Evolution of DNA and RNA as catalysts for chemical reactions

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In vitro selection from combinatorial nucleic acid libraries has provided new RNA and DNA molecules that have catalytic properties. Catalyzed reactions now go far beyond self-modifying reactions of nucleic acid molecules. The future application of *in vitro* selected RNA and DNA catalysts in bioorganic synthesis appears promising.

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Abbreviations
dNTP deoxyNTP
NTP nucleoside triphosphate
TSA transition-state analog

Introduction

Combinatorial nucleic-acid libraries have found increasing use for the isolation of molecules with new binding properties or novel catalytic activities. Although the seven types of natural ribozymes catalyze only hydrolysis and transesterification reactions at internucleotide phosphodiester bonds, this restriction is not given in the search for artificial catalysts from synthetic combinatorial libraries. Meanwhile, an impressive range of RNA-catalyzed and DNA-catalyzed reactions has been described, including Diels–Alder reactions, glycosidic bond formations, alkylations, acylations, and amide bond formations [1,2].

The field of RNA and DNA catalysis has been subject to numerous review articles, most of which use either an evolutionary perspective and relate the results to a hypothetical prebiotic 'RNA world', or discuss the work with respect to the structure, function and catalytic bandwidth of RNA [2–5]. In this article, we will review the progress made in 1999 in the field of artificial ribozymes and deoxyribozymes. Naturally occurring ribozymes, their variants, and aptamers are not within the scope of this article. Finally, we will apply a chemist's perspective and discuss artificial ribozymes with respect to their potential application as catalysts in bioorganic synthesis and biotechnology.

Selection methodology

Artificial ribozymes and deoxyribozymes are selected from synthetic combinatorial RNA or DNA libraries, which are generated by solid-phase synthesis using mixtures of nucleoside phosphoramidites rather than individual monomers. The huge complexities of these libraries (typically in the order of 10^{15}) can only be deconvoluted because nucleic acid libraries are genetically encoded (i.e. they carry the information for their own enzymatic replication). Functional sequences can be enriched in a selection (or partitioning) step and then amplified by known enzymes (template-dependent polymerases). This property enables an iterative enrichment of active species over several rounds of selection and amplification until they dominate the resulting library.

Two strategies have been worked out to isolate new catalysts. In the first strategy, RNA or DNA molecules are selected that bind with high affinity to a transition-state analog (TSA). These binders (aptamers) are then screened for catalysis of a reaction proceeding via the respective transition state. Despite numerous attempts, this approach has produced few new catalysts, and rate acceleration has typically been only ~100-fold. The second strategy, direct selection, enriches RNA or DNA molecules that combine catalyst and substrate properties. The library is incubated with a potential reactant; library members that become covalently attached to this reactant, and thereby either acquire a new functional group or alter a characteristic property, are isolated. This approach was initially used to isolate catalysts for modifying reactions of RNA (phosphorylation or alkylation of RNA), but several examples of reactions on non-nucleotide reactants are now available. Conversion of two free reactants with multiple turnover, however, is still hard to obtain.

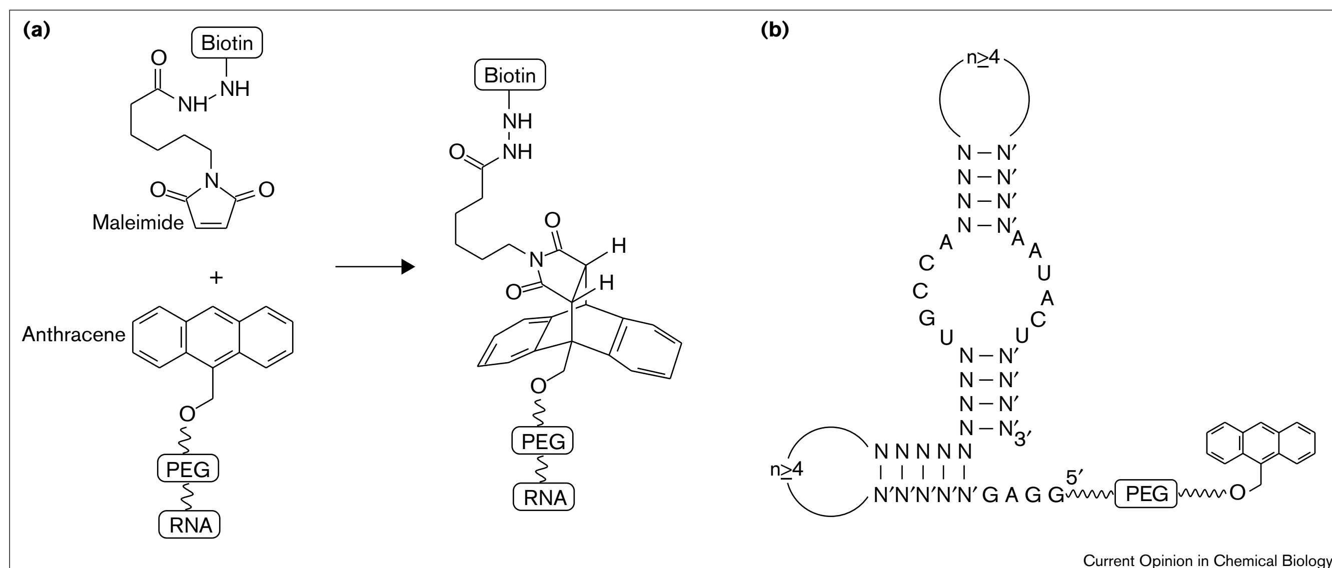
Reactions catalyzed by nucleic acids

Nucleic acids, especially RNA, appear to be versatile catalysts capable of accelerating a broad range of reactions.

C–C bond formation

Unmodified RNA has been shown to catalyze the formation of C–C bonds by Diels–Alder reactions [6••]. In the selection, each molecule of a combinatorial RNA library (2×10^{14} species, 120 randomized positions) was coupled by a long, flexible polymeric tether to anthracene (which acted as the Diels–Alder diene), while the other reactant (maleimide as the dienophile) was biotinylated (Figure 1a). Biotinylated reaction products were isolated using immobilized streptavidin, and the attached RNAs were enzymatically amplified. After 10 rounds of selection and amplification, 16 independent sequence families were found to significantly accelerate the Diels–Alder reaction. Of these 16 families, 13 contained a small motif, which was found to be responsible for catalysis (Figure 1b). A 49-mer RNA containing this motif accelerates the reaction about 18,500-fold. The nucleotide sequences in the bulge region were mostly conserved, whereas the helical stems were variable. The ribozyme displays Michaelis–Menten-type kinetics, and its metal-ion requirements are remarkably adaptable. The motif

Figure 1

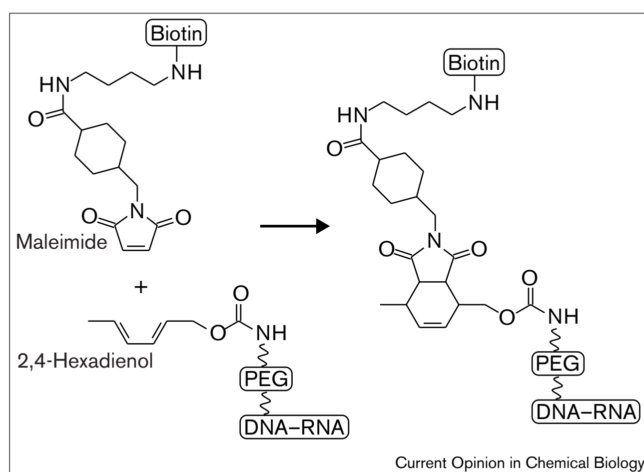


A 49-nucleotide RNA catalyst for the Diels–Alder reaction. **(a)** Diels–Alder reaction of the aromatic diene anthracene and the dienophile biotin maleimide. The anthracene is covalently coupled to the RNA by poly(ethylene glycol) (PEG). **(b)** Proposed secondary structure motif of the *in vitro* selected Diels–Alderase ribozymes. The loops contained four or more nucleotides without any sequence constraints. N, any nucleotide; N′, nucleotide complementary to Watson–Crick base-paired N.

was also rationally converted into a *trans*-system in which a 38-mer catalyst acts with multiple turnover on external anthracene–tether–oligonucleotide substrates. This work demonstrates the potential of small, selection-derived RNA molecules for the control of comprehensive, synthetically relevant organic transformations.

A previously described, chemically modified, copper-dependent Diels–Alderase that accelerates the reaction of an acyclic diene with a maleimide dienophile about 800-fold was characterized in more detail [7] (Figure 2). In the selection, the RNA had been transcribed in the presence of 5-(4-methylpyridylcarboxamide)uridine, so that all uridine residues were modified with a pyridyl whose ring nitrogen was in the *p*-position. Using a variety of analogs, it was then established that the pyridyl nitrogen at this position is an absolute requirement for catalysis. The catalyst exhibits high specificity for the dienophile used in the selection; other maleimide dienophiles showed markedly reduced activity, and a fumarate dienophile was not converted at all. A cyclic diene was not accepted as substrate. Product inhibition studies showed that the RNA recognized functional group components of both the diene and the dienophile. These results suggest that the pyridyl-modified RNA can form an intricate metal/ligand-dependent active site capable of highly specific molecular recognition.

Figure 2

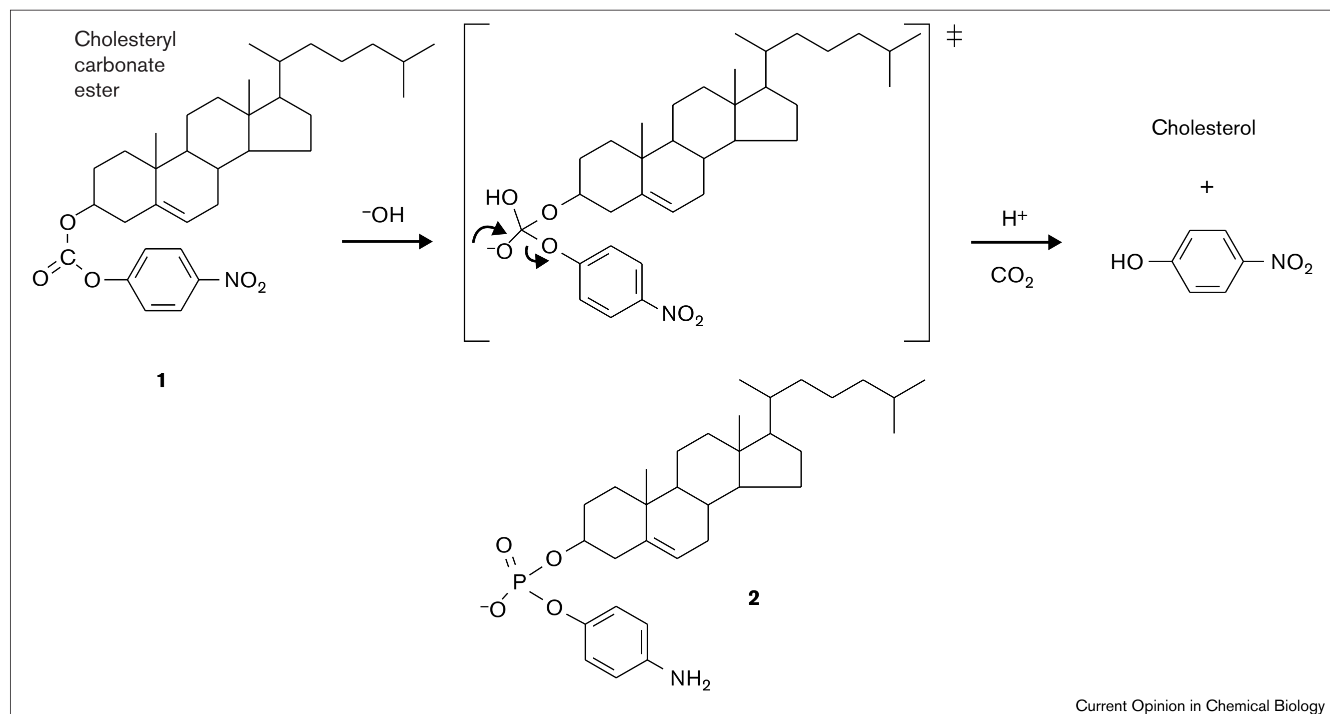


RNA-catalyzed Diels–Alder reaction of a 2,4-hexadienol acyclic diene and a biotinylated maleimide dienophile. The diene is covalently coupled to the RNA via poly(ethylene glycol) (PEG) and a short DNA strand.

Carbonate ester hydrolysis

The first successful selection by TSA-screening for a reaction involving cleavage of a covalent bond has been reported (Figure 3) [8•]. RNAs were selected that bind with submicromolar activity to the immobilized phosphate ester TSA **2**. Of the 11 TSA-binding RNAs, 2 were catalytically active and hydrolyzed the corresponding cholesteryl carbonate ester **1**. Rate acceleration for one species was 110-fold. Both binding and catalysis appear to be rather specific for **1**, as demonstrated with a variety of analogs. TSA **2** strongly inhibits hydrolysis of **1**, suggesting that the binding pocket for **2** is the catalytic site.

Figure 3



Hydrolysis of a cholesteryl carbonate ester. Cholesteryl carbonate ester **1** gives cholesterol and *p*-nitrophenol by hydrolysis. The analog **2** of the transition state was used to select for RNA binding. Out of these aptamers, RNAs were found to catalyze the hydrolysis reaction.

RNA aminoacylation

RNA can catalyze aminoacylation and other acyl transfer reactions. Recently, more self-aminoacylating ribozymes with interesting properties have been reported [9••,10]. A small 29-mer RNA itself is able to accelerate two related reactions: it first converts to aminoacyl-RNA, and then to peptidyl-RNA by using a second molecule of activated amino acid (Figure 4) [9••]. Small ribozymes that catalyze more than one reaction can be viewed as important molecules in an early 'RNA world' from which evolution then generated more specialized catalysts with higher rate accelerations. One of these more highly evolved RNA catalysts was reported to accelerate its own conversion to phenylalanyl-RNA faster and with higher substrate specificity then present-day aminoacyl-tRNA synthetases, natural protein enzymes that catalyze the same reaction, thereby giving credibility to the possible existence of an ancestral translation system relying entirely on ribozyme catalysis [10].

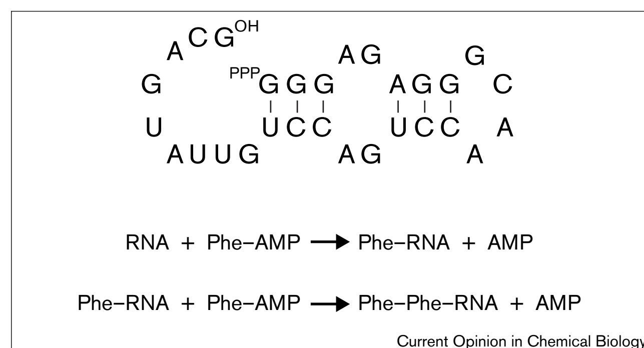
Peroxidase activity

A previously described hemin-binding 18-mer DNA oligonucleotide with peroxidase activity, which was assumed to form a guanine quadruplex structure, was then compared with the corresponding RNA oligonucleotide version, as well as with unrelated DNA quadruplexes [11]. Even though hemin binding of the RNA oligo was 30-fold weaker, catalysis was nearly identical to the DNA part. An unrelated DNA quadruplex that binds hemin as strongly as the DNA catalyst showed only minor peroxidase activity. Obviously, not every cofactor-binding site also qualifies as a catalytic site.

DNA phosphorylation

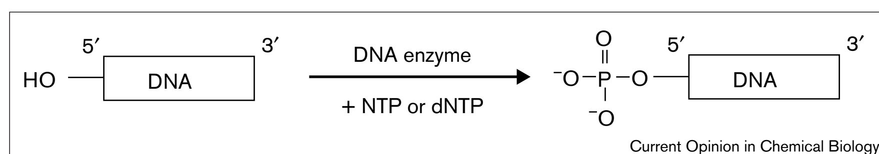
Phosphorylation of RNA molecules, in a way similar to that of polynucleotide kinase enzymes, has been known to belong to the catalytic repertoire of artificial ribozymes [12].

Figure 4



Ribozyme-catalyzed aminoacyl-RNA and peptidyl-RNA synthesis. A 29-nucleotide RNA enzyme (top) is able to successively form the aminoacyl ester Phe–RNA, and then peptidyl-RNA (Phe–Phe–RNA) by using phenylalanine adenylate (Phe–AMP) as substrate.

Figure 5



DNA phosphorylation catalyzed by DNA enzymes. The 5′-hydroxyl group of a DNA molecule is phosphorylated by using any of the NTPs or dNTPs.

Recently, a comprehensive study investigated such capabilities for deoxyribozymes (Figure 5) [13••]. From a random pool of single-stranded DNA, those members were selected that could transfer a phosphate from an external nucleoside triphosphate (NTP) or deoxynucleoside triphosphate (dNTP) to their own 5′-hydroxyl group. Only phosphorylated DNAs are susceptible to enzymatic ligation of a suitable oligonucleotide, which distinguishes reacted DNAs both during electrophoresis and selective polymerase chain reaction (PCR) amplification. Nearly 50 individual DNAs with polynucleotide kinase-like activity were isolated, each making use of one or more of the eight standard NTPs or dNTPs. While some DNAs absolutely required one specific nucleotide, others were flexible. Reselection from a biased pool produced DNAs that could differentiate 100-fold between the related NTP and dNTP substrate. This finding shows that DNA's chemical simplicity does not preclude it from distinguishing between closely related organic compounds. An optimized DNA enzyme operates with a rate enhancement of nearly one-billion-fold over the uncatalyzed rate of ATP hydrolysis. As the authors were able to convert the self-phosphorylating DNAs into true *trans*-acting enzymes, the deoxyribozymes could be of great utility for the sequence-specific phosphorylation of DNA or RNA sequences.

Miscellaneous

RNA that contains only three different types of nucleotide can substantially catalyze chemical reactions [14•]. Starting from a previously described RNA ligase ribozyme containing roughly equal proportions of all four nucleotides, *in vitro* evolution yielded a ribozyme that completely lacked cytidine. The catalytic rate was about 10^5-fold faster than the uncatalyzed rate of template-directed RNA ligation.

As an example of 'molecular ecology', the evolution of resistance was reported [15]. Continuos *in vitro* evolution of an RNA ligase ribozyme was carried out in the presence of a DNA enzyme capable of cleaving, and thereby inactivating the ribozyme. The evolved population retained its ligase activity, but with a 2000-fold reduction in vulnerability to cleavage.

In another experiment, dual-catalytic RNAs were selected from random sequences [16]: the RNA promotes cleavage at one site and template-directed RNA ligation at another site, suggesting two different conformations surrounding one metal-ion-binding site. Remarkably, except for two conserved, unpaired nucleotides, all other nucleotides appear to be involved in standard Watson–Crick-type duplexes.

Tremendous progress has been made with artificial regulation systems for ribozyme activities [17•,18••,19••]. Sophisticated systems ('aptazymes', allosterically regulated ribozymes that act as molecular switches) have been described in which a self-cleaving ribozyme could be switched on or off by small effector molecules. This was achieved by fusing an effector-binding domain to the ribozyme (Figure 6). The systems were either rationally engineered, or obtained by *in vitro* selection, and could also contain additional modules facilitating conformational changes ('communication modules'). In one example, a 5000-fold activation of a hammerhead ribozyme was obtained upon addition of a cyclic nucleotide cofactor. These systems could find many practical uses, including highly specialized reagents for monitoring the presence and concentration of different analytes, and *in vivo* applications of specifically regulated gene-therapy systems [20].

Conclusions and future perspectives

It is now evident that DNA and RNA can accelerate a broad range of chemical reactions (for recent compilations, see [1,2]). Various types of bond forming and breaking reactions, mostly at phosphorus or carbon centers, are well documented. Some of the described catalysts rival the performance of protein enzymes, catalytic antibodies or synthetic catalysts. Thus, the functional capabilities of nucleic acids make their use in bioorganic synthesis very attractive.

The most powerful features, however, are related to the iterative search and evolution strategies that can only be applied to genetically encoded libraries. It is actually possible to develop functional catalysts for chemical reactions without prior structural knowledge by iterative screening and deconvolution of vast combinatorial nucleic acid libraries. Direct selection with linker-coupled reactants [6••,8•] has expanded the scope of RNA catalysis and could — in principle — enable a search for catalysts of virtually any bond forming or breaking reaction. This represents a very powerful approach to catalyst discovery.

Another important feature is molecular evolution. A once-enriched catalyst can be further evolved and improved by adding the element of mutation, combined with an altered selection pressure. Counter selection can be applied to suppress the enrichment of unwanted catalytic activities or

to fine-tune the properties of the enriched populations of catalysts. Thereby, catalytic performance can be improved, substrate specificity altered, or reaction conditions changed [13••]. In addition, a wide variety of functional groups can be appended to DNA or RNA to help perform catalytic tasks that cannot be solved by unmodified DNA or RNA [21].

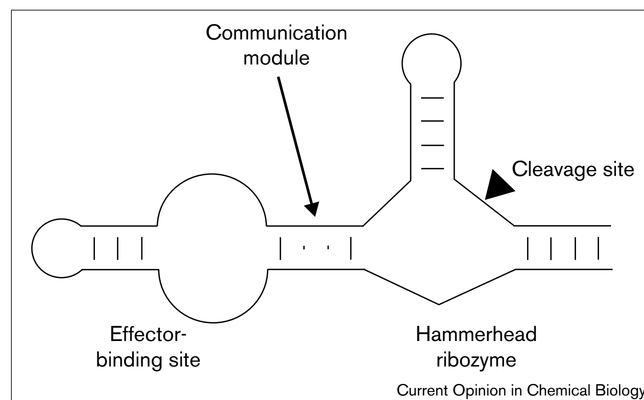
We therefore envision the use of RNA and DNA as highly selective catalysts for synthetically relevant transformations. Regioselectivity and chemoselectivity are well documented for several artificial ribozymes and, as a chiral polymer, RNA can exquisitely differentiate between chiral targets. We anticipate that the excellent enantioselectivity in substrate recognition shown by nucleic acids will finally allow their use as enantioselective catalysts in organic synthesis; this use has recently been demonstrated for catalytic antibodies, which were utilized to elaborate the complex natural product epothilone [22]. Acceleration of energetically disfavored chemical reaction pathways is also an attractive target, which might significantly shorten some multi-step organic syntheses.

In vitro evolution could be used to generate catalysts for combinatorial syntheses. The broad applicability of natural enzymes is often limited by their evolutionarily obtained substrate specificities. Counterselection can be used to purposely emphasize or de-emphasize certain positions in the substrate molecules, which could then be utilized to introduce a variety of substituents. For a broader utilization of nucleic acid catalysts, a number of problems must still be solved and, to date, there is no documented example of DNA or RNA catalysts being used in preparative organic chemistry.

The reaction kinetics of most *in vitro* selected ribozymes are quite slow (especially under multiple-turnover conditions), and conversion of two free reactants with turnover still represents a major problem. These limitations could be addressed by new selection techniques involving fast kinetic methods (quenched flow), selection in micelles, or a combination with high-throughput screening [23].

Another problem is compatibility with typical conditions of organic synthesis. Currently, no example exists of DNA or RNA catalysis in pure organic solvents, even though numerous systems involve 2–20% water-miscible organic co-solvents in aqueous buffers (for example, see [6••]). Nucleic acids are highly charged and are therefore unlikely to dissolve and fold in unpolar solvents. To make RNA or DNA more compatible with organic building blocks and solvents, however, counterions could be exchanged against tetraalkylammonium or similar ions. The use of derivatized nucleotides or post-synthetic modification to introduce hydrophobic sidechains into the nucleic acids should not only increase the solubility in organic solvents, but also enable better interactions with hydrophobic targets.

Figure 6



Schematic view of an allosteric ribozyme, the Hammerhead ribozyme.

The application of *in vitro* selected nucleic acids in organic synthesis could benefit from the technological developments and corresponding cost reductions made with respect to the use of nucleic acids as drugs. While 10 years ago, a DNA synthesis at a 100 mg scale was a costly endeavor, therapeutically relevant DNA oligonucleotides can now be synthesized at a multi-kilogram scale. RNA, too, can be produced in very large batches, enabling the potential use of *in vitro* selected oligonucleotides as catalysts in organic synthesis.

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