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Synthetic PEGylated Glycoproteins and their Utility in Gene Delivery

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Abstract

PEGylated glycoproteins (PGPs) were synthesized by co-polymerizing a Cys-terminated PEGpeptide, glycopeptide and melittin peptide. Compositionally unique PGPs were prepared by varying the ratio of PEG-peptide (20%-90%) and melittin (0-70%) with a constant amount of glycopeptide (10%). The PGPs were purified by RP-HPLC, characterized for molecular weight and polydispersity by GPC-HPLC and SDS-PAGE, and for composition by RP-HPLC following reduction to form monomeric peptides. PGPs formed DNA condensates of 200-300 nm in diameter that were administered to mice via the tail vein. Biodistribution studies confirmed their primary targeting to liver hepatocytes with a DNA metabolic half-life of 1 hour. Upon stimulation by hydrodynamic dosing with saline, PGP DNA (5 μ g) mediated luciferase expression in the liver detected by bioluminescence imaging (BLI) after 24 hours. The level of gene expression mediated by PGP DNA was 5000-fold less than direct hydrodynamic dosing of an equivalent amount of DNA and was independent of the mol percent of melittin incorporated into the polymer, but dependent on the presence of galactose on PGP. The results establish the ability to prepare three-component gene delivery polymers that function in vivo. Further design improvements in fusogenic peptides for gene delivery and for the simultaneous use of a nuclear targeting strategy will be necessary to approach levels of expression mediated by the direct hydrodynamic dosing of DNA.

Keywords

gene delivery; melittin; glycopeptide; biodistribution; polymerization

Introduction

Glycoproteins are a class of biological molecules composed of a protein backbone modified with N or O-glycans (1). Their functions are quite varied and often involve the interaction of the terminal sugars on the glycan with endogenous lectins on mammalian cell surfaces (2).

One of the earliest non-viral gene delivery systems was able to target DNA to internalize into hepatocytes via the asialoglycoprotein receptor (3,4). This delivery system was composed of an asialoglycoprotein covalently linked to polylysine. Refinement of this delivery system has been on-going by many groups since this first demonstration. The ligand has been simplified by utilization of a cluster of the monosaccharide galactose (5-7), neoglycopeptides (8) or purified Nglycans (9-11). Polylysine has been substituted by PEI (12), chitosan (13), dendrimers (14), and synthetic peptides (10,15-18) containing clusters of Lys or Arg residues.

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In many cases, the polymers have been modified by polyethylene glycol (PEG) (10,19-22) to render them more biocompatible for use as in vivo gene delivery agents. In all, these refinements have led to more sophisticated non-viral gene delivery systems that are smaller, more homogeneous, more modular, and better equipped to achieve sub-cellular targeting than their predecessors.

Two key steps to enhancing the expression of a receptor mediated non-viral gene delivery system in vivo are 1) DNA escape from the endosome (23-26) and 2) nuclear targeting (27). Several studies have reported the utility of fusogenic peptides to enhance endosomal escape and gene expression in vitro (21,25,28) but fewer studies have reported their use in vivo (29, 30) and none have reported the use of melittin in vivo. In part, the difficulty in demonstrating the utility of fusogenic peptides in vivo relates to the synthetic challenge in incorporating these into the gene delivery system so that they can be properly released within the endosomal compartment of the target cell. A further complication with testing the efficacy of fusogenic peptides in vivo is the need to simultaneously achieve efficient targeted delivery of DNA to cells and nuclear targeting of DNA, to observe appreciable gene expression in animals.

Ongoing studies from our lab have reported the development of peptides possessing terminal Cys residues that bind DNA and polymerize through disulfide bond formation (31,32). The resulting polypeptide DNA condensates mediate enhanced in vitro gene transfer, presumably by triggered release of the DNA following intracellular reduction of disulfide bonds (33). To test the utility of this approach in vivo, we have reported the use of a Cys-terminated glycopeptide and PEG-peptide that co-polymerize on DNA, direct its biodistribution through receptor-mediated targeting to hepatocytes in mice, and produce measurable but low-level gene expression in vivo (11,22).

In the present study, we have advanced this delivery system to generate ternary peptide copolymers composed of PEG-peptide, glycopeptide and a fusogenic melittin peptide. A detailed account of the polymerizable melittin peptide used in this study was recently reported (34). The resulting PEGylated glycoproteins (PGPs) are prepared with a precise stoichiometry of peptide components and have the ability to bind to DNA and mediate targeted gene delivery in vivo. Entry into hepatocytes requires careful control over the particle size of DNA condensates (35). Once inside the cell, the reduction of disulfide bonds should lead to the rapid dissociation of the carrier from the DNA and release of melittin in the endosome where it can function to facilitate DNA escape to the cytosol.

However, without the ability to target DNA to the nucleus, endocytosed DNA remains largely in the cytosol where it is incapable of mediating significant gene expression in quiescent hepatocytes. To stimulate gene expression, we report the utility of hydrodynamic dosing (36) of saline 5 minutes after a conventional dose of formulated DNA. This approach has allowed us to utilize bioluminescence imaging (BLI) to compare the efficiency of PGP DNA relative to the direct hydrodynamic dosing of an equivalent amount of DNA.

Materials and Methods

General Methods

Substituted Wang resin for peptide synthesis, N-terminal Fmoc protected amino acids, 9-hydroxybenzotriazole, and diisopropylethylamine were obtained from Advanced ChemTech (Lexington, KY). *N,N*-Dimethylformamide, trifluoroacetic acid (TFA), acetic acid anhydride, acetonitrile, and piperidine were purchased from Fisher Scientific (Pittsburgh, PA). Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP), thiazole orange, polylysine of varying molecular weight and bovine testes β -galactosidase (EC 3.2.1.23) were obtained from Sigma Aldrich Co. (St Louis, MO). PEG standards were purchased from Tosoh Corp. (Tokyo, Japan).

D-luciferin and luciferase from *Photinus pyralis* were purchased from Roche Applied Science (Indianapolis, IN). Inactivated “qualified” fetal bovine serum (FBS) and Seebblue® pre-stained peptide standard were from Invitrogen Co. (Carlsbad, CA). BCA assay kit, Blocker™ BSA in TBS (10×) and 1-Step™ NBT/BCIP were from Pierce (Rockford, IL). Precast 4-15 % gradient SDS-PAGE gels (Ready Gel®) were purchased from BioRad (Hercules, CA). GS-1 lectin was from EY Laboratories Inc. (San Mateo, CA). Protran® (pure nitrocellulose transfer and immobilization membrane, 0.2 μm) was from Schleicher & Schuell BioScience (Bassel, Germany). pGL3 control vector, a 5.3 kb luciferase plasmid containing a SV40 promoter and enhancer was obtained from Promega (Madison, WI, USA). pDNA was amplified in DH5α strain of *Escherichia coli* and purified on an endotoxin-free Qiagen megaprep column (Valencia, CA) according to the manufacturer's instructions.

Peptide Synthesis

Peptide synthesis was carried out on an Apex 396 from Advanced Chem Tech (Louisville, KY). Peptide purification was performed using a semi-preparative (10 μm) C₁₈ RPHPLC column from Vydac (Hesperia, CA). Preparative HPLC was performed using a computerinterfaced HPLC and fraction collector from ISCO (Lincoln, NE). Electrospray mass spectrometry (ES-MS) was performed using an Agilent 1100 LC-MS system.

Cys-terminated melittin peptide, (**M1**) CKKKIGAVLKVLTTG LPALISWIKRKRQKKKC, was synthesized using standard Fmoc procedures as described previously (34).

DNA binding peptide (**P1**) (Acm)CKKKKKKKKKCKKKKKKKKKWC(Acm) (acetamidomethyl) was synthesized using standard Fmoc procedures starting from Fmoc-Cys (Acm)-Wang resin, except that N-capping with diisopropyl ethylamine and acetic anhydride was included after each coupling cycle to avoid truncated sequences. The peptide was cleaved from the resin and deprotected (except for the Acm protecting groups on cysteines) in 95% TFA. The peptide was then purified to homogeneity by injecting 1 μmol onto a semi-preparative RPHPLC column (2 × 25 cm) eluted at 10 ml/min with 0.1% (v/v) TFA and a gradient of acetonitrile (5-25% over 30 min) while monitoring tryptophan absorbance at 280 nm. The major peak eluting at 22 min was collected and pooled from multiple injections, concentrated by rotary evaporation, lyophilized, and stored dry at -20 °C. The purified peptides were reconstituted in 0.1% TFA, quantified by tryptophan absorbance ($\epsilon_{280\text{ nm}} = 5600\text{ M}^{-1}\text{ cm}^{-1}$) and characterized on an Agilent 1100 LC-MS resulting in m/z, 2706.6 / 2706.1 (found / calculated).

An Acm-protected PEG-peptide was synthesized by reacting **P1** with PEG-maleimide (molar ratio 1:1.5) in 0.1 M sodium phosphate buffer (pH 7.0) at RT for 3h. The PEG-peptide was purified by injection of 2 μmol onto semi-preparative RP-HPLC (2 × 25 cm) eluted at 10 ml/min with 0.1% TFA and a gradient of acetonitrile (5-65% over 30 min) while detecting Abs_{280 nm}. Final deprotection was accomplished by dissolving 5 μmol in 1 ml of cold TFA followed by the addition of silver tetrafluoroborate (20 mol eq. per Acm group) and anisole (10 eq. per Acm group) followed by reaction at 4°C for 1 hr with stirring. The deprotected PEGpeptide was precipitated as the silver salt in 60% (v/v) diethyl ether. The precipitate was centrifuged then re-dissolved in 0.1 M acetic acid containing DTT (40 eq. per Acm group) and reacted at RT for 3 hr. Following centrifugation to remove insolubles, the deprotected PEG-peptide was purified on a semi-preparative RP-HPLC eluted as described above. The isolated PEG-peptide was freeze-dried and reconstituted in 0.1% TFA and reanalyzed by analytical HPLC to determine purity. The PEG-peptide was characterized by MALDI-TOF by spotting 1 μl (1 nmol) in α-cyano-4-hydroxycinnamic acid (30 mg/ml) on the target. A broad peak with an average mass of 8077 m/z was observed, consistent with the anticipated mass of the conjugate.

Acm-protected glycopeptide was synthesized by conjugating iodoacetamide triantennary *N*-glycan (37) and **P1** at a molar ratio of 1:1.25 in 0.2 M Tris buffer (pH 8.0) for 12 h at RT. The resulting glycopeptide was purified by a semi-preparative RP-HPLC column (2 × 25 cm) eluted at 10 ml/min with 0.1% TFA and a gradient of 5-20% acetonitrile over 30 min. The peak corresponding to the glycopeptides was pooled and lyophilized. Final deprotection of the Acm groups was performed as described above. After purification on semi-preparative RP-HPLC, the glycopeptide was concentrated, lyophilized and reconstituted in 0.1% TFA, then quantified by Abs_{280 nm} ($\epsilon_{280 \text{ nm}} = 6930 \text{ M}^{-1} \text{ cm}^{-1}$) and characterized by LC-MS resulting in *m/z*: 4773.5 / 4775.0 (found / calculated).

Synthesis of PGPs

Cys-terminated melittin peptide, glycopeptide and PEG-peptide were combined in a predetermined ratio resulting in 200 nmols of total peptide in 200 μl of 0.1% TFA. The peptides were vortexed and freeze-dried, then dissolved in 16 μl of 0.1 M sodium phosphate pH 8 containing 4 μl of DMSO and reacted at RT for 48 hrs.

The progress of the polymerization was monitored using Ellman's method (38) to monitor free thiols. An aliquot (2 μl) was combined with 1 ml of DTNB solution (10 mM DTNB in 100 mM sodium phosphate pH 7.3 containing 0.5 mM EDTA) at times ranging from 1 min to 48 hrs. The absorbance was determined at 412 nm relative to a blank of DTNB solution.

PGPs were purified on a semi-preparative RP-HPLC column (2 × 25 cm) eluted at 10 ml/min with 0.1% TFA and a gradient of 20-55% acetonitrile over 30 min. The primary peak was collected, concentrated by freeze drying, dissolved in water and quantified by Abs_{280 nm} ($\epsilon = 6900 \text{ M}^{-1} \text{ cm}^{-1}$). Agalatosyl PGP was prepared by treating the PGP 3 with β -galactosidase as reported previously (22). The agalactosyl PGP3* was characterized by compositional analysis as describe below, during which LC-MS verified the complete removal of galactose from the glycopeptide.

RP-HPLC purified PGPs (10 nmol) were prepared in 100 μl of 50 mM Tris pH 7.5 to which 50 nmol equivalents of TCP was added in 10 μl of water and reacted for 3 hrs at RT. The product was chromatographed on analytical RP-HPLC eluted 1 ml/min with 0.1% TFA and 5-65% gradient of acetonitrile over 30 min. The glycopeptide, PEG-peptide and melittin peptide were quantified against primary standards of each peptide to allow calculation of the peptide composition of each PGP.

The molecular weight of each PGP was determined GPC-HPLC on an Agilent 1100 HPLC system equipped with a refractive index detector and a Shodex OH Pak SB-800 HQ column (7.8×300 mm). Freeze dried PGPs were dissolved in the mobile phase (10 v/v% methanol in water prepared in 0.5 M sodium chloride and 50 mM sodium phosphate pH 7) and 100 μl (10 nmol) was applied to the column. The average molecular weight (MW) was calculated relative to the elution time of polylysine standards of 3.5, 20.4, 27.4, 66.7 and 84.0 kDa and PEG standards of 24, 50, 107, 140 kDa.

PGPs (8 nmols) were dissolved in non-denaturing sample loading buffer containing 8 M urea, then loaded onto a 4-15% gradient SDS-PAGE without a stacking gel and run for 1.5 hrs at 90 V. PGPs bands were visualized by staining with coumassie followed by destaining. PGPs were also stained for PEG using Dragendorff's method (39). PGPs were likewise stained for galactose following transfer onto a nitrocellulose membrane in transfer buffer (10 mM NaHCO₃, 9.9 mM Na₂CO₃, 0.1 % SDS, pH 9.9). The membrane was blocked overnight in 2.5 % BSA in TBS (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride), washed 3 times with TBST (0.1 % Tween 20 in TBS) and once with lectin buffer I (1 mM CaCl₂ and 1 mM MgSO₄ in TBS). The membrane was then incubated with 1 ml of alkaline phosphatase-

conjugated GS-1 lectin (1 µg/ml) for 1.5 h. The unbound lectin was removed by washing with TBST three times and the membrane was treated with 5 ml of lectin buffer II containing 100 µg/ml of nitro blue tetrazolium and 200 µg/ml of 5-bromo-4-chloro-3-indolyl phosphate.

DNA Formulation, Biodistribution and Gene Expression

PGP binding to DNA was monitored by a fluorophore exclusion assay (17). PGP DNA condensates (2.5 µg in 50 µl) were prepared at 0, 0.1, 0.15, 0.3, 0.6 and 1.2 nmol of peptide monomer per µg of DNA then added to 950 µl of HBM (0.27 M mannitol, 5 mM Hepes, pH 7.5) containing 0.1 µM thiazole orange. The fluorescence of the intercalator dye was measured using an LS50B fluorometer (Perkin Elmer, UK) by exciting at 500 nm while monitoring emission at 530 nm with the slit width set at 15 and 20 nm, respectively. Fluorescence blanks were subtracted from all values before data analysis.

The particle size of peptide DNA condensates was determined by quasi-elastic light scattering at a scatter angle of 90° on a Brookhaven ZetaPlus particle sizer. Condensates were prepared at a DNA concentration of 50 µg/ml in 400 µl of HBM at a stoichiometry of 0.3 nmol of peptide per µg of DNA corresponding to a charge ratio of approximately 2:1 for each. The mean diameter and population distribution were computed from the diffusion coefficient using a unimodal cumulate analysis supplied by the manufacturer. The zeta potential was determined as the mean of 10 measurements in 5 mM Hepes pH 7.4.

Plasmid DNA was radiolabeled with ¹²⁵I as described previously (40) resulting in ¹²⁵IDNA with a specific activity of 250 nCi/µg. PGP DNA biodistribution studies were conducted in ICR mice. Mice were anesthetized and ¹²⁵I-DNA (2.5 µg, 0.75 µCi) condensed with 0.8 nmol PGP in 50 µL of HBM were dosed i.v. via the tail vein. After 5, 15, 30, 60, 120, 240, 360, 480 min, mice were sacrificed by cervical dislocation and the major organs (liver, lung, spleen, stomach, kidney, heart, large intestine, and small intestine) were harvested, rinsed with saline, and weighed. The radioactivity in each organ was determined by direct γ-counting and expressed as the percent of the dose in the target organ (10). The distribution of liver targeted DNA between hepatocytes and Kupffer cells was determined following collagenase perfusion as described previously (10).

PGP DNA condensates were prepared for in vivo gene expression by combining 15 µg of pGL3 in 150 µl of HBM (0.27 M mannitol, 5 mM Hepes, pH 7.5) with 4.5 nmol of PGP in 150 µl of HBM while vortexing, resulting in DNA condensates possessing a calculated charge ratio (NH₄⁺:PO₄⁻) of 2. Triplicate mice (20 g) were administered a 100 µl tail vein dose (5 µg of DNA, 1.5 nmol of PGP) and after 5 min, a 1.8 ml dose of PBS was hydrodynamically delivered (36) via the tail vein to stimulate gene expression. After 24 hrs the mice were dosed i.p. with 80 µl (2.4 mg) of D-luciferin then anesthetized with isoflurane gas, and imaged after 4 min on an IVIS Imaging System (Xenogen, Alameda, CA). The luminescence in liver was quantified (photons/sec/cm²/steradian) by Living Image software and is presented with a pseudo-color overlay of luminescence intensity on grayscale images. The photon intensity in liver was converted into pg of luciferase via a standard curve that was reported previously (41).

Results

The present study prepared three Cys-terminated peptides used as monomers to polymerize into PEGylated glycoproteins (PGPs) with utility for gene transfer. This strategy would allow polymeric peptides to undergo reduction into their monomeric components after cellular internalization (42). We have previously utilized DNA as a template to polymerize either one or two Cys-terminated peptides into polymers (22,31). However, template polymerization becomes inherently more difficult as the number of peptide components increase. This is especially true if each peptide component possesses a different binding affinity for DNA. Thus,

when preparing polymers that possess a PEG-peptide, glycopeptide and a melittin peptide it became desirable to polymerize, purify and characterize the resulting PGPs prior to the formation of DNA condensates. The component PEG-peptide, glycopeptide and melittin peptide utilized in this study and the resulting PGPs are illustrated in Figure 1.

To establish optimal polymerization conditions for multi-component PGPs, a model peptide (CWK₁₇C) was used (31). The results obtained were similar to those reported by Seymour and colleagues using a single peptide (43). The polymerization reaction was monitored by RP-HPLC with the full-length polymer eluting later relative to monomeric and intermediate length peptides. The polymerization progressed further toward completion when the peptide was concentrated to 10 mM in 100 mM sodium phosphate buffer pH 8. More dilute peptide solutions polymerized more slowly and to a lesser degree of completion. Lowering the pH to 7 or 6 caused the reaction to slow whereas raising the pH to 9 or 10 did not increase the rate. Likewise, phosphate buffer concentrations higher than 0.1 M did not influence the rate of reaction. When applying the polymerization conditions described above to a two or three component polymerization to form PGPs, the same trends were observed except that the limiting solubility of the melittin peptide (**M1**) necessitated the addition of 20 v/v % DMSO, which also facilitated oxidation to form cystines.

The time course of the optimized reaction to form PGPs was monitored by Ellman's reaction to detect residual thiols (Fig. 2). The results indicate that the reaction was >95% complete after 48 hrs at RT. Analysis of the reaction by RP-HPLC established that the glycopeptide, PEG-peptide and melittin peptide were consumed during the 48 hrs of reaction with the formation of a major peak (Fig. 3). PGPs were preparatively purified to remove minor earlier or later eluting shoulder peaks.

A calibrated GPC-HPLC was used as the primary means to measure the approximate size of each PGP. Each purified PGP appeared as single peak on a Shodex column eluted with 10% methanol and 0.5 M sodium chloride (Fig. 4A-E). When using commercially available polylysines as calibrants, the average molecular weight of PGP 1 - PGP 4 was determined to be 83-106 kDa (Table 1). The average molecular weight of PGP 5 was significantly lower (35 kDa) than the other PGPs, most likely due to the larger ratio of **M1** in the polymer (Table 1). The average molecular weight of PGP 1-4 was estimated to be approximately 50 kDa when using PEG standard to calibrate the GPC-HPLC (Table 1). Based on the monomer composition and molecular weight of each monomer, the average degree of polymerization (dp) for PGP 1-4 was found to be 10-13 monomer peptides based on polylysine standards or dp 5-6 based on PEG standards.

Gradient SDS-PAGE was used as a secondary measure of molecular size. As illustrated in figure 4, PGPs 1-4 each showed a high molecular weight band of greater than 250 kDa based on protein standards stained with coumassie (Fig. 4, Lanes A-E), as well as Dragendorff's stain (Lanes F-J) for PEG and by lectin overlay (Lanes K-O) to detect for galactose terminated N-glycans. Although the PAGE analysis established that each PGP is polymeric and contains both PEG and N-glycan, the molecular weight by PAGE appears to be overestimated. As with GPCHPLC, the PAGE analysis of PGP 5 established that it is substantially less polymeric than the other PGPs (Fig. 4, lane E, J, O).

Each purified PGP was also characterized for the precise composition of Cys-terminated peptide in the polymer. Reduction of each PGP followed by RP-HPLC analysis led to the quantitative analysis of each peptide component (Fig. 5A-E'). The results summarized in Table 1 indicate that melittin peptide was slightly over incorporated and the glycopeptide was slightly under incorporated relative to the input ratio. However, these results clearly indicate the potential to manipulate the PGP composition.

PGPs were individually combined with plasmid DNA and the binding stoichiometry was measured by fluorophore displacement assay (not shown). Based on this experiment, the PGP to DNA stoichiometry was set at 0.3 nmol (based on peptide monomer) per μg of DNA corresponding to approximately a 2:1 N:P ratio. The particle size of each PGP DNA condensate ranged from 200-300 nm whereas the zeta potential was + 0.5-6 mV (Table 1).

The biodistribution of tail vein dosed PGP ^{125}I -DNA revealed each targeted the liver as the primary target site (Fig 6). Nearly 50% of the dose accumulated in the liver within 5 min following i.v. dosing. Control experiments reported previously (10), established that naked ^{125}I -DNA also accumulates with 50% of the dose recovered in the liver after 5 min but is rapidly metabolized and eliminated from the liver with a half-life of 30 min. Likewise, previously published biodistribution studies also established that 65% of the liver targeted dose of naked ^{125}I -DNA was recovered from Kupffer cells and only 35% recovered from hepatocytes (10). In contrast, each PGP ^{125}I -DNA resulted in a slightly longer liver metabolic half-life of 1 hr (Table 1). Detailed analysis of the liver targeting established that PGP DNA condensates mediated specific targeting with 60% of the dose recovered with hepatocytes and only 40% with Kupffer cells (Table 1). A control PGP that lacked galactose (PGP3*) was deficient in specific targeting, resulting in the recovery of 62% of dose from Kupffer cells and 38% from hepatocytes (Table 1).

To determine the influence of melittin concentration on gene transfer efficiency, each PGP DNA sample was dosed i.v. tail vein in mice. A conventional dose of 5 μg of DNA administered in 100 μl failed to produce luciferase gene expression after 24 hours as measured by a Xenogen CCD camera (not shown). In contrast, a hydrodynamic dose of 5 μg of naked plasmid DNA in 1.8 ml of normal saline delivered via the tail vein in 5 sec to 20 g mice produced 1×10^{10} light units in triplicate mice at 24 hours post DNA administration (Fig. 7).

To evaluate if hydrodynamic dosing could stimulate the gene expression from DNA delivered by a conventional dose, a hydrodynamic dose of 1.8 ml of normal saline was administered in five seconds via the tail vein at 5 min post conventional dose administration. Luciferase expression was then measured by a Xenogen CCD camera at 24 hrs post administration of DNA. The results indicate that PGP DNA gained access to the liver cells and that upon stimulation by hydrodynamic dose the DNA gained access to the nucleus. A control experiment of conventionally dosed naked DNA followed by a hydrodynamic dose of saline established the complete absence of gene expression (Fig 7, con 1). This result suggests that a conventional dose of PGP DNA results in hepatocyte internalization and perhaps endosomal release, with the nuclear localization of DNA stimulated by a hydrodynamic dose of saline. Likewise, a control experiment that utilized PGP3*, which was devoid of galactose, failed to mediate specific targeting of plasmid DNA to hepatocytes (Table 1) and greatly diminished the influence of the hydrodynamic dose of saline to stimulate gene expression (Fig. 7, PGP3*).

The magnitude of expression mediated by PGPs 1-5 DNA was approximately 5,000-fold less than direct hydrodynamic dosing of an equivalent amount of DNA (5 μg). Surprisingly, the level of expression mediated by PGP DNA was independent of the amount of melittin incorporated into the PGP. The expression mediated by PGP1, which was a copolymer of PEG-peptide and glycopeptide, was indistinguishable to that mediated by PGP2-5.

Discussion

Receptor mediated gene delivery holds promise as being a safe and efficient means of targeting DNA into specific cells following i.v. dosing. For non-viral gene delivery to reach its full potential, many biological barriers must be overcome from the point of dosing to crossing the nuclear membrane of the target cell. We have systematically studied receptor mediated gene

delivery aimed at targeting the liver and in particular, hepatocytes as a model system to discover solutions to the barriers that exist for all receptor mediated gene delivery systems. To this end, the asialoglycoprotein receptor on hepatocytes has been useful since its cell trafficking and ligand specificity are well understood (44,45).

We have previously demonstrated specific targeting to hepatocytes in mice via the asialoglycoprotein receptor mediated by a triantennary glycopeptide (10). We have also demonstrated the necessity for PEG to block protein binding and the recognition of DNA condensates by the reticuloendothelial system (10,22,46). Binary peptide copolymers were prepared from glycopeptides and PEG-peptides using plasmid DNA to conduct a template polymerization (22). One of the primary advantages of this approach is the triggered release of DNA upon reduction of the disulfide bonds within the cell. In order to cause endosomal escape, we have recently reported the design of synthetic Cys-terminated melittin peptides that bind and polymerize on DNA and mediate gene transfer in vitro (34). In the present study we have selected a melittin analogue that mediated potent gene expression in vitro to incorporate into a co-polymeric gene delivery system in an attempt to enhance gene expression in vivo.

To incorporate all three peptide components into a polymer and control their mol ratio, we found it most efficient to co-polymerize concentrated mixtures of monomeric peptides followed by RP-HPLC purification of the resulting PGPs. The advantages of this approach over template polymerization are that it is not necessary that each monomer possesses DNA binding affinity, the resulting polymer can be characterized for size and composition, and more than three peptide components can be co-polymerized to further increase the efficiency of gene delivery.

The de novo synthesis of PGPs 1-5 is a significant advancement toward more sophisticated gene delivery carriers that not only achieve primary targeting in vivo, but may potentially achieve secondary targeting by lysing endosomes and releasing DNA intracellularly. The molecular weight and compositional analysis of each PGP demonstrates that the polymerization results in the incorporation of 6-13 monomer peptides and the input ratio of monomer peptides closely reflects the ratio of peptides incorporated into the PGPs. At low melittin ratios, PGPs are higher molecular weight and somewhat more homogeneous whereas at high melittin ratios the opposite is observed. Still, each PGP was able to bind to plasmid DNA and form condensates that were of sufficiently small size to mediate specific targeting to the liver.

One of the difficulties in developing and testing targeted gene delivery systems in vivo is that even if cell-type specific targeting is achieved, appreciable gene expression will not be observed unless the DNA gains access to the nucleus. This is indeed the case for PGP DNA which fails to mediate measurable gene expression relative to direct hydrodynamic dosing of identical amounts of DNA (Fig. 7).

Since hydrodynamic dosing is able to deliver a small fraction of a 5 μ g DNA dose to the nucleus of mouse hepatocytes resulting in a high level of gene expression (36), we rationalized that it may also stimulate the movement of cytosolic DNA to the nucleus. We tested this by measuring the luciferase gene expression after administering the PGP DNA by conventional small volume dosing (100 μ l), followed after 5 min by a hydrodynamic dose of saline that occurred after the DNA condensate had time to bind and perhaps internalize into hepatocytes via receptor mediated endocytosis. We found that a delay 15 or 30 min prior to hydrodynamic stimulation resulted in unreliable stimulation of gene expression. The resulting expression profiles clearly establish that some of the hepatocyte targeted DNA could be stimulated to enter the nucleus resulting in its expression. The mechanism by which this occurs is unknown and could be

similar to that by which hydrodynamic dosing delivers DNA to the nucleus (47), although the overall magnitude of gene expression still does not compare with direct hydrodynamic dosing.

Despite finding that hydrodynamic dosing could stimulate gene expression, we found that there was no correlation between the amount of melittin in PGPs and the magnitude of gene expression. This could result from the inability of melittin to release from the polymers during reduction or that this particular melittin analogue is not fully active in endosomal lysis in hepatocytes when entering via receptor mediated endocytosis. In fact, the melittin analogue used in this study was found to maintain nearly full hemolytic potency at pH 7 relative to natural melittin, but lost most of this activity at pH 4 (34). It is also possible that the lack of correlation may result from the use of hydrodynamic stimulation which could mask the influence of melittin *in vivo*.

In conclusion, we have developed a modular gene delivery system by generating PGPs that function to deliver 5 µg of DNA *in vivo* leading to measurable gene expression in liver following hydrodynamic stimulation. However, given that hydrodynamic stimulation was essential to observe gene expression *in vivo*, the results point toward the need to develop more sophisticated delivery systems that are able to deliver the cytosolic DNA to the nucleus *in vivo* without the need for artificial intervention.

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Figure 1. Structure of Cys-Terminated Peptides and PEGs

The structure of Cys-terminated glycopeptide, PEG-peptide and melittin peptide are shown. Following polymerization for 48 hours, the resulting PGPs, illustrated as a representative structure, were isolated by RP-HPLC and characterized for composition and molecular weight. R represents the side chains of melittin amino acids (34).

Figure 2. Kinetic Analysis of Polymerization Reaction

The time course of the PGP polymerization reaction was measured by monitoring the number of residual thiols by Ellman's reaction. The results indicated that the reaction was >95% complete after 48 hrs.

Figure 3. RP-HPLC Analysis of PGP Formation

The time course of polymerization of Cysteine-terminated glycopeptide, PEG-peptide and melittin peptide was monitored by RP-HPLC. The results indicate that PGPs elute slightly later than the monomeric components on RP-HPLC eluted with a gradient.

Figure 4. Molecular Weight Characterization of PGPs

The average molecular weight of each PGP was determined by GPC-HPLC and estimated by gradient SDS-PAGE. Panels A-F (upper) and lanes A-O (lower) represent (A, F, K) PGP1: 90% PEG-peptide : 10% glycopeptide, (B, G, L) PGP2: 80% PEG-peptide : 10% glycopeptide : 10% melittin, (C, H, M) PGP3: 70% PEG-peptide : 10% glycopeptide : 20% melittin, (D, I, N) PGP4: 40% PEG-peptide : 10% glycopeptide : 50% melittin, (E, J, O) PGP5: 20% PEG-peptide : 10% glycopeptide : 70% melittin. The gradient SDS-PAGE gel was stained with coumassie to detect protein (lanes A-E), with iodobismuthate to detect PEG (lanes F-J) and lectin overlay to detect N-glycan (lanes K-O).

Figure 5. Compositional Analysis of PGPs

The composition of each PGP was determined by RP-HPLC following reduction of the polymer with TCEP. Panels A-F illustrate the purified PEGylated glycoprotein prepared with mol composition of (A) PGP1: 90% PEG-peptide 10% : glycopeptide, (B) PGP2: 80% PEG-peptide : 10% glycopeptide : 10% melittin, (C) PGP3: 70% PEG-peptide : 10% glycopeptide : 20% melittin, (D) PGP4: 40% PEG-peptide : 10% glycopeptide : 50% melittin, (E) PGP5: 20% PEG-peptide : 10% glycopeptide : 70% melittin. Panels A'-F' illustrate the recovery of the three peptide components from reduction with TCP. The precise composition determined relative to standard curves is indicated in Table 1.

Figure 6. Biodistribution Analysis of PGP DNA

The biodistribution analysis of each PGP- ^{125}I -DNA condensate is compared following tail vein dosing in triplicate mice. The results establish the liver as the major target site. The liver metabolic half-life of PGP DNA is approximately 1 hour whereas naked ^{125}I -DNA dosed via the tail vein produced a similar targeting efficiency to liver but only a 0.5 hour half-life. Each formulation demonstrated specific targeting to hepatocytes as indicated in Table 1 whereas a control formulation where galactose had been removed produced much less targeting to hepatocytes.

Figure 7. In Vivo Gene Expression of PGP DNA Condensates

Luciferase expression was measured by BLI 24 hours following an i.v. tail vein dose of PGP DNA (5 μ g) in 100 μ l in triplicate mice and a subsequent hydrodynamic dose of 1.8 ml of saline administered 5 min later to stimulate nuclear uptake and gene expression. The insets illustrate representative BLI images for each treatment group. Control 1 illustrates the result of tail vein dose dosing 5 μ g of DNA in 100 μ l of saline followed 5 min later by a hydrodynamic stimulation dose resulting in no detectable luciferase expression. Likewise, PGP3* has been modified to remove galactose, greatly reducing the specific targeting and the liver mediated gene expression. Alternatively, PGP 1-5 demonstrate that the gene expression is independent of the amount of melittin incorporated into the PGP.

Overall helicity of peptides in phospholipid bilayers measured from molecular dynamics simulations and circular dichroism experiments.

Table 1

Structure	Monomer Composition (mol%) ^d	Measured Composition (mol%) ^b	Molecular Weight (PLL) ^c , (PEO) ^d	Particle Size ^e	Zeta Potential ^f	t _{1/2} ^g hr	PC:NPC ^h
PGP 1	0 : 90 : 10	0 : 91.7 : 8.3	106 kDa, 50 kDa	221 ± 8	+0.5	1.1	60 : 40
PGP 2	10 : 80 : 10	12.8 : 82.5 : 4.7	109 kDa, 51 kDa	210 ± 13	+5	1.2	59 : 41
PGP 3	20 : 70 : 10	24.4 : 70.3 : 5.3	99 kDa, 47 kDa	227 ± 11	+6	1.2	65 : 35
PGP 4	40 : 50 : 10	44.8 : 46.4 : 8.8	83 kDa, 46 kDa	232 ± 1	+6	1.3	60 : 40
PGP 5	70 : 20 : 10	73.2 : 19.8 : 7.0	35 kDa, 21 kDa	295 ± 7	+5	1.1	63 : 37
PGP 3 ^{wt}	20 : 70 : 10	12.6 : 80.9 : 6.5	99 kDa, 47 kDa	227 ± 11	+6	1.0	38 : 62

^aRepresents the input mol ratio of Cys-terminated melittin, PEG-peptide and glycopeptide.

^bRepresents the measured mol ratio of Cys-terminated melittin, PEG-peptide and glycopeptide for each purified PGP.

^cValues are the calculated MW based on polylysine standards.

^dValues are the calculated MW based on PEG standards.

^eThe mean particle size determined at a stoichiometry of 0.3 nmol of PGP per µg of DNA. The value represents the mean diameter (nm) based on unimodal analysis.

^fThe zeta potential of PGP DNA condensates at a stoichiometry of 0.3 nmol of PGP per µg of DNA.

^gThe metabolic half-life of PGP ¹²⁵I-DNA in triplicate mice. The results are derived from Fig. 6.

^hThe PC: NPC ratio of DNA targeted liver.

ⁱRepresents a control PGP3 in which galactose has been removed.