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Tunable biohybrid hydrogels from coacervation of hyaluronic acid and PEO-based block copolymers

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Abstract

Accurately tuning the macroscopic properties of biopolymer-based hydrogels remains challenging due to the ill-defined molecular architecture of the natural building blocks. Here, we report a biohybrid coacervate hydrogel, combining the biocompatibility and biodegradability of naturally occurring hyaluronic acid (HA) with the tunability of a synthetic polyethylene oxide (PEO) -based ABA-triblock copolymer. Coacervation of the cationic ammonium or guanidinium-functionalized copolymer A-blocks with the anionic HA leads to hydrogel formation. Both mechanical properties and water content of the self-healing hydrogels can be controlled independently by altering the copolymer structure. By controlling the strength of the interaction between the polymer network and small-molecule cargo, both release rate and maximum release are controlled. Finally, we show that coacervation of HA and the triblock copolymer leads to increased biostability upon exposure to hyaluronidase. We envision that noncovalent crosslinking of HA hydrogels through coacervation is an attractive strategy for the facile synthesis of tunable hydrogels for biomedical applications.

KEYWORDS

biomaterials, block copolymers, coacervates, hyaluronic acid

1 | INTRODUCTION

Hydrogels are one of the most promising materials for biomedical applications[1] with applications ranging from drug delivery[2,3] to cell culture[4] and regenerative medicine.[5] To apply these materials in a clinically relevant setting, the ability to control their properties while retaining biocompatibility and injectability is important. Additionally, facile material synthesis and tunable properties are vital. Despite the many efforts to develop functional hydrogel systems for drug release, only a handful of systems have been commercialized.[6,7] To accelerate the translation of such materials into the clinic, the hydrogels preferably consist of commonly used, FDA-approved materials.[7] Additionally, their preparation should allow for facile and independent tailoring of various properties to meet specific needs.

Hydrogels from biologically derived materials have received ample attention due to their inherent natural biodegradability. In addition, these materials have attractive biocompatibility and carry functional groups that can be used for further modification. Examples of

these materials include crosslinked alginate,[8] chitosan,[9] and other polysacharides.[10] However, the biological origin of these materials is also a major disadvantage, leading to ill-defined materials with poor properties, batch-to-batch variation, and large microscopic heterogeneity.[5]

In contrast, fully synthetic hydrogels, such as physically and covalently crosslinked polyethylene glycol, poly-*N*-isopropyl-acrylamide and polyvinyl alcohol,[8,11,12] allow for great control over material properties,[13–15] high material purity, and limited batch-to-batch variation.[16] Despite the advantage of high control over the material properties, the lack of inherent biodegradability and compatibility has strained the implementation of synthetic hydrogels in biomedical applications.

To address the downsides of synthetic or biopolymer-derived systems, hybrid hydrogel systems have been developed that combine the advantages of biologically derived materials with the tunability of synthetic materials.[17,18] Hyaluronic acid (HA), one of the major components of the extracellular matrix,[19] is a particularly attractive biomolecule to incorporate in a biohybrid scaffold, due to its inherent biocompatibility, biodegradability, and ease of chemical modification. As a result, this FDA-approved material has attracted interest as a suitable material for biomedical applications. Several HA-based systems are presently being applied for clinical use.[19,20]

HA hydrogels are most commonly prepared via covalent crosslinking.[20,21] While robust materials are obtained, this strategy often limits general applicability, because new materials can only be prepared by chemical synthesis. This means, variations of the materials' properties, for example, by variation of crosslinking density, each time require covalent reactions. Since these often require harsh conditions, more benign crosslinking methods are required. Even though new attempts to realize such benign covalent strategies (e.g., by using Michael reactions) have been reported,[22–24] the resulting materials may still suffer from incomplete crosslinking or residual small-molecule by-products. Thus, facile methods that are based on the simple mixing of aqueous solutions of hydrogel building blocks are highly desirable to tune materials' properties. Such strategies can be realized best through noncovalent crosslinking strategies. Conversely, this strategy is not as well explored for the crosslinking of HA, although this method might enable facile and highly modular material synthesis without the need for hash conditions or toxic reagents. Moreover, the use of noncovalent crosslinks to prepare the final material allows for extensive purification of the supramolecular building blocks to obtain the purity required for biomedical applications and no side products are generated during the crosslinking. HA hydrogels have been prepared by grafting host–guest molecules, such as β-cyclodextrin and adamantane[25] and cucurbit[8] urils.[26] Noncovalent crosslinking of HA with ionic interactions has, to the best of our knowledge, only been reported using sodium trimetaphosphate.[27] Therefore, a need to expand the scope of facile noncovalent crosslinking in HA hydrogels has to be addressed.

Here, we introduce an HA-based hydrogel that is crosslinked in a simple, noncovalent fashion by the formation of coacervate complexes[28] between anionic HA and the cationic endblocks of synthetic X-PEO-X triblock copolymers (e.g., ammonium: A-PEO-A; guanidinium G-PEO-G) (Figures 1 and 2). This approach enables us to tune the material properties by precise modification of the synthetic copolymer structure, that is, changing block sizes and chemical nature of the endblocks. In addition, the biobased HA ensures biodegradability.

To demonstrate the modularity and the potential of this approach for applications in the development of biomaterials, we first show that the complex coacervate domains are in charge balance. Second, the dependence of the mechanical properties and water content of the material on the polymer structure of the X-PEO-X triblock copolymer is discussed. In addition, we show that the noncovalent nature of the crosslinks induces self-healing properties. Next, it is shown that the release of small molecules from the gels can be controlled by tuning the strength of the interaction between cargo and polymer network. Lastly, the hydrogels are shown to have improved stability against hyaluronidase, but the material remains biodegradable.

2 | EXPERIMENTAL

2.1 | Materials and synthesis

HA sodium salt (M_w = 400.000–800.000 g·mol^{-1}) was provided by Allergan Inc. All other materials were bought

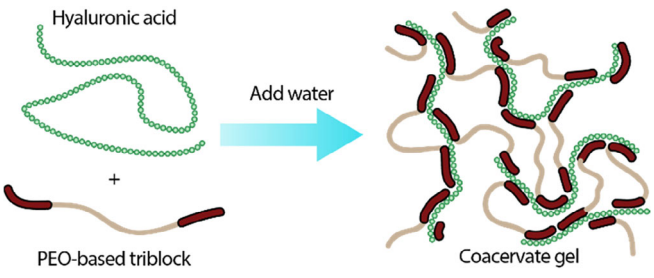

FIGURE 1 Coacervate hydrogels can be prepared by simply dissolving the solid components, hyaluronic acid and the PEO-based triblock copolymer in water [Color figure can be viewed at wileyonlinelibrary.com]

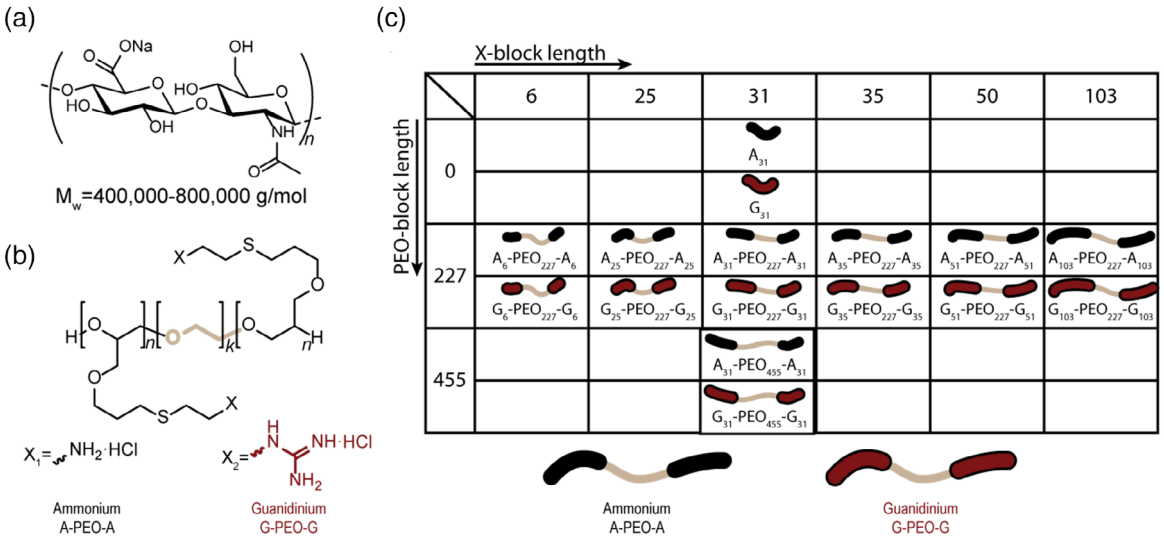

FIGURE 2 The library of polymers used in this study: (a) the chemical structure of hyaluronic acid; (b) the chemical structure of the X-PEO-X copolymer platform, which can be functionalized with different X-groups; (c) different triblock copolymer structures were used to study the properties of the HA/X-PEO-X triblock copolymer coacervate hydrogels. By changing for both X-groups the lengths of both the end X-blocks and the middle PEO-blocks, all system parameters are varied. HA, hyaluronic acid [Color figure can be viewed at wileyonlinelibrary.com]

from Sigma. PEG-based triblock copolymers were synthesized in a multistep procedure and characterized according to an earlier described procedure.[28] Starting from α-ω-hydroxy-PEG of varying molecular weights as a macroinitiator, PAGE-b-PEG-b-PAGE triblock copolymers with varying molecular weights of the PAGE endblocks were synthesized by anionic ring-opening polymerization of allyl glycidyl ether. These polymers were then functionalized through a light-induced thiol-ene click reaction with cysteamine to yield the amino-functionalized polymers. The resulting product was purified by dialysis and lyophilization. The amino-functionalized polymers were converted to guanidinium-functionalized polymers using 1*H*-pyrazole-1-carboxamidine hydrochloride, as reported earlier.[29] Dialysis against deionized water afforded the guanidinium-functionalized polymers. Additional details can be found in the Supporting Information.

2.2 | Mechanical characterization

Rheological measurements were carried out on a TA ARES-LS1 rheometer with a 25 mm parallel plate geometry with a gap of 0.500 mm at ambient temperature. Gels were prepared in two syringes connected via a Luer connector. In the preparation, the water and solid materials were thoroughly mixed by pushing the material back and forth between the syringes. All samples, contained in the syringes, were centrifuged to remove any air bubbles before being applied to the rheometer. After application of the gel to the rheometer, any excess gel was removed, such that the gel measured had a diameter of 25 mm and height of 0.5 mm. In a typical rheology experiment, gels of the desired concentrations were subject to a dynamic frequency sweep of $f = 0.005–80$ Hz and $\gamma = 2\%$. Measurements were performed in duplicate or triplicate.

Strain recovery measurements were done by carrying out a dynamic time sweep (5 Hz, 2% strain, 30 min), after which the gels were immediately subject to periods of increasing deformations (25–50–100% strain, 2 Hz, 5 min). After each period of deformation, the recovery of the moduli was monitored with an additional dynamic time sweep (5 Hz, 2% strain, 30 min).

2.3 | Biodegradablity characterization

To investigate the biodegrabalitiy of the gels, gels were prepared in a syringe and diluted with hyaluronidase solution to yield 15 wt% gels. These materials were then prepared for rheological measurements by centrifuging as described previously. The samples were kept on ice to prevent the rapid enzymatic degradation before being applied to the rheometer.

2.4 | Determination of equilibrium water content

HA (25 mg) and the amount of X-PEO-X triblock copolymer that is required for charge balance were dissolved in

1 mL MilliQ in two syringes that were connected with a Luer connector. The gels were mixed several times per day by pushing the plunger back and forth multiple times. After 3 days, the weight of the wet gels was determined and the samples were lyophilized, after which the weight of the remaining solid material was determined. From this, the water content of the swollen gel was calculated.

2.5 | Small-molecule release

Charge-balanced gels (15 wt% solid in MilliQ) were prepared by dissolving HA, PEO-based triblock copolymer and dye in MilliQ and mixed several times in the connected syringe setup. The gels (800 mg) were placed in a small glass cup and gently centrifuged to give an equally spread out gel film. The glass cups were placed in a glass vial, which contained an inset to secure the cup (See Figure S5). PBS (75 mL, 37°C) was gently poured on top of the gel. Then, the samples were placed in a shaking incubator (New Brunswick Scientific Innova 44) and mildly agitated (25 rpm, 2″ stroke) at 37°C. Aliquots (150 μl) were taken at various time intervals. The aliquots were transferred to a 96 well plate and directly measured with a Tecan M220 Infinite Pro plate reader. Using a calibration curve, the absorbance was converted to release.

3 | RESULTS AND DISCUSSION

To obtain stable complex coacervate domains, the material should be in charge balance, that is, a 1:1 molar ratio between the anionic carboxylate groups on HA and the cationic groups on the X-PEO-X endblocks is required for coacervation.[30,31] To investigate the influence of charge stoichiometry on gel formation of the HA/X-PEO-X system, mixtures of two polymers with varying weight ratios were prepared. For this, HA and an X-PEO-X copolymer with guanidinium-functionalized endblocks (G_{25}-PEO_{227}-G_{25}) were dissolved in excess water (3 wt% total polymer concentration). Three samples were prepared at HA/G_{25}-PEO_{227}-G_{25} weight ratios of 1:0.8 (excess of HA), 1:1.18 (charge balance), and 1:1.4 (excess of G_{25}-PEO_{227}-G_{25}). In all gel forming mixtures, a liquid phase was expelled and a hydrogel phase could be observed (Figure 3a). ^{1}H-NMR spectroscopic investigations (Figure 3b) of the expelled aqueous solution indicated that for the case with excess HA, only HA is expelled to the solution phase and, conversely, for the case with excess G_{25}-PEO_{227}-G_{25}, exclusively PEO-based triblock is expelled. For the charge-balanced sample, only trace amounts of either polymer could be detected in the supernatant. These results strongly suggest that excess charges are not incorporated in the coacervate domains, indicating that charge balance is required. The ability of the gel phase to self-regulate their stoichiometry, even in the presence of excess charges, demonstrates the robust character of this crosslinking method. Additionally, the expulsion of excess water in the charge-balanced sample shows that the gel phases equilibrate to a minimum polymer content when formed from dilute solutions.

To prove the existence of a crosslinked hydrogel network, rheological measurements were performed on the HA/G_{25}-PEO_{227}-G_{25} gel. The frequency sweep of the HA/G_{25}-PEO_{225}-G_{25} gel shows a crossover frequency, f_{cross}, where the storage modulus, G', equals the loss modules, G'' (Figure 4), thus suggesting viscoelastic behavior due to physically crosslinked polymer network. Consequently, the preparation of charge-balanced solutions of HA/X-PEO-X provides a facile and robust method to prepare hydrogels.

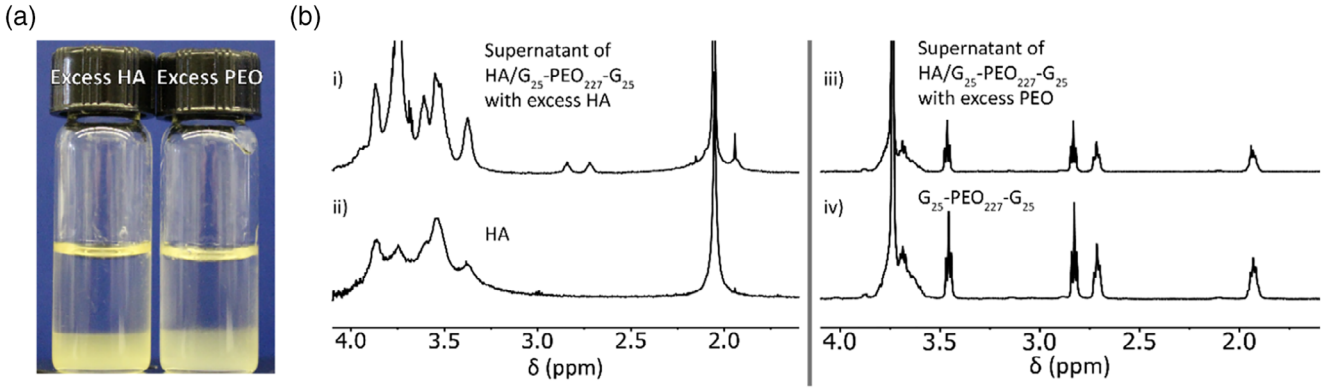

FIGURE 3 HA/X-PEO-X hydrogels form at charge balance and regulate their water content: (a) upon preparation of hydrogels in dilute solutions, a separation between hydrogel and supernatant is observed; (b) 1H-NMR spectroscopy indicated that gels that were prepared with excess HA (i) only expel HA (compare with spectrum ii). Gels prepared with excess triblock copolymer (iii) exclusively expel the triblock copolymer (compare with spectrum iv) [Color figure can be viewed at wileyonlinelibrary.com]

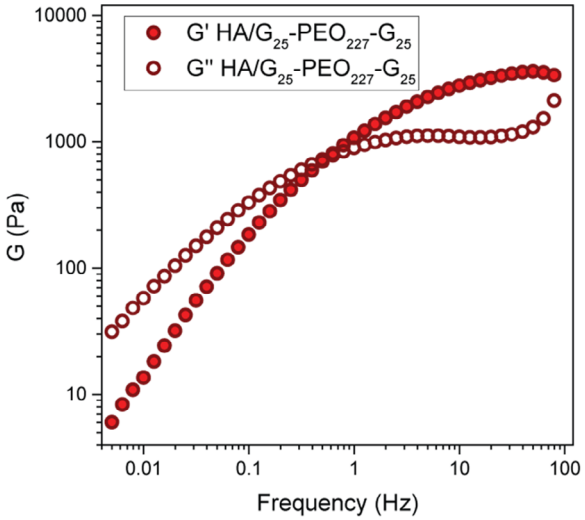

FIGURE 4 Frequency sweep for 15 wt% HA/G25-PEO227-G25 coacervate hydrogels. The crossover frequency, fcross, at 0.55 Hz confirms the viscoelastic nature of the material [Color figure can be viewed at wileyonlinelibrary.com]

It is suggested that the noncovalent crosslinks enable the hydrogels to relax stress by reorganizing the network when stresses are applied at a low rate. Thus, at low deformation rates below the crossover frequency, the material behaves like a viscous liquid, indicated by a loss modulus that is higher than the storage modulus. Conversely, when the stress is applied at a high rate, the supramolecular crosslinks cannot reorganize and the material behaves as an elastic solid, similar to the behavior observed in covalently crosslinked hydrogels.

After demonstrating the viscoelastic properties, we proceeded by studying the tunability of the mechanical properties of the HA/X-PEO-X hydrogels. The ability to control the hydrogel's mechanical properties is important for tailoring a material to a specific application. For covalently crosslinked materials, this can typically be done by varying the crosslinking density: a higher intermolecular crosslinking density leads to an increased modulus.[13] In our approach, alteration of the crosslinking density by changing the feed ratio of the X-PEO-X crosslinker is hindered by the observed stoichiometric self-regulation of the HA-PEO hydrogels. However, our modular approach allows an alternative strategy to control the mechanical properties of the material. This is based on varying the molecular structure of the X-PEO-X triblock copolymer, that is, changing the respective block lengths and nature of the ionic group.

To demonstrate this modularity, charge-balanced HA-PEO hydrogels were prepared with the various synthesized X-PEO-X triblock copolymers. We specifically prepared hydrogels at a fixed total polymer content to prevent any deviating artifacts. Such deviations might arise from changes in overall polymer concentration upon changing the structure of the X-PEO-X block copolymer. For example, increasing the PEG midblock increases the M_w of the X-PEG-X triblock. If charge balance with a fixed HA content is ensured, the overall polymer mass in a hydrogel sample would increase with the PEG midblock. To circumvent this effect, the total polymer concentration in all gels is held constant. With this, we can determine the sole influence of the polymer structure on the mechanical properties of the hydrogel. To test the mechanical properties, frequency sweeps were performed on all samples at a total polymer content of 15 wt % ($c_{HA} + c_{X\text{-}PEO\text{-}X} = 15$ wt%). To compare and achieve quantitative insights into the mechanical properties of the material, a common parameter needs to be identified that is relatively insensitive to shifts in the spectra upon change the copolymer structure. For this, we used the value of the storage modulus G' at 5 Hz. At this frequency, all investigated materials behave like viscoelastic solids and G' is relatively weakly dependent on the frequency (Figure 4). Thus, possible shifts of the mechanical spectrum upon variation of the copolymer structure are minimized.

To investigate the extent to which the HA/X-PEO-X hydrogels have tunable mechanical properties, we first investigated the influence of changing the endblock length on both the mechanical properties and the hydrogel composition (Figure 5). For this, the following structural parameters were varied in the BCPs: First, the endblock length for ammonium (A) and guanidinium (G) functionalized X-PEO-X polymers was varied for a fixed PEO_{227} midblock (Figure 5a). We observe that increasing the length of the endblocks increases G' for both the A-PEO-A and G-PEO-G series with a PEO_{227} midblock. Interestingly, the more basic G-PEO-G copolymers do not show increased mechanical stability with respect to the A-PEO-A copolymers, suggesting that despite their increased basicity, the ionic crosslinks are, however, weaker. We speculate this effect is caused by the loosely organized solvent mantle that solvates the guanidinium residues and decreases the binding energy.[32] Additionally, by increasing the X-PEO-X endblock length at a constant overall polymer concentration (15 wt%), the HA:X-PEO-X mass ratio shifts to higher values, indicating an increase in HA concentration at high X-block lengths (Figure 5b,c). Concomitantly, the concentration of the PEO midblock decreases. To investigate whether the change in mechanical properties is due to a change in the stabilizing interactions in the coacervate crosslinks or a change in hydrogel composition, several additional experiments were performed.

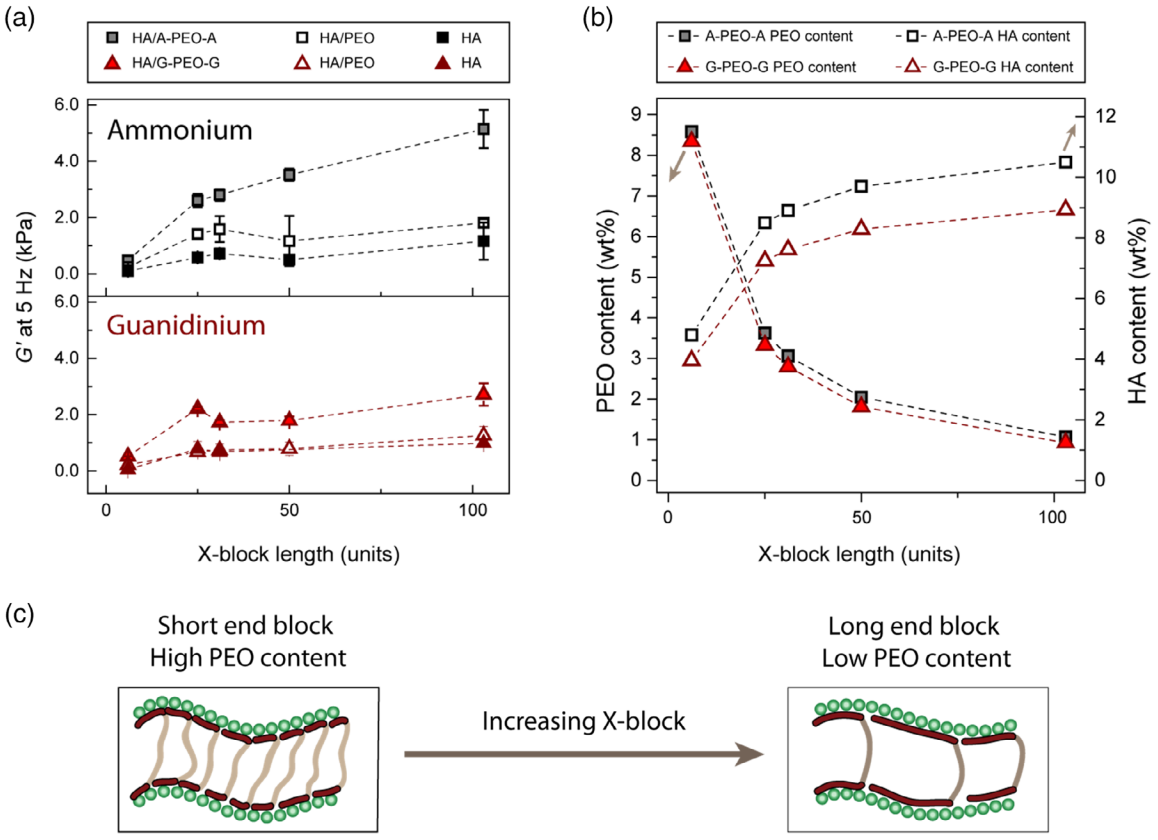

FIGURE 5 The mechanical properties of 15 wt% HA/X-PEO-X hydrogel are dependent on the end-block length. (a) Increasing the X-block length at a constant PEO-midblock length of 227 units leads to an increase in G' at 5 Hz. Control experiments with equal compositions of HA and PEO homopolymer and equal concentrations of HA confirm that the coacervate domains contribute to the mechanical integrity; (b) an increase in the X-block length leads to more charges per triblock copolymer and hence to a higher amount of HA and a lower amount of PEO midblock in the hydrogel; (c) cartoon representation of the effect of increasing the endblock length [Color figure can be viewed at wileyonlinelibrary.com]

To investigate the contribution of chain entanglements of the high-molecular weight HA to the mechanical properties of the HA/X-PEO-X hydrogels, control experiments were conducted on pure HA solutions with an equal concentration of HA as in the hydrogels and on solutions of HA with pristine PEO in the same concentrations as the corresponding hydrogels (Figure 5a). By comparing both control experiments with the HA/X-PEO-X hydrogels, a clear stabilizing effect of the coacervate crosslinks can be observed, leading to a significantly higher mechanical stability. Additionally, because the highly hydrated PEO midblocks bridge the less hydrated coacervate domains,[33] these PEO blocks will introduce flexibility to the polymer network. As a result, an increase in X-block length and concomitant decrease in PEO content (Figure 5b) will reduce the flexibility in the polymer network, leading to an additional contribution to increase in G' at higher X-block lengths.

Similar considerations are valid for the samples with varied PEO midblock lengths and fixed endblocks (X_{31}-PEO-X_{31}) (Figure 6): An increase in PEO midblock length corresponds to a decrease in HA content and an increase in the flexible PEO bridges, leading to a decrease in G'. This is illustrated by the fact that the highest moduli for both ammonium and guanidinium endblocks are obtained for HA gels crosslinked by the respective homopolymers.

In summary, we observe that the gel stiffness (G') increases with HA content and decreases with increasing PEO content. This can be tuned by varying the endblock length: an increasing M_w of X corresponds to more HA and less PEO. Therefore, the gels get stiffer, that is, have higher storage moduli. In contrast, increasing the midblock length results in more PEO and less HA; therefore, the gels get softer, that is, have lower storage moduli G'. This demonstrates good control over the mechanical properties by tuning of the synthetic part of the hybrid hydrogels.

In addition, the mechanical properties are expected to show self-healing behavior, which is of particular interest in the design of injectable materials. Since the crosslinking coacervate domains of the HA/X-PEO-X gels

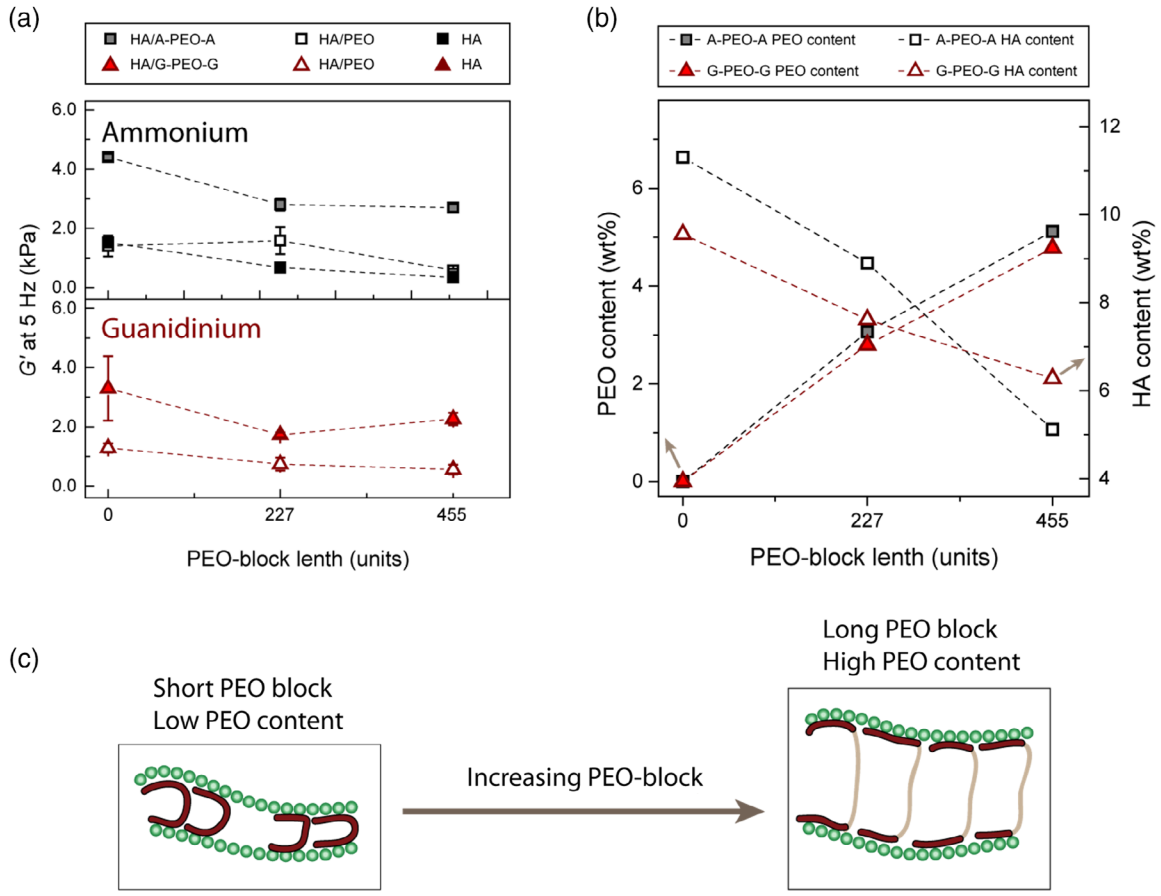

FIGURE 6 Increasing the PEO midblock length for fixed endblocks (X_{31}-PEO-$X3_1$) at a fixed total wt% hydrogel material of 15 wt% leads to (a) a decrease in mechanical stability, (b) a decrease in HA content, and an increase in the hydrogel; (c) cartoon representation of the increase in PEO content as the PEO endblock length is increased [Color figure can be viewed at wileyonlinelibrary.com]

consist of dynamic, noncovalent interactions, these materials reform their coacervate domains after externally induced breakage. The fact that the hydrogels are prepared in and extruded from syringes for the rheology measurements already gives a first indication that the coacervate hydrogel is self-healing. To further study the HA/X-PEO-X material's ability to regain mechanical integrity after rupture, an HA/G_{31}-PEO_{227}-G_{31} gel was subject to dynamic time sweeps in which increasingly larger oscillatory strains were applied.[34,35] Figure 7 shows that after the period of high strain, which caused a considerable drop in G', the modulus recovers almost instantly for strains of 25%, 50%, and 100%. This strongly supports the hypothesis that the coacervate HA/X-PEO-X hydrogels are self-healing, thus highlighting their potential for use in injectable materials that require mechanical integrity *in vivo*.

Besides mechanical properties, the equilibrium water content and minimal polymer concentration are other

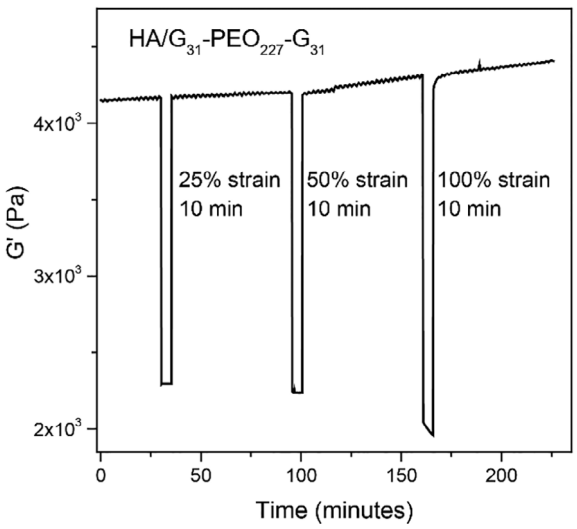

FIGURE 7 The coacervate hydrogels are self-healing. Upon increasingly large deformations the material recovers its mechanical integrity almost instantaneously

important parameters that determine the suitability of hydrogels in biomedical applications. However, for the coacervate hydrogels, the polymer network self-regulates its water content (*vide supra*). Hence, tuning the water content by the concentration of the feed solutions offers only limited tunability. Since the coacervate domains are characterized by a fixed water content,[33] it is hypothesized that it is the hydration of the PEO domains which governs the ability of the biohybrid gels to swell and controls their equilibrium water content. Therefore, changing the amount of PEO in the gel by changing the polymer structure, might allow control over the water content of the gel.

To test this, the equilibrium water content was determined for selected HA/X-PEO-X hydrogels with varying block copolymer structures. Several hours after preparing the hydrogels with excess water, a stable two-phase system was observed. To ensure that the material is also equilibrated at the microscopic level, the gels were mixed several times per day over the course of 3 days, after which the equilibrium water content was determined. In agreement with our assumption, we found that for increasing X-endblock lengths, that is, decreasing PEO contents, the equilibrium water content decreases for both the ammonium and the guanidinium functionalized triblocks, as depicted in Figure 8a. In addition, Figure 8b demonstrates that the equilibrium water content increases with the length of the PEO midblock if the endblocks are constant. As such, the equilibrium water content of the gel can be controlled by controlling the PEO content of the gels (Figure S4). As such, these results suggest that the hydration of the PEO governs the equilibrium water content of the polymer network.

Having shown that the physical properties of the hydrogel can be controlled, we were interested to test the potential of our materials for biomedical applications. Here, we focused on hydrogels as reservoirs for therapeutically active compounds that can be released over prolonged periods from the networks. However, the release of small molecules from un-functionalized hydrogels often shows "burst" release behavior, that is, a fast release of the majority of embedded compounds. In contrast, introducing physical interactions such as electrostatics between drugs and hydrogels networks enables sustained release profiles.[36]

Since the coacervate hydrogel integrity relies on ionic interactions, it is envisioned that the HA/X-PEO-X gels can exploit this interaction for the sustained delivery of charged drugs. In these hydrogels, the charged cargo can be incorporated into the coacervate domains through electrostatic interactions with either HA for cationic drugs or for the copolymer cationic X-groups for anionic drugs. The gel can be kept in charge balance in the presence of the ionic cargo by replacing a fraction of the charged polymer with the cargo. It is hypothesized that thereby the functional payload can be incorporated into the scaffold and be an integral part of the polymer network. This way, the release may, additionally, promote the material's degradation and clearance of the residual gel matrix.

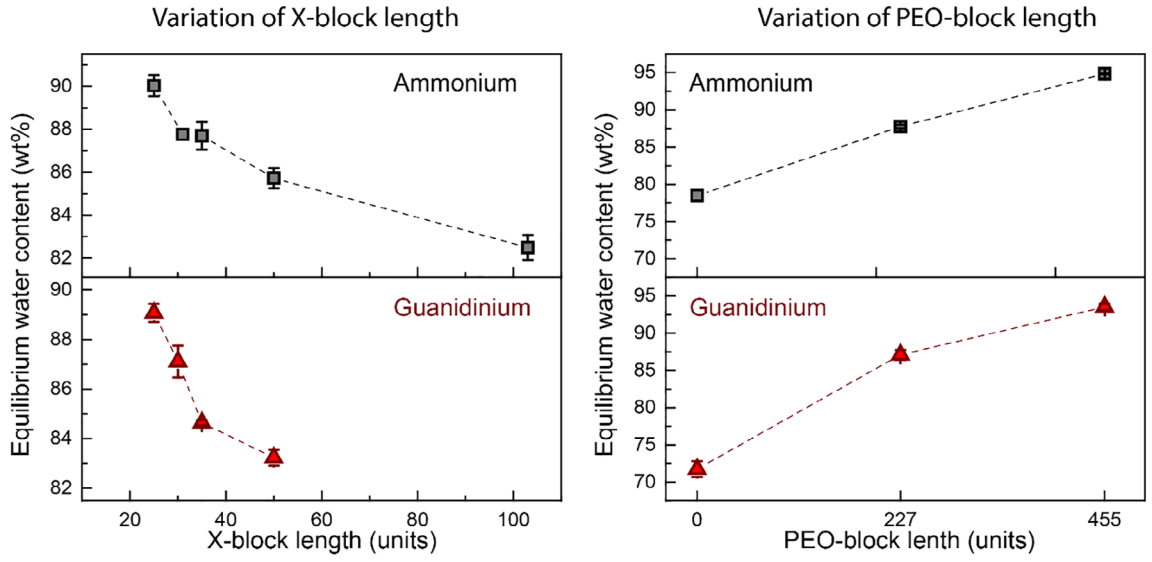

FIGURE 8 The equilibrium water content of HA/X-PEO-X hydrogels can be tuned by changing the copolymer structure, which governs the content of water rich PEO domains in the polymer network; (a) increasing the X-block size, leading to lower PEO content, leads to lower water content; (b) increasing the midblock length, which increases the PEO content of the polymer network, leads for both X-groups to an increase in water content [Color figure can be viewed at wileyonlinelibrary.com]

To study the release of charged small molecules, HA/G_{35}-PEO_{227}-G_{35} (15 wt%) gels were loaded with charged dyes (1 wt% w.r.t. total polymer content) while maintaining charge balance. Anionic fluorescein and cationic methylene blue were investigated as model compounds for charged drugs. The loaded gels were incubated in excess PBS buffer at 37°C and the cumulative release of the respective dye was measured in the supernatant. The resulting release profiles are shown in Figure 9. Interestingly, no reshaping or degradation of the gel material during the release was observed.

For fluorescein, the release reaches 30% after approximately 10 days. This release rate is comparable to the rate reported for host–guest-mediated release from β-cyclodextrin functionalized HA gels, thus suggesting strong interactions between cargo and network.[37] The cumulative release rate is independent of the initial loading of the gels, which suggests that the release is determined by the equilibrium constant between released and complexed fluorescein.

Conversely, for methylene blue, the release is less sustained. The respective hydrogels release approximately 50–90% of their cargo within 24 hr. Also, the cumulative release of methylene blue is dependent on the loading, with higher loadings leading to lower relative amounts. This suggests that, as the methylene blue is released, the resulting anionic charges in the polymer network increasingly stabilize the remaining methylene blue cargo. This stabilization leads to a decreasing amount of additional absolute release when the amount of loaded cargo in the gel is increased. As a result, a decrease in the cumulative relative release from the hydrogel is observed. It is assumed that an increasing stabilization of the cargo correlates with an increasingly negatively charged network upon release. A resulting multivalent effect of the resulting free anionic carboxylate groups can efficiently stabilize the delocalized positive charge of the methylene blue cargo. It is assumed that this effect also contributes to the observed gel stability upon release, that is, the absence of macroscopic gel degradation or dissolution.

Having demonstrated the ability to load ionic dyes into the coacervate domains, we assumed that tuning the strength of the respective ionic interaction between cargo and the X-PEO-X triblock copolymer might allow for control over the release profile. Here, it is assumed that a higher pK_a difference between the ionic groups in polymer and cargo results in a stronger ionic interaction and thus in a slower release profile. To test this, the pK_a of the ionic groups in the polymeric scaffold and in the cargo were varied in two experiments. First, the release of the sulfonate dye Orange II from HA/G_{35}-PEO_{227}-G_{35} gels was measured. It is proposed that the lower pK_a of the sulfonate moiety, which is between 2 and 3,[37] as compared to the fluorescein pK_a of 4.2,[38] will lead to stronger interactions with the cationic polymer, resulting in a lower release. The release profile of Orange II from HA/G_{35}-PEO_{227}-G_{35} gels, shows a maximum release of 10% (Figure 10a). After the burst release in the first 24 hr, further sustained release is minimal. This is in stark contrast to the sustained release (over 10 days) of fluorescein. This highlights the stronger interaction between the guanidinium groups of the polymer and the sulfonate groups in Orange II than with the carboxylate groups in fluorescein.

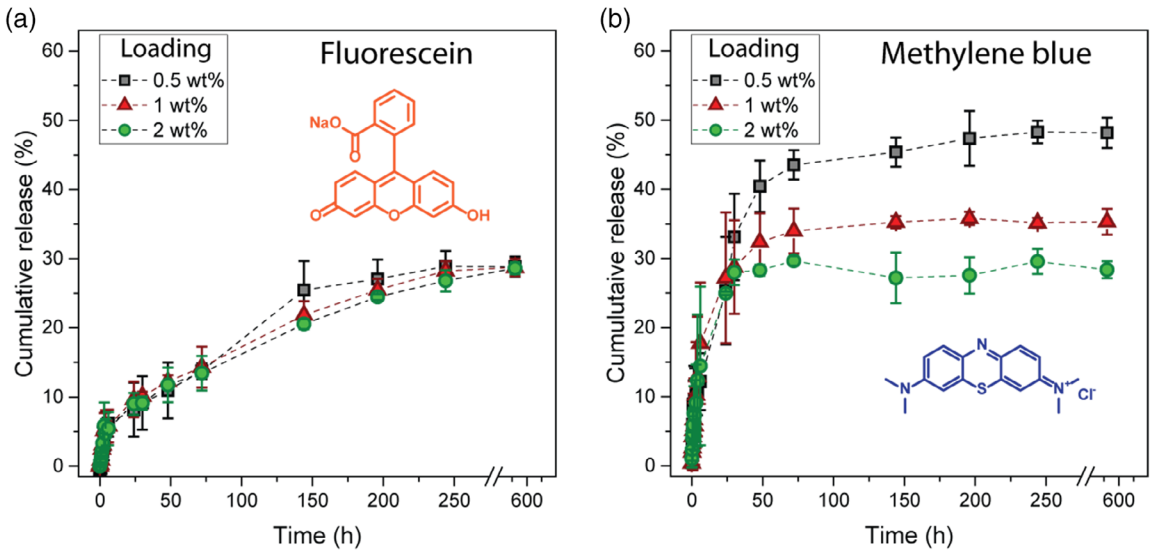

FIGURE 9 HA/G_{35}-PEO_{227}-G_{35} show sustained release of both anionic and cationic cargo for various cargo loadings, ranging from 0.5 wt% to 2 wt%; (a) anionic fluorescein is released up to a maximum of 30%, independent of the gel loading; (b) higher loading of cationic methylene blue leads to lower relative release [Color figure can be viewed at wileyonlinelibrary.com]

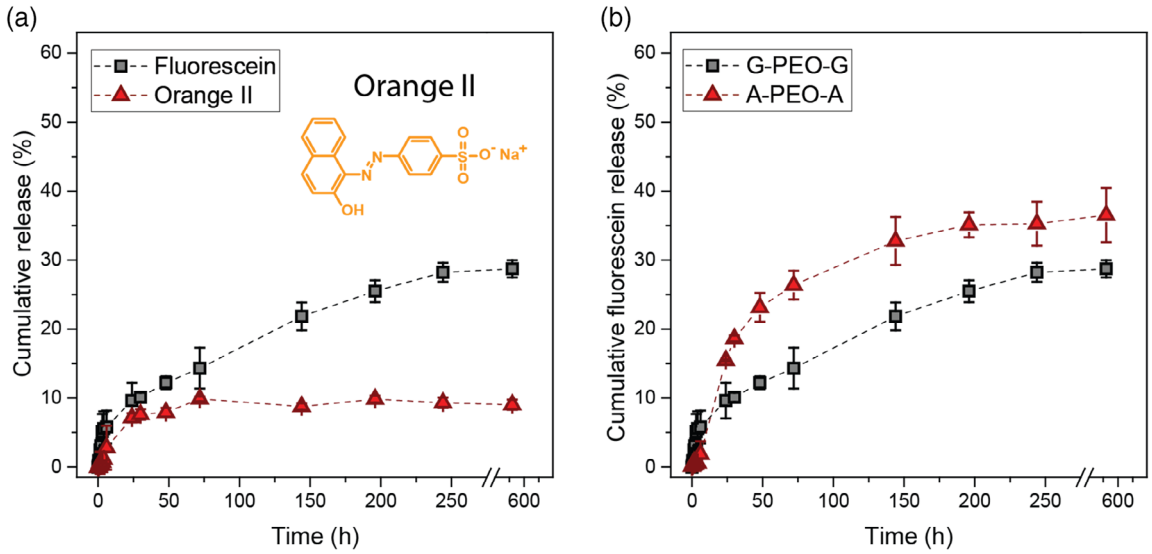

FIGURE 10 The release of ionic small molecules from HA/X-PEO-X hydrogels can be controlled by tuning the strength of the interaction between the polymer network and the small molecule; (a) the sulfonate dye Orange II is released at lower rate and percentage than fluorescein; (b) fluorescein is released faster and to a larger extent from HA/A_{35}-PEO_{227}-A_0 gels than from G_{35}-PEO_{227}-G_{35} [Color figure can be viewed at wileyonlinelibrary.com]

Second, the release of the fluorescein from HA/G_{35}-PEO_{227}-G_{35} was compared with the release from HA/A_{35}-PEO_{227}-A_{35}. Here, the ammonium groups, with typical pK_a values between 10 and 11, have a lower pK_a than the guanidinium groups, which have pK_a values between 13 and 14. This is expected to cause a weaker interaction (lower difference in pK_a values) with the carboxylate groups of the fluorescein cargo, thus leading to higher relative release. The results of these experiments are given in Figure 10b. HA/A_{35}-PEO_{227}-A_{35} gels loaded with fluorescein reach a maximum release of 35% after 200 hr. Both the release rate and maximum release are higher for the HA/A_{35}-PEO_{227}-A_{35} gels than for the HA/G_{35}-PEO_{227}-G_{35} gels, thus supporting the assumption that a lower pK_a difference can be used to increase the release. From these experiments, it becomes obvious that the release profile can be tailored by controlling the interaction between the cargo and the polymeric scaffold. We expect that the modularity of the X-PEO-X platform can be exploited to achieve comparable release profiles for different cargo molecules.

In a last series of experiments, the biodegradability of HA/X-PEO-X hydrogels was investigated. Biodegradability is highly desired for materials that are temporarily implemented in the body. To facilitate clearing of the material from the body, it is desirable that the material loses its mechanical integrity in vivo, yet remains its structure long enough to fulfill its function. HA, as one of the principal components of the coacervate hydrogel, is readily degraded by hyaluronidases into monomeric saccharides in vivo.[39] Hence, it is expected that the HA/X-PEO-X gels will also be degraded upon exposure to hyaluronidase. However, we hypothesize that the coacervation of HA with X-PEO-X will decrease the availability of the HA-backbone to the enzyme and hence decrease the rate of degradation when compared to pristine HA.

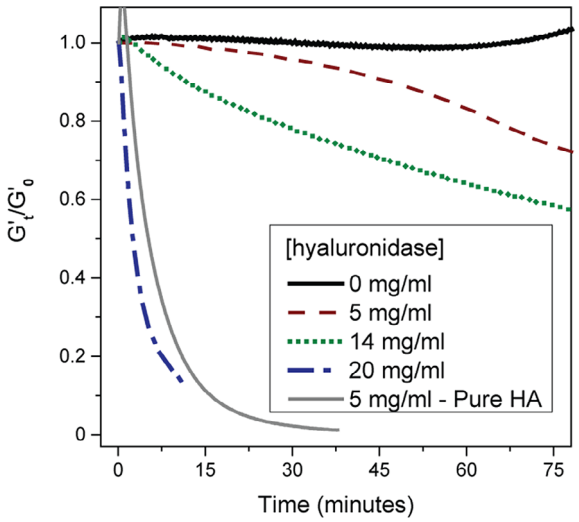

FIGURE 11 HA/G_{31}-PEO_{227}-G_{31} gels show increased stability when exposed to hyaluronidase when compared to pristine HA. HA, hyaluronic acid [Color figure can be viewed at wileyonlinelibrary.com]

To test this hypothesis, HA/G_{31}-PEO_{227}-G_{31} hydrogels with various amounts of incorporated hyaluronidase were prepared. Subsequently, the mechanical integrity was monitored by rheology. Figure 11 shows the normalized time-dependency of the storage modulus of HA, and

HA/G_{31}-PEO_{227}-G_{31} gels with various enzyme concentrations. Pure HA degrades very rapidly at low hyaluronidase concentrations. The HA/G_{31}-PEO_{227}-G_{31} gels, however, show a much lower relative decrease in G' for similar enzyme concentrations. The required enzyme concentration to achieve a similar rate of degradation is significantly higher. This is evidenced by the sample containing 20 mg/mL hyaluronidase, which has a decay rate similar to the sample of pure HA containing 5 mg/mL. These results suggest that the rapid degeneration of HA *in vivo*[40] can be slowed down by complexing the polymer in a coacervate domain. Due to the improved stability of the hydrogels, lower levels of free polyelectrolyte will be present *in vivo*, alleviating possible toxic side effects of the polycations.[41] As such, the HA/X-PEO-X platform proves to be an interesting candidate for further development into a modular, tunable drug release platform.

4 | CONCLUSIONS

To address the need for a versatile noncovalent crosslinking strategy to prepare tunable HA-based hydrogels, a versatile coacervation approach was developed. This mild, facile and scalable crosslinking method is based on the ionic interactions between the natural biopolymer of anionic HA and the cationic X-end groups of synthetic X-PEO-X triblock copolymers. It was demonstrated that using well-defined synthetic block copolymers as crosslinkers allows control over the mechanical properties and equilibrium water content of the gels. Precise tuning of these hydrogel properties can be achieved by varying the block copolymer structure (X- and PEO-block lengths). Due to the ionic nature of the crosslinks, the hydrogels are self-healing, as confirmed by the very rapid recovery after repeated large deformations. In addition to the ability to precisely adjust their physical properties, it is shown that the modularity of the X-PEO-X triblock copolymer platform allows for tunable and sustained release of ionic cargo from the gel. Controlling the strength of the interaction between cargo and scaffold, by changing the nature of the charged moiety on the polymer, allows for the tuning of the release properties of the material. HA/XPEO-X coacervate gels have shown to be biodegradable but have prolonged lifetimes when compared to pristine HA. Therefore, these materials are excellent candidates for applications that require a prolonged lifetime in vivo.

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