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Hydrolytic Stability of Methacrylamide and Methacrylate in Gelatin Methacryloyl and Decoupling of Gelatin Methacrylamide from Gelatin Methacryloyl through Hydrolysis

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Gelatin methacryloyl (GelMA; GM) is a promising nature-derived photocurable material that can mimic the extracellular matrix because GelMA features tailorable mechanical properties, proteolytic degradation, and good cell adhesion. GelMA contains not only methacrylamide but also methacrylate. However, the hydrolytic stability of methacrylamide and methacrylate groups of GelMA in aqueous solutions has not been scrutinized. Here, the structural change of GelMA through hydrolysis is investigated for the first time. The structural change of hydrolyzed GelMA is quantitatively identified using colorimetric and ^{1}H NMR methods. The methacrylate groups decompose markedly at high pH solutions, but the methacrylamide groups remain stable. Further, pure gelatin methacrylamide is successfully decoupled from GelMA for a better understanding of GelMA structure and future use for biomedical applications.

1. Introduction

Gelatin is a collagen-derived protein product, which has been used in food, pharmaceutical, and chemical industries for hundreds of years.[1] Besides, recent research reports have placed increasing attention on harnessing gelatin as biomimetic scaffolds for tissue engineering because of its outstanding biocompatibility and biodegradability.[2–6] Also, gelatin inherits cell-bonding motifs (e.g., Arg-Gly-Asp), proteolytic cleavage sites, and modifiable functional groups (e.g., hydroxyl, amino, and carboxylic groups) from parent collagen, which makes it an attractive candidate for a tunable versatile hydrogel platform mimicking the extracellular matrix.[7,8]

However, the main drawback of gelatin can be its poor mechanical properties like many other natural materials; thus several kinds of cross-linking strategies have been developed including using cross-linking agents such as glutaraldehyde, carbodiimide, or genipin.[9–11] Among these methods, the strategy of functionalizing gelatin via the reaction with methacrylic anhydride (MAA) has proved to be effective owing to the advantage of easy spatiotemporal gelling control over other strategies.[7,12–15] The resulting product, gelatin methacryloyl (GelMA; GM), possesses highly tunable physical properties and retains its innate bioactivities whereas its synthesis process remains simple and cost-effective.[7,16,17] GelMA can be easily cross-linked using photoinitiators (e.g., I2959) under 365 nm UV light in a short time, which has little effect on cell viability.[7] Therefore, it has been applied to a variety of bioapplications such as 3D cell culture,[18] 3D bioprinting,[19,20] and regeneration of various kinds of tissues.[7,21]

GelMA contains both methacrylate and methacrylamide because hydroxyl groups as well as amino groups may react with MAA; especially, highly substituted GelMA has a higher portion of methacrylate compared with lowly substituted GelMA.[16,22,23] Despite a wide range of bioapplications of GelMA, the structural stability of methacrylamide and methacrylate groups of GelMA in aqueous solutions has been little known, which would be important because it could affect the structural change and physiochemical properties of GelMA in aqueous environments. We hypothesized that the structural stability of methacrylamide and methacrylate in GelMA could be identified via hydrolysis owing to their potentially different stability in aqueous solutions.

Here, we made GelMA undergo hydrolysis treatments with different pH and durations, and identified the structure and stability of hydrolyzed GelMA by employing some analytic methods such as a 2,4,6-trinitrobenzene-sulfonic acid (TNBS) assay, a Fe(III)-hydroxamic-acid-based assay, and ^{1}H NMR

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spectroscopy.[20,22,23] In addition, we successfully decoupled pure gelatin methacrylamide from gelatin methacryloyl containing methacrylamide and methacrylate groups for further characterization.

2. Experimental Section

2.1. Materials

Gelatin (type B, 250 bloom), sodium carbonate, sodium bicarbonate decahydrate, sodium hydroxide, acetohydroxamic acid, hydroxylamine, sodium dodecyl sulfate, and alanine were purchased from Aladdin (Shanghai, China). Methacrylic anhydride (MAA), iron(III) perchloride, 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (I2959), and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were purchased from Sigma-Aldrich (Shanghai, China). Deuterium oxide (D_2O) and 2,2,3,3-D_4 (D, 98%) sodium-3-trimethylsilylpropionate (TMSP) were obtained from Cambridge Isotope Laboratories (Andover, USA). All the reagents were used as received.

2.2. Synthesis of Highly Substituted Gelatin Methacryloyl

GelMA synthesis was conducted similarly according to the previous report.[16,17] In order to prepare highly substituted GelMA containing both methacrylamide and methacrylate, a feed ratio of around 0.2 mL of MAA to 1 g of gelatin was employed. In short, 150 g of gelatin was dissolved in 1200 mL of a 0.25 M carbonate–bicarbonate (CB) buffer solution (7.98 g of sodium carbonate and 21.53 g of sodium bicarbonate decahydrate in 1 L of distilled water (DI water), and the pH of the gelatin solution was adjusted with 5 M sodium hydroxide or 6 M hydrochloric acid to 9.3. Subsequently, 31.6 mL of MAA (94%) was added to the gelatin solution in a time-lapse manner. The reaction proceeded at 50 °C under magnetic stirring at 500 rpm for 1 h. Then the pH of the solution was readjusted to 7.4 to stop the reaction. After being filtered, the GelMA solution was dialyzed against distilled water at 50 °C in a Millipore TFF system (Darmstadt, Germany) equipped with Pellicon 2 cassette including 10 kDa Biomax membrane, lyophilized and stored at −20 °C until further use.

2.3. Preparation of Hydrolyzed Gelatin Methacryloyl

GelMA samples were hydrolyzed at different alkaline pH (pH 9, 10, 11, and 12) for different durations to accelerate their hydrolysis. For example, GelMA hydrolyzed at pH 12 for 1 h (GM-12-1) was prepared by dissolving 5 g of GelMA in 50 mL of DI water and then by adjusting the pH of the GelMA solution with 5 M sodium hydroxide or 6 M hydrochloric acid to 12. Subsequently, the obtained solution was stirred at 50 °C for 1 h, followed by readjusting the pH to 7.4 to stop the reaction. The final solution was dialyzed using a PALL Minimate TFF system (Ann Arbor, USA) with a capsule (10 kDa MWCO) at 50 °C, lyophilized and stored at −20 °C until further use.

2.4. Fe(III)-Acetohydroxamic-Acid-Based Assay for Quantification of the Methacrylate Groups in GelMA

2.4.1. Standard Curve

A series of acetohydroxamic acid solutions in DI water (5.0×10^{-3}, 2.5×10^{-3}, 1.25×10^{-3}, 6.25×10^{-4}, 5×10^{-4}, and 2.5×10^{-4} mol L^{-1}) was prepared and mixed with a Fe(III) solution (0.5 mol L^{-1} iron(III) perchloride in 0.5 mol L^{-1} hydrochloric acid) at a 1:1 v/v ratio, according to the literature.[23] UV–vis absorption spectra of the resulting solutions were recorded from 420 to 700 nm in a microplate with 200 µL of a solution in each well. Absorbance at 500 nm (A500) was plotted against the concentration of acetohydroxamic acid and least square linear fitting was performed to obtain the working curve.

2.4.2. Sample Preparation for UV–vis Absorption Spectrum Measurements

One-hundred microliters of a hydroxylamine hydrochloride solution (0.5 mol L^{-1} in DI water) was mixed with 100 µL of 1 M NaOH, and then 200 µL of a GelMA solution (50 mg mL^{-1}) was added to the mixed solution. The resulting mixture was vortexed for 30 s and allowed to react at room temperature for 10 min. After that, 550 µL of 0.5 M HCl was added to acidify the mixture, and 50 µL of a Fe(III) solution (0.5 mol L^{-1} iron(III) perchloride in 0.5 mol L^{-1} hydrochloric acid) was added to the mixture solution. After the resulting mixture was vortexed for 30 s, UV–vis absorption spectra of the solution were recorded as aforementioned. The amount of methacrylate groups in GelMA samples was quantified using the working curve.

2.5. TNBS Assay for Quantification of the Methacrylamide Groups in GelMA

TNBS assay was performed as previously described.[16] Briefly, GelMA and gelatin samples were separately dissolved at 1.6 mg mL^{-1} in 0.1 M sodium bicarbonate buffer. Then, 0.5 mL of each sample solution was mixed with 0.5 mL of a 0.1% TNBS solution (in 0.1 M sodium bicarbonate buffer), and then the mixture was incubated at 37 °C for 2 h. Next, 0.25 mL of 1 M HCl and 0.5 mL of 10 w/v% sodium dodecyl sulfate were added to stop the reaction. The absorbance of each sample was measured at 335 nm. The alanine standard curve was then plotted to determine the amino group concentration, with sample solutions prepared at 0, 0.8, 8, 16, 32, and 64 µg mL^{-1}.

2.6. ^{1}H NMR Measurements for Quantification of Methacrylamide and Methacrylate Groups in GelMA

Twenty milligrams of each of gelatin, GelMA, and hydrolyzed GelMA samples was separately dissolved in 800 µL of deuterium oxide containing 0.05% TMSP as a chemical shift reference similarly according to the literature.[22] ^{1}H NMR-spectroscopy of samples was conducted at 27 °C on a Bruker Avance-I 400 MHz spectrometer (Weinheim, Germany). Phase

and baseline corrections were made on all NMR spectra prior to the integration of specific signals for quantification of the contents of methacrylate and methacrylamide in GelMA. The molar amount of methacrylate and methacrylamide in GelMA was quantified using the following formulas.

The amount of methacrylate (mmol g^{-1})

$$= \frac{\int \text{Methacrylate (the peak at about 6.1 ppm)}}{\int \text{TMSP(at 0 ppm)}} \times \frac{9H}{1H} \times \frac{n \text{ mmol (TMSP)}}{m \text{ g (GelMA)}};$$

The amount of methacryloyl (mmol g^{-1})

$$= \frac{\int \text{Methacryl(the peaks at 5.6 – 5.8 ppm)}}{\int \text{TMSP(at 0 ppm)}} \times \frac{9H}{1H} \times \frac{n \text{ mmole (TMSP)}}{m \text{ g (GelMA)}};$$

The amount of methacrylamide = The amount of methacryloyl – The amount of methacrylate

2.7. 2D-NMR

1H 13C-HSQC (Heteronuclear Single Quantum Coherence) NMR Spectroscopy: For 2D-NMR spectroscopy of GelMA and its completely hydrolyzed GelMA (GM-12-1), around 70 mg of each sample was dissolved in 933 µL of deuterium oxide including 0.05% TMSP as a chemical shift reference. NMR spectra were collected at 40 °C on a Bruker Avance-I 400 MHz spectrometer. Phase and baseline corrections were made on all NMR spectra to present real absorption signals.

2.8. Compression Measurements

For compression tests, GelMA derivatives (GM, GM-10-1, GM-11-1, and GM-12-1) at 20 w/v% containing 0.5 w/v% I2959 were prepared, and slabs of GelMA hydrogels with a diameter of 4 mm and a thickness of 4 mm were fabricated under UVA light in the spectrum of 320–390 nm (INTELLI-RAY 600 UV chamber equipped with a 600 W metal halide type lamp, Uvitron International Inc., West Springfield, USA,) with 37.5 mW cm^{-2} for 5 min. The hydrogels were tested using a universal mechanical testing machine (UTM2102; Shenzhen, China). The speed of the crosshead was 0.25 mm·s^{-1}. The compression strain and stress were recorded until the hydrogel was crushed. The compressive modulus was determined as the slope of the stress–strain curve from 0 to 0.15 strain.

2.9. Circular Dichroism Analysis

Circular dichroism (CD) experiments, covering the UV spectral range from 260 to 180 nm, were conducted using Chirascan Plus (Applied Photophysics, Leatherhead, UK). The samples (0.2 mg mL^{-1}) were first stored at 4 °C for 2 h before use to get a strong signal of triple-helix structure. The acquisitions were performed at 4 °C after 300 µL of a solution was loaded in a quartz cell with an optical path length of 1 mm.

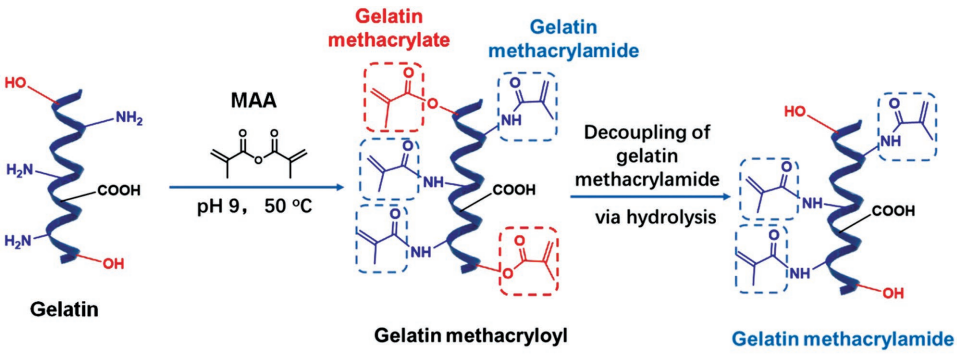
2.10. Statistical Analysis

Statistical analysis was conducted using the Microsoft Excel statistical analysis software. Comparisons between two samples were made using a two-tailed pair Student's *t*-test. A one-way ANOVA was performed to test for differences among at least three groups. The standard deviation was calculated and presented for each group (mean ± standard deviation). *p* values less than 0.05 were considered statistically significant, and the value of *n* denotes the number of samples or the number of experimental runs.

3. Results and Discussion

3.1. Synthesis of Highly Substituted GelMA Containing Methacrylamide and Methacrylate

Highly substituted GelMA containing methacrylamide and methacrylate groups was prepared similarly according to our previous reports (**Scheme 1**).[16] As a result, GelMA with an 85% yield was successfully synthesized under the reaction conditions (a feed ratio of 150.0 g of gelatin to 31.6 mL of MAA, 0.25 M carbonate–bicarbonate buffer, an initial pH of 9.3, a reaction temperature of 50 °C, and a reaction time of 1 h). As presented



Scheme 1. Highly substituted GelMA was synthesized via the reaction of gelatin with MAA. The obtained GelMA contained methacrylate as well as methacrylamide groups. The structural and compositional change of gelatin methacryloyl was investigated through hydrolysis. Furthermore, pure gelatin methacrylamide was successfully decoupled from gelatin methacryloyl.

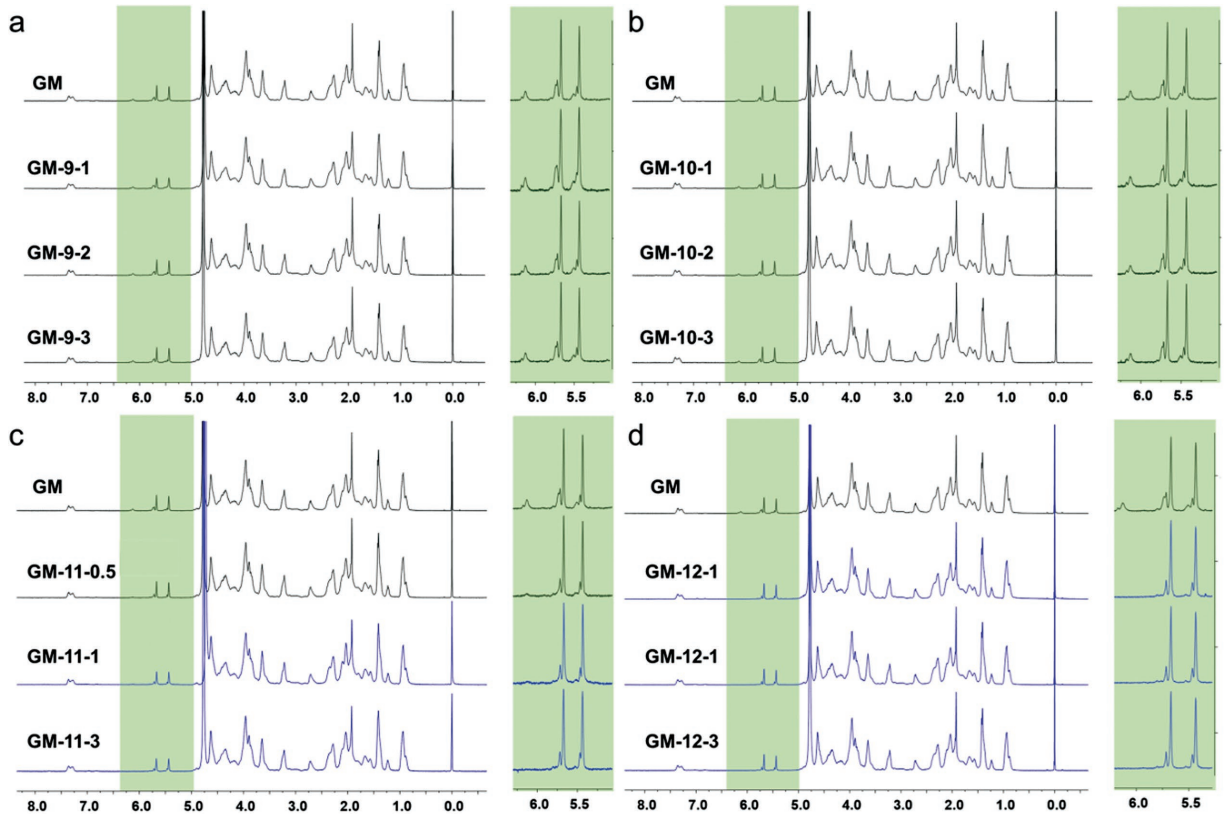


Figure 1. ^{1}H NMR spectra of GM (GelMA) and hydrolyzed GM at different pH for a different duration. a) Hydrolyzed GM at pH 9 for 1–3 h. b) Hydrolyzed GM at pH 10 for 1–3 h. c) Hydrolyzed GM at pH 11 for 0.5–3 h. d) Hydrolyzed GM at pH 12 for 1–3 h. The acrylic peaks between 5.4 and 6.1 ppm appeared unchanged for hydrolyzed GM at pH 9 and 10 over the hydrolysis period. On the other hand, the acrylic peak at 6.1 ppm reduced abruptly for hydrolyzed GM at pH 11 even for half an hour. Hydrolyzed GM at pH 12 exhibited no peak at 6.1 ppm within 1 h. Nevertheless, the sharp main peaks at around 5.4 and 5.7 ppm seemed intact across the hydrolysis treatments.

in the ^{1}H NMR of GelMA (**Figure 1** and Figure S1, Supporting Information), the presence of the acrylic peaks from 5.4 to 6.2 ppm indicates the successful conjugation between gelatin and MAA for gelatin methacryloyl; the product of GelMA contained not only methacrylamide but also probably methacrylate groups, judging from the additional specific peaks appearing at around 5.7 and 6.1 ppm.[16,22]

3.2. Quantification of Methacryloylation of GelMA via Colorimetric Methods (TNBS and Fe(III)-Hydroxamic-Acid-Based Assays) and ^{1}H NMR Spectroscopy

For quantification of methacryloylation of GelMA, methods such as TNBS, a Fe(III)-hydroxamic-acid-based assay, and ^{1}H NMR spectroscopy are generally utilized.[16,22,23] The TNBS assay generally offers the precise information of chemical conjugation of MAA to primary amino groups (lysine and hydroxyl lysine groups) by measuring the remaining amino groups, resulting in quantifying only the amount of methacrylamide groups in GelMA as illustrated in Figure S2, Supporting Information.[17] By contrast, the Fe(III)-hydroxamic-acid-based assay can quantify the methacrylate groups in GelMA, via determining the concentration of Fe(III)–*N*-hydroxymethacrylamide complex from the UV–vis absorption spectra, as seen in Figure S3, Supporting Information.[23] Both the colorimetric methods (TNBS and Fe(III)-based methods) are relatively precise; however, they cannot offer clearly the information of the presence of impurities. On the other hand, ^{1}H NMR spectroscopy using a known concentration of an internal standard reference (3-timethylsilylpropionic-2,2,3,3-D4, acid sodium salt: TMSP) can provide the quantitative information of methacrylamide and methacrylate groups in gelatin methacryloyl simultaneously; one proton (1H) of the acrylic protons (CH_2=C(CH_3)COO−) in methacrylate appears at around 6.1 ppm, and the other proton (1H) of the acrylic protons (CH_2=C(CH_3)COO−) in methacrylate and one proton (1H) of the acrylic protons (CH_2=C(CH_3)CONH−) in methacrylamide are overlapped between 5.7 and 5.6 ppm, each integral of which can lead to quantification of methacrylate and methacryloyl (methacrylate and methacrylamide), respectively, based on the TMSP integral (9H at 0 ppm).[22] In addition, ^{1}H NMR spectroscopy can provide the important information of the presence of impurities. However, the accuracy of quantification of methacrylamide and methacrylate groups using simple ^{1}H NMR with TMSP may depend on the quality of the NMR instrument. Therefore, in this study, the degree of methacryloylation of synthesized GelMA was compared by employing two colorimetric methods (TNBS and Fe(III)-hydroxamic-acid-based assays) and ^{1}H NMR spectroscopy (**Figure 2**). The molar amount (mmol)

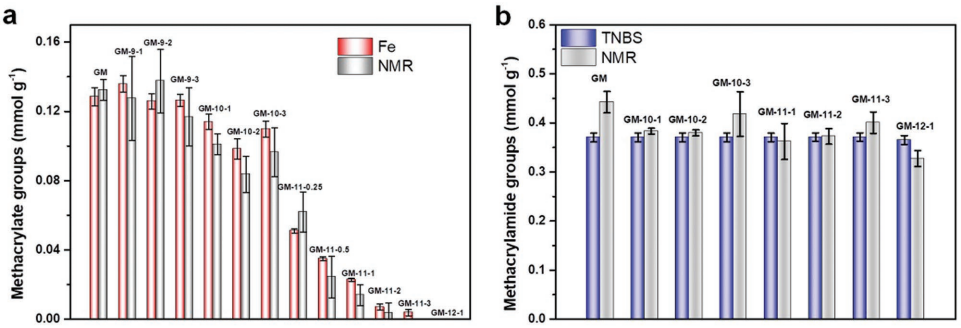


Figure 2. Quantification of methacrylate and methacrylamide groups in GelMA and hydrolyzed GelMA. a) Quantification of the methacrylate groups using the TMSP-based NMR method ($n = 3$) and Fe(III)-hydroxamic-acid-based method ($n = 3$). The amount of the methacrylate tended to decrease as the pH and duration of hydrolysis increased. The methacrylate groups remained quite stable at pH 9 and were hydrolyzed marginally at pH 10 whereas they were markedly hydrolyzed at pH 11 and completely decomposed at pH 12 even for 1 h. b) Quantification of the methacrylamide groups using the TMSP-based NMR method ($n = 3$) and TNBS method ($n = 3$). The methacrylamide groups remained intact across the hydrolysis conditions.

of methacrylamide in GelMA (1 g) quantified from ^{1}H NMR and TNBS was 0.442 ± 0.022 and 0.370 ± 0.009 mmol g^{-1}, respectively, whereas that of methacrylate in GelMA (1 g) from ^{1}H NMR and the Fe(III)-hydroxamic-acid-based assay was 0.132 ± 0.006 and 0.128 ± 0.005 mmol g^{-1}, respectively. Consequently, synthesized GelMA exhibited both methacrylamide and methacrylate groups in its structure. GelMA contained 2.8 (from the colorimetric methods) or 3.5 (from ^{1}H spectroscopy) times a higher molar amount of methacrylamide than that of methacrylate groups because amino groups in gelatin were much more reactive to MAA than hydroxyl groups in gelatin.[23] Besides, regarding impurities, no peaks of methacrylic acid (the main by-product of GelMA synthesis) appearing at around 5.3 5.7, and 1.8 ppm were detected owing to the complete dialysis.

3.3. Hydrolytic Stability of Methacrylamide and Methacrylate in GelMA

For testing the structural stability of GelMA, hydrolysis treatments (different pH solutions [pH 9–12], an incubation period up to 3 h, and a temperature of 50 °C) were employed to accelerate the hydrolysis process, as summarized in **Table 1**. 1H NMR spectra of hydrolyzed GelMA samples were recorded to verify their structural change (Figure 1). GM-A-B denotes GelMA hydrolyzed at pH A for B hours, and Gel-12-1 stands for gelatin hydrolyzed at pH 12 for 1 h. For GM-9-1, GM-9-2, and GM-9-3, the acrylic peaks between 5.4 and 6.1 ppm of all hydrolyzed samples at pH 9 appeared intact just as those in pristine GelMA, which indicates that both of the methacrylamide and methacrylate groups in GelMA are stable at pH 9. In the ^{1}H NMR spectra of GM-10-1, GM-10-2, and GM-10-3 samples, the acrylic peaks of the hydrolyzed samples at pH 10 appeared little changed, meaning that methacrylate groups as well as methacrylamide groups seem not particularly labile under these hydrolysis conditions. On the other hand, in GM-11-0.5, GM-11-1, and GM-11-3 samples hydrolyzed at pH 11 for 0.5, 1, and 3 h, respectively, one peak appearing at 6.1 ppm as well as a small peak at around 5.8 ppm tended to reduce markedly as hydrolysis was prolonged, whereas the main sharp peaks at 5.4 and 5.7 ppm remained unchanged. In GM-12-1 sample hydrolyzed at pH 12 for 1 h, the peak at 6.1 ppm completely disappeared and the small peaks appearing at around 5.5 and 5.8 ppm were slightly decreased; however the main sharp peaks at 5.4 and 5.7 ppm appeared intact. It has been reported that the main sharp peaks at 5.4 and 5.7 ppm belong to the acrylic protons of methacrylamide groups whereas the overlapped small peaks at around 5.5 and 5.8 ppm, and the additional peak at 6.1 ppm are ascribed to the methacrylate functionalization of hydroxyl groups.[22] In summary, the methacrylamide groups in GelMA appeared unchanged across the hydrolysis conditions whereas the methacrylate groups were susceptible to hydrolysis especially at pH 11 and 12.

Furthermore, the structural and compositional change of methacrylamide and methacrylate groups in GelMA during hydrolysis was quantified using the aforementioned colorimetric

Table 1. Details of the hydrolysis conditions for structural analysis of GelMA (GM).

No.	Code-pH-h	Sample	Hydrolysis	pH	Time [h]
1	Gel	Gelatin	N	–	–
2	GM	GM	N	–	–
3	GM-9-1	GM	Y	9	1
4	GM-9-2	GM	Y	9	2
5	GM-9-3	GM	Y	9	3
6	GM-10-1	GM	Y	10	1
7	GM-10-2	GM	Y	10	2
8	GM-10-3	GM	Y	10	3
9	GM-11-0.25	GM	Y	11	0.25
10	GM-11-0.5	GM	Y	11	0.5
11	GM-11-1	GM	Y	11	1
12	GM-11-2	GM	Y	11	2
13	GM-11-3	GM	Y	11	3
14	GM-12-1	GM	Y	12	1
15	GM-12-2	GM	Y	12	2
16	GM-12-3	GM	Y	12	3
17	Gel-12-1	Gelatin	Y	12	1

GM-A-B denotes GelMA hydrolyzed at pH A for B hours, and Gel-12-1 stands for gelatin hydrolyzed at pH 12 for 1 h.

and ^{1}H NMR methods as displayed in Figure 2. The results from the colorimetric and ^{1}H NMR methods exhibited similar trends; the colorimetric methods provided values with relatively smaller standard deviations than the ^{1}H NMR method. Therefore, the quantitative values described in the following statements were obtained from the colorimetric methods unless otherwise mentioned. As for the molar amount of the methacrylate groups of hydrolyzed GelMA, hydrolyzed GelMA samples (GM-9-1 [0.136 ± 0.005 mmol g^{-1}], GM-9-2 [0.126 ± 0.004 mmol g^{-1}], and GM-9-3 [0.126 ± 0.004 mmol g^{-1}]) at pH 9 for 1–3 h retained almost the similar molar amounts of the methacrylate as found in pristine GelMA (0.128 ± 0.005 mmol g^{-1}), whereas GM-10-3 (0.110 ± 0.005 mmol g^{-1}) hydrolyzed at pH 10 for 3 h exhibited a slight decrease in the molar amount of the methacrylate groups, compared with that of pristine GelMA (0.128 ± 0.005 mmol g^{-1}). However, the molar amount of the methacrylate groups in hydrolyzed GelMA at pH 11 decreased abruptly as the hydrolysis proceeded. In GM-11-0.5 (0.035 ± 0.001 mmol g^{-1}), more than half of the methacrylate groups in GelMA already decomposed. Even in GM-11-3, the presence of the methacrylate groups could not be detected via ^{1}H NMR spectroscopy, but a marginal molar amount of the methacrylate (0.004 ± 0.002 mmol g^{-1}) was measured only by the Fe (III)-based colorimetric method. GM-12-1 contained no amount of the methacrylate groups, which were completely hydrolyzed. As for the quantification of the methacrylamide groups in hydrolyzed GelMA, the amounts of the methacrylamide of the hydrolyzed GelMA samples were close to that of pristine GelMA, regardless of the hydrolysis conditions, especially based on the results of the TNBS colorimetric method (e.g., GM (0.370 ± 0.009 mmol g^{-1}), GM-10-1 (0.370 ± 0.009 mmol g^{-1}), GM-11-3 (0.371 ± 0.009 mmol g^{-1}), and GM-12-1 (0.365 ± 0.009 mmol g^{-1})). ^{1}H NMR results also showed that GelMA and hydrolyzed GelMA contained similar amounts of the methacrylamide but with relatively larger error bars. These quantitative results are consistent with observations in ^{1}H NMR spectra, suggesting that methacrylamide groups can be stable across the hydrolysis treatments whereas methacrylate groups can undergo hydrolysis to a certain degree in a solution with pH above 10 and degrade completely at pH 12 for 1 h. It is because hydroxide ions as a reactive nucleophile in a higher pH (base-mediated hydrolysis) are more available than in a lower pH and thus can increase the rate of hydrolysis of methacrylate groups in GelMA. Also, methacrylamide groups in GelMA are less electrophilic and reactive than methacrylate groups in GelMA owing to greater resonance stabilization of the C–N bond of amide groups. In addition, the hydrolysis treatment with elevated pH may potentially degrade the backbone of GelMA, leading to a series of chain scission and an increase in *N*-termini of its fragments. However, the remaining amino content of GelMA was close to that of each of hydrolyzed GelMA samples based on the TNBS results (Figure 2), indirectly suggesting that the backbone degradation of hydrolyzed GelMA seemed to be marginal under the current hydrolysis conditions.

In addition, CD spectra of Gel (gelatin), GM (GelMA), and hydrolyzed GM provided the information of their secondary structure at different temperatures as depicted in Figure S4, Supporting Information. GM and hydrolyzed GM samples displayed similarly a marked rise in the intensity at 199 nm at 4 °C, compared with gelatin, indicating that the methacryloyl functionalization of gelatin as a main factor could promote random coil conformations. The difference between GM and hydrolyzed GM samples at 199 nm was marginal. The triple-helix contents of GM and hydrolyzed GM at 222 nm were close to one another, indicating that GM and hydrolyzed GM could retain a similar amount of the triple-helix formation at 4 °C. However, they dropped significantly compared with gelatin. At 37 °C, all samples showed almost the same patterns in the CD spectra and exhibited an increase in the intensity at 199 nm compared to those at 4 °C. The triple-helix contents of Gel, GM, and hydrolyzed GM at 222 nm decreased significantly compared with those at 4 °C for all of them appeared to have a random coil structure at 37 °C.

3.4. 2D-NMR Spectra of GelMA and Hydrolyzed GelMA, and Decoupling of Gelatin Methacrylamide from GelMA

^{1}H ^{13}C-HSQC-NMR data offered convincing evidence of the structural and compositional change of hydrolyzed GelMA samples as presented in **Figure 3** and Figure S5, Supporting Information. 2D-NMR of pristine GelMA displayed two sets of separated acrylic signals (CH_2=C(CH_3)COO− and CH_2=C(CH_3)CONH−) from methacrylate and methacrylamide as well as an additional signal (at ^{1}H 5.5 and ^{13}C 74.5 ppm) of the γ-CH of the methacrylated hydroxyproline; the specific signal appearing at around ^{1}H 6.1 and ^{13}C 127.7 ppm corresponded to one (C−H) of the acrylic bonds (CH_2=C(CH_3)COO−) in the methacrylate groups whereas the signal appearing at around ^{1}H 5.7/^{13}C 127.7 ppm was attributed to the other (C−H) of the acrylic bonds (CH_2=C(CH_3)COO−) in the methacrylate groups, whose proton peak appeared closely with one proton (at ^{1}H 5.7/^{13}C 120.7 ppm) of the acrylic bonds (CH_2=C(CH_3)CONH−) of the methacrylamide groups in ^{1}H NMR. Further, the signal (at ^{1}H 5.5 and ^{13}C 74.5 ppm) of the γ-CH of the methacrylated hydroxyproline stood closely with the other signal (at ^{1}H 5.4 and ^{13}C 120.7 ppm) of the acrylic bonds (CH_2=C(CH_3)CONH−) of the methacrylamide groups in the ^{1}H chemical shift. The results were consistent with the previous literature.[22] More interestingly, 2D-NMR of hydrolyzed GelMA (GM-12-1) showed that the two distinguished signals belonging to the methacrylate groups as well as the signal of the γ-CH of the methacrylated hydroxyproline vanished after hydrolysis at pH 12 for 1 h, whereas other signals attributed to the methacrylamide groups remained intact, as seen in Figure 3b. These outcomes clearly indicate that gelatin methacrylate is much more susceptible to hydrolysis than gelatin methacrylamide. In addition, it is noteworthy that pure gelatin methacrylamide was successfully decoupled from GelMA containing methacrylamide and methacrylate through the hydrolysis process.

GelMA has been extensively tested for biomedical applications including drug delivery systems, tissue regeneration, and 3D bioprinting.[7,13,15] Promising results have been reported.[24,25] However, the biocompatibility and toxicity of GelMA and its degradation products including a small molecule of methacrylate have not been thoroughly investigated for clinical application.[13] Methacrylic acid (the main hydrolyzed product of gelatin methacryloyl) was found

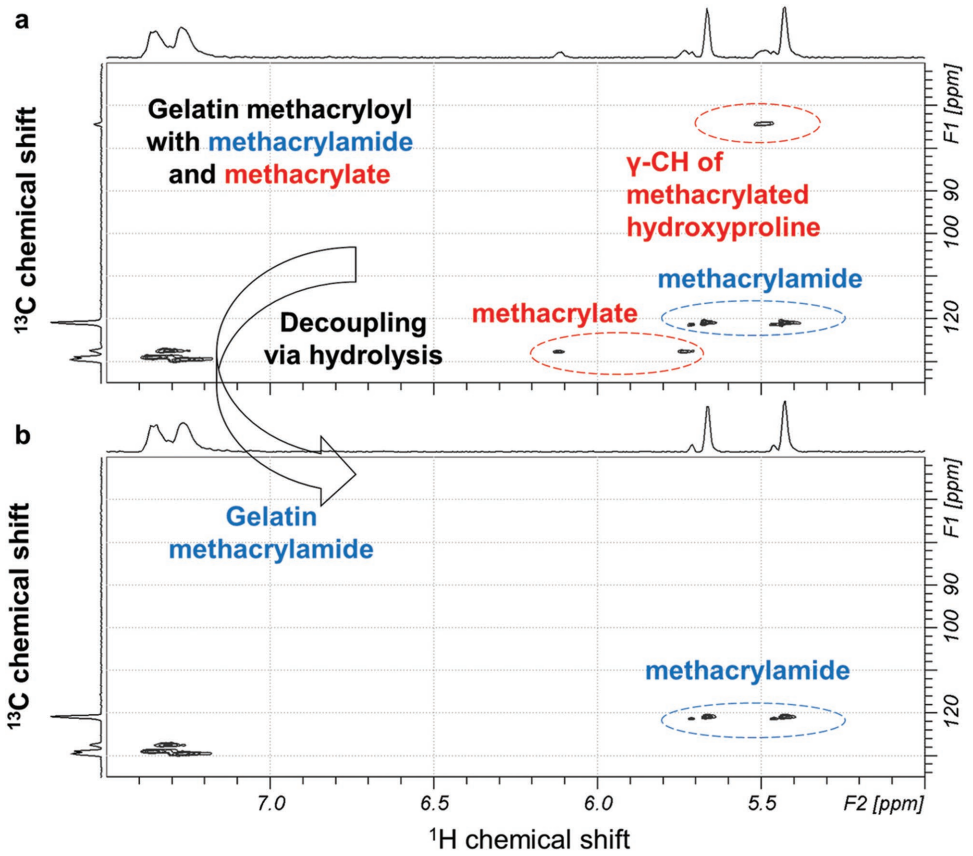


Figure 3. 2D-NMR, zoomed ^{1}H ^{13}C-HSQC-spectra in D_2O covering the signals of the acrylic groups of the methacrylate and methacrylamide as well as those of the phenylalanine groups in GelMA and hydrolyzed GelMA. a) 2D-NMR of GelMA exhibited the distinctive chemical shifts of the methacrylate and methacrylamide as well as the chemical shift of γ-CH of the methacrylated hydroxyproline. b) In 2D-NMR of hydrolyzed GelMA (GM-12-1) at pH 12 for 1 h, the respective signals from methacrylate groups and γ-CH of the methacrylated hydroxyproline completely disappeared. Pure gelatin methacrylamide was successfully decoupled via hydrolysis from GelMA.

to be a highly corrosive chemical, whose oral LD_{50} for rats ranged from 277 to 2260 mg kg^{-1}.[26] Also, in poly(ethylene glycol)-PEG-based hydrogel systems, PEG-diacrylamide with hydrolytically stable amides was used for tissue engineering applications in replacement of PEG-diacrylate with hydrolytically unstable ester bonds.[27] One study revealed that the implant of PEG-diacrylamide-based hydrogels exhibited superior in vivo biocompatibility to that of PEG-diacrylate-based hydrogels in a rabbit deep corneal stromal pocket model.[28] Furthermore, the purity of GelMA might matter for GelMA to be pushed for FDA approval and be used in the pharmaceutical biomedical industry in the future. The decoupling of gelatin methacrylamide from GelMA via hydrolysis might be useful to obtain pure gelatin methacrylamide from gelatin methacryloyl.

3.5. Mechanical Properties of Hydrogels of GelMA and Hydrolyzed GelMA

The mechanical properties of hydrogels of GelMA and hydrolyzed GelMA were investigated with a compressive test. GM, GM-10-1, GM-11-1, and GM-12-1 were selected, and each hydrogel was prepared by curing each solution of 20 wt% containing 0.5 wt% I2959 under UVA light (320–390 nm) with an intensity of 37 $mW \cdot cm^{-2}$ for 5 min. As presented in **Figure 4**, GM-12-1 hydrogel (0.18 ± 0.01 MPa) exhibited a lower modulus than pristine GM hydrogel (0.21 ± 0.01 MPa; $n = 5$, $p < 0.005$).The compressive modulus of hydrogels of hydrolyzed GelMA samples decreased with moving samples from GM-10-1 to GM-12-1 ($n = 5$, one-way ANOVA; $p < 0.00001$), which means that hydrogels of hydrolyzed (especially above pH 11) GelMA became softer and softer. The breaking compressive strain of hydrogels of hydrolyzed GelMA increased slightly with varying samples from GM-10-1 to GM-12-1 hydrogels ($n = 5$, one-way ANOVA; $p < 0.00001$). The strain (0.59 ± 0.02) of GM-10-1 hydrogel was very close to that (0.58 ± 0.01) of GM hydrogel ($n = 5$, $p = 0.39$). However, the strain values of GM-11-1 hydrogel (0.66 ± 0.04) and GM-12-1 hydrogel (0.69 ± 0.03) were higher than that (0.58 ± 0.01) of GM hydrogel ($n = 5$, $p = 0.04$, and 0.03, respectively). The compressive strength at break of hydrogels of hydrolyzed GelMA samples displayed an increasing trend as samples varied from GM-10-1 to GM-12-1 hydrogels. The compressive strength values of GM-10-1, GM-11-1, and GM-12-1 hydrogels were 0.64 ± 0.07, 0.69 ± 0.09, and 0.81 ± 0.18 MPa, respectively whereas that of GM hydrogel was 0.54 ± 0.10 MPa. However, the strength value of each hydrogel of hydrolyzed GM samples was not significantly different from that of GM hydrogel ($n = 5$, $p > 0.05$). Thus, it could

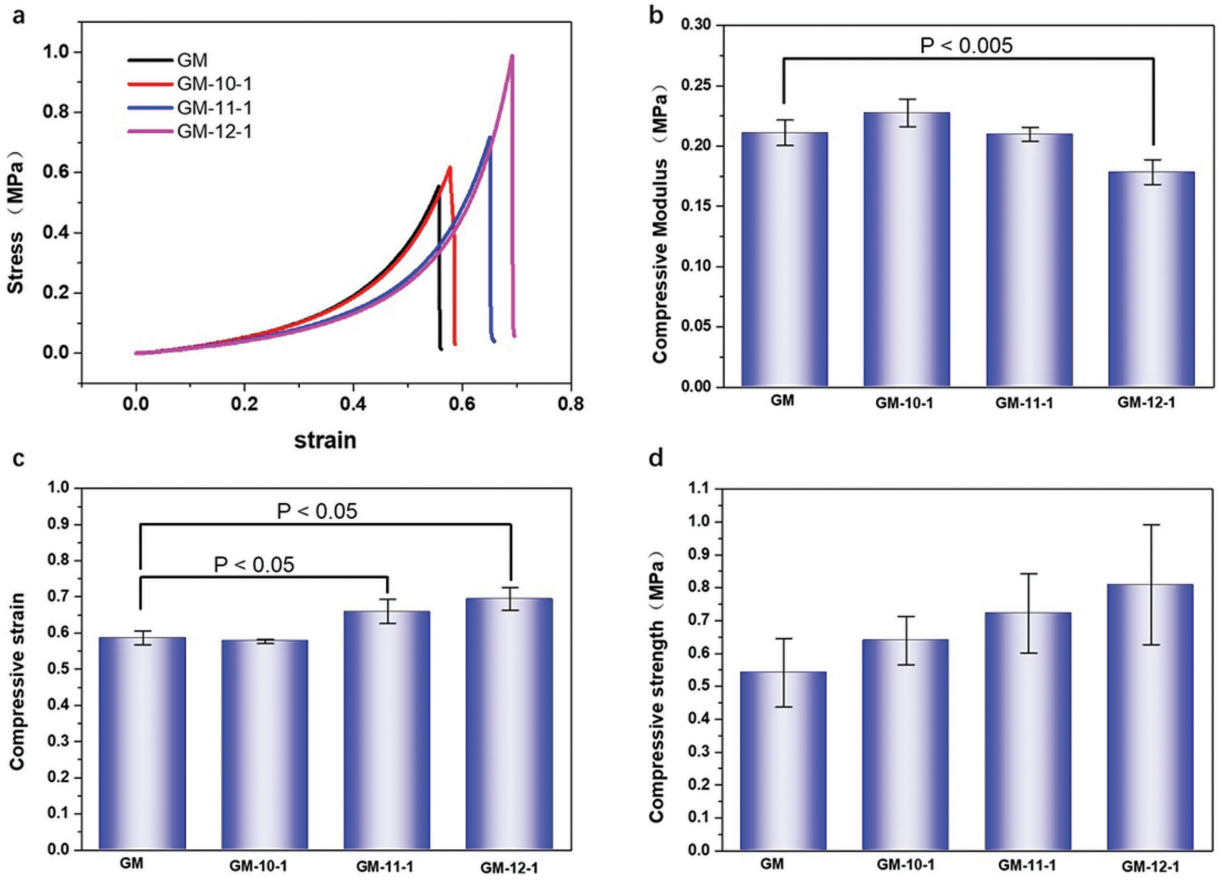


Figure 4. Mechanical properties of hydrogels prepared from GM, GM-10-1, GM-11-1, and GM-12-1. a) Compression curves of GM, GM-10-1, GM-11-1, and GM-12-1 hydrogels. b) The compression modulus of hydrogels of hydrolyzed GM tended to reduce in the following order: GM-10-1 > GM-11-1 > GM-12-1 hydrogels ($n = 5$, one-way ANOVA; $p < 0.00001$). GM-12-1 hydrogel showed a lower modulus than pristine GM hydrogel ($n = 5$, $p < 0.005$). c) The compressive strain value of each hydrogel of GM-11-1 and GM-12-1 increased compared with that of GM hydrogel ($n = 5$, $p = 0.04$, and 0.03, respectively). d) The compressive strength of hydrogels of hydrolyzed GM samples tended to increase in the following order: GM-10-1 < GM-11-1 < GM-12-1 hydrogels. However, the strength value of each hydrogel of hydrolyzed GM samples was not significantly different from that of GM hydrogel ($n = 5$, $p > 0.05$). Overall, hydrogels of hydrolyzed GM could be softer and tougher than pristine GM hydrogel because hydrolyzed GM samples have a lesser amount of methacrylate, possibly leading to a lower cross-linking density and a larger mesh size.

be speculated that the mechanical properties of hydrogels of hydrolyzed GelMA were very closely related to the molar amount of the methacrylamide and methacrylate in GelMA. GM-12-1 had no more methacrylate groups compared with GM containing both methacrylamide and methacrylate groups, probably resulting in hydrogels with a lower cross-linking density. Consequently, hydrogels of hydrolyzed GelMA samples might have a larger mesh size owing to a lower concentration of methacryloyl groups than pristine GelMA hydrogels, which could render hydrogels of hydrolyzed GelMA less brittle and more compliant.

4. Conclusions

In conclusion, we successfully synthesized highly substituted GelMA containing methacrylamide and methacrylate groups, and quantified the structural change of methacrylamide and methacrylate groups in hydrolyzed GelMA by harnessing quantitative methods such as ^{1}H NMR spectroscopy and colorimetric assays (Fe-hydroxamic-acid-based method and TNBS). Methacrylate groups in GelMA were labile to degrade especially in high pH solutions (pH 11 and 12) whereas methacrylamide groups remained stable even in high pH solutions. In addition, we demonstrated that pure gelatin methacrylamide could be completely decoupled from GelMA through hydrolysis. Hydrogels of decoupled gelatin methacrylamide were more compliant than those of gelatin methacryloyl.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

gelatin methacrylamide, gelatin methacrylate, gelatin methacryloyl, hydrolysis, structural stability

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