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Multilayer petal-like enzymatic-inorganic hybrid micro-spheres [CPO-$(Cu/Co/Cd)_3(PO_4)_2$] with high bio-catalytic activity

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ABSTRACT

CPO, a versatile enzyme, has limitations in industrial applications due to its poor stability and the difficulty of reuse. The preparation of stable immobilized enzymes with high catalytic activity is therefore desirable, but remains a challenge. A facile preparation of a series of enzyme-inorganic hybrid micro-spheres [chloroperoxidase (CPO)-$(Cu/Co/Cd)_3(PO_4)_2$] and its application in the decolorization of crystal violet is reported in this work. All the hybrid micro-spheres show a multilayer petal-like structure. The formation of hybrid micro-spheres were proposed via four stages: crystallization, in-situ coordination, self-assembly and size growth. The process was entropy-driven, and there was a competition between precipitation of phosphate and coordination of M^{2+} with amide groups of CPO. The introduction of excess of chloride ions retarded the phosphate from precipitating by forming $[MCl_4]^{2-}$ complexes, while simultaneously promoting the coordination of M^{2+} with amide groups. These hybrid materials showed high bio-catalytic activity in the decolorization of crystal violet. The decolorization efficiency reached 99.66%, 98.44% and 98.05% just within 3 min using CPO-$Cu_3(PO_4)_2$, CPO-$Cd_3(PO_4)_2$ and CPO-$Co_3(PO_4)_2$ respectively. They also have good thermal stability, and can keep 52.89% catalytic activity after 8 cycles of use.

CPO-inorganic hybrid micro-spheres [chloroperoxidase (CPO)-$(Cu/Co/Cd)_3(PO_4)_2$] is promising in the treatment of waste water containing crystal violet. It also has potential application for treatment of other soluble organic dyes in industrial wastewater.

1. Introduction

Chloroperoxidase (CPO), a glycosylated heme-thiolate protein secreted by the fungus *Caldariomyces fumago*, exhibits peroxidase, catalase and cytochrome P450-like activities and shows sensitive recognition toward chiral substrates. Because of its specific active sites, CPO catalyzes a wide range of reactions such as oxygen insertion (sulfoxidation and epoxidation), halogenation and hydroxylation reactions (Kiljunen and Kanerva, 2000; Manoj and Hager, 2008; Fernandez-Fueyo et al., 2015; Kaup et al., 2007). CPO can be therefore widely used in the preparation of chiral drugs (Gao et al., 2015), biotransformation (Zhang et al., 2011), biosensing (Ribeiro et al., 2014), and dye degradation (Zhang et al., 2016a).

However, this versatile enzyme has limitations in the large-scale industrial applications due to its poor stability at elevated temperatures and in the presence of organic solvents, as well as the difficulty

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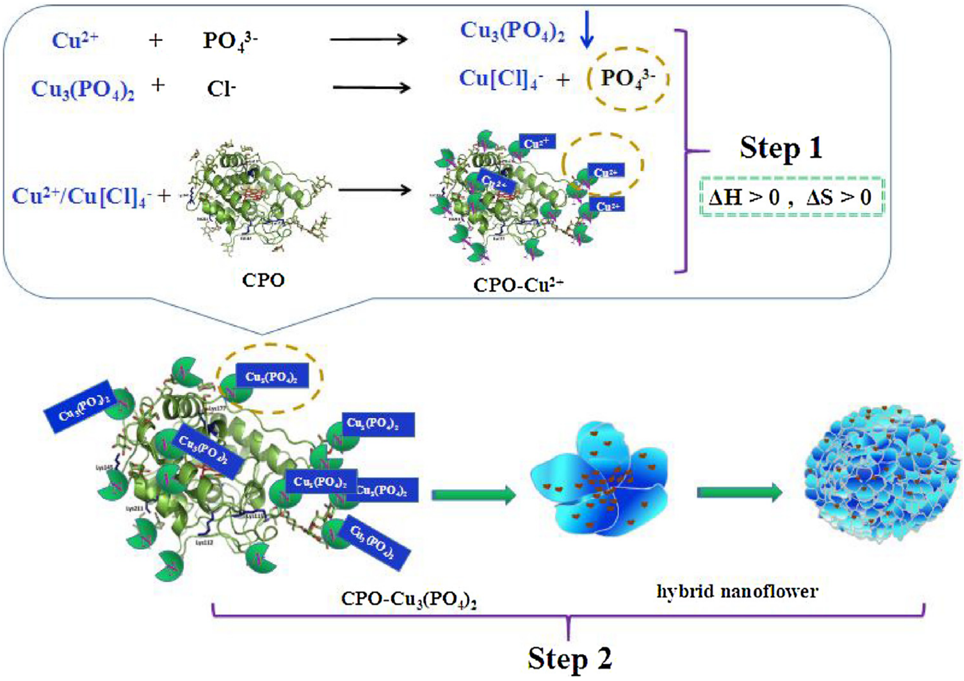

Scheme 1 – The growth mechanism of hybrid materials.

of its separation from reaction system to be reused. In recent years, much work has focused on the immobilization of CPO on solid supports to improve its operational stability and allow it to be reused. Many strategies have been proposed for the immobilization of CPO, including entrapment (Le et al., 2015; Bruns and Tiller, 2005; Lee et al., 2009), covalent immobilization (Bayramoglu et al., 2008; Aburto et al., 2005; Montiel et al., 2007; Wang et al., 2009), and cross-linking (Perez et al., 2009; Jung et al., 2009; Roberge et al., 2009). However, enzyme inactivation is generally inevitable during these immobilization processes due to mass transfer limitations of substrate and/or product in the immobilization matrix and the changes of conformation at active site of enzyme (Sheldon and van Pelt, 2013). The development of immobilization methods and support to obtain stable immobilized enzymes with high catalytic activity is therefore desirable, but remains a challenge.

Recently, enzyme-inorganic hybrid materials, and especially flower-shaped micro or nano crystals, have attracted increasing attention because of their high activity and their potential applications in catalysis, protein analysis, and biosensors (Ge et al., 2012; Mann, 2009; Sun et al., 2014; Wang et al., 2013; Lin et al., 2014a, 2014b; Liu et al., 2017). The activity of the first reported laccase-incorporated $Cu_3(PO_4)_2$ nanoflower exhibited an increased activity compared with free laccase (Ge et al., 2012). The activity of these enzymes embedded in the inorganic crystal increased due to the following factors: (1) the components were combined by assembly, which had a weak allosteric effect on the enzymes; (2) a high surface area was induced; and (3) there was a synergistic catalytic effect between the enzyme and the inorganic metals (Yin et al., 2015; Zhang et al., 2016b).

Inspired by these investigation, a series of CPO-based hybrid microspheres with a multi petal-like structure were prepared in this work, using Cu(II), Cd(II), and Co(II) phosphate as inorganic compound respectively due to their similar electron configuration. These obtained CPO-$(Cu/Co/Cd)_3(PO_4)_2$ hybrid materials were applied in the decolorization of crystal violet. The formation process and growth mechanism of the hybrid materials were studied and illustrated using scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD) measurements, and microcalorimetry measurements. The factors that had influence on the morphology of the hybrid micro-sphere were investigated. The relationship between the morphology and enzymatic performance of the as-prepared hybrid materials were evaluated by measuring the decolorization efficiency of crystal violet.

2. Results and discussion

2.1. *Morphology of CPO-$(Cu/Cd/Co)_3(PO_4)_2$*

The obtained hybrid materials were all flower-like, with a dense multi-layer petal structure. Their morphology was correlated to the metal species, anion type, and concentration of CPO. Figs. 1–4 show SEM images of the CPO-phosphate hybrid materials. Fig. 1 shows that the CPO-$Cu_3(PO_4)_2$ displayed a different morphology from pure $Cu_3(PO_4)_2$. The $Cu_3(PO_4)_2$ precipitate was a large bulk structure (Fig. 1a), while the CPO-$Cu_3(PO_4)_2$ were flower-like spheres (Fig. 1b), showing hierarchical structures (Fig. 1c).

When $CuSO_4$, $Cu(CH_3COO)_2$, or $CuCl_2$ was employed as Cu^{2+} source to prepare the CPO-$Cu_3(PO_4)_2$ hybrid materials in phosphate buffer, it was found that anions (SO_4^{2-}, CH_3COO^-, Cl^-) influenced the growth process and the morphology. Fig. 2 showed that the formation of multi-layer petal-like scaffold of CPO-$Cu_3(PO_4)_2$ was slower by using $CuCl_2$ (Fig. 2c) than that by using $CuSO_4$ or $Cu(CH_3COO)_2$ (Fig. 2a,b). Then Fig. 3 indicated that the CPO-$Cu_3(PO_4)_2$ showed different morphology when prepared by $CuSO_4$, $Cu(CH_3COO)_2$ or $CuCl_2$. CPO-$Cu_3(PO_4)_2$ had a dense multi-layer structure prepared using $CuSO_4$, something like hydrangea macrophylla (insets). The CPO-$Cu_3(PO_4)_2$ prepared using $Cu(CH_3COO)_2$ or $CuCl_2$ have more dense multi-layer structure, something like allium giganteum (insets), and their flower-like spheres were larger.

When $CoCl_2$ or $Cd(CH_3COO)_2$ was used to prepare the CPO-$(Co/Cd)_3(PO_4)_2$ hybrid materials, different flower-like morphologies were observed. Fig. 4 showed that the morphology of CPO-$Cd_3(PO_4)_2$ was butterfly like, while CPO-$Co_3(PO_4)_2$ formed flower-like sphere, but with more dense petals and a higher specific surface volume than that of CPO-$Cu_3(PO_4)_2$. The morphology of $Co_3(PO_4)_2$ (a) and $Cd_3(PO_4)_2$ (b) particle was showed in Fig. S1. These results showed that the morphology of CPO-phosphate hybrid materials was highly dependent on the type of inorganic constituents.