Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmaceutics and Biopharmaceutics 57 (2004) 447–455
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Investigation of the cytotoxicity and insulin transport of acrylic-based copolymer protein delivery systems in contact with caco-2 cultures

Aaron C. Foss[1], Nicholas A. Peppas*

Departments of Chemical Engineering, Biomedical Engineering, and Division of Pharmaceutics, The University of Texas at Austin, Austin, TX, USA

Received 8 December 2003; accepted in revised form 2 February 2004

Abstract

Microparticles or nanospheres of hydrogels of crosslinked poly(methacrylic acid) grafted with poly(ethylene glycol) as well as crosslinked poly(acrylic acid) grafted with poly(ethylene glycol) were prepared for use as oral insulin delivery carriers. The copolymer carriers were synthesized by precipitation/dispersion polymerization that led to gel nanospheres or by bulk polymerization and subsequent size reduction of thin films to obtain gel microparticles. The cytotoxicity of these copolymers was investigated in contact with Caco-2 cell cultures using a metabolic assay to measure the effect of the presence of copolymers on the cell viability. The copolymers were found to exhibit no cytotoxic effect on the cell cultures. Insulin-loaded formulations were also tested for cytotoxicity and insulin transport studies across cell monolayers. The copolymers were shown to open the tight junctions between cells, increasing the available area for diffusion across the cell monolayer, and thus increasing the permeability of insulin across the monolayer.

Keywords: Acrylic-based copolymer; Copolymer nanospheres; Poly(ethylene glycol); Caco-2 cell culture; Insulin transport

1. Introduction

The harsh conditions of the gastrointestinal tract often denature an orally administered protein formulation before it reaches the blood stream. In the case of oral insulin delivery devices, less than 0.1% of the orally dosed insulin reaches the blood stream intact [1]. Clearly, to make use of the ever-expanding library of proteins, proteins must be administered through injections.

Unfortunately, injections are often painful, leading to low patient compliance [2]. Research has focused on alternate ways of delivering proteins and other unstable therapeutic agents, including oral delivery. By minimizing injections, patient compliance could increase and the treatment regime could be made less intrusive, improving the efficacy of the treatment.

Numerous scientific obstacles must be overcome in order to create an oral protein delivery system. The greatest barriers are the harsh conditions of the stomach and the transport barrier, present in the intestine. The stomach has an acidic environment full of proteolytic enzymes. Sensitive proteins are often denatured or destroyed in the stomach. The small amount of protein that makes it through the stomach intact must then be transported across the lining of the intestine to get into the bloodstream. This transport process is greatly reduced by the mucus and no significant insulin can be transported across the cell layers.

Previously, we have shown that copolymers of methacrylic acid (MAA) containing pendent chains of poly(ethylene glycol) have unique properties as carriers for transmucosal drug delivery [3–10]. In this work, we have extended the previous studies by developing a group of copolymer hydrogels capable of protecting proteins while they are in transit through the stomach, and then aiding in increasing protein transport across the cellular barrier in the upper small intestine. Our present studies were conducted with insulin-loaded systems. The family of copolymers studied contained either methacrylic acid or acrylic acid (selected, for their pH-sensitive nature and ability to bind

* Corresponding author. Departments of Chemical Engineering, Biomedical Engineering, and Division of Pharmaceutics, The University of Texas at Austin, 1 University Station C0400, Austin, TX 78712-0231, USA. Tel.: +1-512-471-6644; fax: 1-512-471-8227.
E-mail address: peppas@che.utexas.edu (N.A. Peppas).
[1] Present address: Boston Scientific, 1 Scimed Place, Maple Grove, MN 55311, USA.

0939-6411/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2004.02.008

calcium) and was grafted with poly(ethylene glycol) selected for its ability to stabilize and protect proteins.

2. Materials and methods

2.1. Synthesis of P(MAA-g-PEG) and P(AA-g-PEG) copolymer hydrogels

Synthesis of copolymer hydrogel carriers was carried out under nitrogen using UV-initiated free-radical polymerization. Mixtures of methacrylic acid (MAA, Polysciences Inc., Warrington, PA) and poly(ethylene glycol) monomethylether monomethacrylate (PEGMA with PEG of molecular weight of 1000, Polysciences Inc., Warrington, PA), or of acrylic acid (AA, Polysciences Inc., Warrington, PA) and PEGMA were prepared.

The monomers were mixed in molar ratios of 4:1, 2:1, 1:1 or 1:2 of MAA:EG or AA:EG repeating units. Tetraethylene glycol dimethacrylate (TEGDMA, Polysciences Inc., Warrington, PA) was added as the crosslinking agent in the amount of $X = 0.01$ moles of TEGDMA per mole of total monomers. Thus, a network prepared with a nominal crosslinking ratio of $X = 0.01$ is said to be 1% crosslinked. 1-Hydroxylcyclohexyl phenyl ketone (Irgacure-184, Ciba-Geigy Corp., Hawthorne, NY) was used as the free-radical initiator and added in the amount of 0.5 wt% of the monomer mixture.

2.2. Synthesis of copolymer gel nanospheres

Dilute solutions of the monomer mixture were prepared in deionized water. Depending on the monomers involved and the monomer feed ratio used, most dilutions were around 1 vol% monomer mixture in deionized water. The monomer solution was purged for 20 min with nitrogen to remove all dissolved oxygen and polymerized under UV light at an intensity of 138 mW/cm^2 for 20 min. As discussed before [7,8] nanospheres were prepared by a unique surfactant-free precipitation polymerization process. The resulting nanospheres were subsequently washed using a dialysis tubing (molecular weight cut-off of 12,000–14,000, Spectrum, Rancho Dominguez, CA) against deionized water until all unreacted monomer and other small molecular weight oligomer and polymer chains were removed.

D(+)-Trehalose was added in the amount of 1 g trehalose per g of copolymer gel nanospheres and the mixture was frozen overnight and freeze-dried to remove all water present. The resulting dry copolymer gel nanospheres were stored in a desiccator until use.

2.3. Synthesis of copolymer gel microparticles

Copolymer microparticles were prepared by crushing thin films prepared by UV polymerization. A solution of 50 wt% monomer in deionized water and ethanol was purged with nitrogen to remove all oxygen present. The solution was then pipetted between two glass microscope slides with Teflon® spacers of 0.9 mm separating the slides, and irradiated at 138 mW/cm^2 for 20 min. The resulting film was removed from the slides and washed in deionized water for 5 days to remove all unreacted monomer.

The copolymer films were then dried in a vacuum oven and crushed with mortar and pestle. The resulting dry copolymer gel particles were sieved to obtain microparticles in the size range of 150–220 μm, which were stored in a desiccator until use. The polymerization kinetics, copolymer composition, and structural analysis of these PEG-containing systems have been discussed before [11–15].

2.4. Caco-2 cell studies

All cell studies were conducted with Caco-2 cells. Caco-2 cells spontaneously differentiate and possess tight junctions, which are characteristic of the cells lining the intestine [16,17]. Thus, they serve as a good model for studying the effect of copolymer carriers on the tight junctions of the small intestine. The properties of the Caco-2 cell line are passage number dependent [18]; thus, all studies were carried out with passage numbers 60–80.

Caco-2 cells were cultured in 96-well plates (CoStar, Corning Incorp., Corning NY) with Dulbecco's modified Eagle's medium (DMEM, Sigma, St Louis, MO). After 7 days, the cell culture reached 90% confluency. At that point all DMEM were removed and replaced with Hank's balanced salt solution (HBSS, Sigma, St Louis, MO). Ranges of concentrations of copolymer gel particles were prepared in HBSS and placed in contact with the cell cultures. The pH of HBSS was 7.4, thus causing the gel particles to be in their highly swollen state [19]. Cell viability studies of nanospheres and microparticles of both P(MAA-*g*-PEG) and P(AA-*g*-PEG) were performed. While these studies were carried out at pH 7.4, we have shown before that they are relevant to the conditions of the upper small intestine [9].

The 96-well plate was incubated for 2 h at 37 °C. Then, the HBSS with copolymer gel particles was removed and the cell culture was washed three times with 200 μl of fresh HBSS to remove all remaining copolymer gel particles. A sample of 100 μl of HBSS and 20 μl of CellTiter 96® aqueous solution cell proliferation assay (Promega, Madison, WI), an NADPH reactive assay, were placed in each well and incubated for 90 min. The NADPH produced by the living cells reacted with the reagents of the assay, resulting in a colorimetric change at 490 nm. The colorimetric change was measured via UV-absorption by a microplate reader (Elx 800 NB Bio-Tek Instruments, Winooski, Vermont).

2.5. Preparation of Transwell plates

Caco-2 cell cultures were grown in the apical chambers of 6-well Transwell plates (Costar, Corning Inc., Corning NY) with DMEM media for 21 days. The membranes in the plate separating the apical and basolateral chambers had a pore size of 4.0 μm. The formation of the tight junctions between adjacent cells was monitored by measuring the resistance across the cell layer with an electrical resistance probe. After 21 days of growth, the resistance reached an equilibrium value, which indicated the monolayer's tight junctions had formed [20,21].

2.6. Transepithelial electrical resistance studies

Transwell plates were used for transepithelial electrical resistance studies (TEER). All DMEM growth media were removed and replaced with HBSS. The resistance across the cell monolayer was then measured to detect any difference in TEER value between different cell media. A sample of 10 mg of copolymer gel particles was added to the apical chamber of each well. Particles of four different copolymer gel compositions were investigated. The first system studied was nanospheres of crosslinked P(AA-*g*-PEG) with an initial feed monomer ratio of 2:1 AA:EG. P(MAA-*g*-PEG) nanospheres with a feed monomer ratio of 1:1 MAA:EG were also studied in addition to copolymer gel microparticles of P(AA-*g*-PEG) with a monomer feed ratio of 2:1 AA:EG. The final copolymer gel particles were P(MAA-*g*-PEG) microparticles with a monomer feed ratio of 1:1 MAA:EG. The control study used a blank well with cells and media subjected to identical conditions as the experimental wells.

The resistance across the cell monolayer was measured at given intervals to determine the extent to which the cell tight junctions opened [17,22].

2.7. Protein transport enhancement studies

Transwell plates were also used for the protein transport studies. The chamber on the apical side of the monolayer was filled with 2 ml of HBSS containing 10 mg of copolymer and 0.4 mg of insulin (Insulin from bovine pancreas, Sigma, St Louis, MO). Four copolymer systems of: P(AA-*g*-PEG) microparticles with monomer feed ratio of 2:1 AA:EG; P(AA-*g*-PEG) nanospheres with monomer feed ratio of 2:1 AA:EG; P(MAA-*g*-PEG) microparticles with monomer feed ratio of 1:1 MAA:EG; and P(MAA-*g*-PEG) nanospheres with monomer feed ratio of 1:1 MAA:EG were studied.

The Transwell plate was kept at a constant 37 °C for the duration of the experiment. A sample of 10 μl from the basolateral chamber of the Transwell was taken at given time intervals. A sample of 10 μl was taken from the apical chamber of the Transwell cell every hour. The insulin concentrations of the samples were analyzed via insulin ELISA (Bovine Insulin EIA, ALPCO Diagnostics, Windham, NH).

3. Results and discussion

Synthesis of the copolymer carriers for insulin delivery was conducted via two techniques. The first method was a precipitation/dispersion polymerization to prepare gel nanospheres [6,7]. The second was an in situ free-radical polymerization leading to thin films, which were subsequently crushed to obtain gel microparticles [4,5].

In the present research we conducted studies with hydrogels with a wide range of compositions as described by the AA:EG and MAA:EG molar ratios in order to evaluate their suitability as carriers for protein delivery [23–28]. This range included four monomer feed ratios of 4:1, 2:1, 1:1 and 1:2 acid to EG repeating units. The monomer feed ratio played a dominant role in the physical properties of the gel network.

As the monomer feed ratio of carboxylic acid groups was increased, the equilibrium-swelling ratio increased and approached that of a pure MAA network. Lowman and Peppas [29] concluded that the ability of the copolymer gel network to hydrogen bond, and thus complex at lower pH, went through a maximum at a monomer feed ratio of 1:1 MAA:EG. Thus, the copolymers studied here were prepared with a wide range of comonomer feed ratios of MAA to EG to examine the different characteristics of the copolymer gel networks with different compositions. It was also important to maintain an adequate amount of PEG in the copolymer gel network because PEG acts as a mucoadhesion promoter to the copolymer gel carriers as observed and discussed by Sahlin et al. [30]. P(AA-*g*-PEG) was also examined as a possible protein carrier for oral delivery.

3.1. Preparation of copolymer nanospheres and microparticles

The mechanism for insulin release from copolymer nanospheres was based on the complexation/decomplexation process due to hydrogen bonding and the associated increase of the network mesh size. The pH-sensitive nature of the copolymer nanospheres was evident when the pH of the environment was increased and the copolymer gel nanosphere mixture became transparent. From previous studies on crosslinked P(MAA-*g*-PEG) and P(AA-*g*-PEG) gel networks, it is known that as the pH of the swelling medium increased, the swelling ratio of the network increased. All copolymer network carriers studied became transparent at high pH values. The change in gel opacity as the pH was increased was caused by the hydrogen bonding complexation between the etheric oxygen of PEG and the carboxylic acid group of MAA or AA which was disrupted at high PH values leading to incorporation of more solvent into the copolymer network. This transition of the gel particles from

a phase-separated structure to a one-phase system was a unique characteristic of these complexation hydrogels.

Residual monomers present in the post-reaction mixture along with small chain oligomers were removed from the gel nanospheres mixture by partitioning with deionized water. The newly formed copolymer gel nanospheres were placed in cellulose tubing with a molecular cut-off weight of 12,000–14,000 and then placed in a deionized water bath. Washings were analyzed via UV to ensure all unwanted materials were removed from the copolymers. D(+)-Trehalose was added as a good cryoprotectant [31] and particle stabilizer.

The method of gel microparticle preparation required the use of solvent to create a favorable environment for the formation of hydrogen bonding complexation. A 50/50 wt% ethanol/water solvent was shown by Lowman and Peppas [32] as a good solvent for preparing these films.

3.2. Cell viability

The main goal of the cytotoxicity studies was to determine the cell viability of Caco-2 cell cultures in the presence of the copolymer micro- or nanospheres. The results of the cell proliferation assay were expressed in terms of the amount of NADPH produced by the cells still functioning in the culture. These were compared to the data from control cell cultures to calculate a ratio of NADPH produced, by averaging the UV absorption of wells that contained a given copolymer gel particle concentration and dividing it by the average UV adsorption of the control cell culture. This ratio was a measure of the gel particles' effect on the cell culture's viability.

As the half-life of NADPH was short, on the order of seconds, only NADPH produced by cells still viable in culture was detected. Residual NADPH from dead cells was removed with the polymer, or decayed and did not react with the tetrazolium compound to produce a colorimetric change. The results of these studies could be used to compare the behavior of microparticles of P(MAA-*g*-PEG) and P(AA-*g*-PEG) with different monomer feed ratios.

Fig. 1 shows the cell viability versus particle concentration as expressed by an NADPH production ratio for the set of P(MAA-*g*-PEG) crushed microparticles. Four different P(MAA-*g*-PEG) copolymers were synthesized with varying monomer feed ratios, of 4:1, 2:1, 1:1, 1:2 MAA:EG repeating units.

The P(MAA-*g*-PEG) copolymer microparticles caused no significant decrease in cell viability with the exception of the P(MAA-*g*-PEG) microparticles with 4:1 MAA:EG feed ratios. The P(MAA-*g*-PEG) copolymer gel microparticles of 1:2, 1:1 and 2:1 MAA:EG caused less than a 15% decrease in NADPH production compared to control cultures. All these copolymer microparticles would be suitable as oral insulin delivery devices since even at the highest concentration studied, they did not exhibit any effect on the cell cultures. The P(MAA-*g*-PEG) copolymer microparticles

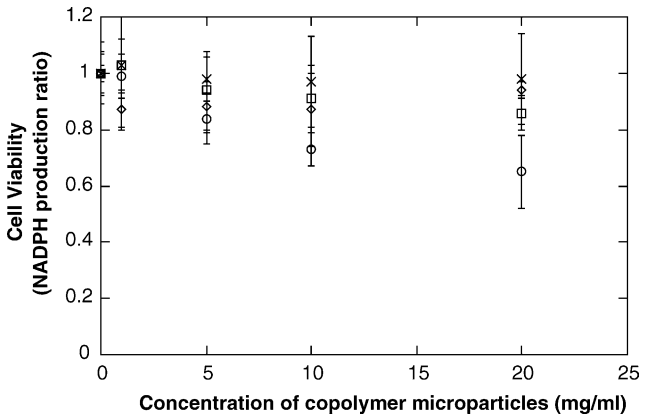

Fig. 1. Viability of Caco-2 cell culture after 2 h of contact time with various microparticles of P(MAA-*g*-PEG) with monomer MAA:EG feed ratios of: 4:1 (○), 2:1 (□), 1:1 (↓), 1:2 (⊆). Cell viability was measured by NADPH production compared to control cell culture with no copolymer present. Studies conducted with Caco-2 cell cultures ($n = 6$).

with a 4:1 MAA:EG monomer feed ratio exhibited a reduction in NADPH production of nearly 35% at the highest concentration of gel microparticles. As shown in Fig. 1, the cell viability, or ratio of NADPH production, approached unity as the concentration of copolymer gel microparticles decreased.

While the error bars of the data points overlapped for the P(MAA-*g*-PEG) microparticles with 1:2, 1:1 MAA:EG feed ratio and on occasion for those prepared from the 2:1 MAA:EG monomer feed ratio, it was observed that the gel microparticles of P(MAA-*g*-PEG) with feed monomer ratio of 1:2 MAA:EG consistently caused the lowest decrease in NADPH production. These results could be explained by the fact that the carboxylic acid group in MAA could bind calcium, an important ion in cell function [16,17], thus disrupting the calcium concentration necessary for cell function. The P(MAA-*g*-PEG) copolymer gel

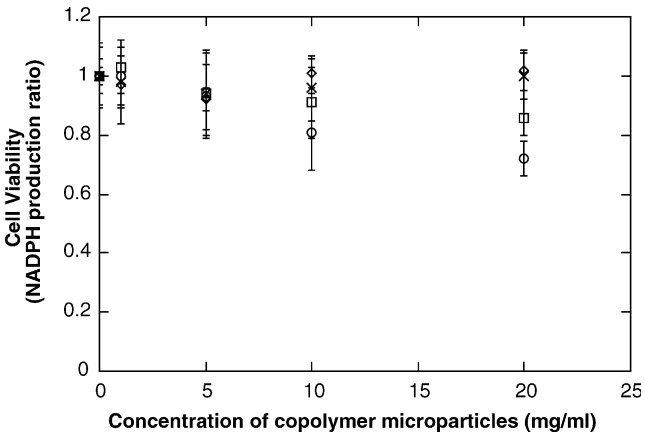

Fig. 2. Viability of Caco-2 cell culture after 2 h of contact time with various microparticles of P(AA-*g*-PEG) with monomer feed ratios of AA:EG = 4:1 (○), 2:1 (□), 1:1 (↓), 1:2 (⊆). Cell viability was measured by NADPH production compared to control cell culture with no copolymer present. Studies conducted with Caco-2 cell cultures ($n = 6$).

microparticles with 1:2 MAA:EG monomer feed ratio had the lowest calcium-binding ability.

Fig. 2 shows the cell viability for the set of P(AA-*g*-PEG) microparticles. Four different P(AA-*g*-PEG) copolymers were studied with monomer feed ratios of 4:1, 2:1, 1:1, 1:2 AA:EG repeating units.

The results indicate that at the highest copolymer gel microparticle concentration the P(AA-*g*-PEG) gel microparticles prepared with a monomer feed ratio of 1:1 and 1:2 AA:EG repeating units exhibited no cytotoxicity. The P(AA-*g*-PEG) gel microparticles prepared with monomer feed ratio of 2:1 AA:EG repeating units caused only a minor decrease in NADPH production, suggesting a slight effect on the cell viability. The P(AA-*g*-PEG) gel microparticles with monomer feed ratio of 4:1 AA:EG exhibited a more pronounced effect on the NADPH production, causing a decrease in NADPH production of nearly 30% at the 20 mg/ml gel microparticle concentration. These results indicate that the presence of large amounts of acrylic acid repeating units in complexation hydrogels with preponderence of AA of EG units, leads to highly acidic local microenvironments due to the ionization of the AA units. Therefore, hydrogels that do not exhibit a stoichiometric ratio of AA:EG may cause some cell cytotoxicity. The reader is drawn to a comparison of these results with those of Torres-Lugo et al. [9] who noted that in P(MAA-*g*-EG) the ratio of MAA:EG did not affect cell viability. Clearly, the presence of the additional $-CH_3$ in the structure has two effects on the physicochemical and cellular behavior of these hydrogels. First, it stabilizes the hydrogen bonds forced between $-COOH$ and $-O\text{-}CH_2\text{-}CH_2$-units, and then it provides protection of the cells.

The results of Figs. 3 and 4 show the cell viability of nanospheres of P(MAA-*g*-PEG) and P(AA-*g*-PEG) prepared with different monomer feed ratios. In Fig. 3 we plot the cell viability versus the concentration of P(MAA-*g*-PEG) gel nanospheres, prepared by a precipitation/

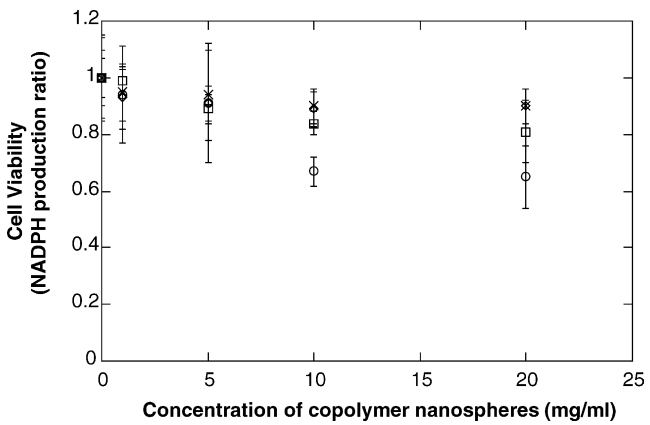

Fig. 3. Viability of Caco-2 cell culture after 2 h of contact time with various nanospheres of P(MAA-*g*-PEG) with monomer feed ratios of MAA:EG = 4:1 (○), 2:1 (□), 1:1 (↓), 1:2 (⊆). Cell viability was measured by NADPH production compared to control cell culture with no copolymer present. Studies conducted with Caco-2 cell cultures ($n = 6$).

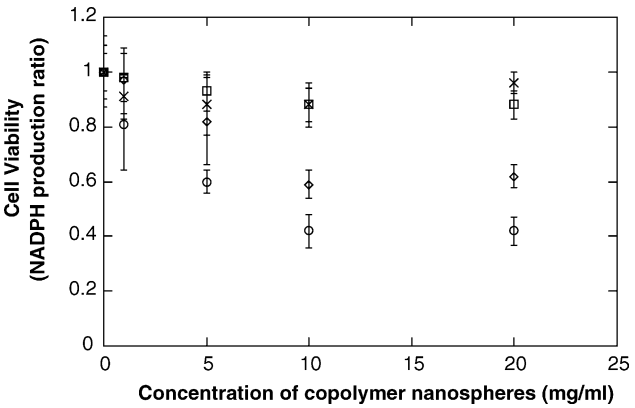

Fig. 4. Viability of Caco-2 cell culture after 2 h of contact time with various nanospheres of P(AA-*g*-PEG) with monomer feed ratios of AA:EG = 4:1 (○), 2:1 (□), 1:1 (↓), 1:2 (⊆). Cell viability was measured by NADPH production compared to control cell culture with no copolymer present. Studies conducted with Caco-2 cell cultures ($n = 6$).

dispersion polymerization technique, in contact with Caco-2 cell cultures. Cell viability was expressed as a ratio of NADPH production with respect to the control. Similar to the gel microparticles, the P(MAA-*g*-PEG) gel nanospheres with monomer feed ratios of 1:2 and 1:1 MAA:EG repeating units caused little decrease in the NADPH production of the cell culture. Both gel nanospheres caused only a 10% loss in NADPH, even at the highest concentration of nanospheres that was 20 mg/ml. The P(MAA-*g*-PEG) gel nanospheres with a monomer feed ratio of 2:1 MAA:EG caused only slightly more of a decrease in NADPH production, but the cell viability remained above 80%. The P(MAA-*g*-PEG) gel nanospheres with a monomer feed ratio of 4:1 MAA:EG caused a significant loss in NADPH production.

The P(MAA-*g*-PEG) gel nanospheres with a monomer feed ratio of 4:1 MAA:EG had a diameter of 3 μm in their fully swollen state as measured by photon correlation spectroscopy (PCS). P(MAA-*g*-PEG) gel nanospheres with monomer feed ratios of 2:1, 1:1 and 1:2 MAA:EG had diameters of 1.5, 1.1 and 0.5 μm, respectively, in their fully swollen states. At a pH of 2.0, all copolymer gel nanospheres had a diameter near 0.2 μm.

The larger decrease in cell viability observed at high concentrations of P(MAA-*g*-PEG) gel nanospheres with a monomer feed ratio of 4:1 MAA:EG was attributed to its higher content of MAA in the network. The exact mechanism by which the increased MAA caused a decrease in NADPH production is unclear. It could be related to the larger nanosphere size when compared to the other P(MAA-*g*-PEG) gel nanospheres at the conditions of the study. It could also be that the higher amount of MAA, which bound ions in solution, disrupted the local ionic equilibrium and caused a loss in NADPH production. While the mechanism was unclear, the results indicated the P(MAA-*g*-PEG) gel nanospheres with a monomer feed ratio of 4:1 cause a loss in NADPH production at concentrations of 10 and 20 mg/ml. All other monomer

feed ratio P(MAA-*g*-PEG) nanospheres caused no significant NADPH production loss. Thus, as long as the monomer feed ratio was kept at or below 2:1 MAA:EG, the copolymer gel carriers could be expected to cause no decrease in cell viability, as measured by NADPH production.

Fig. 4 shows the cell viability versus the concentration of P(AA-*g*-PEG) gel nanospheres, prepared by a dispersion polymerization technique, in contact with Caco-2 cell cultures. The P(AA-*g*-PEG) gel nanospheres with monomer feed ratios of 1:2 MAA:EG repeating units caused little decrease in the NADPH production of the cell culture. It caused only a 10% loss in NADPH even at the highest concentration of nanospheres that was 20 mg/ml. Unlike the P(MAA-*g*-PEG) gel nanospheres, the copolymer with a monomer feed ratio of 1:1 AA:EG caused a significant decrease in the production of NADPH at concentrations of 10 and 20 mg/ml. The P(AA-*g*-PEG) gel nanospheres with a monomer feed ratio of 2:1 MAA:EG caused no decrease in NADPH production. The P(AA-*g*-PEG) gel nanospheres with a monomer feed ratio of 4:1 MAA:EG caused a significant loss in NADPH production.

The P(AA-*g*-PEG) gel nanospheres with a monomer feed ratio of 2:1 AA:EG caused no significant loss in cell viability. The lack of effect on the cell viability meant the P(AA-*g*-PEG) gel nanospheres with a monomer feed ratio of 2:1 AA:EG could be good candidates for oral insulin delivery devices. This conclusion is based on the fact that copolymers with increased amounts of AA show an increased response to pH changes, since AA can disassociate at high pH values.

3.3. Transepithelial electrical resistance

It is well known that the transepithelial electrical resistance (TEER) measures the resistance to electric current across a layer of cells. In these studies, a monolayer of Caco-2 cells was grown on a porous membrane and the TEER value of the cell monolayer was measured while the culture was in contact with copolymer gel carriers in cell media. The integrity of the tight junctions between the cells could influence transport across the cell monolayer via the paracellular route [33–35]. The P(MAA-*g*-PEG) gel microparticles were shown by Torres-Lugo et al. [36] to decrease the TEER values depending on the concentration of copolymer gel microparticles. The decrease in TEER value was attributed to the opening of the tight junctions via the calcium ion binding done by the P(MAA-*g*-PEG) gel microparticles. Calcium ions in solution have been shown to be very important to the integrity of the tight junctions. Any significant loss of calcium concentration opens the tight junctions [37,38]. Madsen and Peppas [39] showed the P(MAA-*g*-PEG) gel copolymers to have a significant calcium-binding ability.

The TEER studies in this work investigated the four best candidates that were selected after the cytotoxicity studies. Each copolymer gel carrier was placed in the medium in contact with the cell culture. The TEER value was measured

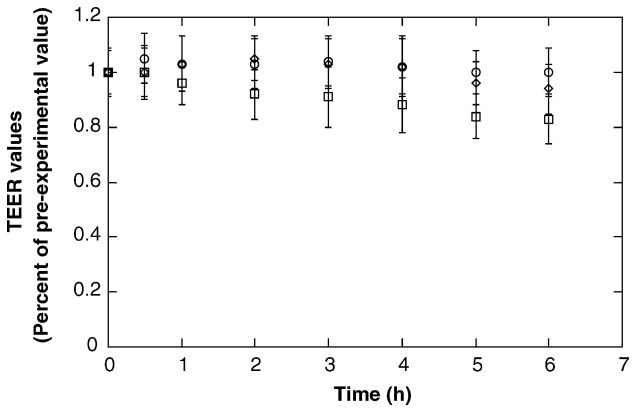

Fig. 5. TEER values of Caco-2 cell culture monolayers in contact with P(MAA-*g*-PEG) with a monomer feed ratio of 1:1 MAA:EG nanospheres (□), microparticles (↓) and control (○) as a function of time. TEER values expressed as a ratio of measured resistance value to pre-experimental resistance value ($n = 3$).

as a function of contact time to record the different effects caused by the copolymer gel carriers. Fig. 5 presents the results of the TEER experiments conducted with P(MAA-*g*-PEG) gels with monomer feed ratios of 1:1 MAA:EG repeating units. A control study was also plotted as a reference on Fig. 5. As each experiment used different cellular monolayers, the initial TEER value for each Transwell was measured and compared to the experimentally measured TEER values. The TEER value of the cellular monolayer did not decrease below 80% of the initial TEER during the 6 h studied in any of the cases shown in Fig. 5. The P(MAA-*g*-PEG) gel nanospheres with a monomer feed ratio of 1:1 MAA:EG caused a decrease in the TEER values more quickly than the P(MAA-*g*-PEG) gel microparticles. Most likely, nanospheres had a shorter calcium ion binding time which affected the opening of the tight junctions and led to subsequent decrease in TEER values.

Fig. 6 presents the results of the TEER experiments conducted with P(AA-*g*-PEG) gel carriers with monomer

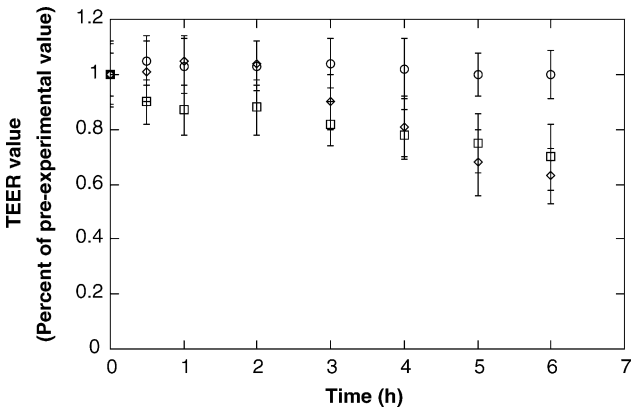

Fig. 6. TEER values of Caco-2 cell culture monolayers in contact with P(AA-*g*-PEG) with a monomer feed ratio of 2:1 AA:EG nanospheres (□), microparticles (↓) and control (○) as a function of time. TEER values expressed as a ratio of measured resistance value to pre-experimental resistance value ($n = 3$).

feed ratios of 2:1 AA:EG repeating units. It was observed that the P(AA-*g*-PEG) gel carriers with monomer feed ratios of 2:1 AA:EG both caused a reduction in the TEER value by nearly 30–35% after 6 h. This result was much larger than the 10% observed with the P(MAA-*g*-PEG) gel carrier in Fig. 5. As in Fig. 5, the copolymer gel nanospheres caused a quicker reduction in the TEER values, but after a few hours the microparticles produced a similar effect on the TEER values.

The ability of the copolymer gel carriers to increase the transport of insulin across the Caco-2 cell monolayer was investigated by conducting insulin transport studies. Torres-Lugo et al. [36] identified the mechanism of insulin transport across a Caco-2 cell monolayer as paracellular transport. Borchardt et al. [40] had previously shown that chelating calcium in solution caused a significant increase in paracellular permeability via the opening of the tight junctions.

Insulin concentration in the apical chamber was measured every hour to monitor the insulin there. Small samples were taken from the basolateral chamber for insulin analysis. The 10 μl sample of the total 2.5 ml was deemed small enough to not affect the overall insulin concentration in the basolateral chamber. The studies were conducted with 3 wells for each copolymer gel carrier system. The insulin permeated through the cell monolayer to the basolateral chamber versus time was plotted and the slope, dQ/dt, was determined. Then, the insulin permeability could be calculated from Eq. (1).

$$P_m = \left(\frac{dQ}{dt}\right)\left(\frac{1}{AC_o}\right) \quad (1)$$

Here, P_m was the permeability of the insulin across the Caco-2 cell monolayer. A was the surface area of the membrane the cells were grown on and C_o was the concentration of insulin in the apical chamber. The surface area of the membrane was 4.71 cm^2. The concentration of insulin in the apical chamber was 0.207 ± 0.005 mg/ml.

The permeability of insulin across the Caco-2 cell monolayer in the presence of the copolymer gel carriers studied is presented in Table 1. The control experiment was a transport study performed without copolymer added to the apical chamber. It must be noted that these results and the associated analysis of Eq. (1) are only approximate as they refer to steady state conditions in the apical and basolateral sides. Considering the very low permeability values for these large proteins, the variation in drug concentration in the apical side is neglible.

Table 1
Permeability of insulin across Caco-2 cell monolayers in contact with various copolymeric carriers

Copolymer system	Permeability × 10^{10} (cm/s)
P(MAA-*g*-PEG) microparticles monomer feed ratio 1:1 MAA:EG	1.93 ± 0.13
P(MAA-*g*-PEG) nanospheres monomer feed ratio 1:1 MAA:EG	1.62 ± 0.16
P(AA-*g*-PEG) microparticles monomer feed ratio 2:1 AA:EG	2.12 ± 0.12
P(AA-*g*-PEG) nanospheres monomer feed ratio 2:1 AA:EG	1.94 ± 0.13
Control	1.11 ± 0.08

$n = 3$, ± = 95%CI.

The presence of these copolymer gel carriers increased the permeability of insulin across the monolayer. The P(AA-*g*-PEG) gel microparticles with monomer feed ratios of 2:1 AA:EG repeating units caused the greatest enhancement of insulin transport, nearly doubling that of the control value. The P(MAA-*g*-PEG) gel nanospheres had a smaller impact on the transport of insulin across the monolayer than all other copolymer gel carriers. The P(MAA-*g*-PEG) gel microparticles exhibited greater enhancement of insulin transport compared to P(MAA-*g*-PEG) nanospheres as shown in some previous work [41–46].

The copolymer gel particles of P(AA-*g*-PEG) had a greater effect on the insulin permeability than the P(MAA-*g*-PEG) particles. This increased effect on the permeability could be explained by their quicker swelling dynamics and higher equilibrium-swelling ratio. The hydrogen bounding complexation in the P(AA-*g*-PEG) system was not as strong as the P(MAA-*g*-PEG) due to the lack of the methyl group to stabilize the hydrogen bonding that occurred [22]. Thus, the P(AA-*g*-PEG) copolymer probably bound calcium faster and caused a quicker opening of the tight junctions than the P(MAA-*g*-PEG) gel carriers [47–53].

4. Conclusions

We studied the behavior of P(MAA-*g*-PEG) and P(AA-*g*-PEG) nanospheres and microparticles prepared by free radical polymerization of comonomer feed ratios of 4:1, 2:1, 1:1 and 1:2 MAA:EG or AA:EG units. The cell viability studies indicated that all copolymer gel carriers studied had a negligible effect on the NADPH production of Caco-2 cell culture. This suggests that these materials would be good candidates for oral insulin delivery devices. Cell viability studies indicated that gel carriers with high MAA:EG or AA:EG monomer feed ratios, those of 4:1 MAA:EG or AA:EG, caused a significant decrease in NADPH production and would not be a suitable candidate for an oral insulin delivery device. The best candidates for an oral insulin delivery system were the P(MAA-*g*-PEG) gel carriers prepared with monomer feed ratios of 1:1 MAA:EG and the P(AA-*g*-PEG) gel carriers prepared with monomer feed ratios of 2:1 AA:EG.

TEER studies determined the copolymer gel carriers' ability to open the tight junctions between cells in a Caco-2 cell monolayer. P(AA-*g*-PEG) gel carriers had a greater ability to disrupt the tight junctions of the Caco-2 cell monolayer than the P(MAA-*g*-PEG) gel carriers.

Insulin transport across Caco-2 cell monolayers showed that all copolymer gel carriers contributed to an increase in insulin permeability. The copolymer gel carrier with the greatest effect was the P(AA-*g*-PEG) gel microparticles with a monomer feed ratio of 2:1 AA:EG prepared by in situ polymerization of thin films that were crushed to obtain microparticles.

Acknowledgements

This work was supported in part by NIH grant EB 000246.

References

[1] F. Banting, H. Best, Pancreatic extracts, J. Lab. Clin. Med. 7 (1922) 464–468.
[2] J.R. Campbell, New Opportunities in Diabetes Management, Miles Inc, Terrytown, NY, 1993.
[3] A. Lowman, N.A. Peppas, A complexation/decomplexation mechanism in ph-responsive copolymer networks, Polym. Prepr. 38 (1) (1997) 622–623.
[4] A. Lowman, N.A. Peppas, Complexation graft copolymers as oral drug delivery systems, Polym. Prepr. 38 (2) (1997) 56–57.
[5] A. Lowman, N.A. Peppas, Pulsatile drug delivery based on a complexation/decomplexation mechanism, in: S.M. Dinh, J.D. DeNuzzio, A.R. Comfort (Eds.), Intelligent Materials for Controlled Release, ACS Symposium Series, Washington, DC, vol. 728, 1999, pp. 30–42.
[6] M. Torres-Lugo, N.A. Peppas, Preparation and characterization of poly(methacrylic-*g*-poly(ethylene glycol) nanospheres, J. Nanoparticle Res. 4 (2002) 73–81.
[7] D.N. Robinson, N.A. Peppas, Preparation and characterization of ph-responsive poly(methacrylic acid-*g*-poly(ethylene glycol) nanospheres, Macromolecules 35 (2002) 3668–3674.
[8] C. Donini, D.N. Robinson, P. Colombo, F. Giordano, N.A. Peppas, Preparation of nanospheres for methacrylic monomers for pharmaceutical applications, Intern. J. Pharm. 245 (2002) 83–91.
[9] M. Torres-Lugo, M. Garcia, M. Record, N.A. Peppas, pH-Sensitive hydrogels as gastrointestinal tract absorptions enhancers: transport mechanisms of salmon calcitonin and other model molecules using the caco-2 cell model, Biotechnol. Progr. 18 (2002) 612–616.
[10] M. Torres-Lugo, M. Garcia, M. Record, N.A. Peppas, Physicochemical behavior and cytotoxic effects of P(MAA-*g*-EG) nanospheres for oral delivery of proteins, J. Control. Release 80 (2002) 197–205.
[11] R.A. Scott, N.A. Peppas, Highly crosslinked, PEG-containing copolymers for sustained solute delivery, Biomaterials 20 (1999) 1371–1380.
[12] J.H. Ward, A. Shahar, N.A. Peppas, Kinetics of living radical polymerizations of multifunctional monomers, Polymer 43 (2002) 1745–1752.
[13] R.A. Scott, N.A. Peppas, Kinetics of copolymerization of PEG-containing multiacrylates with acrylic acid, Macromolecules 32 (1999) 6149–6158.
[14] R.A. Scott, N.A. Peppas, Compositional effects on network structure of highly crosslinked copolymers of PEG-containing multiacrylates with acrylic acid, Macromolecules 32 (1999) 6139–6148.
[15] N.A. Peppas, Y. Huang, M. Torres-Lugo, J.H. Ward, J. Zhang, Physicochemical foundations and structural design of hydrogels in medicine and biology, Ann Rev. Biomed. Engng 2 (2000) 9–29.
[16] G. Borchard, H.L. Luessen, A.G. de Boer, J.C. Verhoef, C.M. Lehr, H.E. Junginger, The potential of mucoadhesive polymers in enchancing intestinal peptide drug absorption. III: effects of chitosan-glutamate and carbomer on epithelial tight junctions in vitro, J. Control. Release 39 (1996) 131–138.
[17] R.T. Borchardt, G.M. Pauletti, F.W. Okumu, Effect of size and charge on the passive diffusion of peptides across caco-2 cell monolayers via the paracellular pathway, Pharm. Res. 14 (1997) 164–168.
[18] R.T. Borchardt, T.J. Raub, I.J. Hidalgo, S.L. Kuentzel, Characterization of the caco2 cell line as an intestinal epithelial model system, Fed. Am. Soc. Exp. Biol. J. 2 (1988) A734.
[19] R.T. Borchardt, I.J. Hidalgo, T.J. Raub, Permeability properties of an intestinal epithelial model system (caco-2 cell), Fed. Am. Soc. Exp. Biol. J. 2 (1988) A733.
[20] R.T. Borchardt, K.M. Hillgren, A. Kato, In-vitro systems for studying intestinal drug absorption, Med. Res. Rev. 15 (1995) 83–109.
[21] H. Yu, T.J. Cook, P.J. Sinko, Evidence for diminished functional expression of intestinal transporters in caco-2 cell monolayers at high passages, Pharm. Res. 14 (1997) 757–762.
[22] A.M. Lowman, M. Morishita, M. Kajita, T. Nagai, N.A. Peppas, Oral delivery of insulin using pH-responsive complexation gels, J. Pharm. Sci. 88 (1999) 933–937.
[23] J. Klier, N.A. Peppas, Complex-forming hydrogels sensitive to physiological conditions, Proc. Adv. Biomed. Polym. 1 (1989) 107–109.
[24] C.L. Bell, N.A. Peppas, Complexation in P(EG-*g*-MAA) hydrogels, Polym. Prepr. 34 (1) (1993) 831–832.
[25] N.A. Peppas, K.B. Keys, M. Torres-Lugo, A.M. Lowman, Poly(ethylene glycol) containing hydrogels in drug delivery, J. Control. Release 62 (1999) 81–87.
[26] M. Torres-Lugo, N.A. Peppas, Molecular design and in vitro studies of novel pH-sensitive hydrogels for the oral delivery of calcitonin, Macromolecules 32 (1999) 6646–6651.
[27] N.A. Peppas, P. Bures, W. Leobandung, H. Ichikawa, Hydrogels in pharmaceutical formulations, Eur. J. Pharm. Biopharm. 50 (2000) 27–46.
[28] A.M. Lowman, N.A. Peppas, Hydrogels, in: E. Mathiowitz (Ed.), Encyclopedia of Controlled Drug Delivery, Wiley, New York, NY, 1999, pp. 397–418.
[29] A.M. Lowman, N.A. Peppas, Molecular analysis of interpolymer complexation in graft copolymer networks, Polymer 41 (2000) 73–80.
[30] J.J. Sahlin, N.A. Peppas, Hydrogels as muco- and bioadhesive materials: a review, Biomaterials 17 (1996) 1553–1561.
[31] M. Joshi, A. Misra, Dry powder inhalation of liposomal Ketotifen fumarate: formulations and charaterization, Intern. J. Pharm. 223 (2001) 15–27.
[32] A.M. Lowman, N.A. Peppas, Solute transport analysis in pH-responsive, complexing hydrogels of poly(methacrylic-*g*-ethylene glycol), J. Biomater. Sci. Polym. Ed. 10 (1999) 999–1009.
[33] S. Yee, In vitro permeability across caco-2 cells(colonic) can it predict in vivo (small intestinal) absorption in man-fact or myth, Pharm. Res. 14 (1997) 763–766.
[34] B. Gumbiner, Biochemistry and assembly of epithelial tight junctions, Am. J. Physiol. 253 (1987) C749–C758.
[35] R.T. Borchardt, P.S. Burton, R.A. Conradi, N.F.H. Ho, A.R. Hilgers, How structural features influence biomembranes permeability of peptides, J. Pharm. Sci. 85 (1996) 1336–1340.
[36] M. Torres-Lugo, H. Ichikawa, N.A. Peppas, Cytotoxicity and transport enhancement of proteins through cell monolayers using novel pH-sensitive hydrogels, Proc., World Meet. APV/APGI 3 (2000) 201–202.
[37] A.W. Sedar, J.G. Forte, Effect of calcium depletion on the junctional complex between oxyntic cells of the gastric glands, J. Cell Biol. 22 (1964) 173–188.
[38] F. Lacaz-Vieira, Calcium site specificity, early Ca + 2-related tight junction events, J. Gen. Physiol. 110 (1997) 727–740.

[39] F. Madsen, N.A. Peppas, Complexation graft copolymer networks: swelling properties, calcium binding, and proteolytic enzyme inhibition, Biomaterials 20 (1999) 1701–1708.
[40] R.T. Borchardt, G. Knipp, N. Ho, C. Barsuhn, Paracellular diffusion in Caco-2 cell monolayers: effect of perturbation on the transport of hydrophilic compounds that vary in charge and size, J. Pharm. Sci. 86 (1997) 1105–1110.
[41] B. Kim, N.A. Peppas, Synthesis and characterization of pH-sensitive glycopolymers for oral drug delivery systems, J. Biomater. Sci. 13, Polym. Ed. 13 (2002) 1271–1281.
[42] O. Sipahigil, M. Torres-Lugo, N.A. Peppas, FTIR spectroscopic analysis of protein/carrier interactions in novel protein delivery systems, STP Pharma 12 (2002) 345–350.
[43] B. Kim, K. La Flamme, N.A. Peppas, Dynamic swelling behavior of pH-sensitive anionic hydrogels used for protein delivery, J. Appl. Polym. Sci. 89 (2003) 1606–1613.
[44] B. Kim, N.A. Peppas, Analysis of molecular interactions in P(MAA-*g*-EG) hydrogels, Polymer 44 (2003) 3701–3707.
[45] B. Kim, N.A. Peppas, PEG-containing hydrogel microparticles for oral protein delivery applications, Biomed. Microdevices 5 (2003) 333–341.
[46] B. Kim, N.A. Peppas, In vitro release behavior and stability of insulin in complexation hydrogels as oral drug delivery carriers, Intern. J. Pharm. 266 (2003) 29–37.
[47] H. Ichikawa, N.A. Peppas, Novel complexation hydrogels for oral peptide delivery: in vitro evaluation of their cytocompatibility and insulin-transport enhancing effects using caco-2 cell monolayers, J. Biomed. Mater. Res. 67 (2003) 609–617.
[48] J.E. López, N.A. Peppas, Cellular evaluation of insulin transmucosal delivery, J. Biomater. Sci., Polym. Ed. (2004) in press.
[49] J.E. López, N.A. Peppas, Effect of PEG molecular weight and microparticle size on oral insulin delivery from P(MAA-*g*-EG) microparticles, Drug Dev. Ind. Pharm. (2004) press in press.
[50] N.A. Peppas, Devices based on intelligent biopolymers for oral protein delivery, Intern. J. Pharm. (2004) in press.
[51] R. Langer, N.A. Peppas, Advances in biomaterials, drug delivery, and bionanotechnology, Am. Inst. Chem. Eng. J. 49 (2003) 2990–3006.
[52] K. Nakamura, R.J. Murray, J.I. Joseph, N.A. Peppas, M. Morishita, A.M. Lowman, Oral insulin delivery using P(MAA-*g*-EG) hydrogels: effects of network morphology on insulin delivery characteristics, J. Control. Release 95 (2004) 589–599.
[53] M. Morishita, T. Goto, N.A. Peppas, J.I. Joseph, M.C. Torjman, C. Munsick, K. Nakamura, K. Takayama, A.M. Lowman, Mucosal insulin delivery systems based on complexation polymer hydrogels: effect of particle size on insulin enteral absorption, J. Control. Release (2004) in press.