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Efficient and controllable synthesis of highly substituted gelatin methacrylamide for mechanically stiff hydrogels†

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We report an effective and novel method to controllably produce highly substituted gelatin-MA with nearly 100% degree of substitution despite the use of a very low concentration of methacrylic anhydride (MAA). The method is based on sequential time-lapse loading of MAA after pH adjustment in a carbonate–bicarbonate buffer system. Rheological studies indicate that hydrogels formed from more highly substituted gelatin-MA exhibit significantly improved mechanical stiffness.

Gelatin is a natural protein extract that is derived from collagen and can form hydrogels with excellent material properties such as biocompatibility and biodegradability.[1] It is found in high abundance, easily obtained and inexpensive compared to other protein-based biomaterials.[1–3] However, gelatin typically forms physically-crosslinked hydrogels only under a limited set of gelatin concentrations and temperatures, and the fabricated hydrogels have weak mechanical strength.[1,4]

Numerous chemical strategies have been developed to optimize the formation of gelatin hydrogels and corresponding mechanical properties.[4–8] The addition of a small molecule chemical reagent (*e.g.*, glutaraldehyde) to cross-link the gelatin molecules increases the mechanical strength of the hydrogel network.[1,7] Alternatively, gelatin can be reacted with methacrylic anhydride (MAA) or glycidyl methacrylate in order to form a photoreactive gelatin derivative.[9–21] Photopolymerizable gelatin derivatives undergo quicker and more homogeneous crosslinking compared to the aforementioned chemical methods involving small molecules.[5–7,14] Successful conjugation of gelatin molecules with photoreactive agents is a critical step in the hydrogel fabrication process.

Gelatin methacrylamide (gelatin-MA) is a popular type of photopolymerizable gelatin-based derivatives that has been widely utilized in hydrogel scaffolds and 3D bio-printing.[10,11,14,15,17,18,22–26] Its synthesis was pioneered by van Den Bulcke *et al.* in 2000, and is based on the reaction of free amino groups of lysine/hydoxylysine residues in gelatin molecules with methacrylic anhydride (MAA) in phosphate-buffered saline (PBS) under physiological pH conditions.[18] While the reaction requires one MAA molecule per one free amino group, a 13–44 fold molar excess of MAA over free amino groups is typically used for gelatin-MA synthesis.[10,18,27,28] Even so, the degree of methacrylamide substitution can greatly vary depending on the specific type of gelatin extract. Type A gelatin is obtained from acid treatment at pH 1–2 and has an isoelectric point (IEP) around pH 7–9, while type B gelatin is produced from alkali treatment at pH 12–13 and has an IEP around pH 5–6. When using type A gelatin, the highest attainable degree of substitution (DS) is reported to be between 70 and 85%.[9,13,16,17] On the other hand, Hoch *et al.* have reported nearly complete conversion of type B gelatin into gelatin-MA by maintaining the pH around 7.0–7.4.[19,29] However, it still required a high molar excess (10–20 fold) of MAA over free amino groups of type B gelatin and also caused side reactions such as conversion of hydroxyl groups of gelatin. Hence, current schemes are unable to fully convert type A gelatin into gelatin-MA and have low efficiency (high MAA concentration) for both types of gelatin. One possible reason for the inefficient chemical synthesis of gelatin-MA is that a reaction by-product, methacrylic acid, lowers the solution pH during the reaction. In turn, the free amino groups of gelatin become ionized, which inhibits the reaction with MAA. Maintaining a high pH may help to improve the efficiency of the gelatin reaction with MAA owing to more availability of free amino groups as seen in Fig. 1.

In light of the reported findings obtained with types A and B gelatin, we hypothesize that maintaining a pH above the corresponding IEP of the gelatin type would provide an optimal condition for highly efficient conversion of gelatin into gelatin-MA. The goal of this study is to develop a suitable reaction

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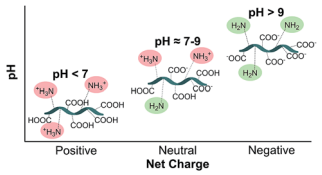

Fig. 1 Schematic illustration of how solution pH affects the protonation stage of free amino groups on type A gelatin.

scheme to convert type A gelatin into gelatin-MA. To this end, a sodium carbonate–bicarbonate (CB) buffer system was utilized that provides a suitable buffer range around pH 9.0, which is above the corresponding IEP of the gelatin molecules (~pH 7–9) and was maintained during the reaction. By comparing the results with gelatin-MA synthesis reactions performed in conventional PBS conditions around pH 7.4, we demonstrate significant improvement in the efficient and controllable synthesis of type A gelatin-MA with a near-100% DS. The highly substituted gelatin-MA also proves to be a superior material for fabricating mechanically stiff hydrogels.

First, we investigated the effect of buffer system on the solution pH during the course of the reaction between gelatin and MAA as presented in Fig. 2 and Table 1. Two buffer systems were selected: PBS (a neutral buffer) and CB (an alkaline buffer). In each case, 1 mL of MAA was initially added to 10 g of gelatin, which is a relatively low feed ratio compared to the conventional feed ratio of MAA (6–20 mL) to type A gelatin (10 g) in order to obtain gelatin-MA with a degree of substitution around 70–85%.[9,13,16,17,27,28] Type A gelatin has approximately 2.86 mmol of lysine per 10 g according to the literature;[30] hence, the reaction molar ratio of MAA (6.31 mmol per 1 mL) to lysine (2.86 mmol per 10 g gelatin) is around 2.2 to 1 in our reaction scheme. As depicted in Fig. 2B, the sequential loading of MAA (0.167 mL at each step) every 30 min at 50 °C for 3 h was applied and the solution pHs were monitored as the reactions progressed. All buffer solutions decreased in pH as reaction proceeded due to an increase in the amount of methacrylic acid generated as a by-product. After gelatin was dissolved in PBS at pH 7.4 and CB buffer at pH 9.7, the pHs of PBS and CB buffers dropped to 5.3 and 7.8, respectively. The final pHs of PBS and CB were 3.7 and 5.3, respectively, after 3 h reaction.

Reducing the pH below IEP (7–9) of type A gelatin can cause remaining unreacted free amino groups to be ionized into positive charged amino groups which are not reactive with MAA. In order to further promote the reaction, we adjusted the solution pH back to the optimal buffer range in each system before adding MAA, leading to regeneration of the reactive free amino form. The sequential loading of MAA (0.167 mL at each step) after pH adjustment every 30 min at 50 °C for 3 h was applied and solution pHs of the PBS and CB systems were monitored and readjusted to 7.8 and 9.0, respectively, every 30 min as seen in Fig. 2C.

The pHs of the two buffer systems dropped more rapidly compared to the same reactions without pH adjustment, indicating that MAA could be consumed more quickly due to the reaction with regenerated free amino groups and continual hydrolysis of MAA at pH maintenance.

Next, in order to calculate the DS in the gelatin-MA products, we conducted the 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay for determination of unreacted free amino groups in gelatin. Reactions in PBS without pH adjustment resulted in a DS of 51.81 ± 0.16% while similar reactions in CB buffer resulted in a DS of 76.24 ± 0.54%, as presented in Table 1.

With pH adjustments, significant improvements were observed for both buffer systems, with a DS of 80.35 ± 0.49% for PBS (at pH 7.8) and a DS of 97.20 ± 0.28% for CB buffer (at pH 9.0). ^{1}H-NMR analysis was also used to verify the extent of the DS. The results are in agreement with the aforementioned values as depicted in Fig. 2D. There were minimal side reactions with hydroxyl groups of gelatin other than amino groups in all experimental groups, while the spectra of the gelatin derivatives at $5.6 \leq \delta \leq 6.1$ ppm appeared when a 10–20 : 1 molar ratio of MAA to type B gelatin was employed in PBS.[19]

The dependence on MAA concentration was also investigated in CB buffer with pH 9 adjustment, as presented in Fig. 3. The concentrations of MAA added to gelatin (10 g per 100 mL, 10% w/v) ranged from 0.125 to 2% v/v, which correspond to a range of molar ratios of amino groups to MAA from 1 : 0.275 to 1 : 4.4. All the gelatin solutions reacted with different amounts of MAA at pH 9 adjustment in CB buffer. The pH of each

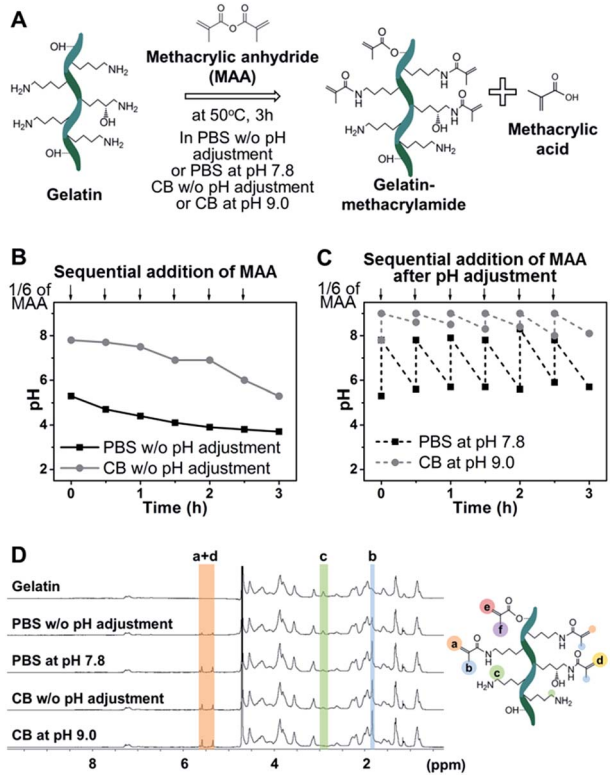

Fig. 2 (A) Schematic illustration of gelatin-MA synthesis. (B) Change in solution pH of PBS and CB buffer systems during reaction progress (without pH adjustment). (C) Change in solution pH of PBS and CB buffer systems during reaction progress (with pH adjustment at each MAA addition step). (D) ^{1}H-NMR verification of gelatin-MA conversion based on DS values.

Table 1 Comparison of gelatin-MA preparation methods in feed ratio, buffer system, pH and DS

Group	Conventional method	PBS w/o pH adjustment	PBS at pH 7.8	CB w/o pH adjustment	CB at pH 9.0
Gelatin (% w/v)	10	10	10	10	10
MAA (% v/v)	6–20	1	1	1	1
MAA (mL)/gelatin (g)	0.6–2/1	0.1/1	0.1/1	0.1/1	0.1/1
Molar ratio (MAA/amine)	13–44	2.2	2.2	2.2	2.2
Buffer	PBS	PBS	PBS	CB	CB
pH adjustment	No	No	Six times at pH 7.8	No	Six times at pH 9.0
DS from NMR (%)		47	87	78	100
DS from TNBS (%)	70–85	51.81 ± 0.16	80.35 ± 0.49	76.24 ± 0.54	97.20 ± 0.28

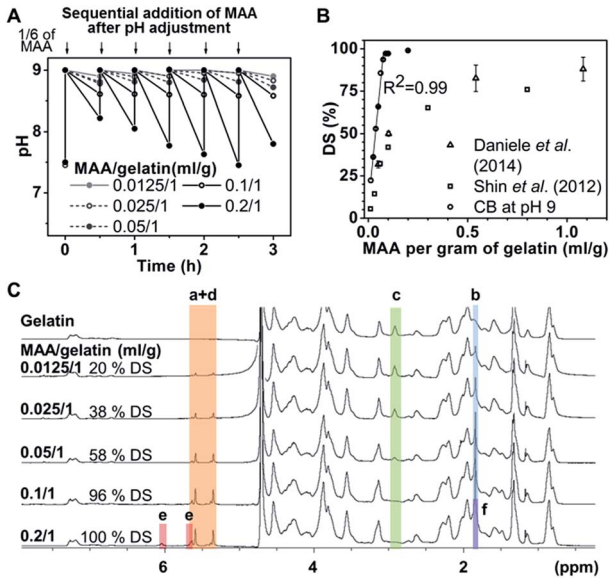

Fig. 3 (A) Change in solution pH of CB buffer system as a function of MAA/gelatin feed ratio. (B) DS *versus* MAA/gelatin feed ratio in comparison with previous studies. (C) ^{1}H-NMR verification of DS values.

reaction solution dropped in proportion to the amount of MAA added which is directly associated with the production of the methacrylic acid by-product. The DS results achieved with our improved scheme are presented in Fig. 3B alongside results obtained by the conventional scheme in past studies.[13,17]

In the conventional method using PBS, it was difficult to control the DS beyond 80–90% because a higher feed ratio of MAA to gelatin might produce more by-product (methacrylic acid) and cause protonation of the remaining free amino groups. Even a feed ratio of 0.6 mL (MAA)/g (gelatin) −2.0 mL (MAA)/g (gelatin) (13–44 molar excess of MAA over free amino groups) was limited to producing type A gelatin-MA with a DS of 90% or less with the conventional method. By contrast, using our method, the DS of gelatin-MA increased up to around 97% or more in a nearly linear and controllable manner based on the feed ratio—a more than three-fold improvement in DS over the conventional method in a similar range of feed ratios.[17]

Indeed, the feed ratio from 0.0125 to 0.1 mL (MAA)/1 g (gelatin) led to type A gelatin-MA with DS ranging from 22.32 ± 1.27 to 97.20 ± 0.27% (*cf.* ESI Table S1†). The feed ratio of 0.1 mL (MAA) to 1 g (gelatin) corresponding to a 2.2-fold molar excess of MAA over free amino groups yielded a near-100% DS. The feed ratio of 0.2 mL (MAA) to 1 g (gelatin) caused free amino groups to be completely consumed and additionally some hydroxyl groups on amino acids reacted with MAA, as interpreted from the ^{1}H-NMR data showing peaks at between 6.1 and 5.7 ppm (Fig. 3C). Therefore, type A gelatin-MA with a DS ranging from 20% to 100% could be tailored within a low concentration of MAA (1% v/v) with a negligible degree of side reactions of hydroxyl groups of gelatin with MAA.

Similarly, pH effect of gelatin-MA synthesis on DS was studied, resulting the highest DS at pH 9 in CB buffer solutions (*cf.* ESI Table S2†). This is considered to be due to faster hydrolysis of MAA at pH 10 and pH 11, decreasing the efficacy of the reaction.

Finally, gelation tests were conducted on gelatin-MA hydrogels by using a rheometer with UV curing system. Gelatin-MA samples (30% w/v in distilled water) containing 1% I2959 photo-initiator were placed on a glass substrate and then irradiated with UV light. As shown in Fig. 4A, gelatin-MA solutions immediately started to form gels and were cured within 50 seconds. There was a strong positive correlation between the storage modulus of each gelatin-MA hydrogel and the DS of the corresponding gelatin-MA monomers as shown in Fig. 4B; gelatin-MA with 22.32, 36.18, 66.02 and 97.20% DS exhibited 0.7 ± 0.1, 5.3 ± 0.9, 25.9 ± 1.4 and 83.2 ± 4.8 kPa storage moduli, respectively. A higher DS of gelatin-MA could result in a greater crosslinking density in UV curing, which led to a hydrogel with a higher storage modulus. These results are

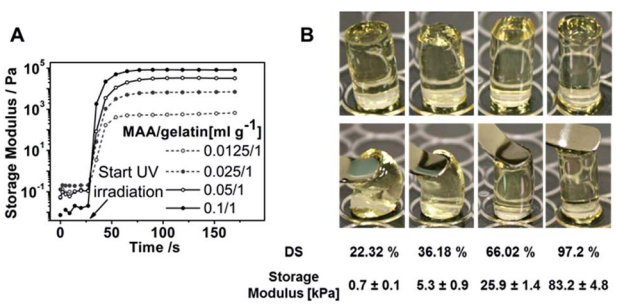

Fig. 4 (A) Rheological analysis of storage modulus on UV irradiated gelatin-MAs with different MAA/gelatin ratios. (B) Demonstration of fabricated gelatin-MA hydrogels with different DS values.

consistent with the previous reports.[17,18] Our method enables precise control of mechanical properties in order to prepare a range of soft to stiff protein-based hydrogels.

In summary, we report the efficient and controllable preparation of gelatin-MA with near-100% DS, offering a mechanically improved polymeric material for protein-based hydrogel platforms. The carbonate–bicarbonate buffer system with pH adjustment scheme enabled us to use an appreciably smaller molar excess of MAA while enabling excellent control over the degree of substitution as compared to the conventional PBS system. Looking forward, there is significant potential to utilize highly substituted gelatin-MA for hydrogel-based tissue engineering and drug delivery applications.

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Notes and references

1 J. B. Rose, S. Pacelli, A. J. El Haj, H. S. Dua, A. Hopkinson, L. J. White and F. R. A. J. Rose, *Materials*, 2014, **7**, 3106–3135.
2 E. Jain and A. Kumar, *Nat. Protoc.*, 2013, **8**, 821–835.
3 D. Olsen, C. L. Yang, M. Bodo, R. Chang, S. Leigh, J. Baez, D. Carmichael, M. Perala, E. R. Hamalainen, M. Jarvinen and J. Polarek, *Adv. Drug Delivery Rev.*, 2003, **55**, 1547–1567.
4 J. Ratanavaraporn, R. Rangkupan, H. Jeeratawatchai, S. Kanokpanont and S. Damrongsakkul, *Int. J. Biol. Macromol.*, 2010, **47**, 431–438.
5 C. Fukaya, Y. Nakayama, Y. Murayama, S. Omata, A. Ishikawa, Y. Hosaka and T. Nakagawa, *J. Biomed. Mater. Res., Part B*, 2009, **91**, 329–336.
6 A. Bigi, G. Cojazzi, S. Panzavolta, N. Roveri and K. Rubini, *Biomaterials*, 2002, **23**, 4827–4832.
7 A. Bigi, G. Cojazzi, S. Panzavolta, K. Rubini and N. Roveri, *Biomaterials*, 2001, **22**, 763–768.
8 H. W. Sung, D. M. Huang, W. H. Chang, R. N. Huang and J. C. Hsu, *J. Biomed. Mater. Res.*, 1999, **46**, 520–530.
9 X. Zhao, Q. Lang, L. Yildirimer, Z. Y. Lin, W. Cui, N. Annabi, K. W. Ng, M. R. Dokmeci, A. M. Ghaemmaghami and A. Khademhosseini, *Adv. Healthcare Mater.*, 2015, DOI: 10.1002/adhm.201500005.
10 J. Visser, D. Gawlitta, K. E. Benders, S. M. Toma, B. Pouran, P. R. van Weeren, W. J. Dhert and J. Malda, *Biomaterials*, 2015, **37**, 174–182.
11 R. Levato, J. Visser, J. A. Planell, E. Engel, J. Malda and M. A. Mateos-Timoneda, *Biofabrication*, 2014, **6**, 035020.
12 E. Kaemmerer, F. P. Melchels, B. M. Holzapfel, T. Meckel, D. W. Hutmacher and D. Loessner, *Acta Biomater.*, 2014, **10**, 2551–2562.
13 M. A. Daniele, A. A. Adams, J. Naciri, S. H. North and F. S. Ligler, *Biomaterials*, 2014, **35**, 1845–1856.
14 T. Billiet, E. Gevaert, T. de Schryver, M. Cornelissen and P. Dubruel, *Biomaterials*, 2014, **35**, 49–62.
15 T. Billiet, B. van Gasse, E. Gevaert, M. Cornelissen, J. C. Martins and P. Dubruel, *Macromol. Biosci.*, 2013, **13**, 1531–1545.
16 W. Schuurman, P. A. Levett, M. W. Pot, P. R. van Weeren, W. J. Dhert, D. W. Hutmacher, F. P. Melchels, T. J. Klein and J. Malda, *Macromol. Biosci.*, 2013, **13**, 551–561.
17 H. Shin, B. D. Olsen and A. Khademhosseini, *Biomaterials*, 2012, **33**, 3143–3152.
18 A. I. van Den Bulcke, B. Bogdanov, N. de Rooze, E. H. Schacht, M. Cornelissen and H. Berghmans, *Biomacromolecules*, 2000, **1**, 31–38.
19 E. Hoch, T. Hirth, G. E. M. Tovar and K. Borchers, *J. Mater. Chem. B*, 2013, **1**, 5675–5685.
20 M. Sivakumar, P. R. G. Radhakrishnan and H. Kothandaraman, *J. Appl. Polym. Sci.*, 1991, **43**, 1789–1794.
21 B. F. Pierce, G. Tronci, M. Rossle, A. T. Neffe, F. Jung and A. Lendlein, *Macromol. Biosci.*, 2012, **12**, 484–493.
22 F. P. W. Melchels, W. J. A. Dhert, D. W. Hutmacher and J. Malda, *J. Mater. Chem. B*, 2014, **2**, 2282–2289.
23 S. Pedron, E. Becka and B. A. Harley, *Adv. Mater.*, 2015, **27**, 1567–1572.
24 A. L. Rutz, K. E. Hyland, A. E. Jakus, W. R. Burghardt and R. N. Shah, *Adv. Mater.*, 2015, **27**, 1607–1614.
25 Y. C. Chen, R. Z. Lin, H. Qi, Y. Yang, H. Bae, J. M. Melero-Martin and A. Khademhosseini, *Adv. Funct. Mater.*, 2012, **22**, 2027–2039.
26 P. Occhetta, R. Visone, L. Russo, L. Cipolla, M. Moretti and M. Rasponi, *J. Biomed. Mater. Res., Part A*, 2015, **103**, 2109–2117.
27 C. B. Hutson, J. W. Nichol, H. Aubin, H. Bae, S. Yamanlar, S. Al-Haque, S. T. Koshy and A. Khademhosseini, *Tissue Eng., Part A*, 2011, **17**, 1713–1723.
28 P. Kim, A. Yuan, K. H. Nam, A. Jiao and D. H. Kim, *Biofabrication*, 2014, **6**, 024112.
29 E. Hoch, C. Schuh, T. Hirth, G. E. M. Tovar and K. Borchers, *J. Mater. Sci.: Mater. Med.*, 2012, **23**, 2607–2617.
30 R. Kale and A. Bajaj, *J. Young Pharm.*, 2010, **2**, 90–94.