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# **Biobased pH-responsive and self-healing hydrogels prepared from O-carboxymethyl chitosan and a 3-dimensional dynamer as cartilage engineering scaffold**

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1 Biobased pH-responsive and self-healing hydrogels prepared from O-carboxymethyl chitosan  
2 and a 3-dimensional dynamer as cartilage engineering scaffold

3  
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17

18 Abstract:

19 Novel dynamic hydrogels were prepared from O-carboxymethyl chitosan (CMCS) and a water  
20 soluble dynamer Dy via crosslinking by imine bond formation using an environmentally friendly  
21 method. Dy was synthesized by reaction of Benzene-1,3,5-tricarbaldehyde with Jeffamine. The  
22 resulting soft hydrogels exhibit a porous and interconnected morphology, storage modulus up to  
23 1400 Pa, and excellent pH-sensitive swelling properties. The swelling ratio is relatively low at  
24 acidic pH due to electrostatic attraction, and becomes exceptionally high up to 7000% at pH 8  
25 due to electrostatic repulsion. Moreover, hydrogels present outstanding self-healing properties as  
26 evidenced by closure of split pieces and rheological measurements. This study opens up a new  
27 horizon in the preparation of dynamic hydrogels with great potential for applications in drug  
28 delivery, wound dressing, and in particular in tissue engineering as the hydrogels present  
29 excellent cytocompatibility.

30

31 Keywords: O-carboxymethyl chitosan; Imine chemistry; Dynamic hydrogel; Self-healing; pH  
32 responsive; Cytocompatibility

33

## 34 1. Introduction

35 In the past decades, hydrogels have been widely studied as biomaterials for various  
36 applications in drug delivery, wound dressing and tissue engineering due to their outstanding  
37 properties such as biocompatibility (Qu et al., 2018), biodegradability (Ghobril & Grinstaff,  
38 2015), mechanical properties (Van Vlierberghe, Dubruel, & Schacht, 2011), and stimuli-  
39 responsive properties (Zhang, Tao, Li, & Wei, 2011). Hydrogels consist of a cross-linked  
40 network (Lv et al., 2018; Su et al., 2015) which can absorb large amounts of water (Yang, Wang,

41 Yang, Wang, & Wu, 2018) or physiological fluids (Dimatteo, Darling, & Segura, 2018) while  
42 maintaining their three-dimensional structural integrity. Hydrogels can be classified into two  
43 categories according to the preparation approach: chemical gels or irreversible gels, and physical  
44 gels or reversible gels (Iftime, Morariu, & Marin, 2017; Stewart et al., 2017). Chemical gels are  
45 formed by irreversible covalent bonding, whereas in physical gels, the polymeric chains are held  
46 together by chain entanglements and/or supramolecular hydrophobic, ion-pair or hydrogen  
47 bonding interactions.

48 Dynamic hydrogels or dynagels are a dynamic system on both the molecular and  
49 supramolecular levels (Burdick & Murphy, 2012; Marin et al., 2014). They are able to reversibly  
50 exchange their components (Sreenivasachary & Lehn, 2005), responding to external stimuli such  
51 as pH (Zeng et al., 2017), ions (Arnal Hérault, Banu, Barboiu, Michau, & van der Lee, 2007;  
52 Rotaru et al., 2017), and temperature (Zhang, Jin, Li, Zhang, & Wu, 2018). Among the various  
53 dynamic reactions (Zhang & Barboiu, 2015a), imine bond formation is considered as the most  
54 promising strategy to generate dynamic materials with modulable properties. In fact, imine  
55 chemistry allows to implement reversible rearrangements of the components in a multivalent  
56 material which can bind bioactive molecules, cells or present self-healing properties (Chao,  
57 Negulescu, & Zhang, 2016). This dynamic constitutional framework is composed of linear  
58 and/or multi-armed components reversibly interconnected via imine bonds and containing  
59 stimuli-responsive functional groups (Zhang & Barboiu, 2015b).

60 Chitosan (CS), a polysaccharide obtained from alkaline hydrolysis of chitin found in the  
61 exoskeleton of crustaceans, presents remarkable properties such as biocompatibility,  
62 biodegradability, low toxicity, low cost or immune-stimulatory activity (Ali & Ahmed, 2018).  
63 Chitosan is a good candidate for in-situ dynamic reversible crosslinking via its amino groups

64 present along the polymer chain with aldehydes (Marin, Simionescu, & Barboiu, 2012), alginate  
65 (Qin et al., 2019), or gelatin (Qiao, Ma, Zhang, & Yao, 2017), resulting in the formation of pH-  
66 responsive and biodegradable hydrogels. The water solubility of chitosan depends on many  
67 factors, in particular pH of the medium, chain length and degree of acetylation (Varum, Ottoy, &  
68 Smidsrod, 1994). The carboxymethylation of the D-glucosamine moieties of chitosan generates  
69 O-carboxymethyl chitosan (CMCS) which is readily soluble in water at neutral pH, thus allowing  
70 uses in tissue engineering, drug delivery, wound dressing and food industry.

71 Crosslinking of CS or CMCS with aldehydes via imine bond formation along polymeric chains  
72 proceeds with very low yield in aqueous medium, but is significantly improved in hydrogels or  
73 in solid state films with dynamic properties (Marin et al., 2012). It is well known that no  
74 continuous cross-linked networks are formed when monoaldehydes (Iftime et al., 2017; Marin, et  
75 al., 2012) or dialdehydes (Yu et al., 2017) are used for cross-linking. The resulting hydrogels  
76 exhibit good swelling behaviors, but disordered micro-structure and weak mechanical strength.  
77 In contrast, dynamic hydrogels exhibiting pH and temperature-responsive swelling behaviors,  
78 strong mechanical performance, and self-healing behavior have been obtained by using 3-armed  
79 (Deng et al., 2015) or 4-armed aldehydes (Huang et al., 2016). Nevertheless, the toxicity of  
80 aldehydes, and especially of glutaraldehyde (Bhatia, 2010; Ghobril & Grinstaff, 2015) restricts  
81 their use for biomedical applications, and imposes the necessity of finding new biocompatible  
82 crosslinking agents.

83 Jeffamine is a polyetheramine composed of poly(propylene oxide) (PPO) and/or poly(ethylene  
84 oxide) (PEO) blocks with primary amino groups attached to the chain ends. It is widely used as a  
85 macromonomer to prepare PEO-based hydrogels as its water solubility facilitates reaction in  
86 aqueous medium (Zimmermann, Bittner, Stark, & Mülhaupt, 2002). In this work, bifunctional

87 Jeffamine ED-2003 was linked to benzene-1,3,5-tricarbaldehyde via imine formation, yielding a  
88 constitutional dynamer Dy that can be used as a water soluble cross-linking component. CMCS  
89 based hydrogels were then prepared by mixing CMCS and Dy aqueous solutions through a  
90 “green” synthetic route. The chemical structures of the hydrogels, their morphological,  
91 rheological and swelling properties, as well as their self-healing behaviors were evaluated and  
92 discussed. The cytocompatibility of hydrogels was assessed by co-culture in the presence of  
93 human mesenchymal stromal cells (MSCs) to evaluate their potential as scaffold in cartilage  
94 engineering.

95

## 96 2. Experimental section

97 2.1 Materials: Benzene-1,3,5-tricarbaldehyde (BTA) from Manchester Organics and O,O'-Bis(2-  
98 aminopropyl) PPO-*b*-PEO-*b*-PPO (Jeffamine<sup>®</sup> ED-2003, Mn  $1.9 \times 10^3$ ) from Sigma Aldrich  
99 were used without purification. CMCS (Mn  $2 \times 10^5$  Da, degree of deacetylation 90 %, degree of  
100 carboxymethylation 80 %) was purchased from Golden-shell Biochemical Co., Ltd. Methanol  
101 (96%), citric acid ( $\geq 99.5\%$ ), disodium hydrogen phosphate dodecahydrate ( $\geq 99\%$ ), boric acid  
102 ( $\geq 99.5\%$ ), borax ( $\geq 99\%$ ) were of analytical grade, and obtained from Sigma Aldrich.

103 2.2 Synthesis of dynamer Dy: Typically, BTA (162 mg, 1 mmol), Jeffamine (1.90 g, 1 mmol) are  
104 added in 30 mL methanol, and the reaction mixture was stirred at 70 °C for 4 h. After  
105 evaporation of the solvent, 20 mL Milli Q water was added, yielding a homogeneous dynamer  
106 solution of  $5 \times 10^{-2}$  M as calculated from the remaining aldehyde groups.

107 2.3 Preparation of CMCS-Dy hydrogels: CMCS (1.06 g, 5 mmol calculated from D-glucosamine  
108 units) was dissolved in 50 mL Milli-Q water at room temperature, yielding a transparent solution  
109 of  $1 \times 10^{-1}$  M. CMCS and dynamer solutions were mixed at different ratios to a total volume of

110 12 mL, followed by ultra-sonication for 1 min to remove trapped bubbles. Gelation then  
111 proceeded at 37 °C for 24 h, yielding a CMCS-based hydrogel.

112 Freeze-drying was performed as follows so as to conserve the original structure. As-prepared  
113 hydrogels were placed in small vials and immersed in liquid nitrogen (-196 °C) for instantaneous  
114 freezing. The vials were then placed in a 500 mL round-bottomed flask which was fixed on  
115 LABCONCO® freeze dryer. The hydrogels were freeze-dried for 24 h before analyses.

116 2.4 Characterization: <sup>1</sup>H NMR spectroscopy was carried out using Bruker NMR spectrometer  
117 (AMX500) of 300 MHz. CDCl<sub>3</sub> or D<sub>2</sub>O was used as the solvent. 5 mg of sample were dissolved  
118 in 0.5 mL of solvent for each analysis. Chemical shifts were recorded in ppm using  
119 tetramethylsilane (TMS) as internal reference. The morphology of freeze dried hydrogels was  
120 examined using scanning electron microscopy (SEM, Hitachi S4800). The samples were  
121 subjected to gold coating prior to analysis. Fourier-transform infrared spectroscopy (FT-IR) was  
122 performed with Nicolet Nexus FT-IR spectrometer, equipped with ATR diamant Golden Gate.

123 2.5 Structural stability of Dy: The stability of the dynamer Dy was evaluated in D<sub>2</sub>O under  
124 neutral and acidic conditions since imine bond formation is reversible at low pH. D<sub>2</sub>O solutions  
125 at pH of 1, 3, and 5 were prepared by addition of trifluoroacetic acid. NMR spectra were  
126 registered just after dynamer dissolution and after 7 days.

127 2.6 Rheology: The rheological properties of hydrogels were examined with Physical MCR 301  
128 Rheometer (Anton Paar). Hydrogels prepared in Milli-Q water were placed on a cone plate  
129 (diameter of 4 cm, apex angle of 2 °, and clearance 56 μm). Measurements were made in the  
130 linear visco-elastic range as a function of time, strain, or frequency.

131 2.7 Swelling: The swelling behavior of hydrogels was evaluated in buffer solutions at various pH  
132 values. Solutions from pH 1 to pH 7 were prepared using 0.1 × 10<sup>-3</sup> M citric acid solution and

133  $0.2 \times 10^{-3}$  M disodium hydrogen phosphate solution, whereas solutions of pH 8 and 9 were  
134 prepared using  $0.2 \times 10^{-3}$  M boric acid solution and  $0.5 \times 10^{-4}$  M borax solution. Freeze-dried  
135 gels were immersed in a buffer, and taken out at different time intervals. The swollen hydrogels  
136 were weighed after wiping surface water with filter paper, freeze-dried for 24 h, and weighed  
137 again. The swelling ratio and mass loss ratio of hydrogel were calculated according to equation  
138 (1) and equation (2), respectively:

$$139 \quad \text{Swelling ratio \%} = \frac{(M_s - M_d)}{M_d} \times 100 \quad (1)$$

$$140 \quad \text{Loss ratio \%} = \frac{(M_0 - M_d)}{M_0} \times 100 \quad (2)$$

141 Where  $M_0$  is the initial mass of xerogel,  $M_s$  is the wet mass of the swollen hydrogel, and  $M_d$  is  
142 the dried mass of the swollen hydrogel after lyophilization.

143 2.8 Self-healing experiments: Various hydrogel samples were prepared in Milli-Q water, and in  
144 pH = 7 and pH = 8 buffers. Some of them were dyed yellow with 5  $\mu$ L of lucigenin, or dyed red  
145 with Rhodamine. 3 different approaches were applied to examine the self-healing behavior of  
146 hydrogels: 1) a hole with diameter around 3 mm was punched at the circle center of the sample;  
147 2) samples were split into two pieces, and then a yellow piece was put together with a transparent  
148 piece immediately at 37 °C ; 3) injection of transparent and red samples on the surface of a Petri  
149 dish to observe color changes.

150 2.9 Cell cultures and Cytotoxicity: 2 mL of CMCS at 100 mmol and 1 mL of dynamer at 50  
151 mmol were mixed. Human MSCs isolated from adipose tissue (AT-MSCs) or from bone marrow  
152 (BM-MSCs) were added at a concentration of  $1 \times 10^6$  cells / mL. As control, AT-MSCs or BM-  
153 MSCs were seeded on 96-wells TCPS (Tissue Culture Polystyrene System) plates at  $5 \times 10^3$  cells  
154 per well, or were embedded in 3 mg / mL rat collagen type I hydrogel (Corning) at  $1 \times 10^6$  cells /  
155 mL. 50  $\mu$ L of cell laden solution were loaded in each well of 96 wells ultra-low adhesion plates

156 (Corning). After 2 h gelation in an atmosphere at 37 °C, 5 % CO<sub>2</sub> and 95 % of humidity, 100 μL  
157 of proliferative medium (αMEM containing 10% fetal calf serum, 100 μg/mL  
158 penicillin/streptomycin, 2 × 10<sup>-3</sup> M glutamine, 1 ng / mL of basic fibroblast growth factor) were  
159 added on the top of the hydrogel. MSCs were cultured for 7 days with medium change at day 3.  
160 After 1 or 7 days culture, the cell viability was analyzed by confocal microscopy (Leica) after  
161 staining the live cells in green and the dead cells in red using the live/dead assay kit (Invitrogen).

162

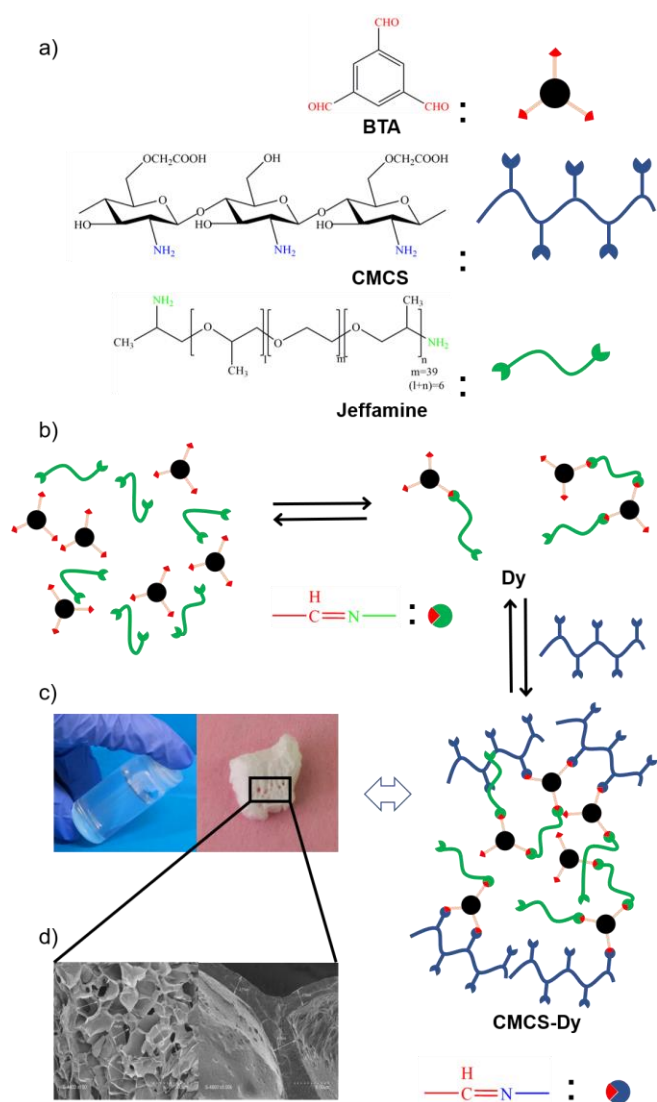
### 163 3. Results and discussion

164 3.1 Synthesis of dynamer: A water soluble dynamer Dy was first synthesized by reaction of BTA  
165 as the core structure and bifunctional diamine, Jeffamine<sup>®</sup> ED-2003 (Mn=1900) as the water-  
166 soluble linker at a molar ratio of 1:1 via reversible imine bond formation, as shown in Scheme 1.  
167 Thus, it remains in average one aldehyde group per molecule of BTA for further cross-linking  
168 reaction with the amine groups of CMCS.

169 <sup>1</sup>H NMR spectroscopy was used to monitor the formation and stability of imine bonds during the  
170 synthesis of the dynamer. Fig. 1 presents the <sup>1</sup>H NMR spectrum of the dynamer mixture obtained  
171 after 4 h reaction at 70 °C. Three signals of aldehyde groups are observed in the 10.1-10.3 ppm  
172 range, corresponding to different degrees of substitution in trialdehyde. Signals **a** at 10.21 ppm, **b**  
173 at 10.15 ppm, and **c** at 10.09 ppm belong to non-substituted, mono-substituted, and di-substituted  
174 trialdehydes, respectively. The molar ratio of signals **a**, **b** and **c** is 1:6.6:11.5, as determined from  
175 the peak integrations. These findings indicate formation of a dynamer with various free aldehyde  
176 groups, which is beneficial for subsequent crosslinking with amino groups of CMCS by imine  
177 formation. Signal **d** in the range of 8.0-8.7 ppm is assigned to the imine and aromatic protons,  
178 signal **e** around 3.7 ppm to the methylene and methine protons, and signal **f** around 1.2 ppm to

179 the methyl protons of Jeffamine, respectively (Catana et al., 2015). The presence of residual  
180  $\text{CHCl}_3$  and  $\text{H}_2\text{O}$  is detected at 7.3 and 1.8 ppm, respectively.

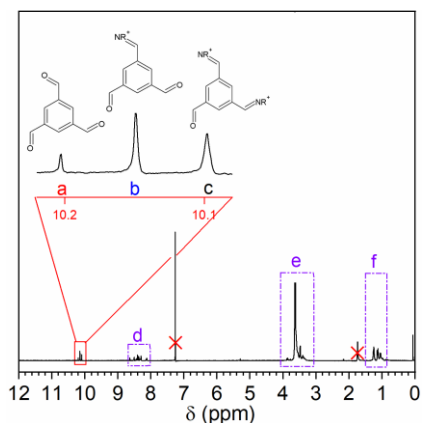
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182

183 **Scheme 1.** Synthesis route of CMCS-based dynamic hydrogel: a) chemical structures of BTA,  
184 CMCS and Jeffamine; b) synthesis of the dyanmer Dy by reaction of equimolar BTA and  
185 Jeffamine, and synthesis of hydrogel by imine formation between Dy and CMCS; c) images of as  
186 prepared hydrogel and freeze dried hydrogel; and d) SEM images of freeze dried hydrogel.

187



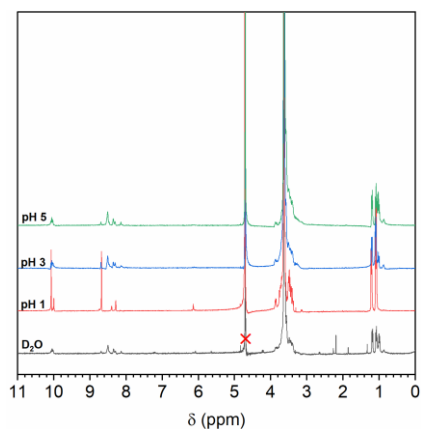
188

189 **Fig. 1.**  $^1\text{H}$  NMR spectrum of the dyanmer Dy obtained by reaction of BTA and Jeffamine in  
 190  $\text{CDCl}_3$ .

191

192 The effect of reaction time on the formation of dyanmer was investigated. No difference was  
 193 observed on the  $^1\text{H}$  NMR spectra of samples up to 72 h reaction (Fig. S1, Supporting  
 194 Information), thus implying that equilibrium was reached after 4 h reaction. Therefore, the  
 195 dyanmer obtained after 4 h reaction was selected for further studies. Moreover, the dyanmer  
 196 apparently remained unchanged for a week in pure  $\text{D}_2\text{O}$  and in acidic  $\text{D}_2\text{O}$  at pH 3 and 5, while  
 197 became highly hydrolyzed in strongly acidic medium at pH 1 (Fig. 2). The spectra obtained in  
 198  $\text{D}_2\text{O}$  and at pH 3 and 5 remain unchanged even after 7 days (Fig. S2, Supporting Information). In  
 199 contrast, major changes are observed on the spectrum of dyanmer at pH 1. The signal **a** (10.21  
 200 ppm) belonging to free aldehydes becomes much more intense, indicating that imine bonds are  
 201 hydrolyzed back to aldehydes. Therefore, the dyanmer Dy seems stable at neutral and slightly  
 202 acidic pH, but unstable at strongly acidic pH. As previously observed for PEGylated networks,  
 203 Jeffamine chains could have a protecting effect against the hydrolysis of imine bonds, favoring  
 204 the imine formation in slightly acidic or neutral media (Catana et al., 2015).

205



206

207 **Fig. 2.**  $^1\text{H}$  NMR spectra of the dynamer Dy in pure  $\text{D}_2\text{O}$  and acidic  $\text{D}_2\text{O}$  at  $\text{pH} = 1, 3,$  and  $5$

208

209 3.2 Synthesis of CMCS-Dy hydrogels: CMCS based hydrogels were prepared via a ‘green’ and  
 210 environmentally friendly method, as shown in Scheme 1. The free aldehyde groups of Dy react  
 211 with the amine groups of CMCS to form imine bonds in water, leading to a three-dimensional  
 212 network of CMCS based hydrogel. A series of hydrogels were obtained by mixing  $1 \times 10^{-1}$  M  
 213 CMCS and  $5 \times 10^{-2}$  M dynamer aqueous solutions to a total volume of 12 mL. Gelation  
 214 proceeded at  $37^\circ\text{C}$  for 24 h. The D-glucosamine to dynamer molar ratio varied from 1:1 to 8:1,  
 215 as shown in Table 1.

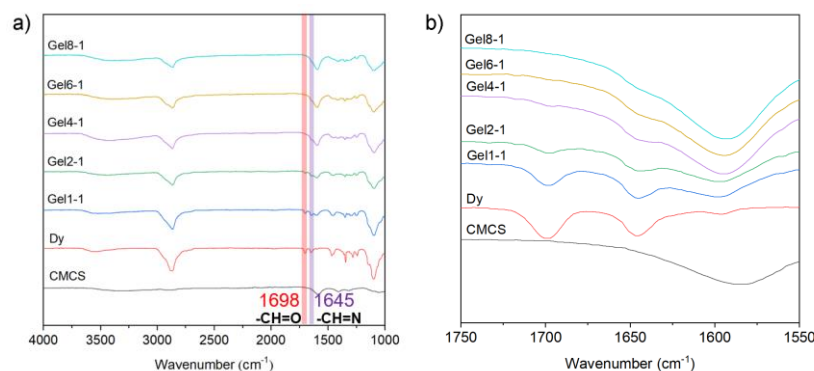
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217 **Table 1.** Molar and mass composition of CMCS-Dy hydrogels <sup>a)</sup>

Sample	D- glucosamine/Dy molar ratio	CMCS <sup>b)</sup>			Dy			Total polymer concentration [w/v %]
		[mmol]	[mg]	[w/v %]	[mmol] <sup>c)</sup>	[mg] <sup>d)</sup>	[w/v %]	
Gel1-1	1:1	0.4	85	0.7	0.4	810	6.8	7.5
Gel2-1	2:1	0.6	127	1.1	0.3	608	5.0	6.1
Gel4-1	4:1	0.8	170	1.4	0.2	405	3.4	4.8
Gel6-1	6:1	0.9	191	1.6	0.15	304	2.5	4.1
Gel8-1	8:1	0.96	204	1.7	0.12	243	2.0	3.7

218 a) Hydrogels are prepared by mixing CMCS and dynamer solutions at different ratios to a total volume of 12 mL;  
 219 b) The concentration of CMCS solution is 100 mM. Calculations are made on the basis of the average molar mass of 212 g/mol  
 220 obtained for D-glucosamine, taking into account the degree of deacetylation of 90 % and the degree of carboxymethylation of  
 221 80 %;  
 222 c) The concentration of Dy solution is 50 mM calculated from the remaining aldehyde groups. In a typical reaction, 1 mmol BTA  
 223 (162 mg) reacts with 1 mmol Jeffamine (1900 mg) to form a dynamer. As BTA has 3 aldehydes and Jeffamine 2 amines, there  
 224 remains theoretically 1 mmol of aldehydes in the dried dynamer. Addition of 20 mL water yields a dynamer solution of 50 mM.  
 225 d) The amount of Dy in solution is obtained from the initial quantities of BTA and Jeffamine.  
 226

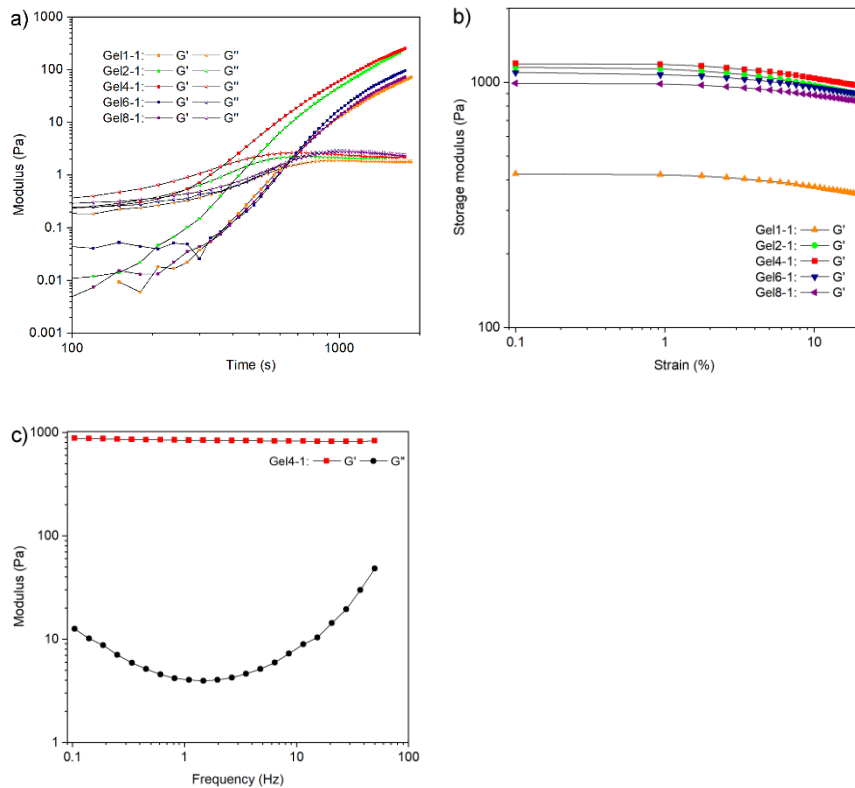
227 FTIR was used to confirm the formation of imine bonds during the synthesis of the dynamer  
 228 Dy and CMCS based hydrogel. As shown in Fig. 3, the characteristic bands of aldehyde and  
 229 imine bonds are observed at 1698 and 1645  $\text{cm}^{-1}$  on the spectra of Dy, respectively. The dried  
 230 gels present all the characteristic bands of CMCS and dynamer: a large band in the 3200-3500  
 231  $\text{cm}^{-1}$  assigned to free OH and  $\text{NH}_2$  groups, an intense band at 1595  $\text{cm}^{-1}$  assigned to carboxyl  
 232 groups of CMCS, and two strong signals at 2850 and 1100  $\text{cm}^{-1}$  attributed to C-H and C-O  
 233 stretching in the dynamer, respectively. With increasing D-glucosamine to dynamer molar ratio,  
 234 the aldehyde band progressively disappears at ratios above 4:1, while the imine band merges  
 235 with that of carboxyl groups at 1595  $\text{cm}^{-1}$  which turns more intense. These findings confirm that  
 236 hydrogels are formed because of imine formation between aldehyde and amine groups.  
 237



238  
 239 **Fig. 3.** a) FT-IR spectra and b) enlarged view of the 1550-1750  $\text{cm}^{-1}$  wavelength range of  
 240 CMCS, dynamer Dy and freeze-dried CMCS-Dy hydrogels.

241  
242 3.3 Rheological studies. The rheological properties of hydrogels were investigated under various  
243 conditions. CMCS and Dy aqueous solutions were mixed in situ on the plate of rheometer, and  
244 changes of the storage modulus ( $G'$ ) and loss modulus ( $G''$ ) were followed as a function of time  
245 at 37°C (Fig. 4a). For all samples, at the beginning of experiment the storage modulus is lower  
246 than the loss modulus ( $G' < G''$ ), which illustrates a liquid-like behavior of the starting mixture.  
247 After an induction time, both  $G'$  and  $G''$  begin to increase,  $G'$  increasing faster than  $G''$ . A cross-  
248 over point between  $G'$  and  $G''$  is detected, indicating sol-gel transition. As shown in Figure 4a,  
249 the gelation time decreases from 600 s for Gel1-1 to a minimum of 360 s for Gel4-1, and then  
250 increases to 660 s for Gel8-1. In fact, gelation occurs by crosslinking via imine bonds formation  
251 and is thus dependent on the ratio between amine groups of CMCS and aldehyde groups of the  
252 dynamer. In Gel1-1, there are less amine groups than aldehyde ones, as calculated by taking into  
253 account the degree of deacetylation of 90 %. Thus, gelation is relatively slow. Gelation is  
254 progressively improved for Gel2-1 and Gel4-1, as the concentration of amine groups increases.  
255 Gelation is not optimal for Gel2-1, since unreacted aldehyde groups are detected by FTIR after  
256 24 h at 37 °C (Fig. 3). In contrast, optimal imine bond formation is achieved for Gel4-1 as  
257 aldehyde groups are no longer detectable. Nevertheless, with further increase of D-  
258 glucosamine/dynamer ratio to 6:1 and 8:1, the gelation becomes longer as there are less aldehyde  
259 groups available for imine formation.

260



261

262

263 **Fig. 4.** a) Storage modulus ( $G'$ ) and loss modulus ( $G''$ ) changes as a function of time after mixing  
 264 CMCS and Dy aqueous solutions at various ratios at 37 °C, strain of 1%, and frequency of 1 Hz;  
 265 b)  $G'$  changes as a function of applied strain for all hydrogels at 25 °C, and frequency of 1 Hz;  
 266 and c)  $G'$  and  $G''$  changes of Gel4-1 as a function of frequency at 25 °C, and strain of 1%. All  
 267 hydrogels are prepared in Milli-Q water.

268

269 Rheological measurements performed at 25 °C illustrate the viscoelastic behaviors of as prepared  
 270 hydrogels. The storage modulus of all gels slightly decreases (less than 20 % of the initial value)  
 271 when increasing the strain up to 20 % (Fig. 4b), indicating that the hydrogels are stable in this  
 272 strain range with viscoelastic behavior. On the other hand, the modulus increases with increasing  
 273 D-glucosamine to dynamer molar ratio from 1:1 to 4:1, reaching a maximum value of *c.a.* 1200  
 274 Pa at 4:1. In contrast, higher D-glucosamine to dynamer ratios of 6:1 and 8:1 result in decrease in

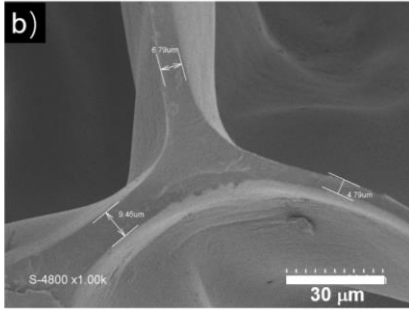
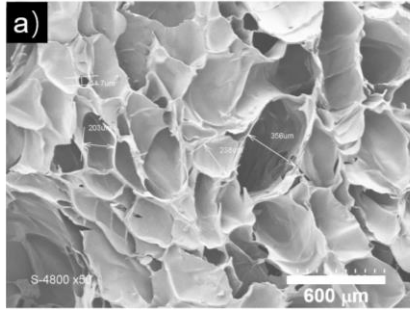
275 modulus because there are less aldehydes available for crosslinking in Gel6-1 and Gel8-1  
276 compared to Gel4-1. These findings well agree with storage modulus ( $G'$ ) and loss modulus ( $G''$ )  
277 changes versus time in Fig. 4a, confirming that optimal crosslinking is achieved with Gel4-1. In  
278 order to investigate the stability of the hydrogels, a frequency sweep over a range from 0.01 to  
279 50 Hz was carried out at a fixed strain of 1 %. Taking Gel4-1 as an example (Fig. 4c), the storage  
280 modulus  $G'$  is always much higher than the loss modulus  $G''$ .  $G'$  remains nearly unchanged,  
281 whereas  $G''$  exhibits some fluctuations with increasing frequency. The other hydrogels exhibit  
282 similar behaviors (Fig. S3, Supporting Information). These rheological results well corroborate  
283 with the formation of highly stable covalent networks, in contrast to physical hydrogels whose  
284 storage and loss moduli are dependent on the frequency (Li, El Ghzaoui, & Dewinck, 2005;  
285 Zhang et al., 2010). It is generally admitted that hydrogels with  $G'$  below 2000 Pa are 'soft'  
286 materials suitable for specific tissue engineering applications (brain, cartilage, muscle, etc).

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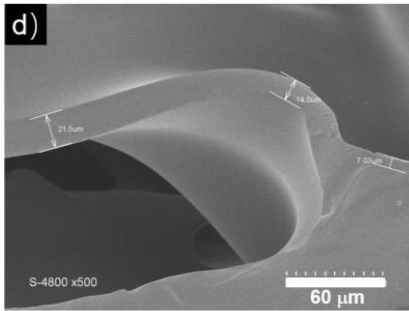
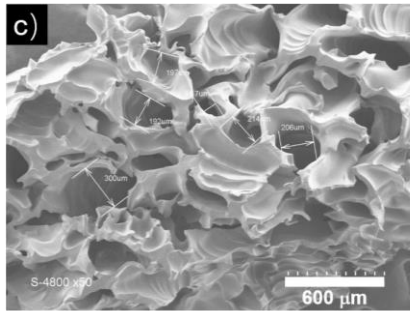
288 3.4 Morphology and swelling studies: Scanning electron microscopy (SEM) was used to  
289 qualitatively assess the microstructure of the freeze-dried hydrogels. As shown in Fig. 5, all  
290 samples exhibit a sponge-like structure with open and interconnected pores. Gel4-1 apparently  
291 exhibits the most uniform porous structure with mean pore size around 150  $\mu\text{m}$  and mean wall  
292 thickness of *c.a* 3  $\mu\text{m}$ , whereas the other samples, in particular Gel1-1 and Gel8-1, present larger  
293 and irregular pore size and larger wall thickness. These findings well agree with the optimal  
294 imine formation or crosslinking of Gel4-1 since higher crosslinking leads to smaller pore size  
295 and wall thickness.

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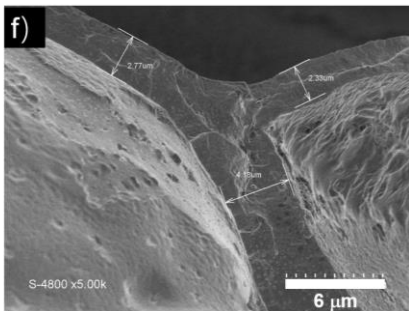
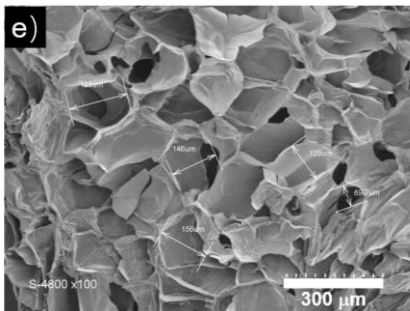
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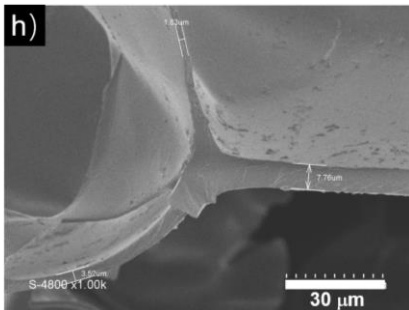
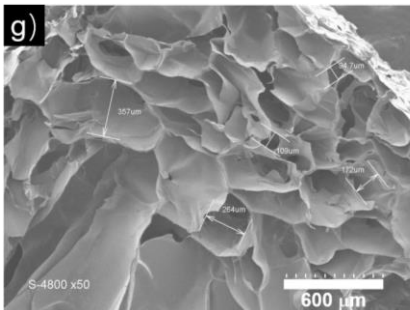
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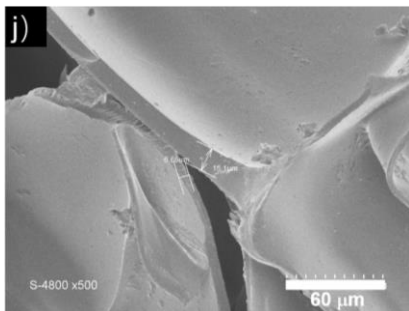
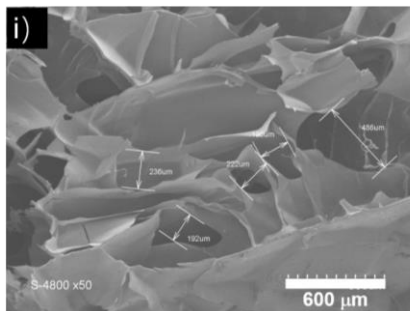
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301



302 **Fig. 5.** SEM images of freeze dried hydrogels: (a, b) Gel1-1; (c, d) Gel2-1; (e, f) Gel4-1; (g, h)  
303 Gel6-1; (i, j) Gel8-1.

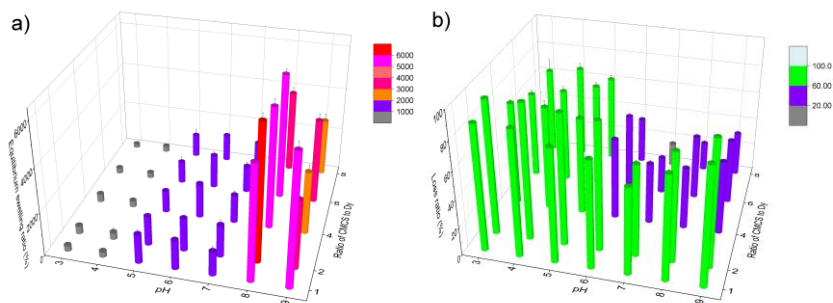
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305 The swelling behaviors of hydrogels are of major importance for the applications as drug  
306 carrier or as tissue engineering scaffold. The five samples exhibit similar swelling behaviors at a  
307 given pH value in the pH range from 3 to 9. The highly pH-sensitive swelling ratios are below  
308 1000 % for acidic media (gray bar, pH 3/4), between 1000 % and 2000 % for neutral media  
309 (violet bar, pH 5/6/7), above 2000 % and up to 6000 % for alkaline media (orange bar, pink bar,  
310 reddish orange bar, magenta bar, and red bar, pH 8/9) as shown in Fig. 6a. Interestingly, when  
311 immersed in slightly alkaline medium at pH 8, the swelling ratio of Gel4-1 dramatically depends  
312 on the immersion time (Fig. 6c). It increases from 3130 % (green bar) after 1 h to 7050 % (red  
313 bar) after 48 h immersion. In more alkaline medium at pH 9, the variation of the swelling ratio is  
314 attenuated, from 2500 % (blue bar) after 1 h to 3500 % (green bar) after 48 h immersion (Fig. 6c).  
315 In contrast, this exceptional time dependent swelling behavior of Gel4-1 rapidly reaches an  
316 equilibrium at 1 h for pH 3-7.

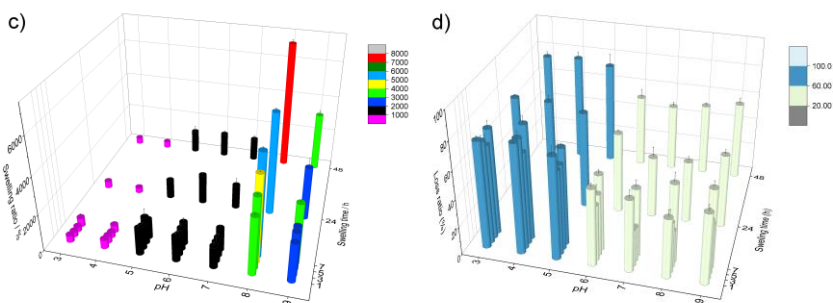
317 Mass loss could occur after swelling of hydrogels at various pH values, resulting from the  
318 diffusion and washing away of non-crosslinked species, including those initially present or  
319 formed by hydrolysis of imine bonds under acidic conditions. Thus the mass loss ratio reflects  
320 the crosslinking degree and the stability of hydrogels. Obviously, when immersed in acidic  
321 media at  $\text{pH} \leq 5$  for all hydrogels or in the whole pH range for Gel1-1 and Gel2-1 with low  
322 CMCS and high Dy contents (Fig. 6b), the mass loss ratio is above 60 % (green bar). Loss ratios  
323 below 60 % (violet and gray bars) are obtained only in neutral or alkaline media ( $\text{pH} \geq 6$ ) for  
324 Gel4-1 with optimal crosslinking, and Gel6-1 and Gel8-1 with decreasing Dy content (Table 1).

325 These findings indicate that higher Dy content and acidic medium are conducive to the mass loss  
326 of hydrogels during swelling. It is thus supposed that unconnected or incompletely connected Dy  
327 is predominant in the soluble fraction. Dy rings or homopolymers could be formed during  
328 reaction of BTA and Jeffamine (Scheme 1). These species may escape coupling with CMCS in  
329 the hydrogel preparation procedure. This assumption is consistent with IR analysis showing the  
330 presence of the aldehyde band for Gel1-1 and Gel2-1, and its absence for Gel4-1, Gel6-1 and  
331 Gel8-1 samples (Fig. 3). The reaction conditions could be improved by reducing the time and/or  
332 lowering the temperature to minimize the formation of these species.

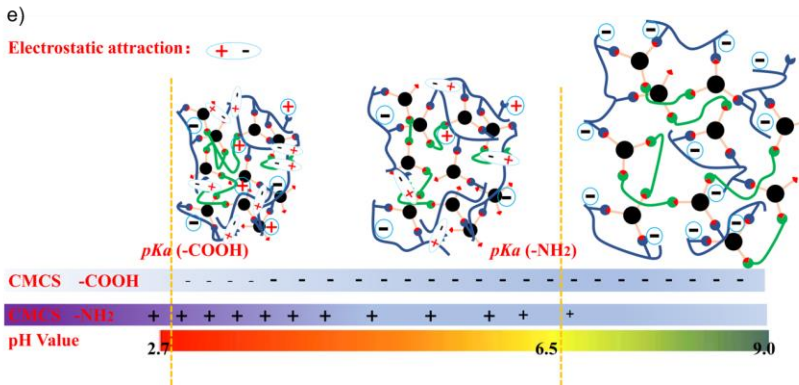
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336

337 **Fig. 6.** a) Equilibrium swelling ratios, and b) Mass loss ratios of Gel1-1, Gel2-1, Gel4-1, Gel6-1,  
 338 and Gel8-1 at various pH values for 24 h, c) Swelling ratios, and d) Mass loss ratios of Gel4-1 at  
 339 different pH values as a function of immersion time, e) Schematic presentation of the swelling  
 340 behavior of freeze-dried hydrogels immersed in buffers at various pH values.

341

342 The mass loss ratio of Gel4-1 also varies with immersion time at different pH values: in the  
 343 range of 20-60 % (milk white bar) up to 48 h in neutral / alkaline media at  $pH \geq 6$ , and above 60%  
 344 (dusty blue bar) in acidic media at  $pH=3-5$  probably because of the partial hydrolysis of imine  
 345 bonds (Fig. 6d). These results indicate that freeze dried hydrogels could be interesting for uses in  
 346 physiological environment owing to higher swelling and better stability.

347 The pH dependent swelling behaviors of hydrogels could be explained by the electrostatic  
 348 interactions due to the presence of amino and carboxyl groups along CMCS chains. In fact, the  
 349  $pK_a$  of amino and carboxyl groups is 6.5 and 2.7 (Lv et al., 2018), respectively. Thus, at acidic  
 350 pH 3 and 4, there is strong electrostatic attraction between negatively charged  $-COO^-$  and  
 351 positively charged  $-NH_3^+$  groups, which results in shrinkage or low swelling ratio of hydrogels  
 352 (Fig. 6e). With increasing pH up to 7, there are less protonated  $NH_3^+$  and ionized  $-COO^-$  groups,  
 353 leading to lower electrostatic attraction and higher swelling. In contrast, at pH 8, the  $NH_2$  groups  
 354 are not charged, while the electrostatic repulsion between the charged  $-COO^-$  groups along

355 CMCS chains leads to strong swelling. However, at pH 9, the electrostatic repulsion between the  
356  $\text{-COO}^-$  groups is counterbalanced by the  $\text{OH}^-$  ions in solution. Consequently, the swelling is  
357 attenuated as compared to that observed at pH 8.

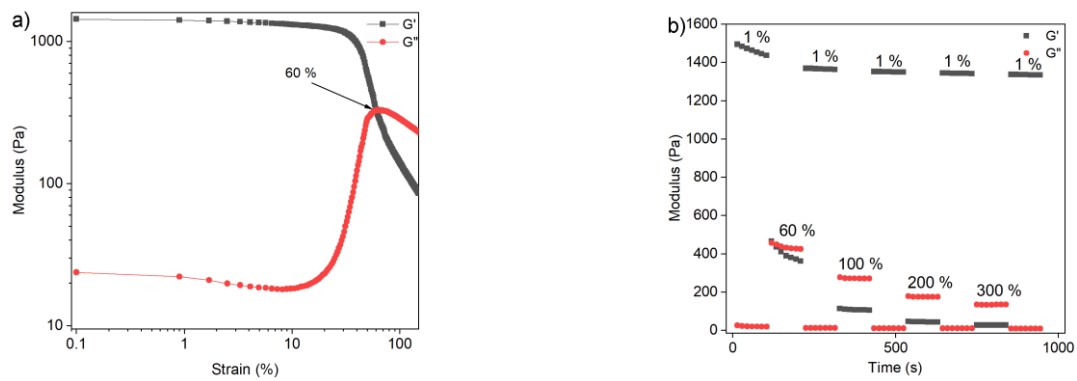
358 Changes of the micro-structure of hydrogels were observed by using SEM after 24 h swelling  
359 at two pH values. At pH 4, all freeze-dried hydrogels strongly shrink with reduced pore size and  
360 pore number (Fig. S4, Supporting Information). Noticeably, the pore size of Gel4-1 decreases  
361 from *c.a* 150 to 100  $\mu\text{m}$ , and the wall thickness increases from *c.a* 3.5  $\mu\text{m}$  to 15  $\mu\text{m}$ , reminiscent  
362 with the contraction of hydrogels due to electrostatic attraction at acidic pH (Figure 6e). In  
363 contrast, expansion of the porous structure is observed at pH 8 (Fig. S5, Supporting Information).  
364 The pores wall shows a cracked structure, with the thickness strongly decreasing from *c.a* 3.5  $\mu\text{m}$   
365 to 200-600 nm due to strong swelling of hydrogels provoked by electrostatic repulsion at basic  
366 pH (Fig. 6e).

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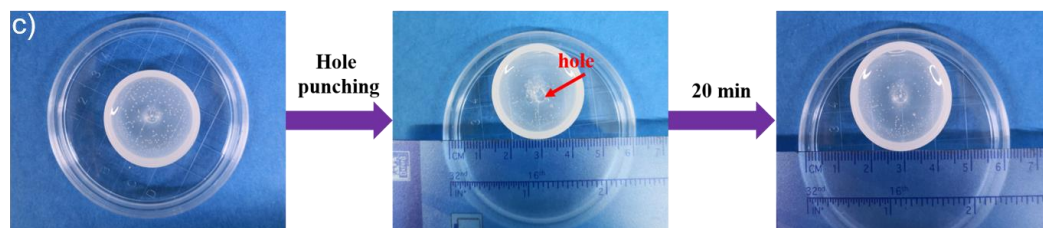
368 3.5 Self-healing: CMCS-based hydrogels present interesting self-healing behaviors as evidenced  
369 by rheological recovery tests at fixed frequency of 1 Hz and at 37°C. Gel4-1 hydrogels were  
370 prepared in Milli-Q water, and in pH 7 and pH 8 buffers in order to examine the self-healing  
371 behavior under different swollen conditions. Gelation was realized at 37°C for 24 h. As shown in  
372 Fig. 7a, both the storage modulus ( $G'$ ) and loss modulus ( $G''$ ) of Gel4-1 in Milli-Q water slightly  
373 decreases until a strain of 20%. Beyond,  $G'$  dramatically decreases, whereas  $G''$  rapidly increases.  
374 A crossover point of  $G'$  and  $G''$  values is observed at a strain of 60%. Similar profiles are  
375 observed for Gel4-1 at pH 7 with a crossover point at 35% (Fig. S6a, Supporting Information). In  
376 contrast, Gel4-1 at pH 8 exhibits lower storage modulus because of its highly swollen state as

377 shown in Figure 6a. A crossover point of  $G'$  and  $G''$  is detected at 55% at pH 8 (Fig. S6c,  
378 Supporting Information).

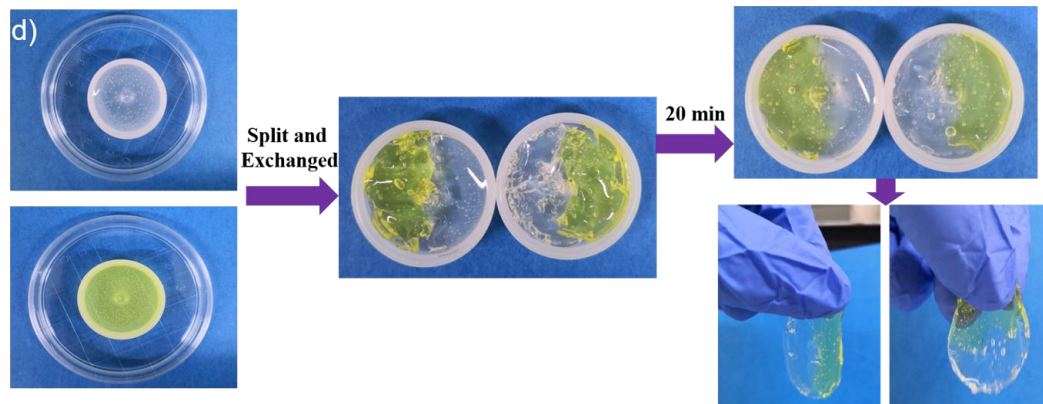
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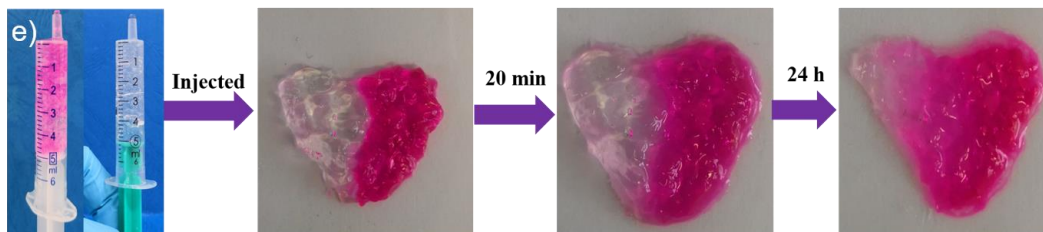
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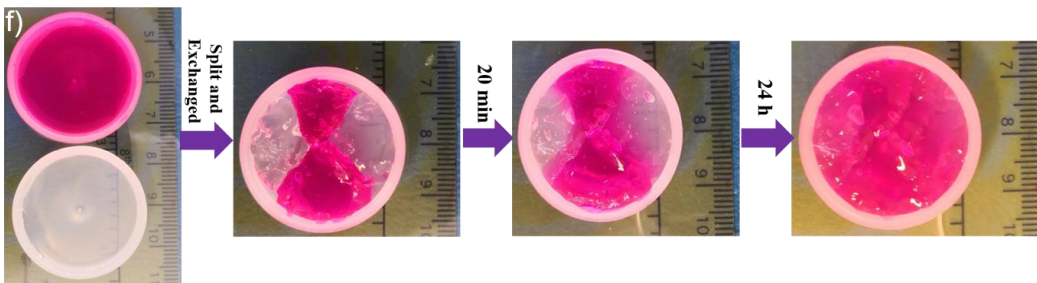
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385 **Fig. 7.** a) Modulus changes as a function of strain of Gel4-1 prepared in Milli-Q water; b)

386 Modulus changes of Gel4-1 prepared in Milli-Q water with alternatively applied high and low

387 oscillatory shear strains at 37°C; c-f) Self-healing macroscopic approaches using hydrogel

388 samples prepared in Milli-Q water (c-d), at pH 7 (e) and at pH 8 (f) , see text for details.

389

390 Based on the strain amplitude sweep results, continuous step strain measurements were

391 performed to examine the rheological recovery behavior of Gel4-1. At 1 %, Gel4-1 in Milli-Q

392 water behaves as a hydrogel since  $G'$  is largely superior to  $G''$ . As the oscillatory shear strain

393 increases from 1% to 60% and is maintained at 60% for 105 s (Fig. 7b),  $G'$  becomes lower than

394  $G''$ , indicating the destruction of hydrogel structure. Both  $G'$  and  $G''$  immediately recover their

395 initial values when the strain is back to 1%. Modulus recovery is observed when larger strains

396 (100, 200, and 300%) and small strain (1%) are alternatively applied. Similar phenomena are

397 also observed for Gel4-1 prepared in pH 7 and 8 buffers (Fig. S6b, S6d, Supporting Information).

398 Therefore, it could be concluded that dynamic hydrogels exhibit rapid recovery (self-healing)

399 behavior probably due to the reconstruction of reversible imine bond linkage when they are

400 subjected to alternatively applied high and low oscillatory shear strains.

401 The self-healing behavior of Gel4-1 was further evidenced with four different macroscopic

402 approaches using one transparent hydrogel sample and another one incorporating yellow

403 lucigenin or red Rhodamine B dyes. First, a hole with diameter of 3 mm was punched at the

404 center of a hydrogel sample prepared in Milli-Q water, and the hole disappeared after 20 min at  
405 37°C (Fig. 7c). In a second approach, transparent and yellow hydrogel samples prepared in Milli-  
406 Q water were cut into two semicircular pieces. They became integrated after only 20 min contact  
407 at 37°C. The merged piece could be then taken off and support its own weight (Fig. 7d).  
408 In a third approach, one transparent hydrogel and another one containing red Rhodamine B dye  
409 prepared in pH 7 buffer were crushed via injection onto a Petri dish using a syringe, and became  
410 integrated 20 min later (Fig. 7e). Almost the whole hydrogel was dyed red after 24 h. Similar  
411 phenomena were observed in a fourth approach for transparent and dyed red hydrogels prepared  
412 in pH 8 buffer (Fig. 7f), demonstrating that the color exchange may be observed via diffusion at  
413 the restored self-healed interfaces between different dynagels at pH 7 or pH 8.

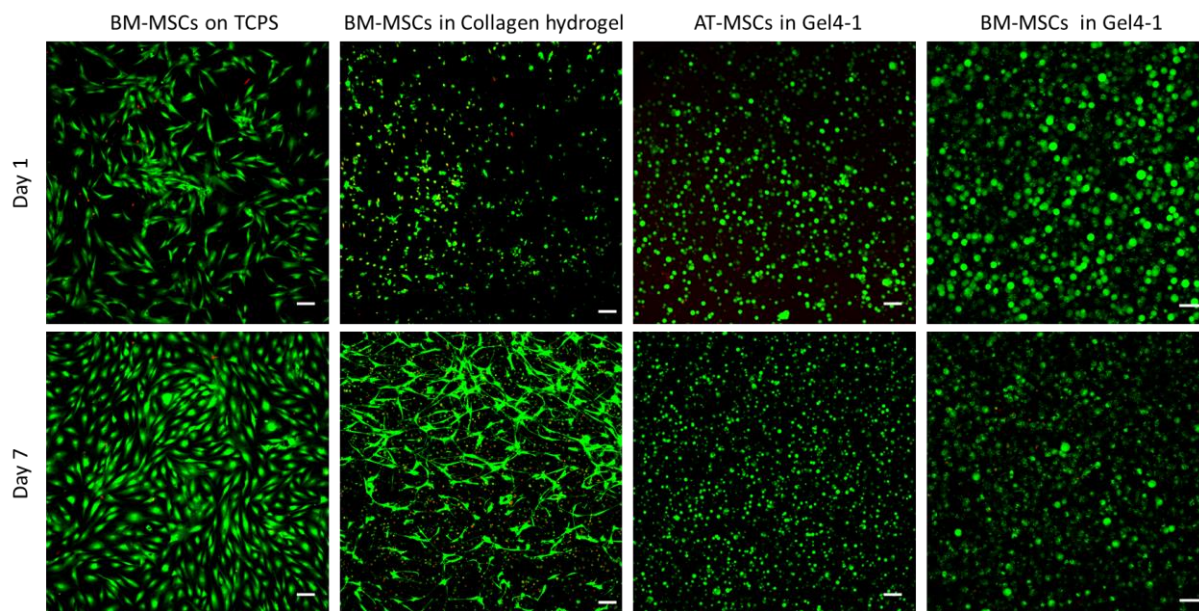
414 These tests strongly demonstrate the outstanding self-healing properties of the dynamic  
415 hydrogels - dynagels via reconstruction of reversible imine bond crosslinking, and migration of  
416 components or constituent exchanges between different hydrogels. Importantly, the use of these  
417 hydrogels with distinct and interchangeable states at different pH conditions would be  
418 advantageous for biomedical applications such as drug delivery and tissue engineering.

419

420 3.6 Cytocompatibility of hydrogels: Human mesenchymal stromal cells (MSCs) isolated from  
421 subcutaneous adipose tissue (AT-MSCs) or from bone-marrow (BM-MSCs) were encapsulated  
422 inside Gel4-1 ( $1 \times 10^6$  cells/mL), and cultured up to 7 days in proliferative medium at 37 °C to  
423 evaluate the cytocompatibility. Compared to control conditions in 2D on TCPS plate or  
424 encapsulated in a type I collagen hydrogel, AT-MSCs and BM-MSCs exhibited a round shape in  
425 Gel4-1 and not a fibroblastic phenotype (Fig. 8). One day after inclusion in the hydrogel, the  
426 large majority of AT-MSCs (95%) and BM-MSCs (99%) were alive as indicated by the green

427 color in confocal microscopy using live/dead assay, whereas only 66% of viability was observed  
428 in type I collagen hydrogel (Fig. 8). The two cell types survived for at least 7 days, as only 1 and  
429 4% of dead cells were quantified for AT-MSCs and BM-MSCs, respectively (Fig. 8). These  
430 findings demonstrate the excellent cytocompatibility of the hydrogel. It is noteworthy that the  
431 size of AT-MSCs and BM-MSCs after 7 days was smaller than that at day 1, suggesting that the  
432 pore size was reduced without affecting cell viability. Three-D reconstruction clearly shows the  
433 homogeneous distribution of MSCs in the whole hydrogel volume, indicating that the gelation  
434 time was compatible with homogenous distribution of the cells without sedimentation (Vid. S7,  
435 Supporting Information).

436



437

438 **Fig. 8.** Cell viability of human AT-MSCs or BM-MSCs in Gel4-1, in comparison with BM-  
439 MSCs in collagen hydrogel or plated on TCPS as control. Cells were labelled using the  
440 Live/Dead assay after 1 or 7 days in culture and imaged using confocal microscopy. Viable cells  
441 were stained in green and dead cells in red. Images are maximal projections of z-axis and scale

442 bars represent 100  $\mu\text{m}$  (TCPS: Tissue Culture Polystyrene Surface; AT-MSCs: Adipose Tissue-  
443 MSCs; BM-MSCs: Bone Marrow-MSCs).

444

#### 445 4. Conclusions

446 Multistate pH-sensitive hydrogels were synthesized via dynamic covalent imine bonding from  
447 two water soluble polymers, *i.e.* O-carboxymethyl chitosan (CMCS) and a cross-linking dynamer  
448 obtained by reaction of amine terminated Jeffamine as connector and Benzene-1,3,5-  
449 tricarbaldehyde as core center. The hydrogel Gel4-1 with D-glucosamine to dynamer molar ratio  
450 of 4:1 exhibits the shortest gelation time and the highest storage modulus, in agreement with  
451 optimal cross-linking or imine bond formation. Freeze-dried gels exhibit interconnected porous  
452 structures and pH-dependent swelling behavior. The swelling ratio is relatively low at acidic pH  
453 3-5 due to electrostatic attraction, while became very high, up to 7000 % at pH 8 due to  
454 electrostatic repulsion. Moreover, hydrogels present outstanding self-healing properties as  
455 evidenced by closure of split pieces and rheological studies. Self-healing occurs autonomously  
456 for different pH-dependent states, being able to reshape or to regenerate a strong chemical gel  
457 from various situations. Last but not least, MSCs encapsulated in hydrogels are all alive after 7  
458 days, in agreement with the excellent cytocompatibility of hydrogels.

459 This concept, exploiting different physical swelling states depending on pH values, results in the  
460 definition of stimuli-responsive dynagels which self-adapt their structure in response to  
461 environmental conditions. These ‘two-in-one’ dynagels may find potential uses in biomedical  
462 applications in particular as scaffold in tissue engineering.

463

464 **ASSOCIATED CONTENT**

465 **Supporting Information.**

466 The following files are available free of charge.

467 NMR spectra of daynamers; rheology data of as-prepared hydrogels; SEM images of freeze dried  
468 as-prepared hydrogels and hydrogels after swelling in buffers (pH4, pH8); rheology data of self-  
469 healing hydrogels made in buffers (pH7, pH8), 3-D reconstruction of MSCs after 7 days culture  
470 in Gel4-1 (AVI).

471

472 **ABBREVIATIONS**

473 CMCS, O-carboxymethyl chitosan; Dy, Dynamer; BTA, Benzene-1,3,5-tricarbaldehyde; PBS,  
474 phosphate buffered saline; TMS, tetramethylsilane; MSCs, human mesenchymal stromal cells.

475

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481

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