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Physicochemical behavior and cytotoxic effects of p(methacrylic acid–g-ethylene glycol) nanospheres for oral delivery of proteins

Madeline Torres-Lugo[a,1], Marcos García[a,2], Rae Record[b], Nicholas A. Peppas[a,b,*]

[a]*NSF Program on Therapeutic and Diagnostic Devices, School of Chemical Engineering, Purdue University, West Lafayette, IN 47907-1283, USA*

[b]*Department of Biomedical Engineering, Purdue University, West Lafayette, IN 47907-1246, USA*

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Abstract

The challenges faced to orally deliver therapeutic agents with unfavorable physicochemical properties, such as proteins, have been the primary motivation for the design and development of novel oral delivery systems that could circumvent biological barriers. In this work, we examined complexation-sensitive hydrogel nanospheres composed of poly[methacrylic acid–grafted-poly(ethylene glycol)] (P(MAA–g-EG)), on a model biological environment. For this purpose, a gastrointestinal cell culture model, the Caco-2 cell line, was employed to investigate the cytotoxic effects of the polymeric carrier and its effects on the cell monolayer integrity. The determination of the cytotoxic effects of the polymer network on the cell monolayer was performed by a colorimetric assay and by the counting of viable cells using the trypan blue exclusion method. Electrophysiological measurements were performed to measure the transepithelial electrical resistance changes in the monolayers in the presence and absence of the nanosphere suspension. The examination of the physicochemical interactions of the P(MAA–g-EG) nanosphere system with Caco-2 cell monolayers revealed that these systems possessed low cytotoxicity and were capable of opening the tight junctions between epithelial cells, therefore significantly reducing the transepithelial electrical resistance.

Keywords: Transepithelial electrical resistance; Cytotoxicity; Oral delivery of peptides; pH-Sensitive hydrogels

1. Introduction

The treatment of diseases not only requires the development and testing of new drugs but also the minimization of their adverse effects without impairing the quality of life of the patient. The field of controlled release has evolved toward the use of these goals as their primary motivation for the design and development of systems that could improve treatment efficacy. The recent use of peptides and

*Corresponding author. School of Chemical Engineering, Purdue University, West Lafayette, IN 47907-1283, USA, Tel.: +1-765-494-7944; fax: +1-765-494-4080.

E-mail address: peppas@ecn.purdue.edu (N.A. Peppas).

[1]Present address: Department of Chemical Engineering, University of Puerto Rico, Mayagüez Campus, P.O. Box 9046, Mayagüez, Puerto Rico 00681-9046.

[2]Permanent address: School of Pharmacy, University of Santiago de Compostela, Santiago de Compostela, Spain.

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proteins as therapeutic agents is a perfect example, and, for this reason, it has gathered special attention.

Currently, therapeutic peptides and proteins are administered mainly by intramuscular or intravenous injections due to their delicate physicochemical characteristics in aqueous solutions and their susceptibility to be degraded by proteolytic enzymes in biological fluids. In most cases, the treatment of diseases using peptidic agents possesses less undesirable adverse effects, but it also reduces the quality of life of the patient due to the psychological stress caused by frequent injections. As a consequence low patient compliance, decreasing treatment efficacy has been observed. Therefore, the development of non-invasive delivery systems, especially for the oral route, is of utmost importance.

In this work, we are mostly interested in the investigation of the feasibility and behavior under physiological conditions of a novel pH-sensitive hydrogel as a possible delivery carrier for peptides and proteins. pH-Sensitive hydrogels are suitable candidates for the oral delivery of peptide drug due to their ability to respond to their environment. For this purpose, we have developed new hydrogels composed of poly(ethylene glycol) (PEG) chains grafted on a poly(methacrylic acid) (PMAA) backbone, henceforth designated as P(MAA–g-EG).

The molecular design of the oral delivery system developed in this work was based on the capability of the PEG tethered chains to promote mucosal adhesion and protein protection and the calcium chelating capability of the carboxylic pendent groups to promote epithelial cell junction opening [1]. In a recent review by Luessen et al. [2], it was demonstrated that the chelating capabilities of the carboxylic acid containing polymers not only promote the opening of the tight junction, but also inhibited the activity of luminal enzymes such as trypsin and α-chymotrypsin. Such capabilities were also observed in studies performed in our laboratory with the P(MAA–g-EG) system [3].

The P(MAA–g-EG) hydrogels are characterized by their ability to remain collapsed at low pH values, similar to those found in the stomach, and to swell at higher pH, such as those encountered in the upper small intestine. These capabilities allow this system to protect the protein from the undesirable conditions found in the stomach and release it in a more favorable environment such as that encountered in the upper small intestine.

In the past decade, cell cultures from human colon adenocarcinoma cells were developed as a new model to study gastrointestinal absorption. The Caco-2 cell line has attracted the interest of the research community due to its ability to differentiate without difficult cell culturing techniques.

The main goal of this work was to investigate the behavior of the P(MAA–g-EG) nanosphere system on a model biological environment. For this purpose, a gastrointestinal cell culture model, the Caco-2 cell line, was employed to investigate the cytotoxic effects of the polymeric carrier and its effects on the cell monolayer integrity.

2. Experimental

2.1. Synthesis of P(MAA–g-EG), poly(methacrylic acid), and poly(ethylene glycol) nanospheres

2.1.1. Materials

Methacrylic acid (MAA, Aldrich, Milwaukee, WI, USA) and methoxy-terminated poly(ethylene glycol) monomethacrylate (PEGMA, Polysciences, Warrington, PA, USA) with a molecular weight of 1000 were used to synthesize P(MAA–g-EG) nanospheres. The monomers were mixed in 4:1, 2:1, 1:1 or 1:2 molar ratios of methacrylic acid–ethylene glycol units. Tetra(ethylene glycol) dimethacrylate (TEGDMA, Aldrich) was incorporated as 0.75% mol of the total monomers and utilized as the crosslinking agent. Irgacure® 184 (Ciba-Geigy, Hawthorne, NY, USA) was employed as the photoinitiator; it was incorporated in approximately 0.1% (w/w) of the monomer mixture.

2.1.2. Preparation of nanospheres

P(MAA–g-EG) nanospheres were prepared by dispersion polymerization. For this process, the monomers were mixed and the initiator dissolved in the monomer mixture. This mixture was then added to deionized water to provide a final concentration of monomer of 0.75% (w/w) and the container sealed. The container was bubbled with nitrogen for 15 min and placed under a UV source (Spectroline, Model SB-125, Westbury, NY, USA) at an intensity of (7

mW/cm^2). The solution was allowed to react for 10 min. The resulting nanospheres were washed in deionized water using a dialysis membrane (Spectra/Por®, Spectrum Labs., Laguna Hills, CA, USA) with a molecular weight cut-off of 300 000. The purpose of this procedure was to minimize the presence of any unreacted monomer or non-crosslinked chains remaining in solution.

PMAA nanospheres were synthesized by combining the MAA monomer with the crosslinker tetra-(EG) dimethacrylate. The concentration of the crosslinker incorporated was 0.75% by mole of the total moles of MAA monomer. The polymerization and washing procedures were similar to those described for P(MAA–g-EG) nanospheres.

The procedure for the preparation of the PEG nanospheres followed a modification of the procedure just described. Methoxy-terminated PEG monomethacrylate with a molecular weight of 1000 was combined with poly(ethylene glycol dimethacrylate) (PEGDMA, Polysciences, Warrington, PA, USA) also with a molecular weight 1000 at a weight ratio of 50:50. To prepare the solvent mixture a combination of surfactants was necessary. The solutions consist of 0.0045 g/dl of Tween 80® (Aldrich) and a 0.0285% w/w solution of sodium dodecylsulfate (SDS) (Sigma, St. Louis, MO, USA). After both mixtures are prepared, the surfactant solution was prepared by mixing SDS (1%, w/w) and Tween 80 (99%, w/w). The solid monomers were dissolved in the surfactant mixture (2%, w/w, total amount of monomer–surfactant mixture). Argon was bubbled for 20 min to remove any dissolved oxygen. The glass vessel was placed in a silicone oil bath and allowed to equilibrate in temperature for 1 h at 80 °C. After temperature equilibration was reached, the vessel was exposed to a UV source (Spectroline, Model SB-125, Westbury, NY, USA) at an intensity of (7 mW/cm^2) and allowed to react for 5 min. The nanospheres were further washed.

These procedures produced nanospheres without agglomeration. P(MAA) nanospheres were treated in the same manner as P(MAA–g-EG) nanospheres, whereas PEG nanospheres were kept in suspension due to their inability to resuspend after freeze drying.

For salmon calcitonin (sCT) loading, a measured amount of nanospheres was swollen and equilibrated in the appropriate HBSS (depending on the type of experiment). After the equilibration period a concentrated solution of sCT was added to the suspension and allowed to equilibrate overnight. For these experiments hydrogels with protein were not further treated since the interest of these experiments was not the release properties of the hydrogel but the possible enhancement that these hydrogels could provoke. Of course, hydrogel nanospheres are capable of incorporating part of the protein in the structure but for these experiments the concentration of sCT was measured in the hydrogel suspension before and after the experiment.

2.2. Caco-2 cell culture

Caco-2 cells were purchased from the American Tissue Culture Collection (Rockville, MD, USA). The cells were cultivated on 75-cm^2 flasks (Beckman Diagnostics, Franklin Lakes, NJ, USA) using Dulbecco's modified Eagle medium (DMEM) (Sigma) containing 10% fetal bovine serum (Life Technologies, Gaithersburg, MD, USA), 1% nonessential amino acids (Life Technologies), 100 units/ml of penicillin, and 100 μg/ml streptomycin (Sigma). Cells were maintained on a controlled atmosphere at 37 °C, 95% relative humidity, and 5% CO_2. The seeding density for cultivation was approximately 2.5×10^5 cells/flask. Culture medium was changed every other day for approximately 5–6 days until cells reached approximately 80–90% confluency. For all the experiments cells with passage numbers between 60 and 80 were utilized.

2.3. Preparation of nanosphere suspensions for cell culture experiments

Nanospheres suspensions were prepared by freeze drying the nanospheres, weighing the necessary amount, and resuspending in Hank's balanced solution (HBSS, Sigma), containing the appropriate concentration of calcium. The suspensions were pH equilibrated by placing the suspension on a dialysis membrane (Spectra/Por® 0.25 ml/cm, Spectrum Laboratories, Laguna Hills, CA, USA) with a molecular weight cut-off of 15 000, and submerging it on freshly prepared HBSS. The HBSS solution was changed several times to ensure that the appropriate pH of 7.4 was achieved. After pH equilibration,

nanosphere suspensions were sterilized by γ-irradiation with a dose of 2.5 Mrad in a Gammacell 220 (US Nuclear).

2.4. Cell proliferation assays

2.4.1. Cell proliferation assay by a colorimetric method

Cells were cultivated on 96 well plates (Costar®, Corning, Corning, NY, USA), with a cell density of 1.4×10^4 cells/cm^2, for approximately 6–7 days until a homogeneous cell monolayer was obtained.

Cytotoxicity experiments were conducted using nanospheres suspensions of all the monomer ratios with concentrations of 1, 5 and 10 mg/ml. The appropriate volume of nanosphere suspension was added to the wells to obtain a final mass per unit area of 0.32, 1.62 and 3.24 mg/cm^2, for 1, 5 and 10 mg/ml, respectively. Monolayers were in contact with the suspension for 2.5 h. Cell viability was determined using Cell Titer 96® (Promega, Madison, WI, USA), a colorimetric method, where the reactants in the assay are bioreduced by the cells through a pathway where NADPH or NADH is involved and transformed into a colored formazan product that absorbs at 490 nm. The assay reagents include 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and an electron coupling reagent phenazine ethosulfate (PES). This bioreduction is conducted exclusively in the presence of metabolically active cells. Therefore, the concentration of the formazan product is proportional to the number of viable cells. The intensity of the color produced by the reaction was measured using a microplate reader (Elx800NB Bio-Tek Instruments, Winooski, VT, USA) at a wavelength of 490 nm. Positive controls consisted of monolayers exposed to HBSS, whereas negative controls contained cells exposed to a solution of 10% (v/v) bleach.

Results for the colorimetric assay were obtained by normalizing the absorbance of experimental wells with that of the controls. The absorbance value of the negative control was subtracted from the absorbance of both controls and experimental wells. The background absorbance produced by dead cells treated with 10% (v/v) bleach was also subtracted.

2.4.2. Cell proliferation assay by cell counting

Cell proliferation assays by cell counting required the cultivation of Caco-2 cells in 6 well plates (9.6 cm^2/well) (Costar®) seeded with a concentration of 1.4×10^4 cells/cm^2 and cultured in DMEM for 7 days or until a complete monolayer was obtained. Experimental conditions such as nanosphere preparation were similar to those described in Section 2.4.1.

Cell monolayers were exposed to nanosphere suspensions for 2.5 h. After the incubation period, with the nanosphere suspension, the cell monolayers were rinsed with a solution of 0.2% (v/v) of EDTA–PBS solution. The monolayers were then subjected to trypsinization to resuspend the cells. Then, cells were treated with trypan blue (Sigma) and counted with an hemocytometer.

2.5. Transepithelial electrical resistance measurements

Cells were seeded on 6 well Transwell® plates (4.71 cm^2/well, pore size 0.4 μm) (Costar®, Corning) with a cell density of 5.25×10^4 cells/cm^2. The plates were cultivated in DMEM for 21–25 days.

Nanosphere suspensions composed of different monomer ratios were prepared with a concentration of 10 mg/ml (3.24 mg/cm^2) as described in Section 2.3. For all these experiments, nanospheres were resuspended in HBSS free of calcium, magnesium and phenol red, henceforth designated as HBSS–CMF.

Cell monolayers were equilibrated for an h prior the initiation of the experiment with HBSS–CMF solution in the apical chamber and HBSS solution containing 200 μmol/l calcium in the basolateral chamber. This solution is henceforth designated as HBSS/200. During this period of time, the electrical resistance of the monolayers was monitored until a constant resistance was measured using a volt meter (World Precision Instruments, Sarasota, FL, USA). This was necessary to minimize the stress on the cell monolayer due to the change in media. The small amount of calcium present in the basolateral side was found to be necessary to minimize any cell detachment and retain the monolayer integrity. After the equilibration period, nanosphere suspensions replaced the HBSS–CMF in the apical side of selected wells.

The TEER values of the cell monolayer were monitored using a voltmeter with chopstick electrodes at different time intervals. The temperature of the wells was kept constant during the experiment since the transepithelial electrical resistance (TEER) values are temperature dependent [4].

Nanosphere suspensions were kept in contact with the cell monolayers for 2.5 h. After this period of time, nanosphere suspensions and experimental media were removed and replaced with fresh DMEM. The TEER values were monitored for up to 24 h.

3. Results and discussion

3.1. Cytotoxicity assays

Cytotoxicity studies included the investigation of the effects of nanospheres composed of different monomer ratios at various concentrations. The size of the nanospheres depended upon the surrounding pH. In the collapsed state (pH~4.7) their size was approximately 475 nm and in the swollen state the size was approximately 1600 nm (pH~7.3). Two different approaches were followed to investigate cytotoxicity, which included an indirect measurement utilizing a colorimetric assay, and the counting of viable cells using the trypan blue exclusion method.

Results for the colorimetric assay, revealed that for all nanosphere concentrations and compositions, the cell monolayers remained more than 95% viable when compared to control indicating that this system appears to possess very low cytotoxicity (see Fig. 1). Several average values for the fraction of NADH+ activity produced a value higher than one, which would indicate a higher viability than to controls. These values can be explained in the basis of the stress produced to the cells upon contact with the hydrogel suspension, thus accelerating their metabolism. The combination of error and low cytotoxicity of the system produced values slightly higher than one for some cases.

Viability experiments were performed at different stages of cell growth due to experimental challenges, particularly the experiment with cell counting. If experiments were performed much later in the growth pathway (i.e. after differentiation has occurred), the trypsination process was not as effective in detaching the cells and many did not remain viable just because of the procedure. This was due to the development of tight junctions and the further adhesion of the cell to the substrate. Since the purpose of both viability experiments was to compare the results obtained with both procedures, experiments were performed at similar growth stages. In order to ensure that similar behavior was encountered during experiments, trypan blue exclusion procedure was also performed at the end of the TEER experiments. At the end of the TEER experiment a solution of trypan blue was added to the monolayer in the apical chamber. After 2 min the solution was rinsed and observed under the microscope. Cells appearing blue were considered dead whereas viable cells did not uptake the dye. A qualitative observation indicated that cells remained viable after the experiment.

To corroborate these results, additional experiments were performed. These experiments were performed by exposing the cell monolayers to nanos-

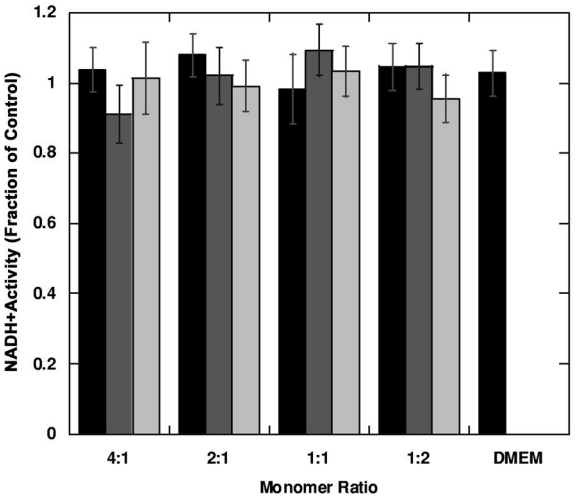

Fig. 1. Fraction of NADH+ activity as a function of the hydrogel monomer ratio obtained from Caco-2 cell monolayers at 37 °C when exposed to P(MAA-g-EG) nanospheres with various concentrations (1 mg/mL, 5 mg/mL, 10 mg/mL) dispersed in HBSS. Results were calculated by the ratio of the absorbance obtained from experimental wells and the absorbance from control wells, which did not contain nanosphere suspension. Each bar represents an average of $n=32$ for control and $n=16$ for experimental wells±one stdv.

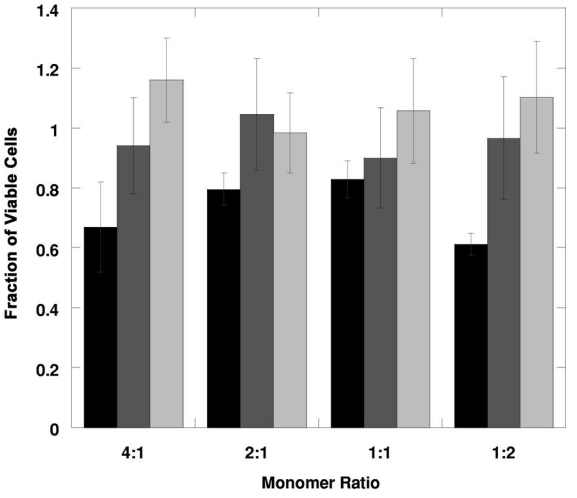

Fig. 2. Fraction of viable cells as a function of hydrogel monomer ratio obtained from Caco-2 cell monolayers at 37 °C when exposed to P(MAA-g-EG) nanospheres with various concentrations (1 mg/mL, 5 mg/mL, 10 mg/mL) dispersed in HBSS. Results were calculated by the ratio of counted viable cells resulting from monolayers exposed to nanospheres and the counted viable cells resulting from monolayers that were not exposed to nanosphere suspensions. Each bar represents an average of $n=3$ for controls and experimental wells±one stdv.

phere suspensions and then counting viable cells using the trypan blue exclusion method. Results from this technique are illustrated in Fig. 2. HBSS was also employed as the experimental medium in this case. In all cases the controls refers to monolayers exposed to experimental media without any nanospheres (i.e. HBSS).

As concentration was increased, an apparent trend of lower cell viability was encountered. When different monomer ratios were compared, no trends could be found. At high concentrations (10 mg/ml), the lowest cell viability corresponded to the 1:2 ratio with 60% of cells viable followed by the 4:1 ratio with 67%. A negative control using 10% (v/v) bleach was also investigated (data not shown). In this case, only cell debris was observed.

However, for these experiments the range in standard deviations was high—from 3 to 20%. The high variability between experiments was due to the detachment and further removal of the cells during washing before performing the assay. Because of the capability of the hydrogels to weaken the tight junctions (see Section 3.2), this detachment is likely to occur preferentially in those wells with high concentration of nanospheres. Therefore, this effect could also result in an apparent increase in cytotoxicity. Detached cells cannot be considered dead because, like during passage operation, a calcium chelator was used to detach the cells from the flask and further seed them on a new one where they will reattach and grow. Therefore, it cannot be concluded that all detached cells were dead, which would indicate a moderate cytotoxicity of the polymer.

3.2. Transepithelial electrical resistance

Electrophysiological measurements were conducted to elucidate the effects of the hydrogel on the cell monolayer when they became in contact. Several experiments were conducted, including the examination of the effects of nanosphere monomer ratio, the effect of particle size by comparing the effects caused by crushed hydrogels and nanospheres, and the examination of each individual component of the system (i.e. PMAA and PEG).

The changes in TEER are an indication of the permeability of ions through the epithelial cell monolayer. It is an indirect measurement of the membrane integrity, by indirectly measuring the opening of the tight junctions. Results for the TEER experiments using P(MAA–g-EG) nanospheres are illustrated in Figs. 3–5. The period of time illustrated in the figures from −60 min to 0 min represents the equilibration period where the cells were allowed to get accustomed to the new medium.

Experimental data illustrate several interesting findings. Cell monolayer appeared to reach a second steady state for the TEER measurement for both controls and experimental wells. This was represented by an initial drop in the TEER after the medium was changed from DMEM to HBSS and the maintenance of a constant resistance throughout the 2.5 h of exposure to nanosphere suspensions or nanosphere-free medium. This maintenance of monolayer resistance is attributed to the presence of the small amount of calcium in the basolateral side. For all experiments, cell monolayers recuperated after the removal of the polymer suspensions and incorporation of DMEM, a calcium rich medium (data not shown). In all cases the cell monolayers recuperated most of their original TEER values in

approximately 5 h after the nanosphere suspensions were removed. After 24 h, all membranes recuperated, demonstrating the reversibility of the process and that the cells were not damaged by the presence of the polymer. These results provide further evidence of the low toxicity of the polymer, since the monolayers were capable of fully recuperating.

Another factor under consideration was the effect of size of the polymer matrix. Crushed polymer films (size<150 μm) were prepared following the procedures described by Torres-Lugo and Peppas [5]. Similar experimental conditions as for the nanospheres were followed. The comparison between the behavior of the crushed particles and microspheres is depicted in Fig. 3. No statistical differences in the TEER values were encountered, indicating that particle size of the hydrogel is not an important factor.

The presence of nanosphere suspensions of various compositions decreased the TEER to approximately 50% of the original value and was significantly lower than controls. The reduction in TEER by the nanosphere suspension was independent of composition, although there were different concentrations of MAA in the structure for each case

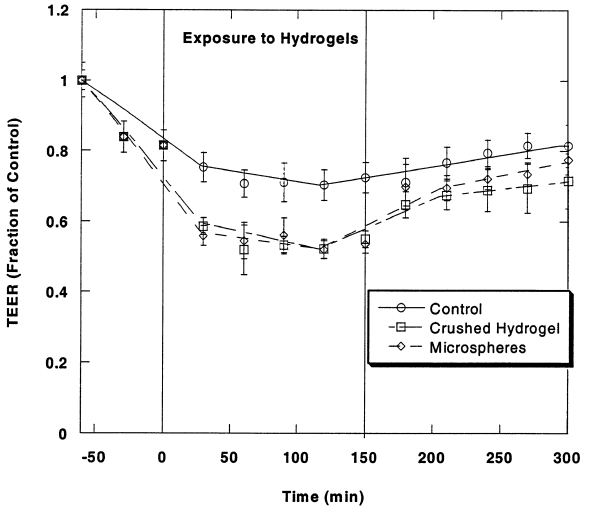

Fig. 3. Fraction of the TEER as a function of time for monolayers exposed to crushed hydrogel films and nanospheres from 1:1 ratio with a concentration of 10 mg/ml in Caco-2 cell monolayers at 37 °C. Apical chamber contained the nanosphere suspension in HBSS–CMF and basolateral chamber contained HBSS/200. Each datum point represents an average of $n=3$ for controls and experimental wells±1 S.D.

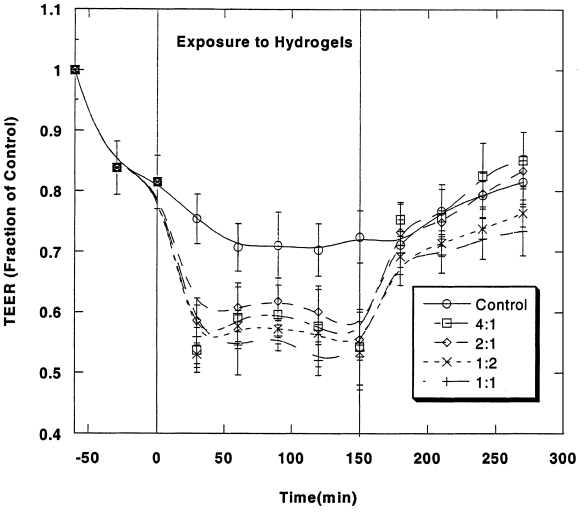

Fig. 4. Fraction of the TEER as a function of time for monolayers exposed to P(MAA–g-EG) hydrogel nanospheres with different monomer ratios with a concentration 10 mg/ml in Caco-2 cell monolayers at 37 °C. Apical chamber contained the nanosphere suspension in HBSS–CMF and Basolateral chamber contained HBSS/200. Each datum point represents an average of $n=3$ for control and experimental wells±1 S.D.

(see Fig. 4). An analogous phenomenon was observed when crushed particles from P(MAA–g-EG) polymer films were tested [6]. A recent investigation by Madsen and Peppas [3] reported the calcium chelating capabilities of P(MAA–g-EG) crushed hydrogel films. Table 1 reports the values of the micromol of calcium chelated per mg of polymer calculated from the data provided in the article. These values revealed that although each monomer ratio was capable of chelating different amounts of calcium the differences were not dramatic. This suggests the possibility that each monomer ratio was capable of chelating enough calcium to open the tight junction to a similar extent.

Table 1
Calcium chelation capabilities of P(MAA–g-EG) crushed hydrogel films with different compositions [3]

Monomer ratio	μmol Ca^{2+}/mg polymer
4:1	2.2
2:1	1.9
1:1	1.7
1:2	1.4

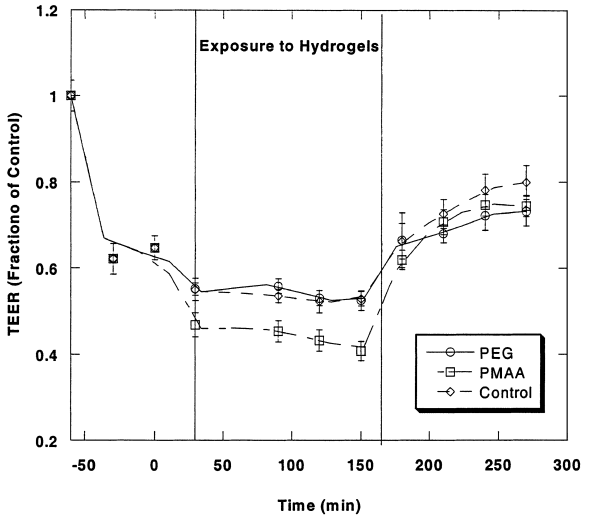

Fig. 5. Fraction of the TEER as a function of time for monolayers exposed to crosslinked PEG and PMAA hydrogel nanospheres with a concentration of 10 mg/ml in Caco-2 cell monolayers at 37 °C. Apical chamber contained the nanosphere suspension in HBSS–CMF and basolateral chamber contained HBSS/200. Each datum point represents an average of $n=3$ for control and experimental wells±1 S.D.

In order to understand if the observed TEER values are due to the presence chelating properties of the MAA or by an unknown effect of PEG in the cell monolayer, TEER experiments were conducted using crosslinked PMAA and PEG nanospheres. These nanospheres were also prepared by dispersion polymerization. As expected, PMAA nanospheres were capable of reducing the TEER to 35% of the original values compared to PEG nanospheres which were unable to reduce the TEER values and were statistically similar to controls (see Fig. 5). Carboxylic acid containing polymers have been previously examined and demonstrated to produce a similar change in TEER values on Caco-2 cell monolayers [1,2,7–10].

4. Conclusions

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. Nanosphere suspensions of various monomer ratios and concentrations were examined for their cytotoxic effects and their ability to open the tight junction by measuring changes in the membrane transepithelial electrical resistance. Cytotoxicity experiments revealed that the nanosphere system possessed low cytotoxic effects as evidenced from the observations obtained from colorimetric cell proliferation assays and viable cell counting assays. These observations were independent of hydrogel composition.

The intimate contact between the hydrogel nanospheres and the epithelial cell monolayer generated changes in the cell monolayer integrity as evidenced by the changes in transepithelial electrical resistance. The chelation capabilities of these hydrogels allowed them to open the tight junctions, and, therefore, the potential to increase the transport of paracellularly transported molecules. These effects were found to be related to the presence of MAA in the hydrogels. However, for the different compositions of P(MAA–g-EG) tested no difference in their capability to produce opening of the tight junctions was found. The reincorporation of calcium to the cell monolayer produced the recuperation of the cell monolayer electrical resistance, indicating that the process was reversible and that the monolayers were not affected by the presence of the nanospheres. This is further evidence that the hydrogel nanospheres possessed low cytotoxicity.

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