Swelling Behavior and Biocompatibility of Carbopol-Containing Superporous Hydrogel Composites

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ABSTRACT: Carbopol-containing superporous hydrogel composites (SPHCc) with fast swelling and high swelling ratio were prepared using free radical copolymerization. Swelling behavior of the SPHCc was studied in various salt and pH solutions and their biocompatibility was evaluated using tissue damage and cytotoxicity studies. The swelling ratio of the SPHCc decreased with the increase of Carbopol/monomer ratio and was sensitive to pH and ionic strength of the swelling medium. The release of insulin from the SPHCc was rapid in 0.01*M* phosphate buffered saline (PBS, pH 7.4). In the jejunum mucosal membrane toxicity studies in rat, no significant morphological damage was observed after application of SPHCc. Besides, no damage of the Caco-2 monolayers was detected after incubation with SPHCc in the trypan blue and propidium iodide tests. On the basis of these results, it was concluded that the SPHCc might be a safe and effective carrier for peroral delivery of peptide and protein drugs. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 104: 2785–2791, 2007

Key words: hydrogels; swelling; biocompatibility; composites; Carbopol

INTRODUCTION

Hydrogels are three-dimensional crosslinked hydrophilic polymers that are able to swell in an aqueous environment without dissolution.[1,2] Because of their high water affinity, environmental sensitivity, and high permeability, hydrogels have been widely used as a carrier for drug delivery systems (DDS).[3–5]

In recent years, peroral delivery systems for macromolecule drugs such as peptides and proteins have drawn much attention because of the development of many macromolecule drugs and lots of disadvantages caused by common administration via parenteral injection routes. Superporous hydrogels (SPH) and SPH composites (SPHC) with croscarmellose sodium (Ac-Di-Sol®) have been developed and used as an appropriate carrier for oral delivery of peptide and protein drugs.[6–9] Because of large numbers of interconnected pores that form open channel structure, SPH and SPHC possess fast swelling and high swelling ratio.[6,7] Because of their high swelling ratio and carboxylic groups in their structure, these polymers have the capabilities of inhibiting proteolytic enzyme and opening the intercellular tight junctions that impede the absorption of hydrophilic macromolecular drugs.[6,10,11] Besides, SPH and SPHC are safe excipients and do not cause any damage to the intestinal mucosal membranes and Caco-2 cell monolayers.[10,11]

Carbopol is the commercial name of a series of highly hydrophilic polyacrylic acid polymer. It has been extensively used as an excipient for DDS because of its well-characterized biocompatibility and low toxicity. Carbopol can swell quickly in water and adhere to the intestinal mucus because the functional groups of —COOH can form hydrogen bridges to interpenetrate the mucus layer. Moreover, Carbopol can inhibit the activity of the dominant enzymes in the gastrointestinal tract because of the carboxylic groups in its structure.[12]

This research intended to prepare Carbopol-containing superporous hydrogel composites (SPHCc) to obtain a carrier for peroral delivery of hydrophilic macromolecular drugs with fast swelling, high swelling ratio, enhanced absorption, and good biocompatibility of SPH and Carbopol. The effects of the pH, Carbopol/monomer ratio, and ionic strength on the swelling behavior of SPHCc were investigated. Insulin was used as a model protein to study drug release from the SPHCc in 0.01*M* phosphate buffered

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saline (PBS, pH 7.4). Finally, the biocompatibility of SPHCc was evaluated through tissue damage and cytotoxitity studies.

EXPERIMENTATION

Reagents and animals

Acrylic acid (AA), acrylamide (AM), *N*,*N*′-methylenebisacrylamide (Bis), ammonium persulfate (APS), and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were obtained from Sigma (St. Louis, MO). AA was distilled under reduced pressure before use. Pluronic® F127 (PF127) was gifted from BASF (Parsippany, NJ). Sodium bicarbonate was obtained from Shanghai Hongguang chemical plant (Shanghai, China). Carbopol 974P was a gift from BFGoodrich (Cleveland, USA). The enteric-coated capsules for rat were gifted from Chaozhou Capsule Plant (Guangdong, China). Cell culture media and supplies were from Gibco (Grand Island, NY).

Male rats weighing 200 ± 20 g (Sprague-Dawley origin), bred and housed in the Animal Centre of Fudan University (China), were used. The rats were fasted prior to experiment with free access to water.

Preparations of the SPHCc

The procedure of preparing SPHCc was similar to that of SPH as described previously.[13] However, various amounts of Carbopol powder were added to the reactive mixture after adding APS and before adding TEMED. The SPHCc were immersed in a mixture of 0.01*M* hydrochloric acid/ethanol (3 : 7, v/v) for 1 day and dialyzed (molecular weight cut off 8000 Da) against absolute ethanol for at least 4 days to eliminate possible residual substances. After purification, the SPHCc were dried up to constant weight.

Morphological examinations

The dried SPHCc were used for morphological examinations with scanning electron microscopy (SEM). To prevent the morphology of porous structures from changing, the dried SPHCc were put in liquid nitrogen and cut to expose their inner structure. The inner surface of the SPHCc were coated with a thin layer of gold alloy and imaged in a SEM (*S*-520, Hitachi).

Swelling measurements

The dried SPHCc were weighed and then immersed in a solution with determined pH (1.0–7.4) or ionic strength (0.0001–1*M*). At various time intervals, the hydrogel was removed from the solution and weighed after excessive solution on the surface was blotted. Data presented in this experiment were the mean values of triplicate measurements. Results were calculated according to the following equation:

$$Q = (W_s - W_d)/W_d,$$

where W_s is the mass of the hydrogel in the swollen state and W_d is the mass of the hydrogel in the dried state.

Insulin loading and *in vitro* release

Insulin loaded SPHCc

Insulin (15 mg) was dissolved in 10 mL of PBS. Twenty milligrams of SPHCc with Carbopol/monomer ratio of 0.072 was placed in 1 mL insulin solution to take up the total amount of the solution. After approximately 30 min, the completely swollen SPHCc loaded with insulin was dried at 25°C under reduced pressure.

In vitro release

The *in vitro* release experiments were carried out by immersing the dried insulin-loaded SPHCc in a glass bottle filled with 25 mL 0.01*M* PBS (pH 7.4) at (37 ± 1)°C. The bottle was shaken at 150 rpm. At predetermined time intervals, 150 μL aliquots of the buffer medium were taken and 150 μL PBS was added to maintain a constant volume. The aliquots were centrifuged at 12,000 rpm for 5 min and analyzed by HPLC.

Analysis of insulin

Samples were measured using HPLC-UV at 220 nm. The mobile phase was composed of acetonitrile-0.03% trifluroacetic acid solution (32 : 68, v/v) at a flow rate of 1.0 mL/min. The column used was a Zorbax 300SB-C18 column (4.6 × 250 mm, 5 μm, Agilent, USA).

Tissue damage studies

Preparation of capsule

To ensure that the enteric-coated capsules could smoothly pass through the pylori of rats, their length should be smaller than 5 mm. Five milligrams of SPHCc was filled into the capsule; the joint between the capsule body and the capsule cap was sealed with the glue for enteric-coated capsules.

Cross-section preparations and light microscopic examinations

Three rats receiving blank capsules without SPHCc were considered as control group and three rats given SPHCc capsules were considered as test group. Both of groups were decapitated 2 h after administration. Another three rats given SPHCc capsules were considered as refresh group and were decapitated 48 h after dosing. The jejunum of the rat was excised and washed with fresh PBS (pH 7.4). The separated jejunum was fixed in 10% neutral formaldehyde solution, alcohol-dehydrated, paraffin-sectioned, and stained with hematoxylin and eosin (HE). Cross sections of the jejunum were examined with light microscopy (Leica, USA).

Cytotoxicity studies

Preparation of Caco-2 cell monolayers

The Caco-2 cell line (human colon adenocarcinoma cell line) was obtained from American Type Culture Collection (Rockville, MD). The cells were cultured at 37°C in an atmosphere of 95% air and 5% CO_2 in Dulbecco's modified eagle's medium. The medium was supplemented with 10% heat denatured fetal bovine serum, 1% nonessential amino acids, 2 m*M* L-glutamine, and 1% penicillin–streptomycin.[14,15] Cells were harvested with trypsin-EDTA and seeded onto polycarbonate Transwell filters (0.4 μm, 4.2 cm^2) at a density of 200,000 cells/cm^2. The culture medium was replaced every 48 h during the first 5 days and every 24 h thereafter. The transepithelial electric resistance (TEER) of the monolayer was monitored with a Millicell®-ERS system (Millipore Corp., USA). After 18–21 days in culture, only confluent monolayers with TEER values above 200 Ω cm^2 were utilized for the following experiments.

Trypan blue test

Prior to each experiment, Caco-2 monolayers were rinsed three times and balanced for 30 min with modified Ringer's solution (MRS, pH 7.4) containing 1 m*M* $CaCl_2$, 5.3 m*M* KCl, 0.4 m*M* KH_2PO_4, 0.5 m*M* $MgCl_2$, 0.4 m*M* $MgSO_4$, 3.3 m*M* NaH_2PO_4, 137 m*M* NaCl, 10 m*M* HEPES-Na, and 25 m*M* D-glucose.[15] SPHCc were completely swollen in MRS and placed on the apical surface of the Caco-2 monolayer for 4 h. As control, Caco-2 cell monolayers were incubated for 4 h without SPHCc. After incubation, SPHCc were removed carefully and both sides of the Caco-2 cell monolayers were rinsed with MRS. 0.1% trypan blue solution in PBS was added to the apical side, and then the plates were placed in the incubator (37°C) for 30 min. The dye was removed and the monolayers were rinsed with MRS. Thereafter, the monolayers were examined with light microscopy for dye exclusion.

Propidium iodide test

After Caco-2 cell monolayers had been incubated in the presence or absence of SPHCc for up to 4 h, SPHCc were removed carefully and both sides of Caco-2 cell monolayers were rinsed with MRS. 50 μg/mL propidium iodide solution in PBS was applied apically for 5 min. The dye was removed and the monolayers were rinsed with MRS. The polycarbonate Transwell filters with Caco-2 cell monolayer were cut carefully from the insert, placed on a round coverslip, and mounted on a heating microscope stage (37°C) of a confocal laser scanning microscopy (CLSM, Olympus, Japan). Propidium iodide was detected at the excitation wavelength of 514 nm and emission wavelength of 617 nm.[11]

RESULTS AND DISCUSSION

Syntheses of SPHCc

The SPHCc were synthesized using a solution polymerization technique with AM and AA as monomers, Bis as a crosslinker, sodium bicarbonate as a blowing agent, APS as an initiator, and TEMED as a catalyst. To obtain SPHCc with well-distributed pores, gelling ought to occur when the foam took place. As described previously,[13] when certain amount of Carbopol was added, the fine and uniform foam was generated and the temperature during the synthesis was decreased, which were advantageous for foam stabilization. PF127 as a surfactant [poly(ethylene oxide)-poly(propylene oxide)-poly (ethylene oxide) triblock copolymer], did not contribute to the chemical structure of the polymer, but could stabilize the generated foam by lowering the film–air interfacial tension and increasing the film viscosity.[6,16] Both PF127 as a foam stabilizer and certain amount of Carbopol worked together to retain most of the gas bubbles, and resulted in the formation of homogenous SPHCc during the fast copolymerization of AM and AA.

SEM

When the Carbopol/monomer ratios were 0.048 and 0.096, the scanning electron microscopic pictures of SPHCc were shown in Figure 1. In the structure of SPHCc, some pores were connected to each other in the inner surface. Furthermore, with the increase of Carbopol/monomer ratio, the size of the pores was reduced. When the Carbopol/monomer ratio was lower, the foam produced was fine and stable; the polymer obtained had connected capillary channels

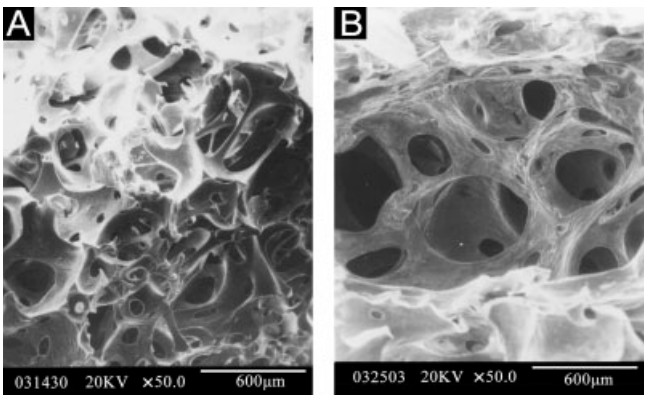

Figure 1 SEM pictures of SPHCc: (A) SPHCc with Carbopol/monomer ratio of 0.048; (B) SPHCc with Carbopol/monomer ratio of 0.096.

with no ''dead end.'' However, when the Carbopol/monomer ratio was higher, bigger gas bubbles were formed. During the synthesis, it was obvious that the bigger gas bubbles were unstable and easy to rupture; during the air-drying process, the individual polymer chains were brought together because of the high surface tension of water, and some of the pores were closed. As a result, the ''dead end'' was formed and the polymer shrank to a smaller and condensed piece. Thus, no higher porosity was expected.[16]

Swelling properties of the SPHCc

Effect of the Carbopol/monomer ratio on the swelling capacity

The swelling behavior of SPHCc with different Carbopol/monomer ratios in double distilled water was shown in Figure 2. From these data, water uptake of SPHCc reached equilibrium state within about 10 min, indicating that the hydrogel could swell to its maximum volume very quickly and possessed a strong absorbing capacity. This was mainly due to capillary wetting of interconnected open pores of SPHCc.[6] As shown in Figure 2, the swelling of the SPHCc decreased as the Carbopol/monomer ratio increased. This could be attributed to the reduction of the porosity as shown in Figure 1.

Effect of the ionic strength on the swelling capacity

The swelling ratio of ionic hydrogels was mainly related to the ionic strength and charge number of the external solution. The swelling kinetics of SPHCc with Carbopol/monomer of 0.072 was shown in Figure 3 as a function of the ionic strength of the swelling medium at pH 7.4. It could be noted that when the ionic strength was no less than 0.01*M*, the swelling ratio of the polymer increased as the ionic strength decreased. When the ionic strength

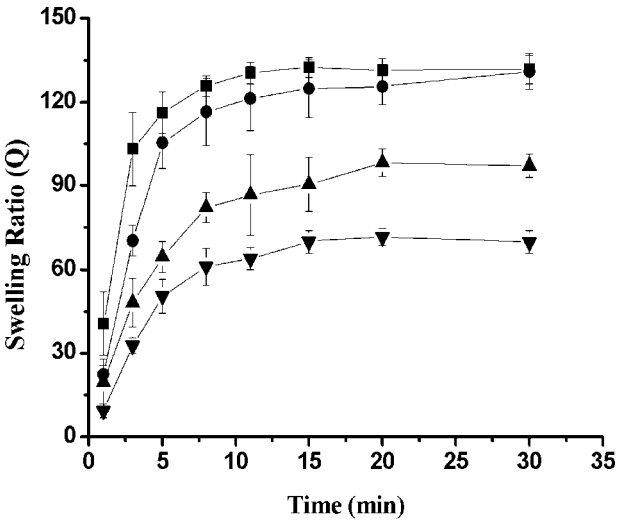

Figure 2 Effect of the content of Carbopol on the swelling kinetics of the SPHCc. (●) 0.024; (■) 0.048; (▲) 0.072; (▼) 0.096.

was no more than 0.001*M*, it did not affect the swelling behavior of SPHCc, and the swelling ratio was similar to that in double distilled water. As the concentration of cations in swelling medium enhanced, a charge screening effect of the additional cations resulted in a nonperfect anion–anion electrostatic repulsion.[17] Therefore, the osmotic pressure resulting from the mobile ion concentration difference between the gel and aqueous phase decreased, and consequently, the swelling ratio decreased.

Effect of the pH on the swelling capacity

Since the SPHCc were composed of acidic groups, which can dissociate or get protonated at some

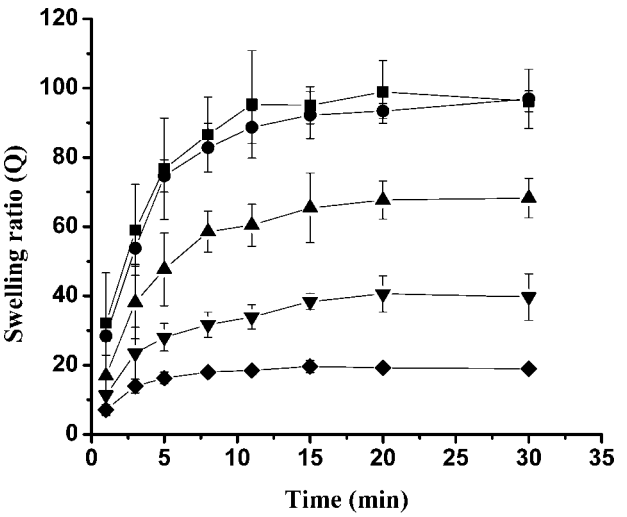

Figure 3 Effect of the ionic strength of swelling medium on the swelling kinetics of the SPHCc with Carbopol/monomer ratio of 0.072. (■) 0.0001*M*; (●) 0.001*M*; (▲) 0.01*M*; (▼) 0.1*M*; (◆) 1*M*.

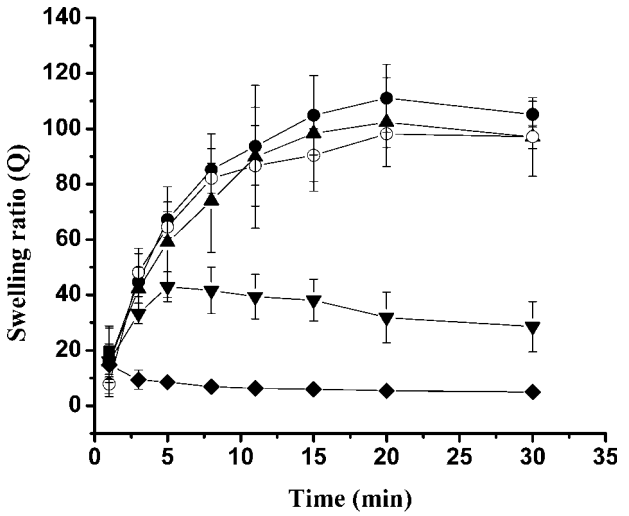

Figure 4 Effect of the pH of swelling medium on the swelling kinetics of the SPHCc with Carbopol/monomer ratio of 0.072. (●) 7.4; (▲) 6.2; (○) 4.9; (▼) 2.0; (◆) 1.0.

suitable pH of the swelling media, the degree of swelling of SPHCc underwent appreciable change with external pH. Figure 4 showed the dynamic uptake of water of the SPHCc in the solutions with pH 1.0, 2.0, 4.9, 6.2, and 7.4. At pH 1.0 and 2.0, a slight swelling capacity of the SPHCc was observed because of protonation of the carboxylic groups and interactions between AA and Carbopol. As pH exceeded 4.9, some carboxylate groups were ionized, and the electrostatic repulsion between $—COO^-$ groups resulted in an enhancement of the swelling capacity. Moreover, the ionization also caused the increase in ion osmotic pressure. These two factors and the capillary wetting of interconnected open pores of SPHCc were thus responsible for a higher degree of swelling in the medium of pH range from 4.9 to 7.4.

In the design of oral delivery of peptide and protein drugs, the formulator must consider that the natural pH environment of gastrointestinal tract varies from acidic in the stomach to slightly alkaline in the intestine. Swelling of the SPHCc in the stomach was minimal and thus the drug release was also minimal, which avoided the degradation of the peptide and protein drugs by the low pH of gastric medium. The extent of swelling increased as the SPHCc passed down the intestinal tract due to increase in pH, and the drug was thereby completely released and absorbed from the intestine.

Release of insulin from SPHCc

The loading capacity of SPHCc with Carbopol/monomer ratio of 0.072 was (4.78 ± 0.13)%. Insulin loading into the gel carriers depended on the mesh size and the hydrophilicity of their network.[18] The average pore size of the SPHCc was larger than 70 μm and insulin could easily diffuse into the polymer network. Larger swelling ratio of the SPHCc due to the porous structure and the highly hydrophilic poly (AA-*co*-AM) as well as Carbopol in the SPHCc contributed to the increased insulin loading.

The cumulative amount of insulin released from SPHCc with Carbopol/monomer ratio of 0.072 was shown in Figure 5. More than 90% of the insulin was released within 30 min and the remaining drug was almost released up to the end of experiment (1 h). SPHCc with Carbopol/monomer ratio of 0.072 was able to swell quickly because of its highly porous structure, so the release rate of insulin from SPHCc was high.

Assessment of toxicity of SPHCc on the jejunum mucosal membrane

The histology of typical cross section of the jejunum was shown in Figure 6. No damage of mucosal membrane was noted based on Figure 6(A) and the villi were intact with light microscopic examinations when no SPHCc was applied to the lumen (control group). It could be inferred from Figure 6(B) that the villi could still remain intact after SPHCc had passed through the intestine. However, a few cells were detached from the tip of the villi. After 48 h, the villi rejuvenated to be intact and no detachment of cell on the top of the villi was observed [Fig. 6(C)].

When the SPHCc were used as carriers for oral drug delivery, it would adhere to the mucosal layer of the intestine for a period of time. It was essential that the polymer displayed low tissue toxicity. When food, drugs, or additives were applied in the lumen, slight alterations would be observed such as

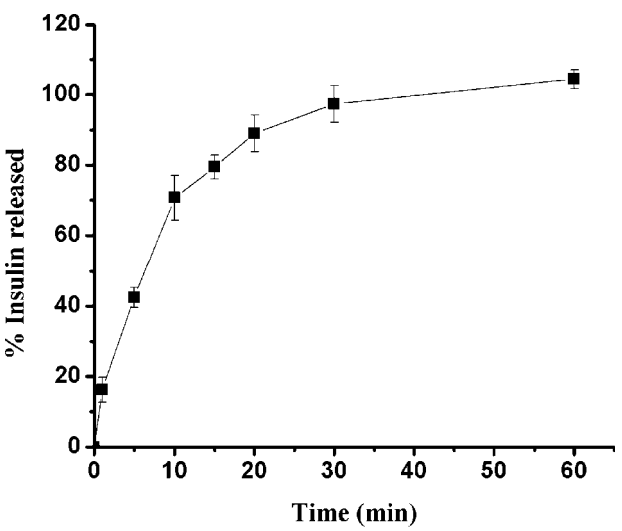

Figure 5 *In vitro* cumulative release of insulin from SPHCc with Carbopol/monomer ratio of 0.072. The release medium was 0.01*M* PBS at pH 7.4.

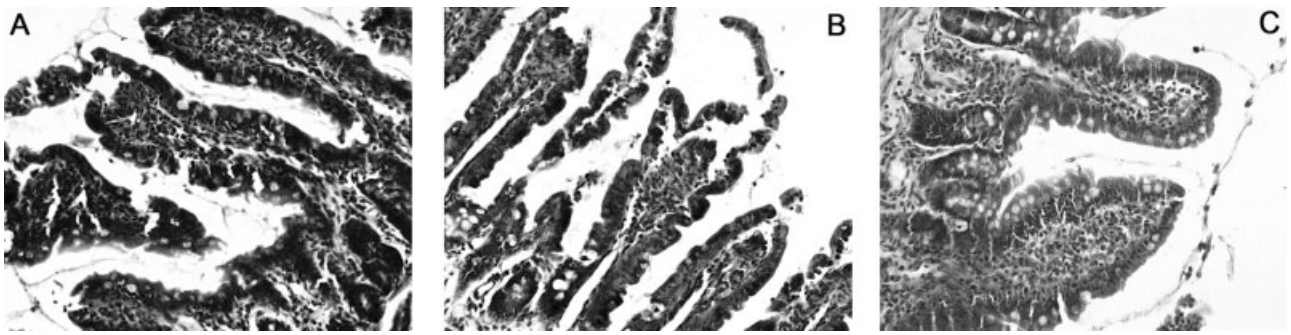

Figure 6 Light micrographs of typical cross sections of the jejunal mucosal membranes (magnification: ×200): (A) control group, (B) test group, (C) refresh group. In this experiment, the SPHCc with Carbopol/monomer ratio of 0.072 was used.

temporary deformation of the villi or some detachment of the cells from the tip of the villi.[19] In the case of test groups, a few cells at the tip of the villi were detached because of mechanical pressure of the fully swollen hydrogel on the intestinal mucosal membrane as well as mucoadhesion resulting from the carboxylic groups in the structure of SPHCc. However, these detachments were not significant when compared with control groups. It could be concluded that the villi in the jejunum lumen and at the mucosal surfaces were intact. It had been reported that the turnover time for the mucus was probably no longer than a couple of hours in the intestine.[20,21] After application of the SPHCc, the intestinal villi regained intactness due to quick regeneration of these detached cells, which demonstrated safety of SPHCc for mucosal application.

Cytotoxicity studies

In the presence of SPHCc, the number of the Caco-2 cells stained with trypan blue was less than 5% of the total monolayer. Compared with controls, no significant difference in cell viability was detected. Therefore, no cell damage occurred when the SPHCc were applied to the Caco-2 cell monolayers.

After incubation with SPHCc polymer for 4 h, the Caco-2 cell monolayers also excluded the propidium iodide and showed no differences with the control group that was applied only with MRS (Fig. 7). Therefore, it could be inferred that no cell damage was brought out and the Caco-2 cell monolayers remained intact meanwhile.

Cytotoxicity of a polymer can be evaluated *in vitro* by incubating polymer samples for prolonged periods in the presence of suitable target cells. If the polymer is cytotoxic, the target cells will be killed and the number of surviving cells will be in inverse proportion to its cytotoxicity. The biocompatibility of SPHCc was evaluated in the Caco-2 cell monolayers generally known as an *in vitro* human intestinal absorption surrogate.[22,23] Two different cytotoxicity tests, including trypan blue exclusion assay and propidium iodide nucleic staining, were used to estimate the cytotoxicity of the SPHCc. Trypan blue dye exclusion assay is the most commonly used method. The assay relies on the alteration in the membrane integrity as determined by the accumulation of the dye in dead cells.[24] In this study, it was observed that the cell membrane remained intact after application of the SPHCc, which could be confirmed by the high dye exclusion using light microscopy. Further evaluation was obtained with propidium iodide staining using CLSM when light microscopy failed to exhibit a clear distinction between dead and living cells. Propidium iodide is one of the most widely used fluorochromes for exclusion of dead or damaged cells. This method relies upon the fact that the membrane-impermeable propidium iodide possesses double positive charge at neutral pH, which could then bind to nucleic acids and become highly fluorescent that is easy to be observed. Dead cells would exhibit a very bright red fluorescence as they rapidly equilibrate with the propidium iodide.[25] In this investigation, much fewer dead cells with red fluorescence after application of the SPHCc were observed when compared with control groups, which

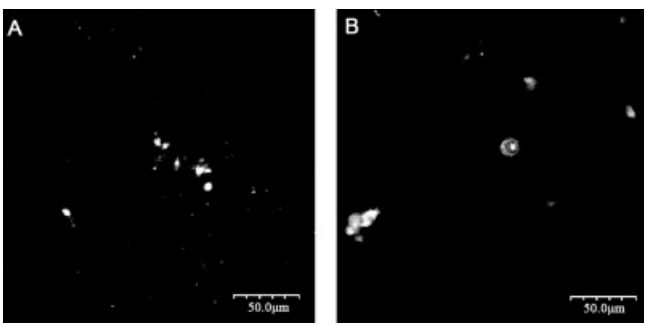

Figure 7 CLSM micrographs of Caco-2 cell monolayers using propidium iodide as dye: (A) cells incubated with MRS solution, (B) cells incubated with SPHCc. In this experiment, the SPHCc with Carbopol/monomer ratio of 0.072 was used.

indicated that the Caco-2 monolayers appeared to be viable after application of SPHCc for 4 h.

As a whole, neither of the assays gave any alarming evidence of cytotoxicity, and thus the SPHCc were considered to be relatively nontoxic after exposure to the cells for a short period of time. The decreased biocompatibility of polymer was correlated with the breakdown of the polymer and the accumulation of degradation products. SPH and Carbopol in the structure of the SPHCc had high crosslinking degree and molecular weight, indicating that SPHCc were difficult to be degraded up to several hours. This partially resulted in the good biocompatibility of the SPHCc.

CONCLUSIONS

In the present study, the SPHCc with superporous structure were synthesized through free radical copolymerization. The SPHCc had fast swelling rate and high swelling ratio because of the high porosity and interconnection among some of the pores within the polymer that approved by the SEM images. The swelling behavior of the SPHCc could be modulated by varying the amount of Carbopol, and it showed the maximum swelling ratio in the pH range of the intestinal tract (4.9–7.4). An increase in the ionic strength of the swelling medium from 0.001*M* to 1*M* resulted in a decrease in the degree of swelling. Insulin release was rapid from the SPHCc in neutral medium, indicating the potential for drug release in the intestine. Histological results revealed that almost no damage was brought about to the jejunum mucosa membrane of the rat after application of the SPHCc. Trypan blue and propidium iodide tests showed similar results that no significant cell damage was observed. The fast swelling, high swelling ratio, pH-sensitivity, and biocompatibility of the SPHCc were applicable in peroral delivery of peptide and protein drugs. The potentiality of SPHCc for the absorption enhancement of hydrophilic macromolecules and the design of a novel peroral peptide delivery system based on SPHCc would be further investigated.

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