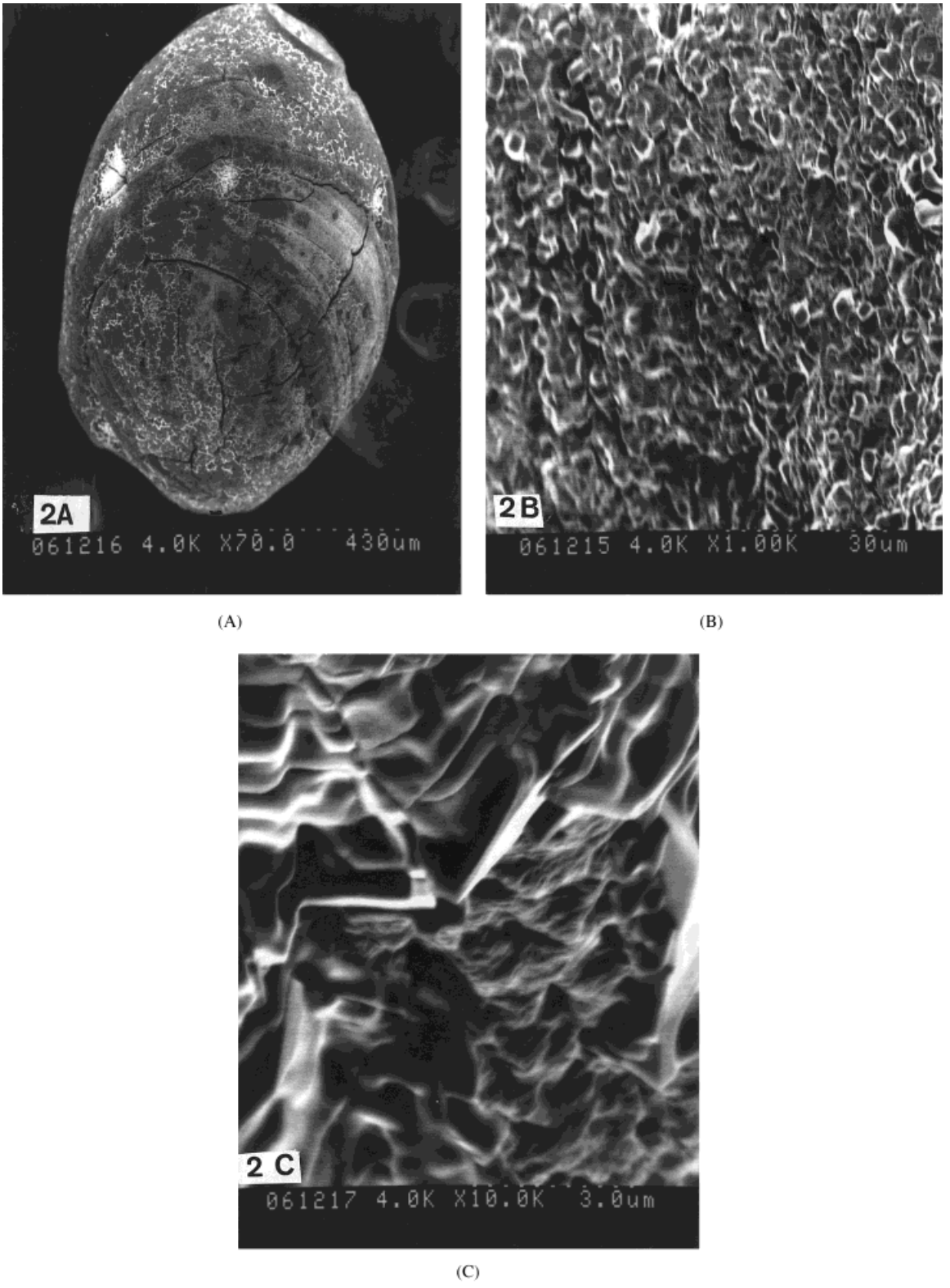


Figure 1 SEM micrographs of hirudin-loaded (A) alginate bead, (B) surface morphology, (C) PEG-grafted alginate bead, and (D) its surface morphology.



(A) (B)

(C)

Figure 2 SEM of hirudin-loaded (A) alginate–chitosan (0.3%) beads, and (B,C) surface morphology.

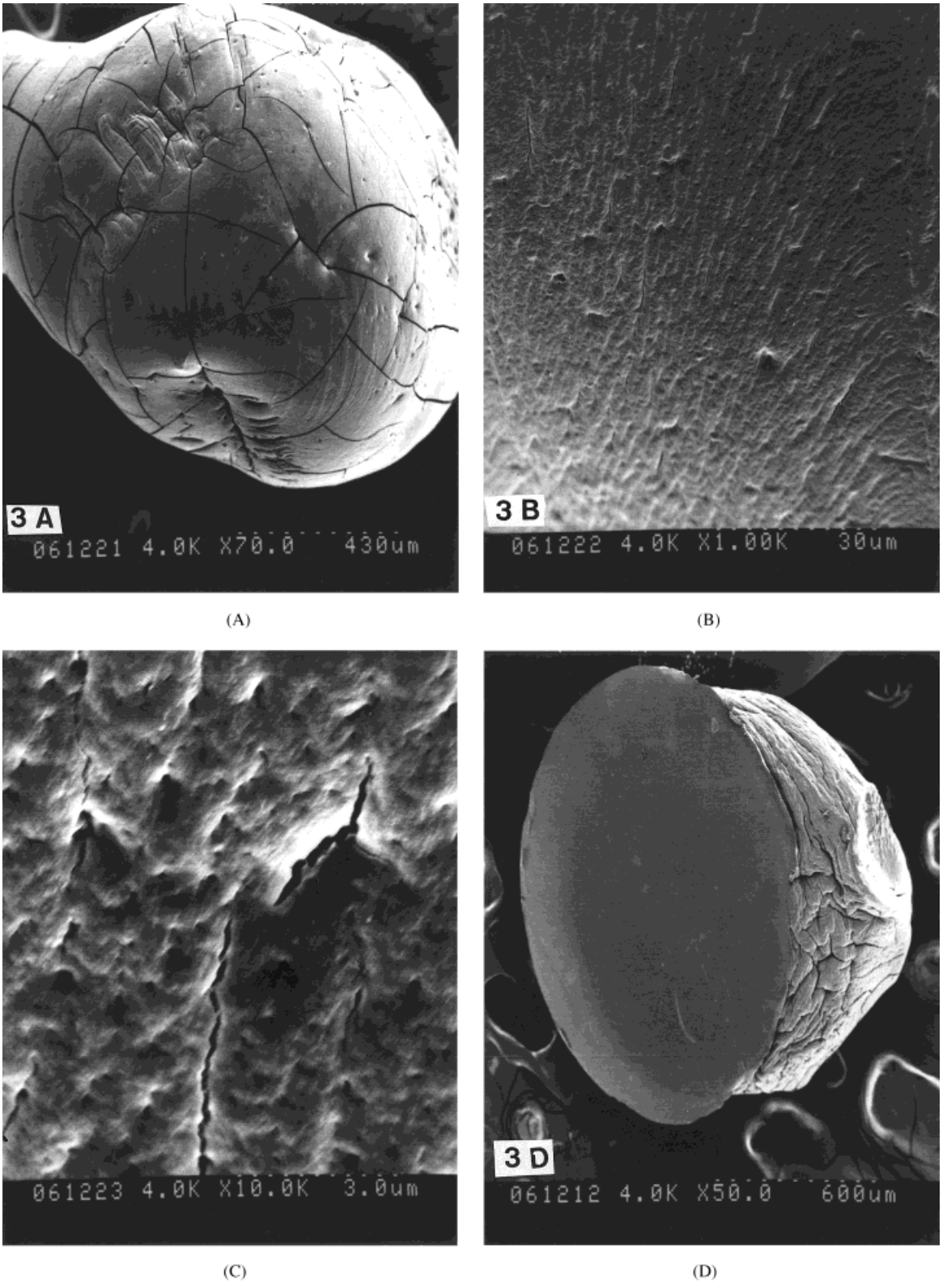


Figure 3 SEM of albumin-loaded (A) alginate–PEG (0.1%) bead, (B,C) its surface morphology, and (D) alginate–PEG (0.5%) bead.

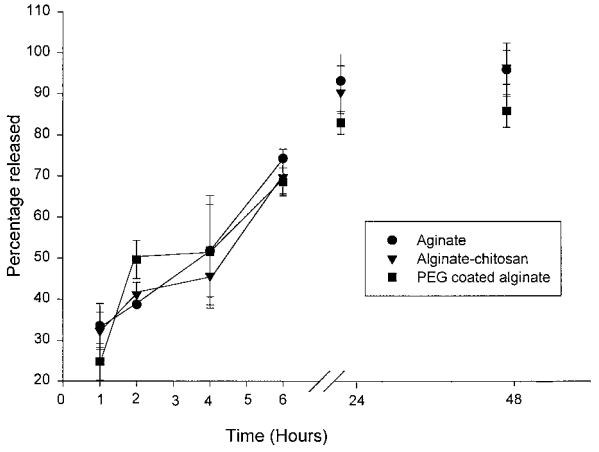


Figure 4 Percentage of hirudin released from alginate beads into Tris-H Cl buffer, pH 7.4. Bar indicates 95% confidence limits.

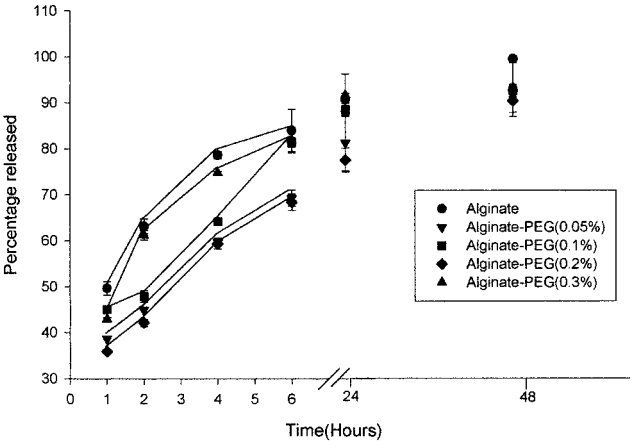


Figure 6 BSA release profile from alginate–PEG (0.1%) beads, into Tris-H Cl, pH 7.4. Bar indicates 95% confidence limits.

release was lower with PEG-grafted alginate beads, compared to unmodified beads. The release of drug was 96% within 48 h, of dissolution, for alginate beads, compared to 86% for PEG surface-modified beads.

The release profile of hirudin from alginate beads after acid treatment (0.1 *M* HCl, 4 h) and the subsequent cumulative percentage release in Tris-HCl buffer, pH 7.4 is depicted in Figure 5. During the low pH treatment for 4 h, between 35–40% payload of the drug was released. But the release rate of hirudin from the acid-treated chitosan–alginate and PEG-grafted alginate beads had been much higher in Tris-HCl buffer, pH 7.4 (Fig. 5). Almost 100% of the entrapped drug was found to be released within 6 h from the modified beads.

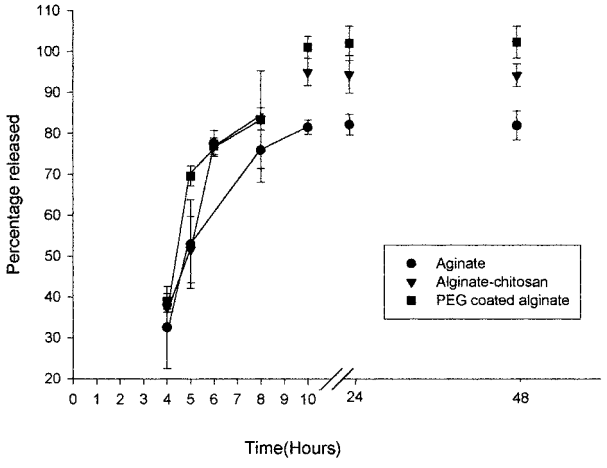


Figure 5 Hirudin release profile into Tris-H Cl, pH 7.4 after acid treatment (0.1 *M* H Cl) for 4 h. Bar indicates 95% confidence limits.

Figure 6 shows the percent albumin release from alginate–PEG beads, as a function of time , in Tris-HCl buffer, pH 7.4. The initial burst release of albumin was substantially reduced by the incorporation of very low levels of PEG in the alginate beads (Fig. 6). The initial drug release was 49.7% within 1 h of dissolution for PEG free beads, compared to 35 to 38% for PEG-modified beads. Further, the microcapsules prepared with the higher concentration (0.3%) of PEG released the BSA faster than the capsules prepared with the lower concentration (0.05%). For example, at 4 h, the capsules prepared with 0.05% PEG released only 59% of entrapped BSA, whereas the capsules prepared with 0.3% PEG released 75% of albumin. In other words, very low levels of PEG had modified the release profile of alginate beads.

As the pH of digestive system varies from a low of ~ 2.0 in stomach to ~ 7.0 and above entering the intestine, the effect of pH on the water of hydration and swelling of beads were studied. Percent water of hydration for 24-h exposed alginate beads in buffer as a function of pH is represented in Figure 7. Significant pH effects were noted for the water content within the beads (Fig. 7), and was low with acidic pH, whereas that with the highest pH, it was higher (~ 60% at pH 1.0 and >90% at pH 7.4). The dry 2% alginate microspheres had ~1 mm in diameter, which expanded to 1.4 in pH 1.0 and 3.1 in pH 7.4 buffers within 24 h (Figs. 8 and 9). The swelling of alginate beads were higher in intestinal pH (7.4) compared to chitosan or PEG-modified alginate beads. For

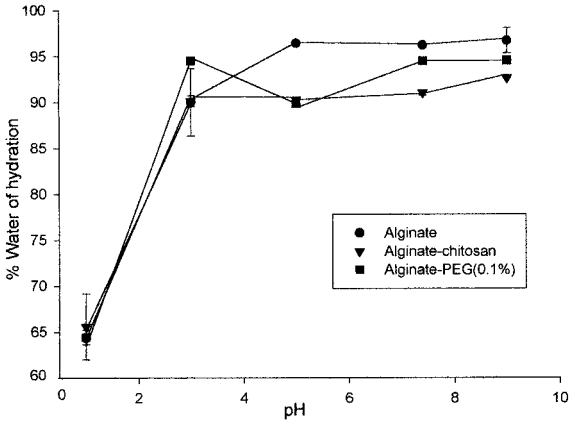


Figure 7 Percent water of hydration of alginate beads (24 h exposed in respective buffers) as a function of pH. Bar indicates 95% confidence limits.

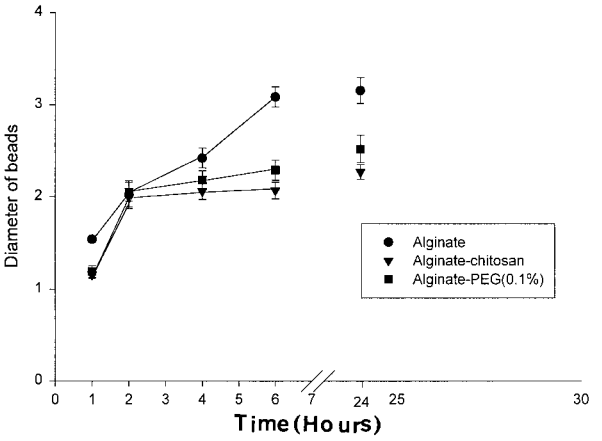


Figure 9 Effect of swelling as a function of time at pH 7.4. Bar indicates 95% confidence limits.

example, at pH 7.4, the maximum expansion factor was 3.16 for the control beads, 2.27 for alginate–chitosan, and 2.52 for beads prepared with PEG.

Table I indicates the amount of hirudin released at specific time intervals from various alginate syatem, and their recalcification times. The plasma recalcification time had increased, owing to the released hirudin from alginate beads. Further, the anticoagulant properties of the released hirudin was higher with a PEG-grafted alginate system compared to bare alginate beads (Table I). In other words, the released hirudin had suggested their anticoagulant properties, demonstrating their specific biological activities.

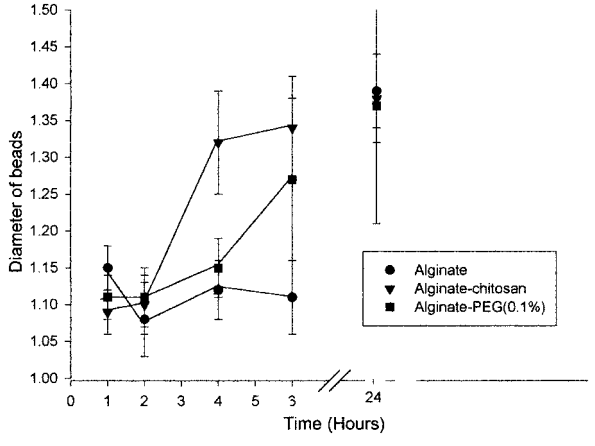


Figure 8 Effect of swelling as a function of time, at pH 1.0. Bar indicates 95% confidence limits.

DISCUSSION

Alginates, chitosan and polyethylene glycol matrices had been reported potentially useful for medical and pharmaceutical applications such as artificial skin, artificial kidney, cell encapsulation, and as drug carrier for target delivery.[1,13,15,29] However, the development of a surface-grafted alginate system for oral delivery of bioactive peptides have hardly been reported. The present results demonstrate that biocompatible PEG-grafted alginate microcapsules provided near zero order, *in vitro* release of hirudin.

Alginates have been used for the oral delivery of various active agents including proteins.[1,11] It is believed that hydrophilic drugs attract water inside the matrix owing to Donnan equilibrium leading to swelling of the matrix.[30] The diffusion and dissolution-based mechanism for release of hydrophilic drugs from such polymers, having interconnected pores filled with water phase is well established.[12,31] The present scanning electron microscopic studies (Fig. 1) of alginate beads reveal that their surfaces have open macro- and microporous channels. Hence, it is assumed that the initial burst release observed (Fig. 4) with hirudin through an alginate matrix may be due to the quick diffusion of the molecule through their water-swollen open channels. However, as can be seen in Figure 1(C,D), the open channels of alginate beads had been filled with PEG, and subsequently a constant drug release (Fig. 4) was achieved.

Chitosan, a hydrogel based on a natural polysaccharide, has been widely investigated for ther-

apeutic drug targeting. The electrostatic interaction of carboxyl groups of alginate with the amine groups of chitosan and the resulting entanglement of the polymer forms a membrane that encloses the active material.[12] It is evident that the chitosan coating had slightly reduced the hirudin delivery in this system (Fig. 4), which may be due to the closure of open channels of alginates via skinny membranes of chitosan structures [Figs. 1(B) and 2(B)].

At low pH alginates did not swell (Fig. 8), but a reversal of shrinkage took place at alkaline pHs (Fig. 9). It seems the low pH conditions strengthened the microcapsule membrane by enhancing the interaction between the negatively charged alginate and the chitosan. Hirudin release was low ($\sim$ 35%) in acid-treated (Fig. 5) alginates. However, an increase in the dissolution rate of beads was observed in Tris-HCl pH 7.4 after acid treatment. This phenomenon is in agreement with the reports of TGF-β_1 release from alginate by Mumper et al.[10] It is assumed that upon acid treatment, the hydrolysis of alginate takes place, which simultaneously reduces the Ca^{++} concentration within the beads, resulting in increased dissolution at neutral pH. These observations were taken in Tris-HCl buffers as reported in our earlier studies.[11] However, the physiological conditions involve substantial level of sodium and phosphate, both of which are known to have an effect on the stability of alginate beads through interaction with calcium, more dissolution studies with phosphate buffered saline may be interesting.

The coagulation of plasma was inhibited with hirudin release (Table 1). It is well understood that the hirudin acts via inhibiting thrombin for its anticoagulant activity.[21] Previous studies have shown a high potency of hirulog in inactivating both free and clot-bound thrombin *in vitro*, and in inhibiting clot formation in a baboon model of experimental thrombosis. In addition to preventing thrombus formation, local thrombin inhibition may also prevent intimal thickening and restenosis.[32] Thus, it is conceivable that the oral release of hirudin from alginate microbeads appears to be appealing for inhibiting cardiovascular complications. There are a lot of limitations to this *in vitro* coagulation assay (Table I) model for comparing biological activity of released hirudin to that on *in vivo* conditions. The biological activity of the drug in an *in vitro* situation represented by cumulative release in a batch reactor may not be comparable with activity that would be generated *in vivo* by a continuous release into an infinite sink conditions. However, the proposed *in vitro* assay might be helpful in explainimng the anticoagulant effect of released hirudin.

Sugano et al.[33] have indicated a significant reduction (25–30%) in plasma cholesterol level of rats fed with 2–5% chitosan, with no effect on growth. They also observed that more cholesterol exists as high-density lipoproteins than very low-density lipoproteins.[33,34]. High-density lipoproteins protect the vessel wall from atherosclerotic plaques, while low-density lipoproteins contribute to plaque formation.[35] Hence, oral intake of hirudin through the chitosan–alginate matrix could both prevent thrombin formation and alter lipid levels in patients beneficially.

Encapsulation with semipermeable membranes has been used to attain the slow, and thereby prolonged, release of therapeutic peptides.[1,4] The protein is encapsulated in a core material that is covered by a biocompatible, semipermeable membrane. PEGs are used for improving the blood compatibility of polymers and are capable of preserving biological properties of proteins.[17] BSA release was substantially modified with the incorporation of low levels of PEG in the alginate microcapsules (Fig. 6). We can speculate that PEG may be a possible candidate to be used with alginates for developing an oral microcapsule system for bioactive molecules. However, further *in vivo* studies are needed to confirm these observations, and the biocompatibility of the encapsulated proteins.

CONCLUSION

This work has demonstrated that chitosan–alginate microbeads may be used as a vehicle for delayed release of a protein drug. The incorporation of biocompatible PEG may protect the protein from degradation, and subsequently, their bioavailability. The pH of the extracellular solution has a significant role in capsule swelling and their protein delivery profile. The released hirudin can inhibit local thrombin generation, and subsequently, they may modulate the surface-induced thrombosis and restenosis. We can, therefore, speculate that chitosan/PEG–alginate system is a good candidate for oral delivery of newer bioactive antiplatelet and antithrombotic peptides. More detailed degradation studies, bioactivity, and biocompatibility of the encapsulated peptide are needed to find applications.

The authors wish to express their thanks to NIH HL 1880 and the Biomedical Engineering Institute for their financial support.

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