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Horseradish Peroxidase-Mediated *In Situ* Forming Hydrogels from Degradable Tyramine-Based Poly(amido amine)s

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ABSTRACT: Horseradish peroxidase (HRP)-mediated crosslinking of poly(amido amine) (PAA) copolymers was successfully applied in the preparation of *in situ* forming degradable hydrogels under physiological conditions. PAA copolymers containing different amounts of tyramine residues (termed as pAEEOL/TA) could be synthesized through Michael-type addition between methylenebisacryamide and amine mixture of 2-(2-aminoethoxy) ethanol and tyramine (TA). Depending on the amount of TA residue, the HRP, and H_2O_2 concentration, the gelation times could be varied from about 50 to 350 s. The swelling and degradation experiments indicated under physiological conditions the pAEEOL/TA-based hydrogels are completely degradable within 6–8 days. Rheological analysis revealed that storage modulus of the hydrogels increased from 2500 to 4100 Pa when increasing HRP concentrations. Importantly, pAEEOL/TA copolymers have low cytotoxicity. Moreover, NIH 3T3 (mouse embryonic fibroblast) cells exposed in the degradation products of pAEEOL/TA-based hydrogels retained high cell viability, implying that the hydrogels are cyto-biocompatible. *In vitro* release of methylene blue and IgG protein from pAEEOL/TA-based hydrogels could be effectively sustained by encapsulation of the drug in the hydrogels. The results indicate that HRP-crosslinked, degradable pAEEOL/TA-based hydrogels are promising for biomedical applications. J. Appl. Polym. Sci. 000: 000–000, 2012

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INTRODUCTION

Hydrogels are three-dimensional, crosslinked networks of water-soluble polymers.[1] Because of their favorable physical and biological properties, such as high water content, acceptable biocompatibility, and low inflammatory response, a large number of hydrogels have been studied widely for biomedical applications, such as controlled drug delivery and tissue regeneration.[2] In particular, *in situ* forming hydrogels, also referred to as injectable hydrogels, have received much more attention in recent years because they gain more advantages over conventional preformed hydrogels.[3,4] For example, liquid gel precursors can be crosslinked *in situ* to fill irregular shaped defect tissues via a minimally invasive surgical procedure. Moreover, different components, such as genes, drugs, and growth factors, can be readily incorporated into flowing gel precursors and homogeneously distributed in hydrogels after gelation.[5,6]

Several chemically crosslinking approaches, such as free radical polymerization reaction and Michael-type addition, have been developed for the preparation of *in situ* forming hydrogels in the past decades. Free radical-initiated photopolymerization is one of the most used methods to yield *in situ* forming hydrogels that normally have stable crosslinking networks and exhibit high mechanical strengths.[7] This method is, however, limited by a few inherent disadvantages. For example, photoinitiators and chemical crosslinking agents are typically cytotoxic, which could seriously harm the cells in the surrounding tissues.[8,9] As an alternative method, Michael-type addition provides a new route to give *in situ* forming hydrogels through Michael-type addition between a nucleophile and an electrophile. Hubbell and coworkers first described on the synthesis of poly(ethylene glycol) (PEG)-based hydrogels formed *in situ* through Michael-type addition between thiolated multiarmed PEG and PEG diacrylate (or PEG divinylsulfone).[10,11] Moreover, Michael-type addition was also applied to produce *in situ* forming hydrogels from the

Table I. Poly(amido amine)s Containing Different Amount of Tyramine Residues

Polymer no.	Code	Feed ratio AEEOL/TA (mol/mol)	Composition AEEOL/TA[a] (mol/mol)	M_w[b] (kDa)	M_w/M_n[b]	Yield (%)
1a	pAEEOL90/TA10	90/10	91/9	6.6	1.3	17
1b	pAEEOL80/TA20	80/20	83/17	6.9	1.5	23
1c	pAEEOL70/TA30	70/30	72/28	7.2	1.7	10

[a]Determined by ^{1}H-NMR, [b]Determined by gel penetration chromatography.

other water-soluble polymers, such as chitosan, dextran, hyaluronic acid, and Pluronic F127.[12] More recently, horseradish peroxidase (HRP)-mediated crosslinking using hydrogen peroxide as an oxidant was investigated to prepare novel *in situ* forming hydrogels.[13,14] It was shown that HRP-mediated crosslinking enables easier control of gelation rate and mechanical strength by varying the HRP concentration when compared with Michael-type addition reactions.[14] Besides, a major advantage of HRP-mediated crosslinking is of milder gelation conditions (e.g., physiological conditions). As such, HRP-crosslinked *in situ* forming hydrogels have been studied widely for biomedical applications. The current HRP-crosslinked hydrogels are mainly prepared from natural polysaccharides, such as dextran and hyaluronic acid. Because these hydrogels are generally nondegradable or degradable slowly under physiological conditions, they are largely applied for tissue engineering. For example, Jin et al. investigated HRP-crosslinked fast, *in situ* forming dextran-tyramine (TA) hydrogels for cartilage tissue engineering.[15,16] Only few studies have appeared on HRP-crosslinked hydrogels for drug delivery. For example, a recent work reported by Kurisawa and coworkers illustrated that HRP-crosslinked, biodegradable hyaluronic acid-TA hydrogels may serve as carriers for protein delivery.[17]

Poly(amido amine)s (PAAs) are a family of peptidomimetic polymers, which can be synthesized by Michael-type addition reaction between bisacrylamides and primary or bis-secondary amine compounds.[18] This reaction allows the availability of a number of PAA polymers with different functional groups in polymeric main chain or side chain. It has been documented that PAAs typically have high solubility, low cytotoxicity, and good degradability under physiological conditions. Thus, these features make PAAs valuable materials for biomedical applications.[18,19] For example, Ferruti and coworkers synthesized PAA copolymers with pendant cyclodextrin residues, which can form a complex with acyclovir for anti-viral therapy.[20] Duncan and coworkers recently reported on a group of PAA-doxorubicin conjugates via an acid-sensitive linker for doxorubicin release at an endosomal pH.[21] Ferruti et al. further prepared preformed PAA-based hydrogels via Michael-type addition and applied the hydrogels for cell culture.[22] However, to the best of our knowledge, no study was yet reported on *in situ* forming PAA-based hydrogels.

The purpose of this study is to prepare HRP-mediated *in situ* forming hydrogels from PAAs. Because PAAs are degradable under physiological conditions, it is expected that the PAA-based hydrogels may have better degradability as compared with those nondegradable polysaccharide-TA hydrogels reported previously. To this end, the PAA copolymers containing different amounts of tyramine residues (denoted as pAEEOL/TA) were synthesized through Michael-type addition reaction between methylenebisacryamide (MBA) and the amine mixture of 2-(2-aminoethoxy) ethanol (AEEOL) and TA. It was found that HRP-crosslinked hydrogels from the pAEEOL/TA could be formed *in situ* under physiological conditions. The properties of the hydrogels were investigated in terms of gelation time, swelling/degradation, and mechanical modulus. Cytotoxicity of the degradation products of the hydrogels were evaluated against NIH 3T3 cells *in vitro*. Moreover, *in vitro* release behaviors of methylene blue (MB) and IgG protein from pAEEOL/TA-based hydrogels were examined under physiological conditions. The results of this study indicate that HRP-mediated *in situ* forming degradable hydrogels based on the pAEEOL/TA are promising for biomedical applications such as drug delivery.

EXPERIMENTAL

Materials

MBA, TA, and AEEOL were purchased from Sigma-Aldrich. MB and IgG protein were ordered from DingGuo Biotech. Co. (Shanghai).

Synthesis of Poly(amido amine) Copolymers Containing Different Amounts of Tyramine Residues (pAEEOL/TA)

The pAEEOL/TA copolymers were synthesized by Michael-type addition reaction between the MBA and amine mixture of AEEOL and TA at different mole ratios (i.e., 90/10, 80/20, and 70/30, Table I). In a typical experiment, pAEEOL80/TA20 copolymer was synthesized by adding MBA (4.13 g, 26.8 mmol), AEEOL (2.25 g, 21.4 mmol), and TA (0.74 g, 5.4 mmol) into a brown reaction flask and dissolved in methanol/water mixture (12 mL, 1/2, v/v) as a solvent. The reaction was performed in the dark at 45°C under nitrogen atmosphere and was allowed to proceed for 5 days yielding a viscous solution. Next, 10 mol % of excess AEEOL was added to consume any unreacted acrylamide groups and stirring was continued for 2 days at 45°C. The resulting solution was diluted with 10 mL water, acidified with 4*M* HCl solution to pH 5, and then dialyzed through dialysis membrane (MWCO 3500 g/mol) for 3 days. The copolymer was collected as a solid powder after freeze-drying. Yield: 1.2 g (17%). The other two PAAs, i.e., pAEEOL90/TA10 and pAEEOL70/TA30, were synthesized in a similar procedure aforementioned.

Polymer Characterization

^{1}H-NMR (300 MHz) spectra were recorded on a Varian Inova spectrometer (Varian, USA). The signal of solvent residue was used as a reference for the ^{1}H-NMR chemical shift and was set at δ 4.79 for D_2O. The molecular weight and polydispersity of

PAA were determined by gel penetration chromatography relative to polyethylene oxide (PEO) standards as described previously.[23]

Gelation Time

Hydrogel samples (0.25 mL, 10 wt %) were prepared in vials at room temperature. As a typical example, freshly prepared phosphate buffered saline (PBS) solution of H_2O_2 (25 μL of 0.4 wt % stock solution, i.e., a final concentration of 12 m*M*) and HRP (25 μL of 0.25 mg/mL stock solution, i.e., a final concentration of 7.5 U/mL) were added into a PBS solution of pAEEOL70/TA30 (200 μL, 12.5 wt %), and the mixture was gently vortexed. The gelation time was determined by the vial tilting method.[24,25] Samples were regarded as hydrogels when no flow occurred within 0.5 min.

Swelling and Degradation

Hydrogel samples (0.25 mL) were prepared in vials according to the procedure described in section 2.4 and accurately weighted (W_i). Subsequently, 2 mL of PBS solutions were applied on top of the hydrogels and then incubated at 37°C. At regular time intervals, the buffer solution was removed from the samples and the hydrogels were weighted (W_t). The swelling ratio is expressed as W_t/W_i. The medium was refreshed after weighing and the experiments were performed in triplicate.

Rheological Analysis

Rheological experiments were performed with a Bohlin rheometer (Malvern, UK) using parallel plate (40 mm diameter, 0°) configuration at 37°C in the oscillatory mode. A PBS solution of pAEEOL/TA was mixed with a PBS solution of HRP and H_2O_2 and then quickly applied to the rheometer. The upper plate was immediately lowered to a measuring gap size of 0.5 mm, and the measurement was started. The evolution of the storage (G') and loss (G'') modulus was recorded as a function of time. A frequency of 0.5 Hz and a strain of 0.1% were applied to maintain a linear viscoelastic regime.

Drug Loading and *In Vitro* Release

The model drug, MB or fluoresceinisothiocyanate (FITC)-labeled IgG (FITC-IgG) protein, was loaded into PAA hydrogels by first mixing the drug with PAA in PBS solution and then with HRP and H_2O_2 in PBS solutions. As a typical example, 1 mg/mL of MB stock solution in PBS buffer was freshly prepared. To a PBS solution of PAA (100 μL, 25 wt % stock solution), 100 μL of MB stock solution was added and mixed by vortexing. Then, freshly prepared PBS solution of HRP (25 μL of 150 U/mL stock solution) and H_2O_2 (25 μL of 0.4 wt % stock solution) was added to the PAA solution and the mixture was gently vortexed. The hydrogels loaded with MB were kept in dark at 37°C for 24 h to complete the crosslinking process. Next, 3 mL of release medium (PBS) was added on the top of the hydrogel, and it was gently shaken in the incubator at 37°C. The release experiment was performed in triplicates. At a different time interval, 3 mL of PBS was taken out and replaced with 3 mL of fresh PBS. The concentration of released MB in the PBS was determined spectrophotometrically at the absorption of 665 nm using a Cary 50 UV–visible spectrophotometer (Varian). MB concentration was calculated using a calibration curve derived from a series of MB solutions at different concentrations. For the FITC-IgG protein, the concentration of released IgG in the PBS was determined using a fluorescence plate reader (Thermo scientific Fluoroskan Asecnt) with excitation and emission wavelengths of 485 and 538 nm, respectively. FITC-IgG concentration was calculated using a calibration curve derived from a series of FITC-IgG solutions at different concentrations.

Cytotoxicity Evaluation of pAEEOL/TA Copolymer

To evaluate cyto-compatibility of pAEEOL/TA copolymers, NIH 3T3 cells were exposed in the Dulbecco's Modified Eagle Medium (DMEM) complete medium containing pAEEOL70/TA30 copolymer at varying concentrations for 2 h and then incubated in fresh DMEM complete medium for another 46 h. The viable cells were determined by Alamar Blue assay according to the manufacture's standard protocol. Cells cultured without treatment as a blank control was taken as 100% cell viability. All tests were carried out in triplicate. Briefly, NIH 3T3 cells were cultured in DMEM complete medium containing 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (Invitrogen). The cells were then seeded at 1×10^4 cells per well in a standard 96-well plate for at least overnight. The pAEEOL70/TA30 solution (PBS buffer, pH 7.4) was sterilized through a filter (Millipore) with a pore size of 0.22 μm. Next, the cells were exposed in the pAEEOL70/TA30 solution at different polymer concentrations at 37°C in a humidified atmosphere containing 5% CO_2. Cell viability was determined by Alamar Blue assay using the manufacture's standard protocol. Briefly, the cells were washed with fresh PBS and 200 μL of 1× Alamar Blue-RMPI 1640 medium (without phenol red) was added to each well. After 4 h of incubation, 180 μL of the medium in each well was transferred to a 96-well plate for reading. Absorbance density (OD) was then recorded using a plate reader (Thermo Scientific) at the wavelength of 570 nm. Cell viability was calculated with the following equation: cell viability (%) = $(OD_{sample} - OD_0)/(OD_{control} - OD_0) \times 100$, wherein OD_{sample}, $OD_{control}$, and OD_0 represent the OD value of the medium with the gels, the medium without the gels, and 1× Alarm Blue-RMPI 1640 medium as a blank, respectively.

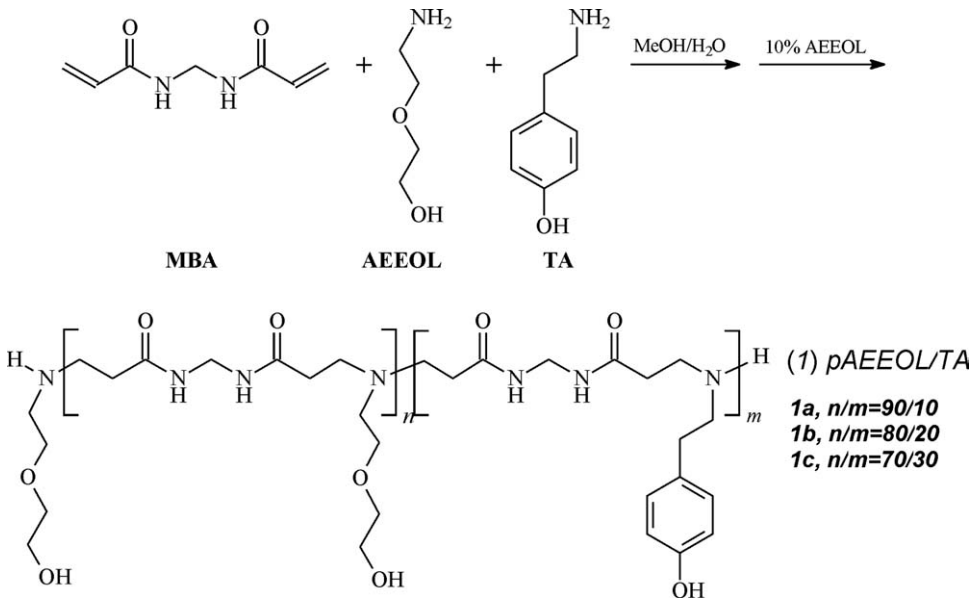
Cytotoxicity of the Degradation Products of pAEEOL/TA-Based Hydrogels

HRP-crosslinked pAEEOL/TA hydrogels were allowed to incubate in PBS at 37°C for 7 days until the disappearance of the hydrogels, which are regarded as the degradation of the hydrogels. The solution was sterilized through a filter (Millipore) with the pore size of 0.22 μm. NIH 3T3 cells were then exposed in the DMEM complete medium containing the degradation products at different concentrations for 2 h and then incubated in fresh DMEM complete medium for another 46 h. The viable cells were determined by Alamar Blue assay according to the manufacture's standard protocol. Cells cultured without treatment as a blank control was taken as 100% cell viability. All tests were carried out in triplicate.

RESULTS

Synthesis and Characterization of Poly(amido amine) Copolymers

Three PAA copolymers (pAEEOL/TA) containing different amount of TA residues were synthesized by Michael-type



Scheme 1. Synthesis of poly(amido amine) copolymers containing different amounts of tyramine residues.

addition reaction between the MBA and the amine mixture of AEEOL and TA (Scheme 1). The reaction proceeded at an equal mole ratio of the MBA and the amine monomer (AEEOL and TA) in the methanol/water mixture as a solvent.[23] During the reaction process, the reacting mixture became more viscous over time. To terminate the reaction, 10 mol % excess of AEEOL was added into the reaction system to consume any unreacted acrylamide groups. As a result, the pAEEOL/TA polymers have AEEOL or TA residue as amino end groups. These copolymers were eventually isolated in their HCl-slat form after exhaustive dialysis (MWCO 3500) and then freeze-drying. They are readily soluble in water (at least 10 wt % solubility) or methanol, but not in chloroform. The ^{1}H-NMR spectra of the copolymers were in full accordance with the expected structures (Figure 1). In addition, the composition ratio between MBA and TA in the copolymers was determined by comparing the integrals of the ^{1}H-NMR signals at δ 4.6 and δ 6.8, respectively, attributed to the proton (HNCH_2NH) in the MBA moieties and the aromatic protons (HOC=C*H*) in the TA moieties. It was found that the compositions of these copolymers are in good accordance with the AEEOL/TA feed ratios (Table I). Besides, the absence of any proton signal between 5 and 7 ppm means that no unreacted acrylamide group is present in these copolymers. Gel permeation chromatography measurements showed that the weight-average molecular weight of these copolymers ranged from 6.6 to 7.2 kDa with a relatively narrow polydispersity (1.3–1.7). Thus, these results indicate the successful availability of TA-based PAA copolymers.

Gel Formation and Gelation Time

PAA-based hydrogels could be successfully prepared via the HRP-mediated coupling reaction of phenol residues in pAEEOL/TA copolymers in PBS buffer at pH 7.4 and ambient temperature. The enzymatic crosslinking of these copolymers was investigated, wherein different HRP or H_2O_2 concentrations as well as polymer concentrations were applied. The gelation time was determined by the vial tilting method as described previously.[14] Figure 2(a) shows the gelation times of the hydrogels based on the pAEEOL/TA copolymers having different amount of TA moieties. It was found that the times of these gels decreased with increasing amounts of the TA moieties in the pAEEOL/TA copolymers at a constant concentration of HRP (15 U/mL) and H_2O_2 (12 m*M*) as well as polymer concentration (10 wt %). The shortest gelation time was observed for the hydrogel from the pAEEOL70/TA30 copolymer. Moreover, the concentrations of HRP and H_2O_2 have an effect on the gelation times. As a typical example, as the HRP concentration was increased from 3 to 15 U/mL, the gelation times of the pAEEOL70/TA30-based hydrogels remarkably decreased from about 360–50 s [Figure 2(b)]. On the other hand, at a constant HRP and polymer concentration, increasing the H_2O_2 concentrations from 8 to 24 m*M* led to an increment in the gelation

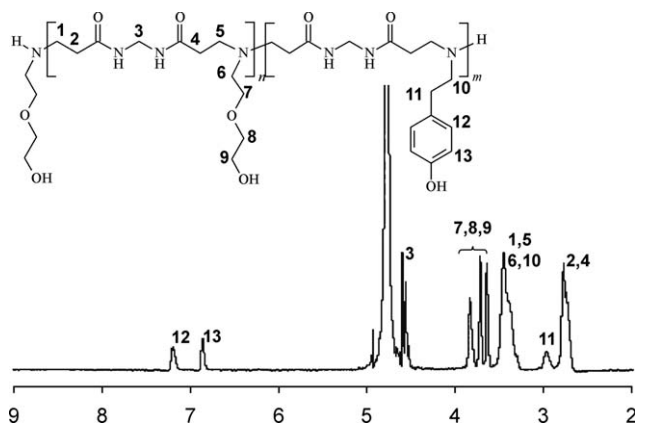


Figure 1. ^{1}H-NMR spectra (D_2O, 300 MHz) of pAEEOL70/TA30 copolymer.

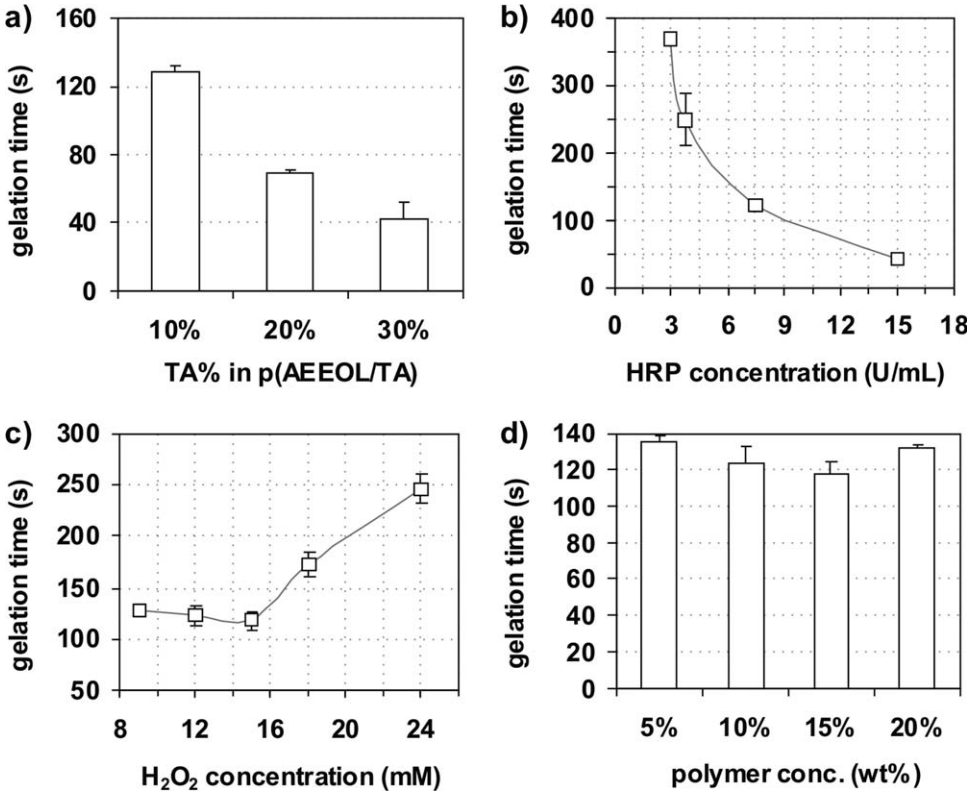


Figure 2. Gelation time of HRP-crosslinked pAEEOL/TA-based hydrogels in PBS buffer (20 m*M*, pH 7.4) under various conditions: (a) Gelation time of 10 wt % pAEEOL/TA-based hydrogels from the pAEEOL/TA copolymers containing different amounts of TA with the same concentration of HRP (15 U/mL) and H_2O_2 (12 m*M*); (b) gelation time of 10 wt % pAEEOL70/TA30-based hydrogels with 12 m*M* H_2O_2 as a function of HRP concentration; (c) gelation time of 10 wt % pAEEOL70/TA30-based hydrogels with 7.5 U/mL HRP as a function of H_2O_2 concentration; (d) gelation time of pAEEOL70/TA30-based hydrogels with the same concentrations of HRP (7.5 U/mL) and H_2O_2 (12 m*M*) as a function of polymer concentration.

time [Figure 2(c)]. Figure 2(d) shows the gelation times of pAEEOL70/TA30-based hydrogels as a function of polymer concentration. Keeping a constant HRP and H_2O_2 concentration, increasing the polymer concentrations from 5% to 20% has little effect on the gelation times. A fast gelation was observed for these hydrogels with the gelation time of 120–140 s.

In Vitro Degradation Study

The degradation behaviors of pAEEOL/TA-based hydrogels under physiological conditions were investigated by determining the swelling ratio as a function of time.[12] Figure 3 reveals the swelling ratios of the hydrogels from the pAEEOL70/TA30, prepared at a constant polymer concentration of 10 wt % but different HRP and H_2O_2 concentrations. In general, all these hydrogels revealed increased and then decreased swelling ratios and, eventually, they were completely degraded within a few days. Moreover, the HRP and H_2O_2 concentrations have an effect on the degradation times. For example, increasing the HRP concentrations from 3 to 15 U/mL slightly increased the degradation time from 5 days to 6 days [Figure 3(a)]. In addition, increasing the H_2O_2 concentrations from 12 to 18 m*M* appreciably prolonged the degradation time from 6 days to 8 days [Figure 3(b)]. The longest degradation time (8 days) was observed for the hydrogel prepared at a HRP concentration of 7.5 U/mL and a H_2O_2 concentration of 18 m*M*. These results show that the degradation behaviors of pAEEOL/TA-based hydrogels can be adjusted by varying the HRP and H_2O_2 concentrations.

Rheological Analysis

The mechanical properties of PAA-based hydrogels were examined by oscillatory rheology experiments. Gel precursor solutions of pAEEOL70/TA30 were prepared at a polymer concentration of 10 wt %. Then, the solution was immediately applied to a rheometer and time sweep were preformed at 37°C to record the storage modulus (G') and loss modulus (G'') in time. As indicated in Figure 4, the storage and loss moduli of the hydrogels increased in time because of HRP-crosslinking reaction [Figure 4(a)]. The crosslinking reaction is considered as complete when the storage modulus reaches its plateau value. Moreover, it appears that the HRP and H_2O_2 concentration has a direct effect on the mechanical properties of these pAEEOL70/TA30-based hydrogels. Keeping the same concentration of H_2O_2 (12 m*M*), the plateau values of the hydrogels increased from 2500 to 4100 Pa when increasing HRP concentrations from 3.75 to 7.5 U/mL [Figure 4(b)]. However, keeping the same concentration of HRP (7.5 U/mL), the plateau values of the hydrogels significantly decreased from 4100 Pa to 2100 Pa when increasing H_2O_2 concentrations from 12 to 16 m*M* [Figure 4(c)].

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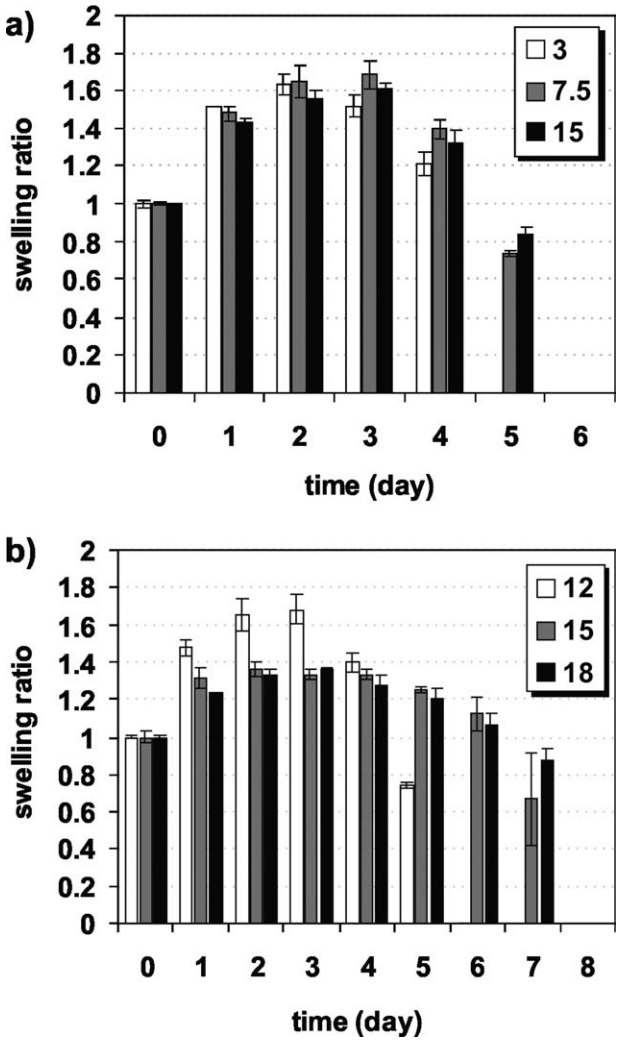

Figure 3. The swelling and degradation profiles of 10 wt % HRP-crosslinked pAEEOL70/TA30-based hydrogels prepared at a H_2O_2 concentration of 12 m*M* and different HRP concentrations (a) or at a HRP concentration of 7.5 U/mL and different H_2O_2 concentrations (b).

In Vitro Cyto-Biocompatibility of pAEEOL70/TA30-Based Hydrogels

The cytotoxicity of pAEEOL70/TA30 copolymer was evaluated against NIH 3T3 fibroblasts *in vitro*. Figure 5(a) shows the cell viability of the cells exposed at different polymer concentrations. It was found that this copolymer had low cytotoxicity (>90% cell viability) up to a tested concentration of 1000 μg/mL. To ascertain whether the degradation products of pAEEOL70/TA30-based hydrogels are cyto-biocompatibility, NIH 3T3 cells were exposed in the degradation products of the hydrogels that were prepared with a constant H_2O_2 concentration (12 m*M*), but different polymer and HRP concentrations. As revealed in Figure 5(b), the degradation produces of these hydrogels also revealed low cytotoxicity (>80% cell viability) up to a tested degradation product concentration of 2.5 mg/mL. These data illustrate that HRP-crosslinked hydrogels from the pAEEOL70/TA30 copolymer possess relatively good cyto-biocompatibility.

Model Drug Release from HRP-Crosslinked PAA-Based Hydrogels

To examine the possibility of HRP-crosslinked pAEEOL70/TA30 hydrogels for drug delivery, MB and IgG protein as model drugs were, respectively, encapsulated into the hydrogels (10 wt %) during gelation process. Figure 6(a) exhibits typical examples of these hydrogels incorporated with MB or FITC-labeled IgG. For comparison purpose, pAEEOL70/TA30-based hydrogels

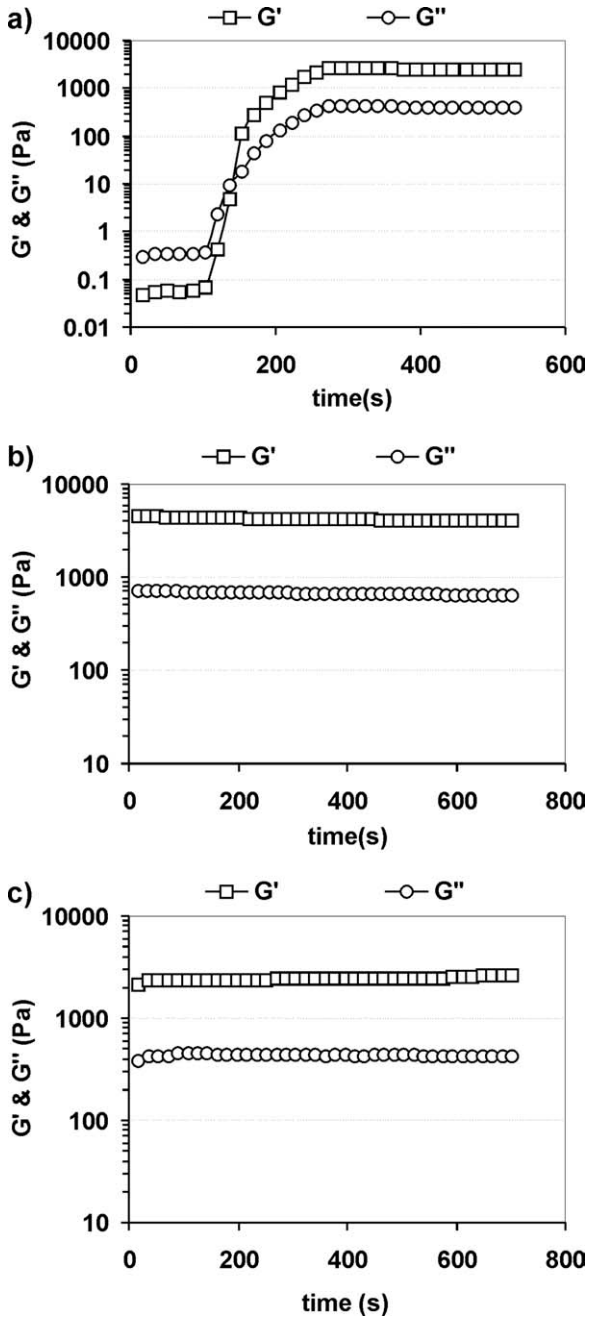

Figure 4. The storage modulus and loss modulus of HRP-crosslinked 10 wt % pAEEOL/TA-based hydrogels prepared at the HRP concentration of 3.75 (a) and 7.5 U/mL (b) when keeping the same H_2O_2 concentration of 12 m*M* or at the HRP concentration of 7.5 U/mL and H_2O_2 concentration of 18 m*M* (c).

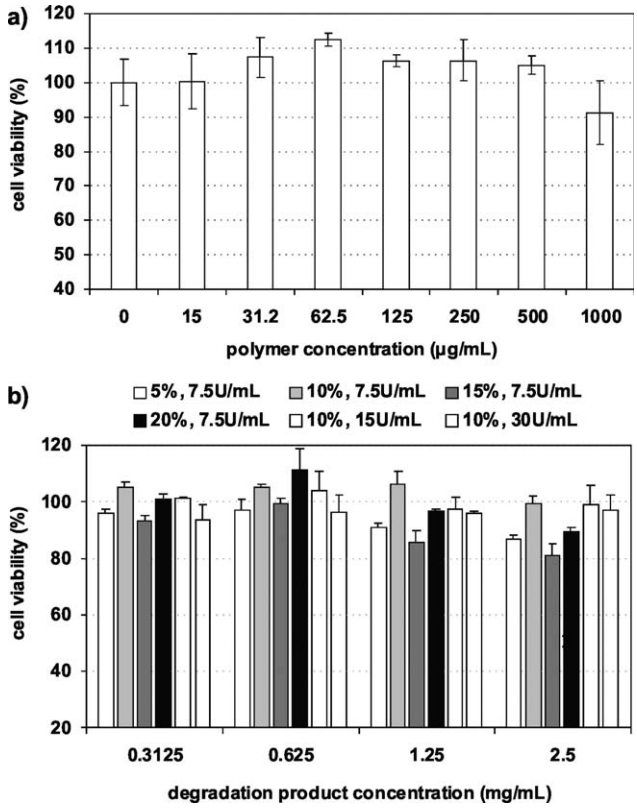

Figure 5. (a) Cell viability of pAEEOL70/TA30 copolymer exposed in NIH 3T3 cells at a function of polymer concentration; (b) cell viability of the degradation products of HRP-crosslinked 10 wt % pAEEOL70/TA30-based hydrogels as a function of the degradation product concentration. The hydrogels were prepared at different polymer and HRP concentrations and the same H_2O_2 concentration of 12 m*M*.

encapsulated with MB were prepared at different HRP concentrations ranging from 3 to 15 U/mL and a constant H_2O_2 concentration of 12 m*M*. *In vitro* release profiles of MB and IgG protein were studied in PBS buffer at pH 7.4 and 37°C. The concentrations for released MB and FITC-labeled IgG can be determined by UV/vis spectrum and fluorescence spectrum, respectively. It was found that the pAEEOL70/TA30-based hydrogels could mediate efficient and sustained MB release [Figure 6(b)]. In addition, a slow MB release was observed when increasing HRP concentrations. For example, at a HRP concentration of 15 U/mL, about 90% of loaded MB was released out within 52 h. However, at a HRP concentration of 3.5 or 7.5 U/mL, almost 100% of loaded MB was already released out within the same time. It is worth pointing out that the MB release from these hydrogels is proportional to the square root of time [Figure 6(c)], indicating that the release kinetics is close to first order. On the basis of above results, FITC-labeled IgG protein was also encapsulated in pAEEOL70/TA30-based hydrogel prepared at a HRP concentration of 15 U/mL and a H_2O_2 concentration of 12 m*M*. The release profile of IgG protein revealed first-order release kinetics within the first 3 days and then a slightly accelerated release [Figure 6(d)]. Taken together, these data indicate that HRP-crosslinked pAEEOL/TA hydrogel can serve as carrier system for drug delivery.

DISCUSSION

PAA is a family of peptidomimetic and degradable polymers.[18] The PAA polymers with various functional groups can be readily synthesized through Michael-type addition between appropriate bisacrylamide and primary amine compounds. Thus, in this study, the pAEEOL/TA can be successfully obtained with the MBA and the mixture of AEEOL and TA at varying molar ratios (Table I). In the reaction, the mixture of water and methanol was used as a solvent to make the AEEOL and TA homogeneous in the reaction system. After 5 days' reaction, an excess of AEEOL was added to ensure that all potentially cytotoxic acrylamide end groups are consumed. The monomers, MBA and AEEOL, are chosen because they are hydrophilic. As such, the pAEEOL/TA copolymers are well soluble in PBS buffer at pH 7.4. Moreover, the TA residues in the copolymers provide phenol groups for HRP-mediated crosslinking.

HRP-catalyzed crosslinking reaction has emerged as an effective approach for the fabrication of *in situ* forming hydrogels based on phenol-containing polymers. In this reaction, HRP can catalyze oxidation of the phenol groups in the TA residues of pAEEOL/TA copolymers using H_2O_2, resulting in the formation of coupled phenols. The hydrogels from the pAEEOL70/TA30 copolymer showed shorter gelation time as compared with those from the pAEEOL80/TA20 and pAEEOL90/TA10 copolymers [Figure 2(a)]. This faster gelation process may be likely attributed to a higher amount of TA residues in the pAEEOL70/TA30, which leads to increased possibility for the coupling of phenol groups in the TA residues. Moreover, the gelation times of pAEEOL70/TA30-based hydrogels decreased with increasing HRP concentrations, but increased with increasing H_2O_2 concentrations [Figure 2(b,c)]. This result is most likely due to an increment in the rate of H_2O_2 decomposition and the production of phenoxy radicals for coupling when increasing HRP concentrations. However, the oxidation of HRP by an excessive amount of H_2O_2 could induce an inactivated HRP, thus decreasing crosslinking efficiency.[26] A similar outcome was also reported by Jin et al., who found that increasing HRP concentrations decreased the gelation times of dextran-TA hydrogels and, on the other hand, increasing H_2O_2 concentrations adversely elongated the gelation times.[14] It should be noted that a suitable gelation time is important when *in situ* forming hydrogels are applied for various biomedical applications. For example, a relatively fast gelation is needed to cause the payloads like drugs to be loaded efficiently and distributed homogenously in hydrogels. A slow gelation, however, allows for sufficient diffusion of flowing gel precursor into defect tissues before hydrogel formation. Because the gelation time of pAEEOL/TA-based hydrogels can be adjusted by varying HRP and H_2O_2 concentrations, these hydrogels offer the possibility for various biomedical applications. The concentration of HRP and H_2O_2 may influence not only the gelation time but also other hydrogel properties. For example, the degradation time and storage modulus of the pAEEOL70/TA30 hydrogels increased with increasing HRP concentrations [Figures 3 and 4(b)]. This is likely due to the availability of a higher crosslinking density as a result of a higher HRP concentration. This result was also shown in HRP-crosslinked dextran-TA or hyaluronic

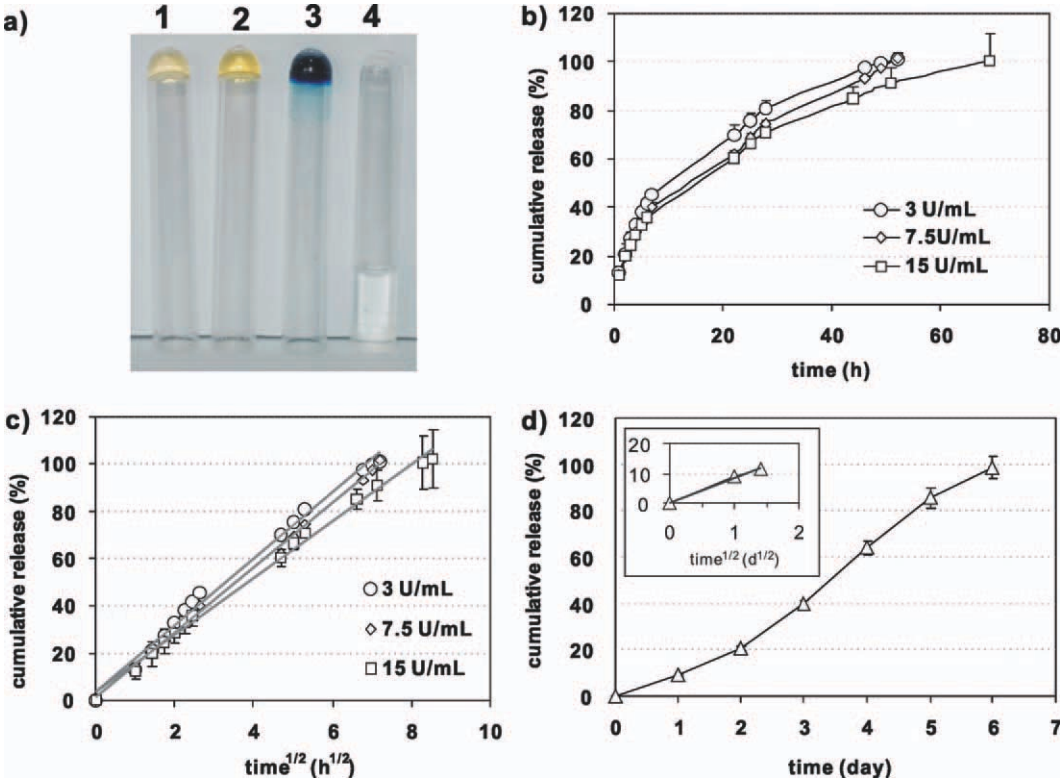

Figure 6. (a) Schematic demonstration of pAEEOL70/TA30-based hydrogel (1), the hydrogel encapsulated with FITC-IgG protein (2) and methylene blue (3), and the PBS buffer (4); (b) cumulative release of methylene blue from HRP-crosslinked 10 wt % pAEEOL70/TA30-based hydrogels, prepared at different HRP concentrations and a H_2O_2 concentration of 12 m*M*; (c) cumulative release of methylene blue for these pAEEOL70/TA30-based hydrogels as a function of the square root of time; (d) cumulative release of FITC-IgG protein from HRP-crosslinked 10 wt % pAEEOL70/TA30-based hydrogels prepared at a final HRP concentration of 15 U/mL and a final H_2O_2 concentration of 12 m*M*. Insert shows the cumulative release as a function of the square root of time. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

acid-TA hydrogels.[16,27] However, an excess amount of H_2O_2 may cause the inactivation of HRP, thus giving rise to decreased crosslinking efficiency. This may explain that increasing the concentration of H_2O_2 leads to reduced storage modulus of the pAEEOL70/TA30 hydrogels [Figure 4(c)].

Cyto-biocompatibility is important for *in situ* forming hydrogels when they are applied for biomedical applications. Unlike natural polysaccharides, the pAEEOL/TA copolymers are synthetic biomaterials. It is, thus, critical to identify whether they are cyto-biocompatibility. It was shown that the cytotoxicity of PAAs depended on their chemical structures.[22] Moreover, it appeared that AEEOL-based PAAs could have low cytotoxicity. For example, reducible PAAs from the cystaminebisacrylamide and AEEOL showed lower cytotoxicity than 25-kDa polyethylenimine.[23] Also, the degradation of PAAs by chemical hydrolysis of amide bond in the polymers under physiological conditions may contribute to the low cytotoxicity.[18] These reasons may explain why pAEEOL70/TA30 copolymer has low cytotoxicity and also the degradation products of HRP-crosslinked pAEEOL70/TA30 hydrogels are low cytotoxic [Figure 5(a,b)].

HRP-crosslinked hydrogels from the pAEEOL70/TA30 copolymer display better degradability as compared with those from nondegradable polysaccharide-TA conjugates. This result certainly benefits from degradability of the copolymer under physiological conditions. Because the hydrogels are totally degradable within a few days, they might be applicable for drug delivery rather than tissue engineering. As expected, HRP-crosslinked pAEEOL70/TA30 hydrogels can mediate sustained release under physiological conditions after encapsulation of MB and IgG protein in the hydrogels. The release kinetics of MB is close to first order [Figure 6(c)], implying a typical diffusion-controlled release behavior from a well-swollen hydrogel. The release profile of IgG protein is however more complex, i.e., a first-order release followed by a slightly accelerated release after 3 days [Figure 6(d)]. This acceleration is most likely because of the degradation of the hydrogel after 3 days. Kurisawa et al. have shown that HRP-crosslinked hyaluronic acid-TA hydrogels may used for the delivery of the protein such as lysozyme and, importantly, the protein still keeps its original bioactivity. It appears that the H_2O_2 as an oxidant has a martial effect on the stability of protein, most probably due to quick conversion of H_2O_2 into water by HRP enzyme. In this study, HRP-crosslinked pAEEOL/TA hydrogels are applied for sustained protein release, and thus, they have a high possibility to deliver therapeutic proteins for human protein therapy. This is the subject of current studies ongoing in our group.

CONCLUSIONS

We have demonstrated that PAA copolymers containing different amounts of TA residues (denoted as pAEEOL/TA) can be synthesized through Michael-type addition between the MBA and the amine mixture of AEEOL and TA. HRP-crosslinked hydrogels from pAEEOL/TA copolymers can be formed *in situ* under physiological conditions. These hydrogels displays a few favorable properties such as fast gelation and good cyto-biocompatibility. Besides, they possess better degradation profiles as compared with those nondegradable polysaccharide-TA hydrogels. *In vitro* release of MB and IgG protein from HRP-crosslinked pAEEOL70/TA30 hydrogels can be sustained without burst effect. The results of this study indicate that HRP-crosslinked hydrogels from the pAEEOL/TA have high potential as carrier systems for drug delivery.

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