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Kinetic Insights of DNA/RNA Segment Salts Catalyzed Knoevenagel Condensation Reaction

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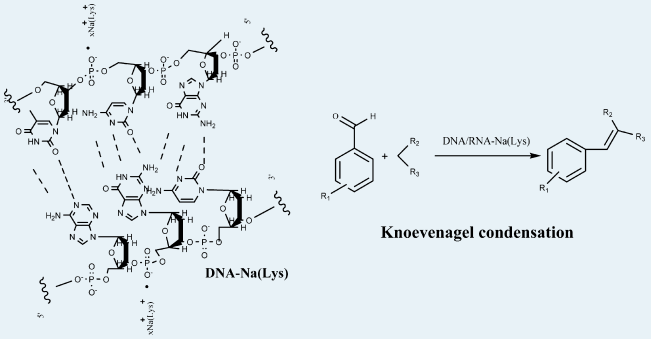
Supporting Information

ABSTRACT: In this work, we demonstrated that (i) salts of RNA/DNA segments were capable of catalyzing Knoevenagel condensation at physiological pH 7.0 with efficiency comparable to one of the best enzymes, porcine pancreatic lipase (PPL); and (ii) a broad scope of substrates could be successfully used in this reaction. Velocity of catalysis was positively correlated with the content of GC nucleosides in DNA/RNA; while the 3D-organization in DNA segments largely contributed to the elevated turnover number of catalysis. An insight into the reaction mechanism (based on quantitative analysis of kinetics) elucidated general similarities between DNA, RNA, and PPL in the substrate binding mechanisms.



KEYWORDS: *Knoevenagel condensation, DNA segments, RNA, kinetic, lipase*

Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA)[1] are traditionally regarded as the carriers of information. Yet, they were also discovered as catalysts since Tarasow et al.[2] found that RNA could catalyze a reaction of carbon−carbon bond formation. Jäschke's group developed anthracene conjugated ribozymes, which mediated the Diels−Alder reaction.[3] Though chemically and structurally dissimilar to enzymes, the ribozymes specifically interacted with their substrates and products,[4] changed active site conformations,[5] and were allosterically activated.[6] Michaelase represents another example of ribozymes reported to be capable of catalyzing the C−C bond formation reaction.[7] Similarly, DNA with either conjugated anthracene or in combination with the copper complex accelerated the Diels−Alder reaction.[8,9] As reviewed elsewhere,[10] these self-assembled DNA-based asymmetric catalysts have demonstrated high enantioselectivity in a variety of carbon−carbon or carbon−heteroatom bond-forming reactions. DNA as a catalyst has also been successfully applied to an aldol reaction[11] and Henry[12] and Michael reactions.[13] It was recently demonstrated that salmon testes DNA (st-DNA) in complex with copper catalyzed Michael addition reactions.[14] The mechanisms of DNA/RNA-mediated catalysis are apparently not identical if considering different reactions performed under different conditions. Yet, the nucleotides contain a variety of functional groups including those of ribose/deoxyribose sugars, phosphate, and organic bases. These groups are capable of H-bonding, metal coordination, $\pi-\pi$ interactions, etc., which often endows the oligo-nucleotide molecules with catalytic or enzyme-like activity.[7−14] For example, Izquierdo et al.[15] found that the catalytic activity of st-DNA in a Michael reaction could be associated with the basic nature of their nucleotides.

Knoevenagel condensation is a nucleophilic addition with great potential for synthesis of drug intermediates,[16] where basic catalysts (such as piperidine,[17] amino acids,[18] and enzymes[19]) are widely used. We recently found that the condensation of aromatic aldehydes and malonitrile can be also catalyzed by pH-neutral amino acid salts (e.g., Lys•HCl).[20] Interestingly, there are also several reported examples of spontaneous synthesis of ylidenemalonitriles and the Knoevenagel condensation of aryl aldehydes with nitroacetonitrile in water in the absence of any catalyst.[21]

To maintain the normal biological functions, thousands of different reactions (affecting human health) take place in the human body at physiological pH (7.0) and mild conditions. It is known that malonaldehyde and alkanones are secondary degradation compounds from fatty acid oxidation, which can result in the generation of toxic compounds in the human body via Knoevenagel condensation and other reactions at physiological pH.[22] In addition, the role of nucleotides and amino acids as pre-enzyme catalysts (functional in nonaggressive solvents at neutral pH) is of fundamental interest for evolutionary biochemistry.[1,2] Herein we explore the potential of neutralized RNA/DNA as catalysts, which accelerate Knoevenagel condensation. Ethanol/water mixtures were used

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to mediate the reaction and increase the solubility of substrates. A broad spectrum of substrates was analyzed, and the extensive kinetic study allowed a more accurate quantitative comparison of the catalysts. The analysis and characterization of products were detailed in a recent publication.[20] The experimental procedures, theory, and analysis of the reactions and kinetic study were described in the Supporting Information.

The commercial DNA/RNA in lyophilized acid forms exhibited nearly no catalytic activity when dissolved in the medium (yields of ≈3% in 4 h). When the nucleotides were neutralized to pH 7.0 by either NaOH or basic amino acids (L-lysine, L-arginine, or L-histidine) the reaction was dramatically accelerated. The yields became comparable to those of porcine pancreatic lipase (PPL, one of the best enzymes for Knoevenagel condensation) and pH-neutral amino acid salts Arg•HCl and Lys•HCl (Figure 1). Still, the catalytic efficiency

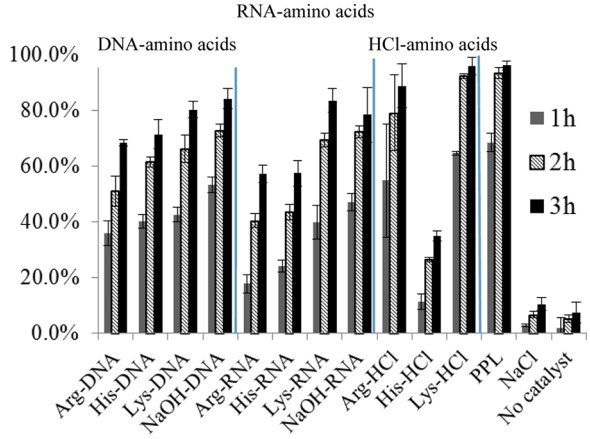

Figure 1. Catalytic activity of DNA, RNA, and amino acids-HCl. The concentrations used: 10 g/L of DNA/RNA salts neutralized by Arg/His/Lys/NaOH to pH 7.0; 0.0188 mol/L of Arg•HCl, 0.0608 mol/L of His•HCl, 0.026 mol/L of Lys•HCl, 0.0212 mol/L of NaCl (the same molar amount of Arg, His, Lys, and NaOH as the corresponding DNA salts was maintained, Table S1 in the Supporting Information); 10 g/L of PPL. DNA was represented by short crude oligonucleotides (<50 bp, degraded). Reaction condition: 0.2 M benzaldehyde, 0.3 M ethyl cyanoacetate, ethanol medium with 4% water added, 40 °C, 500 rpm.

of DNA-Arg and DNA-Lys was somewhat slower than that of Arg-HCl and Lys-HCl, if calculating it per single amino acid. Increased activity of the neutralized oligo-nucleotides was not associated with the ionic strength because no catalytic activity was observed in the solutions of NaCl (Figure 1).

The catalytic versatility of neutralized DNA/RNA was examined using the substrates with substituted groups, and the results were compared to the analogous experiments with PPL (Table 1). The aromatic aldehydes (A_x) with different electron-withdrawing groups had a more active electrophilic center of the carbonyl group and presented higher reaction velocities in comparison to the substrates bearing the electron-donating groups. The apparent rate coefficients are shown in Table 1A, where the highest value was obtained in the reaction $A_4 + B_0$ (Entry 4). On the contrary, the reaction $A_6 + B_0$ (Entry 6) yielded the lowest apparent rate coefficient. The Hammett equation has been one of the most widely used means for the study and interpretation of organic reactions. Its σ-constants are obtained simply from the ionization properties of organic acids in solution, and they can frequently predict the reaction rates from a linear plot.[23] We show the Hammett plot of the Knoevenagel condensation of para-substituted benzaldehydes with ethyl cyanoacetate catalyzed by DNA fragment sodium salt and PPL (Figure 2). It is evident that neither DNA nor PPL presented a good linear correlation between σ-constants and the logarithmic ratios of rate constants ($\log(k/k_0)$), as would be expected for a simple chemical catalyst.[24] This result indicates a complex mechanism of catalysis performed by DNA/PPL, where the formation and dissociation of temporary ligand-catalyst complexes apparently occur. Despite some occasional deviations, there was still a clear tendency that $\log(k/k_0)$ of DNA/PPL mediated reactions increases with increasing σ-constant (Figure 2). We observed earlier a similar result in Lys and Lys•HCl catalyzed reactions.[20] Yet, the order of efficient substrate combinations in DNA•NaOH-catalyzed reactions was different from the one obtained with Lys, Lys•HCl, or PPL. Steric effects, associated with the binding of substrates to the 3D-organized molecules (DNA, PPL), might be the reason. Therefore, interpretation of the Hammett plot cannot be straightforward. Electron-withdrawing groups in the second substrate (methylene compound B_x) increased velocity of condensation (Table 1B).[20,25] The maximal rate was attained in the reaction $A_0 + B_1$ (Entry 1).

The intrinsic catalytic potential of a nucleotide apparently depends on the composition of its base-pairs.[26] In this context, we proportionally varied the composition of the mixture of the individual nucleotides from primarily A-dT (A-U) to predominantly C-G. The reaction velocity increased as the CG content increased (Figure 3).[27] This acceleration is likely to be caused by the electronic effect of amine/imine compounds (better represented in C-G pairs). When analyzing the individual nucleosides, cytidine exhibited the highest activity, followed by thymidine deoxyriboside (Table S2). Within an RNA structure, the properly positioned cytosines and adenines can potentially serve as general acid or base catalysts if their ring nitrogen atoms N_1 and N_3 are protonated (Table S2(A)).[28] The same general acid or base catalysis can also take place in our study with the degraded DNA (crude oligonucleotides, < 50 bp). The DNA salts were not expected to be exclusively in the duplex form because of their partial degradation, a pH-neutral environment and the elevated temperatures (40 or 50 °C) of our catalytic experiments. Unpaired bases are apparently less frequent in the long native DNA molecules (700 bp), but the presence of "hairpin" and "cruciform" structures provides various possibilities for the unusual binding patterns between the DNA molecule and the substrates. We might speculate whether the molecular basis of catalysis is associated with the functional groups like R−NH_2, R−OH, and R−C═O, which possibly participate in the formation of transition state complexes during the substrate binding. Close positioning of the two substrates and/or destabilization of their electronic densities might cause spontaneous condensation, not unusual in such reactions.

Water is expected to play a pivotal role in achieving high catalytic efficiency, because (i) the proper water/ethanol ratio provides a solvent with intermediate polarity important for the solubility of substrates (Figure S1(A));[20] (ii) hydration of the nucleotide bases affects their protonation patterns and 3D-structure.[29] Different optimal water contents were observed for different catalysts (Figure S1(B)). A relatively sharp optimum around 50% was found for short DNA/RNA fragments (<50 bp). The 700 bp DNA•NaOH fragments attained maximal

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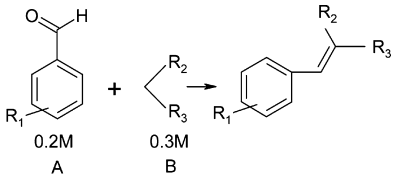
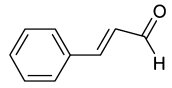
Table 1. Condensation Reaction of Substituted Aromatic Aldehydes (A) and Active Methylene Compounds (B)



(A) Apparent Rate Coefficients[a] Obtained in the Reactions $A_x + B_0$ (Various A) Catalyzed by DNA-NaOH and PPL[b]

Entry(A_x) R1	k_{DNA} min^{-1}	k_{PPL} min^{-1}	Entry(A_x) R1	k_{DNA} min^{-1}	k_{PPL} min^{-1}
none	0.0060	0.0122	6 *p*-$N(CH_3)_2$	0.0003	0.0030
1 *p*-CN	0.0273	0.0981	7 *p*-MeO	0.0005	0.0128
2 *p*-NO_2	0.0494	0.0277	8 *o*-MeO, *p*-MeO	0.0012	0.0210
3 *p*-Cl	0.0320	0.0295	9 *p*-CF_3	0.0034	0.0190
4 *o*-Cl, *p*-Cl	0.0575	0.0981	10 *p*-CH_3	0.0014	0.0025
5 *p*-$phCH_2O$	0.0013	0.0084	11* 	0.0040	0.0084

(B) Coefficients for $A_0 + B_x$ (Various B)

entry(B_x)	k_{DNA} min^{-1}	k_{PPL} min^{-1}
0 R_2 = −CN, R_3 = −COOEt (pK_a 13.1)	0.0038	0.0090
1 R_2 = R_3 = −CN (pK_a 11.1)	0.0450	0.0799
2 R_2 = R_3 = −COOEt (pK_a 16.4)	0.00003	0.00002

[a] $k_{app} = -\log_{10}(A_t/A_0)/t$, where t = time. Catalyst: 50 mg of DNA-NaOH or PPL in a 5 mL reaction mixture, 40 °C. [b] *Entry 11: the substrate is *trans*-cinnamaldehyde, not joined to the R1 group.

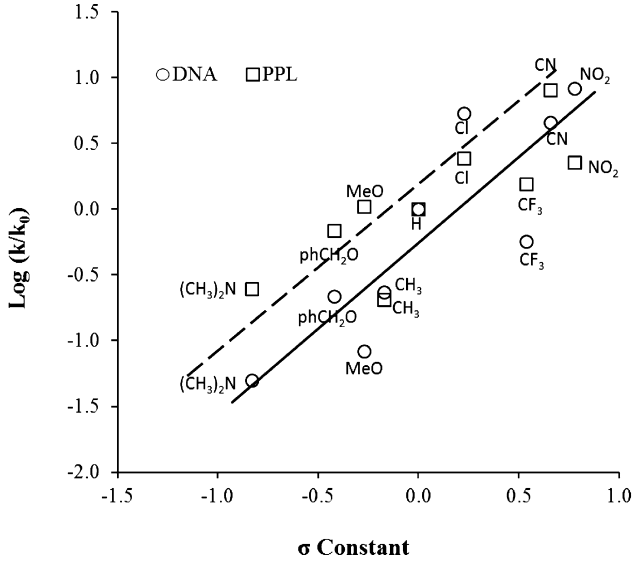


Figure 2. Hammett plot for the Knoevenagel condensation of *para*-substituted benzaldehydes with ethyl cyanoacetate catalyzed by DNA fragment sodium salt (circles) and PPL (squares). The reaction conditions correspond to those in Table 1.

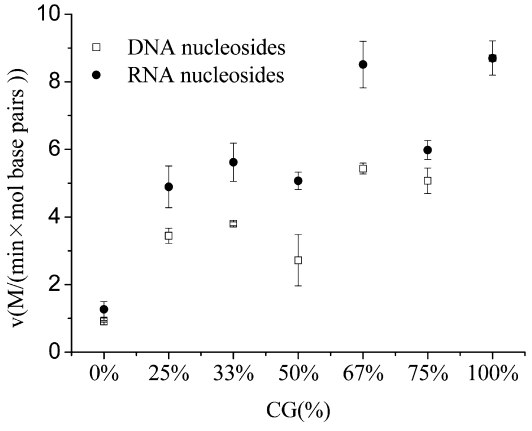


Figure 3. Dependency of the catalytic activity of the nucleoside mixtures on CG concentration (stipulating different proportions of AdT (AU) and CG). General reaction condition: 0.055 mmol bases mixtures (as detailed in the Supporting Information), 0.24 M benzaldehyde, 0.36 M ethyl cyanoacetate, medium of 4% water added to ethanol, within 2 h, 40 °C, 500 rpm.

reaction velocity at a lower water content of 5%−25%. A very broad optimum was found for the enzyme PPL (5%−50%).

Spectroscopic analysis is known to be an effective tool to examine the individual interaction between substrate and catalyst.[30,31] The UV−vis absorption curve of DNA•NaOH +A_0 was different obviously from the curve of DNA•NaOH in intensity and peak shifting, indicating the formation of the

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Table 2. Kinetic Constants of a General Velocity Equation of a Bisubstrate Reaction[c,d]

	700 bp DNA-NaOH	DNA-NaOH	DNA-Lys	RNA-NaOH	RNA-Lys	Lys-HCl	PPL[a]
V_{max} (M/min)	0.182	0.042	0.034	0.030	0.035	0.225	0.32
K_{sA} (M)	≤0.03	0.037	0.039	0.111	0.124	0.364	≤0.07
K_{sB} (M)	≤0.04	0.060	0.049	0.177	0.110	0.271	≤0.05
K_{mA} (M)	1.02	0.463	0.317	0.239	0.374	1.93	0.707
K_{mB} (M)	1.36	0.750	0.397	0.380	0.334	1.440	0.50
Catal. (M)[b]	0.014	0.028	0.021	0.027	0.021	0.250	2.6×10^{-5}
k_+ (min^{-1})	13.35	1.500	1.619	1.119	1.658	0.899	≈12300
Adj. R^2	0.822	0.934	0.817	0.898	0.824	0.894	0.813

[a]The fitting parameters for PPL are not precise, because variation of K_{sA} within the range of $0.07-1 \times 10^{-5}$ M gives nearly the same R^2. [b]The catalyst concentration corresponds to (i) a single nucleotide in the chains of DNA and RNA, (ii) individual Lys in Lys-HCl, and (iii) the whole molecule of PPL, see also discussion in the main text. [c]Corresponding, for example, to a rapid-equilibrium random binding mechanism. [d]Deoxyribonucleic acid sodium salt was from herring testes (type XIV, ca. 700 bp), DNA was from herring sperm (degraded, crude oligonucleotides <50 bp), ribonucleic acid was from torula yeast (Type VI) ; DNA without other notation refers to the degraded, crude oligonucleotides <50 bp. The relative standard errors of parameters were on average as follows: $\pm 0.53 \cdot V_{max}$, $\pm 2.3 \cdot K_{sA}$, $\pm 2.2 \cdot K_{sB}$, $\pm 0.84 \cdot K_{mA}$. $\pm 0.84 \cdot K_{mB}$. Nomenclature: K_{sX} describes the dissociation of X from the catalyst (usually E+X↔EX), K_{mX} describes the corresponding Michaels constant. All constants are related to each other according to the ratio $K_{sA}/K_{mA} = K_{sB}/K_{mB}$ in the case of a random binding mechanism. The values of K_{sB} were assessed from this assumption. Adj. R^2 stands for coefficient of determination adjusted for the number of parameters in the model. Reaction conditions: 50 °C and optimal water concentration in ethanol solvent. Water concentration is, probably, a "hidden" component of all rate and dissociation constants.

DNA•NaOH-A_0 complex; whereas the curves of DNA•NaOH and DNA•NaOH+B_0 are much similar, indicating a different type of catalyst-substrate interactions (Supporting Information, Page P8). The possibility of the independent interaction of substrates A_0 or B_0 with DNA•NaOH or RNA•NaOH was established by monitoring the intrinsic fluorescent response of the catalysts toward addition of either A_0 or B_0. Detectable changes of fluorescence took place in the approximate ranges of concentration 0−0.6 mM for substrate A_0 and 0−1 mM for substrate B_0 (Supporting Information, Pages S10−S11), indicating the independent binding of A_0 or B_0 with a catalyst could take place without the presence of the other. The responses of fluorescence quenching as a function of quencher concentrations at different temperatures were also analyzed by the Stern−Volmer equation (Figures S3 and S4). For both DNA•NaOH and RNA•NaOH, substrate A_0 presented a bigger quenching constant than B_0 at the same temperature; and the constant decreases as temperature increases, indicating a ground-state complex formation between A_0 and catalysts. Substrate B_0 demonstrated a different fluorescence quenching behavior for DNA•NaOH (Ksv decreases as temperature increases) and RNA•NaOH (Ksv increases as temperature increases), suggesting the former is static quenching and the latter belongs to dynamic quenching.[30] This also indicates the interaction may vary among different substrate-catalyst combinations. The above observations are inconsistent with UV−vis spectra analysis that substrate A_0 and B_0 may engender different interactions with catalysts, which might be related to their molecular structure differences.[31]

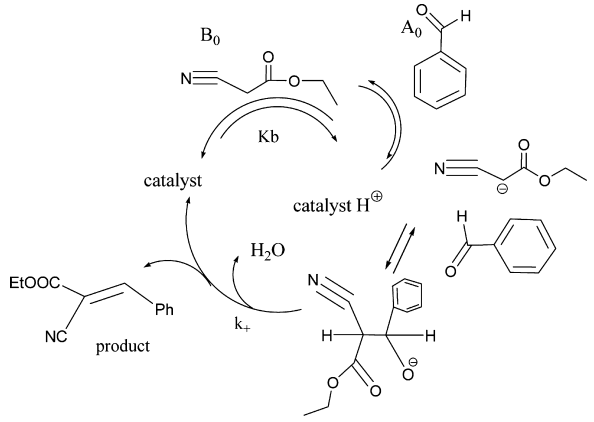
However, it should be emphasized that fluorescence analysis is incapable of delineating the interaction between catalyst and bisubstrates, cooperation and sequential collisions,[30−32] because the reaction may occur when catalyst/A_0/B_0 are simultaneously presented. Meanwhile, the reaction velocity recorded at a single combination of the two substrates does not provide detailed insight into the functioning of a catalyst.[32] Therefore, we investigated the probable substrate-binding mechanisms using various combinations of substrates A_0 and B_0, which is the particular interest of this work. The DNA/RNA-catalyzed reactions were compared to those in the presence of lipase PPL and amino acid Lys•HCl (Table 2). Approximation of our data was performed by the general velocity equation (eq 1) of a bisubstrate reaction, which includes the largest set of parameters.[32]

$$v = \frac{V_{max}}{1 + \frac{K_{mA}}{a} + \frac{K_{mB}}{b} + \frac{K_{sA}}{a}\frac{K_{mB}}{b}}; \qquad V_{max} = k_+ \cdot e_0 \tag{1}$$

This equation contains four parameters, where K_{mA} and K_{mB} correspond to the Michaelis constants of the substrates A and B, respectively; K_{sA} is the substrate constant of A; and V_{max} is the maximal velocity. The latter parameter can be expressed via e_0 (the total concentration of catalyst E) and k_+, which is the turnover number (i.e., the maximal number of catalytic conversions per time unit per active site).

The full equation (eq 1) usually corresponds to either the mechanism of fast-equilibrium random binding (Scheme 1) or

Scheme 1. Proposed Kinetic Mechanism DNA/RNA-Salts Appears To Progress via the Shown Catalytic Cycle



the steady state ordered binding scheme. Very small values of some constants might provide additional information about possible cancelation of the elements in the denominator. In such a way, alternative mechanisms can be hypothesized, e.g. fast-equilibrium ordered binding, ping-pong, etc. The experimental data were presented in 3D-coordinates (initial velocity v as a function of A- and B-concentrations) and subjected to computer fitting (Figure 4). The coefficients of best

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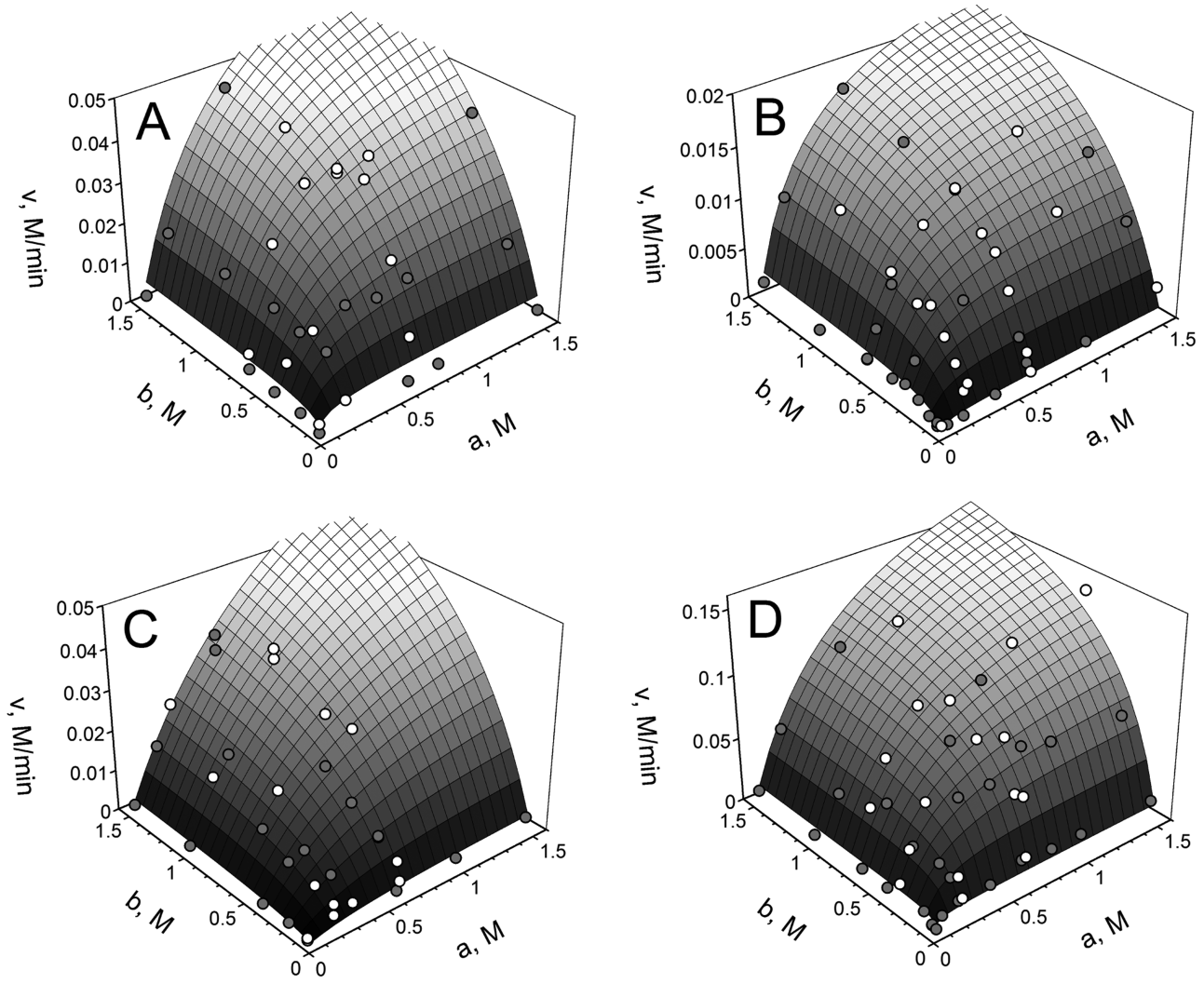

Figure 4. Dependence of the reaction velocity v on the substrate concentrations (a_0, b_0) for different catalysts: (A) DNA-NaOH fragments of 700 bp; (B) DNA-NaOH fragments of <50 bp; (C) Lys-HCl; (D) PPL.

approximation are shown in Table 2. The goodness of fit was assessed via coefficient of determination R^2 adjusted for the number of parameters.

All parameters of Table 2 had measurable values and did not tend to zero (or negative values). We, therefore, hypothesize the random binding to be a plausible model. It stipulates assembly of the reactants into a ternary complex (e.g., A•E•B; E stands for catalyst), where the sequence of ligand binding is unimportant. In our case, the attachment of one substrate apparently hinders the binding of the other (e.g., $K_{sA} < K_{mA}$), probably, because A and B compete for nearly the same specific site on the catalyst. The mechanism can be also interpreted as a variant of the ordered binding with several steady state steps but not the fast equilibrium ordered binding. In the current work we did not pursue precise identification of the model but used the kinetic analysis as a tool to quantify and compare the main parameters of different catalysts.

An important characteristic of any complex catalyst is its turnover number k_+ or k_{cat} (equal to V_{max}/e_0). The meaning of k_+ is not absolutely straightforward if the concentration of the active site (e_0) is not established. The usual assumption of 1 catalytic site per whole molecule unnecessarily favors large molecules. For example, k_+ of 1 bp DNA becomes 10-fold less than k_+ of the identical 10 bp DNA because $e_0(1_{bp})/e_0(10_{bp}) = 10$, even though each base pair has the same catalytic potency. In the absence of any additional information, we ascribed activity to each individual nucleotide (amino acid) within the joined chain, when making a comparison between the catalysis of widely different masses. The turnover numbers of both DNA and RNA salts were slightly higher than the k_+ value of simple catalyst Lys•HCl (see Table 2). Efficiency of each nucleotide within 700 bp DNA•NaOH increased if compared to a shorter chain of 50 bp DNA•NaOH. Substitution of NaOH by Lys provided only marginal effects. The enzyme PPL had the highest turnover number of ≈12300 min^{-1} (Table 2), if calculating k_+ per the whole enzyme molecule composed of 450 amino acids. Yet, k_+ of PPL was reduced to 27 min^{-1} (if calculating its value per single amino acid). This is only ≈2 times higher than k_+ of a nucleotide within 700 bp DNA•NaOH. Comparison of k_+ for the whole molecules of ≤50 bp DNA (<100 nucleotides) and PPL (i.e., two molecular species of similar complexity and size) gave the difference by 2 orders of magnitude (k_+ = 12300 min^{-1} and 150 min^{-1}, respectively). Calculation of k_+ per 52 kDa of mass (the mass of PPL, approximately equal to 80 bp DNA) gives the values of 12300 min^{-1} (PPL), 120 min^{-1} (52 kDa within DNA of 50 bp), and 1070 min^{-1} (52 kDa within DNA of 700 bp). Disregarding the evaluation method of k_+, it is visible that a higher potential for self-organization in the long DNA

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fragments gives the higher catalytic potencies approaching those of enzymes.

The presence of either Na^+ or basic amino acids associated with phosphates of DNA and RNA chains had apparently little effect on the turnover number as long as nucleotides remained neutralized. We have, therefore, tested if any conformational changes take place in DNA/RNA upon the binding of amino acids. We found that (i) all the DNA/RNA salts remained in B-form according to CD spectra, while RNA had a little blue shift; (ii) DNA•Lys, DNA•Arg increased fluorescence emission and showed little sensitivity to solvent polarity when dissolved in 55% ethanol or water; (iii) ^{1}H NMR and CD spectra of DNA•NaOH and DNA•Lys verified that Na^+ and Lys indeed interacted electrostatically with phosphate groups (see details in Spectrum Study in the Supporting Information).

Combining the current knowledge with the generally accepted Knoevenagel condensation mechanisms,[14−19,28] we can propose the following pathway (Scheme 1). First, a ternary complex A•E•B is formed via a fast random binding or according to the ordered steady state mechanism (both described by eq 1). The substrates are located very closely and partially hinder the binding of each other to catalyst E (because $K_{mA} > K_{sA}$). At the second step, a proton is transferred from ethyl cyanoacetate to the catalyst (e.g., DNA/RNA-salt) and enolate ion is formed. Then another substrate benzaldehyde accepts the proton and simultaneously becomes connected to enolate ion with formation of a carbon−carbon bond. Finally, the Knoevenagel adduct is released from the catalyst. An alternative kinetic scheme (also compliant with eq 1) suggests a temporary binding of the active hydrogen compound (B) to the catalyst followed by abstraction of proton. Finally, a direct collision with benzaldehyde (with a very weak or no complex formation) leads to the condensation. A comparison of kinetics for simple and complex catalysts (planned in the future) might shed some light on this subject.

In conclusion, we have demonstrated that RNA/DNA oligonucleotides can effectively catalyze the Knoevenagel condensation reaction at physiological pH. The best achieved efficiency was comparable to the enzyme PPL. Velocity of catalysis was positively correlated to the content of GC nucleosides. A long strand of 700 bp DNA•NaOH exhibited higher turnover number than 50 bp DNA•NaOH, DNA•Lys, and Lys•HCl, which stresses the importance of higher 3D-organization. The deoxyribo- or ribophosphate backbone was not directly involved in catalysis. Quantitative characterization of kinetics elucidated general similarities of DNA, RNA, lipase PPL, and Lys in the substrate binding mechanisms (compliant with general equation of a bisubstrate reaction). This work demonstrated a novel group of catalysts and illustrated possible mechanisms of catalysis relevant to evolutionary biochemistry of simple molecules.

■ ASSOCIATED CONTENT

Supporting Information

Details on DNA/RNA salt preparation, pH influence assumption, the kinetic and spectral characterization data for catalysts. This material is available free of charge via the Internet at http://pubs.acs.org.I

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Notes

The authors declare no competing financial interest.

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