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Quantum Dot-Conjugated Hybridization Probes for Preliminary Screening of siRNA Sequences

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Abstract: In the present study, we describe the design and fabrication of quantum dot-conjugated hybridization probes and their application to the development of a comparatively simple and rapid procedure for the selection of highly effective small-interfering RNA (siRNA) sequences for RNA interference (RNAi) in mammalian cells, for example, siRNAs with high accessibility and affinity to the respective mRNA target. A single-stranded siRNA was conjugated with a quantum dot and used as a hybridization probe. The target mRNA was amplified in the presence of Cy5-labeled nucleotides, and Cy5−mRNA served as a hybridization sample. The formation of siRNA/mRNA duplexes during a comparatively short hybridization time (1 h) was used as a criterion for the selection of highly effective, target-specific siRNA sequences. The accessibility and affinity of the siRNA sequence for the target mRNA site were determined by fluorescence resonance energy transfer (FRET) between a quantum dot (donor) and a fluorescent dye molecule (Cy5, acceptor) localized at an appropriate distance from each other when hybridization occurred. The FRET signal was observed only when there was high accessibility between an antisense siRNA and a sense mRNA and did not appear in the case of mismatch siRNAs. Moreover, the amplitude of the FRET signal significantly correlated with the specific effect of siRNA on the expression of the target mRNA and protein, determined in native cells by RT-PCR and immunoblot analysis, respectively.

Introduction

RNA interference (RNAi) is a powerful sequence-specific gene silencing induced by a double-stranded RNA (dsRNA), providing a powerful tool for gene function analysis quickly and easily. This mechanism is mediated by small interfering RNAs (siRNAs) produced from long double-stranded RNAs of exogenous or endogenous origin by an endonuclease called Dicer (ribonuclease III type). The resulting siRNAs are ∼21−23 base pairs (bp) and serve as guide sequences to induce target-specific mRNA cleavage by an RNA-induced silencing complex.[1−3] This mechanism is extremely potent and requires only few siRNA molecules per cell to silence homologous mRNA expression.

RNAi is conserved in plants and invertebrates.[4−6] The introduction of a long dsRNA (>50 bp) in mammalian cells results in the induction of interferon response and the nonspecific inhibition of translation. The only way to induce RNAi in mammalian cells is to introduce synthetic siRNAs (<30 bp) that can be either delivered exogenously or expressed endogenously from RNA polymerase III promoters. The discovery of this mechanism in mammalian cells (in 2001[7]) enhanced the expectations that the problems associated with gene therapy, especially in cancer, viral infections, and inherited genetic disorders, could be fully solved. RNAi has also been used in obtaining tissue-specific knockdown mice for studying gene functions in vivo.[8] Combined with genomics data, RNAi gene silencing may enable the functional determination of any gene expressed in a cell or a pathway. However, it is too early to predict how useful this technology will be because many questions regarding the estimation of the efficiency of siRNA substances exist.

The gene silencing effect of siRNAs depends predominantly on their access and affinity to the complementary site of the target mRNA. Since the accessibility of siRNA is related to the secondary structure of the target mRNA, it is necessary, although costly and time-consuming, to design and synthesize many siRNA sequences and to test their antisense activity. Appropriate sequences are selected from databases (e.g., http://www.ambion.com/techlib/misc/siRNA_finder.html), and the

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algorithms are continuously improved; however, generally, only one of 10−15 selected siRNAs manifests a real antisense effect in native cells. In addition, the algorithms are improved predominantly on the basis of the increasing amount of experimental results in the siRNA field (a basis for development of siRNA library), and websites do not give recommendations regarding the application of the selected siRNA sequences. Significant progress was made in 2004 with the study of Ui-Tei and colleagues.[9,10] By extensively analyzing the relationship between siRNA sequences and their RNAi effect using 62 targets of four exogenous and two endogenous genes in three mammalian cell types and in *Drosophila* cells, the authors formulate several roles for the selection of highly effective siRNA sequences for mammalian RNAi. The results have been used in the development of the siDirect—a software system for computing highly effective siRNA sequences with a maximum target specificity for mammalian RNAi (http://design.RNA.jp). Despite the fact that this software may significantly restrict the choice, it usually recommends approximately 100−300 appropriate siRNA sequences per gene (restricted to 15−20 sequences after the blast homology test), but in some cases of poor mRNA target specificity (the sequences have homology with approximately 4−5 genes), the success of finding more effective sequences still depends on trial and error.

The development of screening procedures for the prediction of siRNA efficiency and the selection of potent siRNA sequences (before the start of experiments on cells or animals) remains an important priority.

In 2004, another approach to the preliminary screening of functional siRNA sequences was reported—a method of generating an enzymatically synthesized library of siRNA hairpin cassettes obtained from a dsDNA source.[11−13] A brief description of the method with analysis of its advantages and shortcomings was given by Singer et al.[14] The method is laborious and expensive, but it ensures highly effective siRNAs for every gene.

In the present study, we describe a comparatively simple and rapid procedure for the secondary screening of most potentially effective siRNA sequences (preliminarily restricted using databases) for RNAi in mammalian cells, for example, siRNAs with high accessibility and affinity to respective mRNA targets. The preliminary screening of siRNA sequences with high accessibility and affinity to the target mRNA can also be considered as a guarantee to minimize side effects as a result of homology with other genes. Briefly, a single-stranded siRNA was conjugated with a quantum dot and served as a hybridization probe. The target mRNA was amplified in the presence of Cy5-labeled nucleotides, and Cy5−mRNA served as the hybridization sample. The formation of siRNA/mRNA duplexes during a comparatively short hybridization time (1 h) was used as criterion for the selection of highly effective, target-specific siRNA sequences. The accessibility and affinity of the siRNA sequence to the target mRNA site were detected by fluorescence resonance energy transfer (FRET) between a quantum dot (donor) and a fluorescent dye molecule (Cy5, acceptor), which were localized at an appropriate distance apart when hybridization occurred. The FRET signal was observed only in the case of good accessibility and high affinity between an antisense siRNA and a sense mRNA, and was not observed in the case of mismatch siRNAs. Using siRNA sequences with an already known RNAi efficiency and investigating the relationship between their antisense effect and the amplitude of FRET signal, we attempted to determine whether this FRET-based methodology can be used to quantitatively predict the efficiency of siRNA sequences before starting cell transfection and conventional analyses. This would be useful for the secondary selection of the most potent siRNA sequences and for the restriction of the number of sequences to a few siRNAs that are most suitable for the strong down-regulation of the target gene.

In the past several years, FRET has been involved in many biochemical analyses and applied to the development of simple fluorescence detection techniques, including PCR with real-time FRET measurements,[15] DNA hybridization analyses and the formation and dissociation of hairpin structures,[16,17] the elucidation of the dynamics of telomerization or DNA replication,[18] and investigation of interactions between proteins, nucleic acids, and small molecules.[19] Our study extends the limits of FRET analysis in life science research.

Experimental Procedures

A. Preparation of a QD-Conjugated Hybridization Probe. A.1. Synthesis of CdSe QDs. CdSe/ZnS QDs were synthesized, according to the literature (with slight modification).[20,21] Briefly, selenium powder (0.7896 g) was added to trioctylphosphine (TOP, 7.4 g), and the mixture was heated to 150 °C for the preparation of the TOP−Se stock solution. Separately, CdO (0.0254 g) and stearic acid (0.456 g) were heated to 150 °C in argon atmosphere in a three-neck flask. After CdO was completely dissolved, the solution was cooled to room temperature, TOPO and hexadecylamine (HDA, 5 g of each) were added, and the mixture was heated to 300 °C. At this temperature, the TOP−Se stock solution was rapidly injected into the reaction chamber (containing cadmium precursor) to start nucleation, and the temperature of the chamber was fixed at 240 °C for 10 min to ensure additional growth of CdSe nanocrystals. After cooling to 30 °C, the reaction mixture was dissolved in approximately 30 mL of chloroform. The nanocrystals obtained were purified from nonreacted precursors by precipitation with methanol as described by Manna et al.[22] The precipitates were collected using a 0.1 μm VPDF Millipore filter at 700 rpm and redissolved immediately in the same amount of chloroform (containing HDA). To prepare ZnS shell-coated QDs, CdSe nanocrystals were mixed with 5 g of TOPO and 5 g of HDA, and chloroform was evaporated completely. The solution was heated to 220 °C in argon atmosphere. A capping solution, containing 0.1 mM zinc stearate and hexameth-

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yldisilthiane in 6 mL of TOP, was added slowly to the reaction chamber for 10 min. The reaction mixture was cooled to 50 °C, and CdSe/ZnS nanocrystals were purified by the procedure described above.[22] The obtained CdSe/ZnS QDs were highly fluorescent (quantum yield ~75%) and monodispersed.

The nanocrystals were characterized by UV−vis absorption and photoluminescence spectroscopies. UV−vis absorption and PL spectra were recorded using a Hitachi U-4100 spectrophotometer and a Hitachi F-4500 fluorescent spectrometer, respectively.

Water-soluble COOH-functionalized nanocrystals were obtained by encapsulation with an amphiphilic triblock copolymer (~100 kD) consisting of a polybutylacrylate segment, a polyethylacrylate segment, and a poly(methacrylic acid) segment (purchased from Sigma). The polymer/QD ratio was 10:1 because molecular geometry calculations have indicated that at least four polymer molecules are required to completely encapsulate one QD. The procedure for the water solubilization of CdSe/ZnS nanocrystals has been described in detail by Gao et al.[23] The stable encapsulation of QDs with a triblock copolymer (e.g., no aggregation) was achieved. The encapsulated QDs were finally dissolved in PBS (consisting of 150 mM $Na_2HPO_4 \cdot 12H_2O$, 150 mM KH_2PO_4, 136 mM NaCl, 2 mM KCl) using Vivaspin filters to replace the solvent. The quantum yield of the water-soluble CdSe/ZnS QDs was ~37%.

In the present study, all experiments were conducted with CdSe/ZnS QDs with a PL maximum at 580 nm ($\lambda_{ex} = 420$ nm). The thermostability of QDs was verified under hybridization and PCR conditions. The quantum yield did not change significantly under the following conditions: 1 min at 60 °C, 30 s at 90 °C, 56 PCR cycles; 1 h at 65 °C; however, it decreased slightly (from 37 to 32%) over a period of 8 h at 65 °C.

A.2. Conjugation of CdSe Quantum Dots with Single-Stranded siRNA. Amino-modified single-stranded 21−23-mer oligoribonucleotides (ss/siRNAs) were purchased from Fidelity Systems Inc. (USA) and Proligo (Japan). They consisted of oligoribonucleotides conjugated covalently with a 22-, 31-, 39-, or 72-atom noncharged amino linker.

COOH-functionalized QDs were conjugated with NH_2-modified ss/siRNAs using carbodiimide chemistry. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride (EDAC, Molecular Probes), was used as a zero-length cross-linker. The conjugation protocol is simple and takes approximately 4 h.

Briefly, 500 μL of COOH-functionalized QDs (0.1 μM in 40 mM PBS, pH 7.3; QD concentration was calculated by Yu et al.[24]) was incubated with 250 μL of NH_2-modified ss/siRNAs (2 μM in distilled water) and 250 μL of freshly prepared EDAC (60 mg/mL in distilled water). The incubation was carried out for 3 h with shaking in the dark at room temperature. QD−siRNA conjugates were purified by two-step ultrafiltration (Omega Nanosep filters) to remove nonconjugated QDs first and nonconjugated siRNAs second. Aliquots of the nonconjugated siRNA fraction were used for detecting their concentration by OliGreen (Molecular Probes) and the subsequent calculation of the percentage of siRNA conjugated with QD. The efficiency of conjugation was ~68% (it was possible to enhance the efficiency of conjugation up to 95% using streptavidin-coated QDs and biotin-modified ss/siRNAs). The QD−siRNA conjugates were characterized by gel electrophoresis with fluorimetric detection, using commercially available FITC-ss/siRNA (Qiagen) as a standard. The QD:siRNA molar ratio was calculated to be approximately 1:3 to 1:8, depending on the length of the siRNA amino linker. The QD−siRNA conjugates were finally resuspended in 100 μL of 50 mM PBS (pH 7.3). All reagents were prepared in RNase/DNase-free water, and the conjugation was carried out in glass tubes.

Detailed information on QD conjugation with oligonucleotides, used in our study, can be found in EviTags protocols (Evident Technologies, www.evidenttech.com).

B. Preparation of a Cy5-Labeled Hybridization Sample. B.1. mRNA Isolation and Amplification. mRNA was isolated from bcr-abl positive K-562 cells ($1-5 \times 10^6$ cells), using an oligo(dT)-cellulose spin column and QuickPrep mRNA purification kit (Amersham Pharmacia Biotech). The isolated mRNA was essentially free of DNA and protein.

Total mRNA (500 ng) was subjected to linear amplification (using MMLV−RT and T7 polymerase) in the presence of Cy5−CTP (for Cy5-labeling) following the instruction of the Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies, with slight modifications). The amplification was carried out for 6 h, and the consistent cRNA yield was calculated at 200−250X amplification, depending on the sample.

The amplified and Cy5-labeled bcr-abl mRNA was purified on a second spin column, containing 1000 nm white carbonyl microspheres (Interfacial Dynamics Co.) conjugated with a 42-mer oligonucleotide, which was antisense to the 3′ end sequence of bcr-abl mRNA (excluding poly(A) fragment). The oligonucleotide was initially attached to a 39-atom amino linker (Fidelity Systems Inc., USA) and then conjugated with carbonyl microspheres using carbodiimide chemistry (cross-linker, EDAC, Molecular Probes). Briefly, 250 μL of 4 mg/mL white carbonyl microspheres was washed twice with 2 mL of MES buffer (Sigma, pH 6.0) on a 0.1 μm Millipore filter (using low-speed centrifugation at 600*g* for 5 min). The activated microspheres were finally dissolved in 500 μL of MES to obtain a solution of 2% solids (2 mg/mL). Four hundred and fifty microliters of microspheres was mixed with 150 μL of oligonucleotide (12 μM in MES), 150 μL of freshly prepared EDAC (60 mg/mL in MES), and 150 μL of freshly prepared *N*-hydroxysulfosuccinimide, sodium salt (NHSS, Molecular Probes, 68 mg/mL in MES). The incubation was carried out for 4 h at room temperature, followed by washing with MES (pH 6.0) on a 0.1 μm Millipore filter (Amicon) using low-speed centrifugation (600*g* for 5 min) to remove nonconjugated oligonucleotide and byproducts. This conjugation procedure was repeated twice to saturate carbonyl microspheres with the oligonucleotide. Finally, the microspheres were washed with PBS (pH 7.3) and resuspended in 900 μL of the same buffer. Forty microliters of this suspension was used for the detection of oligonucleotide concentration in the microspheres, measured by OliGreen (Molecular Probes). In parallel, the concentration of the nonconjugated oligonucleotide was also measured to clarify the result. The efficiency of conjugation of the amino-modified oligonucleotide with carbonyl microspheres was calculated to be 68%. All the reagents used were prepared in RNase/DNase-free water, and the conjugation was carried out in glass tubes.

The total mRNA was added to the spin column containing the antisense oligonucleotide conjugated with 1000 nm carbonyl microspheres and incubated with gentle shaking for 30 min at room temperature. The column was subjected to low-speed centrifugation (350*g* for 2 min), and the liquid, containing nonbound mRNA, was discarded. The bcr-abl mRNA, retained on the column, was eluted from the matrix with an elution buffer [10 mM Tris-HCl (pH 7.5) and 1 mM EDTA; prewarmed at 68 °C). The bcr-abl mRNA from the eluate (0.5 mL) was precipitated with 50 μM of 5 M potassium acetate (pH 5.0), 10 μL of 20 mg/mL glycogen in DEPC-treated water, and 1 mL of 95% ethanol for 30 min at −20 °C. The precipitated bcr-abl mRNA was resuspended in RNase/DNase-free water, and its concentration was determined spectrophotometrically at 280 nm.

B.2. Cy5−mRNA/QD−siRNA Hybridization and FRET Analysis. Twenty-five microliters of bcr-abl Cy5−mRNA (2.5 μg of mRNA) was incubated with 25 μL of QD−ss/siRNA (containing approximately 0.4 μM QD and 3 μM ss/siRNA) for 1 h at 65 °C in the dark. The short hybridization time allows the detection of siRNA/mRNA duplexes only if the hybridization is extremely effective. Usually, the standard

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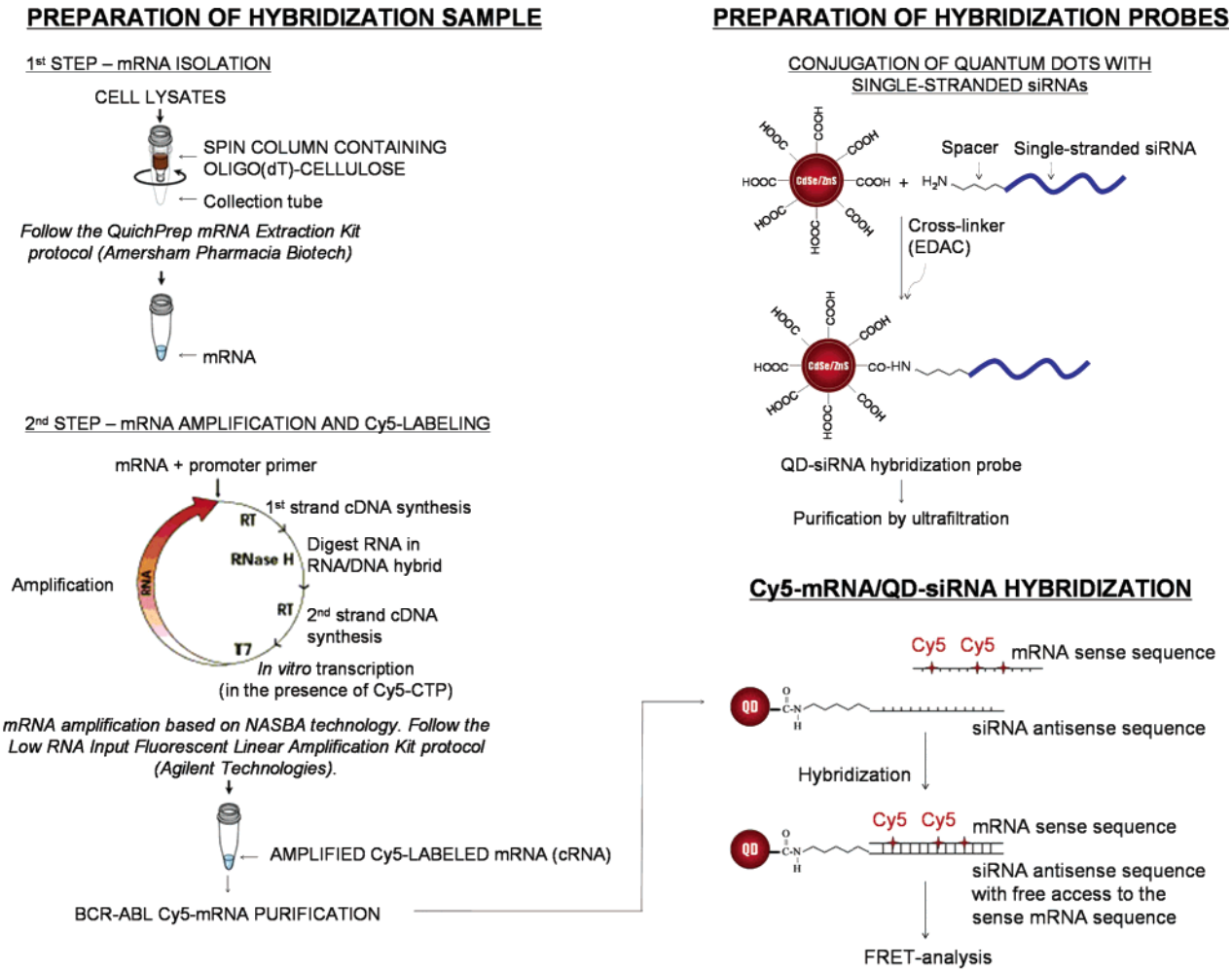


Figure 1. Scheme of experimental design.

protocols for the hybridization of short oligonucleotides with native mRNA require at least 4 h at 65−70 °C. The reaction mixture was transferred to microcuvettes and subjected to FRET analysis. The sample was excited at 420 nm, and the emission spectrum was recorded at 490−700 nm. The changes in both λ_{em}^{max} of QD (∼580 nm) and λ_{em}^{max} of Cy5 (∼660 nm) were monitored. In the absence of hybridization, Cy5 showed no fluorescence at λ_{ex} = 420 nm. The Cy5 fluorescence at ∼660 nm was observed only when hybridization took place, which provided a possibility of selecting siRNA sequences with high accessibility and affinity to bcr-abl mRNA.

In a parallel experiment, the efficiency of hybridization was analyzed using PicoGreen (Molecular Probes), strictly following the protocol of the dsDNA Quantification kit and using a nonlabeled bcr-abl cDNA (corresponding to bcr-abl mRNA) and ssDNAs (corresponding to ss/siRNAs) to avoid the influences of QD and Cy5 on PicoGreen fluorescence (λ_{ex} = 480 nm, λ_{em}^{max} = 520 nm).

Results and Discussion

Basic Principles and Experimental Design. The experimental design is schematically shown in Figure 1.

Cy5−mRNA, which was isolated from cell lysates (obtained from bcr-abl positive K-562 cells) using oligo(dT)-cellulose chromatography on a spin column, was used as a hybridization sample. The total mRNA was amplified using NASBA technology in the presence of Cy5−dCTP. The target Cy5−mRNA (corresponding to bcr-abl oncogene) was purified on a second spin column, containing 1000 nm microspheres conjugated with the 42-mer oligonucleotide, which was antisense to the 3′ end of bcr-abl mRNA (excluding poly(A) fragment). QD−siRNAs (antisense to bcr-abl mRNA) were used as hybridization probes. The target Cy5−mRNA was subjected to hybridization with QD−ss/siRNAs for 1 h at 65 °C. This short hybridization time enabled us to find siRNA sequences with extremely high accessibility and affinity to bcr-abl mRNA. After 1 h incubation, the Cy5−mRNA/QD−siRNA mixture was analyzed by spectrofluorimetry to estimate the FRET capacity between QD and Cy5. The sample was excited at 420 nm, and the emission spectrum was recorded at 500−700 nm. The emission maximums of QD and Cy5 were observed at ∼580 and ∼640 nm, respectively. At a 420 nm excitation wavelength, Cy5 showed no fluorescence. At this excitation wavelength, Cy5 fluorescence can be observed only as a result of FRET from QD to Cy5 if both fluorophores are at an appropriate distance. If QD−siRNA and Cy5−mRNA are at low concentrations (e.g., on the nanomolar or micromolar scale), FRET can be observed only in the case of effective sample/probe hybridization. Thus, the FRET signal can be used as a criterion for screening siRNA sequences with high accessibility and affinity to the respective target mRNA. However, above some critical QD−siRNA and Cy5−mRNA concentrations (even in the micromolar scale), FRET can also be observed as a result of the random events between both fluorophores, and appropriate controls have to be used to

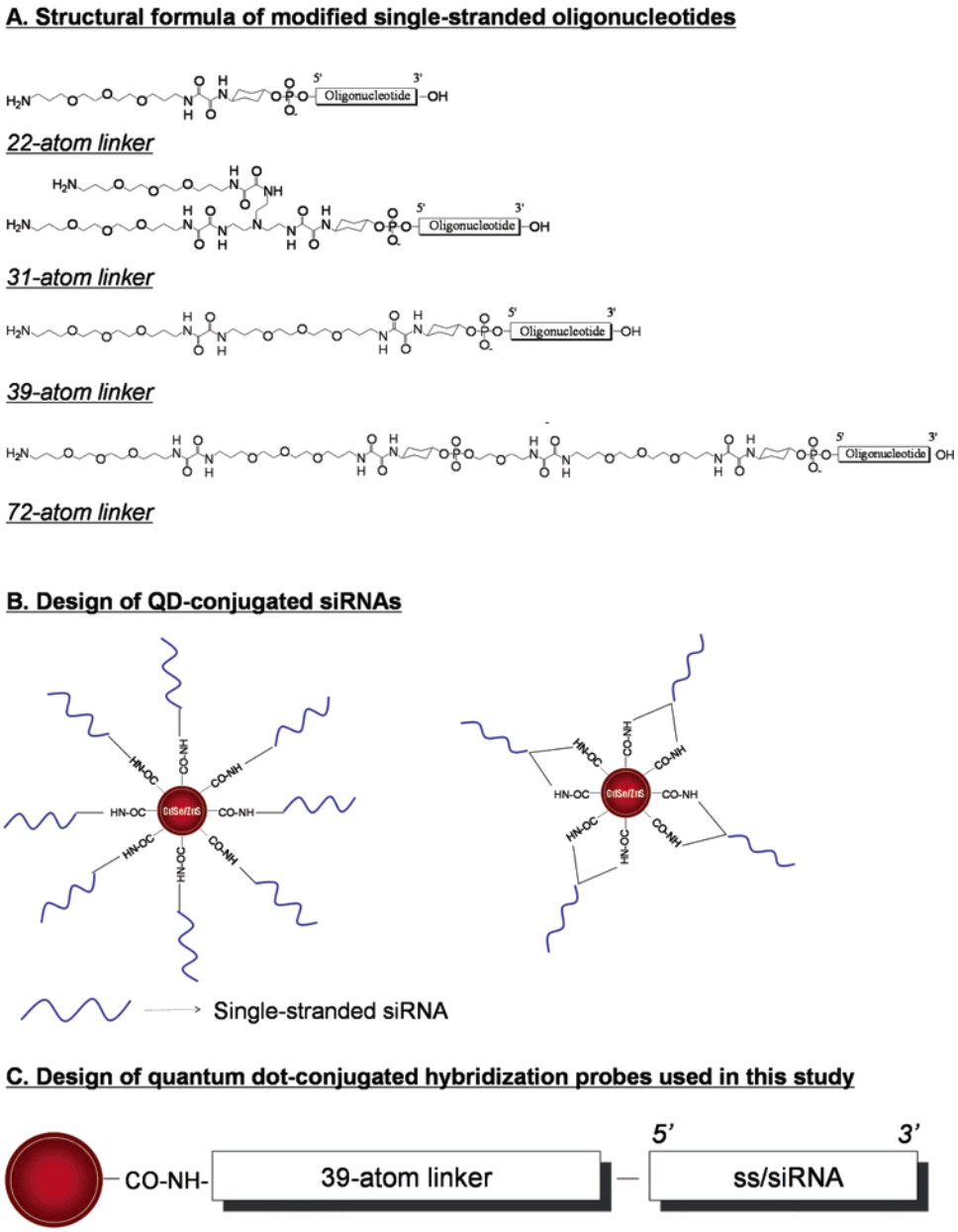


Figure 2. Design of quantum dot-conjugated single-stranded siRNA hybridization probe.

distinguish specific signal from nonspecific signals. In our study, a QD-labeled mismatch siRNA was used as a negative control.

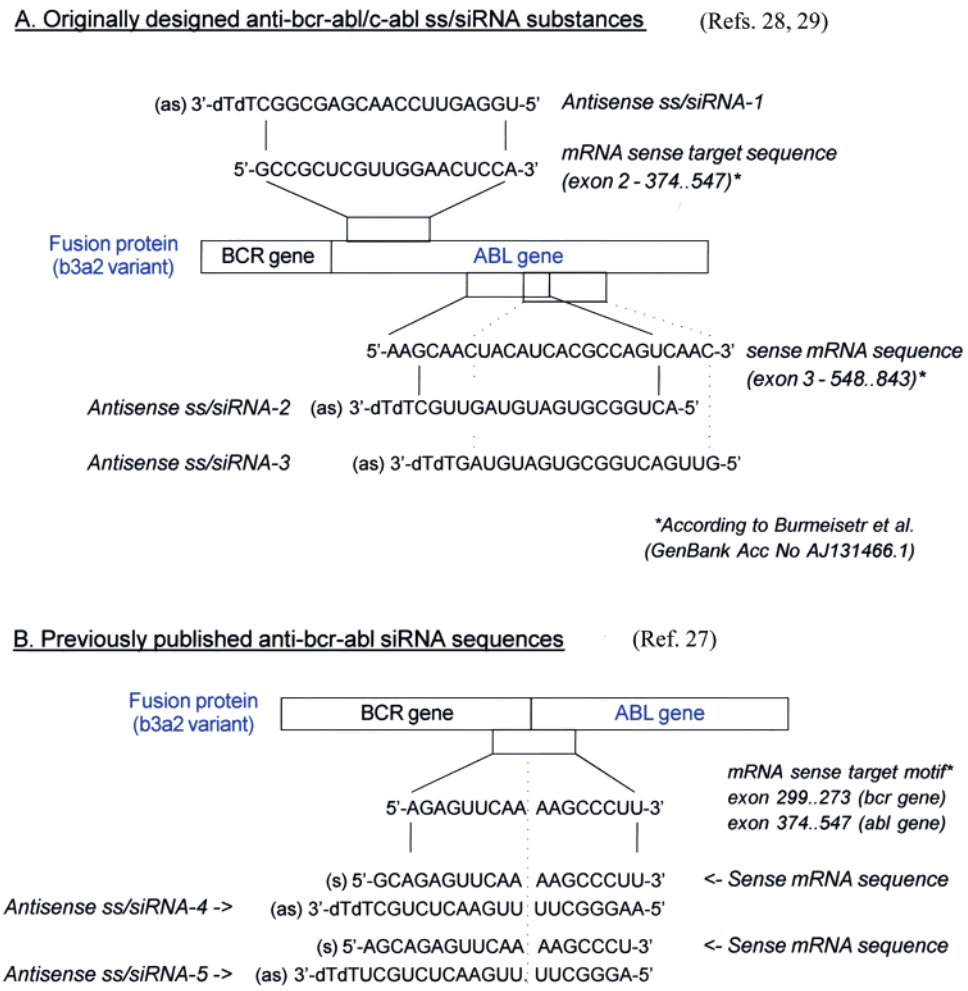
Design of QD−siRNA Hybridization Probes. The design of QD-conjugated single-stranded siRNAs is shown in Figure 2. Amino spacers of different lengths were used to functionalize siRNA, by attaching an NH_2 group at the 5′ end (Figure 2A).

The selection of spacer length was aimed to facilitate the conjugation between QD and siRNA (overcoming the negative charge of both components), as well as to avoid the influence of QD (which is a comparatively large particle) on hybridization. At the same time, the spacer has to ensure an appropriate distance between QD (attached to the hybridization probe) and Cy5 (attached to the hybridization sample) for effective FRET to occur.

NH_2-functionalized ss/siRNAs were conjugated with COOH-functionalized CdSe/ZnS QDs using EDAC as a zero-length cross-linker and carbodiimide chemistry (Figure 2B). QD−siRNA conjugates were purified by two-step ultrafiltration, the details of which are described in Experimental Procedures. The efficiency of conjugation was about 60−77% and was found to increase with spacer length. The QD:oligonucleotide ratio was calculated to be 1:3.4, 1:3.9, 1:6.2, and 1:8.5 in the case of 22-, 31-, 39- and 72-atom spacers, respectively.

In preliminary experiments, we used two single-stranded oligonucleotides with randomly selected complementary sequences (a 22-mer ribonucleotide conjugated with QD and a 22-mer ribonucleotide with incorporated Cy5) as standards to clarify the efficacy of hybridization and FRET capacity between the QD-conjugated probe and the Cy5-labeled sample. It was found that the use of QD conjugated to a 39-atom spacer as a hybridization probe resulted in an optimal hybridization efficacy with a high FRET capacity. In all additional experiments, we used QD-conjugated hybridization probes containing 39-atom spacers (Figure 2C).

Accessibility and Affinity of QD−siRNA Sequences to Cy5−mRNA Target− FRET Analysis. The essential require-



siRNA sequence	Mode of treatment	Bcr-abl mRNA, (% from control)	Bcr-abl protein, (% from control)	Ref. No
siRNA-1	6 days*	45.7±11.2[a]	73.4±14.7[c]	14, 15
siRNA-2	6 days*	8.3±5.1[a]	44.5±10.5[c]	14, 15
siRNA-3	6 days*	9.9±5.6[a]	51.8±9.8[c]	12, 13
siRNA-4	24 hours**	24.8±3.7[b]	-	13
siRNA-5	24 hours**	32.4±1.2[b]	-	13

Figure 3. Anti-bcr-abl siRNA sequences and their RNAi efficiencies in bcr-abl mRNA and oncoprotein in K-562 cells. Table: (∗) Three repetitive siRNA transfections (single dose = 60 nM) at 2 day interval, using lipofectamine. The analyses were carried out 48 h after the third transfection. (∗∗) One siRNA transfection (single dose = 0.5 μg), using electroporation. The analyses were carried out 24 h after the transfection. (a, b, and c) The data shown are mean ±SD from seven (a), four (b), and five (c) independent experiments. Controls are representative of K-562 cells treated with mismatch siRNAs.

ment for FRET is that the emission spectrum of the donor showed overlap with the absorption spectrum of the acceptor. In this context, QDs are the most appropriate donors because their emission maximum can be discretely varied with their size. Using size selection, it is possible to obtain QDs with an emission maximum corresponding exactly to the excitation maximum of the acceptor. In our study, the λ_{em}^{max} of QD was lower (~580 nm) than the excitation maximum of Cy5 (~640 nm). Thus, it was possible to minimize the recovering between QD emission spectrum and Cy5 excitation spectrum and to facilitate data analysis and quantification. FRET requires also close proximity of the donor (quantum dot) and acceptor (Cy5), within 1−10 nm of each other.[30] For most FRET pairs, this distance is about 4.5−5.5 nm for 50% FRET efficiency. According to Gao et al.,[23] the QDs used in our study have to consist of an approximately 5 nm core (2.5 nm radius), a 1 nm TOPO-cap, and a 2 nm thick polymer layer. Thirty nine atom linkers used in our hybridization probe (Figure 2C) would ensure ~3.8 nm distance

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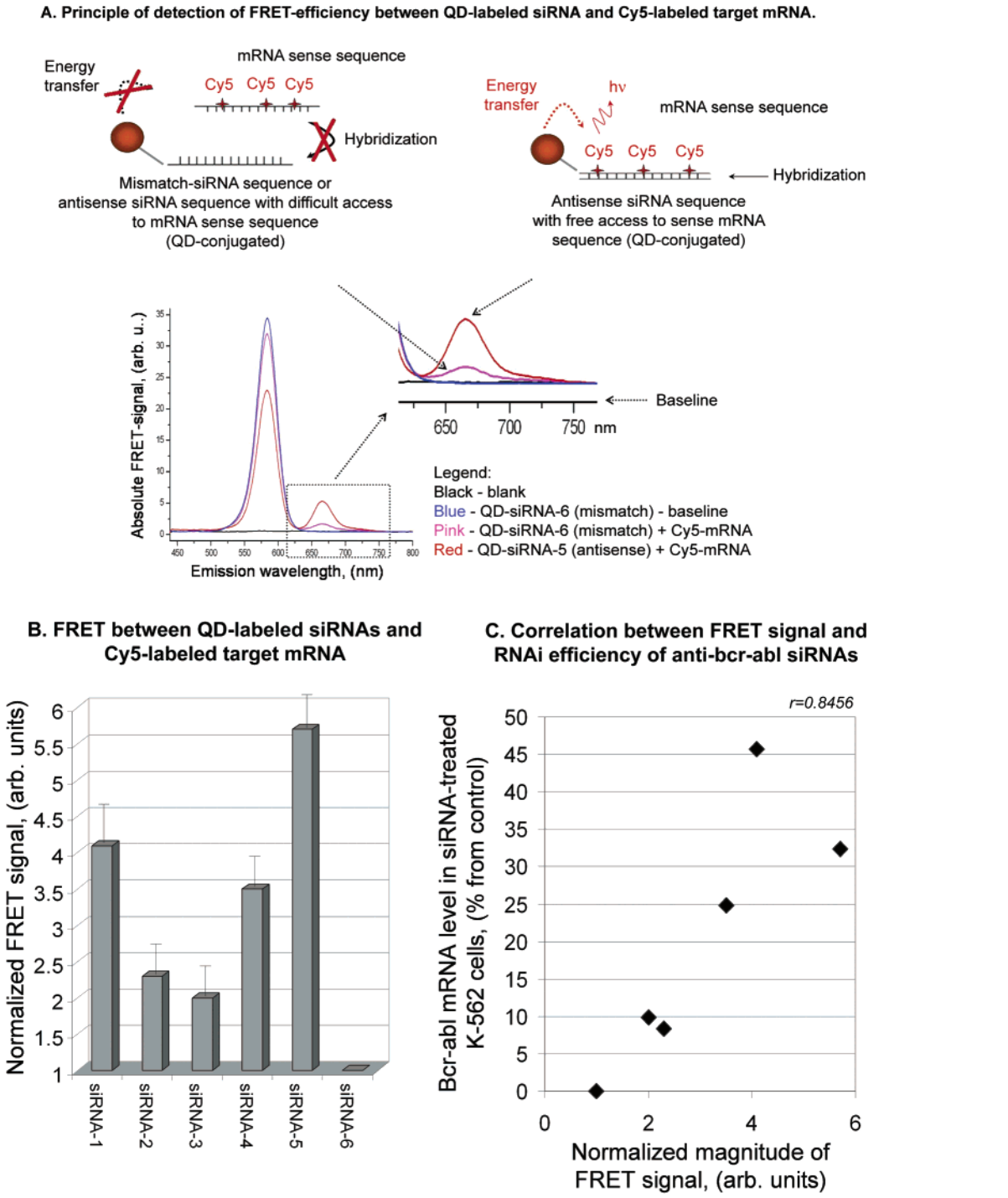

Figure 4. Efficiency of interaction between siRNA hybridization probes and bcr-abl mRNA hybridization sample—FRET analysis. (A) Typical fluorescent spectra of nonhybridized and hybridized QD-labeled siRNA probe and Cy5-labeled mRNA sample (λ_{ex} = 420 nm). 1: QD−siRNA-6 (mismatch). 2: QD−siRNA-6 plus Cy5−mRNA. 3: QD−siRNA-5 plus Cy5−mRNA. (B) FRET between QD-labeled siRNAs and Cy5-labeled target mRNA. *The results on histograms represent the mean ±SD from seven independent experiments for siRNA-1, -2, and -6, and from six independent experiments for siRNA-3, -4, and -5.* (C) Correlation between FRET data (obtained in this study) and effect of anti-bcr-abl siRNAs on the level of target mRNA (obtained from the literature), *r* is the correlation coefficient.

from the donor to the acceptor, which is beyond the typical Forster radius for FRET pairs. In this case, the theoretical FRET efficiency for a single donor−acceptor pair would be less than 20%. However, by increasing the number of acceptors (Cy5) in the hybridization sample (Cy5−mRNA), the FRET efficiency can be increased significantly, up to 40−50%.[19,31] Twenty-two and 31 atom linkers are most appropriate for effective FRET in a single donor−acceptor pair. However, the efficiency of hybridization is significantly lower than that in the case of 39- and 72-atom linkers, which reflects most negatively the absolute FRET signal and the sensitivity of the method.

The siRNA sequences used in our study are shown in Figure 3A,B. They were selected from published reports on RNA interference of the bcr-abl oncogene in Philadelphia-positive

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Table 1. Efficiency of siRNA/mRNA Hybridization Analyzed Using PicoGreen

oligodeoxynucleotide sequence corresponding to	normalized PicoGreen fluorescence at 520 nm (arbitrary units)[a]
siRNA-1	5.2 ± 2.8
siRNA-2	4.5 ± 2.6
siRNA-3	2.7 ± 1.1
siRNA-4	5.8 ± 2.1
siRNA-5	5.5 ± 2.9
siRNA-6 (mismatch)	0

[a] Data are normalized to PicoGreen fluorescence of ss/cDNA.

K-562 cells, and their RNAi efficiency is shown in Figure 3C.[25−29] A mismatch siRNA sequence (ss/siRNA-6) was used as a negative control (3′-dTdTCUGUCCGACUCUGACAAAC-5′). This sequence cannot hybridize with the mRNA target and can be used in detecting the baseline of QD-mediated Cy5 excitation as a result of random events. All data from hybridization and FRET analyses (Figure 4) were normalized to data for this control sample.

Figure 4A shows typical photoluminescent spectra of QD and Cy5 in the absence (mismatch siRNA) or presence (antisense siRNA-5) of hybridization. The mismatch control showed a weak Cy5 fluorescence at ~660 nm, but no hybridization was detected (Table 1). This is indicative of the FRET capacity between both fluorophores as a result of nonspecific random events. All spectra were recorded using approximately 300 nM mRNA and 3 μM siRNA. The use of 2-fold decreasing in mRNA and siRNA concentrations completely abolished the nonspecific FRET signal of the mismatch siRNA (data are not shown).

For the antisense siRNA sequence, the Cy5 fluorescence at ~660 nm increased markedly, which is indicative of effective FRET between the fluorophores (Figure 4A). The histograms in Figure 4B show the mean ±SD data from the FRET analysis of each QD−siRNA sequence, normalized to the data for the mismatch control. The normalized magnitude of FRET-based Cy5 fluorescence increased in the following order: siRNA-5 > siRNA-1 > siRNA-4 > siRNA-2 = siRNA-3. All QD−siRNA sequences in Figure 4B were applied at equal concentrations to mRNA target. The sensitivity of the method was calculated to approximately 40 nM mRNA and 120 nM siRNA. Below these concentrations, it was impossible to detect the FRET signal. The optimal mRNA/siRNA ratio for the effective FRET between QD and Cy5 was 1:6 (mol:mol).

The data from hybridization analyses (carried out in parallel using PicoGreen and nonlabeled oligonucleotides and cDNA corresponding to bcr-abl mRNA) are presented in Table 1.

Since the concentration of single-stranded fragments in the samples was considerably higher than that of double-stranded DNA duplexes, the noise was high and the standard deviations were ±40−50% from the mean values. Despite the restrictions of hybridization analysis used in our study, the results demonstrate that, for mismatch siRNA, we could not expect hybridization, while for antisense siRNAs, the efficiency of hybridization with the target mRNA was comparatively the same for siRNA-1, -2, -4, and -5, and a little bit lower for siRNA-3.

There was a very good positive correlation ($r = 0.8456$) between the FRET data obtained in our study and the effect of siRNA sequences on the level of bcr-abl mRNA determined by RT-PCR analysis (Figure 4C). It is necessary to note that the RT-PCR data have been obtained in different studies, using different transfection methods and experimental protocols, and the comparison with our FRET is only one example.

Conclusions

The present study describes a design and a fabrication of quantum dot-conjugated hybridization probes and their application to the development of a comparatively simple and rapid procedure for preliminary screening of highly effective siRNA sequences for RNA interference (RNAi) in mammalian cells, for example, siRNAs with high accessibility and affinity to the respective mRNA target. A single-stranded siRNA was conjugated with a quantum dot and used as a hybridization probe. The target mRNA was amplified in the presence of Cy5-labeled nucleotides, and Cy5−mRNA served as a hybridization sample. The formation of siRNA/mRNA duplexes during a comparatively short hybridization time (1 h) was used as a criterion for the selection of highly effective, target-specific siRNA sequences. The accessibility and affinity of the siRNA sequence for the target mRNA site were determined by fluorescence resonance energy transfer (FRET) between a quantum dot (donor) and a fluorescent dye molecule (Cy5, acceptor) localized at an appropriate distance from each other when hybridization occurred. The described protocol is comparatively rapid (requires about 4 h) and can be used for the secondary restriction of potentially effective siRNA sequences to obtain a few siRNAs most suitable for the strong down-regulation of the target gene, after their preliminary screening using databases. The results showed that the amplitude of the FRET signal correlates significantly with the specific antisense effect of siRNA sequences on the level of the sense mRNA, as determined by RT-PCR analysis. Presumably, this methodology is also appropriate for quantitatively predicting the efficiency of siRNA sequences before the cell transfection and conventional analyses.

The commercial availability of such products can markedly facilitate the screening of truly effective siRNAs and significantly shorten the time-consuming siRNA screening procedures. Appropriate QD-labeled hybridization probes and the proposed FRET-based methodology are also applicable to microarray data validation.

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