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Protoenzymes: the case of hyperbranched polyesters

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Enzymes are biopolymeric complexes that catalyse biochemical reactions and shape metabolic pathways. Enzymes usually work with small molecule cofactors that actively participate in reaction mechanisms and complex, usually globular, polymeric structures capable of specific substrate binding, encapsulation and orientation. Moreover, the globular structures of enzymes possess cavities with modulated microenvironments, facilitating the progression of reaction(s). The globular structure is ensured by long folded protein or RNA strands. Synthesis of such elaborate complexes has proven difficult under prebiotically plausible conditions. We explore here that catalysis may have been performed by alternative polymeric structures, namely hyperbranched polymers. Hyperbranched polymers are relatively complex structures that can be synthesized under prebiotically plausible conditions; their globular structure is ensured by virtue of their architecture rather than folding. In this study, we probe the ability of tertiary amine-bearing hyperbranched polyesters to form hydrophobic pockets as a reaction-promoting medium for the Kemp elimination reaction. Our results show that polyesters formed upon reaction between glycerol, triethanolamine and organic acid containing hydrophobic groups, i.e. adipic and methylsuccinic acid, are capable of increasing the rate of Kemp elimination by a factor of up to 3 over monomeric triethanolamine.

This article is part of the themed issue 'Re-conceptualizing the origins of life'.

1. Introduction

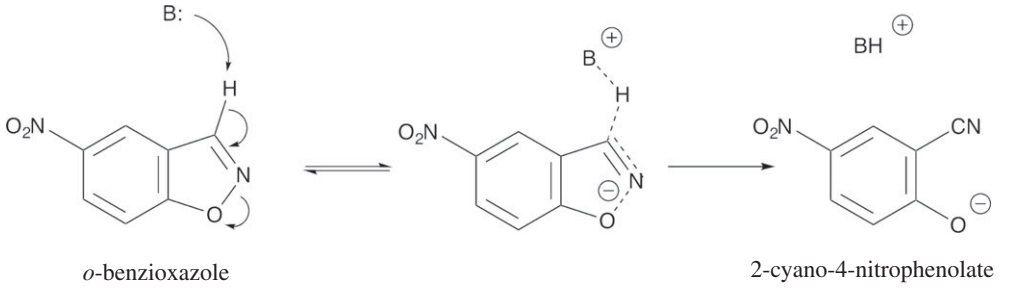
Enzymes are macromolecular catalysts that govern biochemical transformations. The catalytic properties of enzymes are unmatched by other catalysts [1,2]. Enzyme-catalysed reactions proceed orders of magnitude faster than uncatalysed ones; enzymes exhibit unparalleled specificity towards both the substrates and the reaction products. Oparin [3] argued that the emergence of life is inseparable from the emergence of enzymes. The presence of enzyme-like catalysts might indeed explain the transition from complex disorganized prebiotic molecular soup into well-orchestrated reaction networks ubiquitous in biology.

The structure of enzymes is characterized by high degrees of organization and complexity. They comprise organic, mineral or metal cofactors, agents that actively participate in reaction mechanisms, supported by intricately folded globular protein or RNA macromolecules. The function of such a macromolecular scaffold is to specifically bind, orientate and encapsulate substrates while creating a microenvironment conducive to reaction progression. When considering enzyme-like prebiotic catalysts, the abundance of certain small molecule, inorganic cluster and metal cofactors is conceivable; however, long functional proteins and RNA molecules are unlikely to have been present at the early stages of chemical evolution. Herein we consider the concept of the protoenzyme, an enzyme-like structure that uses alternative simpler polymeric scaffolds that could have preceded biopolymeric ones.

The concept of an alternative polymer-based protoenzyme was first effectively explored by Sidney Fox as part of his proteinoid theory that focused on the formation of microsphere structures upon thermal condensation of a-amino acids [4–6]. When pure amino acids are heated, diketopiperazines and small amounts of polypeptides are formed [7,8]. Fox, however, had discovered that dry heated mixtures of amino acids rich in glutamic acid [9,10] yield high molecular weight (up to 20 000 Da) polypeptide-like materials. While biological polypeptides are characterized by a $-[N-C_\alpha-\text{carbonyl C}]_n-$ sequence, proteinoids containing aspartic and glutamic acid possibly contain amide linkages engaging C_β and C_γ as branching points. Proteinoid theory has been extensively criticized by the scientific community due to unsubstantiated claims of linearity [9] and non-randomness of amino acid sequences, and later, more outrageously, life-like behaviour of these thermal polypeptide-like macromolecules [6]. Nevertheless, in several papers Fox and co-workers have documented the catalytic properties of proteinoids, mostly towards hydrolysis reactions [11–13]. Critics pointed out that the demonstrated catalytic activity was tenuous and was surpassed by certain small molecules [8]. Furthermore, Fox and co-workers did not provide a mechanism by which proteinoids could act as catalysts. Proteinoid theory was largely dismissed [8] and rarely revisited in the context of prebiotic chemistry. The biomaterial aspect of the theory, however, had received a small following in pharmaceutical research [14,15].

Despite the shortcomings of the proteinoid theory, it brought forward the idea of non-biological polymers playing a role in the origin of life. An intriguing notion of random polymers serving as templates for prebiotic reactions was first put forward by Mardsten Scott Blois, a biophysicist at Stanford University, USA, in his presentation at 'The origins of prebiological systems' conference organized by Fox in 1965 [16]. Blois was studying the structure of melanin at the time; in particular, he was interested in the comparison between the biological pigment and the tarry polymer produced upon thermal treatment of histidine-rich amino acid mixtures [17]. Blois realized that the synthetic 'melanin' was a heterogeneous polymer, and he argued that such a material had a multitude of potential stereospecific sites capable of binding different molecules and an array of functional groups to facilitate prebiotic reactions [16]. These properties effectively describe synthetic 'melanin' as an enzyme-like agent. Blois dedicated the latter part of his career to melanoma research and health informatics, but his ideas about chemical evolution have never been followed up.

Insertion of a catalytic unit into an abiological polymer to generate systems that mimic an enzymatic properties scaffold is a well-established approach within the synthetic chemistry community [18]. The most well-known example is the so-called 'dendrizymes'. Dendrizymes feature a catalytic core scaffolded by dendrimers, regular branched polymers



Scheme 1. The Kemp elimination reaction. Eliminative cleavage of *o*-benzisoxazole is shown to be sensitive to the effects of the medium.

[19]. The topology of dendrimers enforces globular structure and allows for a controlled microenvironment surrounding the core similar to protein structure, properties that enable artificial enzyme design. Liu & Breslow [20] showed that pyridoxamine covalently bound to poly(aminoamine) dendrimers displayed 1000-fold higher reactivity towards transamination with pyruvate in aqueous solution.

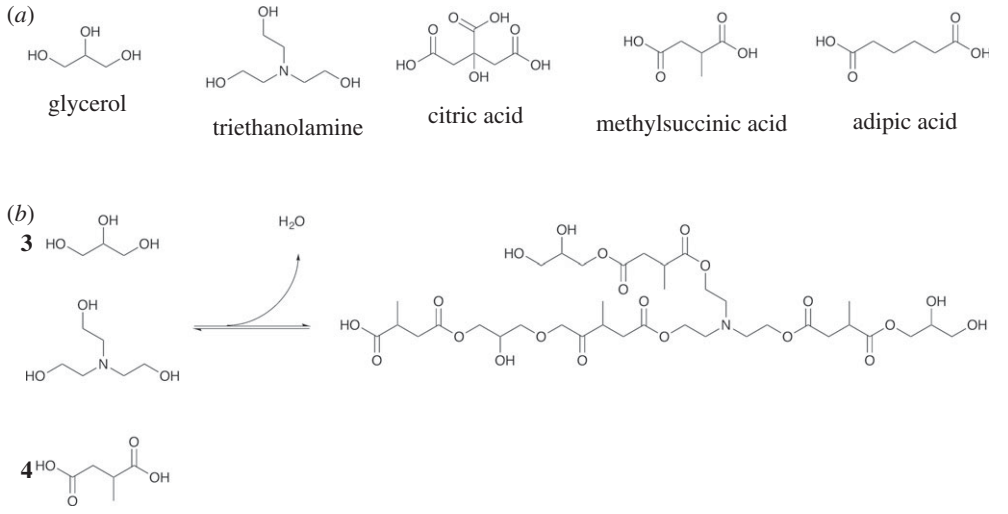
Hyperbranched polymer (HBP) is a term used to describe irregular (highly branched) polymers that form through random reaction of polyfunctionalized monomers with themselves and with other polyfunctionalized monomers. HBPs retain a lot of dendrimer properties [21,22]. The structure of HBPs results in a multitude of end groups that can bind catalysts and substrates, as well as control the polarity of the intramolecular microenvironment. In contrast to dendrimers, HBPs do not contain a distinct core, have less defined intramolecular cargo space and a broad distribution of molecular structures and sizes. Although intrinsically less organized, the advantage of HBPs over dendrimers is their straightforward, often one-pot, synthesis, whereas dendrimers require multi-step procedures. Our previous study has demonstrated the synthesis of hyperbranched polyesters (HBPEs) from citric acid and glycerol under prebiotically plausible conditions of solventless mild heating [23].

Herein we describe a proof-of-principle study demonstrating the catalytic potential of HBP mixtures with a wide distribution of structures and molecular weights. As a catalytic assay reaction, we employ Kemp elimination (scheme 1), base-catalysed ring opening of benzisoxazole by cleaving an NO bond along with deprotonation of carbon to result in *o*-cyano-phenol derivatives [24,25]. The reaction is particularly sensitive to medium effects: its rate reversely correlates with the solvent's polarity. The effect is explained by the base catalyst activation through desolvation in a less polar medium and, to a smaller extent, by the stabilization of the transition state through dispersion forces [26–28]. If HBPs are capable of providing hydrophobic pockets within their globular structure, their presence should increase the rate of Kemp elimination in aqueous solution. This approach has been previously implemented by Hollfelder *et al*. [27], who have correlated the rate of Kemp elimination with the medium effect provided by hyperbranched polyethyleneimine alkylated with different combinations of alkyl groups. For the study, we synthesized HPBEs containing triethanolamine to act as a catalytic core for the Kemp reaction as well as glycerol and citric, adipic and methylsuccinic acid (scheme 2*a*) to provide microenvironments with varying degrees of hydrophobicity. A representative structure of the HBPE formed upon polyesterification of glycerol, triethanolamine and methylsuccinic acid is depicted in scheme 2*b*.

2. Material and methods

(a) Materials

Citric acid (ACS reagent, Fisher Scientific), glycerol (99%, Sigma-Aldrich), triethanolamine chloride (98%, Sigma-Aldrich), adipic acid (99%, Sigma-Aldrich), methylsuccinic acid



Scheme 2. (*a*) Multi-functional alchohols and carboxylic acids used in the study to synthesize HBPEs. (*b*) Schematic of glycerol, triethanolamine and methylsuccinic acid polyesterification and the synthesis of HBPE.

(99%, Sigma-Aldrich), Bis-Tris (98%, Sigma-Aldrich), Bis-Tris hydrochloride (99%, Sigma-Aldrich) and 5-nitro-1,2-benzisoxazole (95%, Ark Pharm, Libertyville, IL, USA) were used as received.

(b) Hyperbranched polyester synthesis

The polyesterification procedure was adapted from Malmström *et al*. [29] In a typical procedure glycerol (0.92 g, 10 mmol), triethanolamine (1.49 g, 10 mmol), citric acid (1.92 g, 10 mmol) and pTSA (34.5 mg, 0.2 mmol) were placed in a three-necked flask furnished with a gas inlet, a drying tube and a stirrer. The flask was then positioned in an oil bath preheated to 110°C. The mixture was left to react for 12 h under Ar flow. Upon cooling, 1 g of the resulting product was placed into a beaker containing 10 ml of deionized water; the beaker was subsequently sonicated for 30 min. The polymer only partially dissolved. The soluble fraction was dialysed against deionized water using 0.5–1 kD float-A-lyzer membrane (Spectrum Labs, Rancho Dominguez, CA, USA) to remove unreacted reactants and the catalyst. The resulting solution was lyophilized to yield a white flaky solid. Only this fraction was analysed and used in the catalyst assay. The same procedure was followed for the synthesis of adipic- and methylsuccinic acid-containing polymers; 1.46 g and 1.32 g of adipic and methylsuccinic acids, respectively, were used instead of citric acid.

(c) Mass spectrometry analysis

Mass spectrometry analysis was accomplished by a direct infusion into a triple quadrupole (TQ) detector (Waters Corporation, Manchester, UK) operating in positive mode. The ionization was achieved by a corona discharge; the sampling cone voltage was set at 15 V. The source and desolvation temperatures were set to 150°C and 250°C, respectively; the nitrogen desolvation flow rate was set to 500 l h^{-1}. The mass spectrometer was calibrated from *m/z* 80–2000 using a 0.5 mM citric acid aqueous solution.

(d) Nuclear magnetic resonance analysis

^{1}H Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 spectrometer at 25°C. The samples were dissolved in D_2O, the pH was adjusted with DCl and NaOD and three ^{1}H spectra were collected employing 30° inversion pulses with an 11 s

acquisition time and 1 s recycle delay. In the polymer NMR spectra (electronic supplementary material, figure S1) the signals associated with triethanolamine, glycerol and acid units were fairly well separated. The polymer molecular formulae were determined through the integration of the appropriate regions.

(e) Kemp elimination assay

The aqueous samples containing 50 µM 5-nitro-1,2-benzisoxazole, 5 mM triethanolamine, either free or as a polymer unit, and 200 mM Bis-Tris buffer at pH 7 were thoroughly mixed in a UV–vis cuvette. Upon mixing, the samples were immediately placed into a Tecan Infinity® M200 spectrometer for time-lapse measurements. The temperature of the cuvette was controlled at 26°C. Spectra between 270 and 500 nm were collected at each time point. Values at 380 nm, the maximum absorbance wavelength of the product, which was consistent throughout the experiment, were used for the analysis (electronic supplementary material, figure S1). The concentration of the product was calculated using the Beer–Lambert law. The extinction coefficient of the product was determined to be 18.1 mM^{-1} cm^{-1} by measuring the absorbance of *p*-nitrophenol in 200 mM Bis-Tris buffer. Initial reaction rates were fitted assuming pseudo first-order kinetics.

3. Results

In our previous report, we have described a simple prebiotically plausible synthesis of HBPE [23]. The polymers were prepared by direct condensation via prolonged solventless heating of citric acid and glycerol at 85°C. The polymerization proceeded without an added catalyst, but it had probably been facilitated by acidic conditions ensured by the citric acid. For the purposes of our current study, we intended to incorporate tertiary amine groups in the form of triethanolamine to serve as an active site for Kemp elimination. The addition of an equivalent of triethanolamine had significantly decreased the acidity of the solventless reaction mixture, consequently mere heating at 85°C failed to produce an appreciable amount of polyesters. We have, therefore, opted for conditions with a higher temperature of 110°C, the use of *p*-toluenesulfonic acid as a catalyst and active removal of water according to common synthetic direct condensation esterification procedures [29].

The synthesis of HBPE using the above method has yielded a structurally diverse mixture of products, as evidenced by the mass spectra depicted in figure 1. The three-component nature of the polymers and the formation of adducts, common with the electrospray ionization methods, made it impossible to equivocally identify all the peaks. As the mass spectra in the case of all the samples are devoid of ions with masses above 1000 *m/z* and isotopic patterns characteristic of doubly charged ions have not been identified, it is reasonable to assume that the polymer mixtures do not contain species larger than 7- or 8-mers.

Figure 2 presents the kinetic profiles of the Kemp elimination reaction as catalysed by the free and polymer-scaffolded triethanolamine. The molecular formulae (table 1) of the polymeric samples were determined using ^{1}H NMR (see electronic supplementary material) to ensure a consistent concentration of triethanolamine, free or a polymer unit, in all samples. The assays were performed at high excess of buffer and catalyst allowing the use of a pseudo-first-order kinetic model to analyse the result. Hollfelder *et al*. [27] have previously used the Michaelis–Menten model to analyse the kinetic data from Kemp elimination catalysed by alkylated polyethyleneimine polymers. In our case, however, highly buffered conditions were required to counteract the unreacted carboxylic acids still capping the polymers. Under such conditions, the background reaction was not negligible, and the free ligand assumption of the Michaelis–Menten model [30] was not satisfied. The 100-fold excess of the catalyst ensured the validity of the first-order assumption. The calculated rate constants are provided in figure 2. The citric acid/glycerol-scaffolded triethanolamine provides only a slight rate increase over unpolymerized

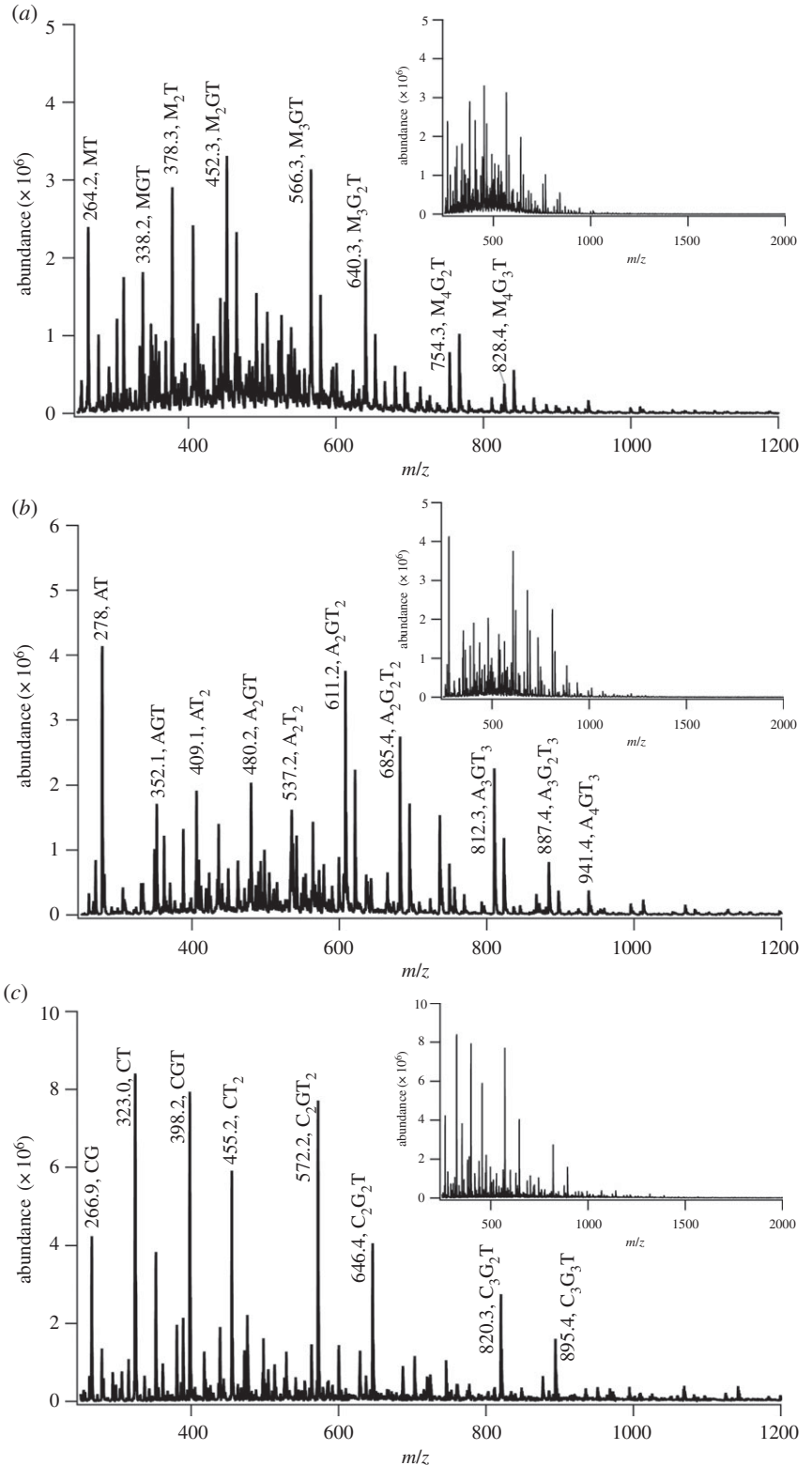


Figure 1. Mass spectrometry analysis of HBPEs formed upon polyesterification of glycerol (G), triethananolamine (T) and (*a*) methylsuccinic acid (M), (*b*) adipic acid (A) and (*c*) citric acid (C). All labelled species correspond to $[M + H]^+$ ions. Insets show full recorded *m/z* range.

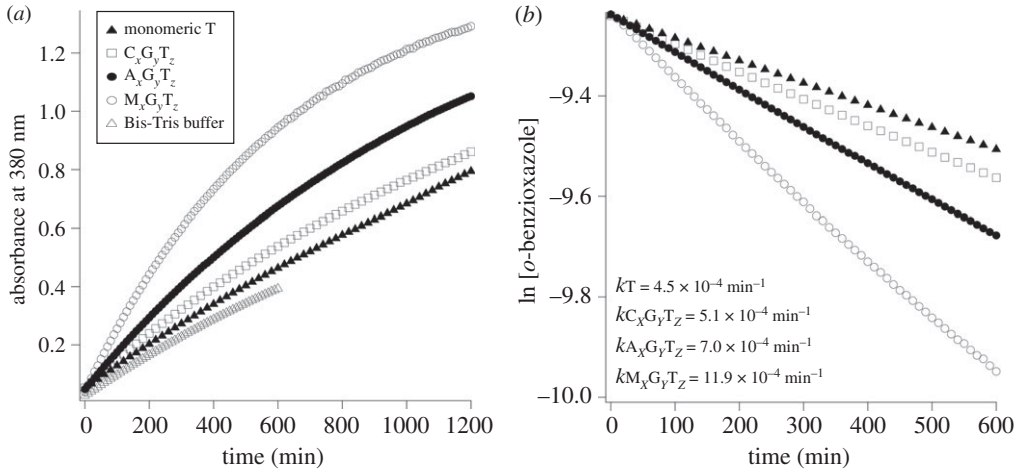


Figure 2. Kemp elimination assay. (*a*) Plot of absorbance at 380 nm versus time for Kemp reactions catalysed by the Bis-Tris buffer, unpolymerized triethanolamine (T) and HBPEs formed upon polyesterification of glycerol, triethanolamine and citric acid ($C_xG_yT_z$), adipic acid ($A_xG_yT_z$), methylsuccinic acid ($A_xG_yT_z$). Increasing absorbance indicates the production of 2-cyano-4-nitophenolate. (*b*) Plot of ln[*o*-benzioxazole] versus time along with the derived rate constants according to the integral representation of the first-order kinetics equations.

Table 1. Composition of the HBPE catalysts.

polymer	molecular formula
citric acid (C)/glycerol (G)/triethanolamine (T)	$C_{2.67}G_{3.42}T_1$
adipic acid (A)/glycerol (G)/triethanolamine (T)	$A_{1.71}G_{1.06}T_1$
methylsuccinic acid (M)/glycerol (G)/triethanolamine (T)	$M_{1.59}G_{1.69}T_1$

triethanolamine, while adipic and methylsuccinic acid polymers accelerate the Kemp reaction by factors of 2 and 3, respectively.

4. Discussion

In the origin of life field, extensive effort is dedicated towards identifying the prebiotically feasible formation of biomolecules—biopolymers in particular. Despite certain successes, the spontaneous formation of long peptides, polynucleic acids and polysaccharides remains elusive. Functionality of biopolymers strongly depends on their monomer unit sequencing in contemporary biology, which is achieved by specific enzymatic machinery and proofreading genetic mechanisms [31,32]. Such mechanisms are unlikely to have been perfected in nascent biology. Our model provides an alternative approach concentrating on functionality over structure. A number of model prebiotic experiments have generated tarry polymeric materials as major products. In addition to the studies by Fox and Blois described above, examples include the Miller–Urey experiment [33], HCN polymerization [34] and Maillard-type reactions [35]. Furthermore, intractable polymeric mixtures have been found in meteorites and are believed to be the source of the brown coloration of the Titan surfaces [36] as pictured by the Cassini–Huygens lander [37]. Structural studies of the above heterogeneous polymers have proved to be notoriously difficult. While our study concentrated on a simplified subset of such structures, we have demonstrated that disordered HBPEs possess catalytic activity.

Our results show that random short HBPEs with a wide distribution of molecular weights are capable of catalysing Kemp elimination in an aqueous solution. A base catalyst scaffolded within these HBPEs has shown higher efficiency towards the Kemp elimination reaction than that observed for the identical base in solution. The rate of Kemp elimination is further amplified in the presence of HBPEs possessing hydrophobic moieties. The citric acid/glycerol/triethanolamine HBPE has demonstrated only an insignificant Kemp elimination rate acceleration compared with monomeric triethanolamine; adipic acid- and methylsuccinic acid-containing catalysts nearly doubled and tripled the rate, respectively. Adipic acid and methylsuccinic acids contain an aliphatic chain segment and a side methyl group, which are, correspondingly, more conducive to the formation of hydrophobic pockets capable of modulating the environment of the Kemp elimination process. A more rigorous spectroscopic analysis is required to verify that the reaction is taking place in the pockets rather than on the polymer surface. These studies are currently underway in our laboratory.

The rate acceleration achieved in our experiment might seem marginal compared with the function of enzymes. Enzymes are composed of peptide strands counting hundreds of carefully sequenced monomers. The HBPEs used in our study are only up to seven or eight monomer units long. Furthermore, it is possible that only a small fraction of the HBPE mixture is catalytically active. HBPEs with the triethanolamine unit buried within the globular polymer structures are expected to be more active than the structures decorated with the triethanolamine amine unit on the periphery. In this study, only the HBPE mixtures were considered; no separation or purification was attempted. Our ongoing studies are considering a more targeted synthetic approach towards HPBE formation as well as possible synergistic effects of the catalyst mixture.

5. Conclusion

We have demonstrated that short disordered HBPEs are capable of forming hydrophobic pockets suited to providing a microenvironment in aqueous solution conducive to the facilitation of Kemp elimination. While the rate acceleration achieved by the disordered polymers is not on a par with enzymes, such polymers are more likely to have been present on prebiotic Earth than long-structured biomacromolecules. The notion that easily formed disordered, or 'messy', polymers could have performed the functions of biopolymers at the early stages and were later replaced with more efficient structures is an attractive area of study to explore in the context of chemical evolution. The exploration space involving the potential of HBPEs of a wide range of compositions and a wide range of reactions is enormous, and it appears reasonable to assume that within this space exciting surprises will be discovered.

Data accessibility. The supporting data for this article are in the electronic supplementary material.

Authors' contributions. I.M. and G.D.C. conceived of and designed the study and drafted the manuscript. I.M. carried out the experiments and performed the data analysis.

Competing interests. We declare we have no competing interests.

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