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Temperature modulated solubility and activity alteration for oligo-(*N*-isopropylacrylamide)–iron tetrasulfonatophthalocyanine conjugates as a new mimetic peroxidase

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Iron tetrasulfonatophthalocyanine (FeTSPc) was covalently bound to the terminus of a temperature sensitive oligomer, oligo-*N*-isopropylacrylamide (ONIPAAm), to form a new mimetic enzyme (ONIPAAm–FeTSPc) to mimic the peroxidase activity of horseradish peroxidase. This FeTSPc-based mimetic enzyme exhibits a lower critical solution temperature (LCST) of 32 °C in neutral solution. It precipitates from water above the LCST and redissolves when the solution temperature is lowered below the LCST. The peroxidase activity of this mimetic enzyme was studied based on its catalytic effect on the reaction of *p*-hydroxyphenylpropionic acid and H_2O_2. The results show that the peroxidase activity of the new mimetic enzyme is higher than that of the free FeTSPc. The possibility of its application in the analytical field was also tested by the determination of H_2O_2 and ONIPAAm–FeTSPc; the detection limits are 8.2×10^{-9} and 1.7×10^{-9} mol L^{-1}, respectively.

1 Introduction

Horseradish peroxidase (HRP) is one of the most commonly used enzymes in bioanalytical chemistry.[1–3] However, natural enzymes are expensive and unstable in solution. Therefore, the use of stable mimetic peroxidases as substitutes for HRP in fluorescence reactions have increased markedly in recent years.[4–8] Since natural HRP has a porphine skeleton in its active center, the studies on the mimetic peroxidase have been mainly focused on metalloporphine complexes so far, involving some natural substances[4,5] and synthesized complexes.[6–8] More recently, we found that a synthesized iron tetrasulfonatophthalocyanine complex (FeTSPc), just like HRP, exhibited very significant catalytic activity for the peroxidation reaction between *p*-hydroxyphenylpropionic acid and hydrogen peroxide,[9] and it can be used as an efficient substitute for HRP.

It is well known that the catalytic active site of a natural enzyme is surrounded by a protein matrix, which gives the active site a hydrophilic or hydrophobic environment and considerably affects the enzyme structure and activity. Some studies have shown that the activity of both synthetic mimics and natural enzymes increased accompanied by the control of the environment of the catalytic active site.[10–12] Here, in order to mimic the environment of the catalytic active site of HRP, we modified the synthesized FeTSPc complex with a water-soluble, temperature-sensitive oligomer.

Poly-*N*-isopropylacrylamide (PNIPAAm) is a well-known water-soluble polymer showing unique, reversible hydration–dehydration changes in response to small temperature changes.[13] It has the property of precipitation when the temperature is raised to above the lower critical solution temperature (LCST) and redissolution when the temperature is lowered below the LCST. The special thermo-sensitive characteristic of PNIPAAm has been successfully applied to immobilized enzymes,[14,15] immunoassays[16,17] and separation processes.[18,19] Li *et al.*[20] recently reported the mimicry of peroxidase by immobilization of hemin on a temperature-sensitive hydrogel. However, the immobilization of biomolecules, such as enzymes, antibodies and antigens, on temperature-sensitive polymer chains has mainly utilized copolymerization reactions with an active acrylate as coupling reagent. In this case, proteins randomly coupled to copolymers are capable of aggregation during preparation, and moreover, conjugation of the copolymer with proteins may inhibit the mobility of the polymer chains.[21] In order to avoid these problems in copolymerization, Okano *et al.* synthesized temperature-sensitive ogliomers with reactive end groups and used them to conjugate to biomolecules.[12,21]

In this work, a terminal carboxy group was introduced into the oligo-*N*-isopropylacrylamide (ONIPAAm) molecule by using mercaptoacetic acid as a chain-transfer agent, and FeTSPc was covalently conjugated to the terminal of the oligomer with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI) as a difunctional reagent. It was found that the LCST property of the modified mimetic peroxidase, FeTSPc–ONIPAAm, was retained, showing an LCST of 32 °C in neutral solution, and FeTSPc–ONIPAAm precipitates from water when the temperature is raised above 32 °C and redissolves when the temperature is lowered below it. The use of an organic oligomer matrix to mimic a protein matrix and FeTSPc to mimic the active site of natural HRP is expected to lead to the synthesis of an efficient mimetic peroxidase, and our experimental results showed that the peroxidase activity of FeTSPc–ONIPAAm mimetic enzyme is higher than that of the free FeTSPc.

2 Experimental

2.1 Materials

FeTSPC was synthesized and purified according to Weber and Busch.[22] *N*-Isopropylacrylamide (NIPAAm), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and *p*-hydroxyphenylpro-

pionic acid (*p*-HPPA) were purchased from TCI-EP (Tokyo Kasei, Tokyo, Japan) and used without further purification. Mercaptoacetic acid, ammonium peroxodisulfate (APS) and hexane-1,6-diamine were obtained from Shanghai Chemical Reagent Co. (Shanghai, China). *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI) was purchased from Sigma (St. Louis, MO, USA). HRP (150 u mg^{-1}) was obtained from Shanghai Buoao Biotech (Shanghai, China). Hydrogen peroxide (H_2O_2, 30%) was supplied by Shanghai Taopu Chemical Factory (Shanghai, China), and a stock standard solution (3% H_2O_2) was standardized by titration with a standard solution of $KMnO_4$. The stock standard solution of *p*-HPPA (0.01 mol L^{-1}) was stored at 4 °C protected from light. An Na_2CO_3–$NaHCO_3$ buffer solution (pH 10.8) at a concentration of 0.1 mol L^{-1} and a phosphate buffered saline (PBS) solution (pH 7.4) at a concentration of 0.01 mol L^{-1} were used. All the reagents were of analytical-reagent grade. Distilled, deionized water was used throughout.

2.2 Apparatus

A Hitachi (Tokyo, Japan) 650-10S spectrofluorimeter equipped with a plotter unit and a 1 cm quartz cell was used for recording and making fluorescence measurements. The absorption spectrum was performed on a Beckman (Fullerton, CA, USA). DU-7400 UV/VIS diode array spectrophotometer. All pH measurements were made with a PHS-301 pH meter (Analytical Instrument Factory, Xiamen, China). A TGL-16G supercentrifuge (Anting Scientific Instrument Factory, Shanghai, China) and an SHZ-88 water-bath vibrator (Taichang Experimental Instrument Factory, Jiangshu, China) were used.

2.3 Preparation of ONIPAAm containing a terminal carboxy group

A 4 mL portion of PBS (pH 7.4) containing 60 mg of NIPAAm, 30 mg of APS, 10 μL of TEMED and various volumes of mercaptoacetic acid were mixed by vibration in a 25 °C water-bath for 60 min to form a series of ONIPAAms with reactive end carboxy groups. When the polymerization reaction was finished, the ONIPAAm was precipitated and separated by centrifugation at 37 °C for 10 min (10000 rpm), then the precipitate was dissolved in 4 mL of cold PBS (pH 7.4). The above-mentioned procedure, *i.e.*, precipitation, separation and dissolution, was repeated three times. Finally, the synthesized oligomer containing a terminal carboxy group was dissolved in 4 mL of cold PBS buffer solution (pH 7.4), and stored at 4 °C.

2.4 Preparation of ONIPAAm–FeTSPc mimetic enzyme

FeTSPc was covalently conjugated to the terminal carboxy group of ONIPAAm with hexane-1,6-diamine and EDCI as coupling reagents as follows. 0.2 g of FeTSPc and 0.35 g of PCl_5 were mixed and ground for 5 min in a ventilated cabinet, 5 mL of anhydrous acetone were added and the mixture was stirred for 5 min before rapid filtration. A 0.5 mL volume of the filtrate was added dropwise to 2 mL of 4×10^{-4} mol L^{-1} hexane-1,6-diamine solution, then the mixture was stirred at 25 °C for 6 h to form FeTSPc–hexane-1,6-diamine.

A 4 mL volume of ONIPAAm solution (prepared as described in Section 2.3) was mixed with 0.2 mL of 6.1×10^{-2} mol L^{-1} EDCI solution and the mixture was incubated for 30 min at 25 °C with magnetic stirring. A 2 mL volume of FeTSPC–hexane-1,6-diamine solution was added and the mixture was stirred at room temperature protected from light for 6 h, then 10 μL of ethanolamine were added to terminate the reaction. The mixture was incubated for 10 min at 37 °C to precipitate the ONIPAAm–FeTSPc conjugate and the resultant precipitate was separated by centrifugation for 10 min at 37 °C (10 000 rpm) and dissolved in 10 mL of cold PBS (pH 7.4) solution, then the solution was incubated for 10 min at 37 °C to precipitate the polymer again. This procedure was repeated three times to purify the precipitate of ONIPAAm–FeTSPc mimetic enzyme, and the mimetic enzyme finally obtained was dissolved in 6.0 mL water and kept at 4 °C in the dark. The concentration of ONIPAAm–FeTSPc was quantified by measuring its absorption at 329 nm.

2.5 Measurement of LCST

ONIPAAm and ONIPAAm–FeTSPc mimetic enzyme rapidly precipitate from the solution when the temperature is raised to above their LCSTs, and this inevitably results in a large increase in the light scattering intensities of the oligomer solutions. Therefore, the change in light scattering intensity was utilized to measure LCST in this work. In the measurement of the LCST, light scattering intensities of ONIPAAm and ONIPAAm–FeTSPC solutions at 490 nm at various temperatures were measured with a Hitachi 650-10S spectrofluorimeter.

2.6 Measurement of peroxidase activity of mimetic enzyme

The peroxidase activities of free FeTSPc and ONIPAAm–FeTSPc was measured with an initial reaction rate method. Into a 10 mL calibrated flask were transferred 2 mL of 0.1 mol L^{-1} Na_2CO_3–$NaHCO_3$ buffer solution (pH 10.8), an appropriate amount of *p*-HPPA and 100 μL of 1×10^{-5} mol L^{-1} ONIPAAm–FeTSPC (or free FeTSPc) solution. The mixture was diluted to volume and 2.0 mL were transferred into a 1 cm quartz cell immediately. While stirring, the relative fluorescence intensity–reaction time curve was recorded simultaneously for 60 s with the addition of 20 μL of 5.0×10^{-5} mol L^{-1} H_2O_2 solution ($\lambda_{ex}/\lambda_{em}$ = 324/409 nm). The data were treated with the Michaelis–Menten equation.

2.7 Determination of equilibrium fluorescence intensity

Into a 10 mL calibrated flask were transferred 2.0 mL of 0.1 mol L^{-1} Na_2CO_3–$NaHCO_3$ buffer solution (pH 10.8), 500 μL of 1.0×10^{-3} mol L^{-1} *p*-HPPA, 100 μL of 1.0×10^{-4} mol L^{-1} ONIPAAm–FeTSPc and a known volume of H_2O_2 solution. The mixture was diluted to volume with water and allowed to stand for 30 min at room temperature. The relative fluorescence intensity of the product was measured at 409 nm with excitation at 324 nm.

3 Results and discussion

3.1 Preparation of ONIPAAm–FeTSPc conjugate

Thiol compounds having functional groups as telogens are known to be effective in introducing functional groups on the end of growing polymeric chains and regulating polymer molecular weight by radical telomerization *via* a chain-transfer reaction.[12,21] Here, a series of ONIPAAms with one carboxy end group per oligomer chain were prepared using APS and TEMED as initiation agents and mercaptoacetic acid as a chain-transfer agent.

Carboxy end groups of ONIPAAms were activated using EDCI and sulfonate groups of FeTSPcs were activated with PCl_5. The activated ONIPAAm and FeTSPc molecules were

coupled using hexane-1,6-diamine as a coupling agent. The resulting ONIPAAm–FeTSPc conjugate showed water solubility at room temperature, and it also exhibited temperature-sensitive characteristics similar to those of ONIPAAm itself. The ONIPAAm–FeTSPc mimetic enzyme conjugate was purified by repeated precipitation, washing and redissolution. The reliability of the synthesis of the mimetic enzyme was tested. The results indicated that the synthesis of these substances showed good reproducibility.

In order to prove that FeTSPc was covalently bound to NIPAAm oligomer in ONIPAAm–FeTSPc mimetic enzyme, we prepared two kinds of solution. One was a solution of ONIPAAm–FeTSPc conjugate prepared according to the procedure described in Section 2.4, and the other was prepared in a similar way but in the absence of EDCI, a difunctional agent for coupling FeTSPc–hexane-1,6-diamine and ONIPAAm. Both of the solutions were then thermally precipitated and centrifuged as described above, and the precipitates were dissolved in PBS (pH 7.4, 0.01 mol L^{-1}). It was found that about 80% of FeTSPc remained in the ONIPAAm–FeTSPc conjugate solution, whereas there was only 0.3% of FeTSPc remaining in ONIPAAm from the mixed solution of ONIPAAm and FeTSPc. These experiments demonstrated that FeTSPc was chemically conjugated to ONIPAAm in the ONIPAAm–FeTSPc conjugate but not absorbed. Furthermore, the FeTSPc molecule was bound to the end of ONIPAAm because ONIPAAm has only one reactive end group per oligomer.

3.2 Characteristics of ONIPAAm and ONIPAAm–FeTSPc mimetic enzyme

The oligomer chain length was controlled by varying the ratio of chain-transfer agent to the monomers. Short chain length oligomers were obtained using large amounts of chain-transfer agent. When the ratio varied from 0.099 to 0.010, the molecular weights of the resulting oligomers were in the range 1300–7500.[21] In this work, the molecular weight of the obtained ONIPAAm which was conjugated to FeTSPc is about 7500 (the molar ratio of mercaptoacetic acid to monomer was 0.010); however, it is an average molecular weight, not a single molecular weight.

The phase transition behavior of the prepared ONIPAAms in aqueous solution was studied based on their light scattering changes at various temperatures (Fig. 1). It can be seen that all of the oligomers dissolved in water when the temperature was lower than 31 °C, and the light scattering intensities of the solutions were very low, whereas if the temperature was raised above 32 °C, the oligomers rapidly precipitated from the solution, and this resulted in a large increase in the light scattering intensities of the oligomer solutions. This indicated that all the ONIPAAms exhibit LCST near 31–32 °C. In addition, the LCST of ONIPAAms seems to be related to the concentration ratio of mercaptoacetic acid to the monomer during the polymerization reaction. The LCST of ONIPAAm shifts to lower temperature with increasing concentration of mercaptoacetic acid, and this phenomenon may arise because a small molecular oligomer was obtained in the presence of high mercaptoacetic acid concentrations. In this work, concentration ratio of 0.01 (mercaptoacetic acid : NIPAAm) was chosen to prepare ONIPAAm and ONIPAAm–FeTSPc mimetic enzyme.

The phase transition characteristic of ONIPAAm–FeTSPc mimetic enzyme is also shown in Fig. 1. It can be seen that the conjugated mimetic enzyme precipitated with increasing temperature and also showed an LCST at about 32 °C. This indicated that conjugated FeTSPc has no or little effect on the phase transition characteristic of ONIPAAm. The phase transfer characteristic of ONIPAAm–FeTSPc shows some advantages: first, the mimetic enzyme can be purified by simple precipitation. Second, since the mimetic enzyme can be reversibly redissolved by cooling, it may be concentrated and recycled. Finally, although it is impossible to enhance the catalytic activity of ONIPAAm–FeTSPc when the temperature is increased above 32 °C, the precipitated mimetic enzyme may be used directly as an immobilized mimetic enzyme.

In order to probe other characteristics of ONIPAAm and ONIPAAm–FeTSPc conjugate, the UV/VIS absorption spectra of ONIPAAm, FeTSPc and ONIPAAm–FeTSPc conjugate in aqueous solution were studied (Fig. 2). It can be seen that ONIPAAm had no absorbance. The ONIPAAm–FeTSPc conjugate showed two main absorption bands with maxima located at 329 and 691 nm. In addition, two faint shoulders can be observed around 451 and 632 nm. In comparison with the spectrum of free FeTSPc, which showed two bands at 329 and 634 nm, the main difference is that the ONIPAAm–FeTSPc conjugate showed a new absorption band at 691 nm and a new faint shoulder band at 451 nm. In addition, the conjugate showed only a faint shoulder band at 632 nm. However, for the absorption band appearing at 329 nm, there was no obvious difference between ONIPAAm–FeTSPc conjugate and free FeTSPc. This indicated that some functional groups of FeTSPc were changed when it covalently bound to NIPAAm oligomer. Therefore, the ONIPAAm–FeTSPc conjugate is expected to have a different mimetic peroxidase activity.

The concentration of FeTSPc in ONIPAAm–FeTSPc conjugate was determined with an FeTSPc calibration curve, because our experimental results showed that the difference

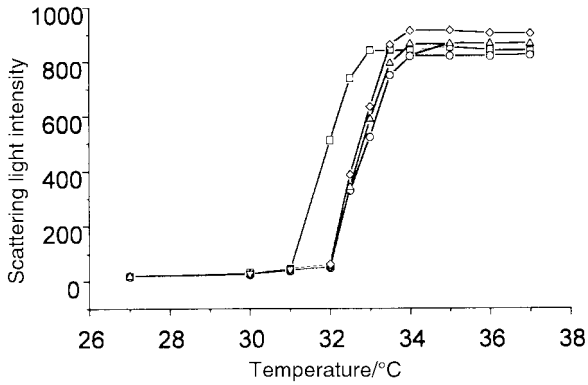


Fig. 1 Temperature dependence of optical scattering for aqueous solution of a series of ONIPAAms with first a fixed molar ratio of mercaptoacetic acid to monomer: (□) 0.5, (Δ) 0.1 and (○) 0.01; (◇) mimetic enzyme (mercaptoaceticacid:monomer = 0.01).

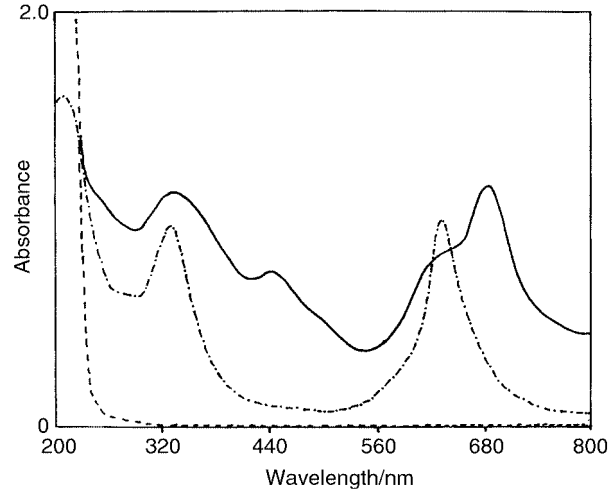


Fig. 2 Absorption spectra of ONIPAAm (dashed line), FeTSPc (dot–dashed line) and ONIPAAm–FeTSPc conjugate (solid line).

between the UV/VIS absorption (at 329 nm) of the solutions containing FeTSPc, ONIPAAm and other reagents before and after conjugation could be neglected.

3.3 Peroxidatic activity of ONIPAAm–FeTSPc mimetic enzyme

Metal phthalocyanines show wide catalytic activities in many fields, such as electroreduction on the surface of an electrode[23] and chemiluminescence.[24] More recently, we synthesized FeTSPc (Fig. 3) and reported its peroxidatic activity in fluorescence analysis.[9,25] It is well known that the catalytically active center is surrounded by a protein matrix in the natural enzyme. Therefore, the catalytic activity of an enzyme depends not only on the catalytic site, but also on the environment of its active center. FeTSPc exhibits some catalytic and peroxidatic activity, but, it only mimics the active center of HRP, and the environments of the catalytic active center of free FeTSPc and HRP are very different. Therefore, to some extent, free FeTSPc cannot precisely mimic HRP. For the ONIPAAm–FeTSPc conjugate, the catalytically active center is surrounded by the polymer matrix, just like the protein matrix in HRP, which may provide the best arrangement for the catalytically active center. Additionally, the polymer itself may serve as a nitrogen or oxygen proximal ligand to the iron center of FeTSPc. Therefore, we suggest that ONIPAAm–FeTSPc mimetic enzyme exhibits higher peroxidatic activity, which is closer to that of HRP.

Just like HRP, ONIPAAm–FeTSPc shows high peroxidatic activity for the fluorogenic reaction of *p*-HPPA and H_2O_2. Under the catalysis of ONIPAAm–FeTSPc, *p*-HPPA, a classical substrate which had been applied to HRP-catalyzed H_2O_2 reactions, was oxidized by H_2O_2 to a fluorescent product, bi-*p*,*p*′-hydroxyphenylpropionic acid, which exhibited strong fluorescence at 409 nm with an excitation at 324 nm. The catalyzed reaction mechanism is assumed to follow the reaction in Scheme 1.

The kinetic profiles for the rate of fluorescence development with the use of HRP, ONIPAAm–FeTSPc and free FeTSPc as catalyst were studied and the results are shown in Fig. 4.

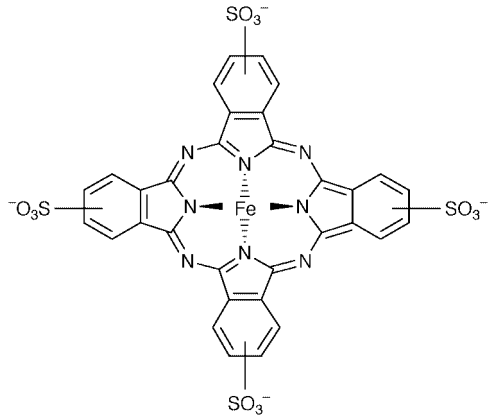
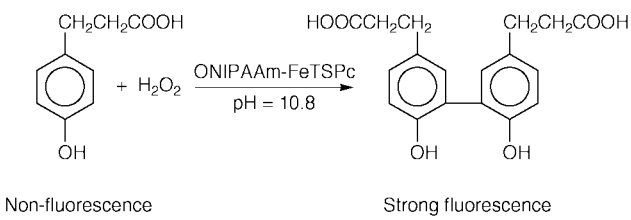


Fig. 3 Molecular structure of FeTSPc.



Scheme 1 Mimetic enzymatic reaction of H_2O_2 and *p*-HPPA with ONIPAAm–FeTSPc as catalyst.

Although Fig. 4 clearly shows that the kinetic profiles of the three catalytic systems have distinct differences, ONIPAAm–FeTSPc and free FeTSPc show high peroxidatic activity similar to that of HRP. The initial rate with ONIPAAm–FeTSPc is lower than that with HRP, but higher than that with free FeTSPc. This indicates that the oligomer matrix in the ONIPAAm–FeTSPc mimetic enzyme can enhance the catalytic activity of FeTSPc.

In order to study the effect of the oligomer on the catalytically active site in ONIPAAm–FeTSPc mimetic enzyme, the catalyticactivities of ONIPAAm–FeTSPc, free FeTSPc and HRP were studied by the initial rate method. The kinetic data obtained with ONIPAAm–FeTSPc, free FeTSPc (5.0×10^{-7} mol L^{-1} in the final solution), HRP (7.5×10^{-8} mol L^{-1} in the final solution), H_2O_2 (1.0×10^{-4} mol L^{-1} in the final solution) and different concentrations of *p*-HPPA were applied to the Michaelis–Menten equation:[26]

$$1/v = (K_m/V_{max}) \times 1/[S] + 1/V_{max}$$

The Michaelis–Menten constant K_m and the maximum rate V_{max} were obtained from Lineweaver–Burk plots ($1/v$ *vs.* $1/[S]$) (Fig. 5), and the transformation constant K_{cat} was obtained from $V_{max} = K_{cat}[E_0]$, where $[E_0]$ is the initial concentration of the mimetic enzyme. The K_{cat} value gives the maximum number of molecules of substrate which can be transformed into the product by one enzyme molecule in unit time, and thus indicates the enzymatic activity: the greater the K_{cat} value, the greater is the enzymatic activity. These catalytic parameters are summa-

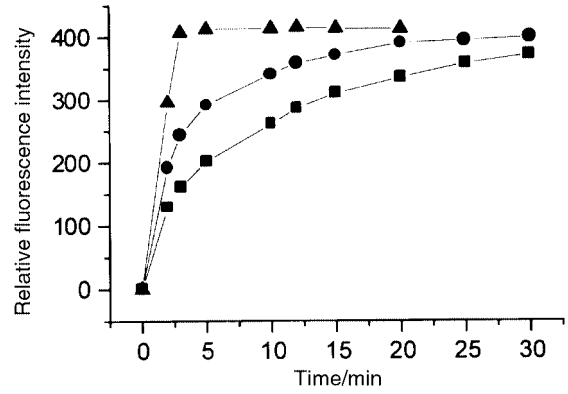


Fig. 4 Kinetic profiles of the reaction between 2.5×10^{-6} mol L^{-1} H_2O_2 and 1.0×10^{-3} mol L^{-1} *p*-HPPA in the presence of 2.0×10^{-6} mol L^{-1} FeTSPC (■), ONIPAAm–FeTSPc (●) and 5.0×10^{-7} mol L^{-1} HRP (▲).

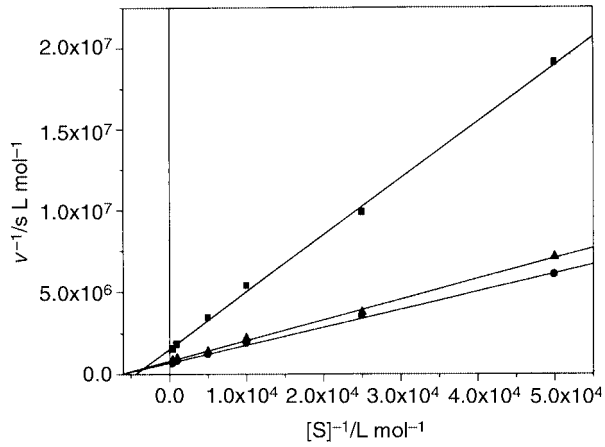


Fig. 5 Lineweaver–Burk plots for the free FeTSPc (■), ONIPAAm–FeTSPc (●) and HRP (▲) with *p*-HPPA as a substrate. The concentrations of FeTSPc, ONIPAAm–FeTSPc and HRP are 5.0×10^{-7}, 5.0×10^{-7} and 7.5×10^{-8} mol L^{-1}, respectively. H_2O_2 concentration = 1.0×10^{-4} mol L^{-1}.

rized in Table 1. It can be seen that the K_m values were 1.54×10^{-4}, 2.27×10^{-4} and 1.26×10^{-4} mol $L^{-1}$for ONIPAAm–FeTSPc, free FeTSPc and HRP, respectively. This indicates comparable binding of H_2O_2 to both mimetic enzymes just like HRP. While the catalytic activities of both mimetic enzymes are lower than that of HRP, the K_{cat} ratio of ONIPAAm–FeTSPc to free FeTSPc is about 2:1, *i.e.*, the catalytic activity of FeTSPc more than doubled when bound to the oligomer.

3.4 Recycling of ONIPAAm–FeTSPc mimetic enzyme

The recovery of ONIPAAm–FeTSPc and re-usability were also examined. Since ONIPAAm–FeTSPc conjugates precipitate from solution above the LCST, these molecules can be easily separated from reaction mixtures by simple removal of the supernatant. The catalytic reaction of H_2O_2 (1.0×10^{-5} mol L^{-1}) and *p*-HPPA (5.0×10^{-4} mol L^{-1}) was carried out for 25 min at 25 °C. The ONIPAAm–FeTSPc was precipitated and separated from the product by centrifugation at 37 °C for 10 min (10000 rpm). The recovered conjugate was re-used in a subsequent reaction. The catalytic activity of recovered mimetic enzyme in each cycle is shown in Fig. 6. It can be seen that the mimetic enzymatic activity remains at about 94% after the eighth cycle. This indicates that during the process of conjugate separation and recovery by changing the temperature, only a small amount of the mimetic enzyme was denatured.

3.5 Optimum conditions for the ONIPAAm–FeTSPc catalyzed reaction

The effect of pH on the catalytic reaction was examined and the results are shown in Fig. 7. It can be seen that the fluorescence intensity increased substantially with increasing pH, and maximum fluorescence was obtained when the final pH was in the range 10.8–13. Generally, there are two pH values that are important for the final analytical sensitivity in the enzyme-catalyzed fluorescence reaction: one is the pH for the enzyme-catalyzed reaction and the other is the pH yielding the greatest fluorescence intensity of the products. Our experimental results show that the optimum pH for the ONIPAAm–FeTSPc–HPPA system differs from those of the HRP–HPPA and FeTSPc–HPPA systems; the common element in these systems is that the final measurement pH must be ⩾10 for the best results. The optimum pH for the actual oxidation process in the HRP–HPPA system is 5.8, and that in FeTSPc–HPPA system is 1–7; typically the reactions are carried out around these pHs and base is then added to render the product fluorescent.[27] However, the ONIPAAm–FeTSPc system behaves differently: the reaction itself occurs best at a pH about 10.8, which is the same as the optimum pH for the final fluorescence measurement (Fig. 7). This indicates that the optimum pH for the product fluorescence is not affected by the reaction pH, *i.e.*, the catalytic reaction and final fluorescence measurement can be performed at the same pH value. This would significantly simplify the analytical procedure.

The effect of various buffer solutions on the ONIPAAm–FeTSPc–HPPA reaction system was also examined and the results showed that the peroxidase activity of ONIPAAm–FeTSPc in NH_3–NH_4Cl and Na_2CO_3–$NaHCO_3$ buffer systems was much higher than that in a glycine–NaOH buffer system, although their optimum reaction pHs were very similar. In this work, Na_2CO_3–$NaHCO_3$ buffer (pH 10.8) was adopted.

The influence of reaction time on the fluorescence development with the use of ONIPAAm–FeTSPc as catalysts was investigated (Fig. 4). The fluorescence intensity reached an approximately constant and maximum value after reacting for 25 min, and remained unchanged for at least 5 h. Here, the equilibrium method was chosen, and a reaction time of 25 min was adopted.

The effect of the concentration of *p*-HPPA on the catalytic reaction was also tested. The results showed that the fluorescence development of the system was maximum and constant when the final concentration of *p*-HPPA was in the range 2.0×10^{-4}–1.0×10^{-3} mol L^{-1}. In this work, a final concentration of *p*-HPPA of 5.0×10^{-4} mol L^{-1} was adopted.

3.6 Analytical performance

The possibility of using the proposed mimetic enzyme in the detection of both H_2O_2 and catalyst was studied. The calibration graph for the determination of mimetic enzyme was constructed under the optimum conditions described above with concentrations of *p*-HPPA and H_2O_2 in final solution of 5×10^{-4} and 2×10^{-4} mol L^{-1}, respectively. The results showed that a good linear relationship was observed between the relative fluorescence intensity and mimetic enzyme concentration, and the linear range extended from 3.0×10^{-9} to 4.0×10^{-8} mol L^{-1} ONIPAAm–FeTSPc. The detection limit (3σ, $n = 9$) was 1.7×10^{-9} mol L^{-1}. The correlation coefficient was 0.9986 and the relative standard deviation was 1.5% ($n = 6$) for determining 3.0×10^{-8} mol L^{-1} mimetic enzyme.

For the determination of H_2O_2, the concentration of *p*-HPPA and mimetic enzyme in final solution were 5.0×10^{-4} and 1.0

Table 1 Catalytic parameters with free FeTSPc, ONIPAAm–FeTSPc and HRP as catalyst and *p*-HPPA as a substrate

Mimetic enzyme	K_m/ 10^{-4} mol L^{-1}	V_{max}/ 10^{-6} mol L^{-1} s^{-1}	$[E_0]$/ 10^{-7} mol L^{-1}	K_{cat}/ s^{-1}
FeTSPc	2.27	0.65	5.0	1.3
ONIPAAm–FeTSPc	1.54	1.39	5.0	2.8
HRP	1.26	1.20	0.75	16

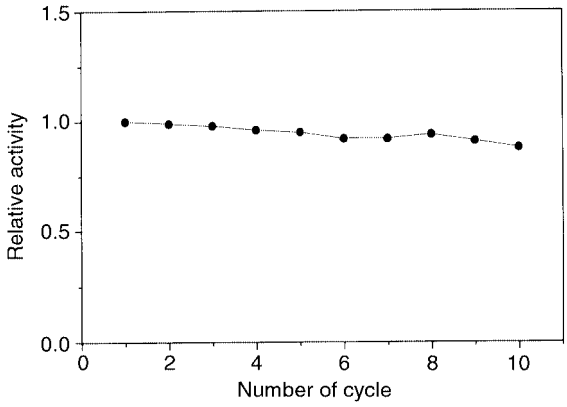


Fig. 6 Relative peroxidatic activity of ONIPAAm–FeTSPc re-used in repeated cycles of precipitation–dissolution.

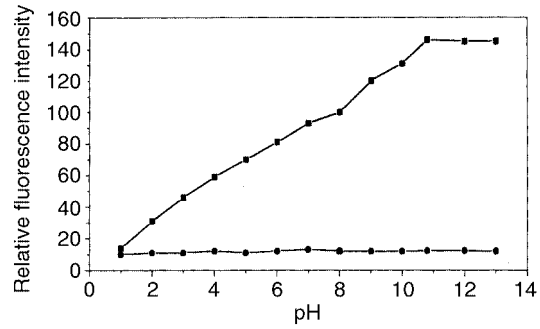


Fig. 7 Effect of pH on the fluorogenic reaction (■) with and (●) without ONIPAAm–FeTSPc as catalyst. Concentrations: $H_2O_2 = 1.0 \times 10^{-6}$, mimetic enzyme $= 1.0 \times 10^{-6}$ and *p*-HPPA $= 5.0 \times 10^{-4}$ mol L^{-1}.

$\times 10^{-6}$ mol L^{-1}, respectively. A good linear relationship was observed over the range 2.0×10^{-8}–1.5×10^{-6} mol L^{-1} H_2O_2. The detection limit (3σ, $n = 9$) was 8.2×10^{-9} mol L^{-1}.

4 Conclusion

ONIPAAm–FeTSPc, a water-soluble, thermally precipitated mimetic enzyme, was synthesized by a simple method. It not only mimics the catalytically active center of HRP but also mimics the environment of the active center. Therefore, compared with free FeTSPc, the proposed mimetic enzyme showed higher peroxidatic activity. The other advantage of ONIPAAm–FeTSPc is that the pHs for the product fluorescence and the catalytic reaction are the same, and the oxidation reaction and final fluorescence measurement can be performed at the same pH without any pH adjustment. Furthermore, with its LCST characteristic, ONIPAAm–FeTSPc is expected to provide not only a homogeneous catalytic reaction but also a simple heterogeneous separation process. This mimetic enzyme can be simply recovered and re-used by phase transfer, and this will be very useful in bioanalytical chemistry.

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