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Preintercalation of Layered γ-Zirconium Phosphate for Preparation of Immobilized Hemoglobin

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Abstract: Layered γ-Zirconium phosphate **(** γ-ZrP**)** preintercalated with butylamine, tetra (n-butylammonium)hydroxide, dimethylamine respectively, or only ultrasonificated, for preparation of immobilized hemoglobin were investigated in this report.

Keyword: γ-Zirconium phosphate, preintercalation, exfoliation, hemoglobin immobilization.

Nowadays, intercalation chemistry remains a distinctive subject of considerable interest because of its encouraging application in many fields such as heterogeneous catalysis, nonlinear optics, solid-state protonic conductors and specific absorbents[1]. Layered zirconium phosphate owns special merits to be used as host matrix for protein. γ-Zirconium phosphate is known for its large interlayer distance (1.22 nm), rigid layer and small surface density of active sites on the side of a layer compared with α-Zirconium phosphate. Till now, the background influence due to different amines, such as butylamine[2,3] (BA), dimethylamine[4] (DMA) and tetra (n-butylammonium) hydroxide[5] (TBA), intercalation behavior on the immobilization of protein has not yet been clarified. So far **γ-**ZrP has not been used for immobilization of protein by intercalation. It is necessary to take a fundamental research on the preintercalation with amines for immobilization of Hb in the galleries of γ-ZrP**.**

Experimental

γ-ZrP synthesized based on the procedure described in literature[6] was dispersed in phosphate buffer solution. After the addition of amine in a given molar ratio of amine to γ-ZrP, the mixture was ultrasonificated for 20 min. If γ-ZrP was not processed with amine, the mixture was directly ultrasonificated for 20 min. The pH was adjusted to desired value with KOH and HCl solution. The layered phosphate was then contacted with Hb solution (10 mmol/L) and shaken for 4 h. The enzyme-matrix conjugate was collected by centrifugation (12000 rpm) and the concentration of Hb in the supernatant was measured by visible absorption at 405 nm and the Hb retained on the matrix could

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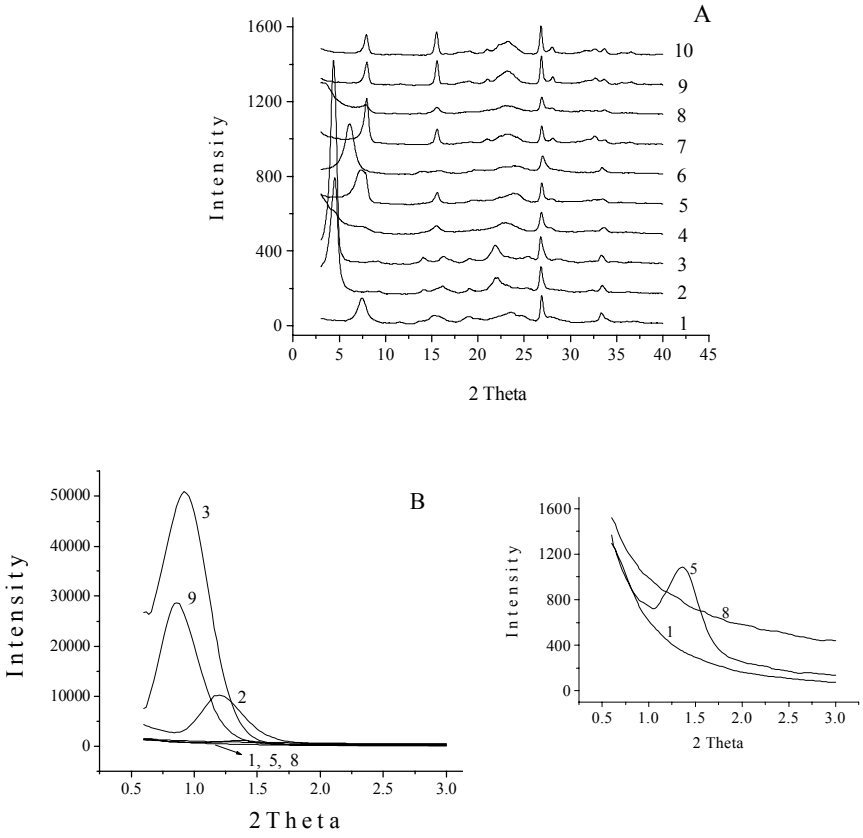
be calculated. The resulted complex was then sequentially washed with phosphate buffer, 1 mol/L NaCl solution and finally with buffer again (the times of washing was dependent on the desportion behavior of Hb

Figure 1 XRD patterns from 3° to 40° (A) and from 0.5° to 3° (B)



(1) γ-ZrP (2) BA (1:5 m.r.)-γ-ZrP, (3) BA (1:10 m.r.)-γ-ZrP, (4) Hb-BA (1:5 m.r.)-γ-ZrP, (5) DMA (1:2.5 m.r.)-γ-ZrP, (6) DMA (1:10 m.r.)-γ-ZrP, (7) Hb-DMA (1:10 m.r.)-γ-ZrP, (8) TBA (1:2.5 m.r.)-γ-ZrP, (9) γ-ZrP pretreated with ultrasonification only, (10) Hb-γ-ZrP. All intercalated γ-ZrP samples were prepared in low ionic strength.

on different matrices). Dry products of immobilized Hb on γ-ZrP were obtained by lyophilizing the samples.

Although it was a simple acid-base reaction between the host and the guest-amine, different systems displayed wide intercalation behavior, which would influence the amount and the enzyme activity of the immobilized Hb. We not only studied the adsorption, desorption during washing, catalytic activity and thermal stability of immobilized enzyme, but also discussed the effects of pH, ionic strength and

concentration of amine on the intercalation and immobilization behavior of these systems. The changes in the interlayer distance were examined by X-ray powder diffraction (**Figure 1**). In this experiment, an approximate measurement of the degree of enzymatic activity was the ratio of the concentration of the catalytic reaction product under different concentrations of substrate (guaiacol) to the quantity of the protein-matrica complex.

Table 1 The effect of different concentration of amine on Hb immobilization

Amine	Ionic strengh	The molar ratio of γ-ZrP to amine	Concentration of Hb in supernatant (μmol/L)
Butylamine	low	1:2.5	0.109
		1:5	0
		1:10	0
	high	1:5	0.948
		1:10	0.025
Dimetylamine	low	1:2.5	0.163
		1:5	2.65
		1:10	4.30
	high	1:2.5	4.26
		1:5	4.08
		1:10	4.62
Tetra (n-butyl-ammonium)hydroxide	low	1:1	4.33
		1:2.5	1.80
		1:5	1.31
	high	1;1	1.47
No amine prcess (ultra-sound treatment only)	low		3.17
	high		4.96

Results and Discussion

The driving forces for protein immobilization mainly included two kinds of contribution[7]. The first one was electrostatic interaction, in which the $-NH_2$ groups of protein reacted with P-OH groups of γ-ZrP by exchange with protonated amine[8]. In the case of γ-ZrP ultrasonificated only, the protein directly intercalated into γ-ZrP by neutralizing interlamellar P-OH groups. The fact that the adsorption could be altered through pH or electrolyte concentration proved the existence of electrostatic interaction. On the other hand, nonelectrostatic driving forces also contributed to the adsorption, especially in the case of Hb-BA-γ-ZrP**.** Increment of BA intercalated into interlayer region was accompanied by d-enlargment (**Figure 1**) without exfoliation because the prevalent hydrophobic interaction arose from hydrocarbon chain. γ-ZrP intercalated with BA, which finally formed bilayer, could be considered as a hydrophobic sponge[1] to absorb hemoglobin molecule. **Table 1** demonstrates not only a larger Hb adsorption in low ionic strength but also an increment of Hb uptake with increasing amount of BA preintercalated into γ-ZrP. In the case of TBA, being strong base with large cations in low ionic strength, it initially produced intercalation compound, by addition of excess base, cation-cation repulsion caused delamination of the solid which then transformed into unilamellar colloids[5]. The intercalation of TBA could not be observed from XRD patterns (**Figure 1**) due to the formation of an amorphous phase. Because TBA was a

good "leaving group" with its low charge density[5] and could be displaced by proteins, so the immobilization of Hb on the surface of single sheet was mainly driven by electrostatic interaction. DMA displayed a richer behavior than others. Being an alkylamine, it gave an effect on the intercalation of Hb as BA did[4] in spite of the narrow expansion of the interlayer distance. It also exfoliated γ-ZrP in a little degree than TBA did. Some experimental facts, for example, the disappearance of low angle reflection peak of DMA (1:10 m.r.)-γ-ZrP (For simplicity, the number followed by acronym (m.r.) was used to express the molar ratio of γ-ZrP to amine thereafter.) (**Figure 1**) and the lower Hb loading (**Table 1**), provided evidences to the supposition of the presence of contracted interlayer distance when the addition of DMA increased to some extent. The binding of Hb could be not only in the galleries but also on the outside of the stacks of lamellas. It fell in between the cases of BA and TBA. The intercalated water molecule in γ-ZrP pretreated with ultrasonification only formed a hydrophilic atmosphere. The observation of largest loading of Hb on BA-γ-ZrP and almost the smallest Hb loading of Hb-γ-ZrP (**Table 1**) were consistent with the fact that protein tends to adsorb on hydrophobic surface[2].

Due to the largest degree of interlayer distance enlargement and the hydrophobicity of the butyl group, the superiority of BA was obvious. Bulk immobilization with better activity retention has been achieved by preswelling the starting material γ-ZrP with BA. Another striking advantage that has also been found in this experiment was its protective effect on pH and thermostability of the protein upon immobilization. Comparing with α-ZrP, the large dimension of the interlayer region and the rigidity of the layers make γ-ZrP a good precursor to produce novel well-designed functional materials in intercalation manner. It also presents a potential protein immobilization technique[9].

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