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Osmotic Pressure Triggered Rapid Release of Encapsulated Enzymes with Enhanced Activity

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In this study, a single-step microfluidic approach is reported for encapsulation of enzymes within microcapsules with ultrathin polymeric shell for controlled release triggered by an osmotic shock. Using a glass capillary microfluidic device, monodisperse water-in-oil-in-water double emulsion droplets are fabricated with enzymes in the core and an ultrathin middle oil layer that solidifies to produce a consolidated inert polymeric shell with a thickness of a few tens to hundreds of nanometers. Through careful design of microcapsule membranes, a high percentage of cargo release, over 90%, is achieved, which is triggered by osmotic shock when using poly(methyl methacrylate) as the shell material. Moreover, it is demonstrated that compared to free enzymes, the encapsulated enzyme activity is maintained well for as long as 47 days at room temperature. This study not only extends industrial applications of enzymes, but also offers new opportunities for encapsulation of a wide range of sensitive molecules and biomolecules that can be controllably released upon applying osmotic shock.

1. Introduction

Enzymes are macromolecular biological catalysts that can accelerate chemical and biochemical reactions. Compared to traditional chemical catalysts, enzymes have many advantages, including high substrate specificity and catalytic efficiency, mild operational conditions, and compatibility to the environment.[1] Therefore, enzymes are used widely in diverse industrial segments, such as food, personal care, leather, paper, textile, and detergent.[2] In fact, the industrial enzyme market was valued at $4.2 billion in 2014, and is projected to reach $6.2 billion by 2020, according to a recent report.[3]

In spite of much progress and success in enzymatic industrial applications, one of the major barriers that prevent wider use of enzymes is their insufficient stability under storage and processing conditions. Unlike natural physiological environments, where enzymes have evolved to function, different formulation variables, including pH, solvents composition, metal ions, and surfactants, affect enzyme stability through unfolding their native structures that consequently deactivates the enzyme.[4] Thus, to advance enzyme applications in industry requires effective strategies to protect the enzymes from denaturation upon long-term storage and confer operational stability under adverse industrial conditions.

To enhance the stability of enzymes in these industrial environments that are incompatible with enzymes, two general criteria must be fulfilled. First, protection of the enzyme under processing condition must be satisfied. Second, the protected enzyme should be controllably released at predefined conditions. Existing protective methods include immobilizing enzymes to a support, cross-linking of enzyme aggregates using a bifunctional reagent, and encapsulation.[5] Among these methods, immobilization or cross-linking of enzymes suffer from several drawbacks, including lack of enzyme conformation flexibility, and enzyme inactivation through conformational changes during the immobilization and cross-linking reactions.[6] Ideally, effective protection of enzymes should have minimal or no impact on the enzymes themselves. From this perspective, vectoring enzymes within an innocuous environment, such as encapsulation within a carrier material like a polymeric membrane or a network matrix, is of particular interest.[7] It is achieved by physically enclosing enzymes in a

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membrane capsule under mild condition, thus avoiding negative influence on the enzyme structure. In addition, encapsulation also provides confinement without total loss of enzyme freedom but restricts unfolding movements.[8] One method to encapsulate enzymes is an emulsion-based technique. The method involves the formation of water in oil emulsion, followed by an interfacial reaction to produce microcapsules with a stable shell. However, the specific activity and release rate of encapsulated enzymes using emulsion techniques decrease due to strong interactions between enzymes and reactive membrane materials.[9] A second method is based on hydrogels or sol–gel technique, and involves mixing enzymes and precursors to obtain homogenous mixtures, followed by gel formation.[10] Nevertheless, the release of the encapsulated enzymes within dense gels is mostly prohibited or requires additional disintegrants.[11] Mesoporous silica as template is also used to encapsulate enzymes followed by layer-by-layer assembly and core removal.[12] This method is limited by low loading efficiency and harmful interactions between charged shell materials and enzymes. Therefore, developing a new technique to effectively encapsulate and controllably release enzymes with retention of activity is still needed to extend the applications of enzyme in industry.

Here, we describe a single-step microfluidic approach for encapsulating enzymes within monodisperse microcapsules with an ultrathin inert polymer shell that can be triggered to release by applying an osmotic shock. Using a glass capillary microfluidic device, we fabricate monodisperse water-oil-water (W/O/W) template double-emulsion drops with an ultrathin middle oil layer. We dissolve enzymes in an aqueous buffer solution and use it as the inner water phase. After drop formation, the ultrathin middle oil layer will solidify as the middle phase oil diffusing out to produce a solid inert polymer shell with a thickness of a few tens to hundreds of nanometers, which can act as an effective barrier to protect the encapsulated enzymes. More importantly, by selecting a suitable shell polymer, the ultrathin shell can be easily ruptured to release encapsulated enzymes by adding water to the microcapsules, which triggers an osmotic shock to the microcapsules. In addition, we test the activity of the released enzymes and demonstrate that, compared to free enzymes in buffer and detergents, the activity of the released enzymes is well-maintained over a long period.

2. Results and Discussions

2.1. Fabrication and Characterization of Microcapsules with Ultrathin Shell

We use a glass capillary microfluidic device to generate W/O/W double emulsion drops with an ultrathin middle layer.[13] The device consists of two tapered cylindrical glass capillaries that are inserted into opposite ends of a square capillary. In addition, a smaller tapered capillary is inserted into the left cylindrical capillary with hydrophobic inner surface to simultaneously inject a second immiscible fluid, as illustrated in **Figure 1**a. We use the smaller capillary to inject the inner phase consisting of enzymes with buffer and $CaCl_2$ in 10 wt% poly(vinyl alcohol) (PVA) aqueous solution. The buffer is used to stabilize the enzyme, while $CaCl_2$ is used to increase the osmolarity of the inner phase. 10% PVA enhances the viscosity of the inner phase solution and the stability of the double emulsion drops.[14] The middle phase, which is a hydrophobic polymer in dichloromethane (DCM), is injected through the interstices between the smaller capillary and the left tapered cylindrical capillary. The outer phase is injected from the left through the interstices between the left cylindrical and the square capillaries; it is essentially the same solution as the inner phase, but without enzymes. The W/O/W double emulsion drops are formed at the tip of the left injection capillary, as shown in Figure 1b and Movie S1 in the Supporting Information. After formation, as-prepared double emulsion droplets are collected in the collection phase, which has the same composition as the outer phase. The diffusion of DCM into the surrounding water causes solidification of the polymers in the middle phase, leading to stable capsules with a solid shell, as shown in Figure 1c.

Microcapsules obtained are further characterized using confocal fluorescence microscopy and scanning electron microscopy (SEM), as shown in **Figures 2** and 3. The optical images

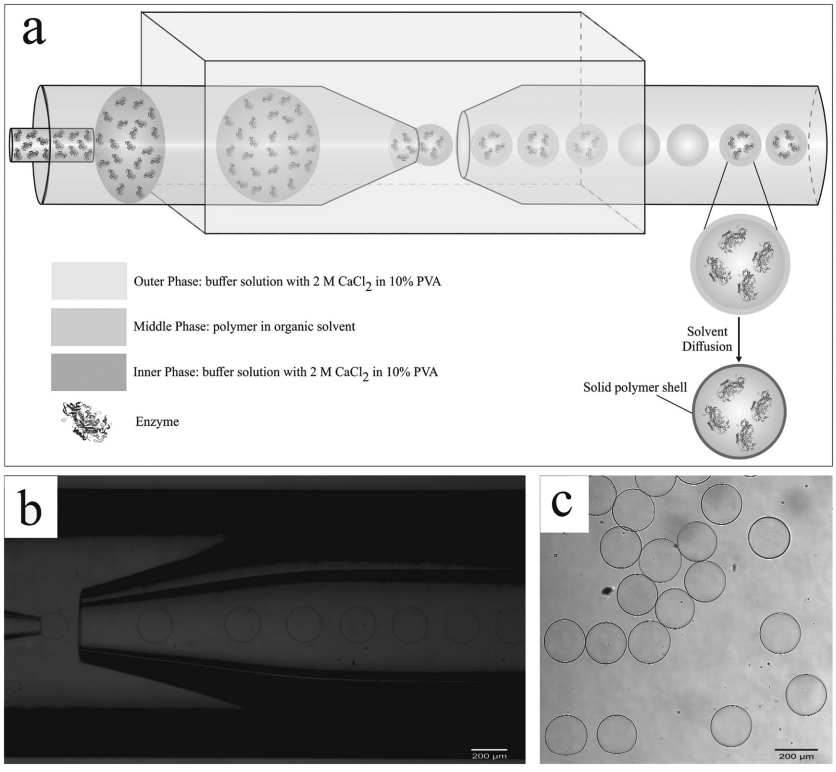

Figure 1. a) Schematic illustration of encapsulation of enzymes using microfluidics. b) Optical image showing production of microcapsules at the tip of the injection capillary. c) Optical image of as-prepared microcapsules.

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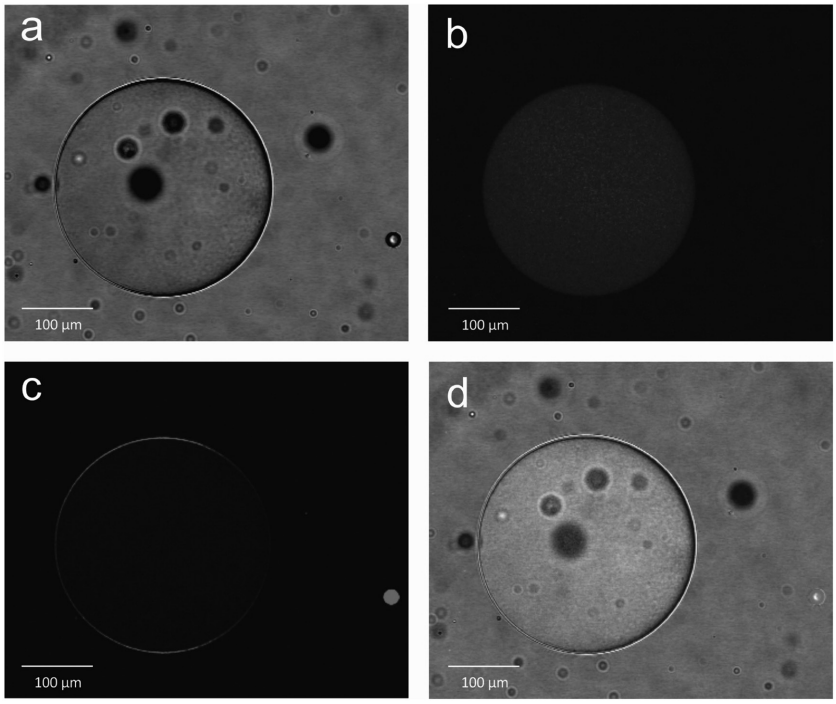

Figure 2. a) Transmission image of microcapsule labeled with FITC–dextran in inner phase and Nile red in shell; b,c) fluorescence images of FITC–dextran in inner phase and Nile red in shell, respectively. d) Overlay of transmission and fluorescence images.

show that the microcapsule is spherical and intact (Figure 2). Furthermore, the microcapsule contains the inner phase and the middle phases. The confocal fluorescence microscopy image of the microcapsule shows the presence of the inner phase stained in green with fluorescein isothiocyanate (FITC)–dextran and the middle phase stained in red by Nile red (Figure 2b,c). By employing two different dyes, the inner phase (green) and the ultrathin middle phase (red) are clearly distinguished in the confocal fluorescence images. The overlay image in Figure 2d clearly shows the core–shell structure, indicating that all the contents of the inner phase, including FITC–dextran or amylase, are located within the microcapsule structure. Thus, microcapsules are successfully prepared using this methodology.

We also use SEM to characterize the structure of the microcapsules, as shown in **Figure 3**. To prepare SEM samples, microcapsules are dispersed on a sample stage and dried under vacuum.

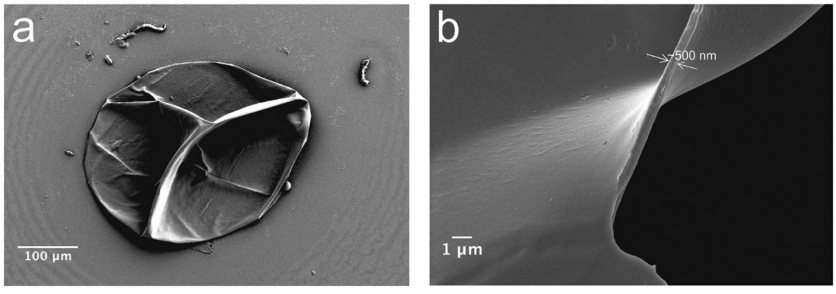

Figure 3. a) SEM image of a collapsed microcapsule after dry. b) SEM image shows the thickness of the microcapsule shell is about 500 nm.

We observe the collapsed structure of the dried microcapsule, as shown in Figure 3a, further confirming the hollow structure of the microcapsules, rather than a solid structure. From a collapsed microcapsule with a broken shell, the thickness of the shell is measured to be about 500 nm (Figure 3b). The ultrathin shell enables extremely high loading volume ratios of actives within the microcapsules.

2.2. Release of Microcapsules Triggered by Osmotic Shock

To trigger the burst release of the microcapsules, we apply an osmotic shock by adding large amounts of deionized (DI) water, which rapidly reduces the external osmotic pressure. The mechanism of osmotic pressure triggered release has been proposed in previous reports.[15] The microcapsules initially swell as water diffuses into their core, driven by the osmotic pressure difference. Ultimately, the swelling ruptures the microcapsule shell to release its cargo material,[16] as shown in **Figure 4**a The total process is completed within tens of seconds to a few minutes. The relatively rapid response arises from the ultrathin shell structure for two reasons: first, the ultrathin shell allows faster diffusion of water across the shell. Second, the ultrathin structure renders the shell not strong enough to sustain the osmotic shock. In practice, DI water is added rapidly to microcapsules suspension, and the final volume does not exceed 50 times the volume of the initial microcapsule suspension. We use confocal microscopy to monitor the process. A spherical microcapsule with labeled inner phase and middle phase suspended in the collection phase is shown in Figure 4b. After the addition of water, the microcapsule swells and then ruptures to release the contents of the inner phase (Figure 4c), followed by a shrinking of the solid polymer shell after completing release of contents (Figure 4d). Movie S2 in the Supporting Information shows the whole process of the burst release of a microcapsule. Interestingly, after burst release, instead of remaining inflated, microcapsules shrink and deflate, as shown in Figure 4d. The reason for the deflation of the capsule after ejection of their content is due to hydrophobic property of shell polymers. These hydrophobic polymers tend to shrink to minimize surface tension in aqueous environment.

To identify microcapsules that can quickly rupture under osmotic shock condition, we utilize different polymeric materials to fabricate microcapsules. We use polycaprolactone (PCL), poly (methyl methacrylate) (PMMA) and polystyrene (PS) with different molecular weights or concentrations dissolved in DCM,

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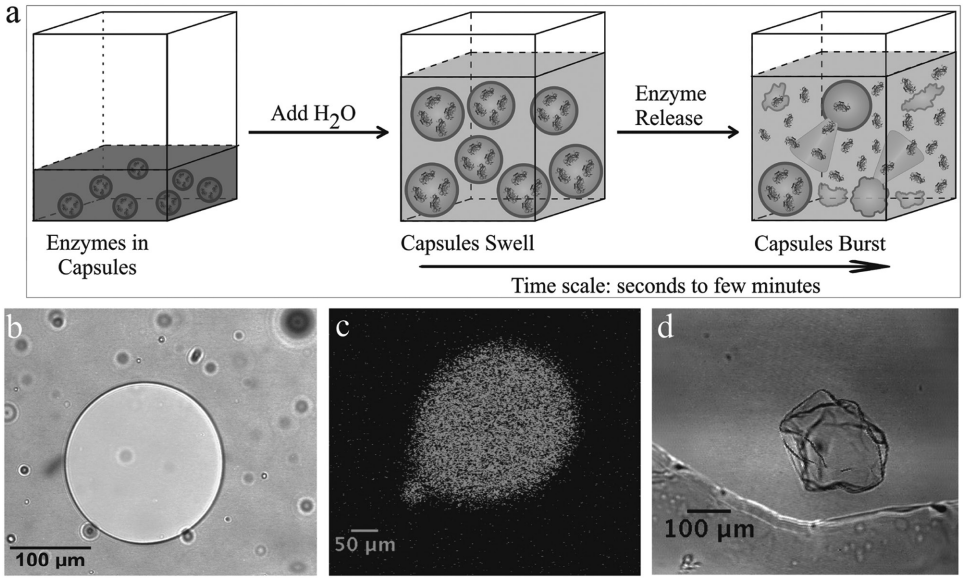

Figure 4. a) Schematic illustration of osmotic pressure triggered release of encapsulated enzymes. b) Confocal fluorescence image of microcapsule. c) Fluorescence image showing release of inner phase content of microcapsule after adding water. d) Shrunk solid polymer shell residue after release of content.

and then use this solution as the middle phase. After obtaining microcapsules using these polymers, we apply osmotic shock to these capsules to investigate their rupture. Although the as-prepared microcapsules are of the same size and composition in each case, we cannot achieve that all capsules break under the same condition, instead, we can only obtain fraction of ruptured capsules, indicating not all capsules response the same to the osmotic shock. The reason is because although all the microcapsules seem to be very similar, at the nano or even microscale, the shell structure of each capsule is not perfect and uniform. There may be more defects formed in some capsules' shell during solidification process of the middle oil phase. As a result, we can only get fraction of capsules ruptured. Similar results have been reported in previous studies on rupture of microcapsules.[17] We calculate the fraction of ruptured microcapsules by counting the number of intact microcapsules before and after water addition. The results of this study are shown in the **Table 1**.

To determine the optimal polymer concentration to fabricate the microcapsules, we use polymer concentration within the range of 2.5%–5.0% and evaluate the microcapsules triggered release response under osmotic shock condition. For all the three polymers, when the concentration is less than 2.5%, there is no stable microcapsule obtained. We observe that the fraction of ruptured microcapsules under osmotic shock decreases with increasing polymer concentrations as well as molecular weights, as shown in the Table 1. For example, when the concentration of PCL increases from 2.5% to 5.0%, microcapsules become more resistant to osmotic shock and remain intact, which results in 100% unruptured capsules in the case of 5.0% of PCL in DCM. For PS, when the molecular weight increases from 298 to 465 kDa, the ruptured fraction decreases from 45% to 31%. This is because although the molecular weights of these polymers are above their entanglement molecular weight, previous studies in polymer science demonstrate that the strength of the polymer can further increase as the molecular weight increases, and eventually reaches a constant level at sufficiently high molecular weight.[18] We believe the molecular weights of these polymers are in the middle range, which is larger than the critical entanglement molecular weights, but not high enough. Thus, a larger molecular weight results in microcapsules with higher mechanical stability. For different polymers with the same concentrations, the ruptured ratio is related to the mechanical properties of the polymers. PCL has a huge elongation at break of 500%,[19] resulting in high stability of microcapsules. For PS and PMMA, although both have similar elongation at break of only 2%–5%,[19] the π–π stacking between benzene rings on PS chains greatly enhance intermolecular interaction, leading to a stronger polymer shell. Therefore, capsules made of PMMA demonstrate better response upon applying osmotic shock. We can find the highest release efficiency, over 90%, is achieved by preparing microcapsules using PMMA with molecular weight of 120 kDa and concentration of 3%. This optimized composition is used for the following experiments.

Table 1. Fraction of ruptured microcapsules with different shell materials.

Polymer	Molecular weight [kDa]	Polymer concentration in DCM	Fraction of ruptured microcapsules
PCL	45	2.5%	50 ± 8%
		3.0%	50 ± 11%
		5.0%	None
PMMA	120	2.5%	90 ± 9%
		3.0%	91 ± 7%
PS	298	3.0%	45 ± 10%
	465	3.0%	31 ± 8%

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2.3. Stability of Encapsulated Enzymes

To test the stability of encapsulated enzymes, we choose amylase as a model enzyme, which is one of the most commonly used enzymes in industry. Here, we want to clarify that our strategy is not limited to amylase, but is versatile to any other enzymes. The versatility and robustness of the encapsulation within microcapsules have been well explored in many previous studies,[20] due to the unique liquid core and solid shell configuration of microcapsules. Various chemicals, biomolecules, or nano/micromaterials have been encapsulated within microcapsules. Particularly, in this strategy, the whole process of encapsulating enzymes followed by solvent diffusion to obtain inert and solid shell is under very mild condition without bringing in any interference to the enzymes. Thus, versatile enzymes can be encapsulated without losing activities.

The activity of amylase is measured using EnzChek Ultra Amylase Assay Kit (Life Technologies) with an incubation time of up to 47 d at room temperature. The assay kit contains a starch derivative labeled with a fluorescent dye at a concentration that the initial fluorescence is quenched. The starch derivative is digested by amylase; digestion relieves the quenching and yields highly fluorescent fragments. All the amylase samples are diluted about 10^6 times using the buffer solution in the assay kit to the same concentrations before adding the starch derivative. Thus, the accompanying increase in fluorescence is proportional to amylase activity. To effectively distinguish the protection of microcapsules to amylases, we place all amylase treatments in a harsher environment that is in an open space at room temperature, instead of storing in a fridge.

We first compare the activities of amylases in the same solution with and without encapsulation. Encapsulated amylase and free amylase in the same formulation as the inner phase solution are stored at room temperature for 1 d and then measured their activities using the assay kit. The initial amylase that is stored in the original bottle in fridge is measured as a control. The results are shown in **Figure 5**.

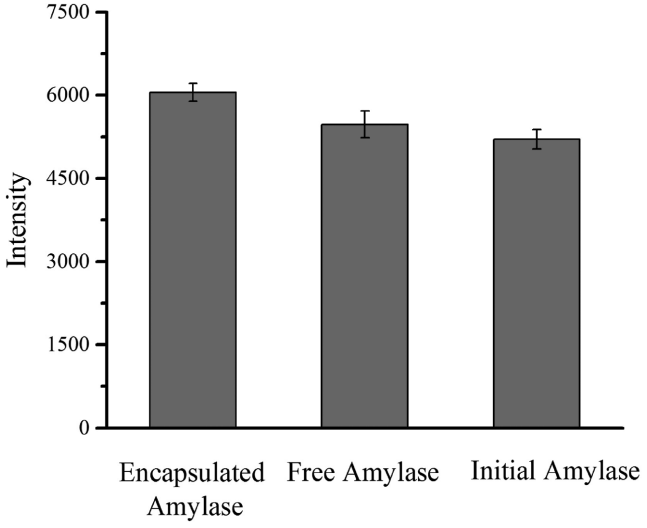

Figure 5. Activities of encapsulated amylase and free amylase in the same solution, after storing at room temperature for 1 d. Initial amylase stored in the fridge as a control.

Interestingly, we find although the encapsulated amylase is suspended in the inner phase solution that is the same as the formulation where the free amylase is suspended, their activity after encapsulation is higher than that of the free amylase, as shown in Figure 5. As we all know, enzymes including amylase are sensitive to their environment. Any changes of environmental conditions, such as temperature, humidity, gases, or even light, will affect enzymes' activity. While after encapsulation, enzymes within microcapsules are under a localized optimized condition. The protection of microcapsules will greatly minimize or even prevent harmful effects of environmental changes on encapsulated enzymes. Besides, even though there are 10% PVA in the inner phase formulation to increase viscosity, the activities of both encapsulated amylase, and free amylase in the same formulation are slightly higher than that of the control. The reason is probably due to chloride ions from the inner phase formulation, which can bind to amylase to switch it to the more active state, as studied in previous report.[21] These results indicate that the inner phase formulation can slightly activate amylase, and PVA as a nonionic surfactant has minimal or no effect on amylase's activity, which is also confirmed in a control experiment showing similar activities of amylase in bulk solution with and without PVA, as shown in Figure S1 in the Supporting Information. More importantly, encapsulating amylase within microcapsules provides effective protection to amylase, resulting in higher activity of the encapsulated amylase. In addition, it is also necessary to point out that considering the versatility and mild condition of encapsulation, our strategy of encapsulation provides protection not only to amylase, but also to any other enzymes.

Long-term stability of enzymes during storage is very important for enzymes' applications. To test the long-term protection of our encapsulation strategy, we monitor the activity of encapsulated amylase up to 47 d. For comparison, free amylases in 75×10^{-3} M 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer solution and detergent (Xtra) are also selected, both of which can better mimic real conditions of enzymes in industrial uses. All amylase treatments are stored at room temperature. The activities of the amylase treatments with same concentration are shown in **Figure 6**.

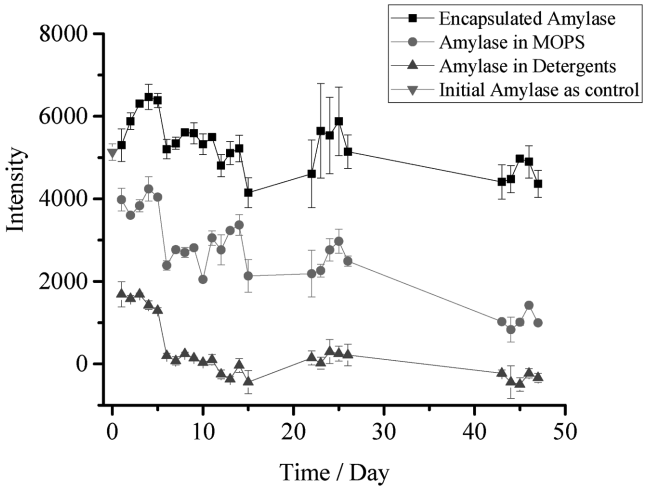

Figure 6. Activities of amylases as a function of incubation time.

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The trend of amylase activity decreases with increasing storing time, as shown in Figure 6. Similarly, due to the activation of chloride ion, the fluorescence intensities increase for amylase in capsule and free amylase in buffer in the initial 5 d, which is consistent with previous results in Figure 5. More significantly, during the whole time period, the activity of encapsulated amylase (black) over time is always higher than that of amylases in bulk buffer solution (red) and in detergent (blue), as shown in Figure 6. Specifically, after an incubation period of 47 d at room temperature, the encapsulated amylase retains over 85% of its initial activity. While, for free amylase dispersed in detergent, the interaction between amylase and detergent leads to denaturation of amylase, resulting in 70% decrease in activity after incubation for 1 d and almost complete loss of activity after 10 d of incubation. Free amylase in MOPS buffer solution maintains about 20% of its initial activity at the end of 47 d. These results demonstrate that encapsulating amylase within microcapsules can effectively stabilize amylase and maintain its activity for a long period within harsh environmental conditions.

3. Conclusion

In conclusion, we successfully demonstrate the concept of enzyme encapsulation for controlled release triggered by an osmotic shock. Amylase is encapsulated into monodisperse microcapsules with ultrathin inert polymer shells during a one-step double emulsion process using a glass capillary microfluidic device. The encapsulated amylase can be easily released by adding water to trigger an osmotic shock. Through carefully designing microcapsule membranes, we have achieved the best-triggered release system using PMMA (120 kDa, 3%) as shell material. Enzymatic activity measurements indicate that the encapsulated amylase activity is maintained well over 85% of the initial activity after incubation for 47 d at room temperature. While, the free amylase in buffer loses more than 80% of the initial activity after 47 d, and the free enzyme kept in detergent completely loses its activity only after 10 d at room temperature. Our study provides a new approach to protect and release enzymes on-demand upon applying osmotic shock. This not only will extend applications of enzymes in modern industry, but also offers new opportunities for encapsulation of a wide range of sensitive molecules and biomolecules and simply controlled release upon applying osmotic shock.

4. Experimental Section

Chemicals: The model protein used in this study was amylase purchased from Sigma-Aldrich, USA. The polymers used were PVA (87%–89% hydrolyzed, M_w 13,000–23,000; Sigma-Aldrich, USA), PCL (M_w 45,000; Sigma-Aldrich, USA), PMMA (M_w 120,000; Sigma-Aldrich, USA), and PS (M_w 298,000 and 465,000; Sigma-Aldrich, USA). 3-MOPS buffer (75×10^{-3} M) at pH 7.2 was used to stabilize amylase. DCM (Sigma-Aldrich, USA) was used as organic solvent. Calcium chloride (Sigma-Aldrich, USA) was used to stabilize the amylase and to increase the osmotic pressure. All the reagents were used as received and were of analytical grade.

Fabrication of Microfluidic Device: A glass capillary microfluidic device was used to fabricate microcapsules. The device was built on a glass slide, and consisted of a square glass tube and two cylindrical glass tubes, as previously reported.[13] Briefly, two cylindrical capillaries were pulled with a Flaming/Brown micropipette puller (Model P-97, Sutter Instrument Co., USA) to obtain tapered tips which were then polished to the desired diameters using sand paper. The tapered capillary with small tip (with diameter of 80 μm) was coated with hydrophobic trimethoxy(octadecyl) silane (Sigma-Aldrich, USA) on its inner wall, which was then inserted into a second square capillary whose inner dimension was slightly larger than that of the outer diameter of the tapered capillary and was used as the injection capillary. In addition, a smaller tapered capillary was inserted into the injection capillary to simultaneously inject a second immiscible fluid. Another tapered circular capillary with large tip was coated with 2-[methoxy(polymethyleneoxy) propyl]-9-12 trimethoxysilane (Gelest Inc., Netherlands) to make the capillary wall hydrophilic and was inserted into the square capillary at the other side to confine the flow near the injection tip.

Microfluidic Encapsulation of Amylase: Microcapsules were produced from double emulsion droplets with ultrathin shells using the microfluidic device employing a biphasic flow. Emulsion phases of the W/O/W emulsion were pumped into the glass capillary devices with syringes using Harvard pumps (Harvard Apparatus Hollston, USA). Syringes were attached to the inlets of the glass capillary device with plastic tubing (PE5 0.86 × 1.32 mm, Scientific Commodities Inc., USA). Various formulations with different types and concentrations of polymers were investigated and used to prepare microcapsules. To prevent osmotic stresses, both inner and outer phases were composed of PVA 10% in water (w/w), MOPS (75×10^{-3} M), and $CaCl_2$ (2 M), but in inner phase solution, 1% (v/v) amylase was added to the solution. Different polymers, including PCL, PMMA, and PS with different molecular weights and concentrations in DCM were used as middle oil phases. The inner and middle phases flowed at a rate of 400 and 600 μL h^{-1}, respectively. The flow rate of the outer phase is 4000 μL h^{-1}. The monodisperse W/O/W double emulsion drops with ultrathin shells were formed.

Measurement of Amylase Activity: To measure the activity of amylase, the authors used EnzChek Ultra Amylase Assay Kit (Life Technologies), which was very sensitive and could be used to detect amylase activity down to a final concentration of 2 mU mL^{-1}. The authors first prepared an amylase standard curve between 0 and 20 mU mL^{-1}, in triplicate, and then prepared dilutions of these three amylase samples to be analyzed. The authors diluted these samples sufficiently to ensure that the concentration of amylase for all these three samples were the same and the activities of these samples were within the range of the standard curve. The concentration of amylases in capsule-free states could be calculated with the initial amount of amylase and the volume of the solution. The concentration of encapsulated amylase was calculated according to the volume of inner phase and the total volume collected as well as the initial concentration of amylase in inner phase. To detect the amylase activity, the authors took 10 μL of solution from each amylase sample. Particularly, for encapsulated sample, the microcapsule suspension was gently vortexed to get homogenous microcapsule suspension before taking 10 μL of suspension. All samples were diluted to get the same concentrations of amylase using the optimal buffer. In their experiments, the authors needed to dilute all these samples about 10^6 times to get suitable activities within the range of the standard curve. This large dilution ratio ensured that the effect of initial environments for all three samples was negligible for the activity measure of amylase. And this dilution process also triggered the release of encapsulated amylase. With this procedure, the authors obtained the real activities of amylases in these three samples.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

encapsulation, enzymes, microfluidics, osmotic pressure, triggered release

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