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Autotransfecting Short Interfering RNA through Facile Covalent Polymer Escorts

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Supporting Information

ABSTRACT: Short interfering ribonucleic acids (siRNAs) are important agents for RNA interference (RNAi) that have proven useful in gene function studies and therapeutic applications. However, the efficacy of exogenous siRNAs for gene knockdown remains hampered by their susceptibility to cellular nucleases and impermeability to cell membranes. We report here new covalent polymer-escort siRNA constructs that address both of these constraints simultaneously. By simple postsynthetic click conjugation of polymers to the passenger strand of an siRNA duplex followed by annealing with the complementary guide strand, we obtained siRNA in which one strand includes terminal polymer escorts. The polymer escorts both confer protection against nucleases and facilitate cellular internalization of the siRNA. These autotransfecting polymer-escort siRNAs are viable in RNAi and effective in knocking down reporter and endogenous genes.

RNA interference (RNAi) has altered the landscape of both basic research to examine gene function pathways[1] and therapeutic paradigms.[2] RNAi may be initiated by delivery of exogenous short interfering RNA (siRNA) to cells. These are typically delivered in short 21−23-mer duplexes and other forms that are processed by the cellular machinery.[1,3] The duplexes interact with the cellular RNA-induced silencing complex (RISC), which eventually uses one strand from the duplex, termed the guide or antisense strand, to silence a target mRNA. Barriers to the use of exogenous siRNA duplexes are their susceptibility to degradation and their cell impermeability.[4] Although chemical modifications can overcome the lability of the native sugar−phosphate backbone toward hydrolysis and nucleases,[5] cell permeability still presents a significant challenge.[2a,3]

Delivery of exogenous siRNA has therefore required complexation with transfection reagents that enhance cell permeability and provide additional protection to the RNA duplex from nuclease degradation.[6] Nonviral transfection reagents have relied on the formation of a nonspecific polyplex between cationic lipid nanoparticles[6a,7] or polymers[7b,8] and the anionic siRNA. Although widely studied for siRNA delivery, these materials have several practical limitations, such as their reliance on ionic interactions to prepare the polyplex, which can be destabilized during circulation or in media.[6b,9]

Alternative methods for siRNA delivery rely on direct covalent modifications of the 5′- and/or 3′-terminus of siRNA with lipid groups,[10] small molecules such as biotin and folate,[11] peptides,[12] nanoparticles,[6c,13] carbon nanotubes,[14] nanostructured DNA,[15] or poly(ethylene glycol) (PEG).[16] Modification of siRNA with linear PEG[17] or brush PEG[18] has been accomplished using disulfide formation or Michael-type addition between thiol and maleimide groups. While the disulfide linkage allows for release of the siRNA duplex following cellular internalization, the generation of redox-sensitive thiols and disulfides that can undergo undesired side reactions or premature degradation poses challenges in the synthesis and purification of the polymer−siRNA conjugates. These conjugates have enhanced stability, though some require additional transfection agent,[18a] limiting their overall utility as a stand-alone siRNA delivery system.

Here we describe straightforward access to siRNA polymer constructs that are stand-alone siRNA delivery vehicles. In the architecture described here, the sense or passenger strand is conjugated to the polymer, with the guide strand simply hybridized to the passenger−polymer conjugate. We reasoned that a suitable polymer directly conjugated to just the passenger strand of the siRNA duplex could confer both desirable properties of nuclease resistance and cell permeability to the ensemble. These stabilized and autotransfecting siRNAs would potentially permit the guide strand to effectuate an RNAi response.

In evaluating rapid and efficient methods for conjugating the RNA,[19] we chose the copper-catalyzed azide−alkyne cycloaddition (CuAAC) or "click" reaction.[20] This reaction has seen widespread use in the preparation of a diverse range of bioconjugates, including protein−polymer hybrids[21] and DNA and RNA conjugates.[19,22] Click conjugation of small molecules and lipids to siRNA have been reported,[23] and the resultant triazole linkage is biocompatible.[24] We therefore considered efficient click conjugation of polymers to both 5′- and 3′-termini of an RNA (Figure 1). An extended RNA sequence that included the sense or passenger strand of an siRNA duplex (**p-RNA**) and

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alkyne groups at both termini was synthesized using standard commercially available reagents.

To generate a series of well-defined azide-terminated polymers for click conjugation to the bisalkyne-functionalized **p-RNA**, we used activators generated by electron transfer atom transfer radical polymerization (AGET ATRP).[22b,26] We probed the abilities of three biocompatible polymers to confer both nuclease resistance and cell permeability to siRNA. The polymers were PEG−methacrylate−$pOEOMA_{475}$ ($\mathbf{P^M}$); $pOEOMA_{300}$-*co*-MEO_2MA ($\mathbf{P^T}$), a temperature-responsive copolymer that is more hydrophobic than $\mathbf{P^M}$ (the lower critical solution temperature for $\mathbf{P^T}$ in water is ca. 39 °C); and $pOEOMA_{475}$-*co*-DMAEMA ($\mathbf{P^N}$), a copolymer containing amino groups that can be cationic at neutral pH. These monoazido-functional polymers all had number-average molecular weights (M_n) of ∼21,000 and narrow molecular weight distributions ($M_w/M_n < 1.2$) [Figure S1 in the Supporting Information (SI)]. Polymers with similar compositions have been successfully used in unconjugated polyplexes and mixtures with siRNA for delivery and have favorable cytocompatibility properties.[8c,27]

These azido-terminated polymers and bisalkyne-terminated **p-RNA** were conjugated under conditions optimized for oligonucleotide click reactions.[19,22b,f,25] A 20-fold molar excess of azido-terminated polymer relative to RNA was used to ensure click conjugation of both termini without RNA degradation. The pseudoligandless reaction was performed in Tris buffer (pH 7.5) with 0.6% acetonitrile as a cosolvent. Following a 90 min reaction, a simple purification step using a 30,000 molecular weight cutoff centrifugal filter device removed the catalyst and excess unreacted polymers as previously shown,[22f] providing $\mathbf{P^xEp\text{-}RNA}$ (x = M, T, N) (Figure 1A). This represents the first report of click conjugation of polymers to RNA. Following bisconjugation of the polymers to the **p-RNA** termini, the complementary 21-mer guide strand (**g-RNA**) was annealed, yielding the three polymer-escort duplex siRNA conjugates ($\mathbf{P^xEp\text{-}siRNA}$, x = M, T, N).

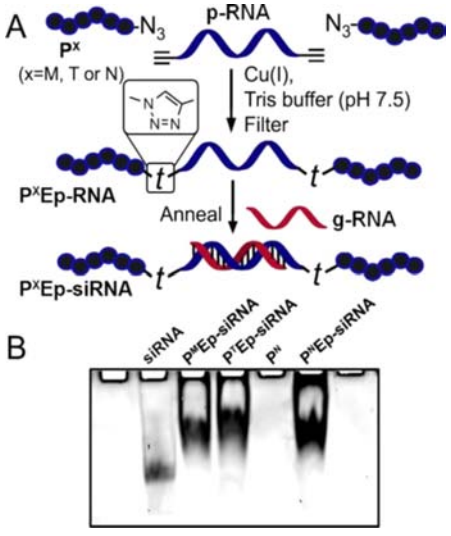

Figure 1. Synthesis of $\mathbf{P^xEp\text{-}siRNA}$. (A) The passenger strand with bisalkyne termination, **p-RNA**, was conjugated to azido-functionalized polymers $\mathbf{P^x}$ (x = M, T, N) and annealed to the guide strand, **g-RNA**, to form $\mathbf{P^xEp\text{-}siRNA}$ (x = M, T, N). (B) A nondenaturing polyacrylamide gel (Tris, pH 8.5) and EtBr staining confirmed that siRNA as well as $\mathbf{P^xEp\text{-}siRNA}$ included duplex RNA; polymer $\mathbf{P^N}$ alone was not stained.

To confirm the presence of both strands and the integrity of the complexes, we visualized the annealed polymer conjugates by ethidium bromide (EtBr) staining on a nondenaturing polyacrylamide gel (Figure 1B). The polymer alone was not stained by EtBr, as exemplified by polymer $\mathbf{P^N}$ (lane P^N), whereas the siRNA duplex alone and the polymer-escort conjugates were stained. The conjugates also displayed retarded migration through the gel. The gel used Tris borate buffer at pH 8.5, which was well above the pK_a of PDMAEMA (∼pH 7.4) to ensure that even $\mathbf{P^NEp\text{-}RNA}$ would enter the gel. No such retarded mobility was observed when the siRNA duplexes were simply mixed with but not conjugated to the polymers (Figure S2), indicating that polyplex formation was unlikely. The uniform band of the visualized RNA conjugated in $\mathbf{P^xEp\text{-}siRNA}$ suggested that the RNA was bisconjugated with flanking polymer escorts and homogeneous rather than a mixture of mono- and bisconjugated RNA. Further, no free siRNA band was observed, indicating that the click conjugation was efficient and that high-purity conjugates were prepared.

The covalent polymer modification at both the 5′- and 3′-termini of the **p-RNA** was expected to render it highly resistant to exonuclease. However, whether this resistance would be conferred to the **g-RNA** strand that is simply hybridized within the construct had to be evaluated. We therefore incubated the three **PEp-siRNA**s with ribonuclease A (RNaseA), which can rapidly degrade both single- and double-stranded RNA. We found that while siRNA (duplex) was almost completely degraded by RNaseA, all of the **PEp-siRNA**s remained intact even after 2 h (Figure 2). This result suggests that the flanking

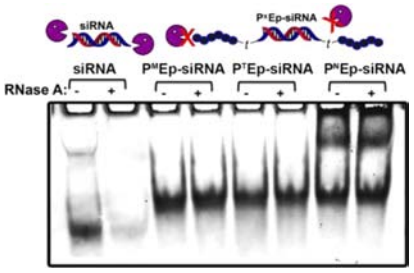

Figure 2. Nuclease stabilty of $\mathbf{P^xEp\text{-}siRNA}$ (x = M,T, N) compared with unmodified siRNA. Samples were incubated with (+) and without (−) RNaseA for 2 h, run on a nondenaturing polyacrylamide gel, and stained with EtBr.

escort architecture can be used to sequester and protect not only the directly conjugated **p-RNA** strand but also the hybridized **g-RNA** sequence of the siRNA duplex from nuclease-mediated degradation.

The protective power of even just one covalent polymer escort also conferred **PEp-siRNA** with resistance to in vitro processing by the endonuclease dicer (Figure S3). Dicer processing is required to convert long RNA duplexes into canonical 21-mer duplexes with overhangs to help their loading into RISC. However, dicer processing is not required for cleavage of the target mRNA.[28] Cleavage of the target mRNA was mediated by argonaute, for which the 21-mer **g-RNA** within the **PEp-siRNA** would be suitable and sufficient if the **g-RNA** was accessible to RISC loading.

Thus, while the **PEp-siRNA**s were stable toward RNaseA and dicer in vitro, dissociation of **g-RNA** from the **PEp-siRNA** would be necessary in vivo for entry into RISC to induce an RNAi response. We therefore determined the efficiency of the **PEp-**

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siRNA conjugates in RNAi-mediated knockdown of a target mRNA (Figure 3). *Drosophila* S2 cells transfected with firefly

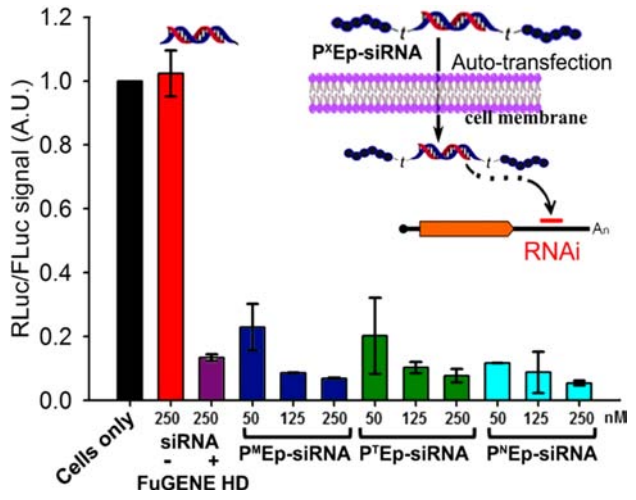

Figure 3. Silencing activity of **P^{x}Ep-siRNA**. The graph shows the RLuc signal relative to the control FLuc signal. Following transfection of the reporter plasmids, S2 cells were treated with siRNA without (−) or with (+) FuGENE HD for transfection or 50, 125, or 250 nM **P^{x}Ep-siRNA**s. The luciferase activity was measured after 24 h; "cells only" was a control well without siRNA. Error bars represent standard deviations from three separate experiments.

luciferase (FLuc) and *Renilla* luciferase (RLuc) plasmids allowed the evaluation of RNAi-mediated knockdown in a dual-luciferase assay. To assess the **PEp-siRNA**s, the hybridized **g-RNA** was chosen on the basis of a published report on targeting of the 3′-untranslated region (3′-UTR) of RLuc mRNA.[22b] FLuc provides an internal control for transfection efficiency and protein production against which the knockdown of the RLuc signal can be compared. Following initial transfection of the Fluc and RLuc reporter plasmids with FuGENE HD, a control duplex siRNA (30 pmol; 250 nM) was transfected after 3 h using an additional amount of FuGENE HD. This resulted in knockdown of the RLuc signal (Figure 3, purple bar) measured after 24 h. In the absence of the additional FuGENE HD, the effect of the control siRNA was negligible (Figure 3, red bar), indicating that after initial transfection of the plasmids, no residual FuGENE HD remained and little nonspecific internalization of siRNA occurred. In stark contrast, all three **PEp-siRNA**s required no transfection reagent and resulted in effective knockdowns. Each **PEp-siRNA** was tested at concentrations of 50, 125, and 250 nM (corresponding to 6, 15, and 30 pmol of RNA, respectively) and evaluated 24 h after addition. Each **PEp-siRNA** resulted in greater knockdown activity than an equivalent or even half the amount of siRNA delivered through the transfection polyplex. Knockdown of the RLuc signal comparable to that with transfected siRNA could be achieved with just one-fifth the amount of RNA (6 pmol; 50 nM) in **P^{N}Ep-siRNA**, which incorporates a positively charged DMAEMA in the copolymer segment (see Figure S1C for chemical composition).

The success of the covalent polymer escorts for auto-internalization and release of the **g-RNA** that was effective in RNAi prompted us to test this architecture for the knockdown of an endogenous gene in human embryonic kidney 293 (HEK293) cells. Lymphocyte-specific protein tyrosine kinase (Lck) is a member of the Src kinase family that is important in signal transduction events, particularly in T-cells.[29] As **P^{N}Ep-siRNA** was the most effective in the S2 cells, we used the **Lck-P^{N}Ep-siRNA** construct (see Table S1 in the SI for sequences). This was simply added to the medium with HEK293 cells. Compared with untreated cells, we observed specific and reproducible knockdown of Lck protein with **Lck-P^{N}Ep-siRNA** without any transfection agent (Figure 4). In contrast, actin, serving as an

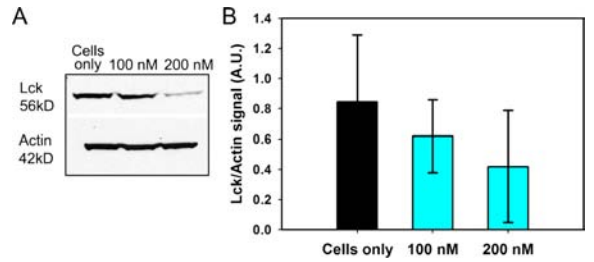

Figure 4. Knockdown of an endogenous gene using **P^{N}Ep-siRNA**. (A) Western blot analysis of Lck knockdown in Hek293 cells that were plated (cells only) or transfected with 100 or 200 nM **Lck-P^{N}Ep-siRNA**. After 48 h, cell lysates were analyzed for total Lck and actin as a loading control. (B) Graph of densitometric quantitation with the Lck signal normalized to actin. Error bars represent standard deviations from three independent experiments.

internal control for gene expression, was unaffected, as assayed by Western blotting (Figure 4A). Quantitation of the relative protein expression levels indicated that the polymer-escort siRNA architecture is indeed viable in human cells to knock down expression of an endogenous gene.

Given the viability of these autotransfecting siRNAs in RNAi across different cell types, we envision a variety of improvements to the architecture to boost its efficacy and investigate the mechanisms related to internalization and action. Constructs that include a 5′-phosphate and other chemical modifications for added stability of the **g-RNA** strand while enhancing release from the duplex as well as modifications that enhance the RNA polymer synergy are being designed for further studies.

In conclusion, we have demonstrated that the flanking **PEp-siRNA** "escort" architecture provides a robust RNAi agent. These **PEp-siRNA** hybrids were obtained readily and efficiently by a simple postsynthetic click reaction, filtration, and annealing. The **PEp-siRNA** architecture simultaneously confers nuclease resistance and cell permeability to the RNA. While nonspecific polyplexes with RNA or even disulfide-linked polymer or nanostructure siRNA conjugates in the reducing cellular environment release the siRNA duplex, the polymer escorts likely remain covalently conjugated to the passenger strand via the triazole linkage. Thus, rather than releasing the entire duplex siRNA upon internalization, **PEp-siRNA** retains the ability to deliver only the hybridized guide-strand RNA as the payload for effective RNA silencing. This has significant implications for RNAi, as it simplifies the design and could avoid off-target effects that may arise from the passenger strand. This siRNA architecture that uses polymer escorts is highly amenable to customization and inclusion of other polymer-associated moieties for multimodal delivery of therapeutic agents.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials and methods, including polymer and RNA synthesis and compositions. 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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