Carbohydrate Polymers 77 (2009) 612–620

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Preparation and characterization of a novel pachyman-based pharmaceutical aid. II: A pH-sensitive, biodegradable and biocompatible hydrogel for controlled release of protein drugs

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ARTICLE INFO

Article history:
Received 7 January 2009
Received in revised form 3 February 2009
Accepted 3 February 2009
Available online 11 February 2009

Keywords:
Smart polymer
Hydrogels
pH-responsive
Pachyman
Crosslinking
Protein delivery

ABSTRACT

In this investigation, the fabrication, physico-chemical and biological characterization of a novel smart hydrogel had been evaluated for its potentials in effective controlling protein delivery. The hydrophilic pachyman-based hydrogel was generated facilely by crosslinking hydrosoluble carboxymethyl pachyman (CMP) with epichlorohydrin (ECH). The ECH concentration possessing maximum (99.7%) encapsulation efficiency and the most appropriate swelling characteristics was found to be 1.25% (w/v). The resultant hydrogel exhibited swelling ratios most favorable for drug release in simulated intestinal media. It could release two model protein drugs (bovine serum albumin and lysozyme) in the controlled manner and with full preservation of the protein stability and enzymatic activity. Importantly, the ECH-CMP hydrogel was confirmed to be biocompatible and biodegradable. From these findings, we were able to conclude that the synthesized pachyman-based hydrogel would be a promising delivery carrier candidate for site-specific delivery of protein drugs.

1. Introduction

Hydrogels are three-dimensional, hydrophilic and un-dissolving polymeric networks capable of imbibition large amounts of water or biological fluids (Elvira, Mano, Roman, & Reis, 2002; Kopecek, 2007; Peppas, Bures, Leobandung, & Ichikawa, 2000; Wichterle & Lim, 1960). Since the introduction of hydrogels as novel materials possibly suitable for a broad spectrum of biomedical applications, their research has become a fast-developing and exciting research field (Hoffman, 2002; Van Tomme, Storm, & Hennink, 2008).

Over the past decade, a number of applications involving stimuli-sensitive hydrogels have arisen tremendously due to the great potential for intelligent materials as matrices, actuators and transducers (Ferreira, Vidal, & Gil, 2000; Holtz & Asher, 1997; Hu, Chen, Wang, Zheng, & Li, 1998; Kim, Nayak, & Andrew, 2005; Langer & Tirrell, 2004; Liu, Li, & Asher, 1999; Lutolf et al., 2003; Mawad, Foster, & Lauto, 2008; Shimoboji et al., 2002; Szepes, Makai, Blümer, Mäder, & Szabó-Révész, 2008). Owing to the fascinating properties of the stimuli-sensitive hydrogels which can sense environmental changes and induce structural changes by themselves, it is certain that they offer many future applications such as suitable materials for the design of intelligent biomaterials and self-regulated drug delivery systems.

Advances in biotechnology have led to the discovery of numerous therapeutic proteins and the use of these proteins as therapeutics opens up new hopes of treating many serious diseases. However, the clinical application of proteins is still challenging, because of their unique properties, such as low stability, short circulation half-life and large molecular size (Lin & Metters, 2008). A well-known and ideal approach for protein delivery is inclusion of the protein drugs in a hydrogel matrix. Particularly, natural polymers are getting the most increasing favor of scientists in fabricating pH-sensitive hydrogels, for getting the desirable controlled release of protein drugs (Chen, Tian, & Du, 2004; Gehrke, Uhden, & McBride, 1998; Jain, Yap, & Irvine, 2005; Lao, Sun, Matsumoto, Mulchandani, & Chen, 2007; Leonard, De Boisseson, Hubert, Dalencon, & Dellacherie, 2004; Sui, King, & Murphy, 2008).

Pachyman, a naturally occurring β-D-glucan, is the basic ingredient of the sclerotium of *Poria cocos* (*P. cocos*) and one of the most important traditional medicines in China and Japan (Chihara, Hamuro, Maeda, Arai, & Fukuoka, 1970; Wang, Zhang, & Dong, 2004; Wang, Zhang, Li, Hou, & Zeng, 2004). It possesses numerous superiorities such as natural resourceful, multifunctional, toxicologically harmless and of low cost as well. Nevertheless, due to the drawback of insolubility in water, its bioactivities and applications

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doi:10.1016/j.carbpol.2009.02.003

were limited to some extent. Thereby, to date, the leading applications of pachyman are still retained in common health-promoting foods and cosmetics additives (Heinen & Waldmann-Laue, 2008; Kung & Kong, 2006; Murad, 1998). Being a major and promising medicinal material in China, hereby, further studies for wide applications of pachyman are still necessary to take full advantage of this native product.

The limitation of pachyman motivated our work in developing new and alternative routes to extend its applications. It is well-known that polysaccharides show a number of peculiar physicochemical merits such as biocompatible, biodegradability and high water-affinity, etc. which make them suitable for widespread applications in drug delivery systems (Elmowafy, Awad, Mansour, & El-Shamy, 2009; Fahmy & Fouda, 2008; Na et al., 2007; Tang, Du, Hu, Shi, & Kennedy, 2007; Vimala, Sivudu, Mohan, Sreedhar, & Raju, 2009; Wang, Dong, Du, & Kennedy, 2007; Wang, Du, Luo, Lin, & Kennedy, 2007). For the first time, our group makes our effort to exploited novel applications of pachyman related to pharmaceutics. Our previous work has already advanced its potential into the field of conventional drug delivery (Xiao et al., 2007). In continuation, a novel hydrogel system was improved aiming to the introduction of pachyman into the controlled drug delivery in this investigation.

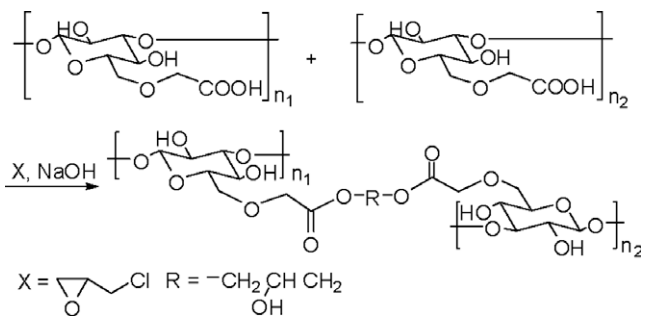
The target of the current study was to exploit novel pH-sensitive pachyman-based hydrogel for the effective protein controlled release system. The present paper pronounced properties clearly suggested that the resultant pachyman-based hydrogel could be developed as a promising and an attractive delivery vehicle for protein drugs.

2. Materials and methods

2.1. Materials

Fresh sclerotium of *P. cocos* was cultivated in LuoTian County (Hubei Province, China). BCA Protein Assay Kit was obtained from Pierce Biotechnology (USA). Bovine serum albumin (BSA), epichlorohydrin (ECH), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin–streptomycin solution, were purchased from HyClone (USA). Dimethyl sulfoxide (DMSO, Sigma) was distilled under vacuum at 78 °C/12 mmHg. All other chemicals were used as received from Sigma–Aldrich (USA), unless otherwise noted.

BALB/c mice of both sexes weighing 20 ± 2 g were purchased from the Animal Centre of Wuhan University (China). Animal experiments were performed in compliance with the local ethics committee and according to the Guiding Principles for the Care and Use of Laboratory Animals in Wuhan University.



Scheme 1. Synthesis scheme of ECH-CMP polymer.

2.2. Synthesis of pachyman-based polymer

Crosslinked carboxymethyl pachyman (ECH-CMP) were prepared by crosslinking carboxymethyl pachyman (CMP) with epichlorohydrin (ECH) as the crosslinking agent (Scheme 1).

Firstly, a series of CMP with various degree of substitution (DS) were prepared basing on our earlier technique (Xiao et al., 2007), detailedly, process parameters such as reactants concentrations, pachyman: solvent ratio, duration of reaction and alkali concentration were studied with respect to their DS systematically, the optimization process began by varying only one parameter at a time, keeping others constantly (data not shown). The product which showed the best water-solubility was selected for the next crosslinking step.

Secondly, under an alkaline condition, various amounts of ECH were added to the uniform CMP aqueous solution at the final concentration of 0.25%, 0.3%, 0.4%, 0.5%, 0.75%, 1.0%, 1.25%, 1.5%, 1.75% and 2.0% (w/v) at 50–55 °C. With the effort to get the final product presented the suitable swelling ratio (Q_s) for subsequent protein delivery, the optimization of the crosslinking reaction was also accomplished by varying every reaction condition roundly (data not shown).

Eventually, the products obtained were purified by washing adequately with DI water and extracted with acetone finally, for the elimination of the ECH traces and of the water from the polymeric network.

2.3. Characterization of CMP and ECH-CMP

CMP and ECH-CMP were identified by infrared spectroscopy (IR, Spectrum One, Perkin Elmer, USA). The DS values of CMP were estimated from potentiometric titration (Muzzarelli, Tanfani, Emanuelli, & Mariotti, 1982). All measurements were performed in quadruple.

2.4. Hydrogel preparation

A homogeneous ECH-CMP polymer solution was obtained under simulating physiological pH (pH 7.4). The solution was stirred overnight. The mixture was vortexed and centrifuged to remove the air bubbles trapped during the stirring process. The air bubble-free solution was poured into a shallow dish of 2.5 cm in diameter, and dried in air at room temperature for 72 h. The gel was thoroughly immersed in DI water for 48 h. Water was changed at a 4 h interval. The hydrogel was finally dried under reduced pressure.

Crosslinking densities of the ECH-CMP hydrogels were evaluated by determining the modulus of elasticity in compression as described elsewhere (Ulbrich, Dusek, Ilavsky, & Kopecek, 1978).

2.5. Scanning electron microscopy

The surface morphology of the ECH-CMP hydrogels was determined using a scanning electron microscope (JEOL JSM-5600 LV, Japan).

2.6. Swelling characteristics of ECH-CMP hydrogels

The swelling profiles of the hydrogel were determined by both one-step and two-step swelling characterization (as shown in Supplementary materials S1). The sample which possessed the best swelling characteristics and suitable for protein drug delivery among all studied groups, was subsequently selected for the study hereinafter.

2.7. Biocompatibility test

In vitro cytotoxicity and *in vivo* acute oral toxicity test of the hydrogel was conducted to evaluate the biocompatibility of the hydrogel (as shown in Supplementary materials S2).

2.8. Hydrogel degradation

The *in vitro* biodegradability of the ECH-CMP hydrogel was determined by both chemical hydrolysis and enzymatic hydrolysis (method shown in Supplementary materials S3).

2.9. In vitro loading and release of proteins from ECH-CMP hydrogel

The drug loading and release profiles from test hydrogels were studied under simulated gastric and intestinal (GI) media by employing BSA (as shown in Supplementary material S4) and lysozyme (as shown in Supplementary material S5) as model protein drugs, respectively.

2.10. Stability of the released BSA

The stability of the released BSA was determined by analyzing the conformation of the released BSA using Jasco J-810 spectropolarimeter (Jasco, Japan) (Ramkissoon-Ganorkar, Liu, Baudys, & Kim, 1999). The SDS–polyacrylamide gel electrophoretic (PAGE) analysis was also employed to assess the structural integrity of BSA in the supernatant.

3. Results and discussion

3.1. Preparation of water-soluble CMP

Biological macromolecules, nevertheless, offer superior polyfunctionality and tissue-biocompatibility compared to synthetic macromolecules. It has been proven that CMP is nontoxic, *in vivo* in testing with intraperitoneal, oral, or subcutaneous treatments (Xiao et al., 2007). Thus, it was preferentially chosen as the precursor compound.

The fabrication of CMP was conducted basing on the typical etherification manner (Iovine & Ray-Chaudhuri, 1978). Purified pachyman was first activated to make the O–H bond nucleophilic in the basification step, with the favor of alkaline reagents as excellent catalysts. Secondly, by means of an etherification step, the hydrophobic pachyman was efficiently transformed into hydrosoluble CMP by introducing hydrophilic carboxymethyl groups onto the polymeric chain. It was observed that a minor proportion of water and room temperature are the most advantageous for the first basification step.

It makes great sense to study the reaction conditions of the polysaccharide etherification. As the DS of the polysaccharide derivative not only relies too much on the reaction parameters, but also it has remarkable impacts on the solubility and swelling property of the products. The optimum condition for the derivation was generalized in Table S1 (as shown in Supplementary material). Table S2 (as shown in Supplementary material) reveals the interdependent relationships between DS and the solubility of CMP. It can be concluded that the solubility of CMP improves gradually with the increment of the DS, due to the gradual introduction of the hydrophilic carboxymethyl groups into the polymeric chain. When the DS reached to 0.42, the product was able to dissolve in water and gave a clear transparent solution. Finally, CMP presented the best water-solubility was selected for the following crosslinking process.

3.2. The crosslinking of CMP

Water-soluble polymers can be converted into hydrogels using bis (or higher) functional crosslinking agents which react with functional groups of polymers via addition reactions (Hennink & van Nostrum, 2002). As depicted in Scheme 1, ECH-CMP was prepared simply by CMP treatment with epichlorohydrin (ECH) in a basic medium. ECH was employed as a convenient base-catalyzed crosslinker. In the synthesis of ECH-CMP, CMP was used as a polyether polyol and an alcoholate (CMP-ONa) formation occurred in the presence of NaOH. The chlorohydrin fragments formed are transformed to epoxy group by dehydrochlorination and finally, crosslinked CMP was obtained. Each ECH molecule reacted with two hydroxyl groups from different CMP molecules, yielding one hydroxyl group, in this manner, two neighboring polysaccharides chains were attached to form a network.

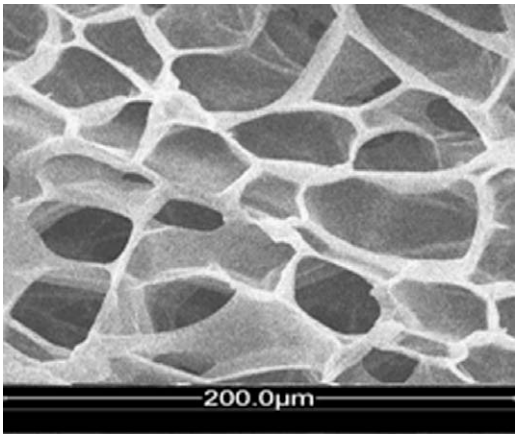


Fig. 1. Scanning electron micrograph of surface morphology of ECH-CMP hydrogel.

In the IR spectra, compared with pachyman and CMP, the FTIR spectrum of pachyman exhibited the bands at 890, 1250 and 3400 cm^{-1} which owing to the saccharide structure. These bands did not change when the CMP was crosslinked by ECH. The spectra of ECH-CMP exhibited the relatively low intensity of the –OH vibration band at 3400 cm^{-1}, much higher intensity of the $–CH_3–$ or $–CH_2–$ vibration bands at 2925 cm^{-1}, besides, stronger peaks at 1325 and 1062 cm^{-1} appeared corresponding to the –CO– groups. This resultant spectrum confirmed the crosslinking reaction had taken place between the hydroxyl groups of CMP.

3.3. Hydrogel characterization

The ECH-CMP hydrogel can be formed under room temperature and physiological pH, allowing the safe incorporation of bioactive molecules for a broad range of medical applications.

It is generally known that the crosslinker plays a crucial role in the formation and mechanical properties of a hydrogel. The ECH-CMP hydrogel can swell considerably in aqueous medium with sufficient tight structure but without any dissolution. The equilibrium swelling degree (Q_s) is one of the most important parameters to evaluate the mechanical properties of a hydrogel. Fig. 2 shows that, in the beginning, the Q_s generally evidenced a tendency of increasing with the enhancive of the ECH amount. One may nevertheless observe that, when at higher ECH concentration (above 1.25%), a decrease in the swelling degree of the hydrogel occurred. This result seemed contradicted the literature data, which predicted a decrease in the swelling degree of a hydrogel when raising the quantity of the crosslinking agent.

In order to explain this phenomenon, we should take into account the helicoidal structure of pachyman (Terry & Anatole, 1975). Presumably, during the crosslinking process, this structure was destroyed by the influence of the basic medium, of temperature and time. Thus, a part of the hydroxylic groups, which stabilized the helicoidal structure by hydrogen bonds, took part in the

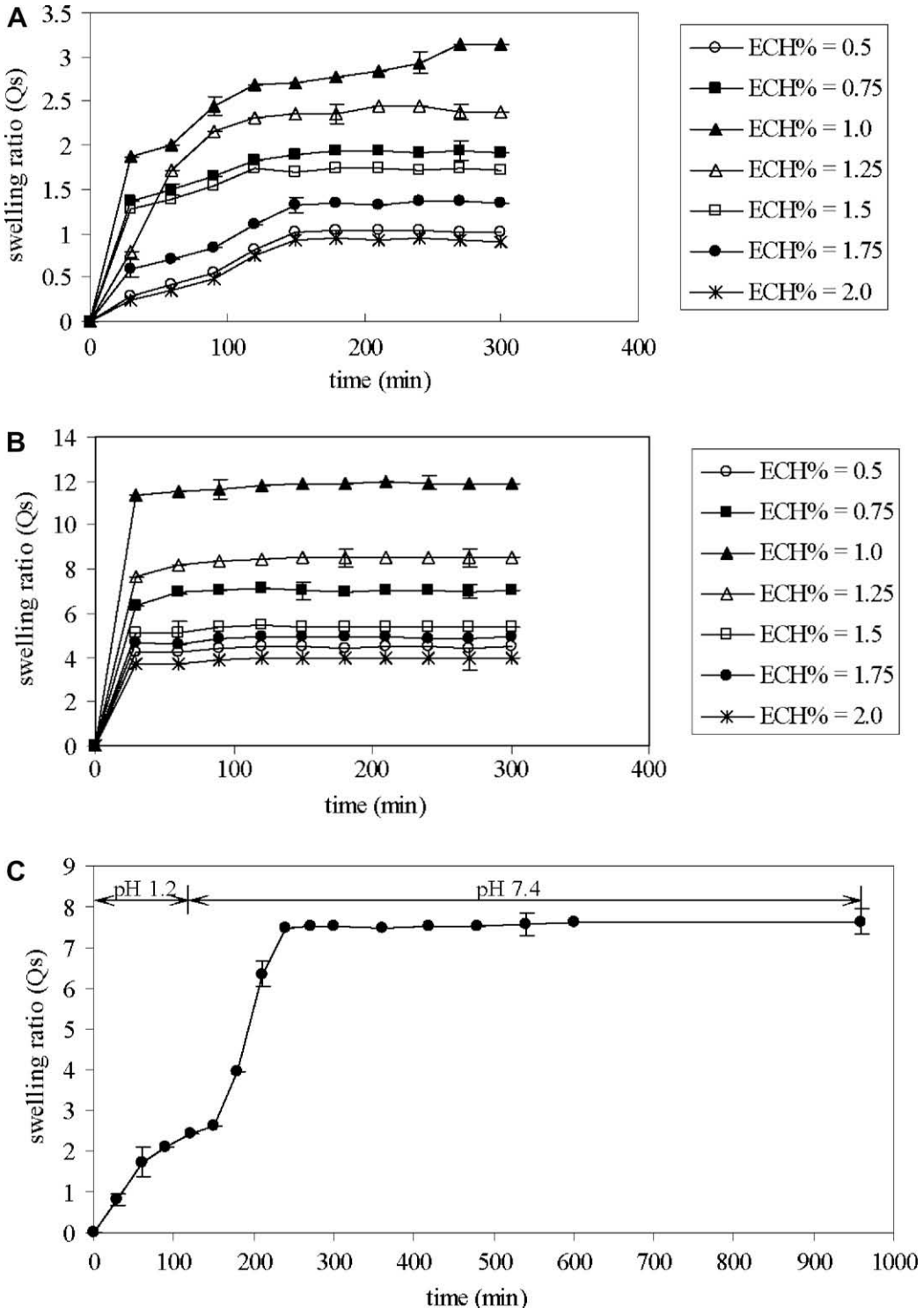


Fig. 2. Swelling profiles of ECH-CMP hydrogels: (A) one-step swelling characteristics with different weight ratios of ECH at pH 1.2, (B) one-step swelling characteristics with different weight ratios of ECH at pH 7.4, (C) two-step swelling characterization.

crosslinking reaction and another part was available to interact with water in the swelling process. Thus, a less compact network was created as compared to the initial one, with bigger net-blanks, which allowed greater "unbounded" water absorption. Nevertheless, a greater extent of crosslinking of test hydrogels resulted in a greater restriction of the mobility of the polymer chains and thus limited the swelling ratio of test gels. Therefore, after the destruction of the helicoidal structure, the equilibrium Q_s tended to decrease with increasing ECH concentration because the advance of the crosslinking reaction produced a compact network, which led to the reduction of the free volume in the network, thus restrained the expansion and the penetration of the network.

Besides, crosslinking of the polymer chains can be achieved by means of either chemical or physical crosslinking (Hennink & van Nostrum, 2002). Although it is believed that physically crosslinked hydrogels are advantageous for macromolecular delivery systems compared to chemically crosslinked hydrogels, in our comparative study (unpublished data), the Ca^{2+} physically crosslinking CMP

hydrogel showed much lesser water-swellability and weaker gel intensity than the ECH-CMP crosslinking gel, which resulted in the very low entrapment efficiency and burst release of entrapped protein drugs. The employment of the crosslinker (ECH) could considerably enhance the gel strength, which could be beneficial for ensuring the maximum entrapment efficiency and the controlled drug release.

Altogether, water-soluble CMP was effectively transformed to a highly water-swellable polymer via a gentle chemically crosslinking condition. In addition, the chains of 2-OH-1,3 propylene introduced were comparatively short, which might be favored for the formation of the hydrogel with suitable pore sizes. Moreover, the crosslinking process was achieved by adding small amounts of a toxicologically acceptable organic solvent and conducted via a mild condition, which might guarantee the tissue-biocompatibility of the hydrogel.

3.4. Swelling characteristics

Crosslinking effect by ECH considerably improved the mechanical strength of CMP. The resultant hydrogel presented far increased swelling capacity along with high resistance to rapid dissolution in aqueous medium.

From the swelling ratio studies of different ECH and CMP combinations (Fig. 2), the 1.25% concentration of ECH was found to be better with a well-suited Q_s of ~2.4 at pH 1.2 and ~8.5 at pH 7.4 while all other groups showed either high swelling at both pH 1.2 and 7.4 or even too low swelling at pH 7.4 which were not desirable. The rapid swelling exhibited by other groups would tend to limit their efficiency for controlled drug release at intestinal pH. Besides, an exorbitant swelling ratio of the hydrogel might fail to provide adequate retention of loaded proteins at the lower end of the gastric pH range (pH 1.2), due to the increased chance of entrapped protein drugs for a rapid release in the intestinal pH and subsequent denaturation by proteolytic enzymes. On the other hand, with increasing the Q_s of the hydrogel, gel disintegration was observed at pH 7.4. Moreover, the encapsulation efficiency studies (Table 1) also supported the ECH content of 1.25%, as it showed the most encapsulation efficiency in the preliminary study. The extensive swelling capacity of the hydrogel can be attributed to its highly porous nature (Fig. 1) where the capillary forces help the diffusion of solvent into the hydrogel. The porous nature of the hydrogel makes it less dense and provides more surface area. As a result, the hydrogel formed by 1.25% amount of ECH was selected for further pH-sensitive and protein release studies.

Table 1
Percentage entrapment efficiency of hydrogels with different ECH amounts.

ECH (%)	Solubility characteristics[a]	Crosslinking density (mol/m^3)[b]	Entrapment efficiency (%)
0.25	Soluble	–	–
0.3	Soluble	–	–
0.4	Partial soluble	–	23.6
0.5	Partial soluble	38.9 ± 9.2	35.3
0.75	Nonsoluble and swollen	10.6 ± 2.9	36.4
1.0	Nonsoluble and swollen	8.7 ± 1.2	58.9
1.25	Nonsoluble and swollen	9.5 ± 2.0	99.7
1.5	Nonsoluble and swollen	32.2 ± 7.1	90.2
1.75	Nonsoluble and swollen	36.1 ± 8.6	83.6
2.0	Nonsoluble and swollen	40.2 ± 10.2	43.7

[a] Determined by naked eyes.
[b] Data were expressed as means ± SD of four experiments.

3.5. pH-sensitivity

In general, the matrix swelling in gastric and intestinal fluids is of determinant relevance for the drug liberation. As expected, the hydrogel underwent obvious changes in swelling that were mediated by external pH stimuli. It exhibited desirable pH-sensitivity which was very low swelling at pH 1.2 (gastric environment) and moderate swelling at pH 7.4 (intestinal environment).

According to the two-step swelling profile (Fig. 2C), the swelling ratio of the ECH-CMP hydrogel at pH 1.2 was about 2.5 while it was approximately 7.6 at pH 7.4. At low pH (pH 1.2), the swelling ratio of the hydrogel was limited due to formation of intermolecular hydrogen bonds. At pH 7.4, the carboxylic acid groups on the hydrogel became progressively ionized (–COO–). In this case, the hydrogel swelled more significantly due to a large swelling force created by the electrostatic repulsion between the ionized acid groups. The electrostatic attraction between opposite charged molecules is an adjustable driving force for structured material construction.

In conclusion, the swelling ratio of test hydrogel presented similar swelling patterns in two-step and the one-step swelling profiles (Fig. 2C, A and B, respectively). The ECH-CMP hydrogel was capable of undergoing changes in gel volume in response to pH changes. Thus, it was chosen for the succedent protein loading and release assays.

3.6. Biocompatibility

3.6.1. In vitro cytotoxicity test

One of the major requirements of the polymeric vectors for use in drug delivery is the absence of cytotoxicity. An *in vitro* MTT assay was performed using the HUH 7 cell line and Hacat cell line (Fig. 3E). As compared to the control, for all concentrations studied, the viabilities of both the two cell lines were typically 100%, but they were dose dependent. Moreover, as illustrated in Fig. 3A–D, the cells attached and spread, without any morphological alteration. Thus, it can be deduced that the ECH-CMP hydrogel was well tolerated by cells.

3.6.2. In vivo biocompatibility test

Oral administration of mice with ECH-CMP hydrogel up to 15 g/kg b.w. had no toxic effect. No death occurred during 14-day observation period, and no toxic response was found in mice. Table S3 shows several clinical signs should be observed in oral acute toxicity test, but not found in the mice treated with the hydrogel. Since the highest tested dose caused no mortality, it was concluded that the maximal tolerance dose (MTD) of ECH-CMP hydrogel were higher than 15 g/kg b.w. in BALB/c mice.

Thereby, the synthesized ECH-CMP hydrogel presented excellent tissue-compatibility, ensuring that the residual reagents and crosslinker which were mainly responsible for the cytotoxicity of the products, could be completely removed. The absence of toxicity for ECH-CMP hydrogel was encouraging for further protein loading and release experiments.

3.7. Biodegradation of the hydrogel

Taking into account the presence of potentially degradable linkage in the structure of ECH-CMP hydrogel, such as glucosidic and ester, chemical and enzymatic degradation studies were performed in simulated physiological conditions. Fig. S1 shows that the hydrogel underwent a negligible chemical hydrolysis with a small dry weight loss. On the contrary, the hydrogel was partially degraded (approximately 18%) after 24 h of incubation with dextranase and esterase. Therefore it is probable that, after an initial surface erosion, enzymes diffused into the polymeric

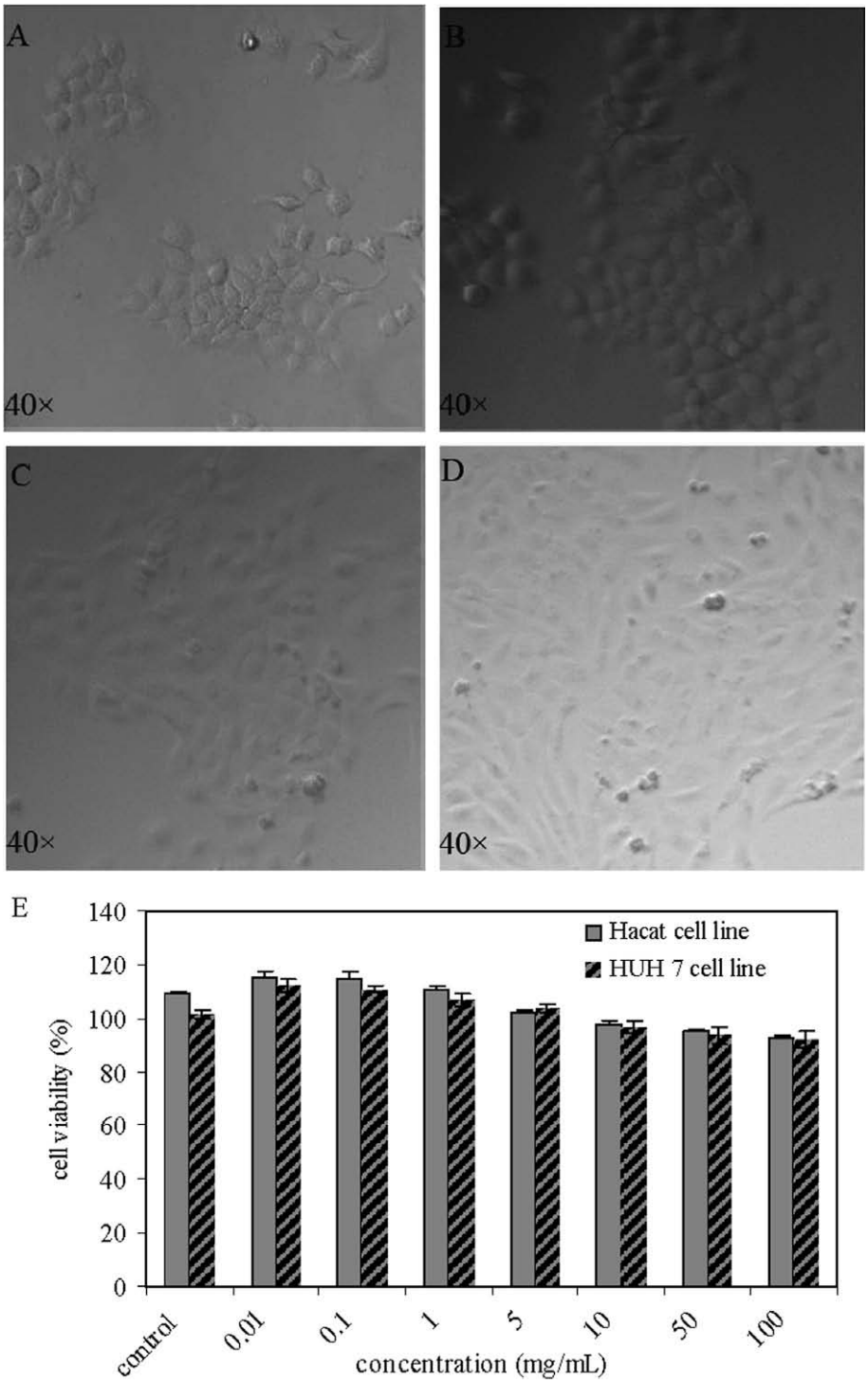


Fig. 3. Microphotographs of HUH 7 cells after 3 days in culture on ECH-CMP: (A) the control, (B) ECH-CMP; microphotographs of Hacat cells after three days in culture on ECH-CMP: (C) the control, (D) ECH-CMP; (E) *in vitro* cytotoxicity of the ECH-CMP as a function of polymer concentration towards HUH 7 and Hacat cell lines.

network, thus causing a degradation of the more internal sites with a consequent pronounced increase in the swelling. It is also evident that the mixture dextranase/esterase is more effective than each single enzyme (data not shown).

3.8. In vitro drug loading and release profile

The most challenging task in the development of protein pharmaceuticals is to deal with instabilities of proteins in the harsh environment of the stomach. Protein encapsulation processes that require the use of organic solvents or heating might potentially physically modify or denature the therapeutic proteins. Encapsulation processes that require chemical bond formation among the encapsulation reagents might unintentionally chemically modify the therapeutic proteins. However, our protein loading process was desirable as the encapsulation of proteins was performed avoiding any organic solvent, high temperature, unfavorable pH and other harsh environmental conditions. The conditions were benign sufficiently as the resulting hydrogel physically entrapped the protein drug.

Fig. 4A and B shows the BSA release profiles of the test hydrogels at pH 1.2 and 7.4. The amount of BSA released at pH 1.2 was very low in both one-step and two-step release studies, only about 14% BSA was released from the test hydrogel, whereas that released at pH 7.4 increased significantly (95.6% and 88.2% for one-step and two-step release profiles, respectively). The favorable BSA release performance could be attributed to the pH-sensitivity of the hydrogel. Swelling of such hydrogel in the stomach was min-

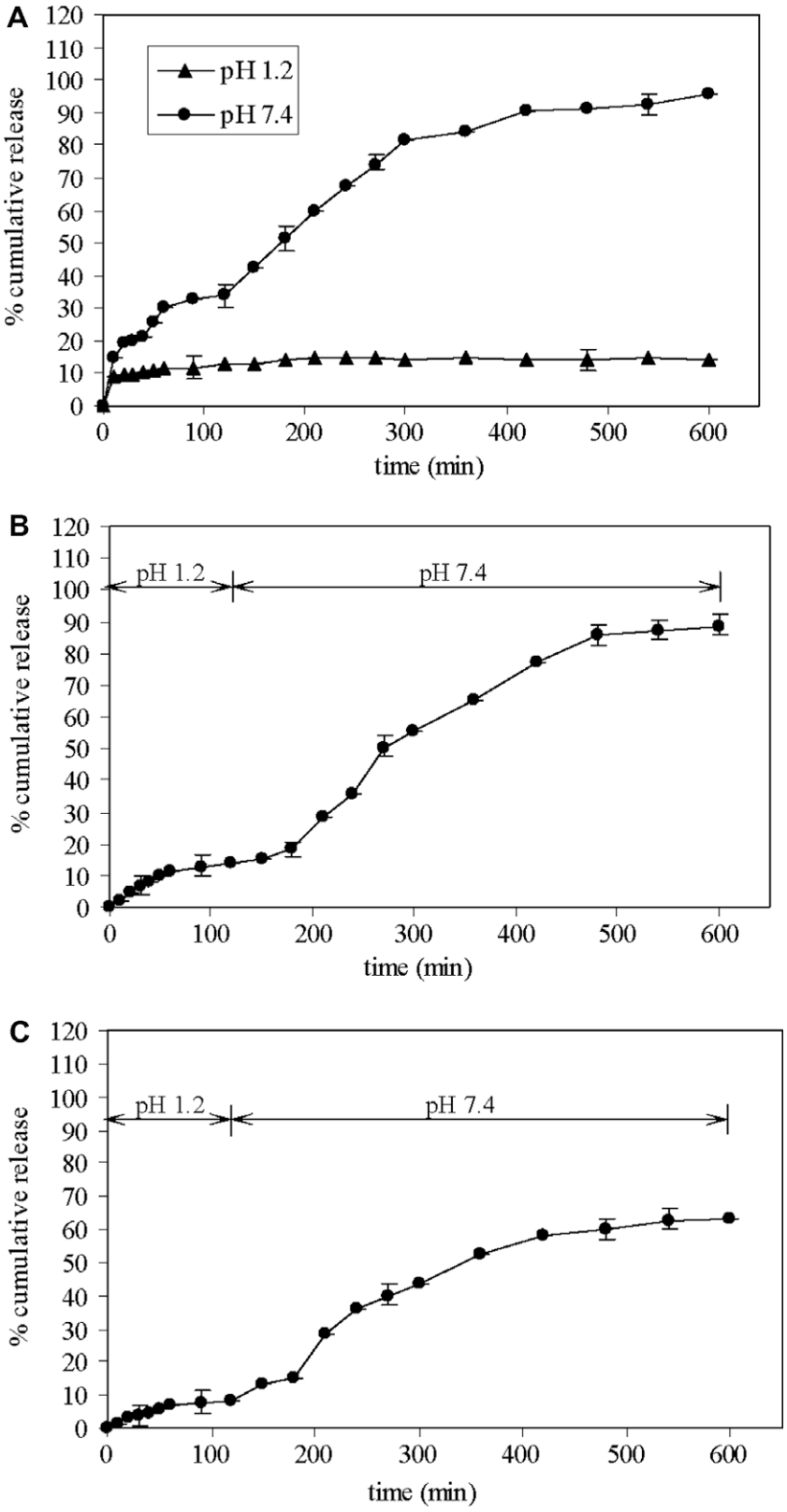


Fig. 4. Release profiles of model drugs from ECH-CMP hydrogel: (A) BSA release profile in pH 1.2 and pH 7.4, respectively, (B) BSA release profile in pH 1.2 and subsequently in pH 7.4, (C) lysozyme release profile in pH 1.2 and subsequently in pH 7.4.

imal and thus the drug release was also minimal. Due to increase in pH, the extent of swelling increased as the hydrogel passed down the intestinal tract, the hydrogel swelled and the controlled release of protein was effected. Because of the high matrix porosity of the hydrogel (Fig. 1), the capillary forces could reinforce the diffusion of solvent into the hydrogel, thereby the protein release from the hydrogel matrix occurred mainly due to the diffusion of the drug though the pores of the swelled matrix in the intestinal pH.

The release of BSA was predicted to be largely due to diffusion and accelerated by the weight loss of the gel. However, this argument cannot be fully justified when comparing the release kinetics (Fig. 4B) with the weight loss profile (Fig. S1), at least at the initial stage of the drug release. In the first 6 h, more than 65% of the BSA was released from the gel while only a negligible amount of the gel matrix was lost. This suggests that the BSA release was dominated by diffusion and not by gel erosion in the early stage of the release. The high BSA diffusion might be due to the large amount of water present in the gel, and the low erosion rate might be due to the intermolecular interaction of the crosslinking network. Approximately 18% of the dry weight was lost after 6 h, thus it is presumed that after drug release from swollen hydrogel, swollen gels began to erode from outside to inner and the drug molecule was released through erosion of polymeric matrix.

Expectably, the synthesized ECH-CMP hydrogel could also release lysozyme in the controlled manner with full preservation of its enzymatic activity (Fig. 4C and Table 2). Fig. 4C shows the two-step release profile of lysozyme. The release profile of lysozyme was similar to BSA. The amount of lysozyme released at pH 1.2 was very low (only about 8.3%), whereas that released at pH 7.4 increased significantly (63%). Nevertheless, the cumulative release of lysozyme was low compared to BSA. This might due to precipitation of lysozyme, as the release media contained quite some precipitates after the release experiment.

Table 2
Enzymatic activity of immobilized and free lysozyme.

Lysozyme treated	Remaining activity[a] (U/mg lysozyme)
Initial lysozyme	890 ± 90
Released from hydrogel	916 ± 60
Lysozyme free in solution	920 ± 76

[a] Data were expressed as means ± SD of three experiments.

3.9. BSA structural integrity

Conformational change and aggregation of proteins have been important subjects to be solved when considering formulations, since exposure of BSA to the ionic solution and crosslinking agents could affect protein structure and stability (Foster, 1977). The circular dichroism (CD) spectra (Fig. 5A) shows that there was a slight change in the conformation of the loaded BSA when released at pH 1.2 as compared to the standard BSA, owing to the stability of proteins at pH 1.2 is poor (Peppas, Huang, Torres-Lugo, Ward, & Zhang, 2000). In contrast, no significant conformation change was noted for the loaded BSA released at pH 7.4 (at pH 1.2 for 2 h and then at pH 7.4). This result suggested that the secondary structure of BSA was preserved after the loading procedure despite prolonged contact of BSA with the polymer and release into a buffer solution at pH 7.4.

Moreover, Fig. 5B demonstrates the SDS–PAGE results of the released BSA and commercial BSA. It was observed that the released BSA solution had distinct band presented at 66 kDa, indicating that the integrity of the released protein was largely maintained. No bands corresponding to lower molecular weights were detected, suggesting that the released BSA did not undergo hydrolysis.

Totally, the ECH-CMP hydrogel provided a benign environment for encapsulation of protein which was advantageous for the maintenance of the stability of protein. Probably, proteins loaded into the hydrogel led to enhanced stability of the protein because of the shielding of protein domains susceptible to proteolytic attack.

4. Conclusions

In order to tailor the fire-new and desired application for natural pachyman in acceptable drug carrier, to combine the advantages of the synthetic and natural polymers and at the same time maintain the favorable properties of natural polysaccharides, a novel stimuli-responsive, biocompatible and biodegradable pachyman-based hydrogel with the capability of controlled protein release was synthesized via the crosslinking method. Moreover, by adopting the protein-friendly preparation method, they were capable of incorporating considerable protein amounts easily without causing denaturation of protein molecules. Remarkably, the crosslinking made the hydrogel more stable and the swelling and drug release were in the satisfactorily controlled manner. Importantly, the stability and enzymatic activity of the released proteins were fully retained. The analysis about the drug release indicated

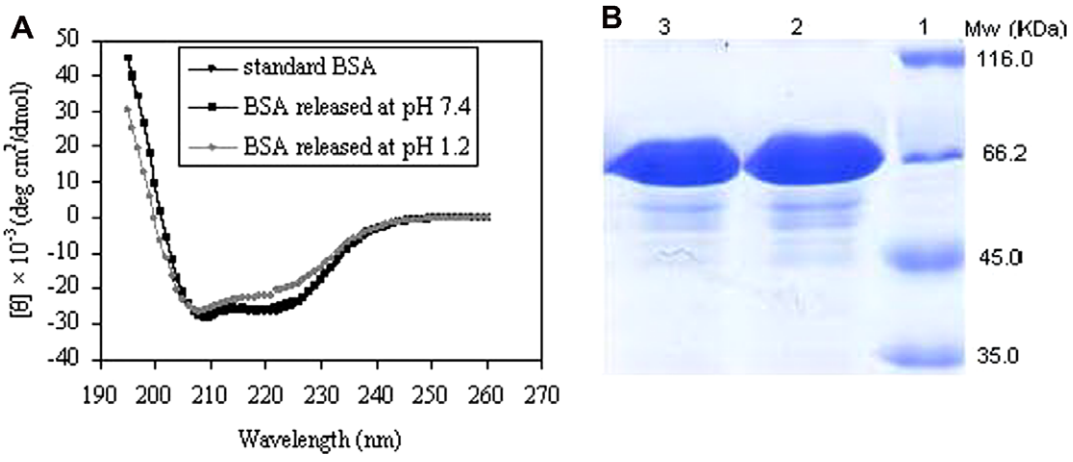


Fig. 5. The stability of the released BSA from ECH-CMP hydrogel: (A) CD spectra of the standard BSA, the loaded BSA when released at pH 1.2, and the loaded BSA released at pH 7.4 (at pH 1.2 for 2 h and then at pH 7.4); (B) coomassie-stained SDS–PAGE gel of the *in vitro* released BSA. Lanes 1, 2 and 3 are, respectively, the molecular weight markers, BSA standard, BSA released from the ECH-CMP hydrogel.

that drug release mechanism was a combined process of drug diffusion and erosion of the drug-loaded matrix wherein the diffusion played the predominant role.

It is interesting to conclude that the proposed pachyman-based hydrogel could be considered as a potent candidate for protein delivery. It is also hoped that this work is a stimulus to open up interesting perspectives for the pachyman-based polymeric drug carriers, which would revitalize and widen the applications of pachyman as well as its derivatives. Promising applications of pachyman in pharmaceutical nanotechnology can also be foreseen in our following study (unpublished data).

Acknowledgments

The authors are grateful to the Ministry of Education of Luotian Town, Hubei Province, for providing high quality *P. cocos*. This work was funded by the Chen-Guang Foundation of Scientific and Technologic Council of Wuhan, Hubei Province (Grant No. 20055003059-21) and the Major Programs of the Ministry of Science and Technology of Hubei province (Grant No. 2006AA301B22).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2009.02.003.

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