Hydrogels

A Synthetic Toolbox for the In Situ Formation of Functionalized Homo- and Heteropolysaccharide-Based Hydrogel Libraries


Dedicated to Professor Gerhard Bringmann on the occasion of his 65th birthday

Abstract: A synthetic toolbox for the introduction of aldehyde and hydrazido groups into the polysaccharides hyaluronic acid, alginate, dextran, pullulan, glycogen, and carboxymethyl cellulose and their use for hydrogel formation is reported. Upon mixing differently functionalized polysaccharides derived from the same natural precursor, hydrazone cross-linking takes place, which results in formation of a hydrogel composed of one type of polysaccharide backbone. Likewise, hydrogels based on two different polysaccharide strands can be formed after mixing the corresponding aldehyde- and hydrazido-modified polysaccharides. A second line of these studies paves the way to introduce a biomedically relevant ligand, namely, the adhesion factor cyclic RGD pentapeptide, by using an orthogonal click reaction. This set of modified polysaccharides served to create a library of hydrogels that differ in the combination of polysaccharide strands and the degree of cross-linking. The different hydrogels were evaluated with respect to their rheological properties, their ability to absorb water, and their cytotoxicity towards human fibroblast cell cultures. None of the hydrogels studied were cytotoxic, and, hence, they are in principal biocompatible for applications in tissue engineering.

Introduction

Gel-like biomaterials play a key role as supports in regenerative medicine.[1–4] Hydrogels based on biomacromolecules have emerged as important scaffold materials, particularly in the field of tissue engineering.[5–11] For clinical applications, biocompatibility is crucial to avoid immunogenic responses. Besides polyactic acid, several polysaccharides such as alginate (1) and hyaluronic acid (2) are important biomacromolecules that have been widely investigated as scaffold materials with advantageous properties concerning immunogenicity as well as options for further functionalization.[12–15] Several polysaccharides are widely used as blends to adjust their physical, mechanical and biological properties.[16–18]

The biocompatibility of these scaffold materials can be improved by attaching additional (bio)functional groups to the biomaterial.[19] By establishing a chemically defined modification protocol, one is able to extend the biological or physiochemical properties of such biomaterials. Particularly interesting derivatizations are fluorescent labels, adhesion factors and drugs, for example, with antibacterial or inhibitory properties against biofilms.[20] As a result, bioconjugates are created that synergistically combine the properties of their individual components and furthermore allow biological and/or chemical limitations and disadvantages of the individual starting materials to be overcome. A well-established peptide-based ligand (L) that induces cell adhesion is the tripeptidic sequence RGD ([R = arginine, G = glycine, D = aspartic acid]. It strongly interacts with integrins on the cell surface when forced into the correct conformation, especially when it is part of a cyclic pentapeptide architecture (commonly cyclic RGDFK or cRGDFK).[21, 22] Recently, complex RGD assemblies have been constructed by orthogonal chemoselective ligation.[23, 24]

Herein, we disclose a synthetic toolbox for the flexible creation of modified polysaccharide strands, based on readily available polysaccharides. These polysaccharides are equipped with a clickable functional group such as aldehyde or hydrazide.[14, 25] This toolbox is based on our preliminary investigations of new biomaterials for use in tissue engineering.[13, 15] and, here, we extend the concept in a comprehensive study on the six polysaccharides alginate (1), hyaluronic acid (2), dextran (3), pullulan (4), glycogen (5), and cellulose (6; Figure 1).

In principle, the chemistry chosen should allow either of the two functional groups (aldehyde and hydrazide) to be introduced into any of the chosen polysaccharides. Furthermore, a biologically relevant ligand (L), namely, cRGDFK (7), was plan-
ned to be attached to the polysaccharide strand by utilizing a second click reaction. The first reaction between two clickable strands provides hydrogels, and the second reaction introduces the biologically relevant ligand. The order of the two reactions can also be reversed. This concept allows creation of functionalized hydrogels in a toolbox fashion based on one or two different polysaccharides, and provides a maximum number of opportunities (Figure 2). It was also planned to unravel the scopes and limitations of the individual biomaterials with respect to further functionalization, hydrogel formation, and biocompatibility.

Results and Discussion

Concepts of difunctionalization of polysaccharides and hydrogel formation

The synthetic studies commenced with polysaccharides 1 and 2 (Figure 1), as these are well-established biomaterials. In fact, they have been used as drug carriers, dermal fillers, detergents, and wound-healing supports.\[5, 26–28\] Among many newly developed chemoselective ligations, click reactions such as the copper-mediated 1,3-dipolar Huisgen cycloaddition,\[35, 36\] and hydrazone formation relying on carbonyl functionality,\[7, 37\] have seen widespread use. However, toxicity aspects associated with metal-mediated processes hamper their use in biomedical applications.\[38–40\] Therefore, metal-free Huis- gen cycloadditions were developed that overcome this problem.\[41, 42\] Some of these reactions can even be performed at room temperature under aqueous conditions, a prerequisite when modified hydrogels are generated from the precursor biomacromolecules in vivo in a tissue environment.\[43\] For example, Rutjes et al. developed a metal-free oxanorbornadiene-based ligation protocol that yields trifluoromethyl-substituted triazoles (Scheme 1, left).\[14, 25, 44, 45\]

We chose hydrazone formation\[7\] with hydrazides (e.g., 13) and aldehydes (e.g., 12) for the condensation of two macromolecular strands consequently leading to the formation of hydrogels (click II, Scheme 1).\[46\] This method is supposed to be compatible with the 1,3-dipolar cycloaddition retro-Diels–Alder concept reported by Rutjes and co-workers. We found that, compared to other copper-free methods, the polar character of the oxabicyclo[2.2.1]heptadiene unit favors click attachment to polar surfaces, as found in polysaccharides.

The two routes for creating functionalized hydrogels are exemplified in Scheme 1. Carboxylate-containing polysaccharides are coupled with the 7-oxabicyclo[2.2.1]heptadiene building block 8 to furnish polysaccharide derivative 9. The copper-free click reaction (click I) with azido-modified ligands then provides functionalized polysaccharide 11. Alternatively, the 1,2-diol unit at C2 and C3 in the sugar rings is subjected to oxidative cleavage conditions to yield modified polysaccharide 12, which contains aldehyde groups suited to hydrogel formation. In parallel, carboxylate-containing polysaccharides are transformed into the corresponding acyl hydrazine 13, the second reaction partner for hydrogel formation. Hydrogel 15 can be prepared by two approaches. First, modified polysaccharide 11 is subjected to the oxidative 1,2-cleavage protocol followed by mixing with hydrazide 13. Alternatively, oxidized polysaccharide 12 is decorated with the biomedically relevant group L (click II). The resulting oxidized and functionalized polysaccharide 14 can then form a hydrogel in the presence of acyl hy-
drazine 13 (click II). The combination of two orthogonal click reactions[46] as pursued here has occasionally been termed double click[47] when conducted simultaneously and sequential click[48–49] when performed in a stepwise manner.

Functionalization of alginate

This sequential click strategy[50–52] can be utilized for polysaccharides that contain a carboxyl group. This was first probed for modifying alginate with cyclic pentapeptide 7,[53] which was attached to the carboxylate terminus at C-6 of 1. The process involved amide formation with the amino-functionalized oxanorbornadiene 8 to yield the corresponding modified alginate 9. As a key step, the Rutjes ligation of 9 with 7 afforded cRGDK-functionalized alginate 16.[14] This was directly oxidized to dialdehyde 17 in one pot by using aqueous NaIO₄. Finally, fast hydrazone formation and subsequent hydrogelation occurred when dialdehyde 17 and the alginate-derived hydrazide 13, also obtained from alginate 1, were mixed in water to form material 18 (Scheme 2).

Alternatively, cRGDK-functionalized hydrogel 18 can be obtained by a double-click strategy. After formation of alginate derivative 9 by using Rutjes reagent 8, NaIO₄-mediated diol cleavage at C2–C3 can be performed in one pot to yield modified alginate 12 (Scheme 3). Remarkably, this process revealed that the oxanorbornadiene system in 9 is stable under these oxidative conditions. According to the added amount of NaIO₄ and oxanorbornadiene 8, the degree of oxidation and functionalization can be individually varied. Dialysis and lyophilization provided 12 as a storable cotton-like material, which underwent hydrogel formation with hydrazide 13.

Next, the two click processes were conducted in one pot (Scheme 3). Alginate derivative 9 was first transformed into dialdehyde 12 and then treated with azido-modified cyclic RGDfK 7. In practice, this transformation must go to completion before an aqueous solution of hydrazine-modified alginate 13 is added. Depending on the degree of aldehyde and hydrazide functionalization of the two modified alginates, spontaneous hydrogelation occurred within a few minutes.[50–52,54–56]

Chemical modification of dextran, pullulan, glycogen and carboxymethyl cellulose

As described above, 1 and 2 are well suited for establishing a chemo-orthogonal strategy and introducing the cyclic RGDfK pentapeptide and a functional group (aldehyde or hydrazide). For both polysaccharides the carboxylate group at C6 serves
as a starting point for functionalization. We included other polysaccharides, such as dextran (3), pullulan (4), glycogen (5), and carboxymethyl cellulose (6), in these studies. However, except for 6, none of the other polysaccharides has a carboxylate group, so we had to alter the synthesis that was developed for alginate 1. While the aldehyde group required is still accessible through periodate oxidation, the hydrazine moiety had to be introduced by an alternative approach. In addition we had to assure further derivatization with a (bio)functional group L.

Scheme 4 shows an overview on introducing a hydrazine group (route A), a hydrazine group and a ligand L (route B), an aldehyde group (route C) and an aldehyde group and a ligand (route D) into polysaccharides 3–6. The missing carboxylate group is introduced by carboxymethylation. The synthetic routes are exemplified for polysaccharides that bear 1,4-glycosidic linkages. Furthermore, all or a substantial number of 6-hydroxyl groups are free for further derivatization. This is the case for polysaccharides 4–6. Only the dextrans have a backbone composed of 1,6-glycosidic bonds. The hydroxyl group at C3 may be prone to carboxymethylation; however, this does not affect the principal synthetic routes A–D.[57, 58]

In all cases carboxymethylation had to be repeated up to six times to assure a sufficient degree of functionalization. We use the following nomenclature exemplified for individual carboxymethylated batches of dextran: “Dex 100 stage 6” represents dextran (MW 100 kDa) after six repetitive carboxymethylations (see Supporting Information for further details and analyses).

Hydrazide component 19 was prepared by carboxymethylation (CICH₂CO₂H, 2 mNaOH, 62 °C, RT, 1.5 h) followed by hydrazide formation (EDC, HOBt, DIPEA, H₂N₂ (1 m), THF, H₂O, RT, 4 d; Scheme 4A). If an additional (bio)functional group L is to be introduced, the synthesis starts with partial carboxymethylation (CICH₂CO₂H, 2 mNaOH, 62 °C, RT, 1.5 h) by using an aliquot of the alkylating agent followed by amide coupling with Rutjes amine 8 (EDC, HOBt, DIPEA, 8, H₂O, 1 d, RT). The ratio between carboxymethylated polymer and the amine was chosen to be only 1% with respect to monomeric carbohydrate units. The outcome of this approach was judged by 1H NMR spectroscopy (for details, see Supporting Information). Next, reaction with organic azide 10 in an aqueous solution yields functionalized polysaccharide 20 after stirring for 4 d at 40 °C (Scheme 4B).

Then, the partially modified polysaccharides are transformed into the hydrazide as described for route A. This protocol yields the desired bifunctionalized polysaccharide 21.

The next step involves straightforward formation of the aldehyde groups by oxidative 1,2-diol cleavage (NaIO₄, H₂O, RT, 12 h; Figure 3). The cleavage commonly occurs at C2 and C3 of the pyranose rings (Scheme 4, route C). To introduce an aldehyde group and additionally a (bio)functional ligand (L), the partially oxidized polysaccharide 22 was reductively coupled with amine 8 (NaCNBH₃, H₂O, RT, 12 h). The resulting functionalized polysaccharide 23 was then coupled with azide 10 (H₂O, 40 °C, 4 d; Scheme 4D). For the reduction, NaCNBH₃ was employed in excess to guarantee quantitative reduction of all remaining aldehyde groups. Finally, intact pyran rings were treated with a second portion of periodate to create new aldehyde groups.

Thus, modified polymers 19–25 were prepared from the polysaccharides dextran (four different commercial dextrans with average molecular weights of 100, 150, 250, and 500 kDa were used) and pullulan (100 kDa). The functionalization of glycogen (5) was also probed. The synthesis had to be terminated at the stage of oxidation and hydrazide formation, because the hydrazides obtained did not form hydrogels in the presence of other modified polysaccharides. The mixtures only yielded undefined precipitates. Concerning carboxymethyl cellulose (6), we started with a commercial sample, which was transformed

into the corresponding hydrazide. This polysaccharide turned out to be suited for hydrogel formation, as shown in Figure 4.

Polysaccharides 3 to 5 were carboxymethylated as described above. Higher degrees of functionalization were obtained when the precipitated polymer was taken up repeatedly up to six times and subjected to the same reaction conditions in the presence of CICH₂CO₂H. For quantification, commonly 1 g of these polysaccharides were first protonated (MeOH/HNO₃ 9:1) followed by filtration, and the analytical procedure was finalized by titration against a 0.2 M solution of NaOH. The degree of carboxymethylation was determined to be about one per monosaccharide unit after six iterative steps of functionalization (Figure 5). Combustion analysis served as a second analytical tool for determining the degree of carboxymethylation (see Supporting Information).

Further modifications of these polymers were achieved by two routes. First, hydrazides were directly prepared from the carboxylates under standard coupling conditions [EDC, HOBt, DIPEA, H₂N₂ (1 M in THF)]. The 2,4,6-trinitrobenzenesulfonic acid assay (TNBS assay, Figure 6) and combustion analysis served to determine the degree of substitution and hydrazide formation (see Supporting Information).

Next, modification of carboxymethylated polymers with cRGDFK (7) was pursued. Prior to that step the Rutjes building block 8 (see Scheme 1) was introduced, again under standard amide coupling conditions (EDC, HOBt, DIPEA). Amine 8 was employed in substoichiometric amounts in order to guarantee that a fraction of carboxyl groups remained accessible. The degree of substitution was determined by 1H NMR spectroscopy and was found to be about 1% per glycosidic unit (see Supporting Information). Then, the polysaccharides containing the 3-(trifluoromethyl)-7-oxabicyclo[2.2.1]hepta-2,5-diene group were coupled under copper-free reaction conditions with azido-RGDFK 7 at 40 °C to yield RGD-modified polysaccharides. At this stage the degree of functionalization was determined to be about 0.5%, as judged by 1H NMR spectroscopy.

Further hydrazide formation under the conditions described above yielded the cRGDFK-bifunctionalized polysaccharide 21.

To modify the oxidized polysaccharide with the Rutjes linker, the polysaccharides were partially oxidized with NaIO₄ followed by reductive amination with Rutjes amine 8 and copper-free click coupling with the azide-containing 7. The cRGDFK-functionalized polysaccharide was finally transformed into the corresponding aldehyde by a second NaIO₄ oxidation step.
Analysis of functionalized polysaccharides

A sample of the polymeric material was collected after each synthetic step and was analyzed with different physical and spectroscopic methods. Commonly, $^1$H NMR spectroscopy is suited to analyze polymer gels, but we found that the $^1$H NMR signals of all the carboxymethylated polysaccharides were too broad, and this hampered reliable integration for accurately determining the degree of functionalization. Alternatively, classical titration allowed quantification of the number of free carboxylate groups present in carboxymethylated polysaccharides. First, the addition of a 9:1 mixture of MeOH/HNO$_3$ (70%) guaranteed protonation of all carboxylate groups. Under these conditions dextran hydrolysis does not occur. After washing of the polysaccharide with MeOH, the number of free carboxyl groups was determined by titration with NaOH (0.2 m). We found that it is possible to achieve carboxymethylation once per glycosidic unit.

Synthesis of model monosaccharides

To elucidate more details of the outcome of polysaccharide oxidations we used 6-methoxy $\alpha$-d-glucopyranoside ($\mathbf{37}$) as monomeric model for polysaccharides that contain a 1,6-glycosidic linkage. Under these reaction conditions no other side reactions occurred.

Hydrogel formation and mechanical properties

With modified polysaccharides 26–36 in hand, hydrazone formation and subsequent hydrogel formation could be carried out by mixing an aldehyde-containing polysaccharide with a hydrazide-functionalized polymer (Scheme 6). To initiate the gelation process, equal volumes of polysaccharide solutions (typically 20 mg mL$^{-1}$ each) were mixed, as depicted in Scheme 6. Polysaccharides 26–36 were tested under these conditions (Figure 7). After mixing, gelation proceeded within 30 min, after which the hydrogel was freeze-dried. The dry gel was taken up in deionized water to determine the amount of water uptake and degree of cross-linking of each blend. Even though the gel strength was not quantified for all combinations, the results in Figure 7 provide a good overview on the gelation ability of the functionalized polysaccharides. As ex-

This observation was further verified by treating the model monosaccharide methyl 6-O-methyl-$\alpha$-d-glucoside under conditions similar to those under which the polysaccharides were functionalized (CH$_3$COOH, solid NaOH, 62 °C, 1.5 h; Scheme 5). Mainly the different monocarboxymethylated regioisomers and minor amounts of diesterified glucosides were formed.

NMR analysis of polysaccharides functionalized as hydrazides was also not successful, so we used a photometric method for analyzing the degree of functionalization. Treatment of these polysaccharides with TNBS introduced the required chromophore. After incubation for 2 h at 40 °C the amount of chromophore present could be measured at a wavelength of 325 nm.\[60\]
expected, a high degree of functionalization and a high degree of cross-linking lead to increased gel rigidity.

The ability of a gel to absorb water is strongly influenced by its preparation procedure. For instance, a blend (4 mg dry weight) of dextran 100 kDa Ox 0.2 and dextran 100 kDa Hyd (stage 1) that was freeze-dried and reswollen formed a soft hydrogel containing about 1 g of water. In contrast, the same blend (4 mg) that was not freeze-dried before swelling was able to bind only 0.3 g of water. This observation suggests that freeze drying partially destroys the 3D network and yields a less interconnected polymer that then can absorb more water.\(^{[62]}\)

In essence, hyaluronic acid hydrazide (HyaHyd, 1630 kDa, 13), cellulose hydrazide (700 kDa, 36) and all carboxymethylated pullulan hydrazides 30, 31 show the ability to cross-link with all oxidized dextran and pullulan derivatives. This indicates that 1,4-glycosidic linkages and the resulting carboxymethylation in the 6-position leads to the best accessibility of the hydrazide group and therefore allows facile hydrazone formation with the counter-aldehyde.

In contrast to pullulan, gelation of dextran hydrazides (26, 27) provides broader variety with respect to the length and degree of polysaccharide modification. As is demonstrated in Figure 7, increasing the degree of functionalization and, hence, cross-linking leads to a reduced ability of the hydrogel to absorb water. This may be rationalized by assuming that a lower degree of substitution causes a larger pore size and improved swelling properties. To correlate the degree of substitution with the mechanical properties of the resulting hydrogels, a representative set of modified polysaccharides was chosen for determining their rheological properties (Table 1).

The data obtained are in good agreement with the trends observed in the gelation experiments (Figure 7). The best conditions for creating stable hydrogels are found for the following combinations: 1) polysaccharides with a moderate degree of hydrazine functionalization and 2) a second polysaccharide strand containing a very small number of aldehyde groups. Notably, high molecular weight hyaluronic acid hydrazides lead to an increased storage modulus \(G'\) of up to 152 Pa. The increased \(G'\) value may be caused by the high average molecular weight of hyaluronic acid as well as better accessibility of the hydrazide functionality.

### Table 1. Rheological data.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Hydrazide</th>
<th>Aldehyde</th>
<th>(G') [Pa](^{[a]})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dex 250 Hyd 2</td>
<td>Dex 150 Ox 0.5</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>Dex 250 Hyd 2</td>
<td>Dex 150 Ox 0.8</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>Dex 250 Hyd 2</td>
<td>Dex 150 Ox 1.1</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>Dex 250 Hyd 4</td>
<td>Dex 150 Ox 0.5</td>
<td>52</td>
</tr>
<tr>
<td>5</td>
<td>Dex 250 Hyd 4</td>
<td>Dex 150 Ox 0.8</td>
<td>35</td>
</tr>
<tr>
<td>6</td>
<td>Dex 250 Hyd 4</td>
<td>Dex 150 Ox 1.1</td>
<td>42</td>
</tr>
<tr>
<td>7</td>
<td>Dex 250 Hyd 6</td>
<td>Dex 150 Ox 0.5</td>
<td>47</td>
</tr>
<tr>
<td>8</td>
<td>Dex 250 Hyd 6</td>
<td>Dex 150 Ox 0.8</td>
<td>32</td>
</tr>
<tr>
<td>9</td>
<td>Dex 250 Hyd 6</td>
<td>Dex 150 Ox 1.1</td>
<td>17</td>
</tr>
<tr>
<td>10</td>
<td>HyaHyd</td>
<td>Dex 150 Ox 0.8</td>
<td>152</td>
</tr>
</tbody>
</table>

\(\text{[a]}\) Storage modulus.

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### Biocompatibility of functionalized polysaccharides and hydrogels

To determine the biocompatibility of the modified polysaccharides we used them to treat human fibroblast cell cultures for 30 min or 6 h in a cytotoxicity assay based on the quantification of lactate dehydrogenase (LDH) release from damaged cells (see Supporting Information for details). After 6 h of exposure, dextran aldehydes exhibited no significant cytotoxic effects, with only slightly increased values for the highest degree of oxidation (Figure 8A), while dextran hydrazides showed cytotoxicity of 10–15% without a clear correlation to the degree of carboxymethylation (Figure 8B). Notably, the same functionalized dextrans exhibited no cytotoxic effect after hydrogel formation (Figure 8C), most likely because of the reduced number of free functional groups after polymerization. In a direct comparison, these hydrogels displayed the same biocompatibility as a combination of rat collagen type I and Matrigel\(^t\), which are used routinely as scaffold materials for cardiac tissue engineering\(^{[63]}\) purposes.

### Conclusions

A bioorthogonal double-click strategy was successfully applied for preparing new hydrogels based on different polysaccharides, which in some cases were conjugated with cRGDK pentapeptide. Hydrogels did not exert cytotoxic activity and showed the same biocompatibility as collagen/Matrigel\(^t\), which is routinely used as scaffold material for cardiac tissue engineering. The synthetic approach relied on azido–oxanorbornadiene cycloaddition and hydrazone formation. Both metal-free methods showed excellent chemoselectivity, so that both a sequential click strategy and a double-click process could be performed under aqueous conditions. Among the polysaccharides studied, only glycogen and carboxymethyl cellulose did not integrate well into the overall concept of hydrogel formation. In essence, our work paves the way to flexibly create libraries of new polysaccharide hydrogels functionalized with one or two biofunctional groups. Other polysaccharides, such as lentinan and lichenan, can be envisaged for further broadening the toolbox described here.
Experimental Section

The sections below cover synthetic key protocols for preparing carboxymethylated, hydrazide-functionalized and oxidized polysaccharides. Furthermore, general protocols for preparing polysaccharides functionalized with cRGDFK as well as hydrogels are given. Additional synthetic procedures, analytical descriptions including NMR spectra and titrations as well as cytotoxicity tests can be found in the Supporting Information.

Typical procedure for the carboxymethylation of polysaccharides

A solution of the dextran 100 kDa (10 g) in deionized water (150 mL) was treated with 2-chloroacetic acid (20 g, 211 mmol), and sodium hydroxide solution (8 M, 50 mL) was added. The mixture was warmed to 62 °C and kept at this temperature for 1.5 h, cooled to RT and finally neutralized by addition of HCl (6 M). The solution was dropped into methanol (0.8–1.0 L) and finally the pre-
Typical procedure for the oxidation of polysaccharides

A solution of dextran 100 kDa (1 g, 1.0 equiv) in deionized water (100 mL) was treated with sodium periodate (1.0 g, 0.8 equiv) in the dark and stirred for 12 h at RT. The solution was transferred to a dialysis bag and dialyzed for 3 d against deionized water. After lyophilization the oxidized polysaccharide (900 mg) was obtained as a colorless, cotton-like solid. This procedure was applied to pullulan, glycogen and dextrans differing in average molecular weight (100,000, 150,000, 250,000, and 500,000 g mol\(^{-1}\)).

General procedure for hydrogel formation

A solution of the oxidized polysaccharide component (20 mg mL\(^{-1}\)) was mixed at RT with a solution containing an equal amount of hydrazide-modified polysaccharide component (20 mg mL\(^{-1}\)). Gel formation typically occurs within a few seconds after mixing the solutions.

General procedure for modification of oxidized dextran and oxidized pullulan with the Rutjes linker

A solution of the polysaccharide (100 mg, 1.0 equiv) in deionized water (10 mL) was treated with sodium periodate (30 mg, 0.2 equiv) in the dark, and stirring was continued at RT for 12 h. The solution was transferred into a dialysis bag and dialyzed for 3 d against deionized water. After lyophilization the colorless, cotton-like solid (100 mg) was dissolved in deionized water, treated with the Rutjes linker (8, 20 mg), and stirring was continued at RT for 2 h. Then NaCNBH\(_3\) (3 mg) was added and the suspension was stirred at RT overnight. The yellowish solution was filtered through an HPLC filter (0.45 μm pore size), dialyzed against deionized water for 3 d and lyophilized to furnish the modified polysaccharide (80 mg) as a pale yellow, cotton-like solid. A cross-linkable aldehyde component was prepared by oxidizing the modified polysaccharide a second time, which yielded new aldehyde groups. Thus, a solution of the modified polysaccharide (100 mg) in deionized water (10 mL) was treated with sodium periodate (30 mg, 0.2 equiv) in the dark, and the reaction mixture was stirred at RT for 12 h. The solution was transferred to a dialysis bag and dialyzed for 3 d against deionized water and finally lyophilized to furnish the modified polysaccharide (90 mg) as a colorless, cotton-like solid.

General procedure for the modification of carboxymethylated dextran and carboxymethylated pullulan with the Rutjes linker

A solution of the polysaccharide (100 mg, 1.0 equiv) in deionized water (50 mL) was treated with EDC (8.2 mg, 0.1 equiv), HOBt (6.0 mg, 0.1 equiv), and linker 8 (5.3 mg, 0.05 equiv) and stirred for 24 h at RT. The solution was dialyzed for 2 d against NaCl solution (3 × 100 g/5 L, 3 × 50 g/5 L), 2 d against water and, after lyophilization, the product (69 mg) was obtained as a colorless, cotton-like solid.

General procedure for copper-free click reaction with cRGDFK

A solution of the polysaccharide (60 mg) in deionized water (10 mL) was treated with cRGDFK (3.0 mg) and stirred for 4 d at 40 °C. The solution was then transferred to a dialysis bag, dialyzed...
for 3 against deionized water and lyophilized to obtain the product (58 mg) as a colorless, cotton-like solid.

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