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Sequential alkyne-azide cycloadditions for functionalized gelatin hydrogel formation

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ABSTRACT

While click chemistry reactions for biopolymer network formation are attractive as the defined reactions may allow good control of the network formation and enable subsequent functionalization, tailoring of gelatin network properties over a wide range of mechanical properties has yet to be shown. Here, it is demonstrated that copper-catalyzed alkyne-azide cycloaddition of alkyne functionalized gelatin with diazides gave hydrogel networks with properties tailorable by the ratio of diazide to gelatin and diazide rigidity. 4,4′-diazido-2,2′-stilbenedisulfonic acid, which has been used as rigid crosslinker, yielded hydrogels with Young's moduli E of 50–390 kPa and swelling degrees Q of 150–250 vol.%, while the more flexible 1,8-diazidooctane resulted in hydrogels with E = 125–280 kPa and Q = 225–470 vol.%. Storage moduli could be varied by two orders of magnitude (G' = 100–20,000 Pa). An indirect cytotoxicity test did not show cytotoxic properties. Even when employing 1:1 ratios of alkyne and azide moieties, the hydrogels were shown to contain both, unreacted alkyne groups on the gelatin backbone as well as dangling chains carrying azide groups as shown by reaction with functionalized fluorescein. The free groups, which can be tailored by the employed ratio of the reactants, are accessible for covalent attachment of drugs, as was demonstrated by functionalization with dexamethasone. The sequential network formation and functionalization with click chemistry allows access to multifunctional materials relevant for medical applications.

1. Introduction

Biomacromolecules such as proteins and polysaccharides are attractive precursors for biomaterials, as they inherently combine biocompatibility and degradability [1]. However, to overcome their water solubility and to tailor their properties, crosslinking strategies have to be employed. Classically, such crosslinking can adopt one of two strategies: either two functionalized macromers are reacted, or a functionalized macromer is reacted with a bifunctional crosslinker. In the latter case, typically the higher reactivity [2] should lead to higher conversion and the properties of the polymer network may in principle be tailored by adjusting the crosslinker type, length, and amount. Examples for such crosslinkings are based on reactions with highly reactive bifunctional molecules such as diisocyanates [3] or dialdehydes [4], direct crosslinking of present functional groups to give ester or amide linkages [5], as well as polymerization reactions such as photocrosslinking [6]. While in part resulting in polymer networks with tailorable properties, the mentioned reactions are prone to allow side reactions, which increase the complexity of analysis and hamper development of structure-property-relationships. Highly reactive groups may furthermore cause toxicity of the materials or released substances. Because of potential cleavability of the formed bonds through reversibility of the reaction, further synthetic transformations may be challenging and change of mechanical properties may occur prior to an intended hydrolytic degradation. An attractive approach would be to employ a high yielding, chemoselective and irreversible reaction with basically no side products, which corresponds to the click chemistry concept introduced by Sharpless [7]. Examples for such click reactions including the copper-catalyzed alkyne-azide cycloaddition (CAAC) [8], strain-promoted alkyne-azide cycloaddition (SPAAC) [9], Diels-Alder reactions [10], thiol-ene additions [11], thiol-Michael additions [12], and oxime formation [13], initially developed for organic reactions of small molecules, have successfully been employed in polymer [14,15] and hydrogel synthesis [16], though typically in the context of

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synthetic precursors such as polyethylene glycol (PEG) or polyacrylamides.

While click chemistry reactions have been shown to allow tailoring of the mechanical properties of polysaccharide-based networks [17,18], this goal has been considerably more difficult to achieve for gelatin. Gelatin is a proteinaceous mixture produced by partial hydrolysis and deglycosylation of collagen, and gelatin-based materials were successfully used for various medical applications such as drug delivery [19], bone [20] or kidney regeneration [21], as well as ocular tissue engineering [22]. Gelatin network formation by strain promoted alkyne-azide cycloaddition (SPAAC) reaction has been reported employing star-shaped poly(ethylene glycol) as crosslinker, however with no [23] or very limited control about the mechanical properties of the resulting gels [24]. Nitrile oxide-norbornene click reaction of gelatin and PEG likewise only allowed a change of the storage modulus by a factor of 2 at constant concentration [25]. Even though gelatin does not contain free alkyne groups, selective functionalization of free amino groups (e.g. of lysine residues) under suitable conditions is possible [26]. It was hypothesized that by employing CAAC by reaction of alkyne-functionalized gelatin with diazide crosslinkers would allow the formation of gelatin-based networks with tailorable properties by variation of the type and quantity of crosslinker, simultaneously controlling the gelatin-inherent triple helicalization by performing the reaction > 37 °C [3]. It been demonstrated that for potential applications in biomedicine copper can be effectively removed from hydrogels [26,27], though the non-toxicity of the hydrogel needs to be shown. Any unreacted alkyne or azide group may be used in a subsequent conversion thereby giving access to functionalized gelatin hydrogels of interest as drug releasing systems without requiring the change of chemistry involved [28]. Drug attachment to a carrier hydrogel by molecular recognition [29] or covalent linkage [30,31] is of significant interest to control – and slow down – release rates. The strategy to use the same reactions for network formation and drug attachment is especially interesting providing a control over the mechanical properties of the gelatin hydrogels by simple variation of the alkyne:azide ratio or crosslinker type rather than changing the polymer concentration of the overall hydrogel system. Furthermore, this could potentially allow for decoupling of material properties and functions, e.g. stiffness, architecture, and drug loading. For this purpose, 4,4′-diazido-2,2′-stilbenedisulfonic acid and 1,8-diazidooctane were employed as crosslinkers with different length and rigidity. In the following, the functionalization of gelatin with propiolic acid, the formation of networks through reaction with different molar ratios of the diazide crosslinkers, and the thermomechanical characterization of the network properties are described. The non-toxicity of the hydrogels is exemplarily demonstrated. Furthermore, a sequential click chemistry approach is exploited as a tool to understand the network architecture by attachment of fluorescent dyes, and for the attachment of a model drug, dexamethasone, as a proof of concept for the accessibility of functionalized gelatin-based hydrogel networks.

2. Experimental section

2.1. Materials

Gelatin (type A from porcine skin, 300 bloom), propiolic acid (PA), *N,N′*-diisopropylcarbodiimide (DIC), 2,4,6-trinitro-benzensulfonic acid (TNBS), *N,N′*-dimethylformamide (DMF anhydrous), copper (II) sulfate ($CuSO_4$), copper (I) iodide (CuI), sodium ascorbate, 4,4′-diazidostilbenedisulfonic acid disodium salt, 1,8-dibromooctane, 1,12-dibromododecane, and PEG-diazide (M_w = 1108 g·mol^{-1}, PDI = 1.2) were purchased from Sigma Aldrich (Munich, Germany). Tetrahydrofuran (THF, reagent ACS, 99.8%), dimethylsulfoxide (DMSO), sodium azide (NaN_3), and borax decahydrate ($Na_2B_4O_7{\cdot}10H_2O$) were purchased from Merck (Darmstadt, Germany). Dichloromethane (DCM, anhydrous) was purchased from Acros (Geel, Belgium), dexamethasone from Alfa Aesar (Karlsruhe, Germany), and 4-azidophenyl hydrazine from Apollo Scientific (Cheshire, UK). All reagents and solvents were of analytical grade and were used without further purification.

2.2. Synthesis of alkyne functionalized gelatin

Gelatin (1 g, 270 µmol of NH_2 groups) was dissolved in Borax buffer (pH 8.5, 20 mL) at 40 °C. The flask was removed from heating and DMF (40 mL) was added. Separately, in another flask propiolic acid (140 mg, 2 mmol) was dissolved in anhydrous DMF (4 mL), the solution was cooled in an ice bath and diisopropylcarbodiimide (126 mg, 1 mmol) was added. The mixture was stirred for 30 min at 0 °C, followed by the addition of dichloromethane (4 mL) and stirring was continued for additional 10 min. Thereafter, the solution of activated propiolic acid was slowly added to the gelatin solution and the reaction was run for 2 h at room temperature. The product was precipitated in THF (150 mL). Alkyne-functionalized gelatin was purified by dissolution in PBS buffer (pH 7.4, 5 mL) at 40 °C, addition of DMF (5 mL) and precipitation in THF (50 mL). Dissolution in PBS buffer (5 mL) at 40 °C and consecutive precipitation in THF (50 mL) was repeated three times. At the end, the product was washed with cold water, methanol and acetone and dried under high vacuum at 40 °C. The degree of functionalization determined by a spectrophotometric method (TNBS) [32] was determined to be 88 mol%.

2.3. Synthesis of gelatin based hydrogels

The alkyne-functionalized gelatin (500 mg, 119 µmol of alkyne groups) was dissolved in a 1:1 mixture of water and ethanol (10 mL) at 45 °C (above the sol-gel transition temperature at which gelatin chains are in a random coiled state). Then, one of the diazides: 4,4′-diazido-2,2′-stilbenedisulfonic acid, 1,8-diazidooctane, 1,12-dibromododecane, or PEG-diazide was added. The synthetic procedure for the diazidoalkanes is given in the supporting information. Four different alkyne group:azide group ratios were used: 0.5:1, 1:1, 5:1, and 10:1. Afterwards, sodium ascorbate (2 mg, 10 µmol) and $CuSO_4$ (2 mg, 8 µmol) were added as catalyst system and the mixture was stirred vigorously for 10 s, poured in a Petri dish and left overnight. The next day, hydrogels were swollen repeatedly in 0.05 M EDTA solution for copper removal and washed with water.

2.4. Synthesis of 5-(5-azidopentanamido)-2-(3-hydroxy-6-oxo-6H-xanthen-9-yl)benzoic acid (AzFF)

To a solution of fluoresceinamine (0.15 g, 0.43 mmol, 1 eq) in pyridine (2 mL), EDC (0.083 g, 0.43 mmol, 1 eq) and azidopentanoic acid (0.062 g, 0.43 mmol, 1 eq) were added. The reaction was left to proceed overnight at room temperature and under stirring. Then the reaction mixture was poured into cold water (15 mL). The solution was acidified (pH < 2) by adding 5 M HCl. After stirring for 1 h, the precipitate was filtered off, washed with 1 M HCl (3 × 3 mL) and dissolved in a small amount of ethyl acetate (EtOAc). The EtOAc solution was dried over Na_2SO_4, filtered and then concentrated. Addition of hexane (150 mL) led to the formation of the product as orange crystals, which were collected and dried under vacuum (90 mg, 44%). ^{1}H NMR (500 MHz, CD_3OD): δ = 8.25 (mc, 1H, Ph), 7.74 (m, 1H, Ph), 7.04 (mc, 1H, Ph), 6.73 (mc, 1H, Ph), 6.63–6.42 (m, 5H, Ph), 3.21 (mc, 2H, CH_2-N_3), 2.38 (m, 2H, CH_2C(O)NH), 1.63–1.53 (m, 4H, $(CH_2)_2$) ppm. ^{13}C NMR (125 MHz, CD_3OD): 172.5, 171.0, 169.2, 151.8, 151.4, 140.1, 140.0, 128.5, 128.3, 128.2, 126.7, 126.4, 126.0, 123.7, 116.9, 116.2, 114.1, 112.0, 109.5, 101.7, 101.6, 50.3, 37.4, 27.5, 21.0 ppm. FT-IR (ATR): 3307–2600, 2095, 1735, 1670, 1605, 1385, 1255, 1207, 1170, 1111, 912, 846, 786 cm^{-1}. MS (ESI$^+$): calculated for $(C_{25}H_{20}N_4O_6+H)^+$: 473.1479, found 473.3828.