Polymer Journal
https://doi.org/10.1038/s41428-020-0348-3

ORIGINAL ARTICLE

Responsive laminarin-boronic acid self-healing hydrogels for biomedical applications

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Received: 1 February 2020 / Revised: 22 March 2020 / Accepted: 31 March 2020

Abstract

The precise chemical modification of marine-derived biopolymers provides a unique opportunity for fabricating a toolbox of bioactive (bio)materials with modulated physicochemical and biological properties. Herein, the β-glucan laminarin was functionalized with phenylboronic acid (PBA) moieties that impart chemical reactivity toward diol-containing polymers via boronate esterification. The modification, which involved a two-pot reaction, was successfully confirmed by nuclear magnetic resonance spectroscopy. The resultant biopolymer readily established boronate ester-crosslinked hydrogels with poly(vinyl alcohol) (PVA) within seconds under physiological conditions. These hydrogels exhibited improved rheological properties, which were easily tunable, and revealed a rapid self-healing behavior upon rupture. Moreover, boronate ester bonds enabled the fabrication of reactive oxygen species-responsive and shear-thinning gels that can be administered in situ and respond to the oxidation state of the surrounding microenvironment. Importantly, due to the catalyst-free and mild-crosslinking conditions, the generated laminarin-PBA/PVA hydrogels did not show toxicity upon direct contact with preosteoblasts for up to 48 h, and thus constitute a promising platform for tissue engineering and drug delivery applications.

Introduction

Polysaccharides of natural origin represent a unique source of intrinsically biodegradable and biocompatible materials with numerous biomedical applications, including drug delivery systems, 3D/4D bioprinting, soft robotics, bioelectronics, tissue engineering, and regenerative medicine [1–4].

Among the myriad of natural sources available for the sustainable extraction of biorelevant compounds, the sea is undoubtedly one of the most attractive because it constitutes a renewable reservoir of a variety of polysaccharides with fundamental physicochemical features [5]. To date, several types of biopolymers of marine origin, including alginic acid, chitin/chitosan, carrageenan, hyaluronan, and agar, have been widely explored. Due to their chemical versatility and cost-effectiveness, these polysaccharides have been processed into biomimetic biomaterials of diverse forms (e.g., particles, films, fibers, sponges, and hydrogels) and nano-to-macro dimensions [6, 7]. Such biofunctional platforms constitute valuable building blocks for advancing bottom-up tissue engineering strategies that better recapitulate the native bioarchitecture of living systems through biomaterials and cell synergies [8].

Among marine-derived biomaterials, polysaccharides extracted from brown algae, such as alginic acid (alginate) and fucoidan, have numerous applications in the cosmetic, food, and biopharmaceutical industries. In addition, those seaweeds are also a rich source of bioactive laminarans (laminarin/leucosin), which can constitute up to 35% of the dry content depending on the surrounding habitat, species, and extraction procedure [9].

Laminarin is a particularly interesting storage β-glucan that essentially constitutes a food reserve in macroalgae. This biopolymer can be sustainably extracted from different seaweed species, such as *Laminaria digitata*, *Laminaria hyperborea, and Eisenia bicyclis* [9]. In particular, the degree of branching in the backbone of laminarin from *E. bicyclis* generally comprises β-(1,3) and β-(1,6) in the main chain and occasional β-(1,6) side chain branches in the *O*-6

Supplementary information The online version of this article (https://doi.org/10.1038/s41428-020-0348-3) contains supplementary material, which is available to authorized users.

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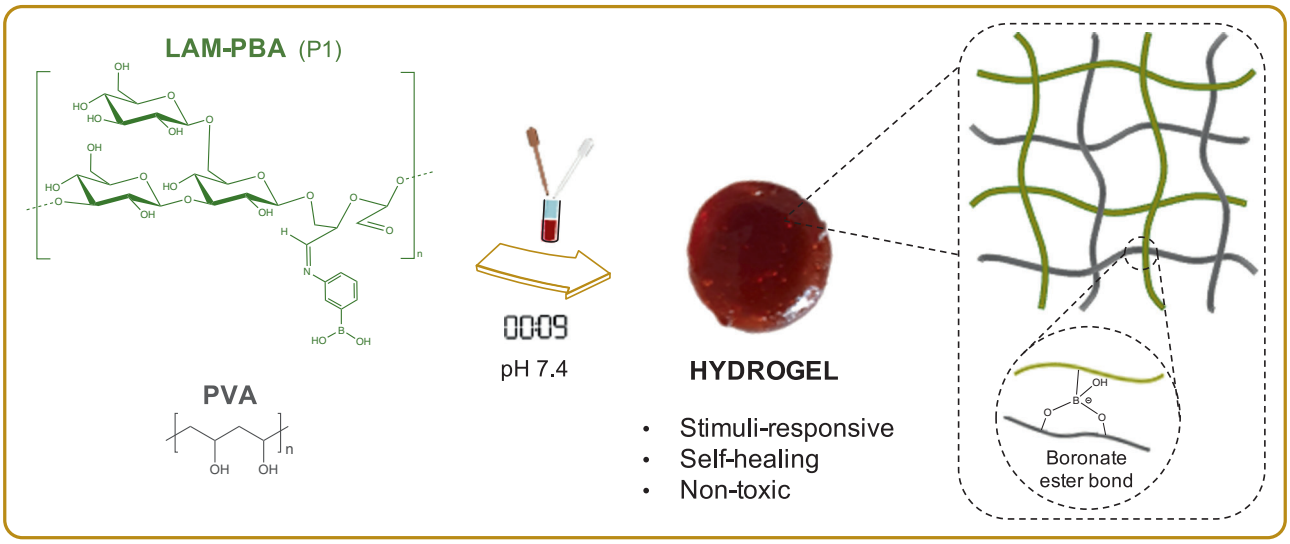

Fig. 1 Schematic representation of hydrogels fabrication via boronate ester crosslinking with PVA

position [10]. This unique structure dictates its water solubility, and a higher degree of branching is more desirable for biomedical applications because it allows dissolution in both hot and cold water [11].

The intrinsic anticoagulant, antioxidant, and immunostimulatory/anti-inflammatory activities of laminarin, which are associated with its biodegradable and chemically versatile backbone, render it a highly attractive biomaterial [12]. However, its lower viscosity and inability to gel compared with alginate has limited its processing into hydrogels, fibers, or particle-based systems. To overcome these limitations and improve its (bio-)functionality/reactivity, laminarin was chemically modified with pendant methacrylic anhydride moieties that react with hydroxyl groups under mild conditions [13]. Using this strategy, the researchers obtained a UV-responsive laminarin-methacrylate derivative that was rapidly photocrosslinked into a mechanically robust and cytocompatible hydrogel. Moreover, this photoreactive derivative has been used for the microfluidic-assisted fabrication of cell-adhesive multifunctional laminarin microparticles [14]. Recent studies have also indicated that chemical modification of the reductive end-sugar of laminarin enables its grafting into hydrophobic polymer backbones and the production of polysaccharide-*b*-polypeptide block copolymers [15]. Hence, the researchers were able to generate small nanoparticles that take advantage of the interaction of laminarin with Dectin-1 receptors for the targeting of immune system cells, such as macrophages. These studies suggest that pristine laminarin constitutes a chemically versatile slate for grafting multifunctional moieties that impart distinctive physicochemical properties.

Taking the above-mentioned results into consideration, biorthogonal and dynamic chemistries can be consider highly attractive strategies for extending the available toolbox of β-glucan-based bioactive materials and exploring new biomedical applications. In addition, it is important to emphasize that its high solubility in organic solvents, including DMSO and DMF, makes laminarin a highly valuable polysaccharide for straightforward chemical modifications [16]. This feature is particularly advantageous because other widely used marine-derived polymers, such as alginate or hyaluronan, require additional and labor-intensive processing into tetrabutyl ammonium salt for organic solvent-based chemical modifications [17].

From this standpoint, biofunctional compounds that allow the development of dynamic and microenvironment-responsive biomaterials provide numerous advantages in comparison with their static, unidirectional photocrosslinkable counterparts [18]. A particularly interesting type of dynamic covalent crosslinking is the formation of reversible boronate ester bonds (in a pH-dependent manner) between boronic acids and *cis*-diol-containing moieties, such as those found in polyols, catechols, and carbohydrates [19]. The typical applications of boronic-functionalized materials include electrochemical and optical sensors, stimuli-responsive hydrogels, insulin delivery systems, and cell culture and capture [20–26]. The polymer networks formed by boronate ester bonds are not permanently rigid but rather transient and can restructure dynamically after disruption, and the interplay between the two functional groups is thus pivotal for self-healing material design [27, 28].

The functionalization of laminarin with boronic acid has yet to be reported, and its successful inclusion is likely to provide innovative applications for this material. Herein, we

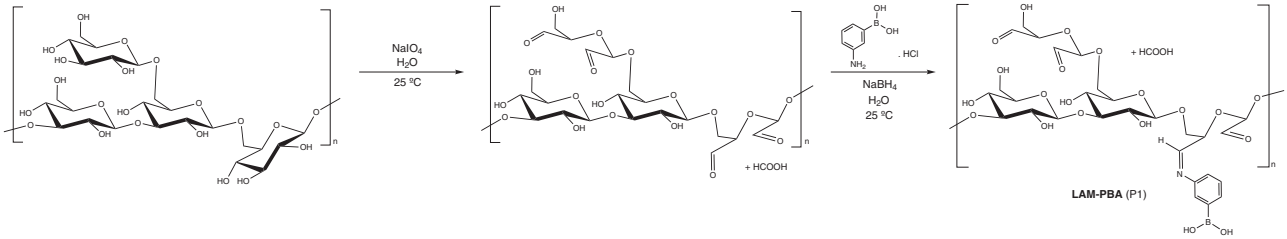

Fig. 2 Functionalization of laminarin with phenylboronic acid (two-pot reaction)

describe the modification of laminarin derived from *E. bicyclis* with boronic acid groups. The resulting biopolymer maintained its high water solubility and enabled conjugation with diol-rich poly(vinyl alcohol) (PVA), a biocompatible and easy-to-handle polymer, via catalyst-free boronate esterification (Fig. 1). This unique crosslinking resulted in the simple and rapid preparation of hydrogels at physiological pH that exhibited self-healing and shear-thinning properties, responsiveness to reactive oxygen species (ROS) and cytocompatibility. The newly synthesized derivative represents a next-generation laminarin-based biopolymer that will have diverse applications in the fields of drug delivery and tissue engineering.

Experimental procedure

Synthesis of LAM-PBA (P1)

Phenylboronic acid-modified laminarin (LAM-PBA) was synthesized via a two-step procedure (Fig. 2). First, laminarin from *E. bicyclis* (500 mg, 0.617 mmol) and sodium periodate (440 mg, 2 mmol) were dissolved in 5 mL of ultrapure water. The mixture was maintained at the room temperature in the dark for 5 h under magnetic stirring, and ethylene glycol (117 µL) was then added to quench the unreacted aldehyde groups. The resulting biopolymer exhibited an oxidation degree of *ca.* 53%, as previously demonstrated [29], was purified by dialysis against water for 3 days at the room temperature and freeze-dried (Telstar LyoQuest). Partially oxidized laminarin (190 mg, 0.234 mmol) and 3-aminophenylboronic acid hydrochloride (76 mg, 0.438 mmol) were then dissolved in ultrapure water (8 mL), and sodium borohydride (166 mg) in methanol was then added to the flask. The reaction was allowed to continue for 8 h in the dark at the room temperature. The mixture was dialyzed and freeze-dried to obtain a pink powder (yield ~89%). Polymer P1 was then characterized by ^{1}H NMR spectroscopy by using a Bruker Advance III spectrometer (Bruker BioSpin GmbH Rheinstetten, Deutschland) operating at 300.13 MHz (University of Aveiro, Portuguese NMR Network-PTNMR). Samples were dissolved in deuterated water (D_2O), placed in 5 mm tubes and spectra were acquired with 256 scans at 298 K. The data were processed using the MestReNova v6.0.2 software.

Hydrogel fabrication

Hydrogels ($P1_5$-$PVA_{2.5/3.7/5}$) were prepared by the mechanical mixing of 10% (w/v) P1 and 5%/7.5%/10% (w/v) PVA solutions (PBS, pH 7.4) at equal proportions to ensure homogeneity. The gelation time at the room temperature was monitored using the vial inversion test, and the gelation process was completed within 10 s.

The microstructure of the gels was examined by scanning electron microscopy (SEM). Prior to examination, the samples were freeze-dried, cross-sectioned, and sputter-coated with gold (Hitachi SU-70, Hitachi Ltd, Tokyo, Japan).

Swelling kinetics and degradation tests

The hydrogels (m_i) were immersed in PBS (pH 7.4) and incubated at 25 °C for 48 h. At predefined time intervals, the samples ($n = 3$) were removed, gently blotted with filter paper and weighed (m_f). The swelling ratio was determined according to the following equation:

$$R(\%) = \frac{m_f - m_i}{m_i} \times 100.$$

In addition, the responsiveness of the gels to ROS was assessed by immersing the disks ($d \approx 10$ mm) in 2 mL of 1 mM hydrogen peroxide (H_2O_2) to calculate the weight loss over time [30, 31].

Mechanical characterization and self-healing evaluation

Rheological studies were performed using a Kinexus lab+ rotational rheometer (Malvern) equipped with a stainless-steel parallel plate geometry. To this end, oscillatory strain amplitude sweep measurements were performed at a frequency of 1 Hz to determine the linear viscoelastic region

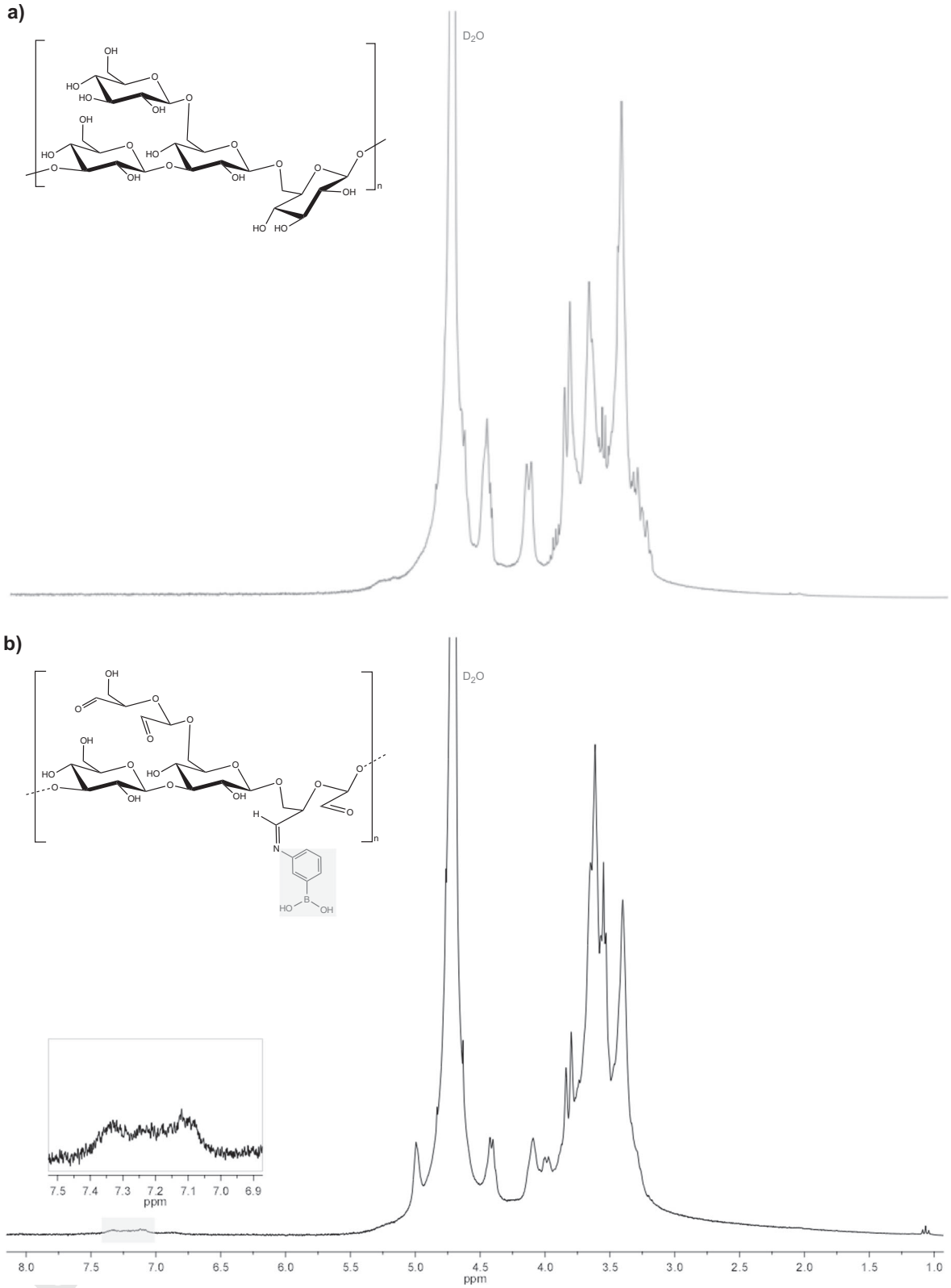

Fig. 3 ^{1}H NMR spectra of **a** laminarin and **b** LAM-PBA (P1) in D_2O

(LVR). Oscillatory frequency sweep measurements were then conducted at a constant strain amplitude of 1% to measure the storage (*G'*) and loss (*G''*) moduli. Shear rate ramp tests were performed to evaluate the shear-thinning

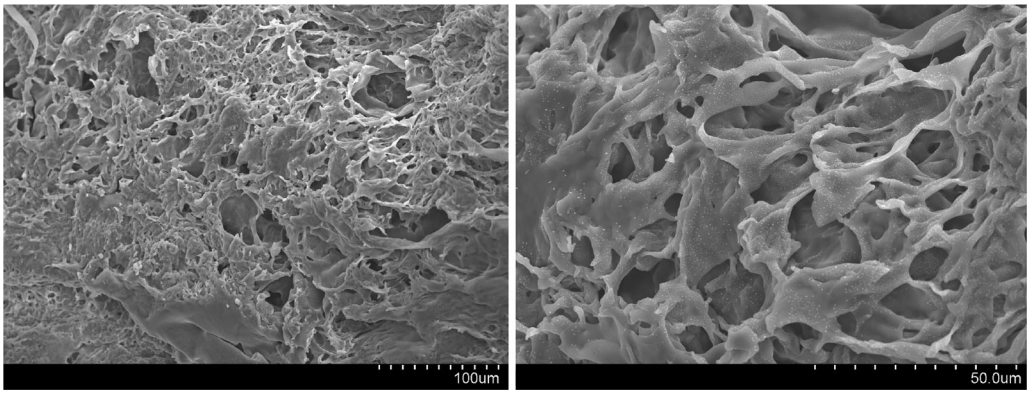

Fig. 4 SEM morphology of freeze-dried $P1_5$–$PVA_{2.5}$ gels: cross-sectional images at two magnifications

profile. Three fresh samples were used for each measurement, and the average results are reported.

The self-healing ability of the hydrogels was quantitatively assessed by dynamic rheology. The samples were cleaved into two pieces and brought back into contact, and the recovery of their moduli was then monitored. The alternate step strain sweep response of the gels was measured at 25 °C and 1 Hz by switching from a strain value of 1% to a value of 100/200%. The self-healing efficiency was calculated as the ratio of *G'* of the healed gels to the original modulus (after two cycles).

Cell culture and cytotoxicity assays

The MC3T3-E1 (ATCC® CRL-2593™) preosteoblast cell line was cultured at 37 °C under a 5% CO_2 humidified atmosphere in α-MEM culture medium supplemented with 10% (v/v) FBS and 1% antibiotic-antimycotic solution. The cells were passaged every 2–3 days once they reached 80–90% confluency and reseeded prior to use.

The MC3T3-E1 cells were plated on a 48-well plate at a density of 5×10^4 cells/mL. Prior to the assay, hydrogels were prepared as described above, sterilized by exposure to UV light for 15 min, and then incubated for 48 h in direct contact with the cells. The metabolic activity was assessed at different time points using the AlamarBlue™ assay. Briefly, the cells were incubated with α-MEM containing 10% (v/v) AlamarBlue™ reagent, and after 4 h, the fluorescence intensity of the medium was detected at excitation/emission wavelengths of 540/590 nm using a multimode microplate reader (Synergy HTX, BioTek Instruments). The cell viability values are presented as percentages relative to the untreated control cells.

Statistical analysis

The data are presented as the mean ± standard deviation (SD) and analyzed using GraphPad Prism software. The statistical significance of the differences was evaluated by one-way ANOVA, and the level of significance was set to a probability $*p < 0.05$.

Results and discussion

Synthesis and characterization of LAM-PBA (P1)

Laminarin was functionalized with a PBA group for the first time via a two-step reaction and characterized by proton nuclear magnetic resonance (^{1}H NMR). Due to its high abundance of hydroxyl groups, laminarin exhibits high solubility in water and polar solvents, and they can be used for the insertion of functional groups. Initially, laminarin was modified via C-3 and C-4 *cis*-diols selective scission with sodium periodate [32]. The successful oxidation of laminarin allowed the synthesis of an amine-reactive derivative, as we previously demonstrated [29]. This approach can be used to functionalize the backbone of laminarin with virtually any biofunctional amine-containing motifs. The chemical composition of polymer P1 could be verified by observing the typical chemical shifts below 5 ppm corresponding to the β-D-glucans backbone [33, 34] and the characteristic aromatic peak of PBA at ~7.0–7.4 ppm, which was not present in pristine laminarin (Fig. 3). This strategy substantially expands the framework of laminarin derivatives that can be synthesized according to the desired physicochemical properties.

Hydrogel preparation

Burgundy-colored hydrogels with different concentration ratios ($P1_5$–$PVA_{2.5/3.7/5}$) were prepared within 10 s of mixing precursor aqueous solutions of P1 and PVA at pH 7.4, and the formation of these hydrogels was driven by the formation of covalent, albeit reversible, boronate ester bonds between the boronic acid residues of laminarin and

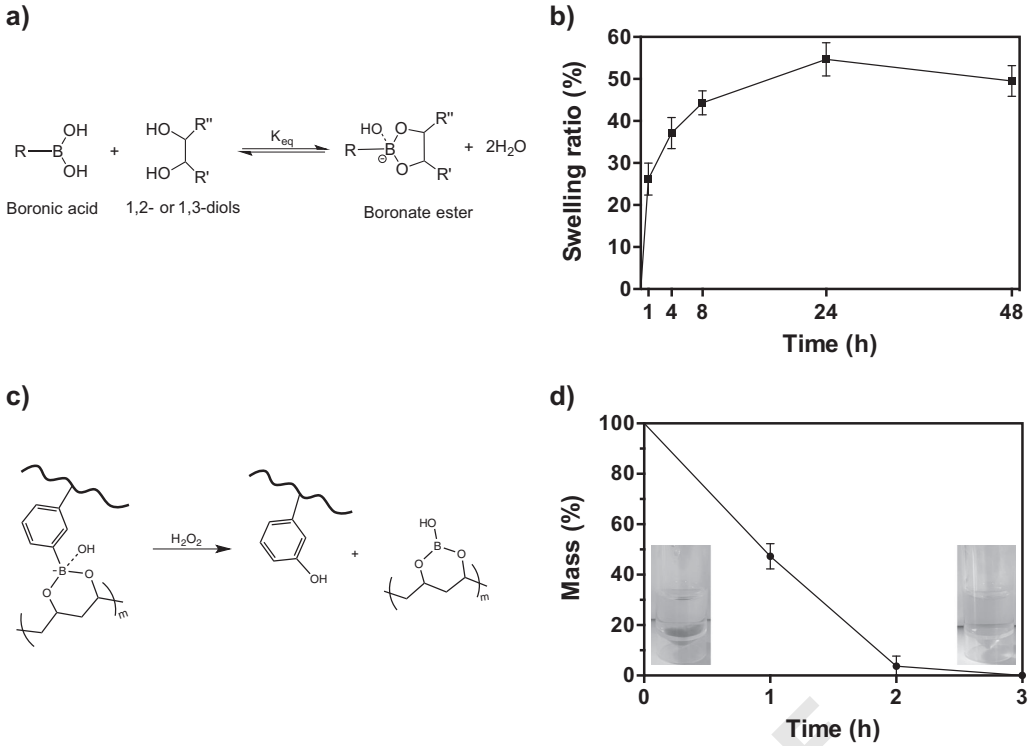

Fig. 5 **a** Mechanism of the complexation of boronic acid and diol for the formation of cyclic boronate ester in aqueous conditions. **b** Swelling ratio of $P1_5$-$PVA_{2.5}$ hydrogel in PBS (pH 7.4) at 25 °C. **c** Schematic of hydrogel degradation upon exposure to H_2O_2. **d** Degradation profile of the gel in 1 mM H_2O_2 solution (mean ± SD from triplicates)

the diols of PVA [28, 35]. Preliminary experiments using different concentrations of the polymers were performed to determine the minimum polymer feed required for the formation of self-standing hydrogels and the $P1_5$–$PVA_{2.5}$ formulation was selected for further experiments (Table S1). SEM images of the $P1_5$–$PVA_{2.5}$ hydrogel, which are depicted in Fig. 4, illustrate a typical micron-sized porous network. Although the formation of boronate ester complexes favorably occurs at pH 9 (pK_a ≈8.8), a sufficient amount of ionizable boronic acid groups that bind to *cis*-diols present on PVA are present at physiological conditions (pH 7.4) (Fig. 5a) [36]. The addition of unmodified laminarin to PVA (under the same experimental conditions) did not result in the formation of any hydrogels, as expected.

Swelling kinetics and stimuli responsiveness

The polymeric network was not in equilibrium with the medium, i.e., the hydrogels were expected to still exhibit additional water uptake after crosslinking, and thus, the swelling kinetics of $P1_5$–$PVA_{2.5}$ gels were assessed for 48 h. As shown in Fig. 5b, the hydrogels exhibited a moderate swelling capacity (*ca.* 55%) and reached equilibrium after 24 h of immersion in PBS (pH 7.4), which indicates that the stability of the hydrogels.

Methods for the selective delivery of therapeutic or diagnostic agents to sites undergoing oxidative stress would prove useful for various diseases characterized by high concentrations of ROS [31]. These species are normally produced during biological processes and exhibit significant oxidative potential, particularly in tumor tissues. In this sense, ROS-responsive materials comprising boronic acid motifs have been explored for the development of fluorescent probes, imaging agents, and oxidation-sensitive carriers [30]. In particular, species such as H_2O_2 can oxidize the boronate ester complex by inserting an oxygen atom in the C–B bond, which induces the formation of borate esters and hydroxybenzyl derivatives (Fig. 5c) [37]. Figure 5d shows the effect of ROS on $P1_5$–$PVA_{2.5}$ gel stability. After 3 h, the hydrogel was completely degraded in the presence of 1 mM H_2O_2, and this degradation was driven by irreversible network rupture.

Mechanical properties and self-healing

We studied the dynamic rheological properties of the hydrogels obtained with representative P1 and PVA concentrations (see Fig. 6a–c). Initially, oscillatory strain sweeps were performed at the room temperature to determine the LVR. We observed an increase in the storage (*G'*) and loss (*G"*) moduli crossover strain value, which is

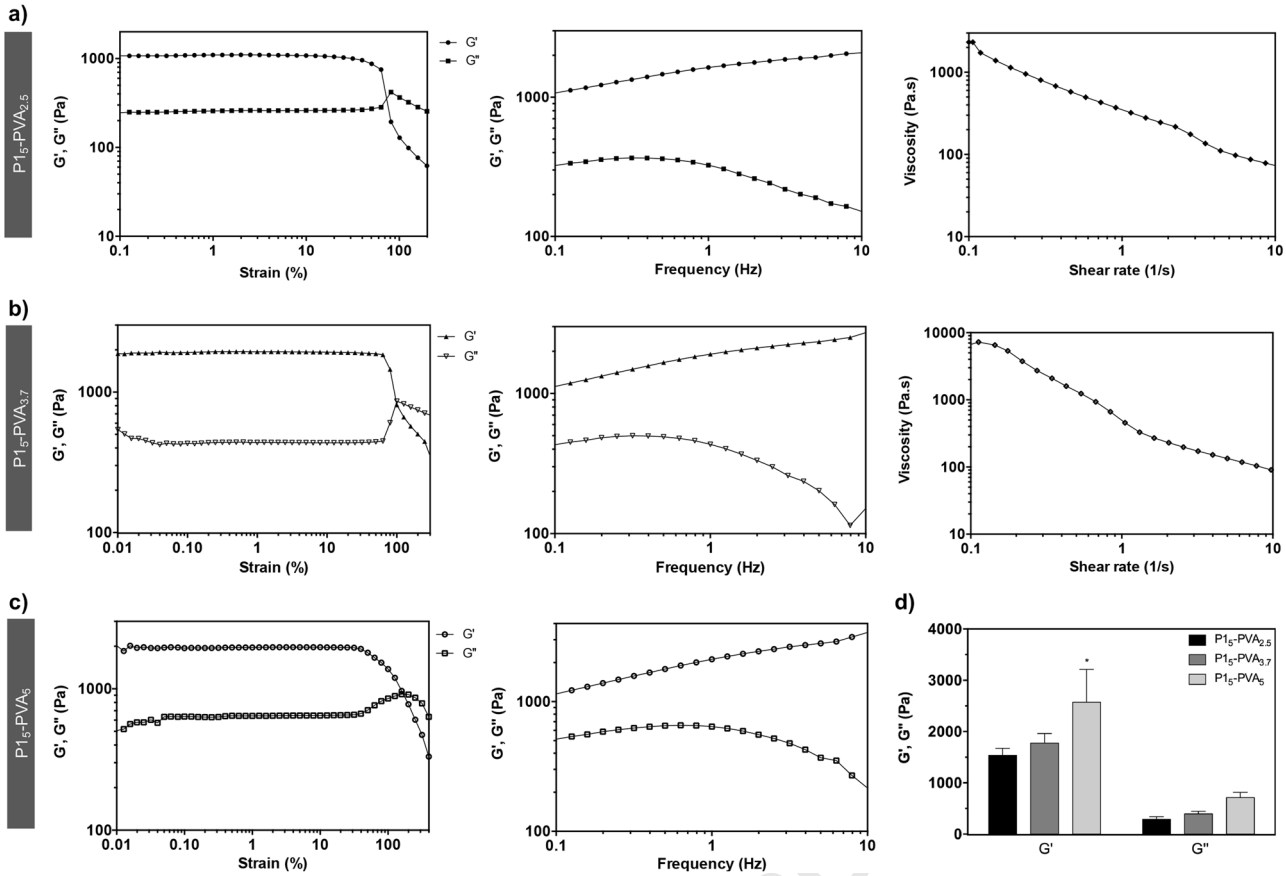

Fig. 6 Rheological properties of **a** $P1_5$-$PVA_{2.5}$, **b** $P1_5$-$PVA_{3.7}$, and **c** $P1_5$-PVA_5. Strain sweep measurements of gels at fixed 1 Hz (left), variation in the *G'* and *G"* moduli with frequency (middle), and shear-thinning behavior at 25 °C (right). **d** Comparison of *G'* and *G"* values of selected gels at 1 Hz as function of the PVA concentration (mean ± SD)

required for disruption of the polymer network, at higher PVA concentrations.

It is well known that the viscoelasticity of a material and mechanotransduction might affect cellular responses [38, 39]. Frequency sweep measurements were thus performed within this region to evaluate the mechanical strength of the constructs. The gels demonstrated a minor frequency-dependent viscoelastic behavior, but the *G'* was greater than the *G"* at all regimes, which is indicative of a gel-like character (elastic component is dominant). The influence of the PVA concentration on the rheological properties of the hydrogels was also evaluated by comparing the *G'* and *G"* values (Fig. 6d). Reducing the concentration from 5 to 2.5% decreased the G' from *ca*. 2500 to 1500 Pa, likely due to the lower crosslinking density, which impacts the mechanical properties; a similar trend was found for *G"*. As expected, the stiffness of the gels improved with increases in the concentration of PVA polymer.

Moreover, as the dynamic boronate esters in the network start to disrupt, the viscosity of the gels ($P1_5$–$PVA_{2.5/3.7}$) decreased with increasing shear rate, following the power law model, which is indicative of a shear-thinning profile.

The self-healing behavior of freshly prepared $P1_5$–$PVA_{2.5}$ gels was visually investigated. To this end, the hydrogels were cut in half, as displayed in Fig. 7a, and after the pieces were directly brought into contact, their healing into one integral piece was observed within 30 min in the absence of any external stimulus, although the cut interface was still vaguely visible. To investigate the hydrogel self-healing capability in more detail, strain sweep measurements were carried out to evaluate the autonomous recovery of their rheological properties (Figs. 7c and S1). At low strain values (1%), the *G'* value was higher than the *G"* value, but the application of a high-magnitude strain (100%) to the gel induced an inversion of the moduli due to disruption of the network (large deformation); after removal of the high strain, both the *G'* and *G"* values of the gel rapidly recovered to their original values without any noticeable loss over time (*ca*. 99% recovery), which differs from other healing mechanisms [40, 41]. These interesting self-healing properties under mild conditions are mainly imparted to the transient dissociation of the dynamic reversible boronate

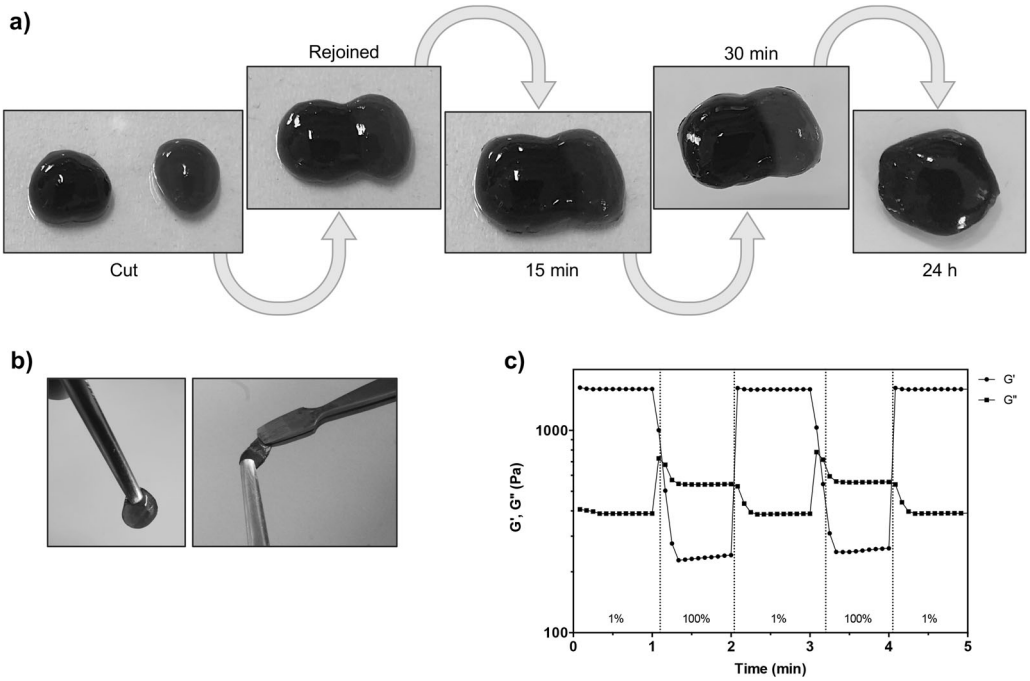

Fig. 7 **a** Hydrogel self-healing behavior: two pieces (with and without green dye for better contrast) were mechanically rejoined, and their recovery process was monitored. **b** The healed hydrogel could maintain its integrity and withstand deformation. **c** A dynamic oscillatory test was employed by shearing the $P1_5$–$PVA_{2.5}$ samples at constant 1 Hz, from 1 to 100% strain (alternate) and by monitoring the variations in the *G'* and *G"* values

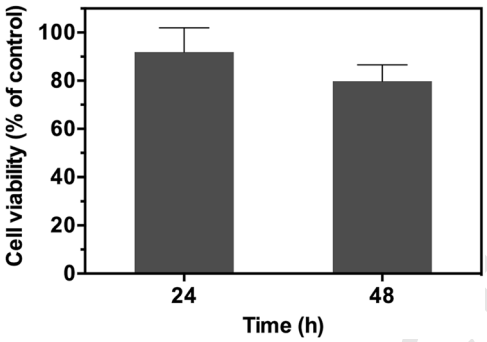

Fig. 8 Metabolic activity of MC3T3-E1 preosteoblast cells incubated with the $P1_5$–$PVA_{2.5}$ gel. The activity was determined using the AlamarBlue™ assay. The cell viability data are shown as percentages with respect to the untreated (control) cells (mean ± SD from triplicates)

ester complexes, which upon reforming, allow the rapid (<1 min) and efficient rearrangement of the polymeric network.

Cytocompatibility evaluation

After the physicochemical and mechanical characterization of the polymeric hydrogels, we performed in vitro toxicity studies to investigate the effect of the $P1_5$–$PVA_{2.5}$ gel on the metabolic activity of MC3T3-E1 cells as a model. As observed in Fig. 8, the gels did not exhibit any toxicity after 48 h, with ~80% of the cells remaining viable.

Conclusion

In conclusion, this study demonstrates the first functionalization of laminarin with PBA moieties through a simple and cost-effective two-pot approach. This biopolymer was then used for the rapid fabrication of versatile hydrogels under physiological conditions based on the dynamic formation of covalent boronate ester bonds between the boronic acids in the backbone and the *cis*-diols of PVA. The 3D constructs exhibited tunable mechanical properties with shear thinning and rapid self-healing characteristics. This platform was found to be cytocompatible for culturing a preosteoblastic model cell line, and its biocompatibility will likely enable the inclusion of different cell types. Furthermore, the hydrogels were confirmed to be responsive to ROS and noncytotoxic, and these findings pave the way for their application in biomedicine as drug delivery carriers and/or bioinks for tissue engineering.

Acknowledgements This work was supported by the European Research Council (ERC-2014-ADG-669858) and project CICECO-Aveiro Institute of Materials, UIDB/50011/2020 & UIDP/50011/2020, financed by national funds through the Portuguese FCT/MCTES. The NMR spectrometer is part of the National NMR Network (PTNMR)

and partially supported by Infrastructure Project N° 022161 (co-financed by FEDER through COMPETE 2020, POCI and PORL, and FCT through PIDDAC). This work was also funded by the Programa Operacional Competitividade e Internacionalização (POCI), in the component FEDER, and by national funds (OE) through FCT/MCTES within the scope of the project MARGEL (PTDC/BTM-MAT/31498/2017). VMG acknowledges funding in the form of a Junior Research contract under the scope of project PANGEIA (PTDC/BTM-SAL/30503/2017).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Graphical Abstract

The functionalization of laminarin with boronic acid groups was described. This biopolymer readily established boronate ester-crosslinked gels with poly(vinyl alcohol) within seconds under physiological conditions. The resultant hydrogels exhibited interesting self-healing properties, reactive oxygen species responsiveness and cytocompatibility.