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## Single-Step Grafting of Aminoxy-Peptides to Hyaluronan: A Simple Approach to Multifunctional Therapeutics for Experimental Autoimmune Encephalomyelitis

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### Abstract

The immune response to antigens is directed in part by the presence or absence of costimulatory signals. The ability to coincidentally present both antigen and, for example, a peptide that inhibits or activates the costimulatory pathway, would be a valuable tool for tolerization or immunization, respectively. A simple reaction scheme utilizing oxime chemistry was identified as a means to efficiently conjugate different peptide species to hyaluronan. Peptides synthesized with an aminoxy N-terminus reacted directly to hyaluronan under slightly acidic aqueous conditions without the need for a catalyst. The resulting oxime bond was found to rapidly hydrolyze at pH 2 releasing peptide, but was stable at higher pH values (5.5 and 7). Two different peptide species, a multiple sclerosis antigen (PLP) and an ICAM-1 ligand (LABL) known to block immune cell stimulation, were functionalized with the aminoxy end group. These peptides showed similar reactivity to hyaluronan and were conjugated in an equimolar ratio. The resulting hyaluronan with grafted PLP and LABL significantly inhibited disease in mice with experimental autoimmune encephalomyelitis, a model of multiple sclerosis. Aminoxy-peptides facilitate simple synthesis of multifunctional hyaluronan graft polymers, thus enabling novel approaches to antigen-specific immune modulation.

### Keywords

Oxime; aminoxy; hyaluronan; conjugation; graft polymer

### INTRODUCTION

The emergence of nanotechnology has heightened appreciation for the effect of ligand valency and density, a physical component of molecular signaling. Although carefully synthesized colloids have become important in this endeavor, researchers have utilized graft polymers to deliver multivalent signals for decades.[1-5] Antigens grafted to keyhole limpet hemocyanin

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represented an early vaccination scheme that is still employed.[6-9] Today, peptides are routinely grafted to polymers or to colloids to target drug delivery, mediate endocytosis, promote tissue growth, modify immune response, and myriad other functions.[10-13] Grafted peptide number and density have now been recognized as key determinants of molecular response for many of these systems.[14-17]

Conjugation reactions to orthogonally graft peptides to biological polymers under benign conditions are, therefore, highly desired tools for mediating multivalent molecular interactions. In particular, current synthetic approaches for conjugating peptides to glycosaminoglycans would benefit from simplified reaction schemes.[18] These fragile biological polymers cannot be exposed to many of the conditions required for traditional conjugation methods (e.g. solvent, pH, temperature) due to stability concerns.[12] In addition, current approaches often utilize multiple steps, such as activation procedures to form reactive intermediates or the addition of linker molecules to facilitate stability or to improve reaction efficiency.[11, 19, 20] These encumbrances coupled with low product yields and lengthy purification procedures make many of the current conjugation schemes inconvenient for translational medicine or assays and impractical for scale-up.[11, 21] Further problems emerge when conjugating multiple different peptides since small differences in reactivity may yield unpredictable molar ratios.[22] Such issues hinder the exploration of multivalent or multifunctional polymers or colloids with defined ligand densities and ratios.

Oxime chemistry has emerged as a promising conjugation scheme that traditionally utilizes the highly specific reaction of aminoxy groups to aldehydes or ketones.[23-26] The reaction can be carried out in water and avoids harsh catalysts or reaction conditions.[27] In addition, aminoxy reactivity is significantly higher than primary amines, thus conferring the desired specificity for conjugation schemes using polypeptides.[18, 25, 28] One drawback to this approach has been the need for available aldehyde or ketone groups to provide a reactive site, a characteristic that usually necessitates engineering the reactant. Recent reports, however, have suggested aminoxy compounds reacted directly to unmodified hyaluronan, thus prompting exploration of oxime chemistry for orthogonal peptide grafting to this glycosaminoglycan.[23, 29, 30]

Hyaluronan is a flexible, highly water soluble polymer that has been translated into a variety of medical products, making it an attractive backbone for multivalent peptide grafting. Additionally, researchers have shown that controlling the size of the hyaluronan scaffold facilitates placement into lymph nodes after subcutaneous injection. Specifically, hyaluronan conjugates between 40 – 80 kDa showed a high degree of accumulation in lymph nodes and minimal systemic exposure [31]. Thus, hyaluronan conjugates in this size range can passively carry drugs (e.g. immune modulators) to lymph nodes as a means to modify immune response. In the first part of this paper, oxime chemistry was confirmed as a unique and simple approach for grafting small molecules or peptides to hyaluronan to create a lymphatic delivery system.

Furthermore, simultaneous delivery of antigen and an immune modulator represents an attractive approach to antigen-specific immunotherapy. LABL is a peptide that inhibits the binding of intracellular cell-adhesion molecule-1 (ICAM-1) [32]. This peptide was also shown to interfere with immune cell adhesion in immortalized and primary cell culture models [33-35]. Coincident delivery of antigen and LABL, therefore, may inhibit the inflammatory immune response and provide 'antigen-specific' therapy for treating autoimmune diseases. Hyaluronan co-grafted with aminoxy-PLP (a multiple sclerosis antigen) and with aminoxy-LABL was administered subcutaneously in the experimental

autoimmune encephalomyelitis murine model of multiple sclerosis to test the efficacy of this oxime grafting scheme.

## MATERIALS AND METHODS

### Peptide Synthesis

Aminoxy peptides were synthesized using 9-fluorenylmethyloxycarbonyl-protected amino acid chemistry on polyethylene glycol-polystyrene resins. The peptides synthesized were *aminoxy*-LABL (*aminoxy*-ITDGEATDSG, *A $\alpha$* -LABL), a cell-adhesion molecule antagonist, and *aminoxy*-proteolipid peptide (PLP) (*aminoxy*-HSLGKWLGHDPKF, *A $\alpha$* -PLP), a known antigen epitope in multiple sclerosis. Peptides were deprotected, cleaved from resin, and isolated by precipitation in ether. Purification was completed using preparatory high performance liquid chromatography (HPLC) followed by lyophilization. Peptide purity was assessed using analytical HPLC and the identity of the synthesized peptide was confirmed by electrospray ionization mass spectrometry.

### Reaction of Aminoxy Molecules to Monomer/Polymer

The reaction conditions were identical for all reactants used (OCMH, OBZ, OPH, LABL peptide, PLP peptide). Aminoxy monomer was mixed with HA in 1:5 excess in acetate buffered solution (pH 5.5). After addition of all reactive species, the pH was measured and adjusted to  $5.5 \pm 0.1$ . For reactions, 1.3  $\mu$ Mol HA was dissolved into 20 mM acetate buffered solution (pH  $5.5 \pm 0.1$ ). Once dissolved, 1.3  $\mu$ Mol *aminoxy*-peptide was added to the solution. When more than one peptide species was added, 0.65  $\mu$ Mol of each peptide was weighed out separately, then both were added simultaneously. After addition of the aminoxy species, the reaction solution pH was adjusted to pH  $5.5 \pm 0.1$ . The solution was mixed for 16 hr at room temperature. After 16 hours, the samples were dialyzed against ddH<sub>2</sub>O to remove unreacted peptide. Dialysis water was changed every 6 hours for 24 hours. After dialysis, the dialysate was frozen at -70 °C and lyophilized. For kinetic experiments, samples were taken from the reaction vessel at predetermined time points and analyzed immediately.

### Fourier Transform Infrared Spectroscopy

Changes in bonding environments during reaction were monitored using a Bruker Tensor 27 FTIR spectrometer equipped with a ZnSe attenuated total reflectance (ATR) plate (Pike Technologies). Fourier transform infrared (FTIR) spectra were collected at room temperature (25 °C). Data were collected over 256 composite scans with a resolution of 4 cm<sup>-1</sup>. The samples were analyzed in 20 mM acetate buffer at a concentration of 3 mg/mL. Spectra from buffer alone were subtracted from reactant and product spectra using the OPUS spectroscopy software and data were further analyzed using GRAMS/AI (Galactic, Inc.).

### Nuclear Magnetic Resonance Spectroscopy

For structural analysis of the various reactants and conjugates, samples were dissolved in D<sub>2</sub>O to a concentration of 10 mg/mL. H<sup>1</sup> and C<sup>13</sup> spectra were acquired on a Bruker 400 MHz spectrometer at 25 °C.

### Gel Permeation Chromatography

The change in molecular weight of HA graft polymer conjugates was determined using a Viscotek GPC max VE 2001 GPC solvent/sample module, VE3580 refractive index detector, and 270 Dual Detector with right angle light scattering. Samples were separated by utilizing a tandem column setup of an Agilent PL aquagel-OH 60 and Agilent PL aquagel-

OH 40 column in series. Columns were heated to 45°C using an Eldex CH-150 heating box. All pullulan standards and graft polymer conjugates were separated in a 0.1 M Ammonium Acetate, pH 5.0, with 0.25 M NaCl Mobile phase for 30 min.

### High Performance Liquid Chromatography

Peptide was quantified by gradient reversed-phase HPLC (SHIMADZU) using a Vydac HPLC protein and peptide C18 column. The HPLC consisted of a SCL-20A SHIMADZU system controller, LC-10AT VP SHIMADZU liquid chromatograph, SIL-10A XL SHIMADZU auto-injector set at 75 µL injection volume, DGU-14A SHIMADZU degasser, sample cooler, and SPD-10A SHIMADZU UV-vis detector (220 nm). The HPLC-UV system was controlled by a personal computer equipped with SHIMADZU class VP Software. Gradient elution was carried out at constant flow of 1 mL/min, from 100% A to 35% A (corresponding to 0% B to 65% B) for 50 min, followed by an isocratic elution at 75% B for 3 min. Mobile phase compositions were (A) acetonitrile-water (5:95) with 0.1% TFA and (B) acetonitrile-water 90:10, v/v) with 0.1% TFA. At the end of each analysis, the cartridge was equilibrated at initial conditions at 1 mL/min flow rate for 5 min with A.

### Induction of EAE and Therapeutic Study

SJL/J (H-2s) female mice, 4 – 6 weeks old, were purchased from Charles River Laboratory and housed under specified, pathogen-free conditions at The University of Kansas. All protocols involving live mice were approved by the Institutional Animal Care and Use Committee. Mice were immunized subcutaneously with 200 mg of PLP139–151 in a 0.2 mL emulsion composed of equal volumes of phosphate-buffered saline (PBS) and complete Freund's adjuvant (CFA) containing killed Mycobacterium tuberculosis strain H37RA (final concentration of 4 mg/mL; Difco). The PLP139–151/CFA was administered to regions above the shoulders and the flanks (total of four sites; 50 µL at each injection site). In addition, 200 ng/100 µL of pertussis toxin (List Biological Laboratories Inc.) was injected intraperitoneally on the day of immunization (day 0) and 2 days post-immunization. The mice received s.c. injections of each sample, equivalent to 100 nMol PLP /100 µL, on days 4, 7, and 10. For all samples and controls, 100 µL of each was injected. Disease progression was evaluated blindly by the same observer using clinical scoring as follows: 0, no clinical signs of the disease; 1, tail weakness or limp tail; 2, paraparesis (weakness or incomplete paralysis of one or two hind limbs); 3, paraplegia (complete paralysis of two hind limbs); 4, paraplegia with forelimb weakness or paralysis; and 5, moribund (mice were euthanized if they were found to be moribund). Body weight was also measured daily.

### Statistical Analysis

Statistical differences were determined by comparing treated groups to the negative control (PBS) for clinical disease score and body weight. A one-way analysis of variance (ANOVA) followed by Fisher's least significant difference was applied to these data. All analyses were performed using GraphPad Software (GraphPad Software Inc.).

## RESULTS

Initial studies focused on confirming reactivity of aminoxy-containing molecules and the glycosaminoglycan hyaluronic acid (HA). The small molecules used were *O*-carboxymethyl hydroxylamine (OCMH), *O*-benzyl hydroxylamine (OBZ), and *O*-phenyl hydroxylamine (OPH). The structures of these molecules and the HA monomer unit are shown in Table 1.

Fourier Transform Infrared Spectroscopy (FT-IR) was used as a preliminary technique to investigate reactivity between these aminoxy compounds and HA. Data showed distinct changes in the FT-IR bonding environments when reacting either OCMH or OBZ (Figure

1). In both product spectra, a new bonding environment appeared at 1750 cm<sup>-1</sup> as well as a distortion of the amide I peak at 1600 cm<sup>-1</sup>. The peak at 1750 cm<sup>-1</sup> is representative of formation of an oxime bond in the product. Additionally, the peak at 1500 cm<sup>-1</sup> disappeared and the peak environment around 1400 cm<sup>-1</sup> was altered in both products, which may suggest a change in the carboxylic acid environment of the HA.

Analysis of products by <sup>1</sup>H NMR showed the appearance of new peaks in the product spectra. The proton spectra for the OCMH conjugate (Figure 2B) and OBZ conjugate (Figure 2C) showed three new peaks at 5.3 ppm, ~6.9 and 7.5 ppm, and 8 ppm when compared to the HA polymer alone. In the OBZ spectra, there was an additional peak environment at 7.3 ppm, however, this was due to the presence of the aromatic ring and not associated with oxime bond formation. The environment at 5.3 ppm is indicative of the hydrogens alpha to the aminoxy O-NH<sub>2</sub>. The doublet environments at ~6.9 and 7.5 ppm are suggestive of the cis- and trans-conformation of the amide methyl group after addition. Lastly, the environment at 8 ppm suggests addition at the carboxylic acid. Peak shifts are supported by previous studies of oxime addition to polymers. [24, 25, 36, 37] These new peak environments were also not present in the OCMH and OBZ control spectra (Supplemental Figure 2).

In addition, <sup>13</sup>C NMR further supported the observations seen in the <sup>1</sup>H NMR. A new peak appeared that was commensurate with an oxime bonding environment (~150 ppm). Also, changes occurred in the carbonyl carbon environments, especially that of the carboxylic acid at ~174 ppm, and ring environments at ~75 ppm corresponding to the grafted side chains (Supplemental Figure 3). Although the data suggested the aminoxy reacted primarily at the carboxylic acid site, the complex spectra for hyaluronan preclude a definitive assignment of the reaction site (Supplemental Figure 4).

Two peptides with aminoxy N-termini were synthesized to further explore this conjugation strategy. During solid phase synthesis, OCMH was simply reacted as the final coupling step to introduce the aminoxy terminus. The peptides synthesized were *aminoxy*-proteolipid peptide (PLP, *aminoxy*-HSLGKWLGHDPKF), a multiple sclerosis antigen and *aminoxy*-LABL (LABL, *aminoxy*-ITDGEATDSG), a cell-adhesion peptide derived from leukocyte function associated-antigen-1 (LFA-1). The peptides were then reacted with HA following the same procedure as with the small molecule probes. Polymers with grafted LABL, or with grafted PLP, were synthesized as well as polymers with both peptide species grafted.

Gel permeation chromatography was used to monitor the increase in apparent molecular weight (MW) of the peptide-HA conjugate products versus the starting HA (Figure 3A). The starting HA and graft polymer products were compared to pullulan standards to calculate final MWs (Figure 3B). An increase in MW was observed that corresponded to the expected products. Grafted PLP showed the greatest shift since PLP (MW= 1594) is larger than LABL (MW=1032). The 1:1 ratio of grafted PLP:LABL appeared midway between the other graft products as expected. Additionally, the theoretical number of peptides per HA polymer was calculated using the estimated molecular weights (Table 2).

The graft polymers were further analyzed to determine the concentration of peptide that was conjugated to the HA polymer. Oxime bonds are known to be labile to hydrolysis in extremely acidic (pH 2) or extremely basic solutions (pH 9). The graft polymer conjugates were incubated in three different pH solutions and the amount of released peptide was determined using HPLC (Figure 4). At pH 2.0, the peptide was released from HA rapidly and was fully released in ~100 min. At pH values closer to neutral, peptide was released much slower.

A mass balance was calculated for the graft polymer conjugates and released peptide based on total reaction times of 8 hrs or 16 hrs (Table 3). A sixteen hour reaction time was sufficient to obtain a conjugation efficiency >90%.

The final ratio of LABL:PLP peptide on the graft polymer conjugate was calculated based on the amount of released peptide. Peptides were grafted close to the targeted 1:1 ratio (Table 4) suggesting similar reactivity of the two aminoxy-peptides.

An animal study was conducted to investigate how the multivalent presentation of a disease-specific antigen (PLP) alongside a co-grafted cell-adhesion inhibitor (LABL) would affect disease progression. HA with grafted LABL and PLP peptides (~1:1 ratio) was injected subcutaneously in the EAE model and significantly suppressed disease when compared to the PBS negative control on days 11-15 ( $p < 0.01$ ). The graft polymer conjugates were also compared to a dose of HA equivalent to the concentration of HA dosed with the graft polymer conjugate. Although HA is a known CD-44 antagonist and can induce an anti-inflammatory affect, HA did not suppress EAE when compared to PBS while the graft polymer significantly suppressed disease progression (day 11 - 14,  $p < 0.05$ ) (Figure 5A)[38, 39]. The graft polymer conjugates were also compared to a physical mixture of free LABL and PLP peptides to test whether the therapeutic effect was simply due to the presence of both peptide species. The graft polymer conjugate provided significantly lower animal scores when compared to free peptides (days 12-18,  $p < 0.05$ ) (Figure 5B).

The clinical scoring results were further supported by corresponding changes in animal weight during the course of disease and a decrease in the incidence of disease for treated mice (Supplemental Figure 5).

As an additional control, HA grafted with both PLP and LABL was compared *in vivo* against a physical mixture of the individual scaffold components: HA polymer (MW 16900), LABL peptide, and PLP peptide. The peptides did not contain the aminoxy reactive group. Instead, each peptide had an unreactive amine terminus. The HA grafted with PLP and LABL again significantly suppressed EAE ( $p < 0.05$ ) when compared to both the mixture of physical components (Day 12-20) and to PBS (Day 12-21), while the physical mixture showed no statistical difference compared to the PBS injections (Figure 6).

The clinical scoring results were further supported by corresponding changes in animal weight during the course of disease and a decrease in the incidence of disease for treated mice (Supplemental Figure 6).

## DISCUSSION

Oxime chemistry has emerged as a powerful tool in conjugation reactions. Using this scheme, small molecules, Pluronic®, and peptides or proteins have been conjugated to polymers[23, 25, 40-42]. Unfortunately, functionalization of both the reactive adduct and the polymer is typically necessary to confer reactivity. These multistep approaches to conjugation can require lengthy and cumbersome synthesis schemes that ultimately produce low yields of the end product. Additionally, when trying to utilize polysaccharide polymers such as hyaluronic acid, functionalization with a reactive aldehyde group can cause the breakdown of the polymer chain leaving a product that is much smaller than desired[43]. Because of these drawbacks, research has relied heavily on other conjugation techniques such as “click chemistry”, thiol functionalization, or amine coupling[11, 44]. These approaches also have drawbacks relating to reaction conditions and purification schemes. Techniques such as carbodiimide chemistry rely on enhancing the reactivity of carboxylic acids and of primary amines. The potential for side products is high if these groups are present in other parts of the reactive species. Simple conjugation schemes that can provide

high reactivity and selectivity, with increased product yield via simplified reaction conditions will be desired as more complex polymer conjugates are designed.

Hyaluronic acid has been used in oxime chemistry schemes. Researchers have targeted either the “ring open” aldehyde of the reducing end of hyaluronic acid, or modified aldehyde side groups added to the polymer[29, 43]. Chemical modifications were introduced to encourage the oxime reaction to proceed through the traditional route of aminoxy addition to an aldehyde or ketone. Here, the reactivity of aminoxy molecules directly to unmodified hyaluronic acid was explored. Hyaluronic acid contains two carbonyl functional groups on each monomer unit, a carboxylic acid and a methyl amide, that may confer reactivity. For example, Gajewiak et al. theorized reactivity through the carboxylic acid in the synthesis of their adherent glycosaminoglycan adducts[23].

Initially, a series of experiments were designed to monitor changes in chemical bonds of the polymer and reactive species. Data acquired from FTIR and NMR showed reactivity between small molecules containing the aminoxy group (OCMH and OBZ) and unmodified HA. This was evident from changes in the carboxylic acid and amide environments, as well as the appearance of new bonding environments in the FTIR data and the presence of new peaks in the NMR. The polysaccharide ring environments in both the FTIR and NMR data were maintained, suggesting that the HA backbone remained unchanged. The proton NMR data showed the appearance of three distinct environments, all suggestive of oxime reactivity at the carbonyl carbon centers on the HA molecule. These data run counter to conventional wisdom, which has suggested that any latent reactivity of aminoxy molecules to hyaluronic acid would proceed through “ring opening” of the polymer backbone structure to yield aldehydes[45-47].

After validating the reactivity of small molecule probes, aminoxy peptide species were conjugated to unmodified HA with a high degree of efficiency. Previous research that utilized conjugation techniques such as carboimide chemistry showed conjugation efficiencies of small molecule or peptide species from 10-50%[48, 49]. Here, data demonstrated that aminoxy chemistry can be used to modify polysaccharides such as HA via a “one pot” reaction with conjugation efficiencies >90%. The approach reported here could have a dramatic impact on the design of graft polymers, enhancing the ability to synthesize multifunctional conjugates.

The synthesis of multivalent graft polymers is of great interest in the area of immune modulation. Previous research by Dintzis and others have shown that antigen display (i.e. valency, number, density) and polymer characteristics (i.e. molecular weight, solubility) can have dramatic effects on the immune response both *in vitro* and *in vivo*[2, 14, 50-52]. In this historical work, immune response was markedly changed by subtle differences in the physicochemical properties of polymers with grafted antigen. More recently, studies investigating priming of T-cells for tumor immunotherapy demonstrated that the co-localization of multiple cell signals could provide an enhanced and directed immune response[53].

Siahaan and others previously demonstrated that the antigenic peptide PLP covalently linked to the cell-adhesion inhibitor LABL suppressed the autoimmune disease EAE [54-57]. By modifying the *N*-terminus of peptides with an aminoxy group, a multivalent graft polymer conjugate displaying both this antigen for multiple sclerosis (PLP) and the cell-adhesion inhibitor LABL was synthesized. HA with both grafted PLP and LABL was tested *in vivo* in the EAE mouse model, which showed the characteristic peak in clinical scores followed by partial remission of clinical symptoms. The graft polymers significantly suppressed EAE compared to PBS and to HA treatment controls. The graft polymer conjugates also showed

significant disease suppression compared to a mixture of the free peptides as well as a mixture of all components: HA, PLP, and LABL. All control studies were conducted at similar HA or peptide concentration as the HA with grafted PLP and LABL. Thus, both peptide signals must be coincidentally displayed along the polymer backbone to provide therapeutic efficacy, a finding supported by reports from Siahaan and others denoting EAE suppression when directly linking PLP and LABL peptides together [54, 56].

## CONCLUSION

A simple approach for grafting aminoxy compounds to HA was proven using chemical and biological studies. Small molecules or peptides containing aminoxy groups were conjugated to hyaluronic acid in a single step under slightly acidic aqueous conditions. Additionally, this oxime chemistry facilitated conjugation of two different peptides (PLP antigen and LABL) to hyaluronic acid in an equimolar ratio. Graft polymers displaying both of these peptides significantly suppressed disease in the EAE mouse model of multiple sclerosis. The “one-pot” reaction scheme presented in this paper offers a simple approach to the synthesis of graft polymers, colloids, or materials that display multiple copies of one or more functional groups. Such an approach may enable new strategies for antigen-specific immune modulation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## ABBREVIATIONS

<b>HA</b>	hyaluronic acid
<b>OCMH</b>	O-carboxymethyl hydroxylamine
<b>OPH</b>	O-phenyl hydroxylamine
<b>OBZ</b>	O-benxyl hydroxylamine

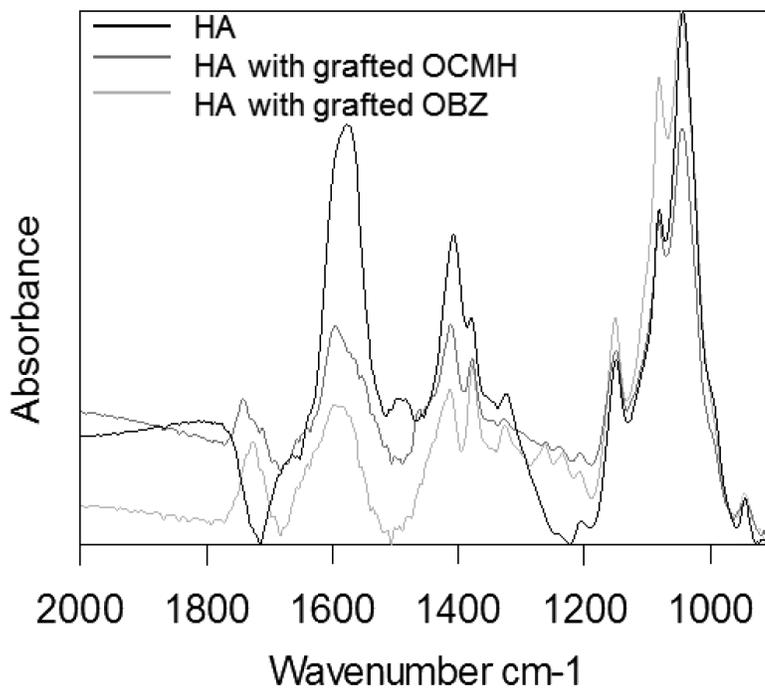
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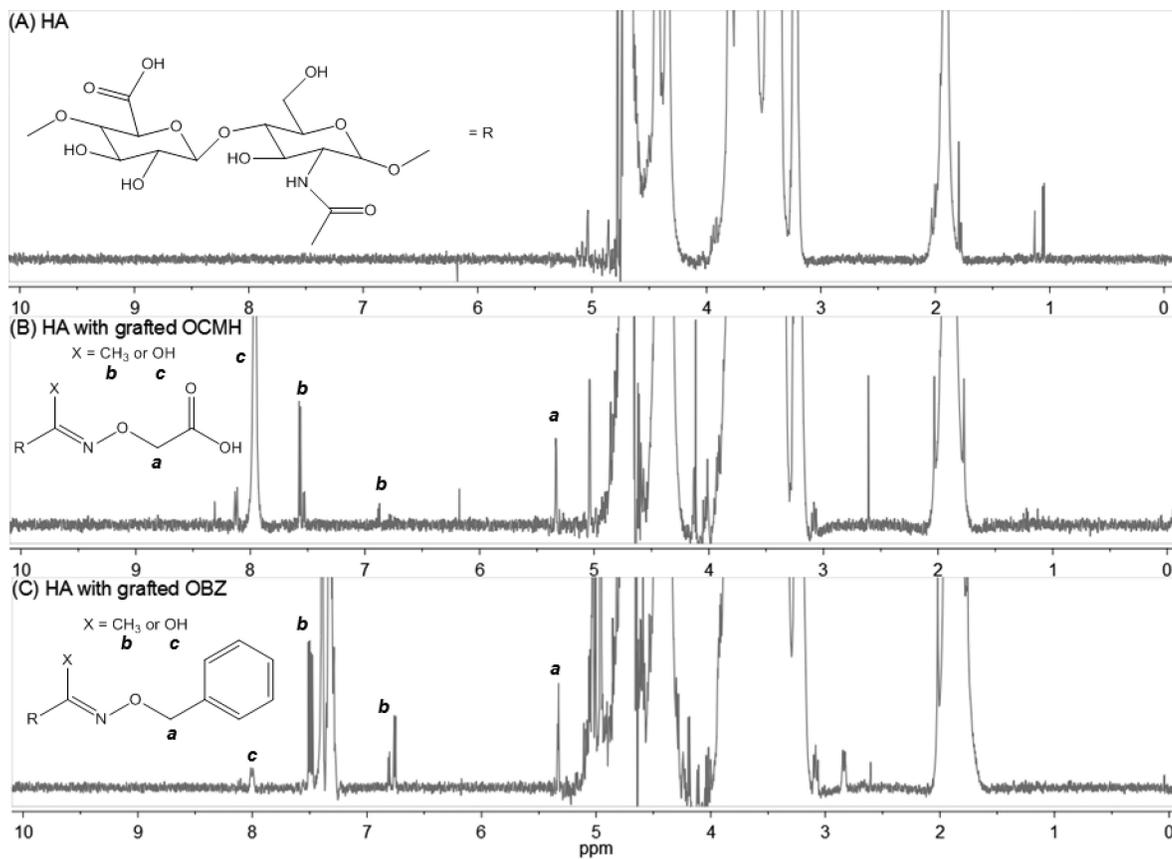
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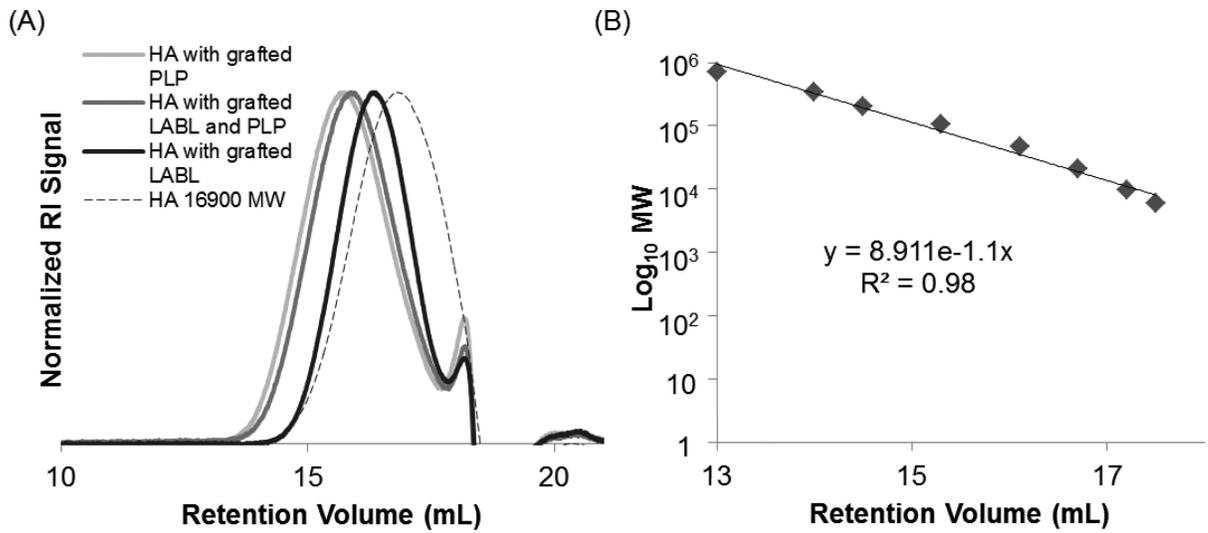
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**Figure 1.** The FTIR spectra of graft polymer products showed new peaks at  $\sim 1750$  and  $\sim 1250$   $\text{cm}^{-1}$  and loss of the peak at  $\sim 1500$   $\text{cm}^{-1}$  when compared to the HA spectra (Supplemental Figure 1).

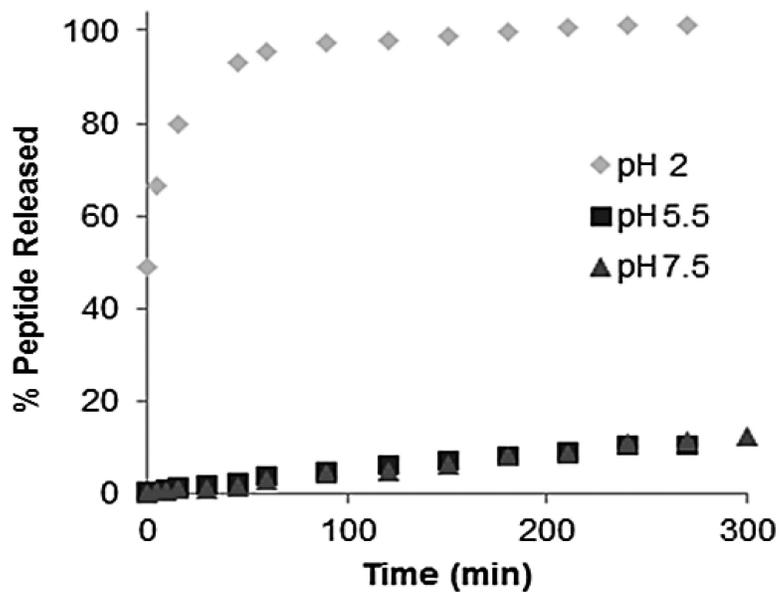


**Figure 2.** <sup>1</sup>H NMR spectra for (A) HA, and for graft polymer products with (B) OCMH or (C) OBZ. The OCMH and OBZ spectra show the appearance of new peaks suggesting oxime bond formation at *a* -5.3 ppm, *b* - ~6.9 and 7.5 ppm, and *c* - 8 ppm. All data was compared to HA and the individual monomer controls (Supplemental Figure 2).

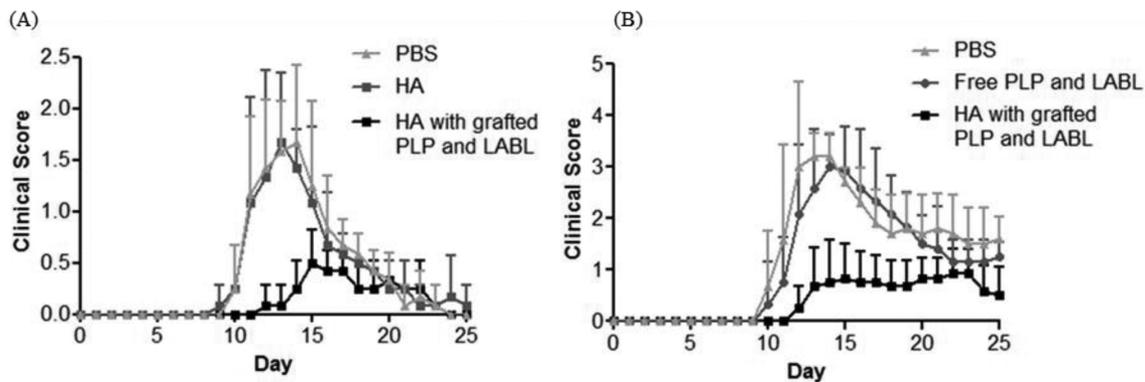


**Figure 3.**

(A) SEC analysis of HA grafted with a single peptide (PLP or LABL) or grafted with a 1:1 peptide mixture showed an increase in MW as compared to unmodified HA. (B) Calibration curve for pullulan standards used to calculate HA polymer graft conjugate product MW.

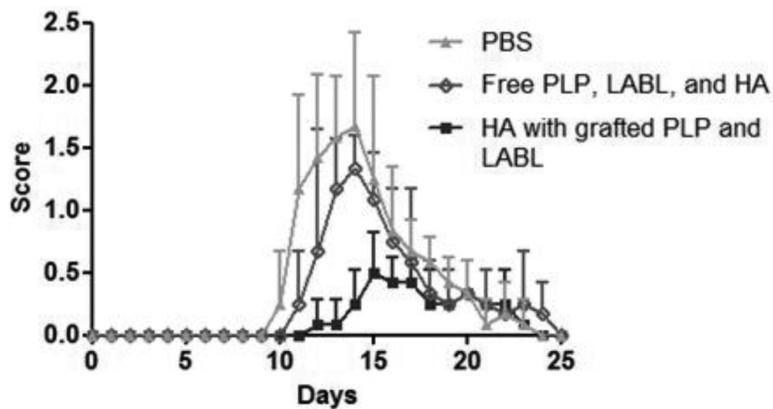


**Figure 4.** The release of LABL peptide from HA was determined in three buffer conditions. Peptide was released rapidly at pH 2.



**Figure 5.**

Graft polymer conjugates were evaluated in the EAE model. (A) HA grafted with PLP and LABL significantly suppressed disease ( $p < 0.05$ ) when compared to both HA polymer (Day 11-14) alone and PBS controls (Day 11-15). (B) A physical mixture of free PLP and LABL peptides showed no effect when compared to PBS control. Conversely, HA grafted with PLP and LABL did significantly suppress disease (Day 12-18).

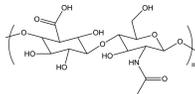
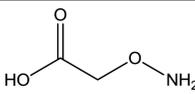
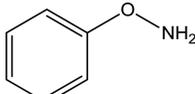
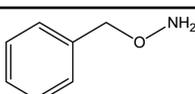


**Figure 6.**

HA grafted with PLP and LABL was compared to a physical mixture of HA, PLP peptide, and LABL peptide. HA grafted with PLP and LABL significantly suppressed disease ( $p < 0.05$ ) when compared to both the physical mixture (Days 12-20) and to PBS controls (Days 12-21). The physical mixture of all components showed no effect when compared to PBS control.

**Table 1**

Structures of hyaluronic acid and aminoxy probes used for testing reactivity.

Molecule	Structure
Hyaluronic Acid Monomer	
O-carboxymethyl hydroxylamine	
O-phenyl hydroxylamine	
O-benzyl hydroxylamine	

**Table 2**

Calculated MWs for graft polymer conjugate products based on SEC.

Sample	Calculated MW *	Theoretical Peptides per HA polymer
HA with grafted LABL	32145	31
HA with grafted PLP	60646	38
HA with grafted LABL and PLP	49080	37

\* Molecular weight estimated from pullulan standards

**Table 3**

Mass balance of grafted HA (31 kDa) and unreacted aminoxy-LABL for the reaction times indicated.

Sample	Area Under Curve	Total Area of Peaks	Reaction Efficiency	Theoretical LABL per HA polymer
Aminoxy-LABL Peptide	8799141	8799141	n/a	n/a
Grafted peptide (8 hr) <sup>*</sup>	5633361	8774823	~64%	26
Unreacted peptide (8 hr) <sup>**</sup>	3141462			
Grafted peptide (16 hr) <sup>*</sup>	8421520	8421520	~96%	41
Unreacted peptide (16 hr) <sup>**</sup>	Not detected			

<sup>\*</sup> Peptide was first hydrolyzed from HA at pH 2

<sup>\*\*</sup> Total unreacted peptide in dialysate washes

**Table 4**

HPLC analysis of peptides released from the HA (31 kDa) graft polymer.

Peptide	Concentration (nMol)	Mol%	Theoretical # Peptide per HA polymer
Aminoxy-LABL Peptide	650	54	22
Aminoxy-PLP Peptide	550	46	19