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Biodegradable Poly(Ethylene Glycol) Hydrogels Based on a Self-Elimination Degradation Mechanism

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Abstract

Two vinyl sulfone functionalized crosslinkers were developed for the purpose of preparing degradable poly(ethylene glycol) (PEG) hydrogels (EMXL and GABA-EMXL hydrogels). A self-elimination degradation mechanism in which an N-terminal residue of a glutamine is converted to pyroglutamic acid with subsequent release of diamino PEG (DAP) is proposed. The hydrogels were formed via Michael addition by mixing degradable or nondegradable crosslinkers and copolymer {4% w/v; poly[PEG-alt-poly(mercapto-succinic acid)]} at room temperature in phosphate buffer (PB, pH=7.4). Hydrogel degradation was characterized by assessing diamino PEG release and examining morphological changes as well as the swelling and weight loss ratio under physiological conditions (37 °C). Degradation of EMXL and GABA-EMXL hydrogels occurred by surface erosion (confirmed by SEM). GABA-EMXL degradation was significantly faster (~3-fold) than EMXL; however, the degradation of both hydrogels in mouse plasma was 12-times slower than in PBS. The slower degradation rate in plasma as compared to buffer is consistent with the presence of γ -glutamyltransferase, γ -glutamylcyclotransferase and/or glutaminyl cyclase (QC), which have been shown to suppress pyroglutamic acid formation. The current studies suggest that EMXL and GABA-EMXL hydrogels may have biomedical applications where 1 to 2 week degradation timeframes are optimal.

1. Introduction

Hydrogels have been used in various biomaterial and biotechnology applications such as tissue engineering [1], artificial organs [2] and drug delivery [3–6] as well as for drug carriers especially for proteins [7]. Biodegradation is considered a critical requirement for most hydrogel applications since surgical removal from the body is painful at best. Degradation occurs by means of labile bonds that are introduced into the hydrogel matrix. A variety of linkages including esters [8], polyesters [9], polyanhydrides [10], imine (Schiff bases) [11], acetal [12], ketal [13], and enzymatically labile peptides [14] have been incorporated into degradable polymeric hydrogels. The hydrogels based on ester and anhydride bonds were designed to be cleaved by simple hydrolysis initiated under acidic or basic pH conditions [15,16]. For example, Harris and Zhao prepared a linear amine reactive PEG crosslinker

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containing two built-in ester bonds. This crosslinker was reacted with branched PEG amines to form degradable hydrogels [17]. There have been many other similar attempts at making degradable hydrogels based on ester mechanisms. Unfortunately, these hydrogels form carboxylic acid degradation products that raise the local acidity of the surrounding tissue, resulting in to scaffold degradation by autocatalysis and the elicitation of a pronounced inflammatory response [18,19]. Acid-sensitive degradable linkers such as acetals, cyclic acetals, ketals and Schiff-base linkages have also been used to prepare degradable hydrogels [12,13,20,21]. These linkers degrade via hydrolysis to produce hydroxyl and carbonyl terminals [20] in a pH dependent manner [22]. Enzymatically cleavable polymeric linkers have been copolymerized with PEG to form degradable gels [23]. Similar linkers have been used for covalently linking drug conjugates to the hydrogel matrix [24]. The rate of degradation of these hydrogels was found to be dependent on both the length of the polymer or copolymer and the concentration of enzyme.

Recently, degradable hydrogels based on self-immolative bifunctional hyaluronan-bisphosphonate conjugates were used for localized delivery and cell specific targeting [25]. This hydrogel degradation process occurs *via* a two-step mechanism. Hydrogel degradation begins with the cleavage of a disulfide bond in the conjugate, followed by spontaneous elimination resulting in the formation of ethylene episulfide, carbon dioxide, and free hydrazide. The conjugate used for this mechanism requires a multistep synthesis and it forms toxic degradation products like hydrazide [26,27].

In the current report, a new class of biodegradable hydrogels based on a unique self-elimination cleavage mechanism has been developed in order to achieve precise control of hydrogel degradation. This self-cleaving mechanism is based on a chemical reaction in which an N-terminal residue of a glutamine in the peptide participates in the displacement of its γ -amino group by its α -amino group. Upon degradation of these hydrogels, PEG-based degradation products are released that are expected to be nontoxic.

2. Materials and Methods

2.1. Reagents

Polyoxyethylene bis (amine) (MW, 3350 Da, DAP), dithiothreitol (DTT), *N, N*-diisopropylethylamine (DIEA) and hydrazine were purchased from Sigma-Aldrich (Saint Louis, MO). *N, N*-dimethylformamide (DMF) and diethyl ether were purchased from Fisher Scientific (Pittsburgh, PA). Benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) was purchased from Novabiochem (San Diego, CA) and 1, 6-Hexane-bis-vinylsulfone (HBVS) was purchased from Pierce (Rockford, IL). Mouse plasma was obtained from Harlan Bioproducts for Sciences (Madison, WI). The crosslinker intermediates **1a** (Dde-Glu(COOH)-Cys(S-StBu)-NH₂) and **1b** (Dde-GABA-Glu(COOH)-Cys(S-StBu)-NH₂) were obtained from EZ Biolabs (Carmel, IN). Sephadex LH-20 was purchased from GE Healthcare (Piscataway, NJ). All other reagents were purchased from Sigma, Aldrich or Fisher Scientific and used without further purification.

2.2. Synthesis of EMXL and GABA-EMXL Crosslinkers

General procedure for the synthesis of 2a/2b—DAP (0.506 g, 1 eq) was dissolved in DMF (5 ml). DIEA (0.172 ml, 7 eq) was added into the reaction mixture and it was stirred for 2 min at room temperature. Compound **1a**/Compound **1b** (0.581 g, 7 eq) and PyBOP (0.5744 g, 7.4 eq) in DMF (10 ml) were added into the reaction mixture, which was stirred at room temperature for 8 h. The product was purified on a Sephadex LH-20 using DMF as eluent. Purified product was poured drop wise into pre-cooled diethyl ether (30 ml) to precipitate the

product. The ether was removed and the flask containing the product was dried under argon gas. The product (**2a/2b**) was characterized by MALDI-TOF-MS.

2a. Yield: 88%. MALDI-TOF-MS. (m/z): calculated: 4350, observed: 4502; **2b.** Yield: 84%. MALDI-TOF-MS. (m/z): calculated: 4516, observed: 4614.

General procedure for the synthesis of 3a/3b—Compound **2a/2b** (0.050 g, 1 eq) and DTT (11.5 eq) were dissolved in DMF (5 ml) at room temperature. Na₂CO₃ (14 μL, 1 eq) was added into the reaction mixture, which was stirred at room temperature for 18 h. The product was purified on a Sephadex LH-20 using DMF as eluent. Purified product was poured drop wise into pre-cooled diethyl ether (30 ml) to precipitate the product. The product was dried under argon gas. The product (**3a/3b**) was characterized by MALDI-TOF-MS.

3a. Yield: 70%. MALDI-TOF-MS. (m/z): calculated: 4174, observed: 4163; **3b.** Yield: 75%. MALDI-TOF-MS. (m/z): calculated: 4340, observed: 4499.

General procedure for the synthesis of 4a/4b—Compound **3a/3b** (0.010 g, 1 eq) and HBVS (0.0676 g, 6 eq) were dissolved in DMF (5 ml). DIEA (0.76 μL, 2 eq) was added into the reaction mixture, which was allowed to stir at room temperature for 8 h. The product was purified on Sephadex LH-20 using DMF as eluent. Purified product was poured drop wise into pre-cooled diethyl ether (30 ml) to precipitate the product. The product was dried under argon gas. The product (**4a/4b**) was characterized by MALDI-TOF-MS.

4a. Yield: 81%. MALDI-TOF-MS. (m/z): Calculated: 4704, observed: 4633; **4b.** Yield: 84%. MALDI-TOF-MS. (m/z): calculated: 4870, observed: 4737.

General procedure for the synthesis of 5a/5b—3% hydrazine in DMF (5 ml) was added to compound **4a/4b** (0.059 g). The reaction mixture was stirred at room temperature for 3 h. The product was purified on a Sephadex LH-20 using DMF as eluent. Purified product was poured drop wise into pre-cooled diethyl ether (30 ml) to precipitate the product. The ether was removed and the product was dried under argon gas. The product (**5a/5b**) was characterized by MALDI-TOF-MS and ¹H NMR.

5a. Yield: 70 %. ¹H(400 MHz, DMSO-*d*₆) δ = 9.0 (6H, s, 2Glu-NH₃⁺), 8.05 (2H, brs, 2PEGCO-NH), 7.9 (2H, s, 2CONH), 7.4 (6H, s, 2CONH₂ and 2SO₂-CH), 6.6 (2H, t, 2 vinyl-CH₂), 6.4 (2H, t, 2 vinyl-CH), 4.4 (1H, m, Cys-α), 4.2 (1H, m, Cys-α), 4.0 (4H, m, 2CH₂SO₂), 3.78 (8H, m, 4SO₂-CH₂), 3.6 (brm, PEG-CH₂O-CH₂), 3.4 (brm, PEG-CH₂), 3.1 (4H, s, CH₂ and Glu-α), 2.8 (4H, t, 2S-CH₂), 1.85 (4H, m, 2Glu-β), 1.8 (4H, brm, 2Glu-γ), 2.1 (4H, brm, Cys-β), 1.7 (8H, m, 4CH₂) and 1.1 (8H, m, 4CH₂). MALDI-TOF-MS. (m/z): Calculated: 4374, observed: 4206.

5b. Yield: 73 %. ¹H(400 MHz, DMSO-*d*₆) δ = 8.0 (2H, brs, 2PEG-NH), 7.8 (2H, brs, 2NH), 7.52 (2H, brs, 2NH), 7.19 (4H, s, 2CONH₂), 6.94 (2H, dd, J = 6 Hz, J = 15Hz, 2SO₂CH), 6.7 (2H, m, 2 vinyl-CH₂), 6.0 (2H, m, 2 vinyl-CH), 5.7 (4H, brs, 2NH₂-GABA), 4.4 (m, 1H, Cys-α), 4.03 (6H, m, Cys-α and 2CH₂-SO₂), 3.8 (4H, m, 2Cys-β), 3.64 (8H, m, 4CH₂SO₂), 3.5 (brm, PEG-CH₂O-CH₂), 3.3 (brm, PEG-CH₂), 3.14 (4H, s, 2S-CH₂ and 2Glu-α), 2.18 (4H, brm, 2GABA-(γ)CH₂), 1.8 (4H, m, 2Glu-β), 1.68 (12H, m, 2CH₂CONH and 4 CH₂), 1.5 (4H, m, Glu-γ) and 1.2 (12H, m, 2 GABA(β)CH₂ and 4 CH₂). MALDI-TOF-MS. (m/z): Calculated: 4540, Observed: 4558.

2.3. Synthesis of Copolymer

Thiol-containing copolymer poly[PEG-alt-poly(mercapto-succinic acid)] was prepared by reacting poly-oxyethylene bis(amine) and *S*-tritylmercapto-succinic acid as reported earlier [6].

2.4. Hydrogel Preparation

Hydrogels were formed *via* the Michael addition reaction between copolymer (SH groups) and crosslinker (VS groups) in 20 mM phosphate buffer (PB) at pH 7.4. Copolymer (4% w/v) was dissolved in PB with gentle shaking. Separately in another vial, degradable (**5a** or **5b**) or nondegradable (HBVS) crosslinker was dissolved in PB with gentle shaking. Addition of copolymer solution to the crosslinker solution formed hydrogels.

A similar procedure was used for the preparation of FITC dextran-loaded hydrogel. FITC dextran was added to the copolymer solution. Hydrogels were formed by adding FITC dextran mixed copolymer solution into the crosslinker solution at room temperature. The gelation time was measured by the vial tilting method [28]. When the sample showed no flow within 5 sec, it was considered as being completely formed hydrogel.

2.5. Scanning Electron Microscopy (SEM)

Morphology of the hydrogels was characterized using SEM. The hydrogel samples were lyophilized and mounted on metal stubs. SEM was also used to analyze the surface morphology of the GABA-EMXL hydrogel during the degradation process. After being taken out of the incubation media at 0, 3, 5 and 8 h, the hydrogel samples were lyophilized and mounted on metal stubs. Samples were sputtered coated (SCD 004; Balzers Union Limited, Balzers, FL) with gold/palladium for 30 sec on a BAL-TEC, SCD 004 sputter coater. The surfaces of the gold/palladium-coated hydrogels samples were observed on an AMRAY-1830I microscope (Amaray, Inc. Pekin, IN) at 20 kV.

2.6. Hydrogel Release Studies

The release of physically trapped FITC-Dextran (20 kDa) from degradable and nondegradable hydrogel depot was studied by fluorescence spectroscopy. FITC-Dextran (20 kDa) loaded hydrogels (200 μ L) were prepared using degradable (**5a** or **5b**) and nondegradable (HBVS) crosslinkers and 4% w/v copolymer. After equilibration, the hydrogels were transferred to flat bottom vials. Hydrogels were completely submerged in 500 μ L phosphate buffered saline (PBS; 20 mM; pH=7.4). To measure the release of FITC Dextran, PBS (500 μ L) were taken at regular time intervals and replaced with an equal volume of fresh buffer. Similar experiments were performed in 500 μ L mouse plasma at 37 °C. Mouse plasma (500 μ L) samples were taken at regular time intervals and replaced with an equal volume of fresh mouse plasma.

Release studies were repeated three times in PBS and in mouse plasma. The concentration of FITC-Dextran in release samples was determined using a plate reader (Tecan Genios, Durham, NC) with an excitation wavelength of 490 nm and emission wavelength of 510 nm.

2.7. Swelling and Weight Loss Experiment

Hydrogels (4% w/v) were transferred to flat bottom vials. Subsequently hydrogels were completely submerged in 500 μ L of PBS (pH 7.4) and allowed to swell at 37 °C. The swollen hydrogels were weighed at regular time intervals after completely removing the buffer. After each weighing the buffer was replenished. Similar studies were performed using mouse plasma. Swelling studies were performed in triplicate in PBS and in mouse plasma (500 μ L). The swelling ratio of each hydrogel was

calculated from the initial hydrogel weight after preparation (W_0) and the swollen hydrogel weight after exposure to buffer at time t (W_t).

$$\text{Swelling ratio}(\%) = \frac{W_t}{W_0} \times 100$$

To determine the weight loss due to degradation of the hydrogel, the original weight of the dry hydrogel sample weighed as W_d . Subsequently, the samples were immersed in PBS and incubated at 37 °C. At different time intervals (3, 6, 8 and 10 h) the samples were removed, rinsed thoroughly with PBS (pH=7.4), lyophilized, and weighed. This value is represented as W_f . The percentage of weight loss was calculated as,

$$\text{Weight loss}(\%) = \frac{W_d - W_f}{W_d} \times 100$$

2.8. Hydrogel Degradation Studies Using Fluorescamine Assay

Fluorescamine is a reagent used for the detection of primary amines in the picomole range. Its reaction with amines is almost instantaneous at room temperature in aqueous media [29]. After hydrogel degradation, DAP was observed as a degradation product, therefore hydrogel degradation behavior was assessed using the fluorescamine assay.

Hydrogels (200 μ L) were prepared using copolymer (4% w/v) and crosslinker (EMXL and GABA-EMXL) and transferred to flat bottom vials. Hydrogels were exposed to PBS (pH=7.4) and incubated at 37 °C. Samples (200 μ L) were taken at regular time intervals and replaced with an equal volume of PBS. Fluorescamine (0.2 mg), dissolved in acetonitrile (0.2 ml), was added to each aliquot and diluted with PBS (pH 7.4) and immediately mixed by vortex. The amount of fluorescamine present in each sample was determined using a plate reader (Tecan Genios, Durham, NC) with an excitation wavelength of 380 nm and emission wavelength of 490 nm. Similar degradation studies were performed in mouse plasma at 37 °C. Mouse plasma samples were extracted using methanol and dried using a vacuum. The extract was redissolved in PBS and the degradation behavior of the hydrogels were measured as previously described. Degradation studies were performed in triplicate.

2.9. Statistical Analysis

Data from all studies were analyzed using GraphPad Prism v.4.0.1 (GraphPad Software, San Diego, CA).

3.0 Results and Discussion

3.1. Design and Development of Degradable Crosslinkers

In the current report, a self-elimination mechanism is proposed for controlling hydrogel degradation. An example of this mechanism involves, luteinizing hormone releasing hormone (LHRH) [30], in which spontaneous cyclization of the N-terminus glutamine residue results in the formation of pyroglutamic acid with the release of ammonia. Previously, the time dependent self-elimination mechanism was observed for glutathione (GSH) [31]. GSH contains the required site γ -carboxyl moieties at the N-terminal residue of Glu and systematic non-enzymatic degradation of GSH at pathological and physiological pH values verified the self-elimination mechanism.

Degradable crosslinkers **5a** and **5b** (Figure 1) based on the self-elimination mechanism were designed and synthesized (Scheme 1) in such a way that each crosslinker has two-vinyl sulfone and two free amino functionalities. In **5b** GABA group was covalently attached to the N-terminus of Glu. Due to simple structural consideration GABA group was chosen as the residue, which could reach the PEG carbonyl functionality for nucleophilic attack. Vinyl sulfone groups were used for the hydrogel formation by reacting with thiol groups of the copolymer. The free amino groups (**5a**: free amino group of Glu; **5b**: free amino group of GABA) initiate the self-elimination mechanism.

Compound **1a** was coupled at both termini of DAP using the coupling reagent PyBOP, yielding **2a**. Cleavage of the *-t*Bu groups from **2a** was performed using DTT in DMF, resulting in compound **3a**. The two free *-SH* groups of **3a** were reacted with HBVS, yielding **4a**. Cleavage of both Dde- groups was performed using 3% hydrazine in DMF, to obtain the degradable crosslinker **5a** (Scheme 1). The MALDI-TOF mass spectra of **2a**, **3a**, **4a** and **5a** are shown in Figure 2.

The **5b** was obtained from **1b** (Scheme 1). A similar process, as mentioned above for the preparation of **5a**, was used for the preparation of the **5b** crosslinker. The MALDI-TOF mass spectra of **2b**, **3b**, **4b** and **5b** are shown in Figure 3.

3.2. Hydrogel Formation and Characterization

3.2.1. Hydrogel Preparation—Hydrogels were investigated for their degradation properties as well as controlled release application. In earlier studies, it was shown that 4% w/v copolymer is an appropriate concentration for hydrogel preparation [4–6] therefore the same concentration was used in the current studies for the preparation of hydrogels.

Hydrogels were formed *via* Michael addition between copolymer (SH groups) and crosslinker (VS groups) in 20 mM phosphate buffer (PB) at pH 7.4. The copolymer poly[PEG-alt-poly(mercapto-succinic acid)] [6] was crosslinked with degradable (**5a** or **5b**) and nondegradable (HBVS) crosslinkers resulting in the formation of hydrogel networks connected by thioether bonds. The gelation times for degradable (EMXL and GABA-EMXL) and nondegradable (NDH) hydrogels were 51 sec, 80 sec and 100 sec, respectively. The slower formation of hydrogels using the **5b** crosslinker was likely due to the steric hindrance from GABA. The gelation times for degradable (EMXL and GABA-EMXL) and nondegradable (NDH) hydrogels are shown in Table 1.

3.2.2. Hydrogel Morphology

Hydrogel morphology was characterized using scanning electron microscopy (SEM). Figure 4 shows an interconnected porous morphology for both the EMXL and GABA-EMXL hydrogels. Figure 4 indicate that the pore sizes in EMXL (panel A,B) and GABA-EMXL (panel C,D) are similar and it ranges from 30 nm to 150 μ m. Morphology of these hydrogels are similar; containing many connective pore structures suggesting their applicability for sustained drug release applications.

3.3. In vitro Release Studies of FITC Dextran

FITC-Dextran (20 kDa) was physically trapped inside the hydrogel matrix and its release from degradable (EMXL and GABA-EMXL) and nondegradable hydrogels was studied at 37 °C in PBS (20 mM, pH=7.4) and mouse plasma. In Figure 5, the release profiles of FITC-Dextran from the hydrogels were shown to be a function of the crosslinkers and incubation medium used for the release studies. The release rate was found to follow first order kinetics.

The total release (~99 %) of FITC-Dextran from EMXL and GABA-EMXL hydrogels in PBS occurred in 10 h and 29.5 h, respectively. In mouse plasma, the release occurred in 72 h from both EMXL and GABA-EMXL hydrogels. FITC-Dextran release from NDH also occurred within 72 h for both PBS and mouse plasma.

These results indicate that the total release of FITC-Dextran from degradable hydrogels was faster in buffer compared to the NDH. In plasma, FITC-Dextran release occurred in the same time frame for both degradable hydrogels and the NDH. The release of FITC-Dextran from degradable (EMXL and GABA-EMXL) hydrogels in the mouse plasma and PBS indicates that the hydrogel pores are substantially larger than the hydrodynamic diameter of the FITC-Dextran, which is 6.6 nm [32]. In buffer, the release from degradable hydrogels was mainly characterized by a combination of diffusion and hydrogel matrix degradation. Further, it was observed that the release profile from the hydrogel in plasma was different from the release profile obtained in buffer. In plasma, FITC-dextran release was mainly determined by diffusion rather than degradation of the hydrogel matrix. Release from the NDH in PBS and plasma was determined by diffusion. The release profile from the degradable hydrogel in PBS was dependent upon degradation, however no effect of degradation was observed in mouse plasma. The differences in the degradation results between the buffer and mouse plasma relate to the presence or absence of enzymes. The detailed degradation mechanism is discussed in the hydrogel degradation mechanism section.

3.4 Hydrogel Degradation Studies

The degradation of the EMXL and GABA-EMXL hydrogels were characterized by assessing DAP release and examining morphological changes as well as swelling ratio under physiological conditions. Hydrogel swelling studies were used to measure the total capacity of hydrogel to absorb the PBS or mouse plasma. This method was the simplest way of characterizing the swelling kinetics and hydrogel degradation behavior. The fluorescamine assay was used to detect primary amines such as DAP, which indicated the degradation of the EMXL and GABA-EMXL hydrogels.

3.4.1. Hydrogel Degradation Studies using Swelling Ratios—The EMXL and GABA-EMXL hydrogels were incubated at 37 °C and the swelling ratio was calculated at regular time intervals as the ratio of the swollen hydrogel weight to the initial hydrogel weight (W_t/W_0). Swelling ratios of the EMXL and GABA-EMXL hydrogels change over time as shown in Figure 6. In mouse plasma, EMXL and GABA-EMXL hydrogels swell in 4–10 h and 2–4 h, respectively. In PBS, EMXL hydrogels swell in 8–10 h and the GABA-EMXL hydrogel swells in 6–7 h. The total degradation of the EMXL hydrogel in PBS and mouse plasma occurred in 29.5 h and 360 h, respectively, whereas GABA-EMXL hydrogel totally degrades in 10 h in PBS and 119 h in mouse plasma. The degradation profile of both hydrogels demonstrates that degradation occurs more rapidly in PBS than in plasma. It is particularly noteworthy that the EMXL and GABA-EMXL hydrogels degraded 12-times slower in mouse plasma than in buffer.

3.4. 2. Degradation Studies using Fluorescamine Assay—Hydrogel degradation profiles are shown as a function of the crosslinkers and medium used (PBS and mouse plasma) for the degradation studies (Figure 7). Complete degradation of GABA-EMXL and EMXL hydrogels occurred in PBS at 10 h and 29.50 h and in mouse plasma at 119 h and 360 h, respectively (Table 1). It was observed that hydrogel degradation followed second order degradation kinetics irrespective of the crosslinkers or medium (PBS or mouse plasma) used.

3.4.3. Surface Morphology of Degraded Hydrogel—To gain insight into the degradation mechanism, the morphology of the GABA-EMXL hydrogel was studied. The

GABA-EMXL hydrogel was selected because it completely degrades within 10 h in PBS (pH 7.4). The surface morphology of the GABA-EMXL hydrogel was examined using a scanning electron microscope at 0, 3, 5 and 8 h. The GABA-EMXL hydrogel at 0 h shows a relatively smooth surface and pore wall, while the degraded hydrogel exhibits irregular porous morphologies (Figure 8). Figure 8B–D confirms that the wall sizes between two pores are shrinking due to their degradation via self-elimination mechanism. After 5 h, the pore walls appeared to be degrading and the surface pore size was increasing compared to the initial pore size. The volume of hydrogel decreased as the bigger surface pore structures collapsed.

These studies show that the pores assume an irregular shape and the diameter of the pores increases as degradation time increases. The emergence of large grooves and extensive porous morphology in the degraded samples (Figure 8B–D) confirms degradation of the hydrogels and suggests that hydrogel degradation begins at the surface via the self-elimination mechanism. The free amino groups that are present on the crosslinkers activate more quickly on the surface due to the medium that was used for the degradation studies. SEM micrographs were consistent with the weight loss experiment (Figure 9) of the GABA-EMXL hydrogel. At 3 h, GABA-EMXL hydrogel had 30% weight loss, after 5 h incubation the weight loss was 70% and after 8 h, 87% weight loss was observed. At 10 h, 100% weight loss of GABA-EMXL hydrogel was observed. This data was also consistent with the degradation data (swelling ratio and fluorescamine assay) of the GABA-EMXL hydrogel. The SEM micrographs and weight loss experiments show that the degradation process occurs more rapidly on the surface of the hydrogel. Therefore the hydrogel degrades via surface erosion through self-elimination mechanism.

3.5 Hydrogel Degradation Mechanism

In the elimination mechanism, the amino groups present on the **5a** and **5b** crosslinkers (Glu and GABA, respectively) attack the γ -carboxyl group of the same molecule and form a five (EMXL) or ten (GABA-EMXL) membered cyclic intermediate (Figure 10). Afterward, the two amide bonds in the intermediates (γ -Glu and PEG) breaks and the hydrogel degrades. In both hydrogels DAP was observed as a degradation product. The degradation products were characterized by MALDI-TOF-MS. The DAP peak was observed at 3582.76 Da as shown in Figure 11. Degradation studies show that GABA-EMXL hydrogel degrades more rapidly than the EMXL hydrogel in medium (PBS and mouse plasma), due to the ring strain of the ten membered cyclic intermediate (Figure 10) that forms during GABA-EMXL hydrogel degradation.

Additionally, two amide bonds are present in the GABA-EMXL hydrogel. It was observed that the amide bond in GABA and α -Glu is more stable than the amide bond in γ -Glu and PEG. Therefore, the degradation of the GABA-EMXL hydrogel occurs via the amide bond cleavage in γ -Glu and PEG from both sides. Otherwise, if the amide bond in GABA and α -Glu were degraded then GABA or cyclic GABA would be released from the hydrogels. Neither product was detected in the mass spectrum (MALDI-TOF-MS) of the degraded GABA-EMXL hydrogel (Figure 12).

Furthermore it was observed that the hydrogel degradation process in plasma might occur via an enzymatic mechanism. Enzymes such as γ -glutamyltransferase, γ -glutamylcyclotransferase and/or glutaminy cyclase (QC) are known for pyroglutamin/pyroglutamate formation [33–35]. QC is present in tissues [36,37], and many other species, (i.e., rat, mice, human) [35,36, 38–40]. γ -glutamyltransferase and γ -glutamyl cyclotransferase enzymes are present in plasma [41,42] and cell membranes of many tissues, including kidney, bile duct, pancreas, liver, spleen, heart, brain and seminal vesicles [43]. It is involved in the transfer of amino acids across the cellular membrane [44] and leukotriene metabolism [45]. It is also involved in glutathione metabolism [46].

Pyroglutamic acid may occur naturally in proteins and peptides as a result of a catalyzed cyclization of the N-terminal glutamyl of glutaminyl residue. For example, L-glutamyl transferase [47] and γ -glutamyl transferase in combination with γ -glutamyl cyclotransferase [48] have been shown to catalyze the formation of L- pyrrolidone carboxylic acid and pyrrolidone carboxyl peptides from glutamine and glutaminyl peptides, respectively. The enzymes that spontaneously convert glutaminyl peptides into pyroglutamyl peptides in mammalian tissues have been well characterized [37]. Elsewhere, it has been shown that γ -glutamyl amino acids can be converted into pyrrolidone carboxylic acid and amino acids by a γ -glutamyl cyclotransferase [49,50]. QC facilitates the formation of N-terminal pyroglutamate (pGlu) formation from glutaminyl precursors which is a post-translational event in the processing of bioactive neuropeptides such as thyrotropin-releasing hormone and neurotensin during their maturation in the secretory pathway [35]. Previously Schilling *et al.* reported that human and papaya QC also catalyzed N-terminal glutamate cyclization. They observed that application of QC and glutamyl cyclase (EC) inhibitors *in vitro* suppressed the formation of pyroglutamic acid [38,39]. Therefore, this might be the reason that the plasma enzymes suppress the self-elimination mechanism; hence it suppresses the hydrogel degradation time. Preliminary *in vivo* studies have shown that subcutaneously injected EMXL hydrogels degraded in 2 weeks in mice (data not shown). Current studies (unpublished) focus on the *in vivo* degradation behavior of these hydrogels.

Conclusions

Degradable PEG hydrogels have been developed with the potential to serve as a drug delivery platform. Hydrogels were rapidly formed under physiological conditions by simply mixing solutions of the crosslinkers and copolymer. Degradation occurs via surface erosion through a self-elimination mechanism where cyclic intermediates were observed for the EMXL (five membered) and GABA-EMXL (ten membered) hydrogels. The results suggest that plasma enzymes (γ -glutamyltransferase, γ -glutamylcyclotransferase and/or glutaminyl cyclase) suppress the self-elimination mechanism resulting in slower degradation in plasma as compared to the buffer. Furthermore, this mechanism produces PEG as a degradation product, which should not affect the local pH (i.e., acidity) or toxicity. This biodegradable self-elimination mechanism based PEG hydrogel has potential for sustaining the delivery of drugs and proteins.

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Fig. 1.
Representation of **5a** and **5b** degradable crosslinkers.

Fig. 2.
MALDI-TOF-MS of **5a** and its intermediates (**2a–5a**). Signals are marked corresponding to peaks for **2a** (A; calculated: 4350, observed: 4502), **3a** (B; calculated: 4174, observed: 4163), **4a** (C; calculated: 4704, observed: 4633), and **5a** (D; calculated: 4374, observed: 4206).

Fig. 3. MALDI-TOF-MS of **5b** and its intermediates (**2b–5b**). Signals are marked corresponding to peaks for **2b** (A; calculated: 4516, observed: 4614), **3b** (B; calculated: 4340, observed: 4499), **4b** (C; calculated: 4870, observed: 4737), and **5b** (D; calculated: 4540, observed: 4558).

Fig. 4. Scanning electron micrographs (SEM) of EMXL (A and B) and GABA-EMXL (C and D) hydrogels. The hydrogels were prepared in PB (20 mM, pH=7.4)

Fig. 5. Cumulative release of FITC-Dextran (20 kDa) from degradable (EMXL and GABA-EMXL) and nondegradable hydrogels (NDH) at 37 °C in (A) PBS (pH 7.4) and (B) Mouse Plasma (mean $\pm$ S.D., n=3).

Fig. 6. Swelling ratios % ($W_t/W_0 \times 100$) profile of EMXL and GABA-EMXL hydrogels at 37 °C in (A) PBS (pH 7.4) and (B) Mouse Plasma (mean $\pm$ S.D. n=3).

Fig. 7. Degradation of EMXL and GABA-EMXL hydrogels at 37 °C in (A) PBS (pH 7.4); and (B) mouse plasma. Hydrogel degradation behaviors were measured using fluorescamine assay (mean $\pm$ S.D., n=3).

Fig. 8. Scanning electron micrograph (SEM) images of the GABA-EMXL hydrogels after incubation in PBS at 37 °C. Samples were removed from incubated hydrogel at regular time intervals (0, 3, 5, and 8 h) and freeze dried. (A) 0 h. (B) 3 h. (C) 5 h. (D) 8 h.

Fig. 9.
Degradation profile of GABA-EMXL hydrogel. Studies were done at 37 °C in PBS (pH=7.4).

Fig. 10. Degradation mechanism of EMXL (A; via five membered cyclic intermediate) and GABA-EMXL (B; via ten membered cyclic intermediate) hydrogels.

Fig. 11. MALDI-TOF-MS spectrum of the degraded hydrogel. The peak for DAP was detected at 3582.76 Da.

Fig. 12. MALDI-TOF-MS of the degraded GABA-EMXL hydrogel. The spectrum shows that there is no signal for GABA ($m/z=103$) or cyclic GABA ($m/z=83$).

Scheme 1.

Synthesis of **5a** and **5b** crosslinkers, a) DAP (3340 Da), PyBOP, DIEA, DMF, RT, 8 h; b) DTT, DMF, Na₂CO₃, RT, 18 h; c) HBVS, DIEA, DMF, RT, 8 h; d) 3% hydrazine in DMF, RT, 3 h.

Table 1

Hydrogel formation and degradation times in PBS and mouse plasma.

Hydrogel Volume	Copolymer	Crosslinker	Gelling Time	Degradation time in PBS (pH 7.4)	Degradation time in mouse plasma
Degradable hydrogel					
200 μ L	8 mg in 132.8 μ L PB	4.7 mg (EMXL) in 66.2 μ L PB	51 sec	29.5 hr	360 hr (~ 15 days)
200 μ L	8 mg in 132.8 μ L PB	5.34 mg (GABA-EMXL) in 66.2 μ L PB	80 sec	10.0 hr	119 hr (~ 5 days)
NDH					
200 μ L	8 mg in 132.8 μ L PB	0.3 mg in 66.2 μ L PB	100 sec	-	-