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Peptide- and Amine-Modified Glucan Particles for the Delivery of Therapeutic siRNA

Jessica L. Cohen^{#†}, Yuefei Shen^{#†}, Myriam Aouadi^{†,‡}, Pranitha Vangala[†], Michaela Tencerova^{†,||}, Shinya U. Amano[†], Sarah M. Nicoloro[†], Joseph C. Yawe[†], and Michael P. Czech^{*,†}

[†] Program in Molecular Medicine, University of Massachusetts Medical School, 373 Plantation Street, Worcester, Massachusetts 01605, United States.

[#] These authors contributed equally to this work.

Abstract

Translation of siRNA technology into the clinic is limited by the need for improved delivery systems that target specific cell types. Macrophages are particularly attractive targets for RNAi therapy because they promote pathogenic inflammatory responses in a number of important human diseases. We previously demonstrated that a multi-component formulation of β -1,3-D-glucan-encapsulated siRNA particles (GeRPs) can specifically and potently silence genes in mouse macrophages. A major advance would be to simplify the GeRP system by reducing the number of delivery components, thus enabling more facile manufacturing and future commercialization. Here we report the synthesis and evaluation of a simplified glucan-based particle (GP) capable of delivering siRNA *in vivo* to selectively silence macrophage genes. Covalent attachment of small-molecule amines and short peptides containing weak bases to GPs facilitated electrostatic interaction of the particles with siRNA and aided in the endosomal release of siRNA by the proton-sponge effect. Modified GPs were non-toxic and were efficiently internalized by macrophages *in vitro*. When injected intraperitoneally (i.p.), several of the new peptide-modified GPs were found to efficiently deliver siRNA to peritoneal macrophages in lean, healthy mice. In an animal model of obesity-induced inflammation, i.p. administration of one of the peptide-modified GPs (GP-EP14) bound to siRNA selectively reduced the expression of target inflammatory cytokines in the visceral adipose tissue macrophages. Decreasing adipose tissue inflammation resulted in an improvement of glucose metabolism in these metabolically challenged animals. Thus, modified GPs represent a promising new simplified system for the efficient

*Corresponding Author University of Massachusetts Medical School, 373 Plantation Street, Worcester, MA 01605, USA. Tel: 1 508 856 2254; Fax: 1 508 856 1617, michael.czech@umassmed.edu.

[‡]Integrated Cardio Metabolic Centre, Department of Medicine, Karolinska Institutet, 141 57 Huddinge, Sweden

^{||}KMEB, Molecular Endocrinology, University of Southern Denmark, DK-5000 Odense, Denmark

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge on the ACS Publications website at DOI: Sequences of siRNA used in the present study; Cytotoxicity data for J774A.1 cells and PECs treated with modified GPs loaded with scrambled siRNA for 1 h, 6 h, and 24 h; Expression of additional inflammatory genes in PECs following treatment of lean mice with modified GPs; *In vivo* toxicity data for GP-EP14 particles, including serum AST, ALT, and IFN- γ .

Author Contributions

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delivery of therapeutic siRNAs specifically to phagocytic cells *in vivo* for modulation of inflammation responses.

Keywords

RNA interference therapy; siRNA delivery; glucan particle; small-molecule amine; amphipathic peptide; macrophage; inflammation

INTRODUCTION

RNA interference (RNAi) is a post-transcriptional biological mechanism wherein double-stranded RNAs can be used to reduce expression of target proteins,¹⁻³ and has emerged as a promising therapeutic strategy for the treatment of many diseases. Synthetic short interfering RNA (siRNA) can activate the endogenous RNAi pathway to achieve highly efficient, sequence-specific gene silencing.^{4,5} Despite great therapeutic potential, the clinical application of siRNA is limited by delivery problems.⁶ Nevertheless, significant progress has been made in the delivery of siRNA therapeutics as evidenced by a number of clinical trials being performed using siRNA.^{7,8} siRNA-based therapeutics are currently being developed for a variety of disease indications, including ocular and retinal disorders, cancer, and viral infections.^{9,10}

Phagocytic cells, such as macrophages, are particularly attractive targets for RNA interference therapy because they secrete factors, including inflammatory cytokines, which play a critical role in the pathogenesis of inflammatory diseases, such as rheumatoid arthritis, atherosclerosis, and Type 2 Diabetes.¹¹⁻¹⁴ Previous studies have reported that gene ablation or neutralization of chemokines and cytokines can alleviate the symptoms of these important human diseases.¹⁵ The potential of this therapeutic strategy is further highlighted by the success of several FDA-approved, anti-inflammatory drugs, which reduce inflammation by targeting chemokines and cytokines primarily produced by macrophages. Anti-inflammatory drugs, such as Humira[®], are currently approved for the treatment of rheumatoid arthritis, plaque psoriasis, Crohn's disease, and ulcerative colitis.¹⁶ However, systemic anti-inflammatory drugs can have serious side effects, including increased risk of infections and cancer.^{17,18} Thus, siRNA delivery systems capable of selectively targeting phagocytic cells present a promising therapeutic approach for the treatment of numerous inflammatory diseases, while avoiding the adverse side effects associated with systemic administration.

Our laboratory has recently developed a technology based on siRNA encapsulated within glucan particles derived from *Saccharomyces cerevisiae* (baker's yeast).^{19,20} We have previously demonstrated that these β -1,3-D-glucan-encapsulated siRNA particles (GeRPs) can potently silence genes selectively in macrophages *in vivo*.¹⁹⁻²⁵ Accumulation of macrophages in the visceral adipose tissue (AT) of obese mice and humans creates a chronic inflammatory state that correlates with impaired glucose homeostasis.²⁶⁻²⁸ We showed that silencing the expression of inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and osteopontin (OPN), in adipose tissue macrophages improves glucose tolerance in obese animals.²² Although the current GeRP formulation showed efficacy, simplifying the

multi-component GeRP system would present a major advantage to their clinical application.

The current GeRP formulation consists of three components – the glucan particles (GPs), the peptide Endoport (EP), and siRNA. EP is an amphipathic peptide composed of leucine and histidine residues. We previously showed that EP is a critical component of the GeRPs and is required for efficient GeRP-mediated gene silencing in macrophages.²⁰ EP serves multiple functions in the overall process of siRNA delivery using the GeRPs, including entrapping the siRNA within the glucan particles and aiding in its endosomal escape. Entrapment of the EP/siRNA complexes in the GPs confers phagocyte-specificity to the gene silencing.²⁰ Thus, while EP/siRNA complexes are able to silence genes in a variety of cell types (including both non-phagocytic and phagocytic cells), the GeRPs provide gene silencing selectively in phagocytic cells, such as macrophages. This unique feature of the GeRP technology is attractive for the development of RNAi therapeutic strategies aimed at treating inflammatory diseases.

A key improvement in the GeRP system would be to decrease the number of components to just two—the siRNA and the delivery vehicle, thus enabling more facile manufacturing and future commercialization. Because of the essential role that EP plays in the GeRPs, we used the properties of EP as inspiration for the design of our new modified delivery vehicles. Since EP contains multiple weak-base histidine residues, the proposed mechanism of action for EP involves conversion of the peptide to its polycationic form in the acidified endosome. This allows co-internalized cargo to pass from the endosome into the cytoplasm of the cell, a process known as the proton-sponge effect.²⁹ Here, we created a small library of small-molecule amines and short peptides to conjugate onto the GPs that would mimic the properties of EP. We hypothesized that covalent attachment of small-molecule amines and short peptides containing weak-base residues to the glucan particle would facilitate siRNA delivery while simplifying the GeRP technology. The protonatable amines and weak-base residues of the peptides should enable siRNA binding to the modified GPs and aid in the endosomal release of siRNA by the proton-sponge effect. These new modified GPs are a simplified delivery vehicle that can directly bind the therapeutic siRNA and deliver the siRNA to phagocytic macrophages *in vivo*. We show here that modified GPs are non-toxic delivery vehicles that can be efficiently delivered to macrophages *in vitro*. Several of the peptide-modified GPs were able to efficiently deliver siRNA and specifically reduce gene expression in peritoneal macrophages in healthy mice following intraperitoneal (i.p.) injection. Importantly, we show that i.p. injections of GPs modified with a 14 amino acid, basic peptide loaded with an siRNA against osteopontin (a chemokine, whose increased expression is associated with insulin resistance and Type 2 Diabetes) reduced its expression by about 80% in the adipose tissue and improved the metabolic phenotype of obese mice. Therefore, the modified GPs represent a simplified delivery system, and provide a useful technology for the efficient delivery of therapeutic siRNAs to phagocytic cells for modulation of inflammation responses.

EXPERIMENTAL SECTION

Small-molecule amines and peptides

Small molecule amines (ethylenediamine and histamine) were purchased from Sigma-Aldrich (St. Louis, MO). All peptides were purchased from 21st Century Biochemicals (Marlborough, MA).

Peptide Sequences—EP14: H₂N-LHLLHHLLHHLHHL-CONH₂

EP5: H₂N-LHHLL-CONH₂

His15: H₂N-HHHHHHHHHHHHHHHH-CONH₂

MethylHis15: H₂N-(Me-H)₁₅-CONH₂

His7: H₂N-HHHHHHH-CONH₂

Leu5: H₂N-LLLLL-CONH₂

Leu15: H₂N-LLLLLLLLLLLLLLLL-CONH₂

Preparation of Glucan Particles (GPs)

β-1,3-D-glucan particles were prepared as previously described.²⁰ Briefly, β-1,3-D-glucan particles were prepared by suspending *Saccharomyces cerevisiae* (100 g of SAF-Mannan; SAF Agri, Milwaukee, WI) in 1 L of 0.5 M NaOH and heating to 80°C for 1 h. The insoluble material containing the yeast cell walls was collected by centrifugation at 10000 rpm for 10 min. This insoluble material was then suspended in 1 L of 0.5 M NaOH, and incubated at 80°C for 1 h. The insoluble residue was again collected by centrifugation (10000 rpm for 10 min) and washed five times with 1 L of water, three times with 1 L of propan-2-ol, and three times with 1 L of acetone. The resulting slurry was placed in a glass tray and dried at room temperature (20°C) to produce 8.1 g of a fine, slightly off-white powder.

Synthesis of Modified GPs

All modified GPs were prepared in a similar fashion. Peptides/small-molecule amines were covalently attached to the GP via the N-terminal amine using reductive amination chemistry. Peptides used in the experiments described were capped on the C-terminus with a carboxamide cap.

Partial Oxidation of GPs—GPs (100 mg) were resuspended in 7 mL of dd-H₂O by sonication. Sodium periodate (22 mg, 0.103 mmol) was dissolved in 3 mL of dd-H₂O and this solution was added to the solution containing GPs. The mixture was stirred for 24 h at 37°C. Oxidized GPs were isolated by centrifugation at 3800 rpm for 10 min, and the resulting pellet was washed with dd-H₂O (2 × 10 mL) by resuspension followed by centrifugation and removal of the supernatant. The samples were used immediately for peptide/small-molecule amine modification.

Synthesis of modified GPs—Oxidized GPs were resuspended in 8 mL of dd-H₂O by sonication. Borate buffer (2 mL, 0.1 M, pH 9.5) and a peptide/small-molecule amine (formulations shown in Table 1, 20 – 165 mg) were added to the GP suspension and mixed overnight at 37°C. The reduction was performed for 72 h at room temperature by adding sodium borohydride (14 mg) to the GP solution. Modified GPs were isolated by centrifugation at 3800 rpm for 10 min and washed thoroughly with dd-H₂O (8 × 30 mL) and ethanol (2 × 30 mL) by resuspension followed by centrifugation and removal of the supernatant. The modified GPs were then flash-frozen and residual water was removed by lyophilization.

Preparation of Fluorescently-Labeled modified GPs—Fluorescently-labeled modified GPs were prepared in the same manner as described above, except that either 5-(((2-(Carbohydrazino)methyl)thio)acetyl)Aminofluorescein (Invitrogen, 1 mg/mL in dd-H₂O) or Cascade Blue hydrazide (Invitrogen, 1 mg/mL in dd-H₂O) was added to the peptide/small-molecule amine solution.

Characterization of Modified GPs

Quantification of Small-Molecule Amine Content—A fluorescamine assay was performed to quantify the incorporation of ethylenediamine on the glucan particles. Ethylenediamine-modified particles were dissolved in DMSO to make final concentrations of 0 – 1 mg/mL. A standard curve was prepared using known concentrations of ethylenediamine. The samples or standards (75 µL) were incubated with 25 µL of a 3 mg/mL fluorescamine solution (Sigma, St Louis, MO) for 15 min at room temperature. Fluorescence was measured at EX/EM = 380 nm/ 470 nm using a microplate reader (Tecan). The percentage of incorporation was calculated based on the standard curve.

Quantification of Peptide Loading—Peptide quantification was performed using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). The peptide-modified particles (10 mg) were incubated in DMSO (1 mL) at 50°C for 2 h to dissolve the particles. The samples were centrifuged to remove insoluble material and diluted with DPBS. The solution was then analyzed for the concentration of peptide using the kit according to the manufacturer's instructions. A standard curve was prepared using known concentrations of peptide. Unmodified GPs (GPs that were not modified with peptides) were analyzed as a control. The signal obtained from the unmodified GPs was subtracted from the reading obtained from peptide-modified particles when calculating the peptide content.

Characterization of Particle Size by Confocal Microscopy—Fluorescently-labeled modified particles were suspended in dd-H₂O at a concentration of 1 mg/mL and the resulting suspensions were mixed with Prolong Gold. Particle suspensions were spotted onto glass microscope slides. Images were obtained using a Solamere CSU10 Spinning Disk confocal system mounted on a Nikon TE2000-E2 inverted microscope. Particle size was determined by measuring the particles in the images.

Zeta Potential Measurements—Zeta potentials of peptide-modified GPs were determined with a Malvern Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK).

Solvents and buffers were filtered through 0.22 μm filters before sample preparation. Zeta potential measurements were performed both in the absence and in the presence of siRNA. A suspension of modified GPs (0.1 mg/mL) was diluted in 1 mL of 20 mM HEPES buffer and sonicated prior to performing the measurement. To load siRNA in modified GPs, siRNA (1 nmole) was mixed with modified GPs (0.1 mg) in HEPES buffer and incubated for 20 min at room temperature. The siRNA-loaded particles were then diluted with HEPES buffer to obtain a final particle concentration of 0.1 mg/mL. The samples were transferred to a 1 mL clear zeta potential cuvette. Zeta potential measurements were collected at 25°C from -150 to +150 mV. For each sample, a total of 30 measurements were collected and analyzed with the Dispersion Technology software (Malvern).

siRNA Binding Assay

The ability of modified GPs to electrostatically interact with siRNA was evaluated in sodium acetate buffer (30 mM, pH 4.8). Either modified GPs (1 mg/mL) were loaded with different concentrations of fluorescently-labeled siRNA (Dy547-siRNA, Dharmacon, Pittsburgh, PA) ranging from 0.2 – 100 μM or Dy547-siRNA (2 μM) was mixed with different concentrations of modified GPs (0 – 1000 $\mu\text{g/mL}$). Particles were loaded with siRNA by incubating in sodium acetate buffer (30 mM, pH 4.8) for 20 min at room temperature. Particles loaded with siRNA were sedimented by centrifugation at 9000 rpm for 5 min and the supernatant was assessed for siRNA content by measuring the fluorescence on a microplate reader. % siRNA binding as well as % free siRNA was calculated relative to the siRNA control that did not contain particles (siRNA alone).

Cell lines and culture

J774A.1 cells were acquired from ATCC (American Type Culture Collection, Manassas, VA). J774A.1 cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (GE Healthcare Life Sciences, HyClone) supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals), 100 $\mu\text{g/mL}$ streptomycin and 100 units/mL penicillin (Thermo Fisher Scientific, Gibco). Cell incubations were performed in a water jacketed 37°C/5% CO_2 incubator.

Peritoneal macrophage isolation

Ten-week old C57BL6/J male mice were intraperitoneally (i.p.) injected with 4% thioglycollate broth (Sigma-Aldrich, St. Louis, MO). Five days following injection, the mice were sacrificed and the peritoneal cavity was washed with 5 mL of ice-cold PBS to isolate peritoneal exudate cells (PECs). Peritoneal fluid was filtered through a 70 μm pore nylon mesh and centrifuged at 1200 rpm for 10 min. The pellet was first treated with red blood cell (RBC) lysis buffer (8.3 g of NH_4Cl , 1.0 g of KHCO_3 and 0.037 g of EDTA dissolved in 1 L of water) and then plated in either 6- or 12-well plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 $\mu\text{g/mL}$ streptomycin and 100 units/mL penicillin. Twenty-four hours after isolation, PECs were treated with modified GPs.

Cytotoxicity assay of modified GPs in J774A.1 cells and PECs

The cytotoxicities of modified GPs (either alone or in the presence of siRNA) were examined by Vybrant MTT cell proliferation assay (Life Technologies). To load siRNA in modified GPs, scrambled siRNA (1 nmole) was mixed with modified GPs (0.1 mg) in sodium acetate buffer (30 mM, pH 4.8) and incubated for 20 min at room temperature. J774A.1 cells were seeded in a 96 well plate at a density of 1×10^4 cells per well and cultured in 100 μ L DMEM containing 10% FBS, 100 μ g/mL streptomycin and 100 units/mL penicillin. PECs were seeded in a 96 well plate at a density of 5×10^4 cells per well and cultured in 100 μ L DMEM containing 10% FBS, 100 μ g/mL streptomycin and 100 units/mL penicillin. After 24 h, the medium was replaced with 100 μ L fresh culture medium containing various concentrations of modified GPs (0 – 100 μ g/mL). One hour, 6 h, or 24 h later, the particle containing media was replaced by fresh culture media. After another 24 h, the medium was changed to fresh culture medium without phenol red, and 10 μ L of a 12 mM MTT stock solution was added to each well. The plate was incubated for an additional 4 h at 37°C in a humidified CO₂ incubator. Following the 4 h incubation, 100 μ L of the SDS-HCl solution was added to each well and incubated overnight. The absorbance of the colored formazan product was recorded at 570 nm using a microplate reader (Tecan) and normalized to the control group with no treatment. An average of three determinations was made.

In vitro internalization of FITC-labeled modified GPs in PECs

PECs were plated in 24-well plates with coverslips at a density of 2×10^5 cells per well and cultured in 500 μ L DMEM containing 10% FBS, 100 μ g/mL streptomycin and 100 units/mL penicillin. Twenty-four hours later, FITC-labeled modified GPs (50 μ g/mL) loaded with scrambled siRNA were added to cells and the plates were returned to the incubator. After 24 hours, particles were replaced by fresh culture media. After another 24 h, cells were washed twice with PBS and fixed with 4% formaldehyde for 15 min. Fixed cells were incubated with rat anti-mouse F4/80 primary antibody (AbD Serotec) followed by goat anti-rat Alexa Fluor 647 secondary antibody (Invitrogen). Cells were mounted in Prolong Gold anti-fade with DAPI (4', 6-diamidino-2-phenylindole) (Invitrogen). Images were obtained using a Solamere CSU10 Spinning Disk confocal system mounted on a Nikon TE2000-E2 inverted microscope.

In Vivo Transfection Experiments

All mice were purchased from Jackson Laboratory. Mice were housed on a 12-h light/dark schedule and had free access to water and food. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School.

Preparation of modified GPs loaded with siRNA—For *in vivo* treatment, modified GPs were loaded with siRNA (Dharmacon; sequences listed in Supplementary Table S1) targeting either F4/80, osteopontin (OPN), or a scrambled (Scr) control sequence. To load siRNA in modified GPs, siRNA (10 nmoles) was mixed with modified GPs (1 mg) in sodium acetate buffer (30 mM, pH 4.8) and incubated for 20 min at room temperature. The siRNA-loaded particles were then diluted with either PBS or sodium acetate buffer to obtain

a final particle concentration of 1 mg/mL and sonicated [15 s at 18 W at room temperature using a Sonicator 3000 (Misonix)] to ensure homogeneity of the particle preparation. Particles were aliquoted into tubes for daily dosing and either flash-frozen in liquid nitrogen and stored at -20°C , or kept at 4°C .

Preparation of GeRPs—GeRPs were prepared as previously described.^{20, 22} Briefly, to load siRNA in unmodified glucan particles (GPs), 3 nmoles siRNA (Dharmacon, Pittsburgh, PA) were incubated with 50 nmoles Endoport (EP) (Gene Tools, Philomath, OR) in 30 mM sodium acetate buffer pH 4.8 for 15 min at room temperature in a final volume of 20 μL . The siRNA/EP solution was added to 1 mg of glucan particles and then vortexed and incubated for 1 h. The siRNA-loaded GeRPs were then resuspended in PBS to obtain a final particle concentration of 1 mg/mL and sonicated to ensure homogeneity of the GeRP preparation. GeRPs were kept at 4°C .

Particle Administration for Knockdown in Lean mice—C57BL6/J male mice (8 weeks old) were i.p. injected once a day for 5 days with 1 mg of modified GPs loaded with 10 nmoles of siRNA. On day 6, mice were sacrificed and the peritoneal cavity was washed with 5 mL of ice-cold PBS to isolate PECs. Peritoneal fluid was filtered through a 70 μm pore nylon mesh and centrifuged at 1200 rpm for 10 min. The pellet was first treated with RBC lysis buffer and then plated in 6-well plates in DMEM supplemented with 10% (v/v) FBS, 100 $\mu\text{g/mL}$ streptomycin and 100 units/mL penicillin. The cells were incubated overnight to enrich for macrophages, then the media was changed. Adherent cells were used for real-time PCR.

Biodistribution of modified GPs in Obese Mice—Genetically obese B6.V-*Lep^{ob}/J* (*ob/ob*) male mice were i.p. injected with either PBS or 1 mg of fluorescently-labeled modified GPs. Twenty-four hours after the injection, mice were sacrificed and tissues were isolated for microscopy.

Isolation of Adipose Tissue Stromal-Vascular Fraction Cells—Adipose tissue stromal-vascular fraction (SVF) cells were prepared from collagenase-digested adipose tissue, as described previously.²² Briefly, visceral epididymal fat pads were mechanically dissociated using the gentleMACS Dissociator (Miltenyi Biotec, Cambridge, MA) and digested with collagenase at 37°C for 45 min in Hank's Buffered Saline Solution (HBSS) (Gibco, Life Technologies, Grand Island, NY) containing 2% bovine serum albumin (American Bioanalytical, Natick, MA) and 2 mg/mL collagenase (collagenase from *clostridium histolyticum*, Sigma). Samples were then filtered through 100 μm BD falcon cell strainers and centrifuged at 1200 rpm for 10 min at room temperature. The adipocyte layer and the supernatant were aspirated and the pelleted cells were collected as the stromal-vascular fraction (SVF). The cells were then treated with RBC lysis buffer, washed in PBS and plated in DMEM supplemented with 10% (v/v) FBS, 100 $\mu\text{g/mL}$ streptomycin and 100 units/mL penicillin.

Particle Administration for OPN Knockdown in Obese Mice—Weight and fasting glucose tolerance test (GTT) were used to randomize the mice into different treatment groups. Genetically obese B6.V-*Lep^{ob}/J* (*ob/ob*) male mice (5 weeks old) were i.p. injected

once a day for 5 days with 1 mg of GP-EP14 particles loaded with 10 nmoles of siRNA. On day 6, mice were fasted overnight. On day 7, GTTs were performed. Following the GTT, mice were sacrificed and various tissues were isolated for further analysis. Samples for RNA extraction were frozen in liquid nitrogen and stored at -80°C .

Metabolic Studies—Glucose tolerance tests (GTTs) were performed on *ob/ob* animals following GP-EP14 treatment. Glucose (1 g/kg) was administered by i.p. injection. Blood samples were withdrawn from the tail vein at the indicated time, and glycemia was determined using glucometers (Alpha-Trak).

Isolation of RNA and Real-Time PCR

RNA isolation was performed according to the TRIzol[®] Reagent protocol (Invitrogen, Grand Island, NY). Tissues were homogenized using the gentleMACS Dissociator (Miltenyi Biotec, Cambridge, MA) in TRIpure Isolation reagent (Roche Applied Science, Indianapolis, IN), and total RNA was isolated according to the manufacturer's instructions. cDNA was synthesized from 0.5–1 μg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. For real-time PCR, synthesized cDNA, forward and reverse primers, along with SYBR Green were run on the CFX96 Realtime PCR System (Bio-Rad). The ribosomal mRNA 36B4 was used as an internal loading control, as its expression did not change over a 24 h period with the addition of siRNA against the genes used in the present study. The expression of each gene within a sample was normalized against 36B4 mRNA expression and expression relative to the control sample using the formula $2^{-(\text{Ct})}$ in which $\text{Ct} = (\text{Ct mRNA} - \text{Ct 36B4})_{\text{sample}} - (\text{Ct mRNA} - \text{Ct 36B4})_{\text{control sample}}$.

Microscopy

The isolated cells were fixed with 4% formaldehyde for 15 min. Fixed cells were incubated with rat anti-mouse F4/80 primary antibody (AbD Serotec) followed by goat anti-rat Alexa Fluor 594 or Alexa Fluor 647 secondary antibody (Invitrogen). Cells were mounted in Prolong Gold anti fade with DAPI (4', 6-diamidino-2-phenylindole) (Invitrogen). Images were obtained using a Zeiss Axiovert 200 inverted microscope equipped with a Zeiss AxioCam HR CCD (charge-coupled device) camera with $1,300 \times 1,030$ pixels basic resolution and a Zeiss Plan Apochromat 63x/1.4 Oil (DIC II) objective or a Solamere CSU10 Spinning Disk confocal system mounted on a Nikon TE2000-E2 inverted microscope.

For tissues, fixed sections were stained with DAPI. Images were obtained using a Zeiss Axiovert 200 inverted microscope equipped with a Zeiss AxioCam HR CCD camera with $1,300 \times 1,030$ pixels basic resolution and a Zeiss Plan NeoFluar 10x/0.3 Ph1 (DIC I) objective.

Toxicity and Immune Response

C57BL/6J male mice (8 weeks old) were i.p. injected once a day for 5 days with siRNA formulated in GP-EP14 particles at a dose of 1.2 mg/kg siRNA/day (1 mg of GP-EP14 particles containing 10 nmoles of siRNA total). Twenty-four hours after the last injection,

mice were sacrificed and blood samples were collected by cardiac puncture. Serum was obtained by centrifuging at 5000 rpm for 10 min.

ELISA assay—Serum cytokine levels were determined using enzyme-linked immunosorbent assay (ELISA) kits. Tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) levels were measured using mouse ELISA kits (Pierce, Rockford, IL) as recommended by the manufacturer.

AST and ALT measurement—To test for liver toxicity, the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity in serum were measured using commercial kits (Abnova, Taiwan) according to the manufacturer's instructions.

Statistical Analysis

The statistical significance of the differences in the means of experimental groups was determined by Student's *t*-test using GraphPad Prism v 6.0c software. For all tests, $p < 0.05$ was considered significant. The data are presented as the means $\pm$ s.e.m.

RESULTS

Design, synthesis, and characterization of amine- and peptide-modified glucan particles (GPs)

The goal of the present study was to simplify the multi-component GeRP system for efficient siRNA delivery. We hypothesized that chemical modification of the glucan particles (GPs) might provide a simplified platform for efficient siRNA delivery *in vivo*. To test this hypothesis, GPs were covalently modified with small-molecule amines and peptides that would directly bind the siRNA as well as facilitate the siRNA release from the endosome to the cytosol. These properties should enable potent siRNA-mediated gene silencing using this delivery technology. We have recently described a method to load siRNA into GPs whereby we first form complexes between siRNA and the amphipathic peptide, Endoport (EP) (Figure 1A). These siRNA/EP complexes can then be loaded and entrapped in the GPs to form the final GeRP system.²⁰ In contrast to the GeRPs, the new amine- and peptide-modified GPs enable loading of the particles with siRNA in a single step simply by mixing the siRNA with the modified GP (Figure 1B).

Small molecule amines and peptides were covalently attached to the GPs via the N-terminal amine using reductive amination chemistry (Figure 1C). The glucan particles were first lightly oxidized with sodium periodate. Amine modification was then performed between the small-molecule amine/peptide and the oxidized glucan particles using sodium borohydride as the reducing agent. Reductive amination is a modular chemistry that allows attachment of a variety of different amine structures. A small library of amine- and peptide-modified GPs (Table 1) was prepared to study the effect of different modifications on siRNA delivery efficiency. Modifications included small-molecule amines, such as ethylenediamine (E) and histamine (H), as well as peptides and combinations of small-molecule amines and peptides. The peptides selected for these studies comprised amphipathic peptide sequences

(based on truncations of the EP sequence) in addition to polyhistidine and poly-leucine peptides.

A fluorescamine assay was used to quantify the coupling of ethylenediamine to the GPs. The amount of ethylenediamine covalently linked to the GPs was approximately 1%. Peptide incorporation was quantified using a bicinchoninic acid (BCA) assay. Using this method, the peptide content in the particles was found to be 0.1 – 0.2 μmol of peptide per mg of GP, corresponding to 1 – 3% of the glucose monomers in the β -glucan structure of the particles.

To determine the ability of the amine- and peptide-modified GPs to electrostatically interact with siRNA, we incubated fluorescently-labeled siRNA with the modified GPs at different concentrations (Figure 2A). Alternatively, modified GPs were loaded with different concentrations of fluorescently-labeled siRNA (Figures 2B-2D). As predicted, the different amine- and peptide-modified particles bind siRNA with varying degrees of strength (Figure 2). Unmodified GPs did not bind siRNA at any of the concentrations tested (Figure 2B).

***In vitro* cytotoxicity and cellular uptake of amine- and peptide-modified GPs**

Several of the new modified GP formulations demonstrated promising siRNA binding abilities. In order to simplify the *in vitro* and preliminary *in vivo* experiments, six representative formulations were selected for further characterization. Initial experiments were performed with three of the peptide-modified particles (GP-His15, GP-His7, and GP-EP14) and two of the small-molecule amine-modified particles (GP-E and GP-H). The siRNA binding curves for particles coupled to two different compounds were similar to those for particles coupled to a single compound. Thus, these particles were not analyzed in these experiments. Unmodified GPs or GeRPs were included in these experiments as a positive control as we have previously reported that GeRPs can deliver siRNA and specifically silence genes in mouse macrophages both *in vitro* and *in vivo*.^{20, 22}

To examine the potential of using the modified GPs to deliver siRNA to macrophages, we first investigated the cytotoxicity of the modified GPs *in vitro*. J774A.1 cells, a mouse macrophage cell line, and primary mouse macrophages (PECs) were incubated with various concentrations of modified GPs (either alone or in the presence of siRNA) and the cytotoxicity was determined by MTT cell proliferation assay. The cytotoxicities of the amine- and peptide-modified GPs were similar to or even less toxic compared to that of the unmodified GPs. The modified GPs showed little or no cytotoxicity at low particle concentrations at all the time points tested (Figure 3 and Figure S1). The majority of the amine- and peptide-modified GPs showed little cytotoxicity at particle concentrations up to 100 $\mu\text{g/mL}$, with the exception of the GP-EP14 particles. GP-EP14 particles displayed dose-dependent toxicity *in vitro*. While low particle concentrations were not cytotoxic, the highest particle concentrations tested demonstrated some cell toxicity (Figure 3 and Figure S1). Cells treated with GP-EP14 particles at a concentration of 100 $\mu\text{g/mL}$ showed a viability of approximately 70%.

Next, we assessed the ability of macrophages to phagocytose amine- and peptide-modified GPs. PECs were treated with fluorescein (FITC)-labeled modified GPs loaded with scrambled siRNA *in vitro* and cellular uptake was analyzed by microscopy (Figure 4). As a

control, PECs were also incubated with FITC-labeled unmodified GPs, the GPs used to prepare GeRPs. PECs were stained with F4/80 antibody, a macrophage specific marker, prior to imaging. Microscopic analysis showed that all of the modified particles (green) were efficiently internalized by F4/80-positive macrophages (red) (Figure 4). Cellular uptake was similar for all of the particles tested. Taken together, these results suggest that amine- and peptide-modified GPs are non-toxic delivery vehicles that are efficiently delivered to macrophages *in vitro*.

Amine- and peptide-modified GPs efficiently deliver siRNA and silence genes in macrophages in lean mice *in vivo*

The ability of amine- and peptide-modified GPs to induce target-specific gene silencing was determined by treating lean mice with modified GPs loaded with either Scr or F4/80 siRNA following the protocol outlined in Figure 5A. Briefly, modified GPs were administered to C57BL/6/J male mice via i.p. injection once daily for five consecutive days. The day after the last injection, PECs were isolated and plated overnight to enrich for macrophages. F4/80 was chosen as our target gene because it is a highly expressed macrophage marker for which our laboratory has a potent siRNA sequence. The expression of F4/80 and other genes of interest were determined by quantitative real-time PCR. Several of the modified GPs were selected for *in vivo* experiments based on their ability to bind siRNA and to be internalized by PECs *in vitro*. The different modified GPs provide varying levels of gene silencing in PECs (Figure 5). Gene knockdown in peritoneal macrophages recovered from GP-His15, GP-His7, and GP-EP14 treated mice is in the range of 30-50%, a modest but significant gene silencing (Figures 5B, 5C, and 5F). In contrast, GPs modified with histamine and ethylenediamine did not result in significant silencing of F4/80 (Figures 5D and 5E). To prove the specificity of the siRNA-mediated knockdown, the expression of several other macrophage and immune cell factors was measured (Figure S2). Expression of these markers (including TNF- α , CD11b, CD11c, IL-10, IL-6, IL-4, OPN, and CD68) was unchanged in PECs from mice treated with modified GPs loaded with F4/80 siRNA compared to modified GPs loaded with Scr siRNA. Thus, several of the modified GP formulations can efficiently deliver siRNA and specifically reduce gene expression in macrophages *in vivo* in lean, healthy mice without affecting off-target genes.

To confirm the internalization of the modified GPs by macrophages *in vivo*, PECs isolated from modified GP treated mice were stained with an antibody against the macrophage marker, F4/80, and analyzed by microscopy. Figures 6A – C show modified GPs (green) in F4/80-positive macrophages (red) thus demonstrating that modified GPs undergo phagocytosis by macrophages *in vivo* in lean mice. In order to quantify the particle uptake, representative microscopy images were analyzed to determine the number of cells containing FITC-labeled modified GPs. Quantification of the particle uptake in PECs indicated that the majority (90%) of F4/80-positive cells contained FITC-labeled modified GPs (Figure 6D).

The modified GP that showed the most significant silencing efficiency, the GP-EP14 particles, was selected for further studies. Liver toxicity of GP-EP14 particles was assessed by measuring aspartate aminotransferase (AST) and alanine aminotransferase (ALT)

activities (markers of liver damage) in serum. No significant changes in either AST or ALT were detected in mice treated with GP-EP14 particles when compared to mice treated with PBS (Figures S3A and S3B). In addition, i.p. administration of GP-EP14 particles in lean mice did not induce the production of pro-inflammatory cytokines, including interferon gamma (IFN- γ) and tumor necrosis factor-alpha (TNF- α) (Figure S3C). No TNF- α expression (<9 pg/mL) was detected in the serum following GP-EP14 treatment.

GP-EP14 particles silence the expression of inflammatory genes in macrophages and improve glucose tolerance in obese mice

We next tested the ability of the GP-EP14 particles to decrease inflammation in the model of obesity-associated inflammation. Accumulation of macrophages in the visceral AT of obese individuals is associated with increased levels of inflammatory cytokines and chemokines that can impair glucose metabolism.²⁶⁻²⁸ For instance, the inflammatory cytokine, OPN, has been shown to play an important role in promoting inflammation and the accumulation of macrophages in the AT during obesity.³⁰⁻³² We have previously shown that silencing the expression of OPN in the visceral AT macrophages in obese mice following i.p. administration of GeRPs improves the metabolic phenotype, i.e. insulin sensitivity, of obese mice.²² Thus, OPN was chosen as a target to test the ability of the GP-EP14 particles to deliver functional siRNA to macrophages in the inflamed AT in obese (*ob/ob*) mice.

In our previous study using the GeRPs, we demonstrated selective delivery of siRNA to macrophages in the visceral (epididymal) AT following i.p. administration in obese mice.²² To study the distribution of GP-EP14 particles following i.p. administration in obese mice, we used FITC-labeled GP-EP14 particles (Figure 7). Five week-old genetically obese (*ob/ob*) mice were administered GP-EP14 particles by i.p. injection and 24 h following the injection various tissues were isolated and analyzed by microscopy. GP-EP14 particles were predominantly observed in cells within the visceral AT and not in the other organs, including the liver, lung, spleen, heart, pancreas, and subcutaneous AT (Figure 7A). These data demonstrate that in obese, insulin-resistant mice, GP-EP14 particles can be used to selectively target the site of inflammation, the visceral AT, while leaving other tissues unaffected.

Next, we evaluated the specific types of cells targeted by the GP-EP14 particles in the AT of obese mice. Visceral AT from mice injected with FITC-labeled GP-EP14 particles was digested with collagenase and the stromal-vascular fraction (SVF), which contains all cells in the AT except adipocytes, was analyzed by fluorescence microscopy. Figure 7B shows F4/80-positive cells (red) containing GP-EP14 particles (green). Taken together, these results show that GP-EP14 particles injected i.p. are specifically internalized by phagocytic cells, such as macrophages, in the visceral AT of obese mice.

To confirm the ability of GP-EP14 particles to deliver functional siRNA and silence genes in the AT of obese mice, five-week-old *ob/ob* mice were administered GP-EP14 particles loaded with either a control Scr or OPN siRNA for 5 days as outlined in Figure 8A. Two days following the last injection, OPN expression was measured in the visceral AT (Figure 8B). Mice treated with GP-EP14 particles loaded with OPN siRNA demonstrated significantly lower levels of OPN expression in the visceral AT than mice treated with GP-

EP14 particles loaded with Scr siRNA. Importantly, the expression of other macrophage and immune cell factors, including F4/80 and TNF- α , were unchanged in the visceral AT of mice treated with GP-EP14 particles loaded with OPN siRNA compared with GP-EP14 particles loaded with Scr siRNA, confirming the specificity of the siRNA-mediated knockdown (Figures 8C and 8D). Consistent with the biodistribution data, no change in OPN expression was observed in other tissues such as the subcutaneous AT or liver (Figures 8E and 8F). Therefore, GP-EP14 particles can selectively deliver siRNA to macrophages and reduce inflammation in the visceral AT *in vivo* following i.p. injection in obese mice.

To determine the effect of GP-EP14 mediated gene silencing on whole-body metabolism, glucose tolerance tests (GTTs) were performed in *ob/ob* mice treated with GP-EP14 particles loaded with either Scr or OPN siRNA (Figure 8A). As shown in Figures 8H and 8I, treatment with GP-EP14 particles loaded with OPN siRNA significantly improved glucose tolerance in obese mice. There was no effect of GP-EP14 particles loaded with Scr siRNA on glucose tolerance (Figure 8I). Importantly, the effect of OPN silencing on glucose tolerance was independent of an effect on weight gain during the particle treatment (Figure 8G). These data suggest that OPN silencing delivered by GP-EP14 particles improves whole body glucose tolerance in *ob/ob* mice and might provide a useful therapeutic strategy for the treatment of insulin resistance and Type 2 Diabetes.

DISCUSSION

A key challenge to realizing the potential of siRNA-based therapeutics is the lack of safe and effective delivery systems.^{6, 8} The major advance reported in this paper is the development of a simplified system to efficiently deliver therapeutic siRNA to phagocytic cells *in vivo* (Figures 5 and 8). This delivery system consists of glucan microparticles that have been functionalized with small molecule amines and peptides. In contrast to the prior GeRP formulation,²⁰ which consists of two separate delivery components and is prepared in multiple steps, the new modified GPs are a single-component delivery vehicle and can be loaded with siRNA in a single step (Figures 1A and 1B). The loading strategy for the modified GPs involves mixing siRNA with glucan particles previously conjugated with small-molecule amines and/or peptides, thus affording a facile and reproducible method of preparing the siRNA delivery system. Since the new modified GPs are a single entity, they offer advantages over the GeRPs in terms of manufacturing and future clinical application.

A critical component of the GeRPs is EP, an amphipathic α -helical peptide, with one face composed predominantly of aliphatic lipophilic amino acids, and the other face composed of basic amino acids.^{20, 29, 33} In the context of the GeRPs, the peptide EP serves the crucial function of entrapping the siRNA within the glucan particles and could contribute to its endosomal escape. It is hypothesized that the weak-base histidine residues of EP assist in the endosomal escape of the encapsulated siRNA by permeabilizing the endosomal membrane upon acidification within the endosome, a process known as the proton-sponge effect.^{29, 34} With this in mind, we designed GPs with surface modifications that would impart the favorable siRNA delivery properties of EP while reducing the number of components needed for efficient delivery.

The present study demonstrates that covalent attachment of small-molecule amines and peptides to the GPs facilitates siRNA delivery while simplifying the GeRP technology. Due to the flexibility of the reductive amination chemistry, we were able to prepare a small library of small-molecule amine and peptide-modified GPs (Table 1). Modifications included small-molecule amines, such as ethylenediamine and histamine, which contain protonatable amines. In addition, we incorporated several peptide modifications based on truncations or other variations of the EP sequence. Shorter peptides represented an attractive alternative because they are known to induce less immunogenic response when compared to longer peptides. Covalent attachment of small-molecule amines, peptides, or combinations of amines and peptides to the GPs enables electrostatic binding of siRNA to this delivery system (Figure 2). In contrast to the unmodified GPs, which do not bind siRNA (Figure 2B), the modified GPs bind siRNA with varying degrees of strength (Figures 2A-D). The binding of siRNA to the modified GPs most likely occurs due to electrostatic interactions between the cationic amines on the modified GPs and the anionic phosphates of the siRNA. Several studies have reported high toxicity of commonly used siRNA delivery vehicles due to their polycationic nature.³⁵⁻³⁷ The small-molecule amine and peptide-modified GPs demonstrated little or no toxicity in both a mouse macrophage cell line (J774A.1 cells) and primary mouse macrophages (PECs) *in vitro* (Figure 3 and Figure S1).

The new modified GPs combine straightforward synthesis and siRNA loading with the additional benefit of phagocyte-specificity conferred by the glucan particle. The GPs are approximately 2 – 4 μm in diameter and are composed primarily of β -1,3-D-glucan.^{19, 20} Previous studies showed that GPs can deliver cargo specifically to phagocytic cells, such as macrophages and dendritic cells.^{20, 38} The specific targeting of the GPs to phagocytic cells is conferred by two distinctive characteristics of the GPs, their size (2 – 4 μm) and their composition (primarily β -1,3-D-glucan). The size of the GPs makes them well-suited for uptake by phagocytic cells, which are efficient at internalizing foreign matter. In addition, the micron-size of the GPs excludes possible uptake by non-phagocytic cells. The surface composition of the GPs facilitates recognition by glucan receptors expressed on phagocytic cells, such as dectin-1.³⁸ Thus, GPs provide the unique ability to deliver therapeutic payloads selectively to phagocytic cells. This key feature of the GPs is particularly attractive for RNAi therapeutic applications where delivery of siRNA to specific cell types is desired. Selectively targeting phagocytic cells, such as macrophages, should reduce the adverse side effects associated with non-specific silencing of genes throughout the body. Characterization of the new peptide-modified GPs showed that the size and zeta potentials of these modified particles are not significantly different from the unmodified GPs. GP, GP-His15, GP-His7, and GP-EP14 particles showed sizes around 2 – 4 μm and had neutral or slightly negative zeta potentials both in the absence and in the presence of siRNA at pH 7.4. The zeta potential results might be explained by the low level of peptide incorporation (1-3% of the glucose monomers in the β -glucan structure of the particles), and that the instrument to measure the zeta potential might not be sensitive enough to detect the change of the charges. In initial experiments, we show that modified GPs are efficiently internalized by macrophages both *in vitro* and *in vivo* (Figures 4 and 6). Particle uptake was similar for the modified GPs and the unmodified GPs (Figure 4).

To demonstrate the ability of the modified GPs to deliver functional siRNA to macrophages *in vivo*, modified GPs were loaded with either Scr or F4/80 siRNA and administered to lean mice (Figure 5A). The various modified GPs induce different levels of gene silencing (Figure 5). GP-His15, GP-His7, and GP-EP14 particles loaded with an F4/80-targeting siRNA significantly reduced F4/80 gene expression in peritoneal macrophages following i.p. injection in lean, healthy mice (Figures 5B, 5C, and 5F). In contrast, GP-Histamine and GP-Ethylenediamine did not induce significant silencing of F4/80 gene expression (Figures 5D and 5E). Silencing F4/80 did not affect the gene expression of other inflammatory markers (Figure S2) demonstrating the specificity of the siRNA-mediated knockdown. While some of the small-molecule amine-modified GPs did not provide significant gene silencing, which might be due to the lack of enough charges for efficient endosomal escape, several of the peptide-modified GPs were able to efficiently deliver siRNA to macrophages *in vivo*. Thus, modified GPs may represent a useful technology for the delivery of siRNA to phagocytic cells.

The peptide-modified GPs provide efficient carriers for siRNA due to their ability to overcome the numerous physiological barriers that impede successful delivery of siRNA *in vivo*. Due to its relatively large size, negative charge, and hydrophilicity, siRNA does not readily cross membranes to enter cells. In addition, inefficient release of siRNA from intracellular membranes into the cytoplasm limits the ability of siRNA to be incorporated into the RNAi machinery and to induce gene silencing. We show here that the modified GPs enable cellular internalization of the siRNA cargo by macrophages both *in vitro* and *in vivo*. Once inside the cells, the siRNA is released from the modified GPs and mediates gene silencing. We hypothesize that the weak-base histidine residues of the peptides become protonated upon acidification of the endosome, leading to disruption of the membrane and enhancing siRNA delivery to the cytoplasm. This mechanism of endosomal escape has previously been shown for Endoport and other histidine-rich peptides.^{33, 34} One of the modified GPs that showed significant silencing efficiency, the GP-EP14 particles, was selected for further studies.

A major limitation for the application of some delivery systems is their toxicity *in vivo*. Several reports have demonstrated potent immune stimulation following nanoparticle-based siRNA delivery.^{39, 40} In addition, siRNA has been shown to induce a toll-like receptor 3 (TLR3) response leading to secretion of IFN- γ .⁴¹ We therefore tested the toxicity of the GP-EP14 particles following i.p. administration. Treatment of healthy mice with GP-EP14 particles did not induce liver toxicity or the production of inflammatory cytokines (Figure S3). The levels of IFN- γ in the serum of particle-treated mice were not significantly changed when compared to mice treated with PBS (Figure S3C). In addition, no TNF- α expression (<9 pg/mL) was detected following GP-EP14 treatment. Thus, GP-EP14 particles can successfully deliver siRNA and mediate gene silencing in macrophages *in vivo* in lean, healthy mice without inducing toxic side effects.

Phagocytic cells, such as macrophages, represent potentially important targets for RNAi therapeutics on the basis of their role in mediating inflammation and immune responses.⁴² Macrophages play an important role in the pathogenesis of many inflammatory diseases, including psoriasis, asthma, rheumatoid arthritis, and inflammatory bowel disease.^{11, 43, 44}

In addition, macrophages contribute to the progression of neurodegeneration, atherosclerosis, fibrosis, cancer, and diabetes.^{42, 45-47} Therefore, development of technology that could deliver siRNA to macrophages would offer a significant advance in the treatment of these and other major human diseases. A number of recent studies have described the delivery of siRNA to phagocytic cells for the treatment of inflammatory diseases.^{22, 48-50} Despite this progress, several major challenges to the clinical translation of siRNA as therapy remain.

We demonstrate the utility of the GP-EP14 particles for the treatment of inflammation-related diseases by reducing inflammatory cytokine expression in a mouse model of obesity and insulin resistance. Obesity rates in the US and worldwide are increasing at an alarming rate, and have resulted in a rise in related health problems such as diabetes and cardiovascular disease.^{51, 52} Several laboratories have shown that macrophages accumulate in the AT of obese rodents and humans, where they secrete a variety of inflammatory cytokines, such as TNF- α and OPN, that can exacerbate insulin resistance.^{26, 28, 53} Previous studies have reported that gene ablation of chemokines or blocking cytokines by injection of antibodies can, in some cases, alleviate insulin resistance.⁵⁴⁻⁵⁶ We have previously shown silencing of TNF- α and OPN expression in macrophages of the visceral AT following i.p. injection of GeRPs in obese mice.²² Importantly, reducing expression of these pro-inflammatory cytokines using the GeRP technology resulted in an improved whole-body glucose tolerance. Thus, AT inflammation may represent a promising target for siRNA-based therapy in patients with obesity and metabolic disease. Using the GP-EP14 particles, we observed a significant 80% reduction of OPN expression in the visceral AT of *ob/ob* mice following i.p. administration of GP-EP14 particles loaded with OPN siRNA compared to GP-EP14 particles loaded with a control Scr siRNA (Figure 8B). Importantly, the GP-EP14 particles silence genes selectively in phagocytic cells in the inflamed visceral AT, with no silencing observed in other tissues such as the subcutaneous AT or liver (Figures 8E and 8F). Thus, the GP-EP14 particles reduce inflammation only in the inflamed tissue while leaving other tissues unaffected. Silencing OPN in macrophages of the visceral AT with the GP-EP14 particles resulted in an improved metabolic phenotype (Figures 8H and 8I).

The modified GPs are simple to prepare and easily loaded with siRNA, two qualities that represent a major advantage for clinical application of a delivery vehicle. In addition, they are a versatile system that can potentially be used to deliver siRNA targeting any gene of interest. Due to their ability to provide efficient siRNA delivery to phagocytic cells with minimal toxicity, we envision the modified GPs as an important step towards further development of this technology for the treatment of a variety of inflammation-related diseases.

CONCLUSIONS

In summary, small-molecule amine- and/or peptide-modified GPs are a simplified system that can efficiently deliver siRNA to phagocytic cells *in vivo*. Modified GPs were synthesized by covalently conjugating small-molecule amines and/or peptides to the GPs. Modification of the GPs facilitated the binding of siRNA to the GPs by electrostatic interaction. The new modified GPs can be loaded with siRNA simply by mixing siRNA with

the modified GPs. *In vitro* experiments showed that modified GPs are non-toxic delivery vehicles and can be efficiently internalized in macrophages. *In vivo* evaluation demonstrated that several peptide-modified GPs can efficiently deliver siRNA and reduce F4/80 gene expression in macrophages of lean, healthy mice with minimal toxicity. Among the formulations evaluated, GP-EP14 particles showed the most significant silencing efficiency. Additionally, GP-EP14 particles can silence the expression of the target inflammatory cytokine, OPN, in the inflamed AT of obese mice. Importantly, reducing the expression of OPN in AT macrophages of obese mice improved the glucose tolerance. Thus, modified GPs, and specifically GP-EP14 particles, may represent a promising new system for the efficient delivery of therapeutic siRNA to phagocytic cells *in vivo* for modulating inflammation responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Hammond SM, Caudy AA, Hannon GJ. Post-transcriptional gene silencing by double-stranded RNA. *Nat. Rev. Genet.* 2001; 2:110–119. [PubMed: 11253050]
2. Tuschl T. RNA interference and small interfering RNAs. *ChemBioChem.* 2001; 2:239–245. [PubMed: 11828450]
3. Ghildiyal M, Zamore PD. Small silencing RNAs: an expanding universe. *Nat. Rev. Genet.* 2009; 10:94–108. [PubMed: 19148191]
4. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21 nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature.* 2001; 411:494–498. [PubMed: 11373684]
5. McManus MT, Sharp PA. Gene silencing in mammals by small interfering RNAs. *Nat. Rev. Genet.* 2002; 3:737–747. [PubMed: 12360232]
6. Whitehead KA, Langer R, Anderson DG. Knocking down barriers: advances in siRNA delivery. *Nat. Rev. Drug Discovery.* 2009; 8:129–138. [PubMed: 19180106]
7. Burnett JC, Rossi JJ, Tiemann K. Current progress of siRNA/shRNA therapeutics in clinical trials. *Biotechnol. J.* 2011; 6:1130–1146. [PubMed: 21744502]
8. Kanasty R, Dorkin JR, Vegas A, Anderson DG. Delivery materials for siRNA therapeutics. *Nat. Mater.* 2013; 12:967–977. [PubMed: 24150415]
9. Davidson BL, McCray PB Jr. Current prospects for RNA interference-based therapies. *Nat. Rev. Genet.* 2011; 12:329–340. [PubMed: 21499294]
10. Rettig GR, Behlke MA. Progress toward in vivo use of siRNAs-II. *Mol. Ther.* 2012; 20:483–512. [PubMed: 22186795]

11. Kinne RW, Bräuer R, Stuhlmüller B, Palombo-Kinne E, Burmester GR. Macrophages in rheumatoid arthritis. *Arthritis Res.* 2000; 2:189–202. [PubMed: 11094428]
12. Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. *Cell.* 2011; 145:341–355. [PubMed: 21529710]
13. Olefsky JM, Glass CK. Macrophages, inflammation, and insulin resistance. *Annu. Rev. Physiol.* 2010; 72:219–246. [PubMed: 20148674]
14. Chawla A, Nguyen KD, Goh YPS. Macrophage-mediated inflammation in metabolic disease. *Nat. Rev. Immunol.* 2011; 11:738–749. [PubMed: 21984069]
15. Kopf M, Bachmann MF, Marsland BJ. Averting inflammation by targeting the cytokine environment. *Nat. Rev. Drug Discovery.* 2010; 9:703–718. [PubMed: 20811382]
16. Lapadula G, Marchesoni A, Armuzzi A, Blandizzi C, Caporali R, Chimenti S, Cimaz R, Cimino L, Gionchetti P, Girolomoni G, Lionetti P, Marcellusi A, Mennini FS, Salvarani C. Adalimumab in the treatment of immune-mediated diseases. *Int. J. Immunopathol. Pharmacol.* 2014; 27:33–48. [PubMed: 24774505]
17. Bongartz T, Sutton AJ, Sweeting MJ, Buchan I, Matteson EL, Montori V. Anti-TNF antibody therapy in rheumatoid arthritis and the risk of serious infections and malignancies: Systematic review and meta analysis of rare harmful effects in randomized controlled trials. *JAMA.* 2006; 295:2275–2285. [PubMed: 16705109]
18. Scheinfeld N. Adalimumab: a review of side effects. *Expert Opin. Drug Saf.* 2005; 4:637–641. [PubMed: 16011443]
19. Aouadi M, Tesz GJ, Nicoloso SM, Wang M, Chouinard M, Soto E, Ostroff GR, Czech MP. Orally delivered siRNA targeting macrophage Map4k4 suppresses systemic inflammation. *Nature.* 2009; 458:1180–1184. [PubMed: 19407801]
20. Tesz GJ, Aouadi M, Prot M, Nicoloso SM, Boutet E, Amano SU, Goller A, Wang M, Guo CA, Salomon WE, Virbasius JV, Baum RA, O'Connor MJ, Soto E, Ostroff GR, Czech MP. Glucan particles for selective delivery of siRNA to phagocytic cells in mice. *Biochem. J.* 2011; 436:351–362. [PubMed: 21418037]
21. Huang W, Ghisletti S, Saijo K, Gandhi M, Aouadi M, Tesz GJ, Zhang DX, Yao J, Czech MP, Goode BL, Rosenfeld MG, Glass CK. Coronin 2A mediates actin dependent de-repression of inflammatory response genes. *Nature.* 2011; 470:414–418. [PubMed: 21331046]
22. Aouadi M, Tencerova M, Vangala P, Yawe JC, Nicoloso SM, Amano SU, Cohen JL, Czech MP. Gene silencing in adipose tissue macrophages regulates whole-body metabolism in obese mice. *Proc. Natl. Acad. Sci. U.S.A.* 2013; 110:8278–8283. [PubMed: 23630254]
23. Jourdan T, Godlewski G, Cinar R, Bertola A, Szanda G, Liu J, Tam J, Han T, Mukhopadhyay B, Skarulis MC, Ju C, Aouadi M, Czech MP, Kunos G. Activation of the Nlrp3 inflammasome in infiltrating macrophages by endocannabinoids mediates beta cell loss in type 2 diabetes. *Nat. Med.* 2013; 19:1132–1140. [PubMed: 23955712]
24. Aouadi M, Vangala P, Yawe JC, Tencerova M, Nicoloso SM, Cohen JL, Shen Y, Czech MP. Lipid storage by adipose tissue macrophages regulates systemic glucose tolerance. *Am. J. Physiol.: Endocrinol. Metab.* 2014; 307:E374–E383. [PubMed: 24986598]
25. Tencerova M, Aouadi M, Vangala P, Nicoloso SM, Yawe JC, Cohen JL, Shen Y, Garcia-Menendez L, Pedersen DJ, Gallagher-Dorval K, Perugini RA, Gupta OT, Czech MP. Activated Kupffer cells inhibit insulin sensitivity in obese mice. *FASEB J.* 2015; 29:2959–2969. [PubMed: 25805830]
26. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AWJ. Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.* 2003; 112:1796–1808. [PubMed: 14679176]
27. Lumeng CN, Saltiel AR. Inflammatory links between obesity and metabolic disease. *J. Clin. Invest.* 2011; 121:2111–2117. [PubMed: 21633179]
28. Heilbronn LK, Campbell LV. Adipose tissue macrophages, low grade inflammation and insulin resistance in human obesity. *Curr. Pharm. Des.* 2008; 14:1225–1230. [PubMed: 18473870]
29. Bartz R, Fan H, Zhang J, Innocent N, Cherrin C, Beck SC, Pei Y, Momose A, Jadhav V, Tellers DM, Meng F, Crocker LS, Sepp-Lorenzino L, Barnett SF. Effective siRNA delivery and target mRNA degradation using an amphipathic peptide to facilitate pH-dependent endosomal escape. *Biochem. J.* 2011; 435:475–487. [PubMed: 21265735]

30. Nomiya T, Perez Tilve D, Ogawa D, Gizard F, Zhao Y, Heywood EB, Jones KL, Kawamori R, Cassis LA, Tschöp MH, Bruemmer D. Osteopontin mediates obesity-induced adipose tissue macrophage infiltration and insulin resistance in mice. *J. Clin. Invest.* 2007; 117:2877–2888. [PubMed: 17823662]
31. Kiefer FW, Zeyda M, Gollinger K, Pfau B, Neuhofer A, Weichhart T, Säemann MD, Geyeregger R, Schleiderer M, Kenner L, Stulnig TM. Neutralization of osteopontin inhibits obesity induced inflammation and insulin resistance. *Diabetes.* 2010; 59:935–946. [PubMed: 20107108]
32. Chapman J, Miles PD, Ofrecio JM, Neels JG, Yu JG, Resnik JL, Wilkes J, Talukdar S, Thapar D, Johnson K, Sears DD. Osteopontin is required for the early onset of high fat-diet induced insulin resistance in mice. *PLoS One.* 2010; 5:e13959. [PubMed: 21103061]
33. Summerton JE. Endo-Porter: A novel reagent for safe, effective delivery of substances into cells. *Ann. N Y Acad. Sci.* 2005; 1058:62–75. [PubMed: 16394126]
34. Summerton JE. Peptide composition and method for delivering substances into the cytosol of cells. 2006
35. Neu M, Fischer D, Kissel T. Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. *J. Gene Med.* 2005; 7:992–1009. [PubMed: 15920783]
36. Zhang S, Zhao B, Jiang H, Wang B, Ma B. Cationic lipids and polymers mediated vectors for delivery of siRNA. *J. Controlled Release.* 2007; 123:1–10.
37. Ballarin-Gonzalez B, Howard KA. Polycation-based nanoparticle delivery of RNAi therapeutics: Adverse effects and solutions. *Adv. Drug Delivery Rev.* 2012; 64:1717–1729.
38. Herre J, Gordon S, Brown GD. Dectin-1 and its role in the recognition of beta glucans by macrophages. *Mol. Immunol.* 2004; 40:869–876. [PubMed: 14698225]
39. Abrams MT, Koser ML, Seitzer J, Williams SC, DiPietro MA, Wang W, Shaw AW, Mao X, Jadhav V, Davide JP, Burke PA, Sachs AB, Stirdivant SM, Sepp-Lorenzino L. Evaluation of efficacy, biodistribution, and inflammation for a potent siRNA nanoparticle: effect of dexamethasone co treatment. *Mol. Ther.* 2010; 18:171–180. [PubMed: 19738601]
40. Tao W, Mao X, Davide JP, Ng B, Cai M, Burke PA, Sachs AB, Sepp Lorenzino L. Mechanistically probing lipid-siRNA nanoparticle-associated toxicities identifies Jak inhibitors effective in mitigating multifaceted toxic responses. *Mol. Ther.* 2011; 19:567–575. [PubMed: 21179008]
41. Kleinman M, Yamada K, Takeda A, Chandrasekaran V, Nozaki M, Baffi J, Albuquerque R, Yamasaki S, Itaya M, Pan Y, Appukuttan B, Gibbs D, Yang Z, Kariko K, Ambati B, Wilgus T, DiPietro L, Sakurai E, Zhang K, Smith J, Taylor E, Ambati J. Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. *Nature.* 2008; 452:591–597. [PubMed: 18368052]
42. Czech MP, Aouadi M, Tesz GJ. RNAi-based therapeutic strategies for metabolic disease. *Nat. Rev. Endocrinol.* 2011; 7:473–484. [PubMed: 21502982]
43. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. *Nature.* 2013; 496:445–455. [PubMed: 23619691]
44. Clark RA, Kupper TS. Misbehaving macrophages in the pathogenesis of psoriasis. *J. Clin. Invest.* 2006; 116:2084–2087. [PubMed: 16886055]
45. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation.* 2002; 105:1135–1143. [PubMed: 11877368]
46. Wynn TA, Barron L. Macrophages: Master regulators of inflammation and fibrosis. *Semin. Liver Dis.* 2010; 30:245–257. [PubMed: 20665377]
47. Qian B-Z, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell.* 2010; 141:39–51. [PubMed: 20371344]
48. Leuschner F, Dutta P, Gorbato R, Novobrantseva TI, Donahoe JS, Courties G, Lee KM, Kim JI, Markmann JF, Marinelli B, Panizzi P, Lee WW, Iwamoto Y, Milstein S, Epstein-Barash H, Cantley W, Wong J, Cortez-Retamozo V, Newton A, Love K, Libby P, Pittet MJ, Swirski FK, Kotliansky V, Langer R, Weissleder R, Anderson DG, Nahrendorf M. Therapeutic siRNA silencing in inflammatory monocytes in mice. *Nat. Biotechnol.* 2011; 29:1005–1010. [PubMed: 21983520]
49. Novobrantseva TI, Borodovsky A, Wong J, Klebanov B, Zafari M, Yucius K, Querbes W, Ge P, Ruda VM, Milstein S, Speciner L, Duncan R, Barros S, Basha G, Cullis P, Akinc A, Donahoe JS, Jayaprakash KN, Jayaraman M, Bogorad RL, Love K, Whitehead K, Levins C, Manoharan M, Swirski FK, Weissleder R, Langer R, Anderson DG, de Fougères A, Nahrendorf M, Kotliansky

- V. Systemic RNAi-mediated gene silencing in nonhuman primate and rodent myeloid cells. *Mol. Ther. @Nucleic Acids*. 2012; 1:e4.
50. Kim S-S, Ye C, Kumar P, Chiu I, Subramanya S, Wu H, Shankar P, Manjunath N. Targeted delivery of siRNA to macrophages for anti-inflammatory treatment. *Mol. Ther.* 2012; 18:993–1001.
51. Flegal KM, Carroll MD, Kit BK, Ogden CL. Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999–2010. *JAMA*. 2012; 307:491–497. [PubMed: 22253363]
52. Flier JS. Obesity wars: Molecular progress confronts an expanding epidemic. *Cell*. 2004; 116:337–350. [PubMed: 14744442]
53. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J. Clin. Invest.* 2003; 112:1821–1830. [PubMed: 14679177]
54. Kamei N, Tobe K, Suzuki R, Ohsugi M, Watanabe T, Kubota N, Ohtsuka Kowatari N, Kumagai K, Sakamoto K, Kobayashi M, Yamauchi T, Ueki K, Oishi Y, Nishimura S, Manabe I, Hashimoto H, Ohnishi Y, Ogata H, Tokuyama K, Tsunoda M, Ide T, Murakami K, Nagai R, Kadowaki T. Overexpression of monocyte chemoattractant protein-1 in adipose tissues causes macrophage recruitment and insulin resistance. *J. Biol. Chem.* 2006; 281:26602–26614. [PubMed: 16809344]
55. Kanda H. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J. Clin. Invest.* 2006; 116:1494–1505. [PubMed: 16691291]
56. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor- α in human obesity and insulin resistance. *J. Clin. Invest.* 1995; 95:2409–2415. [PubMed: 7738205]

Figure 1. Amine- and peptide-modified GPs are a simplified system for siRNA delivery
 Schematic diagrams of loading strategies for (A) GeRPs and (B) modified glucan particles (GPs). (A) The loading strategy for GeRPs involves formation of EP/siRNA complexes, EP/siRNA complex loading in a hydrodynamic volume into the glucan particles, and finally entrapment of the complexes in the particles. (B) Amine- and peptide-modified GPs can be loaded with siRNA by mixing siRNA with particles. Covalent attachment of a small-molecule amine/peptide to the glucan particle facilitates electrostatic binding of siRNA to the delivery system. (C) General method for the covalent coupling of small-molecule amines/peptides to GPs via reductive amination chemistry.

Figure 2. Small-molecule amine- and peptide-modified GPs bind to siRNA

Modified GPs were incubated with fluorescently-labeled siRNA to form electrostatic complexes. Particles loaded with siRNA were sedimented by centrifugation and the supernatant was assessed for siRNA content. (A) Percentage of siRNA binding was plotted against particle concentration ($\mu\text{g/mL}$) to determine the binding affinity of the particles. Error bars have been removed for clarity. % siRNA binding $\pm$ standard deviation for GP-His15 at 0 $\mu\text{g/mL}$, 30 $\mu\text{g/mL}$, 60 $\mu\text{g/mL}$, and 100 $\mu\text{g/mL}$ respectively: 0 ± 0 , 97.5 ± 0.7 , 98.6 ± 0.7 , and 98.9 ± 1.0 . % siRNA binding $\pm$ standard deviation for GP-EP14 at 0 $\mu\text{g/mL}$, 30 $\mu\text{g/mL}$, 60 $\mu\text{g/mL}$, and 100 $\mu\text{g/mL}$ respectively: 0 ± 0 , 96.4 ± 2.1 , 98.7 ± 0.4 , and 98.0 ± 0.5 . (B – D) Percentage of free siRNA was plotted against siRNA concentration (μM). Particles with higher binding affinity have a lower percentage of free siRNA at increasing siRNA concentrations.

A J774A.1 Cells **B PECs**

Figure 3. Modified GPs are non-toxic *in vitro*

Viability of (A) J774A.1 cells and (B) PECs treated with various concentrations of modified GPs for 48 h. Results are mean $\pm$ s.e.m.

Figure 4. Modified GPs are phagocytosed by macrophages *in vitro*

PECs were treated with FITC-labeled modified GPs loaded with siRNA *in vitro* for 48 h. Confocal microscopy shows modified GPs (green) present in F4/80-positive macrophages (red). Nuclei were stained with DAPI (blue) (60x magnification, scale bar: 20 μ m).

Figure 5. Modified GPs can silence genes in macrophages *in vivo* in healthy, lean mice
(A) Time line of modified GP administration and isolation of PECs. Briefly, 8-week old C57BL6/J mice were injected once a day for 5 days with modified GPs or GeRPs loaded with either Scr (black) or F4/80 (grey) siRNA. On day 6, mice were sacrificed and PECs were isolated. Total RNA was extracted and F4/80 expression was measured by real-time PCR in PECs. mRNA expression data from PECs isolated from mice treated with **(B)** GP-His15, **(C)** GP-His7, **(D)** GP-Histamine, **(E)** GP-Ethylenediamine, **(F)** GP-EP14, and **(G)** GeRPs. $n = 5-9$, statistical significance was determined by student's t-test. * $p < 0.05$, *** $p < 0.001$. Results are mean $\pm$ s.e.m.

Figure 6. Modified GPs are internalized by macrophages *in vivo* in healthy, lean mice

Images showing PECs from lean mice administered FITC-labeled modified GPs loaded with scrambled siRNA by i.p. injection. PECs were isolated 24 h following the last injection and stained with an F4/80 antibody. PECs from mice treated with (A) GP-His15, (B) GP-His7, and (C) GP-EP14 (FITC modified-GPs – green, F4/80 – red, DAPI – blue, 63x magnification, scale bar: 20 μ m). (D) Quantification of percentage of PECs containing modified GPs. The %GP+ cells was calculated by counting the number of F4/80+ cells that contained FITC-labeled GPs in a representative field of view. %GP+ cells = (# of F4/80+ cells that contain FITC-labeled GPs)/(total number of cells) $\times$ 100. n = 2-3 fields of view. Results are mean $\pm$ s.e.m.

Figure 7. GP-EP14 particles injected i.p. in obese mice are localized in visceral adipose tissue macrophages

(A) Immunofluorescence microscopy of heart, lung, liver, spleen, kidney, pancreas, visceral AT, and subcutaneous AT from obese mice 24 h after i.p. injection with FITC-labeled GP-EP14 particles (FITC GP-EP14 – green, DAPI – blue, 10x magnification, scale bar: 50 μ m).

(B) Confocal microscopy showing SVF cells isolated from the visceral AT of obese mice treated with either PBS or FITC-labeled GP-EP14 particles. SVF was isolated 24 h after treatment and stained with an F4/80 antibody (FITC GP-EP14 – green, F4/80 – red, DAPI – blue, 63x magnification, scale bar: 10 μ m).

Figure 8. GP-EP14 particles can reduce the expression of inflammatory cytokines specifically in the visceral adipose tissue of obese mice

(A) Outline of GP-EP14 treatment given to obese mice. Briefly, 5-week old *ob/ob* mice were injected once a day for 5 days with 1 mg of GP-EP14 particles loaded with 10 nmoles of siRNA. Forty-eight hours after the last injection, glucose tolerance tests (GTTs) were performed on mice that were fasted for 16 h. On day 7, mice were sacrificed and visceral AT, subcutaneous AT, and liver were isolated. Total RNA was extracted and (B) OPN, (C) F4/80, and (D) TNF- α gene expression was measured by real-time PCR in visceral AT from

mice treated with GP-EP14 particles loaded with Scr (black) or OPN (grey) siRNA, $n = 15$. Expression of OPN in **(E)** subcutaneous AT and **(F)** liver from mice treated with GP-EP14 particles, $n = 15$. **(G)** Total body weight gain in obese mice treated for 5 d with PBS (white) or GP-EP14 particles loaded with Scr (black) or OPN (grey) siRNA. $n = 10$. **(H)** Glucose tolerance tests (GTTs) were performed on mice that were fasted for 16 h. **(I)** Area under the curve (A.U.C) was calculated using GraphPad Prism, $n = 10$, statistical significance was determined by student's t-test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. Results are mean $\pm$ s.e.m.

Table 1

Small-molecule amines and sequences of peptides used in the present studies.

Small Molecule Amines ^a	Selected Peptides ^a
Ethylenediamine (E)	LHLLHHLLHHLHHL (EP14)
Histamine (H)	LHHLL (EP5)
E:H (75:25)	His15
E:H (50:50)	MethylHis15
E:H (25:75)	His7
E:Leu5 (50:50)	His15:Leu5 (50:50)
E:Leu15 (50:50)	His15:Leu15 (50:50)

^a Abbreviations are presented in parentheses. For particles modified with two different amines and/or peptides, the ratio given in parentheses is the theoretical molar ratio of the two compounds.