Protein Refolding Assisted by Periodic Mesoporous Organosilicas

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Herein we report a new strategy for protein refolding by taking advantage of the unique surface and pore characteristics of ethylene-bridged periodic mesoporous organosilica (PMO), which can effectively entrap unfolded proteins and assist refolding by controlled release into the refolding buffer. Hen egg white lysozyme was used as a model protein to demonstrate the new method of protein refolding. Through loading of denatured proteins inside uniform mesoporous channels tailored to accommodate individual protein, protein aggregation was minimized, and the folding rate was increased. Poly(ethyleneglycol) (PEG)-triggered continuous release of entrapped denatured lysozyme allowed high-yield refolding with high cumulative protein concentrations. The new method enhances the oxidative refolding of lysozyme (e.g., over 80% refolding yield at about 0.6 mg/mL).

Introduction

A rapid, large-scale refolding of recombinant proteins from inclusion bodies, which are usually formed by the overexpression of cloned or synthetic genes in *Escherichia coli*, has long been a research interest and will tremendously benefit the bioengineering industry.[1] The formation of inactive protein aggregates, which competes with correct folding especially at high concentrations, has been recognized as the main reason for inefficient protein recovery.[2] In past decades, many methods[3] have been developed to improve refolding efficiency and yield. Among them, batch dilution is still the most widely used and simplest approach. However, to effectively inhibit aggregation the refolding is usually carried out at very low protein concentrations (e.g., <0.1 mg/mL). A procedure to increase the concentration of renatured proteins, known as "pulse-renaturation"[4] or "fed-batch operation",[5] has been developed by stepwise or continuous addition of unfolded proteins to the refolding solution. A limitation of this method is that the presence of denaturant at the same time changes the optimal refolding conditions and eventually destabilizes refolded proteins when the denaturant concentration is too high.

First reported in 1992,[6] ordered mesoporous silicas (OMSs) exhibit unique structural features, such as regular pore geometry, high surface area, and large pore volume.[7] In addition, it is possible to tailor the surface properties for various applications.[8] For example, assembly from organic group bridged silsesquioxanes, $(R'O)_3Si-R-Si(OR')_3$, leads to the formation of hybrid materials known as periodic mesoporous organosilicas (PMOs),[9] which integrate organic groups homogeneously within the framework walls. Applications of OMSs in medical and biological areas, such as protein immobilization,[10] bioactive glasses,[11] and drug release,[12] have attracted a great deal of attention in the last few years. Recently, stimuli-responsive controlled release systems based on functionalized OMSs have been reported.[13] Although such systems have so far been realized only for the release of drugs and fluorescent dyes, it is also possible to release proteins from OMSs in a controllable way.

Here we report a new method to assist protein refolding by the controlled release of unfolded proteins from ordered

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(1) (a) Misawa, S.; Kumagai, I. *Biopolymers* **1999**, *51*, 297−307. (b) De Bernardez Clark, E. *Curr. Opin. Biotechnol.* **2001**, *12*, 202−207.

(2) (a) Goldberg, M. E.; Rudolph, R.; Jaenicke, R. *Biochemistry* **1991**, *30*, 2790−2797. (b) Buswell, A. M.; Middleberg, A. P. J. *Biotechnol. Bioeng.* **2003**, *83*, 567−577.

(3) (a) Rozema, D.; Gellman, S. H. *J. Am. Chem. Soc.* **1995**, *117*, 2373−2374. (b) Maeda, Y.; Koga, H.; Yamada, H.; Ueda, T.; Imoto, T. *Protein Eng.* **1995**, *8*, 201−205. (c) Hevehan, D. L.; De Bernardez Clark, E. *Biotechnol. Bioeng.* **1997**, *54*, 221−230. (d) De Bernardez Clark, E.; Hevehan, D.; Szela, S.; Maachupalli-Reddy, J. *Biotechnol. Prog.* **1998**, *14*, 47−54. (e) Lu, D. N.; Liu, Z.; Wu, J. Z. *Biophys. J.* **2006**, *90*, 3224−3238.

(4) (a) Fischer, B.; Perry, B.; Summer, I.; Goodenough, P. *Protein Eng.* **1992**, *5*, 593−596. (b) Buchner, J.; Pastan, I.; Brinkmann, U. *Anal. Biochem.* **1992**, *205*, 263−270.

(5) (a) Katoh, S.; Terashima, M.; Kishida, H.; Yagi, H. *J. Chem. Eng. Jpn.* **1997**, *30*, 964−966. (b) Katoh, S.; Sezai, Y.; Yamaguchi, T.; Katoh, Y.; Yagi, H.; Nohara, D. *Process Biochem.* **1999**, *35*, 297−300. (c) Katoh, S.; Katoh, Y. *Process Biochem.* **2000**, *35*, 1119−1124.

(6) Kresge, C. T.; Leonowicz, M. E.; Roth, W. J.; Vartuli, J. C.; Beck, J. S. *Nature* **1992**, *359*, 710−712.

(7) Ying, J. Y.; Mehnert, C. P.; Wong, M. S. *Angew. Chem., Int. Ed.* **1999**, *38*, 56−77.

(8) (a) Hoffmann, F.; Cornelius, M.; Morell, J.; Fröba, M. *Angew. Chem., Int. Ed.* **2006**, *45*, 3216−3251. (b) Vinu, A.; Hossain, K. Z.; Ariga, K. *J. Nanosci. Nanotechnol.* **2005**, *5*, 347−371.

(9) (a) Asefa, T.; MacLachlan, M. J.; Coombs, N.; Ozin, G. A. *Nature* **1999**, *402*, 867−871. (b) Inagaki, S.; Guan, S.; Fukushima, Y.; Ohsuna, T.; Terasaki, O. *J. Am. Chem. Soc.* **1999**, *121*, 9611−9614. (c) Melde, B. J.; Holland, B. T.; Blanford, C. F.; Stein, A. *Chem. Mater.* **1999**, *11*, 3302−3308.

(10) (a) Han, Y.-J.; Stucky, G. D.; Butler, A. *J. Am. Chem. Soc.* **1999**, *121*, 9897−9898. (b) Lei, C.; Shin, Y.; Liu, J.; Ackerman, E. J. *J. Am. Chem. Soc.* **2002**, *124*, 11242−11243. (c) Ravindra, R.; Zhao, S.; Gies, H.; Winter, R. *J. Am. Chem. Soc.* **2004**, *126*, 12224−12225.

(11) (a) Yan, X.; Yu, C.; Zhou, X.; Tang, J.; Zhao, D. *Angew. Chem., Int. Ed.* **2004**, *43*, 5980−5984. (b) Shi, Q.; Wang, J.; Zhang, J.; Fan, J.; Stucky, G. D. *Adv. Mater.* **2006**, *18*, 1038−1042. (c) López-Noriega, A.; Arcos, D.; Izquierdo-Barba, I.; Sakamoto, Y.; Terasaki, O.; Vallet-Regí, M. *Chem. Mater.* **2006**, *18*, 3137−3144.

(12) (a) Vallet-Regí, M.; Rámila, A.; del Real, R. P.; Pérez-Pariente, J. *Chem. Mater.* **2001**, *13*, 308−311. (b) Muñoz, B.; Rámila, A.; Pérez-Pariente, J.; Díaz, I.; Vallet-Regí, M. *Chem. Mater.* **2003**, *15*, 500−503. (c) Andersson, J.; Rosenholm, J.; Areva, S.; Lindén, M. *Chem. Mater.* **2004**, *16*, 4160−4167.

(13) (a) Lai, C.-Y.; Trewyn, B. G.; Jeftinija, D. M.; Jeftinija, K.; Xu, S.; Jeftinija, S.; Lin, V. S.-Y. *J. Am. Chem. Soc.* **2003**, *125*, 4451−4459. (b) Giri, S.; Trewyn, B. G.; Stellmaker, M. P.; Lin, V. S.-Y. *Angew. Chem., Int. Ed.* **2005**, *44*, 5038−5044. (c) Mal, N. K.; Fujiwara, M.; Tanaka, Y. *Nature* **2003**, *421*, 350−353. (d) Zhu, Y.; Shi, J.; Shen, W.; Dong, X.; Feng, J.; Ruan, M.; Li, Y. *Angew. Chem., Int. Ed.* **2005**, *44*, 5083−5087.

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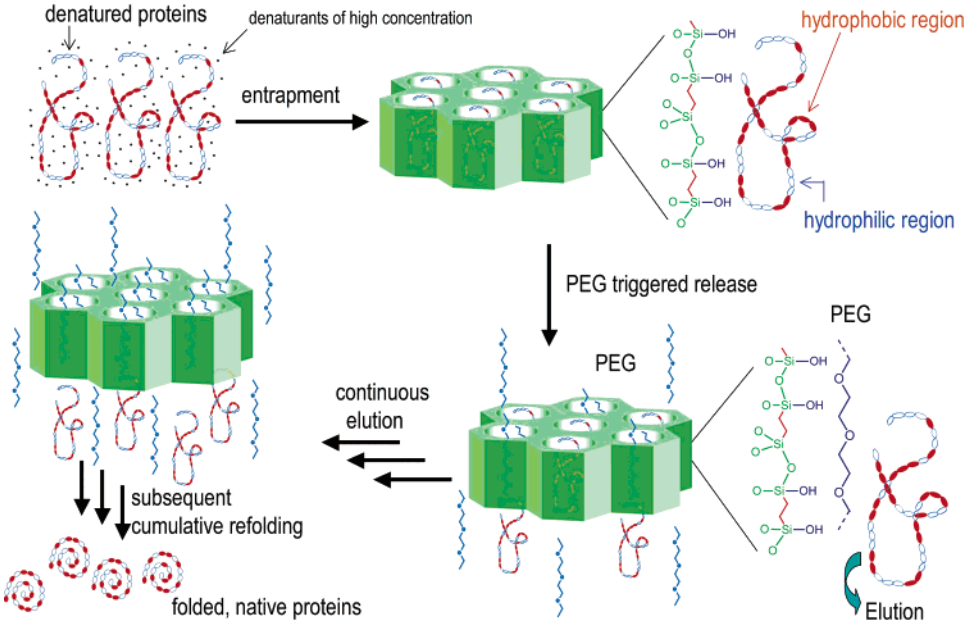
Scheme 1. Protein Refolding Assisted by PMO



mesoporous materials without disturbing the optimal refolding conditions. As illustrated in Scheme 1, the new protein refolding strategy takes advantage of the unique surface and structural properties of PMOs, which can effectively entrap denatured proteins and exhibit a stimuli-responsive controlled release of entrapped proteins into the refolding buffer. Through the loading of denatured proteins inside uniform mesoporous channels tailored to accommodate individual proteins, protein aggregation is minimized. In addition, the continuous release of denatured proteins ensures cumulative refolding. Hen egg white lysozyme (molecular dimensions of $1.9 \times 2.5 \times 4.3$ nm^3)[14] is selected as a model protein to demonstrate a new methodology for protein refolding.

Experimental Section

Reagents. Tetraethyl orthosilicate (TEOS), ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl)aminomethane (Tris, biochemical grade), and urea (biochemical grade) were obtained from Acros. Poly(ethylene glycol) (PEG), nonionic surfactant Brij 76 ($C_{18}H_{37}(OCH_2CH_2)_{10}OH$), and Brij 56 ($C_{16}H_{33}(OCH_2CH_2)_{10}OH$) were purchased from Aldrich. 1,2-Bis-(triethoxysilyl)ethane (BTESE) was received from Gelest. Hen egg white lysozyme (EC 3.2.1.17), dithiothreitol (DTT), reduced and oxidized glutathione (GSH and GSSG), and *Micrococcus lysodeikticus* dried cells were obtained from Sigma. Millipore water was used to prepare all buffer solutions. All other reagents used were of biochemical or analytical grade.

Synthesis of Mesoporous Materials. Ethylene bridged periodic mesoporous organosilicas with two different pore sizes were prepared under acidic conditions using BTESE as the organosilica precursor and Brij 76 and Brij 56 as the templates.[15] Typically, 8 g of Brij 76 (or 7.68 g of Brij 56) was dissolved in an acidic aqueous solution containing 206.8 g of distilled water and 33.2 g of concentrated HCl. When a homogeneous solution was formed after stirring at 50 °C for 1 h, BTESE (14.16 g) was added with stirring at the same temperature. The mixture was stirred at 50 °C for 24 h and then put in an oven at 50 °C for another 24 h under static conditions. The solid was recovered by filtration, washed with water and ethanol, and dried. The template was removed by two consecutive solvent extractions by stirring in EtOH at 60 °C for 5 h using 150 g of EtOH and 2.0 g of concentrated HCl per gram of dry as-made sample. The obtained periodic mesoporous organosilicas were denoted as E-PMO-A (Brij 76) and E-PMO-B (Brij 56). For comparisons, pure siliceous SBA-15 was prepared by the same procedure using Brij 76 as the template and TEOS as the silica source.

Characterization. Powder X-ray diffraction (XRD) patterns were recorded on a Bruker D8 Advance diffractometer with Cu Kα radiation (40 kV, 40 mA). N_2 sorption analysis was performed on a Micromeritics ASAP2010 volumetric adsorption analyzer at 77 K. Prior to the measurement, the samples were degassed at 353 K for 6 h under vacuum. The specific surface area was calculated using the BET method from the nitrogen adsorption data in the relative range (P/P_0) of 0.05−0.30. The total pore volume was determined from the amount of N_2 uptake at $P/P_0 = 0.99$. The external surface area of E-PMO-A was estimated using the high-resolution α_s plot method.[16,17] Nitrogen adsorption data on nonporous silica (LiChrospher Si-1000, $S_{BET} = 26.2$ m^2 g^{-1})[18] was used as a reference to construct the α_s plot. The pore size distribution (PSD) plot was derived from the adsorption branch of the isotherm based on the Kruk−Jaroniec−Sayari modified BJH method.[19] The pore diameter refers to the maximum in the PSD plot.

Denaturation of Protein. Lysozyme at 50 mg/mL was denatured and reduced in 100 mM Tris-HCl buffer (pH 8.5) containing 8 M urea, 30 mM DTT, and 1 mM EDTA. The mixture was incubated at 37 °C for 3 h under N_2. After the mixture reached room temperature, DTT was removed by a HiTrap desalting column (5 mL, Amersham Biosciences). Prior to separation, the column was equilibrated with 25 mL of 100 mM Tris-HCl buffer (pH 8.5) containing 8 M urea and 1 mM EDTA, which was also used as the elution solution for separation. The flow rate was about 1.0 mL/min. The complete reduction of disulfide bonds and removal of DTT were confirmed by thiol titration according to Ellman's method. The lysozyme concentrations were determined by UV absorbance at 280 nm using extinction coefficients of 2.63 and 2.37 cm·mg/mL for native and denatured forms, respectively.[20] All UV absorbance measurements were performed with a Shimadzu UV-3101PC spectrophotometer.

(14) Hartmann, M. *Chem. Mater.* **2005**, *17*, 4577−4593.
(15) (a) Sayari, A.; Yang, Y. *Chem. Commun.* **2002**, 2582−2583. (b) Wang, W.; Xie, S.; Zhou, W.; Sayari, A. *Chem. Mater.* **2004**, *16*, 1756−1762. (c) Burleigh, M. C.; Markowitz, M. A.; Jayasundera, S.; Spector, M. S.; Thomas, C. W.; Gaber, B. P. *J. Phys. Chem. B* **2003**, *107*, 12628−12634.
(16) Rouquerol, F.; Rouquerol, J.; Sing, K. *Adsorption by Powders and Porous Solids: Methods, Principals and Applications*; Academic Press: London, 1999.
(17) Kruk, M.; Jaroniec, M.; Ryoo, R.; Kim, J. M. *Microporous Mater.* **1997**, *12*, 93.
(18) Jaroniec, M.; Kruk, M.; Olivier, J. P. *Langmuir* **1999**, *15*, 5410−5413.
(19) Kruk, M.; Jaroniec, M.; Sayari, A. *Langmuir* **1997**, *13*, 6267−6273.
(20) Wetlaufer, D. B.; Johnson, E. R.; Clauss, L. M. In *Lysozyme*; Osserman, E. F., Canfield, R. E., Beychok, S., Eds.; Academic Press: New York, 1974; pp 269−280.

Table 1. Structural Properties of Mesoporous Materials and Their Adsorption Capacities for D-Lys[a]

materials	template	w_{KJS} (nm)	S (m^2/g)	V (cm^3/g)	C (mg/g)
E-PMO-A	Brij 76	4.8	891	0.83	168
E-PMO-B	Brij 56	4.1	1025	0.80	138
SBA-15	Brij 76	5.3	990	0.96	280

[a] w_{KJS}, KJS pore size; S, BET surface area; V, pore volume; and C, maximum adsorption capacity of denatured protein under current conditions, calculated from the adsorption isotherm.

Adsorption of Denatured Lysozyme (D-Lys) on Mesoporous Materials. With stirring, mesoporous materials (2 mg/mL) were dispersed in D-Lys solutions of various initial concentrations in 100 mM Tris-HCl buffer (pH 8.5) containing 6 M urea and 1 mM EDTA. The amount adsorbed was calculated by subtracting the UV absorbance at 280 nm of the supernatant after centrifugation from that of D-Lys before adsorption.

Elution of Entrapped Protein. D-Lys (0.6 mg/mL) was loaded in 10 mg of mesoporous materials by stirring for 2 h. The protein-entrapped solids were recovered by centrifugation. Elution tests were performed by adding 100 mM Tris-HCl buffer solution (pH 8.5) containing 2 M urea, 1 mM EDTA, and PEG (MW 2000) at various concentrations to the recovered solids with stirring at room temperature. The volume of elution buffer added was determined by the adsorption capacity, giving a mixture of 1 mg of D-Lys per mL of solution. For example, 1.6 mL of elution solution was added to protein-containing E-PMO-A (164 mg of protein per g). At different time intervals, eluted proteins in solution were removed after centrifugation, and the same amounts of fresh buffer solutions were added again.

Protein Refolding. By the batch dilution method, refolding was initiated by a rapid 30-fold dilution of concentrated protein solution into refolding buffer (100 mM Tris-HCl, 1 mM EDTA, 2 M urea, 1.5 mM GSH, 0.3 mM GSSG, pH 8.5) at room temperature. Identical aliquots of the refolding sample were withdrawn and immediately assayed for enzymatic activity. In the case of PMO-assisted refolding, D-Lys (0.6 mg/mL) was first loaded into E-PMO-A (20 mg), as described above. After adsorption for 2 h, protein-containing E-PMO-A (164 mg/g) was recovered by centrifugation. The refolding buffer (3.2 mL of 100 mM Tris-HCl, pH 8.5) containing 2 M urea, 1 mM EDTA, 1.5 mM GSH, 0.3 mM GSSG, and PEG at various concentrations (from 10 to 75 mg/mL) was then added. After the solution was stirred for 20 h, the supernatant was recovered by centrifugation. The concentrations of recovered protein were determined by the Bio-Rad protein assay method. Native lysozyme was used as a protein standard.

Protein Activity Assay. The lysozyme activity was assayed at room temperature by monitoring the decrease in UV absorbance at 450 nm of a *Micrococcus lysodeikticus* cell suspension (0.20 mg/mL in 67 mM sodium phosphate buffer, pH 6.2). Renatured lysozyme solutions were first diluted with 100 mM Tris-HCl buffer (pH 8.5) to the desired concentrations. A diluted protein sample (100 μL) was then mixed with 3.0 mL of the cell suspension. A linear decrease in absorbance was observed, and the slope from 10 to 60 s was used to calculate the refolding yield by comparing to a native lysozyme solution of identical protein concentration. The activity of native lysozyme of equal concentration was 100%.

Results and Discussion

The XRD patterns of E-PMO-A and SBA-15 show the characteristics of an ordered 2D hexagonal mesostructure (Supporting Information). Compared to E-PMO-A and SBA-15, E-PMO-B exhibits less order in the mesostructure, but it also possesses a narrow pore size distribution (Supporting Information). The BET surface area, pore size, and pore volume of template-free samples are summarized in Table 1. E-PMO exhibits both hydrophilic and hydrophobic features as a result of the presence of silanol and ethylene groups on the silica framework (as shown in Scheme 1). These features have been confirmed by both ^{13}C CP and ^{29}Si MAS solid-state NMR spectra (Supporting Information).

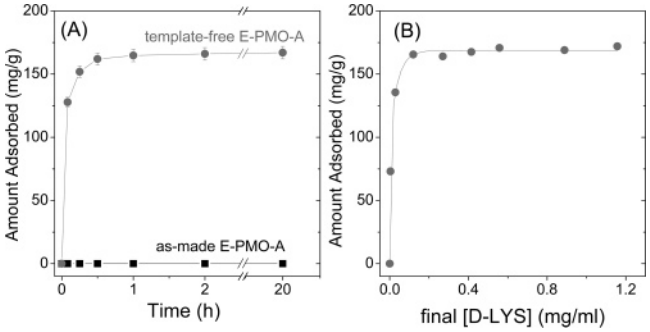


Figure 1. (A) Adsorption kinetics of D-Lys on E-PMO-A with an initial D-Lys concentration of 0.6 mg/mL. At different time intervals, a 1.0 mL aliquot was taken out of the mixture. After centrifugation, the supernatant was used for UV absorbance measurements at 280 nm. The standard deviation has been found to be less than 3%, as indicated by the error bars. (B) Adsorption isotherm of denatured lysozyme on E-PMO-A. E-PMO-A (8 mg) was dispersed in 4 mL of D-Lys solutions of different initial concentrations, and the mixture was stirred overnight to establish adsorption equilibrium.

Hen egg white lysozyme was used as a model protein to study the efficiency of PMO-assisted refolding. The lysozyme was denatured and reduced by 8 M urea and DTT. To avoid the interference of UV adsorption of the protein at 280 nm and the carryover effect[21] on refolding, DTT was removed by a HiTrap desalting column. The adsorption of denatured and reduced lysozyme (D-Lys) was carried out in the presence of 6 M urea. Template-free E-PMO-A quickly adsorbed D-Lys and reached equilibrium within 2 h (Figure 1). The maximum loading capacity was 168 mg of protein per g of E-PMO-A. By comparison, as-made E-PMO-A did not show a noticeable uptake under the same conditions. Considering the small external surface area of template-free E-PMO-A (63 m^2/g, Supporting Information), we conclude that D-Lys was mainly adsorbed onto the internal surface of the mesoporous materials. Compared to E-PMO-A, E-PMO-B exhibited a comparable adsorption capacity whereas SBA-15 showed a much higher loading (Table 1). These protein-loaded mesoporous silicas exhibited no detectable leaching over a period of 24 h in 100 mM Tris-HCl buffer (pH 8.5) containing 2 M urea, as monitored by UV absorbance at 280 nm. This result suggests a strong affinity of D-Lys for the E-PMO and SBA-15 surfaces. At pH 8.5, the lysozyme (isoelectric point pI = 10.8) is positively charged, but the silica surface (pI = 2.0) is negatively charged. Interactions between mesoporous silicas and lysozyme include electrostatic interactions between charged silanol groups on the silica surface and the exposed charge of amino acid residues of lysozyme and hydrophobic interactions between ethylene groups of E-PMO and exposed hydrophobic parts of protein.[22] Therefore, both electrostatic and hybrophobic interactions contribute to the adsorption of D-Lys on E-PMO whereas in the case of pure siliceous SBA-15 electrostatic interactions are dominant. A recent study indicates that electrostatic interactions rather than hydrophobic forces between proteins and silica surfaces are dominant in the bioadsorption of cytochrome *c* on PMO and SBA-15.[23] Compared to SBA-15, PMOs exhibit a lower density of silanol groups on the surface as a result of the presence of integrated organic groups. Thus, D-Lys is expected to show weaker affinity

(21) Lanckriet, H.; Middelberg, A. P. J. *J. Chromatogr., A* **2004**, *1022*, 103−113.
(22) Ricker, R. D.; Sandoval, L. A.; Justice, J. D.; Geiser, F. O. *J. Chromatogr., A* **1995**, *691*, 67−79.
(23) Qiao, S. Z.; Yu, C. Z.; Xing, W.; Hu, Q. H.; Djojoputro, H.; Lu, G. Q. *Chem. Mater.* **2005**, *17*, 4160−4167.

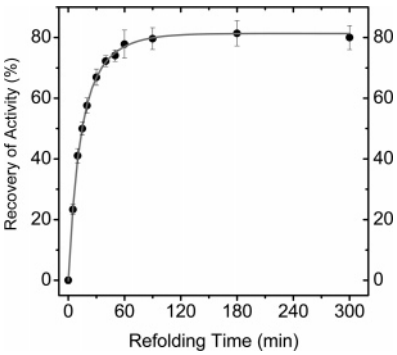


Figure 2. Oxidative refolding kinetics of 0.1 mg/mL D-Lys in the presence of 2.0 M urea and 1.5−0.3 mM GSH−GSSG by the batch dilution method. Refolding was initiated by a rapid 30-fold dilution of concentrated protein solution into refolding buffer at room temperature. At different time intervals, identical aliquots of refolding sample were withdrawn and immediately assayed for enzymatic activity. Error bars show the standard deviation ($n = 3$). The half-time of refolding (~40% recovery) was about 10 min.

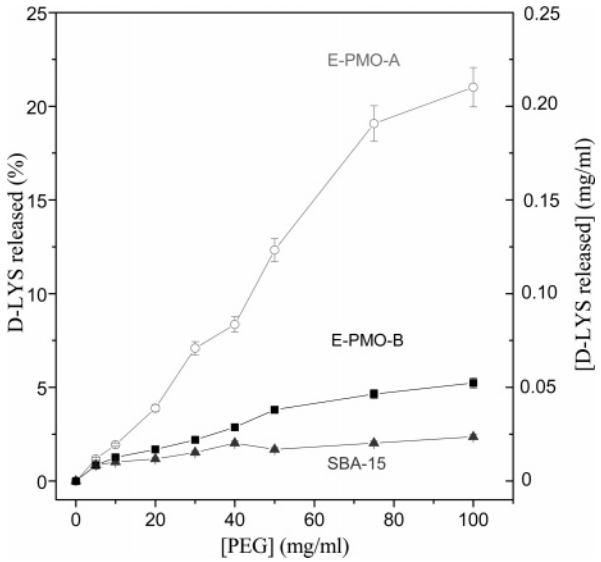


Figure 3. Effects of PEG concentration on the release of D-Lys from mesoporous materials. Amounts of released D-Lys were monitored by the UV absorbance of supernatants after 30 min. The standard deviation has been found to be less than 5%, as indicated by the error bars.

on E-PMO than on SBA-15. All of these factors contribute to the highest adsorption capacity of D-Lys on SBA-15.

Figure 2 shows the refolding kinetics of D-Lys at 0.1 mg/mL in the presence of 2 M urea, 1.5 mM reduced glutathione (GSH), and 0.3 mM oxidized glutathione (GSSG) by the batch dilution method. It is clear that under such conditions D-Lys can quickly renature and achieves high refolding yields (>80%) at low protein concentrations (e.g., below 0.1 mg/mL). Therefore, if the concentrations of released protein can be controlled to be low enough, D-Lys can refold under optimal conditions.

Poly(ethylene glycol) (PEG) was selected as a trigger to release encapsulated D-Lys from the mesopores because it can form strong H bonds with the silanol groups on the silica surface and thus reduce the affinity between D-Lys and E-PMO/SBA-15. As shown in Figure 3, there is an increased release rate with increasing concentration of PEG (MW 2000) in the elution buffer solutions. More proteins can be eluted after a longer time (Supporting Information). Among the three materials studied, E-PMO-A gives the best release results, whereas the amounts of protein released from E-PMO-B and SBA-15 are small under the same conditions.

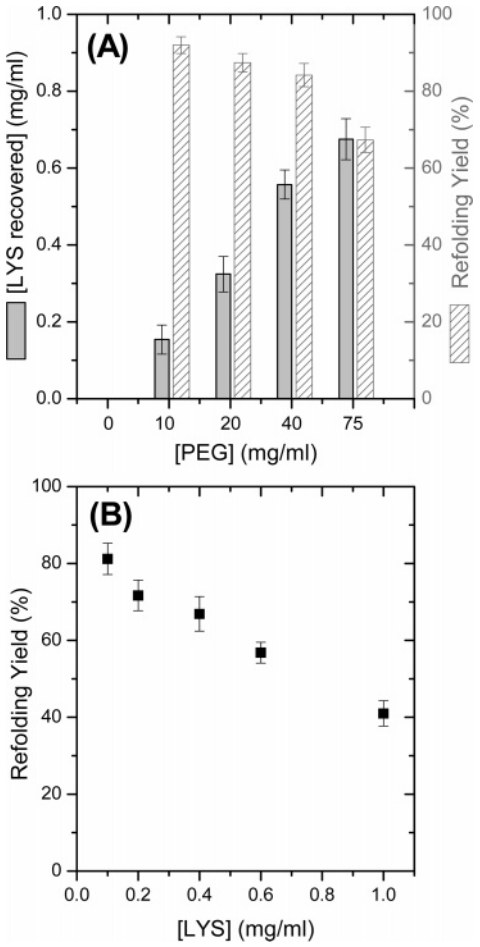


Figure 4. (A) E-PMO-A-assisted refolding of D-Lys. Error bars show the standard deviation ($n = 3$). (B) Effects of protein concentration on the oxidative refolding of D-Lys by the batch dilution method.

As discussed above, too strong an affinity of D-Lys on SBA-15 may be responsible for the slow release rate. Compared to E-PMO-A, E-PMO-B has a smaller pore size (4.1 nm), which is comparable to or slightly larger than the molecular dimensions of the lysozyme and thus may limit the transportation of both PEG and protein. It should be noted that the release rate during the refolding process in the presence of the redox agents would probably not be the same as the results presented here, but the elution rate, total amount, and concentration of D-Lys can be controlled by tuning the PEG concentrations. In addition, the effects of the average molecular weights of PEG (from 600 to 10000) on release have also been studied. Larger molecular weight leads to a faster release rate.

The above results indicate that the pore size and surface properties of mesoporous materials are important for the adsorption and subsequent release of D-Lys. Because of the reluctant release from E-PMO-B and SBA-15, we focus on the folding of D-Lys released from E-PMO-A. The elution and renaturation of D-Lys can be realized concurrently in the presence of the refolding buffer containing both PEG and the redox agents (GSH and GSSG). Consistent with the elution results, an increase in the concentration of refolded lysozyme was found with increasing PEG (MW 2000) concentration (Figure 4A). That is, there is an increase in the recovered lysozyme concentration from 0.16 mg/mL (10 mg/mL PEG) to 0.57 mg/mL (40 mg/mL PEG) with refolding yields above 85%. Compared to the refolding by the batch dilution method (Figure 4B), PMO-assisted refolding under identical conditions exhibits higher refolding yields. At

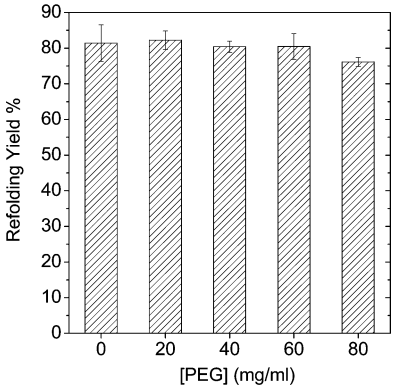


Figure 5. Effects of PEG concentration on the oxidative refolding of 0.1 mg/mL lysozyme in the presence of 2.0 M urea and 1.5−0.3 mM GSH−GSSG. Refolding was initiated by a rapid 30-fold dilution of concentrated protein solution into refolding solutions containing PEG at room temperature. Enzymatic activity was assayed after 6 h. Error bars show the standard deviation ($n = 3$).

higher PEG concentrations, more proteins were released, resulting in higher concentrations of D-Lys. Aggregation may be dominant for refolding at high protein concentrations, whereas a high refolding yield can be obtained only at low protein concentrations because of their different kinetic reaction orders.[2] Therefore, the formation of inactive aggregates at high PEG concentrations reduces the refolding yield. Both incomplete release of entrapped proteins and imperfect refolding (e.g., formation of aggregates) are responsible for <100% recovery, which might be improved by chromatographic procedures.[24,25]

Refolded proteins can be adsorbed on PMOs.[23] However, in the presence of PEG, E-PMO exhibits very little or no adsorption of native lysozyme (Supporting Information). This property ensures that all of the refolded proteins stay in the buffer solution. PEG has also been demonstrated to improve the folding yield for some other proteins.[26] To rule out the possibility of the PEG enhancement effect on the oxidative refolding of lysozyme, a series of refolding experiments were carried out in the presence of PEG by the batch dilution method. As shown in Figure 5, PEG (MW 2000) has little or no effect on the refolding yield of D-Lys under such conditions. Therefore, the improvement of refolding assisted by E-PMO-A can be attributed to the controlled slow but continuous feeding of D-Lys for refolding. Another advantage of the current method is that through the adsorption of unfolded proteins on mesoporous materials denaturant can be easily separated by centrifugation without causing the aggregation of unfolding proteins. The merits of this technique include the reusability of the matrix, the easy separation of proteins from denaturants, and the controllable refolding process. A logical extension of the current method will be in using mesoporous materials as chromatographic beds for protein refolding using a continuous protocol.[25]

Conclusions

A new method to enhance lysozyme oxidative refolding was illustrated by the active entrapment of denatured proteins in PMO and by subsequent PEG-triggered release into the refolding buffer. Unlike the "pulse-renaturation"[4] or "fed-batch operation"[5] method, protein refolding assisted by PMOs does not require the stepwise or continuous addition of denatured proteins containing denaturants to the refolding buffer, which will increase the denaturant concentration to a level at which denaturants interfere with the correct structure formation. Free of this limitation, the new method enables high-yield refolding at higher concentrations (e.g., >1 mg/mL). The rich synthetic chemistry of ordered mesoporous materials, together with various pore geometries and sizes[7] and controllable surface functionalities,[8] makes it possible to develop suitable materials for the adsorption and controlled release of a given protein. This strategy can be extended to the refolding of other proteins.

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Supporting Information Available: XRD patterns, N_2 adsorption isotherms, pore size distribution plots, and ^{13}C and ^{29}Si solid-state MAS NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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(24) Li, M.; Su, Z.-G.; Janson, J.-C. *Protein Expression Purif.* **2004**, *33*, 1−10.
(25) Jungbaue, A.; Kaar, W.; Schlegl, R. *Curr. Opin. Biotechnol.* **2004**, *15*, 487−494.
(26) Cleland, J. L.; Builder, S. E.; Swartz, J. R.; Winker, M.; Chang, J. Y.; Wang, D. I. C. *Bio/Technology* **1992**, *10*, 1013−1019.