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Release behaviour and biocompatibility of drug-loaded pH sensitive particles

Oya Sipahigil [a], Ayla Gürsoy [a,*], Fulya Çakalağaoğlu [b], İmer Okar [c]

[a] *Marmara University, Faculty of Pharmacy, Department of Pharmaceutical Technology, 34668 Istanbul, Turkey*
[b] *Marmara University, Faculty of Medicine, Department of Pathology, 34668 Istanbul, Turkey*
[c] *Marmara University, Faculty of Medicine, Department of Basic Medical Sciences, 34668 Istanbul, Turkey*

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Abstract

The purpose of this work was to investigate the physical properties of drug-loaded poly(methacrylic acid-g-ethylene glycol) {P(MAA-g-EG)} particles, their biocompatibility with the gastrointestinal tract of rats and also the effects of these particles on the tight junctions of the rat intestinal epithelium. Model drugs such as diltiazem HCl, diclofenac Na, ciprofloxacin HCl and isoniazid were used in this study. P(MAA-g-EG) particles were prepared by free radical solution polymerization of methacrylic acid (MAA) and poly(ethylene glycol) (PEG). The loading efficiency of the model drugs in the particles and in vitro release profiles were investigated in pH 7.4 phosphate buffer and in gradually pH changing buffers (pH 1.2, 5.8, 6.8 and 7.4). The stability of free particles and drug-loaded particles was established by Fourier transform infrared spectroscopy (FT-IR) and differential scanning calorimetry (DSC). In conclusion, P(MAA-g-EG) particles controlled the release rate of small molecular weight model drugs according to the pH of the medium. Stability of those particles loaded with drugs did not change in accelerated stability conditions. Histopathological results indicated that loading drugs to the particles prevented cell and tissue damage after 20 h. Free particles showed no change of tight junctions after 2 and 10 h. The results of TEM showed that increasing the amount of P(MAA-g-EG) particles from 100 to 385 mg clearly opened the tight junction, but with serious epithelial cell disruption.

Keywords: P(MAA-g-EG) particles; Histopathology; Tight junction; Diltiazem HCl; Diclofenac Na; Ciprofloxacin HCl; Isoniazid

1. Introduction

Hydrogels are used as carriers for the delivery of drugs, peptides and proteins, as targeting agents for site-specific delivery, or as components for preparation of protein or enzyme conjugates (Peppas, 1997, 1999; Nakamura et al., 2003). Copolymer networks of poly(methacrylic acid) grafted with poly(ethylene glycol) henceforth designated as P(MAA-g-EG), exhibit pH swelling due to the reversible formation/dissociation of interpolymer complexes. In the acidic environment of the stomach, the gels are in a complexed state. As the polymer passes from the stomach into the intestine, the environmental pH increases above the transition pK_a of the gel. Then, the complexes dissociate and the network mesh size rapidly increases leading to the release of the drug (Peppas et al., 2001; Donini et al., 2002).

In this study, diltiazem HCl, diclofenac Na, ciprofloxacin HCl and isoniazid were chosen as model drugs since all were small molecules having different ionic properties at pH 7.4 and all commonly cause gastric irritation (Dukes, 1988).

P(MAA-g-EG) polymers are molecularly designed to contain poly(ethylene glycol) (PEG) chains promoting mucosal adhesion and poly(methacrylic acid) (PMAA) backbones with carboxylic pendant groups (–COOH), which can act as calcium binders leading to epithelial cell junction opening (Madsen and Peppas, 1999). ATR–FT-IR, atomic absorption spectroscopy and Caco-2 cell studies indicated that P(MAA-g-EG) particles could bind to calcium ions which increase the paracellular permeability of epithelial cell monolayers by opening the tight junctions (Madsen and Peppas, 1999; Sipahigil et al., 2002; Torres-Lugo et al., 2002; Foss and Peppas, 2004). These particles are investigated as potential carriers for protein drugs such as insulin and calcitonin (Lowman et al., 1999; Torres-Lugo and Peppas, 1999). Cell culture methods, also known as cytotoxicity tests, can be used to evaluate the toxicity of the hydrogels. The histological analysis of poly[*N*-(2-hydroxy ethyl)-DL-aspartamide]

* Corresponding author. Tel.: +90 216 414 29 65; fax: +90 216 345 29 52.
E-mail address: azgur@superonline.com (A. Gürsoy).

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(PHEA) showed no gross lesion of the stomach or duodenum when observed 24 h. after their oral administration to rats (Giammona et al., 1997).

In this study P(MAA-g-EG) particles were used as carriers of small molecular drugs and also their biocompatibility with the gastrointestinal tract of the rats and their in vivo effect on tight junctions and cells of gastrointestinal system were investigated.

The objectives of this study were the following:

- To evaluate the physical characteristics of P(MAA-g-EG) particles such as loading efficiency, release rates and the stability.
- To characterize the histopathology of the ileum after free particles or pure drugs and drug-loaded particles were administered orally to rats.
- To examine the effect of drugs and particles on epithelial tight junctions in vivo by TEM (Jeol, 1200 EX).

2. Materials and methods

2.1. Materials

Methacrylic acid (MAA) and poly(ethylene glycol) monomethacrylate (PEGMA) were purchased from Polysciences Inc. (Warrington, PA). Tetra(ethylene glycol) dimethacrylate (TEGDMA) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Irgacure 184® (1-hydroxy cyclohexyl phenyl ketone) was purchased from Ciba-Geigy Corp. (Hawthorne, NY). Diltiazem HCl, diclofenac Na, ciprofloxacin HCl and isoniazid were obtained respectively from İlsan İltaş A.Ş., Fako, Koçak and Deva, Turkey as a gift. All reagents used were of analytical grade.

2.2. Preparation of P(MAA-g-EG) particles and drug loading

Particles were prepared by free radical solution polymerization of methacrylic acid (MAA) and methoxy-terminated poly(ethylene glycol) monomethacrylate (PEGMA). The MAA was vacuum distilled at 54 °C/25 mmHg to remove the inhibitor, methoxyethylhydroquinone. PEGMA was used as received. The monomers were mixed in 1:1 molar ratio of MAA/EG repeating units with PEG of molecular weight 1000. The monomer mixture was diluted with a mixture of 50% (w/w) ethanol and deionized water. TEGDMA was added as the crosslinking agent in the amount of 75% of the total moles of the monomers. Irgacure 184® was used as the photoinitiator.

The solution was purged with nitrogen to remove oxygen, which acts as a free radical scavenger. The mixture was then poured between microscope slides (75 mm × 50 mm × 1 mm) (Fisher, Pittsburgh, PA) separated by teflon spacers with an approximate thickness of 1 mm. The monomer mixture in the microscope slides was then exposed to UV light. The films were washed for 7 days in deionized water (changed daily) to remove the solvent and any unreacted monomer. After the washing period, the films were dried under vacuum at 25 °C and pulverized into shapeless crushed particles and the size of the particles was determined using the standard sieves.

Drug loading was accomplished by swelling particles in pH 7.4 phosphate buffer solution (USP XXIV) containing drug (diltiazem HCl or diclofenac Na or ciprofloxacin HCl or isoniazid) by stirring mechanically on an oscillating plate for 24 h. After 0.1N HCl was added for the complexation, drug-loaded particles were filtered from membrane filter under vacuum and lyophilized (Leybold-Heraeus Lyovac GT2). Drug:particle ratios were (1:25) and (5:25).

2.3. Drug release from particles

Drug-loaded particles were placed within a dialysis bag (Cellulose membrane, Sigma Chemical Co., St. Louis, MO, USA) and immersed into a vessel containing the buffer solution. Drug release studies were carried out at 37 ± 0.5 °C under mechanical stirring at 120 rpm in different dissolution media using USP XXIV paddle method. pH 7.4 phosphate buffer solution (USP XXIV) was used as dissolution medium. The gradual pH change method was also utilized for dissolution studies. In this method, at 1.5 h intervals the pH of the dissolution media was changed from pH 1.2 to 5.8, 5.8 to 6.8 and 6.8 to 7.4 by adding 0.5 M NaOH. Drug amounts were determined spectrophotometrically.

2.4. Stability studies

Drug-loaded particles were stored in a stability chamber (Binder 240, Germany) at 40 ± 2 °C and 75 ± 5% relative humidity for 3 months in order to evaluate the effect of accelerated conditions on the release profiles of these systems. FT-IR and DSC analysis were also performed before and after the stability study. FT-IR spectra were obtained with a Nicholet Magna-550 spectrophotometer using KBr disc method. The DSC scans were recorded by a Mettler Toledo apparatus, model TSO 801 Ro, DSC 822. The measurements were made using an aluminium sample pan, in nitrogen atmosphere with a flow rate of 10 ml/min at a heating rate of 10 °C/min.

2.5. Laboratory animals

Wistar rats, 150–250 g, used for the histopathology and transmission electron microscopy studies, were fasted for 24 h before the treatment. Throughout the experiments the rats were housed in iron cages. Approval was obtained by the Ethics Committee of the Faculty of Medicine of Marmara University.

2.6. Histopathological study

After fasting for 24 h with free access to water, rats (n = 6 rats) were lightly anaesthetized with ether. Free particles (100 mg), drugs (0.2 mg diltiazem HCl, 0.4 mg diclofenac Na, 2 mg ciprofloxacin HCl, 1 mg isoniazid) and particles containing the same amount of drugs (drug-loaded particles) were administered to Wistar rats orally with a gavage, separately. Rats were killed after 2, 10 and 20 h for histopathological examination. The stomach, small intestine and colon of the rats were removed from the abdominal cavity and washed with 0.9% (w/v) saline. These tissues were processed routinely and embedded in paraf-

fin. Sections (5 μm) were stained with haematoxyline eosine (H&E). Tissues were fixed at least 24 h in phosphate buffered 10% formalin solution. Microscobic assessment by light microscopy was performed blind on coded slices. Histological damage was scored on a 0–3 scale as follows; 3: necrotic ulcers, 2: haemorrhage or non-necrotic ulcers, 1.5: mild haemorrhage, 1.0: erythema, 0.5: mild erythema, 0: no observable damage (Giammona et al., 1997). Histological damage index was obtained by calculating the mean score for each group.

2.7. Transmission electron microscopy (TEM)

After fasting for 24 h with free access to water, rats ($n = 6$ rats) were lightly anaesthetized with ether. Free particles (100 and 385 mg), drugs (0.2 mg diltiazem HCl, 0.4 mg diclofenac Na, 2 mg ciprofloxacin HCl, 1 mg isoniazid) and ciprofloxacin HCl loaded particles containing 2 mg ciprofloxacin HCl were administered to Wistar rats orally with a gavage, separately. The rats were anaesthetized and were sacrificed after 2 or 10 h. The small intestine was dissected between the pylorus and caecum. After removal of the intestinal content manually, the whole canal was cleared several times with phosphate buffer. After a perfusion with the fixative, the dissected specimens were immersed in the same fixative consisting of 2.5% gluteraldehyde and 4% paraformaldehyde mixture in phosphate buffer for 24 h. A post-fixation process with 1% osmium tetraoxide and 3% lanthanium nitrate in phosphate buffer was performed. The specimens were then dehydrated, cleared and embedded in epoxy resin (Epon 812). Semi thin sections and thin sections were made, stained with uranyl acetate and lead citrate and observed with Jeol transmission electron microscope. For all experimental groups the ileal region was investigated.

3. Results and discussion

3.1. Size and drug loading

Since particles were crushed, they were shapeless and only sieved particles having 180 μm diameter were used throughout the study.

Drug-loading percentage (w/w ± S.D.) of P(MAA-g-EG) particles were 2.09 ± 0.06, 1.79 ± 0.21, 0.93 ± 0.11 and 0.54 ± 0.05 mg for isoniazid, diltiazem HCl, diclofenac Na and ciprofloxacin HCl, respectively. Raising the initial drug concentration up to five times did not change the percentage of loaded drug amount. Among the drug-loaded particles, loading efficiencies of diltiazem HCl and isoniazid were comparatively higher than the loading efficiencies of diclofenac Na and ciprofloxacin HCl. This might be due to the ionic interaction between diltiazem HCl or isoniazid and the polymer because during the drug-loading process in pH 7.4 phosphate buffer, diltiazem HCl and isoniazid were cationic and the polymer was anionic. On the other hand, ciprofloxacin HCl being neutral and diclofenac Na being anionic in pH 7.4 resulted in low drug loading.

In a study of insulin loaded chitosan particles, high loading of insulin was also attributed to the electrostatic interaction between acidic insulin groups and amino groups of chitosan (Pan et al., 2000).

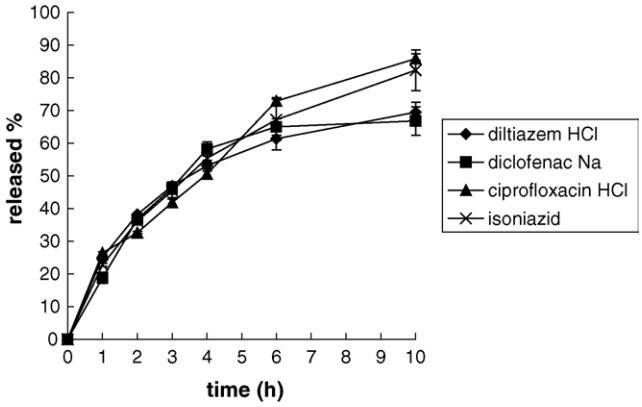

Fig. 1. In vitro release profiles of diltiazem HCl, diclofenac Na, ciprofloxacin HCl and isoniazid from drug-loaded P(MAA-g-EG) particles in pH 7.4 phosphate buffer solution (mean ± S.D.).

However factors other than ionic interaction could be relevant for the drug loading. In a study by Morishita et al. molecular weight, complex nature and shape of the drug molecules (insulin, theophylline, vancomycin and dextran) were found to be influencing factors for loading drugs to P(MAA-g-EG) microparticles. The low loading of theophylline (1.1 ± 0.5%) and vancomycin (14.5 ± 0.3%) was attributable to the fact that the small molecular weight drugs were "squeezed" out of the gel when they were washed with the acidic solution during the loading process, while insulin was sufficiently large to remain entrapped in the gel (Morishita et al., 2002).

3.2. Drug release

As shown in Fig. 1 69.47 ± 1.00% of diltiazem HCl, 66.74 ± 3.11% of diclofenac Na, 85.77 ± 7.64% of ciprofloxacin HCl and 82.27 ± 2.40% of isoniazid were released from P(MAA-g-EG) particles at the end of 10 h in pH 7.4 phosphate buffer solution.

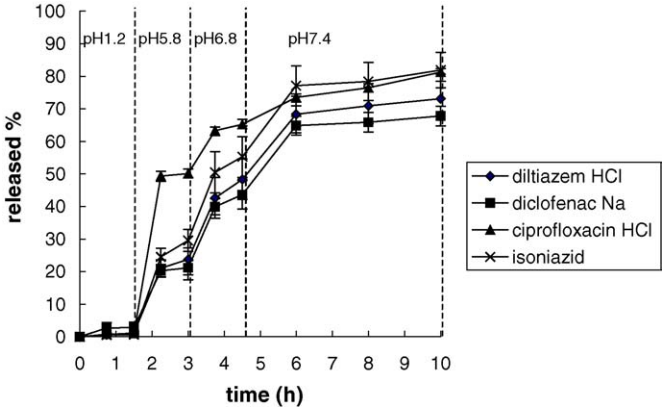

Fig. 2. In vitro release profiles of diltiazem HCl, diclofenac Na, ciprofloxacin HCl and isoniazid from drug-loaded P(MAA-g-EG) particles in gradually pH changing buffer solutions (pH 1.2, 5.8, 6.8 and 7.4) (mean ± S.D.).

Fig. 2 shows the in vitro release profile of the drugs from P(MAA-g-EG) particles in gradually changing pH buffers (pH 1.2, 5.8, 6.8 and 7.4). It is clear that due to the complexation and decomplexation mechanism of the particles at pH 1.2, there was no drug released and as the pH increased, the complexes dissociated allowing the network to swell and rapid drug release occurred. Among the drugs, ciprofloxacin HCl gave a significantly rapid release profile at pH 5.8 and 6.8 compared to other release profiles.

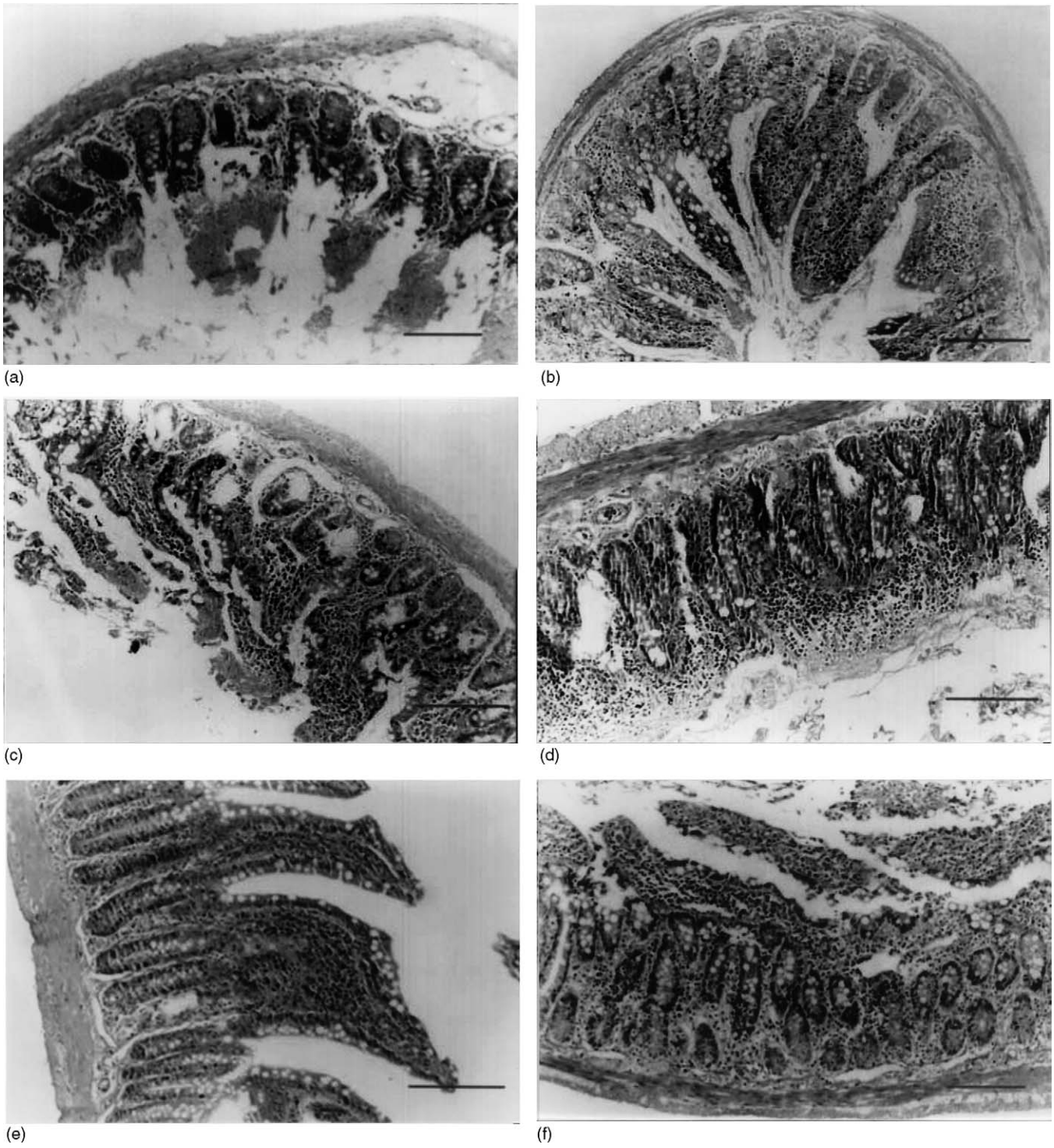

Fig. 3. Histological analysis of the ileal region of the gastrointestinal system taken from the rats: (a) treated 2 h earlier with drug free particles (scale bar: 40 μm); (b) treated 20 h earlier with drug free particles (scale bar: 40 μm); (c) treated 2 h earlier with diclofenac Na (scale bar: 96 μm); (d) treated 20 h earlier with diltiazem HCl (scale bar: 40 μm); (e) treated 20 h earlier with diclofenac Na loaded particles (scale bar: 40 μm); (f) treated 2 h earlier with ciprofloxacin HCl loaded particles (scale bar: 96 μm).

3.3. Stability study

No significant changes were observed in the release rates of the drugs after a storage period of 3 months in accelerated conditions $40 \pm 2\,^{\circ}C$ and $75 \pm 5\%$ relative humidity in the stability chamber ($p > 0.05$). The results of FT-IR and DSC analysis performed before and after the storage period in the stability chamber with the accelerated conditions showed no structural differences of pure drugs (diltiazem HCl, diclofenac Na, ciprofloxacin HCl and isoniazid), particles and the drug-loaded particles (data not shown).

3.4. Histopathological study

The histopathological examination of free particles showed erythema and focal erosion (0.84 ± 0.41) 2 h after oral administration to rats (Fig. 3a), but this result was reduced to mild erythema with focal erosion (0.67 ± 0.00) 10 h after (data not shown) and normal villus structure (0.00 ± 0.52) was observed after 20 h (Fig. 3b). These results indicated damaged cells and tissues in the ileum of the gastrointestinal tract during 2 or 10 h due to the free particles but a recovery from the damage, after 20 h.

Apart from the free particles, pure drugs (diltiazem HCl, diclofenac Na, ciprofloxacin HCl and isoniazid) were also examined histopathologically 2 and 20 h after oral administration to rats. Diltiazem HCl (data not shown) (1.00 ± 0.52) and diclofenac Na (1.17 ± 0.55) (Fig. 3c) showed erythema in the ileum after 2 h but 20 h after the treatment, erythema with diffused erosion was observed indicating a progressive damage caused by diltiazem HCl (1.34 ± 0.52) (Fig. 3d) and mild haemorrhage with diffused erosion and desquamation with diclofenac Na (data not shown) (1.50 ± 0.41) were observed. On the other hand, haemorrhage with focal erosion (1.84 ± 0.41) was obvious 2 h after the administration of ciprofloxacin HCl, but after 20 h only erythema (1.00 ± 0.52) was observed (data not shown). Haemorrhage (1.84 ± 0.41) observed 2 h after the administration of isoniazid reduced to erythema (1.17 ± 0.55) 20 h after the administration of isoniazid (data not shown). Mild recovery from the damage could only be observed 20 h after the administration of drugs.

Hence, diltiazem HCl, diclofenac Na, ciprofloxacin HCl and isoniazid were loaded into the particles and histopathological evaluation was carried out to investigate whether loading drugs to particles could reduce the cell and tissue damage of free drugs. The observation of erythema caused by free diltiazem HCl both after 2 and 20 h increased to haemorrhage (1.81 ± 0.38) (data not shown) 2 h after the administration of diltiazem HCl loaded particles. However after 20 h the result was a significant reduction of the lesion showing no damage (0.34 ± 0.52) (data not shown). Diclofenac Na loaded particles gave similar results with free drug. Both showed erythema (1.17 ± 0.55) 2 h after the oral administration (data not shown). However 20 h after the oral administration of diclofenac Na loaded particles a normal villus structure with mild erythema (0.50 ± 0.41) (Fig. 3e). This judged to be a significant reduction of the mild haemorrhage caused by the free drug. The observation of haemorrhage caused by ciprofloxacin HCl and isoniazid reduced to erythema in ciprofloxacin HCl loaded particles (0.95 ± 0.49) (Fig. 3f) and reduced to mild haemorrhage in isoniazid loaded particles (1.53 ± 0.69) (data not shown) 2 h after the oral administration. The observation of erythema caused by ciprofloxacin HCl and isoniazid after 20 h resulted in a reduction of the lesions showing normal villus structure with mild erythema when ciprofloxacin HCl (0.50 ± 0.41) or isoniazid (0.50 ± 0.41) were loaded into particles (data not shown). It can be concluded that loading these drugs to the particles decreases cell damage to a certain degree after 20 h.

In an histopathological study of oral administration of free diflunisal, a non-steroidal anti-inflammatory drug, characterized by gastric hemorrhage resulted in a significant reduction of gastric lesions when diflunisal was loaded into PHEA microparticles (Giammona et al., 1997). Budesonide (BDS) and Eudragit microparticles containing BDS loaded cellulose acetate butyrate (CAB) cores were administered orally to rats with experimentally inflamed colons. A significant decrease in the score of the histological damage was observed in the rats treated with Eudragit S microparticles containing BDS-loaded CAB cores compared with BDS suspension (Rodriguez et al., 2001).

3.5. Transmission electron microscopy

Minimal disruptions of tight junctions 2 h after the oral administration of free particles (100 mg) to rats is shown in Fig. 4a. Goblet cells were increased in number and enterocytes, paneth and endocryne cells were normal in shape. Ten hours after the oral administration of free particles (100 mg) to rats, minimal disruptions of tight junctions were also observed as shown in Fig. 4b. Goblet cells, enterocytes, paneth and endocryne cells were normal in shape.

Enlargement of interstitial space (tight junction disruption) 2 h after (Fig. 4c) and shrinking of tight junction 10 h after (Fig. 4d) the oral administration of ciprofloxacin HCl to rats were observed. Two and 10 h after the oral administration of diltiazem HCl to rats minimal disruptions of tight junctions were observed and goblet cells were active and their secretion increased while paneth and endocrine cells were in normal shape (Fig. 4e and f). Dilation of interstitial space and minimal disruption of tight junctions 2 h after the oral administration of diclofenac Na to rats (Fig. 4g) and shrinking of interstitial space and tight junctions 10 h after the oral administration of diclofenac Na to rats (Fig. 4h) were observed. Almost no enlargement of interstitial space and minimal disruption of tight junctions was observed 2 h after the oral administration of isoniazid (Fig. 4j) to rats. Shrinking of interstitial space and recovery of tight junction 10 h after oral administration of isoniazid to rats were observed (Fig. 4k).

Among the drugs, only ciprofloxacin HCl (2 mg) showed the most distinctive enlargement of interstitial space, hence ciprofloxacin HCl (2 mg) loaded particles were investigated to see whether any protective effect of the particles could be observed. But no significant difference between ciprofloxacin HCl and ciprofloxacin HCl loaded particles were observed. Disruption of tight junctions 2 h after oral administration of

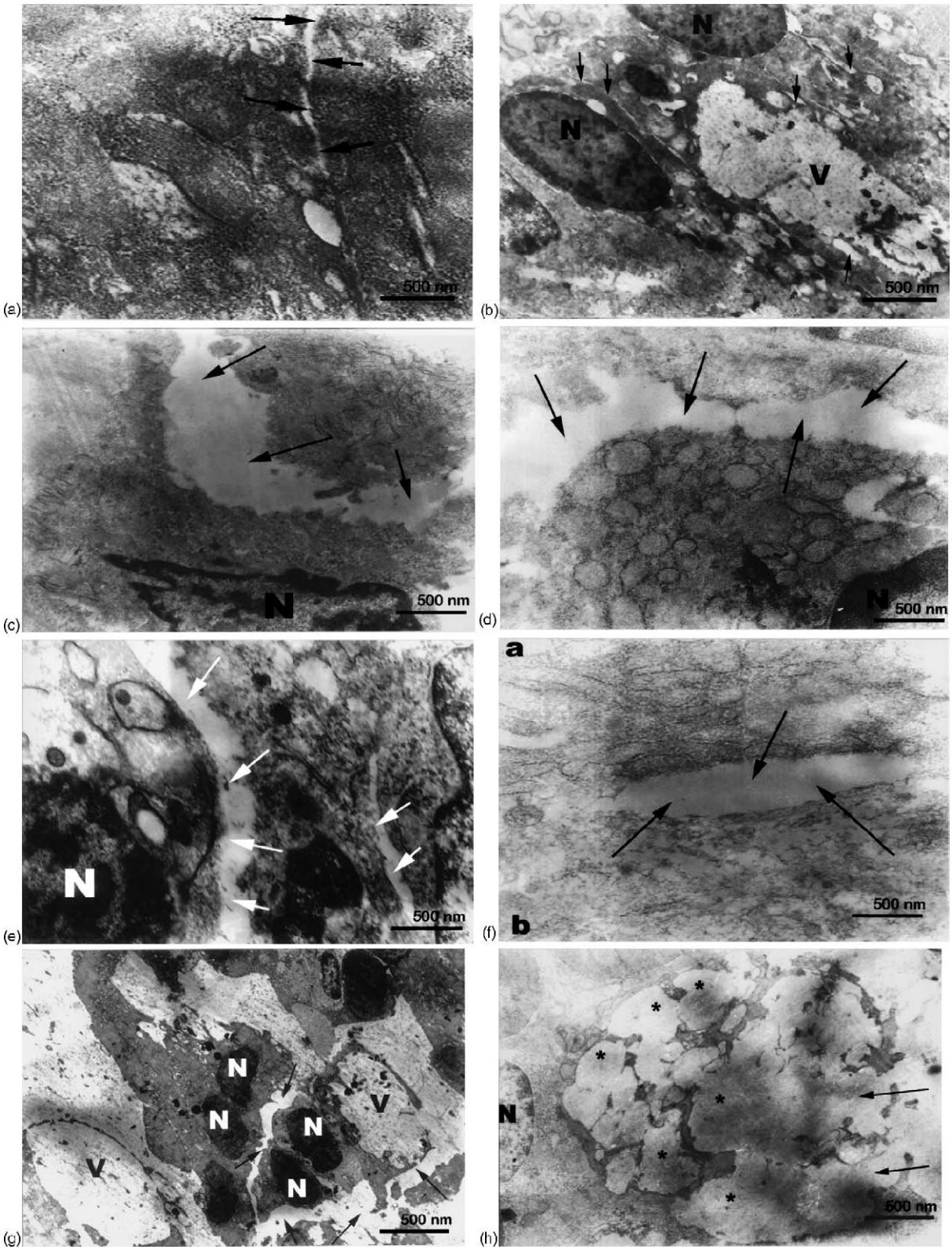

Fig. 4. Transmission electron micrographs of tight junctional complexes of the rats: (a) treated 2 h earlier with drug free particles; (b) treated 10 h earlier with drug free particles; (c) treated 2 h earlier with ciprofloxacin HCl; (d) treated 10 h earlier with ciprofloxacin HCl; (e) treated 2 h earlier with diltiazem HCl; (f) treated 10 h earlier with diltiazem HCl; (g) treated 2 h earlier with diclofenac Na; (h) treated 10 h earlier with diclofenac Na; (j) treated 2 h earlier with isoniazid; (k) treated 10 h earlier with isoniazid; (l) treated 2 h earlier with ciprofloxacin HCl loaded particles; (m) treated 10 h earlier with ciprofloxacin HCl loaded particles; (n) treated 2 h earlier with 385 mg of drug free particles; (*in all the figures* arrows are showing junctional disruption and asterisks are showing foamy disruption except in (n) arrows are showing enterocytes and asterisks showing junctional disruption).

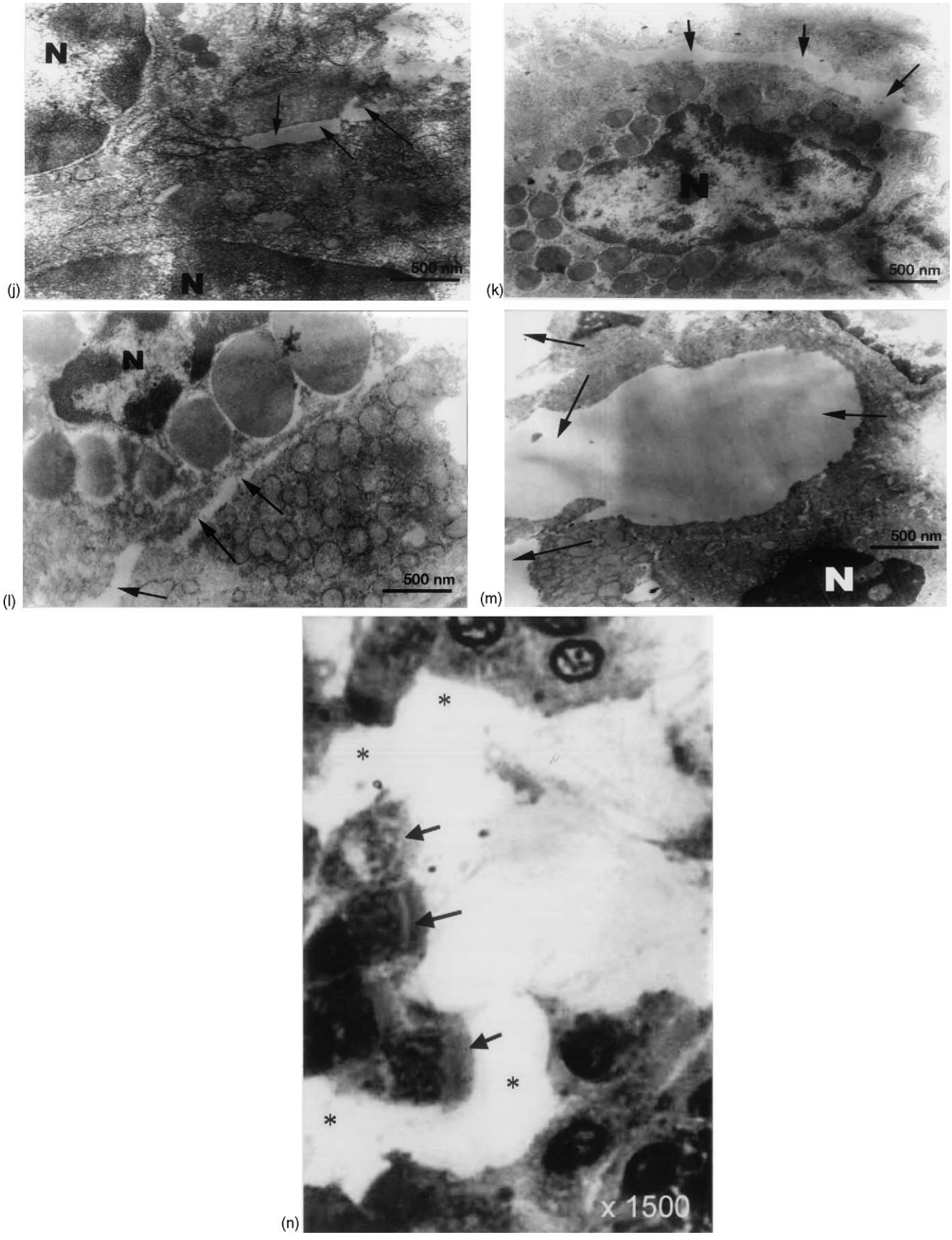

Fig. 4. (*Continued*).

ciprofloxacin HCl loaded particles to rats were given in Fig. 4l. Goblet hypertrophy and increase in secretion were observed. Partial damage to the enterocytes were obvious. As shown in Fig. 4m, 10 h after oral administration of these drug-loaded particles to rats, enhanced enlargement of interstitial space and disruption of tight junction were observed. Goblet hypertrophy as well as partial damage in the enterocytes were observed again.

Since 385 mg free particles were necessary for loading 2 mg ciprofloxacin HCl (loading percentage 0.54 ± 0.05 mg), 385 mg free particles were also investigated using the same procedure in order to observe whether only particles without any drug have the ability to open the tight junctions. Two hours after, the absorptive cells of the small intestine displayed an enlargement, distension and opening of the tight junctional complex. The cell alignment was disrupted. Goblet cells were partially damaged and partially not observed (Fig. 4n). On the other hand, 10 h after the oral administration of 385 mg particles to rats, the distance between the absorptive cells was increased. The cells were damaged and nearby the defects in the tight junctional complex and basal junctional complex was also affected. The lining property of the epithelial had disappeared. It can be concluded that increasing the dose of free particles from 100 to 385 mg increased the disruption of tight junction of the epithelial cells.

In another study, the physicochemical effects of P(MAA-g-EG) nanospheres on epithelial cell monolayers were investigated using the Caco-2 cell line as a model for the gastrointestinal tract. Nanospheres were examined for their cytotoxic effects and their ability to open the tight junction by measuring changes in the membrane transepithelial electrical resistance (TER). The intimate contact between the hydrogel nanospheres and the epithelial cell monolayer generated changes in transepithelial electrical resistance. The chelation capabilities of these hydrogels allowed them to open the tight junctions in Caco-2 cell line used as a model for the gastrointestinal tract (Torres-Lugo et al., 2002; Foss and Peppas, 2004). Apart from P(MAA-g-EG) particles, various anionic and nonionic surfactants (Anderberg et al., 1992), fatty acids (Tengamnuay and Mitra, 1985), methylated cyclodextrins (Martin et al., 1999), calcium chelators such as EDTA (Tomita et al., 1996) and poly(acrylic acid) (Kriwet and Kissel, 1996), positively charged polymers such as chitosan (Junginger and Verhoef, 1998) and trimethyl chitosan (Thanou et al., 1999) have been found to enhance the paracellular permeability of mucosal epithelia by transiently opening the tight junctions.

Although studies on P(MAA-g-EG) particles were carried out using Caco-2 cells, this study, according to the literature, is the only in vivo examination of these particles histopathologically and involving of tight junctions.

4. Conclusions

It can be concluded that P(MAA-g-EG) particles can control the release rate of small molecular weight model drugs according to the pH of the medium. The stability of these particles loaded with drugs did not change in accelerated conditions.

According to the literature, this study is the only in vivo investigation on P(MAA-g-EG) particles histopathologically and for their effect on epithelial tight junction of rats.

Histopathological investigation of free particles showed cell damage in the ileum after 2 h but this effect slowly diminished after 10 h. Normal villus structure with no damage in the ileum of the gastrointestinal system 20 h after oral administration of particles to rats was observed. Hence, the observed cell or tissue damage with free drugs (diltiazem HCl, diclofenac Na, ciprofloxacin HCl and isoniazid) were prevented at the end of 20 h when those drugs were loaded into P(MAA-g-EG) particles. On the other hand, the results of TEM showed that increasing the amount of P(MAA-g-EG) particles from 100 to 385 mg clearly opened the tight junction but with serious epithelial cell disruption.

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