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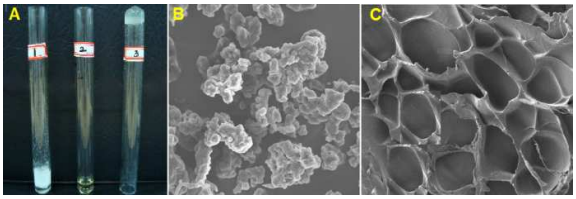

A biocompatible and biodegradable hydrogel based on a natural polysaccharide was prepared, characterized and confirmed to be an effective and "smart" carrier for controlled protein delivery.

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PAPER

A novel pH-responsive hydrogel based on natural polysaccharides for controlled release of protein drugs

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A biocompatible and biodegradable hydrogel based on a natural polysaccharide was prepared and characterized to evaluate its applicability as effective carrier for controlled protein delivery. The hydrogel exhibited significant pH-sensitivity most favorable for protein release in simulated intestinal medium. It is capable of incorporating considerable amounts of protein drugs (encapsulation efficiency up to 97.6 wt%) following a protein-friendly preparation procedure. It is tested that the hydrogel is able to release two entrapped model protein drugs (bovine serum albumin and lysozyme, respectively) in a controlled manner with full preservation of protein stability and enzymatic activity for lysozyme. Moreover, the insulin-loaded hydrogel was effective in reducing blood glucose level in diabetic animal models. Importantly, the hydrogel showed no evidence of cytotoxicity *in vitro* and *in vivo*, rather, it is biodegradable. The synthesized hydrogel shows favorable features as a promising delivery carrier candidate for targeted delivery of protein drugs to the specific sites.

1. Introduction

Polysaccharides have gained rising attention in drug delivery recently due to their favorable features. They are natural resourceful, biocompatible, biodegradable and less expensive. Also, other characters of polysaccharides, such as high water-affinity and ease of chemical modification, facilitate the versatile applications of polysaccharide-based materials in the field of drug delivery [1-11].

Pachyman, a naturally occurring β-(1-3)-D-glucan, is the key component of the sclerotium of *Poria cocos* (*P. cocos*), one of the most important and time-honored herbal medicines in China and Japan [12-15]. Several polysaccharides have shown obvious anti-cancer activities such as *Panax Ginseng* and *Ganoderma lucidum*. However, pachyman fails to show any anti-tumor effect despite the great efforts made in developing pachyman as antitumor agents. This has been attributed to their insolubility in water[16-21]. To date, pachyman has been mainly applied in nutrition supplements and cosmetics additives [22-26]. Further studies are still necessary to explore possible applications of pachyman in drug therapy to take full advantage of this natural resource. Previously, our group has investigated the applications of pachyman in pharmaceutics for the first time [27, 28]. In this study, another pachyman-based hydrogel system with favourable properties was developed and evaluated for the use of drug delivery.

Currently, protein drugs are becoming a quite important class of therapeutic agents along with the rapid development of biotechnology[29-32]. Therapeutic proteins usually have low stability, short circulation half-life and large molecular size. Therefore, suitable delivery systems are needed to guarantee the safe and controlled delivery of protein drugs [32, 33]. Also, protein drugs are commonly administrated parenterally and repeated injections are required. To increase patient compliance and to minimize possible health hazard caused by frequent injection, oral delivery is an ideal alternative route of protein drug administration[34-36].

Among all the delivery systems for protein drugs, "smart" hydrogels have attracted considerable attention because of their perceived "intelligence". Hydrogels can effectively protect protein drugs from degradation in gastrointestinal (GI) tract[29, 37, 38]. Moreover, hydrogels are able to retain a significant amount of water to resemble natural living tissues. They are also inherent biocompatible, offering them good opportunities as protein delivery systems [29, 33, 39-41]. Amongst, sugar-based hydrogels are of particular interest as they show minimal toxicity compared to conventional hydrogels and can potentially serve as a targeting ligand in the human body[4, 42].

In this study, we aim to explore possible application of pachyman as a safe and effective drug carrier for controlled release of protein drugs. The structure of the hydrogel was

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characterized by infrared spectroscopy and the morphology was observed under scanning electron microscope (SEM). Bovine serum albumin (BSA) and lysozyme was used as two different model protein drugs to study the encapsulation efficiency and drug release pattern in simulated gastric and intestinal buffer. Besides, the pH-sensitivity, biodegradability and biocompatibility of the hydrogel were all examined. Finally, the performance of the hydrogel system to deliver active insulin *in vivo* orally was evaluated on model diabetic mice.

Compared to the conventional hydrogel system, the pachyman-based hydrogel showed obvious pH-sensitivity which could protect the loaded protein drugs from degrading by the harsh environment of the stomach. Importantly, since the natural polysaccharide was used as the starting materials, the hydrogel offer superior polyfunctionality, biocompatibility, biodegradability and of low-cost. The experimental results presented have indicated great potential of the pachyman-based hydrogel as a safe, pH-responsive and controllable protein drug delivery vehicle.

2. Materials and methods

2.1 Materials and animals

Fresh sclerotium of *P. cocos* was naturally planted in LuoTian County and supplied by Hubei Hongyuan Medicine Company (Hubei, China). BCA Protein Assay Kit was purchased 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, USA) was distilled under vacuum before used. All other chemicals were of analytical or better grade and used as received from Sigma-Aldrich Chemical Co. (St. Louis, MO, 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 according to the Guiding Principles for the Care and Use of Laboratory Animals in Wuhan University and in compliance with the local ethics committee.

2.2 Hydrogel synthesis

Epichlorohydrin (ECH) crosslinked hydroxypropyl pachyman (ECH-HPP) hydrogel was prepared via a two-step reaction (Scheme 1). Firstly, series of hydroxypropyl pachyman (HPP) with different degree of substitution (DS) were prepared according to our earlier report[28]. ECH-HPP was then synthesized by crosslinking HPP with epichlorohydrin (ECH) as the crosslinking agent. In order to get the desired ECH-HPP hydrogel with proper swelling capacity for protein delivery, different amounts of ECH were added to optimize the crosslinking reaction. The influences of DS on water solubility of the products as well as the correlation between swelling ratio (SR) and crosslinking density (CLD) were investigated in detail by varying the reaction parameters such as amounts of reactants, reaction time and reaction temperature.

2.3 Characterization

The chemical structure of HPP and ECH-HPP were identified by infrared spectroscopy (IR, Spectrum One, Perkin Elmer, USA). The DS values of HPP were estimated basing on the principles given by Jones and Riddick[43]. Size exclusion chromatography combined with multi-angel laser light scattering (SEC-LLS, Viscotek, USA) measurements were carried out to measure the molecular weight distribution of HPP.

Cross-linking densities (CLD) of the ECH-HPP hydrogels were measured by determining the modulus of elasticity in compression as reported previously [44].

2.4 SEM morphology investigation

The surface morphology of the hydrogel was determined using a scanning electron microscope (JEOL JSM-5600 LV, Japan). The hydrogel samples were first equilibrated in distilled water at room temperature, quickly frozen in liquid nitrogen. Afterwards, the samples were freeze-dried under vacuum at -48°C for 3 days until all the solvent was sublimed.

2.5 Swelling characterization

The swelling profiles of the hydrogel with different crosslinking density were determined by both one-step and two-step swelling characterization. For one-step swelling characterization, the swelling ratio (SR) were measured gravimetrically by immersing the gels in two different buffer solutions (pH 1.2, HCl-KCl buffer or pH 7.4, phosphate buffer) in sealed containers at 37 °C, respectively, simulating gastrointestinal (GI) tract environments[45]. Afterwards, the immersed gel was periodically removed from the buffer solution, blotted with filter paper to wipe off the excess surface water, weighed and returned to the same container until swelling equilibrium were observed. This procedure was performed in triplicate at all of the time points. The SR was calculated by the dynamic weight change of the hydrogels in terms of time by the following equation (1):

$$Q_S = \frac{(W_S - W_d)}{W_d} \quad (1)$$

Where W_s is the weight of the gel at equilibrium-swollen state and W_d is the initial weight of the dried test sample.

Also, a two-step swelling characterization of the hydrogel formed by 1.75% ECH was tested by immersing the dried gel in 5 mL of a solution at pH 1.2 (simulated gastric fluid) for 2 h and subsequently transferred into a solution of pH 7.4 (simulating intestinal tract medium) for additional 14 h. The temperature was kept at 37 °C throughout the measurement. At specific time intervals, samples were removed, blotted and their swelling ratios (SR) were calculated by the aforementioned equation (1).

2.6 Biocompatibility test

In vitro cytotoxicity and *in vivo* acute oral toxicity test of the hydrogel were conducted to evaluate the biocompatibility of the hydrogel.

In vitro cytotoxicity of the hydrogel was tested by MTT assay against HUH 7, Hacat and L02 cell lines and the cell morphology were recorded by microscopic examination. In brief, cells (1×10^4) were seeded in 96-well plates and incubated overnight in DMEM. The media was replaced with fresh media containing ECH-HPP polymer solution with different concentrations (0.01, 0.1, 1, 5, 10, 50, 100 mg/mL) and incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37 °C. Cells grown in pure culture

medium served as a 100% cell viability control. Afterwards, cells were washed twice with PBS and 0.2 mL of MTT solution was added to each well. The plate was further incubated for 4 h to allow the metabolism of MTT. Later, 150 μL of DMSO was added to each well and the plate was incubated for 30 min at room temperature. Finally, the cytotoxic activity of the polymer was quantitatively analyzed using the MTT assay to measure the metabolic reduction of 3-(4, 5-dimethylthiazol-2yl)-2, 5, diphenyltetrazolium bromide to formazan by viable cells.

Acute oral toxicity was carried out to test the *in vivo* biocompatibility of the hydrogel. All animal studies were conducted under a protocol approved by the Wuhan University Animal Care and Use Committee. Twenty mice of both sexes were fasted with water allowed to access freely overnight prior to the experiments. The mice were orally injected with ECH-HPP hydrogel suspension twice (4 h intervals) at a dose of 7.5 g/kg b.w.. So, the total dose given to each animal was up to 15 g/kg b.w..The same volume of 0.9% aqueous NaCl was injected p.o. into the negative control mice. Subsequently, all animals were observed for 14 days and the mortality and their general conditions including the energy, activity, hair, feces, behavior pattern, and other clinical signs were recorded. At 14th day, all animals were sacrificed and gross histological examinations of the major organs were performed. Finally, the acute toxic classifications of the ECH-HPP hydrogel were evaluated according to China's criteria of acute classification (Ministry of Health, People's Republic of China).

2.7 Hydrogel degradation

In vitro biodegradability of the ECH-HPP hydrogel was determined by both chemical hydrolysis and enzymatic hydrolysis, respectively. Chemical hydrolysis of the hydrogel was performed in a pH 7.4 phosphate buffer solution. In particular, aliquots exactly weighed of the hydrogel (30 mg) were dispersed in the hydrolysis medium and incubated at 37 °C under continuous orbital shaking (100 rpm). At predetermined time intervals, the gels were separated out from the release medium, washed with DI water and gently blotted. Then, they were freeze-dried for 48 h and weighed again.

Enzymatic hydrolysis were conducted by incubating aliquots of the hydrogel for 24 h under continuous stirring (100 rpm) at 37°C PBS (pH 7.4) containing dextranase (final enzyme concentration 15 U/mL) and esterase (final enzyme concentration 125 U/mL). The enzyme solutions were prepared just before use. The activities of dextranase and esterase have been confirmed by performing the assays reported in the literature [46]. After the treatment, the hydrogel was treated with the same procedure used for the sample recovered after the chemical hydrolysis. Each experiment was performed in triplicate at all of the time points for both chemical hydrolysis and enzymatic hydrolysis.

2.8 Drug loading and release studies

The drug loading and release profile from ECH-HPP hydrogels were studied under simulated GI medium by employing BSA and lysozyme as two model protein drugs, respectively. Firstly, the hydrogel were swelled in BSA or lysozyme solution with a final concentration of 1wt%. After swelling equilibrium, the gel were taken out, dried and reweighed. For confirmation of percentage of drug loaded in hydrogels, the amount of protein solution left in the loading medium was determined by the Micro-BCA assay as described by the manufacturer. The entrapment efficiency (ee) was defined as a percentage of protein mass loaded in the hydrogel to the originally provided protein mass by means of the following equation (2):

$$\text{entrapment efficiency} = \frac{\text{actual protein concentration}}{\text{theoretical protein concentration}} \quad (2)$$

The protein release from the test hydrogels were evaluated by both one-step and two-step releasing study, respectively. For the one-step releasing study, the dried, drug loaded gel was immersed in solutions with pH 1.2 (simulated gastric fluid), pH 6.8 (simulated intestinal fluid) or pH 7.4 (simulated colonic fluid). For the two-step releasing, the gel was firstly immersed in a solution at pH 1.2, 37 °C for 2 h and subsequently transferred into a solution of pH 7.4 for additional 8 h. Specifically, 20 mL of the solutions were taken in a conical flask and were placed in a rotary water bath shaker at 100 rpm under physiological temperature (37 °C). Samples were withdrawn at regular intervals and the release of BSA was calculated by the Micro-BCA assay. With each sampling, the solution was replenished with fresh medium which was pre-equilibrated at 37 °C, maintaining the total volume constant. This procedure was performed in triplicate at all of the time points studied. The percentage of cumulative amount of released protein drugs (BSA or lysozyme, respectively) was calculated and plotted against time.

2.9 Stability of the released BSA

The stability of the released BSA was determinedby analyzing the conformation of the released BSA using Jasco J-810 spectropolarimeter (Jasco, Japan)[47]. Besides, the SDS-polyacrylamide gel electrophoretic (PAGE) analysis was conducted to study the structural integrity of BSA in the supernatant.

2.10 Determination of the enzymatic activity of lysozyme

In order to test whether the activity of the lysozyme loaded in the hydrogel had been kept after releasing, the enzymatic activity of the lysozyme before and after releasing from the hydrogel had been measured according to a literature reported method (be referred to as "Initial lysozyme" and "Lysozyme released from hydrogel", respectively) [48]. Briefly, 200 μL of sample solution containing the released lysozyme was added to 2.0 mL of phosphate buffer solution (pH 7.4) containing 0.6 mg of *Micrococcus lysodeikticus* suspension as the substrate. A decrease in suspension turbidity which was attributed to the degradation of the substrate was recorded with a spectrophotometer in terms of incubation time. The enzymatic activity was calculated from the initial slope of the course of turbidity vs. time. The activity of lysozyme which had been kept dissolved in PBS (pH 7.4) at 37°C for various periods of time was also measured for comparison (be referred to as "Lysozyme free in solution").

2.11 *In vivo* pharmacological response of insulin-loaded hydrogel

Diabetic animal model was built by injecting streptozotocin (STZ)

intraperitoneally in male BALB/c mice. After 3 days of injections, diabetic values were checked. The diabetic mice were fasted overnight prior to treatment and remained fasted for another 12 h during the experiment, only allowed water *ad libitum*. Mice were divided into four groups each containing minimum of 6 mice. The first two groups of the diabetic animals were treated with insulin-loaded ECH-HPP hydrogel orally at two different doses of insulin (50 and 100 IU/kg b.w., respectively). The next two groups of the diabetic mice were used for the comparison study, which were treated with either subcutaneous injection of insulin solution at a dose of 5.0 IU/kg b.w or oral gavage of insulin solution at a dose of 100 IU/kg b.w. Blood samples from the tail vein of the mice were collected in a microcentrifuge tube at specific times after administration and the blood glucose level (BGL) were checked by a glucose meter (Roche).

2.12 Statistical analysis

All the experiments were repeated at least three times and the data were presented as means ± standard deviation. Statistical analysis was performed on Origin 7.0. In all the tests, the statistical significance was set at $p< 0.05$.

3. Results and discussion

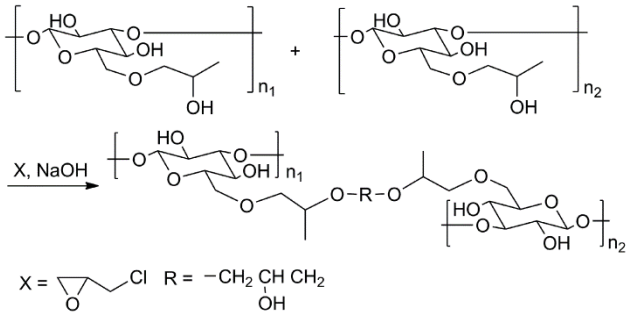
3.1 Synthesis of ECH-HPP

Chemical modification of a natural polysaccharide gives the opportunity to combine the typical biocompatibility of natural polymers with the advantages of synthetic polymers such as chemical versatility, better mechanical properties, etc. Among all of the chemical modifications, chemical crosslinking is a commonly used approach for producing hydrogels. In this study, ECH-HPP hydrogel was prepared by HPP crosslinking with epichlorohydrin (ECH) in a basic medium (Scheme 1). ECH was employed as a convenient base-catalyzed cross-linking agent. It could be claimed that each ECH molecule could react with two hydroxyl groups from different HPP molecules, yielding one hydroxyl group, in this manner, two neighboring polysaccharides chains were attached together to form a network.

Firstly, the hydroxypropyl group substitution reaction of pachyman was conducted base on a typical etherification of polysaccharides[49]. Comparing the FT-IR spectrum of the ECH-HPP to that of pachyman and HPP (Fig. 1), it showed that the FT-IR spectrum of pachyman exhibited the bands at 890 cm^{-1}, 1260 cm^{-1} and 3400 cm^{-1}which could be attributed to the saccharide structure. These bands did not change when HPP was cross-linked by ECH. Besides, the spectra of ECH-HPP 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 2891 cm^{-1}, due to the introduction of isopropyl groups. Moreover, stronger peaks from 1030 cm^{-1} to 1110 cm^{-1} appeared corresponding to the increment of -C-O-C- bands and -CHOH- groups after crosslinking.

It is important to assess the reaction conditions of the etherification of the polysaccharides because the degree of substitution (DS) of the polysaccharides play crucial role on the physical properties such as molecular weights (Mw), solubility and swelling property of the products. As shown in Table 1, optimization of hydroxypropylation of pachyman for obtaining HPP with good water solubility and moderate molecular weight was achieved by varying the reaction conditions including the amounts of reactants, reaction time and reaction temperature. Table 2 reveals the correlations between DS and the solubility of HPP. Overall, it can be concluded that the solubility of HPP improves gradually with the increment of the DS, mainly due to the introduction of the hydrophilic hydroxypropyl groups into the macromolecule chain of pachyman. When the DS reached to 0.62, the product was able to dissolve in water and gave a clear transparent solution, with a Mw of 18,1000 and a low molecular polydispersity (Mw/Mn = 1.12).

The major drawback restricting the application of pachyman is the water insolubility. The reaction process optimized in this study for obtaining water soluble pachyman derivatives may be of great significance in extending the application of pachyman.



Scheme 1. Synthetic procedure of ECH-HPP polymer.

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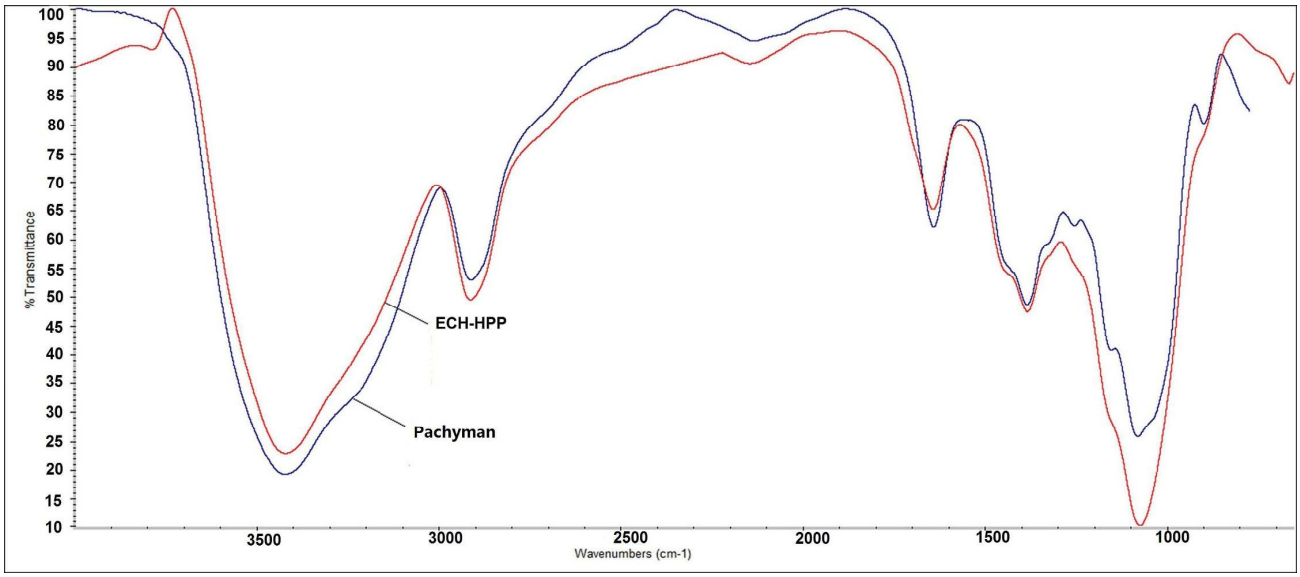

Fig. 1 FT-IR spectrum of HPP and ECH-HPP.

Table 1 Reaction conditions optimized for preparation of HPP

Pachyman : Propylene oxide ratio (g/mL)	Pachyman : Water ratio (g/mL)	Quantity of NaOH (g)	Reaction temperature (°C)	Reaction time (h)	DS[a]	Molar mass distributions[b]		Solubility[c]	
						Mw ($g.mol^{-1}$)	Mw : Mn	Water	DMSO
1: 8	1: 12	12	40	8	0.62	181000	1.12	+[d]	+

[a]DS means the degree of substitution of hydroxypropyl groups to monosaccharide residue of pachyman.
[b]Determined by SEC-LLS chromatograms. [c]+: Soluble.

Table 2 The correlation between the DS and water solubility of HPP

DS of HPP[a]	Solubility[b, c]
0.1053 ± 0.0003	-
0.1356 ± 0.0022	±
0.3412 ± 0.0019	±
0.4672 ± 0.0031	±
0.4966 ± 0.0040	±
0.5190 ± 0.0032	±
0.5708 ± 0.0028	±
0.6233 ± 0.0031	+
0.6275 ± 0.0021	+
0.6502 ± 0.0029	+

[a]Data were expressed as mean ± S.D. of four experiments.
[b]Data were determined by naked eyes.
[c] -: Insoluble in water; ±: Swelling in water; +: Soluble in water.

3.2 Hydrogel characterization

It is well known that the swelling ability of hydrogels plays important role in regulating many of their properties, such as permeability to hydrophilic or hydrophobic drugs, rates of enzymatic or hydrolytic degradation, and mechanical properties [50]. Thus, the swelling behavior of the hydrogel was studied in medium at pH 1.2 and pH 7.4 mimicking the gastric and intestinal liquids respectively.

As shown in Fig. 2A and 2B, in the beginning, the swelling ratio was very small at the ECH amount of 0.4% and 0.5% (vol-%) probably because a relative loose crosslinking network was formed. Later, the swelling ratio (SR) generally showed a tendency of increase with the enhancement of the ECH amount. However, when the amount of ECH increased to 1.75 vol-%, a decrease in the SR of the hydrogel occurred. Although this result is consistent with the earlier report on composite hydrogels based on xanthan and poly(vinyl alcohol)[51], it is contradicted with the literature data which predicted a decrease in the swelling degree

of a hydrogel when raising the quantity of the cross-linking agent. This phenomenon is probably caused by the destruction of the helicoidal structure of pachyman in the basic medium and under heated condition during the cross-linking reaction. As a result, a portion of hydroxyl groups used to stabilize the helicoidal structure by hydrogen bond was involved in the cross-linking reaction while the rest of the hydroxyl groups were able to interact with water in the swelling process. Thus, compared to the initial helicoidal structure of pachyman, a less compact network with greater net-blanks formed, which allowed the absorption of more "unbounded" water. However, when the cross-linking density increases to a higher extent, swelling becomes restrained as a more compact network was constructed, which led to the reduction of the free volume entered into the network and the expandability and the penetrability of the hydrogel network was restricted.

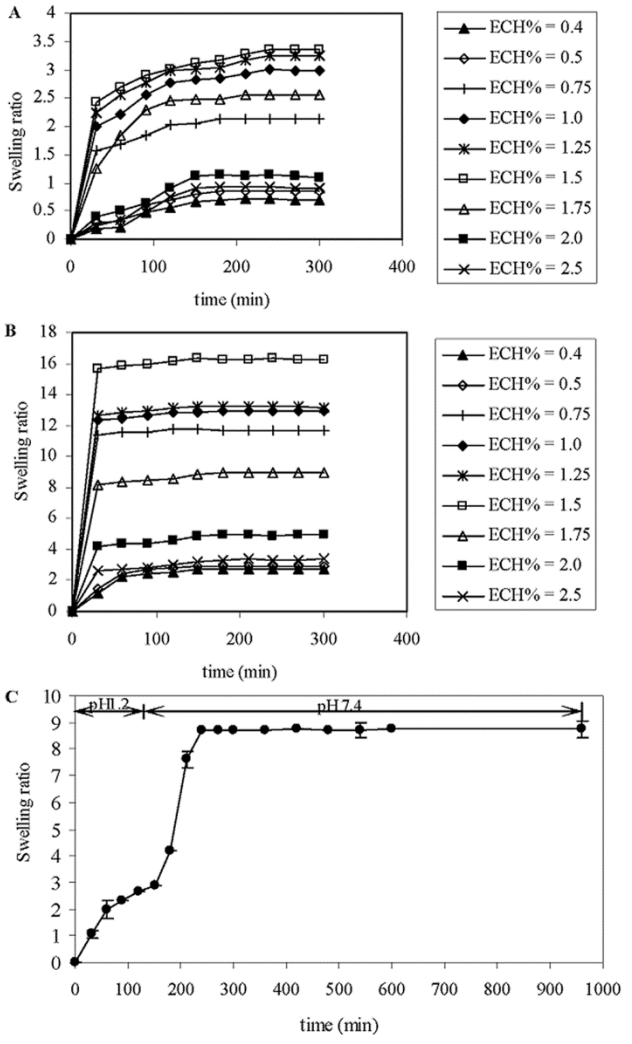

Fig. 2 Swelling profiles of the ECH-HPP hydrogel: (A) One-step swelling characteristics of the hydrogel with different amounts of ECH at pH 1.2; (B) One-step swelling characteristics of the hydrogel with different amounts of ECH at pH 7.4 and (C) Two-step swelling characteristics of the hydrogel at pH 1.2 for 2h and subsequently in pH 7.4 for 14 h.

Besides, comparison of the SEM image of the HPP and ECH-HPP hydrogel (Fig. 3B and 3C) also revealed a continuous and porous structure of the ECH-HPP hydrogel which is in agreement with the formation of a polymer network. The porous nature of the hydrogel makes it less dense and provides more surface area, where the capillary forces help the diffusion of water into the hydrogel. Upon the hydroxypropylation and the crosslinking reaction, it can be clearly seen from Fig. 3A that the starting material, pachyman which was neither water-soluble nor swellable (tube#1) was firstly changed to a transparent water-soluble solution of HPP (tube#2), and finally turned into a highly swellable ECH-HPP hydrogel (tube#3).

From the swelling ratio studies of different ECH and HPP combinations (Fig. 2A and 2B), crosslinking with 1.75 vol% of ECH was found to be the most favourable amount for crosslinking as the ECH-HPP hydrogel synthesized by 1.75 vol% of ECH showed a suitable SR at both pH 1.2 and pH 7.4 (~2.5 at pH 1.2 and ~8.6 at pH 7.4). However, the SR of ECH-HPP hydrogel of all other groups were not desirable for protein drug delivery since some of them showed too high swelling at both pH 1.2 and 7.4 while other groups showed too low swelling at pH 7.4. The rapid swelling of the hydrogel is not favorable for controlled drug release at intestinal denaturation by proteolytic enzymes. Besides, with increasing the SR of the hydrogel, gel disintegration was occurred at pH 7.4.

Moreover, the entrapment efficiency studies (Table 3) also confirmed that the amount of 1.75 vol% ECH to be the best, as it showed the highest entrapment efficiency (97.6%). Thus, the hydrogel formed by 1.75 vol% amount of ECH was selected for further pH-sensitivity and protein release studies.

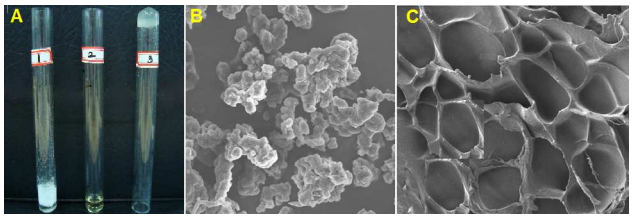

Fig. 3 The surface morphology and photographs of the ECH-HPP hydrogel: (A) photographs of the pachyman in water (tube #1), HPP in water (tube #2) and ECH-HPP hydrogel (tube #3); (B) SEM image of HPP and (C) SEM image of the hydrogel.

3.3 pH-sensitivity

Generally, swelling performance of a hydrogel in gastric and intestinal fluids is of determinant relevance for the drug release. The tested hydrogel presented similar swelling patterns between one-step swelling profiles (Fig. 2A and 2B) and two-step (Fig. 2C) swelling profiles in simulated physiological environment. As expected, the ECH-HPP hydrogel showed obvious difference in swelling that were dominated by external pH changes. Specifically, the swelling ratio of the hydrogel was very low at pH 1.2 (gastric environment) while the swelling ratio was moderate at pH 7.4 (intestinal environment). The swelling ratio of the hydrogel was low at pH 1.2 probably because of the formation of intermolecular hydrogen bonds. However, at pH 7.4, the hydroxy groups on the hydrogel was likely to be progressively ionized (-ONa). Therefore, the swelling of the hydrogel was highly increased probably due to a large swelling force created by the electrostatic repulsion between the ionized

groups. Thus, the ECH-HPP hydrogel could protect the loaded protein drug from degradation by the low pH environment of the stomach.

Table 3 Percentage entrapment efficiency of hydrogels with different ECH amounts

ECH (%)	Solubility characteristics[a]	Cross-linking density (mol/m^3)[b]	% Entrapment efficiency (ee)
0.3	Soluble	-	-
0.4	Partial soluble	5.6±0.3	10.6
0.5	Partial soluble	8.5±1.2	12.3
0.75	Nonsoluble and swollen	9.6±2.2	28.4
1.0	Nonsoluble and swollen	10.2±2.1	47.9
1.25	Nonsoluble and swollen	12.6±3.6	59.8
1.5	Nonsoluble and swollen	15.3±3.2	70.2
1.75	Nonsoluble and swollen	19.6±1.8	97.6
2.0	Nonsoluble and swollen	20.3±2.7	53.7
2.5	Nonsoluble and swollen	19.2±1.9	20.2

[a]Determined by naked eyes. [b]Data were expressed as mean ± S.D. of four experiments.

3.4 Biocompatibility of the hydrogel

One of the key factors of the polymeric vectors for their application in drug delivery is the absence of cytotoxicity. MTT assay has proven to be a sensitive and reliable method for evaluating biocompatibility of polymers [52].

In this study, an *in vitro* MTT assay over the HUH 7 cell line, Hacat cell line and human normal hepatocyte cell line L02 (Fig. 4) was performed to test the biocompatibility of the ECH-HPP hydrogel. As compared to the negative control, for all concentrations studied, the viabilities of all the three cell lines were almost 100% and they were dose dependent. Moreover, as cell morphological analysis shown in Fig. 4A-F, the cells attached and spread regularly compared to the negative control, there was no morphological alteration such as retraction or detachment of cell monolayer on the cells. Thus, it can be deduced that the ECH-HPP hydrogel was well tolerated by cells.

Furthermore, *in vivo* biocompatibility test also confirmed that oral administration of mice with ECH-HPP hydrogel up to 15 g/kg b.w. had no toxic effect. There was no death and no toxic response of all the mice studied during the 14-day study period. The animals displayed full of energy, normal behavior, and free movement. No macroscopic pathological alterations were found in all mice at necropsy. Since the highest tested dose (15 g/kg b.w.) caused no mortality, it could be concluded that the maximal tolerance dose (MTD) of ECH-HPP hydrogel were higher than 15 g/kg b.w. in BALB/c mice.

Thereby, the synthesized ECH-HPP hydrogel presented excellent biocompatibility which was expected since the starting materials, pachyman is a biocompatible natural polysaccharide. It could also be confirmed that all the residual reagents and cross-linker which might lead to the cytotoxicity of the products have been removed completely after the reaction.

3.5 Hydrogel biodegradation

The ability of synthetic biomaterials to degrade in the presence of biological milieu is a crucial property for a wealth of applications[53]. The presence of potentially degradable linkage in the structure of ECH-HPP hydrogel, such as glucoside and ether confer a possible biodegradability of these systems. In order to evaluate whether the degradation could occur, chemical and enzymatic degradation studies were performed in simulated physiological conditions, respectively. Fig. 5 shows that the chemical hydrolysis of the hydrogel was negligible since only a small dry weight was lost. However, approximately 15% of the hydrogel was degraded after 24 h of incubation with dextranase and esterase. Moreover, the degradation rate of the hydrogel lied on the swelling ratio (SR) of the hydrogel. It could be deduced from Fig. 5 that the degradation rate decreased with decreasing of the SR. The reason that the hydrogel with a higher swelling ratio had a faster degradation rate is probably due to the formation of a less compact network when the swelling ratio of the hydrogel is high. It is speculated that after an initial surface erosion of the hydrogel, enzymes diffused into the polymeric network and then more internal sites were degraded.

3.6 *In vitro* drug loading and release profile

One of the most challenging obstacles in developing oral protein formulation is the inactivation or rapid enzymatic degradation in the harsh environment of the stomach. Thus, pH-sensitive drug delivery system has been considered as a superior strategy in oral protein delivery as it can protect the protein drugs from proteolytic degradation in stomach[54, 55]. From the drug release profile in simulated GI tract shown in Fig. 6, it can be deduced that the protein release from the ECH-HPP hydrogel possessed responsiveness against the external pH change under the simulative GI conditions. Lower degree of swelling and slower protein release was observed under low pH conditions (pH 1.2, gastric environment); however, under high pH conditions (pH 7.4, intestinal environment), the hydrogels swelled rapidly and showed a quick release of BSA from the hydrogels.

Specifically, at pH 1.2, only about 12% of the encapsulated

BSA was released from the test hydrogel in the one-step release study, however, at both pH 6.8 and pH 7.4, the protein released significantly higher (89.3% and 96.8%, respectively). So, both at the pH of duodenum andileum, the protein was released in a controlled manner over a period of 10 h. As for the two-step release profile (Fig. 6B), protein release at pH 1.2 was similar to that obtained in one-step swelling. But when the hydrogel were transferred to simulated intestinal fluid (SIF, pH 7.4), the protein released was found to be much higher (about 89%) and a sustained and prolonged release profile was observed. Thus, the ECH-HPP hydrogel was found to be effective in releasing protein in a sustained fashion in intestine, while protecting it from the harsh stomach environment. The favorable BSA release performance from the ECH-HPP hydrogel could be attributed to the pH-sensitivity of the hydrogel. It could be deduced that the protein release from the hydrogel matrix mainly due to the diffusion of the drug through the pores of the swollen matrix in the intestinal pH. Since the hydrogel is highly porous in pH 7.4, the capillary forces could facilitate the diffusion of solvents into the hydrogel.

It was predicted that the release mechanism of BSA was firstly caused by diffusion and further accelerated by the weight loss of the gel. However, this hypothesis cannot be fully established in the early stage of the drug release when considering the weight loss profile (Fig. 5) and the drug release kinetics (Fig. 6). In the first 6 h of the two-step BSA release study, about 57% of the BSA was released from the hydrogel, nevertheless only a negligible amount of the gel matrix (~5%) was lost. This result suggests that at the first stage of the release, the main driving force for BSA release was not gel degradation but diffusion, which might be attributed to the large amount of water entered in the gel. After the first 6 h, the weight loss of the hydrogel was increased to 15% and the swollen gels began to degraded from outside to inner. Therefore, it can be presumed that after the first stage of the drug release, the drug molecule was released from the swollen hydrogel on account of both erosion of polymeric matrix and diffusion.

Moreover, the ECH-HPP hydrogel could also release the other model drug, lysozyme in the controlled release manner and the enzymatic activity of the loaded lysozyme had been fully preserved (Fig. 6C and Table 4). The two-step release profile of lysozyme shown in Fig. 6C revealed that the release pattern of lysozyme was similar to that of BSA. Only about 7.8% of the lysozyme was released at pH 1.2, however, the amount of lysozyme released was significantly increased to 65% at pH 7.4. This favorable controlled release performance of lysozyme could be also attributed to the pH-sensitivity of the hydrogel.

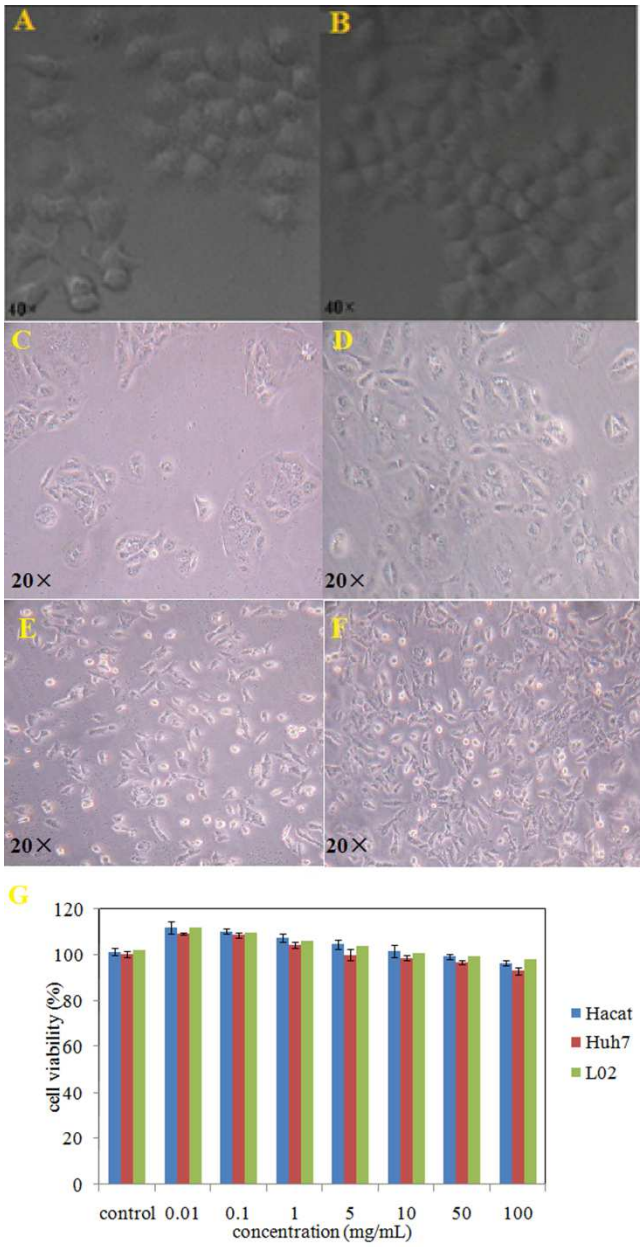

Fig. 4 *In vitro* biocompatibility test of the ECH-HPP hydrogel. Microphotographs of HUH7 cells, Hacat cells and L02 cells after three days in culture on ECH-HPP hydrogel: (A) the control of HUH7 cells; (B) HUH7 cells cultured on the hydrogel; (C) the control of Hacat cells; (D) Hacat cells cultured on the hydrogel; (E) the control of L02 cells; (F) L02 cells cultured on the hydrogel and (G) *In vitro* cytotoxicity of the ECH-HPP as a function of polymer concentration towards HUH7, Hacat and L02 cell lines, respectively.

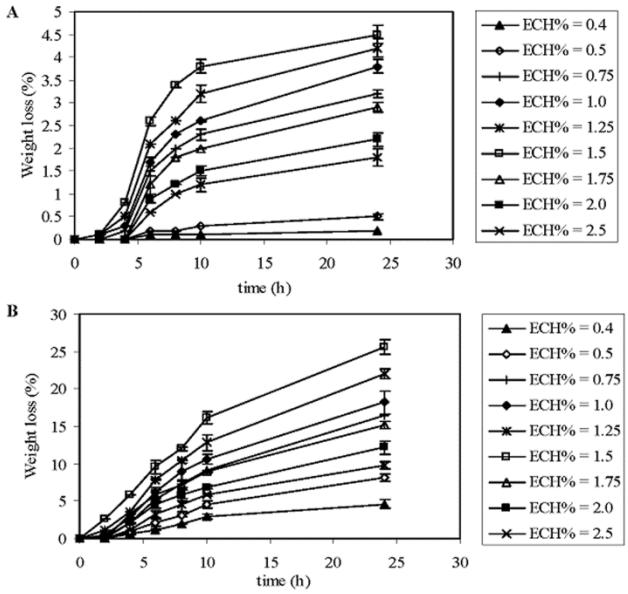

Fig. 5 Weight loss of the ECH-HPP hydrogel as a function of immersion time in PBS (pH=7.4) at 37°C: (A) Chemical hydrolysis and (B) Enzymatic hydrolysis.

Table 4 Enzymatic activity of the released and free lysozyme

Lysozyme treated	Remaining activity[a] (units/mg lysozyme)
Initial lysozyme	890 ± 90
Released from hydrogel	906 ± 39
Lysozyme free in solution	910 ± 69

[a]Data were expressed as mean ± S.D. of three experiments.

3.7 BSA structural integrity

Exposure of BSA to the ionic solution or cross-linking agents could lead to possible detrimental effects on protein structure and stability, such as protein denaturation, aggregation, hydrolysis, and reaction with the cross-linking agents. Therefore, the influence of the hydrogel environment on the integrity of encapsulated BSA was investigated by both SDS-PAGE and circular dichroism (CD) spectra. Compared with the BSA released from ECH-HPP hydrogel and the BSA standard in solution (i.e., a BSA standard), the CD spectra (Fig. 7A) shows that there was a slight change in the conformation of the loaded BSA when released at pH 1.2. This was probably due to the poor stability of proteins at pH 1.2 [56]. However, no significant conformation change was observed for the BSA released from the two-step releasing (at pH 1.2 for 2 h firstly and then at pH 7.4 for 8 h). This result suggested that the secondary structure of the loaded BSA was preserved during the process of loading, prolonged contact of BSA with the ECH-HPP hydrogel and releasing into a buffer solution at pH 1.2 for 2 h and subsequently at pH 7.4 for 8 h.

Moreover, the SDS-PAGE results of the BSA released from the two-step release study also revealed that the integrity of the released protein was highly maintained as the released BSA solution had a clear band presented at 66 kDa which is consistent with the commercial standard BSA (Fig. 7B). Besides, there were no bands representing fragments of lower molecular weights detected, suggesting that the released BSA did not undergo hydrolysis.

Therefore, the ECH-HPP hydrogel is potential as a safe carrier for delivery of protein drugs because it could fully maintain the stability of the protein. This is mainly attributed to the benign environment for the protein encapsulation process and the shielding effect of protein susceptible to proteolytic attack by pH and enzymes.

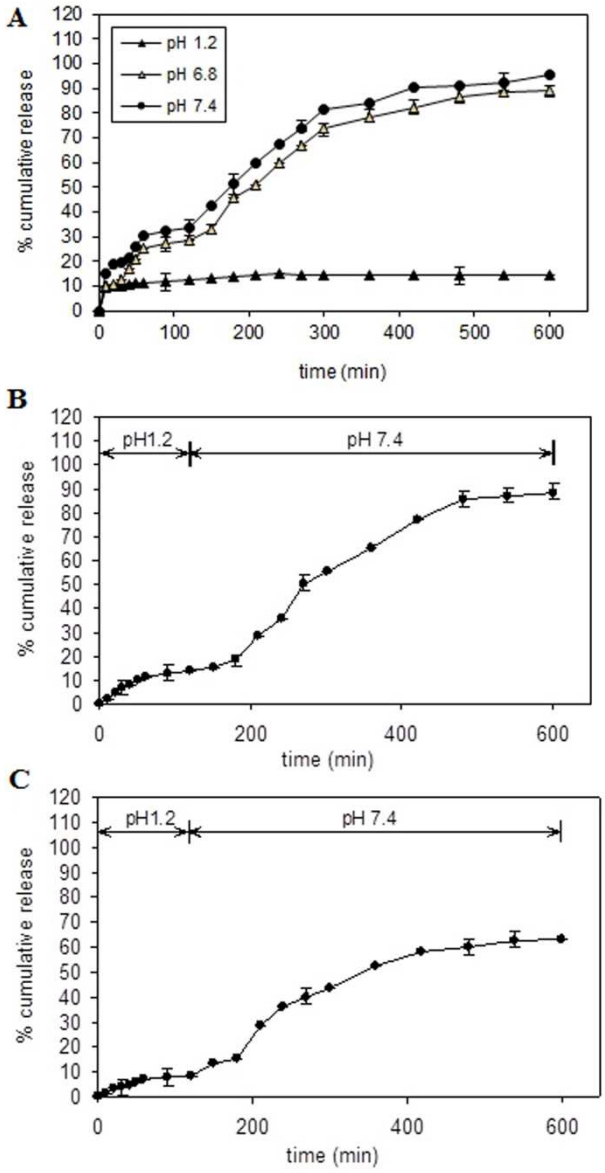

Fig. 6 Drug release profile of the ECH-HPP hydrogel: (A) One-step BSA release profile of the hydrogel in pH 1.2 or pH 6.8 or pH 7.4, respectively; (B) Two-step BSA release profile of the hydrogel in pH 1.2 for 2h and subsequently in pH 7.4 for 8 h and (C) Two-step lysozyme release profile of the hydrogel in pH 1.2 for 2h and subsequently in pH 7.4 for 8 h.

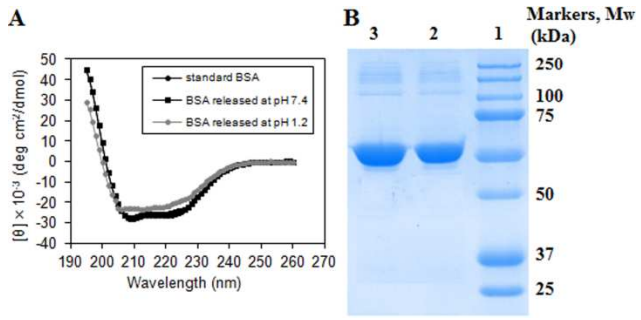

Fig. 7 The stability test of the released BSA: (A) CD spectra of the standard BSA, BSA released at pH 1.2 and BSA released at pH 7.4 (at pH 1.2 for 2 h and then at pH 7.4 for 8 h) and (B) Coomassie-stained SDS-PAGE gel of the released BSA at pH 7.4. Lanes 1, 2 and 3 are,respectively, the molecular weight markers, BSA standard, BSA released from the ECH-HPP hydrogel at pH 7.4.

3.8 *In vivo* evaluation on diabetic animals

In vivo oral hypoglycemic effects of the insulin-loaded ECH-HPP hydrogel were evaluated in diabetic mice in comparison to the control groups, i.e. diabetic mice treated with subcutaneous injection of free insulin solution at a dose of 5.0 IU/kg b.w. or orally injection of free insulin solution at a dose of 100 IU/kg b.w. As shown in Fig. 8, no significant reduction in glycemia was observed with the group treated with orally injection of free insulin solution at the dose of 100 IU/kg b.w., which propably due to the enzymatic degradation of the insulin in the GI tract. However, the diabetic mice which treated with the insulin-loading ECH-HPP hydrogel showed an obvious reduction in glycemia in a dose dependent manner (50 IU/kg b.w. and 100 IU/kg b.w.). Notably, the insulin-loaded ECH-HPP hydrogel at higher dose (100 IU/kg b.w.) could reduce blood glucose level (BGL) to 6 mM after oral administration and sustained at least up to 9 h. In addition, subcutaneous administration of the free insulin solution at the dose of 5 IU/kg b.w. also decreased the BGL significantly within 30 min but subsequently, a rapid increase in BGL was observed after 2 h of the injection. Thus, the synthesized ECH-HPP hydrogel could be considered as a suitable system of oral insulin delivery since it could protect insulin from degradation by GI tract and reducing glycemia in diabetic model at peroral delivery in a sustained way.

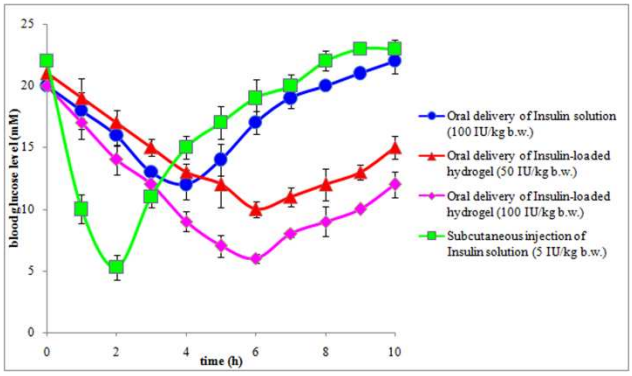

Fig. 8 *In vivo* hypoglycemic effect of the ECH-HPP hydrogel.

4. Conclusions

In the development of therapeutic proteins and peptides, protein drug delivery systems which could be able to retain their integrity and biological activity is one of the key components. Polysaccharides have great structural diversity and are less expensive, natural resourceful, nontoxic and easy to make chemical modifications. These superior properties make polysaccharides quite promising base structures of drug carriers. The present study aims to develop a new polysaccharide-based hydrogel for safe and effective protein delivery. The synthesized ECH-HPP hydrogel has offered the following advantages and shown quite promising as drug carrier for oral protein drug delivery: (1) biodegradability and biocompatibility; (2) pH-sensitivity which provides desirable protective effecton protein drugs at low pH environment of the stomach; (3) efficient loading and controlled release of the proteins entrapped without any loss of their stability and biological activity; (4) protection of insulin from degradation by GI tract and prolonged hypoglycemic effects in diabetic model at peroral delivery and (5) less expense and ease of production and structural modification.

Pachyman is the key component of many herbal medicines in China and Japan. As one of the polysaccharides, it exhibits many favorable features as drug carrier. However, its application in the pharmaceutical area has not been explored extensively. The presented study has provided some initial effort in assessing the application of pachyman as protein drug carrier. More work in investigating the use of pachyman in a nano-system in anticancer drug delivery is currently ongoing (Unpublished results).

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