Cellular Internalization of Therapeutic Oligonucleotides by Peptide Amphiphile Nanofibers and Nanospheres

Didem Mumcuoglu,†,# Melis Sardan Ekiz,†,# Gokhan Gunay,† Turgay Tekinay,†.§ Ayse B. Tekinay,*‡,† and Mustafa O. Guler*‡,†

†Institute of Materials Science and Nanotechnology, National Nanotechnology Research Center (UNAM), Bilkent University, Ankara 06800, Turkey
‡Life Sciences Application and Research Center and §Department of Medical Biology and Genetics, Faculty of Medicine, Gazi University, Ankara 06500, Turkey

Supporting Information

ABSTRACT: Oligonucleotides are promising drug candidates due to the exceptionally high specificity they exhibit toward their target DNA and RNA sequences. However, their poor pharmacokinetic and pharmacodynamic properties, in conjunction with problems associated with their internalization by cells, necessitates their delivery through specialized carrier systems for efficient therapy. Here, we investigate the effects of carrier morphology on the cellular internalization mechanisms of oligonucleotides by using self-assembled fibrous or spherical peptide nanostructures. Size and geometry were both found to be important parameters for the oligonucleotide internalization process; direct penetration was determined to be the major mechanism for the internalization of nanosphere carriers, whereas nanofibers were internalized by clathrin- and dynamin-dependent endocytosis pathways. We further showed that glucose conjugation to carrier nanosystems improved cellular internalization in cancer cells due to the enhanced glucose metabolism associated with oncogenesis, and the internalization of the glucose-conjugated peptide/oligonucleotide complexes was found to be dependent on glucose transporters present on the surface of the cell membrane.

KEYWORDS: oligonucleotide delivery, peptide amphiphiles, glycoconjugates, self-assembly, internalization, glucose transporters

INTRODUCTION

Therapeutic oligonucleotides such as aptamers, CpG oligonucleotides, siRNAs, and antisense oligonucleotides have received considerable attention in the pharmaceutical industry for their ability to selectively inhibit the activity of nucleic acid sequences.1−3 Unlike small molecule drugs, oligonucleotides can be designed to specifically target a broad range of biologically relevant mechanisms, which makes them highly attractive molecules for the treatment of conditions such as cardiovascular diseases, metabolic diseases, cancer, and genetic disorders.1−3 However, oligonucleotide drugs invariably suffer from high degradation rates and short half-lives in the plasma, and although their stability can be improved through chemical modification, their susceptibility to nucleolytic enzymes and rapid clearance by renal filtration and reticuloendothelial activity are nonetheless major issues for the success of their pharmaceutical applications. Consequently, many oligonucleotide-based clinical trials have suffered due to problems associated with low biodistribution and poor pharmacokinetic and pharmacodynamic properties.6,7

Drug delivery systems are able to prevent the exposure of drug molecules to biological environments, greatly increasing their stability and improving their pharmacokinetic and pharmacodynamic properties. As such, delivery vehicles are an ideal means of bypassing the limitations of oligonucleotide-based therapeutic agents. Carrier systems intended for oligonucleotide delivery should be able to protect their cargo from enzymatic degradation and immune system recognition and prevent their reticuloendothelial system clearance and rapid renal excretion. The cellular internalization of oligonucleotide carriers is a critical step in delivering oligonucleotides to their target sites in the body. Here, we investigate the effects of carrier morphology on the cellular internalization mechanisms of oligonucleotides by using self-assembled fibrous or spherical peptide nanostructures.
cleotides has been shown to be increased when cationic lipids,8 polymers,9 and nanoparticles10−12 are used as carrier systems. However, the size and morphology of a carrier system are also important for determining the mechanisms of its internalization; thus, a better understanding of these factors and their impacts is vital for the development of new delivery systems. Nevertheless, the effect of size and geometry in drug delivery has previously been described only for a limited number of materials and cell types, and further investigation is necessary to improve our basic understanding of the carrier internalization process.13,14

Glucose molecules can be utilized for targeting tumor tissue, as cancer cells tend to overexpress glucose receptors to meet their heightened metabolic demands.15 This tendency, which typically accompanies a switch from oxidative phosphorylation to glycolysis for ATP production,16 is known as the Warburg effect17 and is considered to be one of the hallmarks of cancer.18 Consequently, cancer cells consume more glucose than their healthy counterparts and must overexpress certain glucose transporters to increase their glucose uptake. The glucose transporter GLUT1, for example, is overexpressed in many cancer types, including breast cancer,19 renal cell carcinomas (RCCs),20 and colorectal cancer.21 Glucose transporters can also be targeted to monitor cancer activity and progression, as the expression of GLUT1 was found to be correlated with invasiveness of breast cancer22 and inversely correlated with prognosis of cancer.23 Other than the GLUT family, the expression of sodium-dependent glucose transporters (SGLT1 and SGLT2) was also found to be related to cancer survival in pancreatic adenocarcinoma24 and metastasis of lung cancer.25 Glucose-conjugated anticancer drugs have been shown to target tumor tissue based on increased glucose uptake in cancer cells: glufosfamide,26 paclitaxel,27 doxorubicin,28 and tamoxifen29 glycoconjugates were synthesized and tested for cancer targeting, and glucose conjugation was found to enhance the solubility and cellular uptake of these drugs. Many clinical trials also utilized glycoconjugates for targeting tumors and decreasing side effects of chemotherapeutics.30

**Figure 1.** (a) Chemical structures of amphiphilic glycopeptides Lauryl-VVAGKS(β-D-Glc)-Am (Glc-K-PA) and Lauryl-P3GKS(β-D-Glc)-Am (Glc-P-PA) and peptide amphiphiles Lauryl-VVAGK-Am (K-PA) and Lauryl-P3GK-Am (P-PA). (b) CD spectra of PA and PA/AON complexes.
RESULTS AND DISCUSSION

Peptide amphiphile molecules (K-PA and P-PA) and amphiphilic glycopeptides (Glc-K-PA and Glc-P-PA) in Figure 1 were synthesized using solid phase peptide synthesis and characterized by LC-MS (Figure S1). Glc-K-PA and K-PA were designed with a Val-Val-Ala peptide sequence followed by a hydrophilic lysine residue to allow their complexation with oligonucleotides, and this motif was replaced with three proline residues in Glc-P-PA and P-PA. The targeting molecule D-glucose (β-D-Glc) was conjugated onto the peptide sequence by using serine-linked glucose for both Glc-K-PA and Glc-P-PA. Whereas the Val-Val-Ala sequence facilitates β-sheet formation through hydrogen bonding among the peptide molecules, proline residues serve as β-sheet breakers by disrupting β-sheet secondary interactions.35,36

The self-assembly mechanisms of these systems are shown in Scheme 1. When dissolved in an aqueous solution, PA molecules self-assemble into nanostructures that contain hydrophobic amino acid residues and an alkyl tail on their interior and present their hydrophilic sections on their exterior. Glc-K-PA and K-PA molecules, which contain β-sheet forming regions and exhibit a positive charge at neutral pH, form cylindrical nanostructures as a result of their complexation with negatively charged oligonucleotides. In contrast, Glc-P-PA and P-PA possess β-sheet breaking proline residues and form spherical nanostructures upon the neutralization of their lysine side chain with oppositely charged oligonucleotides. In addition, both of the amphiphilic glycopeptides (Glc-P-PA and Glc-K-PA) self-assembled into nanostructures that present the glucose unit on their periphery, further enhancing cell–material interactions.

The secondary structure (Figure 1b), morphology (Figure 2), size (Figure S2), and zeta potentials (Table S1) of the self-assembled peptide–oligonucleotide nanostructures were studied. The secondary structures of the PAs were investigated with circular dichroism (Figure 1b), and Glc-K-PA and K-PA were found to form β-sheet structures in the presence of AONs at physiological pH, as suggested by a positive peak at around 193 nm and a negative peak at around 217 nm. In the absence of AONs, the negative peak at 217 was weakened in the K-PA solution and disappeared entirely in the Glc-K-PA solution, and a negative peak at 197 nm appeared for both PAs, indicating a random coil conformation. On the other hand, the proline-rich PAs Glc-P-PA and P-PA showed mixtures of random coil and poly proline helix (PPII) secondary structures with a strong negative band at 203 nm and weak positive band at 226 nm.37–39 The secondary structures of proline-rich peptides were preserved after their complexation with AONs.

TEM images were acquired to observe the morphologies of nanostructures in the presence of AONs (Figure 2). K-PA and Glc-K-PA molecules formed nanospheres in the presence of AONs with fiber diameters of approximately 10 nm. The presence of AONs appears to allow a complete self-assembly process with β-sheets as the predominant secondary structure, supporting our CD results. TEM images of P-PA/AON and Glc-P-PA/AON complexes, in contrast, suggest that the proline-rich PAs assemble into spherical nanostructures with diameters of 50–100 nm. The hydrodynamic sizes of P-PA/AON and Glc-P-PA/AON nanospheres were determined to be approximately 70 nm after DLS measurements (Figure S2). The complexation was also confirmed with zeta potential measurements (Table S1), as the charge of the system decreased following the addition of the negatively charged AONs onto the PAs.

The biocompatibility of nanofibrous and nanospherical systems was investigated by using the MCF-7 cell line with 24 h of culture (Figure S3). No significant decrease in cell viability was observed at 30 μM; accordingly, PA molecules were used at this concentration for in vitro experiments.

The cellular internalization of the PA/AON nanocomplexes was visualized by confocal microscopy following staining nuclei with TO-PRO-3 and actin filaments with phalloidin (red color shows FAM-AON). The PA/AON complexes were incubated for 4 h (Figure S4) and 24 h (Figure 3) to investigate their
The uptake after 24 h was also quantified with flow cytometry. Error bars show SEM; two independent experiments were repeated with $n = 3$ in each experiment. Student’s $t$ test shows statistical significance with $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Mediated endocytosis, caveolae-mediated endocytosis, and micropinocytosis), and the effect of various types of endocytosis inhibitors (amiloride, dynasore, chlorpromazine, and nystatin) on the internalization of nanofibers and nanospheres was determined through flow cytometry. Amiloride inhibits micropinocytosis, dynasore inhibits dynamin-dependent endocytosis, chlorpromazine inhibits clathrin-mediated endocytosis, and nystatin inhibits caveolae-mediated endocytosis. Nearly no inhibition in uptake was observed for nanospheres (P-PA/AON and Glc-P-PA/AON) in the presence of endocytosis inhibitors, suggesting that nanosphere internalization occurs by direct transduction through the cell membrane (Figure 5). This result is consistent with our observations during live confocal imaging, where spheres entered into cells in the first 5 min of incubation. The smaller size of nanospheres might support their rapid entry into cells. There is a small decrease in the uptake of nanospheres in amiloride- and dynasore-treated cells, which suggested that spheres could enter cells via micropinocytosis and through dynamin-dependent pathways. This rapid and diffusion-mediated internalization is a possible reason for the lack of additional uptake in glucose-conjugated nanospheres.

Nanofiber internalization, on the other hand, was inhibited by amiloride, dynasore, and chlorpromazine, suggesting that nanofiber uptake occurs by micropinocytosis, dynamin-dependent endocytosis, and clathrin-mediated endocytosis (Figure 4). Although Glc-K-PA/AON exhibited a decrease in uptake in response to amiloride treatment, no change was observed in K-PA/AON, which might due to the effect of amiloride on the inhibition of the glucose transporter mechanism. These results show that the internalization of nanofibers is energy-dependent and that they are taken up by cells mostly by endocytosis, whereas nanospheres mostly enter cells passively. This difference in internalization pathway might result from the size and geometry of the self-assembled nanostructures, as membrane wrapping times of nanoparticles were previously shown to depend on their sizes and shapes. According to our results, nanospheres (which have smaller aspect ratios) can pass easily through the cell membrane, whereas high-aspect-ratio nanofibers require energy-dependent pathways for internalization. In addition, nanofiber internalization is slower and must be mediated through endocytic vesicles, whereas nanospheres are able to diffuse through the cell membrane following their internalization. Consequently, the overall internalization of the
nanofibers at 24 h was greater than that of nanospheres, as nanofibers cannot leave the intracellular environment whereas nanospheres can diffuse back out through the cell membrane. Variances were also observed in the internalization mechanisms of glucose-conjugated PAs. In particular, the uptake of Glc-K-PA/AON was increased compared to that of K-PA/AON (Figure 3 and Figure 4) because the glucose moiety increases interactions between nanofibers and the cell membrane. The glucose transporter inhibitors phloridzin dihydrate (also known as phloridzin) and cytochalasin B were used to further analyze the effect of glucose conjugation on the internalization of nanofibrous AON delivery systems. The expression of glucose transporters GLUT1, GLUT2, GLUTS,31 GLUT12,31 and SGLT148 was previously shown in MCF-7 cells. Of these, phloridzin specifically inhibits SGLT1 and SGLT2 and minimally inhibits GLUTs,49 and cytochalasin B inhibits glucose transport50 by inhibiting GLUT transporters (especially GLUT1−4).41 We observed a decrease in the internalization of Glc-K-PA/AON following phloridzin treatment, suggesting that the uptake of the glycopeptide nanofibrous oligonucleotide delivery system is mediated by its interactions with the sodium-dependent glucose transporter SGLT1 (Figure 6). Strikingly, phloridzin decreased the uptake of Glc-K-PA/AON almost to the level of K-PA/AON, demonstrating that glucose conjugation increases cellular internalization by promoting the binding of the nanofiber to glucose transporters. Even in the presence of GLUT and SGLT1 inhibitors, other glucose receptors might still be functional and may contribute to the internalization.23,52 On the other hand, it has been previously shown that the internalization of glucose-conjugated nanoparticles may be facilitated by caveolae- and clathrin-mediated endocytosis.53−55 Although the chemical and biological inhibitors of specific cellular uptake mechanisms provide valuable tools for the study of cellular internalization mechanisms of nanomaterials, their interactions with the nanomaterials might change the overall characteristics of the nanomaterials, such as surface charge, which is an important determinant of cell uptake rate.
In this study, we utilized self-assembled peptide amphiphile nanostuctures with distinct geometries (nanofibers and nanospheres) to observe the effect of the shape of the delivery system on the cellular internalization of oligonucleotides. We observed that the size and shape of the delivery system are important parameters for the cellular internalization of the ODN cargo. Whereas nanofibers were found to enter cells directly, nanofibers were internalized by dynamin- and clathrin-mediated endocytosis. The internalization of nanospheres was a rapid process, whereas the uptake of nanofibers took more time. However, fibers were found to carry more oligonucleotides into cells under long-term observation, as nanospheres are able to diffuse out of the cells despite the rapid uptake. Peptide amphiphiles were also functionalized with glucose units to produce glycopeptide delivery systems, which enhanced AON delivery through glucose transporter-mediated internalization in the nanofiber system (but did not yield any enhancement in performance in the nanosphere system due to differences in uptake mechanisms). Thus, glucose-functionalyzed, self-assembling peptide amphiphiles can be useful for clinical oligonucleotide delivery, although future carrier designs should also consider that the effectiveness of targeting and internalization-enhancing moieties may depend on the size and morphology of the carrier.

EXPERIMENTAL SECTION

Materials. 9-Fluorenylmethoxycarbonyl (Fmoc)- and tert-butoxycarbonyl (Boc)-protected amino acids, except glycy amino acid, [α-(2,4'-dimethoxyphenyl)]Fmoc-aminomethyl[phenoxy]-acetamidonorleucyl-MBHA resin (Rink amide MBHA resin), and 2-((1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), were purchased from NovaBiochem. Fmoc-Ser[Δ]Glc(OAc)4]-OH was purchased from AAPPTec. Lauric acid and Dulbecco’s modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and passaged at cell confluency between 80 and 90% using trypsin—EDTA. In transfection experiments, PA/AON complexes were prepared in ddH2O to have a molar ratio of 30:1 and administered in serum-free medium (1% penicillin-streptomycin-containing DMEM).

Cellular Internalization of PA/AON Complexes. Thirteen millimeter glass coverslips were placed in 24-well plates, and 4 × 10^5 MCF7 cells per well were seeded in standard medium. After 24 h, medium was discarded, and PA/AON complexes were administered in serum-free medium. The AON final concentration was set to 1 μM, and the PA concentration was adjusted accordingly. After incubation, cells were washed with PBS three times, fixed with 4% paraformaldehyde, and stained with phalloidin and TO-PRO-3. Cells were visualized with a laser scanning confocal microscope (LSM 510, Zeiss).

Mechanisms of Internalization. Small molecule chemical inhibitors were used to inhibit specific internalization pathways for investigating the internalization mechanism of PA/AON complexes. Inhibitor concentrations were adjusted in a preliminary experiment, and the maximum dose that did not induce cell death was selected for testing. Thus, 8 × 10^4 MCF-7 cells/well were seeded in standard medium in 24-well plates. After 24 h, medium was removed, and 400 μL of serum-free medium was added. Then, 50 μL inhibitor solutions were administered to a final concentration of 100 μM amlodipine, 20 μM dynasore, 0.2 μg/mL of chlorpromazine, or 10 μg/mL of nystatin. In addition, final concentrations of 1000 μM chloridazon dihydrate and 50 μM cytochalasin B were used in glucose transporter inhibition studies. After 1 h of incubation, PA/AON complexes were administered to have a final concentration of 30 μM PA and 1 μM AON. Cells were incubated for another 24 h and collected in suspensions. After washing with PBS two times and centrifugation at 1500 rpm for 5 min, cells were dissolved in PBS. Cells were then trypsinized, kept on ice, and analyzed with a Guava easyCyte flow cytometer (Millipore). Cells were gated by side scatter channel (SSC) and forward scatter channel (FSC) using a nontreated control. The fluorescence intensity of FAM-AONs was measured with the green channel.
This work is partly supported by TUBITAK 114Z562 and Cinar and A.D. Ozkan for fruitful scientific discussion. D.M. and M.S.E. contributed equally to this work. The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We would like to thank M. Guler for TEM imaging, and G. Cinar and A.D. Ozkan for fruitful scientific discussion. D.M. and M.S.E. are supported by TUBITAK-BIDEB fellowships. This work is partly supported by TUBITAK 114Z562 and 113T045.

REFERENCES

ACS Applied Materials & Interfaces


(33) Yin, L. G.; Dalsin, M. C.; Sizov, A.; Reineke, T. M.; Hillmyer, M. A. Glucose-Functionalized, Serum-Stable Polymeric Micelles from the Combination of Anionic and RAFT Polymerizations. Macromolecules 2012, 45 (10), 4322–4332.


