

Forum Contribution

Novel siRNA-based molecular beacons for dual imaging and therapy

Short interfering RNAs (siRNAs) have become a mainstream tool reliably used to study and silence protein expression. We offer a proof-of-principle demonstration that siRNAs may be modified into a siRNA-based molecular beacon that activates upon binding to sequence-specific mRNA in cells while mediating RNA interference. We successfully demonstrate detection and knockdown of telomerase expression in human breast cancer cells. This probe provides a novel look at siRNA target validation that is not currently possible in live cells and holds promising potential in biological applications for disease detection and therapy based on mRNA expression, such as a telomerase-targeted siRNA probe in cancer.

Introduction

Small interfering RNAs (siRNAs) are composed of complementary RNA strands of 21–23 nucleotides (nts) in length with sense and antisense orientation targeting the mRNA of interest [1, 2]. Shown as powerful methods of gene silencing with high specificity, RNA interference (RNAi) is mediated by these siRNAs, which direct mRNA target cleavage based on Watson-Crick base pairing of the guide strand (antisense) in the RNA-induced silencing complex (RISC) [3–5].

The discovery of RNAi has generated tremendous interest by both biotechnology and pharmaceutical companies to evaluate siRNA applications ranging from animal knockouts [6] to treatment of human diseases [7], such as age-related macular degeneration [6] or HIV by targeting viral replication mechanisms [7]. Presently, siRNA has solely been used therapeutically, while siRNA imaging has been limited to end-labeling with fluorophores to monitor delivery of siRNA into cells. Our hope is to leverage RNAi towards diagnostic and monitoring applications of mRNA by utilizing molecular beacons (MBs).

MBs are highly sensitive and specific fluorescent nucleic acid probes used for monitoring the expression of nucleic acids at the single-cell level. Consisting of four key components – a loop, stem (typically 4–6 bases), a donor and acceptor fluorophore, MBs

rely on a fluorescence resonant energy transfer (FRET) fluorophore pair to generate a signal upon binding to complementary sequences [8]. Studies have shown that MBs can discriminate between targets that differ by only a single nucleotide [9]. Used in a wide range of biomedical applications, MBs offer the capacity to monitor gene expression in single living cells that more traditional forms of analysis do not [10].

We report a proof-of-principle demonstration of siRNAs modified into an siRNA-based MB. A potential probe for cancer detection and therapy was synthesized by creating an siRNA-based MB that detects and targets the hTR sequence for telomerase [11]. Expressed in over 85% of all tumors [12], telomerase is essential for tumorigenesis by preventing cell death through extending existing telomeres using an RNA template located within the enzyme [13–15]. siRNAs have been demonstrated as potential therapeutic agents to treat cancer by silencing the expression of telomerase and inducing cancer cell senescence and apoptosis [11, 16].

We synthesized our novel siRNA-based probe utilizing two short 21-nt RNA complementary strands as the stem and a highly flexible poly(ethylene glycol) (PEG) molecule covalently linked to the RNA strands as the loop of an siRNA-based MB (Fig. 1). Two different PEG lengths were used, M_r 3400 and 5000. A commonly used FRET fluorophore pair, Cy3 and Cy5 fluorophores, was conjugated to the 3' terminus of the antisense and 5' terminus of the sense strand, respectively. Cy3 and Cy5 emission peaks are at 565 and 667 nm. Previous studies have shown that modifications of all termini of the siRNA duplex except the 5' terminus of the antisense strand can be made without significantly affecting gene silencing [17]. After the siRNA probe incorporates into RISC, the anti-guide strand would presumably be cleaved at the ninth position [5], leaving a nine-base pairing stem molecular probe. Longer MB stems have shown to have improved ability to discriminate targets and yield higher signal-to-background ratios than shorter stems [18]. Next the presence of a complementary mRNA would displace the anti-guide strand, resulting in activation of the probe for imaging.

siRNA-based probe synthesis

Modified 21-mer oligoribonucleotide (ss/siRNAs) were purchased commercially (Dharmacon RNA Technologies, Lafayette, CO, USA) utilizing a previously published and validated sequence targeting the telomere repeat

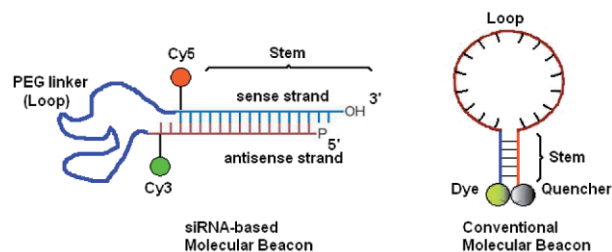


Figure 1. siRNA-based MB in comparison to the conventional MB.

template sequence (hTR) [11]. The modified sense strand sequence is: 5' HS-U*UGUCUAACCCUAACUGAG-TT-3' where U* is a Cy5-dUTP fluorophore-conjugated base. The modified antisense strand is: 3' NH₂-T*T-AACAGAUUGGGGAUUGACUC-5' where T* is a Cy3-dTTP fluorophore-conjugated base. Cy3 and Cy5 are a commonly used fluorophore FRET pair. The sense and antisense oligoribonucleotides were reacted with a maleimide-PEG-NHS ester heterobifunctional linker (3400 and 5000 M_r PEG; Nektar Therapeutics, Huntsville, AL, USA). During the coupling reaction, siRNA antisense and sense strands were added in a twofold molar excess to ensure sufficient coupling onto the heterobifunctional PEG linker. The reaction was allowed to proceed overnight in the dark in RNase-free phosphate-buffered saline, pH 7.4 at 4°C.

siRNA-based probe isolation

The conjugated ssRNA-PEG-ssRNA was then isolated from free ssRNA and PEG by 15% denaturing PAGE. The sample was heated at 90°C for 2 min prior to gel loading, mixed with gel loading buffer, and electrophoresed at 35 mA. The PAGE gel was post-stained with a 0.5 µg/mL ethidium bromide solution. Two distinct bands were seen for each probe. A reference nucleotide sizing marker (Decade Markers, Ambion, Austin, TX) was run in parallel with the bands to determine which band contained the conjugated siRNA probe. The siRNA probe band (determined by nucleotide size) was then carefully excised from the gel and isolated by the crush and soak method. After 48-h elution at 4°C in the dark with 10 mL 1 M NaCl, the sample was centrifuged at 2000 × *g* for 5 min. The elution buffer was ethanol precipitated with a 4-volume excess of cold 90% ethanol/10% 3 M sodium acetate at -20°C for 48 h to isolate the siRNA probe. The solution was centrifuged at 20 000 × *g* for 30 min at 4°C and resuspended in annealing buffer (20 mM KCl, 6 mM HEPES-KOH pH 7.5). Annealing was

performed by heating to 60°C for 5 min, and slow-cooled to room temperature over 30 min. Extinction measurements at 260 nm ($\epsilon = 414\,600 \text{ L/mol cm}$) were performed in a quartz cuvette to determine final siRNA conjugate concentration on a Varian Cary 50 Bio spectrophotometer (Walnut Creek, CA, USA). The probe was then aliquoted into RNase-free tubes and stored at -80°C.

Spectroscopy measurements

All measurements were performed with a constant temperature 1.5-mL stoppered quartz fluorescence cuvette (Starna Cells, Atascadero, CA, USA) on a Horiba Jobin Yvon SPEX FL3-22 Fluorimeter (Edison, NJ, USA) with dual excitation and emission monochromators. Sample temperature was controlled by a circulating temperature water bath through the quartz cuvette. Time-integrated photoluminescence was measured using 488 nm excitation light and emission scans from 525 to 800 nm. Bandpass slits and integration time were set to 3 nm/3 nm and 100 ms, respectively, on the fluorimeter. All values were normalized over time to a rhodamine 6G standard to avoid any artifacts that could arise from possible lamp fluctuations. FRET efficiency between Cy3 and Cy5 fluorophores was calculated by intensity of Cy5 fluorescence/(intensity of Cy3 fluorescence + intensity of Cy5 fluorescence).

Cell culture studies

The human breast cancer cell line SK-BR-3 and normal breast fibroblast cell line CCD-1059Sk were from American Type Culture Collection (ATCC, Manassas, VA, USA). SK-BR-3 cells were cultured (37°C, 5% CO₂) in McCoy's 5A medium with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA, USA). CCD-1059Sk cells were cultured (37°C, 5% CO₂) in minimum essential medium (Eagle) with 10% fetal bovine serum. Both SK-BR-3 cells and CCD-1059Sk cells were plated onto glass chamber slides (Nalge Nunc International, Rochester, NY, USA) for microscopy studies. Cells were plated

into six-well tissue culture plates at a concentration to provide 80% confluence 24 h later prior to transfection using Lipofectamine 2000 (Invitrogen). At that time, the siRNA probe was diluted to a final volume of 500 µL with Opti-Mem Reduced Serum Media. In a separate tube, 30 µL Lipofectamine was diluted with 470 µL Opti-Mem. The two solutions were separately mixed gently and incubated at room temperature for 5 min. The contents were then combined and mixed gently by pipetting and incubated at room temperature for 30 min. The liposome complexes were then added to the culture medium and mixed gently for 30 s. After 44 hours, the cells were trypsinized, counted, and 2000 cells removed for assay of telomerase activity. Cell studies were performed with the 3400-M_r siRNA probe, 5000-M_r siRNA probe, non-targeting Silencer Negative Control no. 1 siRNA (Ambion, Austin, TX), and mock transfection at 100 nM siRNA concentrations.

siRNA imaging

All images were collected using a Zeiss LSM 510 META NLO confocal system mounted on an Axiovert 200M inverted microscope and Plan-Apochromat 63× objective lens (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA). siRNA probes were excited with a 488-nm Argon laser source in live cell imaging. Spectrofluorimeter measurements demonstrated minimal excitation of the Cy5 fluorophore with a 488-nm excitation source, indicating that Cy5 emission generation is due to fluorescence resonance energy transfer from the Cy3 fluorophore. All images were acquired with the same detector, gain, pinhole, and power settings at 1024 × 1024 pixels to allow direct visual comparison between normal and cancer cells. Lambda emission scanning from 510 to 700 nm was performed using the META detector to verify fluorescence intensity changes between Cy3 and Cy5 fluorophores.

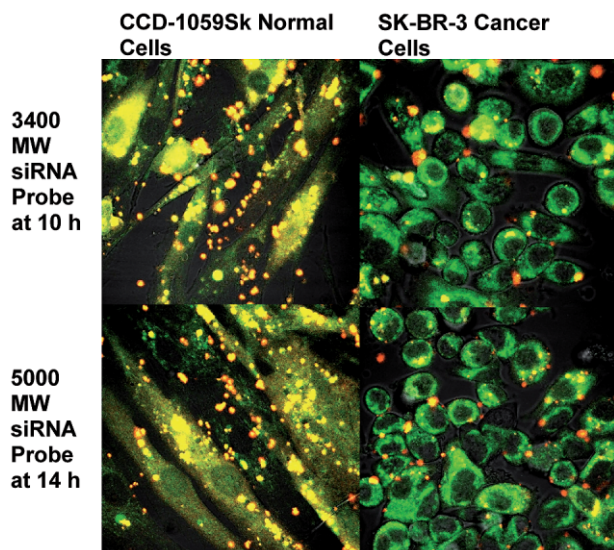


Figure 2. Confocal images at 10 and 14 h post transfection with Lipofectamine 2000 in normal human breast fibroblast and human breast cancer cells transfected with 3400- and 5000- M_t siRNA-based probes targeted against the hTR sequence in telomerase. Microscopy settings were held constant for image comparison. Analysis of the images shows a significant increase in Cy5 fluorescence due to FRET in normal cells that do not express telomerase *versus* cancer cells that do express telomerase.

Telomerase activity

Telomerase activity was assayed using a commercial fluorescence-based TRAPEZE[®] XL telomerase detection kit (Intergen, Purchase, NY, USA), which utilizes a modified telomeric repeat amplification protocol. Lysates (1000 cell-equivalents) were mixed with the TRAPEZE XL reaction mix containing Amplifluor[™] primers, and incubated at 30°C for 30 min. Samples underwent PCR amplification. Amplified telomerase products were quantitated with a SpectraMax M2 fluorescent plate reader. Telomerase activity was calculated by comparing the ratio of telomerase products to an internal standard for each lysate, as described by the manufacturer.

Results and discussion

After synthesis and isolation of the probe, we evaluated the hybridization of the siRNA-based probes upon addition of complementary target strands *in vitro*. After a 20-min incubation at 30°C, a 9.3-fold and 8.2-fold decrease in Cy5 fluorescence occurred for 3400- and 5000- M_t siRNA probes, respectively. In addition, temperature cycling studies between 15 and 90°C

were performed to examine the separation and re-annealing of the siRNA probe based on FRET efficiency (see Supporting information).

We next evaluated siRNA probe targeting to telomerase in live human cells. Cancer and normal cells were transfected with 100 nM siRNA probe using cationic lipid-based transfection agents. The sequence of our siRNA probe targeted the hTR sequence of telomerase mRNA. Cancer cells express high levels of telomerase mRNA, while normal cells minimally express telomerase mRNA. Confocal imaging was performed every 2 h after transfection up to 14 h and then every 4 h up to 38 h post-transfection. Figure 2 demonstrates the activation of the siRNA probe in cancer cells in contrast to normal cells. Interestingly, the 3400- M_t siRNA probe discriminated differences between normal and cancer cells earlier than the 5000- M_t siRNA probe. The ideal time for imaging the siRNA probes appeared to be 6–10 h post-transfection for the 3400- M_t siRNA probe and 10–14 h post-transfection for the 5000- M_t siRNA probe. At 24 h post transfection, no difference was observed between nor-

mal and cancer cells (no FRET observed), presumably due to complete loss of the siRNA probe stem by degradation from intracellular exonucleases.

Lastly, we evaluated the efficacy of the siRNA-based probe at mediating RNAi. A commercial telomerase activity assay was performed on the cells 44 h post transfection with the siRNA probe. Cells appeared healthy after transfection prior to telomerase assay. Figure 3 demonstrates effective gene silencing of telomerase by the siRNA-based probe. This finding is consistent with previous observations of telomerase silencing using this siRNA probe sequence [11]. More importantly, the modifications to siRNA did not inhibit effects of gene silencing in agreement with previous studies [17].

By tethering the anti-guide strand of the siRNA duplex to the guide strand, the two strands are able to re-anneal if no mRNA is present to compete for hybridization. Whereas the vast majority of MBs are constructed using an oligonucleotide backbone comprising the loop and stem, it was necessary to utilize a synthetic PEG linker as the loop for our design since Dicer, an RNAi protein, has been shown to cleave oligonucleotide hairpin beacons such as shRNAs into two separate strands during RNAi [17]. Future work includes evaluating different siRNA sequences to further examine specificity and off-targeting effects of these modified siRNA probes as well as imaging and silencing of other proteins and improving existing fluorophore selection for *in vivo* imaging modalities. Potential future applications of this method could be its use in studying diseases or monitor RNAi. Ultimately, rapid delivery of siRNA probes into cells could provide a useful clinical tool in early detection and therapy of diseases.

Although there have been a substantial number of recent studies evaluating the therapeutic applications of siRNA, we describe here a novel bioengineering modification to siRNAs. In this study, we provide an initial proof-of-principle demonstration that

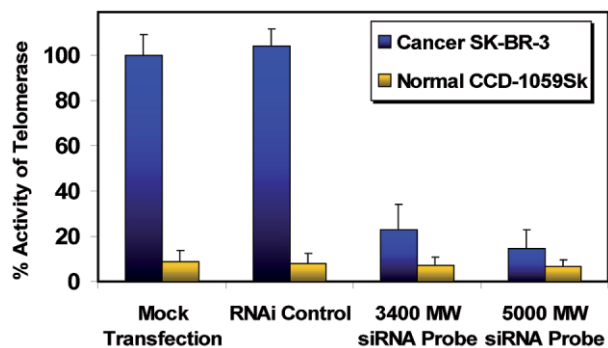


Figure 3. Telomerase activity relative to untreated SK-BR-3 human breast cancer cells after transfection with 100 nM siRNA probe at 44 h ($n=3$). siRNA probe is able to mediate RNA interference and significantly knockdown expression of telomerase in cancer cells.

siRNAs may be modified into a fluorescence-based MB for mRNA detection and silencing. Using siRNA-based probes in the imaging and silencing of telomerase could potentially be useful in detecting and treating precancerous lesions. These modified siRNAs may provide promising new ways to evaluate mRNA expression in diseases and serve as useful reporter/gene silencing tools for target validation in basic science and for dual imaging and therapy in clinical research.

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