Supporting Information

Boronic Acid-Modified Alginate Enables Direct Formation of Injectable, Self-Healing and Multistimuli-Responsive Hydrogel

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1. Materials and characterization methods

Materials

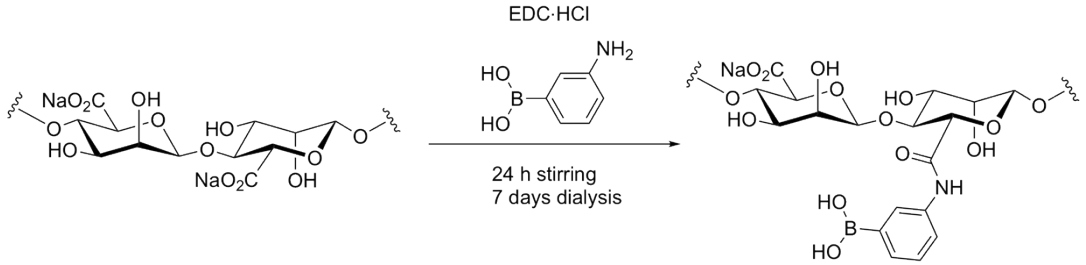
Alginic acid sodium salt (low viscosity, 15-20 cP for 1% in H_2O) and dialysis tubing cellulose membranes (MWCO 14000, LOT 3110) were purchased from Sigma Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from Carl Roth. 3-Aminophenylboronic acid monohydrate was purchased from Alfa Aesar. All chemicals were used as received without further purification.

Characterization methods

Fourier transform infrared (FTIR) spectra were recorded at room temperature using an Excalibur FTS 3000 FTIR spectrometer (Biorad) equipped with a single reflection ATR (attenuated total reflection) accessory (Golden Gate, Diamond). Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker Avance-300 instrument, using D_2O as solvent. UV-vis spectra were recorded using a Varian Cary 50 UV spectrophotometer and quartz-glass cuvettes of 0.5 cm thickness. Wild Makroskop M420 optica microscope equipped with a Canon shot A640 digital camera was used for collecting pictures of the self-healing process. Oscillatory rheology was performed with an AR 2000 Advanced rheometer (TA Instruments). A 1000 μm gap setting and 25 °C were used for the measurements in a plain-plate (40 mm, stainless steel). Rheological measurements involved dynamic strain sweep (DSS) (moduli *vs*. strain), dynamic frequency sweep (moduli *vs*. frequency) and dynamic time sweep (moduli *vs*. time). The self-healing behavior of model gels was investigated by several cycles of a 3-steps loop experiment involving: application of a low shear strain (5% strain, 1 Hz, 5 min; gel state, G' (storage modulus) > G'' (loss modulus); (2) increase of the shear strain until the gel fractures (500% strain, 1 Hz, 2 min; viscous material, $G' < G''$), and (3) return at the same rate to the initial strain % value (5% strain, 1 Hz, 10 min; recovered gel phase, $G' > G''$).

2. Synthesis of conjugates and preparation of hydrogels

Synthesis of phenylboronic acid-modified alginate (Alg-B(OH)$_2$)



Alg-B(OH)$_2$ was prepared by grafting 3-aminophenylboronic acid onto alginate in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) following a previously reported procedure[i] with slight modifications. Sodium alginate (5 g, 25 mmol based on monomer unit) was dissolved in deionized water (500 mL) to which EDC·HCl (4.8 g, 25 mmol) and 3-aminophenylboronic acid (1.95 g, 12.5 mmol) were added. The mixture was stirred at room temperature for 24 h and then dialyzed against distilled water for 1 week, replacing water at least 7 times, and lyophilized. The successful grafting was confirmed by ^{1}H NMR, showing the typical phenyl group signals around 7.5 ppm (Figure S1a). The alginate degree of substitution (DS), *i.e.* the molar ratio of phenylboronic acid unit to the carboxylate groups of alginate, was calculated from the ratio between the integration values of the phenylboronic acid unit those from alginate and taking into account the number of hydrogens responsible for the integrated area (*i.e.* 5H alginate integrated around DHO peak and 4H for the phenyl group). Although the complex interpretation of the polysaccharide signals in these conditions impedes an exact determination of the DS, the calculated value (25%) is in very good agreement with the previously reported one.[ii] The modified biopolymer (Alg-B(OH)$_2$) was further characterized by FTIR showing the expected small band at 1450 cm^{-1} associated to the C-B vibration (Figure S1b). A slight broadening of the alginate signals at ~1410 and ~1300 cm^{-1} (corresponding, respectively, to the O-C-O symmetric vibration and C-C-H + O-C-H deformation of the pyranose rings)[iii] can also be noticed and associated to the B-O vibrations, which normally are

present around 1350 and 1300 cm^{-1}.[iv] Determination of the pKa of the conjugate and native alginate was carried out by acid-base titration at constant ionic strength. Specifically, the corresponding alginate material (1% w/v) was dissolved in 4 mL of 50 mM KCl and the pH was reduced to pH 2.3 by adding 0.1 M HCl. Then, the pH was increased by gradually adding 0.1 M NaOH until pH 12 was reached. The changes in pH during the titration experiment were monitored potentiometrically.

Preparation of Alg-B(OH)$_2$ hydrogels

The desired amount of Alg-B(OH)$_2$ was dissolved in 0.1 M PBS aqueous solution to obtain a final concentration of 3, 4 or 5 % w/v. The addition of 75 µL 1 M NaOH per mL of solution led to immediate formation of the gel (Figure S2 and Figure S4).

Response of Alg-B(OH)$_2$ hydrogel to pH change: After obtaining an Alg-B(OH)$_2$ gel under basic conditions, the pH of the medium was lowered by addition of 200 µL CH_3COONa/CH_3COOH buffer (pH = 5) on top of the gel. The drastic pH change led to the collapse of the gel due to the dissociation of the pH dependent boronate bonds between the polymeric chains. After raising the pH again by addition of 100 µL NaOH, a stable gel was reformed, due to the reversible formation of cyclic boronate esters (Figure S3).

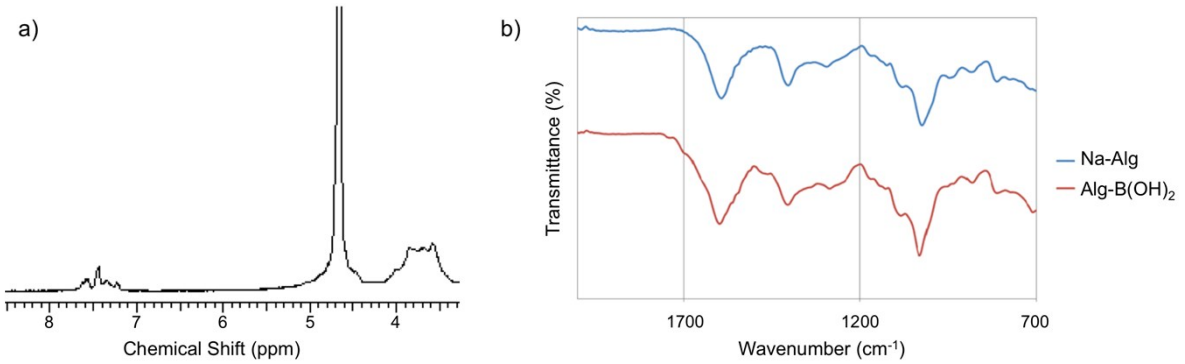


Fig. S1 a) ^{1}H NMR spectrum of Alg-B(OH)$_2$. b) FTIR spectra of Na-Alg and Alg-B(OH)$_2$.

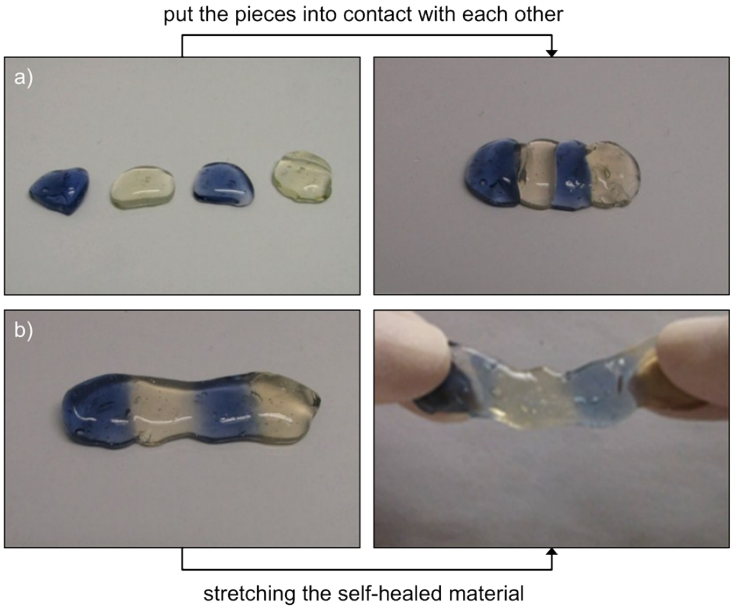


Fig. S2 Self-healing process of dyed and non-dyed pieces of Alg-B(OH)$_2$, 3% w/v, a) before and b) after 30 minutes healing without any external trigger. As observed, complete healing of the cuts was recorded and the gel could be stretched without falling to pieces. The dye used in this experiment was Direct Blue 1.

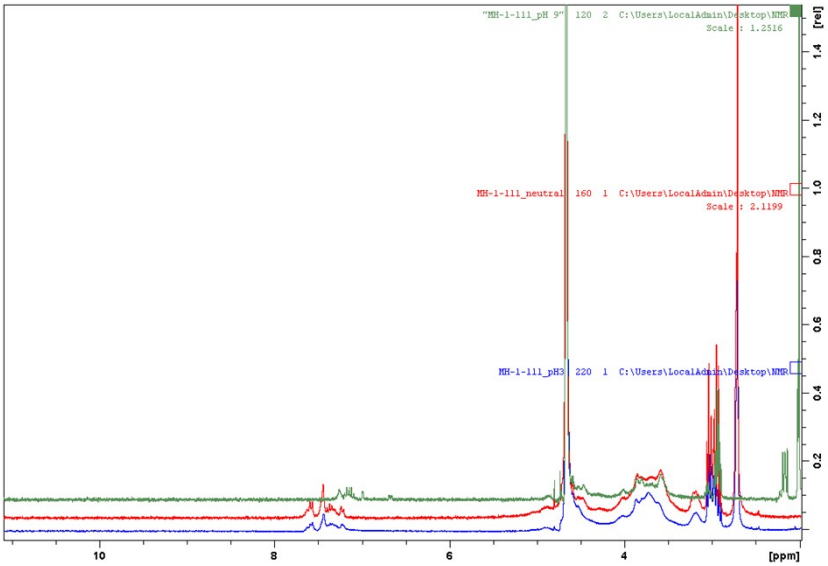


Fig. S3 ^{1}H NMR of Alg-B(OH)$_2$ at 3% w/v under acidic pH (blue line, pH 3), neutral pH (red line, pH 7) and basic (green line, pH 9).

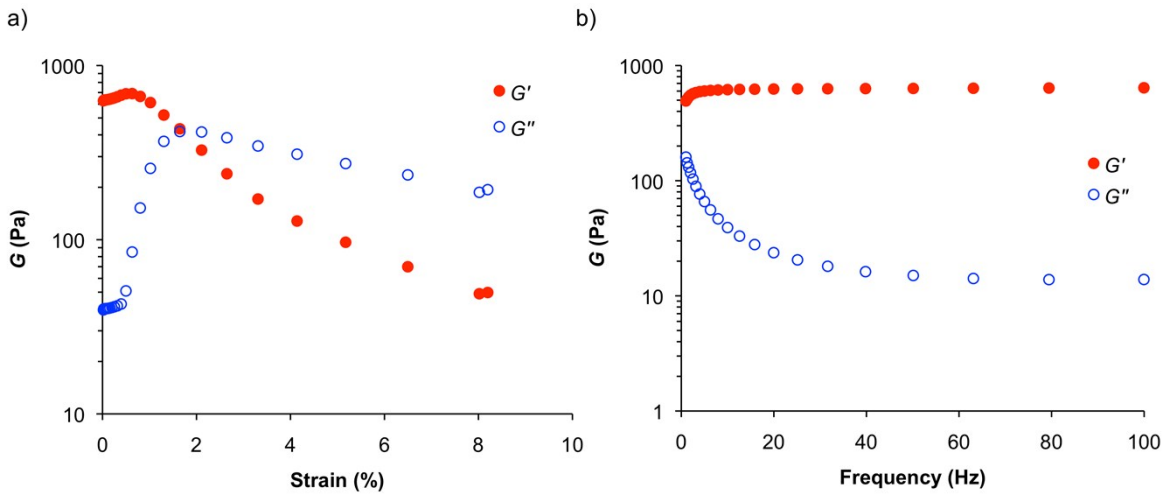


Fig. S4 a) Dynamic strain sweep rheological experiment carried out for the gel made of Alg-B(OH)$_2$ at 3% w/v. b) Dynamic frequency sweep rheological experiment for the same sample.

3. Biological studies

The biocompatibility, injectability, self-healing and cell encapsulation ability of biohydrogels constitute important properties for their potential applications in tissue engineering, cell therapy and others biomedical applications.[v]

Cell culture and materials

HeLa cells were maintained in a humidified atmosphere (5% CO_2) at 37 ºC and cultured in Dulbecco's modified Eagle's medium (DMEM), which was supplemented with fetal bovine serum (FBS, 10%), streptomycin (100 µg mL^{-1}) and penicillin (100 U mL^{-1}). DMEM, phosphate-buffered saline (pH 7.4) and trypsin-EDTA (0.05 %) phenol red were purchased from Gibco. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide was purchased from Sigma-Aldrich.

Cell encapsulation

To explore the cellular viability and cell release of Alg-B(OH)$_2$ hydrogels, we carried out the 3D encapsulation of HeLa cells: HeLa cells were regularly passaged to maintain an exponential growth.

Cells were incubating with Trypsin-EDTA (2 mL) for 5 min at 37 ºC to detach cells from the flask surface. DMEM was added (8 mL) and the mixture was put in a 15 mL centrifuge tube. Cells were centrifuged for 5 min at 1300 rpm. Cells (10×10^6 and 2×10^6) were resuspended in Alg-$B(OH)_2$ solution (15 mg in 500 µL of PBS), respectively. 80 µL of the solution were placed in three molds and 1M NaOH (6 µL) was added. The final mixture was slightly stirred. The 3D encapsulation of cells within alginate hydrogels took place in less than 2 min. Monoliths containing cells were incubated in DMEM (1 mL) supplemented with FBS (10 %) without antibiotics at 37 ºC in a 24-well and 6-well cell culture plates (Figure S5).

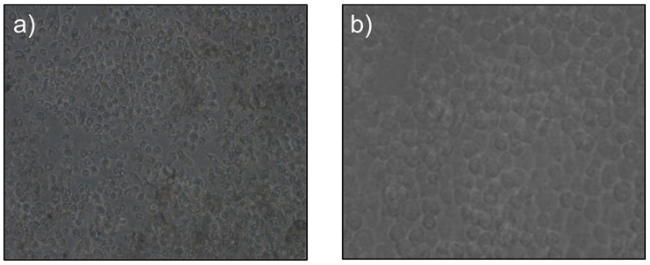


Fig. S5 Release of encapsulated HeLa cells after 24 h (*left*) and 48 h (*right*) of incubation.

Injection experiment

HeLa cells at a concentration of 18×10^6 were suspended in Alg-$B(OH)_2$ solution (32 mg in 1 mL of PBS) following the same procedure described above. 1M NaOH (160 µL) was added and the resultant solution was slightly stirred. Once alginate hydrogels (×2) containing cells was formed, they were extruded through a 21-gauge needle directly into a 24-well cell culture plate. Gels were incubated in DMEM (1 mL) supplemented with FBS (10%) for 24 h.

Cytotoxicity studies

We used the MTT assay[vi,vii] to confirm the safety and compatibility of Alg-$B(OH)_2$ hydrogels by evaluating the mitochondrial function of cultured cells and evaluating the formazan production in cells: After 24 h of hydrogel incubation (encapsulated and extruded hydrogels, respectively), DMEM was removed and cells were washed with PBS (400 µL). Trypsin-EDTA (150 µL) was added and incubated 5 min at 37 ºC. After adding DMEM (850 µL), cell suspensions were centrifuged ((3 rcf × 3 min), 8 min). Then, cells were seeded in a 96-well plate at a density of 5000 cells/well in complete DMEM supplemented with 10% FBS (200 µL). Cells were incubated 24, 48 and 72 h. MTT dye solution (25 µL; 5 mg mL^{-1}) were added at appropriate times and cells were incubated for additional 4 h at 37 ºC. Medium was removed and DMSO (100 µL) was added in order to dissolve formazan crystals. The plate was read at 570 nm in a Promega Glomax Multidetection System instrument. Six replicates were used for each sample and results are shown as mean ± standard deviation (SD). Cell viability is reported as the percentage of non-encapsulated compared to encapsulated cells.

4. References

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