Preparation of a Copper Polyphosphate Kinase Hybrid Nanoflower and Its Application in ADP Regeneration from AMP

Xinzeng Sun, Huanqing Niu,* Jiarui Song, Dahai Jiang, Jing Leng, Wei Zhuang, Yong Chen, Dong Liu, and Hanjie Ying

ABSTRACT: In this research article, we reported a self-assembly approach to prepare a copper polyphosphate kinase 2 hybrid nanoflower and established a cofactor ADP regeneration system from AMP using the nanoflower. First, the structure of the hybrid nanoflower was confirmed by scanning electron microscopy, X-ray diffraction, Fourier transform infrared spectroscopy, and X-ray photoelectron spectroscopy, which indicated the successful loading of the enzyme in the hybrid nanoflower. Moreover, compared to the free enzyme, the hybrid nanoflower exhibited a better performance in ADP production and possessed wider catalytic pH and temperature ranges as well as improved storage stability. The hybrid nanoflower also exhibited well reusability, preserving 71.7% of initial activity after being used for ten cycles. In addition, the phosphorylation of glucose was conducted by utilizing ADP-dependent glucokinase coupled with the ADP regeneration system, in which the hybrid nanoflower was used for regenerating ADP from AMP. It was observed that the ADP regeneration system operated effectively at a very small amount of AMP. Thus, the hybrid nanoflower had great application potential in industrial catalytic processes that were coupled with ADP-dependent enzymes.

1. INTRODUCTION

Cofactor-dependent enzymes such as oxidoreductases, transferases, and kinases are able to catalyze many useful reactions in industry. Cofactors, such as ATP/ADP/AMP, coenzyme A, NAD(P)(H), and FAD(H2), are often very expensive to be used as stoichiometric agents, so that the generation, regulation, and recycling of cofactors have been investigated extensively in recent years for industrial applications. In particular, the ways of efficient regeneration of high-energy cofactors (such as nucleotide triphosphates), which involved in the phosphoryl transfer reactions, have been extensively studied for chemical synthesis.

ATP is most commonly used as a high-energy phosphate donor for kinase reactions. So far, there are four major enzymatic methods for ATP regeneration, catalyzed by pyruvate kinase, acetate kinase, creatine kinase, and polyphosphate kinase (PPK). Among them, because of the high stability and low cost of the inorganic polyphosphate (polyP), ATP regeneration systems using polyP as the substrate in a coupling reaction catalyzed by PPK are attractive for the commercial use of ATP-dependent enzymes. As reported, PPKs can be categorized into group 1 to 3 (PPK1, PPK2, and PPK3) while PPK2s are further subdivided into class I to III based on their functionality. Class I, II, and III of PPK2 allow regeneration of ATP from ADP, ADP from AMP, and ATP from AMP, respectively. A class III PPK2 from *Meiothermus ruber* was selected to convert nucleoside monophosphate into nucleoside diphosphate (NDP) and applied in the cascade reaction of NDP glucose production. Currently, although PPKs have been applied in biocatalysis mainly for recycling ATP from ADP, ADP regeneration systems based on PPKs were less well developed, and their application was relatively scarce.

As biocatalysts in industrial processes, free enzymes have some inherent disadvantages such as instability of catalytic activity, high cost, poor storage stability, and lack of reusability. In order to overcome shortcomings of free enzymes, immobilization technology of enzymes has been studied and improved since the last century. Recently, construction of organic–inorganic hybrid materials has been investigated as a simple and efficient method for enzyme immobilization. Nadar et al. reported a hybrid nanoflower with organic component of glucoamylase and inorganic component of copper phosphate with enhanced thermal and storage stability. The protein molecules and copper ions form complexes, which become nucleation sites for the primary crystals of copper phosphate. The interaction between the protein and copper ions then grows into micron-sized particles with nanoscale characteristics and shaped like petals. The

Received: January 23, 2020
Accepted: April 7, 2020
Published: April 27, 2020
method is simple, green, and consumes low amount of energy. Construction of Cu-based inorganic protein nanoflowers has been applied to immobilize different enzymes including laccase, horseradish peroxidase, soybean peroxidase, trypsin, papain, and so forth. In addition to using copper as the inorganic part, other metal ions have been developed. Han and Liu embedded organophosphorus hydrolase (OPH) into cobalt phosphate through biomineralization and cell surface display technology, which greatly improved the biosensing performance of OPH. Moreover, Fe, Mn, and Ca are also gradually developed and utilized. Also, the inorganic protein hybrid nanoflowers have been used in a diverse range of areas, including enzyme purification, fast detection, and enzymatic catalysis for chemical synthesis.

In this study, a recombinant class III PPK2 from *Arthrobacter* sp (ArPPK2) was used for enzyme inorganic hybrid nanoflower production. First, we produced the copper-ArPPK2 hybrid (ArPPK2-Cu3(PO4)2·3H2O) nanoflower by a self-assembly process, and its properties were characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and X-ray photoelectron spectroscopy (XPS). Moreover, the catalytic conditions for ADP generation, the reusability experiments, and the storage stability of the ArPPK2-Cu3(PO4)2·3H2O nanoflower were investigated. Finally, its potentiality of regeneration of ADP from AMP coupled with ADP-dependent glucokinase (ADP-GK) was investigated. In the large scale of glucose-6-phosphate (G-6-P) production, ATP-glucokinase (ATP-GK), which needs ATP as a phosphate donor, was usually used to phosphorylate glucose. Meanwhile, a process of G-6-P production by ATP-GK coupled with an ATP regeneration system based on acetate kinase exhibited high efficiency. Another type of glucose-phosphorylating enzymes, ADP-GK requiring ADP as the phosphoryl group donor instead of ATP, may have great potential for G-6-P production based on lower cost substrates compared with ATP-GK. Therefore, based on the ArPPK2-Cu3(PO4)2·3H2O nanoflower using a very small amount of AMP, the utilization of ADP regeneration system is potentially attractive for industrial catalytic processes by ADP-dependent enzymes. As far as we know, the self-assembly ArPPK2-Cu3(PO4)2·3H2O nanoflower has not been reported in the literatures and it was used for regenerating ADP from AMP for the first time, providing a cost-efficient approach for ADP regeneration.

### 2. RESULTS AND DISCUSSION

#### 2.1. Synthesis and Characterization of the ArPPK2-Cu3(PO4)2·3H2O Nanoflower

According to the method described in Section 4.3, blue ArPPK2-Cu3(PO4)2·3H2O nanoflowers were obtained by adding CuSO4 solution (120 mM) to phosphate buffer containing ArPPK2 (10 mM, pH 7.4) and incubating at 4 °C for 72 h. The formation mechanism of enzyme inorganic hybrid nanowires is as follows: first, protein molecules form complexes with Cu2+ through the coordination facility of amide groups, which provide a site for nucleation of primary crystals. Later, more crystals combine to form larger agglomerates, forming loose petal-like structures. Finally, the nanoflower is fully formed, in which proteins induce the nucleation of copper phosphate crystals to form a scaffold for the petals and act as the “glue” that holds them together.

We measured the catalytic activities of nanoflowers with different enzyme concentrations (Table 1). Furthermore, the general morphologies of the ArPPK2-Cu3(PO4)2·3H2O nanoflowers were observed by SEM. As reported previously, the enzyme concentration had a certain influence on the nanoflower formation. Therefore, we observed the changes of the nanoflower morphology with different enzyme concentrations. As shown in Figure 1a, the nanoflower with 0.1 mg/mL concentration of ArPPK2 exhibited a very tight spherical structure. As the concentration of enzyme gradually decreased (Figure 1b–d), the diameter of the nanoflower gradually increased and showed a “blooming” trend. When the enzyme concentration decreased to 0.02 mg/mL, the nanoflower showed a beautiful flower-like structure (Figure 1e). This nanoflower presented the size around 10 μm, and it had hierarchical structures with high surface-to-volume ratios. Without the enzyme, the nanoflower cannot be formed (Figure 1f). We speculated that the tighter structures of nanoflowers with the higher enzyme concentrations could cause structural deformation of the ArPPK2 or introduce steric hindrance to the catalytic sites. As shown in Table 1, the nanoflower with 0.02 mg/mL enzyme concentration showed the best catalytic activity. Therefore, the nanoflower with 0.02 mg/mL enzyme concentration was used for further studies.

The XRD patterns in Figure 2a showed the crystallographic structures of the nanoflower and Cu3(PO4)2·3H2O. They were approximately the same and matched with standard Cu3(PO4)2·3H2O (JCPDS 00-022-0548) (see the inset of Figure 2a). It indicated that the incorporation of the enzyme did not impact the crystal structure of Cu3(PO4)2·3H2O.

In addition, we analyzed them by FTIR spectroscopy. As shown in Figure 2b, the peaks at 1047, 990, and 628 cm<sup>−1</sup> could be attributed to P–O vibrations, indicating the presence of phosphate groups. Moreover, the typical amide I and II bands of the enzyme could be observed at 1642 and 1537 cm<sup>−1</sup>, indicating the successful loading of ArPPK2 in the nanoflower. Moreover, no new peaks and significant peak shifts were observed, implying that the ArPPK2-Cu3(PO4)2·3H2O nanoflower was executed in a self-assembly manner without the formation of covalent bond.

To further confirm the composition and chemical configuration of the ArPPK2-Cu3(PO4)2·3H2O nanoflower, XPS analysis was carried out. From the survey spectrum in Figure 3a, there were Cu, C, O, P, and N elements in the nanoflower while the N element only appeared in the sample of nanoflower, indicating that the enzyme was immobilized in the nanoflower. For the Cu 2p XPS spectrum (Figure 3b), the peaks corresponding to Cu 2p<sub>3/2</sub> and Cu 2p<sub>1/2</sub> were observed at 954.54 and 934.48 eV, respectively. In the C 1s spectrum (Figure 3c), three peaks at about 284.7, 285.8, and 287.8 eV were attributable to the C–C, C–O, and C=O groups,

| Table 1. Relative Activity of Nanoflowers with Different Enzyme Concentrations |
|-----------------|-----------------|
| enzyme concentration (mg/mL) | relative activity (%) |
| 0.01 | 93.5 ± 3.34 |
| 0.02 | 100.0 ± 2.45 |
| 0.04 | 95.3 ± 2.12 |
| 0.06 | 89.4 ± 5.47 |
| 0.08 | 82.5 ± 3.92 |
| 0.10 | 67.2 ± 6.69 |

ACS Omega 2020, 5, 9991–9998

https://dx.doi.org/10.1021/acsomega.0c00329
Figure 1. SEM images of nanoflowers with the different concentrations of enzymes: (a) 0.10, (b) 0.08, (c) 0.06, (d) 0.04, (e) 0.02, and (f) 0 mg/mL.

Figure 2. XRD patterns of particles. (a) Cu$_3$(PO$_4$)$_2$·3H$_2$O (black line) and ArPPK2-Cu$_3$(PO$_4$)$_2$·3H$_2$O nanoflower (red line). (b) FTIR spectra of Cu$_3$(PO$_4$)$_2$·3H$_2$O (black line) and ArPPK2-Cu$_3$(PO$_4$)$_2$·3H$_2$O nanoflower (red line).

Figure 3. XPS spectra of the ArPPK2-Cu$_3$(PO$_4$)$_2$·3H$_2$O nanoflower: (a) survey spectrum, (b) Cu 2p, (c) C 1s, and (d) O 1s.
respectively. From the O 1s spectrum (Figure 3d), it showed that two peaks corresponding from 531.1 and 532.6 eV were attributed to the emission of carboxylate groups.

2.2. Time Course of AMP Conversion into ADP and ATP. To better understand the performance of the ArPPK2, the time course of AMP conversion into ADP and ATP with ArPPK2 was explored. As shown in Figure 4a, the free ArPPK2 catalyzes the conversion of AMP to ATP in two phosphorylation steps. ArPPK2 first catalyzes the phosphorylation of AMP and then converts ADP to ATP at a reduced rate. Specially, the ArPPK2-Cu3(PO4)2·3H2O nanoflower lost most activity of phosphorylating ADP to ATP, and ADP was mainly accumulated in the reaction system (Figure 4b). After immobilization, ArPPK2 basically lost the ability to catalyze ADP to ATP, probably because the substrate channel was narrowed after enzyme fixation, making it more inclined to use AMP as the substrate. At the end of reactions, we found that the conversion rate of AMP reached 98.6% for the free ArPPK2 and 97.5% for the nanoflower, respectively. At the same time, we found that the productivity rates of ADP and ATP of free enzyme were 22.4 and 76.2%, respectively, while the productivity rates of ADP and ATP of nanoflower reached 96.7 and 0.8%, respectively. The nanoflower was more
2.3. Optimum Reaction Conditions and Storage Stability of the ArPPK2-Cu₃(PO₄)₂·3H₂O Nanoflower. As shown in Figure 5a,b, although the optimal concentration of Mg²⁺ (20 mM) did not change, the optimal concentration of polyP₆ changed before and after immobilization. The optimal concentration of polyP₆ for the ArPPK2-Cu₃(PO₄)₂·3H₂O nanoflower and free ArPPK2 was three times and two times higher than that of AMP, respectively. The effects of temperature and pH on the free ArPPK2 and the nanoflower are shown in Figure 5c,d, respectively. The optimum temperatures of both free ArPPK2 and the nanoflower were 30 °C. Also, at temperatures from 30 to 55 °C, the ArPPK2-Cu₃(PO₄)₂·3H₂O nanoflower retained higher residual activities than the free ArPPK2 (Figure 5e). The nanoflower displayed a wider application of temperature range, enhancing its practicality in industrial applications. The optimum reaction pH of ArPPK2-Cu₃(PO₄)₂·3H₂O nanoflower was 8, which was as same as that of free ArPPK2. However, the pH range of the ArPPK2-Cu₃(PO₄)₂·3H₂O nanoflower was wider than that of the free ArPPK2 (Figure 5f). Within a certain pH range, the nanoflower structure may provide a suitable buffering environment to retain better residual activity under both acidic and basic conditions.

To determine the storage stability of the immobilized enzyme, the free ArPPK2 and the ArPPK2-Cu₃(PO₄)₂·3H₂O nanoflowers were stored at 4 and 25 °C for 28 days, and the enzyme activities were examined at a fixed time. As shown in Figure 5g, when stored at 4 °C, the enzyme activities showed no obvious differences between the free enzyme and the nanoflower. However, when stored at 25°C for 28 days, 76.0% of initial overall enzyme activity for the nanoflower could be retained while the free enzyme retained 30.2% of initial enzyme activity (Figure 5h). This may be attributed to the encapsulation of the enzyme in the nanoflower, thereby minimizing changes in the enzyme activity site and maintaining enzyme stability during the storage process.27

2.4. Reusability Studies. The reusability of the enzyme is an important factor for economic feasibility. Successive cycles of experiments were performed to evaluate the reusability of the ArPPK2-Cu₃(PO₄)₂·3H₂O nanoflower. As shown in Figure 6, the activities of the recycled nanoflower declined slowly with the increasing cycle numbers, and 71.7% of the initial activity could be maintained after ten catalytic cycles. The decreased catalytic activity after reusing could be because of several reasons such as the enzyme inactivation during the washing process and loss of material during recycling assay.27,28

2.5. ADP Regeneration System Based on the ArPPK2-Cu₃(PO₄)₂·3H₂O Nanoflower for G-6-P Formation. Figure 7a shows the scheme of G-6-P formation by ADP-GK coupled with the ADP regeneration system. As shown in Figure 7b, G-6-P was not observed when only ADP-GK and AMP were added. When ADP-GK and AMP were added, G-6-P was successfully generated. It indicated that ADP-GK could produce G-6-P with ADP but not with AMP. When ADP-GK, free ArPPK2, and AMP were added, G-6-P was successfully synthesized. When the ArPPK2-Cu₃(PO₄)₂·3H₂O nanoflower was used to replace free ArPPK2, the production of G-6-P increased significantly. When reactions were completed, the concentrations of G-6-P produced by free enzyme and the nanoflower were 62.7 and 116.4 μg/mL, respectively, and the control value was 8.4 μg/mL. It suggested that the free enzyme and the nanoflower in the two-enzyme cascade reactions could catalyze regeneration of ADP for at least 7 and 13 times, respectively. These results indicated that the ADP regeneration system employing ArPPK2 operated effectively. The reusability of the ArPPK2-Cu₃(PO₄)₂·3H₂O nanoflower in the double-enzyme cascade reaction for G-6-P formation was also examined. As shown in Figure 7c, about 58.1% of the initial concentration of G-6-P could be produced in the fourth batch, and the nanoflower nearly lost its activity after the sixth batch. This phenomenon may be caused by the high concentration of phosphate in the catalytic system.16 Therefore, we need to further rationally design an efficient salt-tolerant system to solve this problem. Moreover, we expected the application of our ArPPK2-Cu₃(PO₄)₂·3H₂O nanoflower in other ADP-dependent cascade reactions that release AMP.

3. CONCLUSIONS

In this study, we successfully produced the ArPPK2-Cu₃(PO₄)₂·3H₂O nanoflower by the self-assembly approach and initially established the ADP regeneration system based on this nanoflower. First, the morphology, structure, and composition of ArPPK2-Cu₃(PO₄)₂·3H₂O nanoflower were characterized by SEM, XRD, FTIR, and XPS. Moreover, the optimum pH and temperature, storage stability, and reusability of the ArPPK2-Cu₃(PO₄)₂·3H₂O nanoflower were also investigated. The nanoflower showed improved storage stability and retained high catalytic activity after being used for ten cycles. Finally, we assessed the phosphorylation of glucose by utilizing ADP-GK coupled with the ADP regeneration system. Our study showed that the ArPPK2-Cu₃(PO₄)₂·3H₂O nanoflower had great application potential in industrial catalytic processes that were coupled with ADP-dependent enzymes.

4. MATERIALS AND METHODS

4.1. Materials and Chemicals. Escherichia coli cultivation medium (LB) was purchased from Oxoid (The UK). E. coli Rosetta (DE3) and plasmid pET-28a were both purchased from Novagen (The USA). Isopropyl β-D-thiogalactopyranoside (IPTG) was purchased from Shanghai Jinsui Biotechnology Co., Ltd. (Shanghai, China). ATP, AMP, NAD⁺, kanamycin, chloromycetin, sodium hexametaphosphate (polyP₆), Tris, and glucose-6-phosphate dehydrogenase were purchased from Sangon Biotech Co., Ltd. (Shanghai, China).
All other chemicals were of analytical grade from commercial sources and used without further purification.

4.2. Cell Culture and Protein Purification. The *E. coli* Rosetta cells carrying the recombinant plasmid pET-28a-ArPPK2 were incubated in LB medium with 50 μg/mL kanamycin and 34 μg/mL chloromycetin at 37 °C at 200 rpm. When the optical density at OD600 reached 0.6–0.8, 0.2 mM IPTG was added, and the cells were incubated at 25 °C at 200 rpm for 12 h. The gene-encoding ADP-GK from *Pyrococcus furiosus* (UniProt accession number Q9V2Z6) was synthesized by Genewiz Biotech Co., Ltd. (Suzhou, China). The recombinant plasmid pET-28a-ADP-GK was introduced into *E. coli* Rosetta. Also, the recombinant ADP-GK was expressed as previously described.29 The cells were harvested by centrifugation (4 °C, 10,000 g, 10 min). The obtained cells were harvested by centrifugation and resuspended in Tris-HCl (100 mM, pH 8.0), followed by sonication on ice, and the supernatant was collected as the crude enzyme. The crude enzyme was purified with a Ni-NTA His•Bind Resin column.30 Unbound proteins were washed from the column with washing buffer (100 mM Tris-HCl, 25 mM imidazole, pH 8.0). The pure enzyme was eluted from the column with elution buffer (100 mM Tris-HCl, 300 mM imidazole, pH 8.0). The protein concentration was measured according to Bradford.31 The purified proteins were further applied to sodium dodecyl sulfate polyacrylamide gel electrophoresis (12%) and activity analysis.

4.3. Preparation of the ArPPK2-Cu3(PO4)2·3H2O Nanoflower. The synthesis of enzyme inorganic hybrid nanoflowers was performed as reported previously with some modifications.28,32 In brief, 10 mL of phosphate buffered saline (10 mM, pH 7.4) containing different concentrations of recombinant enzymes from 0.02–0.1 mg/mL was mixed with 67 μL of CuSO4 solution (120 mM) at 4 °C for 72 h. The precipitated pellets were centrifuged at 10,000g for 15 min, washed with ultrapure water three times, and then frozen-dried under vacuum.

4.4. Characterization of the ArPPK2-Cu3(PO4)2·3H2O Nanoflower. The sample morphology and microstructure of the ArPPK2-Cu3(PO4)2·3H2O nanoflower were analyzed by SEM (S4800, Hitachi High Technologies Corporation). The material crystal phase was characterized by powder XRD (Bruker D8, Cu Kα radiation) among the range of 5–90° at the rate of 0.02/s. FT-IR (Nicolet-460, Thermo Fisher Scientific) was performed in the range of 400–4000 cm−1 on a KBr pellet.33 The surface properties and composition were conducted by XPS (ESCALAB 250xi, Thermo Fisher Scientific).

4.5. Enzyme Activity Assay. ArPPK2 activity was determined using the following reaction system: 100 mM Tris-HCl (pH 8.0), 2 mM polyP6, 20 mM MgCl2, and 1 mM AMP. Known amount of the ArPPK2-Cu3(PO4)2·3H2O nanoflower and the free ArPPK2 were added into the mixture and incubated at 30 °C for 30 min. Enzyme activity was assayed by measuring its ability of AMP conversion. The concentrations of AMP, ADP, and ATP were measured by high-performance liquid chromatography (HPLC) as described by Chen et al.34

In addition, in order to determine the time course of the conversion of AMP by the free ArPPK2 and the ArPPK2-Cu3(PO4)2·3H2O nanoflower, 50 μL and 0.3 mg/mL of free ArPPK2 and nanoflower with the same enzyme concentration were added into 1 mL reaction solution and incubated at 30 °C, respectively. At selected time points, aliquots were
withdrawn, and the reaction was quenched by addition of 8% trichloroacetic acid, centrifuged, and the supernatant was analyzed by HPLC. The initial concentration of AMP was used as a relative reference. All reactions were performed and analyzed in triplicates.

4.6. Optimum Reaction Conditions and Storage Stability of the Nanoflower. The optimum $\text{MgCl}_2$ and polyP$_6$ concentration were determined in the range of 0.5–70 and 0.25–50 mM, respectively. The optimum pH and temperature were determined in the range of 6.5–9.5 and 25–55 $^\circ\text{C}$, respectively. In addition, for the storage stability assay, the free ArPPPK2 and the ArPPK2-Cu$_3$(PO$_4$)$_2$·3H$_2$O Nano flower was stored at 4 and 25 $^\circ\text{C}$ for 28 days, and the enzyme activity was examined at a fixed time every 4 days. All reactions were performed and analyzed in triplicates.

4.7. Reusability Studies. The obtained ArPPK2-Cu$_3$(PO$_4$)$_2$·3H$_2$O nanoflower was incubated in 1 mL of mixed solution containing 100 mM Tris-HCl (pH 8.0), 3 mM polyP$_6$, 20 mM $\text{MgCl}_2$, and 1 mM AMP at 30 $^\circ\text{C}$ for 30 min. After each cycle, the ArPPK2-Cu$_3$(PO$_4$)$_2$·3H$_2$O nanoflower was recovered by centrifugation at 10,000g for 10 min and washed three times with Tris-HCl (100 mM, pH 8.0) to remove any remaining substrates or products accumulated on the support. The recovered ArPPK2-Cu$_3$(PO$_4$)$_2$·3H$_2$O nanoflower was employed in the next reaction measurement immediately. All tests were performed in triplicates.

4.8. ADP Regeneration with the ArPPK2-Cu$_3$(PO$_4$)$_2$·3H$_2$O Nanoflower. In order to evaluate ADP regeneration, the ArPPK2-Cu$_3$(PO$_4$)$_2$·3H$_2$O nanoflower was coupled with ADP-GK for catalysis. We used the following reaction system to produce G-6-P: 100 mM Tris-HCl (pH 8.0), 20 mM glucose, 20 mM $\text{MgCl}_2$, and 1 mM ADP. Based on this, we formulated a double enzyme-coupled catalytic reaction system: 100 mM Tris-HCl (pH 8.0), 20 mM glucose, 20 mM $\text{MgCl}_2$, 3 mM polyP$_6$, and 1 mM AMP. For testing the reusability of the ArPPK2-Cu$_3$(PO$_4$)$_2$·3H$_2$O nanoflower in the reaction system, after each cycle, the ArPPK2-Cu$_3$(PO$_4$)$_2$·3H$_2$O nanoflower was recovered by centrifugation at 10,000g for 10 min and washed twice with Tris-HCl (100 mM, pH 8.0). Then, the ArPPK2-Cu$_3$(PO$_4$)$_2$·3H$_2$O nanoflower was used for the next cycle immediately. The concentrations of G-6-P were determined by the method according to Chen et al. All tests were performed in triplicates.

# AUTHOR INFORMATION

Corresponding Author
Huanqing Niu — State Key Laboratory of Materials-Oriented Chemical Engineering and College of Biotechnology and Pharmaceutical Engineering, National Engineering Technique Research Center for Biotechnology, Nanjing Tech University, Nanjing 210009, P. R. China; orcid.org/0000-0002-9019-0463; Email: huanqingniu@njtech.edu.cn

Authors
Xinzeng Sun — State Key Laboratory of Materials-Oriented Chemical Engineering and College of Biotechnology and Pharmaceutical Engineering, National Engineering Technique Research Center for Biotechnology, Nanjing Tech University, Nanjing 210009, P. R. China
Jiarui Song — State Key Laboratory of Materials-Oriented Chemical Engineering and College of Biotechnology and Pharmaceutical Engineering, National Engineering Technique Research Center for Biotechnology, Nanjing Tech University, Nanjing 210009, P. R. China
Research Center for Biotechnology, Nanjing Tech University, Nanjing 210009, P. R. China
Dahai Jiang — State Key Laboratory of Materials-Oriented Chemical Engineering and College of Biotechnology and Pharmaceutical Engineering, National Engineering Technique Research Center for Biotechnology, Nanjing Tech University, Nanjing 210009, P. R. China
Jing Leng — State Key Laboratory of Materials-Oriented Chemical Engineering and College of Biotechnology and Pharmaceutical Engineering, National Engineering Technique Research Center for Biotechnology, Nanjing Tech University, Nanjing 210009, P. R. China
Wei Zhuang — State Key Laboratory of Materials-Oriented Chemical Engineering and College of Biotechnology and Pharmaceutical Engineering, National Engineering Technique Research Center for Biotechnology, Nanjing Tech University, Nanjing 210009, P. R. China

Complete contact information is available at:
https://pubs.acs.org/10.1021/acsomega.0c00329

Author Contributions
The manuscript was written through contributions of all the authors. All the authors have given approval to the final version of the manuscript.

Notes
The authors declare no competing financial interest.

# ACKNOWLEDGMENTS

This work was supported by the Jiangsu Synergetic Innovation Center for Advanced Bio-Manufacture (grant number XTE1842), the young investigator grant program of National Natural Science Foundation of China (grant number 21706123), the National Natural Science Foundation of China, General Program (grant number 21878142), the key program of the National Natural Science Foundation of China (grant number 21636003), the Key Research and Development Plan of Jiangsu Province (grant number BE2019001), Jiangsu Government Scholarship for Overseas Studies (grant number JS-2019-053), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).
REFERENCES

(1) Liu, W.; Wang, P. Cofactor regeneration for sustainable enzymatic biosynthesis. Biotechnol. Adv. 2007, 25, 369–384.

(2) Finningar, W.; Cutlan, R.; Snajdrova, R.; Adams, J. P.; Littlechild, J. A.; Harmer, N. J. Engineering a seven enzyme biotransformation using mathematical modelling and characterized enzyme parts. ChemCatChem 2019, 11, 3474–3489.

(3) Cao, H.; Nie, K.; Li, C.; Xu, H.; Wang, F.; Tan, T.; Liu, L. Rational design of substrate binding pockets in polyphosphate kinase for use in cost-effective ATP-dependent cascade reactions. Appl. Microbiol. Biotechnol. 2017, 101, 5325–5332.

(4) Aehbergerová, L.; Nahálka, J. Polyphosphate-an ancient energy source and active metabolic regulator. Microb. Cell Fact. 2011, 10, 63.

(5) Kulmer, S. T.; Gutmann, A.; Lemmerer, M.; Nidetzky, B. Biocatalytic Cascade of Polyphosphate Kinase and Sucrose Synthase for Synthesis of Nucleotide-Derived Derivatives of Glucose. Adv. Synth. Catal. 2017, 359, 292–301.

(6) Kumar, S.; Dagar, V. K.; Khasa, Y. P.; Kuhad, R. C. Genetically modified microorganisms (GMOs) for bioremediation. In Biotechnology for Environmental Management and Resource Recovery; Kuhad, R. C., Ed.; Springer: New Delhi, 2013; pp 191–218.

(7) He, W.; Wamer, W.; Xia, Q.; Yin, J.-j.; Fu, P. P. Enzyme-like activity of nanomaterials. J. Environ. Sci. Health, Part C: Environ. Health Sci. 2014, 32, 186–211.

(8) Patel, R. N. Chemo-enzymatic synthesis of pharmaceutical intermediates. Expert Opin. Drug Discovery 2008, 3, 187–245.

(9) Nadar, S. S.; Gawas, S. D.; Rathod, V. K. Self-assembled organic-inorganic hybrid glucoamylase nanoflowers with enhanced activity and stability. Int. J. Biol. Macromol. 2016, 92, 660–669.

(10) Han, L.; Liu, A. Novel cell-inorganic hybrid catalytic interfaces with enhanced enzymatic activity and stability for sensitive biosensing of paraaxon. ACS Appl. Mater. Interfaces 2017, 9, 6894–6901.

(11) Ocsoy, I.; Dogru, E.; Usta, S. A new generation of flowerlike horseradish peroxidase as a nanobiocatalyst for superior enzymatic activity. Enzyme Microb. Technol. 2015, 75–76, 25–29.

(12) Rai, S. K.; Naromilli, L. K.; Sangwan, R. S.; Yadav, S. K. Self-Assembled Hybrid Nanoflowers of Manganese Phosphate and L-Arabinoisomerase: A Stable and Recyclable Nanobiocatalyst for Equilibrium Level Conversion of d-Galactose to d-Tagatose. ACS Sustainable Chem. Eng. 2018, 6, 6296–6304.

(13) Chen, X.; Xu, L.; Wang, A.; Li, H.; Wang, C.; Pei, X.; Zhao, P.; Wu, S. G. Efficient synthesis of the key chiral alcohol intermediate of Crixotinib using dual-enzyme@CaHPO4 hybrid nanoflowers as assembly of mimetic biominalerization. J. Chem. Technol. Biotechnol. 2019, 94, 236–243.

(14) Zhu, L.; Geng, L.; Zhang, Y.; Wang, R.; Ge, J.; Liu, Z.; Zare, R. N. Rapid detection of phenol using a membrane containing laccase nanoflowers. Chem.—Asian J. 2013, 8, 2358–2360.

(15) Ocsoy, I.; Dogru, E.; Usta, S. A new generation of flowerlike horseradish peroxidases as a nanobiocatalyst for superior enzymatic activity. Enzyme Microb. Technol. 2015, 75–76, 25–29.

(16) Yu, Y.; Fei, X.; Tian, J.; Xu, L.; Wang, X.; Wang, Y. Self-assembled enzyme-inorganic hybrid nanoflowers and their application to enzyme purification. Colloids Surf., B 2015, 130, 299–304.

(17) Lin, Z.; Xiao, Y.; Wang, L.; Yin, Y.; Zheng, J.; Yang, H.; Chen, G. Facile synthesis of enzyme-inorganic hybrid nanoflowers and their application as an immobilized trypsin reactor for highly efficient protein digestion. RSC Adv. 2014, 4, 13888–13891.

(18) Liang, L.; Fei, X.; Li, Y.; Tian, J.; Xu, L.; Wang, X.; Wang, Y. Hierarchical assembly of enzyme-inorganic composite materials with extremely high enzyme activity. RSC Adv. 2015, 5, 96997–97002.

(19) Tran, T. D.; Kim, M. I. Organic-inorganic hybrid nanoflowers as potent materials for biosensing and biocatalytic applications. BioChip J. 2018, 12, 268–279.

(20) Niu, H.; Ding, M.; Sun, X.; Zhuang, W.; Liu, D.; Ying, H.; Zhu, C.; Chen, Y. Immobilization of a polysaccharide kinase 2 by coordinative self-assembly of his-tagged units with metal-organic frameworks and its application in ATP regeneration from AMP. Colloids Surf., B 2019, 181, 261–269.

(21) Yan, B.; Ding, Q.; Ou, L.; Zou, Z. Production of glucose-6-phosphate by glucokinase coupled with an ATP regeneration system. J. Microb. Biotechnol. 2014, 30, 1123–1128.

(22) Ge, J.; Lei, J.; Zare, R. N. Protein-inorganic hybrid nanoflowers. Nat. Nanotechnol. 2012, 7, 428.

(23) Zhang, L.; Ma, Y.; Wang, C.; Wang, Z.; Chen, X.; Li, M.; Zhao, R.; Wang, L. Application of dual-enzyme nanoflower in the epoxidation of alkenes. Process Biochem. 2018, 74, 103–107.

(24) Zheng, L.; Sun, Y.; Wang, J.; Huang, H.; Geng, X.; Tong, Y.; Wang, Z. Preparation of a Flower-Like Immobilized D-Psicose 3-Epimerase with Enhanced Catalytic Performance. Catalysts 2018, 8, 468.

(25) Gao, J.; Hui, L.; Lingyan, P.; Kai, G.; Materials, L. J. A. A.; Interfaces. A biocatalyst and colorimetric/fluorescent dual biosensors of H2O2 constructed via Hemoglobin-Ca3(PO4)2 organic/inorganic hybrid nanoflowers. ACS Appl. Mater. Interfaces 2018, 10, 30441–30450.

(26) Molina, G. A.; Esparza, R.; López-Miranda, J. L.; Hernández-Martínez, A. R.; España-Sánchez, B. L.; Elizalde-Peña, E. A.; Estevez, M.; Bionterfaces, S. B. Green synthesis of Ag nanoflowers using Kalanchoe Daigremontiana extract for enhanced photocatalytic and antibacterial activities. Colloids Surf., B 2019, 180, 141–149.

(27) Cao, G.; Gao, J.; Zhou, L.; He, Y.; Li, J.; Jiang, Y. Enrichment and Coimmobilization of Cofactors and His-Tagged-ω-Transaminase into Nanoflowers: A Facile Approach to Constructing Self-Sufficient Biocatalysts. ACS Appl. Nano Mater. 2018, 1, 3417–3425.

(28) Nadar, S. S.; Rathod, V. K. Encapsulation of lipase within metal-organic framework (MOF) with enhanced activity intensified under ultrasound. Enzyme Microb. Technol. 2018, 108, 11–20.

(29) Koga, S.; Yoshioka, I.; Sakuraba, H.; Takashashi, M.; Sakasegawa, S.; Shimizu, S.; Oshima, T. Biochemical characterization, cloning, and sequencing of ADP-dependent (AMP-forming) glucokinase from two hyperthermophilic archaea, Pyrococcus furiosus and Thermococcus litoralis. J. Biochem. 2000, 128, 1079–1085.

(30) Mitchell, D. M.; Gennis, R. B. Rapid purification of wildtype and mutant cytochrome c oxidase from Rhodobacter sphaeroides by Ni2+-NTA affinity chromatography. FEBS Lett. 1995, 368, 148.

(31) Bradford, M. A. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976, 72, 248–254.

(32) Altightkaynak, C.; Yilmaz, I.; Koksal, Z.; Özdemir, H.; Ocsoy, I.; Özdemir, N. Preparation of lactoperoxidase incorporated hybrid nanoflower and its excellent activity and stability. Int. J. Biol. Macromol. 2016, 84, 402–409.

(33) Hao, M.; Fan, G.; Zhang, Y.; Xin, Y.; Zhang, L. Preparation and characterization of copper-Brevibacterium cholesterol oxidase hybrid nanoflowers. Int. J. Biol. Macromol. 2019, 126, 539–548.

(34) Chen, X.; Song, H.; Fang, T.; Cao, J.-m.; Ren, H.-j.; Bai, J.-x.; Xiong, J.; Ouyang, P.-k.; Ying, H.-j. Enhanced cyclic adenosine monophosphate production by Arthrobacter A302 through rational redistribution of metabolic flux. Bioresour. Technol. 2010, 101, 3159–3163.

(35) Patel, S. K. S.; Otari, S. V.; Chan Kang, Y.; Lee, J.-K. Protein-inorganic hybrid system for efficient his-tagged enzymes immobilization and its application in L-xylulose production. RSC Adv. 2017, 7, 3488–3494.

(36) Chen, Y.; Liu, Q.; Chen, X.; Wu, J.; Xie, J.; Guo, T.; Zhu, C.; Ying, H. Control of glycolytic flux in directed biosynthesis of uridine-phosphoryl compounds through the manipulation of ATP availability. Appl. Microbiol. Biotechnol. 2014, 98, 6621–6632.