Development of artificial ribozymes by in vitro selection has so far, mostly been addressed from the viewpoint of fundamental research. However, such ribozymes also have high potential as selective catalysts in practical syntheses. Immobilization of an active and selective ribozyme is an important step towards this end. A 49-nucleotide RNA molecule that was previously found to stereoselectively catalyze Diels–Alder reactions between various anthracene dienes and maleimide dienophiles was quantitatively immobilized on an agarose matrix by periodate oxidation of the 3′-terminal ribose and coupling to a hydrazide moiety. Typical loadings were 45 pmol μL⁻¹ gel. The specific activity was comparable to that of soluble ribozyme, and high enantioselectivities were obtained in catalyzed cycloadditions. The catalytic matrix was found to be stable and could be regenerated about 40 times with only minimal reduction of catalytic activity. Like the soluble ribozyme, the immobilized catalyst stereoselectively converts various diene and dienophile substrates. By using either natural D-RNA or enantiomeric L-RNA, both product enantiomers were made synthetically accessible with similar selectivities.

Introduction

Enzymes have found increasing use in organic synthesis in recent years. Mild reaction conditions and high stereo-, regio-, and chemoselectivities have prompted researchers to consider enzymes as attractive alternatives to traditional chemical catalysts. High production costs and low stability are some of the disadvantages associated with enzyme usage. For many years, immobilization of biocatalysts on solid materials has been one of the strategies used to overcome these limitations and to improve ease of handling.[1, 2] The main benefits of enzyme immobilization are easy separation of the biocatalyst from the reaction product(s) and reuse of the biocatalyst, advantages that simplify catalyst application, improve reaction technology, and provide the cost advantages essential for establishing an enzyme-catalyzed process in the first place. In several cases immobilized enzymes are even superior to small-molecule catalysts.[3]

It has become evident that, in addition to proteinaceous enzymes, nucleic acid (DNA and RNA) enzymes exist that can catalyze a widespread range of reactions. While the chemistry of the naturally occurring ribozymes is rather limited, this restriction does not apply to artificial ribo- and deoxyribozymes obtained by combinatorial chemistry. Starting from synthetic combinatorial libraries, catalysts for a broad range of chemical transformations ranging from cleavage of amide[4] or carboxylic ester bonds[5] to C–N and C–C bond forming reactions[6–13] or the catalysis of isomerization reactions[14] have been generated by a process termed in vitro selection. A number of other practically relevant organic transformations are currently being studied. Thus, nucleic-acid-based catalysts developed to solve a specific synthetic problem may become interesting tools for organic chemists.[15] A particularly valuable aspect of this approach is the fact that selectivity, reactivity, and reaction conditions of the catalysts can be tuned by the way the combinatorial selection is carried out.

Our research group recently described the isolation of RNA molecules that catalyze the formation of carbon–carbon bonds between anthracene dienes and maleimide dienophiles by [4 + 2] cycloaddition (Diels – Alder reaction).[7] This reaction type is one of the most important C–C bond forming processes available to organic chemists, and since its discovery there has been much interest and success in developing catalytic methods for improving its rate and selectivity.[16–23]

These ribozymes were the first to catalyze a truly bimolecular reaction between two free non-RNA reactants in solution, and we could identify a small secondary structure motif responsible for the catalysis. A 49-nucleotide-long RNA containing this motif acted as an efficient catalyst and accelerated the reaction with high enantioselectivity. A synthetic mirror-image ribozyme...
composed of L-ribonucleotides was found to have the opposite stereoselectivity.[24] The substrate specificity of the ribozyme was studied extensively.[25] The acceleration of the reaction rate was suggested to be primarily due to an entropic effect.[26]

The catalytic properties of these RNA catalysts combined with their small size render them attractive for numerous types of studies. As part of our program to establish ribozymes as selective catalysts in organic synthesis, we report herein the immobilization of Diels–Alderase ribozymes and their initial characterization.

Results and Discussion

While there are various ways to attach RNA molecules to surfaces, most of them have not been investigated in the context of RNA catalysis. One of the most simple and widespread methods is the oxidation of the cis-diol system at the 3'-terminal ribonucleotide by treatment with periodate to give the respective dialdehyde, followed by coupling to a hydrazide-derivatized solid matrix (Figure 1).[27, 28]

Incubation of radioactively labeled Diels–Alderase ribozyme with NaIO₄ solution at 4°C gave quantitative oxidation without detectable degradation within 1 h. After removal of periodate by precipitation, the oxidized RNA was incubated for various time periods with adipic acid dihydrazide agarose, followed by extensive washing. Measurement of ³²P by scintillation counting allowed quantitative assessment of the coupling. After 14 h, 43% of the input RNA was attached to the matrix, while after 20 h the immobilization yield was 55%. Despite concerns about hydrolysis of the RNA on prolonged storage, the best immobilization yields were obtained after 48 h at room temperature (98%). This corresponds to a loading of 49 pmol/L wet gel.

The activity of the immobilized ribozyme was measured by using two substrates that were known to be accepted in solution, namely 9-hexaethylene glycol anthracene (1) and N-pentyl maleimide (2). Since the attachment site (the 3'-terminal nucleotide) was located directly at a junction between two helices that are essential for activity, a reduction of enzymatic performance was anticipated (see Figure 1). However, the immobilized ribozyme performed well in catalysis. 600 pmol immobilized ribozyme converted 9.5 nmol 1 under the reaction conditions used, while the same amount of soluble ribozyme gave 10.7 nmol conversion under the same conditions. Undervativized adipic acid hydrazide agarose was used as a negative control and gave the rate of the uncatalyzed background reaction.

Attachment to the solid phase appeared to have little influence on the accessibility and geometry of the catalytic center, as the enantioselectivity (ee) of the reaction was found to decrease only slightly compared to that achieved with the soluble ribozyme (78±8% ee versus 88±8% ee, measured by chiral HPLC, Figure 2).

For practical use of immobilized enzymes, their long-term stability is an important criterion. To analyze this parameter, one batch of immobilized ribozyme was reused over 40 times with different substrates over a period of 11 months. Between

Figure 1. RNA-catalyzed [4+2] cycloaddition using resin-immobilized ribozyme. HEG, hexaethylene glycol.

Figure 2. HPLC analysis of Diels–Alder reactions of 1 and 2 catalyzed by 49-mer D-RNA (A) and the enantiomeric 49-mer L-ribozyme (B). Samples were analyzed on a chiral column (see the Experimental Section for details). The lower curve in the chromatograms corresponds to the uncatalyzed background reaction.
experiments, the ribozyme-derivatized matrix was stored at –20 °C. Activity and selectivity were measured as described above. After 43 uses, the matrix still had 83% of the activity measured in the first experiment (see Figure 3). No significant differences could be detected in the enantioselectivity of product formation. These results demonstrate good long-term stability.

**Figure 3.** Long-term stability of immobilized L-ribozyme. Relative yields of the ribozyme-catalyzed reaction of 1 and 2 within one hour were recorded over a period of 11 months (see the Experimental Section for details).

As it is important to have selective synthetic access to both product enantiomers in stereoselective synthesis, we repeated this set of experiments with a synthetic mirror-image ribozyme composed of L-ribonucleotides. As already observed with soluble ribozyme, the L-ribozyme has the same activity, but opposite stereoselectivity to the d-ribozyme (Figure 2B).

As expected from earlier studies, the immobilized Diels–Alderase ribozymes accept a wide range of anthracene and maleimide derivatives and convert them stereoselectively (data not shown). Further studies are underway to systematically explore the relationship between substitution pattern, reactivity, and selectivity.

**Conclusions and outlook**

In conclusion, this work demonstrates that Diels–Alderase ribozymes can be covalently attached to a solid phase in a near-quantitative manner by well-known chemistry. Their catalytic activity and selectivity is maintained for many cycles of catalysis. The resin shows excellent long-term stability.

Future work will focus on the establishment of a continuous-flow reactor packed with ribozyme resin, which will allow continuous synthesis. Preliminary data suggest that soft agarose matrices are not the ideal material for these applications because of pressure-associated problems. In addition, the immobilization of Diels–Alderase ribozymes with higher rate accelerations is planned, which may allow higher yields in ribozyme-catalyzed cycloadditions.

Although the activity and selectivity of these immobilized ribozymes are still far from those that have been achieved with small-molecule Diels–Alder catalysts, this work provides a starting point for establishing immobilized nucleic acids as practical catalysts.

**Experimental Section**

**Materials:** The 49-mer RNA (sequence: GGAGCUCGCUCUCCGGC-GAGGCCGUGCAGGUCUCUCCGGAGCAAUCUGGCC) was obtained from Dharmacon (Boulder, CO) and IBA-NAPS (Göttingen, Germany). The mirror-image 49-mer L-ribozyme was generously provided by NOXXON Pharma AG (Berlin, Germany). Chemicals were purchased from Sigma Aldrich and Pharmacia, and used as described. Adipic acid dihydrazide agarose (CarboLink Coupling Gel, Pierce) was used as the solid matrix. Quantitative radioactivity measurements were performed on a Beckmann Coulter LS6000 SC scintillation counter. Schleicher & Schuell Centrex MF centrifugal filters were used as reaction vessels and for separating immobilized ribozymes from the reaction mixture.

**RNA labeling with [32P]:** 49-mer RNA (20 pmol) was dissolved in Buffer A (50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, 10 mM MgCl2, 5 mM dithiothreitol (DTT), 0.1 mM spermidine, 0.1 mM ethylenediaminetetraacetate, pH 7.6) and incubated with γ-[32P]-ATP (1 μL, 370 kBq/μL) and T4 polynucleotide kinase (10 U) at 37 °C for 2 h. Labeled product was purified by denaturating 8% PAGE. Unlabeled RNA was mixed (doped) with [32P]-labeled RNA before use.

**Ribozyme immobilization (example):** Reactively doped 49-mer RNA (600 pmol, 80 000 cpm) was oxidized in NaIO4 solution (200 μL, 25 μM) for 60 min at +4 °C. The RNA was precipitated by treatment with LiClO4 solution (2% w/v in acetone) and collected by centrifugation at room temperature. The remaining pellet was dissolved in Buffer B (0.1 mM sodium acetate, pH 5.5; 60 μL). The resulting solution was mixed with adipic acid dihydrazide–agarose (30 μL) and allowed to incubate in the filter column at room temperature for two days. The ribozyme-derivatized resin was washed extensively with water. Immobilization was quantified by [32P] Cerenkov counting of the washed resin and all supernatants and wash fractions.

Mirror-image L-ribozyme was treated in the same way; the enzymatic [32P]-labeling was done by using an enzyme concentration that had been increased fourfold compared with the D-ribozyme.

**Undervatized Carbo Coupling Gel served as a negative control. All matrices were stored at –20 °C.**

**HPLC analysis of reaction products:** Reaction mixtures were analyzed on a chiral NEA (R) column (YMC Europe, 250 × 4.6 mm) with water/ethanol (60:40) as the eluent, isocratic at 0.8 mL/min and 45 °C, with UV detection at 230 nm. Samples were injected as quenched reaction mixture (68 μL).

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