Published in final edited form as:

J Control Release. 2019 January 10; 293: 36–47. doi:10.1016/j.jconrel.2018.11.007.

Glatiramer acetate persists at the injection site and draining lymph nodes via electrostatically-induced aggregation

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Abstract

Glatiramer acetate (GA) is widely prescribed for the treatment of relapsing-remitting multiple sclerosis, however, the mechanism of action is still not fully understood. We investigated the structural properties of GA and examined alterations to the drug upon injection into the subcutaneous space. First, a variety of biophysical characterization techniques were employed to characterize GA in solution. GA was found to exist as alpha helices in solution with a hydrodynamic radius of ~3 nm in size. To simulate GA behavior at the site of injection, GA was injected into a solution of 1.5 MDa hyaluronic acid (HA). Visible aggregates were observed immediately upon injection and subsequent testing indicated aggregation was driven by electrostatic interactions between the positively-charged GA and negatively-charged HA. *In vivo* testing confirmed GA formed spherical particles in the nano- to micrometer size range, suggesting this mechanism contributes to persistence at the injection site and in draining lymph nodes. The aggregates were found to associate with glycosaminoglycans, suggesting an electrostatic mechanism of induced aggregation like the simulated injection. These novel observations may help explain the complex immunomodulatory mechanisms of GA and adverse injection site reactions seen in patients.

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Declaration of Interest

None

Supplementary Information

Additional synthesis procedures, figures, and data are available under supplementary information.

Supplementary data

Supplementary material

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Keywords

Glatiramer acetate; Copaxone®; SC injection simulation; Multiple Sclerosis

Introduction

Glatiramer acetate (GA), which is the active ingredient in Copaxone®, is one of the most widely prescribed treatments for relapsing-remitting multiple sclerosis (RRMS).¹ After approval, Copaxone® emerged as an important therapeutic option, demonstrating a 30% reduction in the relapse rate of RRMS patients.^{1–2} Compared to other available treatments such as natalizumab (Tysabri®), fingolimod (Gilenya®), teriflunomide (Aubagio®), and dimethyl fumarate (Tecfidera®), Copaxone® has no long-term monitoring requirements, minimal adverse effects, and can be self-administered subcutaneously either at 20 mg per day, or 40 mg three times per week.^{1–2} Copaxone® has minimal if any systemic bioavailability³, however, reactions at the injection site and in draining lymph nodes—such as, injection site mass, erythema, atrophy, and lymphadenopathy—are observed.⁴ Despite the success of Copaxone®, the mechanism of action of GA is still not fully understood.

GA was originally designed as a myelin basic protein (MBP) mimic to induce experimental autoimmune encephalomyelitis (EAE) in mice; however, GA was found to suppress EAE instead.⁵ GA is a heterogenous mixture of copolymers consisting of L-glutamic acid, L-alanine, L-tyrosine, and L-lysine in an approximate molar ratio of 0.14: 0.43: 0.09: 0.34. It has a molecular weight ranging from 2,500 to 20,000 Da, with the average falling between 5,000 and 9,000 Da.² The isoelectric point (pI) of MBP is estimated to be around 10, and since GA is a mimic of MBP, GA is expected to have a large net positive charge.⁶ So far, no defined antigen-specific sequences within this mixture have been identified. Due to its heterogeneous nature, current understanding of GA's physicochemical properties is limited.²

Since GA exhibits a plethora of immunomodulatory effects, controversies among studies exist.^{5, 7–8} GA was reported to bind to MHC class II molecules on the surface of antigen presenting cells (APCs), which was proposed to competitively inhibit the binding and activation of T-cells specific for MBP.^{8–9} Other studies have indicated that polypeptides similar to GA but composed of D amino acids bind to MHC class II molecules, but are not able to ameliorate the disease. This casts some doubt on the importance of competitive inhibition of MBP.^{8, 10–11} In addition, GA-reactive Th2 cells are believed to develop in the periphery, travel to the CNS, and are reactivated in the CNS through cross-recognition of myelin antigens. This ultimately results in the release of anti-inflammatory cytokines.^{5, 8, 12–14} This mechanism is supported by the presence of GA-reactive Th2 cells in the CNS of mice treated with GA, and in some MS patients, cross reactivity between the Th2 cells and myelin basic protein was observed.⁸ Nonetheless, recent studies have demonstrated that GA can suppress autoimmune diseases in a non-CNS antigen-specific manner, which challenges the notion that reactivation in the CNS through cross-recognition of myelin antigens is a requirement.^{8, 15–17} Weber *et al.*¹⁶ have demonstrated that type II monocyte-induced regulatory T cells specific for OVA, which is a nonmyelin antigen, were able to ameliorate EAE without specificity to GA nor recognition with myelin antigens. The

complex immunomodulatory effects of GA suggest a multifaceted mechanism. Additional insight may be garnered by investigating the structure of GA, especially in the context of the subcutaneous space, the injection site.

Pharmacokinetic reports of GA are varied; however, current understanding suggests GA is primarily localized at the site of injection.³ One clinical study investigated subcutaneous injection of a supratherapeutic dose of 60 mg, which reached a maximum blood concentration of 69–605 ng/mL at 15–30 min post-injection.³ The detected GA in systemic circulation was suspected to be in the form of fragments. This indicated that GA has extremely low systemic exposure. Additional clinical data showed 43% of patients suffered from erythema compared to 10% of placebo, 40% of patients had an injection site pain compared to 20% of placebo, and 26% of patients suffered from injection site mass compared to 6% of placebo.⁴ Furthermore, GA does not seem to be able to penetrate the blood-brain barrier into the central nervous system, the location of target tissues in MS and EAE.³ Radioactively-labeled GA was subcutaneously injected into rats and monkeys.¹⁸ The highest amount of radioactivity was detected in the stomach and the thyroid, while the brain exhibited the lowest radioactivity. Thus, local adverse effects and pharmacokinetic studies both point to persistence of the drug at the injection site.

In studies reported here, the physicochemical behavior of GA was studied *in vitro* and *in vivo*. First, the structure of GA was defined as a function of pH and temperature. Subsequent studies utilized hyaluronic acid (HA) to simulate the subcutaneous injection site. GA was observed to form aggregates and precipitate in the presence of HA, and the aggregation mechanism was investigated. Studies in mice confirmed GA also formed spherical aggregates near the injection site and the aggregates associated with glycosaminoglycans in the extracellular matrix. GA persistence at the site of injection and draining lymph nodes was also studied in light of the observed aggregation phenomenon.

Experimental Section

Materials:

Both 20 and 40 mg/mL solutions of Copaxone® 1 mL pre-filled syringes from Teva Neuroscience, Inc. (Kansas City, MO) were donated by the University of Kansas Medical Center. Sodium Hyaluronate of sizes 16 kDa to 1.5 MDa were purchased from Lifecore Biomedical LLC (Chaska, MN, USA). Poly-L-lysine (9 repeating units) and poly-L-Arginine (9 repeating units) were purchased from Biomatik USA, LLC (Wilmington, DE, USA). Poly-L-glutamic acid (50 repeating units) was purchased from Alamanda Polymers Inc. (Huntsville, AL, USA). Sirius Scissor Cartridge packs containing HA based extracellular matrix (ECM) were obtained from Sirius Analytical Inc. (Beverly, MA, USA). Sulfo-Cyanine7 NHS ester was purchased from Lumiprobe (Hunt Valley, MD, USA). PEG amine 5kDa was obtained from Creative PEGworks (Chapel Hill, NC, USA). Immobiline DryStrip gels (IPG strips) and pI markers (pH 5 – 10.5) were purchased from GE Healthcare Life Sciences (Marlborough, MA, USA). For tissue imaging studies, primary antibody rabbit anti-versican monoclonal antibody (Cat#: ab177480) was purchased from Abcam (Cambridge, MA, USA); secondary antibody 18 nm colloidal gold AffiniPure donkey anti-rabbit (Cat#: 711–215-152) was purchased from Jackson ImmunoResearch (West Grove, PA,

USA); Hank's Balance Salt Solution (HBSS) 1X was purchased ThermoFisher Scientific (Waltham, MA, USA); 10% normal goat serum was purchased from MilliporeSigma (Burlington, MA, USA); uranyl acetate, osmium tetroxide, and lead citrate were purchased from Electron Microscopy Sciences (Hatfield, PA, USA). All other analytical grade chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA) and were used as received. To make physiological buffer used for *in vitro* subcutaneous injection simulation, 12.8 g NaCl, 0.18 g MgCl₂ hexahydrate, 0.8 g KCl, 0.4 g NaN₃, 0.4 g CaCl₂ dihydrate, and 4.2 g of NaHCO₃ were dissolved in 2 L of doubled distilled water by stirring. pH was adjusted to 7.4 by sparging CO₂ gas into the solution. Wash buffer used for tissue imaging consists of 0.01% Sucrose/0.1% Saponin diluted in 1X HBSS. The blocking solution consists of 10% normal goat serum diluted in wash buffer. All mice used for the study were maintained in sterile housing under the veterinary supervision of the University of Kansas Animal Care Unit. All procedures were approved by the Institutional Animal Care and Use Committee.

Far-UV Circular Dichroism Spectroscopy:

GA was dialyzed in 10 mM citrate, 20 mM phosphate, and 40 mM borate (CPB buffer) from pH 2 – 12 at 1 pH intervals in 2000 MWCO regenerated cellulose dialysis cassette (22.5–30µm thickness, Thermo Scientific Slide-A-Lyzer). Triplicates of 0.1 mg/mL samples of dialyzed GA were prepared in CPB buffer pH 2 – 12, and the pH was adjusted using 6 M HCl or 6 M NaOH. Circular Dichroism (CD) spectra were obtained using a Chirascan-Plus CD spectrometer (Applied Photophysics Ltd, Leatherhead, UK) equipped with a 150 W Xe lamp, solid-state avalanche detector, and a 6-cuvette position Peltier temperature controller (Quantum Northwest, Liberty Lake, WA). Spectra over a range of 195–260 nm were collected using 0.2 cm pathlength cuvettes, and the temperature was increased from 10 °C to 90 °C at 2.5 °C intervals and an equilibration time of 90 s. Ellipticity values at 222 nm of buffer-subtracted samples from pH 2 – 12 were plotted against temperature. Savitzky-Golay smoothing filter was applied to all spectra using the Chirascan software.

Raman Spectroscopy and Dynamic Light Scattering:

GA was prepared at 10 mg/mL in CPB buffer at pH 2 – 12. A Malvern Helix (Malvern Instruments, Malvern, UK) was used for the combined Raman and Dynamic Light Scattering (DLS) measurements. For Raman, a 785-nm excitation laser was focused on the sample and the spectra were collected for 10 co-additions of 10 s. Background Raman measurements were performed over the same range of temperatures and pH and subtracted from the corresponding sample values. For DLS, a 632 nm laser with 173° backscatter detection was used to minimize multiple scattering effects from high protein concentrations. DLS autocorrelation curves were fit using the CONTIN algorithm and reported as number average diameters. Custom quartz cuvettes containing 25 microliters of sample were placed in a temperature-controlled compartment. Measurements were performed from 20 to 90 °C every 2.5 °C with a 120 s equilibration time between data acquisitions. A screw-in Teflon stopper was used to minimize sample evaporation.

2-Dimensional Gel Electrophoresis:

20 µg of GA was loaded in a 7 cm IPG strip (linear gradient, pH 6 to 11) containing 125 µL of rehydration buffer (urea 8M, CHAPS 2%, Pharmalyte 1% and Bromophenol blue 0.002%). Loading and rehydration steps were performed overnight. Isoelectric focusing was performed in an IPGphor Isoelectric focusing system (Amersham Pharmacia Biotech, Amersham, UK) using the following parameters: constant 300 V for 30 min, gradient 1000 V for 30 min, gradient 5000 V for 1h 30 min and constant 5000 V for 30 min. Isoelectric focusing procedures were calibrated using a high range pI calibration kit (pH 5 – 10.5, GE Healthcare, Chicago, IL). IPG strips were equilibrated for 30 min in 1 mL of equilibration buffer (urea 6 M, Tris-HCl 75 mM, pH 8.8, glycerol 30 %, SDS 2 % and Bromophenol blue 0.002%). The second dimension was performed in an 18% polyacrylamide gel with 30% ethylene glycol. Equilibrated strips were loaded on top of the gel and sealed with Laemmli-SDS buffer containing 0.3% agarose. Separation was performed at a constant 200V during 30 min using tricine running buffer (100 mM Tris-HCl, 100 mM tricine, 0.1% SDS). The gel was stained with Coomassie brilliant blue solution (0.025% coomassie blue in 10% acetic acid).

Images of aggregation as a function of time:

One 1 mL of 20 mg/mL Copaxone[®] pre-filled syringe was injected into a Sirius Scissor cartridge filled with 4 mL of HA (1.5 – 1.8 MDa, 10 mg/mL). The cartridge was placed inside a beaker containing physiological buffer at pH 7.4 on a stir-hot plate at 34 °C. Photos of the cartridge were taken immediately post injection and at 30-min intervals up to 120 min. Precipitates inside the cartridge were abstracted and smeared onto a glass slide. The precipitates were observed using an inverted phase contrast microscope (Olympus IX-83 motorized microscope, Olympus Life Science, Waltham, MA) equipped with a 20x objective at 30-min intervals up to 120 min. The images were collected and analyzed with cellSens software.

Subcutaneous injection simulation release study:

Injection simulation was performed using the Sirius Scissor instrument (Sirius Analytical Inc., Beverly, MA) with the 300 mL chamber filled with physiological buffer and a cartridge containing 10 mg/mL 1.5 – 1.8 MDa HA. Side walls of the cartridge contain regenerated cellulose membranes with 14 kDa MWCO and 150 µm x 50 µm punctures to allow movement of particles. The instrument was setup to run for 3 d, collecting transmittance, pH, and temperature data every 5 s for the first hour, then every 20 s until 12 h, and every 5 min until the end of the experiment. Prior to injection, the pH probes were calibrated and the instrument was equilibrated at 34°C and pH 7.4. A pre-filled 1 mL syringe of 40 mg/mL Copaxone[®] was transferred into a 1 mL Tuberculin syringe with a 25G 5/8 inch needle and was injected into the cartridge with a 1mL syringe adapter. Immediately following injection, 1 mL of physiological buffer was drawn from the chamber and replaced with 1 mL of fresh physiological buffer; this was repeated at 30-min intervals up to 3 h and at 12 h-interval thereafter until the end of the experiment. The concentration of GA released into the chamber buffer was determined using a bicinchoninic acid assay (Micro BCA protein assay kit, Thermo Scientific, Waltham, MA) by constructing a calibration curve with GA

concentrations of 2, 5, 10, 20, 30, and 40 $\mu\text{g/mL}$ in physiological buffer. The concentration at each time point was determined by comparing the optical density (OD) value at 562 nm to the calibration curve in triplicate (data not shown). The concentration values were adjusted to account for GA that was taken out during sampling and then converted to percentage of GA released before plotted as a function of time. The experiment was performed three times to confirm reproducibility.

Precipitate Characterization by Raman Spectroscopy:

The aggregate was generated by adding 100 μL of 40 mg/mL Copaxone[®] to a 1 mL solution of 10 mg/mL 1.5 MDa HA solution. The aggregate was taken out with a micropipette and washed 3 times by adding 1 mL of water to the aggregate and pelleting at 5000 rpm for 5 min with a micro centrifuge (accuSpin Micro, Fisher Scientific, Hampton, NH). The aggregate was smeared onto a quartz glass slide. For reference, a 1 mL pre-filled 40 mg/mL Copaxone[®] sample was dialyzed against water and subsequently lyophilized with a VirTis Freezemobile 12 XL (Warminster, PA) to obtain a solid form of the pure drug. The Raman spectrum of the aggregate, lyophilized GA, and 1.5 MDa sodium hyaluronate powder were attained using a LabRAM ARAMIS Raman microscope spectrometer (LabRAM HORIBA Jobin Yvon, Edison, NJ) equipped with a HeNe laser ($\lambda = 633 \text{ nm}$, a laser power of 17 mW) as an excitation source. The instrument was setup with a 200 μm confocal hole, 150 μm wide entrance slit, 600 g/mm grating, and 100x objective Olympus lens. The quartz slide was mounted onto a high-precision, computer-controlled x-y stage and a spectra range of 400 cm^{-1} to 3600 cm^{-1} was examined. Data processing was done using LabSPEC 5 software (HORIBA Jobin Yvon).

Analysis of precipitation using polypeptides:

Solutions of Poly-L-lysine (9 repeating units), poly-L-arginine (9 repeating units), and poly-L-glutamic acid (50 repeating units) were prepared in water at concentrations of 14.2 mg/mL, 17.2 mg/mL, and 6.73 mg/mL, respectively, to reflect the molar ratio of these amino acids found in Copaxone[®]. To 250 μL of 10 mg/mL 1.5 MDa HA, 50 μL of each polypeptide was injected and the formation of precipitate by each polypeptide was documented.

Fluorescent Imaging of Precipitates:

Fluorescent precipitates were generated by adding 50 μL of 20 mg/mL rhodamine B-labeled GA dissolved in deionized water to a 250 μL solution of 10 mg/mL coumarin-labeled HA dissolved in deionized water. The precipitate was removed with a micropipette and washed 3 times by adding 1 mL of water to the precipitate and pelleting it at 5000 rpm for 5 min with a micro-centrifuge. Unlabeled precipitates used as controls were generated with 20 mg/mL unlabeled Copaxone[®] employing the same method outlined above. The resulting precipitates were placed onto a glass slide and left to dry overnight. The images of the precipitate were obtained using an inverted fluorescence microscope (Olympus IX-83 motorized microscope; Olympus Life Science Solutions, Waltham, MA) with attached 20x and 40x objective; differential interference contrast; 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, Ex: 378–407 nm, Em: 412–454 nm), Fluorescein (FITC, Ex: 458–491 nm, Em: 497–535 nm), and tetramethyl rhodamine isothiocyanate (TRITC, Ex: 540–571 nm, Em: 577–616 nm).

nm) filters. Image analysis was performed using cellSens software. Exposure time for each dye was consistent between all samples to permit comparison.

Effects of HA size and concentration on precipitate formation with GA:

The concentration and size of HA was varied to determine the effect on the formation of precipitate with GA. For the varying HA concentration experiments, 20 μ L of 20 mg/mL GA was added to 1 mL of 1.5 MDa HA at concentrations of 10 mg/mL to 0.156 mg/mL (serial dilutions from 10 to 0.156 mg/mL by halving the concentration per dilution). Visual inspection was performed on each vial to detect precipitation, and each vial was classified as lump, strand, cloudy, or no visible particles. For the varying HA size experiments, the same methodology was applied but using 10 mg/mL HA with sizes ranging from 1.5 MDa to 16 kDa.

In vivo GA migration imaging:

Mice used in study were fed with Harlan 2912 irradiated low chlorophyll diet (Envigo, Indianapolis, IN, USA) for at least one week prior to imaging to reduce organ and skin auto-fluorescence. Two female SJL/J mice (20–25 g, Envigo, Indianapolis, IN) were anesthetized with isoflurane. The method is adapted from Bagby *et al.*¹⁹ Mouse hair near the abdomen and limbs was removed by depilatory cream. One mouse was injected with 10 μ L of 20 mg/mL sulfo cyanine 7-labeled GA at the center of a footpad every hour for 4 h in the following order: right hindlimb at the 1-h time point, right forelimb at the 2-h time point, left hindlimb at the 3-h time point, and left forelimb at the 4-h time point. Similarly, the control mouse was injected with 10 μ L of 20 mg/mL sulfo cyanine 7-labeled 5 kDa PEG at the center of each footpad every hour for 4 h on the opposing limb from the other mouse (i.e. left hindlimb at 1-h, left forelimb at 2-h, right hindlimb at 3-h, and right forelimb at 4-h). For imaging, the mice were anesthetized with 4% inhaled isoflurane in oxygen and placed adjacent to each other and both bright-field and fluorescent images were taken using a MaestroFlex whole body imager (Cambridge Research and Instrumentation, Woburn, MA) employing an excitation filter of 710–760 nm and a longpass emission filter of 800 nm. The mice were not imaged for more than 5 min in each session. Each image focused on one pair of limbs representing one specific time point. This permitted the direct comparison of fluorescence intensity between labeled GA and labeled PEG control. The draining lymph nodes near each site of injection was later resected and imaged to determine whether the GA or PEG is draining towards this lymph node. Images were taken using the auto exposure function (limits pixel saturation to <2%) with a black tape covered background to improve the dynamic imaging range. No changes were made to the f-stop, camera position, and zoom settings throughout the experiment. Measurements of fluorescent intensity were performed by defining a region of interest around the limbs or the resected lymph nodes, and measuring the average relative fluorescent intensity of the same area for both mice injected with either cyanine 7-labeled GA or cyanine 7-labeled PEG.

Tissue Imaging:

One female SJL/J (20–25 g, Envigo, Indianapolis, IN) mouse was anesthetized under isoflurane. The mouse was injected with 20 μ L of 20 mg/mL Copaxone[®] in the right hindlimb, and 20 μ L of 40 mg/mL mannitol in the right fore-limb. The limbs with the

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footpad attached were harvested 30 min post injection. Mouse flexor and extensor muscle tissues were removed proximally and distally from the right (mannitol-injected footpad) forelimb. Tibialis anterior muscle tissue was removed proximally and distally from the right (Copaxone®-injected footpad) hindlimb. Forelimb and hindlimb muscle tissues were immediately immersion fixed in ice-cold 4% paraformaldehyde for 1 h and then cut into small blocks of approximately 1 μ m. Forelimb and hindlimb muscle tissue blocks were washed with Hanks Balanced Salt Solution (pH 7.4; HBSS)/ 0.01% sucrose 4 times, 5 min each, at room temperature (RT) and then permeabilized with HBSS/0.01% sucrose/0.1% saponin 4 times, 10 min each. To prevent nonspecific binding of the antibody, forelimb and hindlimb muscle tissue blocks were immersed in blocking solution for 3 h at RT, shaken, and then incubated with the primary antibody overnight at 4°C. The primary antibody, rabbit anti-versican monoclonal antibody, was diluted in blocking buffer with 0.01% Triton X-100. The secondary antibody (18 nm Colloidal Gold AffiniPure Donkey anti-rabbit) was diluted 1:20 in HEPES (25 mM, pH 7.4)/ 0.01% sucrose/ 0.1% saponin. After washes (5 times, 5 min each) to remove the unbound antibody, the tissue blocks were incubated with the secondary antibody for 3 h at RT, shaken, and then washed with HBSS/0.01% sucrose/0.1% saponin. Forelimb and hindlimb muscle tissue blocks were post-fixed with a Freeze-Substitution Cocktail (FSC; 2% uranyl acetate, 0.2% lead citrate, 2% osmium tetroxide, and 1% p-Phenylenediamine in dried absolute acetone) and placed overnight at -80 °C. On the following day, FSC was changed, filtered, and gradually warmed to -20 °C overnight. The next day, FSC was changed, filtered, and gradually warmed to 4 °C for 4–6 h. After rinsing out the FSC 2 times (5 min each) with dry acetone, the muscle tissue blocks were dehydrated with acetone, hexamethyldisilazane (HMDS; 3:1, 1:1, 1:3) for 1 h per ratio at RT. To complete the dehydration, muscle tissue blocks were immersed in 100% HMDS and placed in a desiccator under vacuum until dry. A second group of muscle tissue samples from the injected forelimbs and hindlimbs (i.e., controls) were dissected and processed for scanning electron microscopy. No immunohistochemical procedures were performed in this second group of muscles. The blocks of muscle tissue were fixed in Karnovsky's fixative (2.5% Glutaraldehyde/2% paraformaldehyde) and diluted in 2 mM cacodylate buffer for 1.5 h at RT. After a 5 min acetone wash, the blocks were post-fixed in 2% osmium tetroxide in dried absolute acetone overnight at RT. The next day, the fixative was replaced by 1% p-Phenylenediamine in acetone for 1 h at RT. The solution was removed and the muscle tissue blocks were stained with 4% uranyl acetate diluted in dried absolute acetone for 1 h at RT. After rinsing the uranyl acetate 2x (5 min each) with dry acetone, the muscle tissue blocks were dehydrated with acetone, Hexamethyldisilazane (HMDS; 3:1, 1:1, 1:3) as described above. For image acquisition, each muscle sample tissue block was placed on the aluminum stub over carbon tape. Only immunolabeled muscles showing charging effects were sputter-coated with less than 5 nm gold palladium using an EMS Quorum 150RS sputter coater. Images were acquired on a Hitachi SU8230, Cold Field Emission Scanning Electron Microscope with a secondary electron detector (SE), at accelerating voltages ranging from 1.0 to 5.0 kV, aperture 2 and a working distance between 7.0 to 8.0 mm. Energy Dispersive Spectroscopy (Oxford Instruments, X-MaxN 80 mm²) was performed to further characterize GA within the forelimb and hindlimb muscle tissue blocks.

Results and Discussion:

Structural Characterization of GA.

To determine whether GA forms higher-ordered structures in solution, biophysical characterization techniques such as Raman and circular dichroism (CD) spectroscopy were performed. Amide I (1640 to 1700 cm^{-1}) and amide III (1210 – 1310 cm^{-1}) regions of the Raman spectrum (for full list of Raman peak assignments, see supplementary Table S1) reflect the peptide backbone structure, which provides secondary structural information (Figure 1A).²⁰ The 1645 cm^{-1} and 1680 cm^{-1} peaks in the amide I region (Figure 1B) represent α -helical and β -sheet structure respectively. The ratios of these two peaks as a function of temperature from pH 2 – 12 were plotted (Figure 1C). As temperature increased, the amide I ratio decreased. As a function of pH, however, the amide I ratio increased as pH increased. A similar trend was observed in CD spectra where the magnitude of the ellipticity at 222 nm , which corresponds to α -helical structure, decreased as the temperature increased but increased as the pH increased (Figure 1D). Both Raman and CD results suggested the presence of α -helical structure that decreases with increasing temperature but increases with increasing pH. Furthermore, an isobestic point was observed in the Amide I spectra (Figure 1B) at around 1657 cm^{-1} , which suggests the existence of an equilibrium that is sensitive to temperature between alpha helices and beta sheets.

Tyrosine regions in the Raman spectra (Figure 1A) reflect tyrosine environments, which can be useful for probing tertiary structural information.²⁰ Thus, tertiary structure was examined by calculating tyrosine ratios of $1600/1615\text{ cm}^{-1}$, and $850/830\text{ cm}^{-1}$ (Figure 2). The tyrosine ratio of $1600/1615\text{ cm}^{-1}$ is indicative of the charge state of tyrosine²¹, whereas the tyrosine ratio of $850/830\text{ cm}^{-1}$ provides information on the hydrogen bonding state of the tyrosine hydroxyl group.²⁰ When the ratio is plotted against temperature, a significant change in the ratio over a small range of temperatures would typically indicate a change in tertiary structure. However, other than the possibility of a very slight inflection point near $55\text{ }^{\circ}\text{C}$, no other significant changes in tyrosine ratios were observed when a temperature ramp from 20 to $90\text{ }^{\circ}\text{C}$ was performed (Figure 2C & D). Nevertheless, it is interesting to note that the $1600/1615\text{ cm}^{-1}$ ratio for pH 12 was significantly higher than the other pH values. This probably reflects the fact that the hydroxyl group at pH 12 is fully deprotonated and thus carries a negative charge. Based on these results, no substantial tertiary structural change appears to occur with GA. This is most easily explained by an absence of significant tertiary structure, but not necessarily secondary structure.

Our results agree with Anderson *et al.*,²² who demonstrated that both Glatopa[®], the generic version of GA, and Copaxone[®] can form α -helical structures in solution. This result may at first appear surprising considering the randomness of the amino acid sequence and heterogeneity of GA amino acid particles. The phenomenon of composition drift during the manufacturing process may offer some explanation.²² The polymerization of GA undergoes random propagation through amino acid N-carboxyanhydrides. More abundant and more reactive amino acids will most likely be the first to react and thus be more concentrated at the beginning of the chain. As these amino acids are consumed, the less abundant and less reactive amino acids will build up near the end of the chain. Next, the de-polymerization

step in GA manufacturing would create fragments that may not adhere to the initial amino acid molar ratio but rather are skewed towards certain amino acid combinations. This may create a higher probability of certain sequences in the overall GA composition and may result in certain fragments (e.g. amphiphiles or alpha helical sequences) forming higher order structure²³.

DLS Particle Sizing Characterization.

To determine the particle size distribution of GA, DLS was performed on GA samples over the pH range of 2–12. Colloidal stability was determined by static light scattering (SLS) based on the scattered light count rate, which correlates to particle size. Based on the scattering plot (Figure 3A), scattering decreased from 20 to 40 °C at pH values of 4 – 10, while it remained low at the extreme pH values of 2 and 12. This could possibly be explained by electrostatic interactions between the peptides. At extreme pH values of 2, GA is overall positively charged, and at pH 12, GA is overall negatively charged. Peptides at the extreme pH values will repel each other, which may have resulted in the small scattering values even at low temperatures. The charge of the peptides at middle pH values of 4–10, however, are less obvious and may have resulted in electrostatic attraction between the positively and negatively charged peptide segments, resulting in the formation of larger entities. This may in turn have led to higher scattering values at low temperatures. These tangled peptides may have then unfolded as the temperature was increased, which resulted in a decrease in scattering. Above 50 °C, significant increases in scattering was observed in GA samples at pH 10 and 12. This may have been caused by aggregation at higher temperatures. This is especially true for the GA sample at pH 10, where visible aggregates were observed at the end of the experiment.

Based on dynamic light scattering (DLS) experiments (Figure 3B), GA at all pH values appeared to have a mean size around 3 nm, which is the expected value based on GA's molecular weight range of 2.5 – 20 kDa. However, the particle diameter appears to decrease from 20 to 40 °C at all pH values. This possibly suggests an initial dissociation of aggregated peptides or collapse when heated. Particle sizes at pH 10 could not be determined due to the increased turbidity caused by aggregation. It is important to note, due to the heterogeneous nature of GA, the polydispersity values of all samples were generally above 0.5.

DLS and particle sizing of Copaxone[®] has previously been performed by Teva Pharmaceutical Industries, Ltd., and was recently described by Conner *et al.*²⁴ as a stable colloidal suspension that form two populations of sizes around 5 nm and 111 nm. Based on the intensity of the DLS signals, two to three populations of GA in solution were observed (Supplementary Figure S1), but reporting DLS values based on a number analysis proved a more accurate description since species that were much larger than 5 nm accounted for less than 1% of the total number of particles.

2D Gel Electrophoresis.

Two-dimensional gel electrophoresis was used to determine the pI and the molecular weight distribution of GA. Isoelectric focusing, the first dimension in a 2D gel, was performed

using immobilized pH gradient (IPG) strips that had a pH range of 6 – 11. Subsequently, SDS-PAGE was performed using a gel that contains 18% total acrylamide (T) and 5% cross linker (C). Copaxone® formed a large smear in the basic regions of the gel and the size distribution based on molecular weight marker spanned from 5 to over 40 kDa (Figure 4). By using pI markers with pI ranges of 5.2 to 10.25 run on a separate gel, the pI of GA at its midpoint was determined to be 9.75 and the range was 9.25–9.82. This result would explain the aggregation seen at pH 10, which is close to the pI of GA. Although the appearance of GA as a smear on the second dimension makes quantitative analysis difficult, qualitatively, it reflects the heterogeneous nature of GA.

***In vitro* Subcutaneous Injection Simulation.**

A novel subcutaneous (SC) injection site simulator system (Sirius Scissor) was used to predict the rate of drug dissipation from the injection site. Since HA is a major component that fills the spaces within the collagen fiber network, HA of sizes ranging from 1.5 to 1.8 MDa are typically used to fill this instrument's cartridge to mimic the extracellular matrix (ECM) found in the subcutaneous space.^{25–26} Here, we observed that immediately after injecting 20 mg of GA into the HA solution, visible precipitates in the micron size range began to form and settled to the bottom of the cartridge (Figure 5A, i). Phase contrast microscope images showed a gradual decrease in size of the precipitates over time (Figure 5A, ii). To estimate the amount of drug released from the injection site, another experiment was conducted by injecting 40 mg of GA into the cartridge containing the HA solution and quantifying the amount of GA released into the simulator chamber over 3 d. Over the first 3 h, approximately 20% of the GA was steadily released from the cartridge into the chamber (Figure 5B), and roughly 70% of the GA was released over 3 d (Figure 5C). Release began to plateau after 48 h. These results suggested that about 30% of GA might still be at the site of injection. Visible precipitates were observed in the cartridge at the end of day 3.

GA underwent a significant physical change from a clear solution to one forming large visible particles in the cartridge. The *in vitro* release study indicated that GA was able to retain at the injection site for days. This may help to explain some of adverse effects associated with GA such as injection site mass, inflammation, erythema and pain, etc.²⁷

Precipitate Characterization.

To characterize the precipitate formed when injecting GA into HA, Raman spectra of the precipitate, lyophilized GA, and HA itself were obtained (Figure 6). The spectrum of the precipitate resembles an amalgamation of the spectra of both GA and HA. The amide I (1640 to 1700 cm^{-1}), amide III (1210–1310 cm^{-1}), and tyrosine (820 to 850 cm^{-1}) bands from lyophilized GA were clearly present in the precipitate. The pronounced C-H band (1390 to 1500 cm^{-1}) and C-C bands (920 to 980 cm^{-1}) from HA were also seen in the precipitate spectrum. This result indicated the precipitate was a heterogeneous aggregate composed of both GA and HA. To confirm this finding, GA was derivatized with rhodamine B (RGA) and 1.5 MDa HA with coumarin (CHA). The precipitate was generated by adding 20 mg/mL of GA to a 10 mg/mL solution of HA. The resulting precipitate was observed under a fluorescent microscope (Figure 7) equipped with DAPI and TRITC filter sets. The precipitate generated using RGA and CHA was clearly fluorescent under both conditions,

indicating the precipitate does contain both GA and HA. The excitation and emission spectra of RGA and CHA can be found in the supplementary information (Figure S2).

The formation of the GA-HA aggregate was suspected to result from electrostatic interaction between GA and HA due to their oppositely charged nature. At physiological pH, GA is predominately positively charged due to the large proportion of lysine residues, while HA is predominantly negatively charged due to the deprotonated D-glucuronic acid. To determine whether electrostatic interactions indeed played a role in the formation of aggregates, positively charged poly-lysine (PLL) and poly-arginine (PLA), and negatively charged poly-glutamic acid (PLG) peptides were added to a 10 mg/mL solution of 1.5 MDa HA. Precipitates only formed with PLL and PLA but not with PLG (Supplementary Figure S4), which suggests electrostatic interactions play an important role in the formation of GA-HA aggregates.

Since HA plays an important role in forming aggregates with GA, we explored the effects of HA size and concentration on the formation of visible GA-HA aggregates. GA at 20 mg/mL concentration was added to solutions of HA at varying concentrations and sizes. The resulting solutions were classified as lump, strand, cloudy, or no visible particles (Table 1). A decrease in concentration or size of HA reduced the size of the GA-HA aggregates; the effect was more prominent when decreasing the size of HA. GA-HA aggregates formed a translucent solution when the size of HA was below 357 kDa. These observations further demonstrate that aggregate formation is dependent on HA.

***In vivo* GA migration imaging Using Cyanine 7 labeled GA:**

To determine whether GA persists at the injection site *in vivo*, whole body fluorescent imaging was performed on mice injected with either cyanine 7-labeled GA (cy7-GA) or cyanine 7-labeled 5 kDa PEG (cy7-PEG) as a control into the footpads of mice. Both GA and 5 kDa PEG were derivatized with cyanine 7 near-IR dye using an NHS ester conjugation. The concentration of cyanine 7 dye attached to GA or PEG was determined using fluorescence calibration curves (Supplementary, Figure S3), and it was determined to be $343 \pm 69 \mu\text{M}$ and $760 \pm 52 \mu\text{M}$ for 20 mg/mL cy7-GA and 20 mg/mL cy7-PEG, respectively. Prior to imaging, 10 μL of either cy7-GA or cy7-PEG at 20 mg/mL concentration was injected into the footpads of a different mouse every hour for 4 h. The footpads were observed under visible light followed by near-IR fluorescent imaging (Ex: 710–760 nm, Em: 800 nm longpass). The images (Figure 8A) and the injection site average fluorescence intensity at a defined area around the limbs (Figure 8B) both demonstrate that while there is no noticeable difference in fluorescence intensity between GA and PEG after 1 and 2 h post-injection, the intensity at the injection site for PEG was noticeably less than GA after 3 and 4 h. This agreed with *in vitro* simulated injection results in which GA was retained in the cartridge during a similar time interval. The draining lymph nodes were resected and examined under near-IR fluorescence to determine if GA or PEG was draining towards the lymph nodes (Figure 9). Both GA and PEG traveled to the lymph nodes within the first hour post-injection, however, cy 7-GA was able to persist in the lymph nodes after 3–4 h, while cy7-PEG drained away.

The *in vivo* imaging studies supported the *in vitro* results by demonstrating that GA does persist at the site of injection in mice despite its intrinsically soluble nature. In addition, GA could travel to the draining lymph nodes and compared to the control, GA remained in the lymph node for over 4 h. For SC injections, the lymphatic system preferentially uptakes particulates with sizes ranging from 10 to 100 nm.¹⁹ Particles less than 10 nm may be absorbed into blood vessels while particles larger than 100 nm have a greater propensity to remain at the site of injection. GA-HA aggregates may have a broad size distribution, ranging from nanometer particles that travel towards the lymph nodes to micron-sized particles that stay at the injection site. Our results may provide a viable explanation for the lymphadenopathy that was observed in 7 to 33% percent of patients in clinical trials.^{27–28} In addition, GA may stimulate lymphocytes at the draining lymph nodes, which could explain some of the immunomodulatory effects reported elsewhere.^{5, 7–8}

Murine tissue imaging:

To further examine the effect of GA near the injection site, the limbs, which were injected with either GA or mannitol, were sectioned and imaged using scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS). GA formed polydispersed spheres that range from 0.1 to over 10 μm in size and were embedded in the extracellular matrix of the tissue near the injection site (Figure 10A, C). These spheres were not observed in the control tissue samples injected with mannitol (Figure 10B). To confirm that the spheres are not inorganic artifacts, EDS images were taken of the larger spheres. The results demonstrated that the spheres are primarily composed of carbon atoms (Figure 10B). To further identify the presence of these spheres further away from the injection site, proximal tibialis anterior muscle tissues were collected and observed under SEM. However, unlike tissues near the injection site, no spherical aggregates were observed (Figure S5). The strand-like structures observed in the SEM images were identified as containing versican through labeling with primary rabbit anti-versican monoclonal antibody, followed by secondary 18 nm colloidal gold anti-rabbit antibody (Figure S5). These results suggest GA can localize at the injection site in the form of nanometer and micron-sized spheres. Although the spherical particles found at the tissue near the site of injection differ in morphology compared to the aggregates seen *in vitro*, the results still indicated that GA can localize at the site of injection through the formation of particles. The morphology and size of the aggregates *in vitro* were highly dependent on the concentration and size of HA (Table 1). Thus, ECM components at the injection site could have induced the morphological differences observed between the *in vivo* and *in vitro* data.

As a potential mechanism of action, GA particles at the injection site could be redirecting the immune response away from the central nervous system (CNS) towards the injection site. With recent advancements in biomaterials, injectable polymers and scaffolds that can shift morphology post-injection have been explored to recruit and modulate host immune cells.^{29–36} Lynn *et al.*³² studied injectable polymers that could self-assemble into immunogenic particles *in vivo* at physiological temperatures. Kim *et al.*³¹ recently developed injectable self-assembling mesoporous silica rods (MSRs) that can form scaffolds *in vivo* to induce a host immune response. Steinman and others at Stanford University have shown amyloid fibrils are therapeutic in EAE, thus further supporting the potential role of

large particles in redirecting autoimmunity.^{37–38} Based on our results, parallels between GA and these self-assembling polymers and scaffolds exist. They form larger structures and persist at the site of injection for an extended period of time, potentially for days. The large precipitates may trigger innate immune responses at the site of injection, which may lead to inflammation and other injection site adverse effects. GA was designed to mimic myelin basic protein (MBP) epitopes and cross-reactivity between GA and MBP has been reported.^{13, 39} GA-specific T-cells has also been observed.⁴⁰ In addition, the antidrug immune response associated with GA may be linked to the formation of particulates at the injection site.^{3, 27}

Conclusion:

A unique mechanism of action of GA was investigated through an improved understanding of biophysical properties and drug behavior at the site of injection. GA is a heterogeneous mixture of randomly sequenced peptides with variable sizes, yet GA was observed to form alpha helices in solution. DLS and 2D gel electrophoresis confirmed GA on average is about 3 nm in size, with a pI of approximately 9.75, and a MW distribution of 5 kDa to over 40 kDa. Through *in vitro* subcutaneous injection simulation experiments, GA was found to form aggregates in the presence of HA. The formation of nanometer and micron-sized particles was demonstrated in mice, where particles were observed in the tissue near the site of injection. In addition to injection site localization, fluorescence imaging experiments in mice showed GA was able to travel to and persist in draining lymph nodes. Although the outcomes observed at the injection site does not directly indicate causation of the observed clinical events, these observations do offer a new prospective on the mechanism of action and may help explain some of its complex immunomodulatory effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We gratefully acknowledge the Macromolecule and Vaccine Stabilization Center, the Immunology Core lab, and the Institute for Bioengineering Research at the University of Kansas for providing instrumentation and training. We would also like to acknowledge Dr. Sharon Lynch at the University of Kansas Medical Center for donating Copaxone®.

Funding Sources

This work was supported by the National Institutes of Health [Grant 5T32GM008359–26, T32 GM008545, GM-115042, MH-106245], and the University of Kansas [Department of Pharmaceutical Chemistry, Stella and Waugh Fellowship].

References

1. Wingerchuk DM; Carter JL, Multiple sclerosis: current and emerging disease-modifying therapies and treatment strategies. *Mayo Clin Proc* 2014, 89 (2), 225–40. [PubMed: 24485135]
2. Varkony H; Weinstein V; Klinger E; Sterling J; Cooperman H; Komlos T; Ladkani D; Schwartz R, The glatiramide class of immunomodulator drugs. *Expert Opin Pharmacother* 2009, 10 (4), 657–68. [PubMed: 19245345]

3. Messina S; Patti F, The pharmacokinetics of glatiramer acetate for multiple sclerosis treatment. *Expert Opin Drug Metab Toxicol* 2013, 9 (10), 1349–59. [PubMed: 23795716]
4. Neuroscience, T., Copaxone US prescribing information. 2018 <https://www.copaxone.com/Resources/pdfs/PrescribingInformation.pdf>
5. Aharoni R, Immunomodulation neuroprotection and remyelination – The fundamental therapeutic effects of glatiramer acetate: A critical review. *Journal of Autoimmunity* 2014, 54, 81–92. [PubMed: 24934599]
6. Jalilian B; Einarsson HB; Vorup-Jensen T, Glatiramer acetate in treatment of multiple sclerosis: a toolbox of random co-polymers for targeting inflammatory mechanisms of both the innate and adaptive immune system? *International journal of molecular sciences* 2012, 13 (11), 14579–14605. [PubMed: 23203082]
7. Kala M; Miravalle A; Vollmer T, Recent insights into the mechanism of action of glatiramer acetate. *Journal of Neuroimmunology* 2011, 235 (1), 9–17. [PubMed: 21402415]
8. Lalive PH; Neuhaus O; Benkhoucha M; Burger D; Hohlfeld R; Zamvil SS; Weber MS, Glatiramer acetate in the treatment of multiple sclerosis: emerging concepts regarding its mechanism of action. *CNS Drugs* 2011, 25 (5), 401–14. [PubMed: 21476611]
9. Fridkis-Hareli M; Teitelbaum D; Gurevich E; Pecht I; Brautbar C; Kwon OJ; Brenner T; Arnon R; Sela M, Direct binding of myelin basic protein and synthetic copolymer 1 to class II major histocompatibility complex molecules on living antigen-presenting cells--specificity and promiscuity. *Proc Natl Acad Sci U S A* 1994, 91 (11), 4872–6. [PubMed: 7515181]
10. Aharoni R; Schlegel PG; Teitelbaum D; Roikhel-Karpov O; Chen Y; Arnon R; Sela M; Chao NJ, Studies on the mechanism and specificity of the effect of the synthetic random copolymer GLAT on graft-versus-host disease. *Immunol Lett* 1997, 58 (2), 79–87. [PubMed: 9271317]
11. Webb C; Teitelbaum D; Herz A; Arnon R; Sela M, Molecular requirements involved in suppression of EAE by synthetic basic copolymers of amino acids. *Immunochemistry* 1976, 13 (4), 333–7. [PubMed: 59690]
12. Aharoni R; Teitelbaum D; Leitner O; Meshorer A; Sela M; Arnon R, Specific Th2 cells accumulate in the central nervous system of mice protected against experimental autoimmune encephalomyelitis by copolymer 1. *Proceedings of the National Academy of Sciences* 2000, 97 (21), 11472.
13. Aharoni R; Teitelbaum D; Sela M; Arnon R, Copolymer 1 induces T cells of the T helper type 2 that crossreact with myelin basic protein and suppress experimental autoimmune encephalomyelitis. *Proceedings of the National Academy of Sciences* 1997, 94 (20), 10821.
14. Neuhaus O; Farina C; Yassouridis A; Wiendl H; Then Bergh F; Dose T; Wekerle H; Hohlfeld R, Multiple sclerosis: Comparison of copolymer-1- reactive T cell lines from treated and untreated subjects reveals cytokine shift from T helper 1 to T helper 2 cells. *Proceedings of the National Academy of Sciences* 2000, 97 (13), 7452.
15. Kantengwa S; Weber MS; Juillard C; Benkhoucha M; Fellay B; Zamvil SS; Gougeon ML; Chofflon M; Lalive PH, Inhibition of naive Th1 CD4+ T cells by glatiramer acetate in multiple sclerosis. *J Neuroimmunol* 2007, 185 (1–2), 123–9. [PubMed: 17306890]
16. Weber MS; Prod'homme T; Youssef S; Dunn SE; Rundle CD; Lee L; Patarroyo JC; Stuve O; Sobel RA; Steinman L; Zamvil SS, Type II monocytes modulate T cell-mediated central nervous system autoimmune disease. *Nat Med* 2007, 13 (8), 935–43. [PubMed: 17676050]
17. Zhang CJ; Zhai H; Yan Y; Hao J; Li MS; Jin WN; Su N; Vollmer TL; Shi FD, Glatiramer acetate ameliorates experimental autoimmune neuritis. *Immunol Cell Biol* 2014, 92 (2), 164–9. [PubMed: 24275856]
18. Ziemssen T; Neuhaus O; Hohlfeld R, Risk-benefit assessment of glatiramer acetate in multiple sclerosis. *Drug safety* 2001, 24 (13), 979–990. [PubMed: 11735654]
19. Bagby TR; Cai S; Duan S; Thati S; Aires DJ; Forrest L, Impact of molecular weight on lymphatic drainage of a biopolymer-based imaging agent. *Pharmaceutics* 2012, 4 (2), 276–95. [PubMed: 24300232]
20. Nemecek D; Stepanek J; Thomas GJ, Jr., Raman spectroscopy of proteins and nucleoproteins. *Curr Protoc Protein Sci* 2013, Chapter 17, Unit17 8.

21. Hildebrandt PG; Copeland RA; Spiro TG; Otlewski J; Laskowski M; Prendergast FG, Tyrosine hydrogen-bonding and environmental effects in proteins probed by ultraviolet resonance Raman spectroscopy. *Biochemistry* 1988, 27 (15), 5426–5433. [PubMed: 3179264]
22. Anderson J; Bell C; Bishop J; Capila I; Ganguly T; Glajch J; Iyer M; Kaundinya G; Lansing J; Pradines J; Prescott J; Cohen BA; Kantor D; Sachleben R, Demonstration of equivalence of a generic glatiramer acetate (Glatopa). *Journal of the Neurological Sciences* 2015, 359 (1), 24–34. [PubMed: 26671082]
23. Habibi N; Kamaly N; Memic A; Shafiee H, Self-assembled peptide-based nanostructures: Smart nanomaterials toward targeted drug delivery. *Nano Today* 2016, 11 (1), 41–60. [PubMed: 27103939]
24. Conner JB; Bawa R; Nicholas M; Weinstein V, Copaxone in the era of biosimilars and nanosimilars *Handbook of Clinical Nanomedicine-From Bench too Bedside*. Singapore: Pan Stanford Publishing Pte Ltd 2014, 1–31.
25. Kinnunen HM; Mrsny RJ, Improving the outcomes of biopharmaceutical delivery via the subcutaneous route by understanding the chemical, physical and physiological properties of the subcutaneous injection site. *J Control Release* 2014, 182, 22–32. [PubMed: 24631859]
26. Kinnunen HM; Sharma V; Contreras-Rojas LR; Yu Y; Alleman C; Sreedhara A; Fischer S; Khawli L; Yohe ST; Bumbaca D; Patapoff TW; Daugherty AL; Mrsny RJ, A novel in vitro method to model the fate of subcutaneously administered biopharmaceuticals and associated formulation components. *J Control Release* 2015, 214, 94–102. [PubMed: 26210441]
27. Insert P.
28. Windhagen A; Maniak S; Marckmann S; Wilkening A; Lindert RB; Heidenreich F; Blasczyk R, Lymphadenopathy in patients with multiple sclerosis undergoing treatment with glatiramer acetate. *Journal of Neurology, Neurosurgery & Psychiatry* 2001, 70 (3), 415.
29. Ali OA; Emerich D; Dranoff G; Mooney DJ, In situ regulation of DC subsets and T cells mediates tumor regression in mice. *Sci Transl Med* 2009, 1 (8), 8ra19.
30. Ali OA; Huebsch N; Cao L; Dranoff G; Mooney DJ, Infection-mimicking materials to program dendritic cells in situ. *Nat Mater* 2009, 8 (2), 151–8. [PubMed: 19136947]
31. Kim J; Li WA; Choi Y; Lewin SA; Verbeke CS; Dranoff G; Mooney DJ, Injectable, spontaneously assembling, inorganic scaffolds modulate immune cells in vivo and increase vaccine efficacy. *Nature Biotechnology* 2014, 33, 64.
32. Lynn GM; Laga R; Darrah PA; Ishizuka AS; Balaci AJ; Dulcey AE; Pechar M; Pola R; Gerner MY; Yamamoto A; Buechler CR; Quinn KM; Smelkinson MG; Vanek O; Cawood R; Hills T; Vasalatiy O; Kastenmüller K; Francica JR; Stutts L; Tom JK; Ryu KA; Esser-Kahn AP; Etrych T; Fisher KD; Seymour LW; Seder RA, In vivo characterization of the physicochemical properties of polymer-linked TLR agonists that enhance vaccine immunogenicity. *Nature Biotechnology* 2015, 33, 1201.
33. Rao SS; Bushnell GG; Azarin SM; Spicer G; Aguado BA; Stoeckl JR; Jiang EJ; Backman V; Shea LD; Jeruss JS, Enhanced Survival with Implantable Scaffolds That Capture Metastatic Breast Cancer Cells *In Vivo*. *Cancer Research* 2016, 76 (18), 5209. [PubMed: 27635043]
34. Rudra JS; Tian YF; Jung JP; Collier JH, A self-assembling peptide acting as an immune adjuvant. *Proceedings of the National Academy of Sciences* 2010, 107 (2), 622.
35. St. John AL; Chan CY; Staats HF; Leong KW; Abraham SN, Synthetic mast-cell granules as adjuvants to promote and polarize immunity in lymph nodes. *Nature Materials* 2012, 11, 250. [PubMed: 22266469]
36. Stephan MT; Moon JJ; Um SH; Bershteyn A; Irvine DJ, Therapeutic cell engineering with surface-conjugated synthetic nanoparticles. *Nat Med* 2010, 16 (9), 1035–41. [PubMed: 20711198]
37. Kurnellas MP; Adams CM; Sobel RA; Steinman L; Rothbard JB, Amyloid fibrils composed of hexameric peptides attenuate neuroinflammation. *Sci Transl Med* 2013, 5 (179), 179ra42.
38. Kurnellas MP; Rothbard JB; Steinman L, Self-Assembling Peptides Form Immune Suppressive Amyloid Fibrils Effective in Autoimmune Encephalomyelitis *In Emerging and Evolving Topics in Multiple Sclerosis Pathogenesis and Treatments*, La Flamme AC; Orian JM, Eds. Springer International Publishing: Cham, 2015; pp 221–232.

39. Chen M; Gran B; Costello K; Johnson K; Martin R; Dhib-Jalbut S, Glatiramer acetate induces a Th2-biased response and crossreactivity with myelin basic protein in patients with MS. *Multiple Sclerosis Journal* 2001, 7 (4), 209–219. [PubMed: 11548979]
40. Aharoni R; Kayhan B; Eilam R; Sela M; Arnon R, Glatiramer acetate-specific T cells in the brain express T helper 2/3 cytokines and brain-derived neurotrophic factor in situ. *Proc Natl Acad Sci U S A* 2003, 100 (24), 14157–62. [PubMed: 14614135]

Highlights

- Glatiramer Acetate (GA) can form secondary structure in solution: α -helices and β -sheets
- Mean size of GA is determined to be around 3 nm, and pI of around 9.75
- GA forms micron sized aggregates in the presence of hyaluronic acid
- GA forms spherical, micron sized aggregates *in vivo* at the site of injection
- GA can localize at the injection site and travel towards the draining lymph nodes

Figure 1.

GA secondary structure characterization using Raman and CD spectroscopy. (A) Overall Raman spectra of 10 mg/mL GA from 20–90 °C at pH 6. Amide regions are highlighted in green and tyrosine regions are highlighted in orange. (B) Zoom-in of the amide I region. (C) Amide I ratio of 1645 over 1680 cm^{-1} , which represents the ratio of α -helix to β -sheet. (D) Plot of CD ellipticity values at 222 nm, which corresponds to α -helices, from pH 2–12 and 20–90 °C at 0.1 mg/mL. The inset shows a representative CD plot at pH 6 and 25 °C. The ellipticity values were not converted to molar ellipticity due to the heterogenous nature of GA.

Figure 2.

Tertiary structural characterization by Raman spectroscopy. Tyrosine bands at (A) 1600–1620 cm^{-1} and (B) 820 – 850 cm^{-1} at temperatures from 20 – 90 °C and pH 6. Tyrosine ratio plots of (C) 1600 over 1615 cm^{-1} , which indicates the charge state of tyrosine, and (D) 850 over 830 cm^{-1} , which provides information on the hydrogen bonding state of tyrosine side chain hydroxyl groups.

Figure 3. (A) Colloidal stability (SLS) plot of GA and (B) mean size of GA particles in solution determined using DLS and number distribution analysis over the pH range of 2 – 12. Particle sizes at pH 10 could not be determined due to the increased turbidity caused by aggregation.

Figure 4. Two-dimensional gel electrophoresis of GA. Isoelectric focusing performed on strips with a pH range of 6–11. Molecular weight markers correspond to 40, 25, 15, 10, 4.6, and 1.7 kDa from top to bottom.

Figure 5.

(A) Formation of GA precipitate in 10 mg/ml of 1.5 – 1.8 MDa HA. The samples were observed over 3 h from (i) the scissor cartridge and by (ii) phase contrast microscopy. Visible precipitates formed immediately following injection of GA into HA. Microscope images were observed under 20x magnification. The scale bar corresponds to 50 μ m. (B and C) The amount of GA released from the cartridge (injection site) into the simulator chamber over (B) the first 3 h and (C) over 3 d. Around 70% of the GA was released over 3 d and release appeared to plateau after 48 h. Error bars represent the sample error from three repeated experiments.

Figure 6. Raman spectra of the precipitate, lyophilized GA, and HA. The image on the right shows the GA precipitate viewed under a Raman microscope.

Figure 7.

Fluorescent microscope images of the precipitates generated using (A) unlabeled GA and unlabeled HA; (B) unlabeled GA and coumarin-labeled HA; (C) rhodamine B-labeled GA and unlabeled HA; (D) rhodamine B-labeled GA with coumarin-labeled HA. Coumarin-labeled HA was visible under the DAPI filter set (Ex: 378–407 nm, Em: 412–454 nm) while rhodamine B-labeled GA was visible under the TRITC filter set (Ex: 540–571 nm, Em: 577–616 nm). The precipitate appears to be a heterogenous complex composed of both GA and HA. The scale bar corresponds to 20 μm .

Figure 8.

(A) Footpad injections of cyanine 7 labeled GA and PEG over 4 h. (B) The average relative fluorescence intensity at the injection site for cyanine 7 labeled GA and PEG plotted as a function of time.

Figure 9.

Resected lymph nodes from mouse injected with (A) cy 7-PEG and (B) cy7-GA observed under fluorescence. (C) the average fluorescence intensity from the resected lymph nodes as a function of time.

Figure 10.

Tibialis muscle of mouse injected with (A) GA and extensor muscle of the forelimb injected with (B) mannitol as a control near the injection site (distal). GA appears as polydispersed spherical particles (A,C,D). Muscle samples injected with (C) GA were evaluated by Energy Dispersive Spectroscopy (EDS), (D) showing the presence of Gold, Osmium, Carbon, Oxygen, and Carbon as elements detected in the muscle sample. Scale bars are (A) 5 μm , (B) 20 μm , and (C,D) 10 μm . Images: (A) 5.0 kV, aperture 2, secondary electron, upper and lower detectors, magnification 1,000x, 8.0 mm WD; (B) 5.0 kV, aperture 2, secondary electron, upper detector, magnification 10,000x, 8.0 mm WD; (C and D) FlexSEM 20.0 kV, aperture 2, secondary electron detector, magnification 4,000x, 9.8 mm. WD.

Table 1:

The effect of HA concentration and size on the formation of visible GA-HA aggregates

Concentration of 1.5MDa HA (mg/ml)	Precipitation?	Notes:
10	Yes	Big Lump
5	Yes	Lump
2.5	Yes	Lump
1.25	Yes	Lump
0.625	Yes	Lump
0.3125	Yes	strand
0.15625	No	No visible particles

Size of HA (at 10mg/ml) in kDa	Precipitation?	Notes:
1500	Yes	Lump
741	Yes	Lump
357	Yes	Cloudy with small lump
67	Yes	Cloudy
28	Yes	Cloudy
16	Yes	Cloudy