Temporary/Permanent Dual Cross-Link Gels Formed of a Bioactive Lactose-Modified Chitosan

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Mounting evidences have recognized that dual cross-link and double-network gels can promisingly recapitulate the complex living tissue architecture and overcome mechanical limitations of conventional scaffolds used hitherto in regenerative medicine. Here, dual cross-link gels formed of a bioactive lactose-modified chitosan reticulated via both temporary (boric acid-based) and permanent (genipin-based) cross-linkers are reported. While boric acid rapidly binds to lactitol flanking diols increasing the overall viscosity, a slow temperature-driven genipin binding process takes place allowing for network strengthening. Combination of frequency and stress sweep experiments in the linear stress–strain region shows that ultimate gel strength, toughness, and viscoelasticity depend on polymer-to-genipin molar ratio. Notably, herewith it is demonstrated that linear stretching correlates with strain energy dissipation through boric acid binding/unbinding dynamics. Strain-hardening effect in the nonlinear regime, along with good biocompatibility in vitro, points at an interesting role of present system as biological extracellular matrix substitute.

1. Introduction

Hydrogels—or simply gels—are in essence water-swollen polymer networks being appropriate for various bioengineering applications, encompassing regenerative medicine and drug delivery sectors. Beside necessary biocompatibility, comprehending hydrogel mechanics results of pivotal importance for potentially moving present networks toward the clinic. Indeed, mechanical stimuli from the external milieu control cell behavior and differentiation, tissue development, or promote the onset of pathologies such as cancer and cardiovascular diseases.[1–6]

Gel design has progressively changed from static to dynamic architecture as a consequence of novel concepts in spatial and temporal complexity of living tissues.[7] Native tissues are in fact seldom static networks; they display dynamics relevant for complex tissue homeostasis. Groundbreaking biomaterials should therefore mimic extracellular matrix (ECM) environment, dynamically respond to cell stimuli and self-remodel polymer mesh following to forces applied by cells, ECM production and neo-tissue formation.

Among dynamic (out-of-equilibrium) matrices, boronic acid containing gels have shown great potential in tissue engineering and, more broadly speaking, in medicine.[8–12] The binding of boronic acids to polyols leads to the formation of transient esters, especially with 1,2- and 1,3-diols.[8] Specifically, the cross-linker behaves as a sticker, with borate residues associating and dissociating in a highly dynamic fashion. Hence, this dynamic material can flow under its own weight. Though endowed with unique features such as self-healing, continuous network rearrangement due to cross-linker binding/unbinding dynamics may affect its final performance. Conversely, gels containing permanent cross-links bear their own weight—without apparent flow—but suffer from marked brittleness, as to hamper ultimate application thereof, especially for load-bearing conditions.

Over the last few years, gels composed of dual cross-links, i.e., "temporary and weak" on one side and "permanent and strong" on the other have attracted great interest. They in fact overcome flaws above reported forming a stretchable and elastic network with respect to the counterparts containing sole permanent or temporary junctions.[13–16] Poly(vinyl alcohol), polyampholytes and alginate/polyacrylamide mixtures represent the typical polymers used hitherto for assembling dual systems. However, none of them possesses inborn tissue-inductive properties. Hence, regenerative medicine longs for innovative networks showing peculiar mechanics that simultaneously could assist and direct cells in repairing injured tissues.

In this contribution we describe for the first time dual cross-link hydrogels based on a lactose-derivative of chitosan, shortly named CTL, containing both temporary and permanent reticulations. CTL is a branched diol-rich polysaccharide endowed with bioactive features in terms of cartilage, bone and nerve regeneration.[17–19] Recently, CTL showed the ability to form dynamic gels in the presence of boric acid as temporary

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cross-linker.[12,20] As permanent cross-linker, here we introduce the natural compound genipin, an aglycone extracted from *Gardenia jasminoides* already used to covalently reticulate chitosans.[21,22] The resulting networks manifest adaptable strength, toughness, viscoelasticity and energy dissipation, along with promising biocompatibility.

2. Results and Discussion

An initial increment of viscosity accompanies the mixing of genipin-boric acid mixture with CTL due to rapid interactions of boric acid with diols located on lateral (grafted) lactose moieties of CTL.[23,24] The addition of mannitol as boric acid competitor ensures the formation of a homogeneous and clear network rather than filamentous CTL aggregates surrounded by a polymer-poor phase.[12]

Kinetics at constant boric acid and glucosamine-to-genipin molar ratio ($R_{D/G}$) but different gelling temperatures allowed following the viscoelastic evolution of CTL-genipin-boric acid mixture over time. Specifically, the loss tangent ($\tan\delta = {}^{G''}/_{G'}$) was recorded as a function of time (**Figure 1**A).[25,26] The continuous decay of loss tangents at different gelling temperatures indicates that CTL-based mixtures switch from an initial viscous-like system toward a more elastic structure. Interestingly, the increase in temperature determines the transition from an almost linear to an exponential time-decay of tanδ, clearly suggesting that the temperature accelerates the formation of permanent junctions in the early stages of gel formation as in chitosan-genipin systems.[22] To derive information about the rate of the process, experimental points were fitted by Equation (1)

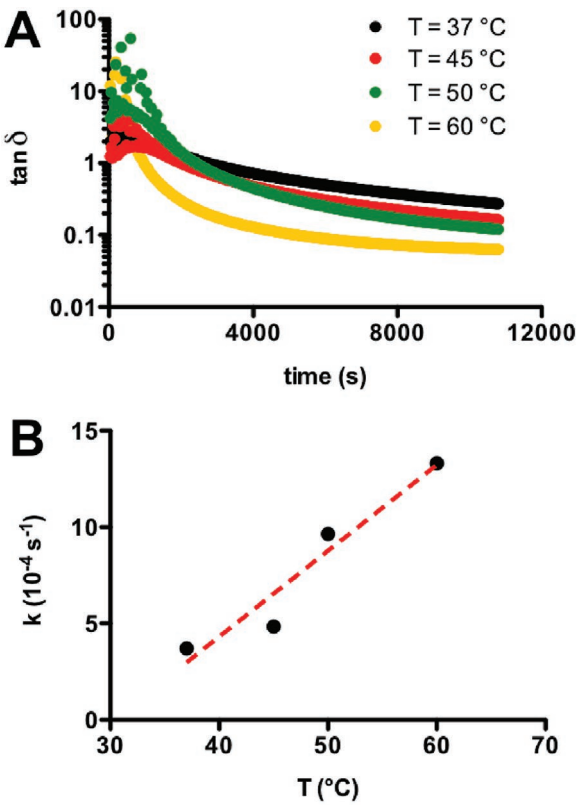

Figure 1. The effect of temperature in modulating the gelling kinetics of CTL-genipin-boric acid system. A) Sample-case dependence of the loss tangent, tanδ (${}^{G''}/_{G'}$), on time for different gelling temperatures, T: T = 37 °C (black dots), 45 °C (red dots), 50 °C (green dots), and 60 °C (yellow dots). B) Dependence of the rate constant, k, on T (°C); dashed line is drawn to guide the eye. The error associated with the calculation of k resulted < 1%. Experimental conditions: [CTL] = 1% w/v, [genipin] = 0.52 × 10^{-3} M and [boric acid] = 8 × 10^{-3} M; phosphate buffered saline (PBS) as solvent, pH 7.4.

$$\tan\delta\ (t) = (\tan\delta_0 - \tan\delta_\infty)\, e^{-kt} + \tan\delta_\infty \quad (1)$$

where tanδ_0 is the value of loss tangent at time zero, tanδ_∞ is the loss tangent at infinite time and k is the rate constant.[20] Figure 1B displays that the increase in temperature (linearly) accelerates the hardening of CTL mixtures. The activation energy of the process was calculated from the slope of the linear fitting of the Arrhenius plot, yielding 49.1 ± 11.6 kJ mol^{-1} (see Figure S1, Supporting Information). The activation energy of the present dual cross-link gel system resulted slightly higher with respect to that found by Sannino and co-workers, likely ascribed to different polymer—i.e., chitosan—and experimental conditions—i.e., acidic pH and elevated genipin amount—investigated.[22]

To comprehend the role played by genipin in modulating gelling kinetics, we next performed a set of time sweep experiments at T = 60 °C and constant boric acid but different glucosamine-to-genipin molar ratio. Figure S2 (Supporting Information) reports the time-dependence of loss tangent. For all $R_{D/G}$ values explored, a slow exponential decay follows an initial and rapid increment of *tanδ*. The former behavior can be safely associated with a true gelation event due to genipin binding by primary amino groups of residual glucosamines in CTL.[27] Conversely, the latter trend resulted quite unexpected and additional investigations are ongoing to shed light on its molecular basis. Equation (1) was exploited to calculate the related rate constants (Figure S2, Supporting Information). A negative (linear) correlation between rate constants and glucosamine-to-genipin molar ratio holds, indicating that the gelation velocity slows down upon decreasing genipin content. Overall, at the end of the process dual cross-link gels appeared from light to dark blue depending on genipin content (Figure S2, Supporting Information). Since cross-linking results almost complete at pH 7.4 (≈96%),[27] the diverse blue gradation depends on the variable cross-linking density throughout CTL-based networks.

After complete gelation, viscoelastic properties of dual cross-link gels were investigated by means of frequency sweep tests. Strikingly, while boric acid-deprived gels behaved as pure elastic networks (elastic modulus independent from frequency, **Figure 2**A), dual cross-link networks at different genipin content manifested viscoelastic properties (Figure 2B–D). For the latter case, the following scaling-law holds at low frequency regime, $G' \propto \nu^n$, where n changed from 0.27 ± 0.01 to 0.04 ± 0.01 in the case of $R_{D/G}$ = 40 and 10, respectively. On the other hand, $G'' \propto \nu^{0.5}$ in the same frequency region for all dual cross-link gels analyzed, in nice agreement with the PVA/glutaraldehyde/borax system.[15]

The viscoelasticity degree of dual cross-link gels was arbitrarily derived from the ${}^{G'}/_{G''}$ ratio at 0.1 Hz,[28] yielding 3, 8, and

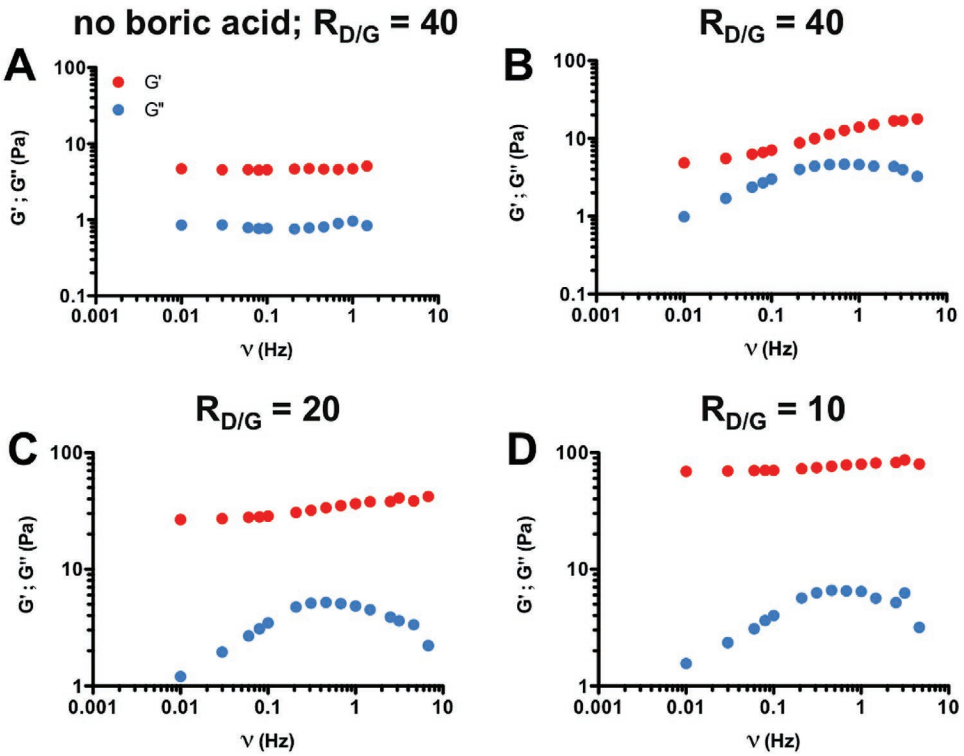

Figure 2. Frequency sweep investigation on CTL-genipin-boric acid dual cross-link gels. Sample-case mechanical spectra as a function of frequency, ν, of A) pure chemical, i.e., boric acid-free, gel network and B–D) dual cross-link gels at different glucosamine-to-genipin molar ratio, $R_{D/G}$. G' (red dots) and G'' (blue dots). Experimental conditions: [CTL] = 1% w/v, [genipin] = 0.13–0.52 × 10^{-3} M and [boric acid] = 8 × 10^{-3} M; phosphate buffered saline (PBS) as solvent, pH 7.4; after complete gelation performed at T = 60 °C, all frequency sweep experiments were performed at T = 37 °C.

18 in the case of $R_{D/G}$ = 40, 20, and 10 gels, respectively. Hence, the higher is the genipin content, the higher is the elastic nature of related gels. Of note, regardless the $R_{D/G}$ values considered, dual cross-link gels display a peak in the viscous modulus at a specific frequency value, ν_{max} (Figure 2). By comparing ν_{max} of networks at different $R_{D/G}$, no straightforward differences emerge among the systems analyzed, being $\nu_{max} \approx$ 0.46 Hz in all cases. Since ν_{max} is associated with the time in which an associated – and transient – cross-linker reverts to its equilibrium position by one or more dissociation steps,[29] we conclude that dual cross-link gels preserved the dynamic nature of CTL-boric acid system even in the presence of stable genipin cross-link points.[12,20] In the present case, the characteristic boric acid relaxation time, τ_x, is calculated from $^1/_{\nu_{max}}$, yielding $\tau_x \approx$ 2s, in line with what found by Mayumi et al.[14] and slightly higher with respect to those of Anseth and co-workers.[11]

We next studied gel dynamics due to temporary cross-links involvement by decoupling viscoelastic moduli into two different contributions resulting from permanent (genipin-based) and temporary (boric acid-based) bonds, respectively. Specifically, the modulus due to permanent cross-links, G_P, was subtracted from the modulus of dual cross-link gels, G_D, to estimate the contribution due to temporary interactions, yielding $G'_T = G'_D - G'_P$ and $G''_T = G''_D - G''_P$.[15] At frequencies lower than ν_{max}, where temporary bonds break to relax the stress, we found that $G'_T \propto G''_T \ \nu^1$ in the case of $R_{D/G}$ = 40 dual cross-link gels, whereas $G'_T \ \nu^{0.1}$ and $G''_T \propto \nu^{0.7}$ for $R_{D/G}$ = 10 counterparts (Figure S3, Supporting Information), suggesting that CTL/genipin/boric acid system displays different dynamics with respect to those showing associative Rouse mode.[14,15,29]

Linear stress–strain dependence was analyzed by means of stress sweep experiments to derive information about gel strength (**Figure 3**A). As expected, the shear modulus lowered upon decreasing genipin amount as a result of reduced cross-linking density, clearly indicating that permanent cross-links are fundamental in assembling more rigid networks. By plotting the elastic moduli as a function of the overall strain applied, all dual cross-link gels manifest strain-hardening behavior at large deformations—albeit to a different extent—regardless the $R_{D/G}$ value investigated (Figure 3B). The magnitude of nonlinear stiffening before network rupture depends upon the overall interactions between boric acid and CTL in the nonlinear region. In this view, the progressive reduction of permanent cross-links renders dual cross-link gels more flexible. Therefore, CTL chains would be more easily stretched and oriented under shear stress, leading to a higher probability of intermolecular associations at large deformations.[30]

To study the extent of linear stress–strain region, we determined mathematically the onset of nonlinear regime by exploiting the model proposed by Erk et al. (2)

$$\tau = G_0 \ \gamma e^{(\gamma / \gamma^*)^2} \quad (2)$$

where γ^* is the main fitting parameter, defined as the critical deformation at which stiffening becomes dominant.[31] The dependence of γ^* on $R_{D/G}$ is reported in Figure 3C. Strikingly, the

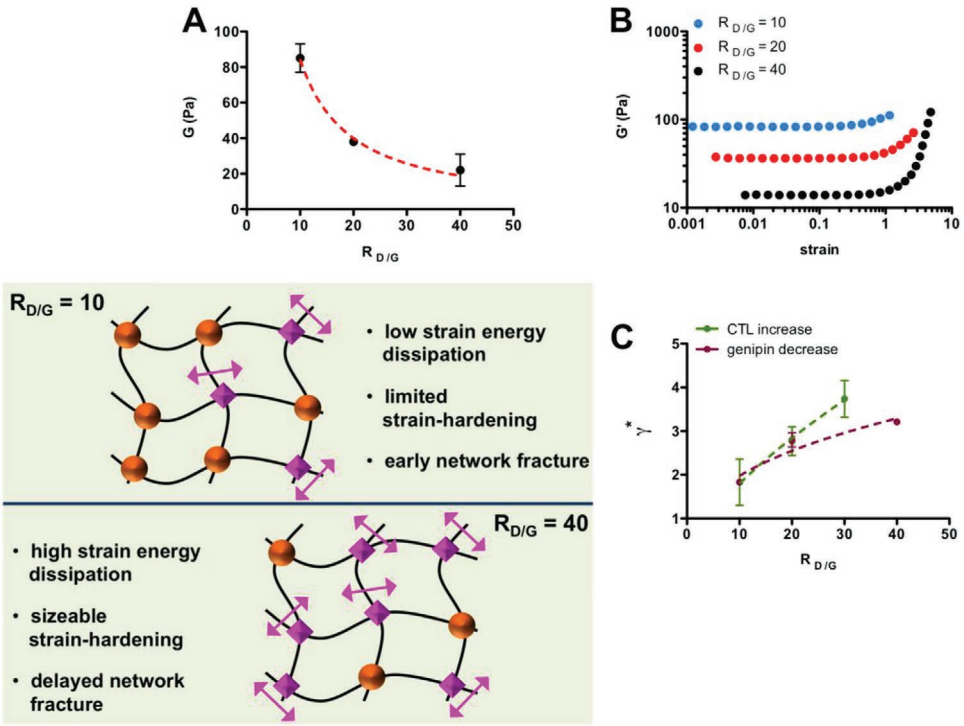

Figure 3. Dual cross-link gels manifest adaptable strength, energy dissipation in the linear stress–strain regime and macromolecular rearrangement—strain hardening–in the nonlinear region. A) Dependence of shear modulus, G, as a function of glucosamine-to-genipin molar ratio, $R_{D/G}$; dashed line is drawn to guide the eye. Data are reported as mean ± s.d., $n = 3$ gels analyzed for each experimental condition. B) Sample-case dependence of the elastic modulus, G', on total strain applied for dual cross-link gels at different $R_{D/G}$: $R_{D/G} = 10$ (blue dots), 20 (red dots), and 40 (black dots). C) Dependence of the critical deformation at which the stiffening becomes dominant, γ^*, on $R_{D/G}$ by varying genipin or CTL amount; dashed lines represent the best fit of experimental points according to the standard power law behavior, $\gamma^* \propto R_{D/G}^n$. Data are reported as mean ± s.d., $n = 3$ gels analyzed for each experimental condition. A–C) Experimental conditions: [CTL] = 1–3% w/v, [genipin] = 0.13–0.52 × 10^{-3} M and [boric acid] = 8 × 10^{-3} M; Phosphate Buffered Saline (PBS) as solvent, pH 7.4; after complete gelation performed at $T = 60$ °C, all stress sweep experiments were performed at $T = 37$ °C. The cartoon resumes the physical characteristics of dual cross-link gels at different $R_{D/G}$. In the case of high genipin content, i.e., $R_{D/G} = 10$, the network behaves as a rigid system. Hence, the strain energy is dissipated by few temporary boric acid-type cross-links and the outcome is the loading of permanent genipin-type cross-links under shear stress. This causes an early exit from linear regime, scarce mesh rearrangement in the nonlinear region and rapid gel fracture (fracture toughness ≈ 20 J m^{-3}). By decreasing the genipin content, i.e., $R_{D/G} = 40$, the contribution due to the boric acid prevails over that of genipin: here, the strain energy is much better dissipated by transient CTL-boric acid cross-links, eliciting the extension of linear regime, sizeable mesh rearrangement at large deformations and delayed gel fracture (fracture toughness ≈ 130 J m^{-3}).

critical deformation scales with the glucosamine-to-genipin molar ratio according to $\gamma^* \propto R_{D/G}^{0.4}$, indicating that the onset of stiffening shifts toward larger deformations. Of note, when genipin was kept constant (i.e., 0.52 × 10^{-3} M) and CTL varied (i.e., 1—3% w/v), a different power law behavior holds, being $R_{D/G}$ exponent equal to 0.7 (Figure 3C), likely due to the increased CTL entanglement. Nevertheless, the increment of γ^* on $R_{D/G}$ upon varying genipin or CTL content emphasizes that a dissipation mechanism is active in damping strain energy in the linear regime. Since no repetition (i.e., polymer motion) can occur in our system due to stable genipin-type cross-links, we therefore conclude that transient (and weak) boric acid-CTL junctions break and re-form upon increasing shear stress thus dissipating strain energy. This phenomenon is magnified at higher $R_{D/G}$, and results in the shift toward larger deformations of network reorganization (onset of strain hardening), unloading of permanent cross-links and, consequently, delay of network fracture (Cartoon in Figure 3).

To test the feasibility of using dual cross-link gels as potential biomaterials, 2D cytotoxicity and cell metabolic activity assays were performed using mouse fibroblast-like NIH-3T3 as cell model. Specifically, well-adherent cells were incubated with dual cross-link gels for 24 h, and the amount of cytosolic LDH enzyme released by membrane-damaged cells quantified colorimetrically (**Figure 4**A). Furthermore, the potential impact of dual cross-link gels on the metabolic activity was measured by evaluating the chemical reduction of the AlamarBlue reagent by cells (Figure 4B). Overall, this set of experiments proved the lack of toxicity of dual cross-link gels toward NIH-3T3 cells, being LDH level and fluorescence intensity generated by gel-treated cells comparable with respect to that of control group. Optical microscopy (qualitative) investigation on cellular monolayers corroborates previous findings, thus categorizing dual cross-link gels as "Reactivity: none, score 0,"[32] with majority of cells exhibiting good morphology and lack of vacuolization.

3. Conclusions

In summary, we have unveiled dual cross-link gels based on the bioactive polymer CTL assembled via temporary (boric

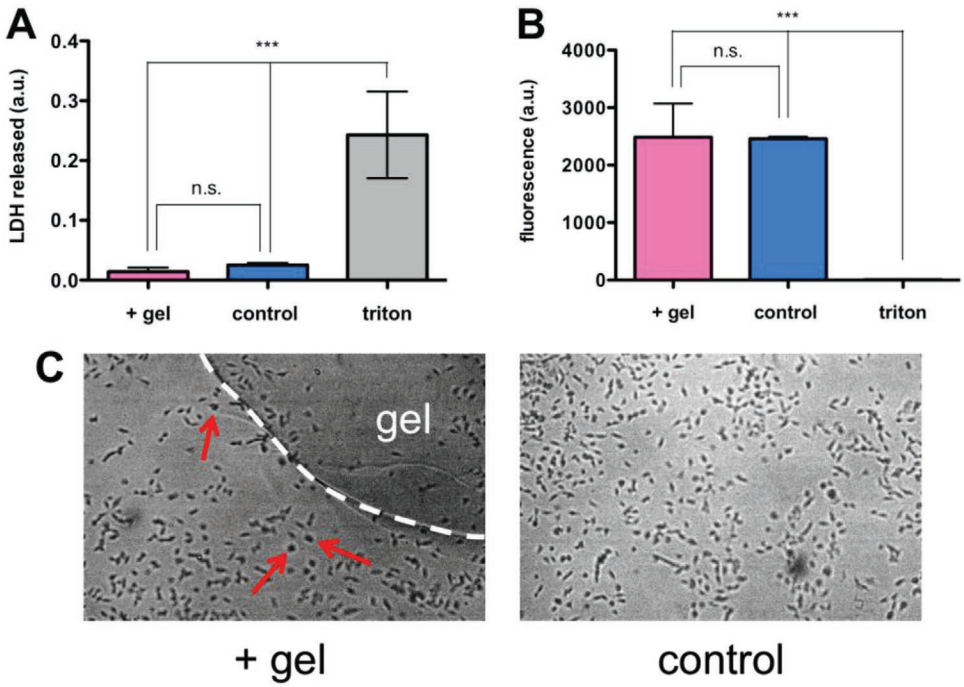

Figure 4. Impact of dual cross-link gels on cell membrane damage, cellular metabolism, and morphology. A,B) Lactate Dehydrogenase—LDH—enzyme quantification and total fluorescence generated by NIH-3T3 cells when incubated with dual cross-link gels. Control group refers to adherent cells without gel, whereas triton group is the positive (death) control. Data are reported as mean ± s.d., $n = 5–6$ replicates for each experimental condition. Statistics: n.s., not significant; ***, $p < 0.001$ (One-way ANOVA followed by Tukey's Multiple Comparison *post hoc* test). C) Optical microscopy of NIH-3T3 cells in the presence or not of dual cross-link gel. Red arrows indicate very few suffering cells (< 20% of total). Images were acquired with a digital camera (40x magnification).

acid-type) and permanent (genipin-type) cross-linkers (Figure S4, Supporting Information). Upon the addition of boric acid-genipin mixture, a two-step CTL gelation occurs: a "rapid" increment of viscosity caused by transient interactions of boric acid with the polyol, followed by a "slow" network strengthening due to genipin-driven reticulation. While the former process can be nicely controlled by the addition of mannitol as boric acid competitor thus avoiding any inhomogeneity,[12] kinetic results have highlighted that the formation of ultimate gels accelerated simply by increasing the genipin content or the gelling temperature. The latter case could provide an advantage in the assembling of injectable materials endowed with different thermo-gelling point to be used for specific clinical settings, for instance in viscosupplementation procedure.

As to the mechanical side, gel strength, viscoelasticity, and linear stretching are modulated by varying glucosamine-to-genipin molar ratio: dual cross-link gels changed in fact from prevalent elastic to more viscous and stretchy upon decreasing genipin content. Strikingly, the greater extension of linear stress–strain regime and the elevated fracture toughness noticed for networks at low permanent cross-linker content was attributed to strain energy dissipation phenomena, in line with what observed for polyampholytes-based gels.[16] Of note, all networks analyzed manifested strain-hardening effect in the nonlinear region, pointing at an interesting role of present system as biological ECM substitute,[33] especially in cartilage and neural tissue engineering sector. The feasibility of using dual cross-link gels as biomaterials is strengthened by the good in vitro biocompatibility. Forthcoming investigations will uncover cell behavior atop 2D or 3D laden gels.

4. Experimental Section

Materials: Hydrochloride form of lactose-modified chitosan, CTL (CAS Registry Number 2173421-37-7) was kindly provided by biopoLife s.r.l. (Trieste, Italy). The chemical composition of CTL was determined by ^{1}H-NMR spectroscopy and resulted: fraction of deacetylated units (F_D) 0.21, fraction of lactose-modified units (F_L) 0.63, and fraction of acetylated units (F_A) 0.16 (see Figure S4, Supporting Information). The calculated molar mass of CTL repeating unit ($MW_{r.u.}$)—given its chemical composition—resulted 403 g mol^{-1}. The physical properties were determined by viscometry: the intrinsic viscosity, [η], of CTL was measured at $T = 25$ °C by means of a CT 1150 Schott Geräte automatic measuring apparatus and a Schott capillary viscometer. A buffer solution composed by 20×10^{-3} M AcOH/AcNa, pH 4.5, and 100×10^{-3} M NaCl was used as solvent.[34] The resulting [η] was 344 mL g^{-1}. Given the molar mass of chitosan used in the synthesis process (325 000 g mol^{-1}) and its repeating unit (168 g mol^{-1}), the molar mass of CTL was estimated to be at around 800 000 g mol^{-1}. Boric acid, sodium chloride, phosphate buffered saline (PBS), mannitol, Triton X-100, AlamarBlue reagent and LDH kit were all purchased from Sigma-Aldrich Chemical Co. (USA). Acetic acid and hydrochloric acid were from Carlo Erba (Italy). Genipin (purity 98%) was from Challenge Bioproducts Co., Ltd. (Taiwan). DMEM (Dulbecco's Modified Eagle's Medium) high glucose, fetal bovine serum (FBS), streptomycin, penicillin and trypsin were from EuroClone (Italy).

Preparation of Dual Cross-Link Gels: CTL solutions were prepared in PBS buffer, pH 7.4. Briefly, CTL (50 – 150 mg) was solubilized in 3.5 mL of deionized water; the pH was then adjusted to 7.4 by adding aliquots of NaOH (1 M). Finally, 400 µL of PBS 10X and deionized water amounts were added to have a final volume of 4 mL. The mixture composed

of boric acid (80×10^{-3} M) and mannitol (160×10^{-3} M) was prepared in PBS 2X buffer, pH 7.4. Genipin (10.4×10^{-3} M) was solubilized in deionized water and diluted to the desired concentration. The final concentration of genipin was selected in order to vary the molar ratio between glucosamine units of CTL (D) and genipin (G), $R_{D/G}$. Mixtures of genipin, boric acid and mannitol were prepared by mixing an equal volume of genipin (water as solvent) and boric acid-mannitol mixture (PBS 2X as solvent), in order to have a final solution of PBS 1X as solvent, pH 7.4.

A typical gel preparation of dual cross-link gels was carried out as follows: 1 mL of genipin-boric acid-mannitol mixture (PBS 1X, pH 7.4) was mixed with 4 mL of CTL solution (PBS 1X, pH 7.4) using Luer-Lock syringe system. The final concentration of genipin resulted in the range $0.13–0.52 \times 10^{-3}$ M, whereas those of boric acid and mannitol 8×10^{-3} and 16×10^{-3} M, respectively.[12] The final concentration of CTL was in the range 1–3% w/v.

Rheological Characterization: Rheological measurements were performed using an HAAKE MARS III rheometer (ThermoScientific) operating in oscillatory shear conditions. The experimental setting used to characterize all samples is the following: titanium plate with 2° cone/plate geometry (Ø = 35 mm) and gap 0.105 mm. Upon addition of genipin-boric acid-mannitol mixture, solutions were mixed to uniform samples and poured atop the plate. Sunflower oil was used to seal the interface between the two plates in order to improve thermal control and limit solvent evaporation. Time sweep experiments were carried out in strain-controlled conditions, with deformation, γ, of 1% kept constant throughout the experiments, frequency, ν, of 1 Hz, t = 3 h and T = 37, 45, 50, 60 °C. The values of storage G' (elastic response) and loss G'' (viscous response) moduli were recorded as a function of time. After time sweep experiments performed at 60 °C, frequency sweep (τ = 1 Pa; ν = 0.01–100 Hz) and stress sweep tests (ν = 1 Hz; 1 Pa < τ < 10 000 Pa) were performed at T = 37 °C.

Cell Culture: Mouse fibroblast-like NIH-3T3 (ATCC CRL-1658) were cultured in Dulbecco's Modified Eagle's Medium High glucose with 0.584 g L^{-1} L-glutamine and 0.11 g L^{-1} sodium pyruvate (EuroClone, Italy), supplemented with 10% v/v heat-inactivated fetal bovine serum (Sigma, USA) and 1% v/v penicillin/streptomycin (EuroClone, Italy), in a humidified atmosphere of 5% CO_2 at T = 37 °C.

Evaluation of Dual Cross-Link Gel Cytotoxicity: Gels for in vitro tests were set up in sterilized conditions. Specifically, CTL powder was UV-sterilized whereas all solutions filtered by means of 0.2 µm pore filters (VWR International). Solutions were also supplemented with 1% penicillin/streptomycin. After mixing, CTL-genipin-boric acid mixtures were poured into 6-well plates and incubated at T = 37 °C for 24 h. Final conditions were: CTL 3% w/v, boric acid 8×10^{-3} M, mannitol 16×10^{-3} M, and genipin 0.52×10^{-3} M. Gels were then cut by means of a disposable 6 mm diameter biopsy punch (Kai Medical, Japan). The height of each resulting gel cylinder resulted ≈4 mm. Cells were plated at a density of 13 500 cells per well using 24-well plates. After adhesion, cell culture medium was discarded and cells rinsed with PBS once. Finally, 2.1 mL of fresh medium were added for each well and gels deposited atop cell layer (V_{medium} /V_{gel} = 19).[35] Triton X-100 (0.1% v/v) was used as positive (death) control, whereas untreated cells were considered as negative control. AlamarBlue as well as Lactate Dehydrogenase (LDH) assay were performed according to manufacturer's protocols after 24 h of incubation. Evaluation of gel cytotoxicity was performed qualitatively and quantitatively according to ISO 10993–5.[32]

Statistical Analysis: Biological results were analyzed by means of a one-way ANOVA (analysis of variance) followed by a Tukey's Multiple Comparison *post hoc* test to evaluate differences among different groups. Differences were considered significant for p values less than 0.05.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceived and designed the experiments: P.S., F.F., E.M., I.D. Performed the experiments: P.S., F.F., A.M., M.C., C.P. Analyzed the data: P.S., F.F., E.M., I.D. Wrote the paper: P.S.

Keywords

adaptable mechanics, bioactive biopolymer, dual cross-link hydrogel, mechanotransduction., tissue engineering

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