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Dual Stimuli Responsive Glycidyl Methacrylate Chitosan-Quaternary Ammonium Hybrid Hydrogel and Its Bovine Serum Albumin Release

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ABSTRACT: A new family of cationic hybrid hydrogels from two new positively charged aqueous soluble precursors, glycidyl methacrylate-chitosan (GMA-chitosan), and 2-(acryloyloxy) ethyl trimethylammonium (AETA), was developed via a simple photo-crosslinking fabrication method. These hybrid hydrogels have pendant quaternary ammonium functional groups on the AETA segments. The chemical composition of GMA-chitosan/AETA hybrid hydrogels were characterized by Fourier transform infrared spectroscopy and their mechanical, swelling, and morphological properties were examined as a function of the composition of the hybrids as well as the effect of pH and ionic strength of the surrounding medium. GMA-chitosan/AETA hybrid hydrogels show a porous network structure with average pore diameter 20–50 μm. The compression moduli of these hybrid hydrogels ranged from 27.24 to 28.94 kPa, which are significantly higher than a pure GMA-chitosan (17.64 kPa). GMA-chitosan/AETA hybrid hydrogel shows pH/ionic strength responsive swelling behavior because of the presence of the positive charge pendant groups. These hybrid hydrogels showed a sustained BSA protein release and a significantly lower initial burst release than a pure GMA-chitosan hydrogel. The two aqueous soluble precursors and the cationic charge characteristics of the resulting GMA-chitosan/AETA hybrid hydrogels may suggest that this new family of biomaterials may have promising applications as the pH responsive protein drug delivery vehicles.

KEYWORDS: drug delivery systems; gels; polysaccharides; photopolymerization

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INTRODUCTION

Hydrogels are networks of synthetic or natural polymer chains that are hydrophilic. On the macroscopic scale hydrogels are solid but they also behave like solutions at the molecular scale; water soluble molecules can diffuse into hydrogels with diffusion constants reflecting the diffusing size and shape.[1] Natural polymers, which are used in designing hydrogels, include polysaccharides and proteins. Among them, chitosan is one of the most studied; chitosan is a linear polysaccharide composed of randomly distributed β-(1–4)-linked D-glucosamine and *N*-acetyl-D-glucosamine units. Because of these characteristics and abundant source, chitosan has attracted a wide range of medical and pharmaceutical applications.[2,3] Very recently, our group developed a new water soluble cationic chitosan-based macromolecular precursor, GMA-chitosan.[4] GMA-chitosan has unsaturated methacrylate groups from glycidyl methacrylate (GMA) grafted onto chitosan backbone as the pendant functional groups in our previous study.[4] Unlike few other published reports of GMA-chitosan,[5,6] our GMA-chitosan precursor has a much higher degree of GMA substitution with high yields, which could provide the required amounts of crosslinkable sites to fabricate hydrogel by a simple photo-crosslinking without the need of a crosslinker. This improved single component hydrogel processing without using crosslinkers or other synthetic polymer coprecursor can maximize the physiochemical advantages of chitosan, such as biocompatibility, biodegradability without the dilution by coprecursors. Another advantage is the use of an aqueous medium for fabricating GMA-chitosan hydrogels to minimize adverse organic solvent effect in biomedical applications.

In the study of drug delivery, stimuli-responsive hydrogels have been produced that exhibits dramatic changes in their swelling behavior, network structure, permeability, and mechanical strength in response to a number of external stimuli, including pH, ionic strength of the surrounding fluid, temperature, presence of specific solute, and applied electrical or magnetic

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fields.[7,8] Stimuli-responsive hydrogels can be used in a wide variety of applications, including tissue engineering, biosensors, and drug delivery devices. Polyelectrolyte hydrogels exhibit pH-dependent swelling behavior that is the function of ionic strength, polarity of the solvent, and temperature. These ionic networks contain either acidic or basic pendant groups. In aqueous media of appropriate pH and ionic strength, the pendant groups can ionize, developing charges on the hydrogel. Depending on the type of ionizable pendant groups, ionization can occur when the pH of the environment is either above or below the pKa of the ionizable group. As the degree of ionization changes due to the change of pH, the number of charges can increase or decrease, resulting in increased or decreased electrostatic repulsions between the chains and resulting in greater or lower swelling ratios. pH-sensitive hydrogel have been most frequently used to develop controlled release formulation; for example, polycationic hydrogels in the form of semi-IPN have been used for antibiotics drug delivery in the stomach for the treatment of *Helicobacter pylori*.[9]

In this study, we reported a new family of cationic hybrid hydrogels based on the two new aqueous soluble precursors: GMA-chitosan and a vinyl monomer having quaternary ammonium cation whose positive charge characteristic is independent of the pH of their surrounding medium. Quaternary ammonium compounds are well-known antiseptics with a favorable safety profile, and have been the components in a variety of personal hygiene products. The cationic charge of primary amine, however, usually has high toxicity, but quaternary ammonium cations grafted on the poly(GMA) shows significantly reduced cytotoxicity.[10] One commercial source of quaternary ammonium cation is 2-(acryloyloxy) ethyl trimethylammonium (AETA), which is a water-soluble chemical with quaternary ammonium group and unsaturated double bond that is photo-reactive. The unsaturated vinyl end group can be used as the reactive site for chemically incorporating quaternary ammonium cation onto polymers like GMA-chitosan having unsaturated double bonds. The resulting hybrid hydrogels of GMA-chitosan and AETA were examined for their swelling and mechanical property as a function of the composition of the hybrids as well as their response to surrounding pH and ionic strength. The bovine serum albumin release property from this new family of GMA-chitosan/AETA hybrid hydrogel was also studied to determine the advantage of this new family of biomaterial for protein release.

EXPERIMENTAL

Materials

Chitosan (75–85% deacetylated) of molecular weight (MW) 50,000–190,000 Da, 2-(Acryloyloxy) ethyl trimethylammonium (AETA) (80 wt % in aqueous solution), bovine serum albumin (BSA) of molecular weight ~66,000 Da were purchased from Sigma Chemical Company (St.Louis, MO). GMA (97%), 4-(*N*,*N*-dimethylamino) pyridine (DMAP, 99%), toluene sulfonic acid monohydrate ($TsOH{\cdot}H_2O$) dimethyl sulfoxide (DMSO) were purchased from VWR Scientific (West Chester, PA). Ethyl acetate, acetone were purchased from Mallinckrodt incorporation (St. Louis, MO) and used without further purification. The DMSO, toluene, isopropyl alcohol, ethyl acetate, and acetone were ACS grade. Potassium hydrogen phthalate, hydrochloric acid (10%,v/v), disodium hydrogen phosphate, sodium hydroxide, sodium dihydrogen phosphate monohydrate were purchased from VWR Scientific (West Chester, PA) to prepare buffer solutions of different pH (i.e., 3, 7.4, 10) and different ionic strength (i.e., 0.05, 0.1, 0.2*M*). MicroBCA kit was purchased from Thermo Fisher Scientific (Waltham, MA). Irgacure 2959 was donated by Ciba Specialty Chemicals.

Synthesis of Glycidyl Methacrylate Chitosan (GMA-Chitosan)

The GMA-chitosan was synthesized by the procedure described in the prior study.[4] The degree of substitution (DS; the amounts of methacrylate groups per 100 chitosan repeat unit) of GMA-chitosan was determined by ^{1}H NMR spectroscopy. GMA-chitosan with DS 37 is used in the further study of the cationic hybrid hydrogel to achieve the consistent physical integrity.

Fabrication of GMA-Chitosan/2-(Acryloyloxy) Ethyl Trimethylammonium (AETA) Hybrid Hydrogel

To fabricate GMA-chitosan/AETA hybrid hydrogels, 0.3 g GMA-chitosan (degree of substitution 37) was dissolved in 2 mL water and mixed well with predetermined amounts of AETA aqueous solution (80 wt %). The weight ratio of GMA-chitosan to AETA could be any ratio with GMA-chitosan contents no less than 67/33 to achieve good structural integrity and proper mechanical strength of hybrid hydrogels; in this study, the weight feed ratio of GMA-chitosan to AETA used are 80/20 and 67/33. Ten milligram Irgacure 2959 photoinitiator was added into the solution and mixed well at room temperature. Every 400 μL mixture aqueous solution was transferred onto a Teflon® mold (12 mm diameter, 6 mm thickness) and irradiated by a long wavelength UV light (100 W, 365 nm) at room temperature for about 30 min until a disk-shaped hydrogel was obtained. The hydrogels were soaked in deionized water for 16 h at room temperature to remove the unreacted GMA-chitosan and AETA residues. The swollen GMA-chitosan/AETA hybrid hydrogel samples were then dehydrated on a Teflon plate in the ambient air at room temperature until the dry weight was constant for further studies, such as FTIR, equilibrium swelling ratio.

A representative chemical structure of GMA-chitosan/AETA hydrogels is shown in Figure 1. Pure GMA-chitosan hydrogels as a control were fabricated by photo-crosslinking 6% GMA-chitosan aqueous solution without the presence of AETA.

Fourier Transform Infrared (FTIR) Characterization of Dried GMA-Chitosan/AETA Hybrid Hydrogel

Dehydrated GMA-chitosan/AETA hybrid hydrogel (dehydrated in ambient air at room temperature) were analyzed by fourier transform infrared (FTIR) using a PerkinElmer (Madison, WI) Nicolet Magna 560 FTIR spectrophotometer with Omnic software for data acquisition and analysis. The spectra of dry GMA-chitosan/AETA hybrid hydrogel were recorded and compared with a pure GMA-chitosan FTIR spectra.

Swelling Ratio (Q_{eq}) Under Different Ionic Strength and pH Environment

The Q_{eq} of dehydrated GMA-chitosan/AETA hybrid hydrogel and the pure GMA-chitosan hydrogel (as control) were

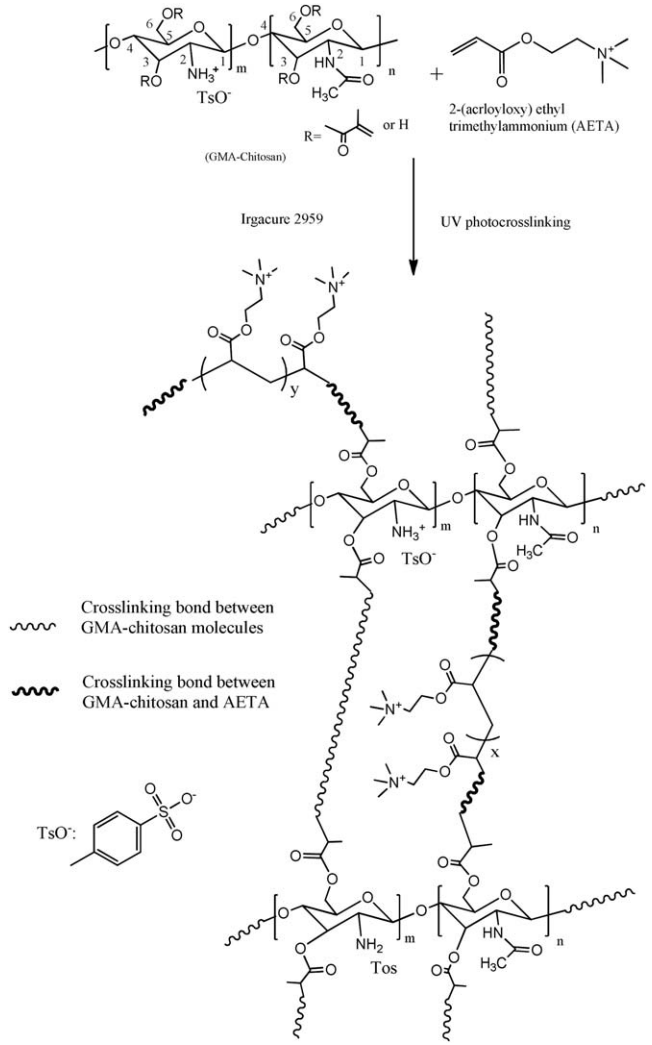

Figure 1. One representative GMA-chitosan/AETA hybrid hydrogel.

performed at room temperature (25°C) by immersing dehydrated hydrogels individually in glass vials containing 15 mL 0.05*M* buffers (pH 3, 7.4, 10) or 15 mL pH 7.4 (0.05, 0.1, 0.2*M*). After 16 h, the GMA-chitosan/AETA hybrid hydrogels reached their swelling equilibrium. The swollen hydrogels were then removed, the excess surface water was wiped and the hydrogels were weighed until a constant weight was obtained. The swelling ratios of the hydrogels were calculated from the swollen and dry weights of the hydrogels according to the following eq. (1).

$$Q_{eq}(\%) = (W_t - W_0)/W_0 \times 100 \quad (1)$$

W_t is the weight of the hydrogel at swelling equilibrium; W_0 is the initial dry weight of the hydrogel before immersion.

The swelling profiles of a hydrogel were determined in triplicate.

Morphological Study of GMA-Chitosan/AETA Hybrid Hydrogel

Scanning electron microscope (SEM) was employed to analyze the interior microstructure of GMA-chitosan/AETA-67/33 hybrid hydrogel. A pure GMA-chitosan hydrogel was examined for comparison. Individual hydrogels were soaked in deionized water at room temperature to reach their swelling equilibrium. Then, the hydrogels were transferred into liquid nitrogen immediately to freeze and retain the swollen structure. The samples were subsequently freeze-dried for 72 h in a Labconco (Kansas City, MO) Freezone 2.5 Freeze drier under vacuum at −50°C, and finally glued onto aluminum stubs and coated with gold for 30 s for SEM observation by Leica Microsystems GmbH (Wetzlar, Germany) 440 SEM.

Compression Mechanical Properties of GMA-Chitosan/AETA Hybrid Hydrogel

The mechanical testing of the GMA-chitosan/AETA hybrid hydrogel and GMA-chitosan hydrogel was performed on a DMA Q800 Dynamic Mechanical Analyzer (TA Instrument, New Castle, DE) in a "controlled force" compression mode. The compressive mechanical property of GMA-chitosan/AETA hybrid hydrogels and GMA-chitosan hydrogel in circular disc shape after reached their equilibrium swelling in deionized water were measured at room temperature (25°C). The hydrogels were mounted between the movable compression probe (diameter 15 mm) and the fluid cup without any liquid media. A compression force from 0.01 to 4 N at a rate of 0.5 N/min was applied on the swollen hydrogel samples at room temperature until fragment of the hydrogels was produced. TA Universal Analysis software was used for mechanical data analysis. Initial compressive modulus and compressive strain at break were used to examine the hydrogel mechanical property. The initial compressive modulus was calculated from the slope of the initial linear portion of the curve. For each type of hydrogel, five samples were used, and their mean value was calculated with a standard deviation.

Enzymatic Degradation of GMA-Chitosan/AETA Hybrid Hydrogel

The enzymatic biodegradation of the circular disk shaped GMA-chitosan/AETA-67/33 hybrid hydrogel and GMA-chitosan hydrogel was evaluated by their weight loss at 37°C in 15 mL lysozyme (1 mg/mL) in 0.05*M* pH 7.4 phosphate buffered saline (PBS) over a period of 10 days. A 15 mL PBS of pH 7.4 served as the control.

The weight of each dry GMA-chitosan/AETA-67/33 hybrid hydrogel or GMA-chitosan hydrogels was measured before immersion. At various immersion intervals, three GMA-chitosan/AETA-67/33 hybrid hydrogels (or GMA-chitosan hydrogels) samples were removed from the immersion solution and dried under vacuum at room temperature till constant weights. The weight loss was calculated according to the following equation: % Weight loss = $(W_o - W_t)/W_o \times 100\%$, where W_o was the initial ($t = 0$) dry weight of hydrogel, and W_t was the dry weight of the hydrogel after incubation at time t. Mean value of was calculated as the weight loss at time t with a standard deviation.

Release Study of Bovine Serum Albumin (BSA) from GMA-Chitosan/AETA Hydrogel

Seventy-five milligram BSA was dissolved in 5 mL deionized water to obtain BSA stock solution of 15 mg/mL concentration. GMA-chitosan/AETA-67/33 precursor solution was prepared in a 20 mL glass vial wherein 300 mg of GMA-chitosan and 187 mg AETA aqueous solution (80 wt %) dissolved in 4.6 mL

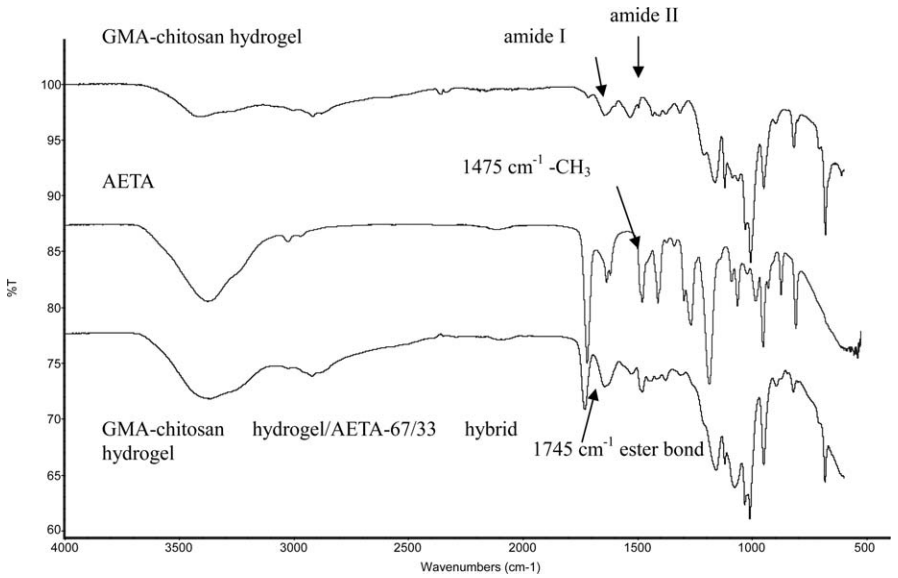

Figure 2. FTIR of GMA-chitosan/AETA-67/33 hybrid hydrogel and its corresponding pure precursors.

deionized water. Two-hundred microliter BSA stock solution and 25 mg Irgacure 2959 were then added into the GMA-chitosan/AETA-67/33 aqueous solution and mixed well after 10 min magnetic stirring. The 400 μL BSA-preloaded GMA-chitosan/AETA-67/33 aqueous solution was transferred to a 20-well Teflon® mold with a micropipette and photo-crosslinked by using a long wavelength UV light (100 W, 365 nm) for about 30 min. After gelation, each BSA-preloaded GMA-chitosan/AETA-67/33 hydrogel sample (with 240 μg BSA loaded) was removed from Teflon® mold carefully and placed in glass vials individually filled with 5 mL 0.05*M* pH 3 buffer or 0.05*M* PBS of pH 7.4 supplemented with 0.02 w/v% sodium azide, and then incubated at 37°C (Julabo, water bath). At each predetermined time interval, 100 μL buffer solution was removed from the vial and diluted to 1 mL by deionized water. Hundred microliter fresh buffer solution (0.05*M* pH 3 buffer or 0.05*M* PBS of pH 7.4) was added back into the glass vial to keep the PBS medium constant during the immersion.

MicroBCA kit (Thermo Scientific, MA) was used to determine the BSA concentration. The protocol in the MicroBCA receipt was followed when using the MicroBCA kit. PerkinElmer (Madison, WI) Lambada 35 UV-Vis spectrophotometer was used to determine the absorption of the samples at 562 nm wavelength. The standard BSA calibration curve was prepared by plotting the average Blank-corrected 562 nm reading for each BSA standard, which is provided in the kit versus its concentration in μg/mL. This BSA standard calibration curve was used to determine the BSA concentration released from the testing hydrogels at a particular time interval. The amounts of BSA released from the testing hydrogels were then calculated from the calibration curve. Samples in triplicate were averaged for each experiment.

RESULTS AND DISCUSSION

FTIR Spectroscopy

Figure 2 shows the FTIR spectra of GMA-chitosan/AETA-67/33 hybrid hydrogel. The sharp carbonyl bands of ester bonds at 1745–1749 cm^{-1} was shown on AETA spectra. The ester bond of methacrylate in GMA-chitosan is about 1725 cm^{-1}. The peak at 1475 cm^{-1} is ascribed to the symmetrical bending vibration of $-CH_3$ of quaternary ammonium group. GMA-chitosan also shows amide (I) band and amide (II) band at 1648–1650 cm^{-1} and 1538–1542 cm^{-1}, respectively due to the incomplete deacetylated *N*-acetyl-D-glucosamine units. The GMA-chitosan/AETA hybrid hydrogel also showed the amide (I) and amide (II) bands, which are absent in the AETA spectrum. The amide groups were from the *N*-acetyl-D-glucosamine repeat unit of chitosan. On the spectrum of the chitosan/AETA hybrid hydrogel, the peak at 1475 cm^{-1} of quaternary ammonium group was shown but absent in a pure GMA-chitosan. The FTIR data demonstrated that AETA was successfully incorporated into the GMA-chitosan hydrogel network upon the UV-crosslinking process. The absorption of ester bond at 1745 cm^{-1} of GMA-chitosan/AETA hybrid hydrogel is strengthened when comparing with a pure GMA-chitosan hydrogel due to the presence of the ester unit in AETA.

Images of GMA-Chitosan/AETA Hybrid Hydrogel After Swelling

Figure 3(A) shows the image of a dehydrated GMA-chitosan/AETA hydrogel in an ambient environment after 48 h. Figure 3(B) shows the optical image of a GMA-chitosan/AETA hydrogel after 16 h swelling in deionized water. The GMA-chitosan/AETA hybrid hydrogel shows high swelling in water and good transparency with no color.

The Influence of Ionic Strength on the Swelling Ratio of GMA-Chitosan/AETA Hybrid Hydrogel

All of the GMA-chitosan/AETA hybrid hydrogels and the pure GMA-chitosan hydrogel control reached their largest swelling ratio after 16 h immersion in deionized water as shown in Figure 4. The swelling ratio of GMA-chitosan/AETA hybrid hydrogel in pH 7.4 buffers at different salt concentrations ranged from 2994% to 1035%, depending on the GMA-chitosan to AETA feed ratio. As the concentration of the salt in

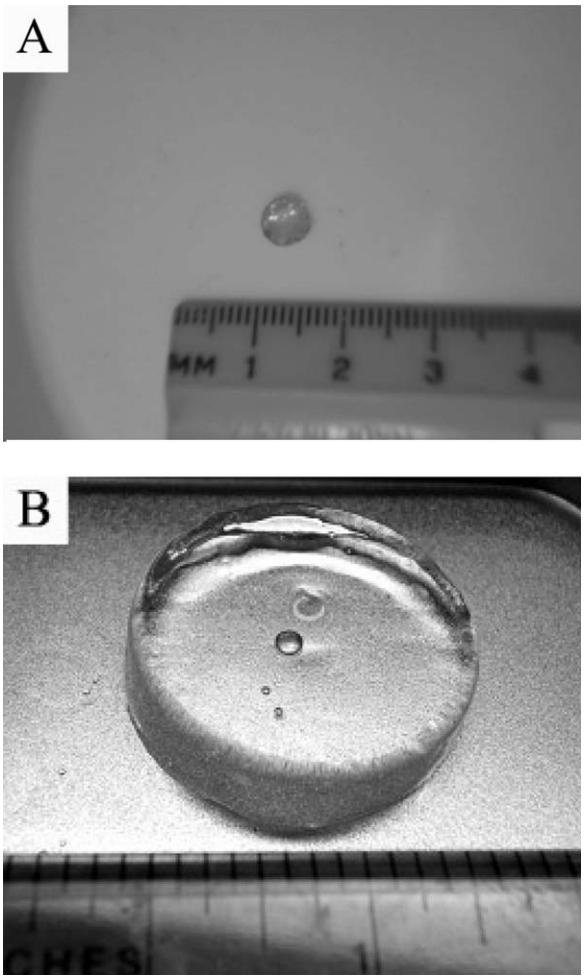

Figure 3. Images of GMA-chitosan/AETA-67/33 hydrogel. (A) Dehydrated GMA-chitosan/AETA-67/33 hydrogel; (B) after swelling in deionized water for 16 h immersion.

buffer increased, i.e., stronger ionic strength, the swelling ratio of the hybrid hydrogel, and the GMA-chitosan based hydrogels decreased. For example, GMA-chitosan/AETA-67/33 hybrid hydrogel achieved 2109%, 1887%, and 1332% swelling ratio in 0.05*M*, 0.1*M*, and 0.2*M* pH 7.4 buffer, respectively, but they were all smaller than the same hybrid hydrogels in deionized water (2675%).

Moreover, GMA-chitosan/AETA hybrid hydrogels having higher AETA contents showed smaller swelling ratios than those hybrids having smaller AETA contents in deionized water and the 0.05*M* ionic strength aqueous environment. For example, the swelling ratio of GMA-chitosan/AETA-80/20 hydrogel is about 2994% in 0.05*M* pH 7.4 buffer, while the data of GMA-chitosan/AETA-67/33 in 0.05*M* pH 7.4 buffer is 2109%. However, higher AETA contents in hybrid hydrogel led to larger swelling ratios in 0.1 and 0.2*M* pH 7.4 buffer.

Many polyelectrolyte hydrogels have the ionic strength responsive swelling behavior.[11–13] For example, in the study of Poly(L-glutamic acid) (PLG)/Polyethylene glycol (PEG) hybrid hydrogels, which have secondary amine groups, Markland et al.[11] reported that an increase in ionic strength of medium resulted in a reduction in the swelling ratio of the PLG/PEG hydrogels at several different pH (2.5–7). Generally, the extent to which a polyelectrolyte hydrogel swells at equilibrium in a buffer solution depends on the amounts of the ionizable groups of the polyelectrolyte and the ionic strength of the immersion medium. An increase in ionic strength of the medium generally decreases the difference in concentration of mobile ions between a hydrogel and surrounding medium, which subsequently reduce the osmotic swelling pressure inside a hydrogel, i.e., reduced swelling ratio.

In 0.1*M* or 0.2*M* buffer medium, the hybrid hydrogels with higher AETA content achieve higher swelling, i.e. GMA-chitosan/AETA-67/33. Consequently, there are more cationic moieties

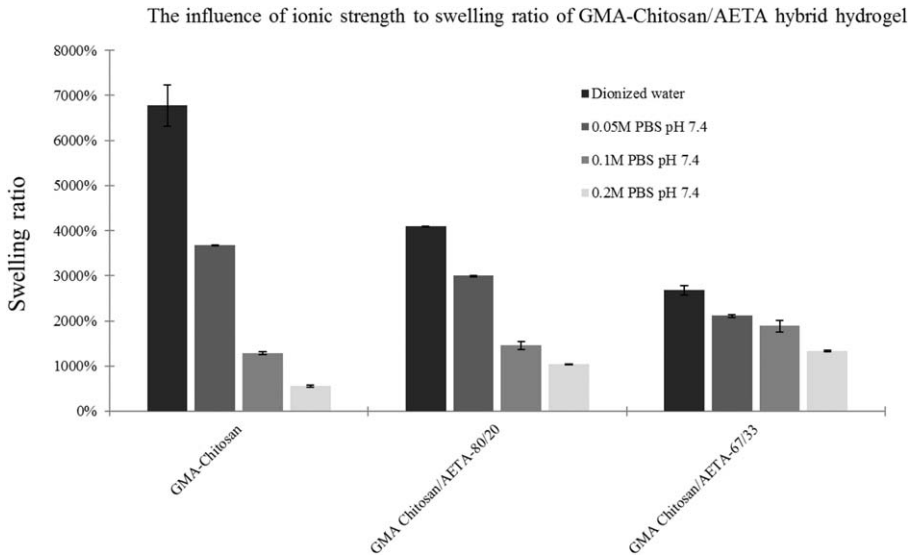

Figure 4. The influence of ionic strength of the immersion media to the swelling ratio of GMA-chitosan/AETA hybrid hydrogel of different GMA-chitosan to AETA contents in deionized water and pH 7.4 at room temperature.

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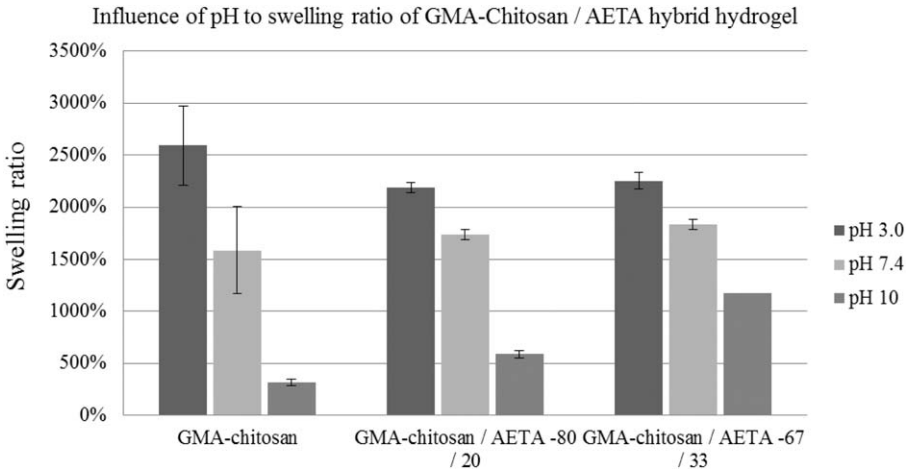

Figure 5. The influence of pH environment to the swelling ratio of GMA-chitosan/AETA hybrid hydrogels of different GMA-chitosan to AETA contents in an ionic strength 0.1*M* aqueous buffer at room temperature.

dispersed in the GMA-chitosan/AETA hybrid hydrogels with higher AETA content. Those cationic quaternary ammonium moiety of AETA is still positively charged (pKa = 10), and the electrostatic repulsion among the quaternary ammonium groups in those hybrids having high AETA contents could result in a higher swelling ratio of the hybrid hydrogel than a pure GMA-chitosan hydrogel. Another factor, which could influence the swelling ratio of GMA-chitosan/AETA hybrid hydrogels is their level of the crosslinking density. Because the molecular weight of the chitosan raw material is 50,000–1,90,000 Da, the GMA grafted onto the chitosan backbone could be relatively "invisible" in the mass of chitosan space due to the shorter spacer between the chitosan backbone and the photo-reactive vinyl end group in GMA pendant group, i.e., lesser level of photo crosslinking. The presence of unsaturated small molecules like AETA in the GMA-chitosan precursor, however, could promote the crosslinking between the methacrylate groups on the GMA-chitosan molecules and AETA. Several published studies have demonstrated that the two component hybrid hydrogel system that contains both water soluble small moiety having vinyl groups and polysaccharide derivative having unsaturated groups is able to achieve a higher crosslinking density than those hydrogels formed by a single component and hence influence the swelling.[11–13] In Markland et al. study[11] about poly(L-glutamic acid)/PEG hybrid hydrogel, the formation of a higher intermolecular crosslinking density resulted in a reduced degree of swelling. An increase in the AETA contents in the GMA-chitosan/AETA hybrid hydrogels could led to higher crosslinking level in a 0.05*M* buffer or deionized water, i.e., smaller swelling ratio of the hybrid hydrogel (2994% swelling in 0.05*M* pH 7.4 buffer) than a pure GMA-chitosan hydrogel (3668% swelling in 0.05*M* pH 7.4 buffer). This higher crosslinking in the GMA-chitosan/AETA hybrid hydrogels was also reflected in their higher mechanical property as described later.

The Influence of pH on the Swelling Ratio of GMA-Chitosan/AETA Hybrid Hydrogels

Figure 5 shows the effect of pH of the medium on the swelling behavior of GMA-chitosan/AETA hybrid hydrogels of different GMA-chitosan to AETA feed ratio at the same ionic strength (0.1*M*). All GMA-chitosan/AETA hybrid hydrogels and the pure GMA-chitosan hydrogels changed their ability to swell when the environmental pH was altered, i.e., lower swelling at a higher pH. For example, GMA-chitosan/AETA-80/20 hybrid hydrogels achieved 2187%, 1732%, and 587% swelling ratios in pH 3, 7.4, and 10 buffers, respectively. In an acidic medium (pH 3), both hybrid hydrogels exhibited similar swelling ratios (2187% for 80/20 and 2253% for 67/33), but a pure GMA-chitosan hydrogels had the highest swelling ratio (2592%). However, this trend disappeared in a basic medium. In a slightly basic medium (pH 7.4), there was no significant difference in swelling among the two types of hybrid hydrogels and the control pure GMA-chitosan hydrogel. In a more basic medium (pH 10), both hybrids showed higher swelling than the pure GMA-chitosan control, particularly the hybrid having higher AETA content, i.e., GMA-chitosan/AETA-67/33 hybrid hydrogel shows the largest swelling (1177%) while the swelling data of GMA-chitosan/AETA-80/20 and pure GMA-chitosan are 587% and 317%, respectively.

The swelling ratio of the hydrogels increases only when the amine groups of chitosan and quaternary ammonium groups of AETA are ionized under the condition of low or acidic pH, i.e., protonation. And the pKa of quaternary amine in the GMA-chitosan/AETA is about 10, while the pKa of amine groups of GMA-chitosan is about 7.3–7.6.[14] The primary amine groups of GMA-chitosan in these hydrogels deprotonized and the quaternary ammonium groups are partially deprotonized with the increasing environmental pH from 3 to 10. So, the osmotic pressure inside the hydrogels decreases as the pH increasing. As a consequence, the decrease in swelling with increasing pH from 3 to 10 can be explained in that a dissociation process of amine groups and quaternary ammonium groups in these hydrogels.

In the same basic environment (pH 10), the swelling of hybrid hydrogels with more AETA contents (1177%) is significantly higher than that hybrid hydrogels having lower AETA contents (587%), and much higher than a pure GMA-chitosan hydrogel

(317%). The reason is because the primary amine groups of GMA-chitosan hydrogel is deprotonated at pH 10, while the quaternary ammonium groups in AETA can remain partially ionized at pH 10. So, the GMA-chitosan/AETA hydrogel interior is able to keep part of positively charge character at this high pH. The osmotic pressure generated by the ionized quaternary ammonium groups led to the higher AETA content hybrid hydrogel achieved higher swelling than a lower AETA content hybrid hydrogel.

The pH effect on the swelling property of other polysaccharide-based hydrogels has also been reported.[15,16] The direction and magnitude of pH effect depend on the type of polysaccharides, particularly their charge characteristic of the ionizable groups. In the Zhong et al.[15] study of anionic maleic-chitosan/PEGDA hybrid hydrogels, they also discovered pH-dependent swelling; but the anionic maleic-chitosan/PEGDA hybrid hydrogel achieved a higher swelling ratio at an alkaline pH than at an acidic condition, opposite to what we observed in the current cationic GMA-chitosan/AETA hybrids. This is because the ionizable groups in maleic-chitosan/PEGDA hybrids is mainly carboxyl group (pKa 1.8–2.4), the osmotic pressure inside the maleic-chitosan/PEGDA hybrid hydrogels is higher at an alkaline condition as carboxyl groups in maleic chitosan are easier to be ionized (deprotonized) in such an alkaline condition.

Morphology of GMA-Chitosan/AETA Hybrid Hydrogel

GMA-chitosan/AETA hybrid hydrogels also have 3D porous network structure upon swelling. On the basis of the SEM images in Figure 6, the GMA-chitosan/AETA hybrid hydrogel network has pores that exhibit irregular shape and thin wall [Figure 6(A,B)], and the diameter of the most pores ranges from 20 to 50 μm. The microstructure of GMA-chitosan/AETA hybrid hydrogel shows no apparent difference from a pure GMA-chitosan hydrogel [Figure 6(C), pore size ranging from 10 to 50 μm).

Compressive Mechanical Properties of GMA-Chitosan/AETA Hybrid Hydrogel

The compression mechanical data of the GMA-chitosan/AETA hybrid hydrogels are shown in Table I. In general, the compression moduli of the hybrid hydrogels are significantly higher than the pure GMA-chitosan control, and no significant different moduli due to different feed ratios of GMA-chitosan to AETA (27.24 ± 3.22 vs. 28.94 ± 4.44 kPa). The compression moduli of the crosslinked GMA-chitosan/AETA hybrid hydrogels were about 59–63% higher than that of a pure GMA-chitosan hydrogel (17.64 ± 5.52 kPa). The addition of AETA coprecursor apparently increased the stiffness of the hybrid hydrogel. The reason might also be attributed to a higher level of crosslinking density of the GMA-chitosan/AETA hybrid hydrogels than a pure GMA-chitosan hydrogel because of the presence of the much smaller AETA coprecursor that could facilitate crosslinking reactions. The effect of feed ratio of the two precursors on the compression modulus of the GMA-chitosan/AETA hybrid hydrogels appeared not apparent, i.e., 28.94 ± 4.44 kPa (GMA-chitosan/AETA at 80/20) versus 27.24 ± 3.22 kPa (GMA-chitosan/AETA at 67/33).

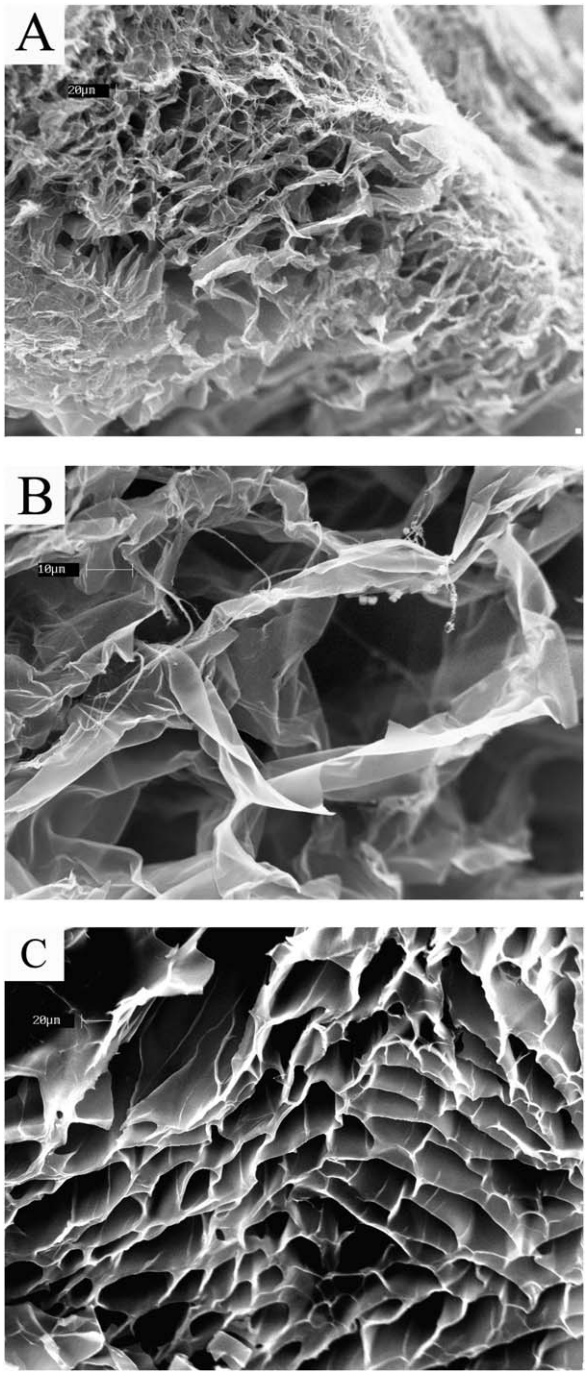

Figure 6. SEM images of GMA-chitosan hydrogen and GMA-chitosan/AETA hybrid hydrogels. (A) GMA-chitosan/AETA-67/33 hybrid hydrogel at 500×; (B) GMA-chitosan/AETA-67/33 hybrid hydrogel at ×2000; (C) GMA-chitosan hydrogel at ×1000.

The compression moduli of the GMA-chitosan/AETA hybrid hydrogels are lower than the Zhong et al.[15] recently reported a new family of anionic maleic chitosan/polyethylene glycol diacrylate (PEGDA) hybrid hydrogels fabricated in the similar manner as the current UV-crosslinking method. The initial modulus of anionic maleic chitosan/PEGDA (MW = 8000) hybrid hydrogel at 1 : 2 feed ratio is 61 ± 1.9 kPa. This value is about 1–2 times higher than the current GMA-chitosan/AETA hybrid hydrogel,

Table I. The Compression Initial Modulus of Swollen GMA-Chitosan Hydrogel and GMA-Chitosan/AETA Hybrid Hydrogel at Room Temperature

Hydrogel samples	Compressive modulus (kPa)
GMA-chitosan	17.64 ± 5.52
GMA-chitosan/AETA-80/20	28.94 ± 4.44
GMA-chitosan/AETA-67/33	27.24 ± 3.22

probably due to the fact that the high composition of PEGDA in maleic chitosan/PEGDA hybrid hydrogel can lead to a higher crosslinking density than that of the GMA-chitosan/AETA hybrid hydrogels. Different from GMA-chitosan/AETA hybrid hydrogel, the mechanical property of the maleic chitosan/PEGDA hybrid hydrogels largely depended on the molecular weight of the PEGDA coprecursor and the content of PEGDA in the hybrid hydrogel. The composition of PEGDA in the Zhong et al. maleic chitosan hybrid hydrogel is higher than 50%, while the AETA coprecursor composition in the current GMA-chitosan/AETA hybrids are no more than 33% for the purpose of retaining more physiochemical characteristics of chitosan in the hybrids through a higher GMA-chitosan composition.

Enzymatic Degradation of GMA-Chitosan/AETA-67/33 Hybrid Hydrogels

The biodegradation behavior of GMA-chitosan/AETA-67/33 hybrid hydrogels was evaluated in terms of their weight loss in both pure PBS control and lysozyme solution of pH 7.4 at 37°C over a period of 18 days. The total weight loss of these hydrogels ranged from as high as 45% to as low as 35% over the period of 18 days, depending on the hybrid versus pure hydrogels and enzymes versus PBS medium. Figure 7 shows that

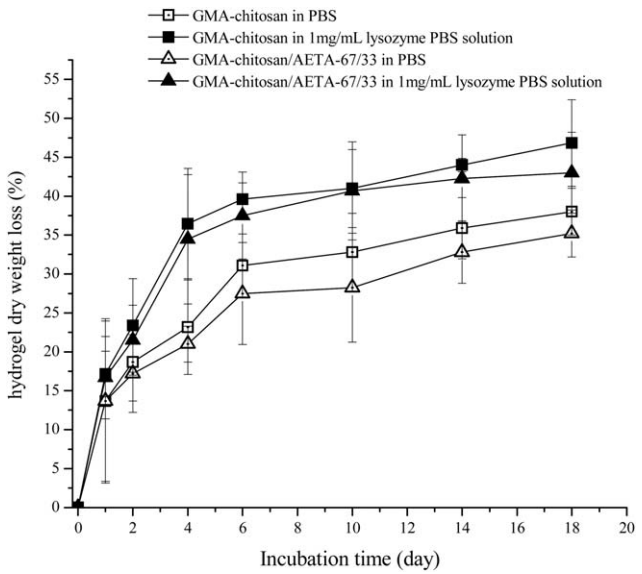

Figure 7. *In vitro* enzymatic degradation of GMA-chitosan hydrogel and GMA-chitosan/AETA-67/33 hybrid hydrogel at 37°C, pH 7.4, 0.05*M* PBS. The solid symbol: degradation of hydrogels with the presence of 1 mg/mL lysozyme; open symbol: degradation of hydrogel control samples in PBS. The DS of GMA-chitosan composition is 37).

GMA-chitosan/AETA-67/33 hybrid hydrogels in the presence of 1 mg/mL lysozyme were degraded faster than the same hybrid hydrogel in the PBS. GMA-chitosan/AETA-67/33 hybrid hydrogels lost 40.7% of original weight in 1 mg/mL lysozyme solution, but 34.2% in PBS after 10 days incubation at 37°C. When compared to the biodegradation profiles of a pure GMA-chitosan hydrogel, GMA-chitosan/AETA-67/33 hybrid hydrogel was degraded slightly slower in PBS (i.e., 1.5–4% less weight loss) and in 1 mg/mL lysozyme solution (i.e., 1.9–4% less weight loss) at the same incubation time.

Lysozyme naturally presents in wound fluid and secretions. Chitosan derivatives are hydrolyzed *in vivo* by lysozyme to oligomers.[17] Lysozyme is able to cleave GMA-chitosan backbone structure at the β(1, 4)linked glucosamine unit and *N*-acetyl-D-glucosamine unit.[18] The biodegradation of GMA-chitosan based hydrogel has two mechanisms simultaneously happened in the presence of lysozyme: (1) the chitosan polymer backbone was cleaved by lysozyme and (2) the ester bonds of methacrylate group on GMA-chitosan was degraded by hydrolysis. The accessibility of the enzyme to polysaccharide-based hydrogel is one key factor to influence the biodegradation rate because lysozyme needs to target *N*-acetyl-D-glucosamine unit of chitosan to proceed the degradation.[18] Lysozyme is a 14.4 kDa protein with an isoelectric point 11.2, which is mainly positively charged in water solution.[19] The presence of electrostatic repulsion between lysozyme and GMA-chitosan/AETA may led to a slower biodegradation of GMA-chitosan/AETA-67/33 hybrid hydrogel than a pure GMA-chitosan hydrogel because the strong positively charged AETA moiety increased the difficulty of lysozyme to target the specific *N*-acetyl-D-glucosamine site of chitosan. The higher crosslinking density of GMA-chitosan/AETA hybrid hydrogel than a pure GMA-chitosan, which is also reflected in the lower Q_{eq} is another factor also contributed to a slower degradation rate in the GMA-chitosan/AETA hybrid hydrogels than a pure GMA-chitosan hydrogel in 1 mg/mL lysozyme PBS solution.

The enzymatic biodegradability of GMA-chitosan/AETA hybrid hydrogels could overcome the poor biodegradability of synthetic pH-sensitive polymers for implantable drug delivery agents or implantable biosensors.[8] The combination of pH-sensitivity and biodegradability of GMA-chitosan/AETA hybrid hydrogel can provide an alternative biomaterial for these applications.

BSA Release from GMA-Chitosan/AETA Hybrid Hydrogel at pH 3 and 7.4

The BSA release profiles from the GMA-chitosan/AETA-67/33 hybrid hydrogels are shown in Figure 8. Both GMA-chitosan/AETA hybrid hydrogels and GMA-chitosan hydrogel show burst releases in the first hour of immersion in a pH 3 and 7.4 media, but the hybrid hydrogels showed significantly lower BSA burst release amounts than a pure GMA-chitosan hydrogel at pH 7.4, i.e., 12.0% BSA release from GMA-chitosan/AETA hybrid versus 32.8% release from a pure GMA-chitosan hydrogel in the first hour. This significantly lower BSA release rate from the GMA-chitosan/AETA hybrid hydrogels persisted over the entire period of study (11 days). At the end of 11 day, 56% BSA released from the hybrids versus 88% BSA released from a pure

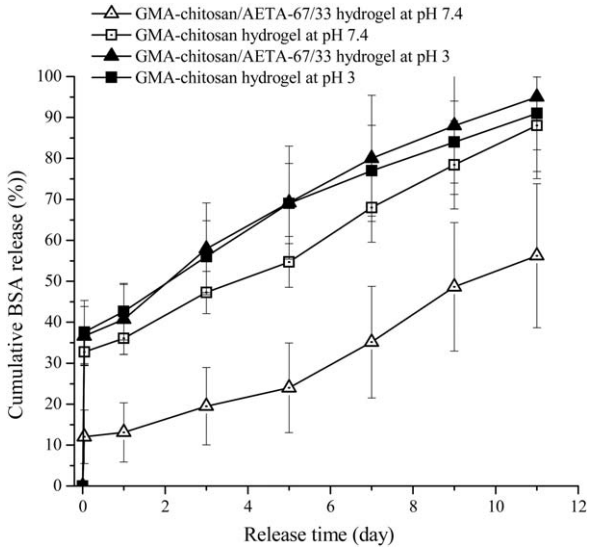

Figure 8. Cumulative BSA release profile from GMA-chitosan/AETA-67/33 hybrid hydrogels and GMA-chitosan hydrogel in buffer solutions at 37°C. The solid symbol: BSA release at pH 3, 0.05*M*; open symbol: BSA release at pH 7.4, 0.05*M*.

GMA-chitosan hydrogel at pH 7.4. However, the BSA release profiles between GMA-chitosan/AETA-67/33 hybrid hydrogels and pure GMA-chitosan hydrogels are very close at pH 3.

This difference in BSA release profiles at pH 3 and 7.4 is related to pH-responsive property of the hybrid hydrogels and the electrostatic interaction between anionic BSA and cationic hybrid hydrogel structure. The isoelectric point of BSA is about 4.7, and in pH 7.4 PBS, BSA is a negative charge protein of 60,000–70,000 molecule weight.[20] The quaternary ammonium group in the AETA segment of the hybrid hydrogels has pKa = 10, i.e., exhibiting strong positive charge in pH 7.4 PBS, i.e., the GMA-chitosan/AETA-67/33 hybrid hydrogel shows positively charge in a pH 7.4 buffer, which could provide stronger eletrostatic attraction to the anionic BSA than a pure GMA-chitosan alone i.e., resulting in a slower and more sustained BSA release from the hybrid hydrogels in this pH level, even though some weak attractions may exist between anionic BSA and cationic GMA-chitosan macromolecules. The cationic nature of GMA-chitosan depends on the amounts of primary amine groups (deacetylation level of chitosan) on the deacetylated chitosan repeat unit, and this primary amine has pKa 7.3–7.6, i.e., a weak cationic in a pH 7.4 medium.

At pH 3, the negative charge character of BSA is largely decreased, hence the electrostatic attraction between BSA and cationic GMA-chitosan/AETA-67/33 hybrid hydrogel also decreased, i.e., the advantage of AETA for electrostatic attraction for BSA disappeared. Hence, the BSA release profile of the GMA-chitosan/AETA-67/33 hybrid hydrogel became similar to that of the pure GMA-chitosan hydrogel at pH 3. The loss of the advantage of electrostatic attraction between BSA and GMA-chitosan/AETA hybrid hydrogels at pH 3 also resulted in a faster BSA release from the hybrid than that of pH 7.4.

pH-sensitive hydrogels have been most frequently used to develop controlled released formulation for oral administration. For example, the pH in the stomach (<3) is quite different from the neutral pH in the intestine and such a difference is able to elicit pH-dependent drug release behavior from GMA-chitosan/AETA hybrid hydrogels. Another advantage of the current photo-crosslinking fabrication method of protein-impregnated GMA-chitosan/AETA hybrid hydrogels is that water soluble protein drug can be preloaded into the hydrogel precursor aqueous solutions before gelation, and hence provides a more homogeneous and uniform loading without the adverse effect of organic solvents. Many other chemically crosslinking methods must follow a postloading method to prevent the undesirable side reactions between hydrogel precursors, crosslinkers, and drugs.[21,22]

For example, the dextran hydrogel crosslinked by epichlorohydrin, phosphorus oxychloride, and *N,N′*-methylenebisacrylamide is only suitable for protein postloading method, because the epoxy group of epichlorohydrin is also able to react with the amine or hydroxyl groups of protein, which can change the protein chemistry structure and lead to a unpredictable release dynamics.[23] A postloading method can also lead to nonhomogeneous and nonuniform drug distribution within the hydrogel matrix as most of the postloaded drugs stay near the surface of the hydrogels. It usually leads to lower drug loading efficiency and shorter drug release period. In our study, GMA-chitosan/AETA hybrid hydrogels and GMA-chitosan hydrogels achieved at least 11 days sustained release of BSA, whereas, the reported release of postprotein loaded dextran-based hydrogels could only last several hours.[24]

CONCLUSIONS

A new family of cationic biodegradable GMA-chitosan/AETA hybrid hydrogels was successfully designed and fabricated in an aqueous medium via UV photocrosslinking. The cationic GMA-chitosan/AETA hybrid hydrogels have 3D porous microstructure and a high capacity of water absorption. By varying the feed ratio of GMA-chitosan to AETA, the swelling (from 297.8% to 3318.2%, depending on pH and ionic strength) and mechanical (compressive modulus, 17.649 ± 0.78 to 180 elling in deonized water rim.103 ± 1.30 kPa) properties of this hybrid hydrogel system could be adjusted. GMA-chitosan/AETA hybrid hydrogels and GMA-chitosan hydrogels achieve their largest swelling ratio in a lower pH PBS solution and lower ionic strength environment because of the balance of osmotic pressure of the hydrogel interior and exterior solution environment. Lysozyme was able to effectively accelerate the GMA-chitosan/AETA hybrid hydrogel biodegradation in aqueous media. A sustained and pH responsive release of BSA can be achieved from GMA-chitosan/AETA hybrid hydrogels in 11 days *in vitro*. This newly developed cationic GMA-chitosan/AETA hybrid hydrogel system offers the advantage in terms of water soluble precursors for preloading therapeutic protein, flexible mechanical property, pH responsive release behavior, and biodegradability.

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