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Dynamic Amphiphile Libraries To Screen for the "Fragrant" Delivery of siRNA into HeLa Cells and Human Primary Fibroblasts

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Supporting Information

ABSTRACT: Dynamic amphiphiles are amphiphiles with dynamic covalent bridges between their hydrophilic heads and their hydrophobic tails. Their usefulness to activate ion transporters, for odorant release, and for differential sensing of odorants and perfumes, has been demonstrated recently. Here, we report that the same "fragrant" dynamic amphiphiles are ideal to screen for new siRNA transfection agents. The advantages of this approach include rapid access to fairly large libraries of complex structures, and possible transformation en route to assist uptake and minimize toxicity. We report single-component systems that exceed the best commercially available multicomponent cocktails with regard to both efficiency and velocity of EGFP knockdown in HeLa cells. In human primary fibroblasts, siRNA-mediated enzyme knockdown nearly doubled from >30% for Lipofectamine to >60% for our best hit. The identified structures were predictable neither from literature nor from results in fluorogenic vesicles and thus support the importance of conceptually innovative screening approaches.

RNA interference (RNAi) has emerged as a powerful method to inhibit the biosynthesis of specific proteins with their complementary small interfering RNA (siRNA).[1] Because of the great potential for applications in biology and medicine, the question of how to deliver siRNA to the cytosol has attracted significant scientific attention.[1] Extensive experience with gene transfection could be adapted partially to yield a high number of nonviral siRNA transporters, including cationic amphiphiles, peptides, polymers, dendrimers, nucleobase-lipid hybrids, and more complex architectures.[1,2] Triggerable amphiphiles[3a] that transform in response to the pH drop in the endosome include Thompson's beautiful vinyl ethers,[3b] acetals,[3c] activated esters,[3d−f] or very elegant ortho-esters[3g] and phosphotriesters.[3h] Pioneering examples for gene transfection with acid-labile hydrazone bridges have suggested that degradation en route into cells could be advantageous to minimize cytotoxicity and maximize oligonucleotide release.[4a] However, other studies have suggested that hydrazone-bridged amphiphiles[4] are inferior to disulfide-bridged amphiphiles.[4b] The unique power of cytosolic disulfide reduction to minimize toxicity and release the substrate is also attracting much attention with cell-penetrating poly(disulfide)s.[5]

Despite these impressive efforts and the enormous potential for applications in biology and medicine, problems concerning their delivery into cells remain, particularly with regard to in vivo applications. They include the frequent need of multicomponent cocktails in practice, variable efficiencies with different cell lines depending on the specific properties of their cellular barriers, and, most importantly, the scarcity of general structure−activity guidelines for rational design. This situation calls for screening approaches.[1d] Dynamic amphiphiles appeared most attractive for rapid access to fairly large libraries. They are amphiphiles with dynamic covalent bonds between their hydrophilic heads and their hydrophobic tails. Dynamic covalent bonds are interesting tools because they can combine the advantages of strong covalent bonds and weak non-covalent bonds, depending on conditions.[6−11] Dynamic amphiphiles have been introduced recently as activators of ion transporters,[7] for slow release of odorants,[8] dynamic micelles, vesicles and gels,[9] and as biosensors[7] or differential sensors[10] that function in lipid bilayers. Here, we show that the same odorant libraries used in "artificial noses" [10] can be used to screen for the "fragrant" delivery of siRNA. Facile access to expanded libraries with hydrazone, oxime and disulfide bridges[11] is used to screen up to 900 amphiphiles, either in model vesicles,[10,11] cells, or both. The screening results for GFP knockdown in HeLa cells disclosed in the following include hits with fairly complex, totally unpredictable structures that can compete with multicomponent systems on the market. They operate as single-component systems, with ability to deliver siRNA into hard-to-transfect human primary fibroblasts, high reproducibility and low toxicity.

Our formal library of 900 dynamic amphiphiles was constructed from 18 heads and 50 tails (Figures 1 and 2). The heads contain one to two ammoniums (**A**) or guanidinium (**G**) cations plus one to six reactive groups to form hydrazone (**H**) or oxime (**O**) bridges with aldehyde or ketone containing tails (**T1**−**T50**). For doubly bridged amphiphiles, preformed disulfide bridges (**S**) were placed in the head part before amphiphile formation.

The synthesis of all peptide dendrons used as scaffolds in the head groups has been reported.[10,11] Most tails were commercially available, some had to be prepared following or adapting straightforward procedures (Scheme S1).[12] Dynamic amphiphiles were prepared by incubation of heads (e.g., **G2H4**) and tails (e.g., **T20**) in DMSO, usually for 1 h at 60 °C (Figure 1). The formation of hydrazone-, oxime-, or disulfide-bridged amphiphiles

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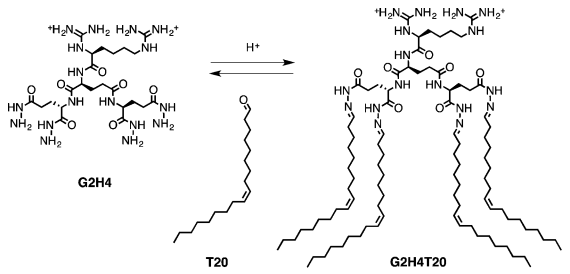

Figure 1. In dynamic amphiphiles (e.g., **G2H4T20**), heads (e.g., **G2H4**) and tails (e.g., **T20**) are connected with dynamic hydrazone (**H**), oxime (**O**) and/or disulfide bridges (**S**, compare Figure 2).

(e.g., **G2H4T20**) was confirmed by ESI-MS as described previously.[10,11] Some amphiphiles were eliminated early on because of poor physical properties. The characterization of most amphiphiles as activators of DNA as cation transporters in fluorogenic vesicles has been reported.[10,11] Several underperforming amphiphiles were also eliminated at this level. The remaining focused library of 160 amphiphiles was tested for cellular uptake.

For robotic library screening, a GFP silencing assay was performed in HeLa GPI-EGFP cells (genetically engineered HeLa cells that stably express GPI-anchored green fluorescent proteins at their plasma membrane). A liquid-handler (Biomeck FX) automatically mixed amphiphiles in serum-containing medium, with a custom siRNA sequence that when properly delivered, has the ability to knockdown EGFP expression (siEGFP). The HeLa GPI-EGFP cells were incubated with this mixture for 72 h and EGFP expression was quantified with a fluorimeter. All experiments were carried out at constant concentrations of siRNA (33.8 nM) and DMSO (0.25% (v/v)) and increasing concentrations of dynamic amphiphiles (Figure 2). No pre-incubation step of siRNA and dynamic amphiphiles in low serum media (i.e., Opti-MEM) was required before transfection.

For comparison, parallel experiments were performed with scrambled siRNA as a negative control. **G2H4T20** was used as the reference in positive control experiments due to its similar transfection efficiency compared with Lipofectamine RNAiMax (Figure S1). EGFP knockdown efficiency was calculated as the percentage of fluorescence decrease observed in cells transfected with siEGFP compared to transfection with scrambled siRNA.

To facilitate high-throughput screening, cell viability was first evaluated as the percentage of fluorescence decrease in samples transfected with complexes made of scrambled siRNA and amphiphiles compared to untreated cells in medium supplemented with 0.25% (v/v) DMSO (see SI for experimental details).[12] Only amphiphiles with cell viability up to 70% were retained for further validation and optimization. In further experiments, nontoxicity of selected amphiphiles was confirmed using a commercially available kit (cytotoxicity detection kit, Roche) that detects cell death by measuring activity of lactate dehydrogenase (LDH) released from cells whose plasma membrane was damaged.

The results from robotic high-throughput screening of our library confirmed that heads and tails alone (33.8 μM final concentration) were neither toxic nor able to transfect siRNA (Figure 2g, ø). The best results were generally obtained for hydrazones with one or two guanidinium heads linked to three or four aliphatic tails, either long unsaturated (**T19**, **T20**, **T21**, **T22**, and **T24**) or shorter saturated alkyl chains (**T12**, Figures 2, S2, and S3). The structures of most of the hits were predictable neither from literature nor from activities in vesicles.[10,11] **G2H4T31** and **A2H4T31** have been previously described to be very active to transport DNA across fluorogenic vesicles[10] but this property was weaker in the HeLa GPI-EGFP assay (Figure 2). Whereas the oleyl tails in **G2H4T20** are well precedented,[1] lauryl tails have been implied as less suitable for cellular uptake.[11,13] Although particularly promising in vesicles,[10,11] fragrant amphiphiles with tails from jasmine and cyclamen **T44** and **T45** did not perform well. We also noticed that minor structural changes down to single carbon homologues could cause large differences in activity. This overall poor predictability confirmed the importance of facile access to large amphiphile libraries as well as methods development for high-throughput robotic screening of siRNA delivery.

The enhanced detergent activity observed in vesicles for the lengthened oxime and disulfide amphiphiles[11] was translated into an increase of the toxicity in cells (Figures 2, S2, and S3). Replacement of the guanidinium head by an ammonium decreased the transfection efficiency of our cationic amphiphiles (Figures 2, S2, and S3). Screening a panel of increasing concentrations for each amphiphiles also allowed overcoming the issue of false negative results due to cytotoxicity. Indeed, some amphiphiles (e.g., **G2H4T24**) were even more active when lowering their concentration (Figures 2 and S2).

After validation and optimization of siRNA transfection conditions in HeLa GPI-EGFP (Figures S4−S6), a time-course assay was performed in order to compare kinetics between active dynamic amphiphiles (i.e., **G2H4T12** and **G2H4T20**) and Lipofectamine RNAiMax. In cells whose siEGFP was transfected with dynamic amphiphiles, a strong fluorescence decrease was already observed 24 h after transfection, well before Lipofectamine RNAiMax whose action started 48 h post transfection. These different kinetics suggested that dynamic amphiphiles take a different or more rapid pathway than Lipofectamine RNAiMax to enter cells (Figure 3a).

siRNA transfection in hard-to-transfect primary human skin fibroblasts was then evaluated using a custom siRNA sequence targeting glyceraldehyde 3-phosphate dehydrogenase expression (siGAPDH).[12] Comparison of a pair of our hits (**G1H3T12** and **G2H4T20**) with Lipofectamine RNAiMax showed a clearly improved knockdown of GAPDH activity, particularly for the dynamic amphiphile **G2H4T20** (Figure 3b). Cell viability of fibroblasts transfected with amphiphile/siGAPDH complexes was confirmed in the same time by measuring LDH activity in cell supernatant (Figure 3b).

The formation of complexes between siRNA and the best dynamic amphiphiles was monitored by routine gel shift assays (Figure S9). According to dynamic light scattering, they have a diameter around 100 nm, and inversion of their ζ potentials occurs at a molar ratio of ~200:1 (Figure S8).[12] The lability of the hydrazone bridges at pH 5.5 was consistent with expectations from the literature.[11,14] In preliminary mechanistic studies, HeLa cells were incubated for 1 h with 40 μM **G1H3T12** and 2 μM of an FITC-labeled DNA oligomer in serum at 37 °C. After washing, mostly punctate fluorescence was observed (Figure S7). This finding was characteristic for the accumulation of DNA in the endosome, although contributions from small aggregates on cell surfaces could not be fully excluded. Weaker diffuse emission from cytosolic areas might suggest that a minor fraction of FITC-DNA can escape from the endosomes or enter cells directly by passive diffusion. Clearly reduced uptake at 4 °C further supported endocytosis as main pathway (Figure S7).

In summary we have developed an easy and straightforward methodology toward the preparation of controlled libraries of dynamic amphiphiles for the screening of single component siRNA transfecting agents. The reported methodology allows the rapid identification of unpredictable hits with similar performance than the actual commercially available cocktails in different

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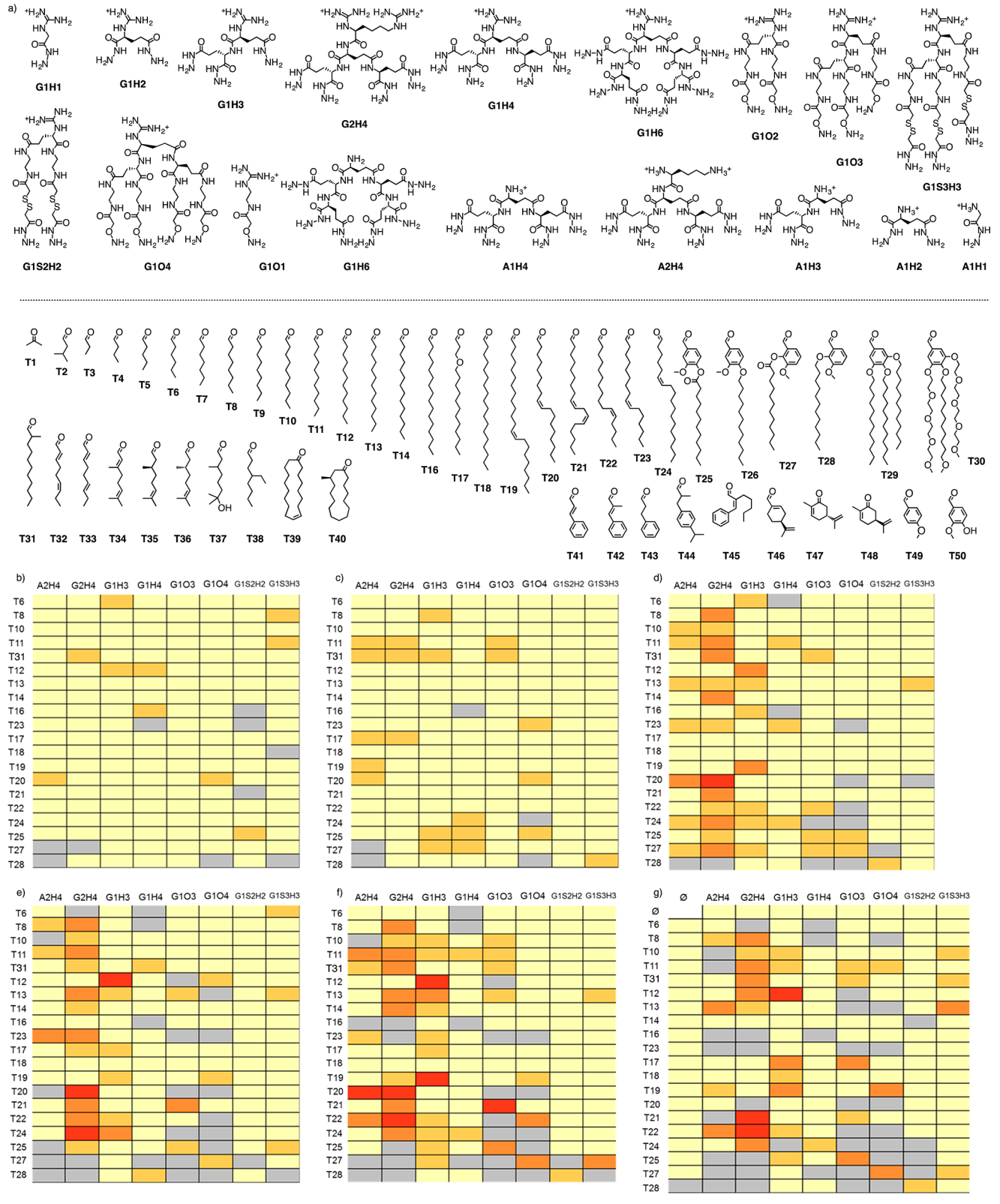

Figure 2. (a) Structure of 18 heads and 50 tails used to build a formal library of 900 amphiphiles. (b–g) *In vitro* screening of 160 dynamic amphiphiles for siRNA delivery. HeLa cells expressing GPI-EGFP were treated with EGFP-targeting siRNA mixed with dynamic amphiphiles. The average percent reduction in EGFP expression is shown after treatment with siRNA (33.8 nM) in the presence of (b) 3.4, (c) 6.8, (d) 13.5, (e) 20.3, (f) 27.0, and (g) 33.8 μM dynamic amphiphiles in the medium (in triplicate). Gray: >50% of cytotoxicity; from yellow to red: 0–20, 20–40, 40–60, 60–80, and 80–100% GFP knockdown.

cells lines. siRNA-mediated enzyme knockdown in human primary fibroblasts almost doubled to >60% (compared to >30% for Lipofectamine RNAiMax). Endocytosis of the siRNA seems to be a major pathway of internalization, but kinetics experiments

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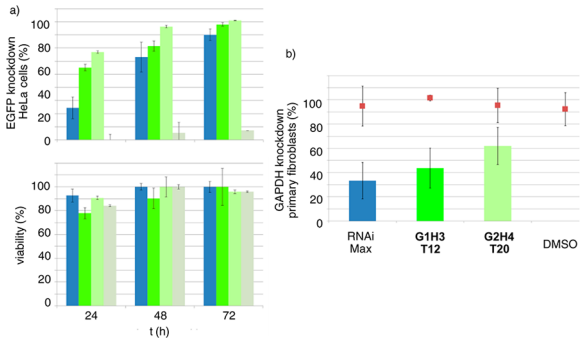

Figure 3. (a) GFP knockdown (top) and cell viability (bottom) 24, 48, and 72 h after forward transfection of EGFP-targeting siRNA in HeLa GPI-EGFP with RNAiMax (blue, bar 1), **G2H4T12** (green, bar 2), **G2H4T20** (light green, bar 3) and DMSO (gray, bar 4). (b) Decrease in GAPDH activity (bars) and cell viability (■) 72 h after forward transfection of GAPDH-targeting siRNA in human primary skin fibroblasts with RNAiMax, **G1H3T12**, **G2H4T20**, and DMSO (left to right).

indicate that siRNA uptake is much faster than with Lipofectamine RNAiMax. Therefore, it is possible that the effective amphiphile population enters the cell by a more direct route than endocytosis. The effortless library preparation and the simplicity of transfection screening certainly certify the potential of dynamic amphiphiles as controlled delivery vehicles.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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