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A New Backbone of Artificial Enzymes Obtained by Cross-linkage of Poly(ethylenimine)

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Abstract: Cross-linkage of the branches of poly(ethylenimine) (PEI) suppresses flexibility of the polymer as revealed by decreased affinity of the amino groups on PEI backbone towards proton or Ni(II) ion. The cross-linkage improves ability of the PEI derivative equipped with β-cyclodextrin to deacylate an ester containing *t*-butylphenyl moiety.

For reproduction of characteristics of enzymatic action such as complex formation with substrates, large rate acceleration, and high selectivity, it is highly desirable to build active sites. To design active sites of artificial enzymes, several catalytic elements are to be placed in proximity and aligned in a productive way. Then, the substrate bound to the active site can interact with the catalytic elements, leading to stabilization of the transition state. Design of such artificial active sites with small molecules would be very difficult. As nature chose polypeptide as the backbone of the active sites, macromolecules have been employed as the skeleton of artificial enzymes. For example, catalytic antibodies use immunoglobulins as the skeleton.[1] In the area of synzymes[2] (synthetic polymers with enzyme-like activities) and molecular imprinting,[3] artificial enzymes are built with synthetic macromolecules.

To design effective synzymes, easy attachment of catalytic groups to the backbone and globular conformation of the polymer are desirable. In addition, water-solubility of the polymer derivatives is also needed to obtain homogeneous synzymes. Branched poly(ethylenimine) (PEI)[2] meets these criteria and has been used as backbones of various synzymes. The PEI used for synzymes has a molecular weight of ca. 60000, containing ca. 1400 monomeric ethylamine residues. About 25 % of the amino groups are primary, about 50 % are secondary, and about 25 % are tertiary. The tertiary amino groups represent the branching points on the polymer skeleton. Thus, PEI is highly branched.

One of the major obstacles to overcome in the design of effective synzymes has been the lack of specific binding sites. As specific binding sites on PEI, we constructed macrocyclic metal centers on PEI which recognized aromatic carboxylates.[4] In addition, we attached β-cyclodextrin (CD) to PEI.[5] In the resulting PEI derivative (CD-PEI), the CD cavity acted as a binding site for hydrophobic moieties.

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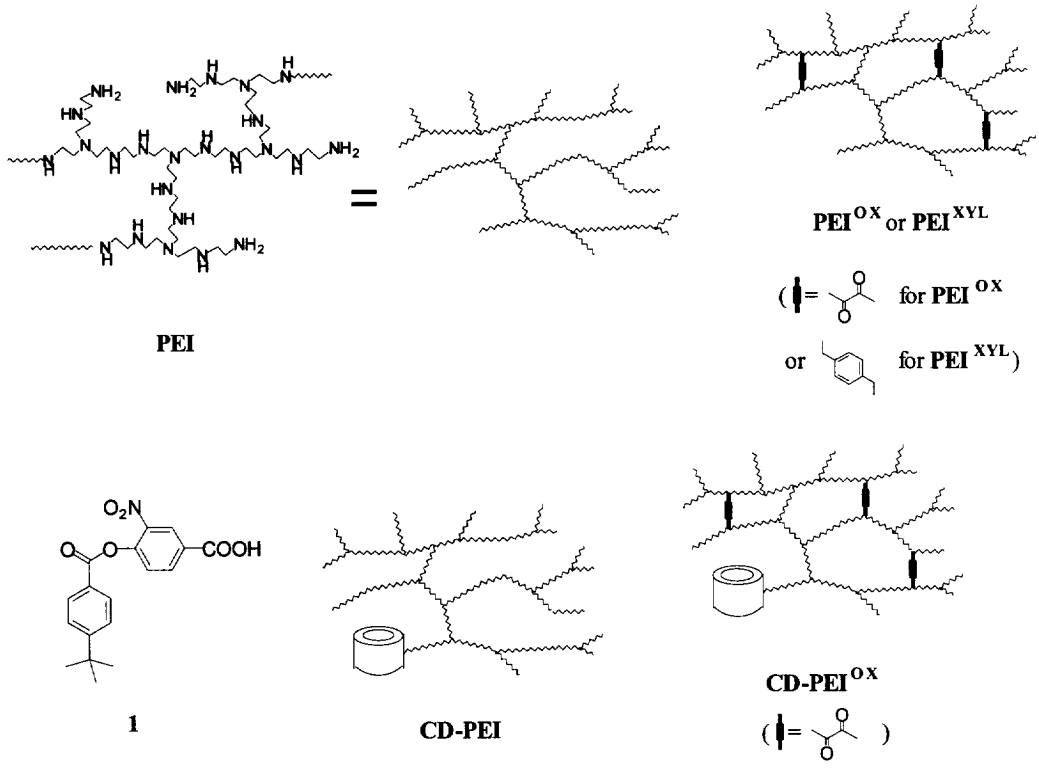

Although PEI has a highly branched structure, its conformation is still flexible enough to relieve structural strains introduced to the polymer backbone.[6] When hydrophobic groups such as lauryl chain are attached to PEI, for example, they form aggregates to minimize exposure to water, leading to the formation of hydrophobic clusters on the polymer.[7] The hydrophobic microdomains attract hydrophobic substrates and, sometimes, stabilize transition states,[8] enhancing the catalytic ability of PEI derivatives.

The flexibility of PEI does not always exert favorable effects on effectiveness of synzymes built on PEI. Even when several catalytic elements are introduced to proximal positions on PEI, for example, conformational changes of the resulting PEI derivative can move the catalytic elements away from one another, hampering collaboration among the catalytic elements. In an effort to develop a method to suppress conformational flexibility of PEI, we have cross-linked PEI with bis(*o*-nitrophenyl) oxalate to obtain PEI^{OX} or with α,α'-dibromo-*p*-xylene to obtain PEI^{XYL} in dimethyl sulfoxide at 25°C. Near quantitative reaction of PEI (8.63 residue mmol) with bis(*o*-nitrophenyl) oxalate (0.265 mmol) in 100 ml dimethyl sulfoxide was confirmed by spectrophotometric measurement of the amount of *o*-nitrophenol released during the reaction whereas that of PEI (26.4 residue mmol) with α,α'-dibromo-*p*-xylene (9.50 mmol) was confirmed by NMR analysis of PEI^{XYL}. Thus, the contents of the cross-linking agents in PEI^{OX} and PEI^{XYL} are 3.1 and 3.6 reside mol %, respectively. PEI^{OX} and PEI^{XYL} were purified by repetitive dialysis.

In Fig. 1, the degrees of protonation of the amino groups at various pHs are compared for PEI, PEI^{OX}, and PEI^{XYL} as well as the analogue of PEI^{XYL} where the xylyl group is introduced by single attachment. The amino groups of PEI^{OX} and PEI^{XYL} resist protonation more strongly than those of PEI. This may be taken to indicate that PEI can change its conformation more readily than PEI^{OX} or PEI^{XYL} to relieve unfavorable

electrostatic interactions between two adjacent ammonium cations. Although no spectrophotometric evidence was obtained for double attachment of the cross-linking agents to PEI, the distinctly different behavior of PEI derivatives containing xylyl groups indicated by curve b and curve d of Fig. 1 supports the double attachment.[9]

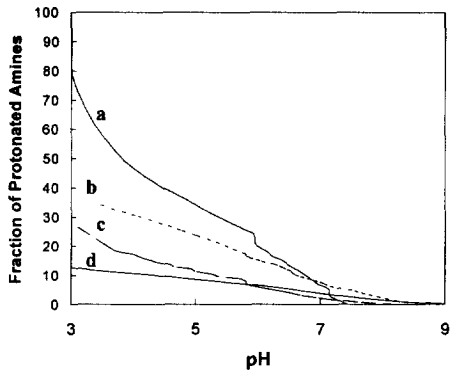

Fig. 1. Fraction of protonated amines for PEI (a), PEI derivative obtained by attachment of α-bromo-xylene (3.0 residue mol %) (b), PEI^{OX} (c), and PEI^{XYL} (d) at various pHs and 25°C.

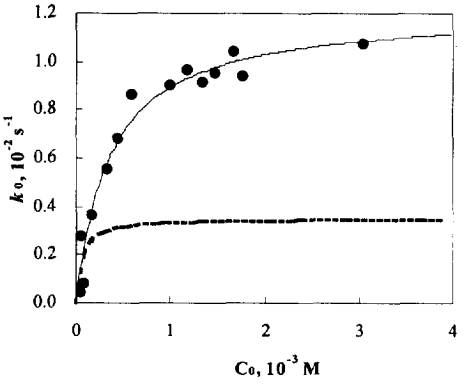

Fig. 2. Plot of k_o (•) against C_o for deacylation of **1** by CD-PEI^{OX} at pH 7.65 and 25°C. Dotted line represents the rate data obtained previously with CD-PEI and **1**.[5a] The rate for deacylation of **1** with CD or PEI was much slower than that with CD-PEI.[5a]

Formation constants (K_f) for the Ni(II) complexes formed on the skeletons of PEI, PEI^{OX}, and PEI^{XYL} (total concentration of Ni(II) ion, 0.121 mM; concentrations of the PEI derivatives, 4.00 residue mM) were measured by a method described previously.[6,10] At pH 7.02 and 25°C, log K_f of 9.78 ± 0.03 and 7.50 ± 0.14 were estimated for PEI^{OX} and PEI^{XYL}, respectively, whereas log K_f of 13.37 ± 0.05 had been reported for PEI[6] previously. The greater K_f for PEI again reflects the higher flexibility of the ethylenediamine moieties of PEI leading to easier participation of more amino groups in Ni(II) binding. The results of Ni(II) binding study as well as those summarized in Fig. 1 demonstrate considerable suppression of conformational flexibility of PEI by cross-linkage of the chains within the PEI molecule.

To check whether the suppressed flexibility of PEI improves the applicability of PEI as backbones of artificial enzymes, CD was attached to PEI^{OX} to obtain CD-PEI^{OX} by the procedure reported previously[5a] for synthesis of CD-PEI. The content of CD in CD-PEI^{OX} was 0.86 residue mol %. Kinetic data for deacylation of **1** in the presence of CD-PEI^{OX} were measured at 25°C and pH 7.65. Under the conditions of C_o (initially added concentration of CD-PEI^{OX} expressed in terms of the concentration of CD moiety) $\simeq$ [*C*], pseudo-first order kinetic behavior was observed. The kinetic data obtained at various C_o are illustrated in Fig. 2. Increase in activity of CD-PEI upon cross-linkage is evident from the curves of Fig. 2. Analysis of the kinetic data according to eq 1 led to the values of k_{cat} = (1.21 ± 0.07) × 10^{-2} s^{-1} and K_m = (3.49 ± 0.76) × 10^{-4} M for CD-PEI^{OX}.

$$k_o = k_{cat}C_o/(C_o + K_m) \quad (1)$$

Kinetic data obtained previously for deacylation of **1** in the presence of CD-PEI led to k_{cat} = (3.53 ± 0.08) × 10^{-3} s^{-1} and K_m = (6.00 ± 0.88) × 10^{-5} M.[5a] As indicated previously with deacylation of **1** by CD-PEI,

the amino group of CD-PEI^{OX} located above the CD cavity would attack the ester linkage of **1** complexed to CD-PEI^{OX}. Comparison of the kinetic parameters for CD-PEI^{OX} with those for CD-PEI reveals that binding of **1** is weaker for CD-PEI^{OX} as judged by the magnitude of $1/K_m$ whereas attack by amino group at the bound substrate is considerably more effective for CD-PEI^{OX} as judged by the magnitude of k_{cat}. The stronger binding by CD-PEI is attributable to the greater flexibility of CD-PEI. This would allow easier adjustment of conformation of the complex formed between the substrate and the polymer leading to relief of steric congestion caused by the complexation. On the other hand, the conformational adjustment may reduce the effective concentration of the nucleophilic amino group of the PEI backbone towards the carbonyl group of the bound ester resulting in the reduction of k_{cat}.

The transition state of the deacylation of **1** is recognized by CD-PEI^{OX} better than by CD-PEI as reflected by k_{cat} whereas the reactant is recognized better by CD-PEI than by CD-PEI^{OX} as reflected by K_m. Since effective recognition of the substrate and more effective recognition of transition states[4b,11]-are the major objective in the area of artificial enzymes, cross-linkage of PEI can improve the utility of PEI as the backbone of artificial enzymes.

Cross-linkage of PEI suppresses conformational flexibility of the polymer. The enhanced rigidity of the polymer skeleton may allow better conservation of the geometry of artificial active sites constructed on the polymer. Design of artificial active sites comprising several catalytic elements on the cross-linked PEI is currently carried out in this laboratory.

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