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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 ($G' = 100-20000$ 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.

Keywords: Click Chemistry, Hydrogel, Polymer Functionalization, Biopolymer, Rheology, Multifunctionality

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 synthetic precursors such as polyethylene glycol (PEG) or poly acrylamides.

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 $>37\text{ }^{\circ}\text{C}$ [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.

Experimental section

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 ($M_w = 1108 \text{ g}\cdot\text{mol}^{-1}$, 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 ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{ H}_2\text{O}$) 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.

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%.

Synthesis of gelatin based hydrogels

The alkyne-functionalized gelatin (500 mg, 119 μ mol 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 μ mol) and CuSO₄ (2 mg, 8 μ mol) 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.

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 5M HCl. After stirring for 1 h, the precipitate was filtered off, washed with 1M HCl (3x3 mL) and dissolved in a small amount of ethyl acetate (EtOAc). The EtOAc solution was dried over Na₂SO₄, 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%). ¹H NMR (500 MHz, CD₃OD): 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, CH₂-N₃), 2.38 (m, 2H, CH₂C(O)NH), 1.63-1.53 (m, 4H, (CH₂)₂) ppm. ¹³C NMR (125 MHz, CD₃OD): 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 (C₂₅H₂₀N₄O₆+H)⁺: 473.1479, found 473.3828.

Synthesis of 2-(3-hydroxy-6-oxo-6H-xanthen-9-yl)-5-pent-4-ynamidobenzoic acid (AIFF)

The alkyne-fluorescein dye was synthesized using a procedure modified from literature [33]. To a solution of fluoresceinamine (0.5 g, 1.44 mmol, 1 eq) in pyridine (6 mL), EDC (0.42 g, 2.17 mmol, 1.5 eq) and 4-pentynoic acid (0.28 g, 2.88 mmol, 2 eq) were added. The reaction was run overnight at room temperature under stirring. Then the reaction mixture was poured into cold water (25 mL). The solution was slowly acidified (pH < 2) by adding 5M HCl. After stirring overnight the precipitate was filtered off, washed with cold water and dissolved in a small amount of EtOAc. The EtOAc solution was dried over Na₂SO₄, filtered and then concentrated. Addition of a large excess of hexane led to the formation of the product as orange crystals, which were collected and dried under vacuum (250 mg, 40%). ¹H NMR (500 MHz, DMSO-d₆): δ = 10.49 (s, 1H, Ar-OH), 10.2 (bs, 1H, COOH), 8.37 (s, 1H, NH), 7.84 (mc, 1H, Ph), 7.28 (mc, 1H, Ph), 7.20 (mc, 1H, Ph), 6.90 (m, 2H, Ph), 6.73-6.57 (m, 4H, Ph), 2.92-2.79 (m, 3H, *H*-alkyne and alkyne-CH₂), 2.60 (mc, 2H, CH₂C(O)N), ppm. ¹³C NMR (125 MHz, MeOH-d₄): 170.9, 169.8, 169.1, 151.7, 151.4, 140.1, 139.9, 128.3, 128.2, 126.8, 126.5, 123.7, 116.9, 116.3, 114.1, 112.0, 109.5, 109.0, 101.7, 101.5, 81.5, 81.1, 68.5, 35.0, 13.5 ppm. FT-IR (ATR): 3500-2400, 2119, 1741, 1673, 1606, 1369, 1253, 1209, 1153, 1111, 850 cm^{-1} . MS (ESI⁺): calculated for (C₂₅H₁₇NO₆+H)⁺: 428.1179, found 428.3010.

Functionalization of gelatin network with 4-azidophenyl hydrazine

In a flask, which contained gelatin-based network (200 mg), oxygen-free DMSO (5 mL) was added, followed by the addition of 6 mL of DMSO, which contained 6 mg of 4-azidophenyl hydrazine and 1.5 mg of CuI. The flask was protected from sunlight with aluminum foil and was shaken at room temperature for 3 days. Thereafter, the product was washed three times with 10 mL of methanol, twice with 10 mL of DMSO and with 0.05 M aq. EDTA solution. At the end, the network was dried in a vacuum oven until constant weight was reached.

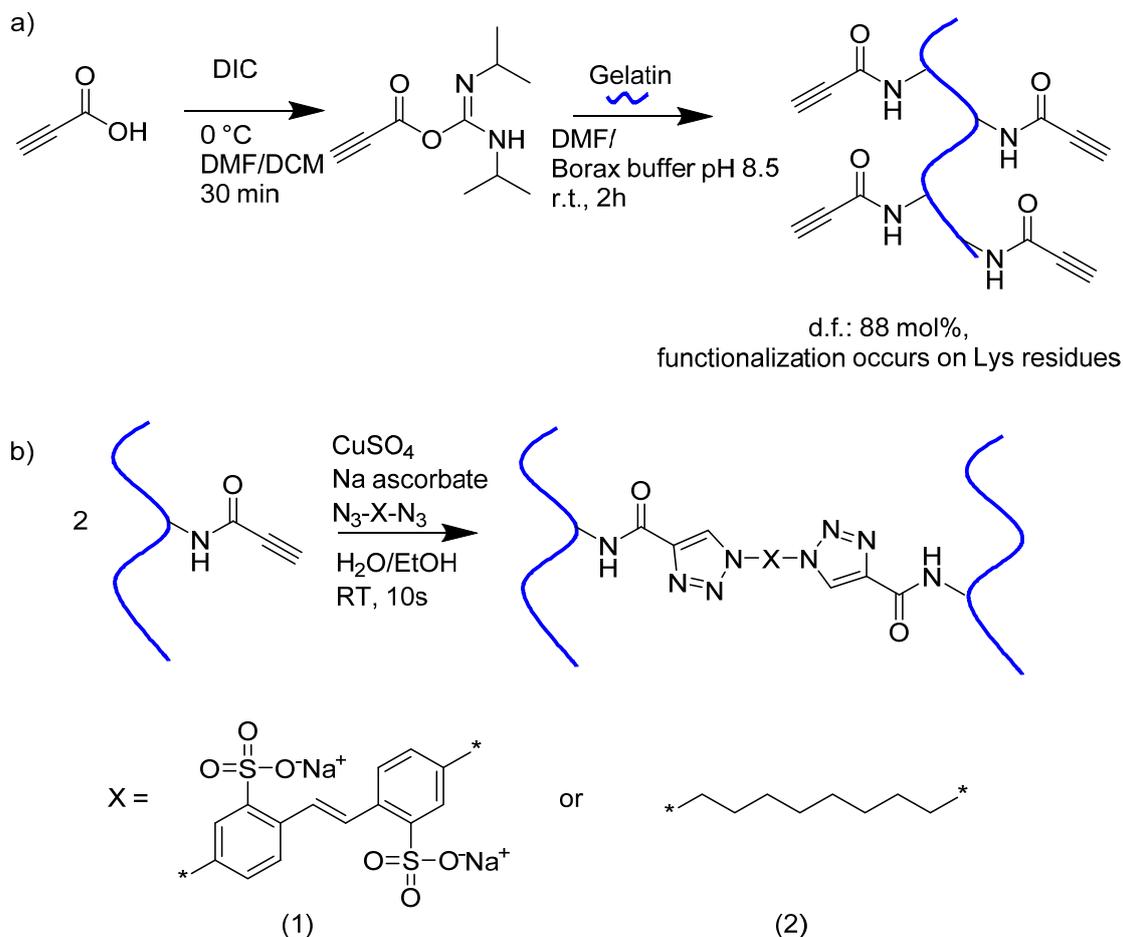
Attachment of dexamethasone to the gelatin hydrogel

In a vial containing gelatin hydrogel functionalized with 4-azidophenyl hydrazine (150 mg) and dexamethasone (35 mg), 5 mL of DMSO was added, followed by the addition of 10 μ L of acetic acid. The reaction was run at 40 °C for 4 days. The hydrogel was washed three times with 10 mL of DMSO, twice with 10 mL of water and with 10 mL of ethanol. The material was dried under vacuum at 40 °C until constant weight was reached.

Synthetic procedure of other materials, as well as the characterization methods and the details of cytotoxicity testing are given in the supporting information.

Results and discussion

The synthesis of the gelatin-based hydrogels was performed by a two-step procedure. The alkyne-functionalized gelatin was obtained through the carbodiimide-mediated coupling of propiolic acid to the amino groups of gelatin (Scheme 1a). This was followed by crosslinking the obtained alkyne-functionalized gelatin by reaction with a diazide using copper (II) sulfate and sodium ascorbate as catalyst (Scheme 1b).



Scheme 1. (a) Functionalization of gelatin lysine residues with propiolic acid; (b) Formal synthesis of gelatin-based hydrogels using the following crosslinkers: (1) 4,4'-diazido-2,2'-stilbenedisulfonic acid (formation of NGs networks) or (2) 1,8-diazidooctane (formation of NGo networks). Please refer also to Scheme 3 for an evaluation of the network structure.

The successful grafting of propiolic acid to the gelatin backbone was confirmed by FT-IR spectroscopy with the appearance of a new peak at 2115 cm^{-1} , which can be attributed to stretching of the $\text{C}\equiv\text{C}$ bond. The degree of functionalization of free amino groups determined by a spectrophotometric method (TNBS) [32] was 88 mol%, which is significantly higher than for the procedure described in literature (44 mol%) [34]. The molecular organization of gelatin chains before and after functionalization was determined by wide-angle X-ray scattering (WAXS). The WAXS spectra of a dry untreated gelatin film showed three peaks at $2\theta = 7.5^\circ$, 20° , and 31° , which correspond to the diameter of the triple helix, the amorphous halo, and the amino acids contact along the axis of single helices, respectively [35, 36]. The same molecular organization was

observed in the films of the alkyne-functionalized gelatin, however with a decreased intensity of the peaks at 7.5° and 31°. The triple helical index decreased from 5.5% (untreated gelatin) to 4.1% (alkyne functionalized gelatin). This change is however in the margin of error of the method (Figure S2, Supporting Information).

Dynamic shear oscillation measurements were used to characterize the viscoelastic properties of gelatin before and after derivatization (Figure 1). Before the heating run, both solutions were equilibrated at the temperature of the rheometer forming a physical gel. The obtained gels showed a high storage modulus (G') at low temperature, but on heating G' and the viscous modulus G'' decreased, and a crossover between G' and G'' occurred. The crossover temperature is attributed to the sol-gel transition temperature and it indicates the transition from an elastic network to a solution. Interestingly, the sol-gel transition in alkyne-functionalized gelatin occurred at a higher temperature than in pure gelatin, and the G' of alkyne-functionalized gelatin was at low temperature higher than that of pure gelatin. In a cooling experiment, the gelation temperature of alkyne-functionalized gelatin was observed at a higher temperature compared to pure gelatin solution. The physical gel formed by gelatin upon cooling can be described as an entangled network of triple helix connected via flexible links. The derivatization of gelatin in some cases hinders the renaturation of triple helices, leading to the formation of physical gels with low mechanical strength. Here, the presence of alkyne moieties in the gelatin backbone gave hydrogels with higher strength and gelation occurred at a temperature where pure gelatin solution has a low elastic modulus. This rheological observation may be related to the putative formation of π -hydrogen bonds between terminal alkynes and hydrogen donor groups (OH, NH₂) [37, 38].

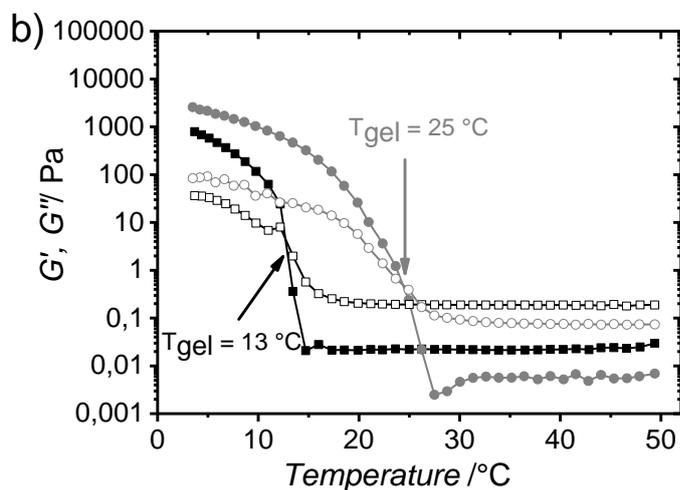
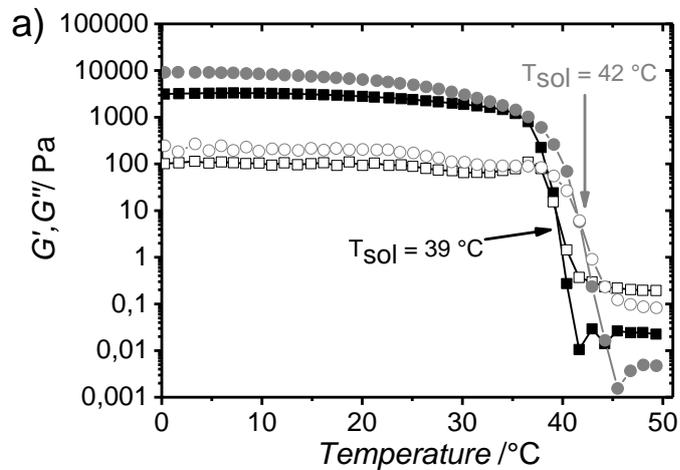


Figure 1. Evolution of the elastic (G') and viscous (G'') modulus as a function of temperature during the heating ramp (a) and cooling ramp (b) for gelatin (G' ■ and G'' □) and alkyne-functionalized gelatin solutions (G' ● and G'' ○).

The influence of molecular rigidity of crosslinker as well as its molar ratio on the properties of hydrogels were investigated by preparing two different networks referred to as NGs_x (crosslinked with the rigid 4,4'-diazido-2,2'-stilbenedisulfonic acid) and NGo_x (crosslinked with the more flexible 1,8-diazidooctane). The x in network abbreviations stands for the alkyne:azide ratio, i.e. a higher x value indicates that a lower concentration of crosslinker was used for the hydrogel formation. WAXS measurements were employed to explore the renaturation of triple helices within the hydrogels. Triple helical regions in gelatin gels act as physical netpoints. Only by

suppressing the triple helicalization it is possible to develop gelatin-gels whose mechanical properties are ruled by the amount and type of crosslinker, rather than the thermal history of the sample [39]. As it can be seen in Figure 2, the peaks at 7.5° and 31° , which correspond to the triple helix and the single helix, respectively, decreased with increased amount of crosslinker, with the rigid crosslinker being more effective in hindering the renaturation of triple helices. It is important to emphasize that at low crosslinker concentrations renaturation of triple helices was not completely suppressed, which can have a measurable impact on the properties of the material. At an alkyne:azide ratio of 10:1, triple helical index of NGo was 3.6% and of NGs_10 was 2.1%, while at a 1:1 ratio the triple helical index was 2.1% (NGo) and 0.8% (NGs).

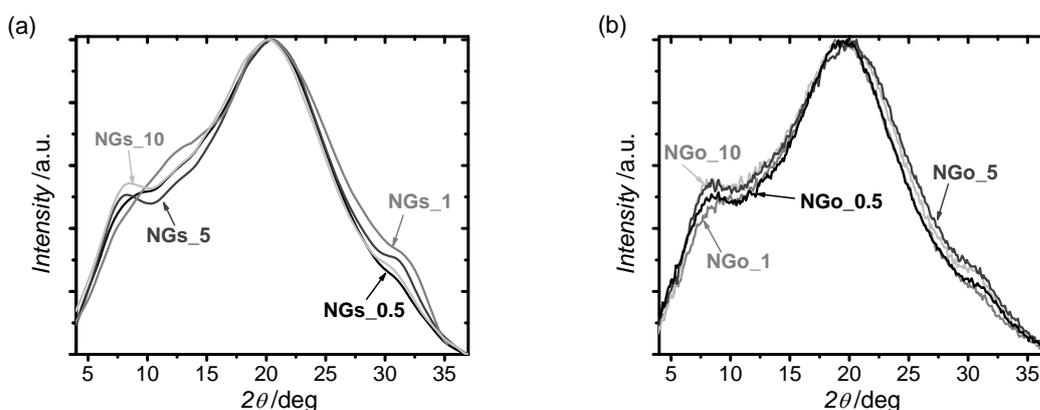


Figure 2. WAXS spectra of dry gelatin networks: (a) NGs and (b) NGo

The glass transition (T_g) of the dry hydrogels was investigated by temperature-modulated differential scanning calorimetry (DSC), and decreased with decreasing crosslinker content. Namely, the T_g of NGs_0.5 and NGs_5 was 73°C and 57°C , respectively, while T_g of NGo_0.5 and NGo_5 was 90°C and 72°C , respectively. Interestingly, the glass transition of NGs was lower than that of NGo systems. It was expected that a rigid crosslinker is more efficient in restricting mobility of gelatin chains and as a consequence T_g should increase [40]. However, the opposite effect was observed. This may be related to the point that 1,8-diazidooctane at lower concentrations did not completely suppress renaturation of triple helices, as shown by WAXS measurements, and since triple helices can act as physical crosslinking points, the mobility of gelatin chains was reduced and as consequence T_g increased.

Further, the effect of the crosslinker type at different alkyne:azide ratios on the swelling and mechanical properties of hydrogels was determined. The hydrogels were allowed to reach equilibrium swelling by incubation in PBS buffer at 37 °C for 24 h. As shown in Figure 3, the degree of swelling of gelatin-based hydrogels increased with decreasing crosslinker amount, probably due to the lower crosslinking density [41]. The swellability was also significantly affected by the crosslinker structure. The hydrogels containing the rigid stilbene had lower swelling ratios ($Q = 150$ - 250 vol.%) compared to those containing 1,8-diazidooctane ($Q = 225$ - 470 vol.%).

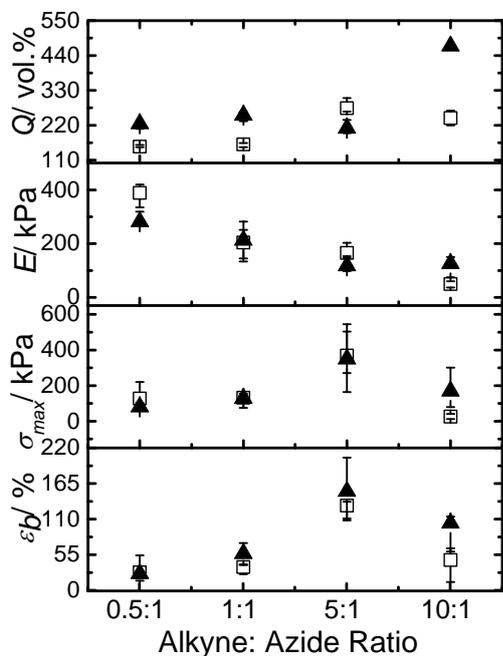


Figure 3. Change in swelling and mechanical properties of gelatin-based hydrogels at different alkyne:azide ratios. Tensile tests were performed at room temperature. From top to bottom: degree of swelling, Q ; Young's modulus, E ; Tensile strength, σ_{max} ; elongation at break, ϵ_b of NGs (□) and NGo (▲).

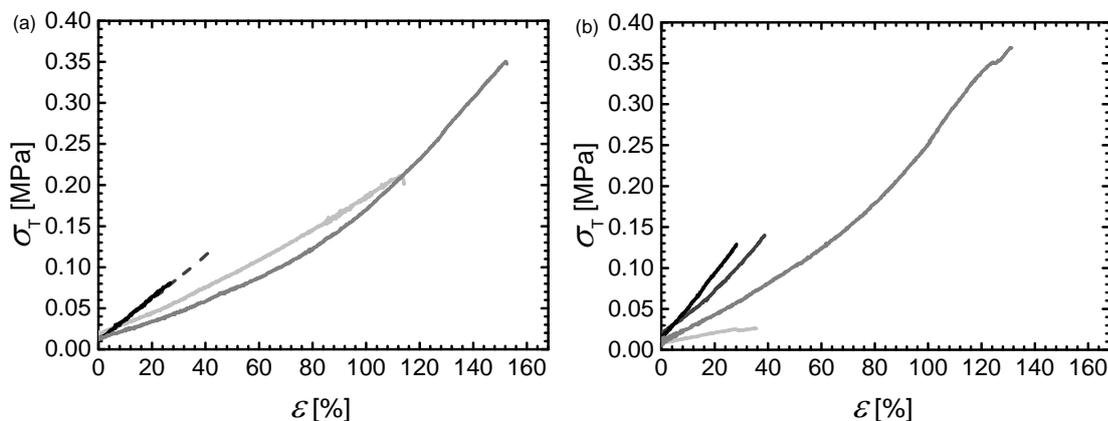


Figure 4. Representative tensile curves of (a) NGo and (b) NGs networks. NGx_0.5: black, NGx_1: dark gray (dashed in (a) for better legibility), NGx_5: gray, NGx_10: light gray,

Mechanical properties of the networks were characterized by tensile tests and rheological measurements. Tensile tests of the swollen materials were performed at room temperature (Figure 3 and 4). It was found that the elastic moduli increased when increasing the crosslinker content (from 50 kPa (37-60 kPa) to 390 kPa (335-420 kPa) for NGs and from 125 kPa (75-150 kPa) to 280 kPa (269-319 kPa) for NGo). Interestingly, NGs networks were stiffer than NGo networks for all alkyne:azide ratios except for the ratio 10:1, for which the elastic modulus of NGo_10 (125 kPa (75-150 kPa)) was higher compared to NGs_10 (50 kPa (37-60 kPa)). The low amount of crosslinker used for the synthesis of NGo_10, combined with its flexible structure, could not completely hinder the physical reorganization of some gelatin chains, which agrees with the results from the WAXS measurements. Therefore, the higher elastic moduli determined in this particular case are the result of chemical and physical (triple helical regions) crosslinking of the gel. The elongation at break ϵ_b (for NGs 30 - 130% and for NGo 25 - 150%) and the σ_{max} (for NGs 130 - 270 kPa and for NGo 80 - 350 kPa) increased with decreasing crosslinker amount, from the ratio 0.5:1 to 5:1, due to increased flexibility of the network chains. However, at the ratio 10:1 a decrease of elongation at break and tensile strength was observed for both networks (for NGs ϵ_b was 45% (13-65%) and σ_{max} was 25 kPa (12-41 kPa), while for NGo ϵ_b was 105% (60-114%) and σ_{max} was 170 kPa (80-211 kPa)), again likely due to the presence of the renaturated triple helices. From the tensile curves (Figure 4) it can be deduced that the hydrogels behave fully elastic as they do not display a yield point. In order to exclude the influence of triple helices on the mechanical properties

of hydrogels in the analyses, rheological measurements were performed at 37 °C. At this temperature unfolding of triple helices occurred and therefore only covalent netpoints were present in the samples. Figure 5 depicts the elastic modulus G' of the swollen hydrogels. G' was higher than the viscous modulus (G'') for all hydrogels in the whole frequency range, which is characteristic for elastic materials. At 37 °C, NGs hydrogels displayed higher G' values than NGo hydrogels at the corresponding alkyne:azide ratios. As NGs_10 had a G' one order of magnitude higher than NGo_10, this supports the interpretation that in tensile tests also the influence of physical netpoints forming triple helices was measured, while in rheology only the covalent netpoints played a role.

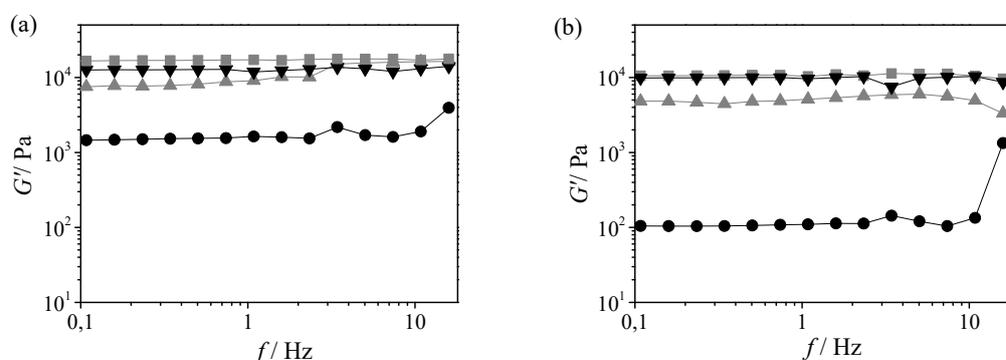


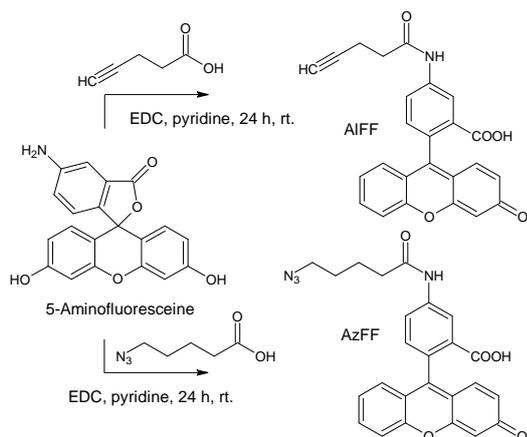
Figure 5. Rheological behavior of hydrogels measured at 37 °C: (a) NGs_0.5 (■), NGs_1 (▼), NGs_5 (▲) and NGs_10 (●); (b) NGo_0.5 (■), NGo_1 (▼), NGo_5 (▲) and NGo_10 (●)

Altogether, the mechanical properties and the swelling of the clicked gelatin hydrogels could be easily tailored by the choice of diazide crosslinker type and rigidity. Such diazides are commercially available in a large variety so that the network formation strategy is highly versatile and can be easily adapted to specific requirements. In Supporting Information Figure S3, the mechanical properties of propionylated gelatin crosslinked with 1,12-diazidododecane or PEG-diazide are reported to highlight this point. Obviously, the observed Young's moduli E and elastic moduli G' are comparable to other hydrogel systems. In this context, the control of the mechanical properties of gelatin-based hydrogels can only be ensured when taking into account and studying the triple helicalization. At the same time, a better control of the crosslinking reaction is given here than in the synthetic strategies given in the introduction [4-6]. In contrast to hydrogels based on

synthetic polymers such as PEG, the gelatin-based hydrogels are hydrolytically as well as enzymatically degradable and offer cell adhesion sites [42].

A cytotoxicity evaluation was exemplarily conducted for NGs_5 by culturing L929 mouse fibroblasts on extracts of the hydrogel and analyzing mitochondrial function, membrane integrity, cell morphology, and endotoxin content (details see supporting information and supporting Figure S5). Altogether, no major differences to a control were observed so that the hydrogel passed the cytotoxicity evaluation.

In the synthesis of small molecules, full and specific conversion of a reactive group is the basis to produce a new and defined chemical entity in high yield [43]. In macromolecular chemistry, mixtures of molecules are produced and handled, varying at least slightly e.g. in molecular weight or sequence structure. Furthermore, the reactivity of several exemplars of one type of functional group along a polymer chain will differ because of coiling of the macromolecule, steric hindrance after conversion of neighboring groups, or, in the case of network formation, because of decreasing flexibility of the polymer chain as well as limited length and conformational freedom of the crosslinker. This means that statistical considerations play an important role for synthesis [44]. While it is unlikely that a complete conversion of all groups can be reached, this may be exploited by subsequential transformations, corresponding to the step-wise use of one and the same type of reaction so that specifically functionalized molecules are produced, which are typically only accessible by orthogonal approaches. Instead of employing different chemical reactions, the different reactivity of groups is used. These considerations were put into praxis by reacting the NGs networks with the azido-functionalized fluorescein (AzFF) or alkyne-functionalized fluorescein (AlFF) in presence of copper (I) iodide as catalyst to evaluate whether the prepared hydrogels contain free alkyne or azide moieties available for the attachment of drugs or other bioactive compounds. The alkyne or azide-functionalized dyes were obtained by EDC mediated coupling of 4-pentynoic acid and 5-azido-pentanoic acid, respectively, with 5-aminofluorescein resulting in the formation of 2-(3-hydroxy-6-oxo-6*H*-xanthen-9-yl)-5-pent-4-ynamidobenzoic acid (**1**) and 5-(5-azidopentanamido)-2-(3-hydroxy-6-oxo-6*H*-xanthen-9-yl)benzoic acid (**2**) (Scheme 2) [45].



Scheme 2. Synthesis of alkyne-functionalized fluorescein (AIFF) and azido-functionalized fluorescein (AzFF) by reaction of 5-aminofluorescein with 4-pentynoic acid or 5-azido-pentanoic acid.

The hydrogels were incubated with the fluorescent dye and CuI for three days at room temperature in DMSO and then extensively washed with PBS buffer, 1:1 methanol/H₂O solution, and water to remove the non-covalently attached fluorescent dye. As a control, gelatin hydrogels were incubated with the fluorescent dye without the copper catalyst, to exclude the possible physical absorption of the dye onto the hydrogel surface. The successful labeling of the free alkyne and azide functionalities was confirmed with confocal laser scanning microscopy (Figure 6).

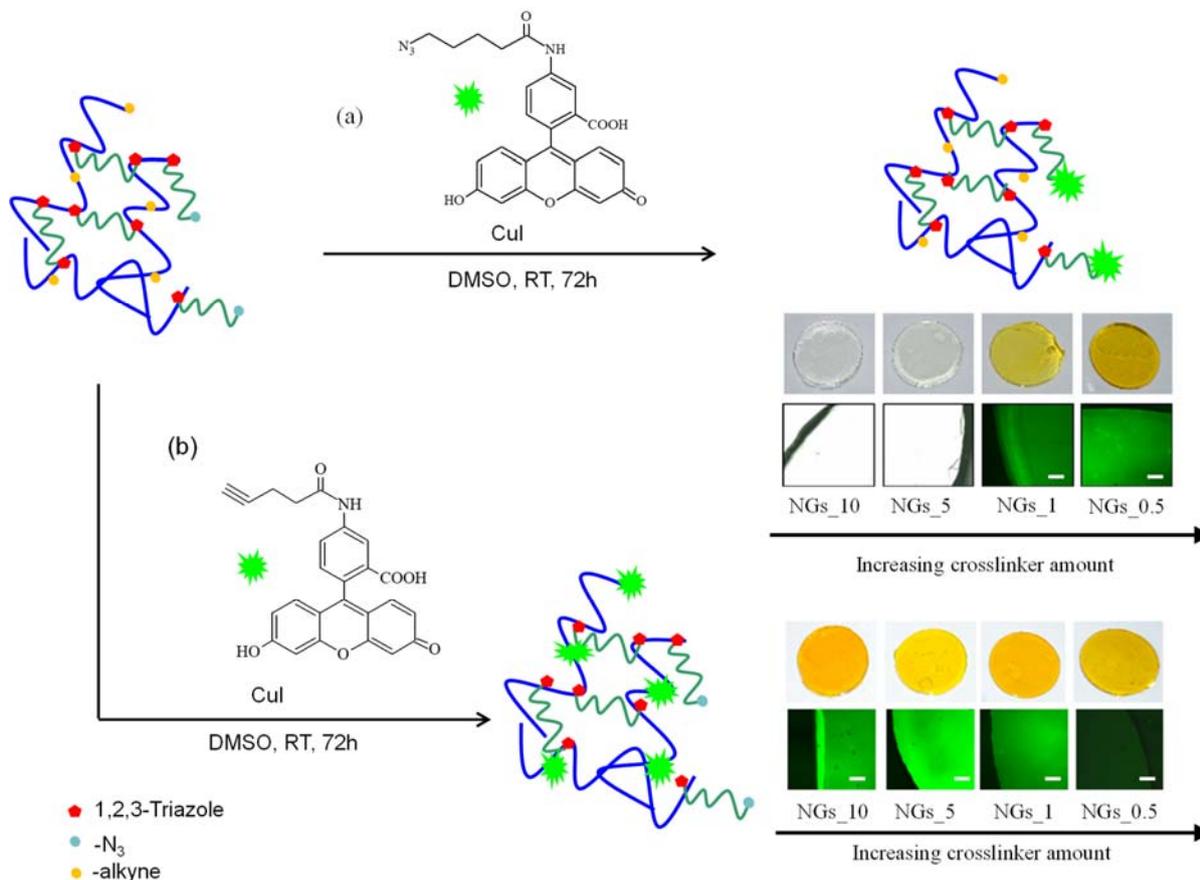
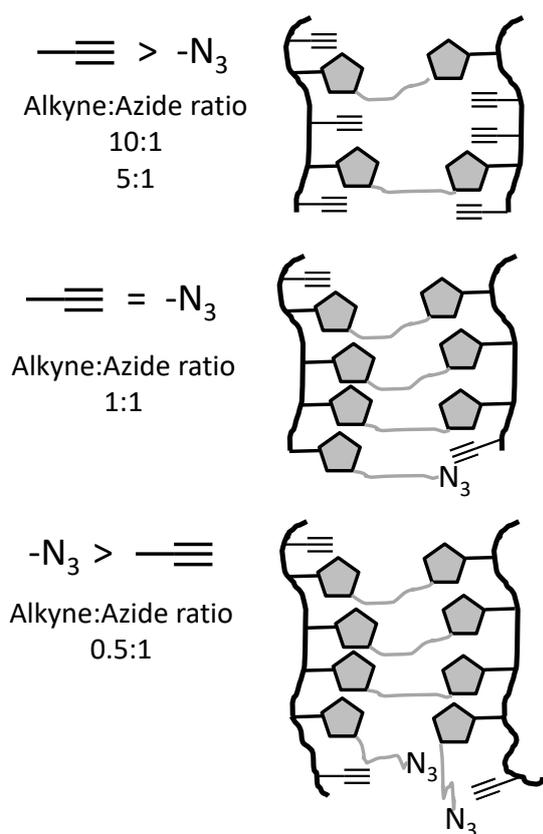


Figure 6. Labelling of NGs networks with (a) alkyne-functionalized fluorescein and (b) azide-functionalized fluorescein. Confocal laser scanning microscopy (CLSM) images: white scale-bars represent 500 μm .

When AIFF was employed, hydrogels NGs_5 and NGs_10 did not show any fluorescence, while moderate and strong labeling was observed for the NGs_1 and NGs_0.5, respectively (Figure 4). This can be explained by the fact that at low crosslinker content (NGs_5 and NGs_10) the bifunctional diazide reacted on both ends with alkyne groups from gelatin and no free azide groups were available for the reaction with AIFF. In contrast, when alkyne:azide ratios of 1:1 or 0.5:1 were employed, free alkyne groups were detected in the structure suggesting that the reaction was not quantitative and that the networks contained dangling chains with free azide groups. Similarly, the presence of free alkyne groups in hydrogels was determined by labeling with AzFF. Fluorescein labeling was observed for all alkyne:azide ratios and the intensity increased with the decreasing amount of crosslinker. Free alkyne groups were expected to be found in networks with

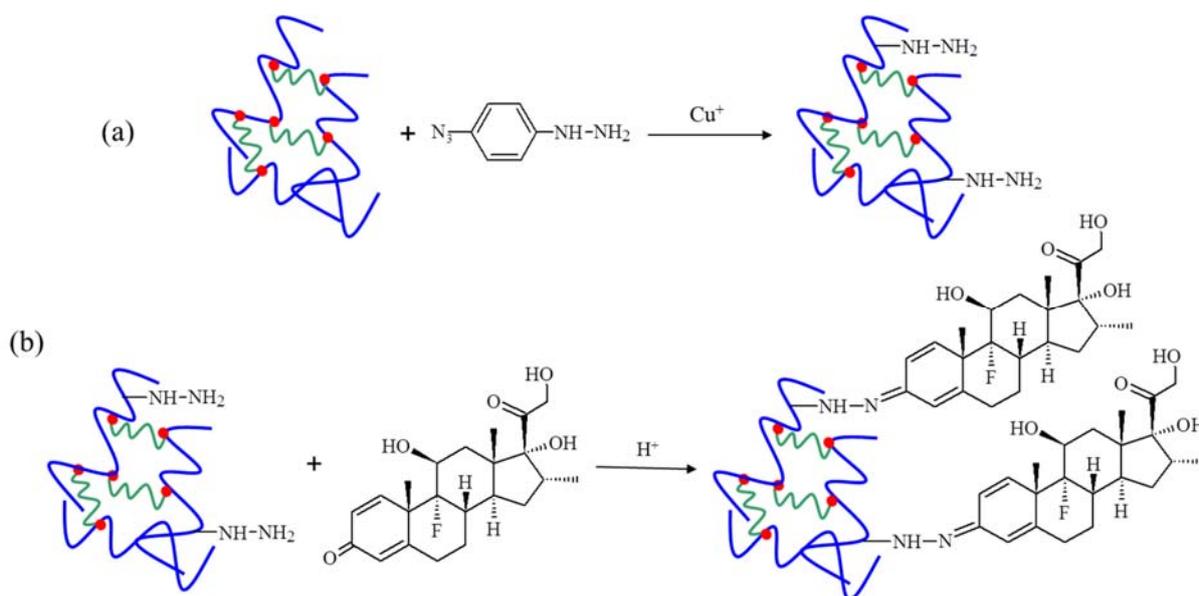
lower crosslinker content (NGs_5 and NGs_10). However, labeling of NGs_0.5 hydrogel with AzFF indicated that the alkyne-azide crosslinking reaction was not quantitative and that not all alkyne groups engaged in the reaction with bifunctional diazide. This may be due to steric hindrance of the reactive group on the backbone as well as inaccessibility due to the limited length of the crosslinker. The fluorescein attachment was quantified by fluorescence spectroscopy. For this purpose, hydrogels were degraded in an alkaline solution (pH 14) at 50 °C. Afterwards, the obtained solutions were adjusted to pH 10 and the fluorescence was measured. 5.0 μmol , 12.9 μmol and 40.2 μmol of AzFF were found per g of dry gel for NGs_0.5, NGs_1 and NGs_10, respectively. For NGs_10, this corresponds to about 19 mol% of the expected value when all theoretically available alkyne groups reacted. For NGs_0.5 and NGs_1 the results show that not all alkyne groups reacted in the network formation step. A potential explanation for a smaller amount of triple bonds available for crosslinking than expected from reaction in the cycloaddition would be the formerly reported alkyne homocoupling in the presence of copper [46, 47]. Test reactions of alkyne-functionalized gelatin in presence of Cu(I) without addition of diazide however did not lead to network formation, so that steric hindrance of the groups might be an explanation of the low yield of the reaction. If also about 19 mol% of the available groups reacted in the fluorescein functionalization of the other networks, this would correspond to about 27 μmol alkyne groups per g in a NGs_0.5 network, and about 69 μmol alkyne groups per g NGs_1 network. Quantification of free azide groups by reaction with AIFF resulted in lower amounts, i.e. 1 μmol per g in NGs_0.5 networks and 0.2 μmol per g in NGs_1 networks, which suggests a low number of dangling chains and efficient crosslinking, even when low amounts of crosslinker were used. The network structures at different alkyne:azide ratios indicating the reactive groups are depicted in Scheme 3. The hydrolytic stability of the networks at 37 °C is exceeding seven weeks [48], which is significantly longer than of PEG-gelatin gels synthesized by SPAAC [24].



Scheme 3. Schematic representation of the network structure elucidated from fluorescent labelling of gelatin-based hydrogels. In all cases, some unreacted alkyne groups are available for further functionalization after the crosslinking step, while azide groups were not detected when using an alkyne excess.

As an example for functionalization of the gelatin networks with a drug, attachment of dexamethasone (DEX) to NGs_1 was chosen. DEX is a clinically important anti-inflammatory glucocorticoid, which is applied to treat e.g. inflammatory skin diseases (such as psoriasis), autoimmune diseases (lupus erythematoses), or brain edema. The systemic application of DEX is, however, subject to unwanted effects such as osteoporosis, hyperglycaemia, hypertension, or peptic ulceration [49]. Furthermore, the attachment of DEX should be seen as example, which is transferable to other drugs with free ketone or aldehyde groups. The modification was achieved in a two-step reaction (Scheme 4). First, a hydrazine group was introduced to the gelatin network through CuI catalyzed alkyne-azide cycloaddition of 4-azidophenyl hydrazine. The amount of attached 4-azido-hydrazine was determined by relating the weight of the unreacted material and the extracted product of the reaction. As a reference, the reaction without catalyst was performed.

NGs_1 contained 2.7 μmol of 4-azidophenyl hydrazine per g of dry hydrogel, meaning that the cycloaddition of this molecule was about 5-fold less efficient than the addition of the AzFF under the same conditions. In the second step, the ketone from DEX was reacted with the hydrazine group of gelatin to form a hydrazone bond. Such attachment of dexamethasone to a polymer backbone has been shown to allow release of the drug under acidic conditions (pH 5-6) in a linear fashion [50, 51], which e.g. exist in inflamed tissues. Both reaction steps were performed in DMSO, as the reagents were soluble in DMSO and gelatin swells in it. To determine the amount of DEX attached to the hydrogel, the hydrogel was treated at 50 °C with acidic solution (pH 2) and the DEX was quantified by HPLC (Figure S3, Supporting Information). Drug loading was determined to be 1 mg per g of dry hydrogel, corresponding to a full conversion of the free hydrazines. In this way, a multifunctional hydrogel drug carrier is accessible [52]. A short release study was performed (Figure S5) to compare the release of DEX when covalently attached to the hydrogel with the release of physically adsorbed dexamethasone. The release of DEX when only physically adsorbed was fast, with 80% of the drug released after only 1 h, and about 94% after 24 h. The release of covalently attached DEX was studied at pH 5, as the hydrazone linkage is stable at pH 7.4 [50]. Here, over a period of 4 weeks, only about 25 wt.-% of the coupled DEX was released, which shows the much slower release for the covalent attached DEX. It should be pointed out that this experiment was intended as illustrative example for the release of a model compound. The drug attachment could also be performed before crosslinking the network, potentially allowing a higher loading, which shows the versatility of the sequential click chemistry approach.



Scheme 4. (a) Introducing hydrazine functionality to the gelatin-based network, (b) Attaching dexamethasone to the gelatin-based network through a hydrazone bond.

Conclusions

Properties of gelatin hydrogels were successfully tailored through alkyne-azide cycloaddition by variation of the diazide crosslinker length, molecular rigidity, and molar ratio. Covalent crosslinking suppressed the renaturation of triple helices except in the case of very low amount of crosslinker. Depending on the molar ratio of crosslinker to reactive groups on the gelatin, the hydrogel networks contained unreacted and accessible alkyne and azide groups, which might be employed for the covalent attachment of e.g. bioactive compounds. This sequential click chemistry approach therefore allows formation of multifunctional biopolymer networks with tailorable properties without the necessity of utilizing protecting groups during synthesis or applying different linking strategies [28].

Acknowledgment

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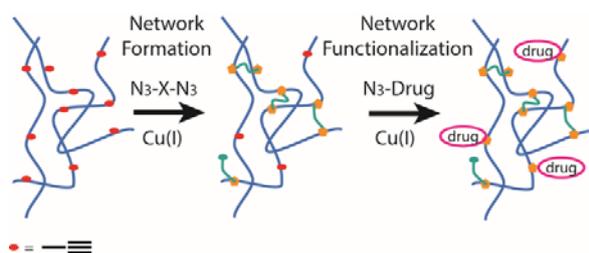
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Graphical abstract

Sequential Alkyne-Azide Cycloadditions for Functionalized Gelatin Hydrogel Formation

by Susanna Piluso, Radovan Vukićević, Ulrich Nöchel, Steffen Braune, Andreas Lendlein, and Axel T. Neffe



Research highlights

- ▶ Tailoring of gelatin hydrogel mechanical properties by click-chemistry is shown
- ▶ The storage modulus G' could be varied by more than 2 orders of magnitude
- ▶ A sequential biopolymer crosslinking and functionalization was enabled
- ▶ The methodology allows the synthesis of non-toxic drug releasing implant materials

-Supporting Information-

Sequential Alkyne-Azide Cycloadditions for Functionalized Gelatin Hydrogel Formation

Susanna Piluso, Radovan Vukićević, Ulrich Nöchel, Steffen Braune,

Andreas Lendlein, and Axel T. Neffe

1. Synthesis of 1,8-diazidooctane

1,8-dibromooctane (1 mmol) and sodium azide (2 mmol) were dissolved in DMSO and the reaction was run at room temperature overnight. Completion of the reaction was checked by ^1H NMR spectroscopy (Figure S1), in which the conversion of the bromo group to the azide was confirmed by a shift of the terminal methylene group from 3.4 to 3.3 ppm. The crude product in DMSO was used immediately after the reaction to avoid potentially dangerous concentration of the short chain aliphatic azide.

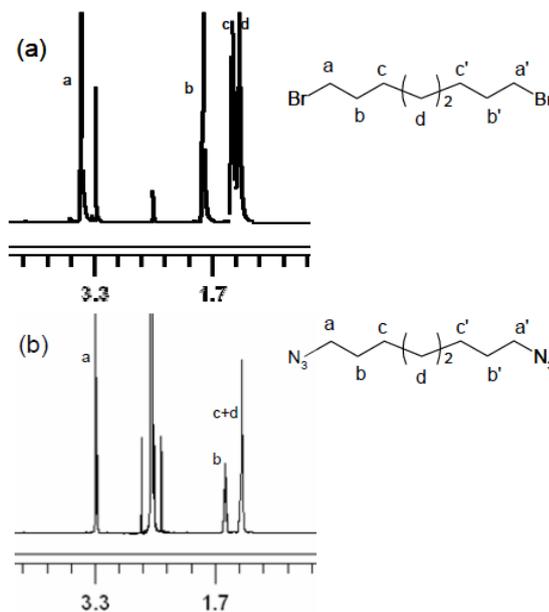


Figure S7. ^1H NMR spectra of (a) 1,8-dibromooctane and (b) 1,8-diazidooctane in DMSO- d_6 . The diazide formation is confirmed by the shift of peak **a** attributed to the methylene group, which is neighbouring the azide.

2. Experimental Methods

2.1 Wide-angle X-ray scattering (WAXS)

Wide-angle X-ray scattering measurements were carried out on a X-ray diffraction system D8 Discover with a 2D Hi-Star detector from Bruker AXS (Karlsruhe, Germany). The X-ray generator was operated at a voltage of 40 kV and a current of 40 mA, producing Cu K α -radiation with a wavelength $\lambda = 0.154$ nm. Integration of the two-dimensional scattering patterns gave the intensity I as a function of the scattering angle 2θ .

The triple helical indices were determined by integrating the corresponding peaks and relating their areas according to $X_{\text{TH}} = 100 * A_{\text{TH}} / A_{\text{Amorph}}$, where X_{TH} is the relative amount of triple helices, A_{TH} is the peak area related to the triple helices, and A_{Amorph} is the peak area related to the amorphous halo.

2.2 Temperature Modulated Differential Scanning Calorimetry (TM-DSC)

The thermal properties of gelatin films were measured using a Phoenix DSC 204 F1 (Netzsch, Selb, Germany). Dried gelatin samples were sealed in a hermetic pan to prevent any loss of moisture during TM-DSC measurements. Two consecutive heating runs from -20 to 150 °C in the first heating run and up to 250 °C in the second heating run were performed with a modulated heating rate with a period of 60 s, amplitude of 0.5 °C, and a heating rate of 5 °C·min⁻¹. Between the heating runs, the samples were quickly cooled at a rate of 10 °C·min⁻¹. The glass transition temperature (T_g) was determined during the second heating run.

2.3 Swelling tests

The swelling behavior of gelatin hydrogels was investigated in PBS buffer. Hydrogel disks (1.4 cm) were immersed in 5 mL of buffer at 37 °C, under mild shaking. After 24 h the swollen hydrogel disks were removed from the solutions and gel surfaces were quickly blotted on a filter paper. The equilibrium swelling ratio was determined as the ratio of the volume of the swollen hydrogel and the volume of the dry material. All experiments were conducted in triplicate.

2.4 Stress-strain tests

Macroscopic mechanical properties of gelatin hydrogels were quantified using a mechanical tester Z2.5 (Zwick GmbH, Ulm, Germany) equipped with a 200 N cell load in tension mode at room temperature. Equilibrium swollen samples were tested with 0.02 N pre-force and 5 mm·min⁻¹ crosshead speed, until break. Tensile moduli were subsequently calculated from the linear region of the stress-strain curve.

2.5 Rheological measurements

Rheological measurements were performed with a stress controlled rheometer MARS II (Thermo Fisher, Karlsruhe, Germany) in parallel plate geometry with a 20 mm upper plate, equipped with a solvent trap to minimize solvent evaporation. Preliminary stress-sweep experiments were carried out to select a stress value in the linear viscoelastic range. All experiments were done in oscillation mode at a frequency of 3 Hz by applying a constant stress of 3 Pa. The temperature was kept constant at 37 °C.

2.6 Imaging with confocal laser scanning microscope (CLSM)

Gelatin hydrogels labeled with alkyne- and azido-functionalized fluorescein were characterized by using a confocal laser scanning microscope (CLSM 510, Carl Zeiss, Germany). The hydrogels were placed between two glass coverslips (Gerhard Menzel GmbH, Braunschweig, Germany) and excited by a 488 nm wavelength argon laser. Images were acquired at 5 fold primary magnification and 70 μm thickness of the optical section. Emission signals were collected in the line mode with an averaging of n = 16 repeats per pixel. The emission (spectral) region was adjusted by using a 505 nm to 530 nm wavelength bandpass filter.

2.7 Fluorescence spectroscopy

Fluorescence spectroscopy data were acquired on a Varian Cary Fluorescence spectrophotometer (Darmstadt, Germany). The hydrogels were degraded in an alkaline solution (pH 14) at 50 °C. The solutions were adjusted to pH 10 and the fluorescence was measured. A calibration curve was determined from the measurements of known concentrations of fluorescein dye and used to quantitatively determine the amount of dye bound to the hydrogels.

2.8 Release experiment and dexamethasone quantification by HPLC

For the release experiments, networks with covalently bound DEX or with physically adsorbed DEX were used. Physical loading was achieved by incubation of the network in a solution of DEX in ethanol ($1 \text{ mg} \cdot \text{mL}^{-1}$) for 24 h and subsequent drying at $30 \text{ }^\circ\text{C}$. About 80 mg of sample were then incubated in $400 \text{ }\mu\text{L}$ of phosphate buffer at $\text{pH} = 5$ (covalently attached) or $\text{pH} = 7.4$ (physically loaded) at $37 \text{ }^\circ\text{C}$. At appropriate time points, $100 \text{ }\mu\text{L}$ each were taken as samples and substituted by fresh buffer. The experiment was performed in duplicate. The overall loading of the hydrogels with covalently attached DEX was determined by incubating the hydrogels at $\text{pH} = 2$ and $50 \text{ }^\circ\text{C}$ for 24 h, and then determining the amount of free DEX. For the physically loaded networks, the samples after the release experiments were incubated in ethanol for 24 h, and the free DEX was determined. The data are shown in Fig. S5.

The quantification of dexamethasone was performed using a HPLC-diode array detection system (Agilent, Waldbronn, Germany). The analytical column was a LiChrospher 100 RP-18 ($5 \text{ }\mu\text{m}$) LiChroCART 125-4 and the mobile phase used was acetonitrile:water (0.1% trifluoroacetic acid in water) - 35:65 v/v. The run time was 10 min, with a flow rate of $1 \text{ mL} \cdot \text{min}^{-1}$ and a column temperature of $30 \text{ }^\circ\text{C}$. The detection was performed with a UV-Vis detector at 240 nm. A calibration curve was determined from the measurements of known concentrations of dexamethasone.

3. Additional Figures

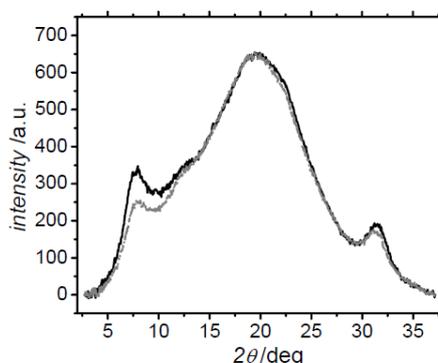


Figure S2. WAXS spectra of dried gelatin (—) and gelatin functionalized with propiolic acid on 88 mol% of the lysine residues (---).

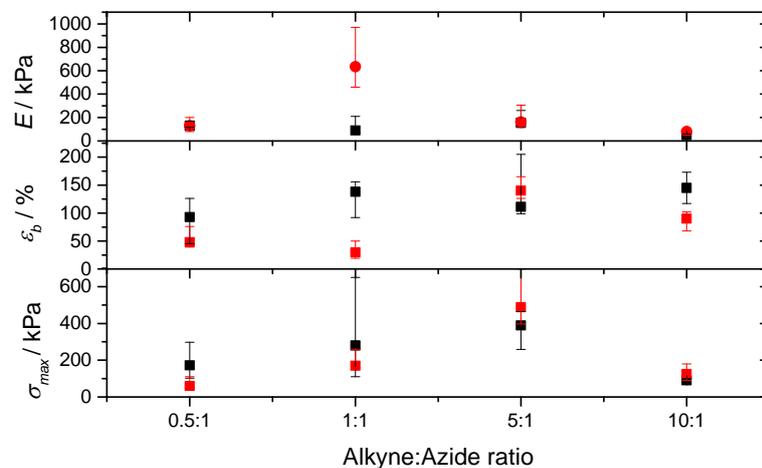


Figure S3. Change in mechanical properties of gelatin-based hydrogels at different alkyne:azide ratios. Tensile tests were performed at room temperature. From top to bottom: Young's modulus, E ; elongation at break, ϵ_b , and Tensile strength, σ_{max} of propionylated gelatin crosslinked with 1,12-diazidododecan ■ or PEG-diazide ●.

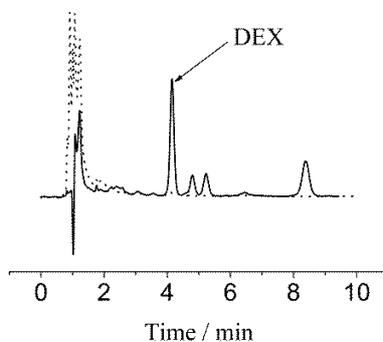


Figure S4. HPLC chromatogram of hydrolyzed hydrogels before (···) and after (—) functionalization with DEX.

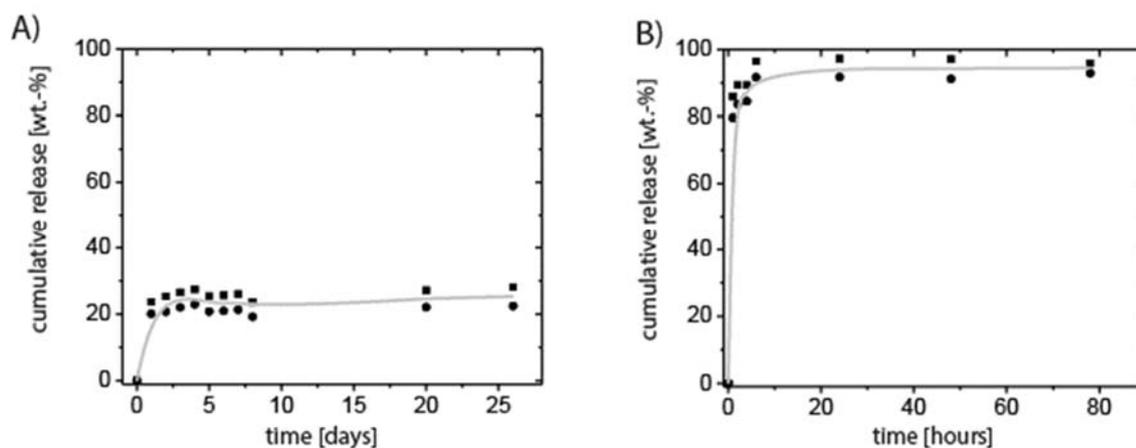


Figure S5. A) DEX release from NGs_1 with covalently attached DEX at pH 5. B) DEX release from physically loaded NGs_1 at pH 7.4.

4. Evaluation of Cytotoxicity

Culture of L929 cells

According to the international standard EN ISO 10993-5, cytotoxicity tests were performed with cells of a 100-day-old male C3H/An mouse (L929 cells; provided by the American Type Culture Collection, ATCC, Wesel, Germany). For cell expansion continuous subcultures of these cells were maintained under standard conditions (humidified atmosphere, 5 vol.% CO₂ in air, 37 °C; NuAire, Incubator, Germany) on polystyrene using minimal essential medium (MEM Biochrom, Berlin, Germany) supplemented with 10 wt.% horse serum (ATCC, Wesel, Germany) and 1 wt.% penicillin/streptomycin/glutamine (Invitrogen, Darmstadt, Germany). Every second day, the culture medium was changed. The cells were subcultured when they reached a subconfluence of about 80% by rinsing them with phosphate buffered saline, and by subsequent trypsinization using trypsin-EDTA solution (trypsin 0.25 wt.% and ethylene-diaminotetraacetic acid 0.53 mM).

Cytotoxicity tests

The hydrogel NGs_5 was tested for cytotoxic effects using *in vitro* cell tests under static conditions in accordance with the supplier's instructions and in conformity with the EN DIN ISO standard 10993-5. The test was performed with hydrogel extracts on L929 cells (indirect test). Sample preparation and the procedure for generating the extract of the materials were performed according to EN ISO 10993 part 12. Hydrogel samples were exposed to serum-free cell culture medium (MEM) under permanent stirring at 37 °C for three days. The resulting extract was then used as cell culture medium for L929 cells. L929 cells were seeded on a polystyrene based cell culture 96-

multiwell plate (Greiner Bio-One, Solingen, Germany). After reaching a subconfluent cell layer of about 50%, the culture medium was replaced by the extract. 48 hours later the viability of the cells, integrity of the cell membrane, mitochondrial activity, and cell morphology were analyzed.

Mitochondrial function studies

As many cell processes require the activity of mitochondria, a measurement of the activity of mitochondrial dehydrogenases helps to establish the level of cell activity. The activity of mitochondrial enzymes of the cells was measured by use of a tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), which when reduced, produces a colored formazan product that is soluble in tissue culture medium (CellTiter® 96 Aqueous Assay, Promega, Germany). The absorbance of the colored formazan product was measured at 492 nm using a SpectraFluor Plus (TECAN, Männedorf, Switzerland) spectrophotometer.

Lactate dehydrogenase (LDH)-release measurement

The activity of the cytoplasmatic enzyme lactate dehydrogenase (LDH) was measured by use of a colorimetric assay in the cell culture supernatant (Cytotoxicity Detection Kit (LDH), Roche, Germany) to evaluate the integrity of the cell plasma membrane at 492 nm using a TECAN SpectraFluor Plus spectrophotometer (TECAN, Männedorf, Switzerland). LDH is located solely within the confines of the cell. The appearance of LDH in the cell culture supernatant therefore indicates that the integrity of the cell membranes has been compromised.

Cell morphology assessment

The morphological phenotype of the cells was evaluated according to the USP 23-NF18 (US Pharmacopeial Convention) and ISO 10993-5 using a phase contrast microscope in transmission (Zeiss, Jena, Germany) based on the cell shape and the organization of the cell layer. The number of cells on the samples was calculated as mean cell number from five different fields of view.

Results

Indirect tests showed that there were no or only slight influences on the integrity of the cell membranes or on the mitochondrial activity of the L929 cells 48 h after being cultured in the hydrogel extracts. There were no differences in the LDH release, indicating that the integrity of the cell membranes remained unchanged. The MTS test showed that the mitochondrial activity was slightly but statistically significantly decreased by 10.9% (paired t-test, two sided: $p < 0.05$). As the absolute values in absorbance differed only by 0.12 (negative control = 1.16, gelatin hydrogel = 1.04, absorbance at 492 nm), this difference was judged to be of rather minor biological relevance, as in typical cytotoxic samples the change of mitochondrial activity can reach levels of more than 75%.

The morphology of L929 cells 48 h after culturing with an extract obtained by stirring the sample in cell culture medium at 37 °C for 72 h (72 h-extract) differed from the morphology of the cells 48 h after culturing with cell culture medium (control) (Fig. S6). After culturing the cells with the eluate, less than 50% of the cells were loosely attached and in some cells vacuoles and granular material were visible. Obviously, some of the cells detached as the cell density was slightly lower.

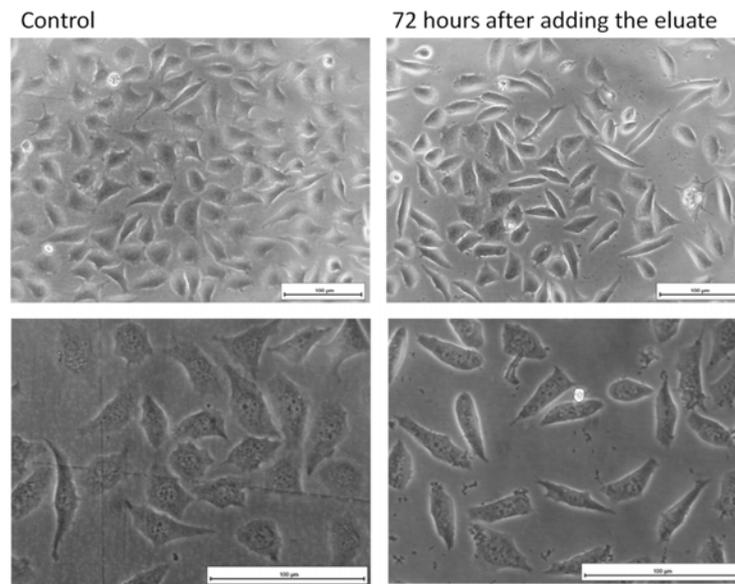


Figure S6. L929 cells 48 h after culturing with culture medium (control, left side) and 72 hours after adding the eluate of the samples (right side); phase contrast microscopy in transmission, primary magnification 1:20 (upper row) and 1:40 (lower row).

The endotoxin load in the sample extract was with $0.0211 \text{ EU} \cdot \text{ml}^{-1}$ less than the FDA limit of $0.06 \text{ EU} \cdot \text{ml}^{-1}$.