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pH-Responsive Hydrogels with Dispersed Hydrophobic Nanoparticles for the Delivery of Hydrophobic Therapeutic Agents

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Abstract

To investigate the delivery of hydrophobic therapeutic agents, a new class of polymer carriers was synthesized. These carriers are composed of two components: (i) a pH-responsive hydrogel composed of methacrylic acid grafted with poly(ethylene glycol) tethers, P(MAA-g-EG), and (ii) hydrophobic poly(methyl methacrylate) (PMMA) nanoparticles. Before the P(MAA-g-EG) hydrogel was crosslinked, PMMA nanoparticles were added to the solution and upon exposure to UV light they were photoencapsulated throughout the P(MAA-g-EG) hydrogel structure. The pH-responsive behavior of P(MAA-g-EG) is capable of triggered release of a loaded therapeutic agent, such as a low molecular weight drug or protein, when it passes from the stomach (low pH) to upper small intestine (neutral pH). The introduction of PMMA nanoparticles into the hydrogel structure affected the swelling behavior, therapeutic agent loading efficiency, and solute release profiles. In equilibrium swelling conditions the swelling ratio of nanoparticle-containing hydrogels decreased with increasing nanoparticle content. Loading efficiencies of the model therapeutic agent fluorescein ranged from 38 – 51 % and increased with increasing hydrophobic content. Release studies from neat P(MAA-g-EG) and the ensuing P(MAA-g-EG) hydrogels containing nanoparticles indicated that the transition from low pH (2.0) to neutral pH (7.0) triggered fluorescein release. Maximum fluorescein release depended on the structure and hydrophobicity of the carriers used in these studies.

Keywords

hydrogels; pH-responsive; nanoparticles; fluorescein; controlled release; drug delivery

INTRODUCTION

Using traditional synthetic techniques for polymer development, but assembling them into new structures or architectures may lead to significant improvements of materials which can be used in biomedical and clinical applications. Of particular interest to us are biomaterials based on pH-responsive hydrogels. Hydrogels are three-dimensional networks, insoluble in aqueous environments due to physical and/or chemical crosslinks, and able to imbibe large amounts of water or biological fluids.¹ A variety of pH responsive, biocompatible polymers

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exhibiting anionic or cationic properties allow these hydrogel networks to be tailored or modified to exhibit desirable physicochemical properties appropriate for the application at hand.² Of particular interest is the synthesis of polymer carriers that are responsive to specific pH ranges, resulting in drastic property changes from the collapsed to swollen state. These drastic changes can be used to achieve local and targeted drug, peptide, or protein delivery.

For these reasons, pH-responsive hydrogels exhibit promising results as polymer carriers for oral delivery of a variety of proteins and peptides which are traditionally administered parenterally or through subcutaneous injections. However, these hydrogels are hydrophilic by design and their translation for use and interaction with hydrophobic, low molecular weight therapeutic agents, is limited and needs to be optimized or modified for greater success. It is proposed that these hydrogels can be balanced with opposing hydrophobic properties which may prove advantageous for the delivery of hydrophobic, low molecular weight therapeutic agents.

Chemotherapeutics are an example of hydrophobic, low molecular weight agents primarily administered intravenously for systemic delivery in the treatment of cancer. The development of oral chemotherapeutics would eliminate the need for intravenous delivery and potentially improve patient comfort,³ be cost-effective,⁴ and give the patient better control over the dosage and environment of the administered chemotherapeutic agent.⁵ A small number of chemotherapeutic agents have been orally delivered and shown promising results such as lower toxicity and clinical efficacy that is equal to or sometimes improved when compared to intravenous treatment.^{6–12,10, 13}

Despite the advantages, there are still obstacles that must be overcome for oral administration of chemotherapeutics including: (i) determination if the chemotherapeutic should transport across the epithelium of the gastrointestinal (GI) tract into the bloodstream (breast or liver cancer) or remain in the GI tract for local delivery (colorectal cancer); (ii) the potential harm to the stomach and GI tract due to the toxicity of the agent; and (iii) the toxic agent being exposed to the low pH and degrading enzymes of the stomach, resulting in reduced activity or inactivation of the toxic agent. pH-responsive hydrogels possess the necessary characteristics for protecting the chemotherapeutic agent and the GI tract from each other, but must be modified with hydrophobic polymers for preferential loading of hydrophobic agents.

In this work, we combined the desirable characteristics of pH-sensitive, hydrophilic networks with hydrophobic nanoparticles to develop amphiphilic polymer structures for bioapplications.^{14–17} Due to their small size, nanoparticles can possess unique characteristics different from their larger parent counterparts and can be easily incorporated into existing polymer structures to elicit new physical and chemical properties.¹⁸ Research in the nanoparticle field has grown significantly in the last decade and a host of established techniques are available to develop nanoparticles of desirable sizes and properties^{19, 20} In our studies, we synthesized and characterized polymer carriers composed of a pH-responsive, hydrophilic polymer, P(MAA-g-EG), prepared from methacrylic acid (MAA) grafted with poly(ethylene glycol) tethers, and hydrophobic poly(methyl methacrylate) (PMMA) nanoparticles (Fig. 1).

The optimal design of P(MAA-g-EG) hydrogels has been determined by our laboratory previously.^{21–25} The carboxyl groups on the MAA moiety form a hydrogen bond with the etheric oxygen of the PEG chain forming a tight, collapsed network in low pH. In neutral pH, the carboxyl groups become deprotonated causing an expanded network due to ionic repulsion. These hydrogels have been used to orally deliver insulin,^{26–28} calcitonin,^{29–31} and

bleomycin^{32, 33} using the pH shift from the stomach (low pH) to the upper small intestine (neutral pH) as the physiological trigger for releasing entrapped drug.

By photoencapsulating PMMA nanoparticles during the UV polymerization using P(MAA-g-EG) hydrogels, we can vary the degree of hydrophobicity of the final polymer carrier and elicit a distribution of physical properties. The effect of PMMA nanoparticles on the loading and release of fluorescein, a model hydrophobic agent, is determined. The transition from the stomach to the upper small intestine may be used as a trigger for release and is further investigated here.

EXPERIMENTAL

Materials

Methacrylic acid (MAA), methyl methacrylate (MMA), tetraethylene glycol dimethacrylate (TEGDMA), 1-hydroxycyclohexyl phenyl ketone (Irgacure[®] 184), ammonium persulfate (APS), 3,3-dimethylglutaric acid (DMGA), Nile Red, dimethyl sulfoxide (DMSO), and ethanol were purchased from Sigma–Aldrich (St. Louis, MO). 10× phosphate buffer solution (PBS) and sodium chloride were purchased from Fisher Scientific (Fair Lawn, NJ). Poly(ethylene glycol) monomethyl ether monomethacrylate (PEGMMA; 1000 g mol⁻¹) was from Polysciences Inc. (Warrington, PA). All chemicals were used as received except for MAA which was vacuum distilled at 54 °C and 25 mmHg (give also in Pa) prior to use to remove the inhibitor hydroquinone. Double distilled water was used in all studies.

Synthesis of PMMA Nanoparticles

A surfactant-free emulsion polymerization (SFEP) technique for the formation of PMMA nanoparticles was adapted for our polymer carrier.³⁴ Monomer MMA and crosslinker TEGDMA were added to a round bottom flask containing water and the thermal initiator APS. MMA and APS were added in the amount of 0.56 M and 8 mM, respectively, based on total solution volume while TEGDMA was added in the amount of 0.6 mol%, based on MMA content. The polymerization was carried out for 3 h at 75 °C in a temperature-controlled water bath under magnetic stirring. Cross-linked PMMA nanoparticles were cooled down to room temperature and dialyzed in water for 10 days with water changed daily to remove any unreacted monomer or thermal initiator. The final nanoparticles were lyophilized until dry.

Synthesis of P(MAA-g-EG) Hydrogels Dispersed with PMMA Nanoparticles

Monomers MAA and PEGMMA were added in a 1:1 molar ratio while the crosslinking agent TEGDMA and the initiator Irgacure[®] 184 were added in the amount of 0.75 mol% and 0.5 wt.% of total monomer, respectively. PMMA nanoparticles were added to this solution in the amount of 1, 2.5, or 5 wt% of the combined weight of MAA and PEG. A 50:50 (w w⁻¹) solution of ethanol in water was added in a 50:50 (w w⁻¹) ratio of total monomer to solvent. The solution was sonicated, purged with N₂, placed between glass slides, and exposed to UV light (Dymax 2000-EC Light Curing System, Torrington, CT) at an intensity of 16 mW cm⁻² for 30 min. The resulting nanoparticle-containing hydrogel was washed for 10 days with water and punched into discs or dried and crushed into particles (75 – 150 μm) for future use. Both particles and discs were dried in vacuum at 30 °C for 1 week. P(MAA-g-EG) hydrogels decorated with 1, 2.5, or 5 wt% PMMA nanoparticles will be identified as 1%-NP, 2.5%-NP, 5%-NP henceforth.

SEM Imaging of PMMA Nanoparticles, P(MAA-g-EG), and 1–5%-NP

Scanning electron microscopy (SEM, Supra 40 VP, Carl Zeiss Inc.) was used to characterize the PMMA nanoparticles and to approximate their nanoparticle diameter, uniformity,

dispersion after being encapsulated in P(MAA-g-EG) hydrogels, and the stability of encapsulation in the swollen P(MAA-g-EG) network. Pure PMMA nanoparticles were dissolved in a small amount of ethanol, dropped onto a carbon taped aluminum stub and the solvent allowed to fully evaporate, all other samples were vacuum dried and placed directly onto carbon taped aluminum stubs. The surface and cross section of P(MAA-g-EG) and 1–5%-NP were imaged in the collapsed and swollen state by placing discs in 0.1 N HCl and 1× PBS (pH 7.4), respectively, for 24 h, flash frozen in liquid N₂, and lyophilized until dry.³⁵ To determine if nanoparticles were being released during swelling, a 5%-NP disc was cycled between the dry and swollen state multiple times and then imaged. All samples were coated with an 8 nm thick layer of Platinum/Palladium for improved image quality. Image analysis of PMMA nanoparticles was performed using ImageJ software and reported diameter sizes are of n=800.

Characterization of PMMA Nanoparticles in P(MAA-g-EG) Hydrogels

Nile Red, a hydrophobic dye, was used to confirm the presence of hydrophobic domains in hydrogels.³⁶ The following protocol was used to stain PMMA nanoparticles: a 20 mg mL⁻¹ stock solution of Nile Red was prepared by dissolving in methanol, 75 µL of this solution was added to 120 mL of 1× PBS and allowed to stir for several minutes. P(MAA-g-EG) and 1–5%-NP discs were soaked in the final working solution for 24 h and then washed several days with water. Canon Digital Photo Professional software was used to enhance luminance, red, green, and blue tone curves were adjusted to correct for white balance to allow the distinction in coloring between the different samples.

The average hydrodynamic diameters, polydispersity indices (PDI), and zeta potential of the PMMA nanoparticles were measured using dynamic light scattering (DLS, NanoZS, Malvern Instruments). Particle size and zeta potential measurements were conducted by resuspending polymerized particles in 1× PBS at 0.1 mg mL⁻¹ and measuring at 25 °C. The values reported are the average of 10 individual runs.

Swelling Behavior of P(MAA-g-EG) and 1–5%-NP

Equilibrium swelling studies were used to study the pH-responsive behavior of P(MAA-g-EG) and 1–5%-NP. Equilibrium swelling behavior was determined by swelling P(MAA-g-EG) or 1–5%-NP discs in 0.1 M DMGA buffers ranging from pH 3.2 to 7.2 which maintained ionic strength using sodium chloride. Polymer discs were placed in the 37 °C DMGA buffers for 24 h, blotted to remove excess solution, and weighed to determine the equilibrium weight swelling ratio.

Loading Fluorescein in P(MAA-g-EG) and 1–5%-NP

Fluorescein served as a model solute with properties similar to traditional chemotherapeutics including similar hydrophobicity, molecular weight, and chemical structure.^{37, 38} Fluorescein was loaded by equilibrium partitioning in the following manner: a fluorescein stock solution was made with 2 wt% DMSO in 1× PBS (pH 7.4) to a final concentration of 0.145 mg mL⁻¹. A 5 mg mL⁻¹ concentration of P(MAA-g-EG) or 1–5%-NP crushed particles to fluorescein stock solution was allowed to stir for 24 h. Then the particles were collapsed by adding 1 N HCl to entrap fluorescein within the network, filtered, and rinsed with water and 0.1 N HCl to remove any surface absorbed fluorescein. A fluorescent plate reader (Biotek Synergy-HT, Winooski, VT), operating at a 485 nm excitation and 590 nm emission wavelengths, determined the concentration levels and calculated the loading efficiency as follows:

$$\text{Loading Efficiency} = \frac{C_o - C_f}{C_o} * 100 \quad (1)$$

where C_o is the initial fluorescein concentration and C_f is the final fluorescein concentration remaining in the solution.

Fluorescein Release Studies

Release experiments were performed on a dissolution apparatus (Distek Dissolution System 2100B, North Brunswick, NJ) operating at 100 rpm and 37 °C. For fluorescein release in neutral pH, a concentration of 0.33 g L⁻¹ of fluorescein loaded microparticles to 1× PBS (pH 7.4) was used. Over the duration of 6 h, samples were taken and replaced with a solution of 1× PBS (37 °C, pH 7.4) to maintain sink conditions. Fluorescein release in low pH was conducted in the same manner as neutral pH except 1× PBS was adjusted to a pH of 2.0 using 1 N HCl.

A two-step pH change from low pH (2.0) to high pH (7.0) was used as a model for the physiological conditions and residence time in the stomach and small intestine.³⁹ Fluorescein loaded microparticles were first placed in 1× PBS at pH 2.0 for 90 min and then 5 N NaOH was added to increase the pH to 7.0 where release continued for 6 h. Samples were obtained as above. The weight of fluorescein released was determined by the fluorescent plate reader and reported as follows:

$$\text{Mass Released} = M_t / M_{\infty} * 100 \text{ replace "Mass" by "weight"} \quad (2)$$

where M_t is weight released at given time and M_{∞} is total weight released.

RESULTS AND DISCUSSION

New polymer carriers composed of a pH-responsive P(MAA-g-EG) hydrogel containing PMMA nanoparticles were synthesized. PMMA nanoparticles were formed using a conventional SFEP procedure. The PMMA nanoparticles were then added to the monomer solution of MAA, PEG, TEGDMA, Irgacure[®] 184, and encapsulated during the free radical UV-initiated polymerization process. The ensuing polymer carriers' swelling, loading, and release profiles were determined and their performance as potential oral delivery devices for hydrophobic agents evaluated.

SEM of PMMA Nanoparticles, P(MAA-g-EG), and 1–5%-NP

Dry nanoparticles were spherical, uniform, and their diameters were determined to be 212 ± 21 nm (Fig. 2). SEM images illustrated nanoparticles were present on the surface and in the bulk of the P(MAA-g-EG) hydrogel (Fig. 3) and existed as small aggregates (1 – 5 μm) resulting in micro-domains of hydrophobicity. These micro-domains are formed by nanoparticles which were not fully dispersed in the aqueous solution before UV polymerization. It has been reported that as a result of the drying process of the nanoparticles, significant hydrogen bonding occurs and makes it difficult to re-disperse them in aqueous media or even organic solvents.⁴⁰

At pH values above the pKa of carboxyl groups on the MMA moiety, the hydrogels form porous networks allowing therapeutic agents to flow in or out based on the diffusional gradient. Fig. 4 shows high porosity of the P(MAA-g-EG) hydrogel sample and how these pores can serve as the transport mechanism for therapeutic agent loading and release. Similar porous networks were imaged for 1–5%-NP, but are not included. Dialyzed nanoparticles were sufficiently washed to remove any residual unreacted monomer and

thermal initiator and thus have no chemical means to be crosslinked into the P(MAA-g-EG) hydrogel, but rather physically restrained. Nanoparticle containing hydrogels were cycled between low and high pH to test whether physically restrained nanoparticles would be released in physiological conditions. SEM images before and after repeated swelling cycles indicate nanoparticles remain intact within the P(MAA-g-EG) (not pictured).

Characterization of PMMA Nanoparticles in P(MAA-g-EG) Hydrogels

The existence of the PMMA nanoparticles in the P(MAA-g-EG) hydrogel was confirmed by soaking in Nile Red for 24 h. P(MAA-g-EG) samples remained clear and translucent while 1–5%-NP samples increased in pink opaqueness with increasing PMMA nanoparticle content (Fig. 5).

Z-Average hydrodynamic diameter in 1× PBS (pH 7.4) was determined to be 199 nm and narrowly distributed (PDI: 0.17). These values support the particle diameter as determined by SEM and confirm that PMMA nanoparticles do not undergo any appreciable swelling in the presence of aqueous solutions. Zeta potential of these nanoparticles measured -56.4 ± 3.5 mV and are considered stable because particles with zeta potentials above 30 mV or below -0 mV maintain their repulsive forces while dispersed.^{41, 42}

Swelling Behavior of P(MAA-g-EG) and 1–5%-NP

Swelling experiments were completed to study the pH-responsive behavior of the polymer drug carriers in simulated physiological fluids and the effects of introducing hydrophobic PMMA nanoparticles into the P(MAA-g-EG) hydrogel. For equilibrium swelling experiments, minimal swelling occurred in gastric conditions (low pH) due to the anionic nature of the P(MAA-g-EG) network and the hydrogen bonding between the carboxyl groups on the MMA moiety and the etheric oxygen of the PEG chain. Until the pKa (4.8 – 4.9) of these carboxyl groups is surpassed, little swelling is expected.^{43, 44}

Equilibrium weight swelling ratios decreased with the increasing amount of PMMA nanoparticle content (Fig. 6). The increasing presence of the PMMA nanoparticles reduces or shields ionic repulsion between deprotonated carboxyl groups and physically reduces the volume for polymer chain movement. Furthermore, as the weight percent of PMMA nanoparticles increases, the space once occupied by a water molecule is replaced by a PMMA nanoparticle and results in the reduction of water uptake into the polymer structure. Each of these items can contribute to the reduction of equilibrium weight swelling ratios. Modifications to the original P(MAA-g-EG) hydrogel have been completed previously and influenced swelling properties similarly. Modifications such as incorporating hydrophobic networks through interpenetrating networks⁴⁵ and grafting moieties for increased mucoadhesion have been completed previously, and have shown to influence swelling properties similarly.^{46, 47}

Loading Fluorescein in P(MAA-g-EG) and 1–5%-NP

The structure and degree of hydrophobicity of the polymer carrier caused different loading efficiencies. Loading efficiencies increased with increasing amount of PMMA nanoparticle content with values of 37 ± 2 %, 41 ± 3 %, 43 ± 1 %, 51 ± 1 %, corresponding to P(MAA-g-EG), 1%-NP, 2.5%-NP, 5%-NP, respectively. The highest efficiency achieved by the 5%-NP hydrogel was due to the highest weight percentage of nanoparticles present in the P(MAA-g-EG) hydrogel. The absence of the hydrophobic nanoparticles resulted in the reduced loading level for P(MAA-g-EG) as compared to the 1–5%-NP. However, the high equilibrium weight-swelling ratio of P(MAA-g-EG) allowed it to still maintain loading levels close to the 1%-NP and 2.5%-NP. Weight percent loading, defined as milligrams of

fluorescein per milligram of polymer, was 1.1 %, 1.2 %, 1.2 %, and 1.4 % for P(MAA-g-EG), 1%-NP, 2.5%-NP, and 5%-NP, respectively.

Fluorescein Release

In vitro release studies completed at constant and two step pH changes were performed and correlated to *in vivo* behavior. In neutral pH, 1–5%-NP and P(MAA-g-EG) reached maximum fluorescein release by 2 h with over 90% of fluorescein released (Fig. 7). Since the media was maintained at a pH 7.4 and the pKa value of MAA is 4.8 – 4.9, the polymer carriers have developed the necessary porous structure for fluorescein to diffuse out into the surrounding solution.

1–5%-NP fluorescein release reached a maximum of 18 % in low pH conditions, while P(MAA-g-EG) released nearly half of its loaded fluorescein (Fig. 8). The presence of the PMMA nanoparticles and its hydrophobic properties allows preferential association with fluorescein to reduce its release in low pH conditions as compared to the P(MAA-g-EG) hydrogel. Fluorescein release performed in low pH conditions for P(MAA-g-EG) and 5%-NP observed the same burst effect for P(MAA-g-EG) (Fig. 9).

After 90 min, the pH was stepped from 2.0 to 7.0 and an immediate increase in the amount of fluorescein released was observed for all polymer carriers and reached a maximum in 2 h (Fig. 8). At pH 7.0, P(MAA-g-EG) exhibited a faster release rate than the PMMA containing hydrogels. This can be explained by the presence of fluorescein at or near the surface of the polymer as a result of the diffusional front already initiated in the low pH conditions. Final fluorescein concentrations ranged from 3.29 – 6.63 $\mu\text{g mL}^{-1}$.

CONCLUSION

By encapsulating hydrophobic PMMA nanoparticles during the UV polymerization of P(MAA-g-EG), we were able to develop hydrogels with amphiphilic properties. The amount of PMMA nanoparticles in the hydrogel systems affected the swelling properties, loading efficiencies, and release profiles.

In equilibrium swelling conditions the swelling ratio decreased with increasing PMMA nanoparticle content. The presence of the PMMA nanoparticles influenced the physical properties of the polymer carriers in several ways: 1) reduces or shields ionic repulsion between deprotonated carboxyl groups, 2) reduces free volume for polymer chain movement of the pH responsive P(MAA-g-EG) network, and 3) reduces water uptake as a result of increased hydrophobicity. Fluorescein loading efficiencies increased with increasing PMMA content and ranged from 38 – 51 % with the 5%-NP achieving the highest.

1–5%-NP release less fluorescein than P(MAA-g-EG) in low pH conditions. Reduction in drug release in low pH conditions is advantageous because it reduces possible toxicity to the stomach wall, avoids degradation or inactivation by stomach secretions such as hydrochloric acid or pepsin, and increases the amount capable of releasing in the small intestine. Fluorescein concentration levels reached 3.29 – 6.63 $\mu\text{g mL}^{-1}$ when the pH shifted from low to neutral. Tumors must be exposed to minimal concentration levels of chemotherapeutics to be effectively destroyed, i.e. 5-fluoruracil requires 0.2 $\mu\text{g mL}^{-1}$ and doxorubicin requires 5 $\mu\text{g mL}^{-1}$.^{48, 49} For these polymer carriers to be effective, an accurate understanding of the loading efficiency and release profiles must be determined so that the administration of a chemotherapeutic agent is appropriate to destroy tumors while minimizing side effects to surrounding healthy tissue.

The P(MAA-g-EG) hydrogel uses the pH shift from the stomach to the small intestine as a physiological trigger for therapeutic release while the PMMA nanoparticles' hydrophobicity preferentially associated with hydrophobic agents. When combined, it is possible these polymer carriers may serve as viable oral carriers for chemotherapeutic agents, improve patient care and comfort, and reduce healthcare costs.

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Figure 1.
Scheme for the development of P(MAA-g-EG) hydrogels dispersed with hydrophobic PMMA nanoparticles.

Figure 2.
Spherical and uniform PMMA nanoparticles (scale bar = 200 nm).

Figure 3. Cross section of P(MAA-g-EG) hydrogel dispersed with PMMA nanoparticles (scale bar = 20 μm ; insert scale bar = 1 μm).

Figure 4.
Porous network formed at neutral pH of P(MAA-g-EG) hydrogel (scale bar = 20 μm).

Figure 5.
From left to right, Nile Red staining of P(MAA-g-EG), 1%-NP, 2.5%-NP, 5%-NP.

Figure 6. pH-Dependent equilibrium weight swelling ratio profile of P(MAA-g-EG) (◆), 1% NP (□), 2.5% NP (▲), 5% NP (○). Curves generated are for $n = 3$ studies.

Figure 7. Fluorescein release in neutral pH of P(MAA-g-EG) (◆), 1%-NP (□), 2.5%-NP (▲), 5%-NP (○) crushed particles (75 – 150 μm). Fluorescein release is expressed as $M_t M_\infty^{-1}$. Curves generated are for $n = 3$ studies.

Figure 8. Fluorescein release in two step pH change from 2.0 to 7.0 of P(MAA-g-EG) (◆), 1%-NP (◻), 2.5%-NP (▲), 5%-NP (○) crushed particles (75 – 150 μm). Fluorescein release is expressed as $M_t M_\infty^{-1}$. Curves generated are for $n = 3$ studies.

Figure 9.

Fluorescein release of P(MAA-g-EG) in pH 2.0 (◇) and pH 7.4 (◆) as well as 5%-NP in pH 2.0 (△) and pH 7.4 (▲). Fluorescein release is expressed as $M_t M_\infty^{-1}$. Curves generated are for $n = 3$ studies.