Metallo-Vesicular Catalysis: a Mixture of Vesicular Cysteine/Iron Mediates Oxidative pH Switchable Catalysis

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Abstract

The design of heme and non-heme active centers, which resemble peroxidase enzymes, has recently received a significant attention. Herein, a cystein-metal complex was designed and encapsulated in a vesicular mixture (1:4; SDS/DTAB) in order to imitate a *chloroperoxidase* (CLP) enzyme via chlorination of thionine at pH 3. This artificial enzyme behaved both as a catalase and CLP at pH 3, and as a peroxidase at pH 1. The shape of the metallo -vesicular catalyst at each pH value was obtained by dynamic light scattering, and was confirmed by transmission electron microscopy (TEM). The different fluidities as interior structure property of catalyst aggregates at pH 3 and 1 were obtained by differential scanning calorimetry (DSC). IR, ^1H and ^{13}C NMR spectroscopies were utilized to investigate the structural properties of the vesicular catalyst. These studies demonstrated that the cysteine/iron (III) center acted as a multifunctional catalyst. In addition, the sulfur and ammonium moieties of L-Cys interacted in the presence of metal ion at pH 1. However, the ammonium side chain was replaced with the carboxylate group at pH 3. Collectively, we engineered two distinct vesicular cysteinate- $\text{Fe}^{3+/2+}$ complexes, which functioned as a pH-switchable catalyst.

Keywords: pH-switchable catalyst; multifunctional catalyst; peroxidase activity; chloroperoxidase activity; catalase activity.

1. Introduction

Chloroperoxidase (CLP) is a versatile heme-based enzyme that behaves differently under various environments. CLP chlorinates substrates at pH 3, and performs as a classic peroxidase by oxidizing substrates at various pH values depending on the types of substrates. It also catalytically decomposes hydrogen peroxide in the absence of an appropriate substrate [1, 2]. The active site of CLP contains a prosthetic heme-iron (III) ligated to the thiol group of a cysteine residue. As with classic peroxidases, the CLP active site contains a polar distal pocket [3, 4]. These structural features enable the enzyme to carry out multifunctional reactions through the formation of a central intermediate, compound I. Formation of the compound I is common among all peroxidases, and the intermediate is considered to be an active form of CLP produced by cleavage of the O-O bond of the hydrogen peroxide through an acid-base mechanism [5, 6].

In the active site of CLP Glu183, which plays a crucial role in pH dependent activity of the enzyme, has multiple actions instead of the two His and one Arg residues present in a classic peroxidase. The carboxylic side chain of Glu takes a proton from incoming H_2O_2 and enables the enzyme to form compound I (Scheme 1) [7-9]. Previous studies have shown that complexes of Fe (III) and cysteine adopt various forms that can perform catalytic reactions. In an acidic environment, Fe (III) reduces L-cysteine to a cystine species that gives rise to distinct ^1H NMR signals [10-12].

Scheme 1- Mechanism of formation of compound I in CLP active site.

Fe (III) has a great potential for carbon bond activation via Fe (III)–H formation. The activation of C–H bond is a significant reaction in modern chemistry, and it is exclusively applied in organic and pharmaceuticals synthesis [13, 14]. Iron has a wide range of oxidative state, including ⁺IV, ⁺V, ⁺VII and ⁺VIII, all of which are labile in an aqueous environment, and mostly appear as oxidant. The rate of carbon bond activation is challenging since the C–H bond

is a strong bond [13, 15, 16]. Fe (II/III), as mentioned above, exhibits an outstanding power in oxidative activation. However, a powerful and robust bio-catalyst that shows activity at a low concentration, as well as improves the rate of transformation, is needed. These can generally be classified in three categories: rate of activity, selectivity and ambiguous mechanism [17-19].

The importance of nanostructures on dynamic functions including catalysts are now well recognized [20]. Catalytic nano-architectonics is the art of assembling the desired functional components according to the scale of catalytic events [21]. Vesicles and micelles, as self assembling aggregates, can be used via nano-architectonics to design and preparation of functional materials through molecular/ atomic dynamic manipulation and control [20]. Micellar or vesicular solutions are introduced as high performing scaffolds that can improve the catalytic behavior of a metallo-biocatalyst from the biomimetic aspect [22, 23]. Many studies have shown the advantages of micelles or vesicles in reactivity and selectivity of oxidative catalysis [22, 24-26]. The surfactant sodium dodecyl sulphate (SDS) forms one of the most interesting micellar scaffold, which efficiently improves heme-based catalysis [22, 23]. The cross-linked micelle is another recent scaffold that appears as a reactive hydrophobic pocket for encapsulation of transition metal-based catalysts with a suitable ligation [27]. An anionic surfactant is not generally able to form a homogenous solution at low pH, but the addition of a cationic compound derives the micellization [28].

The aqueous micellar mixtures are generally characterized by substantial conformational plasticity. Given their inherent structural flexibility, X-ray crystallography is not a suitable approach for studying the conformational or configurationally behavior of reacting reagents [29-31]. In contrast, NMR spectroscopy offers a unique opportunity for structural and dynamic studies of ligand binding and concurrent reactions [32-35].

Several factors can perform switching the function of material as artificial enzyme; like pH [36], temperature [37] , light [38] , etc. [36]. In this study, a pH-switchable multifunctional CLP-like biocatalyst was designed, embodying cysteinate iron (III) via mixed vesicle by taking advantage of the cationic and anionic surfactants, which resist a wide range of pH values. Herein, we have taken advantage of the chemical shifts and coupling patterns due to spin-spin splitting of assigned ^1H and ^{13}C NMR signals to probe the local structural propensities of cysteinate-iron (III) intermediates as CLP active-site.

2. Materials and methods

2.1. Materials

Chloroperoxidase (CLP) was purchased from Fluka. Hydrogen peroxide 30% (V/V), HCl 33% 12 M, potassium chloride and sodium acetate were purchased from Merck. FeCl_3 and thionine were obtained from Sigma. D_2O 99.8% was purchased from ARMAR Chemicals. Acetate buffer 100 mM at pH 3 and phosphate buffer 50 mM at pH 7.2, were prepared using CO_2 -free deionized water. The CLP stock solution was diluted with phosphate buffer (50 mM at pH 7.2) and stored at 4°C without further purification.

L-cysteine was added to the FeCl_3 solution in a 1:5 ratio, in acetate buffer at either pH 1 or pH 3 and mixed for 3 minutes to form self-assembled cysteine/iron complexes for catalyst preparation. Sodium dodecyl sulfate/dodecyltrimethylammonium bromide (SDS/DTAB) mixture was prepared by adding SDS (10 mM) to DTAB (40 mM) in a 1:4 ratio in the aforementioned buffer.

2.2. Methods:

Steady-State Kinetics: UV-Visible spectra of CLP were recorded using a Shimadzu-3100 spectrophotometer. The catalytic activities for chlorination of the enzyme and Fe(III)/L-Cys (10 μ M/50 μ M) mixture were obtained by following the chlorination of thionine (1-50 μ M) at 598 nm based on the method reported by Hager [39]. Thionine is used as a substrate instead of monochlorodimedon (MCD), the common substrate for chlorination, because it is more stable at low pH without showing any changes in its extinction coefficient. The reaction mixture contained 20 mM KCl, 2 mM H₂O₂, 10-50 μ M thionine and 3 nM CLP (or 10 μ M FeCl₃). The concentration of H₂O₂ was kept high (2 mM) in all experiments to obtain first-order kinetics. The progress of reaction was followed at various thionine concentrations (1-50 μ M), and the initial rate was used to draw the Michaelis-Menten diagram. Sequentially, using equation 1, the *Lineweaver–Burk* plots were obtained and used to measure the kinetic parameters (K_m (Michaelis-Menten constant) and V_m (maximum rate)) of chlorination (For more details please see Electronic Supplementary Information [ESI], Fig. S1).

$$\frac{1}{v} = \frac{K_m}{v_m [S]} + \frac{1}{v_m} \quad (1)$$

The Turnover number of CLP and Fe(III)-based mixture for chlorination of thionine were obtained using equation 2. Molar extinction coefficients for thionin is $\epsilon^{598} = 6.02 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

$$V_m = k_{cat} \times E_t \quad (2)$$

To measure catalase and peroxidase activities, hydrogen peroxide and pyrogallol substrates were used respectively. Peroxidase and catalase kinetics were measured as mentioned above. Oxidation of pyrogallol and decomposition of hydrogen peroxide were monitored at 420

nm and 240 nm, respectively. Molar extinction coefficients for pyrogallol and hydrogen peroxide are $\epsilon^{420} = 12 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $\epsilon^{240} = 43.6 \text{ M}^{-1} \cdot \text{cm}^{-1}$, respectively.

Suicide inactivation studies for native and artificial enzymes proceeded in the presence of thionine substrate at 598 nm and excessive amount of hydrogen peroxide at pH 3 as an index. Inactivation kinetic studies were processed as detailed by Ryabov [40]. In this regards, the kinetic of catalytic reaction at different H_2O_2 concentrations (2 mM up to 30 mM) were measured based on Lineweaver–Burk plots and the K_i inactivation obtained using Eq. 3³¹. K_m and V_m as enzymatic reaction parameters were determined for each H_2O_2 injection and the decrease in chlorination was recorded.

$$K_i = \frac{V_m}{K_m} \cdot \frac{1}{\ln[S]_{unreact} - \ln[S]_0} \quad (3)$$

Dynamic Light scattering (DLS, Malvern Instruments, UK) was used to determine the dynamic radii and zeta potential of the designed catalyst. Transmission electron microscopy (TEM) images were recorded on a ZEISS electron microscope operated at 100 kV.

Surface tension measurements were performed under standard conditions using Krüss K100 tensiometer equipped with a Wilhelmy plate and a platinum plate which underwent a flame-dry before each injection. Surface tension of pure water was recorded to calibrate the tensiometer.

Differential scanning calorimetry (DSC) measurements of vesicular Fe/L-Cys containing SDS (1 mM) /DTAB (4 mM) were carried out in a Nano-differential scanning calorimeter II (N DSC II, Calorimetric Scientific Corporation, Model 6100) with a heating rate of $2^\circ \text{C}/\text{min}$ during all scans from 25 to 95°C at pH 1 and 3. Samples were degassed and the heat capacity (C_p) was determined.

Infrared spectra (IR) were recorded on a Bruker Equinox 55 spectrometer equipped with a LiTaO₃ detector on the ATR cell. Samples containing “0.6 M L-cysteine, 0.6 M FeCl₃, 0.1 mM SDS and 0.4 mM DTAB” were prepared in acetate buffer at pH 1 and 3. Aliquots of these solutions (1 μ l) were then loaded on the ATR cell. Final spectra were obtained by subtraction of water absorbing bands. The table for IR assignments is provided in supplementary information.

NMR samples contained “30 mM cysteine, 12 mM Fe³⁺, 40 mM DTAB and 10 mM SDS”. All samples were prepared in D₂O, and pure HCl was added to samples in the NMR tube to reduce the pH. ¹H and ¹³C NMR data were collected with a Bruker Avance 300-MHz NMR spectrometer operating in Fourier transform mode at room temperature. A water pre-saturation pulse sequence (CPMGpr pulse) was used to suppress the water signal.

3. Results and discussion

Transition metals are desirable reagents, which can behave as mediators in the active sites of bio-complexes [41]. In this regard, Fe(III) ions are used as catalytic elements. Many studies have focused on the manipulation of an artificial enzyme that results in a chemo-enzyme, while others have focused on enzyme modifications to create an efficient catalyst. Alternatively, the development of a catalytic reaction may targets the design of the catalytic microenvironment. In this approach, the environmental parameters are changed in order to provide a suitable condition for triggering the reaction. The environmental parameters involved include temperature, pH, ionic condition, and metal salt. Here we provide an aqueous mixture consisting of Fe(III) at low pH to design a proper microenvironment for a catalytic reaction.

Our designed system contained Fe³⁺/cysteine as a catalytic center to mimic the active site of a native CLP. The cysteinate metal complex at pH 3 exhibited CLP-like activity with thionine

as substrate. It also performed both as a catalase, by decomposing H_2O_2 as substrate and as a classic peroxidase by oxidizing pyrogallol. The optimal catalase and CLP catalytic activity occurred at pH 3, however, the optimal peroxidase activity occurred at pH 1 in a new catalytic center.

The cationic and anionic surfactants showed different behavior in aqueous solutions as a result of head groups with opposite electrostatic charge. Consequently, pH dramatically influences the shape of such aggregates by changing the charge of head groups and their hydration [28, 29, 42]. Although, electrostatic forces and hydrophobic interactions play crucial roles in the formation of micelles or vesicles, the steric effects caused by bulky head groups also play an important role in determining the characteristics of the formed nano-structures [29, 30, 43]. Many attempts have been made to use micelles and vesicles to cover the pocket of an artificial enzyme. Such studies have shown that hydrophobic pockets enhance the catalytic activity of a functional center by stabilizing the intermediate complexes [24, 30, 44, 45]. The kinetic parameters (Table 1) demonstrated the pH-dependence of different catalytic activities of the artificial enzyme, and the enhancing effects (decreasing of K_m and increasing of K_{cat}) of adding a specific type of vesicular mixture (SDS/DTAB; 1:4 ratio), that indicates the facile contact between substrate and catalytic center existing in biocatalyst aggregates. The charged surface and hydrophobic interior of SDS/DTAB vesicle provides an organism that the positioning of charged or hydrophobic parts of active-site and substrate can locate next to each other and react at proper direction. The vesicular cysteine/ Fe^{3+} complex (as an artificial enzyme) reproduced 35%, 27% and 9% of the specific activities of the native enzyme for peroxidase, catalase, and chlorination (CLP), respectively.

Table 1. Kinetic parameters of native and designed catalysts

<i>Catalyst</i>	<i>K_m</i> (μ M)	<i>V_m</i> (μ M/min)	<i>k_{cat}</i> (min^{-1})	Specific activity (units/mg)
Native CLP (3 nM) as chloroperoxidase- pH 3	24	0.06	20	420 [39]
Fe(III) (10 μ M) + L-Cys (50 μ M) as chloroperoxidase- pH 3	26	0.022	0.0022	
Fe(III) (10 μ M) + L-Cys (50 μ M) + SDS (1mM) + DTAB (4mM) as chloroperoxidase- pH 3	22	0.03	0.003	38
Native CLP (3 nM) as peroxidase- pH 6	10	0.09	30	760
Fe(III) (10 μ M) + L-Cys (50 μ M) as peroxidase- pH 1	8	0.015	0.0015	
Fe(III) (10 μ M) + L-Cys (50 μ M) + SDS (1mM) + DTAB (4mM) as peroxidase- pH 1	7	19	1.9	266
Native CLP (3 nM) as catalase- pH 3	17.5	30	1000	200000
Fe(III) (10 μ M) + L-Cys (50 μ M) as Catalase- pH 3	28	32	3.2	
Fe(III) (10 μ M) + L-Cys (50 μ M) + SDS (1mM) + DTAB (4mM) as Catalase- pH 3	22	38	3.8	55000

The self-assembly of surfactant molecules constructs various micellar nano-structures [43, 46, 47]. The critical micelle concentrations (CMC) of SDS and DTAB, based on surface tension analysis (ESI, Fig. S2), were 4.8 and 9.1, respectively. Addition of 10 mM SDS to 40 mM DTAB had a great influence on CMC. The SDS/DTAB (1:4 ratio) initiated the aggregation at 3.1 mM at low pH (pH 3), and demonstrated the constructive role of cationic, as well as anionic surfactants when mixed in an aqueous medium (Fig. S2).

Using dynamic light scattering, we demonstrated that the radii or the shapes of the aggregations were pH dependent. As shown in Table 2, Mixing DTAB with SDS produced a large shift in the particle hydrodynamic radii toward the vesicle size [48]. Fig. 1 shows TEM images, which display the pseudo-spherical and lamellar shape of biocatalyst, indicates the vesicular type structure of biocatalyst. As shown at Table 2, addition of the iron-cysteine

complex to the SDS/DTAB vesicles induced swelling in the vesicles mainly at pH 3. This experiment confirmed that the iron-cysteine bound to vesicles near the charged surface.

Figure 1- TEM images of the artificial enzyme at a pH level of 3 (A) and pH 1 (B). The complex was prepared from Fe^{3+} (10 μM), cysteine (10 μM), DTAB (4 mM), and SDS (1 mM).

The size of the aggregates was increased by lowering the pH, in agreement with the structural changes observed in TEM images (Table 2 and Fig. 1). Zeta potential analysis determined that the net charge of the mixed complex was positive (Table 2).

Table 2. DLS parameters for the various species

<i>Species</i>	<i>diameter (nm)</i>	<i>diameter (nm)</i>	<i>Zeta-potential (mV)</i>	<i>Zeta-potential (mV)</i>
	(<i>pH 1</i>)	(<i>pH 3</i>)	(<i>pH 1</i>)	(<i>pH 3</i>)
Fe(III) (10 μM) + L-Cys (50 μM) + SDS (1mM) + DTAB (4mM)	220 nm	56 nm	+13	+61
SDS (1mM) + DTAB (4 mM)	180 nm	54 nm	+12	+36
Pure DTAB (5 mM)	20 nm	18 nm	+30	+27
Pure SDS (5 mM)	60 nm	45 nm	-20	-21

Furthermore, the net surface charge was less positive at pH 1, suggesting that more SDS lies within the vesicular complex at lower pH. The addition of cysteinate iron at pH 1 increased the size of vesicles without changing their zeta potential. This suggests that the cysteinate-iron complex resides within the hydrophobic region of the surfactant. The negatively charged head groups of SDS became neutralized at extremely low pH. This helped to place the head groups next to each other, and also lowered the repulsions between the positive DTAB head groups decreasing the zeta potential of the vesicular complex.

Fig. 2 shows DSC diagrams of SDS/DTAB Fe/L-Cys catalyst at pH 3 and 1. Melting temperature (T_m) is a parameter that is related to the thermal stability of SDS/DTAB Fe/L-Cys biocatalyst structure [49][49][49]. As can be seen in the figure, the T_m for vesicular biocatalyst is 62.5 °C and 39.4°C at pH 3 and 1, respectively, indicating different aggregate structure as well as different aggregate fluidity.

Figure 2-Calorimetric curve obtained from thermal scanning on SDS/DTAB Fe/L-Cys at pH 3 (A) and pH 1(B) after buffer subtraction

Infrared spectroscopy: IR spectra of cysteine/ FeCl_3 at pH 3 and 1 lacked the S-H stretching bands of free L-Cys, consistent with iron binding to the sulfur or Fe-S bond formation (Fig. 3, Fig. 4 and Table S1).

Figure 3- Infrared spectra of free cysteine and the cysteine-iron complex at pH 3. The spectrum of free cysteine shows a band at 2539 cm^{-1} which is characteristic of an SH moiety. The addition of Fe^{3+} eliminates this band, which indicates the formation of a Fe-S bond between Fe^{3+} and cysteine. For more details about IR assignments of L-Cys and Fe/L-Cys see ESI, Table S1.

The addition of Fe^{3+} led to a shift in the NH_3^+ (N-H) stretching mode of cysteine to higher frequency as a result of disrupting the hydrogen bond network of the free amino acids. Moreover, the NH_3^+ stretching mode (in iron/cysteine) showed a strong signal at pH 3, but it was replaced by an NH_2^+ stretching band at pH 1 (Figs 3 and 4).

Figure 4- Infrared spectra of free cysteine and the cysteine-iron complex at pH 1. The spectrum of free cysteine shows a band at 2551 cm^{-1} , which is characteristic of a SH moiety. The addition of Fe^{3+} eliminates this band, which indicates the formation of a thiol bond between Fe^{3+} and cysteine. For more details about IR assignments of SDS and DTAB see ESI, table S1.

The signals at $1730\text{ cm}^{-1}/1490\text{ cm}^{-1}$ corresponded to the asymmetric/symmetric stretching modes of the ring connected carbonyl group. The cysteine N-H bending mode shifted to the higher frequencies upon addition of Fe^{3+} at pH 3, whereas it shifted to lower frequencies upon adding Fe^{3+} at pH 1. These results suggested the presence of an electrostatic interaction between Fe (III) and the ammonium moiety of the ligated amino acid at pH 1. Previous studies have shown the same interactions via NH_3^+ in the presence of metals. However, lack of OH signal and the change in shape of carbonyl (C=O) stretching band suggested electrostatic interaction of the COO^- moiety with Fe^{3+} at pH 3. The COO^- stretching mode overlapped with the NH_3^+ bending mode due to COO^- interaction with an electrophile that shifts it to a lower frequency. These observations suggest that the carboxylate interacts with Fe^{3+} rather than the ammonium group. Given the $\text{pK}_a = 2$ of the carboxylate, the group is expected to be negatively charged at pH 3, and to interact with the positively charged iron ions. However, this capacity is lost at pH 1. The two conformational states appeared to be in equilibrium at both pH values.

IR spectroscopy is used to study vesicular structures, especially aqueous mixture of SDS/DTAB [29, 33]. Aggregation in this system is distinguished by a decrease in the wave number of negative polar head groups. The spectral band assignments are illustrated in Table S2. The addition of SDS to the solution led to the disappearance of the $\text{N}(\text{CH}_3)_3^+$ bending mode (Fig. 5). These results suggested that a strong interaction occurred between the charged groups of SDS and DTAB as indicated by the DLS data. In the presence of DTAB, the stretching mode of S=O shifted dramatically to lower frequencies, consistent with electrostatic interactions between the oppositely charged head groups of SDS and DTAB (Fig. 5 A, B).

A

B

C

D

Figure 5- Infrared spectra of SDS (A) and DTAB (B) did not show any pH-dependent changes. Spectra of the vesicular complexes were obtained at pH 1 (C) and pH 3(D) to investigate changes in the micro-environment. For more details about IR assignments of SDS and DTAB see ESI, table S2.

The electrostatic interaction between S=O and $\text{N}(\text{CH}_3)_3^+$ increases the length of the S=O bond and as a result it absorbs at lower infrared frequencies in the IR spectrum. The most significant change after addition of Fe^{3+} /cysteine complex to SDS/DTAB was the increase in the frequencies of the asymmetric and symmetric S=O bands. These results are attributed to

interaction of the Fe^{3+} /cysteine complex with the SDS head groups, which decreases the electrostatic interactions between the oppositely charged heads. The asymmetric S=O band is sensitive to changes in the types of neighboring head groups, and the obvious change in shape of the spectrum resulted from changes in the microenvironment [28, 33, 34, 50]. The splitting of this band results from electrostatic interactions of Fe^{3+} /cysteine with SDS, perhaps via the ammonium side chain. Fe^{3+} /cysteine contains an overall positive charge with an affinity toward polar S=O head groups. Thus, Fe^{3+} /cysteine is likely located between the negative head groups of SDS over the surface of vesicles. The IR spectrum of the vesicular Fe^{3+} /cysteine mixture exhibited no critical changes in the gauche/trans ratio upon lowering the pH.

^{13}C NMR Spectroscopy: The ^{13}C NMR spectrum of L-Cys is depicted in Fig. 6. Addition of FeCl_3 to the L-Cys solution resulted in signals that shifted up field. The main shifted signal was assigned to $^{13}\text{C}^b$ of the cysteine bond, which is adjacent to the S bound to iron. The signal assigned to $^{13}\text{C}^a$ was shifted downfield as a result of its loose bond change with $-\text{NH}_3^+$ or $-\text{COO}^-$, due to their interaction with metal ion, as suggested by the IR data. The $^{13}\text{C}^c$ signal at pH 1 appeared at its frequency in free L-Cys (Table S3). The cysteine/ Fe^{3+} mixture appeared to represent an equilibrium between bound and free L-Cys, with the equilibrium shifted toward complex formation. The spectra obtained at pH 3 were noisy as a result of apparent exchange broadening, and no signal was observed at the position of $^{13}\text{C}^c$ for free cysteine (Fig 6D). However, a new unassigned signal was appeared (Tables S3 and S4).

Figure 6- ^{13}C NMR spectrum of cysteine (A, C) and cysteine/FeCl₃ (B, D) in D₂O at pH 1 (A, B) and pH 3 (C, D).

^1H NMR Spectroscopy: The ^1H NMR spectrum of FeCl₃/L-Cys exhibited two sets of peaks compared with one set for free L-Cys (Fig. 7 and Tables 3, 4). One set corresponds to the oxidized L-Cys (cystine), and the other signal set is related to the L-Cys/Fe (II) complex. In fact, Fe(III) partially oxidizes the L-Cys into cystine but the remaining L-Cys formed a complex with reduced Fe(II).

Figure 7- ^1H NMR spectra of cysteine (A, C) and cysteine/ Fe^{3+} (B, D) at pH 1 (left side) and pH 3 (right side). The spectra exhibit two sets of $^1\text{H}^a$ and $^1\text{H}^{b2/b3}$ signals.

The $^1\text{H}^{b2/b3}$ (two diastereotopic hydrogens attached to C_β in Fig. 7 B and D) signals were partially shifted by 0.2 ppm as a result of sulfur-iron (S-Fe) bond formation (Tables 3, 4). In fact, such splitting patterns are arisen due to diastereotopicity of $^1\text{H}^{b2/b3}$ in which two sets of doublet of doublets were appeared in the corresponding chemical shifts (2.88-3.05 ppm). The remaining signals corresponded to free L-Cys, which were shifted up field owing to the break-up of hydrogen bond network of L-Cys dimers in the presence of Fe (II) and new bond formation (Scheme 2).

Table 3. ^1H NMR chemical shifts at pH 1

<i>cysteine protons</i>	<i>Chemical shift of cysteine in solution at pH 1</i>	<i>Chemical shift of cysteine/Fe^{3+} in solution at pH 1</i>	
$^1\text{H}^a$	3.91-3.94 ppm	3.99-4.02 ppm	4.09-4.12 ppm
$^1\text{H}^{b2/b3}$	2.88-3.04 ppm	2.86-2.98 ppm	3.01-3.27 ppm

Table 4. ^1H NMR chemical shifts at pH 3

<i>cysteine proton</i>	<i>Chemical shift of cysteine in solution at pH 3</i>	<i>Chemical shift of cysteine/Fe^{3+} in solution at pH 3</i>	
$^1\text{H}^a$	3.88-3.91 ppm	3.95-3.98 ppm	4.07-4.11 ppm
$^1\text{H}^{b2b3}$	2.88-3.04 ppm	2.68-2.82 ppm	2.85-3.10 ppm

Scheme 2- Hydrogen bond network of cysteine in solution at pH 1 resulting mainly from the dimerization of carboxylic acid side chains.

The results obtained from IR spectra also confirmed the lack of S–H vibrational mode. NMR spectra illustrated that a part of L-Cys molecule was oxidized, but there is a remaining concentration of L-Cys bound to Fe (II) via the thiol group. Thus, the lack of the S–H vibrational mode in IR spectra contributes to both oxidation and thiol bond formation of L-Cys (Figs 3 and 4). Furthermore, the 0.1 ppm shift of L-Cys methine proton ($^1\text{H}^a$) at pH 1 and 3 mainly responds

to the electrostatic interaction of ammonium and carboxylic side chains [52, 51, 34] (Tables 3 and 4). The 0.1 ppm shift of L-Cys methine proton ($^1\text{H}^a$) at pH 1 confirmed the interaction of ammonium moiety with metal ion. The same characteristics were observed at pH 3, but in this case the carboxylate side chain replaced the ammonium moiety in the complex. Addition of Fe^{3+} to the amino acid solution broke this network via interactions of Fe^{2+} and L-Cys charged side chains, and caused a 0.2 ppm downfield shift in signals as a result of cystine formation (Figs 7, B and D). This new microenvironment contributes to the 0.15-0.2 ppm up-field shift in signals corresponding to methylene group of L-Cys due to the formation of a complex by interactions between Fe (II) and cysteine (Figs 7, B and D). The spectra demonstrated that the addition of Fe^{3+} did not mainly lead to the oxidation of cysteine to cystine; the ^1H NMR spectrum of cystine exhibited more complicated spin-spin splitting of the $^1\text{H}^{a,b}$ signal. These findings are in good agreement with ^{13}C NMR analysis (Fig. 6).

The addition of cysteine/ $\text{Fe}^{\text{II/III}}$ to vesicular mixture solution: The addition of cysteine/ Fe^{3+} at both pH 1 and 3 led to changes in the ^1H NMR of SDS/DTAB signals (Fig. 8 C, D). The signals related to cysteine were more intense at pH 1 than pH 3.

Figure 8- (Top) ^1H NMR spectra of aqueous mixture of SDS/DTAB vesicles at pH 1 (A) and pH 3 (B). (Bottom) ^1H NMR spectra of aqueous mixture of SDS/DTAB vesicles following the addition of Cys-Fe $^{3+/2+}$ at pH 1 (C) and pH 3 (D).

According to two distinct environment; hydrophobic and hydrophobic parts of vesicle, the difference of ^1H NMR of cysteine signals can be explained by the location of a part of cysteine inside the vesicle hydrophobic pocket at pH 3, whereas the complex is adsorbed to the charged vesicle surface at pH 1 (Scheme 3).

Scheme 3- Schematic interpretation of vesicular Fe-cysteine biocatalyst structures in different pH and three different enzymatic activities.

The cysteinate iron (III) complex mechanism of action: The active site of CLP contains a specific carboxylic moiety, which behaves both as a hydrogen acceptor and a hydrogen donor. This enables the enzyme to form compound I as an initial intermediate for a subsequent chemical reaction. In our designed complex, the carboxyl group of cysteine plays an important role in catalysis at pH 3. The ^1H NMR spectrum obtained during the addition of H_2O_2 to the artificial enzyme showed significant changes in the position of signals corresponding to the $^1\text{H}^a$ and $^1\text{H}^{b2.b3}$ protons of L-Cys (Fig. 9). $^1\text{H}^a$ showed a single signal in the presence of hydrogen peroxide (ESI Fig. S3, Fig. 9). In terms of the mechanism at pH 3, after peroxide binds to the cysteinate- Fe^{2+} complex (Scheme 4), the carboxylic group of cysteine captures a hydrogen from the β -oxygen of H_2O_2 and loses its interaction with iron. In the second step, the loss of hydrogen to distal oxygen of peroxide occurs to provide an appropriate leaving group on the peroxide. However, the carboxylic group becomes protonated at pH 1 (Scheme 4), which enables it to serve as a hydrogen donating agent and the ammonium group of cysteine comes into play as a nucleophile. Chemical shift of the signals corresponding to the methylene (CH_2) and methine (CH) protons along with splitting patterns confirmed the changes that occurred around the chemical microenvironment of L-Cys side chains. In fact, interaction between hydrogen peroxide and Fe (II/III)/L-Cys complex caused a shift toward the low-field due to the de-shielding effect of oxygen bonding (ESI, Fig. S3).

Scheme 4- Mechanistic diagram for designed catalyst at pH 1 (A) pH 3 (B).

These findings confirmed our interpretation that a simple cysteine/iron (II) complex, instead of a cysteine/ Fe^{3+} complex, serves as a catalyst (Fig. S3). Based on recent achievements, the mechanisms of many C–H activating reactions remain unclear [53, 13]. Thus, further research concerning the design of a more robust Fe-based catalyst should contribute to mechanistic studies. The combination of Fe-based catalyst/oxidant ligands may open a window toward the design of carbon bond activating catalyst. The $^1\text{H}^a$ signals of cysteine in the cysteinate- Fe^{3+} complex decreased in intensity when hydrogen peroxide was added. These results are consistent with our proposed mechanism in which polar groups of cysteine play critical roles in the pH-dependent mechanism of the catalyst. All structural experiments showed bidentate binary coordination of Fe^{2+} and cysteine. Mechanistic studies implicated the carboxylic moiety as a central agent during the formation of compound I for chlorination activity. In light of these phenomena, the carboxylic group (COO^-) acts both as a hydrogen acceptor and a hydrogen donor (pH 3) to create oxidized $\text{Fe}=\text{O}$ intermediates, during the chloroperoxidase and catalase activities. However, in case of peroxidase activity, the ammonium moiety takes over at pH 1 (Scheme 3).

Suicide inactivation: Hydrogen peroxide appears as an electron source in oxidative reactions like peroxidase catalysis. H_2O_2 reduces to H_2O during catalytic reaction but high concentrations of hydrogen peroxide is able to destroy the protoporphyrin IX/Fe interactions and inactivate the enzyme via a mechanism called suicide inactivation [54]. Based on the mechanism of CLP enzyme, porphyrin interacts with H_2O_2 and releases molecular water, and forms a compound I intermediate [54]. Compound I is a recognized species that turns to oxo-Fe (III) specie, called compound II. Excessive amount of H_2O_2 cleaves the pyrrole rings and destructs the compound II

structure [40, 55]. Fig. 10 shows high concentration of H_2O_2 (> 10 mM) inactivates the enzyme, which contributes to decreased efficiency.

Figure 10- Inactivation constant (K_i) versus hydrogen peroxide concentration for native CLP (Black) and Fe-based vesicular catalyst (Gray) at pH 3.

Based on previous studies, CLP is inactivated at high concentrations of H_2O_2 (over 7 mM) in the presence of monochlorodimedon (MCD) as substrate [40]. In contrast, Fe/L-Cys/vesicular catalyst resisted high concentration of H_2O_2 , and it revealed the protective role of vesicular scaffold against suicide solvent. The vesicle has a potential for catalyst protection but it would protonate with high level of H_2O_2 . We then imaged the metallo-vesicle solution after exposing to the high concentrations of hydrogen peroxide. DLS analysis indicated that vesicular scaffold radii decreased after addition of H_2O_2 (30 mM) and affected the catalytic ability of our designed catalyst (Fig. 11).

Figure 11- Dynamic radii of vesicular solution (metallo-mixed vesicle involving Fe(III)/L-Cys/DTAB/SDS) versus H_2O_2 concentration.

High-valent species such as oxo-Fe(IV) complex, a reactive intermediate in catalase, peroxidase, and chloroperoxidase reaction, can also catalyze hydroxylation of the C-H bond [15, 13]. If DTAB surfactant is hydroxylated, DTAB vesicles may dissociate during catalase reaction due to loss of hydrophobic interactions, and the stability of the SDS/DTAB micelle tend to disappear in the presence of high concentrations of H_2O_2 . Although, Metallo-mixed vesicle catalyst introduced a robust catalysis, there is much need for a more stable and reactive catalyst.

4. Conclusion

A new pH switchable *chloroperoxidase*, peroxidase, catalase-like biocatalyst system containing cysteine- iron complex encapsulated in a vesicular mixture (1:4; SDS/DTAB) was designed and

found to have good activities, and protection of the catalyst center at higher concentrations of hydrogen peroxide respect to the native CLP enzyme. We chose Fe^{3+} /cysteine as a catalytic center to mimic native CLP, because it provides thiol binding and a carboxylic group. ^1H NMR elucidated the mechanism behind the pH-dependent activity of the artificial enzyme by implicating NH_2 and COO^- as hydrogen acceptors in the first step leading to the formation of pseudo-compound I in the presence of a peroxide substrate. The pH effectively determines the type of catalytic agent by changing the protonation state of the carboxyl group and the iron ligation pattern between pH 1 and pH 3 as elucidated by NMR and IR experiments. However, the important role of pH parameter on each active-site, intermediate structures or the overall shapes of current biocatalyst will result the variety of biocatalyst functions. Scheme 3 interprets a summery for structures and enzymatic functions of vesicular Fe-cysteine biocatalyst in different pH. The present designed biocatalyst provides a low cost and stable multifunctional Fe (II/III) vesicular catalyst.

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pH 3

Catalase activity

Chloroperoxidase activity

pH 1

Peroxidase activity