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Site-Specific Fluorescent and Affinity Labelling of RNA by Using a Small Engineered Twin Ribozyme

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Over the past decade, RNA has become a focus of investigation into structure–function relationships. A large number of methods for structural studies of RNA are available. Application of those techniques often requires decoration of the sample with reporter groups and modifications such as fluorophores, cross-linking reagents, phosphorothioates, affinity tags or ESR spin labels, most desirably at a specific position.[1] We have developed a strategy for RNA modification that relies on a small engineered twin ribozyme that mediates the exchange of a patch of residing sequence of substrate RNA with a separately added synthetic RNA fragment.[2] Here we show that RNA fragments conjugated with fluorescent dyes or biotin are well accepted for strand exchange. Up to 53% of a dye-labelled oligoribonucleotide has been inserted into a 145-mer RNA. Thus, for the first time, specific labelling of a long transcribed RNA at an internal predetermined position is demonstrated.

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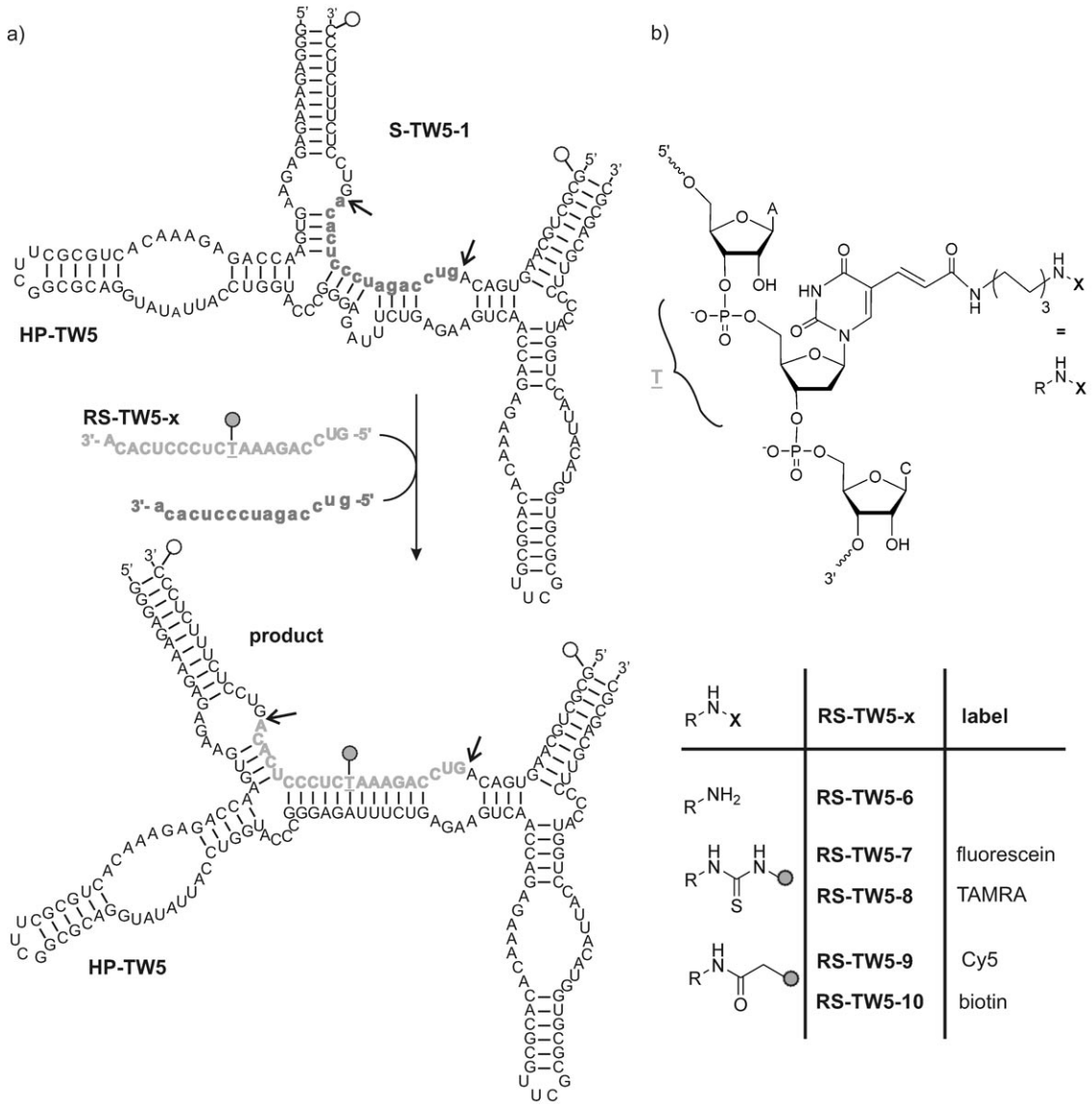
Modified nucleosides can be site-specifically incorporated into RNA by chemical synthesis with modified nucleoside phosphoramidites. While this is a useful strategy for modification of synthetically available RNAs, modification of long transcripts or natural RNA requires alternative techniques. In this case, specific labelling is possible at the two ends by taking advantage of the unique reactivity of the RNA termini.[3] Functionalization at internal sites can be achieved by adding modified nucleoside triphosphates to the transcription mixture.[4] However, the range of modified nucleosides that can be incorporated during transcription is limited by the specificity of the polymerase, and the label becomes distributed over the entire molecule. The recently published procedure of indirect labelling through oligonucleotide hybridization is a useful alternative. However, it is restricted by the availability of specific hybridization sites in the folded state of the molecule.[5]

We have developed a procedure for manipulating at will a chosen patch of a given RNA sequence. A small engineered twin ribozyme promotes, in a strictly controlled fashion, two RNA-cleavage events and two ligations, and thus mediates the specific exchange of RNA patches.[2] The strategy relies on the cleavage/ligation characteristics of the hairpin ribozyme,[6] a small naturally occurring catalytic RNA. Twin ribozymes are derived from tandem duplication of the hairpin ribozyme and thus inherit cleavage as well as ligation activity. Efficient fragment exchange is achieved by destabilization of the duplex between the ribozyme and the RNA patch to be removed (dark grey sequence (lower case letters) in Scheme 1a) and stabilization of the duplex between ribozyme and fragment to be inserted (light grey sequence). After cleavage, the sequence patch (in lower case letters) is readily released from the ribozyme–substrate complex due to the GAUU tetraloop designed to weaken its binding. The new fragment (light grey) contains the four additional nucleotides complementary to the GAUU tetraloop and thus, upon binding into the gap left by dissociation of the cleaved out fragment (dark grey lower case letters), extends the formerly interrupted duplex to 12 base pairs and makes it contiguous. Thus, the structure of the substrate–ribozyme complex is stabilized, and, consequently, ligation of the new fragment to the remaining substrate arms is favoured. In previous experiments, up to 30% of substrate RNA have been transferred into the insertion product.[2a]

We decided to exploit this strategy in a scheme of site-directed RNA functionalization. To this end, a uridine in the centre of the insertion fragment was replaced by an amino-modified deoxythymidine (marked $\underline{\mathrm{I}}$ in Scheme 1, insertion fragment RS-TW5-6). The aliphatic amino group within the RNA chain was treated with either the isothiocyanate (RS-TW5-7 and RS-TW5-8) or *O*-succinimidyl ester derivatives (RS-TW5-9 and RS-TW5-10) of a number of dyes as well as biotin to deliver the modified oligonucleotides shown in Scheme 1b. The fragment-exchange reaction was carried out by using the unlabelled oligonucleotide RS-TW5-6 as well as the four oligonucleotides to which either fluorescein (RS-TW5-7), tetramethylrhodamine (RS-TW5-8), Cy5 (RS-TW5-9) or biotin (RS-TW5-10) had been conjugated to replace the defined patch (lower case letters) of the RNA substrate S-TW5-1. During reaction, the 45-



Scheme 1. Twin-ribozyme-mediated RNA modification. a) Reaction scheme. Substrate RNA is annealed to twin ribozyme HP-TW5 and cleaved at two different sites indicated by arrows. The fragment extending between the two cleavage sites (16-mer, dark grey, smaller case letters) is replaced on the ribozyme by the insertion oligonucleotide RS-TW5-x (20-mer, light grey), which subsequently becomes ligated to the flanking substrate fragments to form the HP-TW5-product complex. T is an amino-modified deoxythymidine that has been labelled with a number of dyes and biotin (cf. b). b) Post-synthetic labelling of the amino-modified oligonucleotide RS-TW5-6 by using fluorescein isothiocyanate, tetramethylrhodamine (TAMRA) isothiocyanate, Cy5 succinimidyl ester or biotin succinimidyl ester to yield the corresponding labelled oligonucleotides RS-TW5-7 to RS-TW5-10.

mer substrate RNA is transformed into a 49-mer product (Scheme 1a); the reaction can thus be followed by fragment-length analysis (Figure 1). The obtained data show that all labelled oligonucleotides (lines 2–5) are inserted equally well as the unlabelled oligonucleotide (line 1). Furthermore, at 25–30%, the product yields are in the same range as that observed for the completely unmodified insertion oligonucleotide in the original system.[2] Next, we addressed the question of whether the approach is suitable for labelling longer RNAs that are inaccessible by chemical synthesis. The 145-mer RNA S-TW5-5 (Scheme 2) was prepared by run-off transcription in vitro. By employing the same strategy as described above, twin ribozyme HP-TW5 was incubated with the long RNA substrate S-TW5-5 in the presence of an equimolar amount of either RS-TW5-7 or RS-TW5-9 (cf. Scheme 1b). In both cases, insertion of the labelled oligonucleotide into the 145-mer RNA was observed with yields of 18% and 11% (Table 1). Conducting the experiment in the presence of a 20-fold excess of two antisense oligonucleotides complementary to the sequence segments of the substrate RNA flanking the ribozyme-substrate complex (Scheme 2) helped to slightly increase yields, even though the effect was not significant (Table 1).

Original experiments were carried out at 37 °C. RNA functionalization in vitro is not confined to physiological conditions and therefore may involve higher temperatures. We carried out reaction of HP-TW5 with substrate S-TW5-5 in the presence

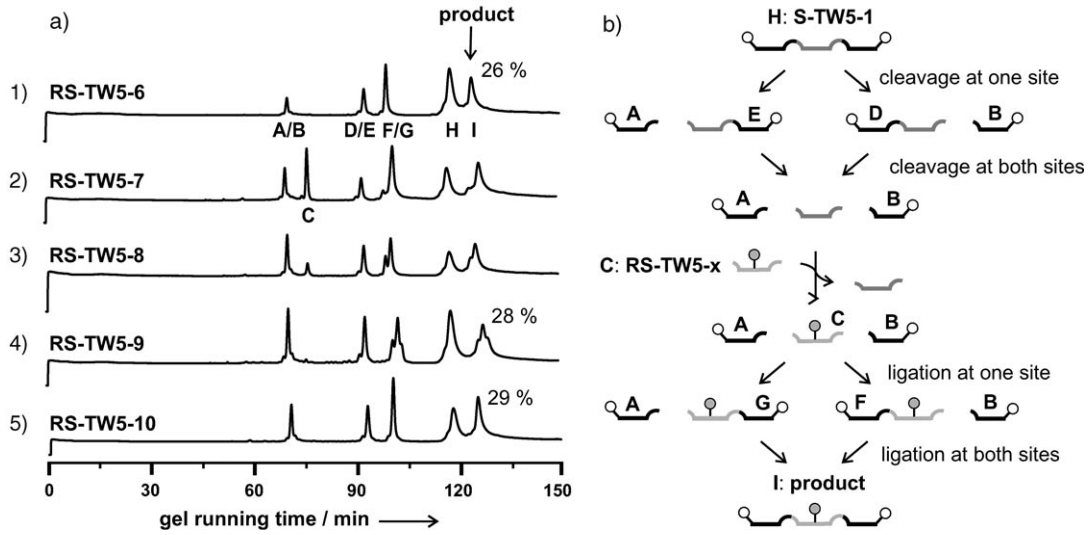
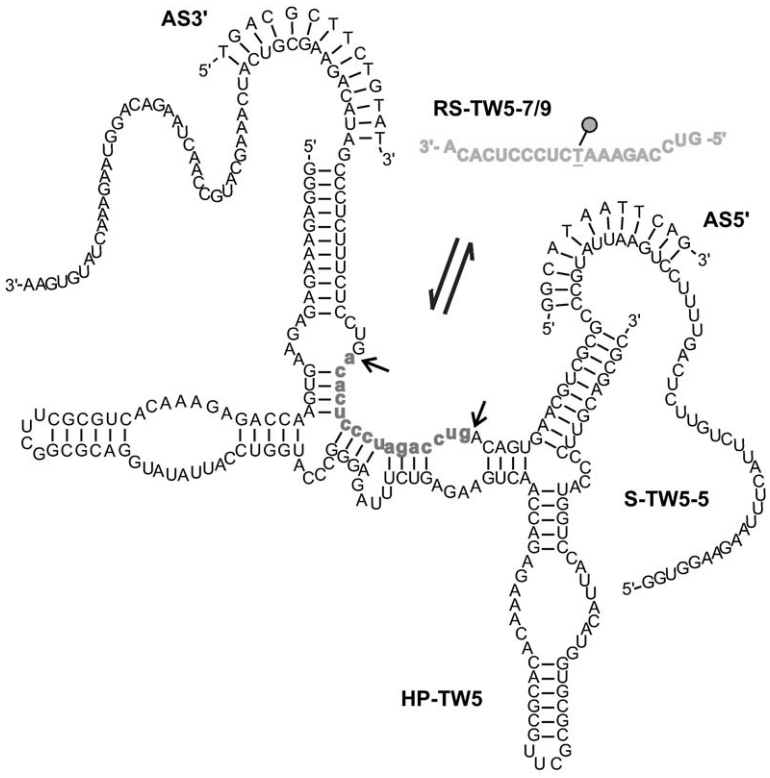


Figure 1. a) Monitoring reactions (fluorescence detection at 488 nm) after 24 h (8 h at 37 °C, then 25 °C). A: 14-mer, B: 15-mer, C: 20-mer, D: 30-mer, E: 31-mer, F: 34-mer, G: 35-mer, H: 45-mer, I: 49-mer. For identity of fragments A to I, see b). Experimental proof for I's being the full-length insertion product was provided previously.[2] Note that fragments differing in length by only one nucleotide are not resolved. This effect is a result of an accelerating influence of the 2′, 3′ cyclic phosphate group on the 15-mer and differential effects of the fluorescein moiety at the 5′- or 3′-end. Yields of labelled product are given for each line. Exact calculation of yields for insertion of oligonucleotides RS-TW5-7 and RS-TW5-8 is not possible due to the fact that the contributions of individual fluorescein and tetramethylrhodamine residues to the overall fluorescence of the 49-mer product cannot be resolved. b) Fragmentation and ligation scheme (cf. Scheme 1 a).



Scheme 2. HP-TW5-mediated labelling of transcript S-TW5-5. S-TW5-5 is a 145-mer transcript. It contains the sequence of the synthetic substrate S-TW5-1 embedded into an arbitrarily chosen sequence. The two antisense oligonucleotides AS5′ and AS3′ that have been added to avoid spurious annealing of the substrate RNA are highlighted in grey.

of insertion fragment RS-TW5-7 (Figure 2 a) at temperatures ranging from 37 to 52 °C. The best results were obtained at 47 °C: 53 % of the added fluorescein-labelled oligonucleotide RS-TW5-7 was inserted to result in transfer of the formerly unlabelled substrate RNA into the fluorescent product (Table 2, Figure 2 b, line 1). We followed the reaction for 1 h, saturation was reached already after 10 min (Figure 2 c). The fragment exchange is supposed to proceed more efficiently at higher temperatures, since the sequence patch that is cleaved out in the first step of the reaction can dissociate more easily. The melting temperature of a model duplex consisting of a part of the substrate sequence (dark grey fragment in smaller case letters) and its complementary strand with the four nucleotide bulge is 38 °C (at 500 nM), while the corresponding full complementary duplex containing the new RNA fragment (light grey) only melts at 73 °C.[2b] Thus, at 47 °C, melting of the cleaved substrate is preferred, whereas binding of the insert is still stable enough to allow ligation to take place. Further increasing the temperature is counterproductive; the yield of final product decreases again (Table 2).

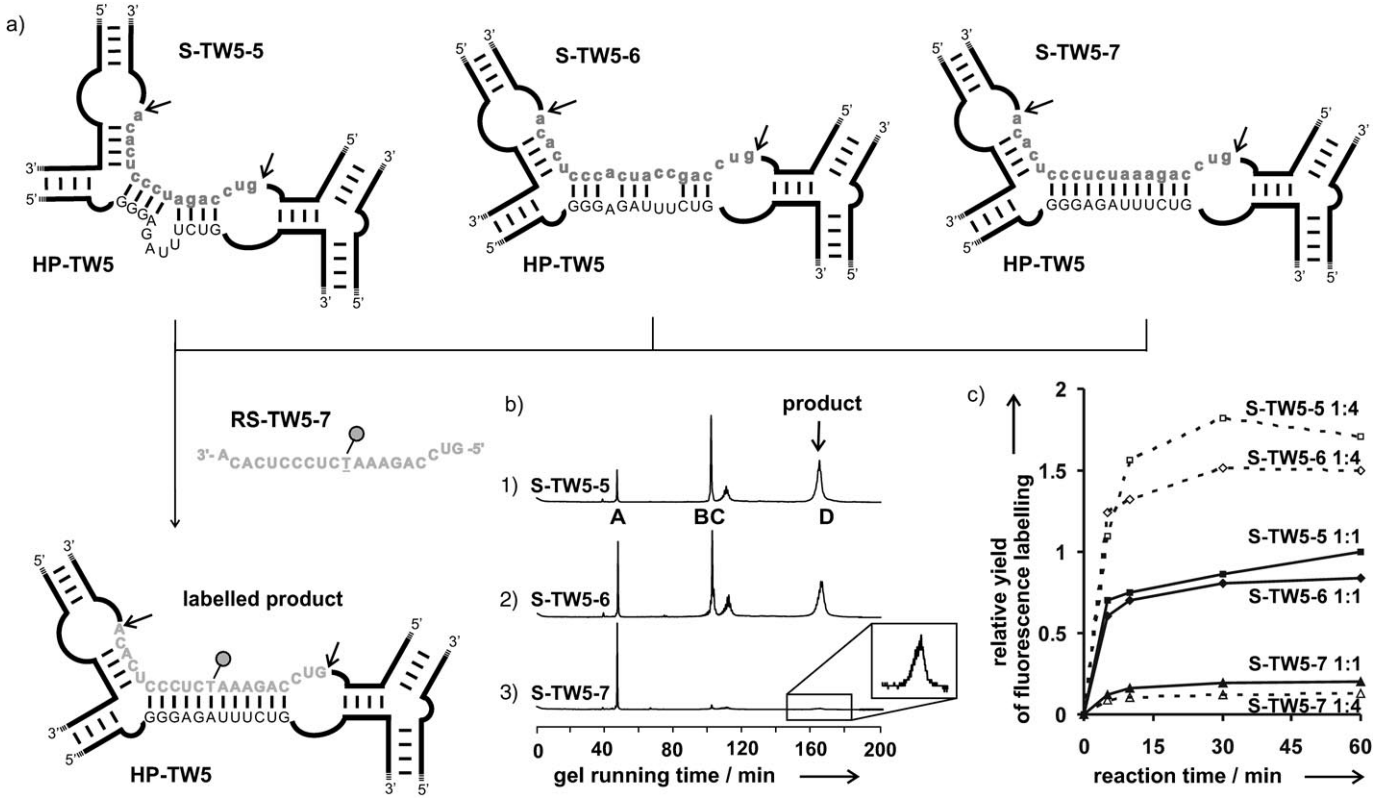


Figure 2. Twin-ribozyme-mediated labelling of transcripts S-TW5-5, S-TW5-6 and S-TW5-7. a) Sequences and secondary structures of twin ribozyme–substrate complexes and of the labelled twin ribozyme–product complex after fragment exchange. Transcripts S-TW5-6 and S-TW5-7 have the same sequence as S-TW5-5 (shown in Scheme 2); the region between the two defined processing sites has been varied as shown. In all three reactions, the same ribozyme–product complex with a contiguous 12 bp helix is formed. b) Monitoring reactions (fluorescence detection at 488 nm) after 1 h of reaction at 47 °C. Reaction of twin ribozyme HP-TW5 with: line 1: S-TW5-5 and RS-TW5-7; line 2: S-TW5-6 and RS-TW5-7; line 3: S-TW5-7 and RS-TW5-7. A: RS-TW5-7 (20-mer), B and C: fragments resulting from single-site ligation (80-mer or 89-mer), D: full-length product (149-mer). c) Time course of reactions. Relative yields of fluorescence labelling with equimolar concentration (——) or fourfold excess (-----) of labelled insertion oligonucleotide over substrate.

Table 1. Yields of insertion of labelled oligonucleotides into a 145-mer transcript (S-TW5-5) in the presence and absence of antisense oligonucleotides (AS).

Insertion oligonucleotide	Label	Without AS[a]	With AS[b]
RS-TW5-7	fluorescein	17.7 ± 1.8 %	24.3 ± 1.6 %
RS-TW5-9	Cy5	10.6 ± 2.3 %	13.5 ± 1.5 %

[a] Average yields of three independent measurements after 3 h of reaction at 37 °C. [b] Average yields of three independent measurements after 3 h of reaction at 37 °C in the presence of a 20-fold excess of antisense oligonucleotides AS5′ and AS3′ (cf. Scheme 2).

Table 2. Yields of insertion of labelled oligonucleotides into substrates S-TW5-5, S-TW5-6 and S-TW5-7 at different temperatures.

$T_{reaction}$	S-TW5-5[a]	S-TW5-6[a]	S-TW5-7[a]
37 °C	24 %	8 %	1 %
42 °C	34 %	26 %	8 %
47 °C	53 %	45 %	11 %
52 °C	38 %	23 %	7 %

[a] Average yields of three independent measurements after 1 h of reaction in the presence of a 20-fold excess of antisense oligonucleotides AS5′ and AS3′ (cf. Scheme 2). Standard deviations do not exceed 5 % yield.

In order to increase the range of application, we modified the approach, as shown in Figure 2 a. The original procedure involved exchanging a 16-mer for a 20-mer oligonucleotide by using additional base pairing and formation of a stable contiguous duplex between the ribozyme and the 20-mer insert to drive the reaction. However, extension of the product RNA can significantly alter the overall structure of the modified RNA due to alternative folding behaviour. In such cases, other strategies need to be employed to keep the folding pattern of the modified product RNA intact. An alternative way to drive the reaction consists of designing a twin ribozyme that forms a duplex interrupted by mismatches upon binding to the substrate RNA in the area of fragment exchange (Figure 2, S-TW5-6). The labelled oligonucleotide to be inserted again forms a contiguous, and thus more stable, duplex, resulting in preferential ligation. The total number of mismatches can be varied and will depend on the acceptance of base modifications without altering the overall structure of the modified product RNA. As can be seen from Figure 2 and Table 2, a scenario involving three mismatches and, consequently, three base mutations in the labelled product RNA at 47 °C allows 45 % of the labelled fragment to be inserted into the final product.

Lastly, we also checked whether two fragments of identical length and sequence could be exchanged. This would be of

valuable interest for labelling RNAs that are sensitive to both extension and base substitution. Reaction of HP-TW5 with S-TW5-7 and an equimolar amount of the oligonucleotide RS-TW5-7 (Figure 2) delivered the labelled product with up to 11% yield (Table 2). Even though the yield is much lower than with substrates S-TW5-5 and S-TW5-6, the obtained result demonstrates that labelling of transcripts is possible without any changes to sequence and overall structure. The reaction can be further driven by an excess of insertion oligonucleotide over substrate. Addition of a fourfold excess of RS-TW5-7 resulted in a twofold increase of the reaction yield with transcripts S-TW5-5 and S-TW5-6 (Figure 2c). In the special case of the exchange of fragments with identical sequence and lengths (transcript S-TW5-7), we were not able to detect an analogous increase of yield due to the low signal intensity and the resulting high error range.

In conclusion, our results demonstrate that a small engineered ribozyme mediates the site-specific modification/functionalization of an arbitrarily chosen RNA molecule and thus paves the way to a wide range of studies in molecular biology, biochemistry and biophysics. One of the most obvious applications is decorating RNA molecules with dyes, specific isotopes or spin labels for FRET, NMR or EPR studies. For example, kinetic and dynamic experiments to understand ribosome function and mechanism[7] by fluorescence resonance energy transfer require the site-specific and stable attachment of fluorescent dyes to ribosomal domains. In the same manner, conformational changes in spliceosomal RNPs[8] could be studied. Furthermore, NMR is a powerful method for studying RNA in solution. However, the restricted resolution of NMR spectra of RNA makes the site-specific introduction of stable ^{13}C,^{15}N isotopes necessary for improving the quality of RNA structures determined by NMR.[9] EPR-based methods have been used to map local dynamics and structural features of RNA, to explore different modes of RNA–ligand interaction, to obtain long-range structural restraints and to probe metal-ion-binding sites.[9b] The site-specific attachment of spin labels is a prerequisite for these studies. Therefore relevant techniques for labelling large RNAs are of major importance.

The twin-ribozyme-mediated labelling reaction can be easily carried out at larger scale without loss of yield (not shown) and thus is capable of producing sufficient material for analysis. Due to its sequence flexibility, the hairpin ribozyme can be adapted to a number of substrates[10] such that hairpin-derived twin ribozymes for modification of a specific RNA sequence may be designed in a rational manner. In addition, the powerful method of in vitro evolution provides a promising alternative to create substrate-specific twin ribozymes. Application of the strategy is not confined to RNA analysis by spectroscopic techniques. Aside from dyes or biotin, virtually any desired modified nucleotide can be inserted into RNA by the twin ribozyme approach. The only restriction is that the modified nucleoside needs to be chemically incorporated into an oligoribonucleotide prior to twin-ribozyme-mediated fragment exchange. Even though the described method is limited by the accessibility of domains within the folded RNA structure, it enriches the arsenal of tools available to functionalize large RNAs.

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