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European Journal of Pharmaceutics and Biopharmaceutics 53 (2002) 161–166

European Journal of Pharmaceutics and Biopharmaceutics

www.elsevier.com/locate/ejphabio

Research paper

Evaluation of superporous hydrogel (SPH) and SPH composite in porcine intestine ex-vivo: assessment of drug transport, morphology effect, and mechanical fixation to intestinal wall

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Received 12 June 2001; accepted in revised form 25 September 2001

Abstract

The objective of this study was to investigate the potential of superporous hydrogel (SPH) and SPH composite (SPHC) polymers to enhance the transport of *N*-α-benzoyl-L-arginine ethylester (BAEE) and fluorescein isothiocyanate-dextran 4400 (FD4) across porcine intestinal epithelium ex-vivo, and to study any possible morphological damage to the epithelium by applying these polymers. In addition, the ability of these polymers to attach to the gut wall by mechanical pressure was examined by using a specifically designed centrifuge model. The transport of BAEE and FD4 across the intestinal mucosa was enhanced 2- to 3-fold by applying SPHC polymer in comparison to negative control. No significant morphological damage was observed by applying these polymers inside the intestinal lumen. Moreover, the SPH and SPHC polymers were able to attach mechanically to the intestinal wall by swelling and did not move in the intestinal lumen even when a horizontal force of 13 gms^{-2} was applied. In conclusion, these polymers are appropriate vehicles for enhancing the intestinal absorption of peptide and protein drugs.

Keywords: Superporous hydrogels; Superporous hydrogel composite; Intestinal drug transport; Peptide drug; Mechanical fixation; Morphology of porcine intestine

1. Introduction

The low bioavailability after mucosal administration of high molecular weight drugs, such as peptides and proteins, limits their therapeutical application. Nevertheless, mucosal administration of these macromolecules (especially peroral administration) attracts the attention of both academia and industries because of several factors, such as convenience and good patient compliance, less sophisticated procedures for preparation and less need of expertise for the administration [1–3]. There are several barriers responsible for the low bioavailability of these hydrophilic macromolecules, such as enzymatic degradation during absorption, the barrier function of the intestinal epithelium and hepatic first-pass effect. Among these barriers, the intestinal epithelium constitutes the major barrier for the absorption of perorally administered peptides and proteins [4]. This epithelium is composed of border cells, mucus-forming goblet cells, Paneth granular cells at the base of the Leiberkühn crypts, and basal granular cells. Of particular interest for drug absorption are the border cells, whose number far exceeds that of the other types of cells. They are high columnar epithelial cells joined at their apical surface by tight junctions. These tight junctions are formed by proteins, including occludin and claudin-1 and -2 [5,6]. Tight junctions are responsible for the restriction of the passage of hydrophilic macromolecules. In general, hydrophilicity and hydrophobicity of the compounds, together with their molecular weight, play an important role in the route of absorption in the gastrointestinal tract. Hydrophobic and small molecules are transported across the epithelial cells (transcellularly), while hydrophilic and large molecules are transported between the epithelial cells where the tight junctions are located (paracellularly) [7–11]. Since peptides and proteins are hydrophilic macromolecules, their transport is mainly hindered by tight junctions. In order to overcome this barrier and open the tight junctions, numerous classes of synthetic and natural absorption enhancers have been used. Examples are chelating agents (e.g. EDTA) [12], surfactants (e.g.

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0939-6411/02/$ - see front matter
PII: S0939-6411(01)00222-3

polyoxyethylene ethers) [13], fatty acids (e.g. sodium caprate) [14], bile salts (e.g. dihydroxy bile salts) [15], salicylates (e.g. sodium-5-methoxy salicylate) [16], acyl carnitines (e.g. palmitoyl carnitine) [17], polyacrylates (e.g. carbomer) [10] and chitosan derivatives (e.g. *N*-trimethyl chitosan chloride) [18]. Different mechanisms of action have been suggested for each one of these enhancers, which are based on a type of physicochemical interaction, either with the proteins of the tight junction structure, or with environmental components (such as extracellular calcium).

Recently, a novel delivery system was developed in our laboratory [19], using superporous hydrogel (SPH) polymers as drug carriers and enhancers which has another mechanism of action. SPH and SPH composite (SPHC) polymers are a new generation of hydrogels. When these polymers are delivered into the intestine, they absorb the intestinal fluids and swell very quickly due to their highly porous structure [20]. Therefore, they attach to the intestinal epithelium mechanically and open tight junctions by mechanical pressure [19].

In this study, the capability of SPH and SPHC polymers were evaluated for their potential to enhance the transport of two hydrophilic model compounds of different molecular weight, *N*-α-benzoyl-L-arginine ethylester (BAEE) and fluorescein isothiocyanate-dextran 4400 (FD4), across porcine intestine ex-vivo. Moreover, the possible damaging effects of SPH and SPHC polymers on the intestinal epithelium, and the ability of these polymers for mechanical fixation in a segment of porcine intestine were investigated.

2. Materials and methods

2.1. Materials

BAEE (*N*-α-benzoyl-L-arginine ethylester HCl, MW 342.8 Da) and FD4 (fluorescein isothiocyanate-dextran, MW 4400 Da) were purchased from Sigma Chemical (Bornem, Belgium). Superporous hydrogel (SPH) and SPH composite (SPHC) were synthesized in our laboratory as described previously [20]. Carbomer (C934P) was donated by BF Goodrich (Cleveland, Ohio, USA). Krebs–Henseleit solution (adjusted to pH 7.2) and 0.9% physiological saline solution containing 0.33% glucose (adjusted to pH 7.2) were prepared freshly and used for the transport studies. All other chemicals were at least of reagent grade.

2.2. Transport of BAEE and FD4 across porcine intestine

Freshly isolated pieces of porcine intestine (jejunum) were obtained from a local slaughter house on the day of the experiment. The intestine was washed with physiological saline (0.9% NaCl) in order to remove faeces and food residues, and then transported to the laboratory in physiological saline containing 0.33% glucose in an ice-cooled box. The intestine was cut into pieces of approximately 20 cm each and kept immersed in physiological saline containing 0.33% glucose prior to transport experiments.

The day before the experiment, a 1.0 mg/ml solution of either BAEE or FD4 was prepared. SPHC polymers were loaded with BAEE or FD4 by immersing 600 mg of the polymers in 10 ml of BAEE or FD4 solution in order to allow complete polymer swelling. Then the polymers were dried in a vacuum oven at 60°C for one day. These dried polymers containing either 10 mg BAEE or 10 mg FD4 were inserted into 20 cm pieces of intestine which were tied up initially at one end with a silk thread. After placing the polymers in the intestine, 20 ml of physiological saline containing 0.33% glucose (pH 7.2) was added into the intestine to allow the polymers to swell and BAEE or FD4 was released from these polymers. The other open end of intestine was also tied with a silk thread to form a sac. In case of negative control, only 10 ml of BAEE or FD4 solution (1.0 mg/ml) was placed in the intestinal sac. The filled sacs were hooked onto a stainless steel support to keep it stretched and immersed in a container filled with 250 ml of oxygenated physiological saline containing 0.33% (w/v) glucose. This solution was mixed very smoothly with a turning paddle (30 rpm) and the container was placed in a water bath at 37 ± 1°C. Samples of 800 μl were withdrawn from the serosal side at time intervals of 0, 15, 30, 45, 60, 75, 90, 120 and 150 min. All the experiments were performed 3–5 times.

2.3. HPLC analysis of BAEE and FD4

BAEE analysis was performed by HPLC-UV_{253}. The stationary phase was a Lichrosorb 7 RP 18 column 100 × 3.0 mm (Chrompack, Middelburg, The Netherlands) accompanied with a RP 18 precolumn. Gradient elution was performed with the following two mobile phases: eluent (A) 90% 0.01 M ammonium acetate buffer pH 4.2 and 10% acetonitrile, and eluent (B) 50% 0.01 M ammonium acetate buffer pH 4.2 and 50% acetonitrile. Gradient elution was performed as follows: 0–3 min 92%A/8%B, with a flow rate of 0.75 ml/min; 3–8 min 50%A/50%B, with a flow rate of 0.75 ml/min; 8–9 min 50%A/50%B, with a flow rate of 0.75 ml/min; 9–11 min 92%A/8%B, with a flow rate of 0.75 ml/min; 11–13 min 92%A/8%B, with a flow rate of 1.05 ml/min; 13–14 min 92%A/8%B, with a flow rate of 0.75 ml/min. The injection volume was 20 μl and retention time of BAEE was 7.0 min. The detection limit for BAEE was 40 ng/ml.

For FD4, the samples were analyzed using HPLC equipped with a size exclusion column, Suprema 30 (Polymer Standard Service, Mainz, Germany). Isocratic elution was performed with 0.05 M ammonium acetate buffer (pH 9.0) as the mobile phase. The injection volume was 100 μl and the flow rate was adjusted to 1.0 ml/min. FD4 was detected with a fluorescence detector at an excitation wavelength of 488 nm and emission wavelength of 520 nm. The

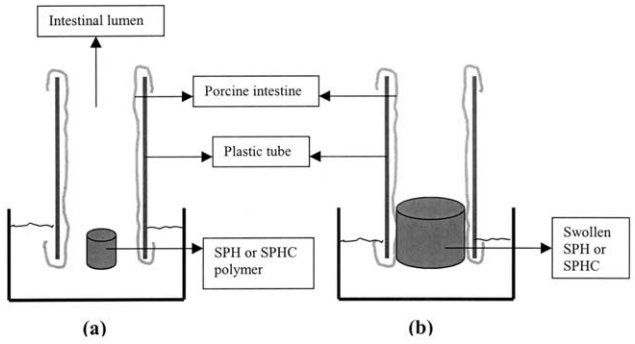

Fig. 1. Schematic picture of the set-up for mechanical fixation studies; (a) placing of SPH or SPHC polymers in the water container while they were inserted in the intestine; (b) swelling of SPH or SPHC polymers.

retention time of FD4 was 7.5 min. The detection limit for FD4 was 25 ng/ml.

2.4. Morphological studies

Before and after performing the transport studies, some segments of the intestine were cut perpendicularly to the lumen axis in pieces of 1 cm. These pieces were kept in 10% formaline until the next day for morphological examination. In order to obtain an accurate view of the intestine under the microscope, the pieces of intestine were fixated in paraffin at 4°C and horizontal cross sections of 3 μm were cut using a microtome (Leica, Rijswijk, The Netherlands). These cross sections were placed on glass slides and dried for 10 min in an oven at 56°C. Thereafter, the sections were stained using a tissue stainer machine (Medite, Burgdorf, Germany) as follows: the slides were placed successively in each one of the following solutions for 90 s: xylene, ethanol 100%, ethanol 70%, ethanol 50%, distilled water, haematoxylin 0.2%, tap water, eosin 1%, tap water, ethanol 100% and xylene. At the end of process, a cover glass was placed over the stained cross sections of the intestine, and visualization was carried out by light microscopy (Axioskop-type microscope; Zeiss, Weesp, The Netherlands).

2.5. Mechanical attachment of SPH and SPHC polymers in porcine intestine

In order to investigate if SPH and SPHC polymers are able to attach mechanically to the gut wall for a certain period of time, a particular experimental set-up was designed. A special centrifuge-like apparatus, able to rotate from 10 to 150 rpm was utilized as a model to introduce a horizontal force to the polymer which was previously placed inside a piece of porcine intestine (jejunum). The lateral movement of the polymer in the intestine as a result of the centrifugation force was considered to show the capability of these polymers to mechanically attach to intestinal surfaces. In this experiment, a 25 cm segment of the jejunal part of intestine was placed inside a plastic tube with the length of 20 cm and internal diameter of 2.7 cm (see Fig. 1). The two ends of the intestine overlapped the outer surfaces of plastic tube and were fixed with a silk thread to the tube. This prevented the intestine from sliding during the centrifugation process. Then a piece of polymer (600 mg) was placed in one side of the intestine and the whole system was immersed vertically in a bowl of water in which the polymer could swell completely (shown in Fig. 1). After swelling of the polymer, the system was placed on the plate of the centrifuge-like apparatus, which was turning horizontally. In each experiment two tubes containing intestine and polymer were placed at opposite ends and the apparatus was started to spin at exceeding frequency (20 rpm to 100 rpm) for 30 min. Additionally, 10 ml of 0.5% (w/v) Carbopol® 934P gel, which shows high mucoadhesive characteristics, was used as a positive control. Erythrosin was added to the Carbopol gel, in order to detect the localization of C934P solution in the intestine after centrifugation.

In order to calculate the amount of horizontal force, which was applied to the polymer, the following equation was used:

$$F = m \times V^2/r$$

in which F is the horizontal force in the centrifuge (N or $kg.ms^{-2}$), m is weight of polymer (kg), r is the radius of centrifuge plate (m) and V is the velocity of centrifuge plate.

In these experiments V was determined to be 2.084 ms^{-1}, resulting in F values of 13.03 gms^{-2}.

3. Results

3.1. BAEE and FD4 Transport studies

The cumulative transport of BAEE across porcine intestine is shown in Fig. 2. In the absence of any polymer, BAEE was transported up to 9.9 ± 0.8% of the applied dose after 120 min. On the other hand, when SPHC polymer was applied into the intestine, BAEE was transported up to 18.1 ± 1.3%, resulting in a 1.8-fold enhancement compared

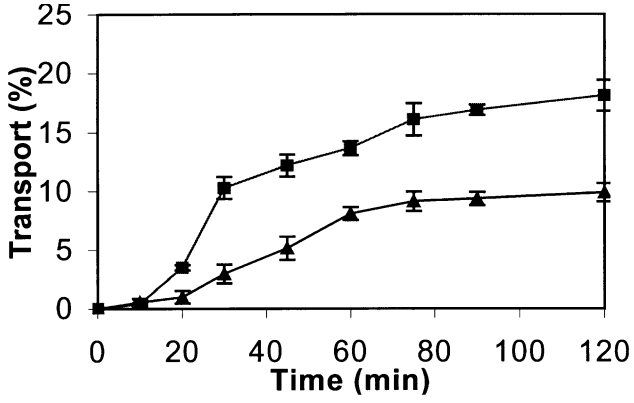

Fig. 2. Transport of BAEE across porcine intestine ex-vivo. Data are expressed as a mean ± SD of 3–5 experiments; (■) SPHC polymer, (▲) negative control.

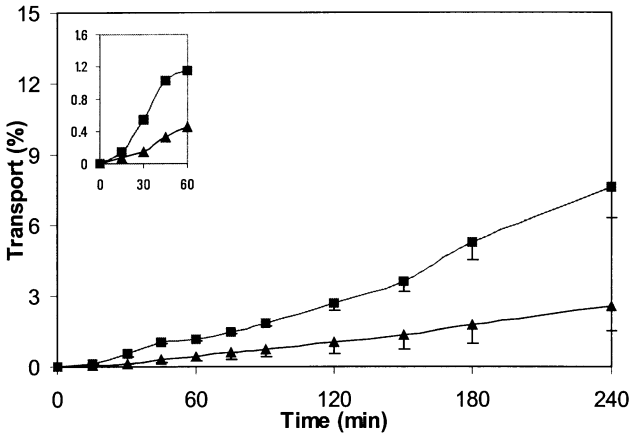

Fig. 3. Transport of FD4 across porcine intestine ex-vivo. Data are expressed as a mean ± SD of 3–5 experiments; (■) SPHC polymer, (▲) negative control.

to the control. Similar results were observed when Krebs–Henseleit solution was used as an experimental medium.

Fig. 3 depicts the cumulative transport of FD4 across the intestinal epithelium. In the presence of SPHC polymer, FD4 was transported up to 7.6 ± 1.2% of the initial dose after 240 min, thus leading to a 2.9-fold enhancement compared to the control (2.6 ± 1.0% at 240 min). Although FD4 was transported at a low rate in the first 60 min for both SPHC polymer and control, it showed the same trend as for BAEE (the first 60 min of FD4 transport is enlarged in Fig. 3). Moreover, the apparent permeability coefficient (P_{app}) values of both BAEE and FD4 were calculated according to approximate surface area in the intestinal lumen and summarized in Table 1. These results showed the same trend in enhancing BAEE and FD4 transport by using SPHC polymer in comparison to control.

3.2. Morphological studies

The light microscopic pictures of jejunal part of the intestine are shown in Fig. 4. A typical cross section of the jejunum before the start of experiment is depicted in Fig. 4A, when the tissue sample was only kept in physiological saline. In this figure the villi are shown by arrow (a) and the epithelial cytoplasm containing rod-shaped mitochondria by arrow (b). It is clear that these villi in the intestinal lumen and at the mucosal surfaces are intact. When food, drugs or additives are applied in the lumen, the morphological structure of the villi should remain intact. Due to absorption processes or physical influences of these compounds on mucosal surfaces, only some slight changes may be observed such as temporary deformation of villi or some detachment of the cells from the tip of the villi [5]. Fig.

Table 1
Apparent permeability coefficients (P_{app}) for BAEE and FD4 across ex-vivo isolated porcine intestine. Data are expressed as a mean ± SD of 3–5 experiments

Substance	Application	$P_{app} \times 10^{-7}$ (cm/s)
BAEE	Control	7.62 ± 0.61
	SPHC	13.98 ± 1.0
FD4	Control	1.45 ± 0.19
	SPHC	2.93 ± 0.96

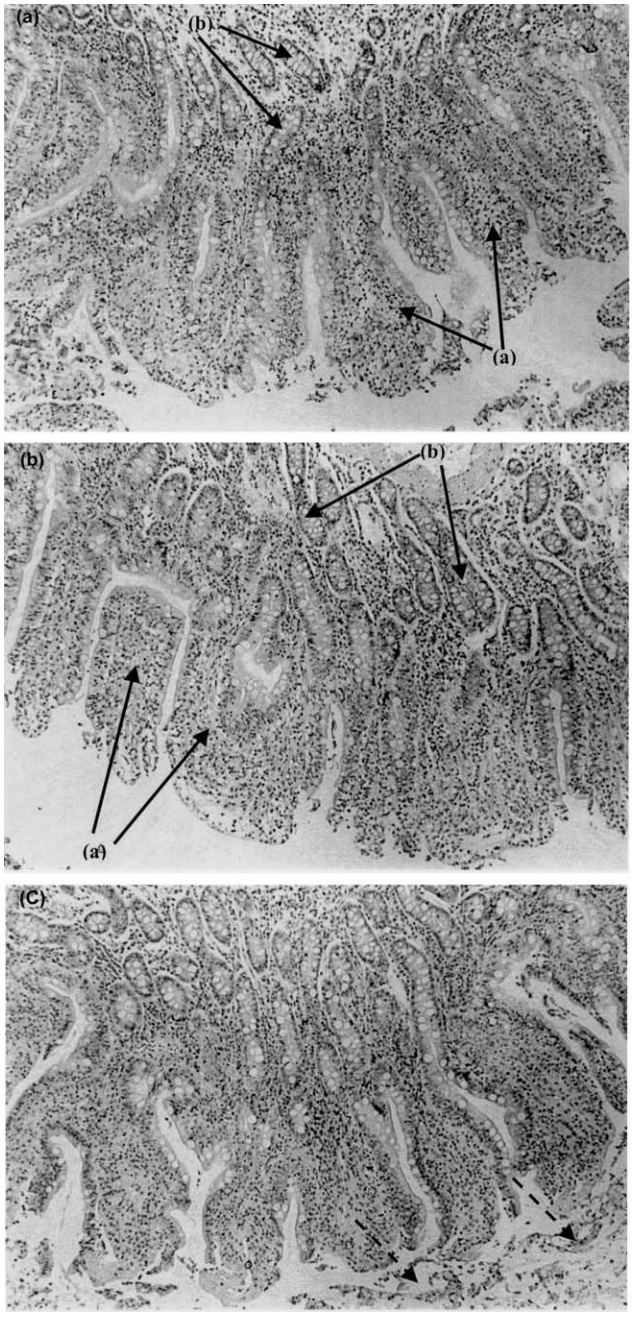

Fig. 4. Light microscopic pictures of typical cross sections of porcine jejunum (magnification: 100×): (A) cross section before starting the transport experiment while the intestine was kept in physiological saline (arrow (a) shows the villi and arrow (b) the epithelium cytoplasm containing rod-shaped mitochondria). (B) cross section after a 4 h transport experiment of FD4 in absence of any polymer (control group). (C) cross section after 4 h transport of FD4 in the presence of SPHC polymer (dashed arrows show some detachment of cells from top of the villi).

4B depicts the mucosal surfaces of the intestinal lumen after an FD4 transport experiment for 240 min, while no polymer has been inserted into the lumen (control group). Only a small change in the shape of the villi was observed, due to the FD4 solution. When SPHC polymer was applied into the intestine, the villi remained intact but, due to swelling of the polymer and subsequent attachment of the polymer to the intestinal epithelium, a very small amount of the cells was detached from the tip of villi (showed by dashed-arrow) (Fig. 4C). The same results were achieved by applying SPH instead of SPHC, and also when the experiments were performed in Krebs–Henseleit solution (not shown).

3.3. Mechanical fixation studies

Using the centrifuge model with the applied horizontal force of around 13 gms^{-2} for 30 min, no movement was observed for either SPH or SPHC polymers within the porcine intestine. On the other hand, the 0.5% (w/v) jelly solution of Carbopol® 934P was moved along the whole length of the porcine intestine and then expelled from the other side of the intestine. Thus, contrary to the well-known mucoadhesive polymer Carbopol® 934P, SPH and SPHC polymers showed improved retentive properties by mechanical fixation at the intestinal wall.

4. Discussion

Our recently developed novel peroral delivery system for peptide drugs consists of two components: (i) the conveyor system which is made of SPH and SPH composite; (ii) the core which contains the peptide drug [19]. The conveyor system has actually two parts: a body made of SPHC and a cap made of SPH. Since the body of the conveyor system is the responsible part for drug transport and mechanical pressure [19], SPHC was mainly evaluated in the present study. Transport studies of BAEE and FD4 as hydrophilic model molecules across the porcine intestine revealed that SPHC was able to enhance the transport of both compounds in comparison to the negative control. However, the total amount of transport for FD4 was less than BAEE, which is due to higher molecular weight of FD4 (4400 Da) in comparison to BAEE (342.8 Da). Since BAEE and FD4 are hydrophilic molecules, their transport across the intestinal epithelium is via the paracellular pathway. This pathway is the main molecular weight dependent passage route in the intestinal epithelium for hydrophilic molecules, and the larger the hydrophilic molecules, the lower its transport across the paracelluar pathway [11,21,22].

The potentiality of SPHC polymer for the observed transport enhancement of BAEE and FD4 can be due to opening of the tight junctions by mechanical pressure and subsequent water influx from the intestinal epithelium into the lumen. This mechanical pressure will embrace the columnar epithelial cells and make a gap between the cells where the tight junctions are located, thereby causing the opening of these junctions. On the other hand, water influx from the interstitial space between the cells into the lumen will force the intestinal cells to maintain the homeostatic pressure; therefore, the cells need to compensate this water loss by opening of the tight junctions in order to facilitate the fast uptake of water molecules and maintaining the homeostasis of the intestinal cells.

From these BAEE and FD4 transport studies, it becomes also clear that in case of polymer application, a rapid increase in the amount of drug transport between 20 and 60 min will be obtained, which is quite appropriate for peroral peptide drug delivery. Actually, in peroral peptide drug delivery it is necessary to have a burst release of drug after a specific lag time, during which lag time the luminal proteolytic enzymes should be deactivated and the tight junctions should be opened. After such a lag time the intestinal environment will be ready for burst release of the drug and absorption into the blood [19].

Changing the buffer medium did not influence the transport characteristics, since in both physiological saline and Krebs–Henseleit solution an approximately 2-fold increase in the intestinal BAEE transport was observed for SPHC in comparison to control group. However, as in these experiments the porcine tissue was kept in physiological saline solution prior to experiment, it is advisable to use physiological saline solution as buffer medium in order to apply minimal stress to the tissue. (And also to keep the ex-vivo experimental conditions close to the physiological condition and constant during the experiments.)

From the present morphological studies, it was concluded that the intestinal villi remained intact for at least 4 h after application of FD4 solution with or without SPHC polymer. In case of the test (i.e. polymer) groups, some cells at the top of the villi (showed by dash arrow in Fig. 4C) were detached due to mechanical insertion of the polymers into the intestinal lumen, and also probably due to swelling of the polymers. However, these detachments were considered to be negligible when compared to Fig. 4A (intestinal tissue before the experiment), and it has also been reported that these cells are able to regenerate very quickly [5,21].

Although much information is available on the transit rates of dosage forms in the gastro-intestinal (GI) tract, there is still limited knowledge available about the movement force in the intestine [23,24]. The peristaltic movement in the intestine is theoretically determined by two forces: horizontal force (in the direction of stomach to rectum) and vertical force (contraction force). Since the horizontal force is more important for the movement of dosage forms along the intestine, this force was applied to these examined polymers in the intestine. The results showed that even with a 13 gms^{-2} horizontal force, both SPH and SPHC attached mechanically to the intestinal wall and keep the dosage form for specific period of time. In contrast to SPH and SPHC polymers, Carbopol® 934P which is known as a potent mucoadhesive polymer [10,25], did not attach strongly enough to the intestine

during the course of the experiment, but moved along the whole piece of intestine. Nevertheless, the issue of mechanical fixation should be further investigated in vivo, for example using γ-scintigraphy.

It has been reported that the GI tract imparts a mechanically destructive force (so-called contraction force) of approximately 1.8 N to various dosage forms [23,26]. This contraction force is strong enough after complete swelling of the SPH and SPHC polymer to both break down the swollen gels and to transport them together with the food content of the gut to the anus. Faeces of pigs fed with these polymers did not allow differentiation between food residues and polymeric residues.

5. Conclusion

The present studies demonstrated that SPHC polymer improves the transport of both BAEE and FD4 as hydrophilic molecules across the porcine intestinal epithelium via the paracellular pathway. No significant morphological damage occurs due to application of the SPHC polymer. Moreover, the mechanical fixation studies showed that SPH and SPHC polymers are able to attach to the intestinal epithelium by mechanical pressure and do not move along the intestine using a horizontally applied force of 13 gms^{-2}.

Acknowledgements

The financial support of Aventis Research & Technology (Frankfurt, Germany) for performing these studies is kindly appreciated.

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