Activation of different split functionalities upon re-association of RNA-DNA hybrids

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Abstract

Split-protein systems, an approach that relies on fragmentation of proteins with their further conditional re-association to form functional complexes, are increasingly used for various biomedical applications. This approach offers tight control of the protein functions and improved detection sensitivity. Here we show a similar technique based on a pair of RNA-DNA hybrids that can be generally used for triggering different split functionalities. Individually, each hybrid is inactive but when two cognate hybrids re-associate, different functionalities are triggered inside mammalian cells. As a proof of concept this work is mainly focused on activation of RNA...
interference; however the release of other functionalities (resonance energy transfer and RNA aptamer) is also shown. Furthermore, in vivo studies demonstrate a significant uptake of the hybrids by tumors together with specific gene silencing. This split-functionality approach presents a new route in the development of “smart” nucleic acids based nanoparticles and switches for various biomedical applications.

Split-protein systems also known as protein fragment complementation assay have been increasingly used for the regulation of enzymatic activities as well as for the rapid detection of proteins, nucleic acids and small molecules\textsuperscript{1–3}. Their use is currently evolving towards biomedical applications\textsuperscript{3}. The splitting of functional proteins into non-functional fragments and their further conditional re-association resulting in a completely restored original function, has allowed for tight control over the functionalities as well as a very high sensitivity for detection. The development of an equivalent technology employing nucleic acids based functionalities may greatly benefit the expanding field of RNA nanotechnology\textsuperscript{4}. In the past several years, there has been a significant increase in interest in using RNA interference (RNAi) for biomedical applications\textsuperscript{5–10}. RNAi is a posttranscriptional sequence specific process of gene silencing employing double-stranded RNAs (dsRNAs) and a set of specific proteins and enzymes\textsuperscript{11–14}. To briefly explain the mechanism, the RNaseIII-like enzyme, Dicer, processes dsRNAs into shorter duplexes (21–23 bp)\textsuperscript{15, 16}. These duplexes, referred to as short interfering RNAs (siRNAs), are then loaded into a RNA-induced silencing complex (RISC) and one of the siRNA strands, called passenger or sense, is discarded. The other strand, called guide or antisense, is used by RISC to recognize the target mRNA for cleavage and translation prevention\textsuperscript{17}. RNAi has become a powerful technique for selective suppression of particular genes of interest in different species showing potential for use in cancer and HIV therapeutics\textsuperscript{5, 6, 10, 18}. Synthetic siRNAs against particular genes of interest can be exogenously introduced into cells to activate RNAi. Moreover, introduction of synthetic asymmetric Dicer substrates slightly longer than siRNAs (25 bp) increases the potency of silencing\textsuperscript{19, 20}. This can be explained by the involvement of Dicer in the process of loading the RISC with siRNAs\textsuperscript{21}. Due to the nature of the enzyme, it is known that Dicer is unable to cleave RNA-DNA (R/DNA) hybrids\textsuperscript{22}. It has also been shown that the substitution of one or both siRNA strands with DNA inactivates RNAi\textsuperscript{23–25}. Therefore, we propose to split the functionality of Dicer substrate siRNAs (or traditional siRNAs) into two R/DNA hybrids (Figure 1), which upon simultaneous presence inside the same diseased cell will recognize each other through toehold interaction within the DNA portion\textsuperscript{26}, re-associate, and release siRNA. Besides allowing for additional control over the RNAi activation, this new approach may also help to overcome some challenges currently associated with the stability and delivery of siRNAs (such as intravascular degradation\textsuperscript{27}). Moreover, any additional functions (such as fluorescent dyes, targeting agents, etc) can be introduced through chemical modifications of the DNA strands in the hybrids thus avoiding interference with the properties of the released siRNAs. Since the modifications of both the 5’- and the 3’-sides of the DNA strands are possible, the number of the additional functionalities can be at least as large as twice the number of DNA strands entering the composition of hybrids.
Design and in vitro studies of R/DNA hybrids

As a proof of principle, we designed several pairs of hybrids which upon re-association release asymmetric Dicer substrates against enhanced green fluorescent protein (eGFP)\textsuperscript{20}, HIV-1\textsuperscript{28}, or glutathione S-transferaseP1 (GSTP1)\textsuperscript{29}. The design rationale of hybrids is the following (Figure 1 and S1): Dicer substrate siRNAs are split between two R/DNA hybrids preventing them from being diced and thus, making them non-functional (S1, step 1). Next, each of the hybrid DNA strands is decorated with a complementary toehold required for hybrid re-association (step 2) resulting in Dicer substrate siRNA release. The complementary single-stranded toeholds in the R/DNA hybrids are designed using Mfold\textsuperscript{30} to avoid any stable secondary structures. In order to exceed a melting temperature ($T_m$) of 37°C, the minimal length of the unzipped toeholds with GC content ≥60% should be at least 12 nucleotides (nts). The $T_m$ for the designed single-stranded toeholds is estimated to be ~40°C using the Wallace rule\textsuperscript{31}. The relative thermodynamic stabilities for the DNA, R/DNA and RNA duplexes can be ordered with the highest for RNA and the lowest for DNA duplexes respectively\textsuperscript{32}. Therefore, the driving force for re-association is the difference in free energies ($\Delta G = \sim -19.5$ kcal/mol, SI, Eq.4) between the initial (hybrids (25 and 27bps) with $\Delta G = \sim -85.4$ kcal/mol, SI, Eq.2) and the final (siRNA (25 bp) and DNA duplex (39bps) with $\Delta G = \sim -104.9$ kcal/mol, SI, Eq.3) states. Free energies of dimerization for the RNA and DNA duplexes were calculated using NUPACK\textsuperscript{33}. All hybrids containing the sense strand will be referred to as “H_s” and hybrids containing the antisense strand as “H_ant”. All sequences are listed in Supporting Information.

Engineered hybrids and Dicer substrate siRNAs were tested for their ability to be processed by human recombinant Dicer as described previously\textsuperscript{8, 34}. Native gel shift assays presented in supporting Figure S2a confirmed previously published observations\textsuperscript{22} that human enzyme Dicer is inactive against individual R/DNA hybrids but cleaves RNA duplexes. Thus, preliminary dicing results support the idea that only re-associated hybrids will be processed by Dicer and further loaded into RISC activating RNAi.

It is known that in a biological context, naked siRNAs can be rapidly degraded by nucleases and therefore, to increase the retention time of the functional siRNAs in the blood stream, chemically modified dNMPs are often introduced\textsuperscript{4, 35}. However, R/DNA hybrids were reported to be well protected in the blood serum\textsuperscript{27}. The relative stabilities measured for R/DNA hybrids are significantly higher than those of corresponding siRNAs (Figure S2b). This feature of the hybrids confirms previous observations and confers them the advantage of potentially longer circulation time in the blood stream.

To introduce the additional functionality of imaging the triggered response to delivery and re-association of hybrids in cells and to study their interactions in vitro, the 3’-side of antisense and the 5’-side of sense binding DNAs were fluorescently tagged with Alexa488 and Alexa546 respectively (Figure 2a). These dyes are commonly used in Förster resonance energy transfer (FRET) studies\textsuperscript{36}. When two fluorescently labeled hybrids are mixed and incubated at 37°C, their re-association places Alexa488 within the Förster distance ($R_0=6.31$nm) of Alexa546. As a result, when excited at 460 nm, the emission of Alexa546 tremendously increases and the signal of Alexa488 drops compared to a control system of
the pre-made fluorescently labeled DNA duplexes unable to recombine (Figure 2b and S3). Titration experiments for different concentrations were carried out to determine the lowest sensitivity concentration (~5nM) of cognate hybrids at which FRET can be recorded (Figure S3).

The kinetics of re-association can also be studied using the same system. In these experiments, hybrids labeled with Alexa488 were incubated at 37°C for two minutes followed by the addition of hybrids tagged with Alexa546. The process of re-assocation through FRET measurements was tracked every 30 seconds (Figure 2c). The experiments were repeated at several different hybrid concentrations (supporting S4b–f).

The reaction of re-association for hybrids consists of two steps and can be represented by the following equation:

\[
2RD \xrightarrow{k_1} RDDR \xrightarrow{k_2} RR+DD
\]

where R and D stand for single-stranded RNA and DNA respectively.

The first step \((k_1)\) is the self-recognition of the hybrids through the zipping of the toeholds leading to the formation of a tetramer. The second step \((k_2)\) is the re-association which yields RNA and DNA duplexes. The mathematical model (SI) predicts that for high initial concentrations of hybrids the second step is rate limiting and the rate is concentration independent while for low initial concentrations the first step becomes rate determining and the rate is concentration dependent. According to that model, we could fit the data at the different concentrations with a single exponential decay (Figure S4) and show that at concentrations lower than ~30 nM the limiting step of re-association is the zipping of the toeholds, while at higher concentrations, the re-association becomes the rate determining step.

To emphasize the importance of the toehold interactions in the process of re-association and siRNA release, a hybrid without a toehold was tested for its ability to recombine (Figure S4h). The results indicate no significant interactions within three hours of incubation at 37°C thus, providing evidence for the crucial role of toehold zipping in the re-association process.

To mimic the transfection conditions in vitro, fluorescently labeled hybrids were separately pre-incubated with Lipofectamine2000 (L2K), a polycationic carrier used in this work for all in vitro transfection experiments, and then the kinetics of re-association were tracked (Figure 2d). Results demonstrated no re-association between hybrids associated with the L2K in solution. Interestingly, the addition of L2K causes a ~10-fold drop in the fluorescence signals for Alexa488 and 546 (Figure 2d and S5a) and provides good protection (<4% degradation) for duplexes against enzymatic activity (Figure S5b).

As an alternative way to track the re-association, another FRET system based on a duplex having Alexa488 quenched by the IowaBlack fluorescence quencher (Figure 2e and S6) was tested. In this case, re-association separates the quencher from Alexa488 restoring its
emission. Consistent with previous findings, experiments with L2K showed no re-association.

**Intracellular studies of hybrid re-association**

The ability of the hybrids to enter and re-associate within cells was assessed through confocal microscopy. Cells were co-transfected with hybrids labeled with Alexa488 and Alexa546 and imaged through confocal microscopy on the next day (Figure 3 and S7). The punctuated inhomogeneous pattern observed in Figure 3a is consistent with an endosomal location of the fluorescent hybrids, as confirmed by colocalization with endosomal markers (Figure 3c–d). The overlap of the Alexa488 and Alexa546 fluorescence indicates a colocalization characterized by a yellow signal. To further check whether FRET occurs within endosomes, Alexa546 sensitized emission was imaged. The sample was excited at 488 nm and the emission of Alexa546 was collected. The FRET signal remaining upon bleed through correction is presented in Figure 3a (1+4 and 5). However, due to the relatively high endosomal concentration of fluorescently labeled hybrids, the observed FRET could also emanate from close proximity of those hybrids that did not re-associate. To address this point, we used a different system; quenched duplexes (Alexa488/IowaBlack). These duplexes only fluoresce when co-transfected with the cognate hybrids thus, providing additional evidence of the intracellular hybrid re-association (Figure 3b and S8).

To assess the intracellular ability of hybrids to release functionalities (siRNAs), experiments with human breast cancer cells stably expressing eGFP (MDA-MB-231/eGFP) were carried out (Figure 4 and S9–10). First, cells were co-transfected with only one hybrid at a time (H_s or H_ant) and three days after, the level of eGFP expression was analyzed with fluorescence microscopy and flow cytometry. The results demonstrated no silencing in eGFP production caused by the individual hybrids (Figure 4a and S10a). However, when cells were co-transfected with separately prepared complexes of L2K and individual cognate hybrids (H_s/L2K and H_ant/L2K), the level of silencing measured three days after was comparable to the silencing resulting from the transfection with control Dicer substrate siRNA (Figure 4a–b and S10b). Based on the kinetic studies for L2K associated hybrids (Figure 2d–e), we can conclude that the re-association of co-transfected hybrids and siRNA release takes place not in the media but in cells.

The release of classical siRNAs (21nts) compared to Dicer substrate siRNAs demonstrated a lower silencing efficiency (Figure S11) agreeing with the reported results\(^\text{20}\). As negative controls, unrelated to eGFP silencing, hybrids designed against HIV-1 were used (Figure S10b).

Furthermore, small internally segmented interfering RNAs reported to have a higher silencing potency\(^\text{37}\), were tested with hybrids (Figure S12). In these experiments, the sense strand was segmented into two shorter RNAs that were used together with a DNA strand to make a segmented hybrid (H_segmented_s). The co-transfection results showed a higher silencing efficiency for segmented siRNA hybrids, which is consistent with published data\(^\text{37}\).
The experiments in which the hybrids were co-transfected on two different days also revealed some silencing (Figure 4c). This is additional evidence for the intracellular hybrid re-association releasing siRNAs.

We then investigated the effect of the lengths of the zipping toeholds in hybrids on the efficiencies of siRNA release. The relative silencing of eGFP was analyzed for different toehold lengths (12, 20, and 30 nts) hybrids co-transfected on the same and on two different days. The results (Figure S13) show no difference in silencing for hybrids co-transfected on the same day. However, when the individual hybrids were transfected with a one day interval, the constructs with 30 nts toeholds silenced the most (Figure 4c) compared to the hybrids with 12 and 20 nts toeholds.

**Hybrid delivery and re-association in vivo**

To assess the delivery of hybrids in vivo, bio-distribution experiments were carried out in athymic nude mice bearing xenograft tumors. Hybrids and siRNAs fluorescently labeled with IRDye700 were delivered to the mice by tail-vein injections and in vivo bio-distribution was first evaluated by fluorescence imaging at different time points (Figure 5a). This pilot experiment confirmed uptake of the fluorescent probe by the tumor and for further quantification, major organs, tumor and blood samples were analyzed ex vivo (Figure 5b and S14). The bio-distribution profile shows a significant uptake of hybrids and siRNAs by the tumor compared to other organs. Also, noticeably, the relative concentration of hybrids in mouse blood after three hours post-injection is almost two times higher than the concentration of siRNAs (Figure 5c). This could indicate increased stability of hybrids in blood compared to siRNA.

In addition, we performed in vivo gene silencing of eGFP expressing xenograft mouse models. Hybrids and siRNAs against eGFP were administrated by one intra-tumoral injection into the mice. The extents of silencing were analyzed ex vivo by measuring the fluorescent intensities of eGFP in tumors (compared to the control animals) 5 and 13 days post-injection (Figure 5d–e and S15). Both injections with hybrids and siRNAs resulted in a significant decrease of eGFP fluorescent intensities. These results are in a good agreement with our multiple in vitro silencing experiments, confirming successful silencing of target genes by hybrids. Notably, the extent of silencing induced by the hybrids technique is higher compared to the one obtained through the standard siRNA approach.

**Release of siRNAs (against HIV-1 and cancer) and aptamers**

To demonstrate the generality of the split RNAi functionality approach, hybrids designed against several HIV-1 and cancer genes were also tested in this study. In the case of the HIV-1 target genes, siRNAs previously described by Low et al. were used to design hybrids and corresponding siRNA duplexes. Two main targets – the protease-coding region found in full-length, genomic RNA that encodes the Gag and Gag-Pol polyprotein precursors (Pro_siRNA), and the env mRNA that encodes the HIV-1 glycoproteins (Env_siRNA, located in gp120) were selected. Results presented in Figure 6 demonstrated dose-dependent viral inhibition with siRNA and hybrids. HIV-1 production was inhibited by 85% with only 20 nM of the Pro_Hybrids. Env_Hybrids, which also binds full-length
mRNA encoding Gag and Gag-Pol, reduced virus production by 65% to 80% (Figure 6a). Levels of gp160 and gp120 were also reduced inside the cell (Figure 6b). Inhibition of HIV-1 gp160 and gp120 reached as high as 76% and 82%, respectively (data not shown). The total amount of Gag (Pr55+p24/p25) was reduced on average by 72% with 20 nM of Pro_Hybrids after recombination inside the cell. Env_Hybrids knocked down up to 75% of cellular Gag (Figure 6b). We also tested different approaches to study the toxicity effect of these siRNAs in our system. Toxicity levels were low, as demonstrated by expression of a co-transfected vector encoding Renilla luciferase (Figure S16). Cellular expression levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were also not significantly affected (data not show). These results clearly demonstrate that hybrids can recombine inside the cell and inhibit HIV-1 through different targets.

As a cancer target gene, glutathione S-transferase P1 (GSTP1) was chosen. GSTP1 is the phase II detoxification enzyme that catalyzes conjugation of glutathione to a variety of electrophilic compounds, including anticancer agents. GSTP1 is believed to play an important protective role in tumor cell pathogenesis and survival and its overexpression in cancer cells has been linked to chemoresistance29. Also, GSTP1 has been proposed to inhibit the mitogen-activated protein kinase (MAPK) pathway through direct interaction with c-jun-NH2-kinase1 (JNK1), decreasing sensitivity of cells to drug-induced apoptosis23. Down-regulation of GSTP1 expression by RNAi could be used therapeutically to sensitize cancer cells to chemotherapy. We show that the endogenous GSTP1 protein expression is effectively down-regulated with hybrids individually co-transfected on two different days. Incubation of the A549 lung adenocarcinoma cells with hybrids resulted in significant (~50%) decrease in GSTP1 protein production (Figure S17).

As an additional example not related to RNAi split functionality, we designed hybrids for the release of an RNA aptamer evolved to specifically bind the malachite green (MG) dye (Figure S18)38, 39. MG is a good fluorescent reporter because it has an extremely low emission in water solutions due to efficient internal conversion. However, its fluorescence increases dramatically upon relocation in a viscous environment or in a MG aptamer binding cage. The use of the MG aptamer was reported for sensing ATP40 and native RNAs41, visualization of co-transcriptional assembly42 and RNA nanoparticle formations43 as well as the laser-mediated inactivation of RNA transcripts38. We deactivated the MG aptamer by splitting it in two cognate hybrids which individually do not enhance the fluorescence of MG. However, re-association of hybrids leads to the release and formation of an active aptamer able to bind MG and induce its fluorescence. This exemplifies the applicability of the hybrids technique to other nucleic acids based functionalities.

We have developed a new strategy to design and engineer programmable R/DNA hybrids capable of undergoing triggered release of embedded split functionalities upon their re-association inside cells. R/DNA hybrids have significantly higher stabilities in blood serum compared to the RNAs and the retention time of hybrids in biological fluids can be further improved through substituting the DNA strands with chemical analogs (e.g. locked nucleic acids (LNA), peptide nucleic acids (PNA), etc) without interfering with RNA function. Moreover, the thermal stabilities of toehold interactions initiating re-association can be fine-tuned by altering their lengths and compositions. The use of hybrids allows intracellular
tracking of the delivery and re-association in real time. In the case of RNAi activation, the hybrids approach allows the triggered release of siRNAs in cell culture and in vivo with a pro-longed effect of gene silencing compared to the siRNA in vivo. In addition, this approach may permit a higher control over the targeting specificity (e.g. if two hybrids are decorated with two different tissue specific recognition moieties). The number of possible additional split functionalities can be increased by introducing a branched hybrid structure. This novel approach opens new routes for further developments in the use of nucleic acids based nanoparticles and switching devices conditionally releasing different functionalities for a broad array of nanotechnological and biomedical applications.

**Methods Summary**

All methods listed below are detailed in Supporting Information.

**RNA and DNA sequence design**

Single-stranded DNA toehold sequences were optimized with the mFold program to minimize the occurrence of alternative secondary structure folds. siRNA sequences for the duplex were used from previous studies. The full list of RNA and DNA sequences used is available in SI.

**Hybrid R/DNA duplexes assemblies and native PAGE**

There is a variety of duplex formation approaches detailed elsewhere and in this work, we used the fastest protocol. Native PAGE experiments were performed as described.

**Recombinant human Dicer assay**

For dicing experiments, samples were incubated for 4 hours at 37°C with recombinant human turbo dicer enzyme kit (Genlantis), containing an ultra-active form of human recombinant dicer enzyme, according to the manufacturer’s suggested protocol.

**Human serum degradation studies**

Aliquots of freshly drawn human whole blood serum (blood was allowed to coagulate, then spun down and supernatant was collected) were immediately used for each new study.

**Fluorescence studies**

To assess the re-association of R/DNA hybrids in vitro, FRET measurements were performed using a FluoroMax3 (Jobin-Yvon, Horiba). For all the experiments, the excitation wavelength was set at 460 nm and the excitation and emission slit widths were set at 2 nm.

**Transfection of human cell-lines**

All in vitro transfections in this project were performed using Lipofectamine 2000 (L2K) purchased from Invitrogen. 10x or 50x solutions of R/DNA hybrids were pre-incubated at 30°C with L2K. Prior to each transfection, the cell media was swapped with OPTI-MEM and prepared 10x or 50x hybrid/L2K complexes were added to the final concentration of 1x.
The cells were incubated for 4 hours followed by the media change (D-MEM, 10% FCS, 1% pen-strep).

**Microscopy**

To assess the re-association of R/DNA hybrids in cells, measurements were performed using a LSM 710 confocal microscope (Carl Zeiss) with a 63×, 1.4 NA magnification lens. All images were taken with a pinhole adjusted to 1 airy unit.

**Endosomal co-localization studies**

To confirm the endosomal location of endocytosed fluorescently labeled R/DNA hybrids in cells, co-staining experiments with several endosomal markers (EEA1 and Rab7) were performed.

**Flow cytometry experiments**

For statistical analysis with flow cytometry experiments, the *MDA-MB-231* 231 (with or without eGFP) cells grown in 12-well plates (1.0×10⁵ cells per well) were lifted with cell dissociation buffer, washed twice with PBS and the level of expression of eGFP was determined by fluorescence-activated cell sorting (FACS) analysis on a FACS Calibur flow cytometer (BD Bioscience). At least 20,000 events were collected and analyzed using the Cell Quest software.

**In vivo experiments**

Animal studies were performed according to the Frederick National Laboratory for Cancer Research (Frederick, MD) Animal Care and Use Committee guidelines. Fluorescence imaging (Maestro GNIR-FLEX, Cambridge Research & Instrumentation, Inc, Woburn, MA) was performed at baseline (pre-injection for determining auto-fluorescence), and at different time points post-injection with hybrid (or siRNA) associated with bolaamphiphilic polycationic carriers[^49], while the animal was anesthetized (1–2% isoflurane in O₂ at 1 L/min flow). For silencing experiments, MDA-MB-231 tumor cells expressing eGFP were used. 5 days post subcutaneous tumor cell injection; the mice were injected intra-tumorally with either siRNA or co-injected with R/DNA hybrids, associated with bolaamphiphilic cationic carriers[^49]. Control mice were injected intra-tumorally with PBS buffer. After 5 and 13 days, mice were sacrificed. Tumors were removed from mice and prepared for imaging. Images were captured using Nikon’s Eclipse 80i microscope, with a QImaging Retiga-2000R camera and Nikon’s NIS-Elements AR Imaging Software.

**HIV-1 inhibition by R/DNA hybrids**

To test the potential for HIV-1 inhibition from hybrids after intracellular re-association, Hela cells were co-transfected with the WT HIV-1 molecular clone, pNL4-3, and the R/DNA hybrids. At 48 h post-transfection, the supernatants were harvested and the reverse transcriptase (RT) activity was measured in an *in vitro* reaction. Levels of RT activity are directly proportional to the amount of released virus. Cell lysates were analyzed by radioimmunoprecipitation assay according to the protocol described previously[^50].
Transfection experiments with anti-GSTP1 R/DNA hybrids and immunoblotting

A549 lung adenocarcinoma cells were transfected with R/DNA hybrid. 24-h after addition of the first hybrid, the complement hybrid was transfected using the same protocol. Cells were collected and processed for immunoblotting using standard protocol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Activation of functionality by two R/DNA hybrids. (a) Cartoon showing the general principle of functionality activation upon re-association of two non-functional units. (b) Schematic representation of R/DNA hybrids re-association resulting in asymmetric Dicer substrate siRNA release. The color code is kept the same throughout the figure.
Figure 2.
Fluorescent studies of R/DNA hybrid re-association in solution at 37 °C. (a) Schematic representations of control DNA duplexes fluorescently labeled with Alexa488 and Alexa546 unable to recombine (upper part) and fluorescently labeled R/DNA hybrids programmed for re-association (lower part). (b) Emission spectra of control DNA duplexes showing no FRET and recombined R/DNA hybrids with increased Alexa546 emission signal. (c) FRET time traces during re-association of R/DNA hybrids labeled with Alexa488 and Alexa546. (d) Fluorescently labeled R/DNA hybrids individually associated with L2K prior to mixing.
were followed by fluorescent time tracing. Please note that L2K forms complexes with hybrids thus, preventing their re-association and the emission signal of Alexa488 (in green) stays above Alexa546 (in red) comparing to (e). (e–f) Schematic representations and FRET time traces (in green) during re-association of R/DNA hybrids labeled with Alexa488 and quencher IowaBlack FQ with schematic representation of corresponding hybrids programmed for recombination. Please note that as well as in (d), L2K forms complexes with quenched hybrids (in blue) and prevents their recombination.
Figure 3.
Re-association and localization of R/DNA hybrids in human breast cancer cells (MDA-MB-231) visualized by confocal fluorescence microscopy. (a) FRET experiments: cells were co-transfected with cognate hybrids labeled with Alexa488 and Alexa546 and images were taken on the next day. (b) Dequenching experiments: cells were co-transfected with cognate duplexes, having one duplex labeled with Alexa488 and IowaBlack FQ. Images were taken on the next day. (c–d) Studying the localization of R/DNA hybrids with commonly used markers for endosomal compartments (c) EEA1 and (d) Rab7.

Image numbers in a–d correspond to: differential interference contrast (DIC) images (1), Alexa488 emission (2), Alexa546 emission (3), bleed-through corrected FRET image (4), 3D chart representation of zoomed fragment indicated by a white box of bleed-through corrected FRET image with the yellow star indicating the correspondence (5), EEA1...
antibody staining (6), and Rab7 antibody staining (7). Images (1+2+3), (1+4), (1+2), (1+3+6), (1+3+7) are superpositions of two or three different images.
Figure 4.
GFP knockdown assays for human breast cancer cells (MDA-MB-231/GFP) which stably express enhanced GFP (eGFP). Three days after the transfection of cells with R/DNA hybrids programmed to release siRNAs against eGFP (H_s and H_ant), (a) eGFP expression was observed by fluorescence microscopy and (b) eGFP expression was statistically analyzed with flow cytometry experiments. As the control, siRNA duplexes against eGFP were used. Please note that the individual R/DNA hybrids cause no decrease in eGFP production (supporting Figure S8). (c) Dependency of toehold lengths in R/DNA hybrids co-
transfected on two different days show their ability to knockdown eGFP expression. R/DNA hybrids containing antisense (H_ant) were transfected one day prior to hybrids with sense (H_s). Three days after, eGFP expression was analyzed.
Figure 5.
In vivo and ex vivo studies of R/DNA hybrids in tumor xenograft mouse model. (a) Time-dependant bio-distribution imaging in vivo. In three hours whole mouse image, fluorescent maximums (in red) correspond to the places of injection (1), tumor (2), and blood withdrawal (3). (b) Relative organ uptakes of fluorescently labeled R/DNA hybrids and siRNAs in tumor-bearing mice three hours post tail-vein injection. A relatively high level of hybrid accumulation occurs in tumor tissue. Error bars denote SD; N=3. (c) The amounts of the fluorescent probe (R/DNA hybrids and Dicer substrate siRNAs labeled with IRDye700) in the mouse blood-stream were measured three hours post-injection. Error bars denote SD; N=4. (d) Ex vivo fluorescent imaging of tumors after 5 and 13 days post-injections in vivo demonstrate comparable levels of eGFP silencing caused by siRNA and R/DNA hybrids. (e) Ex vivo quantification (two animals per each experiment) of eGFP expression after 5 and 13 days post-injection.
HIV-1 expression and production is inhibited by siRNAs and recombinated R/DNA hybrids. (a) HeLa cells were transfected with pNL4-3, with and without siRNAs. Virus supernatant was harvested and the Reverse Transcriptase (RT) activity was measured (this assay estimates the amounts of virus produced by the cells); data are shown normalized to virus controls (VC.1 and VC.2) without siRNAs. Mock represents non-treated HeLa cells. Error bars denote SD; N=4. (b) At 48 h posttransfection, HeLa cells were metabolically labeled with [35S]MetCys for 4h. Cell lysates were radioimmunoprecipitated. Positions of envelope
glycoprotein precursor, gp160, and surface glycoprotein gp120; Pr55Gag (Pr55), CA-SP1 (p25) and CA (p24) are indicated. Quantification of total cell-associated Gag: Pr55+p25+p24. Total Gag in virus control (VC.1) without siRNAs set at 100. Error bars denote SD; N=3.