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Boronic acid-modified alginate enables direct formation of injectable, self-healing and multistimuli-responsive hydrogel.

Asja Pettignano,^{a,b,c} Santiago Grijalvo,^{d,e} Marleen Häring,^a Ramon Eritja,^{d,e} Luca Bernardi,^c Nathalie Tanchoux,^b Françoise Quignard^b and David Díaz Díaz^{*ad}

^a*Institute of Organic Chemistry, University of Regensburg, Universitaetsstr. 31, 93040 Regensburg, Germany. E-mail: david.diaz@ur.de*

^b*Institut Charles Gerhardt, UMR 5253 CNRS/UM2/ENSCM/UM1, Matériaux Avancés pour la Catalyse et la Santé, ENSCM, 8 rue Ecole Normale, 34296 Montpellier Cedex 5, France*

^c*Department of Industrial Chemistry "Toso Montanari", School of Science, Alma Mater Studiorum – University of Bologna, V. Risorgimento 4, 40136 Bologna, Italy*

^d*IQAC-CSIC, Jordi Girona 18-26, Barcelona 08034, Spain*

^e*Biomedical Research Networking Center in Bioengineering, Biomaterials and Nanomedicine (CIBER BBN), Spain*

Abstract. One-step functionalization of alginate with boronic acid groups allowed spontaneous formation of biocompatible hydrogels under basic conditions without additional complementary molecules or crosslinking agents. The dynamic nature of boronate ester bonds formed with vicinal diols present on alginate pyranose rings provided remarkable self-healing, injectable and multi-stimuli responsive properties to the material.

Stimuli-responsive hydrogels are characterized by the ability to respond to external triggers (e.g. pH, temperature, light, specific molecules, etc.), a major property typically associated with their potential use for biomedical applications.^{1,2} In addition, the development of biomimetic gel-based materials with self-healing properties has extended their prospect for new applications. Nature relies on the use of reversible sacrificial bonds that allow for a better adaptation of a biomaterial to specific environments as well as for self-reparation upon damage.³⁻⁶ This has inspired an emerging research area devoted to the fabrication of self-healable hydrogel networks based on dynamic covalent chemistry.⁷⁻⁹ In sharp contrast to covalently crosslinked hydrogels, whose stable bonds do not undergo exchange reactions, gels based on dynamic bonds are able to reform bonds around a damaged zone, allowing the restitution of their functional properties.⁹

Within this context, boronic acids have been extensively studied due to their excellent ability to interact reversibly with diols, forming boronate

esters. The most common interaction occurs with 1,2- and 1,3-diols, forming five and six membered rings, respectively, with a binding affinity following the order *cis*-1,2-diol > 1,3-diol » *trans*-1,2-diol.¹⁰ The formation and dissociation of the boronic ester derivatives can occur both in aqueous and organic media and depend significantly on pH, as well as on the pK_a of the boronic acid–diol pair. Previous studies have demonstrated that the reaction kinetics are fastest in aqueous basic media, where boron is present in its anionic form. The tetrahedral boronate anion, in fact, presents a higher reactivity than its neutral trigonal form with differences in the rate of 10^4 .^{11,12} Nevertheless, even if significantly slower, the reactivity of the neutral boronic acids with diols should not be ignored and the overall equilibria can be illustrated as a cycle (Fig. 1).

More in detail, K_{tet} describes the diol–boronate anion complex and K_{trig} the diol–boronic acid complex formation, with $K_{tet} > K_{trig}$. Taking this into account, the binding affinity of boronic acid with diols is highly dependent on the pH of the medium, with pH values above the pK_a of the boronic acid, favouring the formation of boronate ester. Conversely, the diol–boronic acid interaction is not favoured near the physiological pH and is completely cleaved under highly acidic conditions.¹³

This high reactivity between boronic acids and diols has stimulated the study of boronic acid-containing polymers as therapeutic agents, self-regulated drug delivery systems, nucleotide adsorbents, and sensors for a number of biologically important species including saccharides, glycoproteins and neurotransmitters such as dopamine.¹⁴ Moreover, the use of boronic acid based polymers for biomedical applications is not only possible but largely encouraged by the lack of apparent toxicity or *in vivo* instability issues of these compounds.^{14,15} Furthermore, the dynamic nature of the boronate ester bonds has enabled the development of different self-healing systems, mainly based on the interaction between synthetic polymers bearing pending diols and boronic acid groups.^{1,2,16–18} Nevertheless, considering the high suitability of this dynamic interaction for biomedical applications, the use of biocompatible and biodegradable natural polymers for the preparation of self-healing gels is highly desirable.

Fig. 1 Equilibria involved in the pH-dependent boronate ester formation

Here, we demonstrate the facile preparation of a multistimuli-responsive, self-healing, injectable and biocompatible soft biohydrogel exclusively from a boronic acid-modified alginate (Alg–B(OH)₂) without the need for any external diol source and/or divalent cations

(usually Ca^{2+}) to ionically crosslink the alginate through the well-known egg-model.¹⁹

Alg-B(OH)_2 was prepared by grafting 3-aminophenylboronic acid onto alginate in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride as described in the literature (ESI†). The modified biopolymer was characterized by ^1H NMR and FTIR spectroscopy (Fig. S1, ESI†), which confirmed the successful grafting with a degree of substitution (DS) of 25%.

We hypothesized that the boronic acid pending groups of Alg-B(OH)_2 could reasonably interact in a basic environment with the vicinal diols present on alginate pyranose rings, leading to the formation of crosslinked alginate bearing reversible boronic ester linkages (note that the involvement of hydroxyl groups from different alginate chains in the complexation of boronic units, at least to some extent, should also be considered). Under this scenario, the straightforward synthesis of metal-free and multistimuli-responsive alginate hydrogels could be achieved without the use of additional complementary diol-containing crosslinker molecules and/or divalent cations.²⁰ To our delight, the addition of 1 M NaOH to Alg-B(OH)_2 aqueous solutions in 0.1 M PBS led to immediate gelation (Fig. 2a). Interestingly, the use of Alg-B(OH)_2 at different concentrations (*i.e.* 3, 4 and 5% w/v) revealed a delicate balance between gel stability and self-healing properties. Colourless hydrogels obtained at 4 and 5% w/v were fragile and displayed self-healing properties upon damage (*i.e.* cut), albeit several hours were necessary for the healing. In contrast, softer and stable hydrogels were obtained at 3% w/v and showed complete self-healing within minutes after cutting the bulk gel into two pieces and placing them back in contact. The interfaces between the different pieces visually disappeared during the healing process. Moreover, the restored hydrogel could be stretched without showing any evidence of the damage (Fig. 2b). The efficient fusion of non-complementary interfaces (*i.e.* two gel bodies prepared independently) was also demonstrated by connecting alternate pieces of dyed and non-dyed bulk gels. The resulting gel could also be stretched without falling to pieces (Fig. 3f).

Fig. 2 (a) Gelation of Alg-B(OH)_2 solution upon crosslinking by increasing the pH. (b) Self-healing behavior of the Alg-B(OH)_2 bulk hydrogel prepared at 3% w/v.

From these results, it is evident that the concentration of the conjugate plays a key role in the self-healing properties of the gel, and 3% w/v provides the optimal equilibrium between different phenomena that can influence the overall properties of the gel network (*e.g.*, the lifetime of crosslinks that further affects the self-healing kinetics, generation of transient chain association, size of colloidal aggregates, *etc.*). The self-healing ability of Alg-B(OH)₂ (3%w/v) was further examined by punching a 0.5 cm hole in the middle of a ~ 2 x 1.5 cm gel body. The hole underwent gradual spontaneous reduction in size until its complete disappearance after only 10 min (Fig. 3a–c). The self-healed gel exhibited the same appearance as the fresh one and it could be stretched and hung without any visible sign of damage (Fig. 3d and e). The healing process was better visualized by using gel pieces prepared in the presence of a dye (Fig. 3f and Fig. S2, ESI†).

Fig. 3 (a–c) Self-healing of a 0.5 cm hole in the Alg-B(OH)₂ hydrogel (3% w/v) within 10 min. (d) Previously self-healed sample hanging vertically. (e) Stretching the previously self-healed sample. (f) Stretching a sample prepared by fusion of dyed and non-dyed gel blocks. The dye used in this experiment was Direct Blue 1. (g) Monitoring by optical microscopy the self-healing process of a scratch made on a Alg-B(OH)₂ hydrogel film.

Furthermore, the progressive self-healing of a scratch made on a Alg-B(OH)₂ gel film was monitored by optical microscopy, showing a clear decrease of the scratch size after 7 min and a totally repaired surface within 30 min (Fig. 3g). Oscillatory rheological experiments showed the restoration of the mechanical strength and damping properties of the material after the self-healing process (*vide infra*). It is also important to emphasize that the

macroscopic self-healing process could be repeated many times without any detriment to the functional properties of the material.

As expected, acid–base titration of both Alg–B(OH)₂ and native alginate at constant ionic strength revealed the presence of carboxylic groups with a pK_a of *ca.* 3.3 (as observed in native alginate) as well as a more basic group with a pK_a of *ca.* 9.5 ascribed to the boronic acid moiety (ESI†). Taking into account these values and the dynamic nature of the boronate ester bond, the response of the Alg–B(OH)₂ gel to pH variation was also studied. After obtaining the hydrogel under basic conditions, the medium pH was lowered by adding 200 μ L of sodium acetate buffer (pH 5). This 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 adding 100 μ L of NaOH solution (pH 10), the stable gel was reformed (Fig. 4a). The complexation of boronic units with hydroxyl groups of alginate (*ca.* 5% of the available hydroxyl groups) has been previously investigated.²¹ Moreover, ¹H NMR analysis of the material through a pH gradient (*i.e.*, acidic, neutral and basic pH) showed a gradual chemical shift of signals in the 7–8 ppm region (Fig. S3, ESI†), which is consistent with the borate ester formation at basic pH.¹

Moreover, the stability of the gel network could also be controlled upon addition of competitive saccharide molecules at basic pH. For this experiment, we used fructose due to its elevated binding affinity towards boronic acids.²² When the Alg–B(OH)₂ gel was left in contact with 200 μ L of a 10 mM fructose solution, the gel started to flow after a few minutes and shortly turned into a free-flowing liquid due to the strong interactions between the monosaccharide and the boronic acid crosslinker moieties, leading to the progressive disintegration of the gel network (Fig. 4b).

Fig. 4 (a) Responsiveness of the Alg–B(OH)₂ hydrogel to pH variations. (b) Responsiveness of the Alg–B(OH)₂ hydrogel to the addition of fructose.

The gel nature of the material was confirmed by standard rheological experiments. Specifically, the storage modulus G' (*ca.* 630 Pa) was found to be one order of magnitude greater than the loss modulus G'' (*ca.* 40 Pa) (Fig. S4, ESI†). Besides the observed macroscopic self-healing property of the bulk gel, its thixotropic behavior was also confirmed by a 3-step rheological loop test consisting of (1) application of a low shear strain (5%) at 1 Hz frequency for 5 min, as defined by previous dynamic time sweep experiments (in this step, the sample remained in the gel state, $G' > G''$); (2) increase of the shear strain to 500% to ensure the rupture of the gel (G'

$< G''$) and maintenance for 2min; and (3) reduction at the same rate to the initial shear strain and maintenance for 10 min to stabilize the recovered gel network ($G' > G''$). The loop was repeated three times and revealed the full recovery of the gel strength within a few minutes after each cycle (Fig. 5).

Fig. 5 Rheological loop test showing the thixotropy of the Alg-B(OH)₂ hydrogel. Arrows indicate the increase of the shear stress until 500% ($G' < G''$).

It is worth mentioning that the hydrogel can be lyophilized (freeze-drying), giving a free-flowing powder, which can be subsequently used to reform the hydrogel upon simple addition of water (*i.e.*, an equivalent amount to that removed). This allows convenient storage of the gel precursor formulation for long periods of time while not in use.

Finally, the injectability of the hydrogel, a fundamental requirement for many biomedical applications,^{23–26} was tested and confirmed by immediate re-gelation after flowing the gel without clogging through a 21-gauge needle (Fig. 6a). As expected from the previous experiments, the extruded gel could also be remoulded into any desired shape by gently pressing it for less than 5 min (Fig. 6b). When the injection of the gel was performed into 1 mL of pH 7.4 buffer, the gel successfully reformed at the bottom of the vial and remained stable upon inversion of the vial (Fig. 6c and d).

Fig. 6 (a) Injectability property of the Alg-B(OH)₂ hydrogel. (b) Reshape of the extruded hydrogel into a disc. (c and d) Reformation of the hydrogel upon injection into a vial containing buffer solution (pH 7.4).

Furthermore, in order to assess the potential of the injectable Alg-B(OH)₂ hydrogel for biomedical applications, we explored the cellular

viability and cell release properties.²⁷ For this purpose, we carried out the 3D encapsulation of HeLa cells during the gelation (ESI†). The results from MTT assays showed excellent biocompatibilities with cell viabilities greater than 90% until 72 h incubation when compared to non-encapsulated cells (positive control). No significant differences were observed between 24 and 72 h incubation time.

These results also confirmed the ability of HeLa cells to proliferate after being released through the alginate network (Fig. 7a). Gradual degradation of the hydrogel during incubation at 37 °C (pH 7) allowed cells to be released through the polymeric network without affecting their morphology (Fig. 7b and c; Fig. S5, ESI†). After successful cell encapsulation, we carried out injection studies of cell-encapsulated hydrogels through a needle and evaluated the subsequent cell release of the restored hydrogel (ESI†).²⁸ In vitro MTT studies showed that a significant proportion of cells were able to bear the large stress generated by the injection and self-healing processes (*i.e.* 63% cell viability), indicating preservation of the biocompatible character of the hydrogel (Fig. 7d).

Fig. 7 (a) *In vitro* MTT-based cytotoxicity for Alg-B(OH)₂ hydrogels cultured with HeLa cells at 24, 48 and 72 h of incubation. (b) Non-encapsulated HeLa cells used as a control. (c) Release of encapsulated HeLa cells after 72 h of incubation. (d) Release of HeLa cells after being encapsulated and extruded into a 24-well cell culture plate (24 h of incubation).

In summary, straightforward functionalization of alginate with boronic acid moieties allowed spontaneous formation of a versatile hydrogel under basic conditions. The reaction between the boronic acid groups and vicinal diols present on the pyranose rings of the biopolymer afforded a crosslinked hydrogel without the need for additional complementary molecules.

Extraordinary elasticity, self-healing, injectability and multi-stimuli responsive properties were demonstrated for this material. Undistinguished interfaces were observed after the rapid self-healing of a damaged gel or after the fusion of two gels prepared independently. The dynamic nature of boronate ester bonds permitted the control of the molecular assembly/disassembly by either changes in the pH or addition of a competitive monosaccharide. In addition, the hydrogel showed good biocompatibility upon encapsulation of HeLa cells, of which a significant proportion of cells survived after consecutive gel injection and self-healing processes. The foregoing results indicate that the material could be useful for different biomedical applications. In general, this discovery should inspire the facile preparation of a large variety of new self-healing gels. The impact of higher degrees of substitution in Alg-B(OH)₂ and different alginate compositions (i.e. mannuronate/guluronate ratios) on the gel formation and its functional properties is currently under investigation in our laboratory.

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