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RNA "Traffic Lights": An Analytical Tool to Monitor siRNA Integrity

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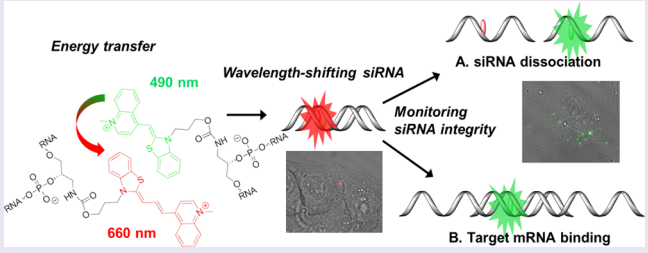
Supporting Information

ABSTRACT: The combination of thiazole orange and thiazole red as an internal energy transfer-based fluorophore pair in oligonucleotides provides an outstanding analytical tool to follow DNA/RNA hybridization through a distinct fluorescence color change from red to green. Herein, we demonstrate that this concept can be applied to small interfering RNA (siRNA) to monitor RNA integrity in living cells in real time with a remarkable dynamic range and excellent contrast ratios in cellular media. Furthermore, we show that our siRNA-sensors still possess their gene silencing function toward the knockdown of enhanced green fluorescent protein in CHO-K1 cells.



RNA interference (RNAi) is a pathway by which a sequence-specific, small interfering RNA (siRNA) leads to the cleavage of complementary mRNA and thus provides a powerful mechanism to control gene expression in eukaryotic cells.[1,2] Besides being an everyday laboratory tool to study genetic functions,[3] RNAi holds great promise in the field of siRNA-based therapeutics.[4,5] However, the poor stability of siRNA in biological media[6] combined with an insufficient delivery to the target cells[7,8] are the major limiting factors for a broad clinical application. Hence, to design better delivery systems for synthetic siRNA, it would be of utmost importance to track the integrity of siRNA. For this purpose, the method of choice is the application of fluorescently labeled siRNA. Various established methods[9−11] like, for example, FISH,[12] are based on a single emission color readout, which bears the risk of wrong positive or wrong negative readout since undesired fluorescence quenching can be caused by cellular components. In contrast, a wavelength-shifting siRNA-sensor overcomes these drawbacks, as its readout focus relies on two distinct emission colors. In literature, only few approaches are reported that are based on the attachment of fluorophores to the 5′- and 3′-termini of the siRNA, which then act as an energy transfer (ET) pair.[13−15] Unfortunately, the sensitivity of these sensors is limited, allowing only to discriminate between 0% and 10% processed siRNA. We hypothesize that siRNA bearing fluorescent base surrogates would be an alternative. Several single and multicolor fluorescent mRNA-sensors were developed on base surrogates, and the question is, can this concept be transferred to monitor siRNA integrity with a much higher sensitivity than possible so far.[16−20]

We recently reported the combination of thiazole orange (TO) and thiazole red (TR) as an interstrand ET pair in DNA (DNA "traffic lights"):[21−23] A distinct color readout by an efficient ET was obtained because both dyes were covalently attached as base surrogates and forced in close proximity to each other by the surrounding DNA architecture. Upon excitation at the TO-typical wavelength of 490 nm (green), a large shift of 140 nm to the TR-typical emission at 670 nm (red) was observed. The goal of this study was to design a doubly labeled siRNA-sensor bearing TO and TR as internal fluorescent labels (Figure 1a). Although DNA "traffic lights" represent a published concept for various applications, for example, in in-stem labeled molecular beacons[22] or DNA aptasensors,[23] the transfer to RNA was an extreme challenge. It was necessary to find a position for the base surrogates that allows, on the one hand, excellent fluorescent properties of the sensor and, on the other hand, does not affect gene silencing efficacy. We envision that the double-stranded siRNA-sensor shows a red-shifted fluorescence signal, while the single-stranded siRNA-sensor, either free or bound to complementary mRNA, shows a green signal (Figure 1b,c). The ratio of the red-to-green (R/G-ratio) fluorescence emission of the siRNA-sensor can consequently be used to describe the integrity of the siRNA. Under certain circumstances, it cannot be ruled out that unspecific siRNA degradation may influence the R/G-ratio: if siRNA cleavage has just initiated, partially degraded double-stranded siRNA-sensor will have a lower melting temperature and hence may dissociate to a partially degraded single-stranded siRNA-sensor. However, if siRNA degradation is progressing, it will result in a significant loss of fluorescence intensity, but hardly influence the R/G-ratio because base surrogates show only a significant fluorescence emission in the vicinity of other nucleotides.[24] We hypothesize that our

Received: December 3, 2012
Accepted: February 12, 2013
Published: February 12, 2013

   dx.doi.org/10.1021/cb3006616 | ACS Chem. Biol. 2013, 8, 890−894

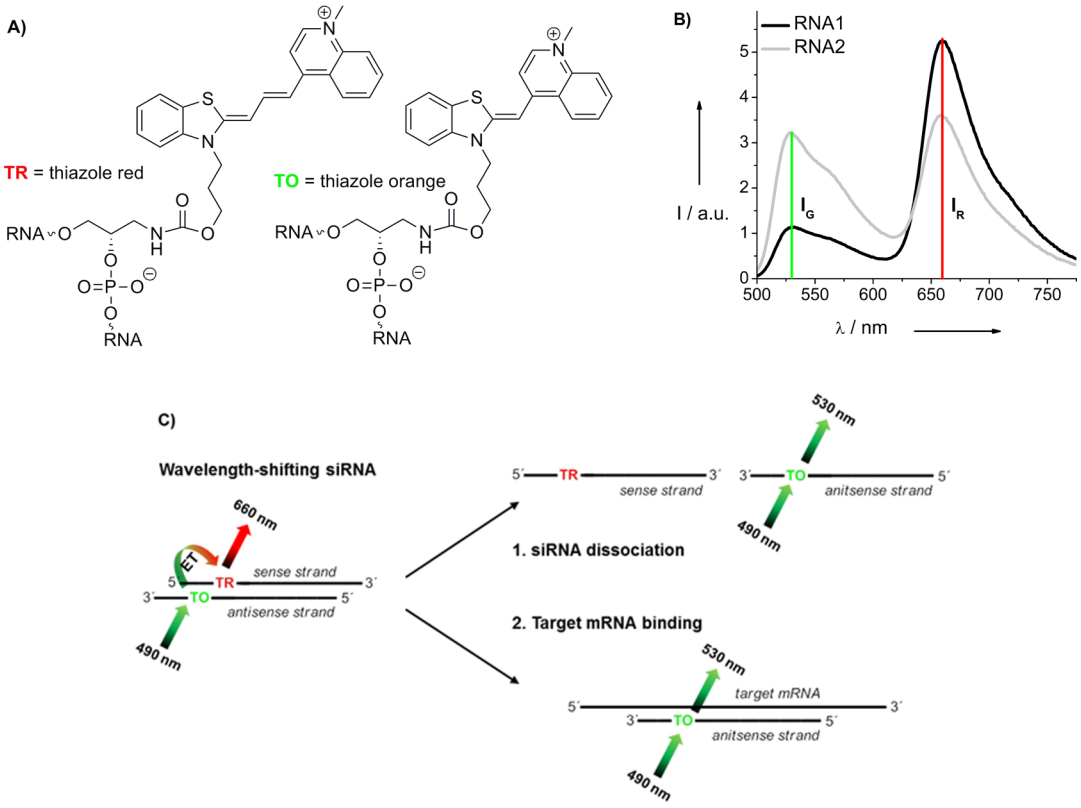


Figure 1. (A) Structure of thiazole orange (TO) and thiazole red (TR) as RNA base substitutions. (B) Fluorescence of **RNA1** and **RNA2**, 2.5 μM in 10 mM Na–P_i buffer, 250 mM NaCl, pH 7, 20 °C, excitation at 490 nm; I_G and I_R indicating fluorescence intensities for the calculation of the R/G-ratio. (C) A schematic representation illustrating the switching of emission color from red to green of a siRNA duplex modified with TO and TR due to (1) dissociation or (2) upon binding to its target mRNA.

proposed concept is superior to others due to the following reasons: (1) the sensor is based on a two-color fluorescence readout, which gives valuable information of the siRNA integrity, and (2) the fluorescent dyes are attached as artificial base surrogates forming an interstrand ET pair, which responds to changes in the environment with a much higher sensitivity than terminally attached fluorophores.

Using our published synthetic building blocks for the incorporation of TO and TR as base substitutions (Figure 1a; Tables S1 and S2 and MS data in Supporting Information),[21] we prepared **RNA1** and **RNA2** via automated solid phase synthesis (Table 1; Tables S1 and S2 and MS data in Supporting Information). For both siRNAs, a sequence consisting of 21 nucleotides was chosen to potentially down regulate the enhanced green fluorescent protein (EGFP) expression. Only the site of modification by the TO/TR ET pair differs: In **RNA1**, the two chromophores were attached close to the 3′-end of the antisense strand, whereas in **RNA2**, a position near the 5′-end was selected. All termini are unlabeled, which still gives the opportunity for potential attachment of bulky residues to facilitate cellular uptake.[6] The UV/vis absorption spectra of **RNA1** and **RNA2** show clearly the presence of both chromophores with well separated signals for TO at 510 nm and TR at 645 nm (see Supporting Information Figure S1). The extinction coefficient for TR is significantly lower in **RNA2**; this may be due to excitonic interactions between both chromophores as previously reported.[23] Consequently, the R/G-ratio (Figure 1b) for **RNA2** of 1.2:1 is lower compared to 4.6:1 for **RNA1**. Notably, R/G-values

Table 1. Sequences and R/G-Ratios of siRNA Duplexes[a]

Duplex	Sequence[b]	R/G-ratio in buffer[c]	R/G-ratio in cell lysate[d]
RNA1	*sense strand (**SS**)* 5`-GCAA**TR**CUGACCCUGAAGUUCAU-3´ 3`-GCCGU**TO**CGACUGGGACUUCAAG-5´ *antisense strand (**AS**)*	4.6:1	6.6:1
RNA2	5`-GCAAGCUGACCCUGAA**TR**UUCAU-3´ 3`-GCCGUUCGACUGGGACU**TO**CAAG-5´	1.2:1	0.9:1
EGFP siRNA[e]	5`-GCAAGCUGACCCUGAAGUUCAU-3´ 3`-GCCGUUCGACUGGGACUUCAAG-5´	-	-
control RNA1	5`-GCAAGCUGACCCUGAAGUUCAU-3´ 3`-GCCGU**TO**CGACUGGGACUUCAAG-5´	-	-
control RNA2	5`-GCAAGCUGACCCUGAAGUUCAU-3´ 3`-GCCGUUCGACUGGGACU**TO**CAAG-5´	-	-

[a]Red-to-green-ratio (R/G-ratio). [b]Sense strands are listed on top; antisense strands are listed below. [c]In 10 mM sodium phosphate, pH 7, 250 mM NaCl, and 2.5 μM RNA. [d]In CHO-K1 cell lysate. [e]Unmodified control siRNA.

determined in cell lysate showed even higher values (6.6:1 for **RNA1**).

Since the R/G-ratio of the dual emission should be applied to measure siRNA integrity, a calibration curve correlating both values was recorded in cell lysate for **RNA1** and **RNA2,** respectively (Figure 2a and Supporting Information Figures S2 and S3). These calibration curves were then used to monitor siRNA integrity in living cells by the red-to-green color change. The obtained R/G-ratios show a clear dependence on the complementary content of double-stranded versus dissociated siRNA-sensor in the samples. That means that a higher amount

dx.doi.org/10.1021/cb3006616 | ACS Chem. Biol. 2013, 8, 890–894

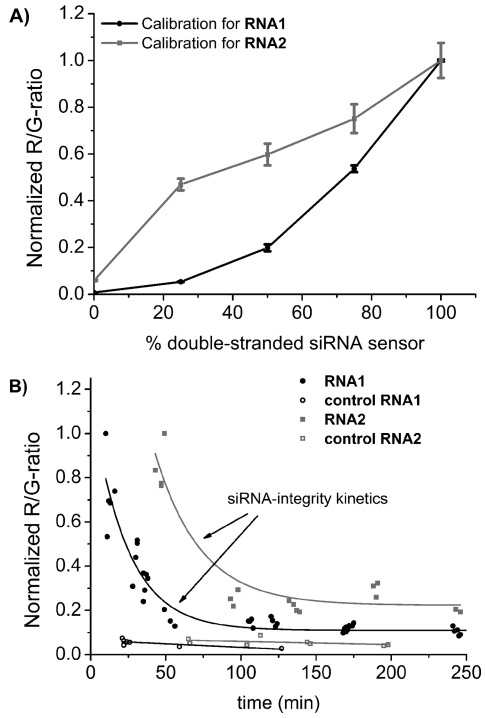


Figure 2. (A) Calibration curve for the amount of intact **RNA1** and **RNA2**; samples containing different amounts of double-stranded siRNA-sensor (see also Supporting Information Figures S2 and S3). The R/G-ratio of 100% double-stranded **RNA1** (**RNA2**) was normalized to 1. Error bars are included. All measurements were carried out in cell lysate. (B) Integrity kinetics of formulations of intact **RNA1**, **RNA2**, and control RNAs (TR modification in **RNA1** and **RNA2** was replaced by G, respectively) in CHO-K1 cells. Cell transfection with biodegradable poly(ethylene imine).[25]

of processed siRNA-sensor corresponds to a lower R/G-ratio. The slope of the calibration curve provides a useful dynamic range from 0% to 50% processed siRNA-sensor. There are only few records about FRET-based siRNA-sensor systems in literature.[13,14] However, the dynamic ranges described therein cover only 0% to 10% processed siRNA-sensor, which makes small changes in higher ranges very difficult to distinguish. Compared to the literature, our result demonstrates an exceptional broad range. We assign this significant improvement to the way how the chromophores were attached to the RNA backbone. Both chromophores were embedded as an internal modification in the RNA double helix. Thus, small changes in the chromophore environment lead to significant changes of the R/G-values as a consequence of orientation and distance dependency of the ET.[23] As a result, a much higher sensitivity regarding siRNA integrity is obtained. Differences regarding the initial slopes of the calibration curves for **RNA1** and **RNA2** can be explained due to an incomplete ET in **RNA2**, which leads to smaller changes in the observed R/G-ratios.

To test the feasibility of the above calibration in standard transfection experiments, CHO-K1 cells were incubated with polyplexes consisting of **RNA1** (or **RNA2**, respectively) and biodegradable poly(ethylene imine).[25] After cell transfection, R/G imaging was performed by confocal laser scanning microscopy at certain time intervals to monitor siRNA integrity. At the beginning of the measurements, 100% double-stranded **RNA1** (**RNA2**) primarily gave a red signal inside the cells. With an ongoing dissociation of double-stranded **RNA1** (**RNA2**), the dyes were separated and a signal change to green was observed. Figure 3 shows the time course of this process inside living cells: Until 15 min after transfection, red emission was recorded

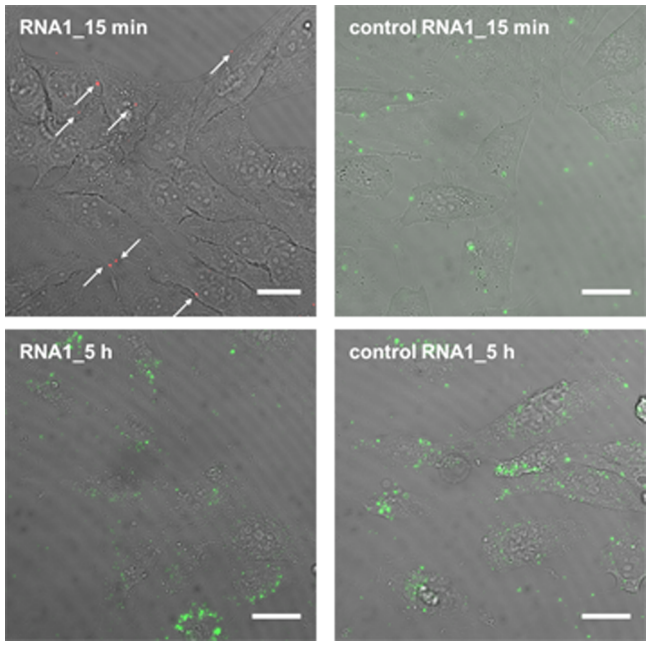


Figure 3. CHO-K1 cells were transfected with polyplexes containing biodegradable poly(ethylene imine) and **RNA1** (left) or **control RNA1** (TR modification in **RNA1** was replaced by G, right). Confocal laser scanning microscopy was performed after 15 min and 5 h, respectively. Double-stranded **RNA1** shows primarily a red signal, whereas 5 h after transfection, dissociated **RNA1** shows a green emission. Bars indicate 20 μm.

primarily. This readout was completely converted into a green emission 5 h after transfection. During this time period, the R/G-ratio dropped continuously, which also indicates that the initially double-stranded **RNA1** was dissociated over the time course of the experiment and may have bound to complementary mRNA (Figure 2b). To demonstrate that the observed color change can be assigned to intracellular integrity, two control RNA duplexes containing a single TO modification (lacking TR) were tested under the same conditions (Figures 2b and 3). The green fluorescence of the control siRNA-sensor remained unchanged during the experimental procedure. Consequently, because the base surrogates show only a fluorescence emission in the vicinity of other oligonucleotides, we propose that unspecific, intracellular degradation of our siRNA-sensors plays a minor role regarding changes of the R/G-ratio. Hence, the developed siRNA-sensor based on a large wavelength-shift from green to red represent a very useful tool to monitor cellular uptake and to follow siRNA integrity in living cells.

To measure the impact of our internal chromophore modifications on the gene silencing efficiency of EGFP expression in CHO-K1 cells, we compared the silencing properties of **RNA1** and **RNA2** with an unmodified RNA duplex (**EGFP siRNA**) (Table 1). These experiments were carried out using biodegradable poly(ethylene imine)[25] as well as lipofectamine RNAiMAX as transfection reagents (Figure 4). **RNA1** as well as **RNA2** show remarkable gene silencing activities, which come up to 75% for **RNA2** compared with the knockdown efficiency of unmodified **EGFP siRNA**. Control experiments and toxicity data support the sequence-specific

dx.doi.org/10.1021/cb3006616 | *ACS Chem. Biol.* 2013, 8, 890–894

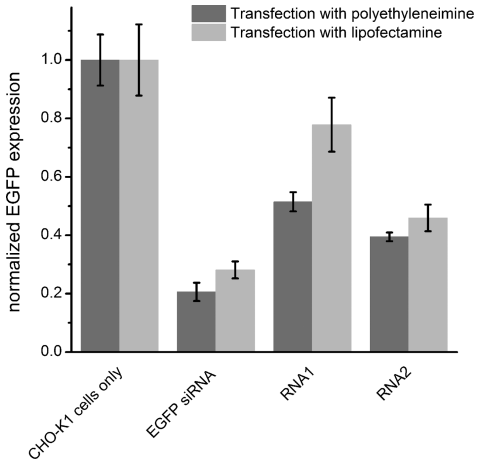


Figure 4. siRNAs containing TO and TR as internal modifications (**RNA1** and **RNA2**) silence the EGFP-gene in CHO-K1 cells at nanomolar concentrations. Error bars show standard deviation. Cell transfection was performed with poly(ethylene imine) and lipofectamine RNAiMAX, respectively.

silencing using **RNA1** and **RNA2** (see Supporting Information Figures S4 and S5, respectively). Even though cell transfection with poly(ethylene imine) shows better results, the same trend for transfection with Lipofectamine was observed for **RNA1** and **RNA2**. This leads to the assumption that internal TO and TR modifications in siRNAs can be tolerated to a certain extent. The better EGFP silencing activity of **RNA2** compared to **RNA1** tells, however, that it is crucial to place the TO/TR pair not in the seed region of the siRNA sequence (for a more detailed comparison of **RNA1** and **RNA2**, see Supporting Information Figure S4). It is more suitable to place the modification on the 3′-side (antisense sequence) of the mRNA cleavage position.

In conclusion, we demonstrated that our DNA "traffic lights" concept of dual fluorescent base surrogates based on TO as green and TR as red emitter can be transferred to RNA. The obtained R/G-ratios of such doubly modified siRNAs provide a large dynamic range and allow monitoring siRNA uptake and integrity in living cells with very good color contrast as the readout. Using these wavelength-shifting RNA "traffic lights", it will become feasible to follow the siRNA integrity inside living cells yielding a complete mechanistic understanding, especially if the probing is combined with single molecule spectroscopy including PALM and STED techniques.

METHODS

RNA Synthesis. Oligonucleotides were prepared with an Expedite 8909 Synthesizer from Applied Biosystems (ABI) using standard phosphoramidite chemistry (Ribonucleotides were 2′TBDMS protected). Reagents and CPG (1 μmol) were purchased from ABI and Glen Research. The synthesis of thiazole orange-modified and trimethine thiazole orange-modified RNA oligonucleotides was performed using a modified protocol. Activator solution (0.45 M tetrazole in acetonitrile) was pumped simultaneously with the building block (0.12 M in acetonitrile). The coupling time was extended to 90 min with an intervening step after 45 min for washing and refreshing the activator/phosphoramidite solution in the CPG vial. The CPG vial was flushed with dry acetonitrile after the coupling. After preparation, the trityl-off oligonucleotide was cleaved from the resin and tac-deprotected by treatment with 1 mL of a 1:1 solution of conc. NH_4OH/methylamine at 65 °C for 20 min. The CPG was removed and the RNA was collected via EtOH precipitation. The TBDMS protecting group was removed in a solution of 1:1 DMSO and $Et_3N{\cdot}HF$ at 65 °C for 2.5 h. The modified RNA was collected through precipitation with trimethyle(propoxy)silane and washed with diethyleether. The oligonucleotides were purified by HPLC on a semipreparative RPC18 column (300 Å, Supelco) using the following conditions: *A* = NH_4HCO_3 buffer (100 mM), pH = 7; *B* = acetonitrile; gradient 0−20% B over 45 min for TO and TR, flow rate 2.5 mL/min; UV/vis detection at 260 and 510 nm for TO-modified oligonucleotides, 260 and 630 nm for TR-modified oligonucleotides. The oligonucleotides were lyophilized and quantified by their absorbance in water at 260 nm on a Varian Cary 100 spectrometer. Duplexes were formed by heating to 90 °C (10 min) followed by slow cooling.

Spectroscopic Measurements. Spectroscopic measurements were recorded in Na−P_i buffer solution (10 mM) using quartz glass cuvettes (10 mm) (2.5 μM DNA and 250 mM NaCl). Absorption spectra were recorded with a Varian Cary 100 spectrometer equipped with a 6_6 cell changer unit. Fluorescence was measured with a Jobin−Yvon Fluoromax 3 fluorimeter with a step width of 1 nm and an integration time of 0.2 s. All spectra were recorded with an excitation and emission bandpass of 2 nm and are corrected for Raman emission from the buffer solution.

Confocal Laser Scanning Microscopy. *Calibration Curves.* Various ratios of singly and doubly labeled siRNA were mixed to obtain 100, 75, 50, 25, and 0% intact siRNA. The calibration solutions were mixed with cell lysate (ratio 3:1), which was obtained as described in ref 13. Cells with disrupted outer cell membrane and intact cell nuclei were applied for the experiments. Subsequently, the fluorescence emission spectra (from 531 to 703 nm) of the samples were recorded after excitation with 488 nm using a Zeiss Axiovert 200 M microscope coupled to a Zeiss LSM 510 META scanning device (Carl Zeiss Co. Ltd., Germany). The fluorescence intensity values were background corrected using siRNA with a TR modification only.

Degradation Kinetics. HeLa cells were incubated with polyplexes consisting of **RNA1** or **RNA2** and biodegradable poly(ethylene imine)[25] at a N/P (nitrogen/phosphor) ratio of 6. At the indicated time points, the fluorescence emission spectra were recorded as described above.

Gene Silencing and Toxicity. CHO-K1 cells stably expressing enhanced green fluorescent protein (EGFP) were applied to measure the knockdown of siRNA. siRNA was delivered either in the form of polyplexes or lipoplexes. Polyplexes were fabricated with biodegradable poly(ethylene imine), and lipoplexes were prepared using lipofectamine RNAiMAX according to the manufacturer's protocol. AllStars Negative Control siRNA (Qiagen) was used as a nontargeting siRNA. The cells were grown in 24-well plates at an initial density of 38 000 cells per well. Twenty hours after seeding, polyplexes or lipoplexes were added to the culture medium. After 5 h, the medium was removed and replaced with fresh culture medium. After 72 h, cells were analyzed by flow cytometry analysis on a FACS Calibur (Beckton Dickinson). The measurements of the mean fluorescence intensity of EGFP and the toxicity after propidium iodide staining was performed as described in ref 26. The values of the mean fluorescence intensity were used to calculate the silencing. The mean fluorescence intensity of EGFP was referred to untreated CHO-K1 cells stably expressing EGFP and expressed as normalized EGFP expression.

ASSOCIATED CONTENT

Supporting Information

Modified oligonucleotides; UV/vis-spectra of **RNA1** and **RNA2**; calibration curve mixtures and corresponding emission spectra; dose-dependent gene silencing; toxicity. 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.

dx.doi.org/10.1021/cb3006616 | *ACS Chem. Biol.* 2013, 8, 890−894

■ ACKNOWLEDGMENTS

Financial support by the Deutsche Forschungsgemeinschaft (BR 3566/2-1, WA 1386/13-1), the University of Regensburg, and Karlsruhe Institute of Technology (KIT) is gratefully acknowledged.

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