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Structural analysis of photocrosslinkable methacryloyl-modified protein derivatives

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ABSTRACT

Biochemically modified proteins have attracted significant attention due to their widespread applications as biomaterials. For instance, chemically modified gelatin derivatives have been widely explored to develop hydrogels for tissue engineering and regenerative medicine applications. Among the reported methods, modification of gelatin with methacrylic anhydride (MA) stands out as a convenient and efficient strategy to introduce functional groups and form hydrogels via photopolymerization. Combining light-activation of modified gelatin with soft lithography has enabled the materialization of microfabricated hydrogels. So far, this gelatin derivative has been referred to in the literature as gelatin methacrylate, gelatin methacrylamide, or gelatin methacryloyl, with the same abbreviation of GelMA. Considering the complex composition of gelatin and the presence of different functional groups on the amino acid residues, both hydroxyl groups and amine groups can possibly react with methacrylic anhydride during functionalization of the protein. This can also apply to the modification of other proteins, such as recombinant human tropoelastin to form MA-modified tropoelastin (MeTro). Here, we employed analytical methods to quantitatively determine the amounts of methacrylate and methacrylamide groups in MA-modified gelatin and tropoelastin to better understand the reaction mechanism. By combining two chemical assays with instrumental techniques, such as proton nuclear magnetic resonance (^{1}H NMR) and liquid chromatography tandem-mass spectrometry (LC-MS/MS), our results indicated that while amine groups had higher reactivity than hydroxyl groups and resulted in a majority of methacrylamide groups, modification of proteins by MA could lead to the formation of both methacrylamide and methacrylate groups. It is therefore suggested that the standard terms for GelMA and MeTro should be defined as gelatin methacryloyl and methacryloyl-substituted tropoelastin, respectively, to remain consistent with the widespread abbreviations used in literature.

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1. Introduction

Biomaterials based on proteins or peptides have been widely studied in the biomedical sciences, partly due to their highly tunable chemical compositions, suitable physical properties, and presentation of bioactive motifs [1]. Specifically, hydrogels based on proteins/peptides are suitable candidates to develop biomimetic matrices that resemble the native extracellular matrix (ECM) for supporting cellular attachment, proliferation, organization, and ultimately tissue regeneration [2,3].

As the most abundant structural protein in ECM, collagen contains various cell-binding motifs (such as the Arg-Gly-Asp, or RGD sequence) and matrix metalloproteinase (MMP)-degradable sequences. Gelatin is a mixture of polypeptides obtained from the partial hydrolysis of collagen. Gelatin is biocompatible and retains the functionality of many of the bioactive peptide sequences found in intact collagen. As a result, gelatin has been widely explored to design and fabricate hydrogels for different tissue engineering and regenerative medicine applications [4,5].

Gelatin is soluble in water at around 40 °C or above, yet can form a physically crosslinked hydrogel at room temperature by partially restoring the characteristic triple helical structures of the parent collagen. However, thermally reversible gelatin hydrogels have some limitations regarding *in vivo* applications, including poor mechanical properties and low transition temperatures [6,7]. Consequently, various chemical crosslinking methods have been developed to generate mechanically strong and stable gelatin based hydrogels [8,9]. Initially, glutaraldehyde [8] and diisocyanate [9] were used as crosslinkers to form stable gelatin based hydrogel network. However, the cytotoxicity of these crosslinkers limited their use as cell-laden matrices for tissue engineering applications [10]. To address these limitations, Van Den Bulcke et al. generated photocrosslinkable gelatin derivatives by reacting gelatin with methacrylic anhydride (MA) [7]. The resulting material was termed as gelatin methacrylamide (GelMA).

Since then, GelMA has gained significant attention in the biomaterials community and has become a widely used biomaterial [4,5,11–18]. GelMA hydrogels possess an attractive combination of properties. For example, they are suitable for both two-dimensional (2D) cell seeding and three-dimensional (3D) cell encapsulation [19,20], compatible with various microfabrication techniques [12,15,21], tunable in regards to their physical properties [20,22], cost efficient, and easy to synthesize.

Despite its widespread applications, functional groups in GelMA molecules have been inadequately studied. As a consequence, GelMA is ambiguously described as gelatin methacrylamide [7,23–27], methacrylated gelatin [11,28–31], and gelatin methacrylate [19–22,32–34] (Fig. 1). This confusing nomenclature can apply to all similarly functionalized protein-based biomaterials, such as MA-modified recombinant human tropoelastin, which is a full-length protein and has been developed as a highly elastic biomaterial for cardiovascular tissue engineering in our group and termed as methacrylated tropoelastin (MeTro) [35–37]. We tested the developed method for structural analysis on GelMA and MeTro because we were among the earliest groups to focus on the biomedical applications of GelMA, and because we first reported the synthesis of MeTro.

In this study, we aimed to more accurately describe the chemical modification of protein-based biomaterials by MA and define the correct chemical nomenclature for these biomaterials based on their functional groups. Here, we report the quantification of relative amounts of the different functional groups in GelMA and MeTro prepolymers, namely, methacrylamide groups and methacrylate groups, using a simple microplate assay method. The results are further confirmed by instrumental techniques such as proton nuclear magnetic resonance (^{1}H NMR) and liquid chromatography tandem-mass spectrometry (LC-MS/MS). Our study provides, to our knowledge for the first time, an understanding of the relative reactivity of amine and hydroxyl groups in gelatin and recombinant human tropoelastin with MA, which allows for structurally correct naming of MA-modified proteins, and provides a solid basis for further structural investigations on modified protein/peptide-based biomaterials.

2. Materials and methods

2.1. Materials

Type-A gelatin from porcine skin (300 bloom), methacrylic anhydride (MA, 94%), deuterium oxide (D_2O, 99.9% in D), sodium hydroxide (NaOH, 98%), acetohydroxamic acid (98%), iron(III) perchlorate hydrate (crystalline, low chloride), hydroxylamine hydrochloride (>99%), hydrochloric acid (36.5–38.0%, BioReagent), and 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's phosphate buffered saline (DPBS) and Fluoraldehyde OPA reagent were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. GelMA synthesis

Synthesis of GelMA with different degrees of methacryloyl substitution was performed according to our previously reported procedures [20]. Briefly, 10 g of porcine skin type A gelatin was dissolved in 100 mL DPBS at 50 °C. At a stirring speed of 300 rpm, various amounts of MA (20 mL for ultra-GelMA, 8 mL for high-GelMA, 3 mL for medium-GelMA, or 0.5 mL for low-GelMA) was added to the gelatin solution and the resulting turbid mixture was stirred at 50 °C for 3 h. Under this condition, hydrolysis does typically not take place [7,20]. The mixture was diluted with additional warm DPBS, and dialyzed against an abundance of distilled water using dialysis tubing (12–14 kDa MWCO, Spectrum Lab Inc.) for seven days at 40 °C to remove methacrylic acid and any other impurities. After dialysis, the resulting clear solution was then lyophilized to afford GelMA as a white solid.

2.3. MeTro synthesis

Synthesis of MeTro with different degrees of methacryloyl substitution was performed according to our previously reported procedures with modifications [35,36]. Briefly, tropoelastin was dissolved in DPBS as a 10% (w/v) solution at 4 °C, followed by the addition of various amounts of MA (for the samples used in this study: 20% (v/v) MA for high-MeTro, 15% (v/v) MA for medium-MeTro, or 8% (v/v) MA for low-MeTro). The resulting mixture was stirred at 4 °C for 12 h before the solution was diluted with DPBS, purified by Amicon Ultra-15 Centrifugal Filter Units (10 K MWCO) repeatedly for 6 times at 4 °C and lyophilized.

2.4. Fluoraldehyde assay

GelMA solutions in DPBS and MeTro solutions in DI water were prepared at 0.5 mg/mL. The solutions were mixed with the Fluoraldehyde OPA reagent solution at a 1/1 (v/v) ratio and allowed to react for 60 s to finish the conversion. Fluorescence intensity of the resulting mixtures was monitored in a microplate reader (excitation/emission = 340 nm/455 nm). Gelatin or tropoelastin was used as the standard while pure DPBS or DI water was used as the blank, respectively. Conversion of amine groups (α) was calculated as follows: