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1 **Injectable biocompatible hydrogels with tunable strength based on** 2 **crosslinked supramolecular polymer nanofibers**

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15 Keywords: Supramolecular polymer bottlebrushes, polymer networks, self-assembly, shear-
16 thinning, stealth properties.

17 **Abstract**

18 Hydrogels based on supramolecular assemblies offer attractive features for biomedical applications
19 including injectability or versatile combinations of various building blocks. We here investigate a
20 system combining benzenetrispeptides (BTP), which forms supramolecular fibers, with the
21 polymer polyethylene oxide (PEO) forming a dense hydrophilic shell around the fibers. Hydrogels
22 are created through addition of a bifunctional crosslinker (CL). Rheological studies revealed that
23 shorter hydrophobic *n*-hexyl spacers (BTP-C₆) lead to stronger hydrogels than the BTP-C₁₂
24 comprising *n*-dodecyl chains. All hydrogels recover rapidly (< 5 s) after deformation in step-strain-



25 measurements. We varied the crosslinker content between 0.1, 1 and 10 mol% and the overall
26 concentration of the gelator. While the shear storage modulus of all BTP-C₁₂ hydrogels remains
27 below 1 kPa independent of the variations, the shear storage modulus BTP-C₆ hydrogels can be
28 tuned from around 0.2 kPa up to almost 8 kPa. Shear rate dependent viscosity measurements
29 further revealed similar shear thinning behavior of all hydrogels, and calculation of extrusion
30 parameters confirms that the hydrogels can easily be injected even through thin cannulas.
31 Accordingly, we injected a fluorescein containing BTP-C₆ sample into chicken breast
32 demonstrating the potential for application as injectable drug depot. Furthermore, BTP-C₆
33 hydrogels prevent adherence of L929 mouse fibroblasts but preserve their relative metabolic
34 activity (>87%) during incubation on the gel when compared to cells growing on adherent surfaces.
35 Our investigations overall reveal that particularly the BTP-C₆ system has attractive features for
36 applications in tissue engineering or as injectable and biocompatible drug depot.

37 Introduction

38 A plethora of examples regarding self-assembly processes of biomacromolecules in nature (e.g.
39 nucleic acids, proteins, and polysaccharides) has inspired the research on supramolecular based
40 hydrogels and their biomedical applications such as tissue regeneration, drug delivery and artificial
41 cell scaffold.¹⁻³ Dissimilar to conventional synthetic hydrogels, which are made of three-
42 dimensional networks of polymer chains linked by permanent covalent bonds, supramolecular
43 hydrogels form through self-assembly of small organic molecules (hydrogelators) by noncovalent
44 interactions.⁴⁻⁹ These intermolecular forces often result in a dynamic system at room temperature,
45 where a liquification (viscous flow) of the supramolecular hydrogels can occur given a sufficient
46 shear force (shear thinning) and the system recovers once the applied force is removed (self-
47 healing).³ The peculiar viscoelastic properties and self-healing ability of supramolecular hydrogels
48 make them promising candidates for applications, such as injectability for tissue engineering or
49 drug delivery.^{1, 2, 10-14}

50 Ureido-pyrimidinone, peptides, benzenetrisamides, and benzenetrisurea are common examples of
51 supramolecular motifs of hydrogels displaying shear thinning behavior.¹⁵⁻²² These systems mainly
52 rely on H-bond interactions and on hydrophilic polymers, mostly polyethylene oxide (PEO), which



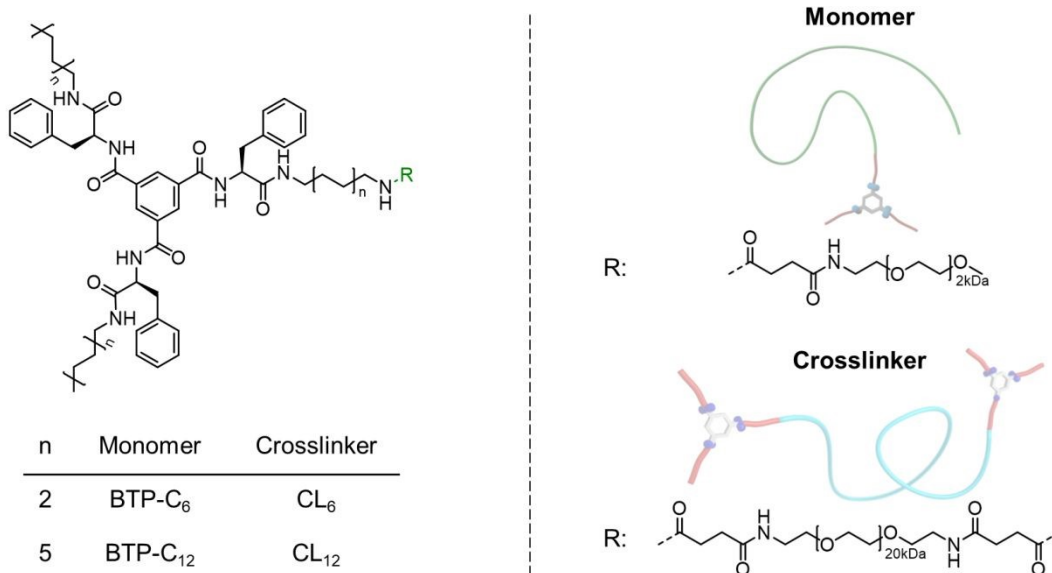
53 are functionalized on their end groups with the corresponding supramolecular motifs. There are
54 different ways these motifs can be utilized. One approach uses the interactions of the end groups
55 in star or branched polymers to form a large crosslinked network in which the end groups form
56 junction sides that act as linkage between the PEO chains.²³⁻²⁶ A different approach utilizes
57 hydrophobic supramolecular motifs such as ureidopyrimidinone, benzenetrisamides and
58 benzenetrisureas (BTU) decorated with hydrophilic groups to obtain amphiphilic molecules, which
59 are able to form fibers through self-assembly.^{16, 22, 27, 28} An advantage of fiber formation, is that
60 through overlapping of the fibers or defects in the structure junction sides are formed, which lead
61 to gel formation.²⁹ Even though some of these systems already form hydrogels without a special
62 crosslinker, the gels become stronger when a homotelechelic polymer with the motif on both end
63 groups is utilized as a crosslinker. Here the crosslinker is integrated during the assembly step to
64 ensure an even distribution throughout the fiber. In respect of biological applications, fast recovery
65 kinetics and the optimum shear force are crucial parameters when designing supramolecular
66 hydrogels.^{30, 31} For instance, fast recovery rates were observed in hydrogels based on peptides.^{19,}
67 ^{32, 33}

68 Previously, our group demonstrated that the BTU motif is able to form hydrogels with a strong
69 shear thinning character enabling 3D printing.²⁸ Although these results are promising, BTUs allow
70 almost no modification of their hydrophobic interactions by varying the spacers nor any changes
71 of the hydrogen bonding units without disturbing the assembly process.³⁴ These aspects limit the
72 adaptability of the hydrogels in terms of mechanical or rheological properties, which are crucial
73 for cell and tissue interactions. Therefore, we explored benzenetrispeptide (BTP) based
74 supramolecular motifs, which form self-assembled fibers when conjugated with PEO chains that
75 can be varied in their length by adjusting the kinetics of the assembly process.^{35, 36} The strong
76 interaction of the BTP allows more variations of the hydrophobic groups as for the benzenetrisurea
77 motif and, for example, *n*-hexyl (C₆) or *n*-dodecyl (C₁₂) chains can both be employed as
78 hydrophobic spacers.^{35, 37} Moreover, recent investigations revealed that the BTP system shows a
79 stronger tendency to form supramolecular fibers compared to the BTU system, even when more
80 complex hydrophilic polymers are utilised.³⁸ The robustness during the fiber assembly and
81 versatility make BTP system a promising candidate for future applications. We conducted multiple
82 rheological measurements on both BTP-C₆ and BTP-C₁₂ based hydrogels varying conditions such



83 as the crosslinker content from 0.1-10 mol% and overall concentration for 25 and 50 mg mL⁻¹.
 84 Furthermore, we examined rheological behavior of the hydrogels at temperatures between 0-80 °C.
 85 To reveal the biomedical applicability for future studies, the injectability and recovery of the gel in
 86 a biological environment were qualitatively assessed by injection of a fluorescein containing BTP-
 87 C₆ hydrogels into muscle tissue (chicken breast sample). In addition, the viability of L929 mouse
 88 fibroblast cells growing on the BTP-C₆ hydrogels was determined.

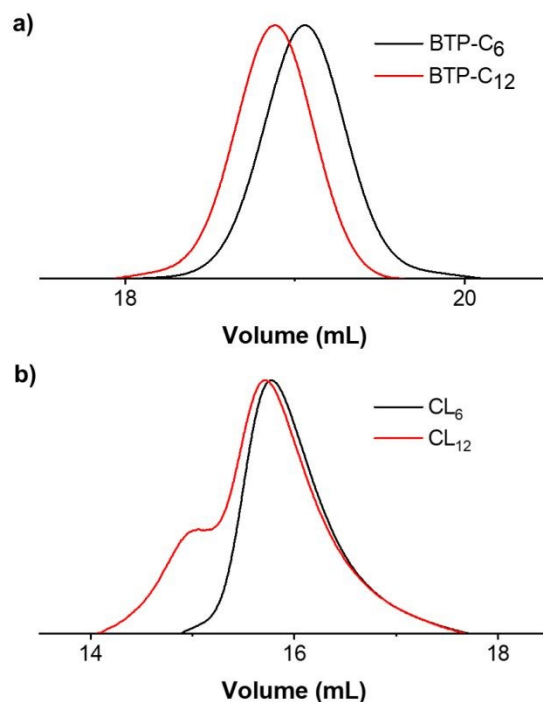
89 Results and discussion



90
 91 **Fig. 1.** Overview of the chemical structures of the prepared benzenetrispeptide based monomers
 92 and crosslinkers.

93 The synthesis of BTP-monomer and crosslinker (CL) units (**Fig. 1**) was performed according to
 94 the procedures established by Gruschwitz and Klein.^{28, 35} For the confirmation of the successful
 95 PEO attachment, ¹H-NMR (see SI) and size exclusion chromatography (SEC) measurements
 96 (**Fig. 2**) were performed. The SEC measurements of the monomer units showed monodisperse
 97 distribution in their elugrams (**Fig. 2a**). Interestingly, a shoulder towards higher molar masses was
 98 observed for CL₁₂ (**Fig. 2b**) although no evidence of side reactions was observed in the NMR
 99 spectra. Therefore, we assume that this occurrence is a result of dimerization of CL₁₂ in the SEC
 100 solvent (DMAc + 0.21wt% LiCl), which indicates poor solubility and strong interaction.





101

102 **Fig. 2.** Overview of SEC measurements, showing elugrams of the BTP-monomer (a) and BTP-
 103 crosslinker units (b). Measured using DMAc (+ 0.21 wt% LiCl) as eluent and a flow rate of 1 mL
 104 min⁻¹.

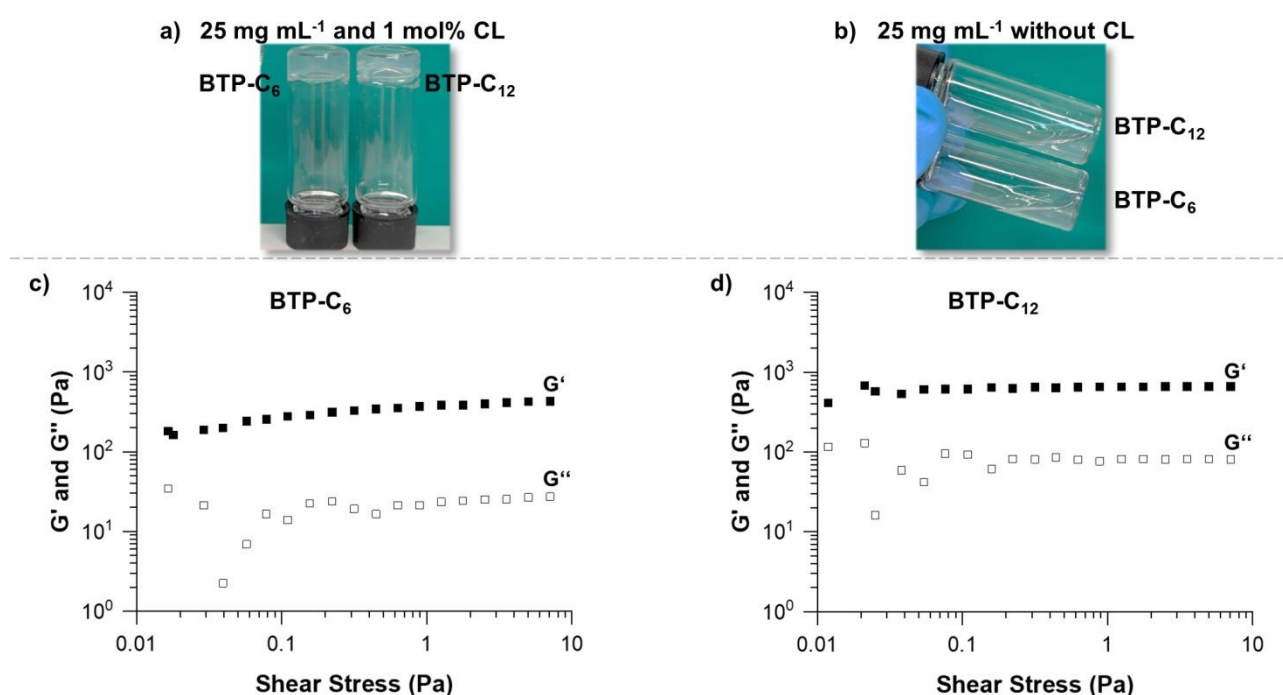
105 Multiple parameters were considered for the rheological investigation to assess the potential of the
 106 BTP based hydrogels for biomedical applications.³⁹ Based on previous experience, the conditions
 107 for the first experiment were set to a sample concentration of 25 mg mL⁻¹ and a crosslinker
 108 concentration of 1 mol%.²⁸ Maintaining comparable weight contents of the supramolecular
 109 building blocks, the difference of molar mass between BTP-C₆ and BTP-C₁₂ causes slight
 110 discrepancies in their final molar concentrations (approximately 0.7 μmol mL⁻¹). For the general
 111 hydrogel preparation procedure, we mixed the BTP-monomer and crosslinker and dissolved them
 112 in THF. To ensure that the system was fully dissolved, the solutions were heated to ~40°C. In case
 113 of the BTP-C₆ system, a small amount of water (<50 μL) was necessary for complete dissolution,
 114 as the system gelled in pure organic solvent (THF) after cooling down. A gradual solvent switch
 115 from THF to water was then performed by slowly adding water (syringe pump at 1 mL h⁻¹) to the
 116 organic mixture reaching a content of 67% water, before the organic solvent was finally removed
 117 by evaporation. This procedure results for both samples BTP-C₆ and BTP-C₁₂ in free standing
 118 hydrogels (**Fig. 3a**). Additionally, we also assessed the capability of the systems to form hydrogels

5



119 in the absence of crosslinker at the given concentrations, but we did not observe any hydrogel
120 formation for both samples (**Fig. 3b**).

121 As the first step of rheological studies, we examined the linear viscoelastic domain of our samples
122 *via* a stress sweep measurement in order to determine how much stress we can apply to our sample,
123 without breaking the gel structure, yet (**Fig. 3c & d** see SI section 2.2 stress sweep measurements
124 of all samples). Shear storage modulus (G') and loss modulus (G'') are stable when subjected to a
125 shear stress from 0.4 to 10 Pa for both samples. Therefore, we choose a shear stress of 1 Pa for
126 further measurements of both samples.

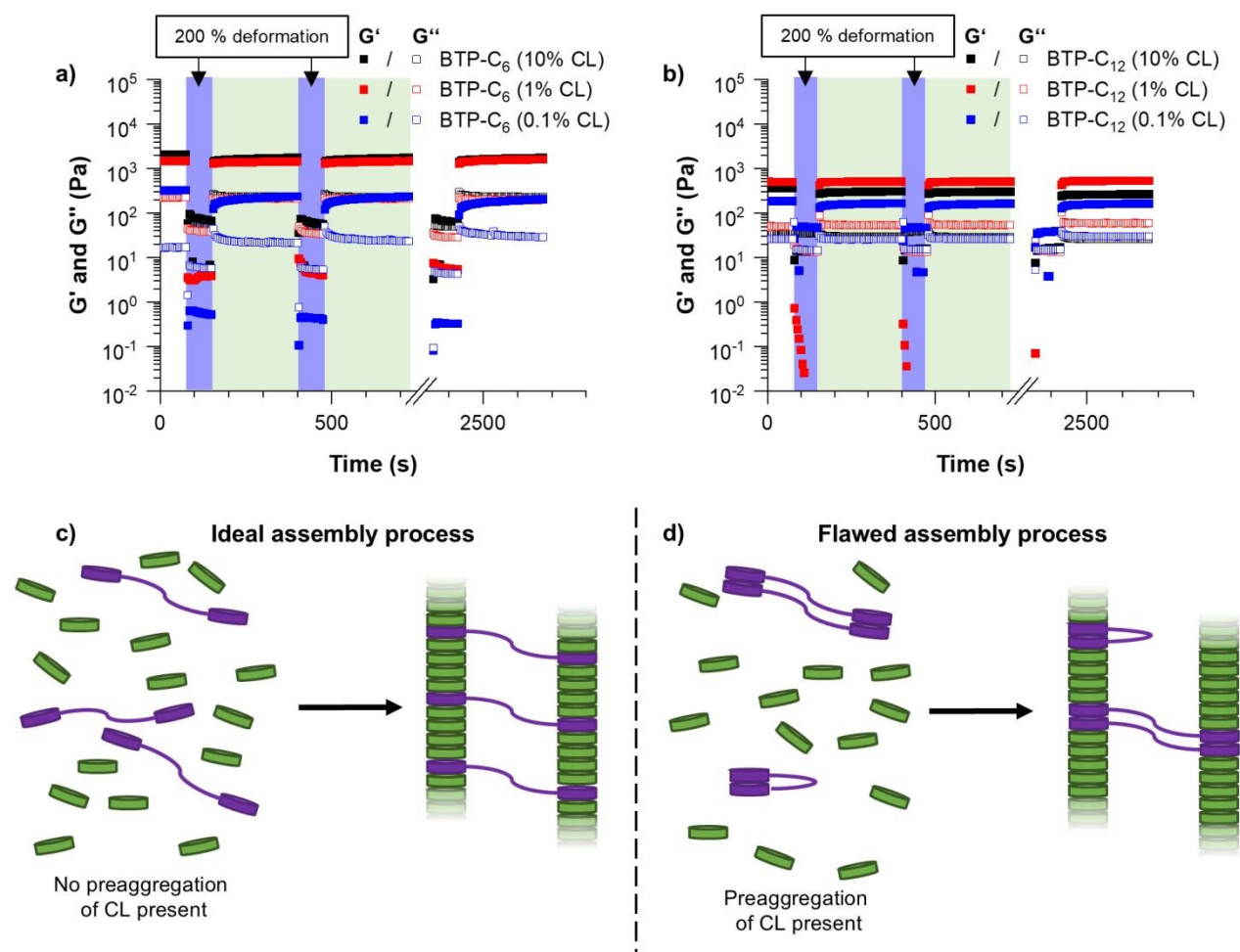


127
128 **Fig. 3.** Images of the hydrogels ($c = 25 \text{ mg mL}^{-1}$) with 1 mol% crosslinker (a) and without
129 crosslinker (b). Stress sweep of the BTP-C₆ (c) and BTP-C₁₂ hydrogels (d) at 20 °C to determine
130 the stress strength usable for further tests.

131 As the first major investigation, we conducted step-strain measurements. During this measurement
132 the sample is multiple times deformed applying a large strain (200 % deformation) and allowed to
133 recover, which yields information on the self-healing properties of the hydrogels, i.e. if the sample
134 recovers and how long it needs to regain its initial strength.⁴⁰ Surprisingly, the BTP-C₆ system with
135 1 mol% crosslinking formed a significantly stronger hydrogel with an average G' value of ~ 1.1



136 kPa (**Fig. 4a**, red squares), whereas the BTP-C₁₂ system resulted in a lower G' value of ~500 Pa
 137 (**Fig. 4b**, red squares) at similar concentrations. We initially anticipated the formation of stronger
 138 hydrogels by BTP-C₁₂ motifs in comparison to BTP-C₆ counterparts due to the longer alkyl chains
 139 present in BTP-C₁₂. A longer aliphatic chain is expected to enhance the hydrophobic interaction,
 140 and thus should induce a higher stability of the self-assembled fibers in the gel. The lower modulus
 141 of the BTP-C₁₂ hydrogel might be related to a preaggregation of CL₁₂ in THF (already observed in
 142 SEC experiments) and, subsequently, an insufficient incorporation into the fiber network or
 143 clustering of the crosslinker during the assembly process (**Fig. 4c & d**).



144
 145 **Fig. 4.** Step-strain measurements of BTP-C₆ (a) and BTP-C₁₂ (b) hydrogels with varying
 146 crosslinker concentrations of 0.1, 1 and 10 mol% at 20 °C. Schematic representation of assembly
 147 formation without (c) and with (d) and preaggregation of the crosslinker.

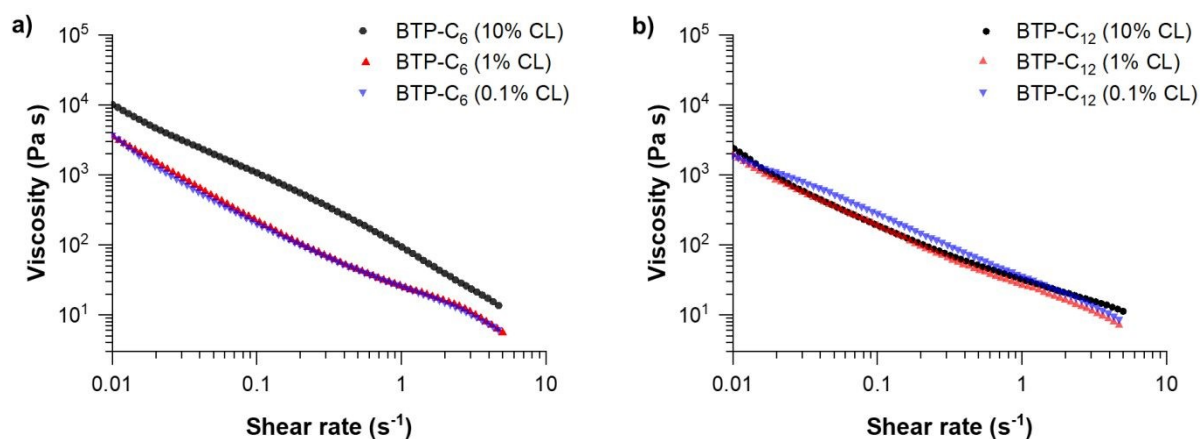


148 Concerning the capability of both systems to reform a gel after shear deformation, the rheological
149 measurements revealed a rapid recovery within less than 5 seconds over several cycles
150 strain/recovery. Only in the case of BTP-C₆ a slight decrease of shear storage modulus was
151 observed after the first strain step, followed by a gradual increase over time during each recovery
152 step. To further verify the robustness of the systems, we performed frequency sweep experiments
153 before and after the step strain measurement, which show only little variation indicating a full
154 recovery of the systems (Fig. S3 & S7).

155 Following these initial investigations on the BTP-C₆ and BTP-C₁₂ systems, we explored the
156 influence of various crosslinker concentrations (0.1, 1, and 10 mol% CL) to evaluate its impact on
157 the mechanical properties of the hydrogels. We examined both systems analogously to the previous
158 measurements. For the BTP-C₆ system, we observed a slight increase in the G' value of the
159 hydrogels from ~1.4 kPa to ~1.7 kPa with 10 mol% CL and a significant decrease in the G' value
160 to ~0.2 kPa for 0.1 mol% CL (**Fig. 4a**, for individual measurements Fig. S3- S5). These findings
161 were in the line with our expectation because a higher crosslinking density leads to a higher stability
162 of the hydrogel and thus a higher G' value.⁴¹ The 10 mol% CL sample featured a similar decreased
163 strength after the first step and gradual improvement as the initially tested sample with 1 mol% CL.
164 In case of the 0.1 mol% CL sample, however, the recovery occurs more slowly and requires several
165 minutes to reach a plateau level again. Surprisingly, the BTP-C₁₂ system revealed lower G' values
166 at higher (10 mol%,) CL content compared to the initial tested sample with 1 mol% CL (~0.5 kPa)
167 (**Fig. 4b**, for individual measurements Fig. S7- S9). This weakening of the gel with increasing CL
168 content supports our hypothesis, that the limited solubility of CL₁₂ causes a decrease in strength
169 compared to the CL₆ system. Furthermore, decreasing the CL content to 0.1 mol% has a less
170 pronounced effect on gel strength (~0.15 kPa compared to ~0.3 kPa for 1 mol% CL) than observed
171 for the BTP-C₆ system. All gels based on BTP-C₁₂ recovered quickly (gel formation < 5 s) and a
172 plateau is rapidly reached for 10 mol% and 1 mol% CL, while the low CL content of 0.1 mol%
173 again causes a slight delay.



174



175

176 **Fig. 5.** Viscosity measurements of BTP-C₆ (a) and BTP-C₁₂ (b) hydrogels with varying crosslinker
 177 concentrations of 0.1, 1 and 10 mol% at 20 °C. For individual measurements with measuring points
 178 below 0.01 s⁻¹ see Fig. S3-5 & S7-8.

179 We further conducted shear viscosity measurements to reveal the effect of the crosslinker
 180 concentration on yield stress and the shear dependent viscosity of BTP-C₆ and BTP-C₁₂ systems
 181 (**Fig. 5**). These are critical parameters to determine the pressure required to induce flow of the gel
 182 and evaluate the injectability of the hydrogels. Therefore, we applied a continuously increasing
 183 shear rate $\dot{\gamma}$ to determine its influence on the viscosity. We evaluated the results by using a power
 184 law fit on the linear regions at higher shear rates according to

$$185 \quad \text{Eq. 1} \quad \eta = K\dot{\gamma}^{n-1}$$

186 These fits allowed us to determine the shear-thinning parameter n and consistency index K for the
 187 tested samples (**Table 1**).^{30, 42} Additionally, we investigated the reversibility of the BTP-C₁₂ system
 188 (1 mol% CL) to assess whether the viscosity of our system remains consistent based on multiple
 189 measurements. Therefore, we performed three consecutive runs by alternating between increasing
 190 and decreasing the shear rate (Fig. S11). The results revealed minimal variations demonstrating the
 191 robustness of our measurement setup and the stability of our system.

192

193



194 **Table 1.** Yield stress and fitted shear-thinning parameter n , consistency index K of BTP-C₆ and
 195 BTP-C₁₂ hydrogels with different crosslinker concentrations. The yield stress was determined at
 196 the highest point before liquification set in during the viscosity measurements. The values for n
 197 and K were obtained by applying a power law fit (Eq. 1) to the linear regression regions of Fig. 4
 198 5a & 5b.

Sample	Crosslinker content (mol%)	Yield stress (Pa)	n	K (Pa s ⁻¹)
BTP-C ₆	10	70.1	0.08 ± 0.01*	122.95 ± 1.86*
	1	32.3	0.14 ± 0.01	25.12 ± 0.36
	0.1	28.22	0.14 ± 0.02	24.31 ± 0.70
BTP-C ₁₂	10	17.7	0.30 ± 0.02	33.35 ± 0.93
	1	13.6	0.16 ± 0.02	25.48 ± 1.31
	0.1	10.3	0.10 ± 0.01	33.89 ± 0.33

199 *Due to the abnormal curve progression of the measurement, the fit was applied to lower shear
 200 rates between 0.02-0.3 s⁻¹.

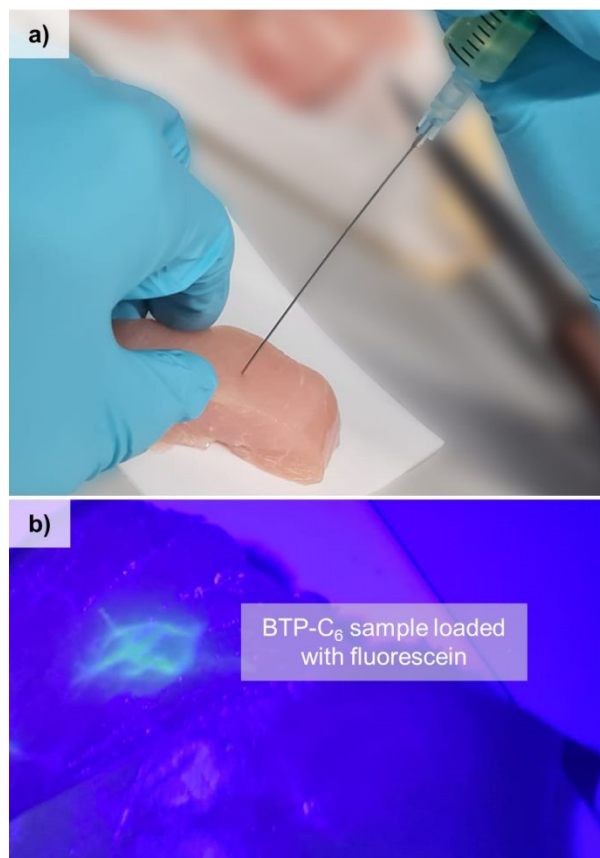
201 For all samples, the calculated values for n are well below 1 which corresponds to a strong shear-
 202 thinning fluid, as the viscosity decreases significantly with increase of the shear-rate. Comparing
 203 the previously reported benzenetrisurea systems, the obtained values for n and K are in a similar
 204 range ($n = 0.15$; $K = 22.97$) proving that this shear-thinning behavior is characteristic for these
 205 types of hydrogelators.²⁸ In addition, both systems indicate significantly lower values compared to
 206 state-of-the-art printable polymer hydrogels based on poloxamer polymers ($n = 0.13$; $K = 406$) or
 207 alginates ($n = 0.31$; $K = 254$).⁴² Among the obtained results (Table 1), lower shear rates between
 208 0.02-0.3 s⁻¹ were applied for BTP-C₆ with 10 mol% CL due to the distinct behavior of the material
 209 with higher shear rates (Fig. 5a). At higher shear rates >0.1 s⁻¹ the value of n is negative ($n = -0.15$
 210 ± 0.01; $K = 91.36 ± 0.59$), which is related to a rather steep slope of the viscosity change with
 211 increasing shear rate. Although such values are unusual, negative n values were also observed in
 212 other studies.^{43,44} A plausible explanation for this anomaly could be that the sample becomes more
 213 fragile with increasing crosslinker content.

214 The obtained values of n and K can also be used to calculate the pressure and force needed to
 215 extrude the hydrogel through a cannula, and so their injectability can be determined.³⁰ Using the
 216 predetermined parameters, we calculated the force needed to extrude the hydrogel through a



217 cannula of certain gauges (see SI Chapter 3 and Table S2). For a gauge 21 cannula, with a length
218 of 50 mm, commonly used for intramuscular injections, and a 1 mL syringe, BTP-C₆ and BTP-C₁₂
219 hydrogels with 1 mol% CL would require 0.62 N and 0.76 N force, respectively, to be extruded
220 with a flowrate of 0.1 mL s⁻¹. A gauge 23 cannula, with a length of 12 mm, commonly used for
221 subcutaneous injection, on the other hand, would require a force of 0.33 N for BTP-C₆ and 0.42 N
222 for BTP-C₁₂ under the same conditions.⁴⁵ In all cases the required forces are considerably lower
223 than the maximum forces considered for manual (40 N) or automatic injection (20 N) of 1 mL with
224 an injection rate of 0.1 mL s⁻¹.⁴⁶ It is noteworthy, that the injection into tissue also affects the
225 necessary force due to the tissues counterpressure, which leads to an increase in the force needed
226 for e.g. subcutaneous injection.⁴⁷ As a proof of concept for the injectability of the hydrogels into
227 muscular tissue, we used a piece of chicken breast and injected the BTP-C₆ gel with a syringe. This
228 test should not only allow practical evaluation of the required injection forces, but also if the
229 hydrogel recovers in such an environment (**Fig. 6**). We used a 1 mol% crosslinked BTP-C₆ hydrogel
230 soaked with 1 mg mL⁻¹ fluorescein, which resembled an easy traceable model compound here. For
231 the injection a gauge 23 cannula with a length of 80 mm and a 2 mL syringe were used, which still
232 allowed an easy and effortless manual injection of the hydrogel. The observed resistance was
233 similar to extrusion of a viscous oil. After sample injection (~0.2 mL) we lightly “massaged” the
234 sample to simulate muscle movement and cut the tissue to reveal the injection site. The hydrogel
235 appears to be distributed between different muscular cords but remains localized in the injection
236 area. It is noteworthy to mention, that neither the massaging nor the cutting of the chicken breast
237 had a strong impact on the distribution highlighting the stability of the gel even when injected into
238 tissue.





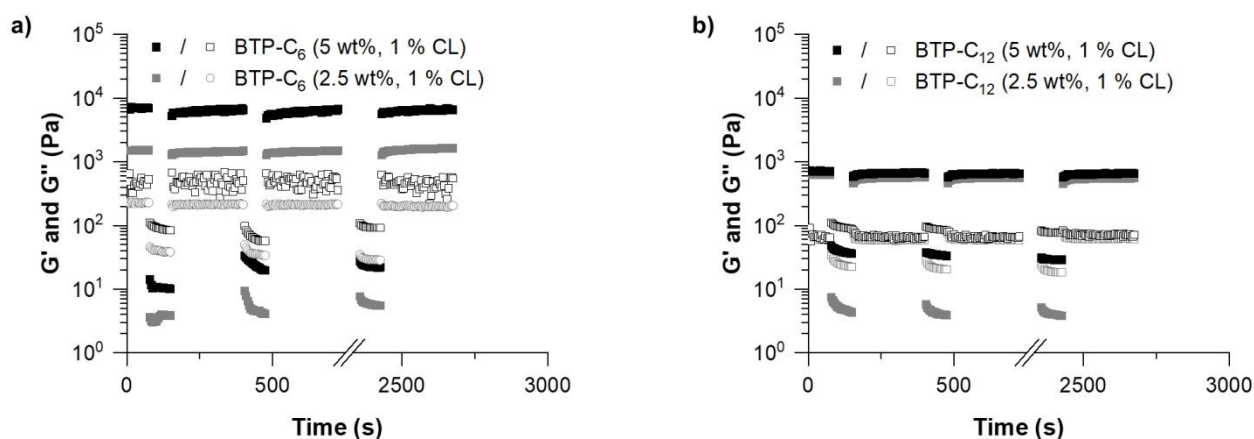
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240 **Fig. 6.** Injection of a BTP-C₆ 1 mol% CL sample (using a G27 cannula), loaded with 1 mg mL⁻¹
241 fluorescein as modal system, into chickenbreast (a). and a crosssection of the injectionside under
242 UV light (b).

243 In order to explore the further potential of the materials, we analyzed the influence of the overall
244 concentration of the gelator on G' value. A higher concentration should lead to a higher density of
245 fibers in the hydrogel, which in return should lead to increased entanglements and, thus, a
246 strengthening of the gel.^{48,49} We therefore kept the CL content at 1 mol% and increased the overall
247 weight concentration of the BTP-system in the hydrogel from 2.5 wt% to 5 wt%, which in previous
248 investigations on the benzenetrisurea system resulted in a noticeable (~three-fold) increase of the
249 gel strength.²⁸ The G' values of the BTP-C₆ system increased to ~8 kPa (**Fig. 7a**), which is almost
250 a six-fold increase compared to the sample with 2.5 wt%. To our surprise, the increase in
251 concentration caused only an increase in strength from 0.3 kPa to ~0.8 kPa for the BTP-C₁₂ system
252 (**Fig. 7b**). We assume that in the latter case the higher concentration of building block and



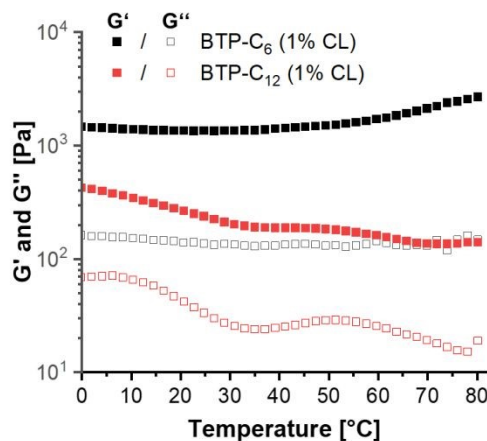
253 crosslinker further impedes the homogeneous distribution of the crosslinker over all fibers, which
254 is in accordance with the observations made for an increased crosslinker content.



255
256 **Fig. 7.** Comparison between the hydrogels with concentrations of 2.5 wt% (grey) and 5 wt% (black)
257 based on BTP-C₆ (a) and BTP-C₁₂ (b) with 1 mol% crosslinker at 20 °C.

258 Following these tests, we further evaluated the impact of increasing temperatures on the hydrogels.
259 Supramolecular interactions typically get weaker at elevated temperatures.⁵⁰ In our tests, the BTP-
260 C₁₂ samples demonstrated such a weakening as the gel strength decreased from ~0.4 kPa at 0 °C to
261 ~0.15 kPa at 80 °C (**Fig. 8**), which is reversible through cooling of the sample (see Fig. S12).
262 Contrary, the BTP-C₆ sample showed an increase in G' from ~1.4 kPa to ~2.7 kPa (**Fig. 8**), which
263 indicates an unexpected thermal stiffening behavior. Such effects are less common for
264 supramolecular assemblies and could be due to changes in the aggregation structure.^{51, 52} For
265 example Chakravarthy *et al.* proposed the formation of a double network at elevated temperatures
266 for their system, which leads to thermostiffening behavior at 37 °C.⁵³ Further investigations into
267 the cause of this phenomenon are currently ongoing, but considering a limited reversibility of the
268 BTP-C₆ sample we refrained from further temperature dependent measurements.





269

270 **Fig. 8.** Temperature sweep measurements from 0 to 80°C of BTP-C₆ (black) and BTP-C₁₂ (red)
271 samples containing 25 mg mL⁻¹ monomer and 1 wt% crosslinker.

272 As the BTP-C₁₂ system was more reliable with temperature changes we further investigated the
273 temperature dependent stress relaxation behavior of the corresponding hydrogels. Isothermal stress
274 relaxation experiments were performed at 5°C, 40°C, 60°C and 80°C (**Fig. 9a**) on the BTP-C₁₂
275 system (containing 1 mol% of crosslinker). The obtained data were normalized after 20 seconds of
276 applied strain (1%, within the linear viscoelastic domain), this delay was necessary to reach a stable
277 strain and reduce signal noise observed at the early stage of the experiments. As expected, the stress
278 decay was faster at high temperatures which we attribute to a faster exchange of supramolecular
279 crosslinker or network reorganization. By analogy with covalent adaptable networks, i.e.
280 thermosets containing covalent exchangeable bonds, stress relaxation behavior of BTP-C₁₂ system
281 can be describe by a multimode Maxwell Model (or KWW model) in which multiple relaxation
282 mode coexist instead of a simple mode.^{54, 55} The obtained data from **Fig. 9a** was accurately fitted
283 (0.993 < R² < 0.998) using the following equation:

284

$$\text{Eq. 2} \quad G(t) = G_o \exp\left(-\frac{t}{\tau}\right)^\beta$$

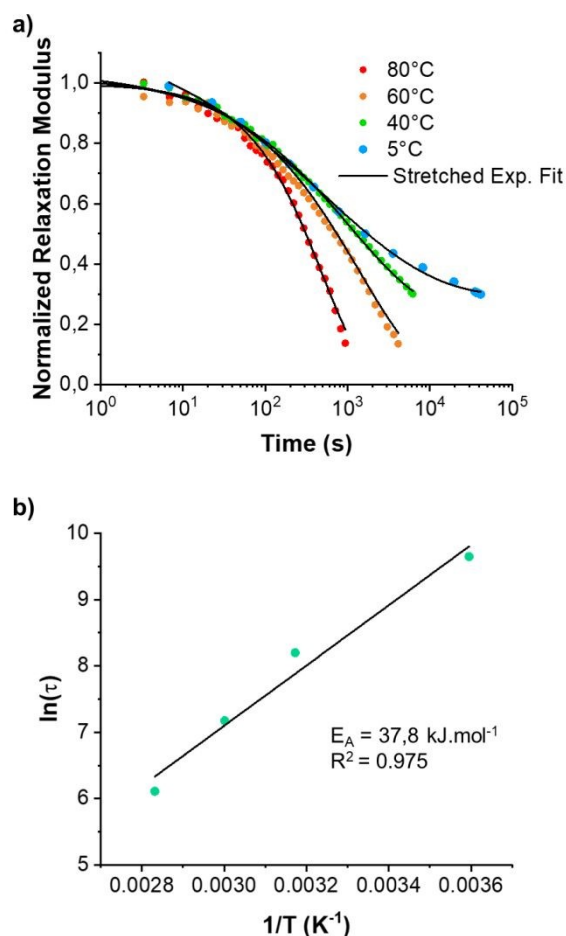
285 Fitting the stress relaxation behavior with this stretched exponential function reveals that at 5°C a
286 broad relaxation time distribution is present ($\beta_{(5^\circ\text{C})} = 0.32$), which gets “narrower” at higher
287 temperatures ($\beta_{(80^\circ\text{C})} = 0.81$). The activation energy $E_A = 37.8\text{kJ}\cdot\text{mol}^{-1}$ (**Fig. 9b**) was extracted from
288 the Arrhenius plot of $\ln(\tau)$ vs $1/T$ where τ is the average relaxation time determined from stretched
289 exponential fitting. These results indicate that the system becomes more dynamic with increasing

14



290 temperature, which is in accordance with behavior of other supramolecular systems. Further studies
291 with regard to the general fiber dynamics are currently ongoing.

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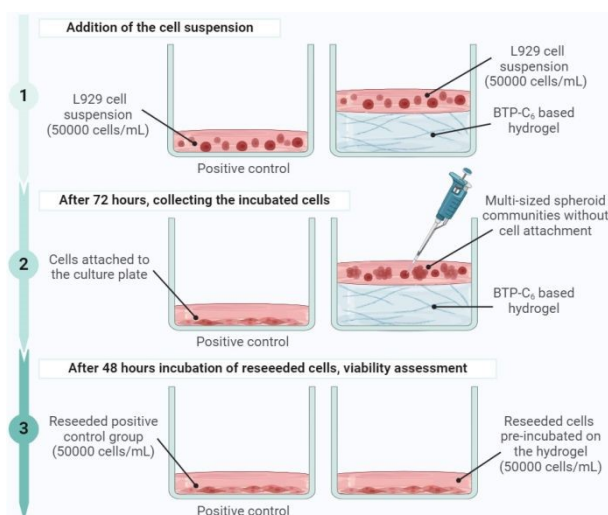
294 **Fig. 9.** Normalized stress relaxation modulus (shear mode) of BTP-C₁₂ (a) at 5, 40, 60 and 80°C.
295 The data was measured consecutively on the same sample and normalized 20 seconds after strain
296 (1%) was applied. The characteristic relaxation times (in s) were plotted in a log plot (b) and fitted
297 using a linear fit to obtain the activation energy (E_A).

298 We finally aimed to assess the biocompatibility of the hydrogels. It is noteworthy to state that
299 instead of using hydrogel extracts, we designed an experimental system incubating L929 mouse
300 fibroblasts directly on a hydrogel layer, which allowed us to further investigate cell-to-hydrogel
301 interactions. Therefore, we tried to cover the bottom of wells in standard cell culture plates with
302 the hydrogel and subsequently seed cells onto the surface. However, following the same procedure



303 for preparing the hydrogels used for the rheological measurements, the BTP-C₁₂ system started to
304 gel at 20 to 30 v% water content which impeded a homogenous coverage of the culture plate surface
305 (Fig. S13). Therefore, we focused on the BTP-C₆ hydrogel with 2.5 wt% and 1 mol% of CL as the
306 exemplary system to evaluate cell interactions and biocompatibility. We added a predetermined
307 amount of BTP-C₆ hydrogel stock solution to a tissue culture (TC) treated 96-well culture plate
308 aiming at a final hydrogel thickness of 1.1 mm after evaporation of the solvent. The aimed thickness
309 ensured that the cells were only in contact with the hydrogel but not the bottom surface of the well.
310 Upon THF evaporation, we obtained intact BTP-C₆ hydrogels in the wells (Fig. S14). To ensure
311 full removal of the solvent and create conditions suitable for cell seeding, we washed the surface
312 of the hydrogels several times with PBS. Finally, we incubated the hydrogels treated with
313 Dulbecco's modified Eagle medium (D10) overnight at 37 °C under a humidified 5% (v/v) CO₂
314 atmosphere. It noteworthy that the thickness and consistency hydrogels did not change over the
315 course of this procedure despite being constantly covered by 100 µL of cell culture medium.

316 Following the preparation of the culture plate with hydrogel, L929 cells (50000 cells/mL per well)
317 were seeded in blank wells without hydrogel (control) and in wells covered with the hydrogels
318 (gel) in triplicate and the experiment was repeated for four different cell passages (P1, P2, P3, and
319 P4) (**Fig. 10**).

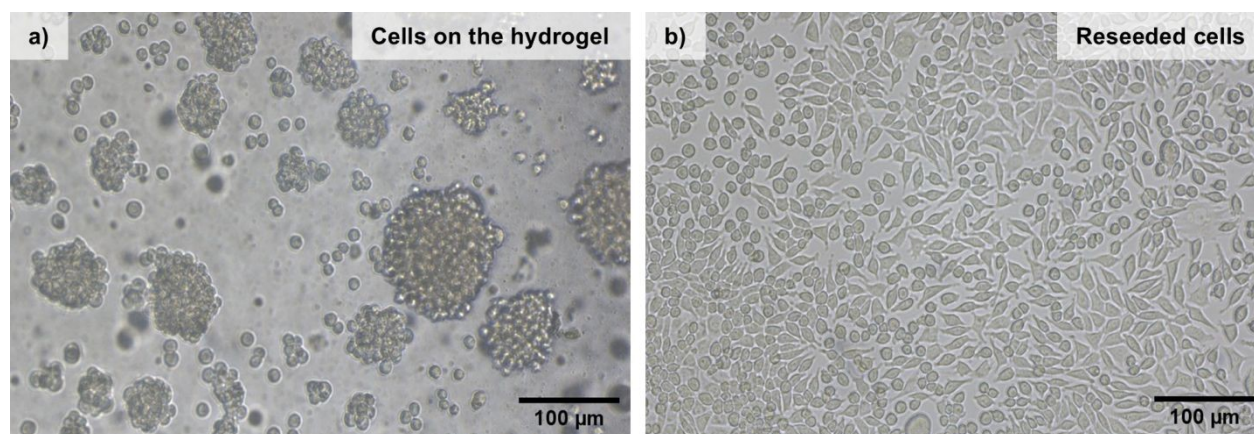


320

321 **Fig. 10.** Graphical representation of the experimental setup for the biocompatibility assessment of
322 BTP-C₆ hydrogel (Created with BioRender.com).



323 The cells were incubated for 72 hours, and afterwards they were imaged by light microscopy (**Fig.**
324 **11**). The cells did not attach to the surface of the hydrogel (**Fig. 11a**) but rather formed multi-sized
325 communities in spheroid shapes based on cell-to-cell aggregations. This is a quite common
326 behavior for the cells growing on the inert surfaces and indicates that the hydration shell around
327 the dense PEO chains on the fibers prevents protein adsorption and further cell interactions.⁵⁶⁻⁵⁸
328 After 72 hours of incubation, the cells were collected and reseeded in a TC treated 96-well culture
329 plate to understand whether the gels had any negative effect on the cell viability, morphology, and
330 attachment ability. Following 48 hours of incubation, the cells pre-incubated on the hydrogels were
331 able to re-attach to the culture plate. (**Fig. 11b**). In addition, there was no significant change in their
332 morphology compared to a control, so we can conclude that BTP-C₆ hydrogel had no detrimental
333 effect on L929 cells.

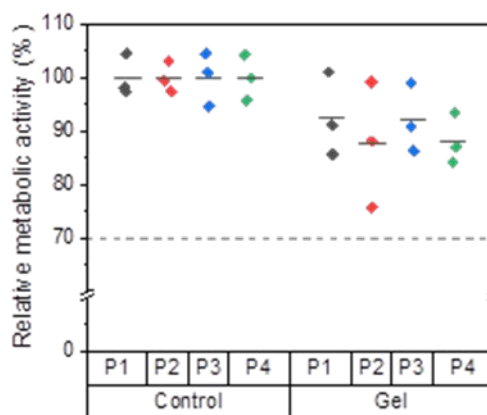


334 **Fig. 11.** Transmitted light microscopy images of L929 mouse fibroblast cells incubated on the
335 BTP-C₆ hydrogel for 72 hours (a; picture taken on the hydrogel) 48 hours of incubation for reseeded
336 cells collected from top of the hydrogel (b). Optical magnification is 10x and the scale bar is 100
337 µm.
338

339 The viability of the reseeded cells was quantitatively investigated *via* PrestoBlue™ assay. For all
340 passages, the mean relative metabolic activity values of the control groups were set as 100%. A
341 cell viability $\leq 70\%$ is considered toxic according to DIN EN ISO 10993-5.⁵⁹ In comparison to the
342 control groups, the cells previously incubated on the hydrogels showed relative metabolic activities
343 in the range of 90% indicating that BTP-C₆ hydrogel had no toxic effect on L929 cells (**Fig. 12**).
344 Independent from the passage numbers, the mean relative metabolic activity was higher than 87%
345 for all gel groups in respect of the control groups and the mean values did not show any significant



346 variation from passage to passage (**Error! Reference source not found.**). In light of both
 347 qualitative and quantitative observations, BTP-C₆ hydrogel can be considered biocompatible and
 348 the formation of multi-sized spheroid on the hydrogel is currently under further investigation.



349
 350 **Fig. 12.** Relative metabolic activity (%) of L929 mouse fibroblast cells growing on the BTP-C₆
 351 hydrogel was determined by PrestoBlue™ assay. The experiments were conducted as triplicate for
 352 four different passages represented as P1, P2, P3, and P4. Calculated mean values were depicted
 353 as lines.

354 Conclusion

355 In conclusion we investigated the rheological properties of hydrogels formed from supramolecular
 356 assembly of benzenetrispeptides with a C₆ and C₁₂ spacer and a conjugated PEO chain. The
 357 introduction of a bifunctional crosslinker induced the hydrogel formation and we found that both
 358 systems display fast recovery rates during step strain measurements. Varying the crosslinker
 359 content had limited effect on the BTP-C₁₂ system, which was related to an inefficient integration
 360 of the crosslinker. However, the BTP-C₆ system could be modified in terms of its shear modulus
 361 over a range from 0.2 kPa up to nearly 2 kPa when increasing the crosslinker content from 0.1 to
 362 10 mol%. Increasing the total concentration of the materials from 2.5 w% to 5 wt% caused another
 363 significant increase of G' for BTP-C₆ system up to 8 kPa, while only a minor increase in G' (from
 364 0.5 to 0.8 kPa) is observed for BTP-C₁₂, which is most likely again due to a limited integration of
 365 the crosslinker. Temperature dependent measurements further revealed a thermo-stiffening
 366 behavior of the BTP-C₆ system, which cannot be clarified at present, while the BTP-C₁₂ hydrogel



367 becomes weaker with the increasing temperature. Stress relaxation measurements of the latter
368 sample revealed an Arrhenius behavior indicating increasing dynamics at higher temperature.
369 Analyzing the shear dependent viscosity further revealed a strong shear-thinning effect of all tested
370 samples, which facilitates effortless injections even through thinnest needles. A fluorescein
371 containing BTP-C₆ hydrogel could therefore be easily injected into a chicken breast as muscle
372 tissue sample applying only a minor force. The hydrogel distributes between muscle cords but,
373 more importantly, is able to reform even within this tissue and remains at the injection side even
374 after massaging the muscle. Direct incubation of L929 mouse fibroblast cells on the hydrogel
375 confirmed that the dense PEO-shell on the fibers prevents an attachment of the cells and spheroid
376 like communities are formed. Nevertheless, reseeded of these cells and comparison to a control
377 resulted in a relative metabolic activity over 87% independent from the passage number, and no
378 signs of changes in cell morphology or behavior were observed. Overall, these experiments prove
379 a high biocompatibility of these injectable supramolecular hydrogels and the tunable shear modulus
380 of the BTP-C₆ system allows adaption of the hydrogel to the specific strength of different soft
381 tissues such as brain, pancreas, or lung resembling it an attractive material for application as drug
382 depot or in tissue engineering.⁶⁰⁻⁶³

383



384 **Methods and Materials**

385 All reagents and solvents were commercial products purchased from Merck, ABCR, Iris BioTech,
386 Rapp Polymere or TCI and were used without further purification. The BTP building blocks were
387 prepared according to procedures described in literature.^{28, 35} Details on the synthesis of the
388 crosslinker are provided in the supplementary information.

389 *Gel preparation*

390 The hydrogels preparation procedure is based on the solvent switch and gel preparation method
391 described in the following.^{28, 36} The gels were prepared by weighing in the lyophilized building
392 blocks and crosslinker in a 20 mL vial with wide neck. 0.75 mL THF were added, the vial was
393 closed, and the solids were dissolved using sonication and light heating. Afterwards the samples
394 were mixed using a magnetic stirrer for 30 min. to the closed vial 1.5 mL of water were added at a
395 speed of 1 mL h⁻¹ using a syringe pump. Lastly the cap was removed to slowly evaporate the THF
396 until the weight of the gel was 1.5 g. Lastly the gel was transferred to the rheometer.

397 *Rheology measurements*

398 The oscillatory dynamic measurements were performed with a Physica Modular Compact
399 MCR102 Rheometer from Anton Paar (Germany). Mechanical properties of the viscoelastic
400 hydrogel material were assayed using a parallel (PP 25 sandblasted)-plate geometry. The linear
401 viscoelastic regime of the samples was obtained from amplitude sweep experiments that were
402 performed from 0.1 to 200% strain at 6.36 rad s⁻¹ angular frequency. Frequency sweep
403 measurements were recorded from 0.1 to 100 rad s⁻¹ at 1% strain. Measurements were performed
404 at 20 °C unless stated otherwise. For measurements above 20 °C paraffin oil was added around the
405 sample and geometries to hinder the sample drying out due to water evaporation.

406 *Biological investigation*

407 L929 cells (CLS Cell Lines Service GmbH, Eppelheim, Germany) were cultured in TC treated cell
408 culture flasks (Greiner Bio-One International GmbH and Labsolute Th. Geyer GmbH & Co. KG)
409 in the presence of Dulbecco's modified Eagle medium (D10) (2 mM l-glutamine (Biochrom,
410 Germany) supplemented with 10% fetal calf serum (FCS, Capricorn Scientific, Germany), 100 U



411 mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin (Biochrom)) at 37 °C under a humidified 5% (v/v)
412 CO₂ atmosphere. Dulbecco's phosphate-buffered saline (1x) (DPBS) and trypsin-EDTA
413 (Capricorn Scientific GmbH, Germany) were used for each cell passaging. The cells with the
414 passage numbers 22 (P1), 23 (P2), 24 (P3), and 25 (P5) showing ≥97% viability were used.

415 For the cell viability assessment, hydrogel stock solution was prepared strictly following the above-
416 mentioned preparation methodology. Under sterile environment, predetermined amount of
417 hydrogel stock solution (57 µL) was added to each well of TC treated 96-well cell culture plates
418 (VWR International GmbH) by aiming 1 to 1.2 mm gel thickness (Fig. S16). After evaporation of
419 THF from the system in a sterile environment, hydrogels formed and a serial of washing with PBS
420 was conducted to adjust pH of the system. Afterwards, 100 µL of DMEM was added in each well
421 containing hydrogel. The plate was incubated overnight at 37 °C under a humidified 5% (v/v) CO₂
422 atmosphere for the next day's cell seeding. The following day, L929 cells were seeded (5000 cells
423 per well) both in the wells without gels (positive control) and with the gels in triplicate. The cells
424 were incubated for three days at 37 °C under a humidified 5% (v/v) CO₂ atmosphere.

425 After 72 hours, control groups were collected by trypsinization. However, the cells growing on the
426 gels were directly collected since the cells did not attach to the surface of the hydrogel. For each
427 sample, the viability and number of the cells were determined by a cell counter (fluidlab R-300
428 anvajo GmbH, Dresden, Germany). 5000 cells from each sample were reseeded in a TC treated 96-
429 well cell culture plate and the cells were incubated for two days at 37 °C under a humidified 5%
430 (v/v) CO₂ atmosphere. After 48 hours, the viability assessment was conducted.

431 Relative metabolic activity of viable L929 mouse fibroblast cells were determined by PrestoBlue™
432 assay (Thermo Fisher Scientific) following DIN EN ISO 10993-5. 10% (v/v) PrestoBlue™
433 solution, which was prepared according to the manufacturer's instructions, was added to each well.
434 Afterwards, the cells were further incubated at 37 °C for 45 min and finally the fluorescence was
435 measured with a multi-plate reader (Infinite M200 PRO Microplate Reader, Tecan, Switzerland)
436 at λ_{EX} = 560 nm/ λ_{EM} = 590 nm. The relative viability (%) of the cells was calculated as in equation
437 3:

438
$$\text{Relative viability (\%)} = \frac{FI_{\text{sample}} - FI_0}{FI_{\text{ctrl}} - FI_0} \times 100, \text{ (3)}$$



439 where FI_{Sample} , FI_0 , and FI_{Ctrl} represent the fluorescence intensity of cell growing on the hydrogel,
440 negative control (cell culture medium without cells), and control cells growing in the native
441 condition (100 % viability), respectively.

442 In addition to the viability assessment, cells were observed with a transmitted light microscope
443 (Axio Observer Vert.A1, Zeiss, Germany) with a 10 \times objective using brightfield imaging.
444 Images were recorded by ZEN lite software (2012, Zeiss).

445 For the statistical analysis, OriginPro2022b software was used. The mean relative metabolic
446 activity of each group was calculated as mean \pm SD. In addition, a one-way analysis of variance
447 (ANOVA) was conducted. At level 0.05, there was no significant difference among mean relative
448 metabolic activity of the passages.

449 **Conflict of interests**

450 There are no conflicts to declare.

451 **Acknowledgments**

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24



1 **Injectable biocompatible hydrogels with tunable strength based on** 2 **crosslinked supramolecular polymer nanofibers**

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15 Keywords: Supramolecular polymer bottlebrushes, polymer networks, self-assembly, shear-
16 thinning, stealth properties.

17 **Data availability statement**

18 The data supporting this article have been included as part of the Supplementary Information

