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Dextrans, Pullulan and Lentinan, New Scaffold Materials for Use as Hydrogels in Tissue Engineering

Pascal Lienig,[a] Samhita Banerjee,[a] Franziska Kenneweg,[b] Thomas Thum,[b] Andreas Kirschning,*[a] and Gerald Dräger*[a]

The development of hydrogels based on dextrans, pullulan and lentinan to be used in biomedical applications including tissue engineering is reported. Despite the fact that selected polysaccharides such as hyaluronic acid are well established, little is known, how these polysaccharides can be chemically modified to create hydrogels under controlled conditions. In this study we present a small library of chemically modified polysaccharides which are used for a divergent approach to achieve biomedical relevant hydrogels. In this case the crosslinking is based on thio ether formation between thiol modified donor and vinylsulfone or maleimide modified acceptor components. Successful synthesis of the linker systems and coupling at the polysaccharides, hydrogel formation takes place under physiological conditions. We extended the study by coupling small molecules like adhesion factors for increasing cell compatibility as well as a dye for further studies. The different hydrogels were studied to their rheological properties, water uptake, their permeability, biodegrability and their cytotoxicity.

Introduction

Gel-like biomaterials play a key role as backbone materials in regenerative medicine.[1,2] Hydrogels based on biomacromolecules are developing increasing importance as scaffold materials due to their natural origin and biocompatible properties, especially in the field of tissue engineering.[3–10] For clinical applications, biocompatibility is crucial to avoid immunogenic rejection reactions. Polysaccharides such as alginate or hyaluronic acid play a significant role in this field, as they possess precisely these desired properties and open up possibilities for further functionalization.[11–14] In addition polysaccharides have also been sometimes used as blends to tailor their mechanical, biological, and physical properties.[15–17]

In recent years, we have expanded the portfolio of polysaccharides for the formation of hydrogels and their use in tissue engineering, both in terms of the choice of natural polysaccharides as well as the use of chemical processes for hydrogel formation.[18] It has been our intention to study the utility of new polysaccharides as biomedical materials and for that known as well as new synthetic chemistry had to be developed. The programme also included search and evaluation of cross-linking strategies that can be performed in an aqueous environment to create hydrogels, preferably directly after the injection of two modified polysaccharides into living tissue. In our previous work, we have mainly used hyaluronic acid,[11,13,19a] polysialic acid[19c] and alginate.[11,12,13,19a] As an additional structural element, we added biofunctional groups such as cyclic RGD (arginine, glycine, aspartic acid) peptides **4** to the polysaccharide scaffolds, which considerably increase affinity or surface recognition of human cells for these hydrogels.[19] The RGD sequence interacts with cell surface proteins, the integrins, provided they can adopt the correct conformation. This is particularly successful if they are part of a cyclic pentapeptide architecture (usually cyclic RGDfK or *c*RGDfK).[20–22]

Such bioconjugates contain and combine the properties of their individual components in synergistically and, moreover, these constructs enable to overcome biological and/or chemical limitations and drawbacks of the individual starting materials.

In the present work, we extend our synthetic toolbox concept towards dextran (**1**), pullulan (**2**), and lentinan (**3**) which so far have not been evaluated in this context. It is important to mention that both dextran and pulllulan are of microbial origin, which is an advantage for tissue engineering applications (Figure 1).

Dextrans (**1**) are high-molecular, branched, neutral polysaccharides that serve as reserve substances for yeasts and bacteria. These glucans have predominantly α-1,6 glycosidic linkages with branches from α-1,3 branches. Pullulan (**2**) is a polysaccharide consisting of maltotriose units with α-1,4 and α-1,6 glycosidic connections. Finally, lentinan (**3**) is isolated from the shiitake mushroom (*Lentinula edodes*) and a glucan with two β-1,6-glycosidic branches for every five β-1,3-glycosidically linked glycose units.

In the concept of hydrogel formation presented here, different functionalized linker elements attached to the carbo-

[a] *P. Lienig, Dr. S. Banerjee, Prof. Dr. A. Kirschning, Dr. G. Dräger
Institute of Organic Chemistry, Leibniz University Hannover
Schneiderberg 1B, 30167 Hannover (Germany)
(+49) 511-762-3011
E-mail: andreas.kirschning@oci.uni-hannover.de
gerald.draeger@oci.uni-hannover.de*

[b] *Dr. F. Kenneweg, Prof. Dr. Dr. T. Thum
Institute of Molecular and Translational Therapeutic Strategies (IMTTS), Hannover Medical School, Carl-Neuberg-Straße 1, 30625 Hannover (Germany)*

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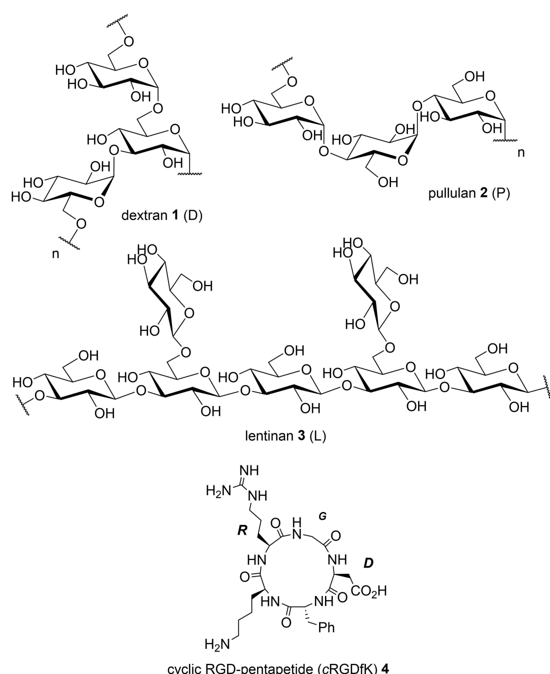
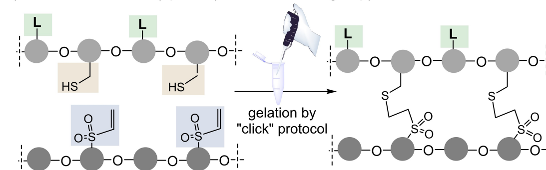


Figure 1. Polysaccharides dextran (**1**), pullulan (**2**), lentinan (**3**) studied in this work and cyclic RGD (**4**) (abbreviations in brackets are used throughout this text; additionally functionalization is abbreviated as follows:,CM = carboxymethylation, VS = vinylsulfone, TPH = thiopropionic hydrazide, TBH = thiobutyric hydrazide, BM = 1-(4-aminobutyl)maleimide, PM = 1-(5-aminopentyl)maleimide, RGD = cyclic pentapeptide with RGDfK element).



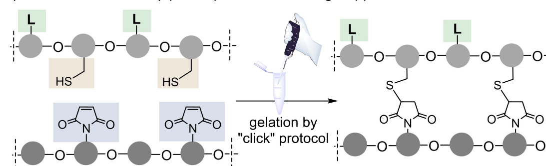
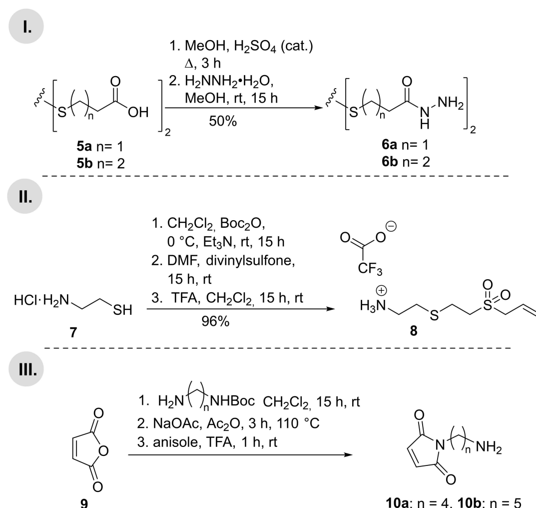


Figure 2. Tool box concept for creating functionalized hydrogels from different polysaccharides (color code used in this work: light brown = donor group, blue = acceptor group, green with L = biofunctional group, e.g. drug, adhesion factor or fluorescent dye).



Scheme 1. Synthesis of linker building blocks (**6 a,b**, **8** and **10**) (Boc = *tert*-butyloxycarbonyl, TFA = trifluoroacetic acid).

hydrate backbone are used, which are suitable for different typer of "click" chemistry leading to crosslinking and hydrogel formation. The concept is designed to be flexible so that either one type of polysaccharide is involved or, alternatively, two different polysaccharide strands are brought together in this process. The concept also allows the attachment of small molecules or bioactive moieties such as dyes or cyclic RGD peptides such as **4** (Figure 2).

Finally, the possibilities and limitations of each biomaterial will be determined with respect to further functionalization, hydrogel formation, and cell compatibility.

Results and Discussion

Chemical syntheses

Synthesis of linker elements: For the functionalization of the three selected polysaccharides, the linker building blocks had to be provided first. For the preparation of the donor component, the polysaccharides were planned to be modified with dithiopropionic and dithiobutyric acid dihydrazide (**6 a,b**).[23] For this purpose, dithiopropionic acid and butyric acid (**5 a,b**) were converted into their corresponding methyl esters. After this, they were reacted with hydrazine monohydrate and methanol, yielding the linker dithiopropionic and dithiobutanoic acid dihydrazides (**6 a,b**) (Scheme 1, case I).

The vinylsulfone group (VS) was chosen as the acceptor component for the proposed click-based hydrogel formations. And here we have chosen a linker concept that leads to a more elongated molecular system of cross-linking. This was achieved protecting cysteamine hydrochloride (**7**) with the Boc-group first which then was coupled with divinyl sulfone to yield the Boc-protected cysteamine vinyl sulfone. Then, the Boc-group was removed again which gave the cysteamine vinylsulfone trifluoroacetate (**8**)[24] (Scheme 1, case II).

Finally, maleimide was also chosen as the acceptor group, and the corresponding linker was prepared by condensation of maleic anhydride (**9**) with mono-Boc-protected diamines.[25] Subsequently, the Boc group was cleaved under acidic con-

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ditions, providing 1-(4-aminobutyl)maleimide (BM) (**10 a**) and 1-(5-aminopentyl)maleimide (PM) (**10 b**) (Scheme 1, case III).

Functionalization of dextran (250 kDa and 500 kDa), pullulan and lentinan: First, we pursued the goal of introducing the carboxymethyl (CM) group, which should allow the polysaccharides to be functionalized with a carboxyl group. This can then be used universally for the coupling of a functionalized linker or serve for the chemoselective introduction of small biorelevant molecules. This was achieved by treating the respective polysaccharide with 2-chloroacetic acid in the presence of sodium hydroxide. The degree of functionalization could be increased by repeating this procedure up to three times. However, ^{1}H-NMR spectroscopic analysis performed after functionalization proved difficult due to marked line broadening. Instead, we used the volumetric method described by Lechner and co-workers (Figure 3).[26]

As expected, the number of carboxymethyl groups (CM) per glycosidic unit increases with each "carboxymethylation" reaction. In total, dextran 250 kDa, dextran 500 kDa and pullulan were subjected to the carboxymethylation protocol up to three times, as the gelation of the TPH derivatives described below already occurred at this stage.

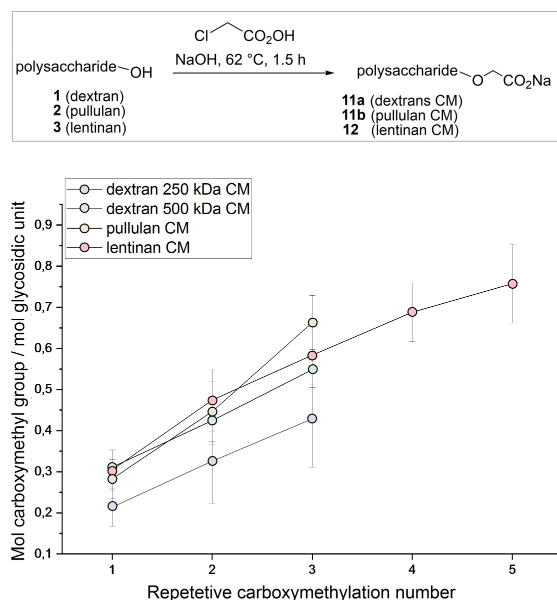

Figure 3. Degree of carboxymethylation of polysaccharides with respect to the number of repetitive reactions[26] (the degree is defined as the number of carboxymethyl groups per glycosidic unit).

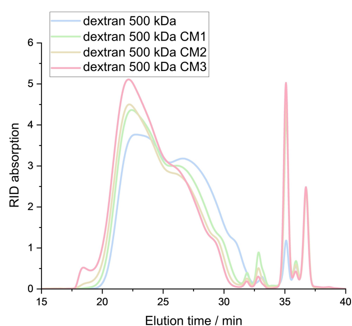

Figure 4. Elugram of dextran 500 kDa and its carboxymethylated derivatives.

In addition, the carboxymethylated polysaccharides were analyzed by gel permeation chromatography (GPC). As an example, the elugrams of dextran 500 kDa and the carboxymethylated derivatives is depicted in Figure 4, the mass average molar mass (M_w) increased at each carboxymethylation step (Table 1, entries 1–4). In the case of dextran 250 kDa and its derivatives, the M_w decreases between entries 7 and 8. This could be due to the reaction conditions employed for carboxymethylation that may result in hydrolysis of some glycosidic bonds during each iteration.

Subsequently, the polysaccharides were further modified by amide coupling, where the carboxymethylated polysaccharides **11** (dextran and pullulan) were first dissolved in water and mixed with a reagent mixture of MES, EDC·HCl, HOBt·H_2O, and linker building blocks **6 a**,**b**. Finally, reductive cleavage of the disufide moiety takes place using DTT (Scheme 2, case I) to afford thiopropionic hydrazide (TPH) (**13 a**, **14 a**) or thiobutyric hydrazide (TBH) (**13 b**, **14 b**) modified polysaccharides. Alternatively, polysaccharides **1**–**3** could be directly modified with divinyl sulfone, resulting in acceptor-functionalized polysaccharides **15 a**,**b** and **16** (case II).[26] Carboxymethylated dextran **11 a** was reacted with the elongated vinylsulfone 8 to furnish modified dextran **17** (case III). Finally, the synthesis of maleimide-modified dextran **18 a**,**b** and lentinan **19 a**,**b** was carried out by reacting the maleimide linker **10 a**,**b** with the carboxymethylated polysaccharides **11, 12** using the EDC method with NHS and MES as additives (case IV). The extent of functionalization of the carbohydrate backbones thus generated was confirmed by ^{1}H NMR spectroscopy, an example is given for the thiol-modified dextran (250 kDa) in Figure 5.

***c*RGDfK modified dextrans (250 kDa and 500 kDa) and lentinan**: The quest for enhancing the affinity of the hydrogel towards encapsulated cells was achieved by attachment of the cyclic RGDfK peptide (L in Figure 2) to the polysaccharide-bound acceptor group. Thus, the carboxymethylated polysaccharides (**11 a**,**b** and **12**) were converted to the corresponding vinyl sulfone derivatives (**20 a**,**b**) followed by EDC-promoted coupling of the *c*RGDfK peptide **4** to yield doubly modified polysaccharides **21 a**,**b** and **22**, resepctively (Scheme 3, case I).

Table 1. Chromatographic results (M_w = mass average molar mass, CM = carboxymethylation, number = repetitive carboxymethylation number).

entry	sample	M_w
1	D500 kDa	254.750
2	D500 kDa CM1	348.850
3	D500 kDa CM2	477.000
4	D500 kDa CM3	687.300
5	D250 kDa	221.450
6	D250 kDa CM1	254.900
7	D250 kDa CM2	308.800
8	D250 kDa CM3	268.850

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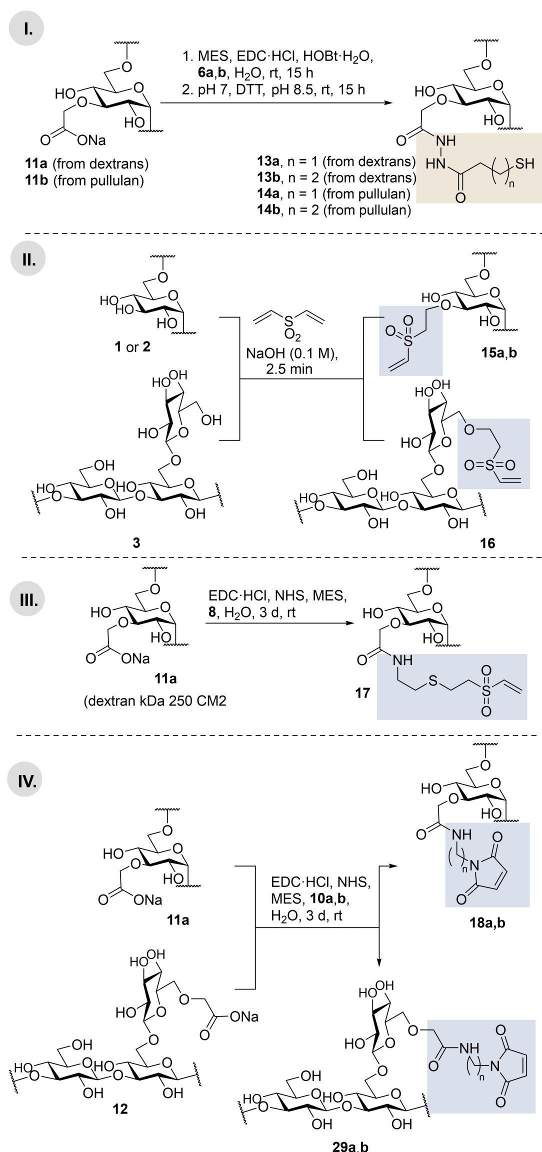

Scheme 2. Modifications of dextrans (250 kDa and 500 kDa) and pullulan by introducing donator or acceptor groups: I = coupling of carboxymethylated polysaccharides with thiol linker **6 a**, **6 b** and **7**; II. & III. = functionalization of polysaccharides with vinylsulfones and IV = functionalization of polysaccharides with maleimide (the location of the functionalization is given only as an example; other positions may also be functionalized) (MES = 2-(*N*-morpholino)ethanesulfonic acid, EDC = 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide, HOBt = 1-hydroxybenzotriazol, DTT = dithiothreitol, NHS = N-hydroxysuccinimide). I.

The degree of substitution was determined photometrically according to the Sakaguchi reaction. The degree of modification was determined to be $1.6\cdot10^{-3}\pm0.04\cdot10^{-3}$% for dextran 250 kDa derivatives and $2.3\cdot10^{-3}\pm0.02\cdot10^{-3}$% for dextran 500 kDa derivatives.[19b] A second line of modification, aimed at the attachment of a small molecule, should allow studies on the biodegradability of the newly formed hydrogels.

This was achieved by introducing a fluorescent dye and establishing a spectrometric fluorescence assay. For this purpose, fluoresceine-labeled dextran **24** (250 kDa) was prepared starting from dextran **20 a** and amino fluoresceine (**23**)

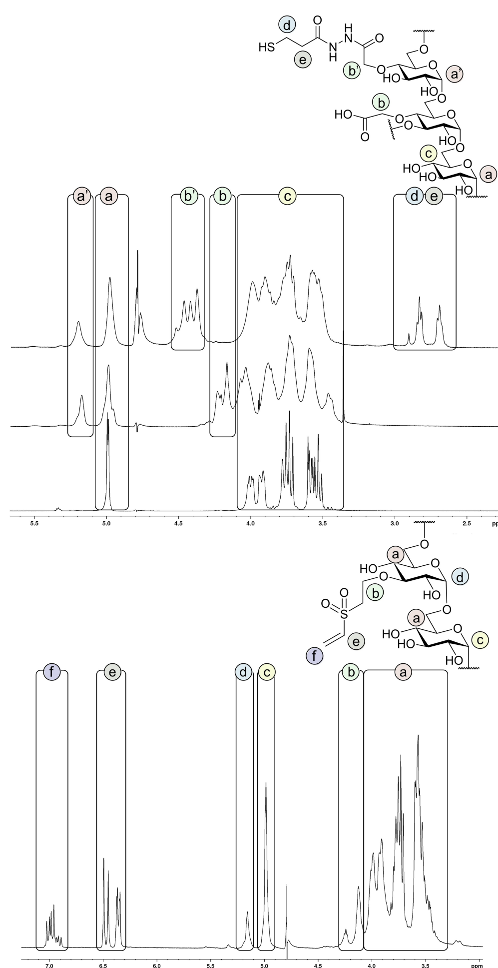

Figure 5. (top) ^{1}H NMR spectra of thiol-functionalized dextran (250 kDa) compared to native and carboxymethylated dextran (250 kDa): a and a' = anomeric protons, b = CH_2 of the carboxymethyl group, b' = CH_2 of thiol-modified carboxymethyl group, c = protons of glycosidic framework, d and e = signals of the methyl groups of thiol-linker; (bottom) ^{1}H NMR spectra of vinyl sulfone modified dextran (250 kDa): a = glycosidic framework, b = $-CH_2CH_2-$ of sulfone linker, c and d = anomeric protons, e and f = olefinic protons.

which contains both the carboxymethyl group and a vinyl sulfone moiety (case II).

Fluorescein-modified dextran 250 kDa TPH (**25**) was synthesized using a one-pot protocol (Scheme 4, case III). Briefly, carboxymethylated dextran 250 kDa (**11 a**) was coupled with amino fluoresceine (**23**) using the EDC protocol, followed by attachment of linker **6 a**. The final reduction with DTT afforded the thiol- and fluorescein-modified polysaccharide **25**.

The degree of thiol modification was determined using the Ellman test (Figure 6).[28] Chemical binding of thiopropionic and thiobutyric acid hydrazide to dextrans (250 kDa and 500 kDa) with dextran CM 1 to 3 was shown to be successful. In contrast, TPH-modified pullulan with a degree of modification greater than 2 resulted in insoluble materials. The degree of substitution is increased with each carboxymethylation step. The TPH-modification of lentinan did not furnish a material with properties for successful gelation.

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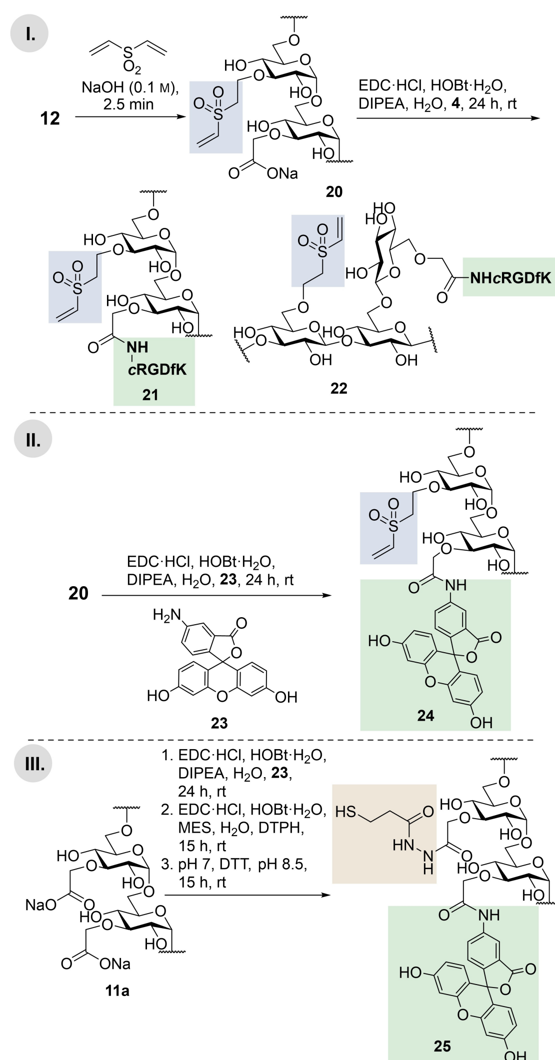
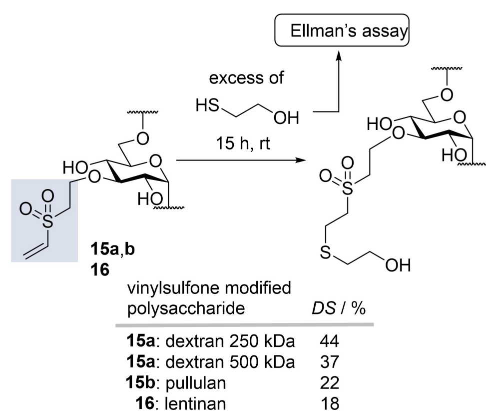


Scheme 3. Synthesis of *c*RGDfK (case I) and fluoresceine- modified acceptor components (case II and III).



vinylsulfone modified polysaccharide	*DS* / %
15a: dextran 250 kDa	44
15a: dextran 500 kDa	37
15b: pullulan	22
16: lentinan	18

Scheme 4. Degree of modification by Ellman's test conducted with vinyl sulfone-modified polysaccharides **15 a,b** and **16**.

The degree of modification of vinyl sulfone-modified polysaccharides **15 a,b** and **16** was determined using an indirect

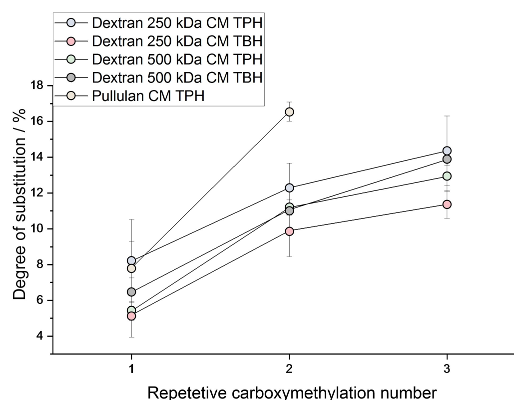


Figure 6. Degree of TPH and TBH modification of dextrans (250 kDa and 500 kDa) and pullulan in relation to the number of carboxymethylation processes.

Ellman assay. The samples were dissolved in an aqueous solution of 2-mercaptoethanol (12 mM). The amount of 2-mercaptoethanol remaining in the solution was determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNBS). A degree of functionalization of 44% for dextran 250 kDa **15 a**, of 37% for dextran 500 kDa **15 a**, of 22% for pullulan **15 b** and of 18% for lentinan **16** was determined analytically. A further increase in the degree of substitution failed to be achieved. This can be explained by the increase in the hydrophobic character of the modified polysaccharides in an aqueous environment.[28] The reason for the low degree of substitution of vinyl sulfone-modified Lentinan (**17**) may be related to its triple helical conformation in aqueous solution. Extending the reaction time from 2.5 to 3.5 min resulted in spontaneous precipitation of the polymers. Under these conditions, for steric reasons, presumably only the side chains are readily accessible for attack by the divinyl sulfone reagent.[29]

Hydrogel formation, swelling and mechanical properties: With the present modified polysaccharides (**13**–**20**), hydrogel formation can occur by thioether formation when a vinyl sulfone- or maleimide-containing polysaccharide is mixed with a thiol-functionalized polymer (see Figure 2). The gelation process was initiated by mixing equal volumes of polysaccharide solutions (20 $mg\cdot ml^{-1}$ for dextran-based hydrogels and 40 $mg\cdot ml^{-1}$ for hydrogels containing lentinan in potassium phosphate buffer pH 7.4). Next, we investigated the swelling properties of the generated hydrogels. Hydrogels were prepared from equal volumes of polysaccharide solutions (20 or 40 $mg\cdot ml^{-1}$). After mixing the precursor solutions, the gels were freeze-dried and then absorbed in water, with the water replaced daily. On the fourth day, the swollen hydrogels were weighed and freeze-dried again to determine the water absorption of each mixture (Figure 7).

Although water uptake was not quantifiable for all combinations, the results suggest a trend. Hydrogels based on maleimide functionalized polysaccharides show higher water absorption than the vinyl suflfone derivatives. However, a decreasing water uptake with increasing number of carboxymethylations is also observed here. In order to determine the

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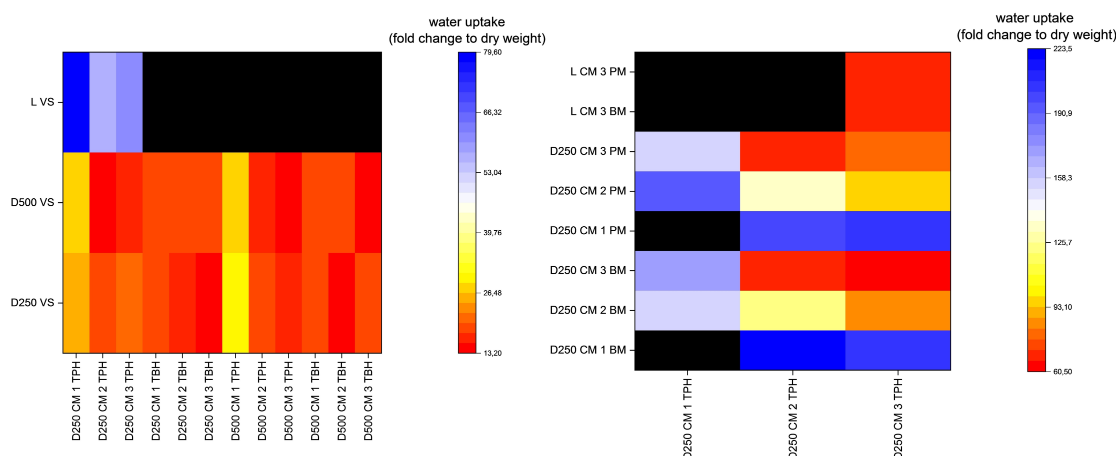


Figure 7. Heat map representation of water uptake of mixtures containing different polysaccharides functionalized with different donor and acceptor groups (black: hydrogels dissolved over the time completely, or combination not testet).

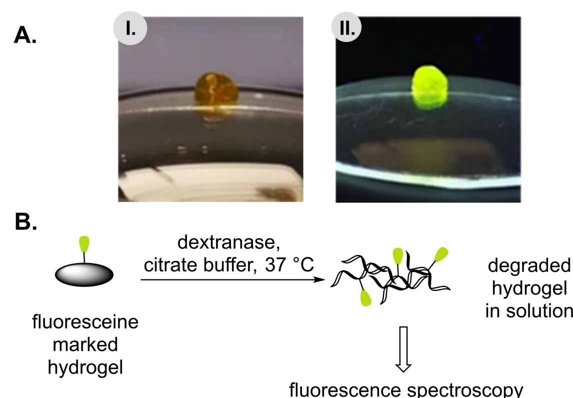
mechanical properties of the new hydrogels, rheological experiments were also performed. In order to obtain an overview of the gelation time and the mechanical strength, a time sweep was performed. For this purpose, the two components were mixed and measured in a rheometer at a time sweep of 45 minutes.

Determination of the storage modulus G' and loss modulus G'' revealed that viscoelastic gels ($G' > G''$) formed and the mechanical strength can be changed by varying the degree of substitution (Table 2). The storage modulus G' increases with each carboxymethylation step of the donor compound. This suggests that a larger degree of modification leads to more donor groups, resulting in a more stable network.

The gelation time decreases with larger degree of modification. Due to the *syn*-orientation of the two carbonyl groups, the bond angle distortion and the ring tension present in the maleimide unit, it is observed that the thiol-Michael reaction proceeds much faster than when using acceptors modified with vinyl sulfone and this explains the shorter gelation times.[28]

Permeability and enzymatic degradation of hydrogels: To investigate the permeability of the new hydrogels, the permeation coefficient P was determined using a vertical Franz^{TM} diffusion cell. Glucose was used as a model molecule for testing permeation through the hydrogels. A dialysis membrane (MWCO 14 kDa) was used as a control membrane. Hydrogels (2 or 4 wt%) were prepared by mixing the compounds (200 μl of each compound) and introducing the liquid into the donor cell of the Franz^{TM} cell. After completion of gelation (1 h), the donor solution (glucose 10 mg·mL^{-1}) was added to the hydrogel, and aliquots of permeated glucose were collected at various intervals over a 48 h period. Glucose concentration was determined by a photometric dinitrosalicylic acid (DNSA) assay. The results are shown in Table 3.

To investigate biodegradability, polysaccharides were labeled with fluorescein (Scheme 2, cases II and III) and then degraded by exposure to dextranase (Scheme 5, case b). For experiments with dextran 250 kDa only, vinyl sulfone and dextran 250 kDa **24** modified with fluoresceine were used as acceptor components. For lentinan hydrogel mixtures, fluorescein-modified dextran 250 kDa CM TPH (**25**) was used as the donor component (Scheme 5, case a). Hydrogels (2 or 4 wt%) were prepared by mixing the compounds (50 μl of each compound) and pouring the liquid into vials.



Scheme 5. A. fluorescein marked dextran and lentinan hydrogel blends without UV irradiation (I.) with UV irradiation (II.); B. enzymatic degradation of fluorescein marked hydrogel.

After complete gelation (1 h), a citrate buffer solution (pH 5.6, 500 μl) containing dextranases (5 U·mL^{-1}) was added, and aliquots of the solution were taken at various intervals over a 24 h period. The results are summarized in Figure 8. Complete degradation of hydrogels was found within 24 h for dextran hydrogels containing 250 kDa. The degradation time of hydrogels increases with the degree of modification of the compounds. For the lentinan hydrogel mixtures, complete degradation was observed only for hydrogels containing lentinan CM PM as the acceptor component. For lentinan modified with vinyl sulfone, 80% degradation was observed within 25 h. Lentinan does not contain a (1→6)-α-ᴅ-glycosidic bond for endohydrolysis. However, degradation of the dextran strain of the donor compound is sufficient to partially degrade the hydrogel. These experiments confirmed the hydrogels reported here show the property of biodegradability.

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Table 2. Gelation time $t_{sol-gel}$ and storage modulus G' of the hydrogels.

entry	donor	acceptor	$t_{sol-gel}$ [min]	G' [Pa]
1	D250 CM1 TPH	D250 VS	2.7	114
2	D250 CM2 TPH	D250 VS	1.9	256
3	D250 CM3 TPH	D250 VS	1.7	477
4	D500 CM1 TPH	D500 VS	4.5	112
5	D500 CM2 TPH	D500 VS	1.4	313
6	D500 CM3 TPH	D500 VS	1.2	459
7	D250 CM1 TBH	D250 VS	11.5	85
8	D250 CM2 TBH	D250 VS	4.9	155
9	D250 CM3 TBH	D250 VS	3.2	186
10	D500 CM1 TBH	D500 VS	6.7	154
11	D500 CM2 TBH	D500 VS	1.8	248
12	D500 CM3 TBH	D500 VS	0.7	372
13	D250 CM1 TPH	L VS	7.1	68
14	D250 CM2 TPH	L VS	3.3	145
15	D250 CM3 TPH	L VS	3.9	911
16	D250 CM1 TPH	L CM4 BM	0.3	17
17	D250 CM2 TPH	L CM4 BM	2.5	147
18	D250 CM3 TPH	L CM4 BM	4.1	243
19	D250 CM1 TPH	L CM4 PM	1.8	107
20	D250 CM2 TPH	L CM4 PM	1.4	85
21	D250 CM3 TPH	L CM4 PM	0.2	10
22	D250 CM2 TPH	D250 CM3 BM	0.17	113
23	D250 CM2 TPH	D250 CM3 PM	0.22	77

Table 3. Permeation coefficient P of some hydrogels.

acceptor	donor	P [10^{-6} cm·s^{-1}]
control		9.3
D250 CM1 TPH	D250 VS	2.8
D250 CM2 TPH	D250 VS	6.0
D250 CM3 TPH	D250 VS	4.6
D250 CM1 TBH	D250 VS	3.8
D250 CM2 TBH	D250 VS	4.0
D250 CM3 TBH	D250 VS	2.9
D250 CM1 TPH	L VS	4.0
D250 CM2 TPH	L VS	4.5
D250 CM3 TPH	L VS	4.9
D250 CM1 TPH	L CM3 BM	3.6
D250 CM2 TPH	L CM3 BM	4.0
D250 CM3 TPH	L CM3 BM	3.3
D250 CM1 TPH	L CM3 PM	3.4
D250 CM2 TPH	L CM3 PM	4.1
D250 CM3 TPH	L CM3 PM	3.0

It is known that the vinyl sulfone as well as the maleimide groups are able to act as Michael acceptors with components present in the environment. IR analysis of lyophilized hydrogels revealed changes (see SI) but these do not provide quantifyable data and we cannot exclude the presence of unreacted Michael acceptor groups in the hydrogel formed.

Cell compatibility of functionalized polysaccharides and hydrogels: To determine the cell compatibility of the functionalized polysaccharides, HEK293t cell cultures were treated for one hour followed by WST-1 (Figure 9). After one hour of exposure, the thiolated polysaccharides showed significant cytotoxic effects. It is shown that the cytotoxicity correlates with the degree of carboxymethylation. The acceptor compounds showed also cytotoxic effects but lower than the donor compounds. For further studies, cells were encapsulated in different hydrogels and incubated for a duration of 24 or 72 hours followed by LDH assay, live dead staining and imaging.

After 24 hours (Figure 9, B), no significant cytotoxic effect was measured for encapsulated cells in vinyl sulfone based hydrogels whereas the maleimide derivatives showed high cytotoxicity in comparison of the untreated samples. These results correlate to the imaging results (Figure 11 cases A–D).

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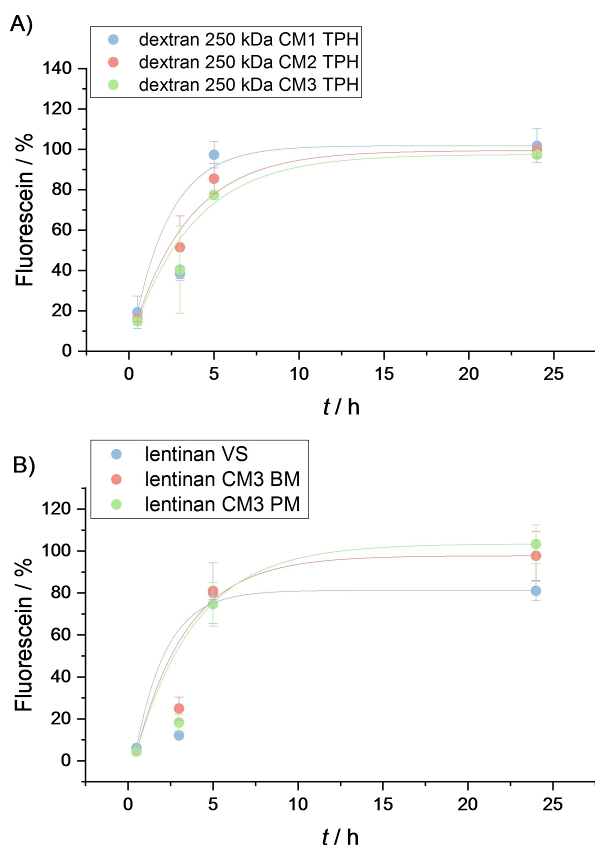

Figure 8. Degradation of hydrogels by dextranase; a) vinylsulfone and fluorescein modified dextran 250 kDa was used as acceptor component; b) fluorescein modified dextran 250 kDa CM2 TPH was used as donor component.

The formation of cell agglomerates may be related to the inhomogeneous mixing of the compounds because of the fast gelation (Figure 10 case A–B). Cells encapsulated in vinyl sulfone based hydrogels show homogeneous distribution threw the hydrogels (Figure 11 cases C–D). To extend the application human cardiac fibroblasts (HCF) were encapsulated also in vinyl sulfone derived hydrogels and LDH assay was performed after 24 and 72 h ((Figure 10) Here the hydrogels showed a slightly cytotoxic effect of the HCF after 24 h which increased by cultivation of 72 h. This results also correlate with the imaging results (Figure 11 case E).

Conclusions

In this report, we describe the use of the polysaccharides dextran, pullulan and lentinan as potential hydrogel materials for use in biomedical applications for the first time. With reference to our earlier work on hyaluronic acid and alginate, we developed a functionalization chemistry that allows in situ hydrogelation, whereby mixtures based on different polysaccharide strands can also be produced. We are thus expanding the portfolio of basic polysaccharide materials for the field of biomedical materials.

As crosslinking method we chose the thiol-click reaction leading to a stable thioether bond. However, we found that thiol-modified lentinan does not provide hydrogels that are stable enough for practical use. Furthermore, we determined several physicochemical properties such as permeability, rheological behavior, and ability to absorb water for the hydrogels were further investigated. The degradability of the different hydrogels was also investigated.

Depending on the type of functionalization, polysaccharides and hydrogels cytotoxic effects were encountered. In the case of HEK293t cells, the vinylsulfone-based hydrogels proved to be a suitable matrix for cultivation for at least three days. However, maleimide-based hydrogels revealed a cytotoxic effect on HEK293t cells. When switching to HFC cell lines, pronounced cytotoxic effects were also observed with the vinyl sulfone-based hydrogels.

Pullulan and dextran therefore appear to be suitable new polysaccharide candidates for further development towards hydrogels for biomedical applications. However, further synthetic investigations are necessary, particularly with regard to the chemistry of cross-linking in order to exclude the presence of unreacted Michael acceptor groups.

Experimental Section

The experimental section below covers synthetic key protocols for preparing carboxymethylated, hydrazide functionalized and oxidized polysaccharides. Furthermore, general protocols for preparing polysaccharides functionalized with *c*RGDfK as well as hydrogels are given. Additional synthetic procedures, analytical descriptions including NMR spectra and titrations as well as cytotoxicity tests are found in the supporting information.

General procedure to prepare TPH/TBH modified polysaccharides

To a solution of the carboxymethylated polysaccharide (100 mg, 1.0 eq.) in water (10 mL) MES (976 mg, 5.0 mmol, 8 eq.), EDC·HCl (178 mg, 0.927 mmol, 1.5 eq.) and HOBt·H_2O (95 mg, 618 mmol, 1.0 eq.) were added and the solution was stirred for 0.5 h at room temperature. Then DTPH (73 mg, 0.306 mmol, 0.5 eq.) or DTBH (82 mg, 0.306 mmol, 0.5 eq) or L-cystine dimethylester hydrochloride (105 mg, 0.306 mmol, 0.5 eq.) was added and the mixture was stirred at room temperature for 15 h. Next, the pH was adjusted to 7.5 by addition of NaOH (2 M) followed by the addition of DTT (477 mg, 3.90 mmol, 5.0 eq.). The pH was then adjusted to 8.5 and the solution was stirred for an additional 15 hours before the pH was then lowered to 3.5 (HCl, 1 M). The mixture was transferred into a dialysis tube and dialyzed for 1 d against brine at a pH of 3.5 ($3\times20\ g\cdot L^{-1}$) and afterwards for 1 d against water at a pH of 3.5. The product was obtained as a cotton-like solid (96 mg) after lyophilization.

General procedure for synthesising VS modified polysaccharides

To a solution of native polysaccharide (200 mg) in NaOH (0.1 M, 10 mL) divinyl sulfone (558 μL, 5.56 mmol, 4.5 eq.) was added with vigorous stirring. The reaction was terminated by adjusting the pH to 3.5 (HCl, 6 M). The mixture was transferred to a dialysis tube and

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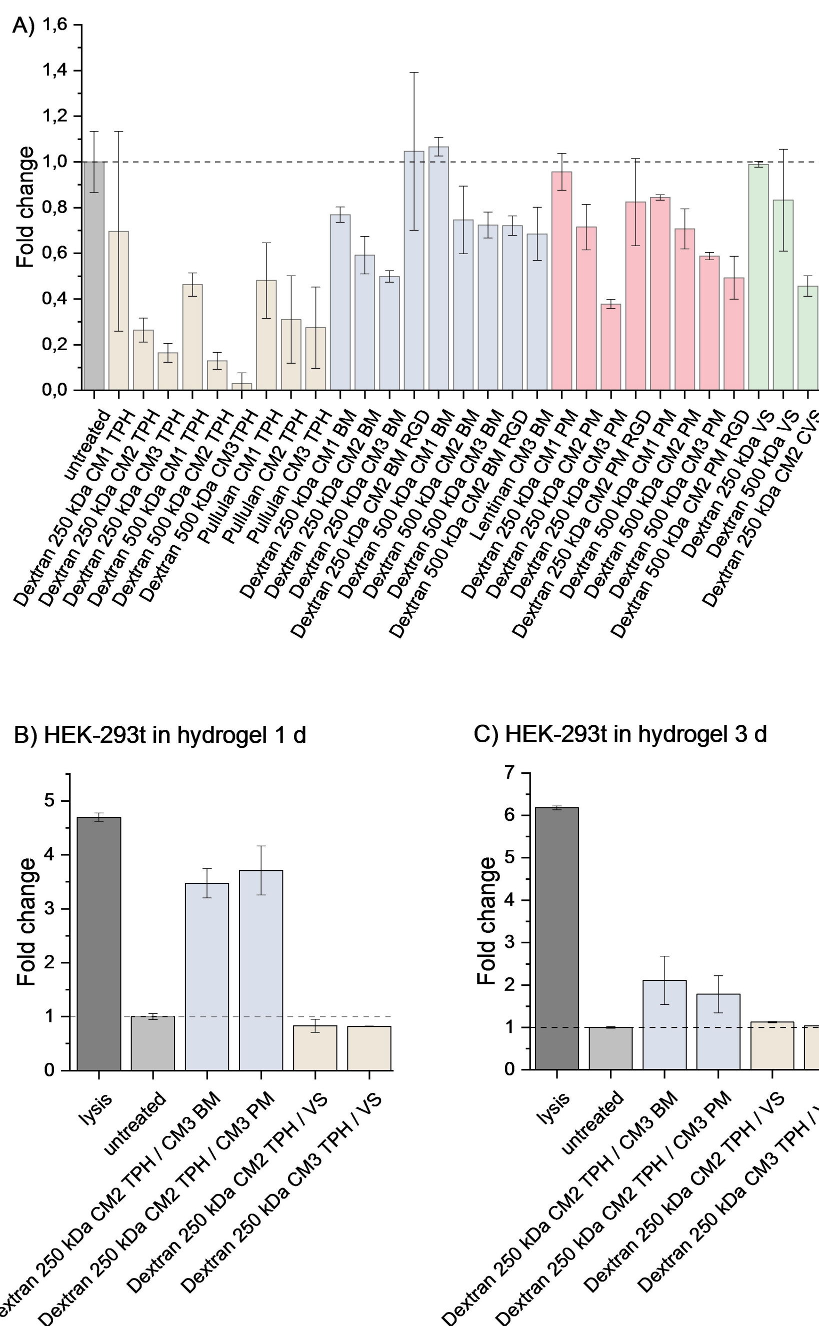

Figure 9. HEK293t cell compatibility tests of A) functionalized polysaccharides tested by WST-1 assay, B) LDH assay of cell encapsulation in hydrogels for 1 d and C) LDH assay of cell encapsulation in hydrogels for 3 d. Data is shown as foldchange of untreated control.

dialyzed for two days in water at pH 3.5. The product was obtained as a cottony solid (190 mg) after lyophilization (see [26]).

General procedure for the preparation of vinylsulfone modified carboxymethylated polysaccharides

To a solution of the carboxymethylated polysaccharide (200 mg) in NaOH (0.1 м, 10 mL) divinyl sulfone (558 μL, 5.56 mmol, 4.5 eq.) was added with vigorous stirring. The reaction was terminated by adjusting the pH to 3.5 (HCl, 6 м). The mixture was transferred to a dialysis tube and dialyzed for two days against water at pH 3.5. The product was obtained as cotton-like solid (184 mg) after lyophilization.

Preparation polysaccharides modified with cRGDfK

First, the polysaccharides were converted to their corresponding carboxymethylated and vinyl sulfone modified derivatives (see above). These modified polysaccharides (100 mg, 0.62 mmol) were dissolved in water (50 mL) and EDC·HCl (8 mg, 0.04 mmol), HOBt·H_2O (6.5 mg, 0.04 mmol), and DIPEA (20 μL, 0.11 mmol) were added. The mixture was stirred at room temperature for 0.5 hours. *c*RGDfK·AcOH (1 mg, 1.66 μmol) was then added and the mixture

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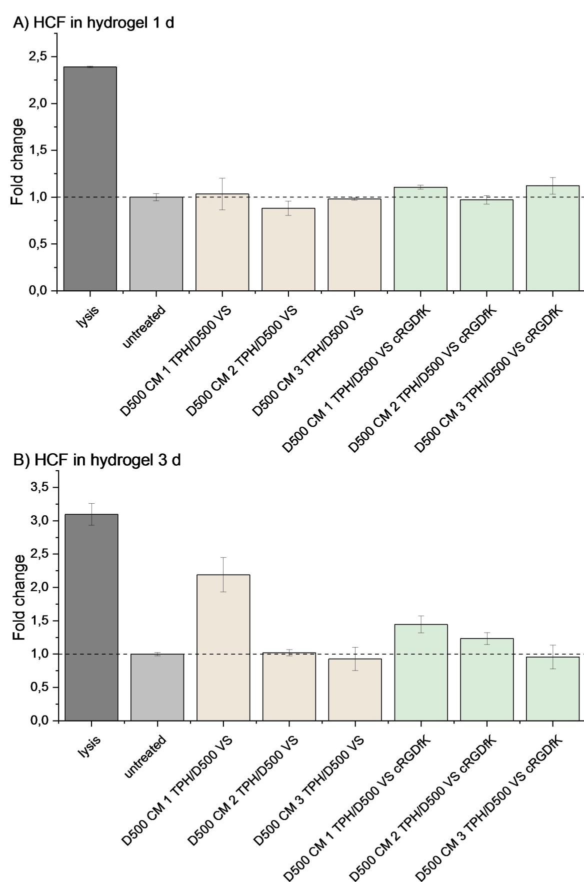

Figure 10. LDH assay of HCF encapsulated in hydrogels for A) 1 d and B) 3 d. Data is shown as foldchange of untreated contro

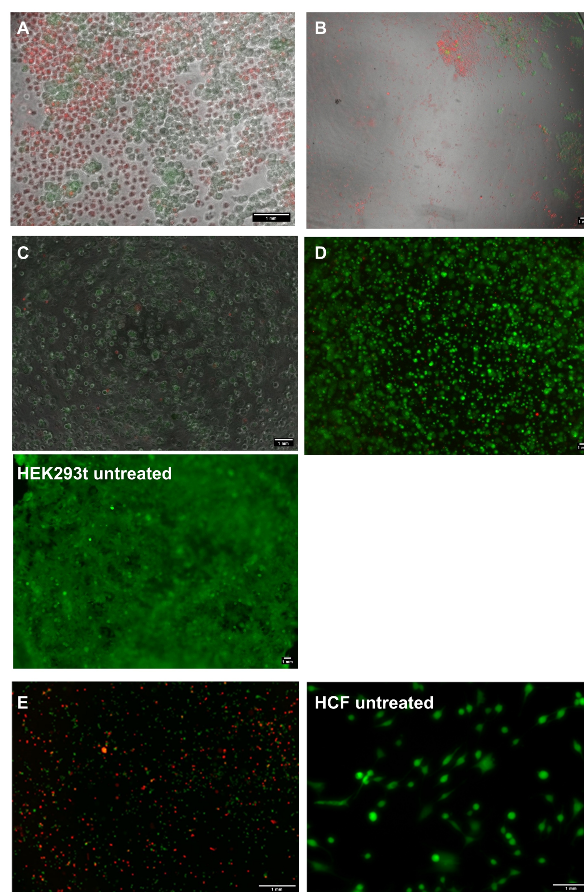

Figure 11. Live dead staining and imaging of A: HEK293t cells encapsulated in D250 CM2 TPH/D250 CM3 BM for 1 d and B: for 3 days; C: HEK293t cells encapsulated in D250 CM2 TPH/D250 VS for 1 d and D: for 3 d; E: HCF encapsulated in D500 CM2 TPH/D500 VS for 1 d. Dead cells are marked red, living cells are marked green.

was stirred at room temperature for one day. The mixture was transferred to a dialysis tube and dialyzed for two days against a NaCl solution ($3\times20\ g\cdot L^{-1}$, $3\times10\ g\cdot L^{-1}$) and two days against water. After lyophilization, the product was obtained as a white, cotton wool-like solid (92 mg).

General procedure to prepare polysaccharides modified with fluoresceine and vinylsulfone

To a solution of vinyl sulfone modified carboxy-methylated polyasaccharide (100 mg, 1.0 equivalents) in water (50 ml) was added EDC·HCl (0.07 eq.), HOBt·H_2O (0.06 eq.), DIPEA (0.19 eq.) and the mixture was stirred at room temperature for 0.5 h. Fluorescein-amine (3 mg, 8.64 μmol) was then added and the solution was stirred at room temperature for 15 h. The mixture was transferred to a dialysis tube and dialyzed against a NaCl solution at pH 3.5 ($3\times20\ g\cdot L^{-1}$) for one day and against water at pH 3.5 for two days. After lyophilization, the product was obtained as a yellow, cotton wool-like solid (80 g).

General procedure to prepare polysaccharides modified with fluoreceine and TPH

To a solution of the carboxymethylated polysaccharide (100 mg) in water (10 ml) was added EDC·HCl (0.07 eq.), HOBt·H_2O (0.06 eq.), DIPEA (0.19 eq.) and the mixture was stirred at room temperature 0.5 h. Fluoresceinamine (3 mg, 8.64 μmol) was added and the solution was stirred at room temperature for 15 h. To the solution were added MES (976 mg, 5.0 mmol, 8 eq.), EDC·HCl (178 mg, 0.927 mmol, 1.5 eq.) and HOBt·H_2O (95 mg, 618 mmol, 1.0 eq.) and the solution was stirred at room temperature for 0.5 h. Then DTPH (73 mg, 0.306 mmol, 0.5 eq.) was added and the mixture was stirred at room temperature for 15 h. The pH was then raised to 7.5 (NaOH, 2 M) and then DTT (477 mg, 3.90 mmol, 5.0 eq.) was added. The pH was adjusted to 8.5 and the solution was stirred for 15 h. The reaction was terminated by adjusting the pH to 3.5 (HCl, 1 M). The mixture was transferred to a dialysis tube and dialyzed against a NaCl solution at pH 3.5 ($3\times20\ g\cdot L^{-1}$) for one day and against water at pH 3.5 for another day. After lyophilization, the product was obtained as a yellow cotton-like solid (93 mg).

General procedure to prepare maleimide modified polysaccharides

To a solution of carboxymethylated polysaccharides (200 mg) in water (100 ml) were added MES (488 mg, 2.5 mmol, 2 eq.), EDC·HCl (567 mg, 3.0 mmol, 2.5 eq.), and NHS (283 mg, 2.5 mmol, 2 eq.), and the mixture was stirred for 0.5 h at room temperature. Either linker 11a or 11b (0.6 eq for dextrans, 2 eq. for lentinan) was then added and the reaction mixture was stirred for 3 d at room temperature. The mixture was transferred to a dialysis tube and dialyzed against a NaCl solution ($3\times20\ g\cdot L^{-1}$, $3\times10\ g\cdot L^{-1}$) for two days and subsequently against water for two days. After lyophilization, the product was obtained as a white, cotton wool-like solid (180 mg).

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General procedure to prepare cysteamine vinyl sulfone-modified polysaccharides

To a solution of carboxymethylated polysaccharides (100 mg) in water (50 ml) were added MES (244 mg, 1.23 mmol, 2 eq.), EDC·HCl (296 mg, 1.54 mmol, 2.5 eq) and NHS (142 mg, 1.23 mmol, 2 eq.) and the mixture was stirred for 0.5 h at room temperature. Then, the TFA salt of cysteamine vinyl sulfone (**8**) (190 mg, 0.617 mmol, 1.0 equivalents) was added, th pH was adjusted to 6.1 (NaOH, 1 M) and the reaction mixture was stirred at room temperature for 3 d. The mixture was transferred to a dialysis tube and then dialyzed against a NaCl solution (3×20 $g\cdot L^{-1}$, 3×10 $g\cdot L^{-1}$) for two days and against water for two days. After lyophilization, the product was obtained as a white, cotton wool-like solid (83 mg).

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Conflict of Interests

The authors declare no conflict of interest (with content to this MS).

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: click chemistry · dextran · hydrogels · lentinan · pullulan

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