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pH-Triggered Adsorption−Desorption of Enzyme in Mesoporous Host to Act on Macrosubstrate

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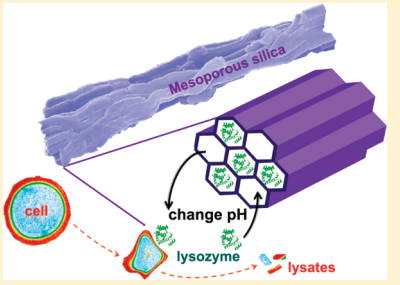
ABSTRACT: Numbers of reports have demonstrated the merits of confining enzyme in mesoporous supports, but have ignored the limitations to the application for biocatalytic transformation of a macrosubstrate. So in this work a strategy based on pH-triggered release and recovery of proteins has been presented to overcome this restriction. By modulation of the pH value, the encapsulated lysozyme was released from the mesopores to act on the macrosubstrate, displaying full bacteriolytic activity, and then was basically readsorbed by another pH modulation. The protein release-recovery is directly confirmed by confocal laser scanning microscopy (CLSM) and X-ray diffraction (XRD) observations. In virtue of substrate-enhanced desorption and privileged readsorption of lysozyme, high bioactivity and good reusability have been achieved.



1. INTRODUCTION

Enzymatic transformations of macrosubstrates are of great interest and profound significance. They are, for example, the hydrolysis of starch by amylases, biodegradation of cellulose and lignin, limited proteolysis by protease, lysis of bacteria cell by lysozyme, and hydrolysis of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA).[1,2] The lysis of a bacteria cell, a substrate in micrometer-size, is very useful in biology,[2] food,[3] and clinical[4] fields. However, a major problem arose when immobilized enzymes were applied to macrosubstrates, in that it was difficult for bulky substrates to access the active sites of enzyme molecules due to the steric hindrance and diffusion resistance generated by the support. Although immobilization onto a nonporous support[5] or external surface[6] caused less diffusion limitation, enzyme loading and retained activity were unsatisfactory. The lysozyme immobilized on polystyrene beads[5] via specific attachment or on extrudate-shaped NaY zeolite[6] by simple adsorption displayed a protein content of 2−3 mg/g support and retained the enzymatic activity in only about 14%. Nanotube silica with a diameter of about 100 nm afforded a relative activity of 85%, but this optimum activity was limited to monolayer adsorption.[7] The protein loading in monolayer adsorption was not higher than 125 mg/g support. When more proteins were adsorbed, the retained activity sharply decreased. Luckarift et al[8,9] reported a lysozyme-silica composite, which demonstrated no significant change in substrate binding. The study also revealed that surface accessible lysozyme in the silica−lysozyme clusters accounted for the lytic activity. The covalent binding of lysozyme to reversibly soluble polymer[10−12] provided an alternative approach. However, incomplete enzyme recovery and enzyme denaturation impeded successive reaction cycles.

Mesoporous materials, with large special surface, high pore volume, and pore dimension comparable to many biological molecules, have been extensively explored[13,14] as supports to encapsulate proteins and enzymes. One prominent superiority of mesoporous supports is the stabilization of encapsulated enzyme, which can be attributed to the "confinement" in mesopores.[15] This confinement can restrain enzyme from unfolding or denaturation, improving intrinsic enzyme stability. In addition, confining enzymes in mesopores facilitates the achievement of operational stability.[16] Mesoporous silica materials also demonstrate merits as enzyme supports in that their surfaces are readily functionalized, which provides opportunity to imitate the diversity of noncovalent forces in biological systems, such as electrostatic attraction, hydrophobic affinity, hydrogen bonding, and $\pi-\pi$ overlapping interactions.[17−19] However, the advantages of mesoporous supports can hardly be exerted on macrosubstrates as a result of substrate exclusion. A number of studies[17,18,20−26] have reported the immobilization of lysozyme in mesoporous silica, but few[18] have implemented the bacteriolytic function. Our previous work[18] found that functionalized mesoporous supports displayed a lysozyme adsorption of up to 700 mg/g support, and the adsorbed lysozyme was well retained in mesoporous supports (below 5% desorption) and restrained from denaturation as well. When the immobilized lysozyme was released by vortex in a large volume of buffer solution, 95% or above relative activity was preserved. Unfortunately, it was hard to recover the released lysozyme because the large volume of buffer solution gave rise to a low population of lysozyme.

Here, we propose a pH-triggered release and recovery strategy, which not only overcomes the diffusion limitations for a macrosubstrate to access the enzyme encapsulated in mesopores, but also facilitates the realization of recyclable bacteriolytic functions. As is well known, electrostatic force, one of the most crucial interactions between proteins and support surfaces, and also between proteins themselves, is

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Table 1. Net Charges of Mesoporous Support, Lysozyme, and Cell Wall at Various pH

	Density of functional groups (number/nm^2) [a]	Net charge (number/nm^2) [b]									
		pH 3.0	pH 4.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0	pH 9.0	pH 10.0	pH 11.0	pH 12.0
SBA-OH	5.7 –OH	-1.1	-4.1	-5.5	-5.7	-5.7	-5.7	-5.7	-5.7	-5.7	-5.7
SBA-C_3H_6-COOH	3.7 -OH + 2.0 -COOH	-0.8	-3.0	-4.8	-5.6	-5.7	-5.7	-5.7	-5.7	-5.7	-5.7
SBA-C_3H_6-$NHCONH_2$	1.9 -OH + 3.8 -$NHCONH_2$	+3.4	+2.4	+2.0	+1.9	+1.9	+1.9	+1.9	+1.9	+1.9	+1.9
lysozyme	-	+14	+12	+10	+9	+8	+7	+6	+5	0	-2
Cell wall	-	-0.6	-1.0	-1.4	-1.4	-1.4	-1.4	-1.5	-1.5	-1.6	-1.6

[a] The density of functional groups is obtained from ^{29}Si MAS NMR and element analysis.[17] [b] The net charge is calculated from the density of functional group and corresponding pK_a. pK_a (Si−OH) = 3.6, pK_a ($-C_3H_6-COOH$) = 4.8, pK_a ($-C_3H_6-NHCONH_2$) = 14.2, pI (lysozyme) = 10.8

sensitive to pH environment. In the system with electrostatic interactions dominating, it is facile to control the sorption of proteins by modulating the pH of the adsorption medium. The pH-triggered release and recovery methodology proposed here no doubt provides a feasible and robust alternative for highly efficient and readily recycled biocatalysts.

2. EXPERIMENTAL SECTION

Materials. Lysozyme from chicken egg white (lysozyme, lyophilized powder, protein ≥ 90%) from Sigma, *Micrococcus lysodeikticus* dried cells from Sigma, fluorescein isothiocyanate (FITC) from Sigma, P123 ($EO_{20}PO_{70}EO_{20}$, molecular weight = 5800) from Aldrich, tetraethylorthosilicate (TEOS) from Sinopharm Chemical Reagent Co. Ltd., and other reagents of analytical purity were all used as received. SBA-15 mesoporous materials (SBA-15) with surface activation and modification were synthesized following the reported procedure.[17,31,32]

Lysozyme Adsorption and Desorption in Buffers. Ten millimolar buffer solutions with different pH were first prepared: citric acid-dibasic sodium phosphate buffer for pH 3.0−6.0, potassium dihydrogen phosphate-dibasic sodium phosphate buffer for pH 6.2−8.0, and sodium carbonate-sodium bicarbonate buffer for pH 9.0−12.0. The buffer concentration employed here (10 mM) is sufficiently low to not affect the lysozyme adsorption according to previous reports.[33,34] Lysozyme solution (1 mg·mL^{-1}) was prepared by dissolving lysozyme in the aforementioned buffers. In the adsorption experiments, 10 mg of mesoporous silica was immerged in 6 mL of lysozyme solution with pH of 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0, respectively. The resulting suspension was oscillated at 298 K for 30 min and centrifuged at 5000 rpm for 10 min. The lysozyme content in supernatant was determined by ultraviolet (UV) absorbance at 280 nm. The amount of lysozyme adsorbed on mesoporous silica solid was calculated by subtracting the lysozyme amount in supernatant from the total lysozyme content. In the desorption experiments, the mesoporous silica solid with lysozyme immobilized at pH 10 was respectively remixed with 6 mL of pH 3.0, 4.0, 5.0, 6.0, and 7.0 blank buffer, respectively, by vortex for 5 s. The mixture was centrifuged to measure the released lysozyme.

Lysis of *M. lysodeikticus* Cell. One hundred twenty milligrams of *M. lysodeikticus* cells was added in 6 mL of buffers and was stirred slowly for 10 min at room temperature. The cell suspension (20 mg·mL^{-1}) was used 1 h after preparation. Four grams of sodium dodecyl sulfate (SDS) was dissolved in 16 g of deionized water at 68 °C to prepare a 20% SDS solution as the protein denaturant.[2] The immobilized lysozyme was mixed with 6 mL of cell suspension by vortex for 5 s and stirred at 298 K. The 100 μL mixture was sampled at intervals, exposed to 100 μL of SDS solution immediately, and diluted by 5 mL of buffer. Then the turbidity at 450 nm was recorded. Lysis activity was calculated from the slope of the time course by linear regression of data points and 1 U was defined as 0.01% of decrease in turbidity per minute.

Readsorption of Lysozyme. The mixture after complete lysis of the cell was adjusted to pH 10.0 using 0.2 mol·L^{-1} sodium hydroxide or 0.24 mol·L^{-1} hydrochloric acid solution, and oscillated at 298 K for 30 min for lysozyme readsorption.

Lysozyme Labeling. FITC was used to label lysozyme as follows: FITC was first dissolved in dimethyl sulfoxide (DMSO) to prepare a 1.0 mg·mL^{-1} FITC solution. For 20 mL of lysozyme solution (1 mg·mL^{-1}, pH 8.0), 500 μL of FITC solution was slowly added under stirring. The conjugation continued overnight in the dark at 4 °C. The unconjugated FITC was removed using dialysis tubing (Viskase, 7000 Da cutoff). The adsorption of FITC-labeled lysozyme was carried out following the same procedure as used for the unlabeled lysozyme. The content of labeled lysozyme in the solution was determined by quantitative analysis of fluorescence intensity with excitation at 488 nm and emission at 520 nm.

Characterization. Scanning emission microscope (SEM) images were taken on a Zeiss Supra 55 VP field-emission scanning electron microscope. Powder X-ray diffraction (XRD) patterns were obtained on a Bruker D8 focus X-ray diffractometer with Cu Kα radiation. Confocal laser scanning microscopy (CLSM) observation was carried out on a Leica TCS SP2 confocal microscope. UV−visible (UV−vis) absorption data were collected on a PGENERAL TU-1221 UV−vis recording spectrophotometer. The fluorescence intensity was collected on a Hitachi F7000 fluorophotometer.

3. RESULTS AND DISCUSSION

In light of our previous work,[17] SBA-15 materials with surfaces activated to generate more silanol group (SBA−OH) or further modified by $-C_3H_6NHCONH_2$ (SBA−$C_3H_6NHCONH_2$) or $-C_3H_6COOH$ (SBA−C_3H_6COOH) are chosen as support candidates. On the basis of the isoelectric point of lysozyme and charge characters of mesoporous surfaces (Table 1), the desorption of lysozyme adsorbed on SBA−OH or SBA−C_3H_6-COOH is supposed to occur at a pH of higher than 11 due to

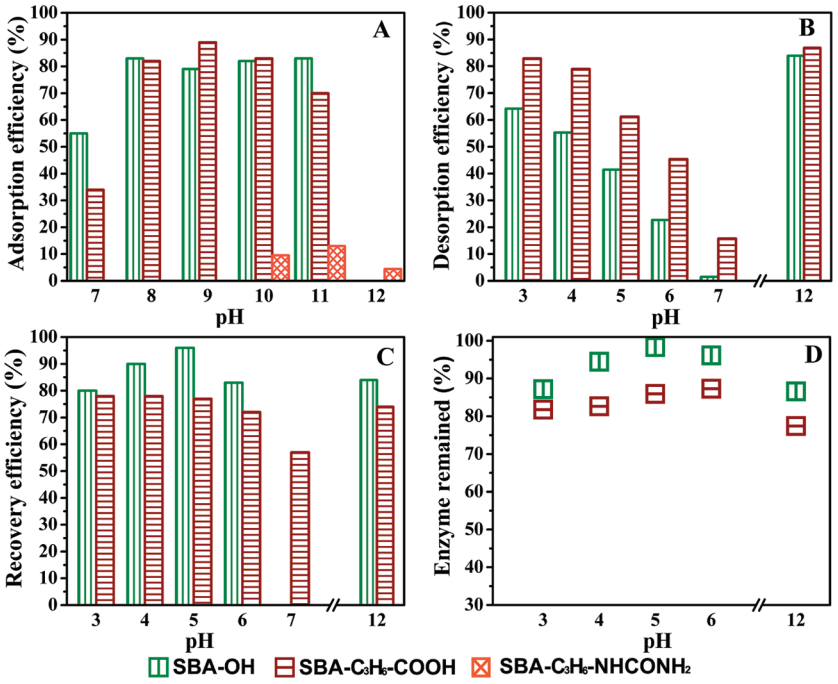

Figure 1. (A) Adsorption efficiency of lysozyme on mesoporous supports at varied pH. (B) Desorption efficiency at varied pH of the lysozyme preadsorbed at pH 10.0. (C) Recovery efficiency at pH 10.0 of the lysozyme desorbed at varied pH. (D) Percentage of lysozyme retained on the supports after one release−recovery cycle.

electrostatic repulsion, and that in SBA-$C_3H_6NHCONH_2$ at a pH of lower than 10. However, on the other hand, even when the surface−protein electrostatic attractions occur, the protein−protein repulsion might cause desorption of the adsorbed lysozyme. To determine the optimum conditions for effective desorption and rapid recovery of lysozyme, therefore, the pH effects are further explored. As reported in our previous work,[17] despite the strong electrostatic interaction between lysozyme and SBA-15 at pH 4.8, the strong lateral repulsion between lysozymes led to quite low adsorbed efficiency on SBA−OH and SBA−C_3H_6COOH. Increasing the pH to 6.8, the adsorbed population significantly increased due to the markedly decreased lysozyme repulsion. So herein the pH effects are to be investigated between pH 7.0 and 12.0. Figure 1A compares the adsorption efficiencies of lysozyme under varied pH between pH 7.0−12.0 on the three supports. SBA−$C_3H_6NHCONH_2$ is observed as not suitable for pH-triggered lysozyme adsorption−desorption because of its quite low adsorption efficiency even though the adsorption time has been prolonged to 3 h. Satisfactory adsorption efficiencies (∼480 mg lysozyme/g support) are achieved in the pH range of 8.0−11.0 on SBA−OH and SBA−C_3H_6COOH. However, when pH decreased to 7.0, the lateral charge repulsion between proteins made a great impact, resulting in lower adsorption efficiency despite the electrostatic attractions between negatively charged supports and positively charged lysozyme (Table 1). According to the results in Figure 1A, a pH of lower than 7.0 or at 12.0 is feasible for desorption of adsorbed lysozyme and the pH range of 8.0−11.0 fits the recovery of desorbed lysozyme using SBA−OH or SBA−C_3H_6COOH as support, as a result of strong surface−protein electrostatic attractions and weak protein lateral repulsions.

As shown in Figure 1B, lysozyme is considerably released from the supports at pH 12.0 as a result of surface−protein repulsions. Eighty-five percent and 88% of adsorbed lysozymes were respectively desorbed from SBA−OH and SBA−C_3H_6COOH at pH 12.0. With pH decreases in turn from 7.0 to 6.0, 5.0, 4.0, and 3.0, the amount of released lysozyme gradually increases, in accordance with increased net charges[27] of lysozyme (Table 1), which enhances protein−protein lateral charge repulsion. The amount of adsorbed lysozyme released from SBA−OH was in 1.5% (pH 7.0), 23% (pH 6.0), 42% (pH 5.0), 56% (pH 4.0), and 65% (pH 3.0), and was in 16% (pH 7.0), 46% (pH 6.0), 62% (pH 5.0), 80% (pH 4.0), and 84% (pH 3.0) from SBA−C_3H_6COOH. Under each pH investigated, more lysozyme was desorbed from SBA−C_3H_6COOH than SBA−OH. Highly efficient recovery in a short time is another important point for effective pH-triggered adsorption and desorption of lysozyme. As can be seen from Figure 1C, readsorption at pH 10.0 recovered the desorbed lysozyme for SBA−OH in higher percentage than for SBA−C_3H_6COOH except for the lysozyme desorbed at pH 7.0. For SBA−OH, more than 80% of lysozyme desorbed at pH 3.0−6.0 was recovered, and the recovered percentage reached 90% or above for the lysozyme desorbed at pH 4.0 and 5.0. For SBA−C_3H_6COOH, less than 80% of desorbed lysozyme was recovered in each case. In one release and recovery cycle, the initial lysozyme was preserved on the support over 80%, as can be seen from Figure 1D. In one cycle of release at pH 5.0 and recovery at pH 10.0, SBA−OH retains lysozyme in 98%. The impact of ionic strength on the adsorption and desorption of lysozyme has also been taken into consideration in this work. For citric acid−dibasic sodium phosphate buffer modulated from pH 3 to pH 10 and back to pH 3 in one cycle, the final total concentration of extra NaCl in the system is as low as 0.014 mol·L^{-1}. This amount of extra salt is not enough to affect the lysozyme adsorption or desorption according to previous reports[33,34] about the effects of ionic strength on the immobilization of a range of proteins on mesoporous silicates. Moreover, the

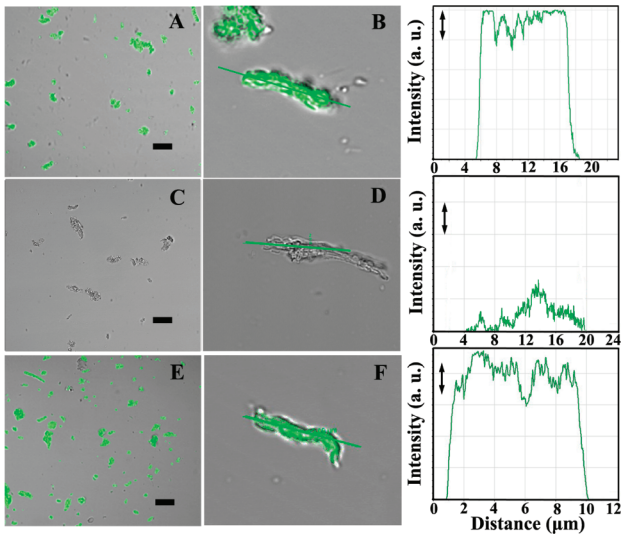

Figure 2. CLSM images and corresponding intensity of SBA−OH with lysozyme encapsulated (A,B), released (C,D), and readsorbed (E,F). The scale bar in the CLSM images (A,C,E) represents 20 μm.

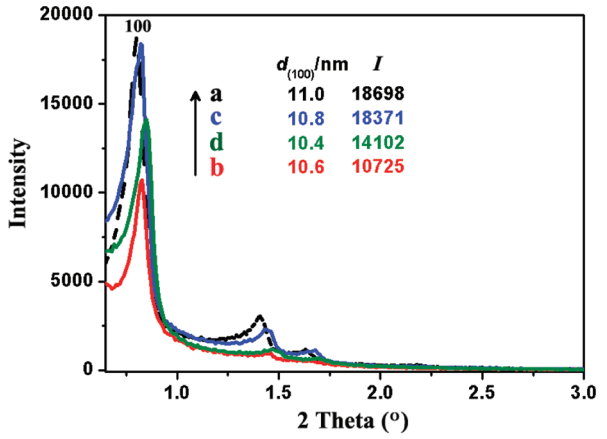

Figure 3. XRD patterns of pristine (a), and lysozyme adsorbed (b), desorbed (c), and readsorbed (d) SBA−OH.

addition of extra NaCl in different loadings into the adsorption system was carried out to investigate its effect on the release of lysozyme. With an additional NaCl concentration of 0.05 mol·L^{-1}, no visible increase in the release of lysozyme (<2%) was detected. When the concentration of added NaCl was increased to 0.15 and 0.33 mol·L^{-1}, the desorption efficiency rose to 11% and 29%. However, from the point of uptake or readsorption of desorbed lysozyme, lower ion strength should be preferred.

In practice, lysozyme should be released in cell suspension. So the effects of cell and lysates on the lysozyme release and recovery have to be taken into consideration. According to a previous report,[28] the cell wall of *M. lysodeikticus* is negatively charged at pH 3.0−12.0 (Table 1). At pH 5.0, the cell wall and lysozyme have opposite charges, and their electronic attraction is supposed to enhance lysozyme desorption. To detect the exact amount of lysozyme desorbed into the cell suspension, lysozyme is labeled by FITC. The quantification of desorbed lysozyme is made through

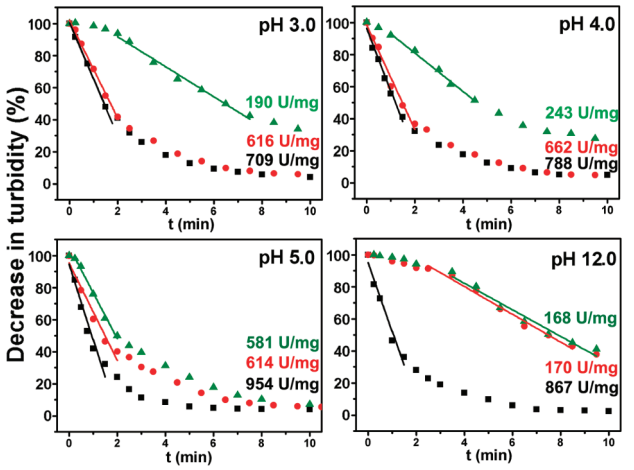

Figure 4. The activity of soluble (■) and immobilized (● first and ▲second cycles) lysozyme in the lysis of *M. lysodeikticus* cell under different pH. The amount of pure lysozyme in either soluble or immobilized form is 4.9 mg.

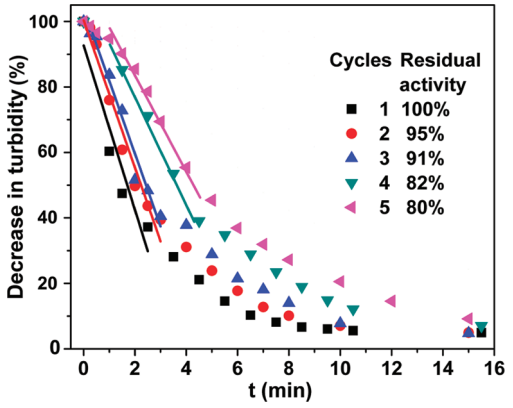

Figure 5. The activity of lysozyme in the lysis of a *M. lysodeikticus* cell under pH 5.0 and the corresponding residual activity at each cycle.

fluorescence measurement without the interference of lysates. The amount of protein released in a suspension of cells was 64%, 153% of that released into the buffer solution in the absence of cell. The counter effect of substrate is observed at pH 12.0 due to the electrostatic repulsion between the cell wall and lysozyme. Twenty-three percent and 70% of lysozyme are released at pH 12.0 in the presence and absence of cell. That is, the release of lysozyme has been significantly facilitated in the presence of cells and lysates at pH 5.0. After complete lysis of the *M. lysodeikticus* cell, lysozyme was recovered at pH 10.0. Fluorescein-labeling is likewise employed to quantify the residual lysozyme in solution. No visible difference was observed between the readsorbed amount with and without lysates, indicative of the specific adsorption of released lysozyme to the protein released from the cells. In one cycle of release at pH 5.0 and recovery at pH 10.0 in the presence of cells and lysates, the protein retention remains 99% of the initial lysozyme loading, almost at the same value (98%) as in pristine buffer solution (Figure 1D). The lysates, including other proteins from the cell, have no negative effect on the readsorption of lysozyme.

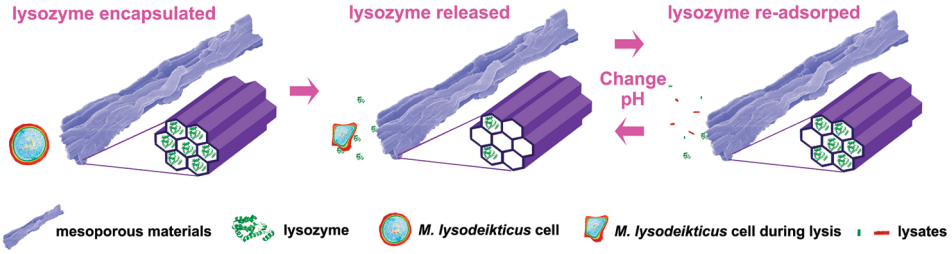

Figure 6. The schematic illustration of pH-triggered adsorption and desorption of lysozyme from mesoporous silica for the lysis of bacteria cells.

In the release−recovery cycle, the location and content of lysozyme in the mesoporous support have been directly observed by monitoring fluorescein-labeled lysozyme using CLSM technique (Figure 2). For comparison, all the CLSM images and intensities are obtained under the same instrument settings. The SBA−OH with lysozyme encapsulated displays uniform fluorescence light in all of the particles. After lysozyme release in cell suspension under pH 5.0, fluorescence light on the support becomes inconspicuous. Correspondingly, the maximum intensity decreases from 255 to around 80. After readsorption of lysozyme at pH 10.0, the maximum light intensity is recovered to 241. The fluorescence intensity change accords well with the above quantitative analysis of proteins. The XRD patterns of SBA−OH with and without lysozyme uptake provide confirming information. As shown in Figure 3, all solids exhibit three well-resolved peaks that can be indexed[29] as (100), (110), and (200) reflections characteristic of a well-ordered mesostructure. However, the solid with lysozyme encapsulated shows much weaker XRD reflections than the pristine support. Such a decrease in reflection intensity was also previously observed[20] and interpreted as a reduction of contrast in density between the silica walls and the pores due to the proteins inside the pores. Lysozyme desorption fully recovers the intensity of XRD reflections, and readsorption of lysozyme reduces the XRD reflections again. The regular change of the XRD intensity well ascertain the reversible desorption and adsorption of lysozyme on SBA−OH support.

The lyophilized cell of *M. lysodeikticus*, a common macrosubstrate for lysozyme,[30] is used to evaluate the activity of lysozyme. As shown in Figure 4, the linear regression of the suspension turbidity indicates that, except the lysozyme released at pH 12.0, all released lysozymes display lysis activity more than 600 U/mg in the first cycle. The activity of lysozyme released from SBA−OH at pH 5.0 is 614 U/mg, as high as 64% of the total activity at pH 5.0 of lysozyme in the SBA−OH. The percentage of released lysozyme determined by fluorescence quantitative analysis is 64%, much larger than the value detected without the cell (42%, Figure 1B), consistent with the observed relative activity. This result not only confirms the fact of substrate-enhanced desorption of lysozyme but also rationally deduces the full preservation of native enzymatic activity. Released at pH 3.0 and 4.0, 65% and 56% desorption of lysozyme was measured in the buffer without cell (Figure 1B) but 87% and 84% of activity were expressed in the presence of cell, further supporting the substrate-enhanced desorption. The substrate-enhanced desorption significantly increases with pH in the order of 3.0, 4.0, and 5.0. In the second cycle for the lysozyme released at pH 5.0, 95% activity of the first cycle is preserved. However, the activity of lysozyme is found to significantly decrease under pH 3.0 and 4.0 in the second cycle. As mentioned above, in the presence of cell as a substrate, the lysozyme desorption amount is implied to significantly increase compared with that without substrate. So the recovery of lysozyme in the second cycle becomes more difficult than that without substrate according to the rule illustrated in Figure 1C, leading to more loss of the lysozyme and thus the decrease of the activity. It reveals that the proper desorption amount of lysozyme is vital, rather than the more the better.

The bioactivity toward the macro-substrate has been carried out in five cycles of release and lysis at pH 5.0 and recovery at pH 10.0. As shown in Figure 5, the bioactivity of lysozyme after five cycles retains 80% of the activity in the first cycle. Effective pH-triggered release and recovery of lysozyme acting on a macro-substrate is schematically shown in Figure 6. The mesopore-encapsulated lysozyme is released efficiently from the mesoporous host, displays full bacteriolytic activity, and then is almost completely recovered.

4. CONCLUSION

In summary, we have demonstrated an effective pH-triggered enzyme release and recovery strategy for the catalytic transformation of macrosubstrates. It takes full use of the merits of mesoporous supports, harvesting higher protein loading and retaining better biocatalytic activity. Substrate-enhanced lysozyme desorption and privileged lysozyme recovery substantially advance recycle efficiency. Although only one enzyme and its macrosubstrate have been involved in this work, it can potentially be extended to other cases by adjusting the release and recovery pH.

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