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Copper Mediated and Copper Free “Click” Decoration of Polysialic Acid for RGD-Modification and Hydrogel Formation

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Summary: This paper describes and compares the copper-catalyzed and copper-free “click” reaction of *N*-azido, *N*-alkynoyl and *N*-oxanorbornadienyl polysialic acid for the formation of hydrogels and cyclic RGD conjugates. To proof the molecular mechanism of the crosslinking reaction, the crosslinking and conjugation reactions were studied in detail on a molecular level by ^{1}H-NMR spectroscopy and mass spectrometry as well as MS/MS experiments identifying the non-hydrolyzable crosslinks.

Keywords: click chemistry; hydrogel; polysialic acid; RGD-peptide

Introduction

Polysialic acid (**1**) is the negatively charged homopolymer of α-2,8-linked sialic acid with 8-200 repeating units.[1] This biopolymer is commonly expressed in the embryonic and adult vertebrate nervous tissue as posttranslational modification of the neural cell adhesion molecule. Here, due to its highly hydrated polyanionic structure, polysialic acid modulates cell-cell interactions.[2] Polysialic acid is stable in mammals due to the lack of the corresponding endosialidases and might therefore serve as valuable scaffold material in tissue engineering since most degradable biopolymers suffer from uncontrolled hydrolysis after implantation.[3] Nevertheless the highly stable and reactive polysialic acid degrading endosialidase NF has been cloned from phage viruses and heterologously expressed allowing the *in vivo* degradation of polysialic acid after injection into the host.[4] This allows for creating a highly stable polysialic acid-based scaffold, which can be selectively hydrolyzed by endosialidase NF injection.

In our previous work, we described the synthesis of polysialic acid based hydrogels which can be completely degraded by endosialidase NF and disclosed that this material shows good biological properties such as perfect cytocompatibility and no cytotoxicity as judged by viability assays with cultured Schwann cells.[5] Since those hydrogels were prepared by base-catalyzed diepoxyoctane cross-linking of an aqueous polysialic acid solution, a large percentage of diepoxyoctane was unspecifically hydrolyzed and did not provide effective cross-linkages. The resulting hydrogel mostly contained the bivalent linker attached to one polymer chain while the other epoxy group was hydrolyzed by water. This encouraged us to design and synthesize polysialic acid derivatives that contain azido (**2**), alkynoyl (**3**) and oxanorbornadienyl (**4**) groups for performing selective Cu-catalyzed and Cu-free “click” cross-linking and grafting reactions (see Figure 1).[6] Especially the Cu-free click chemistry for grafting signal molecules such as cyclic RGD-peptides was already successfully applied to alginate.[7] In this paper, we describe the hydrogel formation and RGD decoration of polysialic acid based on Cu-catalyzed and Cu-free [2 + 3] cycloadditions

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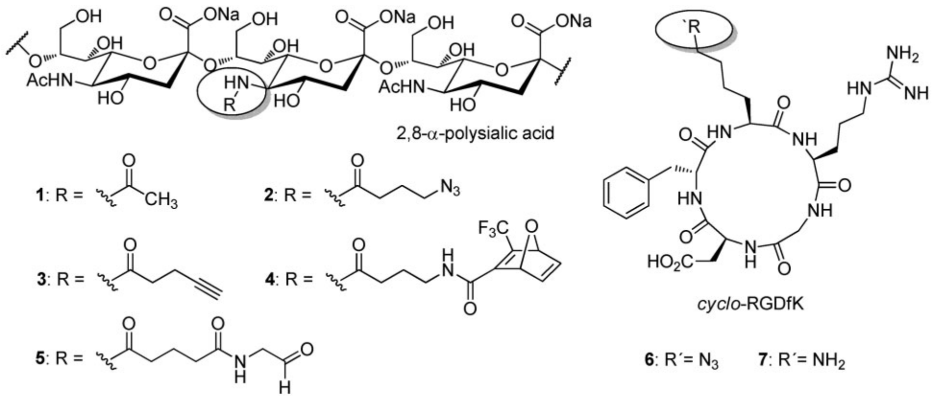


Figure 1.
α-2,8-linked polysialic acid 1 (sodium salt).

of polysialic acid derivatives and azido-modified cyclic RGD peptide **6**.[8] For comparison reasons, RGD containing polysialic acid derivatives were also prepared using a reductive amination strategy between *N*-aldehydo polysialic acid **5** and the more readily available cyclic RGD pentapeptide **7**. The materials obtained were studied by NMR spectroscopy and by electrospray mass spectrometry after acidic hydrolysis to allow a molecular description of the conjugation site. The results obtained should pave the way for further studies describing the mechanical properties of the hydrogels in correlation to the degree of crosslinking.

Materials and Methods

General Remarks

Unless otherwise stated, all chemicals and solvents were purchased in "per analysis quality" from *Sigma-Aldrich*, Steinheim, Germany or *Acros Organics*, Nidderau, Germany and used as received. All dialysis steps were performed using Visking membrane tubes (regenerated cellulose, 0.025 mm membrane thickness, 28.6 mm diameter, obtained from *Roth*, Karlsruhe, Germany) with a molecular weight cut-off of 14.000 g/mol. All modified polymers were exhaustively dialyzed for 3 to 5 days against distilled water. Lyophilization was performed with a *Christ* Alpha 2-4 (Christ, Osterode, Germany) freeze dryer. Mass spectra and HRMS data were recorded on a QTof premier (Waters) equipped with an Acuity UPLC (Waters) and a Waters UPLC BEH C18–2.1 × 100 mm 1.7 μm.

Combustion Analysis of Polysialic Acid-Based Polymers

All combustion analyses (CHN) were performed on a Elementar Vario EL (Version F; Analyse Systeme GmbH; Germany). Due to the hygroscopic properties of polysialic acid and polysialic acid based materials, the measured and theoretical carbon and nitrogen content is strongly fluctuating as shown for commercial α-2,8-polysialic acid (sodium salt; 98% purity; obtained from Nacalai Tesque, Inc., Japan; dried under vacuum prior to analysis):

$$C_{\text{measured}} = 42.18\% \quad C_{\text{theorical}} = 34.02\%$$
$$N_{\text{measured}} = 4.47\% \quad C_{\text{theorical}} = 3.63\%$$

To be able to draw conclusions from the measured data we choose to report the quotient C/N, which was not affected by the degree of water content in the polymer.

$$C/N_{\text{measured}} = 9.44 \quad C/N_{\text{theorical}} = 9.36$$

The theoretical C/N quotient was corrected with respect to the degree of derivatization measured by integration of ^{1}H-NMR spectra.

Polysialic Acid Hydrogel Formation Using Cu-Catalyzed "Click" Chemistry

N-(4-Azidobutyryl) polysialic acid **2**[6] (10 mg, ds = 60%) and *N*-(4-pentynoyl) polysialic acid **3**[6] (10 mg, ds = 60%) were dissolved in distilled water (160 µl). Aqueous $CuSO_4$ (16 µL, 0.1 M) and sodium ascorbate (24 µL in water, 0.1 M) were added. After vigorous shaking, the reaction mixture was kept at room temperature for 2-3 days. A stable hydrogel was formed.

In order to analyze the "click" reaction, the resulting polysialic acid hydrogel (20 mg) was hydrolyzed by acid treatment (1.0 mL, 0.1 M trifluoroacetic acid in water). The heterogeneous system was heated at 80 °C for 1 h and a sample was analyzed directly by UPLC-MS. Besides *N*-(4-azidobutyryl) sialic acid (ESI, $[M-H]^-$: found 377.1320 Da; calcd. for $C_{13}H_{21}N_4O_9$ 377.1309 Da), *N*-(4-pentynoyl) sialic acid (ESI, $[M-H]^-$: found 346.1151 Da; calcd. for $C_{14}H_{20}NO_9$ 346.1138 Da) and *N*-acetyl sialic acid (ESI, $[M-H]^-$: found 308.0997 Da; calcd. for $C_{11}H_{18}NO_9$ 308.0982 Da), the crosslinking triazole **8** (ESI, $[M-H]^-$: found 724.2537 Da; calcd. for $C_{27}H_{42}N_5O_{18}$ 724.2525 Da) could be detected.

Polysialic Acid Hydrogel Formation Using Metal-Free "Click" Chemistry

N-(4-Azidobutyryl) polysialic acid **2**[6] (10 mg, ds = 25-60%) and *N*-oxanorbornadiene polysialic acid **4**[6] (10 mg, ds = 20-25%) were dissolved in distilled water (200 µl). After short vigorous shaking the reaction mixture was incubated at room temperature for 2–3 days to give a stable hydrogel.

To confirm the "click" coupling reaction on a molecular level, the hydrogel (20 mg) was hydrolyzed by acid treatment (1.0 mL, 0.1 M trifluoroacetic acid in water). The heterogeneous system was heated at 80 °C for 1 h and a sample was analyzed directly by UPLC-MS. Besides *N*-(4-azidobutyryl) sialic acid (ESI, $[M-H]^-$: found 377.1302 Da; calcd. for $C_{13}H_{21}N_4O_9$ 377.1309 Da) and *N*-acetyl sialic acid (ESI, $[M-H]^-$: found 308.0988 Da; calcd. for $C_{11}H_{18}NO_9$ 308.0982 Da), the crosslinking trifluoromethyl triazole **9** (ESI, $[M-H]^-$: found 849.2638 Da; calcd. for $C_{30}H_{44}N_6O_{19}F_3$ 849.2613 Da) could be detected.

Preparation of cRGD-Polysialic Acid Via Cu-Catalyzed "Click" Reaction

N-(4-Pentynoyl) poly sialic acid **3**[6] (5 mg, 0.016 mmol) and azido-cRGD **6**[7] (2 mg, 0.003 mmol) were dissolved in distilled water (1 mL). Aqueous $CuSO_4$ solution (40 µL, 0.1 M) and Na-ascorbate solution (60 µL, 0.1 M) were added to the reaction mixture. The reaction was heated under microwave irradiation conditions up to 40 °C for 30 min. Subsequently, trifluoroacetic acid (9.7 µL, 0.1 mmol) was added and the mixture was heated to 80 °C for 1 h. The hydrolyzed products were directly analyzed by UPLC-MS and LC-MS/MS.

Preparation of cRGD-Polysialic Acid Via Metal-Free "Click" Chemistry

N-Oxanorbornadiene polysialic acid **4**[6] (5 mg, 0.015 mmol) and azido-cRGD **6**[7] (2 mg, 0.003 mmol) were dissolved in distilled water (1 mL). The reaction was heated under microwave irradiation conditions up to 40 °C for 60 min. Subsequently, trifluoroacetic acid (9.7 µL, 0.1 mmol) was added and the reaction mixture was heated up to 80 °C for 1 h. The hydrolyzed products were directly analyzed by UPLC-MS and LC-MS/MS.

Preparation of *N*-Aldehydo Polysialic Acid 5

*Preparation of 5-(2,3-Dihydroxypropylamino)-5-Oxopentanoic Acid (**12**)*

A solution of glutaric anhydride (1.60 g, 14 mmol) and 3-amino-1,2-propandiol (1.41 g, 15 mmol) in anhydrous pyridine (10 mL) was stirred at room temperature for 12 h. The pyridine was removed under reduced pressure to give a colorless solution. The solution was diluted in water (10 mL) and extracted with dichloromethane (5 × 10 mL). The aqueous layer was finally concentrated to yield **12** as colorless oil (2.34 g, 11.4 mmol, 82%).

^{1}H-NMR (400 MHz, D_2O) δ: 3.75-3.70 (m, 1H, H-2), 3.52 (dd, J = 12.0, 4.1 Hz, 1H, H-1$_a$), 3.43 (dd, $J = 11.2$, 6.5 Hz, 1H, H-3$_a$), 3.26 (dd, $J = 14.0$, 4.8 Hz, 1H, H-1$_b$), 3.43 (dd, $J = 11.2$, 7.2 Hz, 1H, H-3$_b$), 2.32 (t, $J = 3.4$ Hz, 2H, H-4), 2.24 (t, $J = 7{,}5$ Hz, 2H, H-5), 1.80 (tt, $J = 7.5$ Hz, 3.4 Hz, 2H, H-6) ppm

^{13}C-NMR (100 MHz, D_2O) δ: 178.5 (s, $\underline{C}$OOH), 176.1 (s, NH$\underline{C}$O), 70.1 (t, C-2), 63.2 (t, C-1), 41.6 (t, C-3), 34.7 (t, C-4), 33.1 (t, C-6), 20.1 (t, C-5) ppm.

ESI: calcd for $C_8H_{14}O_5$ [M-H]$^-$: 204.0872; found: 204.0865.

Preparation of N-(1,2-Diol) Polysialic Acid 14

The tetra-*N*-butylammonium salt of γ-amino polysialic acid **13**[6] (1.10 g, 2.2 mmol) was dissolved in anhydrous dimethylformamide (20 mL) and cooled to 0 °C. *N*-Hydroxybenzotriazole (HOBt) (0.76 mg, 5.6 mmol), *N,N*-diisopropylethylamine (DIPEA) (1.1 mL, 6.3 mmol) and EDC•HCl (1.07 g, 5.6 mmol) were added to the reaction mixture followed by carboxylic acid **12** (861 mg, 4.2 mmol) and stirred at room temperature over night. To acetylate the remaining free amino groups the reaction mixture was cooled to 0 °C and acetic anhydride (0.5 mL) was added. After stirring at room temperature for 8 h, the mixture was dried under reduced pressure and treated with sodium hydroxide (4 mL, 1M). After 8 h the mixture was neutralized with hydrochloric acid (1M) and dialyzed (2 d against sodium chloride (0.5 M); 2 d against distilled water). The aqueous layer was lyophilized to yield the desired product as colorless solid (714.0 mg, 1.9 mmol, 87%).

^{1}H-NMR (400 MHz, D_2O) δ: 4.21-4.05 (2H, H-4 & H-6), 3.90-3.71 (2H, H-5 & H-9$_a$), 3.70-3.42 (3H, H-8, H-7 & H-9$_b$) 3.48-3.58 (0.8H, H-1'$_a$ & H-3'$_a$), 3.34 (0.4H, H-1'$_b$), 3.22 (0.4H, H-3'$_b$), 2.66 (1H, H-3$_{eq}$), 2.33 (1.6H, H-4' & H-6'), 2.07 (1.8H, NHCOC$\underline{H}_3$), 1.90 (0.8H, H-5'), 1.72 (1H, H-3$_{ax}$) ppm.

^{13}C-NMR (100 MHz, D_2O) δ: [176.6, 176.4, 174.9, 173.2] (s, 4x$\underline{C}$O), 100.9 (C-2), 77.8 (d, C-8), 73.1 (d, C-6), 70.2 (d, C-2'), 69.0 (d, C-7), 68.3 (d, C-4), 63.3 (t, C-1'), 61.2 (t, C-9), 52.4 (d, C-5), 41.7 (t, C-3'), 39.8 (t, C-3), 35.1 (t, C-4'), 34.9 (t, C-6'), 22.5 (q, NHCOC$\underline{H}_3$), 21.5 (t, C-5') ppm.

Degree of derivatization: 40% (according to integrations of C$\underline{H}$ at 3.34 ppm and NHCOC$\underline{H}_3$).

Preparation of N-Aldehydo Polysialic Acid 5

Diol **14** (713 mg, 1.9 mmol) was dissolved in distilled water (10 mL) and cooled to 0 °C. An aqueous solution of $NaIO_4$ (5 g in 70 mL water) was added to the reaction mixture in the dark ([$NaIO_4$]: 0.3 M) and stirring was continued for 15 min at 5 °C. To quench the reaction, ethylene glycol (25 mL) was added and the resulting mixture was dialyzed against distilled water at room temperature for 4 days. After lyophilization, a colorless, amorphous solid was obtained (470 mg, 1.3 mmol, 68%).

^{1}H-NMR (400 MHz, D_2O) δ: 5.10 (0.4H, C$\underline{H}$(OD)$_2$)*, 4.25-4.09 (2H, H-4 & H-6), 3.95-3.70 (2H, H-5 & H-9$_a$), 3.69-3.51 (3H, H-8, H-7, H-9$_b$), 3.29 (0.8H, C$\underline{H}_2$CHO), 2.66 (1H, H-3$_{eq}$), 2.31 (1.6H, H-1' & H-3'), 2.07 (1.8H, NHCOC$\underline{H}_3$), 1.90 (0.8H, H-2'), 1.73 (1H, H-3$_{ax}$) ppm.

^{13}C-NMR (100 MHz, D_2O) δ: [176.6, 176.4, 174.9, 173.0] (s, 4x$\underline{C}$O), 100.9 (C-2), 88.2 (t, $\underline{C}$H$_2$CHO), 77.7 (d, C-8), 73.1 (d, C-6), 69.0 (d, C-7), 68.2 (d, C-4), 61.2 (t, C-9), 52.4 (d, C-5), 39.8 (t, C-3), 35.0 (t, C-1'), 34.8 (t, C-3'), 22.4 (q, NHCOC$\underline{H}_3$), 21.5 (t, C-2') ppm.

*Hydrated aldehyde in D_2O.

Combustion analysis: C/N = 8.91 (theoretical: C/N = 8.43).

Degree of derivatization: = 40% (according to integrations of C$\underline{H}$ at 5.10 ppm).

Reductive Amination Based cRGD Conjugation of Polysialic Acid

N-Aldehydo polysialic acid **5** (26 mg, 0.07 mmol) was dissolved in distilled water (5 mL) and cooled to 0 °C. Cyclic amino-RGD **7** (5 mg, 0.008 mmol) and one drop of MeOH was added. The mixture was stirred at room temperature for 24 and dialyzed against distilled water (adjusted to pH = 9) for 2 days. The aqueous lays were concentrated and lyophilized to obtain the corresponding Schiff base as a colorless solid (24 mg).

For the reductive amination, the resulting polysialic acid imine (2 mg) was dissolved in distilled water (1 mL). A small amount of $NaCNBH_3$ was added to the solution. The reaction mixture was stirred at room temperature over night. Subsequently, trifluoroacetic acid (9.7 μL, 0.1 mmol) was added. The reaction was heated to 80 °C for 1 h and the hydrolysis products were directly analyzed by UPLC-MS.

Results and Discussion

Polysialic Acid Hydrogel Formation Using Cu-Catalyzed "Click" Chemistry

The copper catalyzed crosslinking of the azide and alkyne containing macromers **2** and **3** resulted in the slow formation of a flexible hydrogel (Figure 2). Upon prolonged incubation in excess phosphate buffer (50 mM, pH 7.2, 37 °C, 24 h) the hydrogel was swelling resulting in a 50% increase of the gel diameter. Still, the gel was intensively colored due to traces of copper and several attempts to remove the copper by treatment with complexation agents or by electrophoresis failed. The gelation time highly depended on the amount of the Cu-catalyst employed, the degree of derivatization of polysialic acid, and the molecular weight of the polysialic acid (Table 1). When polysialic acid with a molecular weight of 45 kDa instead of 15 kDa was used, stable hydrogels could be obtained when a very low degree of derivatization and a low catalyst loading was employed (*entry 8*). After complete acidic hydrolysis of the polysaccharide backbone, polysialic acid dimer **8** was identified by high resolution mass spectrometry showing the inter-chain crosslinkage.

Polysialic Acid Hydrogel Formation Using Metal-Free "Click" Chemistry

Since the removal of residual copper salts turned out to be problematic, copper-free "click" chemistry was employed to crosslink *N*-azido polysialic acid **2** and oxanorbornadiene polysialic acid **4** (Figure 3). The methodology applied here is widely used, but only a few examples use this concept on polymeric systems.[9] The functionalized polysialic acid derivatives were dissolved in water and mixed without any catalyst. Hydrogel formation depended on the concentration of the starting materials, the degree of derivatization, and the reaction time (Table 2). High concentration of starting material resulted in the formation of hydrogels within a short time, which turned out to be quite brittle (entry 1). In contrast, very low concentration of starting materials led to longer gelation times. When the degree of substitution was too low, hydrogel formation was impossible (entry 5).

The optimal conditions for (entry 3) resulted in a stable and flexible hydrogels with identical swelling behavior compared to the Cu-catalyzed approach. Again it was

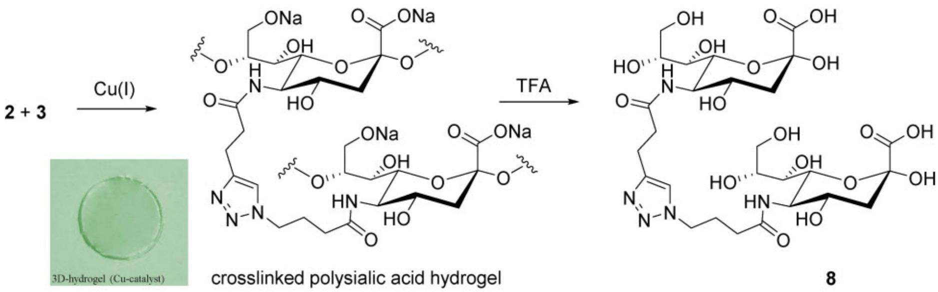


Figure 2.
Polysialic acid hydrogel formation by Cu-catalyzed "click" chemistry (sample size: Ø = 0.9 cm, thickness, 0.1 cm).

Table 1.
Hydrogel formation using polysialic acid derivative **2** and **3**.

Entry	Azide **2**		Alkyne **3**		Catalyst [mol %]		Gelation time
	DS	weight	DS	weight	A	B	
1	60%	10 mg	60%	10 mg	15	10	10 s
2	60%	10 mg	60%	10 mg	12	8	30 min
3	60%	10 mg	60%	10 mg	8	6	24 h
4	60%	10 mg	60%	10 mg	3	2	48 h
5	25%	25 mg	80%	10 mg	3	2	5 d
6	25%	10 mg	25%	10 mg	3	2	7 d
7	80%	10 mg	80%	10 mg	1.5	1	no gel
8	10%	10 mg	10%	10 mg	1.5	1	7 d

If not stated otherwise, polysialic acid derivatives with a M_n of 15 kDa were used; [a] Cu-catalyst (mol%) **A**: Na-ascorbate (0.1 M), **B**: $CuSO_4$(0.1 M); [b] polysialic acid with a M_n of 45 kDa was used

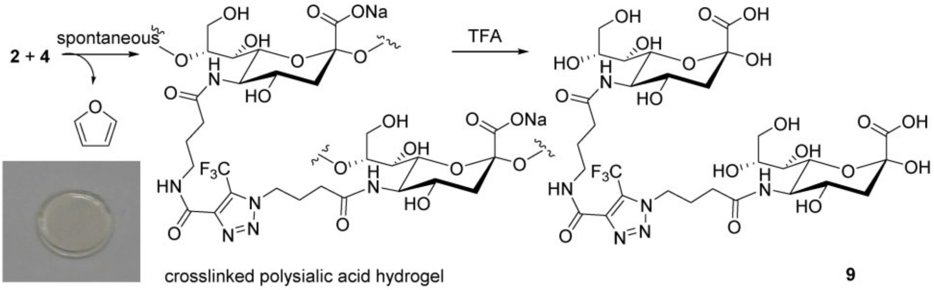


Figure 3.
Polysialic acid hydrogel formation by metal-free "click" chemistry (only one regioisomer is shown).

possible to isolate and describe the inter-chain crosslinkage **9** after acidic hydrolysis of the polysialic acid backbone.

Preparation of RGD-Polysialic Acid Via Cu-Catalyzed "Click" Reaction

The analysis of the cRGD modified polysialic prepared by copper catalyzed [2 + 3] cycloaddition of alkyne **3** and azide **6** was performed by mass spectrometry. After acidic cleavage, the cRGD-coupled sialic acid **10** could be detected and analyzed by LC-MS (Figure 4) and MS/MS spectrometry (Figure 7).

Using high resolution data, various MSMS-fragments could be identified allowing the unambiguous assignment of the structure (see Scheme 2).

Preparation of RGD-Polysialic Acid Via Metal-Free "Click" Reaction

In the second route towards cRGD-modified polysialic acid, metal-free "click" conditions were applied for the coupling of

Table 2.
Hydrogel formation using polysialic acid derivatives **2** and **3**.

Entry	Azide **2** DS	Oxanorbonadiene **4** DS	Volume [μL]	Gelation time
1	25%	25%	90	5 h
2	25%	25%	120	11 h
3	25%	25%	200	3 d
4	60%	25%	200	36 h
5	60%	10%	100	no gel

In all cases, 10 mg of each macromer was used

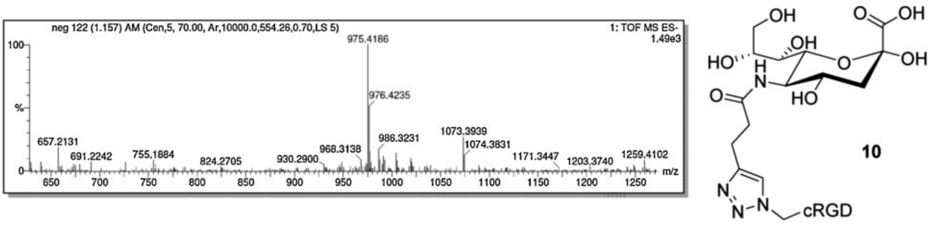


Figure 4.
UPLC-MS spectrum of the cRGD modified sialic acid **10** (calcd for $C_{41}H_{59}N_{12}O_{16}$ [M-H]$^-$: 975.4172; found: 975.4186).

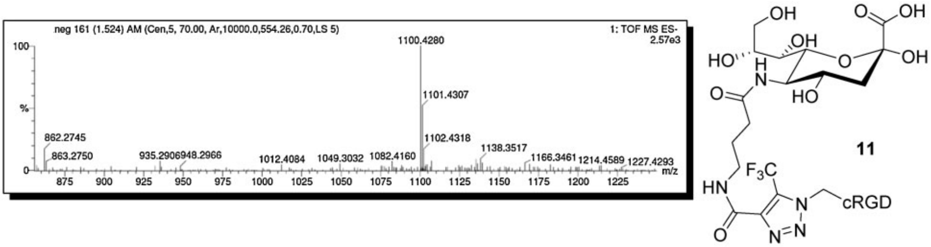
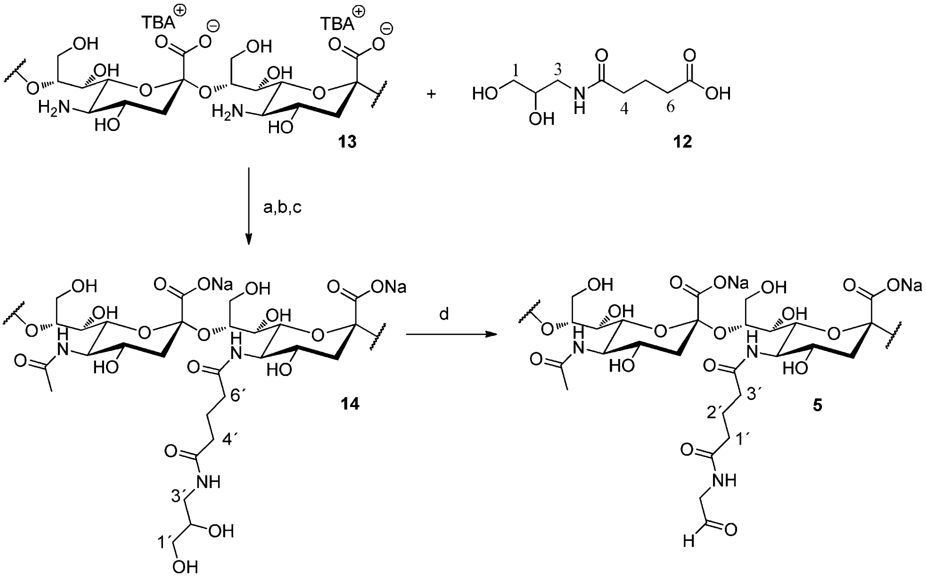


Figure 5.
UPLC-MS spectrum of cRGD-modified sialic acid **11** (calcd for $C_{44}H_{61}N_{13}O_{17}F_3$ [M-H]$^-$: 1100.4260; found 1100.4280; only one regioisomer is shown).

oxanorbornadiene functionalized polysialic acid **4** with azido-modified cyclic RGD peptide **6**. The reaction was carried out in water at room temperature. In contrast to the Cu-catalyzed “click” reaction of *N*-alkyne polysialic acid **3**, the progress of metal-free reaction could be monitored by ^{19}F-NMR analysis.[7] Figure 8 depicts the ^{19}F-NMR



Scheme 1.
Preparation of *N*-aldehydo polysialic acid **5**; a) HOBt, EDC*HCl, DIPEA, DMF, 20 °C, 24 h; b) Ac_2O, 20 °C, 8 h; c) NaOH, 20 °C, 3 h; d) $NaIO_4$, H_2O, 5 °C, 15 min.

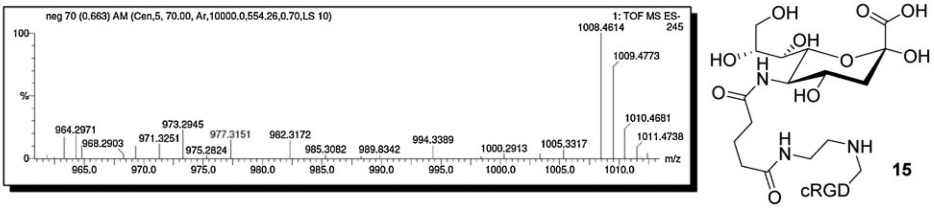


Figure 6.
UPLC-MS spectrum of the amino cRGD modified sialic acid **15**. (calcd for $C_{43}H_{66}N_{11}O_{17}$ $[M\text{-}H]^-$: 1008.4638; found 1008.4614).

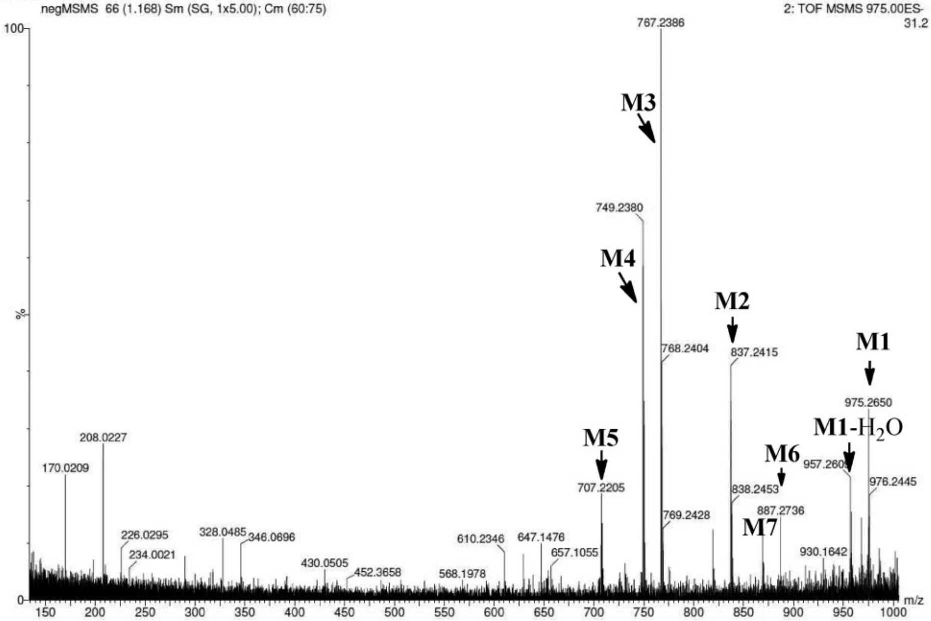


Figure 7.
LC-MS/MS spectra of "click" product **10**.

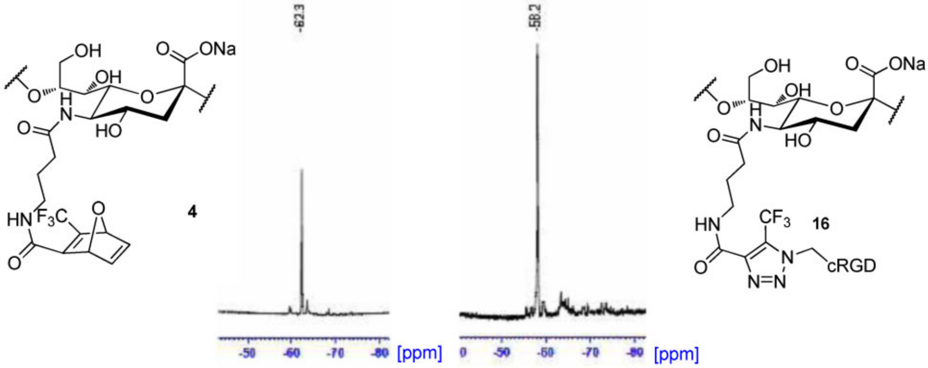


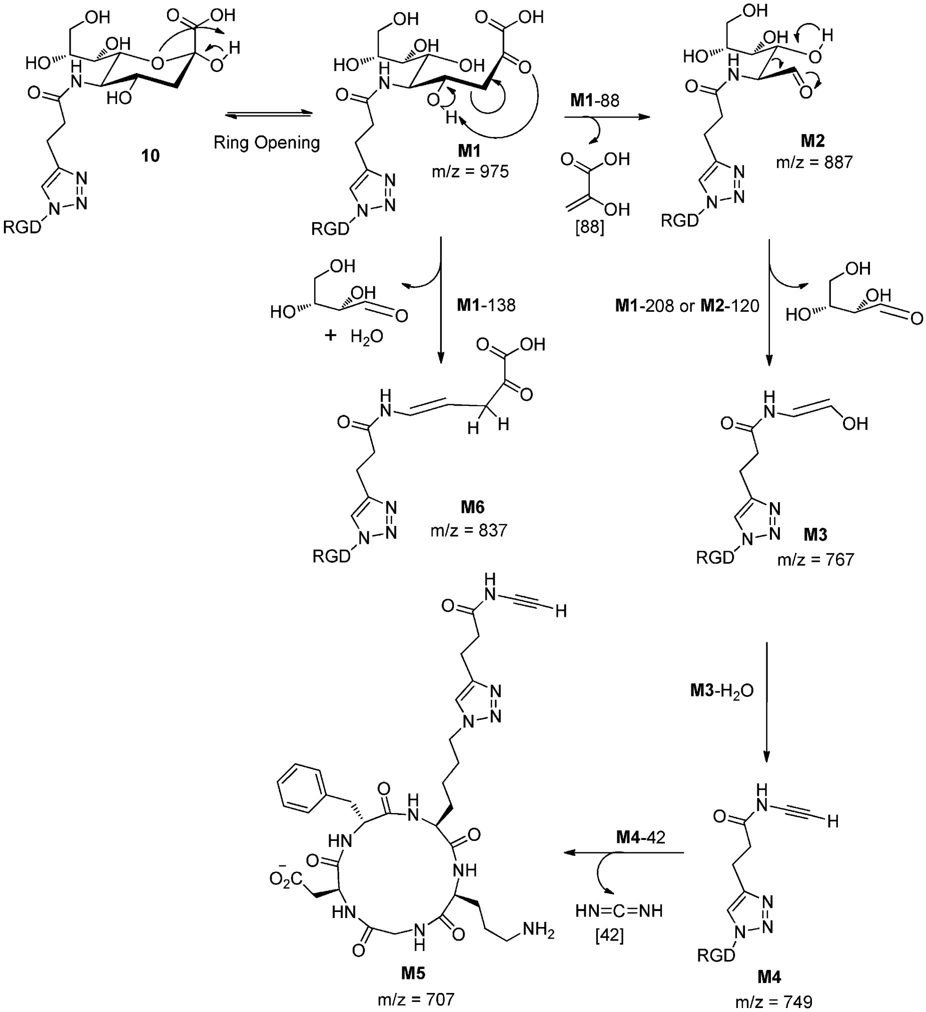
Figure 8.
^{19}F-NMR spectra of oxanorbornadiene functionalized polysialic acid **4** with cRGD conjugate **16**.

spectra of polysialic acid derivative **4** and the corresponding cRGD conjugate. The CF_3 group of the oxanorbornadiene system in compound **4** was detected at -62.3 ppm (*left*), whereas in conjugate **16** this signal is shifted to -58.2 ppm (*right*).

The final proof for the successful conjugation was again obtained by UPLC-MS (Figure 5) and MS/MS analyses of the hydrolyzed conjugate **16**. The MSMS spectrum of the hydrolyzed cRGD-modified sialic acid **11** displayed the same characteristic mass losses as seen for conjugate **10** (data not shown).

Reductive Amination Based cRGD Conjugation of Polysialic Acid

Since several coupling strategies of biopolymer formation involve the reaction of aldehydes with amino functionalized reaction partners, *N*-aldehydo polysialic acid was synthesized. The reaction was performed in analogy to our general route to polysialic acid derivatives.[6] At first, dimethylformamide soluble tetra-*N*-butylammonium salt of deacetylated polysialic acid **13** is *N*-acylated using peptide coupling conditions to introduce a diol functionality (Scheme 1). *N*-Acetylation followed by



Scheme 2.
Proposed fragmentation of "click" product **10** according to LC-MS/MS.

ester cleavage under basic conditions reconstituted the polysialic acid backbone in diol **14**, which was finally transformed to aldehyde **5** by rapid sodium periodate treatment. With this compound in hand, we initiated the conjugation with the lysine side chain present in cRGD-peptide **7**. After extensive dialysis ^{1}H-NMR-signals of the cRGD-peptide moiety were visible but did not provide final confirmation of polymer/peptide conjugation. Therefore, LC-MS as well as MSMS analyses were utilized. and it turned out to be crucial to reduce the acid labile Schiff base before starting acid hydrolysis. After sodium borohydride treatment and acidic hydrolysis, the expected cRGD-conjugated polysialic acid was detected and further analyzed by MSMS (Figure 6). Again the MSMS spectrum of the hydrolyzed cRGD-modified sialic acid **15** displayed the same characteristic mass losses as seen for conjugate **10** (data not shown).

Conclusion

In conclusion, we synthesized two hydrogels based on polysialic acid by employing a) copper-catalyzed and b) copper-free "click" reactions of *N*-azido, *N*-alkynoyl and *N*-oxanorbornadienyl modified polysialic acids. Since it was not possible to remove copper-ions from the hydrogel, the copper-free approach turned to be superior with respect to gelation times as well as hydrogel purity. We disclosed three different routes towards cyclic-RGD conjugated polysialic acid. In each case, the RGD-sialic acid adduct could be isolated and analyzed in detail by LC-MS and MS/MS experiments.

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