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Dendritic Peptide Bolaamphiphiles for siRNA Delivery to Primary Adipocytes

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Abstract

Obesity is a major risk factor for diabetes, heart disease and other health problems. Adipose tissue plays a central role in the development of obesity and obesity-associated diseases. Gene therapy targeting adipose tissue may provide a promising strategy for obesity treatment. However, nucleic acid delivery to adipose tissue or even cultured adipocytes is challenging due to low delivery efficacy and high toxicity of the current cationic lipid based delivery systems, or monoamphiphiles. Herein, we report using dendritic peptide bolaamphiphiles (bolas) to deliver siRNA to primary adipocytes and hepatocytes. The bola consists of two L-Lysine dendrons connected to a fluorocarbon core through disulfide linkages. The Lysine dendrons are functionalized with L-histidine and L-tryptophan to promote endosomal escape and cellular uptake. The bola exhibited over 70% knockdown of GAPDH gene in both primary adipocyte and hepatocytes. Importantly, different from Lipofectamine that significantly reduced genes involved in lipolysis, lipogenesis, fatty acid oxidation and ketogenesis, the bolas had little to no effect on these genes. These results demonstrate the bola as a promising new vector for clinical and experimental applications for delivery of siRNA to metabolic organs.

Keywords

siRNA Delivery; Bolaamphiphile; Adipocyte; Primary Cells

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Introduction

RNA interference (RNAi) is a powerful tool that has many biotechnological applications in the fields of experimental biology, functional genomics, stem cell differentiation, and the treatment of diseases from cancer to obesity to rare hereditary diseases.[1–2] RNAi is a robust mechanism where gene expression in eukaryotic cells is inhibited by specific short double stranded RNA sequences through the cleavage complimentary mRNA.[3] The ability to control gene expression and cure genetic related diseases has made RNAi an attractive therapeutic option as well as a powerful tool in the laboratory to study metabolic pathways. Patisiran, an siRNA based therapeutics developed by Alnylam Pharmaceuticals that treats hereditary ATTR amyloidosis, just finished phase 3 clinical trials and could be approved by the FDA in 2018.[2, 4] This marks the first time an RNAi drug has passed clinical trials, and could lead the way for more RNAi therapeutics to follow.

Obesity is another intriguing clinical target for RNAi based therapeutics. [5–6] Obesity is a major risk factor for not only diabetes, but also cardiovascular disease and cancer.[7] These three devastating conditions account for approximately two thirds of all deaths in the United States and cause enormous suffering, disability, and economic costs.[8] Unfortunately, the options for obesity treatment are very limited. RNAi is an intriguing approach for treating obesity and other metabolic diseases because of its ability to control the expression of proteins, especially proteins that cannot be targeted by small molecules. However, delivery of siRNA to the metabolic organs, specifically adipose tissue, has been challenging.[9–11] Furthermore, cultured adipocytes provide an important tool to study adipocyte biology. Nevertheless, siRNA delivery to cultured adipocyte is also challenging.[12] Different from cancer cells, differentiated adipocytes do not proliferate and have poor nucleic acid uptake efficiency. In addition, the adipocytes are nearly completely filled with lipid droplets with only a ring of cytoplasm. The differentiated adipocytes are therefore fragile and easily damaged by manipulation and chemicals. Previously, electroporation has been used to deliver siRNA into adipocytes. However, this requires a high dosage of siRNA, has very poor transfection efficacy, causes significant damage to the cells and cannot be employed clinically.[13–14] While viral carriers show promise for siRNA delivery to adipocytes, the delivery process is labor intensive and high immunogenicity limits the widespread application.[15–16] As a promising alternative to these delivery methods, synthetic vectors, on the other hand, are versatile, tunable, scalable, and have longer shelf lives.[1, 17–21] Additionally, synthetic vectors have much lower immunogenicity, thus making them more appealing for clinical applications.

Cationic lipids are the most thoroughly studied synthetic siRNA delivery vectors.[22–25] Cationic lipids, or monoamphiphiles, bind to the negatively charged phosphate backbone of siRNA and self-assemble into supramolecular nanoassemblies. The most commonly used cationic monoamphiphile is the commercially available Lipofectamine. While this vector is very effective for *in vitro* siRNA delivery, the low serum stability and high cytotoxicity hampers the potential for *in vivo* and clinical applications.[26] These limitations have led to further exploration of new amphiphile variants for siRNA delivery. One promising amphiphile variant is the bolaamphiphile.[27–29] Bolaamphiphiles are dumbbell-shaped molecules that contain two hydrophilic head groups connected to a hydrophobic core (Figure

1b).[30] Bolaamphiphiles are found in nature within the cell membrane of archaebacteria that live in harsh conditions.[31] Due to its unique molecular architecture, archaebacteria's bolaamphiphile in its cell membranes assembles into monolayers, as opposed to lipid bi-layers of normal cells. Such monolayer assembly increases the membrane stability and enables archaebacteria to survive in these harsh conditions. The robust assembly of bolaamphiphiles makes them attractive structures for synthetic siRNA delivery vectors.

In contrast to lipid monoamphiphiles, bolaamphiphiles have been scarcely studied for siRNA delivery. Previously, bolaamphiphiles have been reported as effective gene delivery vectors.[26, 29, 32–34] Specifically, dendritic peptide bolaamphiphiles were shown to deliver siRNA to multiple cell lines (MDA-MB-231, HEK-293, INS-1 and 4T1 cells) with high efficacy.[28] These dendritic peptide bolaamphiphiles also displayed low cytotoxicity and hemolysis due to the bolaamphiphile architecture. The high delivery efficacy in multiple cell lines and low cytotoxicity of dendritic peptide bolaamphiphiles prompted us to investigate this system for siRNA delivery to primary adipocytes. As noted above, adipocytes are notoriously hard to transfect cell lines and there is a lack of safe and effective delivery systems. Given the high efficiency of siRNA delivery we observed in multiple other cells, we envisioned that our bolaamphiphiles as promising vectors for delivering siRNA to adipocytes. Additionally, we hypothesized that the dendritic peptide bolaamphiphiles will not disrupt the underlying lipid metabolism of the adipocyte cells. As opposed to cationic lipids, such as Lipofectamine, bolaamphiphiles were shown to be less disruptive to the plasma membrane of cells due to the unique dumbbell architecture.[28]

The specific dendritic peptide bolaamphiphile (bola) we chose for this study consists of two L-Lysine dendrons connected to a fluorocarbon core through disulfide linkages (Figure 1a).[28] The dendrons are statistically functionalized with L-histidine and L-tryptophan in 3:1 molar ratio. Each of these features contributes to the high efficacy of the bola: (i) the lysine Dendron provides multivalent interaction for siRNA binding[35] (ii) the fluorocarbon core promotes self-assembly, increased cellular uptake and serum stability[17, 36] (iii) the disulfide bond facilitates stimuli responsive release of siRNA in the cytoplasm[37] (iv) tryptophan improves siRNA binding and cellular uptake[28, 38–39] and (iv) histidine provides pH responsive buffering capacity to enact endosomal escape.[40–42] Herein, we report the delivery of siRNA to differentiated primary adipocytes and hepatocytes without disruption the metabolic function of the cells.

Materials and Methods

Materials

Unless otherwise noted, all reagents were used as received from commercial suppliers without further purification. Protected amino acids were purchased from Advanced Chemtech (Louisville, KY) and Aroz Technologies, LLC (Cincinnati, OH). 1H,1H,12H, 12H-perfluoro-1,12-dodecanediol was purchased from Exfluor Research Corporation (Round Rock, TX). Coupling reagents were purchased from GL Biochem Ltd. (Shanghai, China). Sodium Dextran Sulfate (25 kDa) was purchased from TCI America (Portland, OR) and was used as received. GelRed™ siRNA stain was purchased from VWR (Radnor, PA). All siRNA used in this study was purchased from Ambion (Carlsbad, CA) with *Silencer*®

Select negative control siRNA. The sequences for GAPDH siRNA and Lipofectamine RNAiMAX was purchased from Invitrogen (Carlsbad, CA) and used as a positive control following the manufacturer's protocol. All reactions were performed using HPLC grade solvents unless otherwise noted. All water used in biological experiments was nanopure water obtained from Barnstead Nanopure Diamond (Waltham, MA).

Instrumentation

All compounds were characterized by NMR and ES-MS. ^{1}H NMR spectra were collected at the UC Irvine NMR Facility and recorded at 500 or 600 MHz on Bruker instruments (GN 500 or CRYO600). Mass spectral data (ES-MS) was obtained from the UC Irvine Mass Spectrometry Facility and collected with a Micromass LCT spectrometer. Matrix assisted laser desorption ionization spectral data (MALDI) was obtained from the UC Irvine Mass Spectrometry Facility and collected with a AB SCIEX TOF/TOF 5800 System. ^{1}H NMR chemical shifts were reported as values in ppm relative to specified deuterated solvents. The size and zeta potential of bola/siRNA complexes were measured at 633 nm using Zetasizer dynamic light scattering instrument (Malvern Instruments, Malvern, UK) at 25°C with detection angle of 173°.

DLS Measurements

The size and zeta potential of bola/siRNA polyplexes were measured at 633 nm using Zetasizer (NanoZS) dynamic light scattering instrument (Malvern Instruments, Malvern, UK) at 25°C with detection angle of 173°. The stock vector solutions (5 mg/ml) were diluted to with ddH2O and complexed with 6.5 μL of 40 μM siRNA diluted in with PBS (10 mM phosphate, 10 mM NaCl, pH = 7.4) to give a final [siRNA] of 2.5 μM with N/P ratio of 20 or 45 and a final volume of 100 μL. After a 5-minute incubation, the samples were analyzed for particle size then transferred into a disposable capillary cell for zeta potential analysis.

Synthesis of bola-G0 (1)

1H,1H,12H,12H-perfluoro-1,12-dodecanediol (827 mg, 1.47 mmol, 1 equiv) was charged to 50 mL RBF and placed under an atmosphere of N_2. THF (15 mL) was added along with DIPEA (0.325 mL, 4.04 mmol, 2.75 equiv) and the mixture stirred until completely dissolved. The reaction mixture was cooled in an ice/water bath and 4-nitrophenyl chloroformate (815 mg, 4.04 mmol, 2.75 equiv) added in 3 portions. After 1 h of stirring, the ice bath was removed and the reaction was stirred overnight. The next day, the Boc-Cystamine HCl (2,295 mg, 8.08 mmol, 2 equiv), THF (10 mL), and DIPEA(0.325 mL, 4.04 mmol, 2.75 equiv) were combined in a scintillation vial and mixed until homogeneous. This mixture was added to the reaction mixture at room temperature and stirred overnight. The reaction mixture was then diluted with EtOAc (200 ml) and washed sequentially with 1 M NaOH (5 × 100 mL), 0.5 M HCl (2 x 100 mL), and brine (1 × 100 mL). The organic phase was dried with (Na_2SO_4), the solvent removed in vacuo, and the crude residue purified by CC (0–5% MeOH in DCM) to afford **bola-G0 (1)** (305 mg, 23% over 2 steps). ^{1}H NMR (500 MHz, CDCl3) δ (ppm): 4.65 (t, J = 13.6 Hz, 4H), 3.55 (m, 8H), 2.87 (m, 8H). ESI-MS + (m/z): [M]+H calc., 941.04; found, 941.0.

Synthesis of bola-F10-G1-NH_2 (2)

In a two-dram vial, **1** (0.062 mmol, 1 equiv), Boc-Lys-(Boc)-OH (71.5 mg, 0.136 mmol, 2.2 equiv) and DIPEA (53 μL, 0.308 mmol, 5 equiv) were dissolved in 2.5 mL NMP, followed by the addition of PyBOP (77.0 mg, 0.148 mmol, 2.4 equiv). The reaction was left to stir at room temperature for 24h. After the reaction, the solvent was removed in vacuum. The crude product was purified by dissolution in MeOH and precipitation in water. After purification, the Boc was deprotected in a solution of TFA solution (1 mL TFA, 1.5 mL anisole, 2.5 mL DCM and 0.1 mL TIPS). The deprotection was done in 4h at room temperature, followed by solvent removal in vacuo. The product was then purified by dissolution in a minimum amount of MeOH and precipitation in Et_2O to give **bola-F10-G1 (2)** (87 mg, 87% yield). ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 3.87 (t, J = 5.9 Hz, 4H), 3.72 – 3.63 (m, 2H), 3.47 (s, 4H), 3.00 – 2.81 (m, 16H), 1.91 (s, 4H), 1.77 – 1.68 (m, 4H), 1.56 – 1.47 (m, 4H). ESIMS+ (m/z): [M]+H calc, 1175.24; found, 1175.2.

Synthesis of bola-F10-G2-NH_2 (3)

In a two-dram vial, **2** (0.062 mmol, 1 equiv), Boc-Lys-(Boc)-OH (71.5 mg, 0.136 mmol, 4.2 equiv) and DIPEA (53 μL, 0.308 mmol, 10 equiv) were dissolved in 2.5 mL NMP, followed by the addition of PyBOP (77.0 mg, 0.148 mmol, 5 equiv). The reaction was left to stir at room temperature for 24h. After the reaction, the solvent was removed in vacuum. The crude product was purified by dissolution in MeOH and precipitation in water. After purification, the Boc was deprotected in a solution of TFA solution (Typically, 1 mL TFA, 1.5 mL anisole, 2.5 mL DCM and 0.1 mL TIPS). The deprotection was done in 4h, followed by solvent removal in vacuo. The product was then purified by dissolution in a minimum amount of MeOH and precipitation in Et_2O to give **bola-F10-G2 (3)** (145 mg, 90% yield). ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 4.71 (t, J = 12.6 Hz, 2H), 4.33 (dd, J = 8.6, 5.6 Hz, 1H), 2.96 (t, J = 6.5 Hz, 1H), 2.85 (t, J = 6.6 Hz, 1H), 2.68 – 2.54 (m, 1H), 2.48–2.44 (m, 3H), 2.29–2.18 (m, 2H), 2.06 – 2.93 (m, 4H), 2.91 – 2.77 (m, 4H), 2.08 – 1.34 (br, 18H). ESIMS+ (m/z): [M]+H calc, 1687.62; found, 1687.6.

Synthesis of bola-F10-G2 (4)

In a one dram glass vial were added 30 mg of the TFA salt of the unfunctionalized bola 6 (0.00709 mmol, 1 equiv), boc-His(boc)-OH.DCHA (45.7 mg, 0.0851 mmol, 12 equiv) and boc-Trp(boc)-OH (11.5 mg, 0.0284 mmol, 4 equiv). 1.5 mL DMF was added to dissolve the solids, followed by PyBOP (58 mg, 0.114 mmol, 16.2 equiv) and DIPEA (20 μL, 0.114 mmol, 16.2 equiv). The reaction was left to stir for 24 hours at room temperature. The protected bola was precipitated in an excess amount of deionized water. After removing water 46 completely, the solid was dissolved in 1 mL TFA, 2 mL DCM, 2 mL Anisole and 0.25 mL TIPS. After stirring overnight, the solvent was removed in vacuo, the resulting solid was redissolved in MeOH and precipitated in Et_2O to give the final product, **bola (4)** (52.7 mg, 93% yield). ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 8.56–8.47 (m, 5.0 H, histidine, 72 mol%), 7.53–7.04 (m, 14.2 H, tryptophan, 27 mol%), 5.00–4.90 (m, 16 H), 4.85–4.10 (m, 14 H), 2.67–2.67 (m, 40 H), 1.71–1.06 (m, 36 H).

Synthesis of mf10-PEG2k (5)

To a dried 25 mL RBF was added 3 mL of dry THF followed by the 1,1-H,H-perfluorodecanol (100 mg, 1.0 equiv). The reaction mixture was cooled in an ice/water bath and DIPEA (2.0 equiv) added, followed by *p*-nitro-phenylchloroformate (1.0 equiv). The reaction was allowed to gradually warm to room temperature over 1 hour and the stirred for an additional 5 hours at room temperature. The PEG-amine (0.9 equiv), DCM (2 mL) and additional DIPEA were added and the reaction stirred for 24 h. The reaction mixture was dialyzed against MeOH (MWCO = 1000) until all yellow color was removed (approximately 6 solvent replacements and 36 h) and the product obtained as a white solid **(5)** (28% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 5.34 (s, 1H), 4.16 (t, J = 13.5 Hz, 2H), 3.21 (s, 158H), 2.95 (s, 5H).

Bola + mf10-PEG(2k) coformulation

The bola was coformulated with mf10PEG(2k) using thin film rehydration from ethanol.[43] In a one dram glass vial were added 25 mg of the bola (1 equiv). A solution of 10 mg/ml mF10-PEG(2k) in EtOH (0.05 equiv) was added to the vial and the solvent was removed in vacuo. The Bola + 5 mol% mf10-PEG(2k) was then dissolved in RNase free water and stored in the freezer at a concentration of 5 mg/ml.

General protocol for vector/siRNA complex preparation (OptiMEM)

Prior to complexation all vectors and buffers warmed to room temperature and vortexed. A 1.5 μM solution of siRNA was prepared using OptiMEM as the dilution buffer and the appropriate amount of vector solution (5 mg/ml) required to give the desired N/P ratio diluted with OptiMEM. The vector solution was added to the siRNA solution and gently mixed via pipette to give a complex solution with [siRNA] = 100 nM. After 10 minutes incubation without agitation, this concentrated solution was gently mixed via pipette, further diluted to the desired concentrations with OptiMEM, and immediately added to the cell culture media.

Preparation of complexes of in vivo modified siRNA with vector (OptiMEM)

Prior to complexation all vectors and buffers warmed to room temperature and vortexed. A 40 μM solution of *in vivo* modified siRNA was added to low salt PBS (total final volume 125 uL) and the appropriate amount of vector solution (5 mg/ml) required to give an N/P=45. The vector solution was added to the siRNA solution and gently mixed via pipette to give a complex solution. After 5 minute incubation without agitation, this concentrated solution was loaded into a sterile syringe.

Transfection of 3t3-L1 Adipocytes

3t3-L1 cells were used as a model cell line to study adipocyte delivery. 3t3-L1 cells are fibroblast cells that under proper conditions differentiate into adipocyte like cells, making them an ideal model cell line for the study of siRNA delivery to adipocytes. Confluent 3t3-L1 preadipocytes were treated with DMEM containing 10% FBS, 0.5 mM isobutylmethylxanthine (I7018; Sigma), 1 μM dexamethasone (D4902; Sigma), 2 μg/mL insulin (I0546; Sigma) for 72 hours. The cells were then maintained in DMEM with 10%

FBS. After 6–8 days, 3t3-L1 adipocytes became fully differentiated with lipid droplets easily seen in microscope (Figure S3). After 3t3-L1 cells were fully differentiated, the knockdown effects of the various vectors were assayed. Immediately prior to addition of the complexes the culture media was switched to 150 μL OptiMEM per well. For initial screening, the vector/siRNA complexes were prepared using the general complex preparation protocol in OptiMEM+ 10% FBS. The 5X vector/siRNA complexes were prepared as described previously and 100 μL added to each well to achieve the desired concentration. After 2 hours, 200 uL of OptiMEM+10% FBS was added to the cells. The cells were analyzed 48 hours post transfection to determine the percent knockdown for RNA.

Primary Cell Culture Isolation of Adipocytes

For primary preadipocytes, stromal vascular fraction cells were isolated from inguinal fat pads of 4 weeks old male mice and cultured as described.[44] Primary preadipocytes were cultured in DMEM/F-12 GlutaMAX (Invitrogen) containing 15% FBS. For adipocyte differentiation assay, confluent cultures were exposed to induction DMEM/F-12 GlutaMAX containing dexamethasone (5μM), insulin (0.5μg ml^{-1}), isobutylmethylxanthine (0.5mM), rosiglitazone (1μM) and 10% FBS. Two days after induction, cells were maintained in media containing insulin (0.5μg ml^{-1}) and 10% FBS until they are ready for subsequent experiment.

Transfection of Primary Adipocytes

The transfection efficacy in primary adipocytes was assayed using similar conditions to 3t3-L1 adipocytes. Primary adipocytes were cultured in DMEM/F-12 GlutaMAX (Invitrogen) containing 15% FBS. The vector/siRNA complexes were prepared using the general complex preparation protocol. Immediately before transfection, the media was replaced with 200 uL of OptiMEM+ 10% FBS. The 5X vector/siRNA complexes were prepared as previously described and 100 μL added to each well to achieve the desired concentration. After 2 hours, 200 uL of OptiMEM+10% FBS was added to achieve a final volume of 500 uL. The cells were analyzed 48 hours post transfection to determine the percent knockdown for RNA.

Primary Cell Culture Isolation of Hepatocytes

Primary hepatocytes cells were isolated from 4 weeks old male mice and cultured as described.[44] Primary preadipocytes were cultured in DMEM/F-12 GlutaMAX (Invitrogen) containing 10% FBS until they are ready for subsequent experiment.

Transfection of Primary Hepatocytes

The transfection efficacy in primary hepatocytes was assayed using similar conditions as listed above. Primary hepatocytes were cultured in DMEM/F-12 GlutaMAX (Invitrogen) containing 15% FBS. The vector/siRNA complexes were prepared using the general complex preparation protocol. Immediately before transfection, the media was replaced with 200 uL of OptiMEM+ 10% FBS. The 5X vector/siRNA complexes were prepared as described previously and 100 μL added to each well to achieve the desired concentration. After 2 hours, 200 uL of OptiMEM+10% FBS was added to achieve a final volume of 500

uL. The cells were analyzed 48 hours post transfection to determine the percent knockdown for RNA.

Quantitative RT-PCR

Total RNA was extracted from mouse adipose tissue or cultured cells using Nucleospin®RNA kit (MACHEREY-NAGEL) according to the manufacturer's instruction. One microgram of total RNA was used to synthesize cDNA using Super script III cDNA kit(Invitrogen). The transcripts were quantified with 7900HT Fast Real-Time PCR System (Applied Biosystems) by using Power SYBR Green PCR master mix (ABI)and the CT threshold cycle method. Using primers CAACTACATGGTCTACATGTTC (Forward) and CACCAGTAGACTCCACGAC (Reverse) for GAPDH and CCCTATCACTCCTGCCACAC (Forward) and ACGAAGTGCAATGGTCTTTAGG (Reverse) for TBP. Gene expression levels were normalized to those of TBP and presented relative to the control.

In vivo animal studies

C57BL/6 mice in were purchased from Jackson Laboratories. Mice were fed standard chow. Mouse studies were conducted in accordance with federal guidelines and were approved by the University of California, Irvine. C57BL/6 mice 4 weeks old (~19 g) were injected with *in vivo* modified GAPDH siRNA (Dharmacon) at (N/P = 45) via vein tail, respectively, at a single siRNA dose of 1.5 mg/kg (100 μL solution). After 24 h post injection, the mice were sacrificed and the organs were harvested. The RNA was extracted and quantified as previously described above.

Statistical Analysis

All data were expressed as mean ± SEM. Statistical differences were assessed using unpaired Student's two tailed t tests for two groups and a one-way ANOVA for three groups or more. Statistical significance was assumed at $p < 0.05$.

Results and Discussion

The bola was synthesized using solution-phase coupling chemistry as previously reported.[28] We chose to use a bolaamphiphile with a fluorocarbon core due to the improved serum stability and transfection efficacy as compared the hydrocarbon core variants. Additionally, our previous study showed that bolas with the generation 2 dendron were most effective for siRNA delivery while maintaining low cytotoxicity. The dendrons of the optimized bola are statistically functionalized with L-histidine and L-tryptophan in 3:1 molar ratio. The pH responsive histidine promotes endosomal escape and the tryptophan increases the siRNA binding and cellular uptake.[41] The bola exhibits very strong binding affinity to siRNA, fully complexing siRNA at N/P=8 (the molar ratio of nitrogen in the bola to phosphate in the siRNA).[28] Furthermore, the disulfide linkage in the bola facilitates stimuli responsive release of siRNA upon entering the cytoplasm.[28]

For the bola to be relevant for *in vivo* and clinical applications, the vector must be able to assemble into stable nanocomplexes that are under 200 nm in size.[45] Many self-assembled cationic nanoparticles aggregate in serum and other biofluids, leading to shorter circulation

time and build-up in off-targeted organs and blockage of arteries and veins.[46–47] Self-assembled nanoparticles that bind to siRNA through electrostatic interactions often exhibit low serum stability. Specifically, the positively charged particle electrostatically interacts with negatively charged serum proteins to cause aggregation or release of siRNA.[48] To prevent such interactions and aggregation, hydrophilic stabilizers, such as polyethylene glycol (PEG), are often coformulated with the particle to reduce electrostatic interactions with serum proteins, increase the hydrodynamic radius as well as shield the particle to reduce immune recognition.[47, 49–50]

To stabilize the bola and reduce immunogenic response, a fluorocarbon-modified PEG (fPEG2k) was synthesized to coformulate with the bola (Scheme 2). fPEG2k was coformulated with the bola using thin film rehydration from ethanol at 5 and 10 mole percent relative to the bola. The fluorocarbon tail of the fPEG2k assembles with the fluorocarbon core of the bola to form a spherical nanocomplex shielded by PEG2k. The different coformulations of the bola were then complexed with siRNA and the nanocomplexes were studied using dynamic light scattering (DLS) in high salt phosphate buffered saline (PBS). The bola without fPEG2k coformulation aggregated into particles over 375 nm in just over an hour, and formed micron sized particles within 4 hours (Figure 2). However, with the addition of 5 mol% fPEG2k, the particles formed complexes smaller than 150 nm that were stable for over 4 hours in high salt PBS. This particle size is promising for further *in vivo* and clinical applications because particles below 200 nm exhibit increased circulation time in the blood stream. Additionally, the particles are stable for long periods of time in solution at room temperature, making them more applicable for commercial and clinical applications (Figure 2). DLS was also performed in the presence of serum, to simulate revenant *in vivo* conditions. In the presence of serum, the particle size was slightly larger for all conditions screened (Figure S1). The bola without PEG aggregated into micron sized particles within 4 hours. Coformulation with 5 mol% fPEG2k was able to prevent aggregation for all time points tested, however the particle size was slightly larger than the control with no serum. Presumably, the addition of fPEG2k stabilized the nanoparticles and prevented aggregation in the presence of serum proteins.

The siRNA transfection efficacy of metabolic cells was initially screened in 3t3-L1 differentiated adipocyte cells. Adipocytes play a major role in the development of obesity. However, poor transfection efficiency in differentiated adipocytes hinders obesity and metabolic research in adipocyte cells. Differentiated adipocytes are some of the most difficult cell lines to transfect. Using Cy-3 labeled siRNA, cellular uptake of bola/siRNA complexes was directly observed by imaging the transfected cells (Figure 3c and 3d). To determine the optimal transfection conditions in differentiated adipocytes, the bola was complexed with siRNA at differing N/P ratios (the molar ratio of nitrogen in the bola to phosphate in the siRNA) to find the optimal ratio of bola to siRNA. The bola was complexed with either anti-GAPDH or scrambled siRNA, and then added directly to a 24-well plate of fully differentiated adipocytes. After 48-hours, the mRNA was extracted from the cells and the GAPDH knockdown was assessed via qPCR relative to the scrambled siRNA control. N/P ratios from 45 to 60 were screened to determine the optimal ratio with the highest knockdown efficacy without causing toxicity. In differentiated adipocyte, the siRNA knockdown effect was not saturated until N/P=60. Gene silencing increased from over 45%

to 65% when the N/P ratio was increased from 45 to 60 (Figure 3a). However, at N/P ratio of 60, the cells became visibly distressed, and at N/P 90 increased toxicity was observed along with decreasing transfection efficacy. For the rest of the study, N/P ratio of 45 was used for all *in vitro* transfections.

Next, we repeated the siRNA transfections in differentiated adipocytes with the bola coformulated with fPEG2k to determine if the addition of PEG affected the transfection efficacy. Using the optimized transfection conditions described above, 3t3-L1 differentiated adipocytes were transfected with the bola and the bola + 5 mol% fPEG2k. Both the bola and the bola with 5 mol% fPEG2k exhibited ~70% gene knockdown at [siRNA]=50 nM, similar to commercially available lipofectamine (Figure 3b). PEGylation of delivery vehicles often leads to decreased cellular interactions and uptake.[51] However, we saw no decrease in transfection efficacy. While the precise reason for this is not clear, we propose that this be due to the increased particle stability of the PEGylated bola that causes the nanoparticles to be dispersed in the culture media for longer durations during transfection (Figure S1). Overall, these results indicate that coformulation with 5 mol% fPEG2k improves the stability of the nanoplexes without compromising the transfection efficacy.

We further analyzed the bola's transfection efficacy in primary adipocytes. Effective siRNA delivery into primary adipocytes has pathophysiological merit and potential clinical impact on obesity treatment. Fully differentiated primary adipocyte cells were harvested from C57/Bl6 mice and plated in a 24-well plate. The primary adipocytes were then transfected with GAPDH siRNA, and knockdown was analyzed 48-hours post transfection. The bola exhibited over 70% gene silencing at 50 nM siRNA, comparable to lipofectamine (Figure 4a). At both N/P ratios of 20 and 45, the bola exhibited over 70% knockdown at 50 nM siRNA (Figure S2). The bola also showed increased efficacy compared to the monoamphiphile variant (Figure 4a). The monoamphiphile variant exhibited only 25% knockdown in primary adipocytes. This demonstrates that the molecular architecture, rather than the chemical composition, is critical for the increased efficacy of the bola. The transfection efficacy of the bola was also screened in primary hepatocytes, as they are also heavily involved in metabolism and diabetes. In primary hepatocytes, the bola reduced GAPDH gene expression by over 85% at 50 nM (Figure 4b).

Currently, poor siRNA transfection efficacy in adipocytes is a bottleneck in adipocyte research. Several delivery methods have been reported, but each present a drawback. Viral delivery to adipocytes has been shown to be effective, however viral vectors present a safety risk, are difficult to work with and may affect cellular function of the adipocytes.[52] Electroporation has also been used to deliver siRNA to adipocytes, however this method has low efficacy and imposes significant damage to the cells.[13] Lastly, cationic lipid vectors have been reported to be effective for siRNA transfection to adipocytes. Commercially available Lipofectamine was shown to have between 60–80% knockdown in differentiated adipocytes at 40 nM siRNA dosage.[9, 11, 53] Although Lipofectamine delivers siRNA with good efficacy, as shown below, it also disrupt the lipid metabolism of adipocytes, a common metabolic pathway studied in these cells. This limits the usefulness of lipofectamine and other lipid based vectors in adipocytes.

To confirm the advantages of using our bola vector, we tested the lipid metabolism of the primary adipocyte cells transfected with the bola and lipofectamine. Since adipocytes and hepatocytes are model cell lines to study the role of lipid metabolism in obesity and other metabolic diseases, it is imperative that the delivery vector does not disrupt the lipid metabolism of the cell. The lipid metabolism of cells transfected with the bola was compared to cells transfected with Lipofectamine RNAiMAX as well as untreated control cells (Figure 5a). Lipofectamine RNAiMAX significantly suppressed lipid metabolism of the primary adipocytes. Genes involved in lipolysis, lipogenesis, fatty acid oxidation and ketogenesis were all substantially suppressed. In sharp contrast, bola had little to no effect on lipid metabolism. For all 9 genes assayed, the bola disrupted lipid metabolism much less than Lipofectamine RNAiMAX ($P < 0.05$). The lipid metabolism was also assayed in primary hepatocytes with similar results (Figure 5b).

It is important to note that the unique molecular architecture of the bola allows it to deliver siRNA with high efficacy without negatively affecting lipid metabolism, which is in sharp contrast to normal cationic lipids such as Lipofectamine. The bola has minimal impact to most metabolic pathways of primary adipocytes and hepatocytes. This finding is consistent with our previous report showing that the bola is less cytotoxic and hemolytic than normal cationic monoamphiphiles.[28] We attribute both the lower metabolic disruption and cytotoxicity to the dumbbell molecular architecture of the bola. Because of the low metabolic disruption, the bola shows great promise as a gene delivery vector to primary adipocytes.

Finally, the *in vivo* toxicity of the vector was assessed in eight-week old C57/Bl6 mice. The bola coformulated with 5 mol% fPEG2k and GAPDH siRNA, which formed stable nanoparticles, was injected via tail vein. At the dosages tested (1.5 mg/kg), no toxicity, weight loss or visible signs of distress were observed in the mice. The hepatic and renal functions of the mice were not affected by the bola compared to saline controls (Figure 6). Although GAPDH was reduced by approximately 40% in liver, adipose GAPDH expression was not altered (not shown). Cationic nanoparticles must overcome several barriers prior to targeted siRNA delivery *in vivo*. First, the particles must avoid unfavorable interactions with negatively charged proteins and clearance by the renal system. Next, the particles must be able to penetrate tissues to reach targeted cells. Given the difficulty for cationic nanoparticles to penetrate fat tissues, it is not too surprising that our initial *in vivo* delivery of GAPDH siRNA did not cause GAPDH knockdown in adipose cells. Further studies will include nanoparticle formulation optimization, *in vivo* biodistribution studies, and targeting of adipose tissue. Overall, these results confirm our *in vitro* results that the bola is not cytotoxic and does not disrupt metabolic function of metabolic cells.

Conclusion

In conclusion, the bola is a promising synthetic vector for siRNA delivery to primary metabolic cells. The bola exhibited high transfection efficacy, over 70% knockdown, in both primary adipocytes and hepatocytes. Importantly, in contrary to normal cationic lipids such as Lipofectamine, bola did not disrupt the underlying metabolic function of the cells. Furthermore, colloidal stability of the bola was improved through coformulation with a

fluorocarbon-modified PEG (fPEG2k). The bola coformulated with 5 mol% fPEG2k formed stable formed complexes smaller than 150 nm for over 4 hours in high salt PBS. The formation of stable nanoparticles through coformulation with fPEG2k allowed for the bola to be tested *in vivo* via tail vein injected. *In vivo* tests showed that the bola has low toxicity and did not affect hepatic or renal function. Future studies will access the *in vivo* efficacy and biodistribution of the bola. Since the bola exhibits high gene knockdown without negatively affecting lipid metabolism, we propose that the bola could be a useful tool for loss-of-function assays using RNAi to study different genes' effect on lipid metabolism and diabetes. Adipose tissue plays a major role in the pathophysiology of obesity and diabetes. Excessive fat tissue in obesity has profound detrimental effects on major organs such as heart, liver, kidneys and skeletal muscle. At the molecular level, many genes in adipocytes are involved in glucose and lipid metabolism. Further exploration of the roles of these genes could yield fruitful discoveries impacting the field of obesity and diabetes research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at: http://XXX

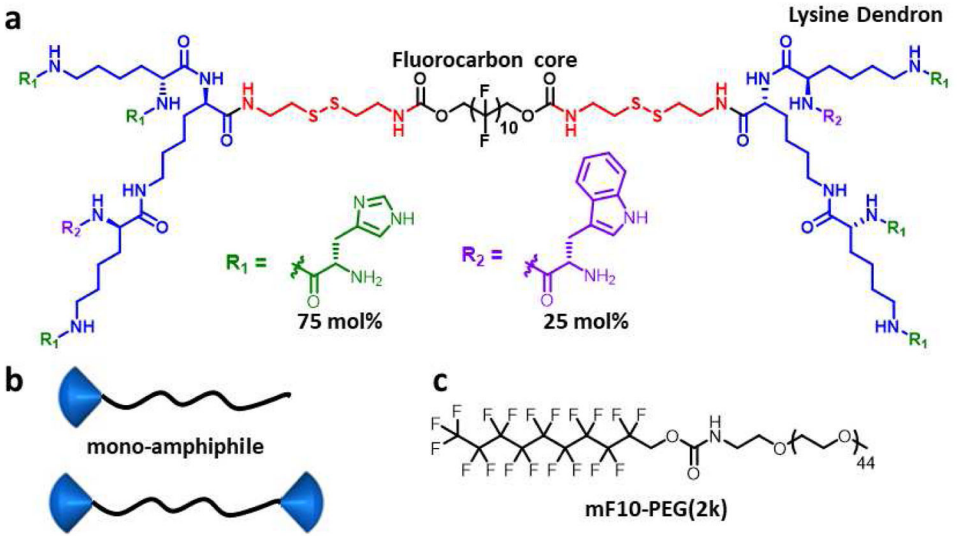


Figure 1.

(a) Structure of dendritic peptide bolaamphiphile (bola). (b) Generic bolaamphiphile and monoamphiphile structure. (c) Structure of fPEG(2k) for coformulation with bola to stabilize nanoparticle assembly.

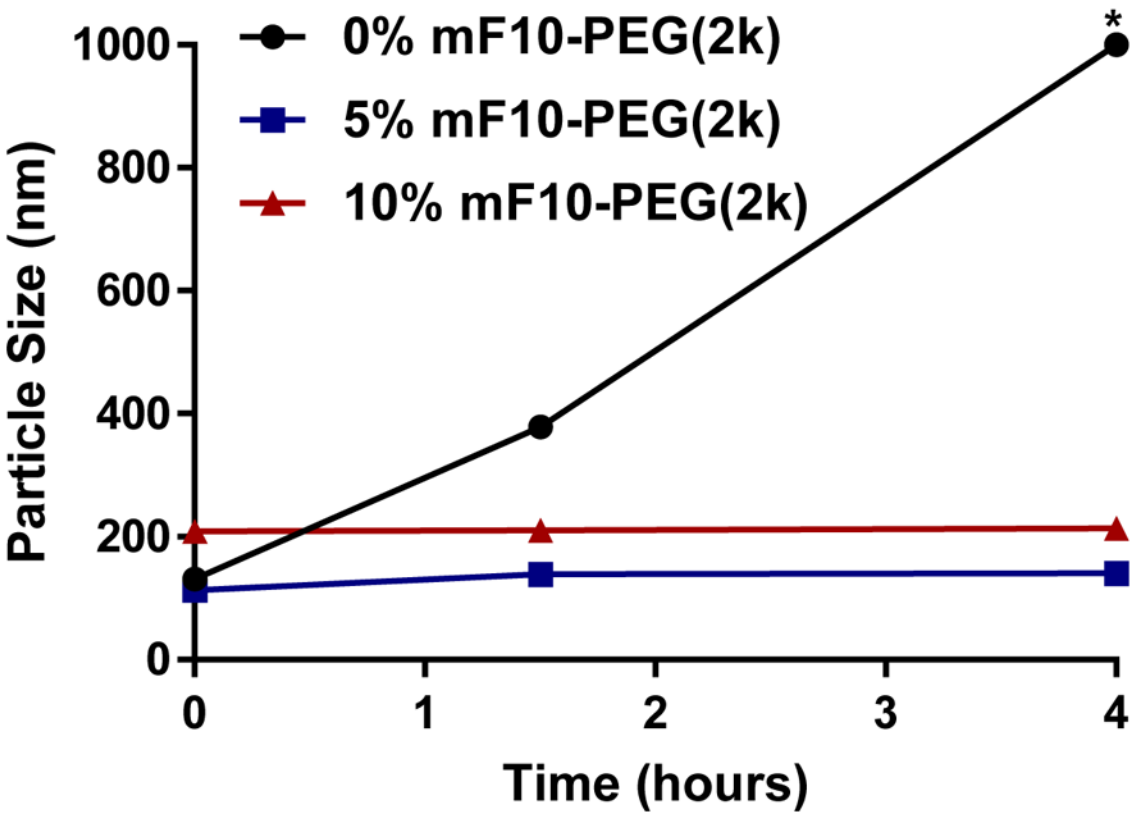


Figure 2.

Colloidal stability of bola/siRNA complexes in PBS with or without mF10-PEG(2k) in the formulation. Particle size was determined via DLS. Vectors were complexed with siRNA at N/P=20. The asterisk (*) indicates that the complex aggregated into micron sized particles.

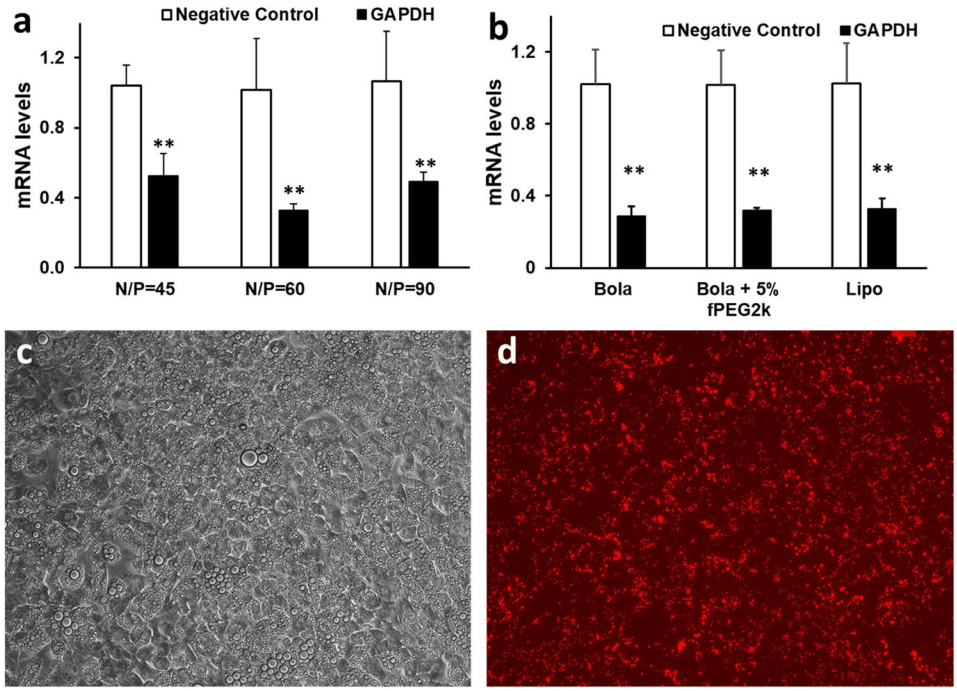


Figure 3.

(a) Transfection to differentiated 3t3-L1 adipocytes with anti-GAPDH siRNA at various N/P ratios. [siRNA]= 50 nM. (b) Screening of transfection efficacy of various vectors in 3t3-L1 differentiated adipocytes with anti-GAPDH siRNA. N/P = 45 and [siRNA]= 50 nM. Lipofectamine RNAiMAX (Lipo) was used as a positive control. qPCR was performed 48 hours post transfection. n=3 per group. *$p<0.05$ **$p<0.01$ (c) and (d) Images to show cellular uptake of adipocytes transfection with Cy3-labeled siRNA: bright field image of the cells (c) and fluorescence image of the transfected cells (d). Transfection performed at siRNA=50 nM and N/P=45.

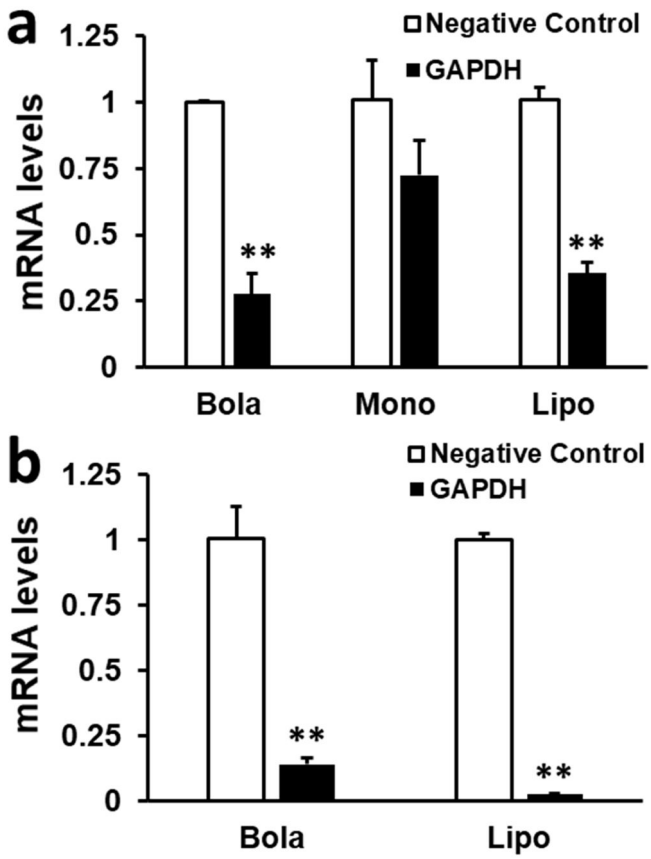


Figure 4.

(a) Transfection to primary adipocytes and (b) to primary hepatocytes with anti-GAPDH siRNA with various vectors at N/P=45 and [siRNA]=50 nM. Lipo = Lipofectamine RNAiMAX. Mono= monoamphiphile structural variant of the bola. Negative control is nontargeting siRNA. qPCR was performed 48 hours post transfection. n=3 per group. **p<0.01 ***p<0.001

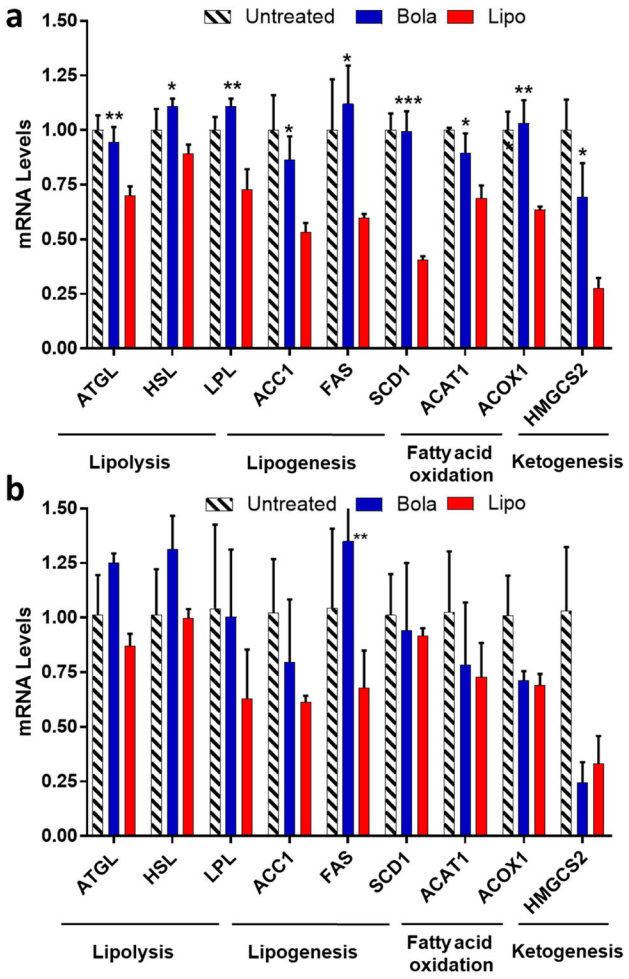


Figure 5.

Effects of bola on lipid metabolism in primary adipocytes (a) and primary hepatocytes (b). [siRNA]=50 and N/P=45. qPCR was performed 48 hours post transfection. n=3 per group. Statistics are between bola and lipofectamine. Bola is never statistically significant from the untreated for primary adipocytes. ($p>0.05$). *$p<0.05$ **$p<0.01$ ***$p<0.001$

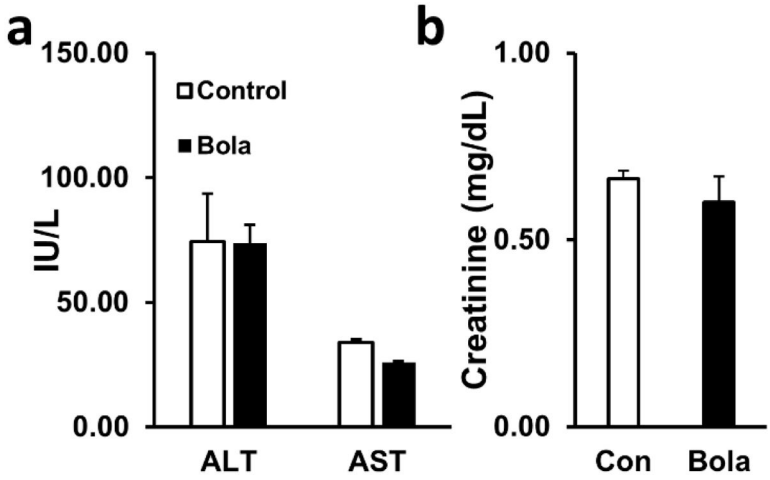
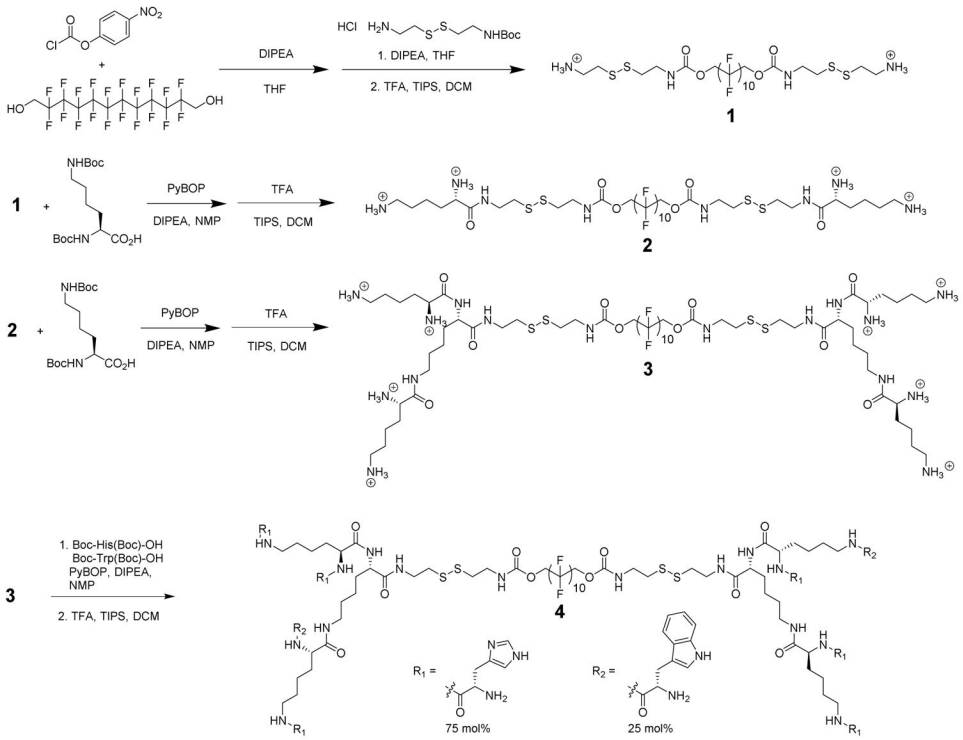


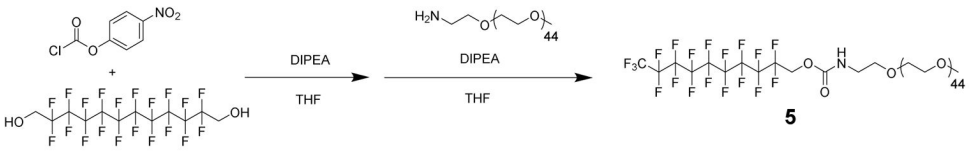
Figure 6.

(a) Hepatic and (b) renal function of mice treated with bola-F10-G2/siRNA. Hepatic function was assayed through the alanine transamidase (ALT) and aspartate transamidase (AST) levels in the blood (IU/L). The renal function was assayed through the creatinine levels in the blood. Eight-week old male C57/Bl6 mice injected with 1.5 mg siRNA/kg complex (N/P=45). 48h later serum was collected and hepatic and renal function were assayed. Control mice were injected with saline. n=3 per group. The *in vivo* toxicity for bola is never statistically significant different from the untreated ($p>0.05$).



Scheme 1.

Synthetic route to the dendridic peptide bolaamphiphile (bola).



Scheme 2.
Synthetic route to mF10-PEG(2k).