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1     **Boronate-ester crosslinked hyaluronic acid hydrogels for dihydrocaffeic acid**  
2                   **delivery and fibroblasts protection against UVB irradiation**

3

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14

15

16    **Abstract**

17    Herein, we exploit the dynamic nature and pH dependence of complexes between  
18    phenylboronic acid and diol-containing molecules to control the release of an anti-  
19    photoaging agent, dihydrocaffeic acid (DHCA), from a dynamic covalent hydrogel  
20    (HG). The HG is prepared by reversible formation of boronate ester crosslinks between  
21    hyaluronic acid (HA) modified with saccharide (GLU) residues and HA functionalized  
22    with 3-aminophenylboronic acid (APBA), part of which is involved in complexation with  
23    DHCA. The hydrogel exhibited increased dynamic moduli and a lower relaxation time  
24    at pH 7.4 in comparison to pH 6, and greater amount of DHCA was incorporated at pH  
25    7.4. Moreover, this hydrogel prolonged DHCA release at pH 7.4 through drug

26 reversible complexation/decomplexation, while the rate of release was fastest in acidic  
27 (skin) conditions. Very interestingly, the incorporation of DHCA into the network  
28 enhances its protection against UVB-induced L929 fibroblast death. Therefore, this  
29 smart hydrogel can contribute to photoaging prevention.

30

31 **Keywords:** hyaluronic acid, dynamic covalent hydrogel, dihydrocaffeic acid, anti-  
32 photoaging, small molecules delivery

33

## 34 **1. Introduction**

35 Human skin is the largest organ and the major protective barrier of the  
36 body. Consequently, it is continuously exposed to external harmful insults, especially  
37 ultraviolet radiation (UV) (Cavinato & Jansen-Dürr, 2017). UVB (280-320 nm) passes  
38 through epidermis and reaches the upper layer of dermis (Britto, Shanthakumari,  
39 Agilan, Radhiga, & Kanimozhi, 2017). Exposure to UVB causes oxidative stress by  
40 simultaneously increasing reactive oxygen species (ROS) production and decreasing  
41 endogenous antioxidants. Oxidative stress induced by UVB promotes a set of  
42 alterations in skin cells, which leads to photoaging, and is a key risk factor to skin  
43 cancer development (Cavinato & Jansen-Dürr, 2017; Dunaway, Odin, Zhou, Ji, &  
44 Zhang, 2018).

45 Several natural antiaging products have emerged, which can counteract UVB-  
46 induced skin damages by modulating some deleterious processes, including oxidative  
47 stress and inflammation (Cavinato, Waltenberger, Baraldo, Grade, & Jansen-Durr,  
48 2017). The active compounds have been incorporated in various delivery systems in  
49 order to improve their efficiency of release in the skin as well as their  
50 photochemoprotective capacity. Micro/nanoemulsions, polymeric nanoparticles and

51 other nanocarriers have been extensively studied (Bagde, Mondal, & Singh, 2018).  
52 However, there are some limitations to using these systems, among them batch-to-  
53 batch variation, shelf stability and cost of production. In addition, the effectiveness of  
54 some formulations has not been studied yet (Bagde et al., 2018; Cavinato et al., 2017).  
55 Thus, there is a need for alternative approaches for the prevention of detrimental  
56 effects of UVB in skin.

57 In this context, hydrogels are interesting candidates as platforms for antiaging  
58 agent delivery. Indeed, as hydrophilic polymer networks (Nie, Pei, Wang, & Hu, 2018),  
59 hydrogels have drawn special attention for pharmaceutical and biomedical applications  
60 due to their high moisture content, soft and flexible structure, transparency,  
61 biocompatibility, biodegradability, non-toxicity and capacity to modify or control drug  
62 release (Kostova et al., 2018; Sahiner et al., 2015; Trombino et al., 2009). Several  
63 natural and synthetic polymers can be used to prepare hydrogels. Hyaluronic acid (HA)  
64 is a natural polymer composed of a repeating disaccharide unit of D-glucuronic acid  
65 and *N*-acetyl-D-glucosamine, linked by  $\beta$ -1,4 and  $\beta$ -1,3 glycosidic bonds (Essendoubi  
66 et al., 2016). HA presents beneficial properties for the skin, such as hydration, skin  
67 repair and wound healing, as well as unique physico-chemical properties, including  
68 viscoelasticity, high water retention capacity and lubrication. Moreover, HA can be  
69 chemically modified to obtain systems responsive to external stimuli. Hence, HA is  
70 widely used in the pharmaceutical and cosmetic fields as an active ingredient and to  
71 develop different topical drug delivery systems (Avadhani et al., 2017; Este, Eglin, &  
72 Alini, 2014; Sahiner et al., 2015). As HA cannot form a hydrogel itself, reversible  
73 crosslinking via dynamic covalent bonds has emerged as an attractive strategy to  
74 obtain HA-based networks (Figueiredo et al., 2019; Lou et al., 2018; Nimmo, Owen, &  
75 Shoichet, 2011). Among dynamic covalent bonds, boric acid and boronic acid

76 derivatives have been widely used to crosslink diol-containing polymers like  
77 polysaccharides as they can readily form reversible borate and boronate esters with  
78 1,2- and 1,3-diol-containing species (Figueiredo, Ogawa, et al., 2020; Mayumi,  
79 Marcellan, Ducouret, Creton, & Narita, 2013; Sacco, Furlani, Paoletti, & Donati, 2019).  
80 In particular, crosslinking via phenylboronic acid (PBA or its derivatives) has been  
81 largely reported as it can be easily grafted on a polymer and can react with saccharides  
82 (or catechols) to form boronate esters that undergo hydrolysis under acidic conditions  
83 (Huang et al., 2018). Therefore, dynamic covalent crosslinks formed between PBA and  
84 saccharide molecules grafted on HA chains can be advantageously used to produce  
85 pH-responsive hydrogels (Figueiredo, Cosenza, et al., 2020; Heleg-Shabtai, Aizen,  
86 Orbach, Aleman-Garcia, & Willner, 2015; Roberts, Hanson, Massey, Karren, & Kiser,  
87 2007; Tarus et al., 2014; Xu et al., 2011).

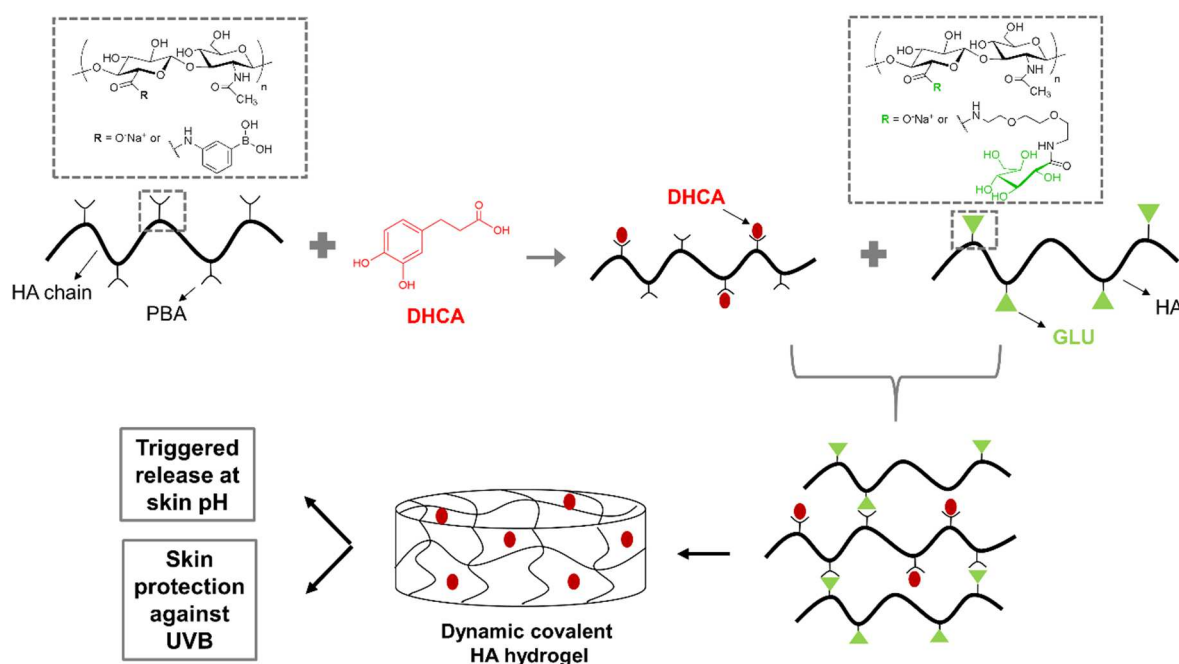
88 This is particularly interesting for epidermal application, since normal human  
89 skin exhibits an acidic pH (ranging from 3.0 to 6.5) (Duffy, Guzman, Wallace, Murphy,  
90 & Morrin, 2017). Dynamic covalent hydrogels offer many other advantages, including  
91 rapid and ease of preparation, flexibility to alter their structure like physically  
92 crosslinked networks while being more stable (Brooks & Sumerlin, 2016; Marco-Dufort  
93 & Tibbitt, 2019; Xu et al., 2011). Therefore, all these properties make such hydrogel  
94 networks attractive candidates for topical application. Accordingly, the purpose of the  
95 present study was to incorporate an antioxidant compound, dihydrocaffeic acid  
96 (DHCA), in a dynamic covalent hydrogel based on HA modified with PBA and  
97 saccharide moieties (Fig. 1), and to evaluate the potential of this smart polysaccharide  
98 network to control DHCA release and enhance cytoprotection produced against UVB  
99 death on fibroblasts.

100 Dihydrocaffeic acid (DHCA) is a phenolic compound with high antioxidant  
101 potential described in the literature (Amić, Marković, Klein, Dimitrić Marković, &  
102 Milenković, 2018; Poquet, Clifford, & Williamson, 2008), and it demonstrated  
103 cytoprotective effect on keratinocytes irradiated with UV (Poquet et al., 2008).  
104 Moreover, our previous studies showed that DHCA decreased some cellular oxidative  
105 harms, matrix metalloproteinases (MMPs) expression and apoptosis by decreasing  
106 ROS production, enhancing endogenous antioxidant defense and inhibiting mitogen-  
107 activated protein kinase (MAPK) pathway on L929 fibroblasts irradiated with UVB  
108 (Oliveira et al., 2019). Therefore, the development of a topical delivery system  
109 containing DHCA is an interesting strategy to prevent photoaging. Interestingly, once  
110 included in the HA-based dynamic covalent hydrogel described above, DHCA may  
111 form dynamic covalent bonds with PBA groups. This is the case as DHCA contains a  
112 catechol in its chemical structure. Thus, the selective entrapment of DHCA through  
113 complexation with PBA could be an attractive alternative to obtain an antiaging product  
114 with a controlled topical release of the active ingredient.

115 Some work have used hydrogels based on HA-catechol to obtain interesting  
116 skin care systems, as reported by Liang and colleagues (Liang et al., 2019). In this  
117 study, the authors developed adhesive hemostatic conductive hydrogels based on HA  
118 modified with dopamine and polydopamine-coated reduced graphene oxide using  
119 hydrogen peroxide/horseradish peroxidase as initiator system. The hydrogels  
120 demonstrated a set of interesting properties, especially for the skin, such as tissue  
121 adhesiveness, self-healing ability, wound healing, antioxidant capacity conferred by  
122 the catechol dopamine, and prolonged drug release.

123 Finally, although other studies have shown the interest of boronate ester-  
124 crosslinked hydrogels for control anticancer drug delivery (Heleg-Shabtai et al., 2015;

125 Huang et al., 2018), the reported process of drug release was based on the hydrogel  
 126 dissociation. This was the case as the active species acted as cross-linkers in the  
 127 polymer network. Conversely, in our case, the anti-photoaging agent DHCA does not  
 128 cross-link the polymer chains. Consequently, the HA network can be obtained without  
 129 the presence of DHCA, and its release from the hydrogel is not expected to promote  
 130 its dissociation.



131  
 132 **Figure 1:** DHCA complexation by HA-PBA and production of HG containing DHCA via  
 133 reversible boronate ester bond formation.  
 134

135  
 136 **2. Experimental section**

137  
 138 **2.1. Materials**

139 Sodium hyaluronic acid (HA, weight-average molar mass ( $M_w$ ): 100 kg/mol) was  
 140 obtained from Lifecore Biomedical. DHCA, 3-aminophenyl boronic acid (APBA),  
 141 Hank's balanced salt solution (HBSS), *N*-acetyl cysteine (NAC), phosphate buffer  
 142 saline (PBS) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were purchased from

143 Sigma-Aldrich. 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride  
144 (DMTMM) and D-glucono-1,5-lactone were provided by Alfa Aesar and Carbosynth,  
145 respectively. Boc-1-amino-3,6-dioxa-8-octanamine (Boc-DOOA) was purchased from  
146 Iris Biotech GMBH. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine  
147 serum (FBS) were purchased from Life Technologies/Gibco Laboratories. Neutral red  
148 was obtained from Interlab. All other solvents and chemicals used were of analytical  
149 grade. Water used in all the experiments was purified by using an Elga Purelab  
150 purification system, with a resistivity of 18.2 M $\Omega$  cm.

151

## 152 **2.2. Determination of the binding affinity ( $K_a$ ) of DHCA for APBA**

153 In order to evaluate the affinity of DHCA for APBA, we determined the  $K_a$  by  $^1\text{H}$   
154 NMR spectroscopy (Bérubé, Dowlut, & Hall, 2008). In this assay, a 1:1 binding  
155 stoichiometry was assumed. The samples were diluted in deuterated PBS 0.01 M pD  
156 6 or pD 7.4, and six different mixtures with a molar ratio of APBA/DHCA ranging from  
157 1 to 2 were obtained (detailed procedure in supplementary material, [Fig. S1 and S2](#)).  
158  $^1\text{H}$  NMR spectra of APBA alone and of APBA in the presence of excess DHCA (to  
159 induce 100% complex formation) were recorded to assign the aromatic proton signals  
160 of bound and unbound APBA. The  $K_a$  was calculated by integration of aryl protons of  
161 the APBA/DHCA complex and of free APBA (equations described in supplementary  
162 material). The  $^1\text{H}$  NMR spectra were recorded on a Bruker spectrometer operating at  
163 400 MHz (Avance III HD model, Bruker BioSpin AG, Fallanden, Switzerland), at room  
164 temperature. Chemical shifts were given relative to external tetramethylsilane (TMS =  
165 0 ppm) and calibration was performed using the signal of the residual protons of the  
166 solvent as a secondary reference.

167

### 2.3. Synthesis of HA-derivatives

HA grafted with APBA moieties (HA-PBA) was synthesized by an amide coupling reaction using APBA, HA and DMTMM as a coupling agent. APBA (93 mg, 0.50 mmol) was added to a water/dimethylformamide (water/DMF, 3:2 v/v) mixture containing DMTMM (212 mg, 0.77 mmol) and HA (306.6 mg, 0.77 mmol), and the pH was adjusted to 6.5 using 1 M aqueous solution of NaOH. The mixture was stirred for 46 h at room temperature. The resulting HA-PBA was purified by extensive dialysis against water (Spectra/Pore, MWCO 6-8 kD) followed by freeze drying (yield: 50%). The degree of substitution (DS, average number of substituting groups per repeating disaccharide unit of HA) of the final product was found to be 0.5 from <sup>1</sup>H NMR analyses (Tarus et al., 2014) using digital integration of the signals at  $\delta$  (ppm) 6.8-7.9 (protons of APBA) and 2.0 (methyl protons in HA). This value was confirmed by that derived from the reaction kinetics using the TNBS method (supplementary material, Fig. S3).

HA grafted with gluconamide moieties (HA-GLU) was obtained in three steps (Figueiredo, Cosenza, et al., 2020). Firstly, a solution of D-glucono-1,5-lactone (80.8 mg, 0.45 mmol) in DMF (20 mL) was dropped in a solution of boc-DOOA (113 mg, 0.45 mmol) in DMF (25 mL) for 3 h. After stirring overnight at room temperature, the solvent was removed under reduced pressure, and the resulting product was dried under vacuum to give boc-DOOA-gluconamide (Fig. S4). In a second step, the boc protecting group was removed by acid treatment of boc-DOOA-gluconamide (56 mg, 0.13 mmol) in trifluoroacetic acid (0.6 mL, 7.8 mmol) for 5 min at room temperature. The pH of the mixture was adjusted to 7.0, and the solvent was removed under reduced pressure, yielding 1-amino-DOOA-gluconamide (Fig. S5). Then, the HA-GLU was synthesized by an amide coupling reaction. 1-amino-DOOA-gluconamide (56 mg, 0.17 mmol) was added to a water/DMF (3:2 v/v) mixture containing DMTMM (207 mg, 0.75 mmol) and

193 HA (300 mg, 0.75 mmol), and the pH was adjusted to 6.5 using 1 M aqueous solution  
194 of NaOH. The mixture was stirred for 65 h at room temperature. The HA-GLU obtained  
195 was purified by dialysis against water (Spectra/Pore, MWCO 6-8 kD) followed by  
196 freeze-drying (yield: 84%). The DS of the HA-GLU conjugate was found to be 0.1 from  
197 the reaction kinetics using the TNBS method (supplementary material, Fig. S3), and  
198 was confirmed by <sup>13</sup>C NMR analyses (Fig. S6).

199

#### 200 **2.4. HPLC analysis of DHCA**

201 Prominence High Performance Liquid Chromatography (HPLC) system  
202 (Shimadzu, Kyoto, Japan), containing vacuum degasser, double micro-volume piston  
203 pump, auto-sampler, thermostatic column compartment and spectrophotometer  
204 detector UV-Vis and LabSolutions Software was used to analyze DHCA. Reversed-  
205 phase chromatography was performed on a C<sub>18</sub> column (150 × 3.0 mm, particle size 5  
206 μm; Shimadzu, Kyoto, Japan). The mobile phase used was 0.01% (v/v) acetic acid in  
207 water and acetonitrile (85:15 v/v), pH 3.8. The injection volume was 15 μL, with a flow  
208 rate of 1 mL/min and column temperature of 25 °C. Elution was monitored for 6 min by  
209 absorbance at 225 nm.

210 The validation of the proposed reversed-phase HPLC procedure was performed  
211 according to International Conference on Harmonization of Technical Requirements  
212 for Registration of Pharmaceuticals for Human Use Q2(R1) (ICH, 2005). The retention  
213 time of DHCA was 2.4 min. The method was specific (Fig. S7) and linear ( $y = 24319x$   
214  $+ 4852.1$ ,  $r = 0.9998$ ) using eight concentrations in the range of 0.1–100 μg/mL of  
215 DHCA. The residue analysis showed that the method presented significant regression  
216 because the calculated F for regression was higher than critical F (110286.7 > 4.04),  
217 and did not present lack of fit, regarding the calculated F for lack of fit was lower than

218 critical F ( $2.33 < 2.39$ ). Moreover, the method presented accuracy and precision (Table  
219 1S). Slight changes in mobile phase constitution (84:16 v/v) and column temperature  
220 ( $24\text{ }^{\circ}\text{C}$ ) did not alter the DHCA quantification (Table S1), indicating that the method is  
221 robust. The limit of detection and quantification were found to be  $0.40\text{ }\mu\text{g/mL}$  and  $1.22$   
222  $\mu\text{g/mL}$ , respectively.

223

## 224 **2.5. Complexation of DHCA by HA-PBA**

225 DHCA was complexed with HA-PBA using a DHCA/PBA molar ratio of 1 in 0.01  
226 M PBS pH 6 or pH 7.4 by stirring at 400 rpm for 30 min at room temperature. Non-  
227 complexed DHCA was removed by centrifugation (7000 rpm, 15 min) using centrifugal  
228 filter devices (Amicon Ultra - 15 mL centrifugal filter unit with Ultracel - 50 K membrane,  
229 NMWL 50 kDa, Millipore, Cork, Ireland). After centrifugation,  $50\text{ }\mu\text{L}$  of DHCA  
230 complexed with HA-PBA (HA-PBA-DHCA), which was retained on the filter, were  
231 diluted in acetonitrile in a ratio of 1:4 (sample:acetonitrile, v/v) and stirred for extraction  
232 of DHCA. An aliquot of the resulted solution was diluted in water in a ratio of 1:5  
233 (sample:water, v/v), filtered in micro filter ( $0.45\text{ }\mu\text{M}$ ) and analyzed by HPLC. The DHCA  
234 complexation efficiency (DCE) was calculated according to equation 1:

$$235 \text{ DCE (\%)} = (\text{weight of complexed DHCA}/\text{initial weight of DHCA in the system}) \times 100\% \quad (1)$$

236

## 237 **2.6. Formation of dynamic covalent HA hydrogels (HG)**

238 The HG were obtained by mixing different volumes of HA-PBA/DHCA complex  
239 and HA-GLU solutions ( $15\text{ g/L}$ ) in 0.01 M PBS pH 6 or pH 7.4, with a PBA/GLU molar  
240 ratio of 1 (Tarus et al., 2014) (Fig. S8). Plain HG were obtained by mixing HA-PBA and  
241 HA-GLU solutions with the same PBA/GLU molar ratio as described above for HG  
242 containing DHCA (Fig. S9).

243

## 244 **2.7. Oscillatory rheometry**

245 The oscillatory rheological analyses of freshly prepared plain HG and HG  
246 containing DHCA produced at pH 6 or pH 7.4 were carried out at 32 °C, using a cone-  
247 plate AR2000 rheometer (TA Instruments, New Castle, USA) with a film of solvent trap  
248 (silicone) to avoid solvent evaporation. The cone used is striated, has a diameter of 20  
249 mm, an angle of 4° and was separated by a fixed distance of 0.118 mm. Oscillatory  
250 strain sweep experiments at 1 Hz were used to determine the linear-viscoelastic range  
251 of the hydrogel networks as shown in Fig. S10. The oscillatory frequency sweep (0.01-  
252 10 Hz) experiments were performed within the linear viscoelastic range (strain fixed at  
253 1.5 %) to determine the frequency dependence of the storage ( $G'$ ) and loss ( $G''$ ) moduli.  
254 The frequency dependences of the  $G'$  and  $G''$  moduli were measured from at least  
255 three replicate samples.

256

## 257 **2.8. *In vitro* DHCA release from HG**

258 DHCA release from HG produced at pH 7.4 was performed in a Falcon cell  
259 culture insert (Corning Life Sciences, Durham, USA), containing a poly(ethylene  
260 terephthalate) track-etched membrane (0.4  $\mu$ M pore size), mounted in a 24 well plate  
261 (Fig. S11). HG containing DHCA (300 mg), or 300  $\mu$ L of DHCA dispersed in purified  
262 water was placed in the donor compartment, and kept in contact with 1 mL of receptor  
263 medium (0.01 M PBS pH 6 or pH 7.4). The system was maintained under stirring (450  
264 rpm) at 32 °C, and an aliquot of 500  $\mu$ L was withdrawn at 0.5, 1, 2, 4, 6 and 8 h, and  
265 replaced with the same volume of receptor medium. The amount of DHCA released  
266 over time was determined by HPLC using the methodology previously validated. All  
267 experiments were carried out in triplicate.

268

## 269 **2.9. Cell viability assay**

270 Fibroblast L929 cell line (NCTC clone 929 [L cell, L929, derivative of Strain L]  
271 ATCC® CCL1™) was maintained and cultured in DMEM supplemented with 2 mM L-  
272 glutamine, 10% (v/v) FBS, penicillin (50 U/mL), and streptomycin (50 µg/mL) at 37 °C  
273 in a 5% CO<sub>2</sub> atmosphere.

274 Neutral red assay (Borenfreund & Puerner, 1985) was used to evaluate the  
275 cytotoxicity of DHCA, plain HG and HG containing DHCA produced at pH 7.4 on L929  
276 cells. The cultured cell suspension was dispensed into a 96-well plate ( $2.5 \times 10^4$   
277 cells/well) and incubated for 24 h. Thereafter, the media was replaced with serum-free  
278 DMEM containing different concentrations of DHCA (7, 14, 21, 28 and 35 µM), of HG  
279 without and with DHCA (amount of hydrogels containing 7, 14, 21, 28, and 35 µM of  
280 DHCA), and incubated for 24 h. PBS was used to wash the monolayers after  
281 incubation, and 200 µL of neutral red solution (40 µg/mL) was added. After 3 h the  
282 supernatant was removed and the cells were fixed with an aqueous solution of 2%  
283 (v/v) formaldehyde and 1% (w/v) calcium chloride, and then 200 µL of a solution of 1%  
284 (v/v) acetic acid and 50% (v/v) ethanol was added. Absorbance was measured at 540  
285 nm (BioTek, PowerWave XS microplate spectrophotometer) after 15 min at 25 °C in  
286 the dark. The percentage of viable cells was calculated relative to untreated cells. The  
287 cytotoxicity analyses were determined in triplicate.

288

## 289 **2.10. Cytoprotection assay against UVB**

290 Neutral red assay was also used to evaluate the capacity of samples to protect  
291 L929 cells against UVB-induced death. L929 cells were seeded into 24-well plates  
292 ( $1.25 \times 10^5$  cells/well) and incubated for 24 h. After incubation, L929 cells were pre-

293 treated for 1 h with DHCA (7, 14, 21, 28, and 35  $\mu\text{M}$ ), HG without and with DHCA  
294 produced at pH 7.4 (containing 7, 14, 21, 28, and 35  $\mu\text{M}$  of DHCA) or 1 mM NAC (as  
295 a positive control) dissolved in serum-free DMEM. Then, the media with treatments  
296 was replaced by HBSS, and cells were irradiated with 600 mJ/cm<sup>2</sup> of UVB, a dose that  
297 causes 50% cell death [OLIVEIRA et al., 2018]. One UVB lamp (Philips, TL 40W/12  
298 RS SLV) at a fixed distance of 20 cm from the surface of the cell culture plates was  
299 used. The irradiation intensity was measured by radiometer (Vilber Lourmat, VLX-3W,)   
300 equipped with a sensor to detect UVB (detects peak of 312 nm) (Vilber Lourmat, CX-  
301 312). After irradiation, cells were incubated for 24 h in serum-free DMEM, and then  
302 were submitted to neutral red assay (Borenfreund & Puerner, 1985). The percentage  
303 of viable cells was calculated relative to untreated and nonirradiated cells. The  
304 analyses were carried out in triplicate.

305

### 306 **2.11. Statistical analysis**

307 Data were expressed as the mean  $\pm$  standard deviation (SD). Statistical analysis  
308 was carried out by Prisma 5.0 software using one-way analysis of variance (ANOVA)  
309 followed by Tukey test. Values of  $p < 0.05$  were considered statistically significant.

310

311

## 312 **3. Results and Discussion**

313

### 314 **3.1. Affinity of DHCA for APBA**

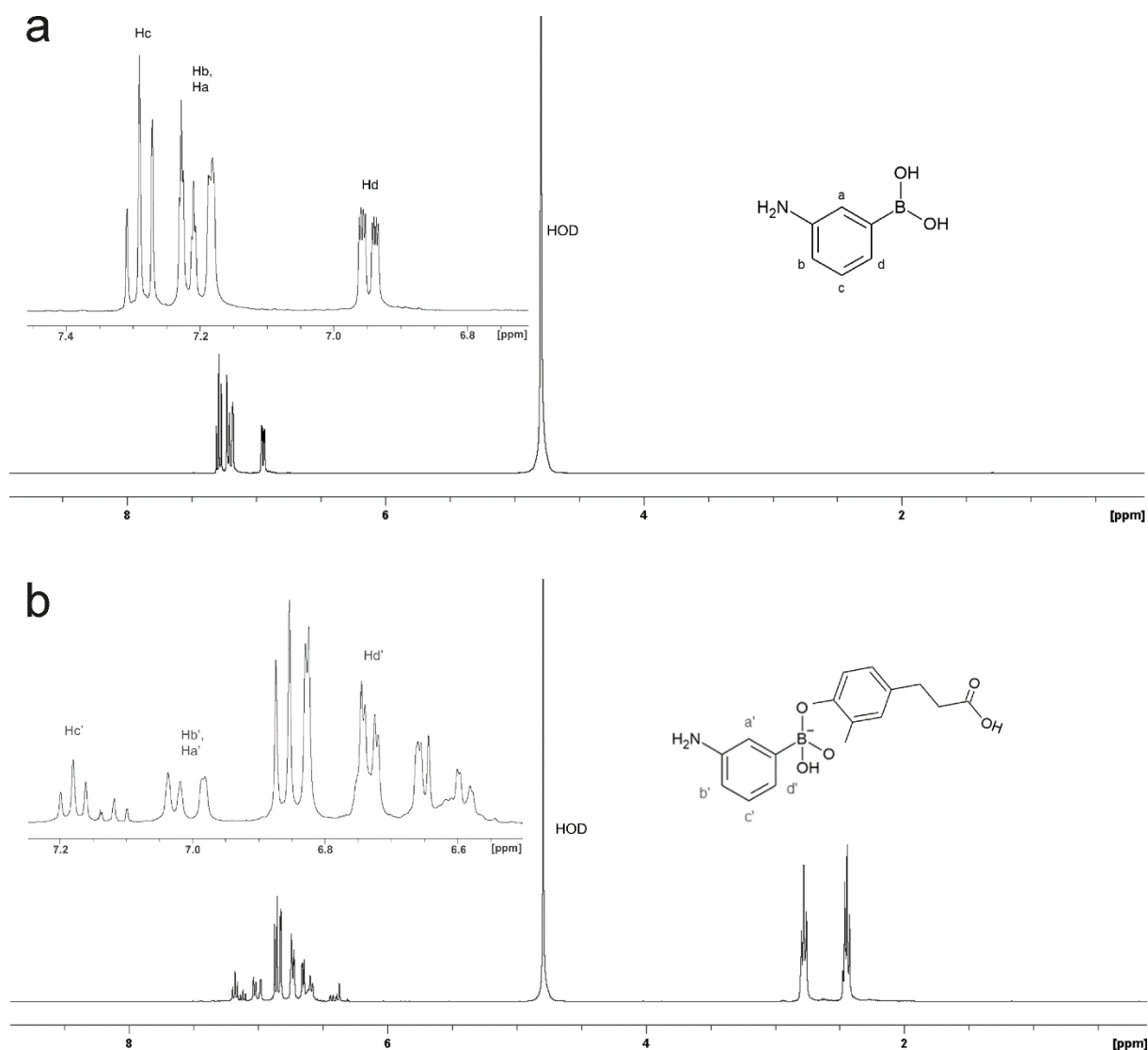
315 To verify the ability to selectively entrap DHCA in the HA hydrogel through  
316 complexation with APBA, we measured the binding affinity,  $K_a$ , of free APBA for DHCA

317 by  $^1\text{H}$  NMR spectroscopy titration. The measurements were performed at pH 6, i.e.  
318 close to the pH of skin, and at pH 7.4, which is the pH of the hydrogel formulations.

319 The  $^1\text{H}$  NMR spectra of APBA alone and in the presence of excess DHCA were  
320 first recorded to easily assign the aromatic proton signals corresponding to bound and  
321 unbound APBA (Fig. 2). Then, several  $^1\text{H}$  NMR spectra of APBA/DHCA mixtures in  
322 which the molar ratio of APBA/DHCA was varied from 1 to 2 were recorded to calculate  
323  $K_a$  as detailed in supporting information (Fig. S1 and S2).

324 The  $K_a$  value between DHCA and APBA was lower in acidic conditions ( $K_a =$   
325  $130.5 \pm 6.1$  L/mol at pH 6) than at pH 7.4 ( $K_a = 1726.7 \pm 28.4$  L/mol). These data are  
326 in line with the fact that the affinity between PBA and diol-containing molecules is pH  
327 dependent (Yan, Springsteen, Deeter, & Wang, 2004). In particular, Springsteen and  
328 Wang (Springsteen & Wang, 2002) found a  $K_a$  of  $150 \text{ M}^{-1}$  and  $830 \text{ M}^{-1}$  of the ester  
329 formed between phenylboronic acid and catechol at pH 6.5 and pH 7.4, respectively.  
330 These data suggest that the pH of skin may be used as a trigger for localized release  
331 of DHCA complexed with APBA in the hydrogel formed at pH 7.4.

332



333 **Figure 2:**  $K_a$  determination between DHCA and APBA by  $^1\text{H}$  NMR spectroscopy titration  
 334 analysis.  $^1\text{H}$  NMR spectra in 0.01 M deuterated PBS pH 7.4 at 25 °C of APBA alone (18 mM) (a)  
 335 and APBA (18 mM) in the presence of excess DHCA (54 mM) (b).  
 336  
 337

### 338 3.2. DHCA complexation by HA-PBA and formation of HG

339 In the present work, two HA derivatives modified with 3-aminophenyl boronic  
 340 acid and with gluconamide moieties (HA-PBA and HA-GLU, respectively) were used  
 341 to obtain a dynamic covalent hydrogel at physiological pH for complexation of DHCA.  
 342 Indeed, we previously demonstrated formation of networks exhibiting a gel-like  
 343 behavior at physiological pH by combining HA-PBA with HA-GLU. Such properties  
 344 were related to the high binding capacity of PBA towards glucose units in the ring-  
 345 opened form (such as gluconamide derivatives, resulting in the formation of boronate

346 ester bonds with slow exchange dynamics. By contrast, HA-PBA alone gives rise to a  
347 viscous solution similar to initial HA, as PBA has nearly no affinity for HA sugars  
348 (Figueiredo, Cosenza, et al., 2020).

349 The HA-PBA and HA-GLU derivatives were prepared by amide bond formation  
350 between the carboxylic groups of HA and amine-functionalized GLU, using DMTMM  
351 as a coupling agent (Figueiredo, Cosenza, et al., 2020). The structures of the HA-PBA  
352 and HA-GLU conjugates were confirmed by  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy,  
353 respectively. Digital integration of the NMR spectra also allowed to assess their degree  
354 of substitution (DS = 0.5 for HA-PBA and 0.1 for HA-GLU). Here, we targeted a DS  
355 value for HA-PBA more than two-fold higher than HA-GLU so that half of the grafted  
356 PBA is used for the selective complexation of DHCA.

357 Formation of the HA-PBA/DHCA complexes was carried out in 0.01 M PBS both  
358 at pH 6 and pH 7.4. The values of DHCA complexation efficiency at pH 6 and pH 7.4  
359 were found to be 50% and 58%, respectively. The difference between the two DCE  
360 values is not so high given the  $K_a$  values found for the APBA/DHCA complex at these  
361 two pH. However, the  $K_a$  values were measured for free APBA, which suggests some  
362 positive impact of HA on the DHCA binding capability to PBA at pH 6.

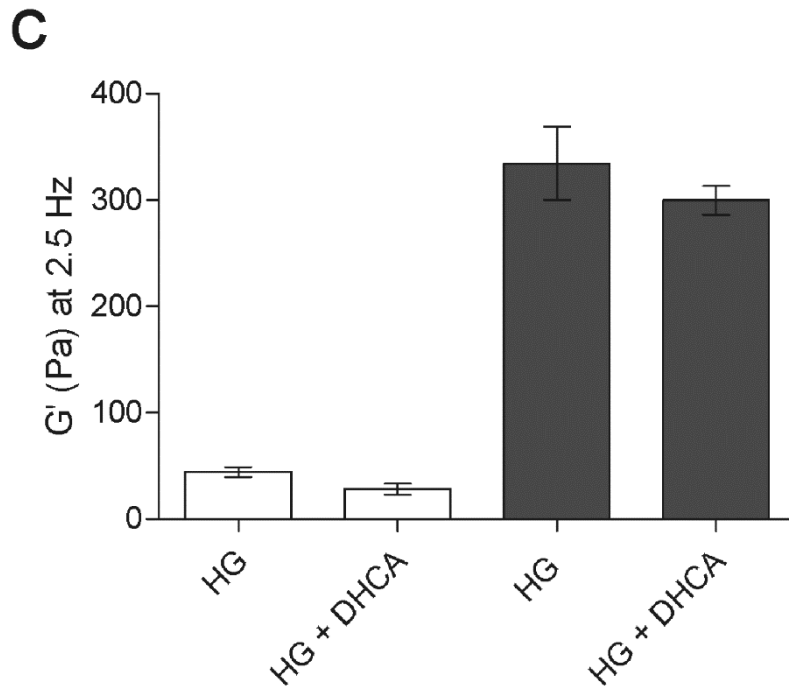
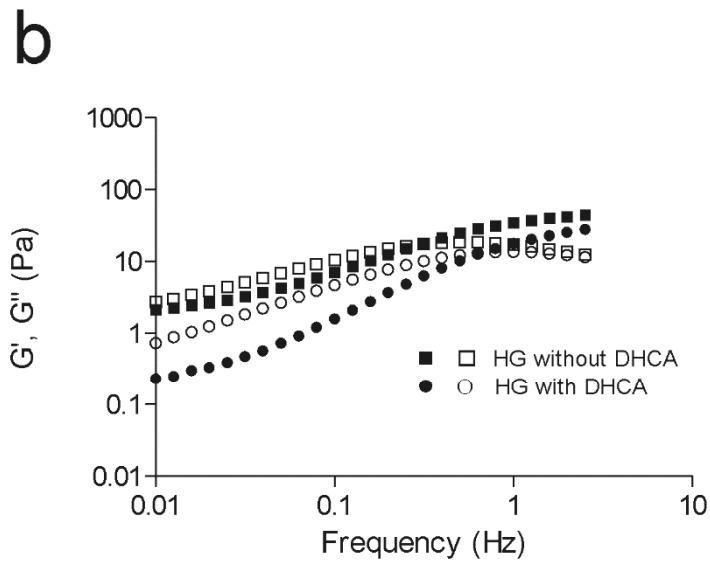
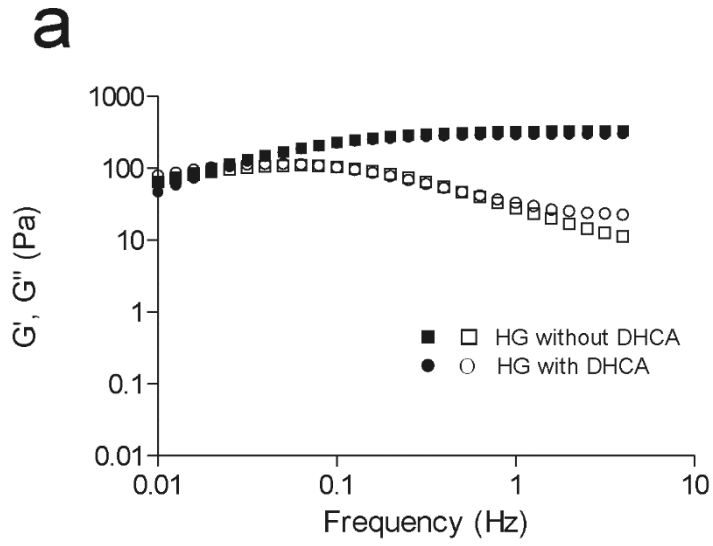
363 Then, the HG at pH 6 and at pH 7.4 were obtained by simply mixing HA-  
364 PBA/DHCA and HA-GLU, through boronate ester bond formation between the  
365 remaining APBA moieties and the GLU molecules grafted on HA (Fig. S8). Plain HG  
366 were also produced by mixing HA-PBA with HA-GLU in the same conditions (Fig. S9).

367 We confirmed hydrogels network formation by dynamic rheological  
368 experiments. In our study, the frequency sweep measurements of the storage (elastic)  
369 modulus ( $G'$ ) and the loss (viscous) modulus ( $G''$ ) were performed to probe the gel-like  
370 properties of the HA-PBA/HA-GLU network with and without DHCA under different pH

371 conditions. These measurements revealed a predominant elastic response for the HA-  
372 PBA/HA-GLU assembly with and without DHCA at pH 7.4, as  $G'$  is higher than  $G''$   
373 within the whole range of frequencies covered (Fig. 3a). These data thus show that  
374 these networks have a gel-like behavior. On the other hand, it was observed that for  
375 both networks produced at pH 6,  $G''$  dominates  $G'$  in a large range of frequency (Fig.  
376 3b). Therefore, a viscous behavior predominates at pH 6. This decrease in the lifetime  
377 of the network ( $G'$ - $G''$  crossover point observed at higher frequency) and in the density  
378 of cross-linking (decrease of the  $G'$  modulus) can be reasonably attributed to the  
379 decrease of affinity of PBA for GLU at pH 6 (Tarus et al., 2014).

380 The difference in HG formation at pH 6 and at pH 7.4 is confirmed by the  
381 analysis of  $G'$  at a fixed frequency of 2.5 Hz (Fig. 3c).  $G'$  values were lower for plain  
382 HG and for HG containing DHCA produced at pH 6 (44.6 Pa and 28.1 Pa, respectively),  
383 than  $G'$  values obtained for those produced at pH 7.4 (305.8 Pa and 296.1 Pa,  
384 respectively). Moreover, it is worth noting that DHCA incorporation in the HG did not  
385 hamper HG formation at both pHs, considering the similar rheological behavior for plain  
386 HG and for HG containing DHCA (no statistically significant difference was found  
387 between  $G'$  for HG produced at pH 6 and at pH 7.4,  $p < 0.05$ ). Taking into account the  
388 better DCE and the most adequate rheological properties of HG produced at pH 7.4,  
389 they were chosen for further biological experiments.

390



392 **Figure 3:** Dependence of storage modulus (filled symbols) and viscous modulus (open  
393 symbols) on frequency of modified HA-hydrogels without DHCA and with DHCA at pH 7.4  
394 (a) and pH 6 (b) at 32 °C, and storage modulus of modified HA-hydrogels without DHCA  
395 (HG) and with DHCA (HG + DHCA) at 2.5 Hz in pH 6 (white bars) and in pH 7.4 (gray bars)  
396 at 32 °C (c), and each column represents the mean  $\pm$  SD ( $n = 3$ ).  
397

### 398 **3.3. *In vitro* DHCA release studies**

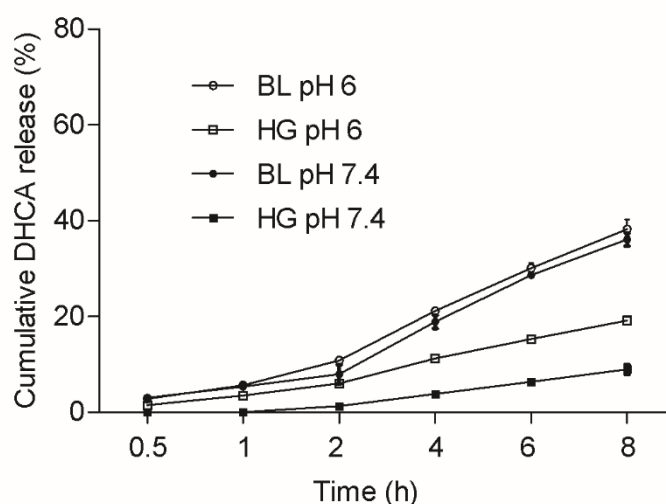
399 Delivery systems for topical administration that provide a controlled drug release  
400 are highly desired, given their potential benefits to the patient. Sustained release can  
401 prevent adverse effects caused by active ingredients present in high concentrations,  
402 supplies the skin for long periods, thereby reducing the number of product  
403 administrations, and decreases drug exposure to external environment. Therefore,  
404 such topical systems promote more safety and comfort for users, in addition to improve  
405 effectiveness of the drug (Costa & Santos, 2017).

406 The *in vitro* release of DHCA from HG was conducted using cell culture inserts  
407 positioned inside wells of a 24-well plate containing receptor media composed by 0.01  
408 M PBS at pH 6 or pH 7.4. These two receptors medium were chosen to emphasize the  
409 different behavior of the HGs in a neutral environment and in a more acidic condition,  
410 similar to the skin environment. For comparison, cell culture inserts containing free  
411 DHCA dispersed in water were also prepared as blanks. Plates were kept under  
412 continuous stirring at 32 °C (to reproduce the skin temperature) (Kregar et al., 2015)  
413 and the receiver solution was collected at pre-determined times for quantification of  
414 DHCA by HPLC. Fig. 4 compares plots of the cumulative release over time of DHCA  
415 solubilized in water (blank) and entrapped in the HG in receptor medium at pH 7.4 and  
416 pH 6. As expected, HG prolonged DHCA release in both receptor mediums, compared  
417 to the blank. In receptor medium at pH 7.4, only 9 % of DHCA was released from the  
418 HG while 36% release was achieved for the blank during 8 h. In receptor medium at

419 pH 6, 38% and 19% of DHCA release was measured for the blank and the HG  
420 formulation, respectively, in the 8 h of testing.

421 The fastest delivery of DHCA at pH 6 can be attributed to two main factors: the  
422 decreased number of boronate ester crosslinks in acidic conditions resulting in the  
423 formation of a loosely crosslinked HA network, and the reduced number of DHCA/PBA  
424 complexes. Both factors facilitate release of DHCA into the surrounding acidic  
425 environment compared to the physiological medium.

426



427 **Figure 4:** *In vitro* DHCA release from distilled water (blank-BL) and from modified HA-hydrogel  
428 (HG) produced at pH 7.4 in 0.01 M PBS pH 6.0 and pH 7.4 at 32 °C. Each symbol represents  
429 the mean  $\pm$  SD ( $n = 3$ ).  
430  
431

### 432 3.4. Cytotoxicity and protective effect of HG against UVB-induced cell 433 death

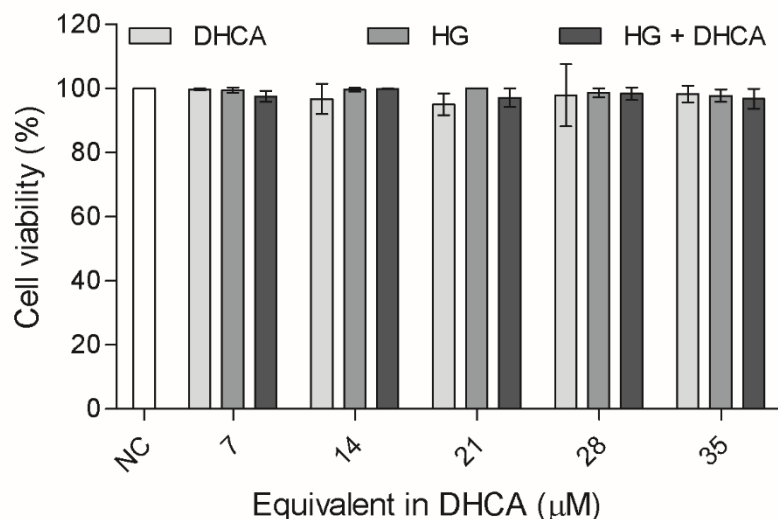
434 Human skin confers physical, chemical, and bacteriological protection for the  
435 organism. Besides, skin prevents dehydration, participates to thermoregulation,  
436 hormone production, contains elements of immune system and expresses external  
437 beauty (Rittie & Fisher, 2015). Hence, the maintenance of a healthy skin presents a  
438 physiologic and social importance. Dermis plays important functions in skin: confers

439 structural and mechanical properties, and offers supplementation of nutrients for the  
440 epidermis (Duque, Bravo, & Osorio, 2017). Fibroblasts are the main cell type of the  
441 dermis, and they produce elastin and collagen fibers, proteoglycans and  
442 glycosaminoglycans, such as HA, which promotes hydration, skin stiffness, resistance  
443 to deformation, skin repair and wound healing (Britto et al., 2017; Duque et al., 2017;  
444 Haydont, Bernard, & Fortunel, 2018). For these reasons, several studies have been  
445 evaluated the detrimental effects of external insults in fibroblasts, including from UV  
446 radiation (Britto et al., 2017; Oliveira et al., 2019).

447         Oxidative stress promoted by UVB causes a set of keratinocytes and fibroblasts  
448 damages, which can result in skin disorders, such as photoaging and skin cancer  
449 (Dunaway et al., 2018). In our previous work, we demonstrated that DHCA is a  
450 promising antiaging agent, which could prevent UVB-induced oxidative stress and its  
451 damages to L929 cells (Oliveira et al., 2019).

452         In order to evaluate the effect of DHCA incorporation in the HG and its ability to  
453 counteract fibroblasts harms promoted by UVB, we firstly verified toxicity and then  
454 cytoprotection against UVB-induced L929 cell death of DHCA, plain HG and HG  
455 containing DHCA. The samples were not toxic to L929 cells in all range of  
456 concentrations evaluated (7 – 35  $\mu$ M equivalent in DHCA), with a cell viability of 98%,  
457 97% and 94% for the highest dose of DHCA (35  $\mu$ M), plain HG (23 mg of HG) and HG  
458 containing DHCA (35  $\mu$ M of DHCA in 23 mg of HG), respectively (Fig. 5).

459



460

461 **Figure 5:** Effect of DHCA, plain modified HA-hydrogel (HG) and HG containing DHCA (7 – 35  
 462 µM of DHCA or hydrogels doses containing 7 – 35 µM of DHCA) in cell viability of L929 cells  
 463 evaluated by neutral red assay after 24 h of treatment. Each column represents the mean ±  
 464 SD ( $n = 3$ ).

465

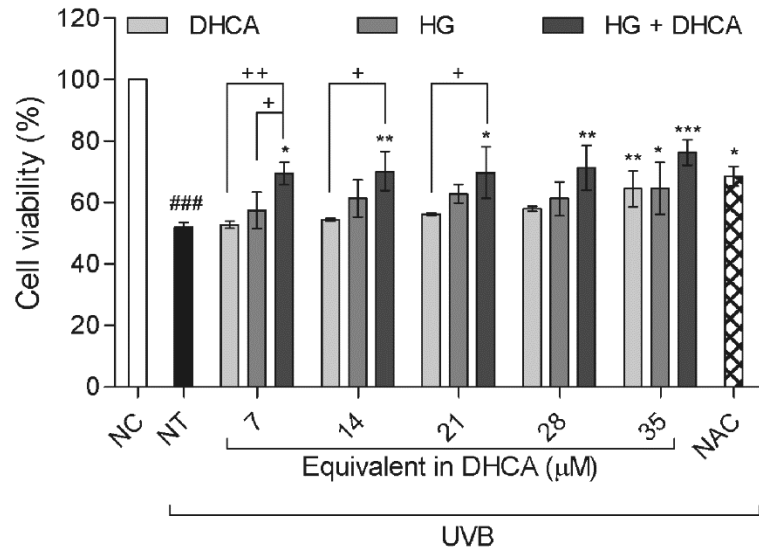
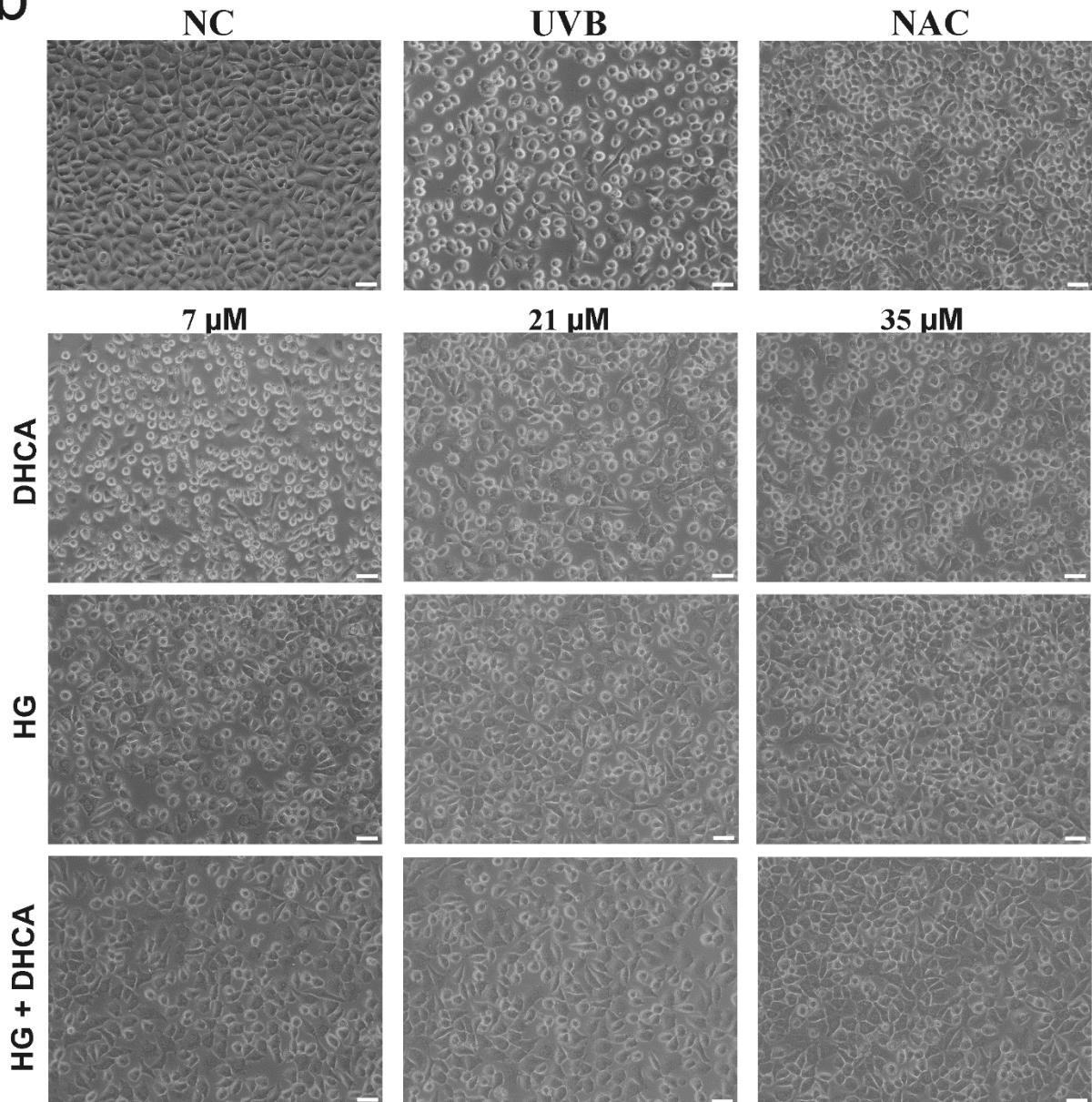
466 As shown by figure 6a, UVB irradiation significantly decreased cell viability at  
 467 600 mJ/cm<sup>2</sup> (cell viability of 52%) compared to nontreated and nonirradiated cells (NC),  
 468 while 1 mM of *N*-acetyl cysteine, the standard antioxidant used, protected UVB-  
 469 induced cell death (cell viability of 69%). As expected, DHCA alone showed a  
 470 cytoprotective effect in a dose dependent manner (significant difference was observed  
 471 between 7 µM and 35 µM of DHCA [ $p < 0.01$ ], as well as between 14 µM and 35 µM  
 472 of DHCA [ $p < 0.05$ ]). A significant increase in the cell viability was obtained only at  
 473 higher dose evaluated (cell viability of 65% at 35 µM of DHCA) (Oliveira et al., 2019).  
 474 Interestingly, plain HG also protected L929 cells against death induced by UVB (cell  
 475 viability of 68% for 23 mg of HG), and the HG containing DHCA provided a significant  
 476 protective effect in all doses used (cell viability of 76% at 35 µM of DHCA in 23 mg of  
 477 HG).

478 Microscopic analyses (Fig. 6b) confirmed the results described above. UVB  
 479 induced a decrease in cell number and changes in cellular morphology. On the other

480 hand, 1 mM NAC and the samples evaluated partially restored cell number, and  
481 improved cellular morphology, especially the HG containing DHCA.

482         These results are in agreement with our previous cytoprotective results for  
483 DHCA alone. This phenolic compound prevented cell death by diminishing oxidative  
484 stress and its cellular damages caused by UVB exposure (Oliveira et al., 2019).  
485 Moreover, we observed a significative protection of plain HG, and that the incorporation  
486 of DHCA in the HG improved the protection against death promoted by UVB, especially  
487 in lower concentrations evaluated. HA, the main component of the HG, have shown  
488 proliferative properties in human dermal fibroblasts by increases expression of  
489 transforming growth factor- $\beta$  (TGF- $\beta$ ), besides to promote collagen preservation  
490 through induction of tissue inhibitor of metalloproteinase 1 (TIMP-1) expression, and  
491 reduction of MMP-1 expression (Monteiro, Tersario, Lucena, Moura, & Steiner, 2013).  
492 Taken together, these HA properties can contribute to the cytoprotective capacity  
493 verified for HG. In addition, the higher cytoprotective capacity of the HG containing  
494 DHCA can also be explained by the beneficial effects of HA on skin cells, which  
495 improves the cytoprotection promoted by DHCA.

496

**a****b**

498 **Figure 6:** Cytoprotective effect of DHCA, plain dynamic covalent HA hydrogel (HG), HG  
499 containing DHCA (HG + DHCA) (7 - 35  $\mu$ M of DHCA or hydrogels doses containing 7 - 35  $\mu$ M  
500 of DHCA) and NAC (1 mM) against L929 cell death induced by UVB. L929 cells were  
501 pretreated for 1 h before UVB irradiation (600 mJ/cm<sup>2</sup>), and after 24 h of irradiation cells were  
502 submitted to neutral red assay (a) or observed using a phase contrast inverted microscope (b).  
503 Each column represents the mean  $\pm$  SD ( $n = 3$ ). ###  $p < 0.001$ : significantly different from  
504 nonirradiated and nontreated cells (NC); \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ : significantly  
505 different from irradiated and nontreated cells (NT); +  $p < 0.05$ , ++  $p < 0.01$ . Scale bars: 50  $\mu$ m.  
506

507

#### 508 **4. Conclusions**

509 In this work, a novel dynamic covalent HA hydrogel containing the antioxidant  
510 and anti-photoaging agent DHCA for skin applications was developed. We  
511 demonstrated the selective entrapment of DHCA in the hydrogel through its  
512 complexation with PBA grafted on HA chains via boronate ester bond formation. The  
513 system developed prolonged DHCA release using the pH as a trigger, which allows an  
514 effective release of DHCA in skin environment. Moreover, the skin beneficial properties  
515 of HA used to produce the hydrogel contributed to improve DHCA protection against  
516 UVB-induced fibroblasts death. In this way, the polysaccharide employed in the  
517 present study provided a set of advantages, considering that it participated in the  
518 dynamic covalent hydrogel production and in the mechanism to control DHCA release,  
519 as well as played an important role in fibroblasts protection against UVB. Therefore,  
520 the potential of a novel smart HA hydrogel produced in preventing skin photoaging for  
521 long periods make it a good candidate for future cosmetic applications.

522

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528

529 **Appendix A. Supplementary material**

530

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