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Polypeptide Grafted Hyaluronan: Synthesis and Characterization

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Abstract

Poly(L-leucine) grafted hyaluronan (HA-g-PLeu) has been synthesized via a Michael addition reaction between primary amine terminated poly(L-leucine) and acrylate functionalized HA (TBAHA-acrylate). The precursor hyaluronan was first functionalized with acrylate groups by reaction with acryloyl chloride in the presence of triethylamine in N,N-dimethylformamide. ¹H NMR analysis of the resulting product indicated that an increase in the concentration of acryloylchloride with respect to hydroxyl groups on HA has only a moderate effect on functionalization efficiency, *f*. A precise control of stoichiometry was not achieved, which could be attributed to partial solubility of intermolecular aggregates and the hygroscopic nature of HA. Michael addition at high [PLeu-NH₂]/[acrylate]_{TBAHA} ratios gave a molar grafting ratio of only 0.20 with respect to the repeat unit of HA, indicating grafting limitation due to insolubility of the grafted HA-g-PLeu. Soluble HA-g-PLeu graft copolymers were obtained for low grafting ratios (< 0.039) with < 8.6 % by mass of PLeu and were characterized thoroughly using light scattering, ¹H NMR, FT-IR and AFM techniques. Light scattering experiments showed a strong hydrophobic interaction between PLeu chains, resulting in aggregates with segregated non-grafted HA segments. This yields local networks of aggregates as demonstrated by atomic force microscopy. Circular dichroism spectroscopy showed a β -sheet conformation for aggregates of poly(L-leucine).

Introduction

Hyaluronan (HA)¹ is a non-branched glycosaminoglycan consisting of disaccharide repeating units of D-glucuronic acid and N-acetyl-D-glucosamine. It exists as a gelatinous mass in human and animal tissues (vitreous body) and as a non-Newtonian viscous fluid in joint cavities (i.e., synovial fluid). It is also present as a gel in the extracellular matrix (ECM) of cells, where it acts as a mechanical stress absorber. It is known that HA functions in many important biological processes including tissue hydration, diffusion of ions, nutrients and oxygen, supramolecular assembly of proteoglycans in the ECM,² cell differentiation and proliferation.³ Because of its unique structure and properties (e.g., biocompatibility and degradability), HA and its derivative hydrogels have broad applications in various technical and medical fields,⁴ such as ophthalmologic surgery, cosmetics, tissue engineering and drug delivery.^{5–10}

Over the past years, various modification strategies like crosslinking¹¹ and esterification¹² have been applied to hyaluronan in order to modify its mechanical and chemical properties to tailor materials for applications such as drug delivery, tissue engineering, joint

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Supporting Information Available: FT-IR spectra of neutralized TBAHA and acrylate functionalized hyaluronic acid (TBAHA-acrylate) and TGA of TBAHA , poly(L-leucine) and TBAHA-g-PLeu graft copolymer. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

lubrication, and cell adhesion and signaling.^{13, 14} Probably, the most widely used method for HA derivatization is coupling of water soluble hydrazides to the carboxylic acid groups of HA at pH 4.75, mediated by carbodiimides, for drug molecule attachment and hydrogel preparation.^{7, 9, 13, 15-19} The functionalization with alkanes using hydrazides to improve rheological property of HA has also been reported.^{19c} Recently, several new methodologies were developed to prepare hyaluronan hydrogels by amidation, and photo-crosslinking.²⁰⁻²² Also, to improve hydrophobicity of HA for hot molding or dissolution in organic solvents, alkanes, silylation and/or acylation have been explored.^{23,24}

Although various HA derivatives have been synthesized by a number of methods, only a few graft copolymers having HA as backbone have been reported using either “grafting onto” or “grafting from” strategies. For example, Ohya *et al.*^{10, 25} prepared HA-graft-poly(N-isopropylacrylamide) (HA-g-PNIPAM) copolymer by “grafting from” polymerization of NIPAM with “iniferter” dithiocarbamate functionalized HA. This graft copolymer served as a tissue adhesion prevention material and hemostatic aid.²⁵ Palumbo *et al.*²⁶ and Pravata *et al.*²⁷ synthesized poly(lactic acid) grafted copolymers of HA (HA-g-PLA) by means of “grafting onto” through esterification of primary hydroxyl groups on the HA backbone, yielding grafting ratios of 1.5-8.0 mole %. Detailed characterization was provided for the graft copolymers, showing hydrophobic interactions in aqueous solution.

Recently, well-defined poly(L-leucine) (PLEu) has been incorporated into block copolymers^{28, 29} because of its hydrophobic nature and ability to adopt secondary structure resulting in a rigid chain conformation. Additionally, Deming *et al.* reported that the incorporation of a PLEu segment into synthetic block copolypeptides significantly affected self-assembly^{30, 31} and the rheological properties of block polypeptides where rigid hydrogel formation was observed even at low concentrations.^{28, 32} Thus, PLEu grafted HA (PLEu-g-HA) may have advantages for mimicking natural processes occurring in proteins and thus have potential use in bio-medical applications. The combination of polysaccharide (HA) and polypeptide (PLEu) should provide tailored biomaterials that can be used in tissue engineering as hydrogels and as drug carriers in delivery system.

In the present work, we describe the synthesis of a new PLEu grafted hyaluronan (PLEu-g-HA) *via* a Michael addition reaction between acrylate-functionalized HA (TBAHA-acrylate) and primary amine terminated PLEu (PLEu-NH₂). Detailed characterization of the products by ¹H-NMR spectroscopy, dynamic light scattering (DLS), static light scattering (SLS), thermogravimetric analysis (TGA), atomic force microscopy (AFM), and circular dichroism (CD) has been performed. The functionalization of HA with acryloyl chloride and the solubility of TBAHA-acrylate and the final graft copolymers are discussed. The conformation of PLEu in the graft copolymer and the morphology of the graft copolymer in aqueous solution are also discussed.

Experimental Section

Materials

Sodium hyaluronates (NaHA) with molecular weights of 74×10^3 and 132×10^3 g/mol were purchased from Lifecore Biomedical (low and medium molecular weight HA were chosen for justification of chemistry applied in our study. It is believed that MW wouldn't be an obstacle if high MW HA is desired). Two separate tetrabutyl ammonium derivatives of HA (TBAHA) were obtained by passing an aqueous solution of NaHA through a column of cation exchange resin (Dowex 50wx8-100, Aldrich), followed by titration with tetrabutylammonium hydroxide (TBAOH, 40 wt% aqueous solution, Fisher) to pH 8 ~ 9, and then lyophilized to obtain solid TBAHA. To clarify, TBAHA-A is derived from 132k NaHA and TBAHA-B is from 74K NaHA. N,N-Dimethylformamide (DMF, Aldrich, HPLC

grade), acryloyl chloride (Aldrich, 98%), and triethylamine (TEA, Aldrich, 99.5%) were stirred over calcium hydride and freshly distilled prior to use. PLeu was obtained from Prof. Nikos Hadjichristidis' laboratory (University of Athens, Greece), and was prepared by ring opening polymerization of the monomer L-leucine *N*-carboxyanhydride ($M_n = 1500$ g/mol, $^1\text{H-NMR}$ in *d*-TFA) *via* high vacuum techniques.³³

Synthesis of graft copolymer

Functionalization of TBAHA (TBAHA-acrylate)—In a typical experiment, a three-neck round bottom flask dried at 150 °C was equipped with inlet and outlet for high purity nitrogen and a magnetic stirring bar. Before sealing the flask with rubber septa, 0.87 g (4.3×10^{-6} moles) of tetrabutylammonium hyaluronate (TBAHA-A) (dried overnight under vacuum at 40 – 50 °C), dry DMF (100 ml) and TEA (3.2 ml) were added to the flask under constant nitrogen purge to form a clear solution. Next, 1.2 ml of acryloyl chloride solution (0.092 M in dry DMF or dry THF) was added drop-wise *via* syringe into the flask, which was subsequently immersed in a water bath. The mixture was left to stir overnight at room temperature. The acrylate-functionalized TBAHA (TBAHA-acrylate) was recovered by precipitating the reaction mixture into a large excess of diethyl ether, and further purified by rinsing with a large excess of ethyl ether several times, and finally dried in a vacuum oven at room temperature (batch process). In addition, a one-pot synthesis to obtain directly the graft copolymer was also developed. In this case, the reaction solution was directly used in the Michael addition reaction for synthesis of the graft copolymer as described below. Small amounts of solution were sampled for characterization, purified by direct dialysis against water, and lyophilized to yield 0.064 g of TBAHA-acrylate.

Michael addition to Prepare Graft Bioconjugates of HA and Poly(L-leucine)—

Grafting of PLeu onto the backbone of TBAHA was performed using a Michael addition reaction between TBAHA-acrylate and PLeu-NH₂ (0.15 g) in the presence of TEA at room temperature. After the contents were combined, the reaction flask was covered with aluminum foil and stirred under N₂ atmosphere for 1 week. Excess/unreacted PLeu was removed by ultracentrifugation ($1.1 \sim 1.4 \times 10^4$ rpm) at 25 °C to obtain a clear, transparent solution, followed by distillation of DMF under reduced pressure at mild temperatures. The viscous residue was again dissolved in water, neutralized with TBAOH and dialyzed against deionized water for three days (molecular weight cutoff of membrane: 3500 g/ml). The dialyzed solution was adjusted to pH ~ 7.0 before the graft copolymer was lyophilized to recover the white polymer solid (0.713 g, yield 74.6 %). An alternate way to purify the graft copolymer was to precipitate the reaction solution into a large excess of ethyl ether. The resulting solid was rinsed several times with ethyl ether, dissolved in water, neutralized, and dialyzed. Unreacted PLeu was removed by centrifugation ($1.1 \sim 1.4 \times 10^4$ rpm). Finally, the copolymer was recovered as a white solid after lyophilization.

Converting the tetrabutylammonium salt to the sodium salt was performed by dialyzing the TBA form of the graft copolymer (0.0323g polymer in 20ml water) against 0.1M NaCl solution for 3 days, and then against deionized water for another 3 days during which the NaCl solution and the deionized water was changed twice daily. The polymer solid was obtained by freeze-drying (0.02 g).

Characterization Methods

$^1\text{H-NMR}$ spectroscopy was performed on Varian Mercury 300 MHz spectrometer with deuterated trifluoroacetic acid (TFA-*d*), D₂O, and dimethylsulfoxide (DMSO-*d*₆) as solvents. Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS) was recorded on Varian Resolutions Pro instrument and data were averaged over 64 scans.

Samples were prepared by placing several drops of aqueous solution onto aluminum foil and allowing it to dry in a hood at room temperature overnight. The thermal stabilities of TBAHA, PLeu, and the graft copolymer, as well as an estimation of PLeu composition in the graft polymer, were evaluated by TGA using a TA Q-50 instrument (temperature range: room temperature to 900 °C), with a 10 °C/min heating rate under nitrogen atmosphere.

Dynamic light scattering (DLS) and static light scattering (SLS) were used to characterize the conformations of NaHA, TBAHA, TBAHA-acrylate and the graft copolymer in 0.1 M NaCl aqueous solution. The hydrodynamic diameters of the polymers at 25 °C were measured on a PD Expert instrument (Precision Detectors) at a scattering angle of 95°. The diameters and polydispersity indices (PDIs) were averaged over 10 repetitive measurements.

Static light scattering (SLS) experiments were conducted using a DAWN® EOS™ instrument having 18 scattering angles ranging from 13° to 147° (Wyatt Technology Corp., Santa Barbara, CA) with an operating wavelength $\lambda = 695$ nm and a He-Ne laser source. The micro-batch mode was used with a normalized scintillation vial (25 mL) where dilutions (for graft copolymer: 2.17×10^{-5} g/mL – 7.61×10^{-5} g/mL) were made from a concentrated stock solution (0.1M NaCl aqueous solution) at 25 °C. The polymer solutions and solvent for SLS were filtered with 0.45 and 0.2 μ m Millipore nylon membranes three times to remove dust. The specific refractive index increments (dn/dc) were determined using an Optilab DSP Interferometric Refractometer (Wyatt Technology Corp.) at $\lambda = 695$ nm, and the dn/dc values in 0.1M NaCl aqueous solution are 0.142, 0.152, 0.148, 0.139 mL/g for NaHA(132K), TBAHA-A, TBAHA-acrylate (A-acrylate) and graft copolymer (A-5), respectively. ASTRA for Windows software was used to collect and process data.

Atomic force microscopy (AFM) experiments were performed using a Nanoscope IIIa Microscope with Multimode Controller (Veeco Instrument) at ambient temperature and humidity. The tapping mode was employed with an antimony-doped Si tip (radius < 10 nm) at a line scanning frequency of 0.5 or 1 Hz. AFM samples were prepared as follows: Mica was pre-hydrated after cleavage at room temperature and humidity overnight. A droplet (~30 μ L) of solution (~ 1-10 μ g/mL) was deposited on the mica surface, allowed to sit for 2 minutes to allow polymer to be adsorbed, and then dried by gently blowing dry N₂ over the sample for 3-5min. Scanning was carried out immediately after the mica surface appeared dry.

Circular dichroism (CD) was used to investigate the secondary structure of poly(L-leucine) in the graft copolymers (A-5, B1) in aqueous solution. CD spectra were recorded on a Model 202, AVIV Instruments Inc. spectrometer under a nitrogen atmosphere. Experiments were performed in a quartz cell with a path length of 0.1 cm, over a range of 190 – 250 nm at 25 °C, and the data were collected and averaged over two scans. The polymer solutions used for CD were prepared in deionized water with a concentration of 0.2 – 0.5 mg/mL.

Results and Discussion

Functionalization of HA with acryloyl chloride (TBAHA-acrylate)

Sodium hyaluronate (NaHA) is a highly hydrophilic polysaccharide, which is soluble in water, but very difficult to dissolve in common organic solvents such as THF, DMF and DMSO. Improving solubility of NaHA in organic solvents is very important for many functionalization reactions. To this end, the dissolution of NaHA in polar organic solvents was enhanced through transformation of the metal counterion (Na⁺) into the non-metal tetrabutyl ammonium (Bu₄N⁺) counterion. This was accomplished by conversion of NaHA first to hyaluronic acid using ion-exchange resin and subsequent neutralization with tetrabutylammonium hydroxide (TBAOH). This method has been extensively applied in the

past for modification of HA.^{10, 22, 24, 25, 34} The obtained tetrabutylammonium hyaluronate (TBAHA) exhibited improved solubility in DMF (~ 0.01 g/mL) compared to NaHA. In order to graft PLeu onto the back-bone of TBAHA, the primary hydroxyl groups of TBAHA were partially functionalized with acrylate. Predetermined amounts of acryloyl chloride were reacted with TBAHA in the presence of triethylamine in DMF, as shown in Scheme 1.

The primary hydroxyl group is known to be more reactive than the secondary hydroxyl groups on HA, thus esterification with acryloyl chloride most likely occurs at these sites. After the esterification, the acrylate functionalized TBAHA was recovered by precipitation in excess ether and the product was thoroughly washed in ether to remove TEA and hydrolyzed analog of acryloyl chloride, acrylic acid. Typical ¹H NMR spectra of TBAHA and acrylate functionalized TBAHA (TBAHA-acrylate) are shown in Figure 1 (Table 1, run 2).

As shown in Figure 1(b), the vinyl protons observed between 5.4 and 6.3 ppm are attributed to the acrylate moiety on the HA backbone, indicating successful functionalization. The percentage of acrylate attached to the backbone was calculated by integration of vinyl protons and methyl protons of the acetamide group at 2.0 ppm in the TBAHA. The material shown in Figure 1(b) has a functionality approximately 0.5, which means that for every two disaccharide repeating units, there is one α , β -unsaturated carbonyl group attached. As the functionality was calculated on the basis of acetamide groups, identifying actual sites of the esterification among the four hydroxyl groups was not possible.

In order to control the degree of functionalization of TBAHA, varying amounts of acryloyl chloride were reacted with TBAHA as summarized in Table 1. The functionality increases as the ratio of acryloyl chloride and hydroxyl groups on HA backbone increases. However, it is difficult to achieve stoichiometric control due to the highly hygroscopic nature of the reagents, even though TBAHA was pre-dried in vacuum.¹ The functionalization efficiency, *f*, was determined using ¹H NMR spectroscopy as described previously. It was observed that at similar *f* values, the samples from two independent reactions showed distinctly different solubility characteristics (Table 1, run 3 and 4). Sample 4 was only swollen (gel-like) in *d*-DMSO. We believe that this difference could have resulted from the conformation of HA and the position of esterification. Although the solubility of TBAHA-acrylate is expected to be much better than its precursor (TBAHA) in organic solvents like DMSO due to the incorporation of hydrophobic functionality (CH₂=CHCO-), solubility apparently depends on the nature of solvent and the aggregation behavior of hydrophobically modified polysaccharides. The results suggest that the dissolution of TBAHA-acrylate is in equilibrium with different types of aggregates in a particular solvent. The nature of the solvent determines the extent of dissolution. For instance, the TBAHA-acrylate forms a viscous gel-like solution in *d*-DMSO or D₂O at high functionalization (Table 1, run 2), but the solution began to readily flow with the addition of tetrabutylammonium fluoride or sodium chloride. The *f* for sample 2 is 1.00 in DMSO in the presence of salts. The same sample in D₂O in the presence of NaOH or *d*-trifluoroacetic acid completely dissolved forming a clear, transparent solution with functionalities of 0.5 and 0.82, respectively. The gel formation in DMSO in the absence of salt is possibly caused by inter-chain hydrogen bonding due to complexation of the acid hydrogen with TEA (detected as signals at 2.5 and 0.9 ppm in Figure 1b), which are broken down by an ionic strength change upon addition of salt or base, although the dialyzed TBAHA-acrylate had been neutralized with TBAOH.

These solubility differences and the presence of different intermolecular aggregates limit accurate quantification of the functionalization.³⁵ All of these observations suggest that the conformation change of HA leading to complex solubility is, at least in part, due to

incorporation of acrylate functional groups into the HA chains, which is further confirmed by light scattering experiments in the following discussion.

Michael addition to form graft conjugates

The graft copolymer PLeu-g-HA was synthesized by Michael addition reaction between the monotelechelic poly(L-leucine) bearing primary amine terminal functional group and the TBAHA-acrylate. Since HA degrades readily under strongly basic or acidic conditions and because the Michael addition reaction between a primary amine and vinyl (acrylate type) group can be conducted under mild conditions, the Michael addition reaction for grafting poly(L-leucine)-NH₂ (PLeu-NH₂) onto TBAHA-acrylate was conducted in DMF in the presence of TEA at room temperature. Similar mild conditions for Michael addition using aprotic or protic solvents at relatively low temperatures have been extensively reported in the literature.^{16, 36-39}

Accordingly, different concentrations of PLeu-NH₂ with respect to acrylate functionalized TBAHA-acrylate were used for the reaction. Table 2 summarizes the reaction conditions and results of the two types of grafting reactions: batch and one-pot synthesis, where the difference is whether TBAHA-acrylate is separated/purified (batch process, A1-2, A4) or used directly (one-pot process, A3, A5-6, B1-2). It is clear that the one pot process gives a much higher yield due to losses during the purification step in the batch process. Precipitating the final reaction solution into ether to recover polymer may cause loss of HA-oligomers and losses may occur during centrifugation of aqueous dialyzed solution.

The highest acrylate containing TBAHA-acrylate (0.013 mol/L) at high [PLeu-NH₂]/[acrylate]_{TBAHA} gave molar grafting ratio of 0.2 indicating a maximum grafting density for the grafting reaction (Table 2, A1). This could be attributed to the insolubility of the modified TBAHA-acrylate as the 0.2 molar ratio PLeu grafted product is not soluble in water or DMF after the purification process. Thus, the reaction ratio of acrylate to hydroxyl groups was reduced and the amount of PLeu-NH₂ used in the reaction was also controlled in order to obtain soluble product. At low ratios of [acryloylchloride]/[OH]_{TBAHA} the acrylate signals were not detected in the ¹H NMR spectra due to low concentration. However, the presence of α , β -unsaturated ester group in the product (TBAHA-acrylate) was observed at 1738 cm⁻¹ in the FT-IR spectrum (Supporting information).

Although quantifying the functionality of HA modification by ¹H NMR spectroscopy is complicated, a moderate control of the functionality was achieved by varying the ratio of acryloyl chloride/TBAHA as shown in Table 1. The graft copolymer synthesized using TBAHA-acrylate containing low concentration of acrylate groups produced soluble graft copolymer. Typical ¹H NMR spectra of purified graft copolymer (A5, Table 2), TBAHA (A), and PLeu in *d*-TFA are shown in Figure 2, illustrating the successful attachment of PLeu chains onto HA. Comparison of the spectra in Figure 2 shows that the characteristic chemical shifts of PLeu and TBAHA can be differentiated from each other, and both are observed in the final graft copolymer: 4.6 ppm (-CO-CH-NH-, PLeu), 0.95-0.82 ppm ((CH₃)₂-CH-, PLeu) and at 2.25 ppm (-NH-CO-CH₃, TBAHA). The grafting ratio was determined from these signals according to their peak areas ((CH₃)₂-CH-in PLeu, -NH-CO-CH₃ in TBAHA), which is defined as number of PLeu chains in every disaccharide repeating unit in TBAHA backbone. For instance, the grafting efficiency, *f*, determined by the grafting mole ratio shown in Figure 2(c) (Table 2, A5) is 0.037. This value corresponds to 3 or 4 poly(L-leucine) chains per every 100 disaccharide repeating units. No difference in grafting efficiency with respect to the molecular weight of HA was observed in this study.

Although the reaction solution was transparent after centrifugation to remove insoluble PLeu, a very pronounced Tyndall effect was observed indicating the presence of aggregates

even in the DMF reaction medium. Thus, the graft copolymer is less soluble in water than in DMF due to the hydrophobic nature of PLeu. At high grafting ratio, the grafted polymer (large aggregates) in water could be removed by ultra-centrifugation, thus leading to low yield. This would also explain the differences observed for entries B1 and B2 (Table 2), where the final yield decreased with increasing grafting ratio when using DMF and the same purification method. This suggests that there exists an upper limit of solubility in DMF for the final graft copolymer due to precipitation during the reaction. Nevertheless, it is shown that the grafting ratio can be moderately controlled by [acryloyl chloride]/[-OH]TBAHA and/or the [PLeu-NH₂]/[acrylate]_{TBAHA}. The highest grafting ratio for a water-soluble product appears to be nominally less than 4.3 molar %, which is supported by the fact that product B2 (Table 2) is not soluble in water. It was reported that a grafting ratio of 7.8 molar % for poly(lactic acid) grafted HA resulted in a dramatic decrease in water solubility,²⁶ which supports this current work since PLeu is significantly more hydrophobic than poly(lactic acid). In fact, the ¹H NMR spectrum of the graft copolymer in D₂O showed no signals attributed to PLeu, suggesting formation of solid aggregates of PLeu. In theory, the formation of such aggregates would be driven by strong hydrophobic interactions between PLeu chains, which is substantiated by light scattering experiments and AFM as discussed in the following. Although other methods like “grafting from” could be used to enhance the grafting efficiency through functionalization of HA backbone with amine groups which can initiate ring opening polymerization of leucine, it will also suffer from the solubility limitation with increasing chain length of PLeu and the mole % of grafting.

Attempts were made to convert the TBA cation back to sodium by dialysis of aqueous solutions of TBAHA-g-PLeu against NaCl solution, followed by dialysis against water to remove excess NaCl. The ¹H NMR spectrum of the graft copolymer product in *d*-TFA showed characteristic signals from PLeu ((CH₃)₂-CH-, 0.96 ppm; (CH₃)₂-CH-CH₂-, 1.67 ppm) chains without residual signals corresponding to TBA (-CH₂CH₂CH₂CH₃, 3.18 ppm). Thus, it is possible to exchange the counter ion, if desired.

Light Scattering Characterization

The grafting of hydrophobic polypeptide onto HA is expected to impart conformational changes in terms of hydrodynamic volume and radius of gyration, which were measured by light scattering experiments. A typical Zimm plot for graft copolymer (A5) in 0.1 M NaCl aqueous solution is shown in Figure 3 below:

Light scattering data (weight-average molecular weight (M_w), hydrodynamic radius (R_H), radius of gyration (R_g), and second virial coefficient (A_2) for PLeu-g-HA graft copolymer (Entry A5, Table 2), as well as precursors TBAHA, TBAHA-acrylate, and parent NaHA in 0.1 M NaCl aqueous solution, are summarized in Table 3.

As shown in Table 3, the molecular weight of TBAHA decreases from that of the parent NaHA possibly due to degradation of HA under acidic conditions⁹ during the ion exchange process, while R_H and R_g do not change significantly because of incorporation of the bulky tetrabutylammonium cation. The values reported in Table 3 for R_H and R_g of NaHA are very close to those previously reported for $M_w = 160$ K NaHA⁴⁰ and indicate a random coil conformation in 0.1M NaCl.⁴¹ It is observed that the functionalization of HA affects the polymer conformation, the M_w of TBAHA-acrylate being 10-fold larger than that of its precursor TBAHA. Similarly, R_H increased from 18.9 nm to 49.8 nm, and R_g increased from 32.6 nm to 80.4 nm further confirming a substantial change in conformation. The second virial coefficient is a parameter characterizing the solubility of a polymer in a particular solvent, or, more formally, it represents the thermodynamic interactions between polymer and solvent. In 0.1 M NaCl aqueous solution, A_2 decreases upon transitioning from

NaHA to TBAHA to graft copolymer, and even becomes negative for TBAHA-acrylate. This indicates that as the grafting process proceeds, the solubility decreases in water due to the incorporation of hydrophobic groups.

These results confirm the association behavior of TBAHA-acrylate chains, which could be prevented by dilution of the polymer solution, as demonstrated by DLS experiments in Figure 4. There is a concentration dependence of R_H of TBAHA-acrylate, experiencing an abrupt R_H decrease when solution was diluted below ~ 0.07 mg/ml. This was not observed for the graft copolymer, where R_H remained relatively constant with variation of concentration (Figure 4, TBAHA-g-PLeu). From ^1H NMR spectroscopy and TGA (PLeu mass percent is about 7.3 %) measurements, it is estimated that there are on average 12 PLeu chains attached to every HA chain, assuming there was no degradation during the reaction (see Supporting Information). The molecular weight of HA-g-PLeu in sodium salt form is calculated to be: $12 \times 1500 + 132000 = 1.5 \times 10^5$ g/mol, which is substantially lower than the value measured by light scattering. Apparently, there exists strong association between the grafted chains, which could account for such a dramatic difference in apparent molecular weight due to the amphiphilic nature of the graft copolymer. This association or aggregation is observed even though the light scattering experiments were carried out at very low concentrations (the lowest concentration was ~ 20 $\mu\text{g/ml}$). The expected critical micelle concentration (CMC) for these graft copolymers in water is significantly lower than this concentration. It is possible that no unimers of PLeu-g-HA graft copolymer exist in aqueous solution due to the strong hydrophobic interactions between the PLeu chains.

Atomic force microscopy (AFM) and Circular dichroism (CD)

Atomic force microscopy (AFM) in tapping mode was previously used to visualize a single HA chain, taking advantage of its interaction with a mica surface, in order to investigate the conformation of HA.^{42, 43} In this work, AFM was used to examine the conformation and morphology of TBAHA-g-PLeu. Figures 5 and 6 show the AFM height images obtained in tapping mode for TBAHA (A) and the graft copolymer (A5), respectively.

It was reported that a single HA chain (sodium form) can be absorbed either as an extended chain or in a condensed conformation on a freshly cleaved mica surface, depending on sample preparation conditions.⁴² In this study, a strongly condensed conformation of single TBAHA chains was observed on pre-hydrated mica (Figure 5a), with apparent heights of ~ 1.6 nm and widths of ~ 29 nm (averaged in Figure 5b); the previously reported values for NaHA were 0.8 nm and 20 nm for height and width, respectively.⁴² This difference may be attributed to the bulky TBA cation, while the more contracted conformation could suggest weaker adsorption of TBAHA on the mica surface.

From SLS and DLS results, it was found that aggregation occurred in aqueous solution with or without salt for the graft copolymer due to its strongly amphiphilic character. The AFM images of TBAHA-g-PLeu in solution without salt could be visualized as aggregates on mica surface (Figure 6). It is interesting to notice that the independent aggregates appear to be connected to each other by partially non-grafted and extended HA chains that form a local network. The connecting chain is about 0.57 nm in height and about 13.1 nm in width, which is in good agreement with that expected for an extended HA chain^{42,43}.

For the aggregates, their size is about 1.3 nm in height and roughly 40.7 nm to 75.6 nm in width (calculation based on averaging area in Figure 6b) suggesting each aggregate self-assembles into a long, thin sheet. However, the width is likely overestimated due to convolution of the scanning tip of AFM.⁴⁴ One explanation for a fully extended chain conformation is adsorption of aggregates on the mica surface and the so-called “combing force” during sample preparation.⁴² It is plausible that the surface interaction of TBAHA-g-

PLEu and hydration characteristics of the mica surface could lead to the formation of such a networked morphology. The aggregated regions are not correlated to the amount of PLEu grafted onto the HA chain and indicate that the intermolecular aggregates are induced by hydrophobic modification of HA. The aggregates formed in aqueous media clearly reflect self-assembly driven by the hydrophobic polypeptide grafted onto the HA backbone.

As mentioned previously, the individual aggregates are more or less like thin sheets, a typical structure for self-assembled polypeptides that is governed by the ability to adopt secondary structure. To this end, circular dichroism spectroscopy (CD) was used to examine the secondary structure of PLEu in the aggregates. In Figure 7, the CD spectra of TBAHA and PLEu-g-HA from two different HA precursors (A and B) are compared. Since TBAHA itself would give a CD signal mainly due to the $n-\pi^*$ transition of the amide chromophore of acetamido group, which minimizes the CD band near 210 nm,⁴⁵ (as shown in Figure 7(A and B) where molar ellipticity $[\theta]$ of TBAHA was calculated with disaccharide repeating unit as residuals). This effect has to be taken into account when analyzing the CD spectra of our HA-g-PLeu graft copolymers. The molar ellipticity of

PLEu was obtained by normalization of TBAHA concentration and subtraction of TBAHA spectrum from graft copolymer spectrum (Figure 7: PLEu in B1 and A5). This simple subtraction was used to obtain an approximate analysis of the secondary structure of the polypeptide, although this could be problematic⁴⁶ because of the potential conformational changes impacting the CD spectra of TBAHA through interaction with polypeptides. From Figure 7, PLEu in both graft copolymers, A5 and B1, show a single negative maximum at about ~215 nm and a positive maximum at 197 nm, characteristic of a typical β -sheet structure, but with different $[\tau]$ for A5 and B1. The percentage of each protein conformation can be calculated by deconvoluting CD spectra into the three basic secondary structures (coil, sheet and helix).⁴⁷ Thus, the percentages of various conformations of PLEu for the graft copolymer were calculated and are summarized in Table 4.

It appears that in the sample A5, the β -sheet is the main secondary structure, with a significant presence of coils. The morphology observed in AFM possibly contains stacks of β -sheets of PLEu²⁹ as well as intermolecular aggregates with partially non-grafted HA segments. In the B1 graft copolymer, PLEu mainly adopts a β -sheet conformation.

Although the traditional graft copolymers such as HA-g-PNIPAM or HA-g-PLA, can form hydrogels due to hydrophobic interaction between grafted branches, none of them can undergo aggregation with secondary structure (β -sheet) which has been demonstrated herein with PLEu grafted HA. And also our graft copolymers provide a possibility of specific interaction between tissues and poly(L-Leucine), which might be of importance in several applications. A detailed rheological characterization of a peptide-modified hyaluronic acid (HA) derivative is under way in our laboratory. The solution morphology of this PLEu modified HA shows a substantial increase in viscosity and has potential use as an associative thickener in biomedical applications. Preliminary results indicate that PLEu modified HA self-assembles under certain aqueous conditions to form long-lived physical networks; this will be the context of a subsequent publication.

Conclusion

A new method to synthesize poly(L-leucine) grafted hyaluronan via a “grafting onto” strategy using a mild Michael addition reaction between amine terminated PLEu-NH₂ and acrylate functionalized HA (TBAHA-acrylate) has been described. The grafting efficiency, f^* of PLEu on TBAHA could be moderately controlled through functionalization of TBAHA (f) and ratio of $[\text{PLEu-NH}_2]/[\text{acrylate}]_{\text{TBAHA}}$ in our study, thus it is believed that the reaction

could be reproduced to get desired grafting ratios. Grafted TBAHA-*g*-PLeu copolymer with a grafting ratio of 3.7 mole % formed intermolecular aggregates and networks in aqueous solution. At higher ratios, the graft copolymers were not soluble in water. The functionalized TBAHA and graft copolymers were thoroughly characterized by ¹H NMR and FT-IR spectroscopies, and TGA. Conformational information, the nature of the aggregates of the graft copolymer in solution, and the secondary structure of PLeu in the aggregates were studied using a combination of DLS, SLS, AFM and CD spectroscopy techniques. The results showed that there exists strong hydrophobic inter- and intramolecular interactions between PLeu chains in the TBAHA-*g*-PLeu copolymer leading to intermolecular aggregates with partially non-grafted HA segments. Thus, it is very possible to form hydrogels with unique secondary structural information rendered by polypeptide, by adjusting the HA backbone molecular weight and grafting ratio, which may be applied in bio-medical applications as injectable associative thickener and can be used in tissue engineering and drug deliver systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. ^1H NMR spectra of (a) TBAHA: tetrabutylammonium hydroxide neutralized product of hyaluronic acid, in D_2O (b) TBAHA-acrylate (run 2): functionalized TBAHA by reacting TBAHA with acryloyl chloride, purified *via* dialysis against deionized water for three days, in D_2O with NaOH 0.2 mg/ml.

Figure 2. ^1H NMR spectra in d -TFA of (a) TBAHA: tetrabutylammonium hydroxide neutralized product of hyaluronic acid (b) PLEu (c) HA-g-PLEu: Michael addition reaction product, purified by centrifugation in DMF and dialysis against deionized water.

Figure 3. Static light scattering Zimm plot of PLeu-g-HA graft copolymer (Entry A5, Table 2) in 0.1M NaCl aqueous solution at 25 °C.

Figure 4. Concentration dependence of R_H determined by dynamic light scattering experiments for NaHA, TBAHA, TBAHA-acrylate, and HA-g-PLeu in reaction A5 (Table 2) in 0.1M NaCl aqueous solution at 25 °C

Figure 5.
(a) AFM height image for TBAHA (A) on pre-hydrated mica under tapping mode; (b) Section analysis of arbitrary part of (a).

Figure 6. (a)AFM height images for graft copolymer (A5) on pre-hydrated mica under tapping mode, circle showing independent local network; (b) Zoom in scanning of (a); (c) Section analysis of arbitrary part of (b), white line.

Figure 7. Circular dichroism spectra of TBAHA (A from M_w 132K and B from 74K NaHA), poly(L-leucine) in graft copolymer (signal obtained by subtracting TBAHA from graft copolymer, PLeu in graft copolymers in reaction B1 and A5, Table 2)

Scheme 1.
Synthesis of functionalized TBAHA (TBAHA-acrylate) and HA-g-poly(L-leucine)

Table 1Reaction of TBAHA with acryloyl chloride in DMF at room temperature.^(a)

run	[TBAHA] 10 ⁻⁴ mol/L ^(b)	[Acryloyl-Cl] /[TBAHA]	[Acryloyl-Cl] /[^(c) -OH] _{TBAHA}	<i>f</i> ^(d)
1	0.42	1323	1	0.84 ^(e)
2	1.06	559	0.42	0.50 ^(f)
3	0.72	331	0.25	0.38 ^(e)
4	0.47	304	0.23	0.38 ^(f)

^(a) ion-exchange and esterification of all reactions were performed step-by-step, while reaction 4 was performed in a one-pot process. The yields were approximately 50 %.

^(b) concentrations of TBAHA calculated from NaHA ($M_w = 1.32 \times 10^5$ g/mol)

^(c) every disaccharide repeat unit of TBAHA has 4 hydroxyl groups

^(d) functionality of TBAHA : acrylate group per disaccharide repeating unit

^(e) ¹H NMR in *d*₆-DMSO

^(f) ¹H NMR in D₂O/NaOH

Table 2

Reaction Conditions and Results for Synthesis of Graft Copolymer HA-g-PLeu

No.	[acryloylchloride] /[-OH] _{TBAHA}	[acrylate] ^(a) mol/L	[PLeu-NH ₂] /[acrylate] _{TBAHA}	Yield (%)	grafting ratio of TBAHA-g-PLeu ^(e) molar ratio, <i>f</i>	mass %
A1	1	0.013 ^(b)	1.79 ^(c)	10	0.200	32.7
A2	0.25	0.011 ^(b)	1.24 ^(c)	NA	0.110	21.1
A3	0.23	0.014	0.12 ^(d)	46	0.013	3.1
A4	0.42	0.006 ^(b)	1.13 ^(c)	33	0.037	8.2
A5	0.02	0.001	1.00 ^(d)	74.6	0.037	8.2
A6	0.04	0.002	0.69 ^(d)	75.6	0.039	8.6
B1	0.08	0.004	0.21 ^(d)	79.3	0.015	3.5
B2	0.26	0.014	0.15 ^(d)	67.5	0.043	9.5

* , A1, A2 and A4 are batch process; A3, A5, A6 and B1, B2 are one-pot process. The precursors HA with M_w 132K for A and with M_w 74K for B were used.

(a) concentration of acrylate group of TBAHA in system, while it is concentration of acryloyl chloride for one-pot process

(b) Calculated using the relationship: $\frac{m_{\text{TBAHA-acrylate}}}{M_n} \times f \times \frac{1}{V}$, in which M_n is molecular weight of repeating unit of TBAHA-acrylate, is assumed to be: $617(\text{TBAHA}) + 55(\text{CH}_2=\text{CHCO}) \times f$ (functionality of TBAHA-acrylate)

(c) ratio of molar of PLeu-NH₂ and acrylate group of TBAHA in batch process

(d) ratio of molar of PLeu-NH₂ and acryloyl chloride added into the solution in one-pot process.

(e) *f*, number of PLeu chains for every disaccharide repeating unit as determined by ¹H NMR in *d*-TFA and mass % = $f \times 1500 / (f \times 1500 + 617)$.

Table 3

Dilute Solution Properties of NaHA, TBAHA, TBAHA-acrylate, TBAHA-*g*-PLEu in 0.1 M NaCl Aqueous Solution

Sample	$M_w/$ ($\times 10^5$ g/mol)	R_H (nm)	R_g (nm)	2nd virial coefficient, A_2 ($\times 10^{-3}$ mol mL/g ²)
NaHA (132K)	1.332	15.9	35.7 $\pm$ 1.6	4.02 $\pm$ 0.12
TBAHA (A)	0.874	18.9	32.6 $\pm$ 1.7	2.81 $\pm$ 0.06
TBAHA-acrylate	8.946	49.8	80.4 $\pm$ 1.0	-0.24 $\pm$ 0.04
TBAHA- <i>g</i> -PLEu (A5)	51.740	155.0	125.7 $\pm$ 1.5	0.05 $\pm$ 0.04
Poly(L-Leucine)	0.015	NA	NA	NA

Table 4Secondary Structure Characterization of Poly(L-leucine) in graft copolymer TBAHA-*g*-PLeu

Conformation (%) Sample	helix	sheet	coil
A5	0	67	33
B1	4	91	5