Aptamer mediated siRNA delivery

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ABSTRACT

Nucleic acids that bind to cells and are subsequently internalized could prove to be novel delivery reagents. An anti-prostate specific membrane antigen aptamer that has previously been shown to bind to prostate tumor cells was coupled to siRNAs via a modular streptavidin bridge. The resulting conjugates could be simply added onto cells without any further preparation, and were taken up within 30 min. The siRNA-mediated inhibition of gene expression was as efficient as observed with conventional lipid-based reagents, and was dependent upon conjugation to the aptamer. These results suggest new venues for the therapeutic delivery of siRNAs and for the development of reagents that can be used to probe cellular physiology.

INTRODUCTION

One of the key problems facing the development of siRNA and other small molecule therapeutics is delivery, both systemic and to specific cell or tissue types (1,2). A variety of small molecules, lipids, peptides and proteins have previously been examined as potential delivery vehicles and vectors for nucleic acids. For example, the non-specific uptake of cholesterol labeled siRNAs has been demonstrated to be effective for delivery to cells grown in culture as well to liver, heart, kidney and lung tissue in mice (3). Similarly, a portion of the HIV-1 gp41 protein fused to a nuclear localization sequence has been demonstrated to be an effective means for the general delivery of siRNAs in tissue culture (4,5).

Peptides have also been utilized for the cell-specific delivery of siRNAs. For example, Shchiffelers *et al.* utilized PEGylated polyethyleneimine and an integrin-binding RGD peptide to direct siRNA uptake to tumor neovalsulature (6). In addition to small peptides, larger, protein-based targeting moieties have also been utilized. For example, the iron-binding protein transferrin has been used to target colloids composed of siRNA and cyclodextrin-containing polycations to transferrin receptor-expressing tumor cells (7). More recently, the tissue-specific delivery of siRNAs has been achieved using fusions between protamine and antibodies. In this system, siRNAs were bound by the basic protamine

and then targeted to tumor cells via antibodies. In one example, by using a fusion to an anti-ERbB2-specific single chain antibody, siRNAs could be targeted to tumor cells expressing the epidermal growth factor receptor ERbB2 (8).

Selected nucleic acid binding species (aptamers) are frequently viewed as non-protein based alternatives to antibodies and are thus also potential targeting agents for the delivery of siRNA cargoes (9). In addition to possessing high affinity and specificity for their targets, aptamers can be synthesized chemically and thus are attractive reagents for use in therapeutic and other applications where quality control is critical. Aptamers targeting surface antigens as well as whole cells have previously been selected (10–13). More importantly, it has recently been reported that nucleic acids selected to bind to a cell surface marker, prostate-specific membrane antigen, can themselves be internalized. These anti-PSMA aptamers have been shown to be capable of carrying a nanoparticle into cells expressing this antigen (14). Building on these findings, we wished to determine whether anti-PSMA aptamers could also deliver functional siRNA molecules to a cell. To do this we have generated an aptamer:streptavidin:siRNA conjugate. Delivery using these aptamer conjugates was found to be efficient and specific for cells expressing the PSMA antigen.

MATERIALS AND METHODS

Aptamer synthesis

The anti-PSMA aptamer (A9) was synthesized by runoff transcription from a double-stranded DNA template bearing a T7 RNA polymerase promoter. All transcription reactions (20 μl) were performed in 40 mM Tris (pH 8.0) 30 mM $MgCl_2$ and 5 mM spermidine, and contained 2 μg aptamer template, 1 μM ATP and GTP, 2 μM $2'$F dCTP and $2'$F dUTP (TriLink Biotech, CA) and 0.5 μl of the mutant T7 RNA polymerase Y639F (15). Reactions were carried out for 8 h at 37°C prior to DNase treatment for 10 min at 37°C. The modified RNA products were purified by denaturing (7 M urea) gel electrophoresis on an 8% polyacrylamide gel. After excision from the gel the RNA was eluted overnight in water and recovered by ethanol precipitation.

Aptamer biotinylation

Purified aptamer (150 nM) was oxidized in 100 mM NaOAc (pH 5.0) and 100 mM $NaIO_4$ for 90 min at room temperature

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in the dark. The reaction was stopped by the addition of an equal volume of 50% glycerol (1 ml) and the RNA was precipitated with ethanol. The oxidized RNA was then reacted with 200 pmol of freshly prepared biotin-hydrazide (Pierce, Rockford, IL) in 500 µL of 100 mM NaOAc (pH 5.0). The reaction was allowed to proceed for 3 h at room temperature and the free biotin hydrazide was removed using a NAP 5 column (Amersham Biosciences, Pittsburg, PA). The modified RNA was gel-purified, and the extent of biotinylation was confirmed by gel-shift analysis on a denaturing (7 M urea, 8% polyacrylamide) gel (16). Typical yields of biotinylated RNA were ~50–60%.

siRNA synthesis

The sequence of the anti-Lamin A/C siRNA used was 5′-GGAACUGGACUUCCAGAAGAACAUCUA (sense) and 5′-UAGAUGUUCUUCUGGAAGUCCAGUUCC (antisense). The sequence of the anti-GAPDH siRNAs used were GAPDH1, 5′-GGCGCUGCCAAGGCUGUGGGCAAGGUC (sense) and 5′-GACCUUGCCCACAGCCUUGGCAGCGUC (antisense); and GAPDH2, 5′-GGCAUUGCCCUCAACGAC-CACUUUGUC (sense) and 5′-GACAAAGUGGUCGUUGA-GGGCAAUGUC (antisense).

All non-biotinylated antisense strands and the biotinytlated GAPDH sense strands were purchased from IDT (Corriville, IA). The 5′-biotinylated sense 27mer RNA (B-RNA) and 5′-biotinylated sense 27mer RNA with a disulfide linker (BSS-RNA) were synthesized on an Expedite 8909 DNA synthesizer (Applied Biosystems, Foster City, CA) using TOM phosphoramidites. All synthesis reagents were purchased from Glen Research (Sterling, VA). The 5′-biotin was added to the RNA using a 5′-biotin phosphoramidite. The 5′-biotin with a disulfide linker was synthesized via the addition of a C6 S-S thiol modification during synthesis followed by the addition of the 5′-biotin phosphoramidite. RNA oligonucleotides were deprotected for 24 h in 3:1 NH_4OH:ethanol at room temperature. Following lyophilization, the RNA was deprotected in NMP/TEA/THF for 1.5 h at 65°C and recovered by butanol precipitation. The bioinylated RNA was purified by reverse phase HPLC on an Xterra C18 MS 2 µm column at 60°C in 100 mM TEAA (pH 7) with a gradient of 5–35% acetonitrile over 30 min. After lyophilization the biotinylated RNA was further purified on a denaturing (7 M urea, 10% polyacrylamide) gel. RNA was eluted from the gel in water and recovered by ethanol precipitation.

Aptamer and siRNA conjugation to streptavidin

Sense and antisense strands of siRNA (40 µM) were annealed in 100 mM KOAc, 30 mM HEPES–KOH (pH 7.4) and 2 mM MgOAc. Samples were heated to 94°C for 30 s and then cooled stepwise in 30 s increments to 72, 52 32, 22, 12 and 4°C. Aptamer:siRNA complexes were assembled by mixing 200 pmol double-stranded siRNA and 200 pmol biotin-aptamer conjugate with 100 pmol of streptavidin (Promega, Madison, WI). The complex was allowed to equilibrate for a minimum of 10 min and was then stored on ice until used. Aptamer:streptavidin:siRNA conjugates were added directly to media at final concentration of 22.5 nM.

Transfections

LNCAP cells (ATCC CRL-1740) and PC3 cells (ATCC CRL-1435) were purchased from ATCC. LNCaP cells were incubated in RPMI 1640 (Gibco) media supplemented with 2 mM L-glutamine, 1.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% FBS. PC3 cells were incubated in HAM's F12K media (ATCC) media supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate and 10% FBS. For transfections and conjugate delivery, cells were grown to 70% confluence in 12 well plates.

For aptamer-mediated siRNA delivery, aptamer:streptavidin:siRNA conjugates were added directly to the media (500 µl) at a final concentration of 45 nM siRNA or 22.5 nM conjugate (average of 2 siRNAs/conjugate). Cells were harvested 72 h after the addition of the conjugate and monitored for gene expression inhibition by real-time PCR.

Alternatively, Oligofectamine (2 µl; Invitrogen, Carlsbad, CA) was mixed with 100 µl of media and added to 100 µl of media containing 22.5 pmol aptamer:streptavidin:siRNA conjugate. After incubating for 10 min at room temperature, the mixture was diluted to 1 ml with media (without FBS) and 300 µl was added to cells. Transfections were incubated for 4 h at 37°C after which the media was removed and replaced with 400 µl of fresh media containing 10% FBS. Cells were harvested 72 h after the addition of the conjugate and monitored for gene expression inhibition by real-time PCR.

Real-time PCR

Total cellular mRNA from each well was extracted with Trizol (400 µl; Invitrogen, Carlsbad, CA). After incubation for 5 min, the samples were diluted with 300 µl water and extracted with phenol/chloroform/isoamylalcohol (700 µl). The RNA was recovered from the aqueous phase by ethanol precipitation. The pelleted RNA was resuspended in 20 µl of water.

Lamin A/C expression was analyzed by real-time PCR. The mRNA for GAPDH served as an endogenous control. For experiments in which both lamin A/C and GAPDH were monitored (Figure 4), 18S rRNA served as the endogeneous control. The primers and probes used were as follows: Lamin A/C forward primer, 5′-ATGATCGCTTG-GCGGTCTAC; lamin A/C reverse primer, 5′-GCCCTGC-GTTCTCCGTTT; lamin A/C probe 5′-FAM-TCGACC-GTGTGCGCTCGCTG-TAMRA; GAPDH forward primer, 5′-GAAGGTGAAGGTCGGAGT; GAPDH reverse primer, 5′-GAAGATGGTGATGGGATTTC; GAPDH probe, 5′-Joe-CAAGCTTCCCGTTCTCAGCC-BHQ; 18S forward primer, 5′-TTGACTCAACACGGGAAACCT; 18S reverse primer, 5′-AGAAAGAGCTATCAATCTGTCAATCCT; 18S probe, 5′-TET-ACCCGGCCCGGACACGGA-TAMRA; INFB forward, 5′-CCAACAAGTGTCTCCTCCAAATT; INFB reverse, 5′-GTAGGAATCCAAGCAAGTTGTAGCT; INFB probe, 5′-FAM-TGTTGTGCTTCTCCACTACAGC-TCTTTCCA-BHQ.

Extracted RNA (~200 ng) was reverse-transcribed in a 10 µl reaction containing 1 µl 10× first strand synthesis buffer (Ambion, Austin, TX), 0.2 mM dNTPs, 0.4 µl RNasin Plus (Promega, Madison WI), 0.4 µl MMLV RT (Ambion, Austin, TX) and 1 µM reverse primers. Reactions were carried out at

42°C for 30 min followed by 10 min at 94°C. Real-time PCR (25 μl) contained 10 mM Tris (pH 8.3) 50 mM KCl, 1.5 mM MgCl$_2$, 0.25 U Taq (NEB, Cambridge, MA), 450 nM forward and reverse primers and 125 nM probe, and 1 μl of the reverse transcription reaction.

All reactions were conducted on either an ABI 7900 or ABI 7300 real-time PCR machine (Applied Biosystems, Foster City, CA) using the following cycling parameters, 95°C for 2 min, followed by 45 cycles of 95°C for 15 s, 60°C for 1 min. The amounts of lamin A/C mRNA in treated and control cells were determined in comparison with the endogeneous GAPDH control mRNA. All data represent the average of three replicates except for those in Figure 5, which are the average of two replicates. Error bars represent ±1 SD from the mean.

RESULTS

Design of aptamer:siRNA conjugates

The exogenous delivery of 21mer and more recently 27mer RNA duplexes has been shown to be a highly effective means of reducing gene expression in eukaryotic cells (17,18). In addition, inhibition of expression has been mediated by the delivery of short hairpin RNAs to cells (19).

Sequence extensions of siRNAs have previously been found to compromise the processing and function of these molecules. Therefore, it was possible that extending siRNA sequences in order to couple them with aptamers might have impeded siRNA function. In contrast, some non-nucleotide modifications of siRNAs have been shown to have little effect on function. For example, a fluorescent reporter appended to the 5′ end of the sense strand of a 21mer siRNA had little or no effect on the ability of the siRNA to inhibit gene expression (20,21). Similarly, fluorescent modifications of a 27mer siRNA still allowed for processing by Dicer and for inhibition of gene expression *in vivo* (18).

Bearing this in mind, we chose to utilize 27mer siRNAs in our design. In addition, we chose to couple the aptamers and siRNAs using a modular strategy in which the RNAs were first biotinylated and then non-covalently joined to one another via the protein streptavidin (Figure 1). Since it was possible that the bulky streptavidin substituent might inhibit siRNA processing *in vivo*, we designed two different biotinylated sense strands, one containing a 5′-biotin (Figure 1; B-siRNA) and one containing a disulfide linkage to biotin (Figure 1; BSS-siRNA). The disulfide linkage in the latter construct should be cleaved upon entering the reducing environment of the cell, releasing the siRNA for processing or fucntion. RNA conjugation to streptavidin and subsequent cleavage of the BSS-siRNAs were confirmed by gel-shift analysis (Figure 2). Only the biotinylated siRNA constructs were shifted in the presence of streptavidin, and only the disulfide-containing BSS-siRNA was released from streptavidin upon treatment with DTT.

Previous studies of nanoparticle delivery had relied upon an anti-PSMA aptamer known as A10 (14). However, another aptamer (A9) appeared to bind more tightly to PSMA (22), and thus we chose to use it for the development of aptamer delivery reagents. The anti-PSMA aptamer A9 delivery
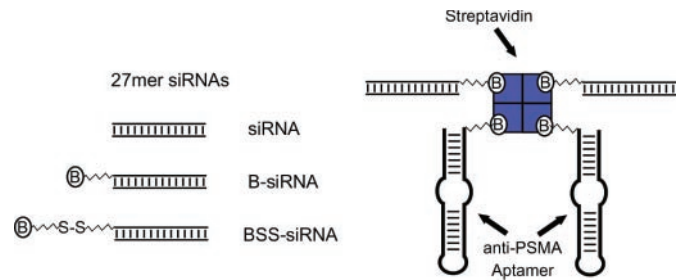

Figure 1. Design of aptamer:siRNA conjugates. 27mer siRNAs (siRNA) were synthesized as either biotin conjugates (B-siRNA), or with a cleavable biotin linker (BSS-siRNA). Conjugates were assembled by incubation with streptavidin and on average each conjugate should have contained two siRNAs and two anti-PSMA aptamers.
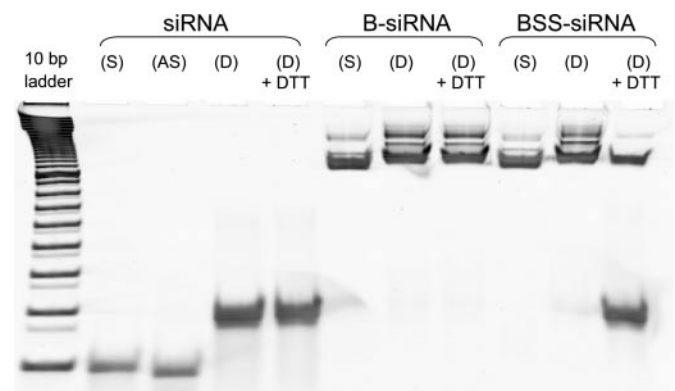

Figure 2. Gel-shift analysis of siRNA:streptavidin conjugates. In the presence of streptavidin, only the biotinylated siRNAs are shifted. The addition of DTT results in the reduction of the disulfide bond in the BSS-siRNA construct, releasing the 27mer duplex from streptavidin.

reagent was synthesized by runoff transcription and was oxidized at its 3′ end using sodium periodate. The resultant dialdehyde was biotinylated by incubation with biotin-hydrazide. A 27mer siRNA was designed based on the 21mer sequence used by Elbashir *et al.* (17) and was used as a 'cargo' for the internalizing, anti-PSMA aptamer. As a positive control, the 27mer was transfected into cells with Oligofectamine and gene inhibition was analyzed by both real-time PCR and western blot analysis (data not shown). Similarly, when the B-siRNA or BSS-siRNA were transfected into cells, the observed level of inhibition was similar to that seen with the unmodified siRNA, confirming that 5′ modification of the sense strand did not significantly affect siRNA function.

The siRNAs and biotinylated siRNAs (sense strands) were synthesized using standard phosphoramidite chemistry. The aptamer:streptavidin:siRNA conjugates were generated by incubating the biotinylated anti-lamin A/C siRNA, biotinylated anti-PSMA aptamer and streptavidin in a 2:2:1 ratio for 10 min.

Cellular delivery of aptamer:siRNA conjugates

Aptamer:siRNA conjugates at a final concentration of 22.5 nM (45 nM siRNA, average of 2 siRNAs/conjugate) were added directly to media containing (PSMA-positive) LNCaP cells. It should be noted that this procedure
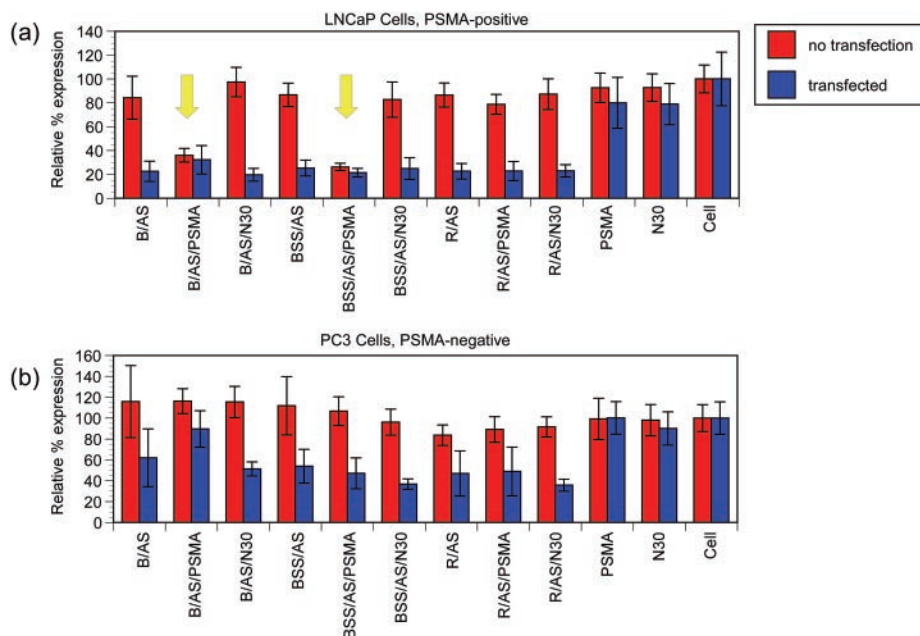
Figure 3. Aptamer-mediated inhibition of expression of lamin A/C. LNCaP cells (**a**) or PC3 cells (**b**) were incubated with streptavidin conjugates containing (i) either no aptamer, a biotinylated anti-PSMA aptamer (PSMA) or a biotinylated random RNA pool (N30) and (ii) either a biotinylated siRNA (B-siRNA), a cleavable biotinylated siRNA (BSS-siRNA) or a non-biotinylated siRNA (siRNA). All samples contained streptavidin. Inhibition of gene expression was only observed for PSMA-positive LNCaP cells when the biotinylated PSMA aptamer and biotinylated siRNAs were connected to one another via streptavidin (yellow arrows). Positive control transfections were carried with same conjugates and Oligofectamine (blue). Data represent the average of three replicates. Error bars represent ±1 SD from the mean.

demonstrates one of the potential advantages of aptamers as delivery reagents: no additional components or preparations were necessary to initiate experiments to assess the inhibition of gene expression. After 72 h, treated cells were harvested, the RNA was extracted with Triazol and the extent of gene inhibition was determined by real-time PCR.

While all of the Oligofectamine transfected samples containing either biotinylated or non-biotinylated siRNA resulted in efficient inhibition of expression (Figure 3a, blue), in the absence of Oligofectamine, significant inhibition of expression was only observed when both the biotinylated siRNA and biotinylated anti-PSMA aptamer were present and joined to one another (Figure 3a, yellow arrows). No inhibition of expression was observed in the absence of an aptamer targeting domain, when the biotinylted aptamer was replaced with a biotinylated random sequence RNA (Figure 3a; N30), or when streptavidin was not included in the reaction mixture (data not shown). Surprisingly, the presence of the bulky streptavidin molecule seemed to have little or no effect on the ability of the attached siRNAs to function; Oligofectamine and aptamer-mediated transfections included identical amounts of siRNA and gave comparable results. The specificity of the anti-PSMA aptamer was further confirmed by carrying out a similar assay with the same anti-lamin A/C siRNA conjugates and PSMA-negative PC3 cells. In these experiments, only cells transfected via Oligofectamine showed inhibition of gene expression (Figure 3b).

Similar results were observed with LNCaP cells when two different siRNAs were used to target GAPDH mRNAs instead of lamin A/C mRNAs (Figure 4; GAPDH1 and GAPDH2). Importantly, the siRNAs directed against GAPDH did not decrease the level of lamin A/C expression, while
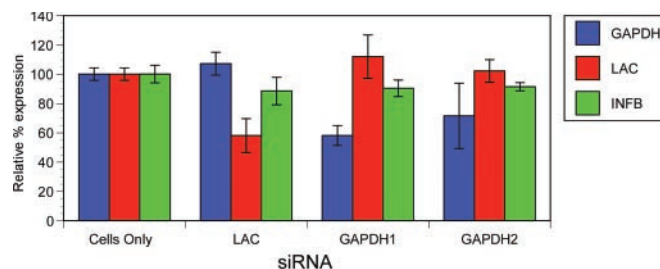

Figure 4. Aptamer-mediated siRNA delivery to LNCaP cells. Cells were incubated with conjugates composed of streptavidin, the anti-PSMA aptamer and either an anti-lamin A/C siRNA (LAC) or one of two anti-GAPDH siRNAs (GAPDH1, GAPDH2) for 72 h prior to RNA extraction. Gene expression for lamin A/C, GAPDH and interferon β (INFB) was assayed by real-time PCR.

siRNAs directed against lamin A/C did not decrease the level of GAPDH expression. In addition, because siRNAs can potentially activate the cellular immune response (18,23,24)? cells were also monitored for the expression of interferon β mRNA (INFB). As shown in Figure 4, exposure to the aptamer conjugates for up to 72 h does not result in an interferon response. As a positive control, cells were exposed to LPS, which resulted in a three order of magnitude increase in interferon mRNA expression (data not shown).

Aptamers mediate fast inhibition of gene expression

PSMA endoctyosis in LNCaP cells is thought to proceed predominantly via clathrin-coated pits. The rate of internalization has previously been measured using antibodies directed against PSMA and with another anti-PSMA aptamer (A10),
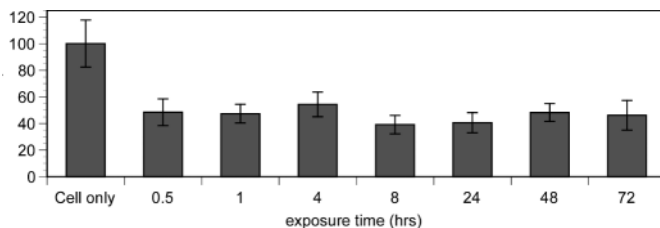
Figure 5. Effect of incubation time on aptamer-mediated gene inhibition by siRNA. LNCaP cells were incubated with aptamer:siRNA conjugates targeting lamin A/C mRNA for the times indicated. After incubation, the aptamer:siRNA conjugates were removed by changing the media. All cells were harvested at 72 h post-transfection.

which binds PSMA with slightly lower affinity than the A9 anti-PSMA aptamer that we have used [a 20 nM K_d for A10 versus 2 nM for A9 (22)]. For both types of targeting agents, internalization was shown to take place within hours of binding (14,25). These results suggested that efficient inhibition of gene expression might be quickly achieved following only a brief exposure to aptamer:siRNA conjugates. To test this hypothesis, we performed a series of reactions in which we varied the incubation time with aptamer:siRNA conjugates from 30 min to 72 h. After incubation the media was removed and replaced with fresh media containing no conjugate. All treated cells were then harvested after 72 h and the extent of gene expression inhibition was determined by real-time PCR. As shown in Figure 5, inhibition was observed after only a 30 min exposure and no increase in the level of inhibition was observed when the exposure time was increased. This rate of internalization is consistent with those previously observed. These results also suggest a reason why aptamer:siRNA conjugates were as effective as Oligofectamine amalgams. Even though the aptamers and siRNAs were essentially 'naked' to serum nucleases in the tissue culture media, they were taken up into the cell so quickly that no real protection (such as being ensconced in Oligofectamine) seemed to be necessary.

DISCUSSION

The development of nucleic acid reagents that can be used to internalize biomolecular cargoes is not only novel, but also extremely practical. Unlike lipid amalgams, nucleic acids can target specific cell types. Unlike peptides, conjugation chemistry between nucleic acid partners can potentially involve either simple hybridization or co-synthesis. While the simplification of conjugation chemistry may not initially seem to be a great advantage, quality control is one of the most time- and labor-intensive portions of pharmaceutical production, and any simplification at this step should result in great savings throughout the process.

We have used streptavdin as a non-covalent linker primarily because of its ease of use and modularity. While streptavidin conjugates and fusion proteins have previously been used as imaging agents (26,27) and for the targeted delivery of therapeutics in whole animals (28,29) as well as in clinical trials (30), such conjugates have also been shown to elicit an immune response (31). Therefore, immunogenicity may limit the use of streptavidin as a delivery vehicle for siRNAs, at least until less immunogenic streptavidin variants can be

identified (32). It may also be possible to conjugate aptamers to siRNAs via linkers other than streptavidin, including by simple co-synthesis or hybridization.

Nonetheless, there are some advantages to modular conjugation via a streptavidin bridge that we have developed here. For example, the use of streptavidin may provide a convenient platform for further dissecting siRNA and microRNA processing machinery *in vivo*. It seems likely that the siRNAs must be removed from streptavidin prior to entry into RNA-initiated silencing complexes. By varying the linker length and chemistry between the biotin moiety and the siRNA, it may be possible to probe the initial processing steps for siRNAs separate from downstream assembly steps and gene regulation.

Importantly, since streptavidin is a tetramer and has four functional binding sites for biotin, internalizing and therapeutic nucleic acids can be mixed and matched. This will be especially important for the future development of siRNA therapeutics and reagents. By using the streptavidin bridge and a judicious mixture of siRNAs, the uniform delivery of multiple different siRNA sequences to a given cell can be ensured. Moreover, it seems that the development of simple transfection reagents for use in systems biology applications may now be a realistic possibility. Aptamers could be pre-appended to streptavidin, and these conjugates in turn mixed with any set or library of biotinylated siRNAs in order to make a one-step, ready-to-use transfection reagent. The simplicity of such a method relative to current procedures (such as frequently irreproducible transfections with lipid reagents or series of complex packaging steps with viral vectors) would greatly expand the use of large siRNA sets in many molecular biology applications.

While our current results are limited to LNCaP cells which naturally express PSMA, the internalization of an anti-PSMA antibody has previously been observed for a number of other cell lines that have been engineered to stably or transiently express PSMA (33,34). Thus it seems likely that the aptamer-mediated delivery of siRNA may prove useful for any cell type that normally expresses PSMA or that can be engineered to express PSMA. Moreover, since aptamers can be selected to bind a wide variety of cells (10–13,35), we envision that additional, cell-specific transfection reagents will be developed and that the method described here for prostate tumor cells may become a general strategy for the specific delivery of siRNA to almost any cell type.

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