COMMUNICATION

ChemComm
www.rsc.org/chemcomm

A polymer supported manganese catalyst useful as a superoxide dismutase mimic

Theeraphon Piacham,[ab] Chartchalerm Isarankura Na Ayudhya,[b] Virapong Prachayasittikul,[b] Leif Bülow[a] and Lei Ye*[a]

[a] *Pure and Applied Biochemistry, Chemical Center, Lund University, Box 124, 221 00 Lund, Sweden. E-mail: lei.ye@tbiokem.lth.se; Fax: 46 46 2224611; Tel: 46 46 2229560*

[b] *Department of Clinical Microbiology, Faculty of Medical Technology, Mahidol University, Bangkok 10700, Thailand*

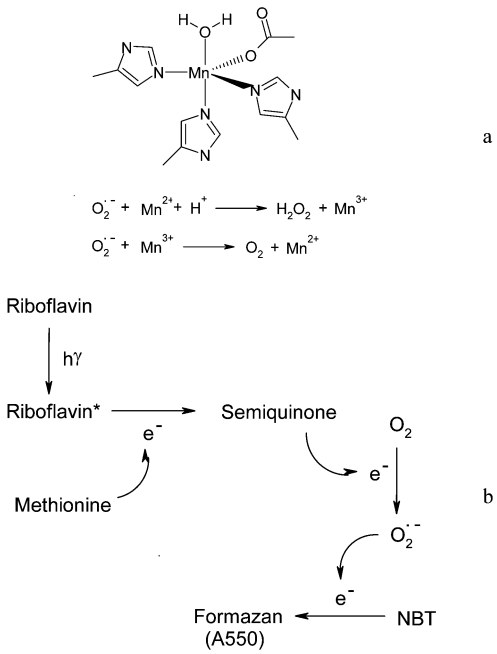
Received (in Cambridge, UK) 13th February 2003, Accepted 1st April 2003
First published as an Advance Article on the web 28th April 2003

Polymer supported manganese was synthesized *via* a template polymerization involving functional monomers to afford a catalyst with superoxide dismutase activity.

Template-directed synthesis of designed molecules has attracted increasing interest due to its inherent simplicity. This strategy has been adopted both in supramolecular chemistry[1] and in molecular imprinting of synthetic polymers.[2] In the latter practice a molecular template is used to generate specific binding sites in cross-linked polymers, in which the distribution of functional groups is dictated by the template molecule. When metal ions are used as templates, they are able to bring appropriately oriented coordination groups into a multi-dentate structure that affords selective binding capability of the polymer towards the metal ion templates.[3] In addition to studying metal ion binding polymers, we have been interested in exploiting the resulting polymers for biotechnological applications, for example as enzyme mimics able to catalyze important biochemical reactions. It is now common knowledge that the catalytic activity of many metalloenzymes depends to a large extent on a correctly co-ordinated metal co-factor located in their active center. For instance, the manganese-based superoxide dismutase (Mn SOD) from human mitochondria contains a Mn(II) coordination sphere and is able to catalyze the disproportionation of superoxide anions ($O_2^{\cdot -}$), an important mechanism in healthy organisms to suppress accumulation of highly reactive superoxide radicals (Scheme 1a).[4]

Synthetic SOD mimics have pharmaceutical potential in treating tissue injury and inflammation.[5] Up to now most SOD mimics have been based on small organic molecules obtained by multi-step synthesis. To investigate the feasibility of building polymer-based metalloenzyme mimics, we started to use the Mn(II) ion itself to assemble a similar coordination sphere in cross-linked polymer matrices. We used 4-vinylimidazole (4V), 1-vinylimidazole (1V) and methacrylic acid (MAA) to produce the necessary Mn(II) binding interactions (Table 1), based on the assumption that these monomers have functional groups similar to those of histidine and aspartic acid, the amino acid residues that form the active metal complex with Mn(II) in the native enzyme. The polymers containing Mn(II) were prepared by a free radical polymerization using excess of a cross-linking monomer, ethyleneglycol dimethylacrylate (EDMA).[6] The SOD activity of the polymers was assayed by measuring inhibition of the photoreduction of nitro blue tetrazolium (NBT) (Scheme 1b), a method slightly modified from that originally described by Beyer and Fridovich.[7] This indirect assay comprised several reactions: The photochemically excited riboflavin was first reduced by methionine into a semiquinone, which donated an electron to oxygen to form the superoxide source. The superoxide readily converted NBT into a purple formazan product. In this way the SOD activity was inversely related to the amount of formazan formed. In our heterogeneous system, we used a metal-free, non-templated polymer as a control to give a background visible absorbance value.

As seen in Table 1, the Mn(II)-containing polymer based on 4-vinylimidazole and methacrylic acid (Mn-P4VM) was able to scavenge the photo-chemically generated superoxide anions, which otherwise could reduce NBT to form a light absorbing formazan. A pre-treatment of the polymer with ethylenediamine tetracetic acid (EDTA) stripped the polymer of Mn(II), which



Scheme 1 (a) Coordination sphere in Mn SOD and the enzyme catalyzed disproportionation of superoxide radicals. (b) SOD activity assay by measuring the inhibition of the photoreduction of nitro blue tetrazolium (NBT).

Table 1 Preparation of polymer supported Mn(II) catalysts and their SOD activity

No.[a]	Polymer	Mn(II)/ μmol	Functional monomer/μmol			SOD activity[b] (%)
			1V	4V	MAA	
1	Mn-P4VM	100	0	300	100	72
2	P4VM[c]	0	0	300	100	0
3	Mn-P1VM	100	300	0	100	89
4	P1VM	0	300	0	100	0
5	Mn-P1V	100	400	0	0	48
6	P1V	0	400	0	0	0
7	Mn-PM	100	0	0	400	62
8	PM	0	0	0	400	0

[a] Entries 1, 3, 5 and 7 were for templated polymers. Entries 2, 4, 6 and 8 were for non-templated polymers. [b] SOD activity was evaluated by the extent of inhibition of the NBT photoreduction.[8] [c] Neither P4VM, nor P4VM pre-loaded with excess Mn(II) showed any SOD activity.10

DOI: 10.1039/b301683h

resulted in a complete loss of SOD activity.[9] This control experiment confirmed that catalysis of superoxide disproportionation was caused by the metal center. However, simply preloading the metal-free polymer (P4VM) with excess $MnCl_2$ did not afford any SOD activity[10] and free Mn(II) in solution only displayed a very low intrinsic SOD activity.[11] Thus it is only metal templated polymer (Mn-P4VM) that produces an enhanced catalytic activity.

Interestingly, when 1-vinylimidazole was used together with methacrylic acid, the resulting metal containing polymer Mn-P1VM showed much higher activity than Mn-P4VM. This may be explained by the fact that in the natural enzyme system, the metal center is coordinated to histidine residues *via* the "pyridine nitrogen" (N-3),[12] which is better simulated by 1-vinylimidazole. To test the importance of the metal center, we also prepared polymers containing other metal ions [Co(II), Fe(III) and Cu(II)] using the same monomer composition as that for Mn-P1VM. When these polymers were assayed, they displayed a decreasing SOD activity in the order of Co(II) (77%) > Cu(II) (55%) > Fe(III) (10%). Catalytic activity of the polymer supported Cu(II) is very low, as compared to that of the mammalian Cu/Zn SOD (almost diffusion-controlled). For the Cu/Zn SOD, a key step in the catalytic reaction is breakage of the imidazole bridge between copper and zinc.[13] This important feature is missing in the polymer supported Cu(II) catalyst.

Our Mn(II)-containing polymer was designed to mimic the coordination center in the native Mn SOD enzyme. The Mn(II) in the polymer was supposed to coordinate with three imidazole and one carboxyl group to maintain catalytic activity. Omitting one of the functional monomers (Mn-P1V and Mn-PM, respectively) resulted in a reduced SOD activity (Table 1).

By varying the amount of polymers used in the assay, we found that approximately 1.2 mg of both Mn-P4VM and Mn-P1VM were needed in order to inhibit 50% of the NBT photoreduction (Fig. 1), which corresponded to 3.7 units of the native SOD enzyme.[14] Repeated use of Mn-P1VM resulted in a reduction of activity, *i.e.* the inhibition of NBT photoreduction decreased from the initial 89% to 79%, which was accompanied by a lowered amount of Mn(II) in the polymer (by 70%) as quantified by ICP-AES analysis. It appeared that among the initial Mn(II) used for polymer preparation, less than 30% were able to form a stable complex with the ligand monomers throughout the polymerisation process, and remained relatively stable in the obtained Mn-P1VM. Further investigation is needed in order to improve the complex stability during polymer preparation, as well as in the obtained polymer supported Mn(II) catalyst.

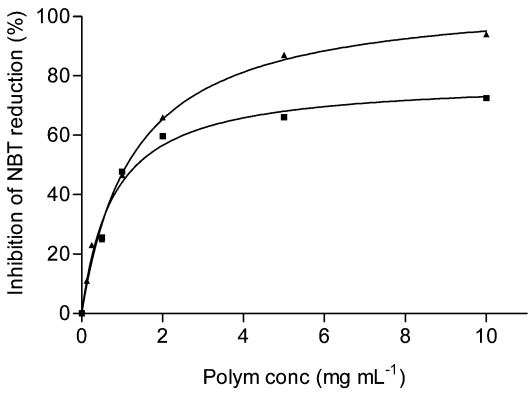


Fig. 1 Inhibition of photoreduction of NBT by increasing the amount of polymer used in the assay. Mn-P4VM (■) and Mn-P1VM (▲).

In summary, we have demonstrated a simple template polymerisation method for the preparation of a polymer supported Mn(II) catalyst that displays favourable SOD activity. By exploiting more sophisticated functional monomers and polymerisation conditions, we expect to greatly improve catalyst stability and activity in the future.

We acknowledge financial support for the integrated program "Biomimetic Materials Sciences (Biomics)" by the Swedish Strategic Research Foundation (SSF). T. P. is grateful for a fellowship from the UMAP Credit Transfer Scheme by the Ministry of University Affairs, Thailand.

Notes and references

1 J.-M. Lehn, *Angew. Chem., Int. Ed. Engl.*, 1988, **27**, 89.
2 (*a*) K. J. Shea, *Trends Polym. Sci.*, 1994, **2**, 166; (*b*) G. Wulff, *Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 1812; (*c*) J. Steinke, D. C. Sherrington and I. R. Dunkin, *Adv. Polym. Sci.*, 1995, **123**, 81; (*d*) M. J. Whitcombe, C. Alexander and E. N. Vulfson, *Synlett*, 2000, 911; (*e*) L. Ye and K. Mosbach, *J. Incl. Phenom.*, 2001, **41**, 107.
3 (*a*) P. K. Dhal, *Tech. Instrum. Anal. Chem.*, 2001, **23**, 185; (*b*) K. Tsukagoshi, M. Murata and M. Maeda, *Tech. Instrum. Anal. Chem.*, 2001, **23**, 245.
4 (*a*) G. E. Borgstahl, H. E. Parge, M. J. Hickey, W. F. Beyer Jr., R. A. Hallewell and J. A. Trainer, *Cell*, 1992, **71**, 107; (*b*) R. Krämer, *Angew. Chem., Int. Ed.*, 2000, **39**, 4469.
5 (*a*) D. P. Riley, *Chem. Rev.*, 1999, **99**, 2573; (*b*) D. Salvemini, D. P. Riley and S. Cuzzocrea, *Nat. Rev. Drug Disc.*, 2002, **1**, 367.
6 For polymer preparation, $MnCl_2$, 4V, 1V, MAA, EDMA (4 mmol) and 2,2′-azobisisobutyronitrile (2 wt% of total monomer) were dissolved in 1 mL of methanol : water (90 : 10, v/v) mixed solvent. The monomer solution was saturated with nitrogen and then polymerized at 60 °C for 24 h. The polymer monolith was manually ground to afford fine particles with sizes of approximately 1–10 μm.
7 W. F. Beyer Jr. and I. Fridovich, *Anal. Biochem.*, 1987, **161**, 559.
8 A stock assay solution was prepared by mixing 27 mL of HEPES buffer (50 mM, pH 7.8), 1.5 mL of L-methionine (30 mg mL^{-1}), 1 mL of NBT (1.41 mg mL^{-1}) and 750 μL of Triton X-100 (1 wt%). To 1 mL of the stock solution in a microcentrifuge tube was added 5 mg of different polymers and 10 μL riboflavin (44 μg mL^{-1}). After vortexing, the mixture was illuminated for 7 min under a PHILIPS Classic Tone lamp (60 W) in a light box, followed by an immediate centrifugation (6000 rpm, 1 min) to sediment the polymer particles. Visible absorbance at 550 nm for the supernatant was measured using a standard UV-VIS spectrophotometer. The extent of inhibition for NBT photoreduction was calculated as: $(Abs_{cont} - Abs)/Abs_{cont}$. Abs_{cont} was the absorbance value at 550 nm obtained from the metal-free polymer.
9 The polymer Mn-P4VM (5 mg) was incubated in 1 mL of 0.1 mM EDTA in HEPES buffer (50 mM, pH 7.8) for 1 h. The polymer was then isolated by centrifugation and tested using the NBT photoreduction method. No decrease in Abs was observed as compared to that with the polymer P4VM.
10 The polymer P4VM (5 mg) was incubated with 50 μg of $MnCl_2$ in 1 mL of HEPES buffer (50 mM, pH 7.8) for 1 h. The polymer was then isolated by centrifugation and tested for SOD activity.
11 The SOD activity of $MnCl_2$ (50 μg) was tested without using the centrifugation step. The Abs_{cont} was the absorbance value at 550 nm obtained in the absence of Mn(II).
12 M. M. Whittaker and J. W. Whittaker, *Biochemistry*, 1997, **36**, 8923.
13 M. A. Houge and S. S. Hasnain, *J. Mol. Biol.*, 1999, **287**, 579.
14 Measurement of the native SOD activity was carried out using the same NBT photoreduction assay. However, the centrifugation step was omitted, and the Abs_{cont} was the absorbance value at 550 nm from the enzyme-free mixture. Here one unit of SOD inhibited the NBT photoreduction by 50%. Using inductively coupled plasma atomic emission spectroscopy (ICP-AES), we found that the initial Mn(II) loading in Mn-P1VM was 5.75 mg g^{-1} polymer. To achieve a similar catalytic activity, the present artificial system (Mn-P1VM) required much more (10^3 fold) manganese as compared to the natural Mn SOD.