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Functional Aptamers and Aptazymes in Biotechnology, Diagnostics, and Therapy

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1. Introduction

Aptamers are single-stranded nucleic acid molecules that possess properties comparable to those of protein monoclonal antibodies, and thus are clear alternatives to long established antibody-based diagnostic or biotechnological products for research, diagnostics, and therapy.[1−4] This class of functional nucleic acids can fold into complex three-dimensional shapes,[5−7] forming binding pockets and clefts for the specific recognition and tight binding of any given molecular target,[8−10] from metal ions and small chemicals to large proteins and higher order protein complexes, whole cells, viruses, or parasites.

Aptamers can be isolated *in vitro* from vast combinatorial libraries that comprise trillions of different sequences, by a process called "*in vitro* selection", or "SELEX", an acronym for "systematic evolution of ligands by exponential enrichment". An *in vitro* selection experiment comprises a number of sequential steps, the first of which is the generation of a nucleic acid library of random sequences. This starting pool of mainly nonfunctional RNA or DNA sequences is generated using a standard DNA-oligonucleotide synthesizer.[11] The design of such libraries involves the synthesis of a short defined sequence, followed by a random region of variable length and another defined sequence at the 5′-end. This pool of synthetic single-stranded DNA (ssDNA) is amplified in the polymerase chain reaction (PCR), generating several copies of each DNA in its double-stranded form. By *in vitro* transcription, a corresponding library of RNA molecules can be generated which can then be used for the *in vitro* selection. If the transcription reaction contains nucleoside triphosphate derivatives that are chemically modified but still are substrates for RNA polymerases, libraries of modified RNAs can be generated in which sequences are equipped with a broad variety of additional chemical functionalities, normally not present in natural nucleic acids.[12−14] Aptamers selected from chemically modified libraries can, in some cases, be completely resistant toward degradation by nucleases. The additional functional groups can lead to ligands with novel

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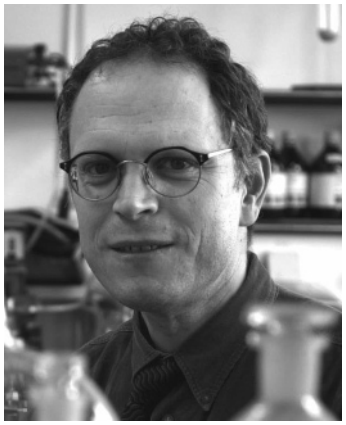

Michael Famulok was born in 1960. He studied chemistry and graduated from the University of Marburg, Germany. From 1989 to 1990 he was a postdoctoral fellow at the Department of Chemistry at MIT. From 1990 to 1992 he was postdoctoral fellow at the Department of Molecular Biology at Massachusetts General Hospital and Harvard Department of Genetics. He began his independent career at the Institute of Biochemistry, LMU Munich, Germany in 1992. Since 1999 he has been Professor of Biochemistry and Chemical Biology at the University of Bonn. Professor Famulok's research interests include *in vitro* selection of combinatorial nucleic acid libraries, evolutive biotechnology, and the application of aptamers and ribozymes for protein function analysis and drug screening.

Jörg Hartig was born in 1974. He studied chemistry at the University of Bonn, Germany. After completing his Ph.D. thesis in the group of Michael Famulok in 2003, he conducted postdoctoral studies from 2003 to 2005 concerning the synthesis and characterization of repetitive sequences at the Department of Chemistry at Stanford University. He is currently Assistant Professor for Chemistry of Biologically Functional Materials at the Department of Chemistry at the University of Konstanz, funded within the Lichtenberg-Program of the Volkswagen Stiftung. His research interests include the development of novel nucleic acid modules for regulating functional RNAs, the characterization of properties and applications of four-stranded nucleic acid motifs such as guanine quadruplexes, as well as the exploitation of such modules for nanobiotechnologies.

physical and chemical properties or can provide additional handles to be utilized for functional improvement. Appropriate modifications can lead to photo-cross-linkable aptamers that covalently attach to their cognate proteins. Alternatively, mixtures of ssDNA or chemically modified ssDNAs can be generated by omitting the transcription step in the SELEX procedure. The selection of DNA aptamers adds the challenge to separate both strands in order to obtain libraries of ssDNA.[15] DNA aptamers are slightly more stable toward nuclease digestion but seem to be less competent to fold into ligand-binding scaffolds. Moreover, RNA aptamers have the advantage of being actively transcribed *in vivo* from suitable templates, whereas DNA aptamers have to be introduced externally.[16] By using combinatorial nucleic acid synthesis, remarkably complex libraries comprising up to 10^{16} different RNA, ssDNA, modified RNA, or modified ssDNA sequences can be produced at once. This number exceeds the diversity of antibodies raised by the immune system by several orders of magnitude.

From this huge diversity, the challenge is to select active molecules from oligonucleotide libraries of such complexity. The high pool complexity ensures the presence of oligonucleotide-structures that are complementary to virtually any shape. Thus, if the pool is incubated with the immobilized ligand, binding sequences will tightly attach to it, so that nonbinding ones can easily be removed. Recently, capillary electrophoresis, among other techniques, has emerged as a convenient and powerful tool to separate bound from unbound sequences.[17−22] The bound material can subsequently be collected, amplified, and used to perform the next selection cycle. Depending on the nature of the ligand, this procedure has to be repeated until the desired ligand-specific aptamers have been enriched. Particular aptamers can then be obtained from the enriched library by cloning and their sequence can be elucidated by standard sequencing protocols.

Besides the possibility to perform the selection itself with modified nucleic acid sequences—which depends on whether the replicating enzymes accept the modified templates and monomer substrates—individual aptamers can also be equipped

Günter Mayer was born in 1972. He studied chemistry and graduated from the University of Munich, Germany. After completion of his Ph.D. thesis at the University of Bonn, he worked as group leader at NascaCell GmbH, Germany, were he headed the department for Combinatorial Biotechnologies. In 2003 he cofounded NascaCell IP GmbH, Germany, a company that focuses on the fee-for-service-based marketing of the aptamer technology. In 2004 he returned to academia, and currently he holds an Assistant Professor position at the University of Bonn. Dr. Mayer's research interests include the exogeneous regulation of aptamer activity with light and the application of *in vitro* selection for the characterization of biomolecules with respect to function, conformation, and identity.

in a modular manner with additional functions[23−25] and therefore can be specifically tailored for many potential applications in biotechnology, molecular medicine, and molecular biology. Moreover, with affinities in the nanomolar to picomolar range, aptamers can bind their target molecules with specificity similar to that of the antigen binding fragment of monoclonal antibodies. As RNA molecules, aptamers can be simply synthesized by the cell's own transcription machinery.[26] Due to their high affinities, they represent excellent candidates for highly specific inhibitors of signal transduction, cell growth, transcription, or viral replication. While nucleic acids in cells and in the blood

degrade rapidly, chemically modified aptamers or aptamers expressed in a certain structural context show dramatically increased stability profiles and are often resistant to nuclease digestion. With the development of appropriate gene transfer systems, the use of intracellular aptamers, or intramers,[16,27] can also be considered in gene therapy procedures to combat infectious diseases and in cancer therapy. An advantage of the nucleic acid-based system would be its greater safety. Being an RNA molecule, provoking cytotoxic immune reactions induced by aptamers have not been observed yet, since the normal protein presentation pathway via T cells does not apply.

Since the first examples of the use of combinatorial nucleic acid libraries for the *in vitro* selection of specific ligand binding RNAs in 1990,[28,29] considerable progress has been achieved in this field. Nucleic acid aptamers for more than a hundred different targets have been described,[30] showing that aptamers can now routinely be obtained for almost every desired target.[30] Aptamer technology is currently in a state in which it demonstrates its potential as a tool for widespread applications in aspects of biotechnology, biomedical research, molecular medicine, diagnostics, and imaging.

In this review, we provide a comprehensive update of some novel and promising developments of *in vitro* selection and aptamer, intramer, and allosteric ribozyme technology. A description of recently discovered natural aptamers, or riboswitches, is also included.

2. Aptamers

2.1. Aptamers as Protein Detecting Reagents

Aptamers have been shown to bind their cognate targets with high specificity and affinity. Dissociation constants can range from micromolar to sub-picomolar values. Moreover, in most cases, aptamers not only bind their cognate protein but also inhibit its function efficiently. Structural data for several aptamer/protein complexes have given insight into the mode of molecular recognition between aptamers and their protein targets that in turn has provided a rationale for the associated inhibitory mechanism. Among them are the crystal structures of the complexes between human α-thrombin and its 15-mer DNA aptamer quadruplex,[31] the structure of human immunodeficiency virus type I reverse transcriptase complexed with its RNA pseudoknot aptamer,[32] the NFκB p50 subunit and its high affinity aptamer,[33] and the structure of the *anti*-RNA polymerase II aptamer FC complexed to RNA polymerase II from yeast[34] (Figure 1). The latter structure, for example, is of relevance for the understanding of gene regulation by natural noncoding RNA molecules (ncRNAs) that bind and inhibit cellular RNA polymerases.[35] Among these is the B2 RNA from mouse, a 178-mer ncRNA that binds Pol II and inhibits transcription of certain genes during heat-shock.[36,37] Because FC and B2 RNAs bind overlapping sites in the Pol II cleft that is also part of the binding site for nucleic acids in an elongation complex, the structure suggested that both the aptamer inhibitor and B2 might prevent DNA entry into the polymerase cleft during initiation. Thus, the template is prevented from reaching the active center and transcription cannot start[34] (Figure 1).

Thus, aptamers represent an interesting class of inhibitory compounds that are readily accessible and can be used for assessing the function of defined protein targets. In fact, owing to the increasing demand for protein inhibitors in the postgenome era, selection routines compatible with automation have been established that allow highly parallel aptamer selections to be performed on several targets at once within a few days.[41−45] The ease by which aptamers can be engineered and chemically modified predisposes them as

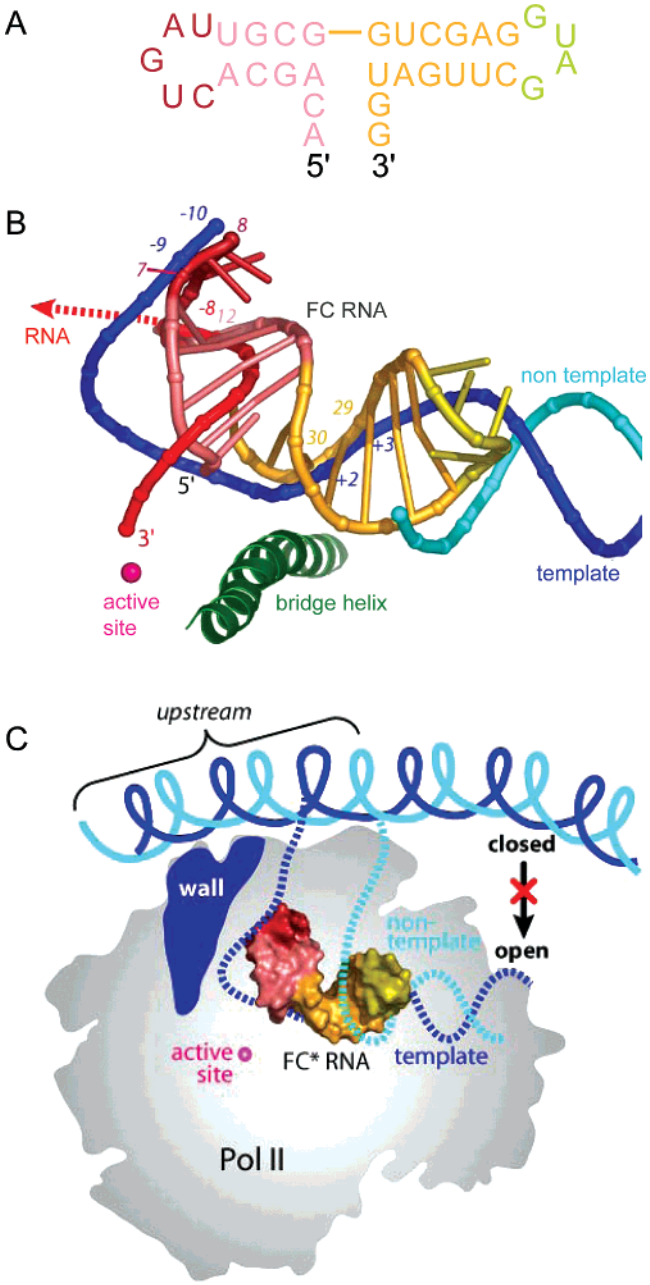

Figure 1. Inhibition mechanism of the anti-Pol II aptamer FC* as proposed by Kettenberger et al.[34] (A) Secondary structure of the FC* aptamer, a truncated version of the FC aptamer,[38] that binds RNA polymerase II from yeast (Pol II) with high affinity. The color code is the same as in parts B and C. (B) Nucleic acids in the Pol II elongation complex share an overlapping but not identical binding site. The Pol II−FC* RNA complex structure was superimposed on the structure of the complete Pol II elongation complex.[39] Phosphate groups at the same location are labeled. The view through this figure is from the side. (C) Inhibition of open complex formation. The upstream region of the DNA promoter in the closed and open complex was modeled on the bacterial RNA polymerase−promoter complex[40] and was extrapolated in the downstream direction. The downstream region of promoter DNA and the template strand in the bubble region of the open complex were modeled according to the complete Pol II elongation complex.[39] FC* RNA is shown as a molecular surface.

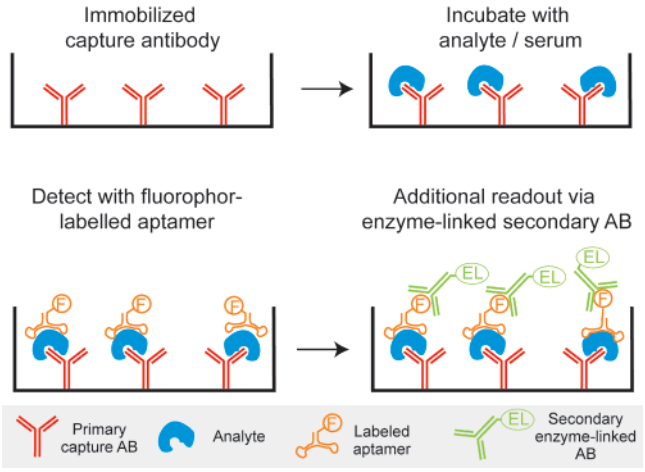

Figure 2. Aptamer-based ELISA technology:[64] A primary capture antibody is immobilized to the plate. After blocking, serum containing the analyte is added. Captured analyte protein can be detected by using analyte-specific aptamers. For detection, the aptamer can be labeled by a fluorophore. In addition, secondary enzyme-linked antibodies that recognize the fluorophore have been used successfully for signal amplification.

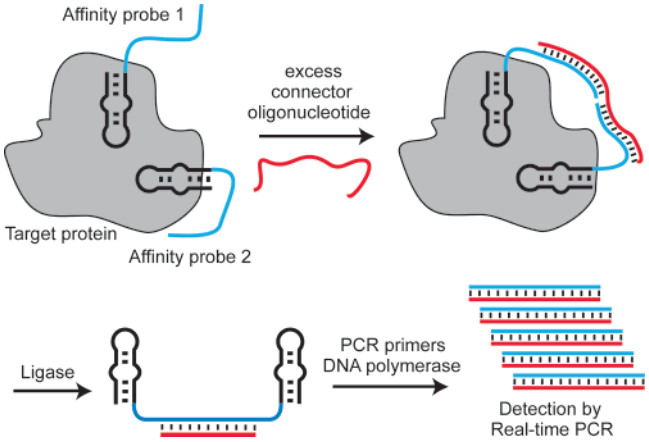

Figure 3. Principle of the proximity ligation assay. Two aptamer-based affinity probes (black) recognize different epitopes of the target protein. The affinity probes are linked to sequences (blue) which are complementary to the connector oligonucleotide (red). After addition of an excess of the connector, ligation of the affinity probes is facilitated due to increased local concentration of both probes. The ligated product is subsequently amplified and detected by PCR, allowing the quantification of minute amounts of target protein.

viable tools for applications that require precise and reliable molecular recognition. Aptamers were employed in western blot assays, FACS analysis, and enzyme-linked assays, showing that they are sophisticated reagents for these purposes. On the other hand, no aptamer has been reported at the present time that is supplied with commercially available diagnostic kits or diagnostic reagents. A plethora of aptamers directed against diagnostically relevant proteins[46−60] (for reviews, see refs 14 and 61−63) bear the potential to be applicable in diagnostic assays. In recent years, various aptamer-based diagnostic assay formats have been developed, some of which will be highlighted in the following chapters.

2.1.1. Aptamers in Standard Diagnostic Assays

There are several diagnostic applications and assay formats in which aptamers have proven their value as diagnostic tools. These comprise western blot analyses, immunoprecipitation (oligonucleotide-precipitation) assays, flow-cytometry, and use as affinity reagents coupled to solid matrices allowing the purification of proteins from crude samples. For such applications, aptamers have been successfully proven and revealed properties equal or sometimes even superior to those of antibodies. Drolet et al. described the use of an aptamer in an enzyme-linked immunosorbent assay (ELISA)-like format (Figure 2) by employing an aptamer that binds to the vascular endothelial growth factor (VEGF) protein.[64]

By this means, they made use of the easy chemical modifiability of aptamers. With a 5′-biotinylated variant of the aptamer, the authors demonstrated that visualization of target bound aptamers in microtiterplates can be achieved by horse radish peroxidase coupled streptavidin (HRP-SA) and enhanced chemiluminescence-based (ECL) detection systems, similar to common antibody applied chemistry. They confirmed that the aptamer provides comparable results to the antibody-based assay. This proved the aptamer as an effective tool for molecular diagnostic applications enabling the measurement of the concentration of its target protein in biological samples. Other publications followed and successfully reported the use of different aptamers binding to L-selectin, tenascin-c, and thyroid transcription factor 1 (TTF1) in identical assays, allowing the specific and sensitive detection of their cognate targets.[65−67] Landegren and coworkers used an ingenious approach to considerably enhance the detection limits of enzyme-linked aptamer-based diagnostic systems.[68−70] Being nucleic acids, aptamers offer a simple but effective route to combine their specific binding properties with the sensitivity of real-time quantitative PCR. Upon binding, two different aptamers that target two distinct epitopes of a protein or protein complex come into close proximity. Subsequently, the free 3′- and 5′-ends of the two aptamers can be ligated by a complementary splint oligonucleotide added to the reaction mixture. Finally, the resulting ligation product can be amplified with two primers specific for each of the aptamer sequences in real time (Figure 3). The authors demonstrated that as few as 400 attomoles of the target protein can be detected by this assay, thereby making it 1000-fold more sensitive than a conventional ELISA.[68] Recently, the technology has been further improved with respect to sensitivity and specificity by using three recognition events in the so-called triple binder proximity ligation assay[71] (3PLA). The general concept of proximity ligation has also been extended to antibody-mediated recognition events.[72]

The compatibility of aptamers with whole cell staining methods has been shown in several publications. For these studies, aptamers targeting different cell surface proteins were used as a fluorescent labeled variant to detect and visualize their target on distinct cells.[73−75] In a study presented by Davis et al., CD4 interacting aptamers were labeled either with fluorescein or phycoerythrin and applied to selectively stain CD4 expressing cells in flow cytometry.[76] In a very similar approach, Ringquist and Parma used an L-selectin binding fluorescein-labeled aptamer to visualize CD62L-positive leukocytes by FACS analysis.[74] Both studies demonstrated that aptamers are able to interact with the target molecule on cell surfaces and can be specifically used to detect the target expressing cells in flow-cytometry approaches. Building on previous results, Blank et al. used flow cytometry to monitor the progression of an *in vitro* selection experiment.[75] Aiming at the identification of new tumor

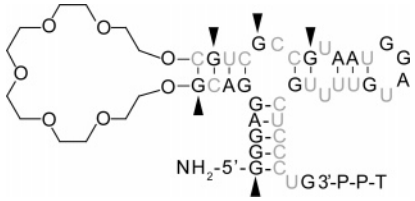

Figure 4. Secondary structure and modification of the 39-mer tenascin aptamer. All pyrimidine residues (gray) contained a 2′-F, 2′-deoxy modification. Purines contain 2′-OCH_3 modifications, except the four obligatory 2′-OH guanosines and the 5′-G (arrows). The 5′-G also contains a 5′-NH_3 group as an anchor to introduce modifications for ^{99m}Tc-labeling. In addition, this aptamer bears a 3′-3′-cap for exonuclease protection. The G6-C13 helix was closed by a non-nucleotide hexaethylene glycol spacer without affinity loss.

markers with a potential application in the diagnosis of glioblastoma brain tumors, the authors developed a SELEX procedure using living cells as targets for SELEX. FACS analysis was applied to monitor the selection of ssDNA aptamers binding to the SV40 transformed endothelial cell line. They demonstrated that the identified aptamers are specific for cryostatic sections of experimentally induced glioblastoma tumor vessels while no interaction with normal endothelial cells was observed.[75] In a further study, the authors used the aptamer as a tool to allow the identification of the native protein target on the cell surface. Coupled to magnetic beads, the aptamer was employed as an affinity reagent for the capturing of the previous unknown protein from cellular fractions. Followed by SDS-PAGE and mass spectrometry analysis, the target protein pigpen was identified as the cognate target of the aptamer. A similar approach was recently followed by Gold and co-workers, where tenascin-c was identified to be the native target after cell-based SELEX experiments.[67]

An impressive example using aptamers for *in vivo* imaging was provided by Charlton et al.[77] They employed a ^{99m}Tc-labeled ssDNA aptamer directed against human neutrophil elastase (hNE) for diseased tissue imaging in a rat model. hNE is a serine protease that plays a crucial role in inflammatory diseases such as septic shock or acute respiratory distress syndrome (ARDS). In the study, the aptamer exhibited an improved signal-to-noise ratio compared to an anti-elastase IgG antibody frequently used in the clinic for *in vivo* imaging of inflammatory sites. A ^{99m}Tc-labeled aptamer that binds tenascin (Figure 4), an extracellular matrix protein which is often used as a tumor marker, was recently shown to be taken up by a variety of solid tumors including breast, glioblastoma, lung, and colon.[78] This result suggests possible clinical applications of labeled aptamers in imaging and therapeutics.

The use of aptamers for western blot analysis was shown in two recent studies.[65,66] Here, biotinylated variants of aptamers targeting either L-selectin or TTF1 were used and allowed to interact with their cognate targets after SDS-PAGE and western blotting followed by incubation with horseradish peroxidase-tagged streptavidin. The studies underline the feasibility of aptamers for the sensitive and specific detection of their target molecules even in crude cell lysates.[65,66,79] Due to the denaturing conditions applied by SDS-PAGE, it is still unclear how the aptamers interact with their target molecules blotted to the transfer membranes. Aptamers are thought to interact with defined three-dimensional structures rather than short peptide epitopes. However, it remains elusive whether the aptamers recognize defined epitopes that are even accessible in the denatured state of the protein (as antibodies do) or the proteins are able to refold at least to some extent into their native structures after blotting. In the latter case, western blot analysis with aptamers will be limited to a small number of proteins capable of refolding after denaturing SDS-PAGE. More data is needed in order to provide a more comprehensive picture about the applicability of aptamers in western blot analysis.

2.1.2. Aptamers as Capture Ligands

Due to their properties of binding with high affinity and specificity to a target molecule, aptamers are well suited for the preparation of affinity columns for protein purification as well as capture ligands on surfaces for biosensor applications.

An example that introduced the applicability of aptamers in affinity chromatography was reported by Romig et al.[65] In this study, the authors used an anti-L-selectin DNA aptamer and attached it to a solid support, thus creating an affinity matrix specific for L-selectin. They applied this matrix for the purification of L-selectin from Chinese hamster ovary cell-conditioned medium and demonstrated that the aptamer capture is sufficient to purify the protein to near homogeneity. Further approaches addressing the purification of proteins using specific aptamers include proteins such as TTF1, tenascin-c, and pigpen. A slightly different application utilizes aptamers as solid supports during HPLC and the selective purification of enantiomers.[80,81] Peyrin and co-workers used aptamers and constructed chiral stationary phases (CSP) for the separation of enantiomers of peptides and small molecules, such as L-tyrosineamide.[80,81] The given examples and other applications of aptamers, such as stationary phases for capillary electrophoresis,[82] illustrate that aptamers are flexible tools suitable for various applications that require sophisticated ligands.

A prominent application of aptamers is their use as capture ligands on the surface of biosensors or on microarrays. In an initial study, Ellington and co-workers demonstrated the general feasibility of aptamers for biosensor applications.[83] In this study, the thrombin/aptamer system was used by covalently immobilizing a fluorescence-labeled anti-thrombin aptamer on a glass support and measuring the changes in the evanescent-wave-induced fluorescence anisotropy of the immobilized aptamer upon interaction with thrombin. A similar approach was recently reported by the Archemix Corporation.[84] They used different fluorescently labeled RNA aptamers and showed specific detection in a muliplex format of the corresponding targets in human serum and cellular extracts.

The thrombin aptamer system was also used by Lee et al. to assemble a fiber-optic microarray biosensor.[85] The thrombin-binding aptamers were bound to silica beads and loaded on the distal tip of an imaging fiber. After incubation with fluorescently labeled thrombin, the interaction could be visualized by interconnection of the imaging fiber with a modified epifluorescence microscope system.[85]

In a different study, Liss et al. constructed a quartz crystal-based biosensor that can be used to detect low levels of IgE in body fluids, in real time.[86] In comparison to antibody-mediated sensing, it turned out that the aptamer-based detection is more robust and stable with respect to regeneration protocols. Since aptamers are relatively small (5−25 kDa) when compared to antibodies (∼150 kDa), they can be coupled on surfaces with higher densities, resulting in a

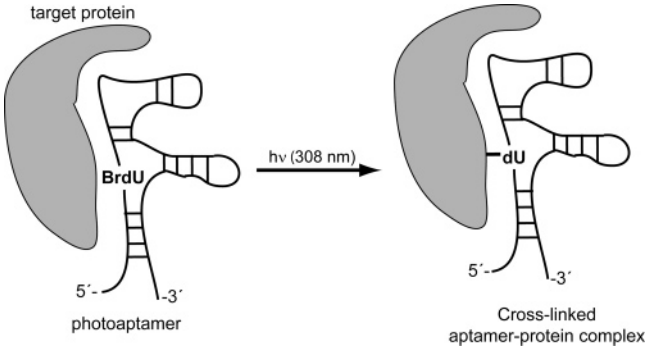

Figure 5. Aptamers containing 5-bromodeoxyuridin groups (BrdU) can be covalently cross-linked with their target proteins by irradiation with UV light (308 nm).

high dynamic detection range. A recent report underlined the repetitive use of aptamer biosensor arrays employing aptamer targeting lysozyme, ricin, and the HIV-1 rev protein.[87] Ellington and co-workers constructed a chip-based microsphere array that has been previously shown to be capable of detecting nucleic acids.[88] Despite the repetitive denaturation of the sensor surface with urea containing EDTA, the signal intensities of anti-ricin aptamer beads were still stable and comparable.[87] Schlensog et al. constructed a biosensor system based on the surface acoustic wave—love waves.[89] Using the thrombin/aptamer and the HIV-1 rev/aptamer as model systems, they demonstrated that the detection limit of the love-wave sensor is 75 pg/cm^2, thus being 40-fold more sensitive than the quartz crystal sensor described above.[86] In a further study, the thrombin aptamer-based SAW sensor was used to monitor and to investigate the mechanisms of ternary complex assembly by heparin, antithrombin III, and thrombin.[90] A similar SAW-based sensor was also shown to be capable of discriminating single mutations in cancer gene fragments.[91]

An attractive alternative approach was demonstrated by Gold and co-workers at Somalogic (Boulder, Colorado). These researchers developed and improved aptamer-based microarray technologies by introducing so-called photoaptamers.[92] These aptamers comprise photoreactive 5-bromodeoxyuridine (BrdU) functional groups, and after binding, these aptamers can be covalently cross-linked to their cognate target molecule by UV irradiation[93,94] (Figure 5). Thereby, harsh washing conditions can be employed to remove background and nonspecifically bound proteins from the array surface. As a consequence, the results obtained with the photoaptamer arrays reveal superior signal-to-noise ratios and low limits of quantification; hence, even low-abundant proteins can be visualized by common protein staining methods.

Recently, Petach and co-workers described the application of a multiplexed photoaptamer array.[95] In this study, the authors examined 17 photoaptamers printed on an array surface. They demonstrated that the array of photoaptamers allows the simultaneous measurement of multiple proteins in serum samples. Interestingly, the study revealed that the detection limit of an analyte protein correlates with the dissociation constant of the photoaptamer.[95] This report represents the first comprehensive study on aptamer-based microarrays and underlines, together with more recent studies,[90,96−99] the feasibility of aptamers for such applications. However, a disadvantage of the cross-linking approach is that an array can only be used once.

The aptamer-based sensor systems we reviewed above were designed and used to allow the detection of biological macromolecules. Complementary approaches are sought that make use of direct changes in fluorescence signaling upon ligand binding, when investigating small molecule probes. In an initial attempt, Ellington and co-workers incorporated a fluorescent label into a RNA library and isolated aptamers that reveal a change in fluorescent signaling upon ATP binding.[100] Another approach was pursued by Yamamoto and Kumar. They used a bimolecular system consisting of a fluorescent donor molecule and a quencher molecule attached to the 5′- and 3′-ends, respectively, of the RNA aptamer. This system allows the visualization of Tat protein binding to the RNA, since complex formation causes the structural rearrangement of the RNA aptamer and hence an increase in fluorescent signaling.[101,102] A similar approach was established by the Stojanovic laboratory. They constructed an aptamer-based sensor for cocaine by exploiting the detailed understanding of a DNA aptamer that was previously shown to be stabilized through ligand binding. Using the donor and acceptor fluorophore system, they constructed an efficient cocaine-specific sensor, in which fluorescence intensity decreases through cocaine binding.[103]

In a recent publication, a more general concept for detection of target molecules was introduced. The authors used a previously selected aptamer for the triphenylmethane dye malachite green (MG).[104,105] Further studies showed that malachite green changes its conformation if bound to RNA,[106,107] together with the finding that fluorescence emission of the dye is enhanced greater than 2500-fold if complexed by the aptamer.[108] In their study, Stojanovic and Kolpashchikov connected already known aptamer sequences for small molecules such as ATP, FMN, and theophylline to the malachite green aptamer.[109] The connection of both domains was carried out by using so-called communication modules, as already known from the generation of ribozymes regulated by ligands, designated as aptazymes (see section 4.1). These short segments are fused between the two aptamer sequences to communicate a binding event from one domain to the other (Figure 6). Specifically, binding of the target molecule induces formation of a stem that, in turn, stabilizes folding of the malachite green aptamer. Hence, fluorescence from aptamer-bound MG is only observed in the presence of the target molecule. The advantage of the approach relies on the possibility to apply these sensors easily in living cells, for example by expression of the RNA constructs. Although the general applicability *in vivo* remains to be proved, this design should enable the detection of a broad range of target molecules. The same MG-binding aptamer was applied by Liu and colleagues to create an artificial genetic switch[110] by combining the malachite green (MG) aptamer[105,111] with an RNA-based transcriptional activation domain.[112] The RNA domain for transcriptional activation showed a 10-fold increased activity in the presence of the cell-permeable dye.

2.2. Aptamers as Therapeutics

It is also feasible to use aptamers directly as drugs. The therapeutic application of aptamers was discussed in several previous reviews,[9,14,115−123] and the past years have seen many potential applications of aptamers as therapeutic agents in model systems.[25,67,124−127] Except for the anti-VEGF aptamer-based drug Pegaptanib sodium or Macugen—which has completed clinical phase III trials[128] and was approved in

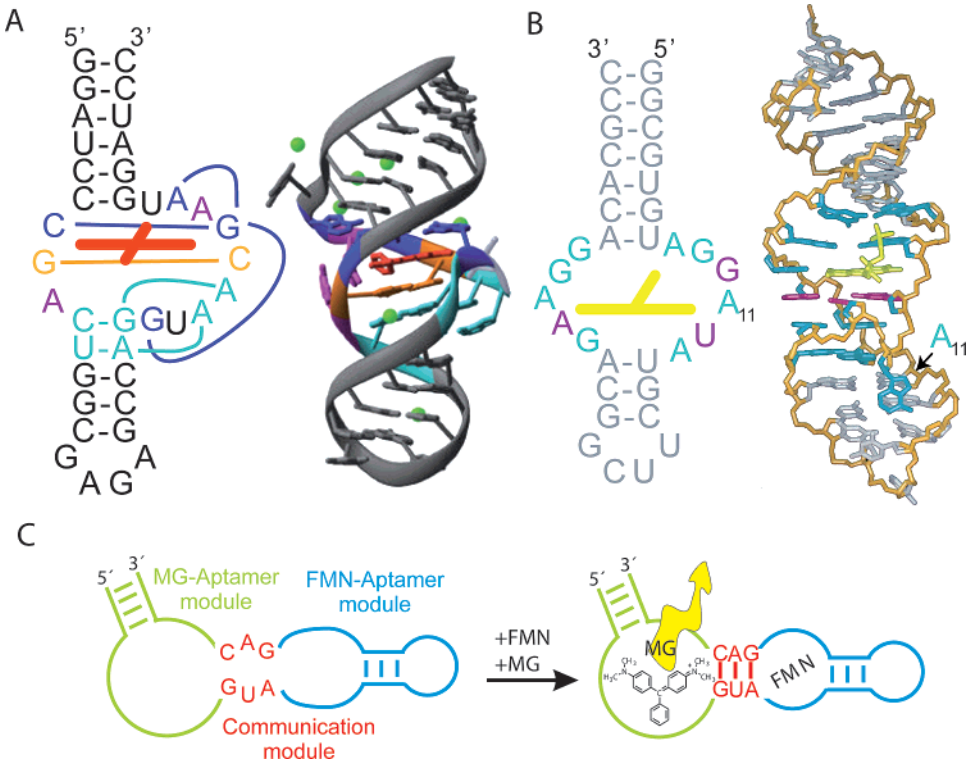

Figure 6. A modular aptameric sensor, composed of the malachite green and flavin mononucleotide (FMN) binding aptamers. (A) Secondary[111] (left) and tertiary[104] (right) structure of the malachite green aptamer. Front view of the tertiary structure: red, bound ligand; orange, canonical G8:C28 base pair; cyan, A26.U11:A22 and A27.C10:G23 base triples; blue, the G24.A31.G29:C7 base quadruple; magenta, the perpendicularly stacking A9 and A30 adenosines; green, bound strontium ions. (B) Secondary[113] (left) and tertiary[114] (right) structure of the FMN aptamer. The view looks into the major groove of the FMN binding site in the center of the complex. Yellow, intercalated FMN; orange, sugar−phosphate backbone; gray, stem and U-U-C-G hairpin bases; blue, internal loop bases except for the G10•U12•A25 base triple (purple). (C) The coupled fluorescent probes for the detection of an aptamer-bound ligand[109] consist of a module that binds to the dye malachite green, a communication unit, and a detector module, which binds to flavin mononucleotide (FMN). The binding of FMN to the detector unit induces helix formation in the communication module. This helix completes the structure required to bind to the dye, which fluoresces only when it is attached to the probe.

2004 by the FDA[129]—no aptamer-based drug has appeared on the market since the discovery of aptamers in 1990. However, during the past few years, considerable progress was made regarding the pharmacology and toxicology of aptamers.[130] Besides the artificial, *in vitro* selected aptamers, RNA and DNA decoy molecules that mimic a transcription factor binding site are used as promising therapeutic molecules.[131−134]

One of the most prominent therapeutic aptamers is Macugen or Pegaptanib, an aptamer directed against vascular endothelial growth factor (VEGF).[135] Retinal function requires a strict control of fluid and electrolytes within cells and the extracellular space. The organization of this control is maintained by the blood−retinal barrier, which is analogous to the blood−brain barrier. The VEGF is a soluble factor, released from ischemic retina, and causes blood−retina barrier breakdown and neovascularization (NV). The level of VEGF is up-regulated in patients with age-related macular degeneration (AMD) and diabetic retinopathy. The inhibition of VEGF would likely inhibit the development of macular edema, and retinal and choroidal NV, leading to loss of vision in patients with AMD or diabetic retinopathy. Several approaches to inhibit VEGF were used in the past few years, including small molecules,[136] peptides,[137] antibodies, and engineered ligands.[138−140] VEGF was one of the first targets for the development of aptamer-based therapies.[24,141−143] A modified version of the anti-VEGF aptamer is a nuclease-resistant, PEGylated, short 2′-F- and 2′-methoxy-modified RNA oligonucleotide that was previously identified by Janjic and colleagues.[142] The ribose bears different modifications at the 2′-position: The pyrimidines now carry a fluorine moiety, and the purines susceptible for derivatization with an *O*-methyl group were identified by a so-called post-SELEX approach. Except for two unmodified adenosine bases, all purine 2′-hydroxy groups are substituted with 2′-*O*-methyl groups. Furthermore, the 2′-*O*-methyl substitution increased the affinity of the aptamer for VEGF by 17-fold. The stability of the aptamer is further enhanced by adding a deoxythymidine to the 3′-terminus via a 3′-3′ linkage. Advantageously, PEGylation of the aptamer reduces its dosing frequency by extending the half-life of the aptamer in body fluids,[144] increases its solubility, and reduces immunogenicity (Figure 7).

Macugen represents the first aptamer-based drug for the treatment of exudative age-related macular degeneration (AMD), and the aptamer succesfully passed through preclinical and clinical studies.[135,145] In the meantime, conditions that allow for the direct selection of 2′-*O*-methyl transcripts, as opposed to the laborious post-SELEX approach, have been developed. Burmeister et al. selected a fully 2′-*O*-methyl-modified aptamer from a library of 3×10^{15} unique 2′-*O*-methyl transcripts for a 23 nucleotide long sequence, ARC245, that binds VEGF with a K_d of 2 nM and inhibits VEGF function in cell culture assays.[146] With the same approach, aptamers to multiple target proteins, including interleukin (IL)-23 and thrombin, have been successfully isolated.[147]

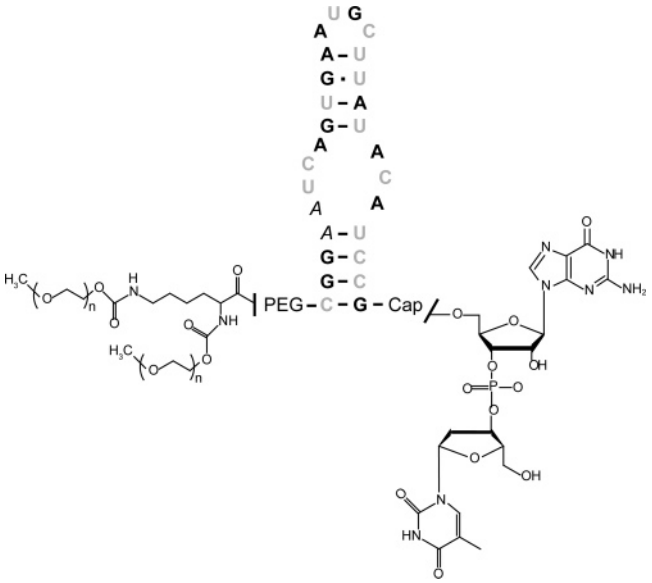

Figure 7. Secondary structure and modifications of the anti-VEGF aptamer pegaptanib (Macugen). Bold gray nucleotides represent 2′-deoxy-2′-fluoro nucleotides whereas bold black nucleotides are 2′-*O*-methyl nucleotides. The two adenosine residues shown in italic black are ribonucleotides. The 5′-position contains a 40 kDa polyethylene glycol (PEG)-linker, where *n* is approximately 450. At the 3′-end, a 3′-3′-dT (Cap) structure was added.

Another aptamer that may be useful in hematology is a 15-nucleotide, G-quadruplex-forming DNA aptamer that efficiently blocks the proteolytic activity of thrombin, a serine protease involved in thrombosis and hemeostasis.[15,148] The anti-thrombin aptamer was one of the first aptamers tested in animal studies in the early 1990s.[149,150] For example, Li et al. demonstrated that the aptamer leads to rapid anticoagulation and thus represents an excellent alternative to replace heparin in a canine cardiopulmonary bypass model.[151] Recent studies involved the analysis of the aptamer and its properties as a thrombus-imaging reagent.[152]

Recently, we developed light sensitive variants of the anti-thrombin aptamer.[153] These aptamers bear a photolabile protecting group, or "cage", at distinct positions that can be efficiently removed by UV-A light at 366 nm. In this way, we constructed light activatable as well as light deactivatable caged anti-thrombin aptamers.[154−156] The latter molecule represents the first anticoagulant with a light sensitive antidote activity (Figure 8). Caged aptamers might enable an excellent way to exogenously regulate aptamer activity in a spatio-temporal manner and thus gain spatio-temporal control of protein function. We also used derivatives of porphyrins that are known to interfere with G-quadruplex formation. We demonstrated that these molecules revert the anticoagulatory activity of the anti-thrombin aptamer in a concentration-dependent manner.[157]

In a different study, Sullenger and colleagues have described the identification of 2′-fluoro-2′-deoxypyrimidine-modified RNA aptamers—a known approach to attain nuclease-resistance of RNAs[12]—that interact with the coagulation factor IXa and antagonize its blood clotting activity.[158,159] The aptamer binds factor IXa with a K_d of 3 nM and exhibits high specificity compared to other coagulation factors. The truncated version of the anti-factor IXa aptamer (9.3t) was PEGylated and successfully established as an inhibitor of blood clotting in standard blood clotting assays and in plasma samples from patients who have developed an immunological response to the commonly used anti-blood clotting drug hep-

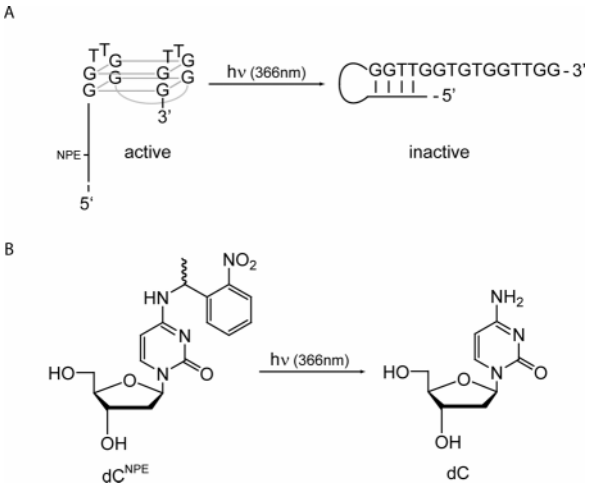

Figure 8. An anti-thrombin aptamer that can be inactivated by light. (A) The G-quadruplex structure of the anti-thrombin aptamer was elongated by an antisense sequence whose base pairing properties are hampered by the site selective incorporation of a caged cytidin nucleobase that contains a photolabile protecting group (NPE: *o*-nitrophenylethyl), representing a temporary mismatch. (B) Upon irradiation by UV-light, the photolabile protecting group is removed and annealing of the complementary sequences occurs, thus rendering the aptamer inactive.

arin (HIT, heparin-induced thrombocytopenia). The authors demonstrated that the aptamer Peg-9.3t significantly prolongs the plasma clotting time. Being a nucleic acid and based on the sequence information of the aptamer, generation of an antidote of the aptamer activity was achieved by applying a complementary oligonucleotide to the aptamer sequence (antidote 5-2). The aptamer anticoagulation activity was efficiently and quickly reversed by the antidote 5-2 when assayed in patient samples under clinical conditions. This study provided an alternative approach for aptamers in drug development and the application of aptamer−antidote pairs whenever regulation of inhibitory activity is needed. The simplicity by which antidotes against therapeutic aptamers can be designed adds significant value to aptamer-based drugs and distinguishes aptamers from other drugs currently used in clinical practice. This is further illustrated by a follow-up study, in which the anti-FIXa-aptamer−antidote pair was shown to work reliably also in two clinically relevant animal models: a porcine anticoagulation model and a murine thrombosis model.[160] Problems associated with moving from *in vitro* studies to applications *in vivo* include rapid clearance from circulating blood and blood nucleases, which would rapidly hydrolyze both aptamer and antidote before they even reached their target in the bloodstream. The strategy to increase the plasma residence time was to attach a cholesterol group[161] to the aptamer (Figure 9).

Indeed, the cholesterol-modified aptamer showed a clearly extended blood residence time as compared to the nonderivatized version and also increased the clotting time in pigs. The antidote neutralized this effect within a short time window. Rusconi et al. subsequently applied a murine arterial injury model and found that the aptamer also prevented occlusive thrombus formation compared to control mice that were treated with a nonfunctional aptamer mutant. Furthermore, the aptamer−antidote pair controlled bleeding, induced by surgical trauma in mice that had been treated with high doses of the aptamer. When the antidote was administered immediately after injury induction, blood loss was significantly reduced and the procoagulant activity of FIX

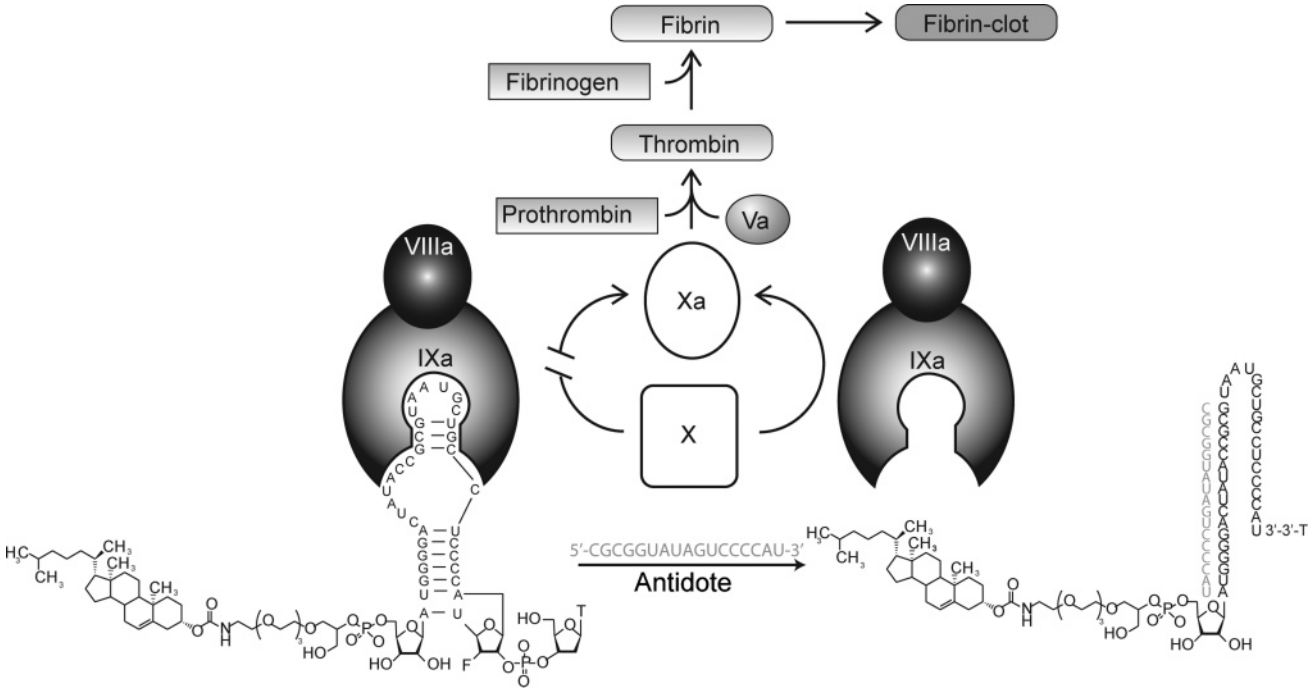

Figure 9. Mechanism of the aptamer−antidote pair. The aptamer is stabilized by a 3′-3′-linked deoxythymidine at the 3′-end and by 2′-fluoro-2′-deoxy modifications at every pyrimidine residue. It carries a cholesterol modification at the 5′-end to increase the plasma residence time. The aptamer binds to activated factor IX (FIXa) and prevents the proteolytic cleavage of factor X (left). In the presence of the antidote (gray sequence), the aptamer is released from FIXa (right). Together with activated factor VIII (VIIIa), FIXa catalyzes the cleavage of factor X to yield activated factor X (Xa), which is required for continuing the blood clotting cascade: with factor Va, Xa cleaves prothrombin to yield thrombin, which catalyzes cleavage of fibrinogen to yield fibrin. Cross-linked fibrin forms the clot.

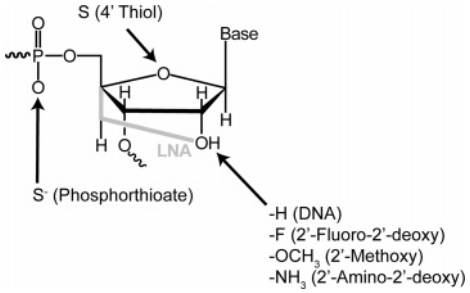

Figure 10. Summary of chemical modifications introduced into aptamers. In LNA, the 2′-O and the 4′-C are bridged by a methylene group (gray).

was immediately restored due to inactivation of the aptamer's capacity to bind FIXa. These results further demonstrate the high potential of aptamer technology for drug development.

The modification of the 2′-hydroxyl group of the sugar ribose is straightforward and sufficient to enhance the stability of a RNA molecule *in vivo* and in living organisms. Other potential sites for chemical modification that may be beneficial for the properties of therapeutic aptamers are the purine and pyrimidine residues,[162−175] sugars,[176−180] and phosphate residues,[181] which all have been shown to be compatible with template-directed enzymatic incorporation into DNA and with all necessary steps required for a complete *in vitro* selection cycle.[175,182] In general, modified nucleotides are increasingly being utilized in all categories of therapeutic oligonucleotides to increase nuclease resistance, target affinity, and specificity.[183] As summarized in Figure 10, 2′-amino,[47,184−187] 2′-fluoro,[124,186,188,189] 2′-methoxy,[147,190] and 4′-thio[180] groups have been used directly in SELEX experiments to increase the stability of aptamers. Stabilization can also be achieved by introducing chemical modifications "post-SELEX",[23,191] for example, by substituting phosphorthioates,[192] locked nucleic acids[191,193] (LNA), or multivalent circular aptamers.[194−196]

Bugaut et al. recently reported an elegant way to enhance the chemical diversity of RNA libraries.[197] They used an RNA library with 2′-deoxy-2′-aminouridine nucleobases and further modified these positions with a set of three different aldehydes in a random fashion. After incubation with the target molecule and elution of the bound RNA species, the aldehydes were removed and the RNA was subjected to RT-PCR and *in vitro* transcription. This approach allows a simple but effective way to further enhance the chemical versatility of nucleic acids without the necessity to be compatible with the enzymatic steps of the *in vitro* selection procedure.

A different approach for aptamer stabilization was introduced by Fürste and co-workers,[198] and independently by Bartel and colleagues.[199] Here, the metabolic stability of aptamers was increased by employing nucleic acids consisting of L-nucleotides. The corresponding L-aptamers, designated as Spiegelmers, have been shown to almost resist degradation by nucleases.[130] In this way, PEGylated Spiegelmers that specifically antagonize gonadotropin-releasing hormone (GnRH) exhibited plasma half-lives of up to 14 h in rats and thus are superior to the use of 2′-F-pyrimidine RNA-aptamers.[200] Wlotzka et al.'s investigation of the preclinical ADME parameters of the anti-GnRH Spiegelmers in rats produced encouraging results. The biological efficacy of the anti-GnRH Spiegelmer was similar to that of the marketed reference compound Cetrorelix (Cetrotide; ASTA Medica AG) albeit 1000 times higher doses of the Spiegelmer were administered.[200,201] Another promising Spiegelmer target is ghrelin, a potent stimulant of growth hormone release, food intake, and adiposity.[202,203] This Spiegelmer was recently shown to reduce obesity in diet-induced obese mice.[204,205] A further advantage of the Spiegelmer and aptamer thera-

peutic approaches is that almost no adverse immune responses were detected in monkeys and rats, which thus indicates that nucleic acids, in the guise of aptamers, are well tolerated.

Besides the well documented and published results depicted above, there are more aptamers and decoy oligonucleotides, sequences that mimic natural DNA binding sites of proteins such as transcription factors, currently under clinical investigation.[128] Among them, the company Aptamera launched clinical trials of a G-quartet-containing aptamer targeting nucleolin[206] for the treatment of cancer. Corgentech started clinical trials using E2F decoy aptamers for the treatment of cardiovascular diseases and the prevention of vein bypass graft failure.[207,208] Next to the decoy approaches, there are several companies, such as Hybridon and Dynavax, currently dealing with immune-stimulatory sequences. These DNA oligonucleotides contain CpG motifs that mimic the presence of bacterial genes and thus induce the activation of toll-like receptor 9 (TLR-9) Th1-cells via toll-like receptor 9 (TLR-9) and interferon-γ mediated pathways.

A potential advantage of aptamers over therapeutic antibodies is that so far there appear to be no indications that aptamers are immunogenic, whereas antibodies can elicit immune responses even within short periods of treatment.[209] A potential disadvantage, rapid clearance via kidneys and correspondingly short circulating time following injection,[14] can be overcome by attaching cholesterol,[210] biotin−streptavidin,[211] or polyethylene-glycol groups[210,212,213] or by anchoring an aptamer to liposomes,[161,214] which have been shown to reduce clearance.

3. Intramers

3.1. Applications of Aptamers *in Vivo*

Aptamers have been used in an increasing number of applications inside living cells and even whole organisms. The promising potential of aptamers and other nucleic acid tools such as ribozymes, antisense oligonucleotides, and small interfering RNAs as therapeutic agents as well as for elucidating the cellular functions of the target molecules is hampered by the fact that nucleic acid uptake into cells is generally very inefficient. Therefore, one of the main future goals remains the development of efficient delivery strategies.[215] Promising techniques are being investigated, among them the use of cell membrane penetrating peptides,[216] finding of efficient but safe[217] retroviral expression vectors for small RNAs,[218−220] and advances such as naked[221,222] as well as liposome-mediated oligonucleotide transfer.[223−226] Intracellularly expressed aptamers (also called intramers) have also been discussed in the recent literature.[16,27,227−230] This section reviews recent studies that employ aptamers in cells and even in whole organisms, taking into consideration the different strategies of aptamer delivery.

3.1.1. Intracellular Expression

Early examples of intracellularly expressed aptamers included inhibition of the special elongation factor SelB in *Escherichia coli*[231] and RNA polymerase II in yeast.[38] In another study, aptamers specific for Ku protein were expressed under the control of a human 7SL promoter in MCF-7 breast carcinoma cells.[232] The Ku protein is involved in the repair of double-strand breaks in DNA. The expressed aptamer is able to block the DNA-binding properties of the Ku protein, thereby sensitizing the cell line to DNA damage. When treated with the anticancer drug etoposide, a compound that inhibits DNA topoisomerase II in a way that generates double-strand DNA breaks, aptamer-expressing cells were more susceptible to undergo apoptosis.

Our group focused on the elucidation of cytoplasmic regulatory proteins that participate in the leukocyte-function-associated antigen-1 (LFA-1)-mediated inside-out signaling cascade. Specifically, we are interested in the family of cytohesins, which belong to the highly homologous class of guanine nucleotide exchange factors (GEFs). The small ADP-ribosylation factor (ARF) GEFs catalyze the GDP/GTP exchange on ARFs. At the present time, four members of the cytohesin family are known: cytohesin 1, cytohesin 2 (also called ARNO: ADP-ribosylation factor nucleotide-binding site opener), cytohesin 3, and cytohesin 4. They take part in integrin signaling, actin cytoskeleton remodeling, and vesicle transport.[233−235] All cytohesins consist of an N-terminal coiled coil domain, a Sec7 domain that contains the GEF activity,[236] a Pleckstrin homology (PH) domain, and a C-terminal polybasic domain.[237,238] The two latter domains enable membrane localization via interaction with phosphoinositides. Although highly homologous, the cytohesins seem to have different biological functions.[234,239] We have selected specific aptamers targeting cytohesins in order to enlighten their different regulatory roles.

When activated by phorbol esters or T-cell receptor stimulation, LFA-1 triggers adhesion of T-cells to ICAM-1, which is present on endothelial cell surfaces. Cytohesin-1 is believed to contribute to the LFA-1 activation by direct interaction with the cytoplasmic domain of the β2-chain (CD18) of the LFA-1 integrin.[237] We have selected an aptamer (M69) that binds to the Sec7 domain of cytohesin 1.[235] Although the aptamer is able to discriminate between large and small GEFs, it binds with similar affinity to cytohesin 1 and cytohesin 2/ARNO. We next expressed M69 in the cytoplasm of Jurkat cells using a transgenic vaccinia virus.[240] Intrameric expression of M69 resulted in inhibition of LFA-1-mediated adhesion of the cells. Moreover, reorganization of the cytoskeleton and cell spreading was also inhibited by M69. Similar results were obtained by dominant negative expression of a cytohesin mutant (E157K) that abolishes GEF activity. These results confirmed an important role of the GEF activity of cytohesin-1 in T-cell spreading.

We have used a 5′-fluorescein-labeled version of the aptamer M69[235] to screen for small molecule inhibitors of cytohesins.[241] Labeling of the aptamer was achieved by *in vitro* transcription in the presence of guanosine-monophosphorthioate, followed by treatment with an electrophilic fluorescein derivative.[242] The screening assay was based on aptamer displacement, followed by fluorescence polarization (Figure 11A). We identified a 1,2,4-triazole derivative, SecinH3 (Figure 11B), that bound to the Sec7-domain of cytohesins and inhibited nucleotide exchange. When applied in human liver cells, SecinH3 allowed implicating insulin-receptor-complex-associated cytohesins in insulin signaling. Mice fed SecinH3 exhibited signs of hepatic insulin resistance and increased plasma insulin, phenomena that are thought to be associated with the onset of type 2 diabetes in humans. In a parallel study, Fuss et al. found that mutations in steppke, the single *Drosophila melanogaster* homologue of cytohesin, led to phenotypes also seen with insulin signaling defects: reduced growth at each stage of development without impaired food uptake.[243] Feeding of SecinH3

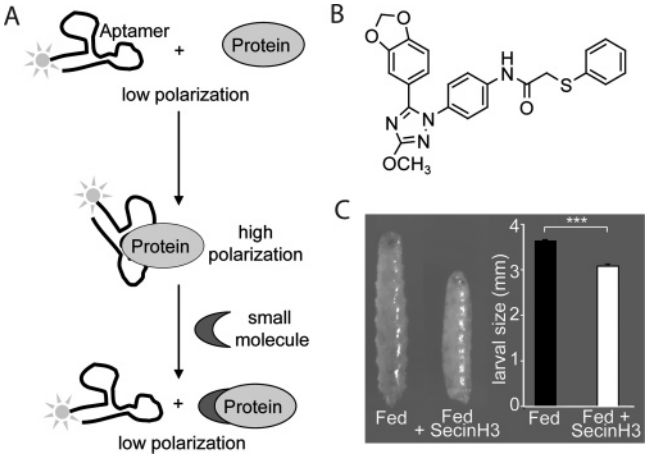

Figure 11. Aptamer displacement screening assay by fluorescence polarization identifies a small molecule with biological function. (A) Schematic for the fluorescence polarization assay used. The fluorescence-labeled aptamer M69 exhibits low polarization in the nonbound state (top panel). When bound to the protein, in this case the Sec7 domain of cytohesin 1, fluorescence polarization becomes high (middle panel). If a small molecule (crescent-shape) displaces the aptamer from the protein, fluorescence polarization is reduced (bottom panel). (B) Chemical structure of SecinH3, the most potent among a series of molecules identified in the aptamer displacement screen, that inhibits cytohesin proteins. Because cytohesins are highly conserved among species from flies to humans, SecinH3 inhibits cytohesins from all these species. (C) SecinH3 induces growth retardation when fed to *Drosophila melanogaster*. The same effect is seen in larvae mutants, in which the Drosophila cytohesin Steppke was mutated. Left: WT larvae fed with (Fed + SecinH3) or without (Fed) the Steppke-inhibitor SecinH3. Right: average size of Fed + SecinH3 ($n = 136$) and Fed ($n = 98$) larvae.[241,243,245]

to flies caused a phenocopy of the mutation and biochemical and transcriptional evidence of loss of insulin signaling (Figure 11C). Thus, the aptamer displacement screen established how to translate information stored within an aptamer into a small molecule.[244] Thereby, chemical space can be explored in a rapid, focused, and modular manner, by indirectly taking advantage of the highest molecular diversity currently amenable to screening, namely that of up to 10^{16} different nucleic acid sequences. Aptamer displacement screens demand small molecules of high initial potency, leading to effective and specific inhibitors that can be used as drug leads or tools in chemical biology.

Cytohesin 1 and 2 are both expressed in T-cells. To investigate whether the homologous proteins inherit different functions, we selected an aptamer specific for cytohesin 2/ARNO.[246] Since the first aptamer (M69) was not able to discriminate between the two cytohesins, we used a counter-selection protocol to exclude sequences that also bind to cytohesin 1. The obtained aptamer (K61) exhibited high affinity toward cytohesin 2 with a K_d of 115 nM, whereas recognition of cytohesin 1 was reduced 35-fold. When tested using an *in vitro* assay, the aptamer did not inhibit the GEF activity of cytohesin 2, presumably because it binds to the coiled-coil-Sec7 interface rather than to the Sec7 domain alone.

Cytoskeletal remodeling and membrane trafficking are thought to be regulated by GTPases of the Rho and ARF families.[247] In addition, the small GTPases of the Rho family participate in serum response factor transcriptional activation, which is stimulated by serum growth factors.[248] We questioned whether the cytohesins also take part in transcriptional activation mediated by the serum response element (SRE)

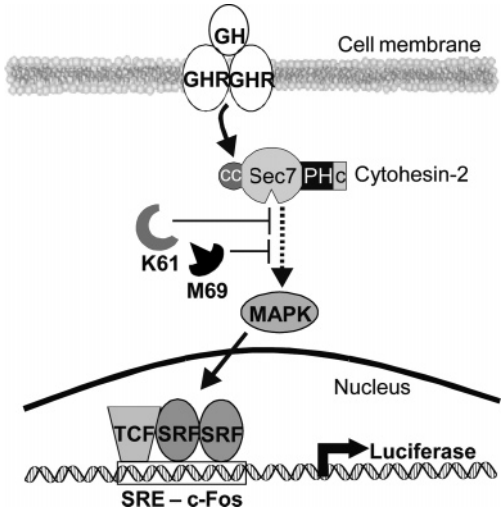

Figure 12. Model for the role of cytohesin-2 as an effector of serum-mediated transcriptional activation via the MAPK pathway. The domain specificity of the aptamers K61 and M69 suggests participation of both the N-terminal and the Sec7 domains of cytohesin-2. GH, growth factors in serum that act on MAPK-activating receptors; GHR, MAPK-activating receptors; CC, coiled-coil domain; TCF, ternary complex factor; SRF, serum response factor. Modified after Theis et al.[246]

by investigating the effects of the aptamer on the expression of a luciferase reporter gene under the control of the SRE promoter in serum-stimulated HeLa cells. Direct transfection of K61 resulted in a concentration-dependent reduction of luciferase expression to basal levels. The effect was aptamer-specific, since overexpression of cytohesin 2/ARNO rescued the serum-stimulated transcription. Furthermore, we found that both K61 and the nondiscriminatory aptamer M69 down-regulate the activation of the MAPK pathway, which is in accordance with the novel activity of cytohesin 2 (Figure 12).[246]

RNA interference was subsequently used to further distinguish both protein functions. A siRNA targeting cytohesin 2 also resulted in down-regulation of MAPK activation, contrasted by the finding that a siRNA against cytohesin 1 expression showed no effect. A possible explanation for this finding could be that cytohesin 2 is involved in transcriptional regulation via SRE rather than cytohesin 1. Taken together, the obtained results further demonstrate that intramers are versatile tools allowing for assignment of novel biological functions to a specific domain or an individual protein within a given protein family.

In another approach, Jeong and colleagues have selected an RNA aptamer that binds to the DNA-binding domain of TCF-1 and inhibits binding of TCF-1 to its specific DNA recognition sequences *in vitro*.[249] To modulate the transcription by TCF/β-catenin complex in the cells, high levels of this aptamer were expressed as intramers in mammalian cells using an RNA expression vector for stable intramer expression. The intramer inhibited TCF/β-catenin transcription activity and modulated the expression of TCF/β-catenin target genes, such as cyclin D1 and matrix metalloproteinase-7. In addition, it efficiently reduced the growth rate and potential to form tumors in HCT116 colon cancer cells. The same group also selected a high-affinity RNA aptamer that associated with β-catenin *in vivo*.[250] Nuclear localized aptamer inhibited catenin-dependent transcription of cyclin D1 and c-myc in colon cancer cells. Its expression as an intramer led to cell cycle arrest and reduced tumor formation.

The aptamer also prevented alternative splicing events induced by stabilized β-catenin. These studies open up the potential that intramers can be applied in gene therapeutic approaches to treat TCF or β-catenin-mediated tumors.

Aptamers have also been expressed in whole organisms.[251] By transforming *Drosophila melanogaster* germ lines with an aptamer construct that binds to the RNA splicing regulator B52, stable expression of up to 10% of the total mRNA level was achieved.[252] Altering the expression levels of the splicing factor causes severe phenotypes in Drosophila. Consequently, when expressed in normal flies, the aptamer proved to be lethal. In mutants overexpressing B52, suppression of all phenotypes was obtained by expression of the aptamer.

3.1.2. Optimization Studies for in Vivo Applications

Although an increasing number of aptamers selected *in vitro* were successfully applied *in vivo*, some studies suggest that the characterized properties exhibited *in vitro* are sometimes not observed when the same RNA sequence is expressed inside cells. For example, a $tRNA^{Gln}$ that was modified with an aptamer specific for glutaminyl-tRNA-synthetase showed 26-fold higher affinity for the cognate synthetase, resulting in increased aminoacylation performance.[253] Nevertheless, the construct failed to support growth when expressed in a knockout strain of *E. coli*, presumably due to increased nuclease digestion compared to the natural $tRNA^{Gln}$.

Expression of aptamers using RNA polymerase III-based cassettes provides a convenient way to produce high levels of small RNAs inside the cell. Nevertheless, problems might arise from expressing the functional RNA in the context of these expression cassettes. Sullenger and co-workers have selected RNA aptamers that bind to the transcription factor E2F1.[254] The family of E2F plays a critical role in controlling cell proliferation. The authors demonstrated that microinjection of the aptamer resulted in blocking the ability to enter the S phase in growth-stimulated fibroblasts.[254] However, when trying to express the aptamer by a RNA pol III construct derived from human methionine tRNA, the activity was substantially reduced. The authors assumed that interaction of the aptamer with the flanking sequences in the expression cassette resulted in misfolding and inhibition of the aptamer. To overcome this effect, they performed a selection with the chimeric tRNA containing randomized flanking sequences. This so-called "expression cassette SELEX" yielded constructs that are transcribed very efficiently and exhibited high activity against E2F1 in human 293 cells.[255] Although a reselection of the expression cassette might not be necessary in all cases, this technique allows for optimization of aptamer function *in vivo*.

In vivo selection to improve aptamer function inside eukaryotic cell nuclei was also carried out by Cassidy and Maher using a previously selected aptamer that binds to NFκB p50 in yeast.[256−258] In contrast to the strategy discussed above, the aptamer itself was reselected by generating aptamer variants containing an average of 2−3 mutations. The resulting library was then screened for optimized binding to NFκB by performing a yeast three hybrid experiment.[258] The authors point out that a combination of *in vitro* and *in vivo* selection enables the generation of highly active aptamers for applications inside cells. Chan et al. used a combined aptamer siRNA approach to gain maximal suppression of NFκB activity in mammalian cells.[259] The combination of both siRNAs and an aptamer that has been shown previously to inhibit the DNA-binding activity the p50 subunit of the NFκB transcription factor in yeast[256−258] showed a strong reduction of NFκB-mediated expression of a reporter gene in mammalian cells, whereas the application of either the siRNA specific for NFκB or the aptamer that targets NFκB alone resulted in incomplete reduction of NFκB acitivty. Cell type-specific delivery of siRNAs into cells was also achieved by using aptamer-siRNA chimeras, as discussed below.[260] These data indicate that the siRNA and aptamer technologies represent complementary approaches to gain maximal control over biomolecule function.

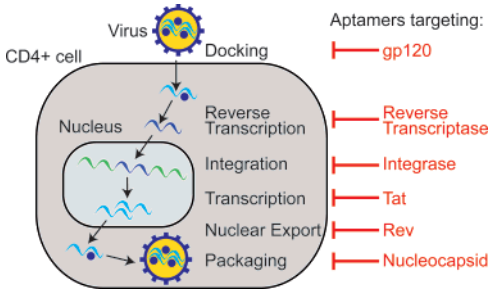

Figure 13. Key steps of the HIV life cycle that have been targeted by aptamers.

The sometimes different behavior of aptamers *in vitro* and *in vivo* implicates that caution should be applied before drawing definite conclusions of biological relevance resulting from *in vitro* studies only. Moreover, a reselection of the aptamer *in vivo* might be necessary if applications inside cells are to be performed.

3.2. Aptamers as Antiviral Agents

The multiple attempts to utilize aptamers to inhibit viral infection and replication have been reviewed recently.[118,261] Aptamers have been selected for a variety of components essential in viral life cycles. Especially, replication of the human immunodeficiency virus (HIV) has been inhibited by anti-HIV aptamers. Figure 13 highlights crucial processes of viral replication that can be inhibited by aptamers targeting HIV proteins. Most of these viral proteins show impaired functions if aptamers are applied *in vivo*. Since assay formats differ considerably in many studies, a comparison of the *in vivo* effectiveness of different aptamers is not facile. Apart from aptamers targeting gp120[262−265] and integrase,[266−269] most studies have focused on the viral proteins HIV RT, Rev, and Tat.

Inhibition of HIV-1 reverse transcriptase by RNA aptamers *in vivo* was demonstrated using a temperature-sensitive DNA polymerase I in *Escherichia coli*.[270] The mutant is not able to grow at 37 °C unless complemented with an exogenous DNA polymerase. If HIV-1 reverse transcriptase is expressed, *E. coli* shows normal growth without the impairment of other functions. Expression of an aptamer specific for HIV-1 RT restores the temperature sensitivity of the strain. The authors note that their methodology allows for easy screening of RT inhibitors and resistant RT mutants.

The first examples of RNA aptamers suppressing viral replication in a human T-lymphoid cell line were demonstrated using RNA motifs that fold into pseudoknot structures and bind to HIV-1 reverse transcriptase with very high affinity and specificity.[32,271−273] Using Jurkat cells stably expressing the anti-HIV-1 RT aptamer in the human initiator $tRNA^{met}$ context, Sczakiel, Restle, and co-workers achieved protection against infection as long as 35 days when low

doses of virus were used.[274] Joshi and Prasad obtained similar results, also showing that viral replication of strains resistant to nucleoside analogue RT inhibitors, non-nucleoside analogue RT inhibitors, and protease inhibitors could be efficiently blocked by pseudoknotted aptamers.[275] Therefore, these aptamers could present an alternative method to target strains that have gained resistance against most HIV therapeutics. This finding is strengthened by a further study of Prasad and colleagues.[276] Most of the HIV-1 reverse transcriptase inhibitors act as template analogues binding to the nucleic acid recognition site of the enzyme. Using a DNA aptamer (RT1t49), they isolated mutants that are resistant to the aptamer when tested *in vitro*. Nevertheless, when they characterized the mutated HIV clones in living cells, they found they were replication deficient due to severe processivity defects. These findings underline that targeting nucleic acid binding interfaces of viruses such as HIV might represent a powerful way to keep the virus in check. In this context, a recent study is noteworthy that employs aptamers against the RNase H domain of HIV-1 reverse transcriptase.[277] The data suggests a different, nontemplate analogue mode of action, since the polymerase activity of HIV RT is not affected. Nevertheless, the investigated aptamers exhibit relatively weak binding affinities in the low micromolar range and have not yet been shown to inhibit RNase H *in vivo*.

Aptamers have also been used to target other important RNA−protein interactions essential in the life-cycle of HIV replication. Among other small RNAs, aptamers for HIV Rev and Tat have been expressed in human cells. In an early study, these aptamers were used as an example to develop nucleus-directed expression cassettes based on the human $tRNA^{met}$ and U6 snRNA promoters.[278] Expression levels, localization, and stability of the expressed constructs were evaluated, complemented by their potential to inhibit viral replication by cotransfection with HIV-1 provirus. Although all HIV-directed small RNAs, such as ribozymes,[279,280] antisense RNAs,[281,282] and Tat-[283] and Rev-specific[284] RNAs, were expressed at moderate to high levels, only a Rev aptamer was able to significantly reduce virus production in this study.

The inhibitory effect of the HIV Rev aptamer was further investigated under the control of the human cytomegalovirus (CMV) promoter in HeLa cells.[285] The authors observed that even the CMV promoter alone is able to act as a decoy resulting in inhibition of HIV replication starting from proviral DNA. The authors suggest that competition of both viral constructs for transcription factors might be the cause of this finding and point out the possibility of misleading results in cotransfection experiments using CMV promoters.

HIV Rev was also targeted by transfection mediated by cationic liposome delivery in HeLa cells. To increase the transfection efficiency, lipofectin was mixed with transferrin before adding the aptamer. Viral replication starting from the DNA provirus was efficiently blocked. A ribozyme targeting the HIV *env* transcript was not able to further improve the effects of the *anti*-Rev aptamer.[286] Similarly, an RNA aptamer targeting the HIV-1 nucleocapsid protein[287] (NC) completely abolished its binding to the stable transactivation response hairpin and psi RNA stem-loops of HIV-1 RNA. When expressed in cells as an intramer, it inhibited the packaging of viral genomic RNA.[288]

The potential of using aptamers for gene therapy targeting HIV was demonstrated by retroviral introduction of a Rev aptamer in human hematopoietic stem cells bearing CD34.[289] These cells have the ability to differentiate into multiple lineages of cell types, including $CD4^+$ T cells and macrophages, the main targets of HIV-1 infection. Normal differentiation of the $CD34^+$ cells was demonstrated in cell culture as well as in a SCID-hu mouse model. Moreover, the Rev aptamer was expressed in end stage cells and rendered thymocytes derived from grafts of the SCID-hu mice resistant to HIV infection.

Aptamers that exhibited 133 times higher affinity toward HIV Tat protein compared to the authentic TAR RNA have been selected and successfully applied to inhibit Tat-dependent trans-activation both in a cell free transcription assay as well as in living cells.[101] The authentic TAR-1 RNA sequence is located in the LTR of the HIV genome. By binding to Tat protein, it significantly stimulates HIV transcription. To evaluate the ability of the selected Tat aptamer to prevent the natural Tat/TAR interaction, HeLa cells were transfected with a LTR-luciferase construct.[290] By expressing the Tat aptamer in addition to the Tat protein, inhibition of trans-activation of transcription was demonstrated. Similar results were obtained by expressing the authentic TAR RNA itself to act as a decoy.[291] Advances in delivery methods such as antibody-targeted liposomes, cationic lipid formulations, and pH-sensitive liposomes as well as pol III-driven transcription of suitable vectors targeting viral infections have been reviewed recently.[223,261,292]

Another example of a viral protein targeted by an aptamer is the nonstructural protein 3 (NS3) of hepatitis C virus (HCV). The protein comprises a helicase and a protease activity, both essential for viral replication. Aptamers for the protease domain of the enzyme have been selected, and the binding mode has been characterized.[293−295] To test the activity of the isolated aptamers *in vivo*, a fusion construct composed of the aptamer and a HDV ribozyme was used, allowing for cleavage of the aptamer from the nascent transcript. The authors claim that this approach is more likely to produce correctly folded aptamers. To further enhance the *in vivo* activity, the aptamer/ribozyme fusion was extended by addition of a cytoplasmic transportation signal. Expression of the trimeric aptamer construct under the control of an RNA polymerase II-dependent chicken-actin globin (CAG) promoter showed significant inhibition of NS3 protease activity in HeLa cells.[296] By simply fusing an oligo-U tail to the 3′-end of the aptamer, the group recently succeeded in generating a bifunctional ligand that also inhibits the helicase function of NS3.[297]

A different strategy to target viral components by aptamers involves targeting viral RNA structures rather than viral proteins. Progress on this relatively new approach has been reviewed recently.[298] Generation of aptamers targeting essential nucleic acid structures complements established techniques such as ribozymes and antisense and RNA interference technologies. The advantage of using aptamers rather than the approaches mentioned above is that highly structured target sites can potentially be targeted. This is of particular significance, since the most interesting viral RNA targets, such as TAR and RRE RNAs in HIV, are highly structured. Moreover, aptamer selection is often successful even without any knowledge of the target structure. In principle, the RNA part of any essential viral protein/RNA interaction represents a promising target for an aptamer selection.

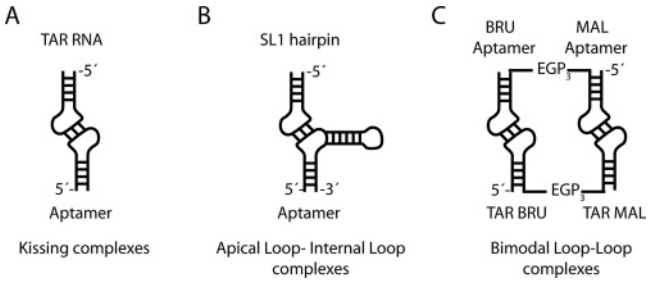

Figure 14. Various kissing complexes found in RNA-binding aptamers. (A) Aptamers that target RNA structures, such as the HIV TAR element, might form so-called kissing complexes. (B) Apical loop−internal loop complexes between RNA hairpins and selected aptamers also form high affinity complexes (SL1: stem loop 1). (C) The high affinity of these complexes can be enhanced by the construction of bimodal kissing complex forming RNA elements. BRU and MAL depict RNA elements within the TAR RNA from HIV. EGP, triethylene glycol phosphate.

One could expect that the selection of a nucleic acid to bind a target nucleic acid should result in finding an antisense oligonucleotide complementary to the target sequence. At least for unstructured targets, antisense sequences should present a sufficient solution. For example, a selection performed against the internal ribosomal entry site (IRES) located in the 5′-UTR of the HCV genome yielded oligonucleotides that are at least in part perfectly matched by Watson−Crick base-pairing.[299] As much as 12 of the 17 nucleotides selected were complementary to the target RNA. When a 2′-*O*-methyl derivative of the selected sequence was tested *in vivo* using a luciferase reporter under the control of the IRES of HCV, half-maximum inhibition of translation was observed at 2.5 nM concentration. The most effective antisense oligonucleotides targeting HCV IRES exhibit IC_{50}'s of around 100 nM, which means that they are 40-fold less efficient than the sequences selected in this study. Obviously, other modes of action participate in the recognition of the target RNA. Indeed, a variety of structurally similar loop−loop interactions, so-called kissing complexes and apical loop/internal loop (ALIL) interactions, have been found to contribute to the binding affinity of aptamers selected for TAR RNA and HCV mRNA[300−305] (Figure 14).

3.3. Aptamers in Animal Models

Aptamers targeting antibodies were successfully applied to prevent the effects of autoimmune responses.[306] In a rat model of myasthenia gravis (MG), an antibody-mediated autoimmune response to nicotinic acetylcholine receptors that causes a neuromuscular disorder associated with muscular weakness and fatigability, injection of a nuclease-stabilized and PEG-conjugated RNA aptamer showed inhibition of the clinical symptoms of MG.

Intravenous and intra-arterially injected aptamers against growth factors have been used to elucidate their roles in a series of diseases. An aptamer specific for platelet-derived growth factor-B (PDGF-B) was successfully applied in cell culture and by intravenous injection in rats to study the factor's role in glomerulonephritis. Glomerular mesangial cell proliferation plays an important role in many renal diseases. PDGF is believed to play a key role in mesangial cell proliferation. To study the effect of PDFG inhibition in renal diseases, the authors applied a modified aptamer to nephritic rats.[125] For this purpose, a previously selected DNA aptamer for PDGF-B (NX1975)[307] was modified for *in vivo* applications by introducing 2′-fluoro- and 2′-methoxy-substitutions, exchanging nucleotide loops by hexaethylene glycol spacers, and attaching the aptamer to 40 kDa PEG. Glomerular cell proliferation as well as glomerular matrix accumulation was dramatically reduced, demonstrating the benefit of direct injection of custom-modified aptamer antagonists into whole organisms. Interestingly, the anti-PDGF-B aptamer proved to be more efficient compared to an antibody recognizing the same growth factor.[308] In a further study, Floege and coworkers investigated the interaction of PDGF-B and transforming growth factor (TGF-β)-related pathways in renal diseases. By utilizing the PDGF-B aptamer, they were able to demonstrate that both pathways are not interconnected in experimental glomerulonephritis.[126]

The same aptamer was recently employed to elucidate whether PDGF-B is involved in the expression of Ets-1, a key factor in neoangiogenesis[309] and the contribution of PDFG-B and its receptors to persistent pulmonary hypertension (PPHN).[310] The aptamer was able to significantly suppress some of the abnormalities caused by PPHN, suggesting that PDGF-signaling contributes to the structural vascular remodeling that takes place during the syndrome. A different aptamer (EYE001) specific for vascular endothelial growth factor (VEGF) was also employed to enlighten the causes of pulmonary hypertension.[311]

In addition to their potential to elucidate the cellular functions of the targeted hormones, aptamers binding to growth factors have been used to point out new methods of cancer treatment. Growth factors involved in pathogenic angiogenesis, such as those discussed above, are promising targets for tumor therapy. When injected intraperitoneally into mice with experimental Wilms tumors, the aptamer EYE001, also known as NX1838, was able to reduce tumor weight up to 84%.[312]

In a further study targeting vascular endothelial growth factor by an aptamer in mice, the role of VEGF in inflammation and ischemia-induced retinal neovascularization was characterized. Interestingly, while potently blocking pathological neovascularization and the accompanying leukocyte adhesion, physiological neovascularization was not affected.[313]

The studies discussed above all have in common that aptamers were administered by direct injection into the organism. Taken together, the results show promising effects, hence proving that aptamers can be applied via this convenient delivery method, at least when extracellular components such as growth hormones and other signaling molecules are targeted.

Recently, delivery of the aptamer EYE001 (NX1838) formulated as poly(lactic-co-glycolic)acid microspheres was studied.[314] A controlled release over a period of 20 days was achieved using this polymeric device. Aptamers have also been shown to exhibit *in vivo* activity when applied by surgically inserting sustained release pellets into the desired tissue. This formulation has the advantage of high concentrations of the aptamer being deliberated over a certain time span at a defined location within the body. Specifically, a nuclease-stabilized aptamer targeting angiopoietin-2 (Ang-2) was administered into the cornea of rats.[124] Ang-2 is believed to antagonize the receptor tyrosine kinase Tie2, which regulates vascular stability. To decipher the different actions of Ang1 and Ang2 related to proangiogenic growth factors, an Ang2-specific aptamer was selected. Delivery of the aptamer into corneal micropockets inhibited

basic fibroblast growth factor-mediated neovascularization. By utilizing the Ang-2 specific aptamer, a proangiogenic effect of Ang2 was demonstrated for the first time. The authors point out that aptamers which block proangiogenic targets might be promising agents in cancer therapy, since angiogenesis is required in many malignant and benign disorders.

3.4. Aptamers as Delivery Molecules

Aptamers that target specific cell surface proteins represent interesting molecules to target a distinct cell type. By this means, one aptamer that binds tightly to the prostate-specific membrane antigen (PSMA), a protein exclusively present on prostate cancer cells, has gained particular interest.[60] This aptamer was used for the localized delivery of siRNA molecules and toxins *in vitro* and *in vivo*. Therefore, chimeric molecules were prepared that bear the PSMA-specific aptamer and a siRNA molecule that specifically targets an mRNA of interest, such as lamin. Chu et al. used biotinylated variants of the aptamer and the siRNAs and prepared streptavidin conjugates, each composed of two aptamers and two siRNA molecules[315] (Figure 15A). The direct administration of the complex to LNCaP cells, a prostate cancer cell line that expresses PSMA, resulted in a reduction of lamin expression. Indeed, expression of lamin was reduced to the same extent as observed in cells that were transfected with oligofectamine. In contrast, cells that do not have the PSMA protein showed no reduction of lamin expression after treatment with the streptavidin-aptamer−siRNA complex. In a different study, McNamara et al.[260] used aptamer/siRNA chimeric RNAs that target the polo-like kinase 1 and BCL2 (Figure 15). The chimeras used in this study solely consist of RNA, without the need for streptavidin-mediated coupling. The chimeras contained a PSMA-binding aptamer domain, a nucleotide-based linker moiety, and a 21-mer siRNA. This construct was designed in a way as to allow digestion by Dicer and further processing by RISC after PSMA-mediated cellular uptake of the chimera. These constructs were shown to efficiently down-regulate the expression of the siRNA targets, and moreover, antitumor activity was observed when subjected to a xenograft model of prostate cancer. This underlines the applicability of aptamer-mediated tumor targeting *in vivo*.

The anti-PSMA aptamer was also used for localizing cytotoxic agents to tumors, thereby minimizing unwanted toxicological side effects. By this means, Farokhzad et al. used a nanoparticle−aptamer conjugate to treat prostate cancer *in vitro* and *in vivo* using a xenograft nude mouse model[316−318] (Figure 15). The nanoparticles contained docetaxel,[319] a chemotherapeutic taxoid drug used in clincal practice for the treatment of patients suffering from prostate cancer. The aptamer-based targeting of docetaxel showed enhanced cytotoxicity toward the cancer cells and reduced systemic toxicity. Similarly, the targeting properties of the anti-PSMA aptamer can also be combined with the toxic characteristics of gelonin, a ribosomal toxin[320] (Figure 15C). These aptamer/toxin conjugates were shown to specifically reduce cell survival of LNCaP cells. Also along these lines, Bagalkot et al. constructed noncovalent conjugates of the anti-PSMA aptamer with doxorubicin and showed that these conjugates can also be applied for the specific inhibition of the viability of prostate cancer cells[321] (Figure 15C). These approaches make use of covalently linked aptamers to either the nanoparticles or the toxins. This further underlines the chemical versatility of aptamers without loss of functionality. Taken together, aptamers represent excellent tools for the localization of cytotoxic agents *in vivo* and thus to reduce unwanted side effects of chemotherapeutics.

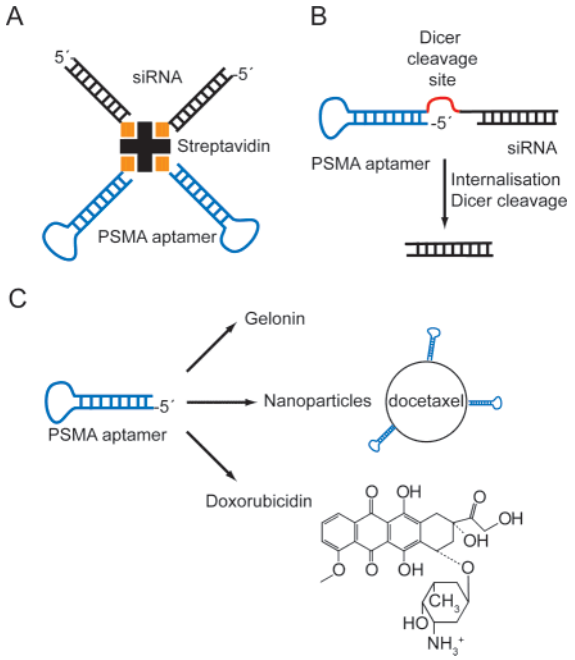

Figure 15. The prostate-specific membrane antigen (PSMA) binding aptamer can be used as a cell type specific delivery agent. (A, B) siRNA molecules can be coupled either via streptavidin−biotin chemistry (A) or directly as a chimeric molecule consisting of an aptamer- and a siRNA-domain to the PSMA aptamer. After PSMA-mediated recognition of the aptamer domain and the receptor-mediated internalization of the aptamer−siRNA complexes, the specific knockdown of the cognate mRNA molecules can be observed. (C) The PSMA aptamer can be covalently coupled via NHS chemistry to toxic proteins, such as gelonin, or to nanoparticles that encapsulate docetaxel. Also, the formation of noncovalent physical conjugates with doxorubicidin has been used for the cellular delivery of cytotoxic agents.

4. Allosteric Ribozymes and Riboswitches

Recently, a growing number of techniques based on ribozymes and aptamers were developed and applied for various purposes in biotechnology, research, and diagnostics and for the development of medical therapeutics. Most of these methods rely on oligonucleotide-regulated ribozymes, also known as aptazymes, reporter-ribozymes, or riboreporters.[322−325] Aptazymes are chimeric molecules that consist of an aptamer domain and a ribozyme module. Just like allosteric enzymes, their catalytic activity is regulated by binding of a small ligand, a protein, or another oligonucleotide to the allosteric domain of the aptazyme, distant from the active site.

4.1. Aptamers and Aptazymes as Molecular Switches

The basis for this concept lies in an intrinsic property of ligand complexation by aptamers, namely adaptive binding which involves different conformational ordering processes, as in the main revealed by structural elucidations of aptamer−ligand complexes.[5−7] Ever since the first NMR[5,114,326−339] and crystal structures[32−34,104,107,340,341] of aptamer−target complexes were resolved, it was clear that

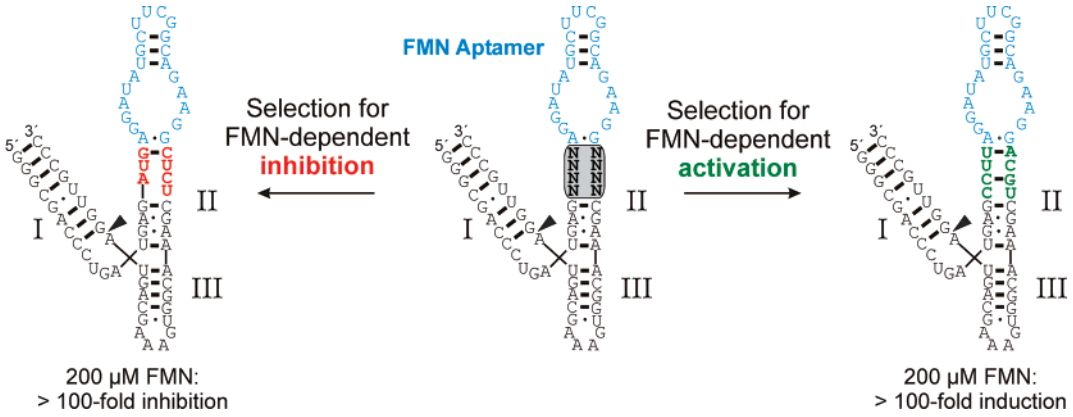

Figure 16. Selection of FMN-dependent aptazymes:[346] The connecting region of an FMN aptamer and a hammerhead ribozyme has been randomized at eight positions. *In vitro* selections were carried out for variants that get activated as well as inhibited by the presence of 200 μM FMN. Both selections yielded a variety of communication modules; one example for each variant is shown.

the binding of a target by an aptamer normally occurs by adaptive recognition. Thereby, aptamers which often comprise unpaired loop or bulge regions that are conformationally heterogeneous in the free nucleic acid acquire a distinct conformation by adaptive folding around the bound target. As a result, complexation of a ligand stabilizes the folding of its aptamer at the binding site, reinforcing the stability of adjacent helical domains. When connected to a weakened but essential helix of a ribozyme or sensor module, ligand binding by the aptamer induces helix stabilization, switching the activity of the ribozyme in the presence of the corresponding ligand.

4.1.1. Aptazymes Based on the Hammerhead Ribozyme (HHR)

The first generations of aptazymes that exploited this concept were based on hammerhead ribozyme (HHR)/aptamer chimeras regulated by small molecules.[342−344] For example, a rationally designed aptazyme regulated by ATP was obtained by fusing a known ATP-binding aptamer[345] adjacent to a hammerhead ribozyme domain.[342,344] In the presence of ATP or adenosine, the cleavage rate of the ribozyme was decreased 180-fold compared to a control reaction in which dATP or other NTPs were used, which do not interact with the allosteric aptamer domain. A detailed investigation of this aptazyme's regulatory mechanism revealed that the observed reduction in catalytic activity of the adjacent ribozyme domain was due to steric interference between the aptamer and ribozyme tertiary structures.[344]

Similarly, two independent studies combined the hammerhead ribozyme with an aptamer that binds flavine mononucleotide[113] (FMN) as the allosteric regulatory module.[343,346] In one study,[343] six variants with different sequences in the essential stem II that connects the aptamer with the hammerhead ribozyme were rationally designed. Stem II is essential for ribozyme activity. Only sequences with a stem II that was shorter than 5 base pairs were found to be sensitive to FMN, indicating that ribozyme activation by FMN occurred by inducing stem II formation. Thus, the connecting stem serves as a communication module between the allosteric FMN binding site in the aptamer and the catalytic domain in the ribozyme. In the second study,[346] stem II was also used as the connecting domain, but this time, the stem II variant with the potential to form four base pairs was randomized in the corresponding eight nucleotides (Figure 16). This small HHR-aptamer library was subjected to *in vitro* selection to identify variants that mediated HHR-dependent RNA cleavage either in the presence or in the absence of FMN. The identified stem II sequences were shown to exclusively allow communication between the modular domains of the HHR construct and, thus, were designated as "communication modules". Using other known aptamers as allosteric sites, the selected communication module was successfully used to engineer aptazymes with other ligand specificities. When the FMN aptamer module[113] was replaced with previously identified ATP[345] and theophylline binding aptamer motifs,[347] new aptazymes regulated by ATP and theophylline, respectively, were obtained. In the meantime, versatile RNA elements that function as communication modules rendering disparate RNA folding domains interdependent have been described.[348] The FMN and theophylline aptamers were also used in a single allosteric HHR to engineer an aptazyme that requires two different small molecule effectors to induce catalytic activity.[349]

An intuitive design of effector-regulated hammerhead ribozymes can also be achieved by using oligonucleotides that regulate catalysis by hybridizing to the ribozyme. In the first example of an allosterically controllable ribozyme,[350] a DNA oligonucleotide complementary to a single-stranded loop in the ribozyme was used to switch on the active state of an otherwise inactive HHR.

There are two types of allosteric ribozymes amenable for screening: First, there are ribozymes that selectively monitor substrate or product formation during an enzymatic reaction. These are used to screen for inhibitors of catalytic activity in an indirect way. For example, a minimized ADP aptamer was used to construct a RiboReporter sensor that detects ADP in a background of ATP and generates a fluorescent signal[351] (Figure 17A). Upon binding to ADP, the sensor undergoes self-cleavage and generates a fluorescent signal. The ADP RiboReporter sensor was used to monitor pERK2 protein kinase activity in a pilot screen of 77 test druglike compounds. The ADP RiboReporter sensor unambiguously identified microplate wells containing a known inhibitor (staurosporine), but no new functional molecules could be discovered in this test screen.

In a similar approach, Nutiu et al.[352] used an allosteric DNA-aptamer that binds adenosine better than 5-adenosine monophosphate (AMP) to report the activity of the alkaline phosphatase-catalyzed cleavage of AMP to adenosine (Figure 17B). This fluorescent reporter was applied for the indirect identification of known small molecule inhibitors of the alkaline phosphatase (ALP).

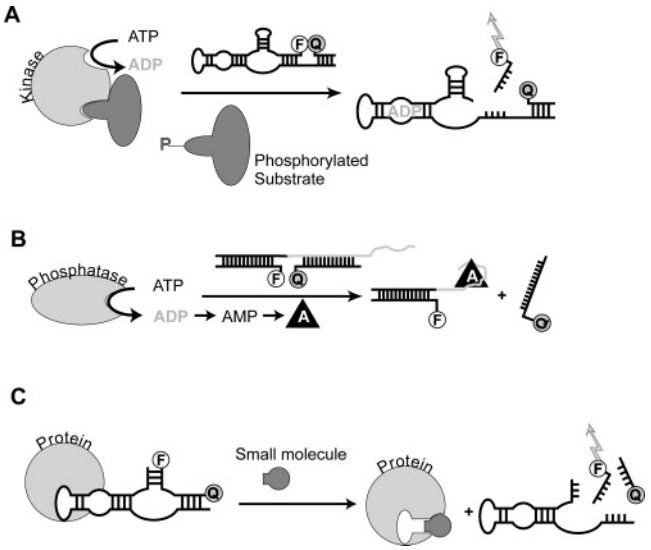

Figure 17. Aptamers and ribozymes for inhibitor screening. (A) A RiboReporter consisting of an ADP-aptamer appended to the hammerhead ribozyme indirectly reports kinase activity by responding to ADP generated in the reaction.[351] ADP binding activates ribozyme cleavage and leads to release of the fluorescence (F)-labeled oligonucleotide, which generates a fluorescence signal. (B) The activity of alkaline phosphatase is reported indirectly by a DNA aptamer that binds to adenosine (black triangle) produced in the reaction.[352,370] Binding of the aptamer to adenosine releases the quencher (Q)-labeled oligonucleotide, and fluorescence can be detected. (C) A protein-dependent hammerhead ribozyme directly reports molecular interactions of the bound protein with inhibitory small molecules.[360] Binding of a small molecule to the protein releases the protein from the ribozyme and thus triggers cleavage of the doubly labeled FRET probe, which causes fluorescence. These reporter ribozymes were used to identify novel small molecule inhibitors of the HIV-1 Rev and HIV-1 reverse transcriptase proteins.

Second, there are ribozymes whose cleavage activity is directly dependent on proteins and peptides. Small molecules or other proteins that disrupt aptazyme−target binding can adversely affect the cleavage activity of these allosteric ribozymes. For example, hammerhead ribozyme-based reporter ribozymes were designed that are either active or inactive upon binding the HIV-1 Rev protein or its arginine rich motif (ARM) peptide epitope.[353] These allosteric ribozymes were used to screen a small library composed of 96 natural antibiotics for molecules capable of disrupting the interaction of the HIV-1 rev-peptide with its complementary RNA binding site, the Rev response element[354,355] (RRE), or an anti-rev aptamer.[356,357] For monitoring the aptazyme activity, a substrate oligonucleotide labeled with a fluorescence donor at the 5′-end and a quencher dye at the 3′-end was used that emitted a fluorescence signal only after cleavage by the ribozyme to allow for efficient real-time analysis of aptazyme performance.[358,359] The screening of the library revealed one compound, the gyrase inhibitor coumermycin A1, that was able to inhibit virus replication *in vivo*. Thus, the small molecule possesses the same characteristics as the aptamer from which it was derived. This study established that it is possible to identify novel small molecule inhibitors for a given protein by using interference with RNA/protein interactions as a basis for screening (Figure 17C).

In a similar study,[360] we fused an HIV-1 reverse transcriptase (RT) binding aptamer[272] to the hammerhead ribozyme. Binding of RT to its aptamer domain induces the formation of a pseudoknot structure.[271] In the absence of the protein effector, the aptazyme remains active, and substrate cleavage can be monitored by an increase in fluorescence. Owing to the specificity of the parent aptamer, the reporter ribozyme only detects RT from the HIV-1 strain and is irresponsive to the homologue RT of HIV-2. This reporter ribozyme was recently used to screen a library of 2,500 small druglike compounds.[361] This screen identified SY-3E4, a substituted *N*,*N*′-diphenylurea derivative, as a new inhibitor of the DNA-dependent, but not RNA-dependent, primer elongation activity of reverse transcriptases from HIV-1 and -2. Other RTs or DNA polymerases either were not targeted or were only marginally blocked by this inhibitor. In addition, SY-3E4 inhibited HIV-1 replication for wild-type and a multidrug resistant strain in a mechanism in which the compound competes with primer/template-complex binding to HIV-1 RT. An analysis of available crystallographic data of HIV-1 and HIV-2 RTs,[32,362−365] constrained by the characteristics of drug resistance mutations[261,275,276,366−369] and the inhibitory profile of the compound, suggested a region in these proteins as a new drugable interface that can be targeted by a small molecule.

There are several other RNA/protein complexes that are important in the life cycle of HIV-1, for example that between the HIV-1 Tat protein and its viral RNA receptor, the *trans*-activation responsive element[371] (TAR). The TAR motif was utilized for rationally designing HHR-based protein-responsive allosteric ribozymes that are regulated by the HIV-1 Tat protein.[372−374]

Allosteric HHRs were also used to monitor the phosphorylation status of proteins.[375] The rational design was based on aptamers that specifically recognized the nonphosphorylated and the phosphorylated forms, respectively, of extracellular regulated kinase 2 (ERK2), a member of the mitogen-activated protein kinase (MAPK) family (the aptamers used in this study differed from previously described aptamers from the same group that inhibit the *in vitro* phosphorylation activity of ERK2[376]). The aptamers were fused to stem II of the hammerhead ribozyme in such a way that the formation of inactive ribozyme conformers was favored in the absence of the respective phosphorylated or unphosphorylated forms of ERK2. In their presence, however, the ribozyme became active in cleaving a substrate RNA. This design bears the potential of monitoring the phosphorylation status of a protein in a solution-phase assay format.

These examples are all based on rationally designed allosteric hammerhead ribozymes. However, allosteric aptazymes can also be isolated directly by *in vitro* selection approaches. This is useful in cases in which no aptamer for a particular ligand is available or in which an aptazyme is sought to perform under special conditions such as limited divalent metal ion concentrations, a certain pH, or unusual ionic strengths.

For example, an RNA library was used to select allosteric ribozymes that responded to cGMP and cAMP.[377] For constructing the library, the entire aptamer domain of the previously described FMN aptazyme[346] was replaced by 25 random nucleotides whereas the "cm+FMN1 communication module"[346] and the ribozyme were left unaltered. This library was subjected to a process called "allosteric selection", which aims for the isolation of aptazymes that self-cleave only in the presence of the desired effector molecule but remain uncleaved in the presence of other compounds. The selection was based on size-separation of cleaved and uncleaved

species by polyacrylamide gel electrophoresis (PAGE), and included negative selection steps in the absence of effector compounds. In this way, allosteric ribozymes were isolated that could be activated 5000-fold by either cGMP or cAMP in a highly specific manner.

Similarly, one of the theophylline-dependent aptazymes[346] was subjected to allosteric selection to isolate allosteric catalysts with new effector specificities.[378] This study showed that related aptamer motifs can be isolated that are capable of binding any of eight related theophylline analogues. Remarkably, one ribozyme variant differed from the parent theophylline aptamer only in a single mutation, which defines a specificity switch from theophylline to 3-methylxanthine.

A related approach, but without exploiting communication modules, was applied by our group. We constructed a HHR-based library replacing stem II of the HHR with 40 random nucleotides.[379,380] This library was successfully subjected to the *in vitro* selection of allosteric ribozymes that depend on the antibiotics doxycycline and pefloxacine. The doxycycline-dependent ribozymes showed inhibition constants as low as 20 nM and revealed remarkable specificity, since almost no regulation by the related antibiotic tetracycline was observed. Recently, allosteric reporter ribozymes that respond specifically to caffeine or aspartame were isolated by allosteric selection.[381]

4.1.2. Aptazymes Based on the Hairpin Ribozyme

The hairpin ribozyme, when annealed to its RNA substrate oligonucleotide, shows a unique secondary structure comprising two domains: A and B.[382−384] Crystallographic and biochemical studies[385,386] revealed that the mechanism of cleavage is characterized by an oscillating reaction pathway starting with a conformation where these two domains are coaxially stacked and aligned in a linear extended fashion. This conformer then folds into a docked structure with domains A and B interacting mainly via noncanonical base pairs, resulting in the ribozyme bending sharply at a site defined as the hinge region.[387−390] Only this docked conformation permits site-specific cleavage of the substrate. Following cleavage, the docked complex unfolds back into the extended structure and the cleaved products dissociate. The hinge region acts as a flexible linker mediating the docking process by stabilizing the active conformation and facilitating the tertiary contacts that define the active site within the interface between the internal loops.[391] This fairly detailed understanding of the molecular mechanism involved in hairpin ribozyme catalysis highlights the hinge region as a promising target domain for controlling the ribozyme's structure and folding pathway by potentially binding effector oligonucleotides. Consequently, a series of ligand regulated ribozymes based on hairpin ribozyme variants have been described.

Our research group, for example, aimed toward the use of allosteric ribozymes for reporting protein−protein interactions.[353] For that purpose, hairpin ribozyme variants were designed that contained sequences complementary to the anti-thrombin DNA aptamer.[15] Upon addition of the aptamer sequence, the aptazymes were specifically inactivated. In the presence of the cognate α-thrombin, the hybridized aptamer was sequestered, leading to activation of ribozyme catalysis by allowing proper folding into its active conformation. As with the hammerhead reporter ribozymes, ribozyme catalysis could be quantified in real time by applying substrate oligonucleotides labeled with a fluorophore and quencher dye. Other proteins, such as the highly related γ-thrombin, which lacked the aptamer binding domain but was otherwise identical to α-thrombin, or factor Xa, another member of the blood coagulation cascade, had no effect. Conversely, in the presence of hirudin, a natural oligopeptide inhibitor of α-thrombin which competes with aptamer binding through its high-affinity interaction with exosite-I,[392] the catalytic activity of the ribozyme remained low, because hirudin led to release of the aptamer from its α-thrombin target so that it hybridizes back to the ribozyme. Thus, the aptazymes were able to report the thrombin−hirudin interaction in a concentration-dependent fashion, thereby representing an effective tool for the analysis of protein−protein interactions.

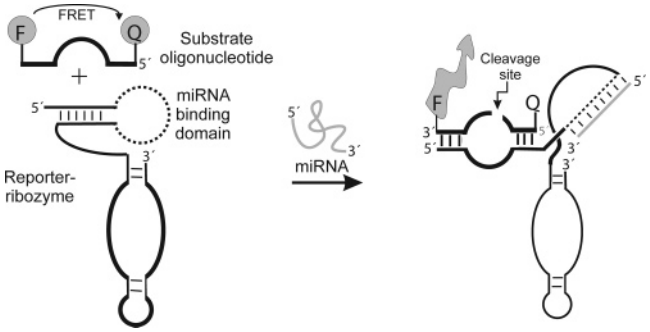

Figure 18. The miRNA-detecting reporter-ribozyme contains a domain (dashed) complementary to a given miRNA. In the absence of the miRNA (gray), the fluorophor (F)- and quencher (Q)-labeled substrate oligonucleotide remains uncleaved and nonfluorescent due to fluorescence resonance energy transfer (FRET). After miRNA binding, the reporter-ribozyme cleaves the substrate. Fluorescence is detected when the cleaved substrate halves dissociate off the ribozyme.

Other examples of engineered hairpin ribozyme variants included reporters for oligonucleotides such as microRNAs[393] (Figure 18), the mRNA leader sequence of the *trp*-mRNA,[394] the RNA-binding site for the bacteriophage R17 coat protein,[395] and sequences of nonspecified function.[396,397] Approaches for the direct detection of oligonucleotides without the requirement of any external labeling become increasingly important because they would allow for the analysis and quantification of a nucleic acid in an organism, cell, or tissue which is incompatible with extra chemical steps such as attachment of fluorophores or radioactive residues.[398]

One of our aims, therefore, was to design allosteric hairpin ribozyme constructs allowing the direct detection of microRNAs[393] (miRNAs). miRNAs are a class of eukaryotic small functional RNA sequences that regulate gene expression.[399−402] We have designed hairpin ribozyme variants by incorporating a new domain C, which is complementary to the target nucleic acid and also contains a short sequence that can partially pair with domain A, thus rendering the ribozyme inactive because domain A can no longer dock to domain B. In this way, ribozyme activity can be induced by externally added miRNAs (Figure 18). This design significantly differs from the approaches with similar goals described before[396,403] (see also another highly sensitive system based on the hepatitis C virus ribozyme[404]), in that here oligonucleotide-induced hairpin ribozymes can be rationally designed,[393] whereas the other approaches require *in vitro* selection of the required regulatory domains.[396,403,404] We designed nine ribozymes that could be activated by different miRNAs. For each ribozyme, fluorescence increased only upon addition of the cognate miRNA whereas addition of noncognate miRNAs resulted in baseline levels of

fluorescence only. The assay had a detection limit of 50 fmol, which is at least 10-fold more sensitive than related molecular beacons.[405,406] Like the MG-aptamer-based system,[109] these reporter ribozymes are also entirely RNA-based and thus could potentially be expressed endogenously, requiring only the addition of the short substrate oligonucleotide.

While the miRNA-sensitive ribozymes could be induced by the external oligonucleotide, slightly modified systems in which the same effector oligonucleotide can serve as either an inducer or a repressor can also be designed rationally.[394] These hairpin ribozyme variants were engineered for detecting proteins such as human thrombin[407] or the trp leader mRNA, the RNA sequence tightly bound by the L-tryptophan-activated trp-RNA-binding attenuation protein (TRAP) of *Bacillus subtilis*.[408] TRAP regulates the expression of genes required for L-tryptophan biosynthesis in response to changes in the intracellular level of L-tryptophan.[409] Comprising 11 identical protein subunits arranged in a single ring, TRAP binds a specific RNA sequence in the nascent *trp*-mRNA transcript leader region, but only when cooperatively activated by 11 bound L-tryptophan molecules.[410−412] As in the case of the miRNA-responsive hairpin ribozymes,[393] variant ribozymes contained an additional domain C, complementary to this mRNA. Ribozyme activity can be switched by hybridizing to the trp leader mRNA and then can be specifically reverted by the TRAP−L-tryptophan complex via sequestration. These reporter ribozymes thus can sense the activity status of a protein controlled by its metabolite molecule and could potentially be applied for the screening of TRAP-binding small molecules. Similarly, these reporter hairpin ribozymes can also be regulated simultaneously by an oligonucleotide effector and the small ligand flavin mononucleotide, if the ribozyme harbors a corresponding FMN aptamer.[413] This example established a new principle for the regulation of ribozyme catalysis in which two regulatory factors (an oligonucleotide and a small molecule) that switch the ribozyme's activity in opposite directions compete for the same binding site in the aptamer domain.

Other oligonucleotide-regulated catalytic nucleic acids are based on RNA-cleaving catalytic DNAs.[414] These DNAs, called "10-23" and "8-17", were obtained by *in vitro* selection.[415] 8-17 was originally selected as a deoxyribozyme, termed "Mg5", that cleaved a ribo-A residue imbedded in a DNA sequence context.[416,417] Later, Mg5 and 8-17 were found to be identical.[418] Based on similar catalytic DNAs, a series of sensor systems for metal ions were developed.[419] Recently, one of these systems was transformed into a colorimetric lead biosensor by using the DNAzyme to assemble gold nanoparticles.[420,421] A similar principle of detection of binding events with gold nanoparticles was constructed which allowed the litmus-test-like detection of adenine and cocaine.[422−424] Similarly, aptamer-modified gold nanoparticles were also applied for colorimetric determination of platelet-derived growth factors and their receptors.[425]

4.1.3. Aptazymes Based on Ligase Ribozymes

The aptazyme approach was broadened by using either other ribozymes or different effector molecules. In this manner, Ellington and co-workers engineered small-molecule-dependent ligase ribozyme variants by using an artificial oligonucleotide-dependent ligase ribozyme[426] (Figure 19).

They used the aptamer domains of ATP[113,345] and theophylline[347] and constructed allosteric ligase ribozymes now dependent on ATP and theophylline, respectively.[427] The

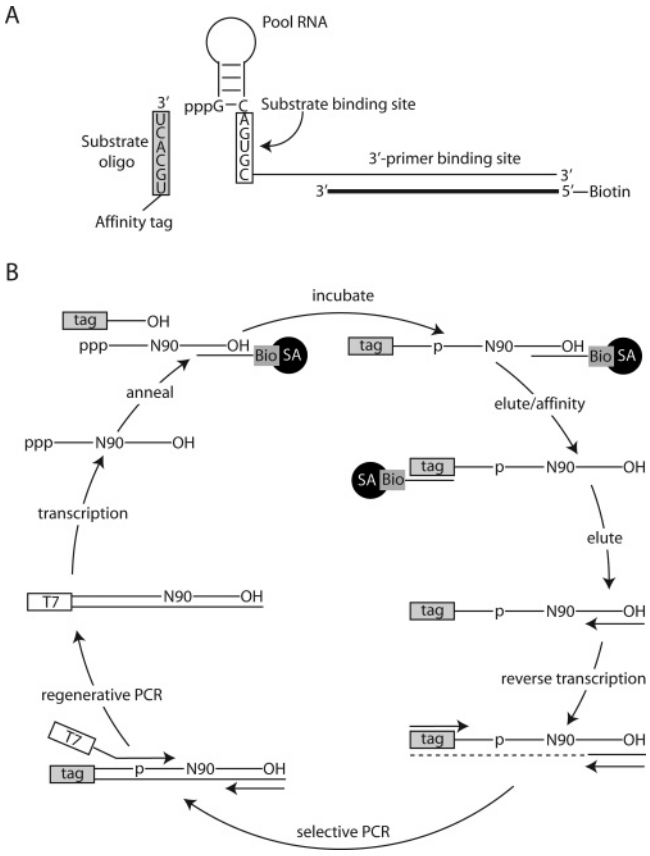

Figure 19. Random pool design and selection scheme for the ligase ribozyme. (A) N90 pool. The substrate oligonucleotide is boxed in gray, and the substrate binding site is boxed white. (B) The selection scheme used to isolate ligase ribozymes from the N90 pool. Five rounds of selection were performed with the pool of N90 RNA molecules, captured on streptavidin agarose (SA; 11 o'clock) with pool-specific primers and substrate oligonucleotides. After equilibration with $MgCl_2$ in buffer, the reaction was initiated by addition of a twofold molar excess of substrate oligonucleotide (gray box, tag). RNA was then eluted from the streptavidin (1 o'clock), and reactive molecules containing the "tag" sequences were affinity purified according to which "tag" sequence was used (2 o'clock). Columns were washed to remove members of the RNA pool that had not reacted with the substrate; ribozymes were eluted with base (3 o'clock). Selected populations were reverse transcribed with SuperScript II reverse transcriptase (4 o'clock). The cDNA was then PCR amplified (6 o'clock). This DNA was used as the input for an additional, regenerative PCR amplification (8 o'clock). The DNA was then transcribed (10 o'clock), and the resulting RNA was used as the input for the next round of selection.

construction of FMN-dependent ligase ribozymes was shown to be less efficient. Thus, *in vitro* selection of FMN-dependent ligase ribozyme variants was applied, similar to a previous selection scheme.[322] In another approach, ligase aptazymes were used as the basis to construct artificial nucleoprotein ribozymes.[428] The resultant nucleoprotein ligases were shown to interact with the cognate protein in a similar way as aptamers do, and moreover, the ligase activity of the ribozymes was activated upon addition of lysozyme or Cyt18. In a second approach, Robertson et al. used the same ligase library to gain HIV-1 Rev-dependent aptazyme ligases.[429] These results may have important implications on the development of ligase-dependent diagnostic arrays for the analysis of the HIV-1 proteome.[99,430] In a different study, Thompson et al. enhanced the aptazyme technology by using group I introns for engineering of theophylline-regulated genetic switches. They demonstrated that the artificial

theophylline-regulated group I intron was able to mediate theophylline-dependent splicing *in vivo*.[325]

4.1.4. Aptazymes Based on the Diels–Alderase Ribozyme

The Jäschke group expanded the aptazyme approach to a ribozyme that catalyzes the Diels−Alder reaction.[431,432] This ribozyme was shown to act as a Diels−Alder catalyst, catalyzing a [4+2] cycloaddition reaction between the diene anthracene and the dienophile maleimide.[433−435] Helm et al. used *in vitro* selection to identify communication modules that enable the allosteric regulation of the Diels−Alder ribozyme in response to the alkaloid theophylline.[436] A rational design approach led to Diels−Alder ribozymes that can be allosterically controlled by three different chemical effectors: the theophylline, the aminoglycoside tobramycin, and a region of the mRNA encoding for the tumor-related kinase Bcr-Abl.[437] Due to the inherent fluorescence of the anthracene moiety, the reactions of the ribozyme and the described aptazymes can be easily monitored by fluorescence spectroscopy. This might allow the direct application of the Diels−Alderase aptazymes as signaling molecules in sensor-based assays.

4.2. Riboswitches: Natural Regulatory Aptamers

The first hints, even though not finally proven, suggesting the existence of natural regulatory aptamers, or riboswitches, came from biochemical and genetic studies in bacteria elucidating the mechanisms of feedback regulation of vitamin and coenzyme biosynthesis pathways. For example, Weglenski and colleagues observed homologies between *in vitro* selected RNA aptamers and RNA sequences in the 5′-untranslated region (UTR) of transcripts of the arginase structural gene (*agaA*) from *Aspergillus nidulans*.[438] It was found that, depending on the growth conditions, transcripts of this gene showed differences in the fairly long and highly structured 5′-UTRs. It was suggested that these UTRs contain putative aptameric binding sites for arginine, which suggested a participation of the amino acid in regulation of agaA-expression via aptameric binding to this 5′-UTR RNA.

The analysis of the transcripts of several operons, encoding for the genes that are mandatory for the biosynthesis of vitamins and coenzymes, such as thiamine, adenosylcobalamine, and flavin-mononucleotide (FMN), revealed evolutionary conserved RNA regions located in the 5′-UTR of these operons.[439−445] Mutational studies illustrated that the conserved RNA regions are necessary for the accurate regulation of various biosynthesis genes.[443,446−452] These findings led to the assumption that either a vitamin or coenzyme-dependent protein or the metabolite itself interacts with the corresponding conserved mRNA region and thus allows the genetic control of protein translation.[453] Nou and Kadner demonstrated that in the presence of adenosylcobalamine the binding of the ribosome to the to the *butB* RNA, an mRNA that encodes for a cobalamine transport protein, is prevented.[454] In accordance to that, Ravnum and Andersson illustrated that cobalamine induces a distinct folding of the mRNA that results in the sequestration of the ribosome binding site (RBS).[455] Finally, Nahvi et al. showed that the *btuB* mRNA selectively binds coenzyme B12 without any further need of a protein factor.[456] The interaction of the mRNA with the coenzyme molecule forced the mRNA into a distinct conformation whereas the RBS is blocked and hence translation initiation is inhibited. The mechanism by which adenosylcobalamine influences gene expression relies

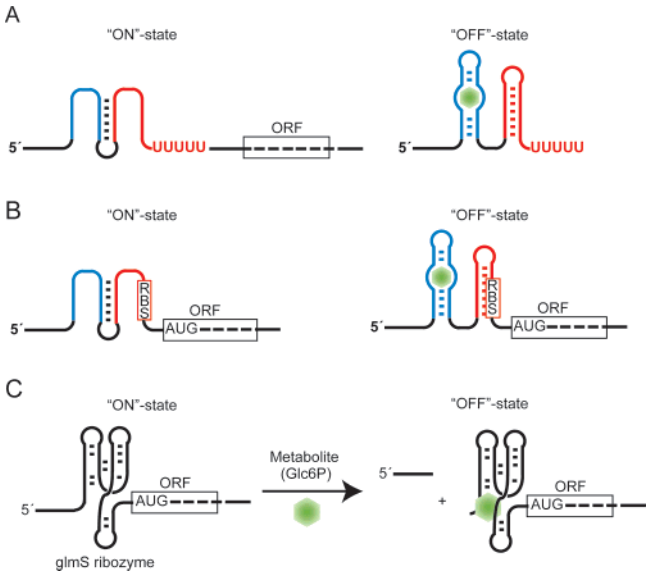

Figure 20. Overview of common principles of riboswitches. (A) Termination of transcription: Upon binding of the regulatory metabolite to the aptamer part of the riboswitch, reorganization of stem-looped regions occurs. As a consequence, within the expression platform of the riboswitch, a transcriptional terminator structure forms and gene expression is shut down. Blue, aptamer platform; red, expression platform; ORF, open reading frame; RBS, ribosomal binding site. (B) Inhibition of translational initiation occurs by a similar mechanism involving refolded hairpin domains. Binding of the metabolite to the aptamer platform results in folding of a stem masking the ribosome binding site (RBS) and, hence, is inhibiting gene expression. Blue, aptamer platform; red, expression platform. (C) Activation of ribozyme cleavage was found in the case of the glmS riboswitch. Glucosamine-6-phosphate (Glc6P) binds to the 5′-UTR of the message and thereby activates ribozyme cleavage, resulting in inhibition of gene expression. The metabolite presumably acts as true cofactor of the cleavage reaction rather than inducing the active ribozyme fold.

on both transcription attenuation and inhibition of translational initiation.[455−457] Since the discovery of the adenosylcobalamine-dependent riboswitch, a series of other distinct riboswitches were identified. Currently, riboswitches regulated by metabolites such as thiamine, FMN, guanine, adenine, *S*-adenosylmethionine, glycine, and lysine are known.[458−467] The overall topology of riboswitches can be divided into two communicating domains: (i) a highly evolutionary conserved aptamer domain, that serves as a specific and high affinity sensor for the metabolite molecule, and (ii) the sequence variable expression platform by which the riboswitch exerts its inhibitory or activation effect[468] (Figure 20). The three-dimensional structures of some riboswitch aptamer domains have been elucidated[469−475] and revealed that the metabolites stabilize secondary and tertiary structure elements that are harnessed by the riboswitch to modulate the synthesis of the proteins coded by the mRNA. In addition, the structures provided insight into how folded RNAs can form precision binding pockets that rival those formed by protein genetic factors.

Riboswitches were found in Gram-positive as well as in Gram-negative bacteria, and it is becoming apparent that eukaryotes also possess these highly conserved elements.[459,476,477] In contrast to the fairly clear topology of the riboswitches, the mechanisms by which they modulate gene expression seem to be rather complex. In addition to the inhibition of gene expression, the discovery of a riboswitch that regulates ribozyme function and the adenine riboswitch that activates gene expression strongly broadened the spectra

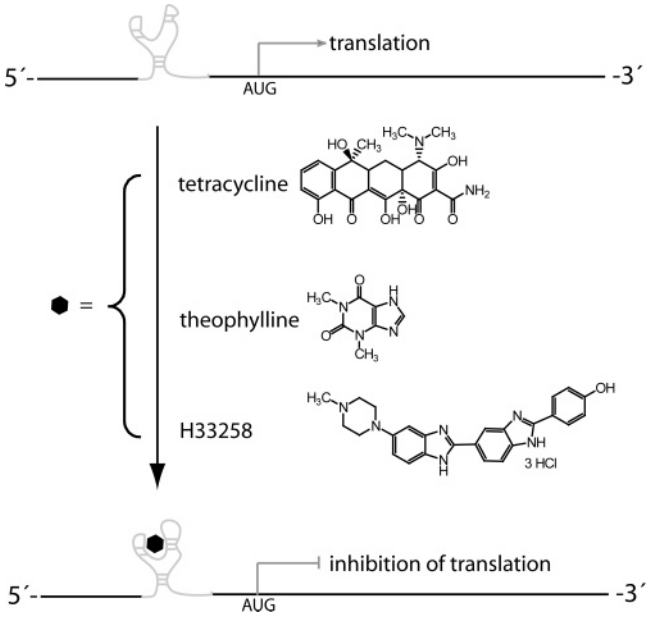

Figure 21. Aptamer sequences can be introduced into the 5′-UTR of reporter genes. Upon the addition of their cognate ligands, e.g., tetracycline, theophylline, or the Hoechst dye H33258, the translation of the reporter is suppressed.

of riboswitch-mediated genetic control.[478,479] Recent findings of the ribozyme-mediated glmS riboswitch point to the possibility that the regulating metabolite, glucosamine-6-phosphate (Glc6P), is actively participating in the cleavage reaction as cofactor instead of inducing the activated structure of the ribozyme;[480,481] see Figure 20C. Recent evidence indicates the use of riboswitches for splicing control in eukaryotes further enhances the portfolio of metabolite-regulated genetic switches.[482] Being regulated by small molecule metabolites, riboswitches may be interesting candidate target molecules for the development of novel antibacterial compounds.[483] Currently, assays that are compatible with high-throughput screening of riboswitch inhibitors are being developed,[484−486] and the first antibacterial small molecule inhibitors that target riboswitches have been reported.[487,488]

4.3. Artificial Riboswitches

Prior to the discovery of riboswitches in nature, the underlying principle was already realized in artificial systems. By introducing aptamers that bind to Hoechst dyes H33258 and H33342 into the 5′-UTR of a β-galactosidase reporter, small molecule-mediated repression of translation was observed in mammalian cells.[489] Translational control elements based on tetracycline and theophylline-controlled aptamer have been realized in yeast.[490−492] In these studies, the potential of aptamer/ligand interactions to suppress translation was demonstrated using reporter genes (Figure 21).

In contrast, Grate and Wilson inserted a malachite green aptamer[111] into the 5′-UTR of a cyclin transcript in *S. cerevisiae*, thereby rendering the cell cycle control responsive to the triphenylmethane dye.[105] Taken together, the approach of inserting small molecule-binding aptamers into transcripts demonstrates the possibility of specifically regulating gene expression on the translational level. Whether the technique possesses further potential toward applications in disease-related therapy remains to be shown.

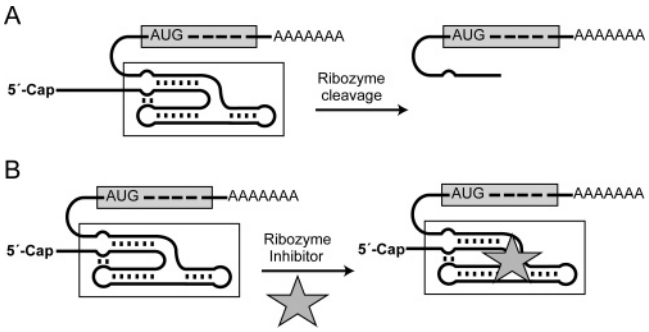

Figure 22. Modulating gene expression with ligand-regulated ribozymes. (A) Ribozyme-mediated inhibition of gene expression. A hammerhead ribozyme derived from *S. mansoni* was introduced into the 5-UTR of an mRNA. The fast cleavage kinetics of the ribozyme results in an mRNA that has lost the 5′-capped fragment, resulting in inhibition of gene expression. Gray box, open reading frame; white box, hammerhead ribozyme. (B) Activation of gene expression by hammerhead inhibitors. Translation of intact mRNA and, hence, gene expression is facilitated by addition of hammerhead ribozyme inhibitors (star). Gray box, open reading frame; white box, hammerhead ribozyme.

Mulligan et al. recently introduced an exogenous control system for regulating gene expression in mammalian cell lines.[493,494] By positioning a modified version of the Sm 1 hammerhead ribozyme from the trematode *Schistosoma mansoni*, termed N79, into different untranslated regions upstream and downstream of a LacZ reporter gene, the expression of this gene was suppressed by the ribozyme's constitutive cleavage activity[495−497] (Figure 22A). However, gene expression could be switched on by adding either an oligonucleotide complementary to the ribozyme or a small-molecule inhibitor of ribozyme activity, thereby interfering with the self-cleavage activity of N79 (Figure 22B). These tailor-made inducers of gene expression were even shown to work in mice. Together with other methods, like intramers[9,16,26,27] or aptazymes,[10,228,244,498] it may be possible to engineer systems for the regulation of *in vivo* gene expression to respond to virtually any small molecule or metabolite.

It is also possible to apply *in vitro* selected aptamers for creating *E. coli* strains that activate protein translation only in the presence of the cognate small molecule.[499] The theophylline aptamer[347] was transformed into a synthetic riboswitch by subcloning it upstream of the ribosome binding site of a β-galactosidase reporter gene that allowed accurate measurement of small changes in β-galactosidase activity in response to theophylline binding. Interestingly, cells that harbored the aptamer could specifically be amplified from a pool containing a 10^6-fold excess of cells expressing a mutant aptamer which do not respond to theophylline. This result suggests that this method might be further used to perform *in vivo* selections for aptamers with riboswitch activities that respond to closely related small molecules from a pool of mutant sequences.

4.4. Natural versus *in Vitro* Selected Aptamers

Comparisons of natural aptamers with *in vitro* selected aptamers reveal distinct differences between the two species. First, the binding motifs of some natural aptamers are longer than the motifs found by *in vitro* selection.[468] Second, the affinities of the natural aptamers for their cognate target molecules are significantly higher than many of those found with artificial aptamers.[323,468] Interestingly, a recent *in vitro* selection study revealed that RNA aptamers with a higher

affinity for GTP are more informationally complex than lower affinity aptamers. Carothers, Szostak, and colleagues have experimentally measured the amount of information required to specify each optimal binding structure in 11 distinct GTP-binding RNA aptamers.[500] This analysis revealed that defining a structure capable of 10-fold tighter binding requires approximately 10 additional bits of information. This increase in information content is equivalent to specifying the identity of five additional nucleotide positions and corresponds to an approximately 1000-fold decrease in abundance in a sample of random sequences. However, the comparison of the binding specificities of these aptamers using a diversity of chemical analogues of GTP revealed distinct patterns of specificity for each individual aptamer, indicating that each aptamer had found a unique three-dimensional solution for GTP binding.[501] This analysis also showed that aptamers that bind their ligand with higher affinity do not necessarily bind this ligand more specifically than a lower affinity aptamer. Thus, additional information needed to improve affinity does not specify more interactions with the ligand. Structural studies carried out with a high-affinity GTP-binding aptamer suggested that the additional components of information in an aptamer leading to tighter binding are primarily utilized for stabilization of RNA tertiary structure with additional RNA−RNA contacts as opposed to increasing the number of specific contacts to the ligand.[502]

Intensive analysis of metabolites and their derivatives binding to natural aptamer domains suggested that the aptamer domains in riboswitches appear to utilize the entire metabolite for high affinity binding.[458,460,466] However, the crystal structures of the thiamine riboswitches revealed that the interaction of the metabolites with the aptamer domains actually depends on the presence of the phosphate groups,[472,473,475] whereas the central thiazole moiety is not recognized by the RNA and can be substituted by a pyridyl moiety, as found in pyrithiamine pyrophosphate.[487] Although the phosphate group is anionic so that the RNA has to overcome electrostatic barriers, it can form such interaction profiles by folding into sophisticated shapes and by positioning divalent metal ions, allowing it to bind, in principle, to almost any desired molecule independent of its overall charge.

In vitro selected aptamers raised against nucleotide cofactors such as ATP revealed a strict prevalence for the nucleotide moiety of the molecules and showed no binding dependence on phosphate groups.[327,503] Indeed, targeting the phosphate group seems to be very challenging and hard to obtain by *in vitro* selection.[503] Nevertheless, a recent example demonstrates that *in vitro* selection experiments can actually be driven in a way that favors triphosphate over nucleotide binding of RNA aptamers.[504] Affinity measurements of the resultant ATP binding aptamer revealed a discrimination factor of 64 over ADP and 1100 over AMP, respectively (Figure 23). This result demonstrates that sophisticated selection protocols can be applied to gain novel artificial aptamers with enhanced specificities and affinities in combination with alternative binding modes. The modular topology of riboswitches, bearing a highly conserved and structured aptamer domain in combination with an expression platform for mediating gene regulatory effects, consequently leads to the desire for developing novel engineered riboswitches on the basis of *in vitro* selected RNA aptamers.

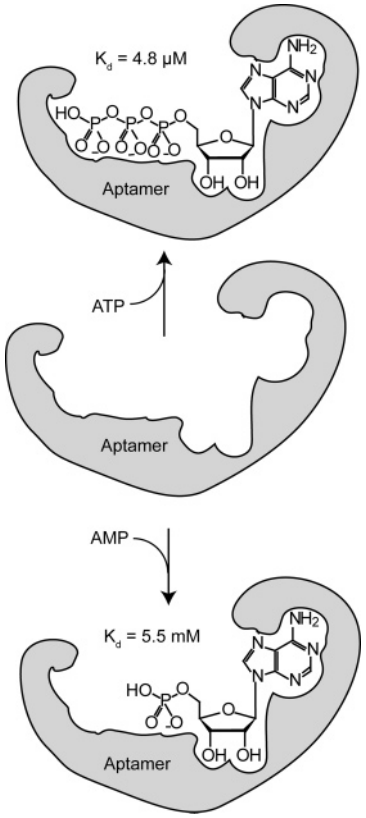

Figure 23. RNA-based ATP aptamer that is able to discriminate between adenosine ligands based on their 5′ phosphorylation state. This aptamer was obtained by applying a selective pressure that demands recognition of the 5′-triphosphate. It binds to ATP with a K_d of approximately 5 μM and to AMP with a K_d of approximately 5.5 mM, a difference of 1100-fold. This aptamer demonstrates that small RNAs can utilize negatively charged moieties for interaction.

5. Conclusion

Aptamer technology has matured over the last 17 years from being a technique to identify ligands for organic dyes and proteins to a wide research field, providing sophisticated inhibitory molecules that allow functional interference in biological systems. The main quality of aptamers might be their flexible applicability. The nucleic acid nature of aptamers allows their synthetic access and, hence, their convenient and versatile modification with functional groups. In this sense, they should be considered to be "chemicals" rather than "biologicals". Aptamers can therefore be employed for various applications ranging from diagnostic to therapeutic assay formats. Although the first therapeutic aptamers are currently emerging, a potential shortcoming that narrows their therapeutic utility to targets on the cell surface or secreted proteins lies perhaps in their nucleic acid nature. The fact that aptamers are intrinsically negatively charged and thus will not easily cross cellular membranes makes it difficult to imagine that they will find equally broad therapeutic application for intracellular target proteins. However, recent developments in the aptamer technology further underline the feasibility of aptamers to streamline the drug discovery process. It is possible to transfer the functional properties of aptamers to small molecules with inherited properties.[241,351−353,361,505,506] In addition, a wide range of delivery systems have been developed to facilitate intracellular delivery of therapeutic nucleic acids in general,[507−517] many of which will likely also work for aptamers.

It is interesting to note that, for virtually all mechanisms currently known to be harnessed by natural riboswitches to

control gene expression, an original precedent of analogous artificial systems that were constructed in the laboratory can be quoted: *in vitro* selected aptamers that affect translation of reporter genes in cells[489] preceded the discovery of similar-acting natural riboswitches.[520] Engineered allosteric ribozymes regulated by small organic molecules antedated the recent discovery of the first catalytic riboswitch regulated by glucosamine-6-phosphate. For decades, scientists have been guided in the construction of synthetic model systems that mimic functional principles applied by the corresponding archetypes of nature. It is rewarding to see that, in the case of riboswitches, engineering of functional nucleic acid molecules inspired the search for and discovery of closely related natural counterparts.

In recent years, aptamers have particularly proven useful in bioanalytical applications. Methods of chemically modifying aptamers have advanced in multifaceted fashion to overcome the shortcoming of their inherent instability or to decorate them with additional functionality. Because aptamers can be selected for almost any target and because of the endless opportunities to chemically couple them to "detectors", there are infinite approaches in which aptamers could become a very useful addition to the biodiagnostics toolkit.

Taken together, aptamers represent versatile tools for the functional characterization of biomolecules, their detection, therapeutic intervention, and the development of small molecules that serve as pharmaceutical lead compounds. Aptamers are now established as key players on these stages, and it can be expected that they will continue to open up entirely new possibilities in the future.

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