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PAPER

Bioorthogonal metal-free click-ligation of *c*RGD-pentapeptide to alginate†

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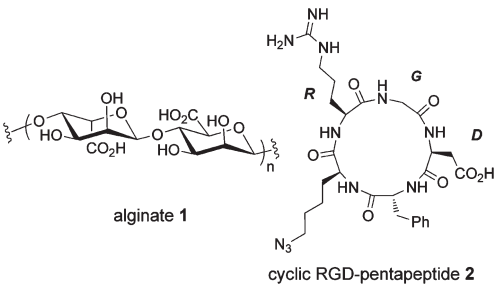
"Click" reactions have become very common and powerful ligation techniques, of which 1,3-dipolar cycloadditions have most frequently been employed. Since metal-mediated cycloadditions are incompatible in biomedical applications due to toxicity issues associated with transition metals like copper, metal-free variants provide important alternatives. The metal-free conjugation process is studied in detail with special emphasis put on the reaction progress. This report unfolds the first aqueous metal-free "click" conjugation of a cyclic RGD-pentapeptide with the biomacromolecule alginate, creating a "smart" bioactive polymer with potential applications in biomedicine.

Introduction

The last decade has seen the development of powerful synthetic methods to link or merge biomolecules with each other or other complex molecular entities. Terms like "click" reactions and chemical ligation are associated with this chemistry that has paved the way to specifically modify complex biological systems at will.[1] In this context, the preparation of peptide–polymer bioconjugates is a very important issue that can be realised by "grafting onto" or by "grafting from" approaches.[2] The most popular "grafting to" technique is the copper-mediated Huisgen-type 1,3-dipolar cycloaddition, first reported by Meldal and Sharpless, respectively.[3] This azido–alkyno cycloaddition is a versatile bioconjugation strategy as both functionalities react chemoselectively, while being rare or not present in nature. These so-called bioorthogonal reactions are insensitive to functional groups in biological systems.[4] As a consequence, "click" reactions are widely employed in biological and biomedical systems.[5] However, metal-catalyzed ligations are not well-suited due to toxicity issues which resulted in the development of several metal-free alternatives[6] that even work in aqueous media.[7] The best studied dipolar cycloadditions within this field rely on cyclooctyne as well as oxanorbornadiene derivatives.[8]

Our approach for decorating biopolymers with bioactive peptides requires components that are soluble in aqueous media which is guaranteed by Rutjes' oxanorbornadiene approach.[9,10] Indeed, this strategy serves multiple aspects for a biocompatible ligation: (i) solubility and reactivity under aqueous conditions, (ii) reaction progress at room temperature, (iii) no additives or catalysts required and (iv) formation of readily removable byproducts. The high reactivity and chemoselectivity of the oxanorbornadiene unit is associated with the vinylic CF_3-substituted olefin, which can undergo Huisgen-cycloadditions with 1,3-dipoles such as azides. Moreover, volatile furan is released as a by-product forcing the equilibrium towards product formation.[9]



Scheme 1 Structures of alginate **1** and azido-derivatised cyclic RGD-pentapeptide **2**.

We utilised Rutjes' method to modify the polysaccharide alginate with a cyclic RGD-pentapeptide for potential applications in regenerative medicine (Scheme 1). Due to specific interactions with integrins on the cell membrane, cyclic RGD-oligopeptides promote the adhesion of cells,[11] including stem cells.[12] To the best of our knowledge, we report the first metal-free ligation of these kinds of peptides with a biopolymer by using a metal-free 1,3-dipolar cycloaddition approach. In order to study the oxanorbornadiene mediated conjugation in detail we chose a model reaction with azido-valeric acid, both for the ligation on the polymeric as well as for the monomeric model level. ^{1}H- and ^{19}F-NMR spectroscopy served as a primary analytical tool. In detail, ^{19}F-NMR spectroscopy allowed us to identify the triazole linkages formed, while ^{1}H-NMR spectroscopy was suited to determine the progress of the reaction. In our hands, ^{19}F-NMR spectroscopy is generally applicable to detect and characterise

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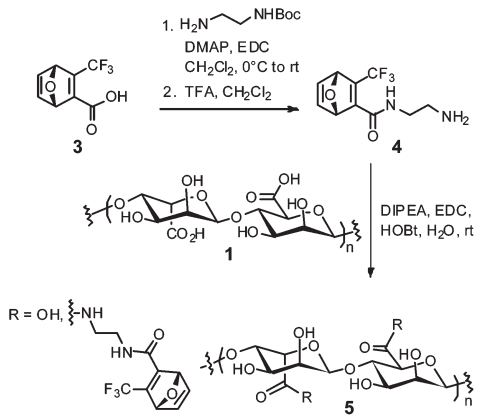
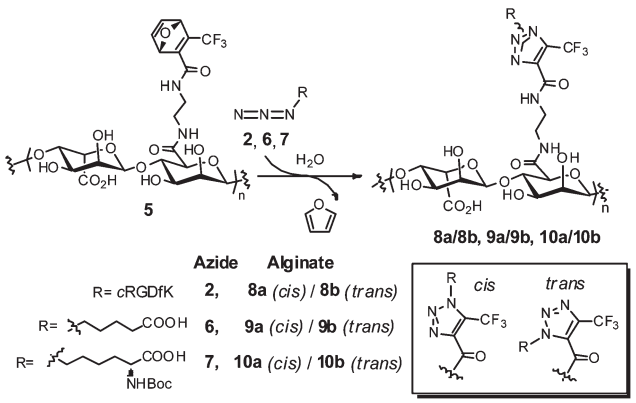


Scheme 2 Synthesis of functionalised alginate **5**.



Scheme 3 Metal-free cycloaddition of azido-*c*RGD **2**, 5-azido valeric acid **6** and Boc-protected ε-azido-lysine **7** yielding triazoles **8–10** (*cis* and *trans* refer to triazole regioisomers).

these fluoro-substituted triazoles, even when formed on polymers.

Results and discussion

Preparation of oxanorbornadiene functionalised alginate 5

Using a slightly modified preparation with respect to Rutjes and co-workers[9] bicycle **3**[13] was transformed into the amino derivative **4** and attached to the carboxylate moiety of alginate **1** (Scheme 2).

Oxanorbornadiene modified alginate **5** was prepared under classical active-ester conditions in an alkaline, aqueous medium. Depending on the added amount of **4**, the degree of functionalisation could be adjusted between 30% and 70%. Analysis of **5** was based on ^{1}H- and ^{19}F-NMR spectroscopy.[14]

The opportunity to adjust the degree of functionalisation in a variable manner is important from a biomedical point of view as it allows us to directly adjust the amount of the alginate bound RGD-peptide. The degree of decoration can either be controlled by the number of oxanorbornadiene functionalities present on the polymer or by the amount of azido *c*RGD-pentapeptide added. As the "click" process does not require additives, excess of unbound azido-conjugates can be simply regained after dialyses.

Preparation of alginate conjugates

Next, three azides, namely valeric acid derivative **6**, Boc-protected azido-lysine **7**[16] and azido-*c*RGD-pentapeptide **2**,[15] were fused with functionalised alginate **5** in aqueous media, yielding decorated alginates **8–10** (Scheme 3). Again, the successful ligation as well as the degree of decoration were proven by ^{1}H- and ^{19}F-NMR spectroscopy in D_2O.[14] The first two azides **6** and **7** served as model compounds for optimising the cycloaddition conditions as well as for establishing the analytical basis of this ligation process.

Due to its low solubility in aqueous solutions azido-*c*RGD-pentapeptide **2**[15] had to be added in sub-stoichiometric amounts. To prevent precipitation of the ligation product, polysaccharide **5** with a low degree of derivatisation (~30%) was used. With intense stirring, the mixture was allowed to react for 4 days in the dark. After dialyses and lyophilisation *c*RGD modified alginate **8** was collected. To the best of our knowledge, this is the first example of a metal-free conjugation of *c*RGD-peptide to a biopolymer alginate under physiologically compatible conditions.

In the case of **7**, only 0.5 equiv. of the azide derivative were added to alginate **5**. ^{19}F-NMR and ^{1}H-NMR analysis of this experiment showed that the expected amount of oxanorbornadiene was consumed and coupled, which in principle paves the way for a stepwise decoration using a second azide.

Detailed study of the ligation

The progress of cycloaddition was studied by ^{1}H-NMR, while ^{19}F-NMR spectroscopy was used to quantify and identify the nature of the cycloaddition products. It has to be stressed that the metal-free oxanorbornadiene cycloaddition generates two regioisomeric triazoles while furan is formed as a volatile byproduct.[9] Furan formation was used to monitor the progress of the cycloaddition and the degree of transformation (Fig. 1a). Simultaneously the oxanorbornadiene unit disappears and both processes served to track the reaction by ^{1}H-NMR spectroscopy using the signal at δ = 2.8 ppm (referring to remaining EDC) for calibration (Fig. 1b and 1c). In the case of 5-azido valeric acid **6** the cycloaddition was monitored over 29 h (Fig. 1b). As can be seen the integrals hardly changed after 25 h, indicating the end of the cycloaddition and almost complete transformation. As the furan by-product is rather volatile it could not serve for exact quantification.

When *c*RGD-pentapeptide **2** was added in sub-stoichiometric amounts, expectedly residual oxanorbornadiene signals originating from **5** could still be detected (Fig. 1c). Using 0.5 equiv. of *c*RGD-pentapeptide **2** the reaction was finished after 4 days when no further increase of furan formation was measured. Compared to azido-valeric acid **6** the reaction of *c*RGD **2** took twice as long, indicating the increased steric demand of the *c*RGD-pentapeptide as well as its lower water-solubility.

In their reports, Rutjes *et al.*[9] provided a detailed analysis of the cycloaddition that commonly provides two regioisomers (here named *cis*/*trans*; Scheme 4, path A). Additionally, they

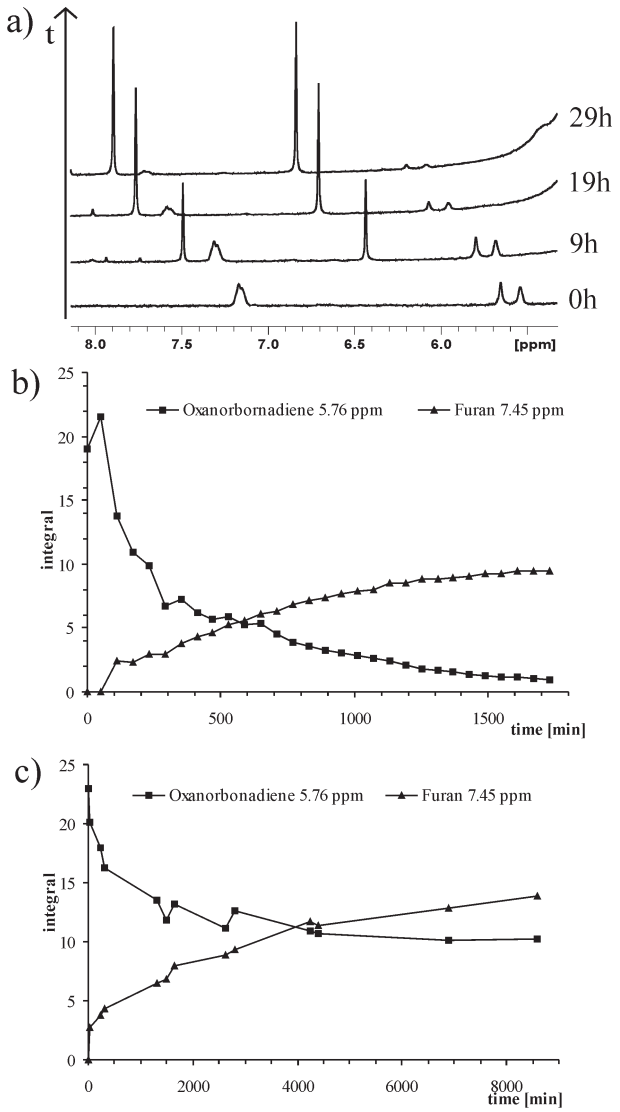


Fig. 1 (a) Ligation progress over time monitored by ^{1}H-NMR spectroscopy (staggered arrangement) exemplified for 5-azido valeric acid **6**: formation of furan (increasing signals) and simultaneous disappearance of an oxanorbornadiene system (decreasing signals). (b) Ligation of **6**: conversion over time; (c) ligation of **2**: conversion over time.

identified another by-product, a trifluoromethylfuran, that must have resulted from an alternative chemoselective azide-initiated cycloaddition with the second olefin (Scheme 4, path B). In principle, this side reaction can be minimised, when methyl-substituted oxanorbornadienes are employed.[10b] In the present case, non-polymeric compounds such as **11** which are formed *via* pathway B can advantageously be removed by dialyses. Still, the trifluoromethylfuran moiety, represented as **12**, is expected to remain at the alginate backbone.

However, ^{1}H-NMR spectroscopy does not allow us to identify the regio- and chemoisomers which are formed upon cycloaddition so we chose ^{19}F-NMR spectroscopy for detecting the trifluoromethyl groups of the different isomers present on the biopolymer (Fig. 2a–c).

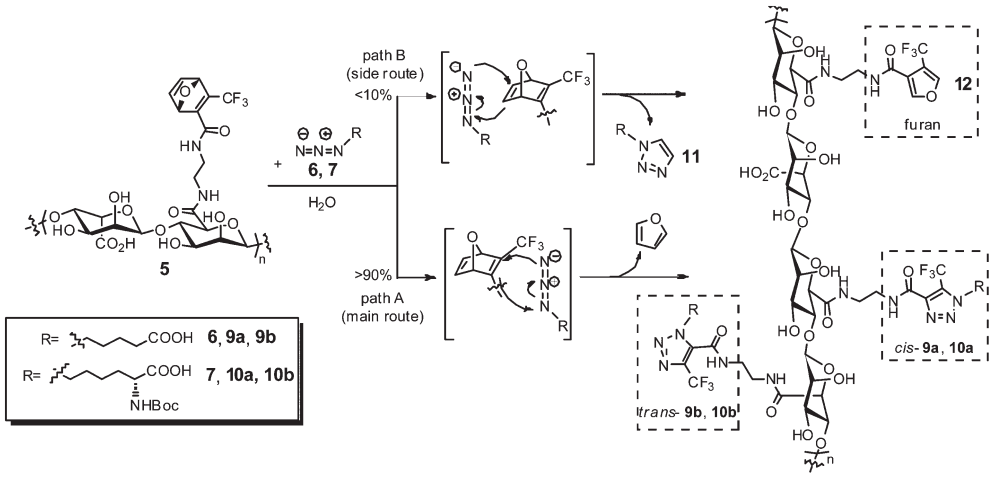
Functionalised alginate **5** gave a ^{19}F-NMR signal for the CF_3 moiety at $\delta = -62.5$ ppm (Fig. 2a). The cycloaddition of azides **6** and **7** resulted in decorated polymers that revealed a new set of ^{19}F signals (Fig. 2b and 2c). The signal at $\delta = -62.5$ ppm completely disappeared when an equimolar amount of **6** with respect to oxanorbornadiene units on alginate was added (Fig. 2b). Substoichiometric amounts of added azide **7** expectedly resulted in incomplete conversion (Fig. 2c). Nevertheless in both cases the same additional signal pattern was found. Likewise, conjugation of cyclic RGDfK-peptide **2** with alginate **5** gave a similar set of ^{19}F signals[14] that only showed marginal chemical shift difference to the ligation products resulting from azide **7**. Specifically, polymer bound ligation products **9** and **10** (Fig. 2b and 2c) revealed new ^{19}F-data at −56.3 ppm (35%) and two signals at −59.1 ppm and −59.3 ppm, respectively, with reduced intensities (together 55%). In addition, a fractional signal at −56.8 ppm (10%) was detected.

As mentioned before, it was not possible to conduct a detailed analysis with these biomacromolecules. Therefore, we prepared model compounds that reflect the mode of cycloaddition products and would help to elucidate the chemical environment of the ^{19}F signals detected on alginates **8–10**. We chose the monosaccharide galacturonic acid as an adequate model compound. Methyl-(allyl-β-D-galactopyranoside)uronate **13** was prepared according to Voss *et al.*[17] Joining the oxanorbornadiene **4** with galacturonic acid **13** was accomplished by first saponification which was followed by coupling under standard conditions. Compound **14** was collected after preparative HPLC purification. In order to clarify the polymer ligation data, 5-azido valeric acid **6** was reacted with **14** under aqueous conditions as described above for alginate (Scheme 5). The cycloaddition products **15a** and **15b** were separated by preparative HPLC and characterised[14] using different NMR techniques (^{1}H-, ^{13}C-, HSQC-, HMBC- and ^{19}F-NMR).

The chemical shifts for the ^{19}F-NMR signals in 5-trifluoromethyl-1,2,3-triazole **15a** ($\delta = -56.3$ ppm, *cis* regioisomer; Fig. 2d) and in 4-trifluoromethyl-1,2,3-triazole **15b** ($\delta = -59.3$ ppm, *trans* regioisomer; Fig. 2e) differed significantly and can favourably be assigned to the signals of the ligation products **9** and **10** (Fig. 2b and 2c). The broader signals observed for the polymer samples are due to longer relaxation times associated with coiled macromolecular backbone structures.

In order to elucidate the nature of the remaining signals (Fig. 2b and 2c) we prepared a second model compound **20** that represents the furan moiety **12** of the undesired pathway B (Scheme 4).[9] The appropriate model compound **20** was prepared in four steps (Scheme 6). Starting from oxanorbornadiene **16**, furan **18** was synthesised by a [4 + 2] Diels–Alder cycloaddition–cycloreversion sequence between **16** and tetraphenylcyclopentadienone **17**.[18] As byproducts, carbon monoxide and tetraphenylbenzene are formed. Trifluoromethyl-substituted furan **18** was first saponificated followed by amide formation with *N*-Boc-ethylendiamine and Boc-deprotection to yield the target furan **20** soluble in aqueous media.

^{19}F-NMR spectroscopic analysis of furan **20** displayed a signal at $\delta = -59.1$ ppm in D_2O. Obviously, the chemical shifts of the trifluoromethyl group in the unfavoured furan by-product and (*trans*)-regioisomer **15b** are very similar. Thus, close inspection of the ^{19}F signals in modified alginates **9** and **10** reveals flanking peaks around $\delta = -59$ ppm (Fig. 2b and 2c). Signal intensities indicate that route B becomes less neglectable when



Scheme 4 Regular cycloaddition provides two regioisomeric triazoles (path A, represented by substructures **9** and **10**); uncommon cycloaddition with an alternative alkene moiety results in the formation of by-products (path B, represented by substructures **11** and **12**) as proposed by Rutjes *et al.* for a non-polymeric system.[9]

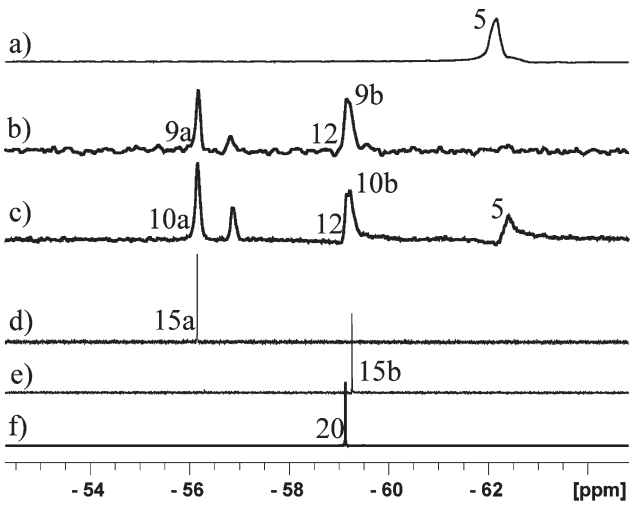
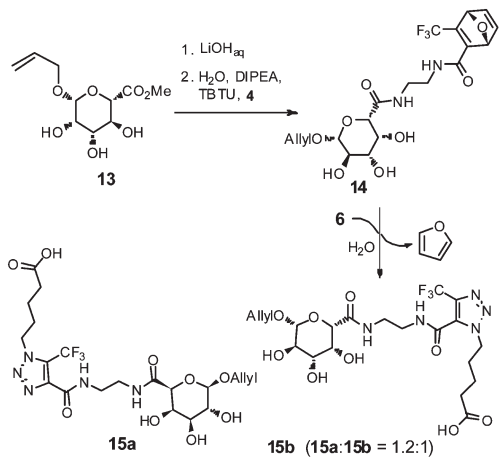
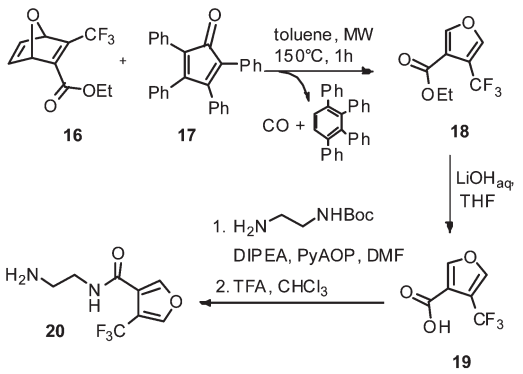


Fig. 2 ^{19}F-NMR spectroscopic data of (a) starting material **5**; (b) addition of 1.0 equiv. **6**; (c) addition of 0.5 equiv. **7**; (d) galacturonic acid *cis*-triazole **15a**; (e) galacturonic acid *trans*-triazole **15b**; (f) furan derivative **20**.



Scheme 5 Synthesis of galacturonic acid derivatives **15a** and **15b**.



Scheme 6 Synthesis of model compound **20**.

switching from solution chemistry to cycloadditions of polymer-bound oxanorbornadiene with azides. Apparently, steric congestion and folding of the polymer backbone do affect the accessibility of both olefinic groups in the oxanorbornadiene system.

Thus, formation of undesired cycloaddition products of type **12** cannot be completely avoided in the case of polymer conjugation. Finally, at this point the chemical environment of the remaining ^{19}F-NMR signal at $\delta = -56.8$ ppm (Fig. 2b and 2c) could not be elucidated.

Conclusions

In summary, we developed a synthetic route for the successful conjugation of *c*RGD-pentapeptides to modified alginate by employing a metal-free 1,3-dipolar cycloaddition approach. The ligation proceeded within 3 days at room temperature in aqueous media. The procedure relies on the initial implementation of the oxanorbornadiene which was followed by a mild, bioorthogonal cycloaddition. Progress of alginate decoration was monitored by

^{1}H-NMR and ^{19}F-NMR spectroscopy. Model compounds were synthesised to evaluate the regio- and chemoselectivity of the cycloaddition that yielded substituted triazoles. So far, this is the first study to analytically follow metal-free "click"-reactions conducted with a biomacromolecule. Finally, the feasibility of a macromolecular metal-free ligation under physiological conditions was proven. Future studies will be directed to develop RGD-modified hydrogels based on different biomacromolecules and study their biocompatibility in the context of regenerative therapies and tissue engineering.

Experimental

General remarks

Unless otherwise stated, all chemicals and solvents were purchased in per analysis quality 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^{-1}. All modified polymers were exhaustively dialysed for 3 to 5 days against distilled water. Lyophilisation was performed with a Christ Alpha 2–4 (Christ, Osterode, Germany) freeze dryer. Melting points (Mp) were determined with an MPA100 OptiMelt instrument (Stanford Research Systems). ^{1}H NMR spectra were recorded at 400 MHz and ^{13}C NMR spectra were recorded at 100 MHz with a BRUKER Avance-400. ^{19}F-NMR spectra were recorded with a BRUKER Avance-III-600. Chemical shift values of NMR data are reported as values in ppm relative to (residual undeuterated) the solvent signal as an internal standard. Multiplicities for ^{1}H NMR signals are described using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet; where appropriate with the addition of b = broad. ^{13}C Multiplicities refer to the resonances in the off-resonance decoupled spectra and were elucidated using the distortionless enhancement by the polarisation transfer (DEPT) spectral editing technique. Multiplicities for ^{13}C NMR signals are reported using the following abbreviation: q = quartet (CF_3). For interpretation of the triazole regioisomers (^{1}H–^{13}C correlations) phase-sensitive HSQC and HMBC experiments were performed. Mass spectra were obtained with a type LCT (ESI) (Micromass) equipped with a lockspray dual ion source in combination with a Waters Alliance 2695 LC system, or with a type Q-TOF premier (Micromass) spectrometer (ESI mode) in combination with a Waters Acquity UPLC system equipped with a Waters BEH C18 1.7 µm column (solvent A: water + 0.1% (v/v) formic acid; solvent B: MeOH + 0.1% (v/v) formic acid; flow rate = 0.4 mL min^{-1}; gradient (*t* [min]/solvent B [%]): (0 : 5) (2.5 : 95) (6.5 : 95) (6.6 : 5) (8 : 5)). Ion mass signals (*m*/*z*) are reported as values in atomic mass units.

Synthesis of the ethylendiamine extended oxanorbornadiene-system (4). Based on the results of Rutjes and co-workers[9] a modified synthesis of 3-trifluoromethyl-7-oxa-bicyclo[2.2.1]-hepta-2,5-diene-2-carboxylic acid **3** was published by Dräger and co-workers.[13]

***tert*-Butyl [2-(-3-(trifluoromethyl)-7-oxabicyclo[2.2.1]hepta-2,5-diene-2-carboxamido)ethyl]carbamate (3a). 3** (100 mg, 0.481 mmol, 1.0 equiv.) and *N*-Boc-ethylendiamine HCl (75.8 µL, 0.481 mmol, 1.0 equiv.) were dissolved in 1 mL of dry methylene chloride under an argon atmosphere at 0 °C. 4-Dimethylaminopyridine (117.5 mg, 0.962 mmol, 2.0 equiv.) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (101.1 mg, 0.529 mmol, 1.1 equiv.) were added and the reaction mixture was stirred overnight at rt. The mixture was washed with brine, the layers were separated and the organic layer was dried over $MgSO_4$. After purification by column chromatography (petroleum ether–ethyl acetate = 1 : 1) **3a** was isolated as a yellowish solid (102.3 mg, 293.9 µmol, 61%). Mp: 145 °C; R_f = 0.45 (PE–EtOAc: 1 : 1); δ_H (400 MHz, $CDCl_3$) 7.37 (dd, *J* 5.3, *J* 1.9, 1H), 7.17 (dd, *J* 5.1, *J* 1.7, 1H), 6.66 (bs, 1H), 5.65–5.64 (m, 1H), 5.62–5.62 (m, 1H), 4.86 (bs, 1H), 3.51–3.41 (m, 1H), 3.35–3.29 (m, 1H), 1.46 (s, 9H); δ_C (100 MHz, $CDCl_3$) 162.7, 157.0, 154.1 (q, $J_{C,F}$ 4.9), 143.8 (q, $J_{C,F}$ 36.3), 143.8, 142.2, 122.2 (q, $J_{C,F}$ 268.4), 86.2, 83.7 (t, $J_{C,F}$ 2.3), 80.2, 41.3, 40.0, 28.5; MS (ESI$^+$): *m*/*z* 371.12 $[M + Na]^+$, calcd for $C_{15}H_{19}F_3N_2O_4Na$ 371.12.

***N*-(2-Aminoethyl)-3-(trifluoromethyl)-7-oxabicyclo[2.2.1]hepta-2,5-diene-2-carboxamide (4).** To a solution of **3a** (6.0 mg, 17.2 mmol, 1.0 equiv.) in 1 mL of methylene chloride, trifluoroacetic acid was added (TFA, 60 µL, excess) successively and stirred for 1 h. All solvents were removed by repeated azeotropic distillation with toluene under reduced pressure. Finally, **4** was isolated as a colourless solid (4.3 mg, 17.2 mmol, quant.). Mp: 155 °C; δ_H (400 MHz, CD_3OD) 7.33 (dd, *J* 5.3, *J* 1.9, 1H), 7.25 (dd, *J* 5.3, *J* 1.9, 1H), 5.70–5.69 (m, 1H), 5.64–5.63 (m, 1H), 3.66–3.59 (m, 1H), 3.53–3.46 (m, 1H), 3.11 (dt, *J* 6.3, *J* 2.7, 2H); δ_C (100 MHz, CD_3OD) 166.0, 155.8 (q, $J_{C,F}$ 5.1), 145.9 (q, $J_{C,F}$ 36.9), 144.6, 143.67, 123.76 (q, $J_{C,F}$ 267.7), 87.1, 84.6 (t, $J_{C,F}$ 2.2), 40.5, 38.34; MS (ESI$^+$): *m*/*z* 249.08 $[M + H]^+$, calcd for $C_{10}H_{12}F_3N_2O_2$ 249.08.

Oxanorbornadiene functionalised alginate (5). Sodium alginate **1** (0.1 g, 0.51 mmol, 1.0 equiv., very low viscosity, ABCR, Germany) was dissolved to homogeneity in 10 mL of $H_2O_{dist.}$ yielding a 1% (w/v) solution. Diisopropylethylamine (166 µL, 1.01 mmol, 2.0 equiv.), 1-hydroxybenzotriazole-hydrate (78.0 mg, 0.51 mmol, 1.0 equiv.) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (146.1 mg, 0.76 mmol, 1.5 equiv.) were added and the reaction mixture was stirred for 1 h. Then, **4** (151.7 mg, 0.61 mmol, 1.2 equiv.) was added and the solution was stirred at room temperature overnight. In order to precipitate the polymer, the aqueous solution was added dropwise to 20 mL of ethanol. After complete settling of the solid, the colourless polymer was filtered off, dissolved in water and purified by dialysis against $H_2O_{dist.}$ for 4 days. The water was exchanged three times a day. After lyophilisation polymer **5** was collected as a colourless, fluffy material (115 mg). δ_H (400 MHz, D_2O) 7.28–7.26 (m, 2H), 5.86 (s, 1H), 5.73 (s, 1H), 4.13–3.56 (m, alginate), 3.43–3.02 (m, 4H); δ_F (600 MHz, D_2O) −62.5.

Azido-*c*RGDfK conjugated to oxanorbornadiene functionalised alginate (8). Oxanorbornadiene functionalised alginate **5** (10.0 mg, 0.025 mmol, 1.0 equiv., 30% derivatisation rate) was dissolved to homogeneity in 3 mL of $H_2O_{dist.}$. Azido-*c*RGDfK **2** (7.8 mg, 12.5 µmol, 0.5 equiv.) was added and the reaction mixture was stirred for 4 days in the dark. Then, the reaction

mixture was directly dialysed against $H_2O_{dist.}$ for 4 days with repeated water exchange (3× per day). After lyophilisation a colourless, fluffy material **8** was isolated (4 mg). δ_H (400 MHz, D_2O) 7.32–7.15 (m, 2H), 4.13–3.56 (m, alginate), 3.43 (s, 2H), 3.29 (s, 2H), 1.61–0.88 (m, 6H); δ_F (600 MHz, D_2O) −56.1, −56.8, −57.5, −62.3.

5-Azidovaleric acid conjugated to oxanorbornadiene functionalised alginate (9). Oxanorbornadiene functionalised alginate **5** (10.0 mg, 0.025 mmol, 1.0 equiv., 70% derivatisation rate) was dissolved to homogeneity in 3 mL of $H_2O_{dist.}$. 5-Azido valeric acid **6** (3.5 mg, 0.026 mmol, 1.0 equiv.) was added and the reaction mixture was stirred for 1 day in the dark. Then, the reaction mixture was directly dialysed against $H_2O_{dist.}$ for 4 days with repeated water exchange (3× per day). After lyophilisation polymer **9** was collected as a colourless, fluffy material (6 mg). δ_H (400 MHz, D_2O) 4.60–4.53 (m, 2H, **9b**), 4.49–4.43 (m, 2H, **9a**), 4.13–3.65 (m, alginate), 3.48–3.43 (m, 2H), 3.06–3.04 (m, 2H), 2.31–2.22 (m, 2H), 1.92–1.79 (m, 2H), 1.57–1.43 (m, 2H); δ_F (600 MHz, D_2O) −56.2 (**9a**), −56.8, −59.1 (**12**), −59.2 (**9b**).

ε-Azido-Boc-lysine conjugated to oxanorbornadiene functionalised alginate (10). Oxanorbornadiene functionalised alginate **5** (10.0 mg, 0.025 mmol, 1.0 equiv., 70% derivatisation rate) was dissolved to homogeneity in 3 mL of $H_2O_{dist.}$. Azido-Boc-lysine **7** (3.4 mg, 0.013 mmol, 0.5 equiv.) was added and the reaction mixture was stirred for 2 days in the dark. Then, the reaction mixture was directly dialysed against $H_2O_{dist.}$ for 4 days with repeated water exchange (3× per day). After lyophilisation polymer **10** was collected as a colourless, fluffy material (7 mg). δ_H (400 MHz, D_2O) 7.29–7.26 (m, 2H), 5.85 (s, 1H), 5.74 (s, 1H), 4.65–4.60 (m, 2H, **10b**), 4.55–4.50 (2H, m, **10a**), 4.17–3.66 (m, alginate), 3.63–3.19 (m, 8H), 3.14 (m, 2H), 1.93–1.85 (m, 2H), 1.63–1.59 (m, 2H), 1.40 (s, 9H); δ_F (600 MHz, D_2O) −56.1 (**10a**), −56.8, −59.1 (**12**), −59.2 (**10b**), −62.3 (**5**).

(Allyl-β-D-galactopyranoside) uronic acid (13a). Methyl-(allyl-β-D-galactopyranoside) uronate **13** was prepared according to Voss *et al.*[17] 0.1 g of **13** (0.40 mmol, 1.0 equiv.) was dissolved in 5 mL $H_2O_{dist.}$ and ~2 mL 1 M $LiOH_{aq}$ (2.02 mmol, 5.0 equiv.) were added until an alkaline pH was obtained. After stirring for 12 h at room temperature, the reaction mixture was neutralised using 1 M HCl_{aq}. A mixture of colourless crystals, monosaccharides and LiCl was isolated after lyophilisation (120 mg). δ_H (400 MHz, D_2O) 6.04–5.94 (m, 1H), 5.38 (dd, *J* 17.3, *J* 1.5, 1H), 5.27 (dd, *J* 9.9, *J* 0.7, 1H), 4.46–4.41 (m, 1H), 4.43 (d, *J* 7.8, 1H), 4.24–4.19 (m, 1H), 4.22 (dd, *J* 0.7, *J* 4.1, 1H), 4.12 (d, *J* 1.4, 1H), 3.68 (dd, *J* 9.9, *J* 3.4, 1H), 3.53 (dd, *J* 9.9, *J* 7.85, 1H); δ_C (100 MHz, D_2O) 174.4, 133.5, 118.8, 101.2, 75.2, 72.9, 70.4, 70.3, 70.0; MS (ESI^-): *m*/*z* 233.07 $[M - H]^-$, calcd for $C_9H_{13}O_7$ 233.06.

Oxanorbornadiene functionalised galacturonic acid (14). 13a (50 mg, 0.21 mmol, 1.0 equiv.) was dissolved in 3 mL of $H_2O_{dist.}$. Then, diisopropylethylamine (70 µL, 0.42 mmol, 2.0 equiv.) and *O*-benzotriazole-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU, 97 mg, 0.25 mmol, 1.2 equiv.) were added and the mixture was stirred at room temperature for 1 h. Afterwards **4** (53 mg, 0.21 mmol, 1.0 equiv.) was added and the reaction mixture was stirred for another 12 h. The mixture was lyophilised and purified by preparative HPLC. (C18-P[A]) (H_2O–MeOH 95 : 5 → 90 : 10 {5 min}, 90 : 10 → 60 : 40 {55 min}, 60 : 40 → 30 : 70 {30 min}, 30 : 70 → 0 : 100 {10 min}, 15 mL min^{-1}). Compound **14** was collected at a retention time of t_R = 68–72 min as a colourless waxy solid (9.8 mg, 21.12 µmol, 10%). δ_H (400 MHz, D_2O) 7.37–7.32 (m, 2H), 6.03–5.92 (m, 1H), 5.85–5.84 (m, 1H), 5.72–5.71 (m, 1H), 5.40–5.27 (m, 2H), 4.47 (d, *J* 7.4, 1H), 4.44–4.36 (m, 1H), 4.23–4.15 (m, 1H), 4.21 (d, *J* 1.4, 1H), 3.72–3.68 (m, 1H), 3.60–3.58 (m, 1H), 3.56–3.52 (m, 2H), 3.47–3.32 (m, 2H); δ_C (100 MHz, D_2O) 170.9, 170.6, 142.9, 142.9, 142.8, 142.7, 133.1, 118.8, 101.3, 85.5, 83.0, 74.5, 72.4, 70.5, 70.1, 69.0, 38.5, 38.2; MS (ESI^+): *m*/*z* 465.15 $[M + H]^+$, calcd for $C_{19}H_{23}N_2O_8F_3$ 465.15.

5-Azidovaleric acid conjugated to oxanorbornadiene functionalised galacturonic acid (15a + 15b). 14 (5 mg, 0.011 mmol, 1.0 equiv.) and 5-azido-valeric acid **6** (2.5 µL, 0.016 mmol, 1.5 equiv.) were dissolved in 1 mL of water. The reaction mixture was stirred at room temperature for 2 days and lyophilised. The solid was purified and separated by semipreparative HPLC (C18-P[A]; 1% aqueous ammonium acetate–MeOH 90 : 10 {80 min}, 50 : 50 {20 min}, 0 : 100 {10 min}). Regioisomer **15a** was collected at a retention time of t_R = 51.17 min, while regioisomer **15b** was collected at a retention time of t_R = 55.65 min. Both products are colourless creamy solids (**15a**: 1.1 mg, 2.0 µmol, 19%; **15b**: 0.9 mg, 1.7 µmol, 15%). δ_H (400 MHz, D_2O) 5.90–5.82 (m, 1H), 5.22 (dd, *J* 17.3, *J* 1.4, 1H), 5.16 (dd, *J* 10.5, *J* 0.7, 1H), 4.60 (t, *J* 7.2, 2H), 4.41 (d, *J* 7.9, 1H), 4.28–4.24 (m, 1H), 4.15 (dd, *J* 3.6, *J* 1.0, 1H), 4.13 (d, *J* 1.1, 1H), 4.11–4.07 (m, 1H), 3.64 (dd, *J* 9.8, *J* 3.4, 1H), 3.60–3.56 (m, 2H), 3.54 (dd, *J* 9.7, *J* 8.1, 1H), 3.40–3.35 (m, 2H), 2.24 (t, *J* 7.4, 2H), 1.90 (q, *J* 8.1, 2H), 1.54 (q, *J* 7.6, 2H); δ_C (100 MHz, D_2O) 180.9, 170.5, 160.9, 141.4, 133.0, 126.9 (q, $J_{C,F}$ 42.8), 118.9 (q, $J_{C,F}$ 269.6), 118.8, 101.3, 74.6, 72.3, 70.5, 70.1, 68.9, 51.3, 38.4, 38.3, 35.1, 28.7, 22.1; δ_F (600 MHz, D_2O) −56.3; MS (ESI^+): *m*/*z* 540.19 $[M + H]^+$, calcd for $C_{20}H_{29}N_5O_9F_3$ 540.19 (**15a**). δ_H (400 MHz, D_2O) 5.93–5.86 (m, 1H), 5.25 (dd, *J* 17.2, *J* 1.5, 1H), 5.19 (dd, *J* 10.5, *J* 1.1, 1H), 4.49 (t, *J* 6.9, 2H), 4.45 (d, *J* 7.9, 1H), 4.36–4.32 (m, 1H), 4.17–4.12 (m, 1H), 4.15 (dd, *J* 3.6, *J* 1.0, 1H), 4.13 (d, *J* 1.0, 1H), 3.66 (dd, *J* 10.9, *J* 3.1, 1H), 3.60–3.55 (m, 2H), 3.49 (dd, *J* 9.8, *J* 8.0, 1H), 3.39–3.33 (m, 2H), 2.18 (t, *J* 7.4, 2H), 1.85 (q, *J* 7.2, 2H), 1.45 (q, *J* 7.8, 2H); δ_C (100 MHz, D_2O) 181.4, 170.5, 158.9, 135.2 (q, $J_{C,F}$ 39.4), 133.0, 131,7, 119.7 (q, $J_{C,F}$ 268.6), 118.6, 101.3, 74.5, 72.3, 70.5, 70.1, 69.1, 49.9, 38.8, 38.3, 35.7, 28.7, 22.1; δ_F (600 MHz, D_2O) −59.3; MS (ESI^+): *m*/*z* 540.19 $[M + H]^+$, calcd for $C_{20}H_{29}N_5O_9F_3$ 540.19 (**15b**).

4-Trifluoromethylfuran-3-carboxylic acid (19). 3-(Trifluoromethyl)-4-ethoxycarbonylfuran **18** was prepared according to Nezis *et al.*[18] **18** (30.0 mg, 0.15 mmol, 1.0 equiv.) was dissolved in 500 µL THF, and 200 µL $LiOH_{aq}$ (1 M) was added. The reaction mixture was stirred over night at room temperature and extracted with ethyl acetate. HCl_{aq} (1 M) was added to the aqueous phase until a pH of 2 was reached. The aqueous phase was extracted with diethyl ether, the combined organic layers

were dried over $MgSO_4$ and the solvent was evaporated under reduced pressure. Compound **19** was obtained as a colourless solid (11.8 mg, 65.6 μmol, 47%). Mp: 118 °C; δ_H (400 MHz, $CDCl_3$) 8.18 (s, 1H), 7.84 (s, 1H); δ_C (100 MHz, $CDCl_3$) 165.9, 151.8, 145.2 (q, $J_{C,F}$ 6.5), 125.3, 121.3 (q, $J_{C,F}$ 267.3), 116.6 (q, $J_{C,F}$ 38.8); δ_F (600 MHz, D_2O) −58.7; MS (ESI^-): *m/z* 178.99 $[M - H]^-$, calcd for $C_6H_2F_3O_3$ 178.99.

***tert*-Butyl (2-(4-(trifluoromethyl)furan-3-carboxamido)ethyl) carbamate (19a).** **19** (15.0 mg, 0.083 mmol, 1.0 equiv.) and *N*-Boc-ethylendiamine (15 μL, 0.094 mmol, 1.2 equiv.) were dissolved in DMF (2 mL). DIPEA (30 μL, 0.182 mmol, 2.2 equiv.) and (7-azabenzotriazole-1-yloxy) tripyrrolidino-phosphonium hexafluorophosphate (PyAOP, 85 mg, 0.163 mmol, 2.0 equiv.) were added and the resulting reaction mixture was stirred for 12 h. The solvent was removed under reduced pressure, the crude product was dissolved in ethyl acetate, washed with water, the layers were separated and the organic layer was dried over $MgSO_4$. Ethyl acetate was removed under reduced pressure until an oily residue was obtained. After purification by column chromatography (ethyl acetate), followed by recrystallisation in chloroform, **19a** was isolated as a colourless solid (22.5 mg, 69.9 μmol, 84%). Mp: 130 °C; R_f = 0.8 (EtOAc); δ_H (400 MHz, $CDCl_3$) 7.93 (s, 1H), 7.78 (s, 1H), 6.88 (bs, 1H), 4.99 (bs, 1H), 3.51 (q, *J* 5.5, 2H), 3.35 (q, *J* 5.5, 2H), 1.41 (s, 9H); δ_C (100 MHz, $CDCl_3$) 160.8, 157.3, 146.7, 144.5 (q, $J_{C,F}$ 6.5), 123.2 (q, $J_{C,F}$ 267.3), 120.1, 115.9 (q, $J_{C,F}$ 34.8), 79.9, 41.3, 39.9, 28.3; δ_F (600 MHz, $CDCl_3$) −58.4; MS (ESI^+): *m/z* 345.10 $[M + Na]^+$, calcd for $C_{13}H_{17}N_2O_4F_3Na$ 345.10.

***N*-(2-Aminoethyl)-4-(trifluoromethyl)furan-3-carboxamide (20).** *t*-Butyl {2-[4-(trifluoromethyl) furan-3-carboxamido]ethyl} carbamate **19a** (5 mg, 0.016 mmol, 1 equiv.) was dissolved in CH_2Cl_2 (2 mL). Trifluoroacetic acid (TFA, 12 μL, 0.155 mmol, 10 equiv.) was added and the resulting mixture was stirred for 2 h at room temperature. All solvents as well as TFA were removed under reduced pressure by azeotropic distillation with toluene (3 mL). After drying at <0.1 mbar **20** was obtained as a colourless solid (2.2 mg, 9.9 μmol, 64%). Mp: 65 °C; δ_H (400 MHz, $CDCl_3$) 8.11–8.10 (m, 1H), 8.08–8.07 (m, 1H), 3.64 (q, *J* 6.0, 2H), 3.35 (q, *J* 6.0, 2H); δ_C (100 MHz, $CDCl_3$) 164.3, 146.7, 145.7 (q, $J_{C,F}$ 6.3), 121.8 (q, $J_{C,F}$ 266.3), 118.2, 115.5 (q, $J_{C,F}$ 43.6), 38.9, 36.9; δ_F (600 MHz, D_2O) −59.1; MS (ESI^+): *m/z* 223.07 $[M + H]^+$, calcd for $C_8H_{10}N_2O_2F_3$ 345.10.

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