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Effect of pH and Phosphate on Trapping Capacity of Various Heavy Metal Ions With Ferritin Reactor in Flowing Seawater

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Abstract

We describe a protein reactor consisting of native liver ferritin of *Dasyatis akajei* (DALF) and a dialysis bag. Our goal was to study a ferritin reactor for its capacity to trap various heavy metal ions (M^{2+}) in flowing seawater. The reactor is sensitive and inexpensive and can be operated by nonprofessional technicians. A positive relationship between the number of trapped M^{2+} with the DALF reactor and its concentration in the flowing seawater was observed. Both the pH in the medium and the phosphate content within the ferritin cavity strongly affected trapping capacity. It was found that the ferritin released its phosphate compound directly with a shift in pH without the need for releasing reagent, which differs from the phosphate release characteristics of horse spleen ferritin, as previously described. This behavior evidently makes the trapping capacity with the ferritin reactor weaken, indicating that this trapping capacity is tightly connected to its phosphate compound. Our study shows that a self-regulation ability of the ferritin shell rather than its phosphate compound plays an important role in controlling the rate and capacity of trapping M^{2+}. The ferritin reactor was constructed to monitor the contamination level of M^{2+} in flowing seawater. Our preliminary data along with fieldwork indicate that the DALF reactor is an analytical means for effectively monitoring the contamination level of M^{2+} in flowing seawater.

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Index Entries: *Dasyatis akajei*; heavy metal ions; ferritin reactor; phosphate effect; seawater; contamination level.

Introduction

Ferritin, which is ubiquitously distributed in animals, plants, and bacteria, plays an important role in iron metabolism in vivo. It can sequester the excess iron in a cell in a nontoxic form and then supply it to the metalloenzymes when needed *(1–3)*. Although ferritins from different species show low sequence identity, they exhibit similar three-dimensional structures. Ferritin is composed of 24 subunits assembled into a spherical shell with 432 symmetries *(2)*. In mammals, ferritin consists of two subunits with different types, called H and L, which are responsible for rapid Fe (II) oxidation and Fe (III) nucleation, respectively *(1–3)*. Ferritin from different tissues or organs has a varied subunit proportion of heavy to light chains. In plants and bacteria, only one type of subunit has been described, which shares the characteristics of both the H and L subunits *(3)*. Unlike the highly conserved protein shell, the inorganic composition within the protein core coming from the different species varies greatly, indicating ratios of phosphate to iron within the protein core of 1:13 (pig spleen ferritin [PSF]), 1:9 (horse spleen ferritin [HSF]), and 1:1.2 (bacterial ferritin of *Azotobacter vinelandii*, AvBF), respectively. In addition, most of the phosphates within the core of mammalian ferritin are believed to coat the surface of the iron core *(2)*. By contrast, the phosphates within the core of bacterial ferritin are found to be heterogeneously distributed, indicating that the phosphate structure within the core is more disordered than that of mammalian ferritin *(4)*.

DALF, the ferritin isolated from the liver of the marine fish *Dasyatis akajei*, is composed of two subunits, H and L. Studies show that DALF differs from other mammalian ferritins in the distribution of phosphate within the protein core, and that it has a phosphate-rich iron core surface (phosphate:iron ratio of 1:2). Otherwise, DALF shares the same characteristics in kinetics of iron release and molecular structure with the other ferritins *(5)*.

In the cell, ferritin sequesters free iron, thus detoxifying it. Previous studies have also found that ferritin has the ability to trap M^{2+}. In addition, Price and Joshi *(6,7)* reported that phytoferritin could bind large amounts of B^{2+}, Zn^{2+}, Cu^{2+}, and Cd^{2+} and act as a metal ion detoxicant. Pead et al. *(8)* found that heavy metal ions such as Cd^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+} were able to bind to apo-HSF, reconstituted HSF, and native HSF. Furthermore, uranium oxide ion (UO_2^{2+}), iron oxide magnetite (Fe_3O_4), manganese oxide (Mn_3O_4), cobalt oxyhydroxide (Co[O]OH), FeS, and CdS could be incorporated within the ferritin nanometer cage under the controlled reaction conditions *(9–14)*. In addition, Huang et al. *(15)* established a reactor of reconstituted PSF and used it to trap Cd^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+} in flowing seawater synchronously.

Normally, the contamination levels of M^{2+} in the effluent from city chemical factories and from other manufacturers are controlled strictly with reference to the contamination standard before drainage. However, this is not always effective for controlling low levels of environmental contamination because of lack of knowledge of the decontamination capacity of rivers and oceans. Further studies indicate that the contamination level rises because the total M^{2+} load exceeds the self-purification capacity of inland seawater *(16,17)*. It is advisable to limit the total amount of metal ion waste from an entire city or an appointed area rather than the individual contamination from an appointed factory, which is a truly effective means for controlling the contamination level in the source of water (or seawater). However, it is difficult to assess this capacity because it requires a perfect technology for continuously monitoring the entire level of contamination in a river or an ocean over the course of a few days or weeks.

Preliminary studies have shown that HSF could trap M^{2+} such as Ni^{2+}, Co^{2+}, Mn^{2+}, and Zn^{2+} and release relatively unstable iron within the protein core *(18)*. Like DALF, mammalian ferritins can withstand a medium with a variable pH range from 2.0 to 10.0, a reactive temperature up to 70°C, and a high concentration of urea or guanidinium chloride *(2,3,15)*. To take advantage of these characteristics, we constructed a reactor to assess the self-purification capacity of seawater and the total M^{2+} amount of environmental load in an individual river or ocean according to the desired time such as the tide and ebb tide.

In the city of Xiamen, China, other researchers have found that M^{2+} (Cu^{2+}, Cd^{2+}) pollution is very serious in the eastern sea area, greatly endangering human health and fish-breeding aquatics *(19)*. On the other hand, previous studies have shown that the ferritins can trap Cu^{2+} and Cd^{2+} ions, as many as 99 and 275 per molecular ferritin *(8)*, which would make them easily analyzed. For this reason we employed Cu^{2+} and Cd^{2+} as a research model to study the trapping capacity of M^{2+} with a DALF reactor.

Previous studies had shown many factors could make ferritin release its iron and phosphate in the core *(5,15)*, so we suggest that alteration of environmental medium in flowing seawater affect stability of iron core. We selected two important factors, inorganic phosphate and pH, for studying this capacity variable in flowing seawater. The results show a positive relationship between the number of trapped M^{2+} with the DALF reactor and its concentration in the flowing seawater. Both the pH in the medium and the phosphate content within the ferritin cavity strongly affected the trapping capacity. It was found that the ferritin released its phosphate compound directly with a shift in pH without the need for a releasing reagent, which differs from the phosphate release characteristics of HSF, as previously described. This behavior caused the trapping capacity of the ferritin reactor to weaken, indicating that this capacity is tightly connected to the phosphate compound. Nevertheless, we strongly believe that once it is improved further the ferritin reactor can be used to monitor continuously the level of M^{2+} contamination in the sea and to evaluate the

decontaminant capacity of M^{2+} contamination in an appointed area during the flood tide and the ebb tide.

Materials and Methods

Fish and Chemicals

The live fish *D. akajei* was purchased from Xiamen Fish Company. DEAE-cellulose 52 was obtained from Waterman, Sephadex G-25 and Sephacryl S-300 HR were products of Pharmacia (Uppsala, Sweden), and other inorganic chemicals were analytical-grade reagents obtained from commercial sources in China.

Isolation and Purification of DALF

The isolation and purification of DALF were done as described previously *(5)*. Briefly, fresh liver was homogenized in 2 vol of distilled water, and then the resultant supernatant was denatured by heat treatment (70°C) for 10 min. Subsequently, the homogenate was quickly cooled to 4°C and centrifuged at 5000*g* for 30 min. The supernatant was precipitated with a solution of 50% saturated ammonium sulfate and refrigerated at 4°C overnight. The next day, the supernatant was centrifuged at 10,000*g* for 30 min and brown sediment was collected. The sediment was then resuspended in 0.025 *M* Tris-HCl buffer (pH 7.0) containing 0.05 *M* NaCl and dialyzed against the same buffer overnight to remove $(NH_4)_2SO_4$. After being fully dialyzed, the crude ferritin extract was purified by anion-exchange chromatography on a DEAE-cellulose 52 column (2×20 cm) and size-exclusion chromatography on a Sephacryl S-300 HR column (1.5×100 cm), respectively. Before the column purified the DALF, the separating system was equilibrated with 0.025 *M* Tris-HCl buffer (pH 7.0) containing 0.05 *M* NaCl and eluted with the same buffer. The purified DALF shows electrophoretic purity before use.

Determination of Chemical Elements and Protein

Protein concentration was determined by the Lowry method with bovine serum albumin of 99% purity as the standard *(20)*. Total Fe^{3+} numbers and other M^{2+} such as Cu^{2+} and Cd^{2+} within the ferritin shell were determined using an atomic absorption spectrophotometer (Analyst 800 Spectrometer; Perkin Elmer) equipped with a carbon furnace after the protein was nitrified. The content of phosphates in the core was determined by an improved phosphomolybdate method described by Cooper *(21)* and Huang et al. *(22)*. Based on the DALF molecular weight of 400 kDa *(5)*, the numbers of the iron, phosphate, and trapped M^{2+} per molecule of DALF can be calculated by spectrophotometry.

Preparation of apoDALF and Reconstituted DALF

apoDALF was prepared by anaerobic reduction with sodium dithionite and chelation of the reduced free ferrous ions with bipyridine followed by

rapid passage through a Sephadex G-25 column (2.0 × 20 cm) to remove the dissolved iron and phosphate *(5)*. Ferritin iron reconstitution solutions were buffered with 25 m*M* Tris, 0.1 *M* NaCl at pH 7.0. For ferritin iron reconstitution, experiments were conducted in the presence of inorganic phosphate; a similar buffer was prepared with 1 m*M* potassium phosphate added. Freshly prepared ferrous ammonium sulfate was added to the apoDALF solution at a ratio of 100 ferrous ions/DALF molecule every 10 min with rapid stirring to supply adequate O_2. The pH in the reaction medium was maintained using 0.05 *M* NaOH. The iron reconstituted in the presence of phosphate required additional phosphate at a P_i:Fe ratio of about 4:1 after ferrous ions were added to the apoDALF solution. After incubating for the desired time, the reconstituted DALF was collected by gel filtration column chromatography (2.0 × 20 cm column of Sephadex G-25 as already described), and then the protein concentration and the contents of iron and phosphate within the protein shell were determined using a spectrophotometer equipped with a computer, respectively *(23)*.

Analysis of M^{2+} Trapped by DALF Reactor

For laboratory experiments, solutions consisting of various heavy metal ions such as Cu^{2+} and Cd^{2+} were prepared using a different pH medium. The DALF within the reactor was loaded on a column equipped with Sephadex G-25 gel exclusion to separate the free metal ions prior to analysis of the elements. The numbers of the chemical ions Cu^{2+} and Cd^{2+} trapped by the DALF in the reactor were determined by atomic absorbance spectrometry, respectively.

For fieldwork, two monitoring sites, Huang Cuo (HC) and Huang Land (HL), in the city of Xiamen, China, were selected to study the capacity of the ferritin reactor to trap M^{2+} because both sites are famous resorts and effluent sites for industrial wastewater. The DALF within the reactor is separated by a normal membrane technology equipped with an obstructing molecular weight of 3000 Daltons. The numbers of ions trapped by the DALF and their concentration in the flowing seawater were determined by atomic absorbance spectrophotometry according to the desired reaction time. In addition, all experimental results shown in Figs. 3–8 were established to be average data for three measurements at the same time.

Design of DALF Reactor and Field Monitoring Equipment

The DALF reactor device shown in Fig. 1 consists of DALF and a dialysis bag of fixing protein, which dissociates M^{2+} in the flowing seawater, enabling it to pass in and out of the bag by diffusion. The DALF reactor is fixed in a reactive container (Fig. 1B). The constant velocity of flow in the container is provided by a magnetic stirrer (Fig. 1D) and a magnetic bar (Fig. 1C). The seawater is circulated from the inlet (Fig. 1E) to the outlet (Fig. 1F) by a micropump equipped with a thermostat.

Figure 2 shows a simple monitoring device for fieldwork. The framework of the device was constructed of stainless rebar steel, and it was covered with a 0.5-cm mesh of iron netting to prevent damage to the DALF

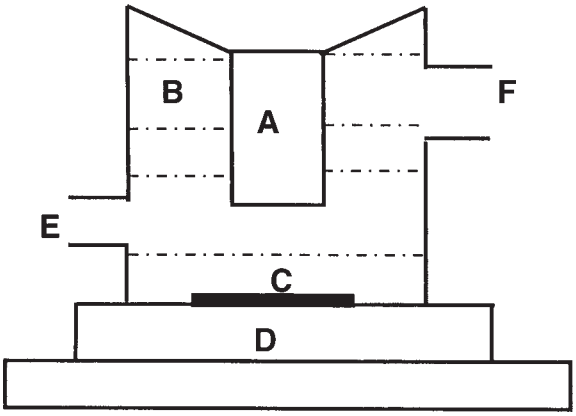

Fig. 1. Ferritin reactor. A, Dialysis bag (DALF + semipermeable membrane); B, container with buffered metal solution; C, magnetic bar; D, stirring motor; E, inlet; F, outlet. The speed of flowing water containing 5% NaCl supplied by a pump was 12 L/min at 25°C. The column volume of the ferritin reactor was 22 (length) × 10 (diameter) mm.

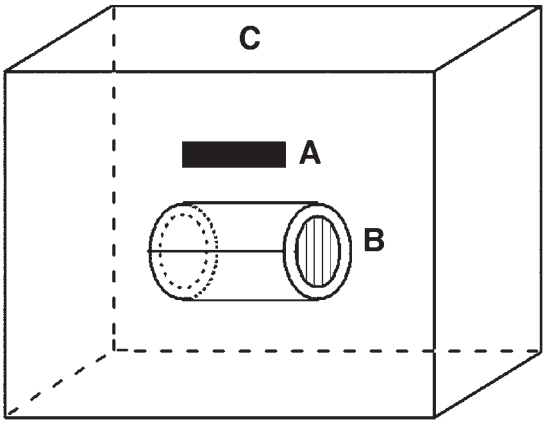

Fig. 2. Simple device for field monitoring. The framework (C) was constructed of steel bars and covered with 0.5-cm iron netting. The DALF reactor (B) was fixed in the center of the cage, with two ends enclosed with semipermeable membrane. A water flow meter (A) was used for measurement of flux passing through the DALF reactor. The buoy system above the cage is not shown. The column volume of the reactor was 33 (length) × 8 (diameter) mm. The container volume containing seawater was 120 (length) × 30 (width) × 60 (height) mm.

reactor by fish and suspended solids in the flowing seawater. The DALF was placed in a polyethylene centrifuge bottle with its bottom removed and a semipermeable membrane enclosing its two ends. The bottle was then mounted securely in the center of the cage. A flow meter and buoy system was equipped to measure the flow velocity of seawater and to determine the depth where the DALF reactor was placed under the seaplane. Accord-

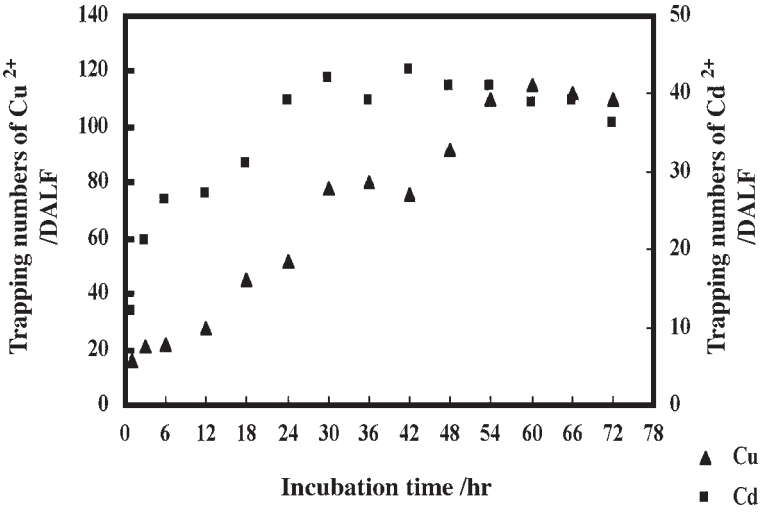

Fig. 3. Kinetics of Cd^{2+} and Cu^{2+} trapped by DALF at pH 7.0. The DALF was incubated in a 0.1 mg/L metal solutions of $CuSO_4$ and $CdCl_2$, respectively, and different incubation intervals were determined. A common biphasic behavior for DALF trapping metal ions was observed. Values are the means of three separate replicates.

ing to the results of the flux of the flowing seawater and the M^{2+} content that the DALF traps, the contamination level of M^{2+} in the flowing seawater was evaluated during the flood tide and the ebb tide or in a few days or weeks.

Results and Discussion

Kinetics of Trapping M^{2+} With DALF Reactor

Figure 3 shows the results of the numbers of Cd^{2+} (right coordinate) and Cu^{2+} (left coordinate) trapped per molecule of DALF at pH 7.0 as a function of incubation time. The solutions containing M^{2+} except for Fe^{2+}/Fe^{3+} were prepared as 0.1 mg/L of $CuSO_4$ and $CdCl_2$ in Tris-HCl buffer (pH 7.0). The incubation time was controlled from 1 to 72 h. The number of M^{2+} trapped with the DALF reactor is observed to have a biphasic behavior with a fast and a slow phase. The turning point of both phases was observed to be 42 h for Cd^{2+} and 60 h for Cu^{2+}. Although there were differences in the time and the trapping numbers, both kinetic curves for trapping Cu^{2+} and Cd^{2+} show a similar trend. In the fast phase, the number of trapped M^{2+} within the DALF reactor increased linearly with time. In the slow phase, the number of trapped M^{2+} leveled off, indicating that the DALF had reached its maximum trapping capacity; that is, ferritin could be saturated by M^{2+} within 2 d of incubation in the flowing seawater. The maximum number of Cd^{2+} trapped by the DALF was 43 Cd^{2+}/DALF, which is much lower than that of HSF as previously described (275 Cd^{2+}/HSF) *(6)*. The maximum number of Cu^{2+} trapped by the DALF was 110 Cu^{2+}/DALF, which is somewhat higher than that of HSF (99 Cu^{2+}/HSF) under similar reaction conditions *(8)*.

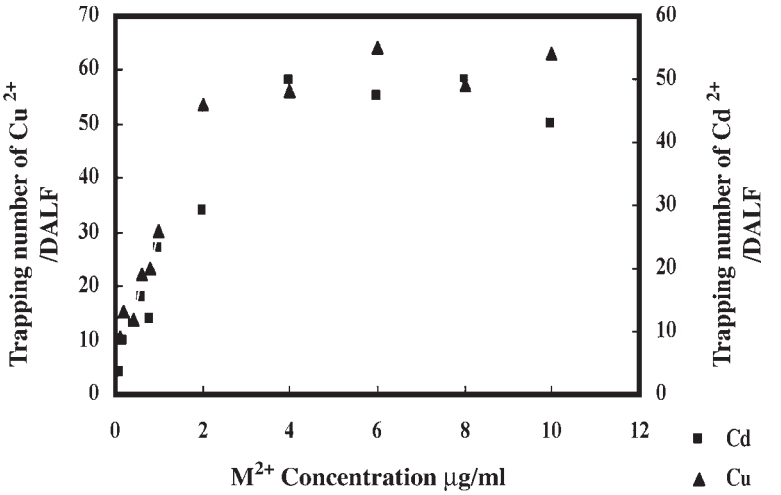

Fig. 4. Effect of metal concentration outside DALF reactor on metal trapped. The numbers of Cu^{2+} and Cd^{2+} trapped by the DALF increased linearly with metal concentration (below 4 and 6 mg/L for Cu^{2+} and Cd^{2+}, respectively) in the medium, but above those concentrations the metal trapped remained nearly constant.

Stability studies were performed to confirm that the DALF in the reactor in the flowing seawater still showed its molecular structure without denaturation in 3 d, which is consistent with the finding of a recent study *(24)*. Even though the capacity of trapping M^{2+} by the reactor was observed to be saturated in 2 d, the monitoring time in the sea might be prolonged by a week because of the low M^{2+} content in the sea except for particular areas with a serious contamination level of M^{2+}.

Effect of Metal Ion Concentration on Trapping Capacity With DALF Reactor

Figure 4 shows the numbers of Cu^{2+} (left coordinate) and Cd^{2+} (right coordinate) trapped per molecule of DALF as a function of its concentration in the flowing seawater. The DALF in the reactor was incubated with Cu^{2+} and Cd^{2+} at desired concentrations ranging from 0.1 to 10 mg/L for 1 h. Then the DALF within the reactor was collected and separated for removal of free ions, and the number of trapped M^{2+} by the ferritin was determined using an atomic absorbance spectrophotometer. As in Fig. 3, the plot in Fig. 4 also shows biphasic behavior. When the M^{2+} concentrations were <6 mg/L for Cu^{2+} and <4 mg/L for Cd^{2+}, the number of M^{2+} trapped in the flowing seawater increased linearly. When the M^{2+} concentrations were above 6 mg/L for Cu^{2+} and 4 mg/L for Cd^{2+}, the trapping number by DALF in the reactor remained nearly constant in 1 h, which indicates that all binding sites for trapping M^{2+} in the DALF had been held by the ions completely in 1 h.

With reference to the results in Figs. 3 and 4, we found that the number of trapped M^{2+} with the DALF reactor depended on the incubation time and

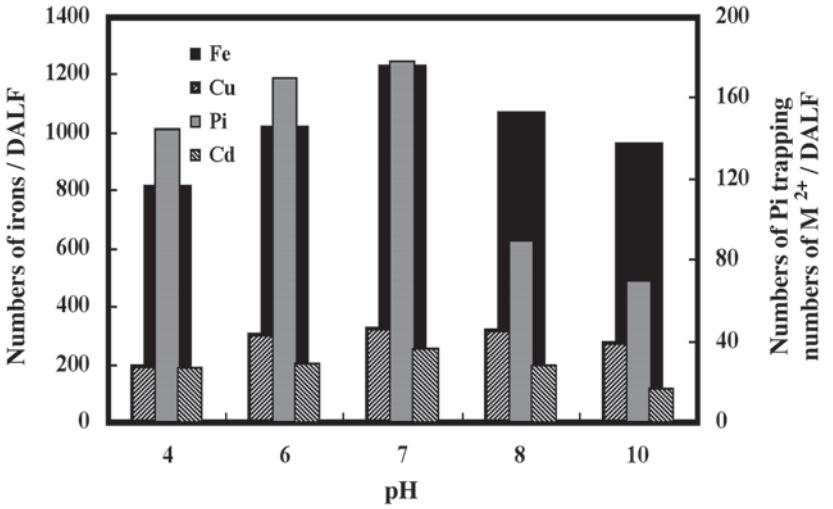

Fig. 5. Effect of pH on content of iron and phosphate in core and DALF trapping capacity. A change in pH caused the release of iron and phosphate and also decreased metal-trapping capacity. At the neutral condition (pH 7.0), the DALF trapped the maximum amounts of Cu^{2+} and Cd^{2+}.

the M^{2+} concentration in the flowing seawater, respectively. Consequently, we used the DALF reactor to monitor the level of M^{2+} contamination in the flowing seawater according to the desired reaction time. Although Figs. 3 and 4 show a saturation phenomenon of trapping M^{2+} in the DALF, the reactor was still employed to analyze the heavy metal pollution level continuously for a few days or a week because of the low M^{2+} concentration in the sea rather than in the laboratory.

Effect of pH on Number of M^{2+} Trapped by DALF Reactor

Huang et al. *(25)* showed that pH changes in the medium could alter the iron core structure within the HSF core and cause ferritin to release its unstable iron and phosphate irreversibly. The variable pH in the medium not only altered the total net charges within the ferritin core, but also affected the conformation of the protein shell including its capacity to self-regulate *(26,27)*. These factors caused the rate of iron release to increment dramatically and compelled the ferritin to release the unstable composition of iron and phosphate within its core. The results in Fig. 5 show the pH dependence of M^{2+} and phosphate in the DALF at different pH values. These results are similar to those of initial studies. In addition, the ratio of iron to phosphate within the DALF core changed with a shift in the pH of the medium, giving the following analytical results for each DALF molecule: 816 Fe^{3+}/145 P_i at pH 4.0, 1019 Fe^{3+}/169 P_i at pH 6.0, 1235 Fe^{3+}/180 P_i at pH 7.0, 1070 Fe^{3+}/90 P_i at pH 8.0, and 960 Fe^{3+}/70 P_i at pH 10.0, respectively. A typical phenomenon of the iron and phosphate iron from the DALF with a shift in pH is observed, demonstrating that H^+ and OH^- ions

in the medium join the core composition of the DALF, causing it to release the unstable iron and phosphate.

Under the physiologic pH condition, the maximum trapping numbers of Cu^{2+} and Cd^{2+} with the DALF reactor were observed. However, this capacity weakened with a shift in pH to acidity or to basicity, respectively, because of the release of iron and phosphate within the ferritin core. The number of trapped Cd^{2+} per molecule of DALF decreased from 36 to 17 when the pH in the medium shifted from 7.0 to 10.0. Moreover, when the pH in the medium shifted from 7.0 to 4.0, the trapping number per molecule of DALF decreased only from 36 to 27. In addition, when the pH of the reactive medium was >10.0 or <2.0, the DALF in the reactor lost its trapping capacity because of protein denaturation or dissociation of its subunit.

To understand the role of phosphate composition within the ferritin core in improving trapping capacity, a reconstituted DALF in the absence of phosphate composition was employed to study the capacity to trap M^{2+}. The results showed that this behavior is still observed. Based on these findings, we believe that the self-regulation capacity of the ferritin shell rather than its iron core plays an important role in the capacity to trap M^{2+}. These binding sites might be located on the interior surface of the ferritin's shell interior. The saturation phenomenon of trapping M^{2+} numbers with the DALF reactor was directly observed once these sites were held by M^{2+}. Further analysis showed that the numbers of these sites decreased gradually, with a shift in pH, which resulted in a weakening of the capacity to trap M^{2+}. Apparently, this capacity is tightly connected to an incremental release of iron and phosphate within the ferritin core. In fact, an increase in the concentration of H^+ or OH^- not only reduced the number of binding sites but also caused the ferritin to release an unstable composition of iron and phosphate. We suggest that these binding sites, rather than the composition of iron and phosphate within the ferritin shell, play an important role in trapping M^{2+}, indicating that this trapping capacity is closely connected to the rate of self-regulation of the protein shell rather than the structure or the composition of the iron core.

Effect of Iron Concentration Within Ferritin Core on Number of M^{2+} Trapped by DALF Reactor

To affirm the aforementioned suggestion about the physiologic role of the binding sites, two experiments involving various concentrations of iron and phosphate within the ferritin core were employed to study the capacity of trapping M^{2+}. Recent studies have shown that Fe^{2+} in the medium can be incorporated into the ferritin core in the presence of O_2 or oxidant without the aid of extra phosphate directly *(23,26)*. Here, reconstituted ferritin having various core sizes consisting of different iron content in the absence of phosphate was developed to study the role of iron in the capacity to trap M^{2+}. Earlier studies showed that the numbers of trapped M^{2+} by holoHSF (native HSF) are greater than those of apoHSF (HSF without iron core) *(8)*,

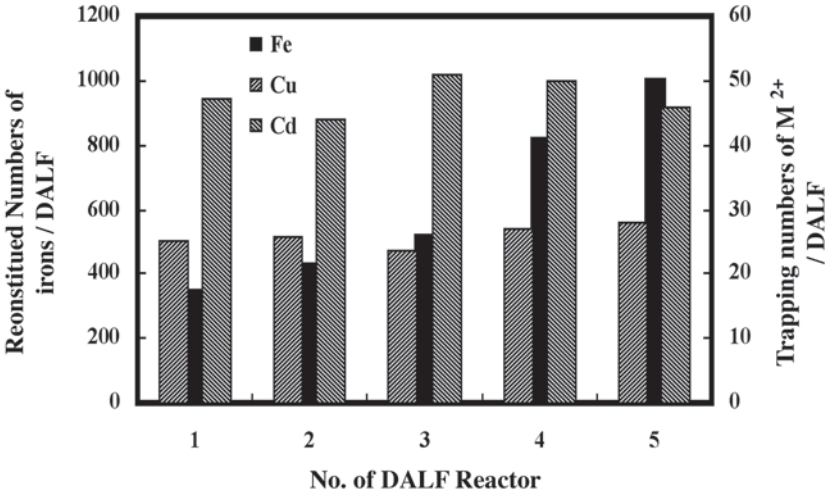

Fig. 6. Effect of reconstituted iron on Cu^{2+} and Cd^{2+} trapping capacity. All the values are the means of three replicates. The results showed that iron in the core had no influence on the trapping of metal iron by DALF.

which means that the ferritin shell is able to trap M^{2+} in the absence of iron core. Huang et al. *(28)* recently observed similar analytical results from DALF.

Figure 6 plots the numbers of Cu^{2+} and Cd^{2+} trapped by DALF with variable iron numbers of reconstituted DALF ($DALF_R$). Identical numbers with $25Cu^{2+}/DALF_R$ and $49Cd^{2+}/DALF_R$ trapped by $DALF_R$ with iron content ranging from apoDALF to $1000Fe^{3+}/DALF_R$ were observed, respectively. This novel behavior shows that the capacity of trapping M^{2+} is independent of iron numbers within the ferritin core. The iron composition within the $DALF_R$ core plays a minor role in improving the capacity of trapping M^{2+}.

According to previous studies, up to 4500 iron atoms could be stored in the 7- to 8-nm cage of the ferritin shell; commonly 1800–2500 iron atoms could be reconstituted *(2,3)*. However, our research group cannot get a reconstituted ferritin core consisting of >1100 Fe^{3+} successfully. Although, the composition of iron and phosphate within the ferritin core and the groups of amino acids on the inside surface of the protein shell was suggested to participate in trapping M^{2+}, we point out that the role of phosphate in trapping M^2 would be more important than that of the numbers of the iron and the groups within the protein shell. The role of phosphate within the ferritin core for metabolizing iron and carrying out other functions such as trapping various heavy metal ions should be not ignored.

Effect of Phosphate on Numbers of M^{2+} Trapped by DALF Reactor

Initial investigations showed that the phosphate within the ferritin core can accelerate iron oxidation and provide the binding sites for iron storage. To study the real role of phosphate in trapping M^{2+}, DALF consist-

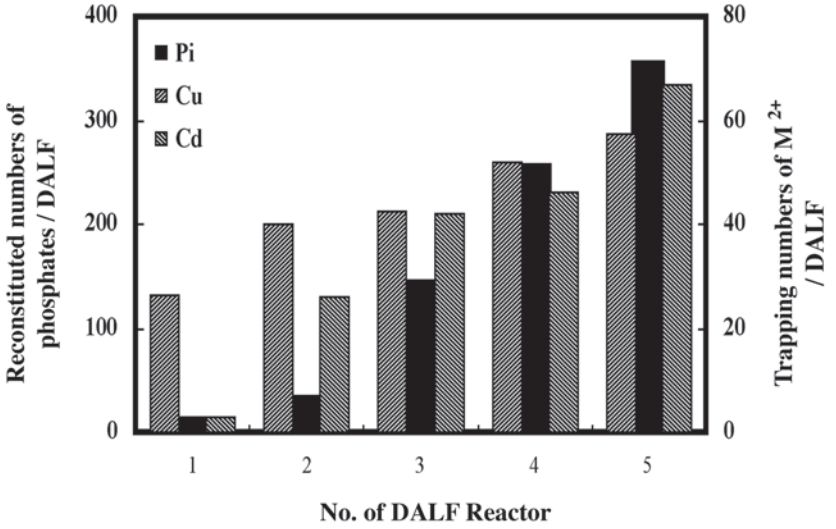

Fig. 7. Effect of reconstituted phosphates on Cu^{2+} and Cd^{2+} trapping capacity. The results indicate that the trapping numbers of Cu^{2+} and Cd^{2+} showed a parallel increase with the phosphate contents in the core, but no linear relation could be found between them.

ing of various reconstituted phosphate numbers within the protein shell was prepared and incubated with M^{2+} (0.1 mg/L of Cu^{2+} and Cd^{2+}) for 24 h, respectively. Finally, The DALF samples were passed through a gel exclusion column to separate the free M^{2+}, and the total numbers of trapped M^{2+} within the reactor were determined by atomic absorbance spectrophotometry. From Fig. 7, it can be seen that the total numbers of trapping Cu^{2+} and Cd^{2+} increased simultaneously with the numbers of phosphates within the DALF core increment. These results were consistent with those previously described, which indicated that holoferritin could trap more M^{2+} than apoferritin *(8)*. Previous studies showed that most phosphates in native ferritin were bound to the surface of the core and that these reconstituted phosphates exhibited the same properties as those of native ferritin *(25–27)*. It was for this reason that we proposed that the phosphate composition within the ferritin core might also serve as binding sites for trapping M^{2+}.

The results in Fig. 7 also show that when the phosphate number within the ferritin core increased from 14 to 357 molecules, the trapping numbers of Cu^{2+} and Cd^{2+} within the protein core increased from 26 to 57 and from 3 to 67, respectively. In addition, the numbers of trapped M^{2+} did not increase linearly with phosphate numbers within the protein shell, meaning that the phosphate composition in the ferritin in part could play a role in formatting a binding site for trapping M^{2+}. Moreover, a factor of self-regulation capacity of the ferritin shell is suggested to play a role in the formation of a rate-limiting step for iron storage and release *(18)*. Thus, we propose that self-regulation capacity, including interaction among the ferritin subunits, may also be a limiting factor for trapping M^{2+} *(22)*.

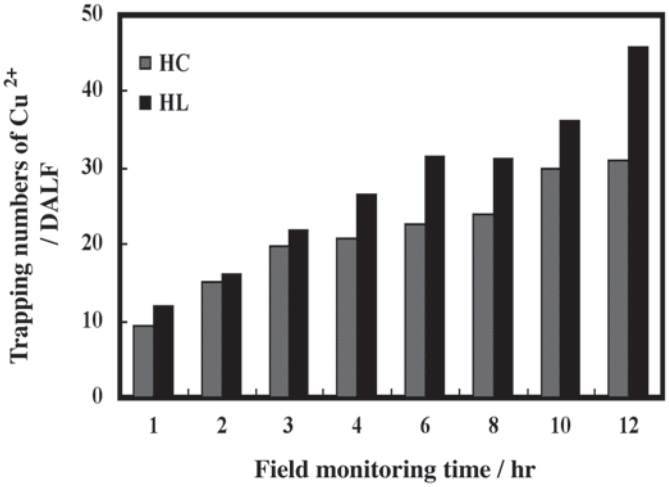

Fig. 8. Field measurement of Cu^{2+} trapped by DALF at two monitoring sites near Xiamen, China. The trapping Cu^{2+} numbers increased with monitoring time. The trapping Cu^{2+} numbers at the two sites were consistent with the Cu^{2+} concentration of seawater, with the average Cu^{2+} concentration in the flowing seawater being 36.6 and 3.0 mg/m^3 at HL and HC, respectively. The seawater temperature was 22–25°C. The NaCl concentration in the flowing seawater was 3.0–6.0% during trapping of M^{2+}.

Monitoring by Ferritin Reactor in Fieldwork

Our knowledge and understanding of the DALF reactor acting to trap M^{2+} was on the basis of experimental studies with a single ion in the laboratory. However, flowing seawater is composed of a complex mixture consisting of M^{2+}, protein, and other organic and inorganic complex, indicating that these factors may affect the capacity of trapping M^{2+} with the ferritin reactor. Nevertheless, the experimental goal here for trapping M^{2+} was to monitor the contamination level of M^{2+} in the field. The tide in the Xiamen inland sea is semidiurnal, with two high waters and two low waters each tidal day. One period with a tidal cycle of 12 h was monitored for M^{2+} content variable in Xiamen inland and showed a diluent capacity of M^{2+} with the flowing seawater during the flood and reflux times.

At each monitoring station, we used three DALF reactors to assess M^{2+} level in the flowing seawater at the same time. All experimental results shown in Fig. 8 are established as the average data for three measurements at the same time. Figure 8 shows the characteristics of DALF trapping Cu^{2+} during the flood tide and reflux tide (12 h). Using the DALF reactor, the numbers of trapping Cu^{2+} increased with the reactive time increments. The maximum trapping capacity of Cu^{2+} was not observed in 12 h, which indicated that the trapping capacity with the reactor was not saturated (compared to Fig. 3). The average Cu^{2+} concentration from the two sites, HL and HC, was 36.6 and 3.0 mg/m^3, respectively. The numbers of trapping Cu^{2+} with the DALF reactor at HL were higher than those at HC. It is clear that the Cu^{2+} concentration in the flowing seawater at HL was higher than

that at HC. It was found that the contaminative headwaters of high Cu^{2+} content in the flowing seawater came from the industrial wastewater at HL.

Conclusion

The ocean, as previously described, has always been considered to have enough capacity to decontaminate all contaminants coming from the mainland. However, a central reason that the ocean has become seriously polluted is that it has lost its capacity to clear away all of the contamination resulting from the daily effluent from chemical factories and other manufactures. Although dissociated M^{2+} in the ocean can be deposited by aquatic and marine sediments, these ions still release back to the seawater, resulting in a new contamination level *(29)*. It is for this reason that an analytical technology for continuously monitoring the level of M^{2+} in flowing seawater that can evaluate the real level of M^{2+} contamination in the sea in a few days or weeks should be developed and improved. There are scarcely any validated analytical methods of chemical and biologic indicators suggested by reference organizations for monitoring trace M^{2+} continuously in the river or sea. Even though various chemical analytical techniques such as atomic absorbance spectrometry and inductively coupled plasma mass spectrometry are capable of measuring the level of M^{2+} contamination quickly and accurately, these methods are rarely used to evaluate continuously the whole level to understand the capacity of decontaminant M^{2+} in the sea or in an appointed area in a few days or weeks. We have shown that the ferritin reactor could be used to monitor the level of contamination continuously once it is improved. Two important factors, phosphate and pH, strongly affected the capacity to trap M^{2+} (Figs. 4–8), and this relationship should be studied before monitoring in the sea in fieldwork according to the desired time.

Previous studies proposed that M^{2+} is able to bind to the ferritin channels along the 432 symmetry axes of the protein shell outside and inside the surface *(2–4,30)*. Our results indicate that ferritin containing a reconstituted iron core in the absence of phosphate shows no effect on the trapping capacity of Cu^{2+} and Cd^{2+}, suggesting that the iron composition is not a binding site for trapping M^{2+}. Moreover, ferritin having a reconstituted inorganic phosphate shows the capacity to be improved greatly. This phenomenon suggests that the phosphate composition within the ferritin core plays an important role in improving the capacity of trapping M^{2+}, as shown in Fig. 5.

Huang et al. *(28)* indicated that added scan potential from the potentiostat could improve the capacity of trapping M^{2+} in the flowing water. A hybrid analytical method of the electrochemistry and the reactor were employed to trap various M^{2+} selectively. It had the advantage of monitoring the level of M^{2+} contamination in the flowing seawater according to the seawater with characteristics of transferring electrons from the physical electrode. Earlier studies showed that ferritin could maintain its natural structure in flowing seawater for 1 wk *(15,24)*. Field monitoring

data demonstrated that chemical ions such as alkali and alkaline earth salts in the flowing seawater had no influence on the capacity of trapping M^{2+} with the DALF reactor as well as with other ferritin reactors *(18)*. These observations were prerequisites for field monitoring by a DALF reactor. It is well known that alkali and alkaline earth salts are found abundantly in seawater. The ferritin reactor may therefore provide a possible means for monitoring M^{2+} contamination in flowing seawater without interference from alkali and alkaline metal ions.

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