Mechanically Tailored Agarose Hydrogels through Molecular Alloying with β-Sheet Polysaccharides

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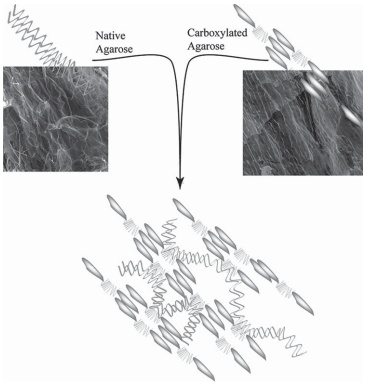
There is mounting evidence that the mechanical property of tissues provides important cues that control cell fate. However, implementation of hydrogels with tunable physicochemical properties is limited due to the challenges associated with crosslinking chemistries. It has been recently shown that mechanically well-defined injectable polysaccharide hydrogels can be engineered by switching their secondary structure from an α-helix to a β-sheet. Based on these findings, a new concept is presented to tailor the mechanical properties of agarose hydrogels via the blending with the β-sheet-rich carboxylated derivative. Using this simple strategy, gels with predictable roughness, fiber organization, and shear modulus ranging from 0.1 to 100 kPa can be formulated. Hydrogels whose mechanical properties can be precisely tailored in vivo without the recourse for chemical reactions are expected to play an important role in implementing mechanobiology paradigms in *de novo* tissue engineering.



1. Introduction

In tissue regeneration, several variables play a role in controlling the fate and evolution of tissue. Numerous studies to date have demonstrated that in addition to soluble growth factors, the physical attributes of the extracellular matrix (ECM) such as stiffness and roughness offer cues that can trigger and/or modulate biological signaling.[1,2] In particular, changes to ECM stiffness has been found to accompany several pathological changes in tissue.[3] Mechanobiology, the interplay between the mechanics of the cell environment and cell biology, offers a new paradigm to affect regeneration. In order to understand this complex interplay between ECM stiffness and biology in the mechanobiology paradigm, there is a need for an easily implementable investigation platform that, in addition to being compatible with traditional cell culture techniques, offers a simple means to realize mechanically well-defined environments. In order to make mechanobiology paradigms clinically applicable, the system needs to possess excellent biocompatibility and be compatible with minimally invasive surgical techniques currently being employed.

Among biopolymers, polysaccharides that are sourced from plants (cellulose), bacteria (gum, xanthan), or

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marine vegetation (agarose, alginate, carrageenan family) have found widespread use in biomedical, cosmeceutical, and food technologies.[4] Polysaccharides are also a major component of the ECM where they play an important role in maintaining the osmotic activity in tissues and sequestration of soluble signals. Due to the diversity in the chemical structure of the sugar monomer repeat units, polysaccharides exhibit many interesting properties including formation of hydrogels. Hydrogels are a tridimensional network of polymer chains that is capable of holding water, and are of particular relevance for tissue regeneration, as they mimic the hydration state of tissues. Agarose, a polysaccharide extracted from marine algae yields strong, porous hydrogels that can be formed under physiological conditions.[5] Due to its cytocompatibility, non-fouling and non-immunogenic attributes, agarose has been exploited as an injectable 3D dermal tissue filler, cell carrier, and as a matrix for de novo cartilage engineering.[6–8] In addition to these benefits, we have recently shown that through controlled carboxylation of the polysaccharide backbone, an α-helix to β-sheet switch in secondary structure can be induced, leading to control over mechanical and physical characteristics of agarose hydrogels. In this system, the evolution of the new β-sheet structure impacts the associative behavior of polysaccharide chains, thereby altering the physicochemical properties of the ensuing gels such as shear modulus and gelation.[9] However, to foster a broad usage of these biomaterials in routine research and drive clinical translation, a simpler way to control gel mechanical properties without resorting to extensive chemical modification needs to be developed.

The blending of polysaccharides has been employed primarily to improve cell–material interactions and cell delivery. For example, it has been shown that agarose/chitosan blend supports tri-dimensional neural growth,[10] and more recently, agarose blended with alginate[11] has been shown to provide a favorable environment for the transplantation of articular chondrocytes.[12] However, blending as a strategy to affect physicochemical properties of hydrogels has not been explored to date.

In metal alloying, the introduction of a secondary metal atom or an atom capable of occupying an interstitial space in the atomic lattice of the primary metal can induce phase transformations and hinder the mobility of atoms within the lattice. With regards to organization of polysaccharide chains, past studies have shown that helical polysaccharides can impact the organization of unstructured (random coil) polysaccharide,[13] and it is well documented that glucomannan, a random polysaccharide can experience cooperative effects in presence of xanthan, which is an α-helical polysaccharide.[14–16] In this context, our recent finding that the mechanical properties of polysaccharide hydrogels can be varied through the induction of a new secondary structure provides a platform to explore concepts of alloying in polymer systems.

Based on the aforementioned considerations, we theorize that the introduction of a β-sheet-rich polysaccharide into an α-helix dominated environment might impact the formation of crosslinks by promoting new associative interactions between the secondary structures and thus impart new physicochemical properties in native agarose. In order to explore this premise, in this study, we demonstrate that the blending of highly carboxylated agarose (CA) with native agarose (NA) is capable of recapitulating much of the diversity in mechanical properties that is achieved through controlled carboxylation of the backbone modification. Furthermore, by comparing the mechanical and structural properties of hydrogels derived from the physical blending of NA/CA with those derived from controlled carboxylation of NA, we propose a model of molecular alloying for controlling the mechanical properties, wherein the interpenetration of polymer strands bearing β-sheet-rich domains with strands predominantly composed of α-helical domains results in the formation of gel points that involve helix–sheet interactions. This paradigm of molecular alloying of secondary structures offers a simple way of establishing mechanically well defined and diverse environments, which is a step in realizing translatable, injectable platforms for implementing mechanobiology paradigms in regenerative medicine.

2. Experimental Section

2.1. Materials

Agarose (type I low melting) was obtained from Merck (Germany), TEMPO ((2,2,6,6-tetramethylpiperidin-1-yl) oxyl), NaOCl (sodium hypochlorite), $NaBH_4$ (sodium borohydrate), NaBr (sodium bromide), and KBr (potassium bromide) were obtained from Sigma–Aldrich (Germany) and used as received. Dialysis membranes with a molecular weight cut off (MWCO) of 12–14 kDa (Spectrum Laboratories, USA) were purchased from Carl Roth (Germany).

2.2. Methods

2.2.1. Oxidation of α-Helical Native Agarose

One gram of NA was transferred into a three-necked round bottom flask, equipped with a mechanical stirrer and pH meter and was dissolved in water at a concentration of 1% w/v by heating to 90 °C. The reactor was then cooled down to 0 °C under vigorous mechanical stirring in order to prevent gelation of agarose and was charged with TEMPO (0.160 mmol, 20.6 mg), NaBr (0.9 mmol, 0.1 g), and NaOCl (2.5 mL, 15% solution). The pH of the solution was then adjusted to 10.8 and maintained throughout

the duration of the reaction. The degree of carboxylation was controlled by the dropwise addition of different volumes of NaOH solution (0.1 M). The reaction was quenched by the addition of $NaBH_4$ (0.1 g) following which the solution was acidified to pH 8 and stirred for 1 h. The modified agarose was then precipitated by the sequential addition of NaCl (0.2 mol, 12 g) and ethanol (500 mL). The product was collected by vacuum filtration using a fritted glass funnel and then extracted using ethanol. The ethanol was removed by extensive dialysis against water for 2 d with a water change every 12 h. The modified agarose was then freeze-dried (Beta 2–8 LD (Christ, Germany)) overnight to yield a white solid. The carboxylation was verified as described earlier[9] by the appearance of bands associated with aliphatic carboxylic acid in the FTIR spectrum (KBr) ($\nu_{C=O}$: 1750 cm^{-1} and NMR (^{13}C: 180 ppm).

2.2.2. Blending of Native Agarose with Carboxylated Analogs

Blends of NA, with CA were prepared by dissolving the polysaccharides in hot deionized water under thorough mixing until a clear solution was obtained, followed by gelation at room temperature before further characterization.

2.2.3. Circular Dichroism

Circular dichroism (CD) spectra were obtained using a J-810 spectropolarimeter (Jasco, Japan) equipped with a Peltier temperature cell (PFD-425S) (Jasco, Japan). Solution of NA or CA (0.15% w/v) was prepared in water by heating to 90 °C for 15 min. Prior to acquisition of the CD spectrum, the sample chamber was cooled to 5 °C for 30 min. The spectrum was recorded as an average of three scans. Spectra and ellipticity values given as 10^3 deg cm^2 $decimole^{-1}$, represent the mean of three independent measurements from different batches normalized to the amount of solubilized material. In order to ensure comparison between all spectra, the same cuvette size (same path length) and identical concentration were used for all measurements.

2.2.4. Rheological Characterization

Rheology experiments were performed with a Physica MCR 301 (Anton Paar, Austria) rheometer equipped with a Peltier thermostat and a plate geometry (PPR25, Anton Paar, Austria). Hydrogels of CA and NA/CA blends were prepared from a 2 % w/v solution in deionized water as follows. The solution was first heated at 90 °C and stirred for 10 min until a clear solution was obtained. The gelation was then initiated on the plate by cooling the solution down from 80 to 5 °C in 30 min followed by equilibration at 5 °C for 30 min to set the gel. Before commencing the frequency sweep, the gel was heated to 37 °C and equilibrated for 30 min and the G' and G'' were recorded as functions of increasing rotational frequency from 0.01 up to 10 rad s^{-1} with a 1% deformation. The G' of the gel was taken at 1 Hz shear frequency.

2.2.5. Scanning Electron Microscopy

Scanning electron micrographs (SEMs) were obtained using a Quanta 250 FEG (FEI, USA) environmental scanning electron microscope (20 kV at 100 Pa). Samples of 2% w/v gels were prepared and 2 mL of this solution was freeze-dried for 24 h under 0.1 mbar vacuum in a 5 mL glass vial. The sample was dissected longitudinally and the interior of the sample was imaged at different magnifications. Images shown here are representative of different areas of several batches of a given composition.

2.2.6. Atomic Force Microscopy

Atomic force micrographs (AFMs) were obtained using a diNanoscope V from Bruker AXS (Germany) in tapping mode with a phosphorous-doped silica RFESP cantilever (Bruker AXS). In order to reduce the adhesion of the agarose gel to the surface, the glass slides were passivated by silanation. Briefly the slides were washed with 0.1 M NaOH, dried in an oven at 60 °C overnight, and then passivated with few drops of dichloromethyl silane (Sigma–Aldrich, Germany) for 10 min. In order to ensure uniform passivation, two slides were sandwiched together. The slides were then washed with water and the excess dichloromethyl silane was removed with soap and the slides were dried at 60 °C in an oven overnight. Agarose samples were prepared as 2% (w/v) solution and 25 μL of the solution was deposited on an unmodified glass slide and a dichloromethyl silane passivated slide was then carefully placed on top of the solution. Two slides 0.5 mm thickness were used as spacer and adjusted between the passivated and the unmodified glass slide, and the whole assembly was cooled to 4 °C for 30 min to induce gelation. The upper glass slide (hydrophobic) was then removed and the thin layer of agarose gel obtained was allowed to stabilize at room temperature for 30 min to avoid any shrinkage or dilatation of the gel during the measurement.

3. Results and Discussion

3.1. Blending NA with CA Yields Hydrogels with Comparable Mechanical Properties as Afforded by Controlled Carboxylation

We have demonstrated that the carboxylation of the NA results in the alteration of the secondary structure of the polymer backbone.[9] This change in secondary structure allows tuning of the mechanical properties of gels so as to match stiffness of diverse mammalian soft tissues. We hypothesized that by introducing carboxylated polysaccharides with β-sheet structures, the organization of polymer chains in the native agarose can be influenced. To examine this premise further, physical blends of NA with CA were investigated.

The mixing of two hydrogels of different secondary structure may either result into a blend of non-interacting particles, each contributing to the mechanical properties of the new hydrogel, or it could lead to a complex material that exhibits new secondary and tertiary structure and may have different mechanical properties compared to its components. According to the law of mass action, the equilibrium between interacting and non-interacting

species, in this case NA and CA, Equation 1a, for a given blend composition depends on the dissociation constant K_d which is given by Equation 1b;

$$NA + CA \rightarrow NA \cdot CA \tag{1a}$$

$$K_d = \frac{[NA][CA]}{[NA \cdot CA]} \tag{1b}$$

Therefore, for a blend of a given composition, the formation of the complex NA·CA depends on K_d and the relative amount of added components NA and CA. Considering, that NA contributes to α-helix content in the complex, and CA contributes to the β-sheet content of the system, and assuming that β-sheet content in NA is negligible, the amount of β-sheet content [β] in every blend can be calculated as per Equation (2);

$$[\beta] = [CA] - [NA \cdot CA] \tag{2}$$

If the two components do not interact (K_d approaches infinity), no formation of complex NA·CA would be observed. Since the source of the β-sheet is CA, in this scenario, the β-sheet content [β] in the blend should be exactly the same as the amount of β-sheet present in the CA, and it should increase linearly with increasing proportion of CA. If the NA and CA polymer chains interact, then one would expect the NA·CA formation to reach a maximum at 1:1 blend. Therefore, combining Equations 1 and (2), assuming a non-zero proportion-dependent contribution of NA·CA, a nonlinear relationship between [β] as CA content is expected as per Equation (3):

$$[\beta] = [CA]\left(1 - \frac{[NA]}{K_d}\right) \tag{3}$$

To verify this prediction, the secondary structure of the blends was analyzed. The secondary structure (e.g., α-helix and β-sheet content) of optically active macromolecules like proteins and polysaccharides can be characterized using circular dichroism (CD).[17] Left and right circularly polarized light interacts differently with the folding of the macromolecule backbone. This difference in absorbance manifests itself as a distinct CD spectrum with positive and negative bands that is indicative of secondary structure. While NA exhibits a characteristic single peak at 185 nm indicating α-helical conformation, CA, which is agarose bearing carboxylic acid groups in 93% of its disaccharide monomer units has two characteristic spectral features in the spectrum that is suggestive of a secondary structure made of two domains. CD signals below 195 nm correspond to an α-helical domain and a positive CD signal at 205 nm is associated with a β-sheet domain.[9] The CD spectra of different blends of NA and CA are shown in Figure 1A. As expected, there is a proportional change to the spectral features corresponding to the β-sheet domains. Interestingly, the maxima of the spectral features associated with α-helical domains undergo a red shift from 186 (100% NA) to 192 nm (100% CA). These spectral features probably arise from the helical disposition of coupled oscillators from different electronic transitions involving n–π^* transitions of OH-chromophores in non-carboxylated domains, but also of π–π^* transitions in carboxylated domains. Because these transitions might result into CD-couplets with positive and negative intensities cancelling each other, the different contributions of different chromophores in the blends might result in an apparent red shift. However, a quantitative analysis of these bands is difficult, because of the low signal-to-noise ratio for signals below 190 nm. In contrast, the CD signal at 205 nm, representing β-sheet domains in CA, is well resolved and allows for a quantitative analysis. According to Beer's law, spectral contributions are proportional to the content of secondary structure in the sample. In order to characterize if the secondary structure domains

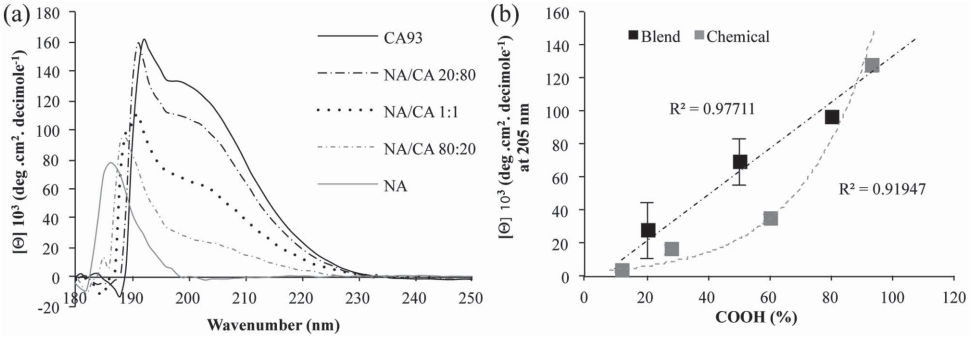

Figure 1. a) Circular dichroism (CD) of 0.15% w/v solution of NA, NA/CA 80:20, NA/CA 1:1 and NA/CA 80/20, b) change in ellipticity at 205 nm, which corresponds to the β-sheet structure, as a function of concentration of carboxylic acid moieties in the blended samples (black) and corresponding chemically modified CA of different percentage of carboxylation (gray).

in NA or CA were either non-interacting (linear proportion dependence) or interacting (non-linear proportion dependence) in different blends, we plotted the ellipticity of the characteristic band at 205 nm against the proportion of each blend (Figure 1B). We observe a clear linear relationship, which according to our model elucidated earlier (Equations (2) and (3)) indicates a lack of molecular interaction of the β-sheet domains of CA with α-helices of NA leading to a new kind of secondary structure.

3.2. Blend Composition Impacts Ultrastructure and Morphology of Freeze-Dried CA/NA Hydrogels

In order to ascertain if blending of CA with NA can recapitulate the morphological changes observed in chemical modified analogues, we analyzed the structure of different freeze-dried blends of NA and CA (0%, 20%, 50%, 80%, and 100% CA) (Figure 2A). It is well established that in NA the fiber organization is random (Figure 2A, I). In contrast, CA has a lamellar organization of fibers, resulting in high-aspect ratio tile-like morphology (Figure 2A, V). In hydrogels composed of chemically modified agarose, the transition from the fibrous to the lamellar morphology upon carboxylation shows graded transitions.[9] Surprisingly in the blends, although there is no co-localization of α-helix and β-sheet domains on the same polymer strand, and the introduction of a β-sheet-rich component promotes a more lamellar organization of fibers. However, as predicted by CD-spectroscopy, the transition is continuous over the different blends and no phase separation is observed. This observation suggests that in the hydrogel a total interpenetration of the CA polymer strands with the NA strands occurs forming a molecular alloy. The altered morphology of the molecular alloy when compared to NA may occur through disruption of the fibrous morphology that is characteristic of helix-helix interactions in NA upon addition of β-sheet containing CA.

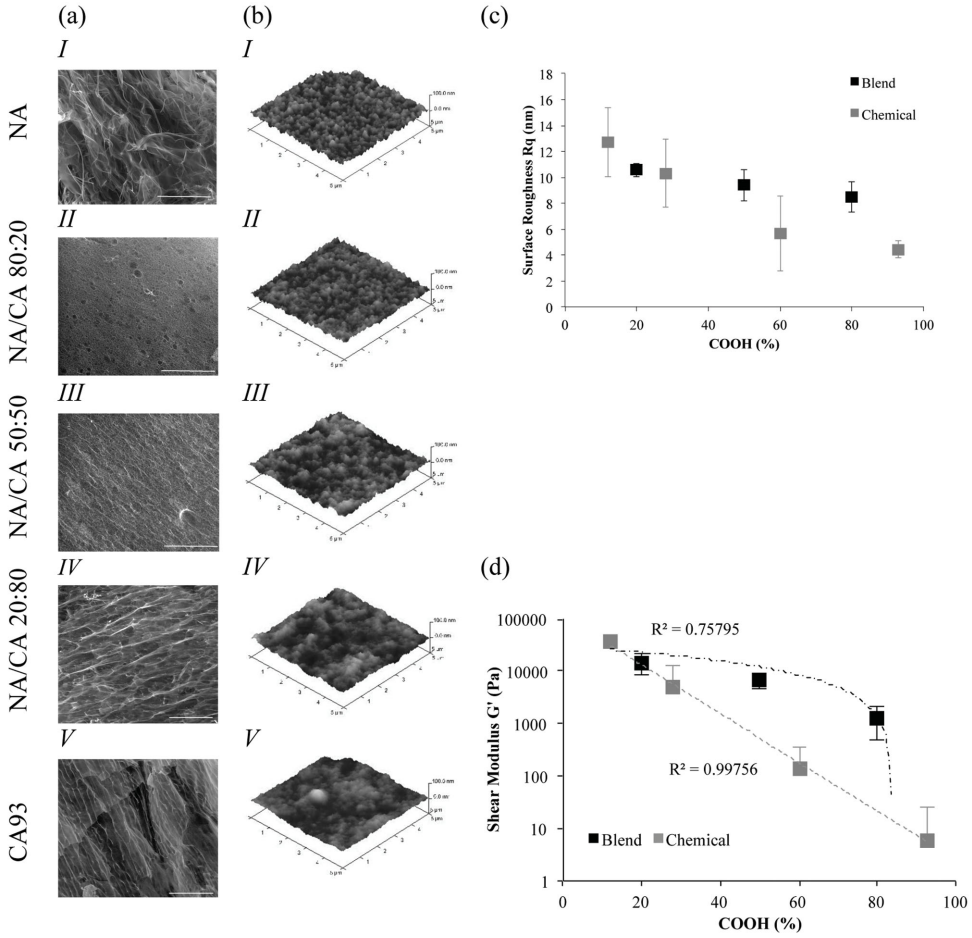

Figure 2. a) Scanning electron micrographs of the inner structure of freeze-dried NA/CA hydrogels blends of various compositions, scale bar 50 μm. b) 3D representation of atomic force micrographs of 2% w/v wet hydrogels obtained under tapping mode. Surface roughness and rheological characterization of 2% w/v hydrogels as a function of carboxylic acid concentration in the blend (black), and in chemically modified CA (gray). c) Surface roughness Rq (in nm) of wet gels measured using atomic force microscopy (AFM), d) shear modulus (*G'*).

3.3. Surface Roughness of Wet Hydrogels is Independent of Blend Composition

It has been shown that cell differentiation may be correlated to mechanical cues of the environment including surface roughness.[1] Since the formation of hydrogels occurs due to association of polymer strands within gelation points, we used tapping mode AFM to measure surface roughness of 2% w/v of the blended hydrogels. The 3D reconstructions of the hydrogel surface of CA and three different blend compositions show that the hydrogel surface is homogeneous at all compositions and does not exhibit any phase separation, which is consistent with the CD and SEM data and further supports the conclusion that there is a molecular alloying of NA with CA polymer strands (Figure 2B). However, this is in contrast to what is observed in chemically modified analogues. The root mean square surface roughness (*R*q) of the different hydrogel blends and of different chemically modified agarose hydrogels are plotted against COOH content in Figure 2C. In chemically modified agarose, increasing carboxylation results in an appreciable reduction in roughness of the wet hydrogel surface from 12 nm (NA) to 4 nm (CA). The evolution of a smoother surface in CA with 93% carboxylation can be attributed to a reduction in the size of gel points with increasing carboxylation.[9] In a blended system, the decrease in *R*q values (10 nm for 20% CA to 8 nm for 80% CA) upon introduction of COOH groups through addition of CA, however, is less pronounced than for chemically modified agarose of comparable COOH content. This provides further evidence in support of a hydrogel network that is composed of an interpenetrated polymer architecture rather than a phase separated one.

3.4. Blending Allows Control Over Rheological Behavior of Hydrogels

As discussed earlier, the blending of β-sheet bearing CA with α-helical NA results in a total reorganization of polymer fibers into lamellar, sheet-like structures. However, since the evolution of such structures in CA is associated with a diminution of hydrogel stiffness, the shear moduli of the three blend compositions were evaluated. We observed that increasing the percentage of CA resulted in a linear decrease in the shear modulus of the gels (Figure 2D). Since we have established that the modification of hydrogel stiffness is a direct consequence of the evolution of β-sheet bearing domains, the linear relationship we observe between shear modulus and COOH content in the blends is in agreement with the results from CD that indicate a linear relationship of COOH content and β-sheet secondary structure in the blends (Figure 1B). However, the reduction in shear modulus is less significant than what is achieved through backbone modification. Since the gels show an anisotropic organization of fibers, one cannot rule out that the mechanical properties may exhibit some anisotropy as well. Although, such effects might be abrogated during shear deformation, this aspect needs to be further investigated.

3.5. The Role of Secondary Structure for Mechanical Properties in NA/CA Hydrogels

Hydrogels are modeled as a network of hydrophilic polymer chains with crosslinking points where these chains interact either through covalent, ionic or H-bond interactions. The mechanical properties of a hydrogel are defined by crosslinks in addition to the polymer backbone. The nature of the crosslink points (physical, covalent, or ionic) defines the strength of the crosslinks, and the deformation behavior of the hydrogel is defined by the distance between the crosslinks (linear density), the volumetric density, and the deformability (elastic, viscoelastic) of polymer network in between the crosslinking points. It is well established that gelation of agarose hydrogels occurs through aggregation of α-helical domains as shown schematically in Figure 3A. Previously, we have shown that this interaction may be severely hampered by the presence of β-sheet-like domains leading to a dramatic decrease of hydrogel stiffness and furthermore that the mechanical properties are proportional to β-sheet content, which was found to exponentially scale with COOH content.[9] We initially hypothesized that the introduction of COOH content by blending NA and CA may invoke similar effects compared to chemical modification. However, in NA/CA blends, the presence of β-sheet domains, although not covalently associated with the α-helix NA, changes the mechanical properties, which is reflected by a linear decrease in shear modulus and a linear increase in β-sheet content for increasing content of COOH in different blends. Interestingly, for backbone-modified agarose, the picture is rather different. Even though the β-sheet content in agarose carrying 28% or 60% COOH introduced by chemical modification is less than what is expected for blends carrying the same amount of COOH (Figure 1B), the impact on shear modulus is dramatically higher in the chemically modified than in the blends. This disparity between dependence of shear modulus in CA homopolymer gels and blends of NA with CA can be rationalized on the basis of a network composed of two structurally distinct associative interactions that lead to crosslink points with different mechanical characteristics as depicted in Figure 3B,C. Crosslinking points composed of only helices have five orders of magnitude higher modulus, than the ones composed of only β-sheet, due to the significantly higher H-bonding in the former. The modulus of such a network would there be dominated by the mechanical properties of the helical aggregates with the weakly associated β-sheet

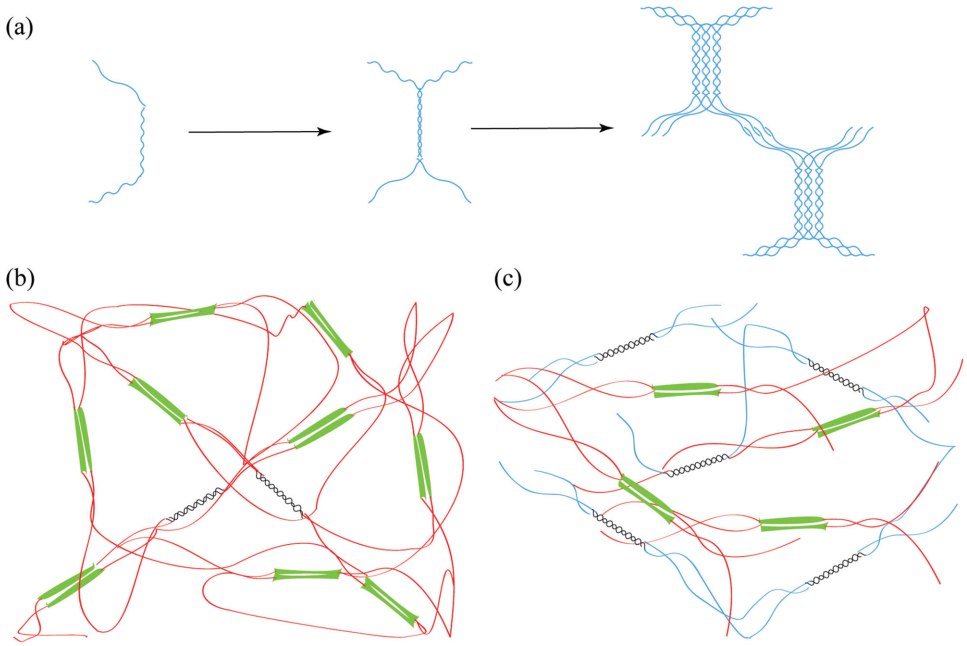

Figure 3. a) Established mechanism of agarose gelation, b) representation of the β-sheet domains inside the CA hydrogel network (red), showing that every polymer strand bears a shear deformable (green) β-sheet domain and some polymer strands bears an α-helix domain (black), c) representation of the β-sheet domains inside the NA/CA blends, where a mix of NA (blue) which bears only α-helical strand and stiff crosslinking points (black) associate with polymer strands of CA (red) bearing a shear deformable β-sheet domain (green).

assemblies providing the viscoelastic component to the network. In the chemically modified hydrogels, CA strands are composed of β-sheet-rich domains with few or no-α-helical domains. Therefore, the few stiff crosslinked points are compensated by viscous interaction of β-sheet on the same polymer strand (Figure 3B). However, in the blend, β-sheet-associated viscous interactions are located only in the CA polymer strand, and as a consequence pure NA strands can form stiff crosslinking points without viscous interaction on the same strands, and still form a molecular alloy through α-helical domain interactions (Figure 3C). This model is consistent with the observation that the blending of CA into NA diminishes the modulus, as the volume fraction of α-helices is reduced, but does not lead to the severe decrease in shear modulus that is observed in CA gels. With increasing CA content, the crosslink points composed of β-sheets are increased at the expense of the α-helical aggregation. However at no blend composition is the network free of α-helical crosslink points. Additionally, in such a network, the electrostatic repulsion between negatively charged β-sheet domains may contribute to the modulation of shear deformation thereby modifying the length and elasticity of interconnecting chains.[9] In a network composed of only rigid crosslink points, the stiffness of the network might be considerably altered by replacement of a few crosslink points with those that can undergo shear deformation. The central premise of the model that α-helix and β-sheet aggregate into discrete domains is confirmed by the CD-results, which clearly point to the absence of any interactions between α-helical and β-sheet-rich domains.

4. Conclusions

We demonstrate herein that the introduction of β-sheet domains into a α-helical environment impacts the mechanical properties and macroscopic organization of polymer chains in a hydrogel. However, this impact strongly depends on the way the β-sheet-rich domains are introduced. The mechanical properties of the gels showed an inverse linear dependency on the concentration of COOH groups in the gel if the NA was blended with different proportions of β-sheet-rich CA, but showed an exponential change if the β-sheet secondary structure was introduced by controlled carboxylation of the NA backbone. Likewise, the amount of β-sheet in the hydrogel blends was linearly proportional to the COOH content, but exponentially proportional to the degree of carboxylation in CA. As a consequence, the β-sheet content in CA is always lower than in blends with the same COOH content. In contrast, the shear modulus for CA hydrogels is always lower than in the blends. The ability to affect the formation of hydrogel crosslink points by blending β-sheet-rich CA with α-helix-rich NA provides a simple means to formulate mechanically well-defined hydrogels, through

molecular alloying and provides a versatile platform to investigate the role of mechanical cues in cellular signaling and organization.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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[1] A. M. Lipski, C. J. Pino, F. R. Haselton, I.-W. Chen, V. P. Shastri, *Biomaterials* **2008**, *29*, 3836.
[2] A. J. Engler, S. Sen, H. L. Sweeney, D. E. Discher, *Cell* **2006**, *126*, 677.
[3] R. W. Tilghman, C. R. Cowan, J. D. Mih, Y. Koryakina, D. Gioeli, J. K. Slack-Davis, B. R. Blackman, D. J. Tschumperlin, J. T. Parsons, *PLoS One* **2010**, *5*, 13.
[4] S. Dumitriu, *Polysaccharides: Structural Diversity and Functional Versatility*, CRC Press, Boca Raton, FL, USA **2004**.
[5] *Thickening and Gelling Agents for Food* (Ed: A. P. Imeson), Springer, Berlin, Germany **1995**.
[6] Y. Suzawa, T. Funaki, J. Watanabe, S. Iwai, Y. Yura, T. Nakano, Y. Umakoshi, M. Akashi, *J. Biomed. Mater. Res. A* **2010**, *93*, 965.
[7] P. J. Emans, L. W. van Rhijn, T. J. M. Welting, A. Cremers, N. Wijnands, F. Spaapen, J. W. Voncken, V. P. Shastri, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 3418.
[8] S. Fernández-Cossío, A. León-Mateos, F. G. Sampedro, M. T. C. Oreja, *Plast. Reconstr. Surg.* **2007**, *120*, 1161.
[9] A. Forget, J. Christensen, S. Lüdeke, E. Kohler, S. Tobias, M. Matloubi, R. Thomann, V. P. Shastri, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 12887.
[10] J. M. Zuidema, M. M. Pap, D. B. Jaroch, F. A. Morrison, R. J. Gilbert, *Acta Biomater.* **2011**, *7*, 1634.
[11] N. Russ, B. I. Zielbauer, K. Koynov, T. A Vilgis, *Biomacromolecules* **2013**, *14*, 4116.
[12] P. Neyret, T. Selmi, L. Barnouin, *E-mémoires l'Académie Natl. Chir.* **2007**, *6*, 5.
[13] M. Tombs, S. E. Harding, *An Introduction to Polysaccharide Biotechnology*, CRC Press, Boca Raton, FL, USA **1997**.
[14] P. A. Williams, D. H. Day, M. J. Langdon, G. O. Phillips, K. Nishinari, *Food Hydrocoll.* **1991**, *4*, 489.
[15] M. Tako, *Biosci. Biotechnol. Biochem.* **1992**, *56*, 1188.
[16] G. Paradossi, E. Chiessi, A. Barbiroli, D. Fessas, *Biomacromolecules* **2002**, *3*, 498.
[17] S. M. Kelly, N. C. Price, *Curr. Protein Pept. Sci.* **2000**, *1*, 349.