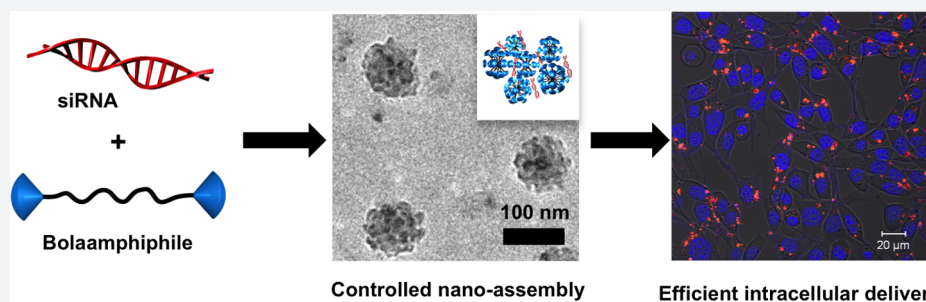


Structure-Based Design of Dendritic Peptide Bolaamphiphiles for siRNA Delivery

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S Supporting Information



ABSTRACT: Development of safe and effective delivery vectors is a critical challenge for the application of RNA interference (RNAi)-based biotechnologies. In this study we show the rational design of a series of novel dendritic peptide bolaamphiphile vectors that demonstrate high efficiency for the delivery of small interfering RNA (siRNA) while exhibiting low cytotoxicity and hemolytic activity. Systematic investigation into structure–property relationships revealed an important correlation between molecular design, self-assembled nanostructure, and biological activity. The unique bolaamphiphile architecture proved a key factor for improved complex stability and transfection efficiency. The optimal vector contains a fluorocarbon core and exhibited enhanced delivery efficiency to a variety of cell lines and improved serum resistance when compared to hydrocarbon analogues and lipofectamine RNAiMAX. In addition to introducing a promising new vector system for siRNA delivery, the structure–property relationships and “fluorocarbon effect” revealed herein offer critical insight for further development of novel materials for nucleic acid delivery and other biomaterial applications.

INTRODUCTION

Since its discovery, RNA interference (RNAi) has demonstrated tremendous utility in a variety of biological applications, including experimental biology,¹ functional genomics,² stem cell research,³ and treatment of diseases.^{4,5} The high potency and specificity of gene silencing induced by small interfering RNA (siRNA) makes this technology particularly appealing for medicinal applications; however, safe and efficient delivery of siRNA into targeted cells remains a major challenge.^{5–7} Much effort has been devoted to the development of synthetic delivery vectors⁸ with a variety of systems being investigated, including lipids,^{9–11} peptides,^{12–15} polymers,^{16–19} dendrimers,^{20,21} and gold nanoparticles.^{22,23} Cationic lipids (such as lipofectamine) and polymers (such as poly(ethylene imine), PEI) have been widely used in a variety of biological studies; however, toxicity and delivery efficiency are often limiting factors for these early vectors.^{24–26} Recently lipid nanoparticles (LNPs)^{8,11,27} and polymeric vectors^{16,28} have demonstrated great promise for therapeutic delivery of siRNA to the liver and solid tumors. Despite major advances, the efficiency of endosomal escape of most vectors is generally low,^{29,30} and interaction of conventional cationic lipids and polymers with the cell membrane can result in membrane disruption, altered cell behavior, and cytotoxicity.^{24,26,31–33} While combinatorial approaches have proven useful for the discovery of new

vectors,^{10,34} a deeper understanding of how structural parameters affect transfection efficiency, circulatory stability, serum resistance, and ultimately *in vivo* efficacy would be of great value for the further development of synthetic siRNA vectors. Toward this goal, a systematic approach based on direct structure–activity correlation should advance our basic understanding and facilitate the rational design of effective new vectors.

Herein we report the rational design of a dendritic peptide bolaamphiphile for safe and efficient siRNA delivery (Figure 1). Unlike regular lipid-like amphiphiles (termed “mono-amphiphile”), which are composed of one or more hydrophobic tails and a single hydrophilic headgroup, bolaamphiphiles (termed “bola”) are dumbbell-shaped molecules having two hydrophilic headgroups connected via a hydrophobic core (Figure 1a,b).³⁵ In nature, bolaamphiphiles are found in cell membranes of certain kinds of extremophile archaeobacteria with the monolayer assemblies, as opposed to the bilayer structures of normal lipids, providing improved stability under harsh conditions.³⁶ The robust self-assembly of bolaamphiphiles makes them attractive for biomaterials applications, with several recent reports of gene delivery using bolaamphiphile-based

Received: June 11, 2015

Published: August 14, 2015

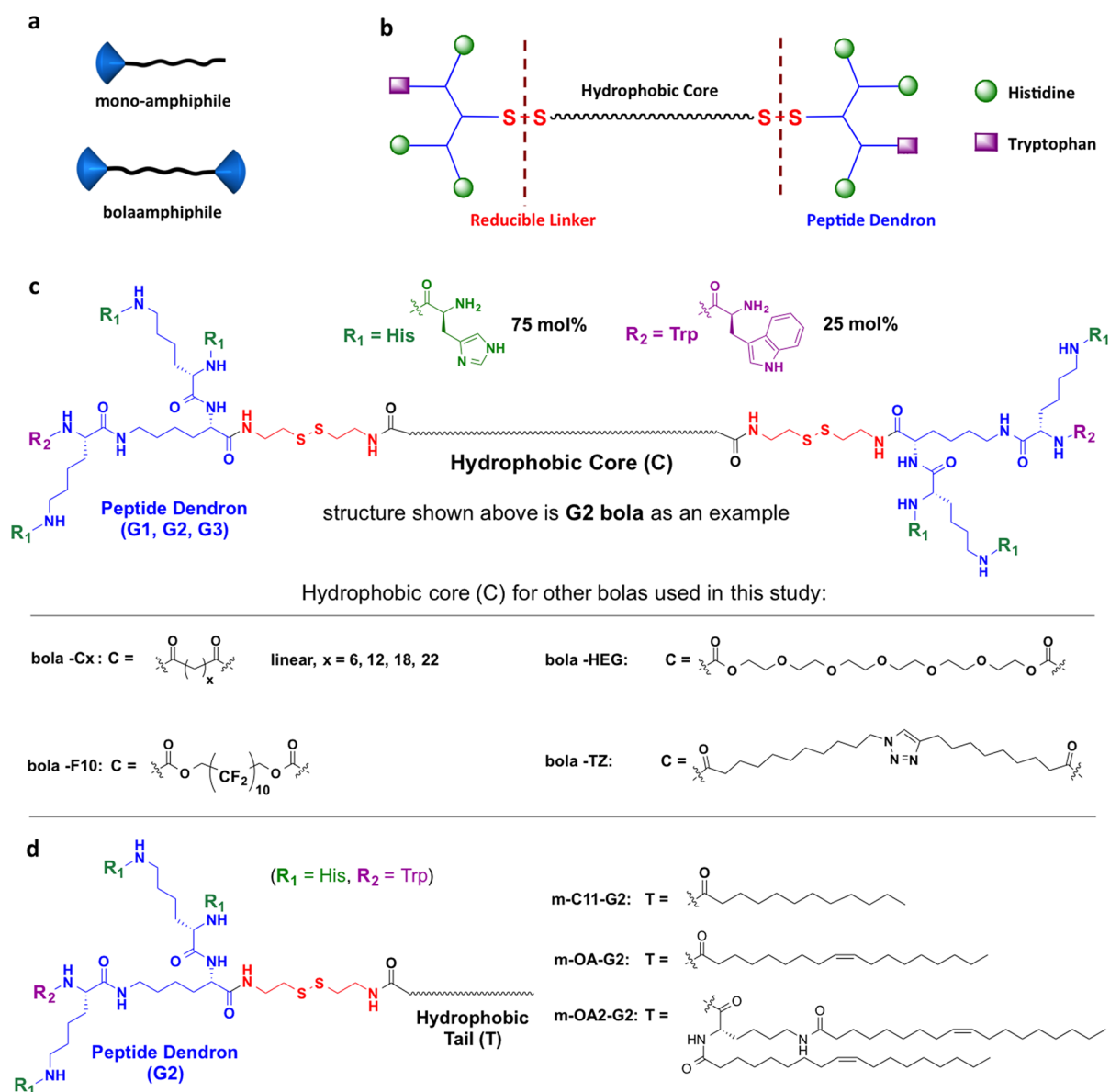


Figure 1. Design concept and structure of bolaamphiphiles. (a) Schematic illustration of mono-amphiphile and bolaamphiphile. (b) Design of dendritic peptide bolaamphiphiles. Bolaamphiphiles are composed of two dendritic peptide headgroups linked to a hydrophobic core by reducible disulfide linkages. The multifunctional dendritic peptide headgroups are functionalized with 75 mol % histidine (His) and 25 mol % tryptophan (Trp) on periphery. (c) Structures of dendritic peptide bolaamphiphiles. The structure of **bola-C18-G2** is shown as an example. The dendritic peptide headgroups range from generation-1 (G1) to generation-3 (G3) dendrons. A series of hydrophobic cores as shown were used in the structure–property studies. (d) Structures of three dendritic peptide mono-amphiphiles used for comparative studies.

vectors.^{37–40} We envision that the unique molecular architecture of bolas can be exploited to disfavor insertion into the cell membrane and offer a more biocompatible alternative to conventional lipids. The use of a hydrophobic core shorter than the native phospholipid bilayer should prevent direct membrane insertion due to unfavorable thermodynamics, with the use of relatively large dendritic headgroups to disfavor a U-shaped conformation (Figure 2a).

The *second* important design component is the choice of dendritic headgroup (Figure 1b,c), for which we chose a lysine-based dendron functionalized with 75 mol % histidine (His) and 25 mol % tryptophan (Trp), the optimal combination for our previous dendronized polymer vector.⁴¹ The dendritic headgroup provides multivalent interactions for efficient binding of siRNA, with the size and valency controllable

through the use of different dendron generations. On the basis of our previous study, Trp improves siRNA binding and cell uptake, while His facilitates endosomal escape due to the pH-responsive nature of the imidazole ring.⁴¹

The *third* key component in our bola design is the hydrophobic core for promoting self-assembly in aqueous solution. As shown in Figure 1c, a variety of hydrophobic cores were studied by systematically changing several molecular parameters such as the length, geometry, and chemical nature. Given the unique self-assembly properties of fluorocarbons and previous reports on fluorinated vectors,^{42–46} we envisioned that the fluorocarbon linker could promote robust self-assembly and also impact the cellular uptake, serum stability, and biodistribution of the complexes. The *final* design element is the disulfide linkages (Figure 1b,c), connecting the dendritic

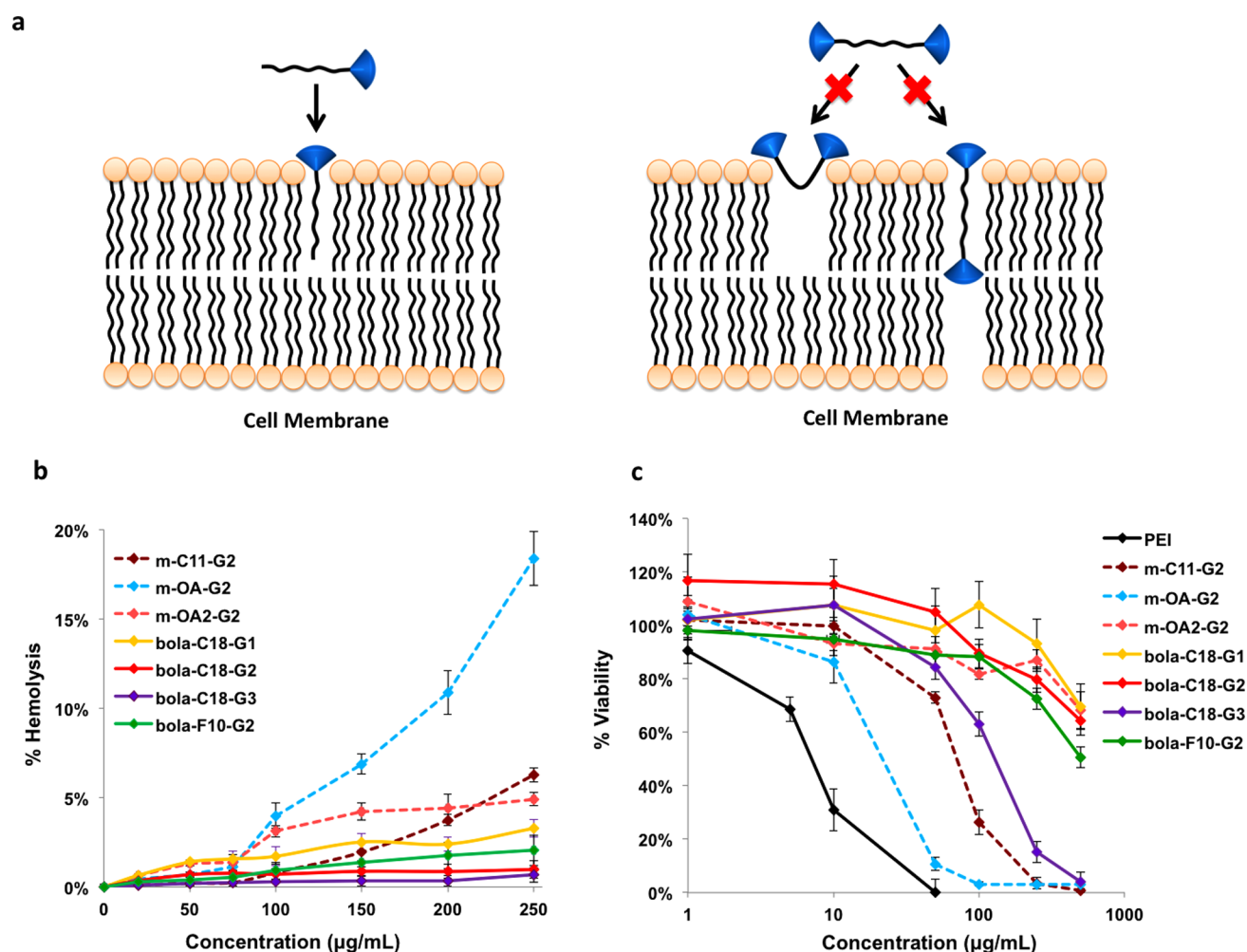


Figure 2. Hemolytic activity and cytotoxicity of bolaamphiphiles. (a) Proposed low membrane disruption character of dendritic bolaamphiphiles in comparison to mono-amphiphiles. Because of the unique molecular architecture, bolaamphiphiles should be more difficult to insert into the cell membrane, causing less membrane disruption. In contrast, mono-amphiphiles can insert into membrane more easily and cause membrane disruption due to their structural similarity to native phospholipids. (b) Hemolytic activity of both mono- and bolaamphiphiles as % hemolysis of bovine red blood cells. (c) Cytotoxicity of both mono- and bolaamphiphiles to NIH 3T3 cells as determined by MTT assay.

peptide headgroups to the central hydrophobic core to provide stimuli-responsive intracellular disassembly of the complexes, facilitating siRNA release in cytosol.⁴⁷

RESULTS AND DISCUSSION

A representative peptide bola structure (**bola-C18-G2**) is shown in Figure 1c. The amphiphiles are named as follows: m- for mono-amphiphile and bola- for bolaamphiphile, followed by the structure of the hydrophobic core (e.g., C18) and then the generation of the dendron headgroups (e.g., G2). Different hydrophobic cores were investigated, including hydrocarbon chains of various lengths (C6–C22) and a fluorocarbon core (F10). The length of the hydrophobic cores was chosen to be significantly shorter than the width of a typical phospholipid bilayer membrane so that bolas will not be able to span across the entire bilayer and cause cell membrane disruption. A control molecule containing a hexa(ethylene glycol) core (HEG) was included as the hydrophilic core should not induce self-assembly in aqueous solution. A hydrophobic core containing a 1,2,3-triazole (TZ) was also synthesized to probe the effect of the kinked backbone. The size of the dendron headgroup was varied from first to third generation (G1–G3). For a direct comparison between mono- and

bolaamphiphiles, three mono-amphiphile analogues were synthesized containing either a C11 alkyl tail (**m-C11**) or one to two oleic acid moieties (**m-OA** or **m-OA2**), with the structures shown in Figure 1d. It should be noted that linear peptide mono-amphiphiles have been extensively studied for self-assembly and biomaterials applications.^{48,49} All molecules were synthesized by solution-phase coupling reactions, and detailed synthesis and characterization data can be found in the Supporting Information.

Our hypothesis of low membrane disruption for bolas was first tested by hemolysis assays. Bovine red blood cells (RBC) were treated with the amphiphiles at various concentrations, and the release of hemoglobin from the RBC was measured as an indicator of membrane lytic activity. Supporting our hypothesis, the mono-amphiphiles exhibited greater hemolytic activity than the bolas (Figure 2b). The hydrophobic tail of mono-amphiphiles significantly influenced their hemolytic activity, as the longer OA amphiphile exhibited 4 times higher membrane lysis than the shorter C11 amphiphile. The double tailed OA2 amphiphile induced a relatively high degree of hemolysis at lower concentrations, but plateaued at higher concentration, possibly due to self-assembly at higher concentration. In sharp contrast, the C18 bolas exhibited very

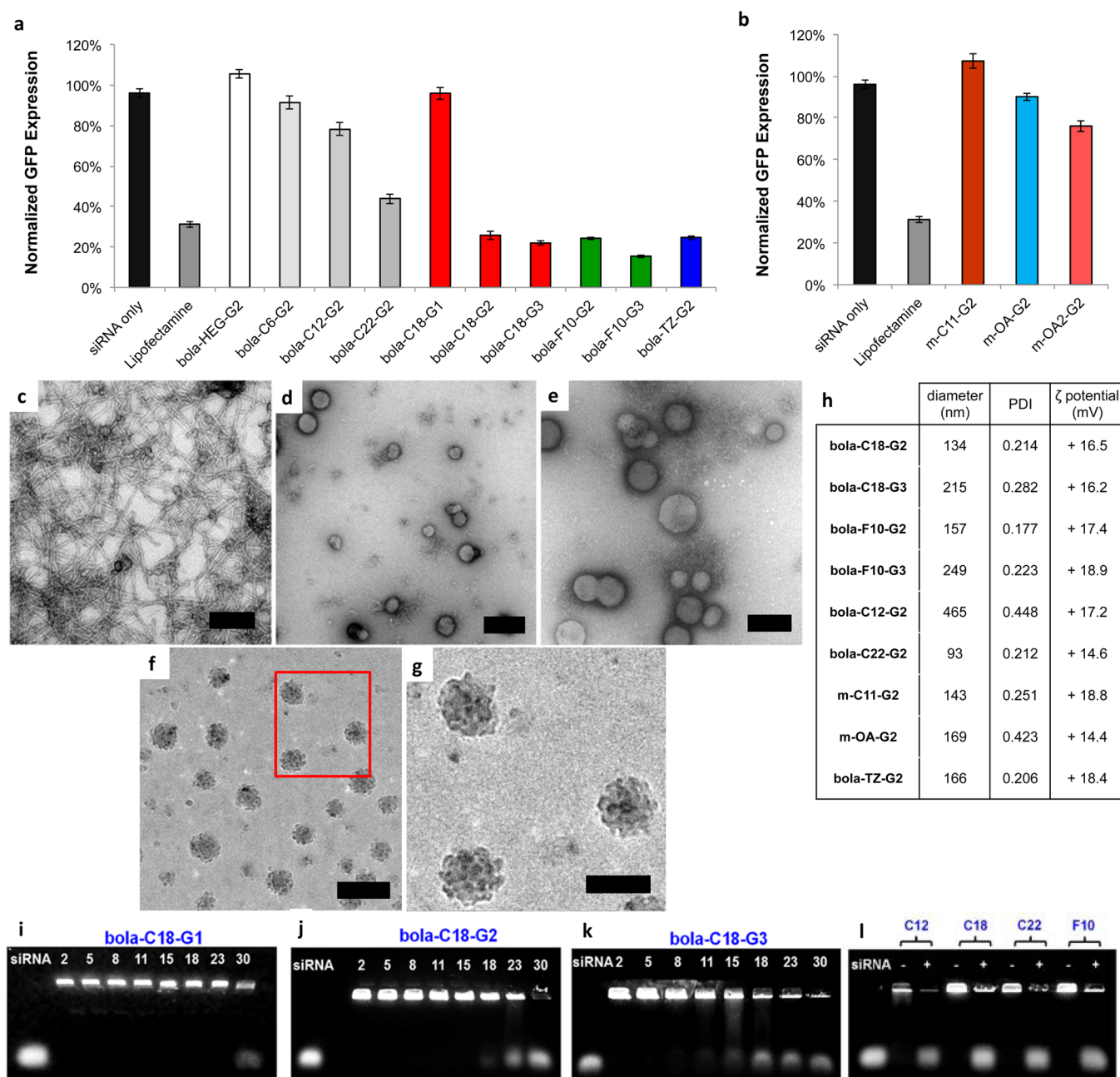


Figure 3. Transfection, TEM imaging, DLS analysis, and gel electrophoresis of different amphiphile/siRNA complexes. (a, b) Initial transfection screening of bola- and mono-amphiphiles in GFP-expressing NIH 3T3 cells. N/P ratio = 45 and [siRNA] = 100 nM. (c–e) Negative-stain TEM (uranyl acetate) images of vector/siRNA complexes: **bola-C18-G1** (c), **bola-C18-G2** (d), and **bola-C18-G3** (e), scale bars = 200 nm. (f, g) Cryo-TEM imaging of **bola-C18-G2** in PBS, scale bar = 200 nm (f) and 100 nm (g). (h) Dynamic light scattering (DLS) particle size and zeta potential analysis of vector/siRNA complexes prepared at N/P = 45 in PBS. (i–k) Dextran sulfate competitive binding assay with different bola complexes (N/P = 45) with the numbers above the wells indicating the S/P ratio (molar ratio of sulfate on dextran sulfate to phosphates on siRNA). (l) Triggered siRNA release by reducing reagent glutathione (GSH). Different **bola-G2** complexes (N/P = 45, named by the core) were treated with GSH (+) or PBS (–) for 60 min before gel electrophoresis.

low hemolytic activity, with **G2** inducing less than 1% hemolysis and **G1** \approx 3% hemolysis at 250 μ g/mL. The higher hemolysis induced by **G1** bola agrees with our proposed headgroup size effect, as the small **G1** dendron provides less steric hindrance to the U-shaped conformation. The different hemolytic activity of these dendron amphiphiles correlates well with their cytotoxicity. As shown in Figure 2c, all **G2** bolaamphiphiles are about 2 orders of magnitude less cytotoxic to 3T3 fibroblast cells than the analogous mono-amphiphiles. The increased cytotoxicity of **bola-C18-G3** is presumably due

to the higher valency of cationic charges of the **G3** dendron. For comparison, branched PEI ($M_n \approx$ 25 kDa), a commonly used polymer for gene delivery studies, induces much greater toxicity than both mono- and bolaamphiphiles, further confirming the safety of bolas for siRNA delivery.

The mono- and bolaamphiphiles were screened for gene silencing activity in NIH 3T3 cells expressing green fluorescent protein (GFP). Initial transfection and gel electrophoresis experiments suggested that while most vectors fully complexed siRNA by N/P = 10 (the molar ratio of the charged amines of

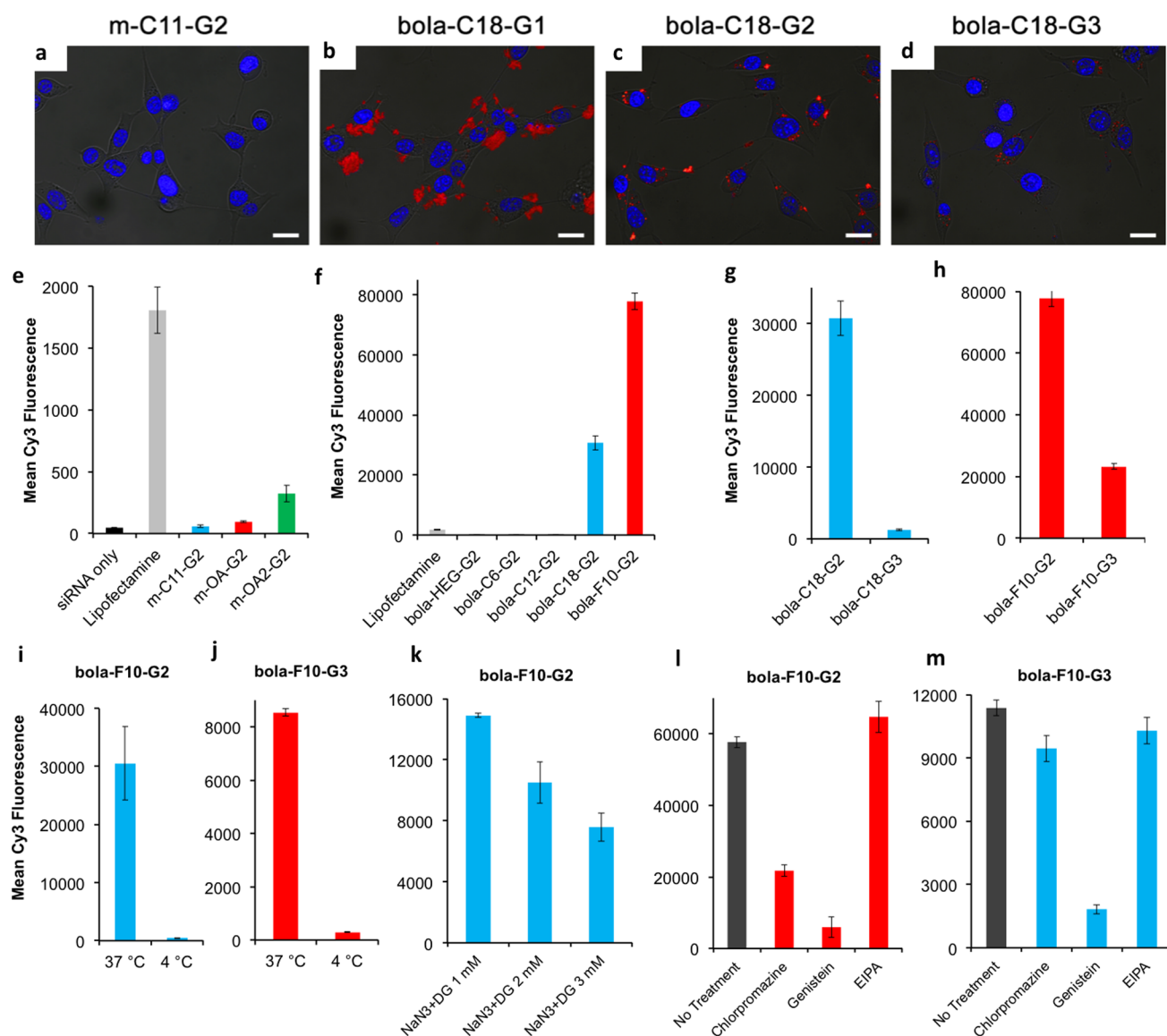


Figure 4. Cell uptake of different amphiphile/siRNA complexes. Cys3-siRNA was complexed with different amphiphiles and transfected to NIH 3T3 cells. (a–d) Confocal fluorescence images of transfected cells (cell nuclei were counter-stained with DAPI, scale bar: 20 μm). (e, f) Cell uptake of siRNA complexes with different mono- and bolaamphiphiles quantified by flow cytometry. (g, h) Cell uptake of G2 and G3 bolaamphiphile vectors. (i, j) Transfection of Cy3-siRNA complexes carried out at 37 or 4 $^{\circ}\text{C}$. (k) Transfection of 3T3 cells pretreated with sodium azide (NaN_3) and 2-deoxy-D-glucose (DG). (l, m) Endocytotic pathway of bola complexes. 3T3 cells were treated with chlorpromazine (30 μM , inhibitor of clathrin-mediated endocytosis), genistein (350 μM , inhibitor of caveolar endocytosis), or ethylisopropylamiloride (EIPA, 30 μM , inhibitor of macropinocytosis) for 1 h before transfection of Cy3-siRNA/bola complexes.

the vector to the phosphates of RNA) the knockdown effect was not saturated until N/P = 30–45, and for all further studies a N/P ratio of 45 was utilized (Figure S1). For comparison to the toxicity and hemolysis assays, **bola-F10-G2** complexes formed at N/P = 45 with [siRNA] = 100 nM have a vector concentration of 110 $\mu\text{g}/\text{mL}$, at which no significant toxic effect was observed (Figure 2c). In general, mono-amphiphiles showed very little gene silencing with the most efficacious, **m-OA2-G2**, inducing only $\sim 25\%$ knockdown (Figure 3b). In contrast, bolaamphiphiles containing the fluorocarbon (F10) or hydrocarbon cores of sufficient length (C18, C22) with G2 or G3 headgroups displayed effective gene silencing. The introduction of triazole ring in the hydrophobic core (TZ) did not significantly change the transfection efficiency. Bolaamphiphiles based on shorter cores (**bola-C6-G2** and **bola-C12-G2**) or bearing G1 headgroups failed to induce a

strong knockdown response (Figure 3a). The vector containing a hydrophilic linker (**bola-HEG-G2**) was completely ineffective at gene silencing, presumably due to the lack of hydrophobic self-assembly.

To understand the correlation between molecular structure and delivery efficiency, transmission electron microscopy (TEM) was utilized to examine the morphology of different amphiphile/siRNA complexes. Figure 3c–e displays TEM images of different **bola-C18/siRNA** complexes stained with uranyl acetate, demonstrating that the size of the dendritic headgroup influences the morphology of the assembled nanostructures dramatically. The compound with the smallest headgroup, **bola-C18-G1**, can pack closely to form twisted nanofibers, similar in morphology to the structures previously reported for well-packed bola assemblies (Figure 3c).³⁵ As the headgroup increases in size, both steric effects and charge

repulsion disfavor the formation of densely packed nanofibrils, with **bola-C18-G2** and **bola-C18-G3** complexes appearing as circular features consistent with assembly into nanoparticles (Figure 3d,e). The larger headgroup of **G3** bolaamphiphiles further reduces the packing efficiency for the hydrophobic core, resulting in larger particles than **G2** bolaamphiphiles. Complexes formed using **G2** bolaamphiphiles containing fluorocarbon (**F10**) or 1,2,3-triazole (**TZ**) cores also displayed nanoparticle morphology (Figure S2a–c), as did both mono-amphiphiles containing a single hydrophobic tail (**m-C11-G2**, **m-OA-G2**, Figure S2d,e). The **m-OA2-G2** complexes formed fibrillar structures similar to those observed in the **bola-C18-G1** images, potentially due to increased hydrophobic interactions from the additional oleate tail enhancing the stability of assembly (Figure S2f). Cryo-TEM was further used to observe the *in situ* morphology of the vector/siRNA complexes in PBS with **bola-C18-G2** chosen for initial analysis. The cryo-TEM images of **bola-C18-G2** (Figure 3f,g) complexes were characterized by nanoparticles with an average diameter of 88 ± 19 nm, which appear to be composed of smaller “granules” approximately 10–20 nm in diameter. Nanoparticles displaying similar “raspberry” morphology have been observed for siRNA complexes of the peptide vector CADY.⁵⁰

The particle size and zeta potential of the complexes were analyzed using dynamic light scattering (DLS) (Figure 3h). Complexes formed from vectors lacking a hydrophobic core or sufficient length (**bola-HEG-G2**, **bola-C6-G2**) did not provide enough signals for analysis, consistent with a lack of assembly. The complexes that formed fibrillar assemblies as observed by TEM (**bola-C18-G1**, **m-OA2-G2**) produced scattering signal, but the data did not correlate when analyzed via standard methods, possibly due to the formation of irregular aggregates or fibrillar networks. For both **bola-C18** and **bola-F10** vectors the particle size of **G2** was smaller than that of the **G3**, while the particle size decreased with increasing length of alkyl core from **bola-C12-G2**, **bola-C18-G2**, to **bola-C22-G2**. The trend is consistent with proposed assembly stability, with smaller headgroup and longer hydrophobic core condensing siRNA into smaller particles and larger headgroup and shorter core into larger particles. The zeta potential of the vector/siRNA complexes was also measured, with all samples displaying a positive surface charge between 15 and 20 mV. Mono amphiphile/siRNA complexes, **m-C11-G2** and **m-OA-G2**, showed similar particle characteristics in DLS, with a slightly larger size than the bola analogue (**bola-C22-G2**).

In our bola design, we hypothesized that the more stable nanoparticles formed by bolaamphiphiles should enhance the siRNA binding strength, which was assessed via a competitive binding assay using the anionic polymer dextran sulfate (DS, $M_n \approx 25$ kDa). Although **m-C11-G2** and **bola-C22-G2** have exactly the same chemical composition, the bola complexes exhibited much higher stability with little siRNA release up to $S/P = 23$ (the molar ratio of sulfate from DS and phosphate from siRNA), while the mono-amphiphile complex started to release siRNA at $S/P = 8$ (Figure S3a,b). Furthermore, the proposed self-assembly model agrees with the stability of different bola complexes. As shown in Figure 3i–k, despite having headgroups with the lowest level of multivalency, **bola-C18-G1** exhibited the strongest siRNA binding with no siRNA release up to S/P of 30, presumably due to the stable fibrillar assembly. The vectors **bola-C18-G2** and **bola-C18-G3**, which demonstrated nanoparticle assembly by TEM, showed siRNA release at the lower S/P ratios of 23 and 15, respectively. The

fluorocarbon vector, **bola-F10-G2**, demonstrated stronger siRNA binding than the hydrocarbon analogue **bola-C18-G2** with no siRNA release until $S/P = 30$ (Figure S3c), again presumably due to the more robust self-assembly induced by the fluorocarbon core. The bolaamphiphiles containing either the shortest hydrocarbon (**bola-C6-G2**) or hydrophilic core (**bola-HEG-G2**) failed to effectively complex siRNA (Figure S3e,g). Stimuli-responsive siRNA release from bola complexes was demonstrated by incubation of the complexes with glutathione (GSH) followed by subsequent gel electrophoresis, which showed effective decomplexation for the GSH treated samples (Figure 3l).

The different self-assembly behavior was corroborated by infrared (IR) spectroscopy. The methylene (CH_2) groups in the alkyl region prefer *trans* conformation in highly ordered packing structure, resulting in lower frequency for their C–H stretching peaks.⁵¹ Figure S4 shows that from **G1** to **G3** bola complexes, both the asymmetric (~ 2930 cm^{-1}) and symmetric (~ 2850 cm^{-1}) C–H stretch peaks shifted to higher frequency, indicating less ordered structure in the alkyl region. This agrees with the proposed self-assembly model, with decreasing packing order from nanofibril to nanoparticle, caused by the enhanced electrostatic repulsion and sterics associated with increasing valency of the cationic dendron.

The cellular uptake of amphiphile-siRNA complexes was investigated by confocal fluorescence microscopy using Cy3 labeled siRNA (Figure 4a–d). The mono-amphiphile (**m-C11-G2**) complexes showed no cellular uptake with no detectable signal from Cy3-siRNA (Figure 4a), while **bola-C18-G1**/siRNA complexes formed aggregates on the cell surface and were not internalized (Figure 4b), in agreement with the observed low gene knockdown for both of these vectors (Figure 3a,b). Complexes formed with both **bola-C18-G2** and **bola-C18-G3** showed significant cellular uptake of siRNA, with **G2** exhibiting higher efficiency (Figure 4c,d). The Cy3-siRNA uptake was further quantified by flow cytometry (Figure 4e–h). Compared to lipofectamine, Cy3-siRNA complexes with all three mono-amphiphiles showed very low cellular uptake (Figure 4e), while **bola-C18-G2** and **bola-F10-G2** exhibited 20–40 times higher uptake than lipofectamine (Figure 4f). Bolas with either shorter alkane cores (**C6** and **C12**) or a hydrophilic **HEG** core could not form stable complexes, and no cellular uptake of Cy3-siRNA was detected (Figure 4f). Notably, cell uptake efficiency was directly related to the size of headgroup. Both **bola-C18-G2** and **bola-F10-G2** showed much higher cell uptake than their **G3** analogues (Figure 4g,h), which could be attributed to the higher stability and smaller size of **G2** complexes.

To further understand the cell uptake process, we chose **bola-F10-G2** and **bola-F10-G3** as representative vectors to study the cell uptake pathway. Figure 4i–k shows that the cell uptake of bola/Cy3-siRNA complexes are energy dependent, with low temperature (4 °C) completely shutting down the uptake (Figure 4i,j) and the metabolic inhibitor $\text{NaN}_3/2$ -deoxy-D-glucose inhibiting uptake in a concentration-dependent manner (Figure 4k). The cell uptake mechanism was further studied by using several small molecule inhibitors to specifically block three common endocytotic pathways: clathrin-mediated endocytosis (chlorpromazine), caveolar endocytosis (genistein), and macropinocytosis (EIPA).^{52,53} As shown in Figure 4l,m, siRNA/**bola-F10-G2** complexes were internalized through both clathrin-mediated and caveolar pathways. The **bola-F10-G3** complexes entered the cell through caveolae-

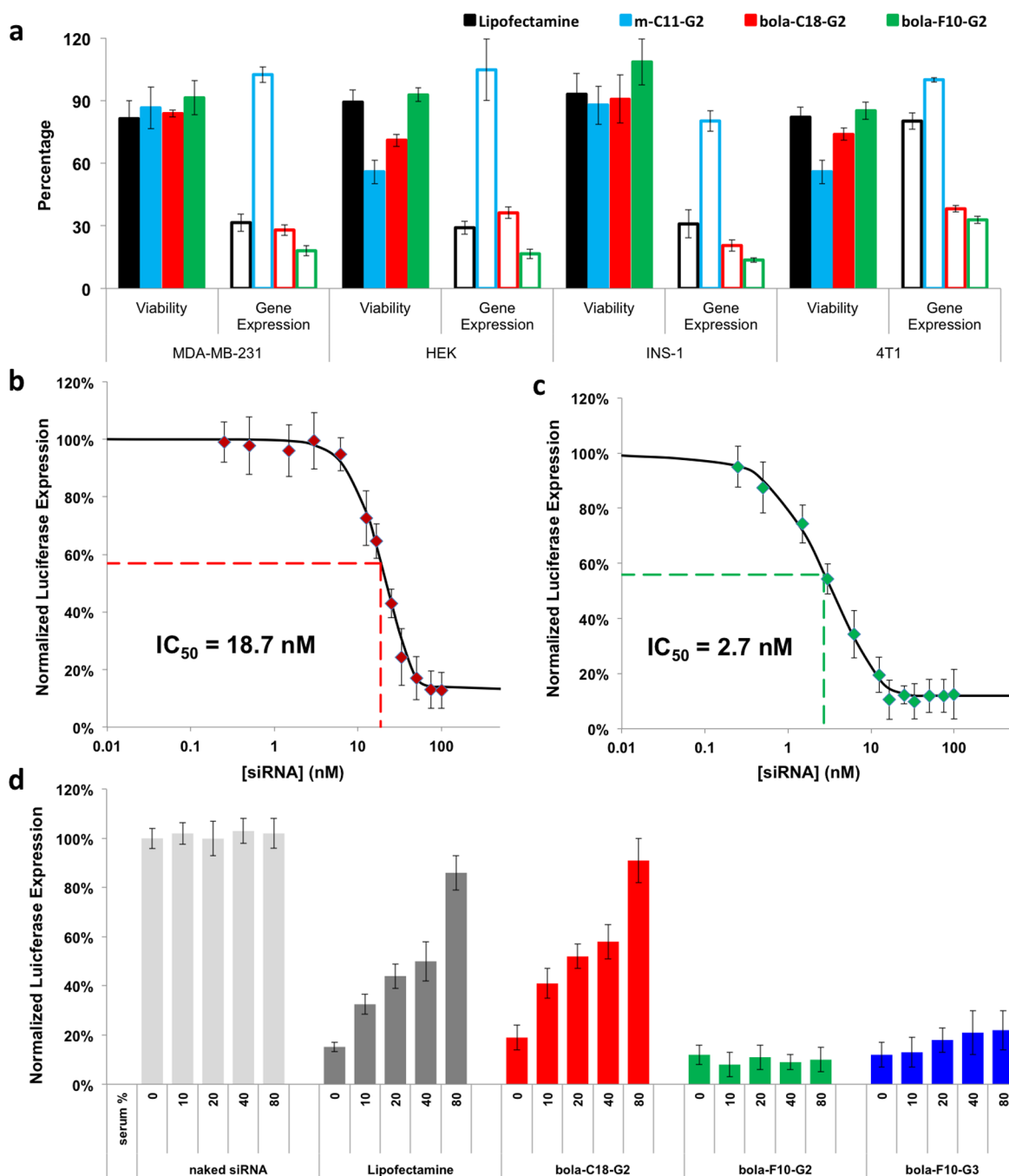


Figure 5. Transfection study in different cell lines and in the presence of serum. (a) Luciferase silencing data for various amphiphiles in different cell lines including MDA-MB-231, HEK 293, β -INS-1, and 4T1. N/P = 45, [siRNA] = 100 nM, viability measured by MTT assay. (b, c) Dose/response curves of **bola-C18-G2** (b) and **bola-F10-G2** (c) vectors in MDA-MB-231 cells. N/P = 45 for all samples, [siRNA] indicated on *x*-axis. (d) Transfection of MDA-MB-231 cells in serum-containing medium. N/P = 45, [siRNA] = 50 nM, final concentration of fetal bovine serum (FBS) in antibiotic-free DMEM transfection media indicated on the *x*-axis.

mediated endocytosis almost exclusively, which can be explained by the larger particle size of the **bola-F10-G3** complexes.^{54,55} The clear dependence of cell uptake on inhibitor concentration (Figure S5) further confirms the cellular uptake pathway.

To demonstrate the general efficacy of these vectors, transfections were carried out in the following luciferase-expressing cell lines: MDA-MB-231, HEK, INS-1, and 4T1 (Figure 5a). Similar to the results obtained from 3T3 cells, mono-amphiphile **m-C11-G2** did not achieve any significant gene knockdown and induced substantial toxicity in HEK and

4T1 cells. In contrast, **bola**amphiphiles **C18-G2** and **F10-G2** both displayed robust luciferase knockdown with minimal cytotoxicity. Notably, for the 4T1 cell line in which lipofectamine failed to induce a strong knockdown response, both **C18-G2** and **F10-G2** effectively silenced luciferase expression.

The previously discussed transfection studies used phosphate buffer (PBS, 10 mM phosphate, 10 mM NaCl, pH = 7.4) for sample preparation (Figure 3a,b and Figure 5a); however, it was observed during optimization that complexes prepared in OptiMEM induced significantly higher knockdown at low concentrations than those prepared in PBS. This optimized

Table 1. Structure–Property Relationship of Dendritic Peptide Amphiphiles for siRNA Delivery

molecular structure	assembly with siRNA	cell uptake	siRNA transfection	serum stability
mono-amphiphiles	nanoparticle/nanofibril	low cell uptake, highly membrane disruptive	low	
bolaamphiphile, small headgroup (G1)	nanofibril	aggregate on cell surface, no uptake	none	
bolaamphiphile, medium headgroup (G2)	smaller nanoparticle	clathrin- and caveolae-mediated endocytosis, high cell uptake	high efficiency	high
bolaamphiphile, large headgroup (G3)	larger nanoparticle	caveolae-mediated endocytosis, low cell uptake	high efficiency	low

protocol was used to further test the transfection efficiency of the vectors in MDA-MB-231 cells, with dose/response curves and IC50 values determined for **bola-C18-G2**, **bola-C18-G3**, **bola-F10-G2**, and **bola-F10-G3**. The fluorocarbon vector **bola-F10-G2** demonstrated highly efficient transfection with an IC50 value of 2.7 nM, while the analogous hydrocarbon vector, **bola-C18-G2**, was less effective with an IC50 of 18.7 nM (Figure 5b,c). For both C18 and F10 cores, the G3 variants displayed higher transfection efficiency than the corresponding G2 analogues, with calculated IC50 values of 1.0 nM and 7.4 nM for **bola-F10-G3** and **bola-C18-G2**, respectively (Figure S6). These results suggest that the fluorocarbon linker greatly enhanced the delivery efficiency, with both F10 vectors displaying IC50 values ~7 times lower than the corresponding hydrocarbon analogues. The toxicity of the bolaamphiphile/siRNA complexes prepared using OptiMEM was assessed via the MTT assay, with the results corroborating the previously observed trend of G3 inducing greater cytotoxicity than G2 for both C18 and F10 linkers (Figure S7).

Although typical *in vitro* transfections are performed in reduced serum or serum-free media, successful transfection in the presence of negatively charged serum components is critical for *in vivo* application. To investigate the serum stability of our vectors, transfections were performed varying the final concentration of fetal bovine serum (FBS) in the transfection media from 0 to 80% (Figure 5d). While lipofectamine RNAiMAX and **bola-C18-G2** were negatively affected even at low serum content, the gene silencing effects of **bola-F10-G2** were not affected by the presence of up to 80% FBS. The analogous G3 variant, **bola-F10-G3**, showed a slight decrease in efficacy at higher FBS concentrations, which can be attributed to less stable assembly and consequentially weaker siRNA binding due to the larger dendritic headgroup. The improved serum stability and efficacy of our fluorocarbon bolaamphiphiles compared to those containing hydrocarbon cores are in agreement with previous reports on the use of fluorinated lipids for siRNA delivery.⁴⁴ With recent reports of fluorination greatly enhancing DNA transfection efficiency of PAMAM dendrimers,^{45,56} we believe this “fluorocarbon effect” may be generally applicable to other amphiphilic vectors.

On the basis of the data presented in this paper, a structure–property relationship can be derived for dendritic peptide amphiphiles for siRNA delivery (Table 1). Compared to the mono-amphiphiles studied, all G2 bolaamphiphiles of similar length displayed stronger siRNA binding, reduced cytotoxicity, and greatly enhanced transfection efficiency. The size of the cationic dendron was observed to have a major influence on the morphology and biological activity of the complexes, with the fibrillar assemblies formed by **bola-C18-G1** affording dramatically less knockdown than the nanoparticle **bola-C18-G2** and **bola-C18-G3** complexes (Table 1). While both G2 and G3 bolaamphiphiles are highly efficient for siRNA transfection, G2 vectors exhibit much higher serum stability than G3 analogues. The use of a fluorocarbon, as opposed to hydrocarbon core

greatly enhanced both the transfection efficacy and serum stability of the complexes, suggesting that further investigation into fluorocarbon-based vectors may prove particularly fruitful.

CONCLUSION

With the high siRNA transfection efficiency in multiple cell lines, excellent serum resistance, and low cytotoxicity and hemolysis, the dendritic peptide bolaamphiphiles presented in this study are promising candidates for further gene delivery applications. Through a rational design approach, we were able to directly link the molecular structure of different vectors to their assembly morphologies and the resulting biological activities (Table 1). The unique bolaamphiphile architecture proved a key factor for improved complex stability, low cytotoxicity and hemolytic activity, and high transfection efficiency. The optimal vector contains a fluorocarbon core and exhibited enhanced delivery efficiency to a variety of cell lines and improved serum resistance when compared to hydrocarbon analogues and lipofectamine RNAiMAX. The high transfection efficiency of the fluorocarbon vectors and the direct correlation from molecular structure to self-assembly behavior to subsequent biologic activity offer critical insight aiding the rational design of new materials for nucleic acid delivery and other biomaterial applications.

METHODS

Amphiphile/siRNA Complex Preparation (PBS). The 5× complex solution for transfection was prepared by pipet mixing of the amphiphile and siRNA solutions. In a typical procedure, 1.5 μM siRNA solution was prepared with PBS buffer (10 mM phosphate, 10 mM NaCl, pH = 7.4). Different amphiphile solutions (5 mg/mL) were diluted with PBS buffer to a final volume of 13.3 μL per well. The 13.3 μL of amphiphile solution was then added to 6.7 μL of 1.5 μM siRNA solution (final 5× siRNA concentration = 500 nM), followed by brief vortexing. The solution was incubated at room temperature for 30 min before addition to the cell culture media.

Optimized Complex Preparation (OptiMEM). The vector/siRNA complexes were prepared by first diluting the vector to the desired concentration using antibiotic-free OptiMEM. The siRNA was diluted to 1.5 μM with OptiMEM, and an appropriate amount of this solution was added to the diluted vector to give a complex solution with siRNA concentration of 500 nM. After 10 min incubation, this complex solution was further diluted to the desired concentration with OptiMEM and used immediately.

Transfection and Flow Cytometry. NIH 3T3 fibroblast cells were seeded at a density of 10 000 cells/well in 48-well plates 24 h in advance. Prior to transfection, the media was replaced with 80 μL of antibiotic-free DMEM, and 20 μL of the previously described 5× complex solutions were added to each well to give 100 nM final siRNA concentration. After 4 h

incubation, the medium was changed back to 250 μ L of DMEM supplemented with 10% fetal bovine serum and cultured for another 48 h. Before the analysis, cells were released from each well by Trypsin and harvested by centrifugation (5 min, 500g). Fluorescence of transfected cells was measured on a Becton-Dickinson LSR II flow cytometer with argon ion excitation laser. For each sample, data representing 10 000 objects were collected as a list-mode file and analyzed using FACSDivaTM software. The normalized GFP expression was calculated by comparing cells treated with anti-GFP siRNA complexes to a control sample treated with complexes prepared with nontargeting siRNA.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acscentsci.5b00233](https://doi.org/10.1021/acscentsci.5b00233).

Experimental details, synthesis and characterization data, gel and TEM images, transfection data, and IR and NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We acknowledge the financial support of the U.S. National Institute of Health (DK098446). We thank Professor Young Jik Kwon for donating the GFP-expressing NIH 3T3 cell, Professor Jennifer Prescher for donating the luciferase-expressing HEK, MDA-MB-231, 4T1, and Professor Yoko Mullen for donating INS-1 cells.

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