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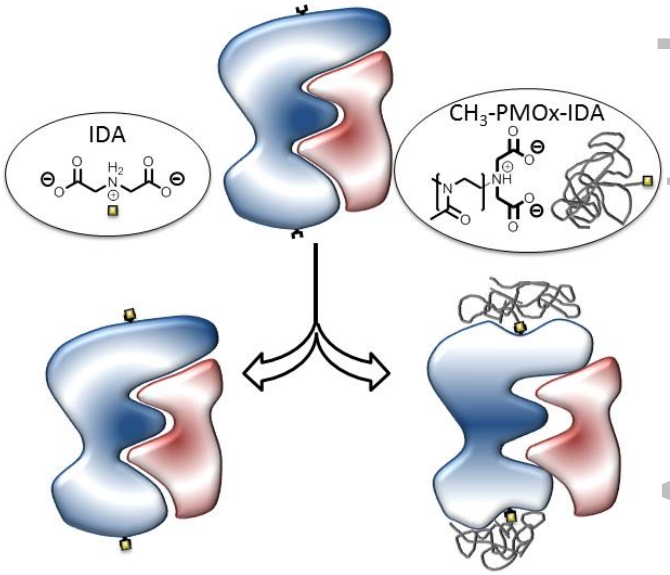
Entropically driven Polymeric Enzyme Inhibitors by End-Group directed Conjugation

M. Hijazi[a], C. Krumm[a], S. Cinar[b] , W.Alachraf[c], Wolf Hiller[d], W. Schrader[c], R. Winter[b], and J.C. Tiller*[a]

Abstract: A new generic concept for polymeric enzyme inhibitors is presented using the example of poly(2-methyl-2-oxazoline) (PMOx) terminated with an iminodiacetate (IDA) function. These polymers are shown to be non-competitive inhibitors for horseradish peroxidase (HRP). Mechanistic investigations revealed that the polymer is directed to the protein by its end group and collapses at the surface in an entropy-driven process as shown by isothermal titration calorimetry. The dissociation constant of the complex was found to be the inhibition constant K_i determined by HRP activity kinetic measurements. Additional experiments suggest that the polymer does not form a diffusion layer around the protein, but might inhibit by inducing minor conformational changes in the protein. This kind of inhibitor offers new avenues towards designing bioactive compounds.

Enzyme-inhibitors are of great importance in fields such as medicine and agriculture. Their specific interactions with single enzymes or enzyme classes are the reason for their application as pharmaceuticals or pesticides. Although much is known about low molecular weight enzyme inhibitors, particularly their mode of action[1] [1b] , there is little known about polymeric enzyme inhibitors. The latter can be classified by inhibitor releasing systems, e.g., for drug delivery[2], inhibitors covalently attached to polymers[3], and macromolecules that act due to their specific backbone structure[4].
The first class is used in a myriad of variations leading to highly improved efficiency in many cases.[5] Only a few examples are known from the second class. For instance, the conjugation of the anionic mucoadhesive polycarbophils such as poly(acrylic acid) with different serine protease inhibitors is able to adhere to the mucosa. The enables the use of less drug and to direct it to the infected tissue.[6] [7] The conjugation of the Bowman-Birk inhibitor (BBI) to the mucoadhesive polymer sodium carboxymethylcellulose (Na-CMC) result in a polymer that protects embedded insulin from enzymatically degradation by serine proteases.[8] Besides modifying the polymer backbone, the polymer end group can be used to couple inhibitors to macromolecules. Human matrix metalloproteinases (MMPs) such as collagenase are inhibited by telechelic poly(2-oxazolines) with *N,N*-dimethyldodecylammonium (DDA) as end groups, which were used for application in dental adhesives.[9] Furthermore, the bacterial topoisomerase II and IV inhibitor ciprofloxacin and the transpeptidase inhibitor penicillin were attached to poly(2-oxazolines). This changed the activity profiles of the antibiotics and increased the hydrolysis stability against penicillinase degradation in cases of the penicillin conjugates. [10] [11] One example for the third class are chitinase inhibitors that are oligo- or polysaccharides with specific binding to the active site of the enzyme.[12] Polyacrylic acid derivatives are able to inhibit the activity of luminal enzymes, e.g. trypsin and carboxypeptidase. The polymers bind to the protein matrix stabilizing ions such as Ca^{2+} in case of trypsin and Zn^{2+} in case of carboxypeptidase, using the specific metal complexing character of their polymer backbone.[13] [14]
In this study, we follow a new concept of polymeric enzyme inhibitors, where a hydrophilic polymer is driven onto the surface of an enzyme by a specific end group that is not an inhibitor by itself. The polymer is then changing the polarity of the surface of the enzyme and/or blocking the active site, resulting in enzyme inhibition (Schema 1).



Schema 1. Schematic representation of the proposed generic concept for enzyme inhibition based on specifically binding anchor groups at inert hydrophilic polymers.

This offers the opportunity to create new enzyme inhibitors using functions that have not yet been considered as inhibitors. In order to prove the concept of this approach, poly(2-oxazoline) was chosen as hydrophilic and biocompatible

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polymer that has only weak interactions with proteins.[15] 2, 2'-Imino diacetate (IDA) was chosen as specific end group that has the potential of binding to bivalent metals that are often found in protein scaffolds.[16] The enzyme horse radish peroxidase (HRP) was chosen as model enzyme, because it contains two calcium ions in the protein structure and is not inhibited by IDA (Schema 1).

The synthesis of the macromolecules was realized by cationic ring opening polymerization of 2-methyl-2-oxazoline followed by terminating the reaction with IDA dimethyl ester (IDA-2Me). Cleaving the ester groups does then lead to the targeted structure (Schema 1 and Figure 1, structure A). A series of single and double side terminated polymers, respectively, with different molecular weights was prepared this way. In addition to ^{1}H-NMR spectroscopy, the structural identity of the polymers was investigated on the example of partially hydrolyzed IDA-PMOx$_{33}$-IDA by electrospray ionization mass spectrometry (ESI-MS).

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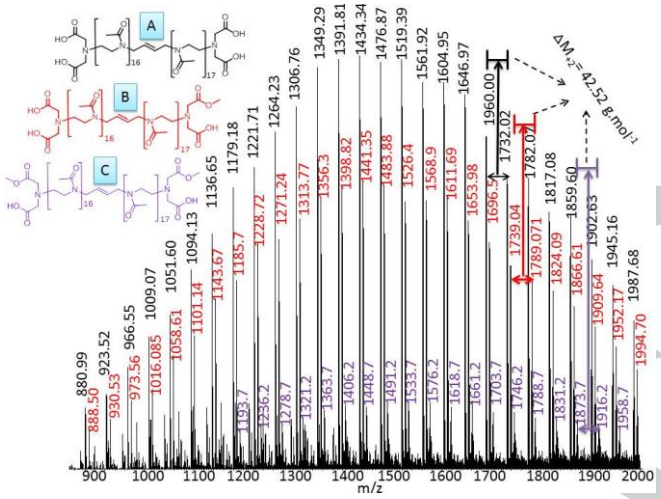

Figure 1. Electrospray ionization mass spectrum of IDA-PMOx$_{33}$-IDA after alkaline hydrolysis at 50 °C for 16 h in the presence of 0.025 M NaOH solution.

As seen in Figure 1, three major generations of polymer masses can be identified. All of them are carrying two positive charges. The distance between the absolute masses is in all cases ΔM+2 = 42.52 g·mol^{-1}, which is exactly one half of the molecular weight of the repeating unit. The masses of the main fraction (56% of all signals) correspond to polymer chains of which the ester end groups are fully converted to carboxylic groups (Figure 1, structure A). The second polymer generation represents polymer chains (35 mol%), which have only one ester group remaining (Figure 1, structure B). The third generation (8 %) represents polymer chains, which still contain two methyl groups (Figure 1, structure C). Determining the overall amount of converted ester groups by the ESI-MS data, results in 86 mol%, which is close to the value of 83% calculated from ^{1}H NMR analysis (Figure S3, Supplements). Thus, the hydrolysis is not changing the PMOx structure. After extended hydrolysis under the same conditions the resulting polymers are fully functionalized with IDA groups (Figure S4, Supplements).

The interactions between HRP and IDA-PMOx$_{33}$-IDA, CH$_3$-PMOx$_{31}$-IDA, and CH$_3$-PMOx$_{31}$-OH, respectively, were investigated by isothermal titration calorimetry (ITC) adding the concentrated polymer solution to the HRP solution. Titration in phosphate buffer showed that addition of IDA-PMOx$_{33}$-IDA to HRP in phosphate buffer results in a strong exothermic signal seemingly indicating a binding at a 1:1 molar ratio (Figure S5A, Supplements). However, a similar titration curve is found when adding the polymer solution to buffer without HRP (Figure S5B, Supplements). Thus, we presume that this signal is due to dissolution of polymer aggregates and overlaps any polymer/enzyme interactions. Thus, the experiments were repeated in water (see Fig. 3). Here, the polymer added to water gives a weak exothermic signal, which can be attributed to the dilution of the polymer solution (Figure S7, Supplements).

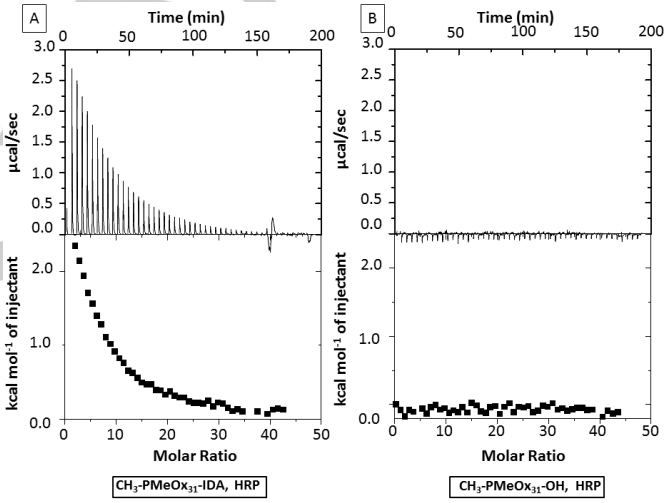

Figure 2. Calorimetric titration isotherm of the binding interaction between PMOx derivatives and horseradish peroxidase (HRP) in water at 25°C. Each peak corresponds to the repeated injection of 6 µl of polymer solution into the reaction cell (V_{cell} = 1.34 ml) in a low concentrated substance solution of 0.03 mmol·L^{-1} HRP. (The heats of dilution of the polymers were determined in a separate experiment and subtracted from this data). The upper part shows the ITC peaks plotted as power (µcal·S^{-1}) against time (min). The lower part shows the resulting integrated heats of the binding process. Left is the titration curve of 6 mM CH$_3$-PMOx$_{31}$-IDA injected into the calorimeter cell containing 0.03 mM of HRP. Right is the titration curve of CH$_3$-PMOx$_{31}$-OH injected into the calorimeter cell containing 0.03 mM of HRP.

The ITC curve of the addition of CH$_3$-PMOx$_{33}$-IDA to HRP indicates an endothermic reaction (Figure 2A). This might be caused by aggregation of polymer and enzyme, where the entropy-driven release of polymer- and enzyme-bound water molecules causes the endothermic effect.[17] Presuming the formation of a 1:1 complex, the dissociation constant was determined to 0.25 mM. In order to see if the interaction between polymer and enzyme is caused by the IDA end group, a PMOx with an OH end group was added to HRP, showing practically no thermal response (Figure 2B). Thus, the IDA end group on the PMOx is directing the polymer to the enzyme

surface. Interestingly, the titration with the double side IDA terminated PMOx (IDA-PMOx_{33}-IDA) shows practically the same ITC curve (Figure S6, supplements) with the same dissociation constant, indicating that one macromolecule might not be able to bind at two different binding sites, simultaneously. Literature-known entropic enzyme inhibitors are often hydrophobic substances, e.g., the anesthetic propofol, that react via hydrophobic interactions with the protein, affording a strong conformational change, which leads to release of water molecules [18] [19]. A similar effect of the here presented IDA conjugated PMOx is unlikely, because the polymer is highly hydrophilic. Circular dichroism (CD) measurements of HRP in the presence of IDA-PMOx_{33}-IDA revealed only a slight change in protein conformation in the presence of the polymer that might lead to inactivation, however, but the polymer does not afford denaturation of the protein (Figure S8, Supplements). This indicates that the entropic effect is most likely due to the surface attachment of the PMOx and the related release of water molecules.[20] To get further insights in the complex formation, DOSY-NMR experiments were performed on a mixture of CH_3-PMOX_{31}-IDA and HRP in D_2O (Figure S13 and Figure S14, Supplements). It was found that the polymer is indeed partially forming aggregates with the enzyme.

The activity of HRP was measured by the oxidation of ABTS with H_2O_2 in the presence of varying amounts of different IDA terminated PMOx (Figure 3).

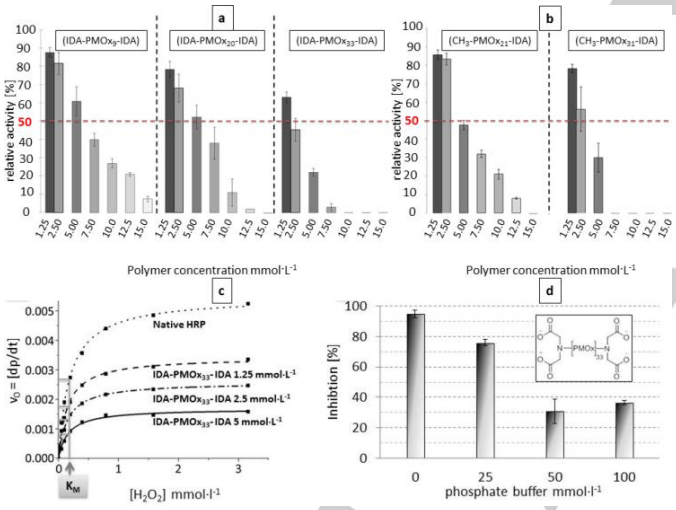

Figure 3. a,b) Inhibition of horseradish peroxidase (HRP) induced by mono- and bifunctional PMOx at varying concentrations (1.25, 2.5, 5, 7.5, 10, 12.5, and 15 mM). c) Kinetics of the HRP inhibition by variation of the H_2O_2 concentration in the range of 0.37-3.3 mM. d) Inhibition of HRP induced by 1 mM IDA-PMOX_{33}-IDA in different phosphate buffer concentrations. The inhibition was measured with ABTS substrate (10 mM) at pH 5.0 for all polymers. All measurements save the kinetics were performed in triplicate, and the error bars are the standard deviation.

As seen in Figure.3a and 3b the presence of the IDA-terminated PMOx inhibits the activity of the HRP. The telechelic IDA-PMOx-IDAs show a similar inhibition compared to the single side terminated polymers, which confirms that only one IDA group per polymer chain binds to the protein. Interestingly, the inhibition increases with greater molecular weight of the polymers, which is unexpected, because the affinity of the IDA group to the enzyme surface should be lower with larger coils attached to IDA. The activity is fully diminished at a concentration of 7.5 to 15 mM IDA terminated polymer. The most active polymer CH_3-PMOX_{31}-DDA is inhibiting HRP by more than 99% at 7.5 mM. No significant inhibition is observed in the same experiment using CH_3-PMOX_{31}-OH as control polymer. The same is found after the treatment of CH_3-PMOX_{31}-OH with NaOH (0.025 M, 59°C, 24h), at this concentration. These results show that only the conjugation of IDA and PMOx results in an enzyme inhibitor. This is consistent with the ITC measurements shown above.

In order to obtain more insights in the nature of the inhibition, the HRP activity was measured in the presence the inhibitor IDA-PMOx_{33}-IDA at different concentrations of hydrogen peroxide (Figure 3c). The presence of the polymer lowers the maximum enzyme velocity V_{max}, but not the Michaelis constant K_M, which is typical for a non-competitive enzyme inhibitor. The inhibition constant K_i was calculated to 1.74 mM ± 0.25 according to equation 1.

$$v = \frac{V_{max}[S]}{[1+\frac{[I]}{K_i}](K_M+[S])}$$

Equation 1. The velocity equation for non-competitive inhibition.[20]

According to the proposed enzyme inhibition mechanism, this value should be equal to the enzyme/polymer dissociation constant of 0.26 mM determined by ITC, which is obviously not the case. Presuming that the presence of phosphate ions influences the binding of the inhibitor to the HRP surface, the activity of the enzyme was measured in media with different buffer concentration in the presence of IDA-PMOx_{33}-IDA. As seen in Figure 3d, the inhibition increases with lower salt concentrations, proofing this presumption to be correct. The K_i value of IDA-PMOx_{33}-IDA in water was found to be 0.25 mM, which perfectly corresponds to the dissociation constant of 0.26 mM measured by ITC. This shows that the formation of polymer/HRP conjugate is solely responsible for the enzyme inhibition.

So far, we could establish that the IDA group directs PMOx to the enzyme surface to inhibit its activity. To shed some light on the possible mode of action, the HRP activity was measured in the presence of Ca^{2+} ions and IDA-PMOx_{33}-IDA (Figure S9, Supplement). Interestingly, adding calcium chloride prior to the polymer does diminish the inhibition from 80 to 28%. This indicates that calcium ions are effective binding competitors for the IDA group. In contrast, when adding calcium chloride to the HRP solution after the polymer, the inhibition was not impaired. This shows that the competitive binding of the calcium ions to IDA does not take place once the enzyme/polymer complex is formed. Therefore, we presume that the binding between polymer and enzyme is primarily entropy-driven as already

indicated by the ITC data. Thus, whereas the IDA group only directs the PMOx via weak interactions to the surface of HRP, subsequent stronger binding of the polymer is induced by dehydration of the interacting interfaces.[17]
According to its non-competitive character, the IDA terminated PMOx does not attach directly to the heme group of the active center. Thus, CH_3-PMOx-IDA attached to the enzyme surface can either influence the protein conformation by changes of surface polarity and/or by blocking the active site. CD spectra do not show strong changes in protein conformation, which supports the latter possibility. The fact that the polymer is capable of fully inhibiting the enzyme is also an indication of blocking the active site. Dynamic light scattering (DLS) data, which show no formation of larger aggregates between HRP and CH_3-PMOx-IDA in water using the same concentration as applied for the ITC measurements, show that the IDA-terminated polymers do not form a shell of many attaching polymers around the protein (Figure S10, Supplement). This suggests specific binding between protein and polymer at one binding site, which could be near the active site. Nevertheless, these are only indicators that are not excluding that a small conformational change of the protein by changed polarity at the surface, found for liposome attaching PMOx,[21] is responsible for the inhibition.

In order to broaden the validity of the shown concept, guaiacol as second substrate was used and the inhibition of HRP in the presence of IDA-$PMOx_{33}$-IDA (1.25-7.5 mM) was investigated. Similar results were obtained when using guaiacol as substrate instead of ABTS (Figure S11, supplements). When fitting the obtained kinetic data with the classic Michaelis-Menten-Model, k_{cat}/K_M (for guaiacol as substrate) changes from 6200 ± 900 to 2100 ± 500 $L \cdot s^{-1} \cdot mol^{-1}$) when adding the polymeric inhibitor, indicating non-competitive inhibition.
In order to test if alkylated IDA group needs to be attached to the polymer terminal for efficient HRP inhibition, the model compound 2,2'-(propylazanediyl)diacetic acid (Pro-IDA) was synthesized. This compound was mixed with the CH3-$PMOx_{31}$-OH in 1:1 molar ratio and tested for HRP inhibition in the guiacol test at the 7.5 mM. No significant inhibition could be observed indicating that only the IDA-terminated PMOx is the inhibitor.
In closing, we have shown in this study that the combination of a non-active polymer and a protein-binding group that is no inhibitor can result in an effective enzyme inhibitor. The ITC data suggest that weak interactions between the end group of the polymer and the protein are sufficient to obtain a strong bond, because the polymer/protein binding is driven by entropy. Thus, this new inhibitor concept offers the chance to find new enzyme inhibitors as pharmaceuticals, pesticides, or antibiotics by combining so far not considered non-inhibiting but enzyme binding functions with hydrophilic polymers. Such conjugates might have different toxicology and immunology profiles compared to currently used systems.

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Keywords: Enzyme inhibitor, Poly(2-oxazoline), horse radish peroxidase, functional polymer end groups

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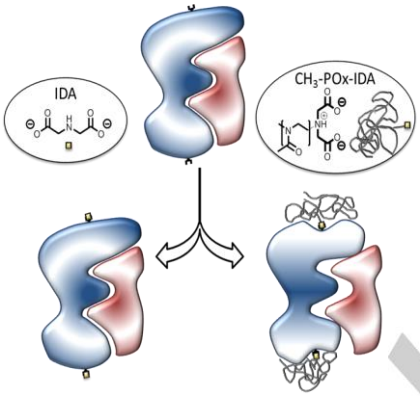

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