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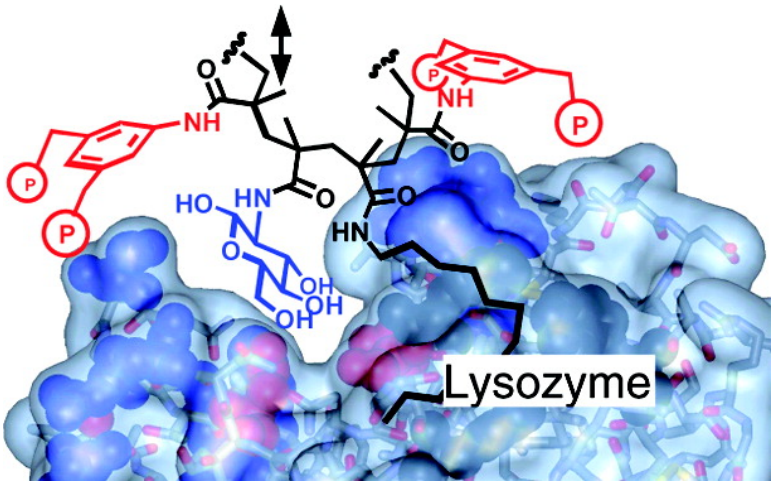


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# A Noncovalent Switch for Lysozyme

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***Abstract:*** A new concept for the external control of protein activity is presented and demonstrated on the example of an artificial Lysozyme switch. Radical copolymerization of selected methacrylamide-based comonomer units tailored for amino acid residues surrounding the active site furnishes polymeric protein hosts that are able to inhibit enzymatic activity in a highly efficient dose-dependent manner ($IC_{50} \approx 1.0$ equiv $\approx 0.7$ $\mu$M). All binding site types on the polymer work cooperatively, using electrostatic attraction, hydrophobic forces, and substrate mimicry. In a native gel electrophoresis, the well-defined 2:1 complex (polymer/protein) migrates to the anode. Even at 250 mM NaCl, a 10-fold polymer excess is able to shut down bacterial cell wall degradation completely. A kinetic investigation points to a competitive mechanism (Lineweaver−Burk plots). CD spectra of pure Lysozyme and its polymer complex are indistinguishable; together with a total lack of preincubation time for maximum inhibition, experimental evidence is thus produced for a preserved tertiary enzyme structure—no denaturation occurs. Addition of the superior complexing agent polyarginine to the enzyme-polymer complex mildly detaches the inhibitor from the protein surface and leads to 90% recovery of enzymatic activity. Thus, Lysozyme could be turned off, on, and off again by consecutive addition of the polymeric inhibitor, polyarginine, and polymer again.

## Introduction

Natural protein regulation is a complex issue—in general, protein function can be turned on and off by several factors. Agonists or antagonists stimulate or attenuate receptors,[1] whereas activators and deactivators are used for allosteric regulation of proteins.[2] Direct enzyme inhibition is brought about by various mechanisms, such as competitive, uncompetitive, noncompetitive, and mixed inhibition.[3] Medicinal chemistry has in the past years extensively exploited these mechanisms to downregulate problematic pathologic protein functions.[4] However, most of these efforts remain restricted to the active site and are often nonreversible such as the so-called suicide inhibitors.[5]

To turn on and off protein function reversibly, allosteric effectors induce a conformational change in the enzyme, which modulates its substrate affinity.[6] As an example, ACTase is down- or upregulated by ATP or CTP, respectively, which bind to a regulatory subunit and thereby shift the catalytic subunit from the inactive T-state to the reactive R-state and vice versa.[7] Alternatively, a reversible covalent modification via phosphorylation takes over the regulation of enzyme activity.[8] Even DNA metabolism can be activated by distant sequences *in trans* and suppressed by polyamines such as spermidine.[9] In recent

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years, chemists have designed various agents that are able to bind to protein surfaces and interfere with protein function.[10]

Very few examples, however, exist for the external addition of artificial compound pairs that are able to turn off and on protein function in a predictable manner. Site-directed mutagenesis has been used to introduce metal binding sites; thus, an artificial cysteine in the hydrophobic binding pocket of staphylococcal nuclease permits external inactivation of the mutant by mercuric or cupric salts and subsequent reactivation by chelating agents.[11] Bioconjugates involving covalent attachment of smart polymers just outside the active site represent another elegant way to control protein activity. Employing azobenzene−NIPAM copolymers on an endoglucanase 12A mutant allowed photoinduced switching between a compact hydrophobic state with a closed active site and an extended soluble state, providing free access to the active site.[12] Similarly, amphiphilic polymer scaffolds have been described, which nonspecifically bind to chymotrypsin, inhibit its peptidase activity, and modulate substrate specificity; very high ionic strenghts again release the protein from the polymer.[13] Recently, synthetic gatekeepers were shown to control the proteolytic activity of the 20S proteasome complex; to this end, a multivalent chelator head with four NTA moieties was used to cross-link His tags introduced at the N-terminal tails of the α subunits around the two openings. Subsequent imidazole addition reactivated the blocked proteasomes.[14]

Our group has pursued another concept, that is, noncovalent protein recognition with designed water-soluble polymers, which carry binding sites for all classes of amino acid residues.[15] Statistic radical copolymerization of methacrylamide-based comonomers thus produced several generations of copolymers, which were optimized for maximum affinity toward one single target enzyme. Thus, Pepsin, Trypsin, and Lysozyme can now be complexed by their respective complementary copolymer in the low nanomolar $K_D$ range (10−90 nM).[16] As a working model, we assume that the linear copolymer undergoes an extensive induced-fit procedure across the protein surface and thus optimizes in a dynamic process its noncovalent contacts to the highest possible number of solvent-exposed residues. If many of these are located near the active site, enzymatic activity will be significantly reduced, because substrate access is hindered.[17] Alternatively, strong noncovalent binding to a protein surface may result in denaturation which will also lead to a lowered enzymatic activity.[18] Since this inhibitory effect relies on weak noncovalent forces, it should, in principle, be reversible.

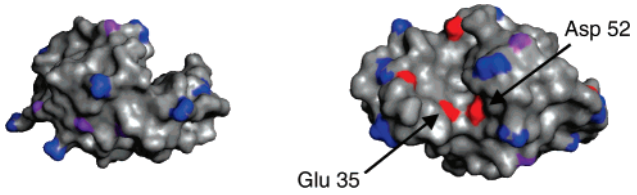


***Figure 1.*** Lysozyme molecule depicted with its Connolly surface as projected from the side and from the top (PyMOL v0.98). Arginine residues are blue, lysines are purple. The catalytically active Asp52 and Glu35 in red are responsible for glycoside cleavage inside the cleft.

Hence, addition of a more powerful complexing agent for the polymer should mildly detach the inhibitor from the protein surface and protein activity should be restored. If polymer recognition is accompanied by an irreversible denaturation, however, the enzyme will not be able to fully recover its original power and the final reaction rate will be lower than in the native state.

We tested this hypothesis with a copolymer that was tailored for Lysozyme recognition (Figure 1). It contains bisphosphonate dianions as molecular tweezers for arginine and lysine, unpolar dodecyl tails for aliphatic and aromatic amino acids, as well as glucosamine moieties, which serve a dual purpose: on one hand, they render the whole polymer, and hopefully also its protein complexes, highly water-soluble; on the other hand, they imitate the enzyme's natural substrate, that is, bacterial cell walls, consisting of hexameric saccharide units. Specifically, Lysozyme cleaves the glycosidic C−O bond between *N*-acetylglucosamine and *N*-acetylmuraminic acid moieties (NAG−NAM).[19] By fluorescence titrations, a 25 nM affinity was determined for copolymer **1**, with an approximate 2:1 stoichiometry (polymer/enzyme).[15]

## Results and Discussion

A standard assay for Lysozyme contains the bacterial substrate "Micrococcus Lysodeicticus"[20] as well as 50 mM phosphate buffer. The bacterial degradation is observed by the decreasing optical density (OD) of the Micrococcus suspension at 450 nm; no UV absorption occurs within the polymer at this wavelength. Enzyme kinetics were determined with a fixed enzyme concentration (1 μM), treated without preincubation with increasing amounts of polymer **1**. Intriguingly, 2 equiv of the polymer sufficed for a total and instant enzyme shut down; the corresponding $IC_{50}$ value is 1.0 equiv (~0.7 μM, Figure 2). SDS-PAGE was used to ensure that, in all cases, the enzyme did not precipitate but was still fully present in solution. Thus, noncovalent attachment of polymer **1** to Lysozyme inhibits its enzymatic activity in a highly efficient, dose-dependent manner. In their elegant work, Kulkarni et al. prepared NIPAM-based copolymers for Lysozyme recovery by affinity thermoprecipitation.[21] These contained multiple acetamido groups in a hydrophilic environment for maximum interaction with the catalytic cleft. High affinities were achieved, although solubility limitations of the polymers prevented to reach full enzyme inhibition, especially with elevated salt loads above 50 mM NaCl (*vide infra*).

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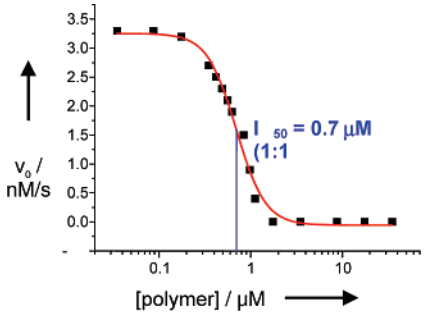


***Figure 2.*** Lysozyme activity determined for 0.7 $\mu$M enzyme treated with increasing amounts of polymer **1**. The polymer conentration at 50% maximum activity indicates the $IC_{50}$ value.

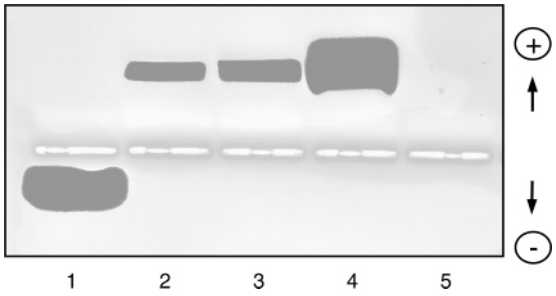


***Figure 3.*** Nondenaturing gel electrophoresis on agarose showing the pure basic (fluorescent) protein being attracted by the cathode, whereas the oppositely charged protein/polymer complex migrates toward the anode.

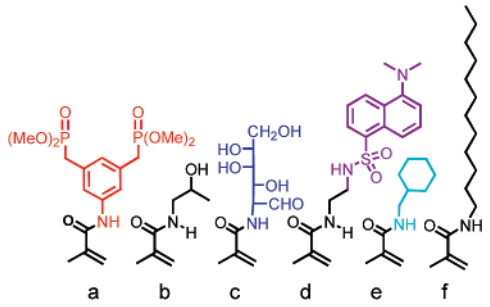


***Figure 4.*** Methacrylamide-based comonomers: (a) bisphosphonate (BP), (b) aminoalcohol (AA), (c) aminosugar, (d) dansyl, (e) cyclohexyl, and (f) dodecyl.

The protein−polymer complex could also be visualized by nondenaturing gel electrophoresis (Figure 3). On agarose, the basic protein migrated toward the cathode, and even the 1:1 complex carried a negative charge excess and was hence transported to the anode. At a 20:1 polymer/protein ratio, the complex spot became darker but did not move any further. Thus, a well-defined aggregate was formed by electrostatic attraction between polymer **1** and Lysozyme in which the bisphosphonate moieties effectively chelated and thereby neutralized lysines and arginines around the active site.

Variation of the comonomer composition within the polymeric enzyme binders revealed the influence of each type of binding site on the inhibition efficiency. Polymers **1**−**6** were examined in parallel experiments with reference to their Lysozyme affinity and inhibition ability (Figure 4). Interestingly, both properties are strongly correlated, as one would assume from the concept of protein surface capping (Table 1). Reduction of the bisphosphonate content in **4** markedly decreases affinity as well as deceleration of bacterial degradation; polymer **3**, lacking all bisphosphonates, does not recognize nor inhibit Lysozyme at all. Clearly, electrostatic attraction dominates

***Table 1.*** Polymer Compositions and Their Corresponding $K_D$ and $IC_{50}$ Values[a]

| | polymers | $K_D$ | ~equiv polymer/ lysozyme | $IC_{50}/\mu M$ |
|---|---|---|---|---|
| **1** | 3 BP, 1 sugar, 1 dodecyl | 30 nM | 1:1 | 0.7 |
| **2** | 3 BP, 1 AA, 1 dodecyl | 50 nM | 9:1 | 6 |
| **3** | 1 sugar | >10 mM | >1000:1 | >1000 |
| **4** | 1 BP, 1 sugar, 1 dodecyl | n.d. | 10:1 | 7 |
| **5** | 3 BP, 1 sugar, 1 cyclohexyl | 1 mM | 27:1 | 19 |
| **6** | 3 BP, 1 sugar, 1 dansyl | 200 nM | 8:1 | 6 |

[a] All experiments were conducted at 0.7 $\mu$M Lysozyme in 50 mM phosphate buffer at pH 7.0. Note the close correlation between protein affinity ($K_D$) and inhibitory power ($IC_{50}$).

substrate as well as inhibitor recognition. Exchange of the dodecyl tail for other hydrophobic moieties such as dansyl **6** or cyclohexyl **5** moderately or significantly lowers protein affinity and likewise leads to reduced inhibition in the same measure. We attribute this difference to the capability of the extended dodecyl alkyl chain to reach out to unpolar amino acid residues in the vicinity of arginines and lysines on the protein surface and engage in hydrophobic and van-der-Waals interactions. Finally, it might be argued that the whole blocking effect originates from the aminosugars imitating the natural saccharide substrate inside the deep cleft. This might indeed contribute to the inhibition mechanism, because polymer **2** with short alcohols instead of sugars binds equally well to Lysozyme but retards enzymatic acticity much less efficiently as polymer **1**. However, a pure sugar carrier such as **3** has no protein affinity nor any influence on enzyme activity. We conclude that each moiety (bisphosphonate, dodecyl, and sugar) contributes to the protein affinity and inhibition capacity of the polymer. Another experiment supports the above-detailed considerations: if the salt content is systematically increased beyond physiological limits (up to 250 mM NaCl), $IC_{50}$ values also rise from 1 to 6 equiv (i.e., 1−4 $\mu$M), underlining the importance of Coulomb attractions. On the other hand, even at this drastic salt load, a 10-fold polymer excess can totally shut down bacterial cell wall degradation.

If the enzyme's catalytic site was noncovalently blocked by the polymer, preventing substrate access, a situation was reached, which is called "competitive inhibition".[22] To verify this assumption, enzyme kinetics can be examined and evaluated in the form of a Lineweaver−Burk plot. If the inhibitor competes with the substrate, maximum enzyme velocity will only be reached at infinite substrate concentrations, indicated by two lines intersecting on the *y*-axis of the plot. This was indeed the case with our polymeric inhibitor: Addition of various polymer amounts to the enzyme solution resulted in proportional deceleration, until the substrate was present in large excess; at this point, the enzyme worked at exactly the same speed as in the native state (Figure 5).

To ensure that the tight grip of the oppositely charged polymer did not lead to a conformational change in the protein, we aquired CD spectra of the pure enzyme and mixtures thereof with various amounts of polymer. Figure 6 shows the normalized CD spectra, which have the same habitus and all show essentially the same curve, with minima at 207 and 230 nm.[23]

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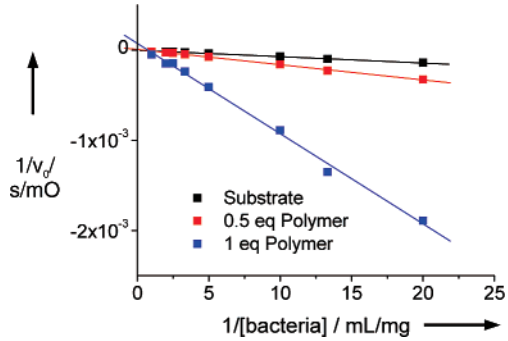


***Figure 5.*** Lineweaver−Burk plots for pure Lysozyme and its complex with 1 or 2 equiv of polymer **1**, displaying the competitive inhibition mode.

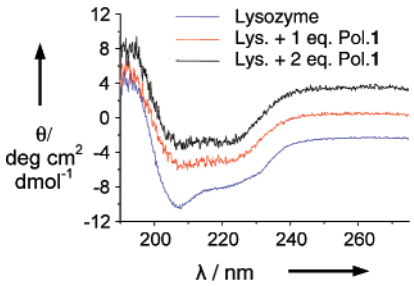


***Figure 6.*** CD spectra of pure Lysozyme and its complex with 1 and 2 equiv of polymer **1**.

Thus, experimental evidence is provided for the fact that Lysozyme's tertiary structure has not been distorted, so that denaturation cannot be responsible for the inhibitory effect of the polymer. This also perfectly agrees with the initially observed fact that inhibition acts instantaneously and does not require a preincubation period.[24]

A fully noncovalent enzyme inhibition offers the possibility of all equilibrium recognition processes: if one binding partner is withdrawn from the equilibrium, for example, by external addition of a more powerful inhibitor complexing agent, the other partner should be released from the host−guest complex.[25] In our case, enzymatic activity should become fully restored, if the bisphosphonate-containing polymer was mildly detached from the protein surface and bound to a perfectly complementary complexing agent of superior affinity. This was indeed accomplished with polyarginine; it detaches polymer **1** from the protein surface and forms an insoluble complex that precipitates quantitatively from buffered aqueous solution. Lysozyme was characterized over a period of 3 min in its bioactive state against *Micrococcus Lysodeicticus* by its characteristic OD decrease. Five equivalents of polymer **1** sufficed for a complete enzyme shut down; after 6 min, 20 equiv of polyarginine were added to this solution, and the enzyme started to work again, with 90% of its original efficiency. Finally, after another 3 min, 100 equiv of polymer **1** were added and the enzyme ceased to work. These four successive phases of the enzyme in its "on" and "off" state are presented in Figure 7. To the best of our knowledge, this is the first time that a natural enzyme is switched off and on again by externally added complexing reagents.

## Conclusion and Outlook

Copolymer **1**, based on methacrylamides, with building blocks tailored for molecular recognition of basic and unpolar amino acid residues as well as for substrate imitation was found to be a potent inhibitor of Lysozyme activity. The subsequent systematic variation of the comonomer content strongly indicated a proportional correlation between affinity and inhibitory potential. In this respect, the bisphosphonate, dodecyl, and sugar units are all contributing to the observed biological effect. Specifically, protein surface recognition by electrostatic attraction and hydrophobic effects is complemented by a substrate mimicry in form of an aminosugar. The protein−polymer complex was stable in the electric field and moved toward the anode as a well-defined negatively charged unit. Even salt loads well beyond physiological limits did not prevent enzyme inhibition with larger amounts of polymer (4−6-fold increase of $IC_{50}$ value). Kinetic experiments revealed a competitive behavior of our polymeric inhibitor. Importantly, no denaturation occurred even at high polymer excess, as documented by CD spectroscopy—this observation is supported by the total absence of a preincubation period. The whole process can be reverted by addition of polyarginine as a superior complexing agent for the bisphosphonate-containing polymer. Successive additions of polymer, polyarginine, and polymer switched enzymatic activity off, on, and off again, with 90% recovery of the enzyme's original activity.

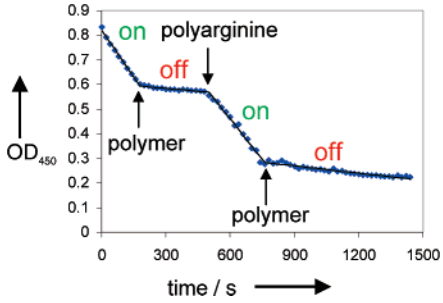


***Figure 7.*** Enzyme switching by successive addition of polymer, polyarginine, and polymer again.

In the future, we will apply this concept of reversible enzyme switching to other proteins of biological and medicinal interest. To this end, a combinatorial optimization of the copolymer composition with respect to protein affinity and, most importantly, inhibitory potential is a *conditio sine qua non*. We are currently pursuing several routes to accomplish this goal.

## Experimental Section

**General Polymerization Procedure.** A solution containing a monomer combination selected from methacrylamide-based bisphosphonate, sugar, alkane, dansylamide as fluorescence label, as well as a catalytic amount of AIBN in DMF was degassed and stirred for 22−24 h at 60 °C. The reaction mixture was diluted with methanol to give a maximum concentration of 5% of the initial monomer mass and added dropwise to 10 times the volume of ethyl acetate. The solid was collected by filtration, washed with ethyl acetate, and dried *in vacuo*.

**Polymer-Analogous Cleavage of Methyl Phosphonates.** The respective polymer (1 equiv) was dissolved in dry acetonitrile (10 mL) under an argon atmosphere. Lithium bromide (2.2 equiv for each dimethylphosphonate group) was added from a stock solution (4.5% in acetonitrile). The reaction mixture was stirred under argon for 72 h at 90 °C. During this period, the polyanionic product precipitated as the poly-(lithiumphosphonate) salt. The solvent was decanted, and the yellowish solid was subsequently washed three times with acetonitrile. Spectroscopically pure product (60−70%) was obtained after filtration and drying *in vacuo*.

(24) Tagore, D. M. Sprinz, K. I.; Fletcher, S.; Jayawickramarajah, J.; Hamilton, A. D. *Angew. Chem.* **2007**, *119*, 227−229.
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**Fluorescence Titrations.** In a fluorimeter, the dansyl group of the polymers was excited at 330 nm, its emission monitored from 400 to 600 nm. All experiments were conducted in 10 mM sodium phosphate buffer (pH = 7.0) in a quartz cuvette. Lysozyme was diluted in the polymer solution (7 $\mu$M), so that there was no change in the overall polymer concentration during the entire titration. 700 $\mu$L of the polymer solution was placed in the cuvette and the Lysozyme solution was added stepwise. Emission intensity changes were recorded and used in a standard nonlinear regression algorithm to calculate the appropriate association constants. The related complex stoichiometries were determined by Job plots from the titration data and taken as the basis for the calculation of binding constants.

**Enzyme Activity Assay. (a) $IC_{50}$ Values.** All Lysozyme activity assays were performed in microplates in 50 mM potassium phosphate buffer (pH = 7.0) with [Lysozyme] = 7 $\mu$M, [Micrococcus Lysodeicticus] = 1 mg/mL, [polymer] = 0.35−350 $\mu$M; 70 $\mu$L of the bacteria suspension were added to a mixture of 10 $\mu$L Lysozyme, 10 $\mu$L polymer, and 10 $\mu$L buffer with a final Lysozyme concentration of 0.7 $\mu$M. The Lysozyme activity was observed by monitoring the OD change at 450 nm over a period of 10 min. From a [polymer]/$v_0$ plot (polymer concentration vs initial enzyme velocity), $IC_{50}$ values were determined at half-maximum $v_0$. **(b) Michaelis−Menten Kinetics.** For Michaelis−Menten kinetics the polymer concentrations were kept constant at 0.7 or 0.35 $\mu$M, respectively, whereas the bacteria concentration varied from 0 to 20 mg/mL. The inhibiton mode was determined from a Lineweaver−Burk plot, that is, 1/[substrate] vs 1/$v_0$.

**Enzyme Switching.** Lysozyme switching was performed by adding to a running enzymatic reaction polymer and polyarginine solutions in alternating order. Specifically, the above-described Lysozyme assay (70 $\mu$L bacteria suspension and 10 $\mu$L Lysozyme) was stopped after 3 min by external addition of a 35 $\mu$M polymer **1** solution (10 $\mu$L, 5 equiv). After another 6 min, a 140 $\mu$M poly-L-Arginine solution (10 $\mu$L, 20 equiv) was injected and the restored bacterial degradation was monitored for 3 min. Finally, the enzymatic reaction was again brought to a halt by addition of 100 equiv of polymer **1**.

**Gel Electrophoresis. (a) SDS PAGE:** The general protocol as delineated by Laemmli was followed with few modifications.[26] Samples for SDS-PAGE were prepared under identical conditions as enzyme activity assays (50 mM potassium phosphate buffer (pH 7.0), [Lysozyme] = 0.7 $\mu$M, [polymer] = 0.35−1.4 $\mu$M). The mixtures were centrifuged (5 min, 13000 rpm), and the sample buffer was added to the solution as well as to the pellet. Electrophoresis was carried out at 20 mA per gel until the bromophenol blue marker reached the bottom of the gel. The gels were stained with 0.25% Coomassie Blue, 50% methanol, 10% acetid acid aqueous solution and destained with 20% ethanol, 10% acetid acid aqueous solution. When bands were clearly visible, the gels were scanned on a flatbed scanner. (**b) Nondenaturing Gel Electrophoresis:** 1% Agarose gels for native gel electrophoresis were prepared in 5 mM potassium phosphate buffer (pH 7.0). Placing a comb in the middle of the gel rack was used to form wells for 30 $\mu$L samples. The samples contained 15 $\mu$L of polymer of varying concentrations (35−700 $\mu$M), 15 $\mu$L of Lysozyme (35 $\mu$M), and 3 $\mu$L of aqueous glycerol (80%). They were incubated for 1 h at ambient temperature and subsequently separated at 30 mA for 1 h. The gels were stained for 15 min with 0.5% Coomassie Blue, 40% methanol, 10% acetid acid aqueous solution, and destained with water overnight. When bands were clearly visible, the gels were scanned on a flatbed scanner.

**CD Spectroscopy.** CD experiments were conducted in a quartz cuvette in 10 mM potassium phosphate buffer (pH 7.0). The samples containing polymer **1** (7 $\mu$M) and Lysozyme in concentrations of 7 or 3.5 $\mu$M, respectively, were scanned from 190 to 275 nm at a constant temperature of 25 °C. As the bisphosphonate and the dansylamide are also absorbing in this frequency range, it was not possible to perform all measurements at the same total concentration. For a better comparison each CD curve was therefore multiplied with a respective normalization factor producing equal absolute values of maximum molar ellipticities.

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**Supporting Information Available:** Full experimental details of polymer synthesis and characterization, fluorescence titration, fluorescence, and UV spectra of the protein polymer complex, gel electrophoresis, enzyme assays, determination of $IC_{50}$ values, and CD spectra of denatured Lysozyme. This material is available free of charge via the Internet at http://pubs.acs.org.

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(26) Laemmli, U. K. *Nature* **1970**, *227*, 680.