Artificial Enzymes

A Simple Protocol for the Modular Assembly of "Millipede" Artificial Enzymes[**]

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Successful strategies for the creation of effective artificial enzymes can be broadly classified into two contrasting approaches.[1] The first involves rational design of macromolecules that present a perfectly engineered array of catalytic functional groups.[2] The smallest miscalculation in orchestrating a tailored active site can however lead to disastrous results. The second approach,[3] which requires selection of a few catalytically active biomolecules from a large pool of randomly generated molecules can be seen in such areas as catalytic antibodies,[4,5] catalytic RNA molecules,[6] and the successful screening developments pioneered by Reetz and co-workers for in vitro evolution.[7] Whilst such selection processes often lead to active catalysts, retrospective structural analysis is then required to obtain a detailed understanding of a possible mode of action. Within this framework, the highly creative and original contribution of Menger and co-workers[8] in recognizing that automated combinatorial derivatization of polymers would produce large libraries of randomly functionalized polymers which could then be screened for catalytic activity is especially significant. Thus, for example, by attaching various combinations of carboxylic acids (some of which contained the well known catalytically active groups of enzymes) to a polyallylamine backbone it was possible to demonstrate significant phosphatase catalytic activity in the presence of a metal ion within the resultant mixture. As emphasized by Menger and co-workers, the goal of this approach, as in many selection systems, is directed towards a polymeric system that serves a useful function, even though any given "combinatorial polymer" is composed of an inseparable mixture of polymeric variations whose structure is impossible to determine.

In light of the above situation, we have therefore sought to develop a simple protocol for the modular assembly of artificial enzymes which combines the positive elements of both rational design and selection, and hence, through programmed variation, provides an exploratory mechanism-based framework for catalyst discovery. The essence of our approach was to concentrate on the most simple but dynamic view of an enzyme as a molecule which can both "hold" and "bite" and also possesses the flexibility to achieve the operation of bringing the "hands" (substrate binding) to the "mouth" (catalytically active group). The idea of attaching nucleophilic groups for solvolysis to a polymeric backbone and thereby creating an artificial enzyme is of course a well-established approach[9] and is also implicit in the aforementioned combinatorial polymers prepared by Menger and co-workers.[8] We reasoned however that the installation of an additional designed and selected unit, whose primary function is to act as a binding region with the role of transition-state stabilization, should considerably enhance the catalytic activity in such polymeric systems. The resultant design in Figure 1 therefore features a polymeric backbone to which separate strands possessing either a designed receptor site based on a transition-state analogue or a catalytically active group are randomly attached. Although this distribution of binding sites and catalytically active groups means that the precise tertiary structure which will evolve is unknown, we considered that the resultant polymer, for which we have coined the name "millipede", would confer a statistical advantage in terms of developing effective regions for catalysis. Thus, any given receptor site could be surrounded by several proximal catalytic groups potentially capable of adopting the necessary stereoelectronic trajectory for attack on the bound substrate (Figure 1).

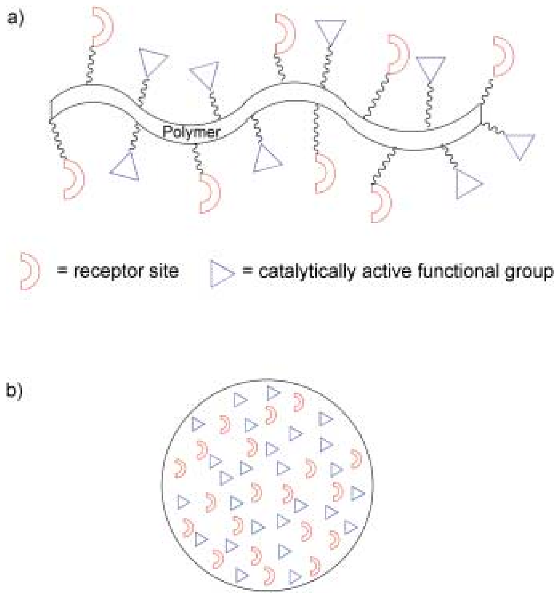

Figure 1. A schematic view of the modular approach to the synthesis of flexible artificial enzymes. a) Linear view: receptor sites and potential catalytic groups are grafted onto a polymer backbone at random intervals. b) Aerial view: each receptor site is surrounded by several catalytic groups, and once a substrate is bound, any one of these may be in the correct position to attack the substrate.

To illustrate this concept, we chose to study the hydrolysis of ester **1** (Scheme 1), with the classical phosphonate ester **4**

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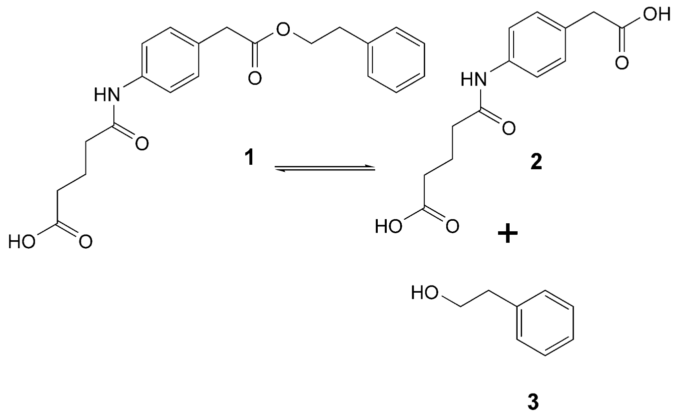
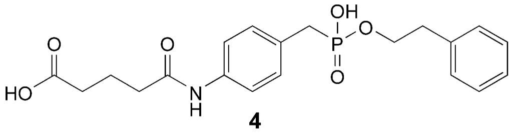


Scheme 1. The hydrolysis reaction chosen for study.

selected as a suitable transition-state analogue (TSA). These compounds were prepared by standard synthetic sequences.



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The most important objective in this approach was to select and prepare a complementary binding unit for phosphonate **4**. Initially, to facilitate this process, **4** was chosen to contain two acidic residues, the carboxylic acid, and the phosphonic acid, together with an additional amide bond all on the acid side of the ester bond in the substrate. Consequently, a suitable binding unit would be bidentate and contain either basic or hydroxy groups at each end capable of binding to the two acidic groups. Dipeptides with hydrogen bonding potential were of course the natural choice. With these thoughts in mind, nine commercially available dipeptides were selected for use in binding studies with phosphonate **4**.

The affinity of the dipeptides for the TSA **4** was then studied by measuring the change in diffusion coefficient (D) upon complexation by using the pulsed field gradient NMR BPPLED technique.[10] The translational diffusion coefficient of a molecule in solution is molecular mass dependent, and on complexation with a host molecule a decrease in diffusion coefficient is observed.[11]

The translational diffusion coefficients of the nine dipeptides and the TSA **4** were measured in D_2O at pD7.[12] The value of D was measured both in free solution, and in mixtures containing one peptide and the TSA in a ratio of 3 mM:30 mM. The diffusion coefficient of free TSA was measured to be $3.38\times10^{-10}\,m^2s^{-1}$. In the presence of the peptides, this value was not expected to change significantly, since even if a stable complex formed, only 10% of the TSA molecules could be involved in binding at any one time. Hence it was the observed change in D for the dipeptides that was of interest.

As revealed in Table 1, the basic dipeptide, Arg–Arg clearly displayed the most significant change in D as might be anticipated from the well-established interaction between guanidine groups and carboxylic acids.[13] The binding constant (K_a) for the interaction between Arg–Arg and the TSA was calculated to be $86\,L\,M^{-1}$ by measuring D at varying concentrations, and using a curve-fitting procedure.[11a,14] This value was more than acceptable to us since hydrogen bonding between two small molecules in water would be interrupted by solvent molecules. Molecular modeling was then undertaken to verify whether the geometries of preferred conformations for both the TSA and Arg–Arg were suitable for a bidentate interaction. As envisaged, the lowest energy complexes showed each guanidine unit of the dipeptide interacting, one with the phosphonate and the other with the carboxylic acid moieties of the TSA. There was no evidence for amide hydrogen bond association. Even though it does not necessarily follow that the dipeptide with the strongest binding interaction with the TSA **4** will provide the most effective unit for catalysis, the ability to rank this affinity using the above NMR protocol[12] provides a valuable method for ordered selection.

Table 1: Observed diffusion coefficients of dipeptides in D_2O at 298 K: D for 3 mM solutions of dipeptide and D' for mixtures containing dipeptide (3 mM) and TSA (30 mM).

Entry	Dipeptide	D [$\times10^{-10}$ m^{2}s^{-1}]	D' [$\times10^{-10}$ m^{2}s^{-1}]	R_d [%][a]
1	H-Arg-Arg-OH	4.10	3.40	17
2	H-Ala-Arg-OH	4.76	4.18	12
3	H-βAla-Lys-OH	4.91	4.39	11
4	H-βAla-His-OH	5.18	4.71	9
5	H-Ser-His-OH	4.89	4.46	9
6	H-Gly-Tyr-OH	4.97	4.64	7
7	H-βAla-Leu-OH	4.97	4.62	7
8	H-Gly-Thr-OH	5.54	5.19	6
9	H-Ala-Gly-OH	6.08	5.72	6

[a] R_d reflects the relative change in diffusion coefficient ($100(D-D')/D$).

With a suitable "receptor unit" in hand, we then had to consider how to incorporate this fragment into a polymer backbone, and also to select a catalytically active group for concomitant incorporation. Based on nature's principles, the obvious choice was to use an amino acid, such as histidine, cysteine, lysine, tyrosine, or serine, which have such a role in the natural proteases.[15]

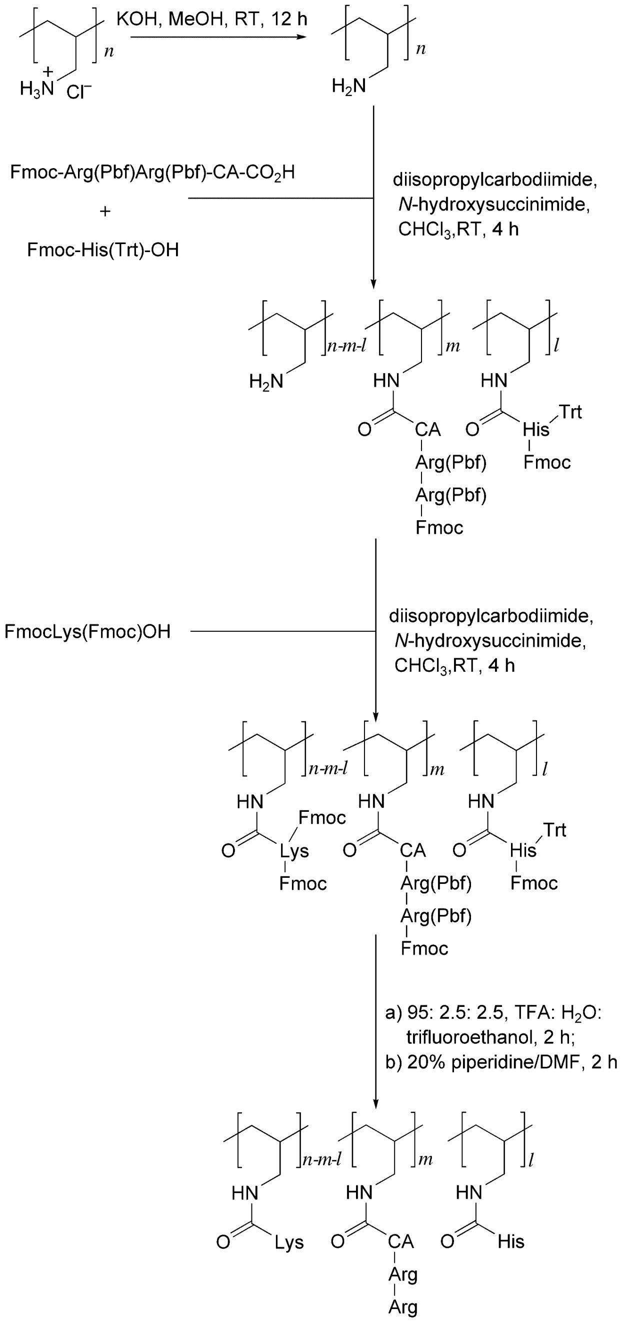
Preliminary studies reported here focus on the use of histidine. As in the combinatorial polymers described by Menger and co-workers,[8] a polyallylamine thread was chosen for the polymer backbone since it was flexible, soluble, and the free amino groups provided an easy method for incorporation of the binding and catalytic moieties into the polymer by standard peptide coupling procedures. It was also decided at this point to lengthen the leg of the millipede by using 6-amino caproic acid as a linker between the polymer and the dipeptides such that the conformational freedom of the binding unit would not be restricted by being placed too close to the polymer backbone. The required protected tripeptide

6-(Fmoc-Arg(Pbf)-Arg(Pbf)-)amido caproic acid was prepared by solid-phase synthesis.

Polymer **5** was then assembled in sequential fashion as shown in Scheme 2. Polyallylamine hydrochloride was converted to the free base by stirring with KOH in methanol.[16] One third of the amino sites were functionalized with 6-(Fmoc-Arg(Pbf)-Arg(Pbf)-)amido caproic acids, and one third with Fmoc-His(Trt)-OH by coupling with diisopropylcarbodiimide and *N*-hydroxysuccinimide. Both of these units were introduced to the polymer at the same time to encourage the desired statistically random distribution. All of the unfunctionalized amino groups on the polymer were then capped with lysine residues. In principle, this third type of leg in the millipede can be chosen to create a more hydrophilic or hydrophobic environment as desired. In addition to the fully armed polymer, three further polymers were also prepared, one containing lysine and the "binding unit" (2:1) **6**, another lysine and the catalytic group (2:1) **7**, with the third being the blank polymer **8** with only lysine residues. In this way the significance of each component can be identified and hence cooperativity in catalysis can be examined. Deprotection of acid-labile groups with 95% trifluoroacetic acid (TFA), followed by base deprotection of the Fmoc groups afforded the fully deprotected polymers in free base form.

Hydrolysis reactions were then performed by adding 15 mg of the appropriate polymer to a 0.5 mM solution of ester **1** in aqueous phosphate buffer at pH 7.[*] The changes in the concentration of both the ester substrate and the acid product **2** were monitored by HPLC.

In blank reactions containing either no polymer, or the blank lysine polymer **8**, no hydrolysis whatsoever was observed. The preliminary kinetic data for formation of the free acid **2** from ester **1** in the presence of polymers **5**–**7** are displayed in Figure 2. The initial relative rates for product formation were calculated to be 1:90:890 for polymers **7**:**6**:**5**, respectively. The fact that polymer **7** embodying the imida-



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Scheme 2. Synthesis of catalytic polymers (CA = $-(CH_2)_5NH-$).

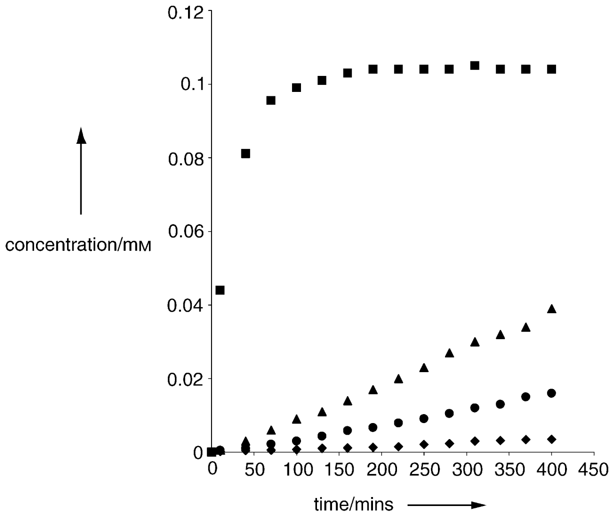

Figure 2. Graph showing rate of formation of product **2** using polymers **5** (■), **6** (▲), and **7** (◆). Also shown (●) is a competition experiment for polymer **6**, when the hydrolysis was performed in the presence of an equimolar quantity of TSA **4**.

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[*] As shown by our diffusion NMR measurements,[12] significant self-association effects are observed for the ester analogue **4** on increasing its concentration. Similar concentration-dependent self-association is also expected for ester **1**, hence a dilute 0.5 mM solution of **3** was used in all our measurements and no variation of substrate concentration was attempted when comparing catalytic properties of polymers **5**–**8** in this study.

zole group alone can function as a catalyst is not surprising since, as we have emphasized, polymer-supported nucleophilic catalysts of this type are known.[9] At first sight, polymer **6** containing the receptor site should function only by binding of the tetrahedral intermediate for ester hydrolysis. However, within the environs of the polymer, positively charged guanidine groups may induce a higher local concentration of hydroxide anions, and this may contribute to the observed rate enhancement and help to explain why the "hands" are more effective than the "teeth". The behavior of polymer **5** clearly indicates that the whole is greater than the sum of the parts, and that, irrespective of mechanistic detail, the initial premise of designing a flexible polymer with separately selected binding regions and catalytic groups offers a valuable strategic approach.

Interestingly polymer **5** appears to demonstrate the often-encountered phenomenon of product inhibition, as implied by the saturation kinetics curve displayed in Figure 2. Such a result can be anticipated since the product diacid is produced in close proximity to the bidentate and highly basic Arg–Arg site. The fact that even with such a challenging substrate, a 25 % conversion to product was achieved in two hours using only 5 mol % of the polymer was a very encouraging observation. We have also verified the binding role of the Arg–Arg unit in polymer **6** using the phosphonate TSA **4**. The change in the diffusion coefficient of the TSA upon binding to the polymer was found to be significantly reduced by 80 % relative to the value of D found in free solution. Using these values K_a was estimated to be $\geq 1500\,\text{L}\,\text{M}^{-1}$, indicating the strong affinity of **4** for polymer **6**. This measurement encouraged us to demonstrate the phenomenon of competitive inhibition of enzyme action. Thus, when the hydrolysis of ester **1** by polymer **6** was attempted in the presence of an equimolar amount of the TSA **4**, the rate of hydrolysis decreased by a factor of 2. A further feature of interest is that, in contrast to combinatorial polymers[8] which can display an induction period of up to 24 h while the polymer "learns to recognize the substrate", polymer **5** did not exhibit this phenomenon. This observation therefore provides additional circumstantial evidence for the effectiveness of binding site incorporation.

In summary, the foregoing study has indicated that this modular "step-by-step" strategy, which combines both design and selection elements for the attachment of separately considered receptor sites and catalytically active groups to a flexible polymer backbone, can be used to construct an artificial enzyme. The method outlined is clearly amenable to a parallel synthesis approach for optimization and can also provide, at every step along the way, detailed information about the relative importance and cooperativity of the groups contributing to the molecular environment of active sites within enzymes.

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