Published in final edited form as:

Anal Biochem. 2012 February 15; 421(2): 477–481. doi:10.1016/j.ab.2011.09.024.

***In vitro* release of organophosphorus acid anhydrolase from functionalized mesoporous silica against nerve agents**

Baowei Chen¹, Saumil S. Shah², Yongsoon Shin¹, Chenghong Lei^{1,*}, and Jun Liu¹

¹Pacific Northwest National Laboratory, Richland, WA 99352, USA

²U.S. Army-Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD 21010, USA

Abstract

We report here that under different physiological conditions, biomolecular drugs can be stockpiled in a nanoporous support and afterwards can be instantly released when needed for acute responses, and the biomolecular drug molecules can also be gradually released from the nanoporous support over a long time for a complete recovery. Organophosphorus acid anhydrolase (OPAA) was spontaneously and largely entrapped in functionalized mesoporous silica (FMS) due to the dominant electrostatic interaction. The OPAA-FMS composite exhibited a burst release in pH 9.0, NaHCO₃-Na₂CO₃ buffer system and a gradual release in pH 7.4, simulated body fluid. The binding of OPAA to NH₂-FMS can result in less Trp exposure of OPAA molecules to aqueous environment. The bound OPAA in FMS displayed lower activity than the free OPAA in solution prior to the enzyme entrapment. However, the released enzyme still displayed the native conformational structure and the same high enzymatic activity as that prior to the enzyme entrapment. The *in vitro* results in the rabbit serum demonstrate that both OPAA-FMS and the released OPAA may be used as the medical measures against the organophosphorus nerve agents.

Keywords

Controlled release; organophosphorus acid anhydrolase; mesoporous silica; organophosphorus nerve agents

Introduction

Functionalized mesoporous silica (FMS) with high affinity for a protein can provide a confined and interactive nanoenvironment increasing protein activity and allow large amounts of protein loading compared to unfunctionalized mesoporous silica or normal porous silica with the same pore size [1; 2; 3; 4; 5; 6; 7; 8]. Mesoporous silicas have a surface area of up to 1000 m² g⁻¹ with ordered pore surface accounting for >95%. The proteins can be electrostatically entrapped in carboxylethyl- or aminopropyl-functionalized mesoporous silica (HOOC-FMS or NH₂-FMS) with rigid, uniform, open nanopore geometry in tens of nanometers. When the proteins are incubated with FMS, they are sequestered in the porous material, based on the electrostatic interaction with the charged functional

© 2011 Elsevier Inc. All rights reserved.

*To whom correspondence should be addressed. Dr. Chenghong Lei, Pacific Northwest National Laboratory, Mail Stop J4-02, P.O. Box 999, Richland, WA 99352, Tel: 1-509-3716962, Fax: 1-509-3717304, Chenghong.Lei@pnl.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

groups. FMS can also protect the proteins because the pore size is sufficiently small to eliminate any invading bacteria (at least 2 μm). Therefore, the protein-FMS composites can be stockpiled. More important, the largely loaded proteins in FMS via electrostatic interactions can have a burst release and/or a long-lasting release under physiological conditions [6].

Organophosphorus acid anhydrolase (OPAA) is a highly efficient enzyme capable of detoxifying G-type nerve agents and speeding up AChE reactivation in vivo [9]. In this work, we will entrap OPAA in NH_2 -FMS via electrostatic interactions and study OPAA release from NH_2 -FMS under physiological conditions which can potentially be used for acute response against OP poisoning and for long-term recovery over time.

Materials and Methods

Materials

Diisopropylfluorophosphate (DFP), 1,3-Bis[tris(hydroxymethyl)methylamino]propan (BTP) were purchased from Sigma Chemical Co. (St. Louis, MO). The OPAA gene was cloned from *Alteromonas* sp. strain JD6.5 into a bacterial expression vector pSE420 and it was expressed and purified as a recombinant enzyme [10; 11]. The purified OPAA in pH 7.2, 10 mM BTP/0.1 mM MnCl_2 was stored at -80°C . It was thawed at 4°C before use. The purity of OPAA determined by SDS-polyacrylamide gel electrophoresis was 90–95%. The original UMS, prepared by using non-ionic block copolymer surfactant as the template, had a pore size of 30 nm measured by the Barrett-Joyner-Halenda method, while the surface area was as great as $533\text{ m}^2/\text{g}$ with an average bead size of 12–15 μm [12; 13]. Normal porous silica (NPS) is uncoated silica bulk material with a pore size of 30 nm with a 12 μm bead size (PolyLC Inc., item #BMSI 1203). A controlled hydration and condensation reaction was used to introduce functional groups into unfunctionalized mesoporous silica (UMS) or NPS [12; 13; 14]. Coverage of 2% (or 20%) HOOC-FMS , $\text{HO}_3\text{S-FMS}$ or $\text{NH}_2\text{-FMS}$ means 2% (or 20%) of the total available silanol groups (5×10^{18} silanol groups per square meter) [12; 13] of UMS or NPS would be silanized with trimethoxysilane with the functional group HOOC , HO_3S or NH_2 [2; 6; 12; 13; 14].

Entrapment of OPAA into FMS

Typically, 0.4 mg of OPAA in pH 7.2, 10 mM BTP/0.1 mM MnCl_2 was used for incubation with 1 mg of FMS in a 1.5 mL tube. The incubation was carried out on an Eppendorf Thermomixer 5436 with a shaking speed 1400 min^{-1} at room temperature ($21 \pm 1^\circ\text{C}$) for 2–3 h. Then, the OPAA-FMS suspension was centrifuged and the supernatant was removed. The resulting OPAA-FMS composite was washed with pH 7.2, 10 mM BTP/0.1 mM MnCl_2 ($5 \times 0.2\text{ mL}$) and then resuspended in 200 μL of the same buffer per mg of original FMS for protein and activity measurement. The concentration of OPAA entrapped in FMS was measured by Bradford method (Bio-Rad) using bovine serum albumin as the protein standard. The protein amount (μg or mg) of an enzyme immobilized with one mg of a porous silica support is defined as the protein loading density (P_{LD}). The protein amounts were measured with three replicates for each sample.

Release of OPAA from FMS

A simulated body fluid (SBF, pH 7.4, 50 mM Tris-HCl buffer containing ion concentrations nearly equal to those of human blood plasma), or pH 9.0, 0.1 M $\text{Na}_2\text{CO}_3\text{-NaHCO}_3$ buffer was used to elute OPAA from FMS. The OPAA-FMS composite was obtained by centrifuge and removing the supernatant (the elution number: 0). Then, a certain volume of the elution buffer was used for each subsequent elution by incubating and shaking OPAA-FMS in the

elution buffer for 5 minutes. The released OPAA was also dialysed in pH 7.2, 10 mM BTP/0.1 mM MnCl_2 when necessary for enzyme activity assay and spectra measurement.

Assay of enzymatic activity

OPAA activity was measured by using a fluoride combination ion-selective electrode (Cat. No. 02-226-11. Denver Instrument Co., CO) to monitor the release of fluoride ion resulting from the OPAA-catalyzed hydrolysis of DFP. Prior to measurement, the electrode was calibrated using NaF standard solutions. 50 μL of 0.3 M DFP was added to 10 mL of pH 7.2, 10 mM BTP/0.1 mM MnCl_2 to monitor for the background hydrolysis of DFP. To measure the OPAA-catalyzed hydrolysis of DFP, 50 μL of 0.3 M DFP was added to 10 mL of the working solution containing an appropriate amount of OPAA in pH 7.2, 10 mM BTP/0.1 mM MnCl_2 so that the 10-minute hydrolysis reaction remained in the electrode calibration range. The amount of fluoride ion released in the presence of OPAA was recorded and the enzyme activity was calculated after the background was subtracted. One unit of OPAA is defined as hydrolyzing 1.0 μmol of DFP to fluoride ion and isopropyl phosphate per minute [15]. The specific enzyme activity of OPAA was defined as units per mg of OPAA. The enzyme activities were measured with three replicates for each sample.

Circular dichroism (CD) measurement

The secondary structures of OPAA in solution and in 20% $\text{NH}_2\text{-FMS}$ were determined with Model 410 Circular Dichroism Spectrophotometer (Aviv Biomedical, Inc., Lakewood, NJ) with a path length of 1.0 cm. Measurements were carried out with 0.05 mg/mL of OPAA in pH 7.2, 10 mM BTP/0.1 mM MnCl_2 . Two scans were accumulated and averaged for each spectrum after the background of buffer solution with or without FMS was subtracted.

Steady-State fluorescence measurement

Fluorescence was measured with a Fluoro Max-2 fluorometer (SPEx, Edison, NJ), using excitation and emission slits of 5 nm. In all cases, control sample (no fluorescent probes present) were used to determine the contribution of background fluorescence or scattered light to the observed spectra. Control samples demonstrated that the background contribution was minimal (i.e., < 6%). The fluorescence emission of tyrosinyl (Tyr) and tryptophanyl (Trp) residues in OPAA was measured with excitation at 278 nm.

OPAA resisting the inhibition of DFP on AChE in vitro

The AChE activity was measured using Ellman spectrophotometric method [16; 17]. The commercial rabbit serum was first diluted with a buffer of pH 7.2, 0.1 M sodium phosphate/0.15 M NaCl to a total protein concentration of 4.2 mg/mL. 0.48 mL of the diluted rabbit serum was incubated with the free OPAA, the released OPAA from FMS, or OPAA-FMS at 21 °C for 30 min. Then, 0.3 M DFP in propanol and the buffer of pH 7.2, 0.1 M sodium phosphate/0.15 M NaCl were added to the mixture and incubated at 21 °C for another 30 min. The final solution contained 1 mg/mL of the serum protein, 0.05 mg/mL of OPAA, 0.77 mg/mL of FMS and 0–180 μM DFP with a total volume of 2.0 mL. The AChE activity was measured by adding 0.2 mL of Ellman assay solution containing 0.32 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 1 mM acetylthiocholine iodide as the substrate. The change in absorbance at 412 nm as function of time was used to calculate the activity of AChE in rabbit serum. Two replicate experiments were carried out.

Results and Discussion

Fig. 1A shows the transmission electron microscopy (TEM) images of 30 nm 20% $\text{NH}_2\text{-FMS}$. There is no significant difference between the TEM images of UMS and their

corresponding FMS. Unlike 3 nm and 10 nm mesoporous silica, the 30-nm mesoporous silica has a large degree of disordering [14], but it still reveals more or less uniform cage-like porous structure [18]. FMS was incubated in the OPAA solution, where OPAA would be spontaneously entrapped in FMSs, thereby avoiding harsh immobilization conditions. UMS and NPS were tested under the same conditions.

Fig. 1B shows P_{LD} of OPAA entrapped in various silica supports pH 7.2, 10 mM BTP/0.1 mM $MnCl_2$. P_{LD} was 131.0 $\mu\text{g}/\text{mg}$ of FMS in 20% NH_2 -FMS. In contrast, P_{LD} of OPAA in 20% $HOOC$ -FMS was only 2.7 $\mu\text{g}/\text{mg}$ of FMS. P_{LD} of OPAA in 2% and 20% HO_3S -FMS was only 27.1 and 13.1 $\mu\text{g}/\text{g}$ of FMS, respectively. The OPAA in UMS displayed a P_{LD} of 33.6 $\mu\text{g}/\text{mg}$, which is more or less close to that in the negatively-charged FMS ($HOOC$ - and HO_3S -) due to the silanol groups of UMS. The calculated pI of OPAA is 5.67. Therefore, at pH 7.2, OPAA is overall negatively charged. The much higher P_{LD} of OPAA in the positively-charged NH_2 -FMS comparing that in the negatively-charged FMS indicates that the binding of OPAA to NH_2 -FMS was mainly resulted from the electrostatic interaction. The OPAA structure is composed of two domains, amino and carboxy domains [19]. The negative charges of OPAA are attributable to a surplus of surface carboxylic residues in glutamates and aspartates [19]. Besides the electrostatic interaction, we believe that functional groups of FMS would offer H-bond and hydrophilic interaction with the charged protein molecules. OPAA, which is hydrophobic regionally [19], might also have the hydrophobic interaction with the spacers (carbon chains: $-CH_2-CH_2-$, $-CH_2-CH_2-CH_2-$) between the functional groups and the mesoporous silica surface. Therefore, the binding strength can be tuned by rational choice of the functional groups (type and coverage) and the spacers in relation to the overall charge and structure of the protein. As expected, in comparison with FMS, NPS (whether functionalized or not) exhibited substantially lower P_{LD} than NH_2 -FMS for OPAA, indicating that the appropriately functionalized mesoporous silica provided a much more favorable microenvironment for enzyme entrapping.

Since the electrostatic interaction is dominated between OPAA and NH_2 -FMS, we tested controlled release of OPAA from NH_2 -FMS by a certain volume of the elution buffers with different pH and ionic strength. Because of the driving force resulted from formation of a new thermodynamic balance and distribution of OPAA in NH_2 -FMS and in the fresh buffer, OPAA can be rapidly or gradually released from NH_2 -FMS along continuous eluting processes depending on the pH and ionic strength of the elution buffers. Fig. 2A shows the controlled release of OPAA from NH_2 -FMS in pH 9.0, 0.1 M Na_2CO_3 - $NaHCO_3$ and SBF that has ion concentrations nearly equal to those of human blood plasma (buffered at pH 7.4 with 50 mM Tris-HCl). A burst release (~80%) of OPAA was observed from NH_2 -FMS with the first volume of the elution buffer in pH 9.0, 0.1 M Na_2CO_3 - $NaHCO_3$, and the gradual releasing with subsequent elutions. Comparing to that in pH 9.0, 0.1 M Na_2CO_3 - $NaHCO_3$, a much smaller burst release and the subsequent gradual release of OPAA from NH_2 -FMS were observed in SBF. The protein contents of the supernatants in between each cycle of shaking-elution-centrifugation were measured. A decreasing P_{LD} was observed along the series of elutions (Fig. 2A). Although, NH_2 -FMS would offer a noncovalent comprehensive interaction (electrostatic, H-bond, hydrophilic and hydrophobic) with the charged amino acid residues of protein molecules, it was the changed pH and ionic strength that made the dominant electrostatic interaction dissociated to different extents and accordingly either the gradual or the burst release would occur.

To question if there was any conformational change when OPAA was entrapped in NH_2 -FMS, we compared the fluorescence and CD spectra of OPAA in NH_2 -FMS and free OPAA in pH 7.2, 10 mM BTP/0.1 mM $MnCl_2$. It is well-known that intrinsic fluorescence of tryptophan residues (Trp) is sensitive to conformational change. Fluorescence emission was monitored at the excitation wavelength of 278 nm, allowing excitation of both tyrosinyl and

tryptophanyl residues. Upon conformational change resulting in a more Trp exposure to aqueous solvent, fluorescence intensity decreases, *verse versa*. Fig. 2B shows the fluorescence spectra of the free OPAA in pH 7.2, 10 mM BTP/0.1 mM MnCl₂, the entrapped OPAA in 20% NH₂-FMS, and the released OPAA from 20% NH₂-FMS in pH 7.2, 10 mM BTP/0.1 mM MnCl₂. Comparing the free OPAA to OPAA-FMS, there was no dramatic emission peak shift but increased emission intensity because of the interaction of OPAA with FMS, which might result in less exposure of tyrosinyl and tryptophanyl residues to the aqueous environment. It is noteworthy that the released OPAA from NH₂-FMS displayed similar fluorescence spectra to that of the free OPAA prior to the entrapment, indicating that the electrostatic interaction of OPAA with NH₂-FMS did not induce permanent change on the OPAA protein structure. To further study the interaction of OPAA with NH₂-FMS, we also measured the CD spectra of the free OPAA in pH 7.2, 10 mM BTP/0.1 mM MnCl₂, the entrapped OPAA in 20% NH₂-FMS, and the released OPAA from 20% NH₂-FMS in pH 7.2, 10 mM BTP/0.1 mM MnCl₂. Although the OPAA entrapped in NH₂-FMS presents minimum CD signal, the released OPAA from NH₂-FMS has a similar profile and intensity as that of free OPAA in the same buffer (Fig. 2C). The specific activity of the entrapped OPAA was lower than that of the free OPAA in solution (Fig. 2D). However, importantly, the specific activity of the released OPAA (101.0 units/mg) from NH₂-FMS was comparable to that of the free OPAA (91.0 units/mg) prior to the entrapment (Fig. 2D). These results demonstrate that the orientation of OPAA in NH₂-FMS might not be favorably constructive, but there was no permanent conformational change for the enzyme entrapped therein. After the samples were stored at room temperature 21±1°C for two months, there were no activity loss for OPAA entrapped in and released from NH₂-FMS. Fig. 2E shows OPAA resisting the inhibition of DFP on AChE in the rabbit serum in pH 7.2, 0.1 M sodium phosphate/0.15 M NaCl. Two replicate experiments showing similar results were carried out. The results indicate that both the released OPAA and the OPAA-FMS displayed a higher efficiency in resisting the inhibition of DFP on AChE in the rabbit serum than the free OPAA prior to the entrapment in FMS. Since the incubation of OPAA-FMS with the rabbit serum was 30 min in the working buffer containing as high as 0.15 M of NaCl, we believe the higher efficiency was ascribable to the released OPAA from FMS due to the high ionic strength. These *in vitro* results in the rabbit serum demonstrate that both OPAA-FMS and the released OPAA may be used as the medical measures against the nerve agents.

In conclusion, the conformational change resulted from the electrostatic interaction of OPAA with NH₂-FMS was not a permanent change, which has been confirmed by the fluorescence and CD spectra and the enzymatic activity measurement. The OPAA-FMS composite exhibited a burst release in pH 9.0, NaHCO₃-Na₂CO₃ buffer system and a gradual release in pH 7.4, simulated body fluid. The burst release of OPAA allows the stockpiled enzyme in FMS to be instantly released for immediate use, that is, after modulating pH to neutral, the instantly released enzyme could be potentially used via an intravenous injection for acute medical responses. The gradual release of OPAA from FMS in the simulated body fluid demonstrates that the OPAA-FMS composite, once injected intramuscularly or subcutaneously at a given body site, could provide a long-lasting local release over a long time for a complete recovery. Further work to adjust the release rate by optimizing the functional group coverage and spacer of FMS is ongoing in this lab.

Acknowledgments

This work was supported by the NIH National Institute of General Medical Sciences (grant number R01GM080987) and the office of Basic Energy Sciences of US Department of Energy (award number KC020105-FWP12152) and the Transformational Materials Science Initiative of Pacific Northwest National Laboratory (PNNL). PNNL is operated for U.S. Department of Energy by Battelle under Contract DE-AC06-RLO1830.

References

1. Takahashi H, Li B, Sasaki T, Miyazaki C, Kajino T, Inagaki S. Catalytic activity in organic solvents and stability of immobilized enzymes depend on the pore size and surface characteristics of mesoporous silica. *Chem Mater*. 2000; 12:3301–3305.
2. Lei C, Shin Y, Liu J, Ackerman EJ. Entrapping enzyme in a functionalized nanoporous support. *J Am Chem Soc*. 2002; 124:11242–11243. [PubMed: 12236718]
3. Lei C, Shin Y, Magnuson JK, Fryxell G, Lasure LL, Elliott DC, Liu J, Ackerman EJ. Characterization of Functionalized Nanoporous Supports for Protein Confinement. *Nanotechnology*. 2006; 17:5531–5538. [PubMed: 21727320]
4. Dunker AK, Fernandez A. Engineering productive enzyme confinement. *Trends in Biotechnology*. 2007; 25:189–190. [PubMed: 17379337]
5. Chen BW, Lei CH, Shin YS, Liu J. Probing mechanisms for enzymatic activity enhancement of organophosphorus hydrolase in functionalized mesoporous silica. *Biochemical and Biophysical Research Communications*. 2009; 390:1177–1181. [PubMed: 19874798]
6. Han YJ, Stucky GD, Butler A. Mesoporous silicate sequestration and release of proteins. *J Am Chem Soc*. 1999; 121:9897–9898.
7. Han YJ, Watson JT, Stucky GD, Butler A. Catalytic activity of mesoporous silicate-immobilized chloroperoxidases. *J Mol Catal B: Enzymatic*. 2002; 17:1–8.
8. Yiu HHP, Wright PA, Botting NP. Enzyme immobilization using SBA-15 mesoporous molecular sieves with functionalised surfaces. *J Mol Catal B: Enzymatic*. 2001; 15:81–92.
9. Herkenhoff S, Szinicz L, Rastogi VK, Cheng TC, DeFrank JJ, Worek F. Effect of organophosphorus hydrolysing enzymes on obidoxime-induced reactivation of organophosphate-inhibited human acetylcholinesterase. *Archives of Toxicology*. 2004; 78:338–343. [PubMed: 14985944]
10. Cheng TC, Harvey SP, Chen GL. Cloning and expression of a gene encoding a bacterial enzyme for decontamination of organophosphorus nerve agents and nucleotide sequence of the enzyme. *Applied and Environmental Microbiology*. 1996; 62:1636–1641. [PubMed: 8633861]
11. Cheng TC, DeFrank JJ, Rastogi VK. *Alteromonas* prolidase for organophosphorus G-agent decontamination. *Chemico-Biological Interactions*. 1999; 120:455–462. [PubMed: 10421483]
12. Liu J, Shin Y, Nie ZM, Chang JH, Wang LQ, Fryxell GE, Samuels WD, Exarhos GJ. Molecular assembly in ordered mesoporosity: A new class of highly functional nanoscale materials. *J Phys Chem A*. 2000; 104:8328–8339.
13. Feng X, Fryxell GE, Wang LQ, Kim AY, Liu J, Kemner KM. Functionalized monolayers on ordered mesoporous supports. *Science*. 1997; 276:923–926.
14. Zhao DY, Feng JL, Huo QS, Melosh N, Fredrickson GH, Chmelka BF, Stucky GD. Triblock copolymer syntheses of mesoporous silica with periodic 50 to 300 angstrom pores. *Science*. 1998; 279:548–552. [PubMed: 9438845]
15. Petrikovics I, Cheng TC, Papahadjopoulos D, Hong K, Yin R, DeFrank JJ, Jaing J, Song ZH, McGuinn WD, Sylvester D, Pei L, Madec J, Tamulinas C, Jaszbetenyi JC, Barcza T, Way JL. Long circulating liposomes encapsulating organophosphorus acid anhydrolase in diisopropylfluorophosphate antagonism. *Toxicological Sciences*. 2000; 57:16–21. [PubMed: 10966507]
16. Ellman GL, Courtney KD, Andres V Jr, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol*. 1961; 7:88–95. [PubMed: 13726518]
17. Nostrandt AC, Duncan JA, Padilla S. A modified spectrophotometric method appropriate for measuring cholinesterase activity in tissue from carbaryl-treated animals. *Fundam Appl Toxicol*. 1993; 21:196–203. [PubMed: 8405782]
18. Liu J, Shin Y, Nie ZM, Chang JH, Wang LQ, Fryxell GE, Samuels WD, Exarhos GJ. Molecular assembly in ordered mesoporosity: A new class of highly functional nanoscale materials. *J Phys Chem A*. 2000; 104:8328–8339.
19. Vyas NK, Nickitenko A, Rastogi VK, Shah SS, Quioco FA. Structural Insights into the Dual Activities of the Nerve Agent Degrading Organophosphate Anhydrolase/Prolidase. *Biochemistry*. 2010; 49:547–559. [PubMed: 20000741]

Fig. 1. (A) TEM images of 30 nm 20% NH₂-FMS at different magnifications; (B) Comparison of porous silica for OPAA immobilization in pH 7.2, 10 mM BTP/0.1 mM MnCl₂.

Fig. 2.

(A) Release of OPAA from NH₂-FMS in two different elution buffers (pH 9.0, 0.1 M Na₂CO₃-NaHCO₃ and pH 7.4, simulated body fluid). The OPAA-FMS composite (with 1 mg of 20% NH₂-FMS) was obtained by centrifuge and removing the supernatant (the elution number: 0). Then, 0.2 mL of the elution buffer was used for each subsequent elution by incubating and shaking OPAA-FMS in the elution buffer for 5 minutes. (B) Fluorescence emission of the free OPAA, the entrapped OPAA in 20% NH₂-FMS, and the released OPAA from 20% NH₂-FMS in pH 7.2, 10 mM BTP/0.1 mM MnCl₂. [OPAA]: 0.05 mg/mL. Excitation wavelength: 278 nm; (C) Circular dichroism spectra of the free OPAA, the entrapped OPAA in 20% NH₂-FMS, and the released OPAA from 20% NH₂-FMS in pH 7.2, 10 mM BTP/0.1 mM MnCl₂. [OPAA]: 0.05 mg/mL. (D) The specific activity of the free OPAA, OPAA entrapped in 20% NH₂-FMS, and the released OPAA from 20% NH₂-FMS. The released OPAA was prepared by first allowing OPAA released from FMS in pH 9.0, 0.1 M Na₂CO₃-NaHCO₃, then dialysed in pH 7.2, 10 mM BTP/0.1 mM MnCl₂; (E) OPAA resisting the inhibition of DFP on AChE in the rabbit serum in pH 7.2, 0.1 M sodium phosphate/0.15 M NaCl. The diluted rabbit serum was first incubated with the free OPAA, the released OPAA from FMS, or the OPAA-FMS at 21 °C for 30 min, then with different concentrations of DFP at 21 °C for another 30 min. The final solution contained 1 mg/mL of the serum protein, 0.05 mg/mL of OPAA, 0.77 mg/mL of FMS. The change in absorbance at 412 nm as function of time was used to calculate the activity of AChE in rabbit serum. AChE activity in rabbit serum without DFP was used as the control (relative activity: 100%), whose absorbance changing rate was 0.045 ± 0.001/min at 412 nm.