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Polypeptide grafted hyaluronan: A self-assembling comb-branched polymer constructed from biological components

Madhuvanthi A. Kandadai^{a,*}, Rajasekhar Anumolu^a, Xiaojun Wang^b, Durairaj Baskaran^b, Leonard F. Pease III^a, Dmitry Bedrov^c, Grant D. Smith^c, Jimmy W. Mays^b, and Jules J. Magda^{a,c}

Rajasekhar Anumolu: al_raj@hotmail.com; Xiaojun Wang: xwang@ion.chem.utk.edu; Durairaj Baskaran: baskaran@utk.edu; Leonard F. Pease: pease@eng.utah.edu; Dmitry Bedrov: d.bedrov@utah.edu; Grant D. Smith: gsmith2@cluster2.mse.utah.edu; Jimmy W. Mays: jimmymays@utk.edu; Jules J. Magda: jj.magda@m.cc.utah.edu

^aDepartment of Chemical Engineering, University of Utah, 50 S. Central Campus Drive, Rm 3290, Salt Lake City, Utah 84112, USA

^bDepartment of Chemistry, University of Tennessee at Knoxville, 552 Buehler Hall, 1420 Circle Drive, Knoxville, Tennessee 37996-1600, USA

^cDepartment of Materials Science & Engineering, University of Utah, 120 S. Central Campus Drive, Salt Lake City, Utah, USA

Abstract

Rheological evidence is provided demonstrating that covalent grafting of monodisperse isotactic poly(L-leucine) branches onto linear hyaluronan (HA) polysaccharide chains yields comb-branched HA chains that self-assemble into long-lived physical networks in aqueous solutions driven by hydrophobic interactions between poly(L-leucine) chains. This is in stark contrast to native (unmodified) HA solutions which exhibit no tendency to form long-lived physical networks.

Keywords

Hyaluronic acid; Polypeptide; Viscoelasticity; Hydrophobic interaction

1. Introduction

Native hyaluronan (HA) is a linear charged polysaccharide that has a ubiquitous presence in the extracellular matrix of the human body, and thus is essentially “invisible” to the body’s immune system [1]. This makes HA an attractive starting material for the synthesis of novel biopolymers for biomedical applications. For example, in recent years, native HA has been chemically modified with cell adhesion peptides for tissue engineering applications [2–3], with cationic polyethyleneimine branches to obtain a vehicle for gene delivery [4], and with hydrophobic bile acid branches for nanoparticle mediated drug delivery [5]. In addition, in

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*Corresponding author: kandadmi@ucmail.uc.edu, Phone: 1-309-533-1411, Fax: 513-558-5791.

Current address: University of Cincinnati, Department of Emergency Medicine, 231 Albert Sabin Way, Suite 1551, PO Box 670769, Cincinnati, Ohio 45267-0769

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order to obtain a potential “viscosupplement” for boosting the viscosity and elasticity of synovial fluid [6–7], native HA has been “hydrophobically modified” via attachment of hydrophobic alkane branches [8]. For this application, since it is important to retain biocompatibility, modification with hydrophobic branches that are purely biological may be an attractive alternative. Wang et al. have recently shown how well characterized monodisperse hydrophobic isotactic poly(L-leucine) branches can be conjugated to linear HA backbone chains [9]. Attachment of hydrophobic branches to linear water soluble polymers has long been used as a means for obtaining commercial polymer additives called “associative thickeners” that are used to boost the viscosity of solutions and suspensions [10]. This boost is thought to occur because the associative thickener self-assembles into a transient network, with contacts between the hydrophobic branches acting as physical crosslinks [10–11]. Thus we hypothesize that by hydrophobically modifying native HA with isotactic poly(L-leucine) branches of appropriate length and grafting ratio, we can convert it into a derivative that self assembles into physical networks in aqueous solutions. This research finding may be somewhat surprising, because isotactic poly(L-leucine) chains are known to be so hydrophobic that they do not dissolve in water unless they are chemically attached to hydrophilic polymers [12–14], and even in this case the isotactic leucine chains tend to collapse into rod-like α -helical conformations [13–15]. This has led some research groups to use less hydrophobic *racemic* poly(leucine) chains as an alternative [14]. Hence if one conjugates isotactic poly(L-leucine) chains to HA, then the value of the grafting ratio and the molecular weight of the branches must be chosen with great care: small enough to retain the native water solubility of the HA backbone, yet large enough to allow intermolecular hydrophobic contacts to occur as needed for network formation. The purpose of this short note is to present proof-of-concept results that demonstrate that it is feasible to construct polypeptide grafted HA chains that self assemble into physical networks. The hope is that this will encourage other research groups to consider polypeptide grafted HA for the various applications that require self-assembling physical networks [5,8].

2. Experimental section

2.1 Polypeptide grafted HA (HA-g-PLeu)

Sodium hyaluronate (Na-HA) is a linear charged polysaccharide with a disaccharide repeat unit consisting of β -D-glucuronic acid and 2-acetamido-2-deoxy- β -D-glucose as shown in Figure 1. Synthesis and characterization of the polypeptide-modified HA derivative (HA-g-PLeu) is described in detail in Wang et al. [9]. The starting material was bacteria produced Na-HA purchased from Lifecore Biomedical (Chaska, MN) with a weight averaged molecular weight of 74 KDa. Monodisperse isotactic poly(L-leucine) chains containing 10 repeat units as shown in Figure 1 were conjugated to approximately 1.5 mole % of the disaccharide units in the native HA backbone.

2.2 Electrospray Differential Mobility Analysis (ES-DMA)

Since poly(L-leucine) grafted hyaluronan (HA-g-PLeu) tends to aggregate into micelles at the lowest concentrations that can be accessed using light scattering [9], the diameter of the coiled unimer was estimated using electrospray differential mobility analysis (ES-DMA) [17–18]. In ES-DMA, one forms nanometer-sized aerosol droplets from dilute macromolecule solutions in such a way that each droplet contains either one polymer chain or one polymer aggregate. For ES-DMA measurements performed at Utah, the HA containing biopolymers to be studied were dissolved at 0.1 mg/ml in 20 mM ammonium acetate (pH 7.85). This solvent is expected to yield droplets with initial diameters of approximately 150 nm. The droplets were quickly dried, given a known charge, and then injected into a differential mobility analyzer which separates the particles on the basis of the charge-to-aerodynamic diameter ratio. The ES-DMA apparatus at the University of Utah can

detect values of the aerodynamic diameter D over the range 3 nm to 250 nm, with a resolution of about 0.2–1.0 nm [17,19].

2.3 Constant stress cone-plate rheology

De-ionized distilled water was used to prepare 0.1 M NaCl which was the solvent used to prepare solutions of Na-HA and HA-g-PLeu for rheological studies at 25 °C. The solvent was chosen to match the conditions at which HA-g-PLeu was characterized in Reference 9, as well as the conditions at which the rheology of Na-HA was studied by Krause et al. [20]. A stock solution of Na-HA or HA-g-PLeu in 0.1 M NaCl was first prepared at the highest polymer concentration of interest (7.5 wt.%), and then the stock solution was diluted down as necessary for rheology tests at lower concentrations. Stock solutions were prepared by gentle mixing at room temperature over two days, and stored at 2 °C until use. Solutions were prepared using either HA-g-PLeu, or a native Na-HA polymer of molecular weight 67 KDa (Lifecore Biomedical). Note that due to sample availability limitations, the molecular weight of the native HA studied by rheology (67 KDa) was similar but not identical to that of the Na-HA starting material (74 KDa) used to synthesize HA-g-PLeu. Linear oscillatory shear measurements, steady shear flow measurements, and creep recovery tests were performed on solutions of native HA or HA-g-PLeu using a constant stress rheometer (model AR550 from TA Instruments) with cone plate fixtures (radius 20 mm, cone angle 2°, sample size 0.6 ml) at a controlled temperature of 25 °C. The preferred method for detecting gel formation is linear oscillatory shear measurements of the elastic shear modulus G' and viscous modulus G'' as a function of oscillation frequency ω at a stress amplitude small enough (0.3 Pa) to avoid gel structure distortion [11,21]. Due to the difficulty in finding a procedure to synthesize HA-g-PLeu [9], only 0.5 grams was available for rheological study. Consequently, it was not possible to study the solution behavior of HA-g-PLeu at a large number of different concentrations. Instead, we searched for the lowest polymer concentration at which HA-g-PLeu will self-assemble into a long-lived network, which we determined to be approximately 5 wt.% (see Results Section). It has recently been shown that protein adsorption at the air interface in cone-plate rheometers can cause spurious rheology measurements due to gel like surface film formation at long times [22]. Therefore, preliminary tests were performed to check for potential hysteresis in the measured viscosity value of HA-g-PLeu solutions due to biopolymer interfacial adsorption. No hysteresis was observed, provided that the duration of the test did not exceed 5–10 minutes [23].

3. Results and discussion

3.1 Estimation of Polymer Coil Overlap Concentration (C^*)

Using ES-DMA, the aerodynamic diameter D was measured to be 10 ± 0.3 nm for native a-HA (67 KDa), and 9 ± 0.3 nm for HA-g-PLeu. The hydrodynamic radius R_h of a single chain of native Na-HA can be estimated from the intrinsic viscosity value $[\eta]$ using the following equation [24]:

$$[\eta] = 10 \pi N_A (R_h)^3 / (3M_v) \quad (1)$$

Where, N_A is Avogadro's number and M_v is the molecular weight of the macromolecule. Using Equation (1) and the Mark-Houwink relation for the intrinsic viscosity of native Na-HA (67 KDa) in 0.1 M NaCl [20], one obtains $R_h = 13.2$ nm. Thus the hydrodynamic diameter ($2 R_h$) is about 2.5 times larger than the aerodynamic diameter D . This is consistent with the findings of Tsai et al., who determined a thickness value for polymer layers adsorbed on gold nanoparticles that was 2–3 times larger when measured using dynamic light scattering (DLS) than when measured using ES-DMA [19]. This difference arises

because polymers are in a liquid environment during DLS measurements thus exhibit expanded random-walk conformations, whereas the same polymers are in a collapsed dried state during ES-DMA measurements [19]. Since the measured D value for both biopolymers is roughly the same and is less than the estimated single coil R_h value, we conclude that 1) it is possible to use ES-DMA to characterize unimers of HA-g-PLeu, despite the very low critical micelle concentration value reported for HA-g-PLeu in Reference 9, and 2) the polymer overlap concentration C^* is roughly the same for the native HA sample and for HA-g-PLeu. For both, we can take C^* to be roughly $1/[\eta]$, or 4.6 mg/ml.

3.2 Rheology

Many of the published correlations for the shear viscosity of native HA solutions are written in terms of the specific viscosity η_{sp} , defined as $\eta_{sp} = (\eta_0/\eta_s) - 1$, where η_0 is the zero-shear-rate-limiting viscosity value of the solution and η_s is the solvent viscosity value [25]. Figure 2 is a logarithmic plot that compares the concentration dependence of η_{sp} of HA-g-PLeu and native Na-HA (67 KDa). The value of η_0 was obtained either by extrapolating steady-shear measurements to zero shear rate, or by extrapolating oscillatory shear measurements of the dynamic viscosity η' to zero frequency [21]. As discussed above, the coil overlap concentration C^* for the native Na-HA sample is estimated as 4.6 mg/ml using the Mark-Houwink relation for the intrinsic viscosity in 0.1 M NaCl [20], and appears on the basis of ES-DMA measurements to be approximately the same for HA-g-PLeu. For native HA, the dependence of η_{sp} on polymer concentration in Figure 2 is consistent with the rheology study of Krause et al. [20]. In particular, η_{sp} exhibits a power law dependence on concentration C with an exponent that is approximately one in the dilute regime ($C < C^*$), approximately two in the semidilute unentangled regime ($C^* < C < 4C^*$), and approximately four in the entangled regime ($C > 4C^*$). The specific viscosity results for HA-g-PLeu below C^* in Figure 2 are similar to those for native HA in Figure 2. However, at concentrations above about 30 mg/ml ($\approx 7 C^*$) in Figure 2, one observes that the specific viscosity curve of HA-g-PLeu departs from that of native HA and has a much stronger concentration dependence. At 50 mg/ml ($\approx 10 C^*$), the zero-shear-rate-limiting viscosity value is 25 times greater for HA-g-PLeu than for the native HA. At 75 mg/ml ($\approx 15 C^*$), the highest concentration studied, the zero-shear-rate-limiting viscosity value is 65 times greater for HA-g-PLeu than for the native HA. We attribute the strong concentration dependence of the viscosity of HA-g-PLeu to a new physical mechanism that occurs with the derivative HA-g-PLeu but not with native HA, namely transient network formation due to hydrophobic associations between poly(L-leucine) branches on different molecules.

The ability of self-assembled HA-g-PLeu networks to store elastic energy was probed in creep-strain recovery tests, in which each sample was subjected to a time-independent shear stress of 0.2 Pa during the creep period for 60 s, followed by 60 s of zero imposed stress in the recovery period. The results obtained for solutions containing 5 wt.% and 7.5 wt.% of HA-g-PLeu in 0.1 M NaCl at 25 °C are shown in Figure 3. The short-time creep behavior is shown in the inset to Figure 3, which also includes results observed for the native HA solution at 5.0 wt%. A purely Newtonian solution will creep indefinitely under an imposed shear stress and exhibit a strain curve of constant slope when plotted against time, with no storage of elastic energy and hence no elastic recoil during the recovery period [21]. This is the behavior observed in Figure 3 (inset) for the native (unmodified) HA solution. In contrast, in Figure 3 the shear strain of the 5 wt% solution of HA-g-PLeu shows an initial elastic jump followed by a slow creep, with almost 67% of the imposed strain recovered after stress removal. This indicates that the 5 wt% HA-g-PLeu solution is a viscoelastic liquid that contains a transient network capable of storing elastic energy. In Figure 3, the shear strain of the 7.5 wt% HA-g-PLeu solution reaches a plateau value during the creep period, indicating that the sample is a soft solid, with complete recovery of the imposed

strain after stress removal. In contrast, no strain recovery at all is observed after cessation of creep for the 5 wt % solution of native HA (67 KDa) [23]. This is a clear indication that the hydrophobic poly(L-leucine) branches are responsible for the elastic recoil observed in Figure 3. Using the initial elastic jump in strain before the onset of creep (Figure 3 inset), one estimates that the elastic shear modulus G is approximately 25 Pa for the 5.0 wt.% solution of HA-g-PLeu, and 125 Pa for the 7.5 wt.% solution of HA-g-PLeu.

Due to the limited amount of sample available, we did not repeat the creep experiments of Figure 3, but instead performed oscillatory shear measurements on freshly-prepared solutions that confirmed the values of G obtained from the creep-recovery tests. Oscillatory shear values of the elastic modulus (G') and viscous modulus (G'') are plotted against oscillation frequency ω in Figure 4, as measured at 25 °C for solutions containing 5.0 wt.% and 7.5 wt.% HA-g-PLeu in 0.1 M NaCl. The high-frequency plateau values of G' in Figure 4 are 24 Pa and 125 Pa for the 5.0 wt.% and 7.5 wt.% solutions of HA-g-PLeu, in excellent agreement with the values calculated from the short-time creep response (Figure 3 inset). In contrast, G' is too small to measure for a 7.5 wt.% solution containing unmodified HA (67 KDa) ($G' < 0.1$ Pa). For a covalently-crosslinked gel, G' exceeds G'' at all ω values, whereas for a physical network, G' exceeds G'' only at frequencies greater than a critical value ω^* approximately equal to the inverse of the average lifetime of network crosslinks. The value of ω^* is thus obtained experimentally as the “crossover frequency”, i.e. the frequency below which the value of G' drops below the value of G'' in plots of G' and G'' vs. ω [11]. In Figure 4, one observes that all frequencies studied, the elastic storage modulus G' of the 7.5 wt.% HA-g-PLeu solution exceeds the viscous loss modulus G'' by a factor of four, that is, the loss tangent $\tan \delta = G''/G'$ is approximately 0.25. At sufficiently low frequencies, one would expect the value of G' to drop below the value of G'' because the contacts between hydrophobic poly(L-leucine) branches are not permanent. There is some indication of this trend near $\omega^* = 0.05$ rad/sec in Figure 4. This is the lowest accessible frequency, because the period of oscillation becomes so long at lower frequencies that polypeptide adsorption at the air interface of the cone-plate rheometer may cause spurious results [22]. Since the lifetime of a physical network is given by the inverse of the crossover frequency of G' and G'' , the lifetime of the network formed by HA-g-PLeu is *at least* 20 s.

4. Conclusion

In conclusion, we have shown that by hydrophobically modifying native Na-HA (74 KDa) with isotactic poly(L-leucine) branches containing ten repeat units at a frequency of 1.5 branches per 100 disaccharide repeat units, we convert Na-HA into a comb-branched derivative that self-assembles into a physical network. This physical network has an elastic shear modulus $G' \approx 100$ Pa, a loss tangent $\tan \delta \approx 0.25$, a network lifetime > 20 s, and exhibits almost complete elastic strain recovery after removal of a shear stress. In contrast, a solution containing unmodified HA chains of similar molecular weight at the same weight concentration exhibits no evidence of elasticity.

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Research Highlights

- Poly (L-Leucine) grafted HA chains self assemble into physical networks.
- This network has an elastic shear modulus ≈ 100 Pa and a loss tangent ≈ 0.25 .
- It has a network lifetime > 20 s.
- It exhibits almost complete elastic strain recovery after removal of shear stress.
- No physical network formation seen in unmodified HA.

Figure 1.

Top: Chemical structure of the HA disaccharide repeat unit containing (a) β -D-glucuronic acid and (b) 2-acetamido-2-deoxy- β -D-glucose. Bottom: Chemical structure of 10 repeat unit isotactic poly(L-leucine)

Figure 2. Concentration dependence of the zero-shear-rate-limiting specific viscosity value of HA-g-PLeu (x) and native Na-HA (Δ), as measured in 0.1 M NaCl at 25 °C. Two sample measurements at each concentration; error bars as shown.

Figure 3.

Shear strain vs. time as measured in creep recovery tests on HA-g-PLeu in 0.1 M NaCl. A constant shear stress of 0.2 Pa was applied at time equal zero and removed at 60 s. HA-g-PLeu concentrations: top curve: 5.0 wt%; bottom curve: 7.5 wt%. Figure Inset: short-time creep behavior under imposed stress of 0.2 Pa. Top curve: 5.0 wt.% native HA. Middle curve: 5.0 wt.% HA-g-PLeu. Bottom curve: 7.5 wt.% HA-g-PLeu

Figure 4. Elastic shear modulus (filled symbols) and viscous shear modulus (unfilled symbols) vs. oscillation frequency, as measured at 25° C for HA-g-PLeu in 0.1 M NaCl at 5.0 wt.% (squares) and 7.5 wt.% (diamonds)