Korean J. Chem. Eng., 38(5), 1066-1077 (2021)
DOI: 10.1007/s11814-021-0775-7

pISSN: 0256-1115
eISSN: 1975-7220

Regenerated coenzyme-based preparation of bienzyme-polymer nanoconjugates and their applications for the synthesis of ethyl (R)-2-hydroxy-4-phenylbutyrate

Yuan Lu*, Hongqian Dai*, Pengpeng Cheng*, Hanbing Shi, Lan Tang*, Xingyuan Sun**,†, and Zhimin Ou*,†**

*College of Pharmaceutical Science, Zhejiang University of Technology, Hangzhou, Zhejiang, China
**The Third Affiliated Hospital of Qiqihar Medical College, Qiqihar, Heilongjiang, China
(*Received 16 January 2021 • Revised 17 February 2021 • Accepted 26 February 2021*)

Abstract−A modular approach was applied for the synthesis of bienzyme-polymer nanoconjugates (nano-BECs) (50-70 nm) consisting of two enzymes (carbonyl reductase and glucose dehydrogenase) conjugated within a single universal polymer scaffold. The amount of the product ethyl (R)-2-hydroxy-4-phenylbutyrate (R-HPBE) with nano-BECs as the catalyst was 533 mM in a dibutyl phthalate-phosphate buffer (dibutyl phthalate-PB) biphasic system, while the amount of R-HPBE was 349 mM using carbonyl reductase-poly(acrylic acid) as the catalyst, indicating that the nano-BECs have an advantage for coenzyme regeneration. Compared with a single aqueous phase, the substrate treatment capacity was improved at the interface of the dibutyl phthalate-PB biphasic system. Under the optimal reaction conditions (35 °C, 40 h, dibutyl phthalate-PB 1 : 1), nano-BECs can completely convert substrate into optically pure R-HPBE (enantiomeric excess (e.e.) >99.9%) in the organic-aqueous system.

Keywords: Asymmetric Reduction, Bienzyme-polymer Nanoconjugate, R-HPBE, Interfacial Biocatalysis, Coenzyme Regeneration

INTRODUCTION

Chiral alcohols are important building blocks for the synthesis of chiral drugs and chemicals. Asymmetric reduction of prochiral ketones via biocatalysis is one of the most effective methods for the synthesis of chiral alcohols [1-4]. Some chiral compounds that are difficult to synthesize by chemical methods can also be prepared by biocatalysis [5,6]. Biocatalysis has been widely used in basic research and industrial production because of its high enantioselectivity, mild reaction conditions, and environmental compatibility [7-9]. Catalysts for the asymmetric synthesis of chiral compounds by biocatalysis include microbial whole cells and enzymes. There are advantages and drawbacks using whole-cell microorganisms or isolated enzymes as biocatalysts in the asymmetric reduction of ketones [10]. Stereoselective carbonyl reductase (CBR) belong to the short-chain alcohol dehydrogenase/reductase family and can stereoselectively catalyze the asymmetric reduction of prochiral ketones to chiral alcohols. CBR for the asymmetric reduction of ketones to prepare chiral alcohols requires coenzymes (NADH or NADPH) as hydrogen donors [11]. Given its high cost, coenzymes must be converted from oxidation form for recycling [12]. Based on the demand for coenzyme regeneration, glucose dehydrogenase (GDH), which helps coenzyme regeneration, is added to provide a continuous coenzyme for asymmetric reduction reactions [13].

Enzymatic catalysis generally requires aqueous media in which the hydrophobic substrates are poorly soluble and even unstable. A straightforward solution to this issue might be the application of an organic-aqueous solvent system [14]. During biotransformation in the water phase, the contact rate of the enzyme and the organic substrate is low. The organic substrate and product are toxic to the enzyme and easily reduce enzyme activity [15]. Therefore, the conversion of the enzyme-catalyzed synthesis of chiral alcohol in a single aqueous phase is not high. Enzyme protein molecules have hydrophilic and hydrophobic structures, so they can easily aggregate and self-assemble at the organic-aqueous interface to form a specific structural arrangement to achieve catalytic reactions at the organic-aqueous interface [16,17]. To increase the conversion, biocatalysis of the organic-aqueous two-phase system is used to synthesize chiral alcohols [18,19]. Organic-aqueous biphasic catalysis is an important class of environmentally friendly processes because it provides a way of heterogenizing the catalysts and substrates/products into two separate and immiscible phases for ready separability [17]. Despite the many advantages of biphasic catalysis, the organic phase in the two phases also has a negative impact on the activity and stability of the enzyme. Therefore, it is expected to achieve the purpose of improving the activity and stability of the enzyme through the molecular modification of the enzyme [20-23].

Coupled polymer-enzyme co-incorporated structures, which provide a favorable microenvironment because of the flexibility and functional groups of the polymer have attracted increased attention [24,25]. Some conventional intermixed structures or self-assembled polymers, such as chitosan, agarose, poly(acrylamide), and poly(propyleneimine), have been widely used in biomedical fields, including protein adsorption, biomolecule immobilization, and controlled drug release [26-29]. Among these functional polymers, poly(acrylic acid) (PAA) is a water-soluble biocompatible polymer owing to its dispersity and capacity [30,31]. Using site-specific attachment strat-

†To whom correspondence should be addressed.
E-mail: 708900599@qq.com, oozzmm@163.com

egies, single enzymes can be conjugated to end groups of polyethylene oxide. These precisely defined single enzyme conjugates are used for biological applications. Single enzymes can also be randomly conjugated within the PAA framework such that amino groups on the enzyme chemically react with carboxylic acid groups on PAA [32]. In this study, amine groups on CBR and GDH were covalently and randomly attached to carboxylic groups on PAA catalyzed by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), resulting in CBR-GDH bienzyme conjugates (BECs). The two types of BECs were used in the asymmetric reduction of ethyl 2-oxo-4-phenylbutyrate (OPBE) to obtain ethyl (R)-2-hydroxy-4-phenylbutyrate (R-HPBE) in the organic-aqueous biphasic system in this study.

R-HPBE is a key chiral intermediate in the production of a variety of angiotensin converting enzyme inhibitors (ACEI) for the treatment of hypertension, such as cilazapril, enalapril, benazepril, ramipril, and quinapril [33]. ACEI is a safe and effective antihypertensive drug that cuts off the renin-angiotensin-aldosterone system and blocks the production of angiotensin II to dilate blood vessels. Owing to the importance of R-HPBE in the synthesis of this class of drugs, different technical processes have been reported for the synthesis of R-HPBE, including asymmetric reduction of 2-oxo-4-phenylbutanoic acid or its derivates [34-36], classical resolution of corresponding racemate [37], and chemo-enzymatic synthesis of the target molecules [38]. Among them, the bioreduction of OPBE with reductase attracts much attention due to its significant advantages such as high conversion, eco-friendliness (neutral pH and room temperature), and remarkable stereoselectivity, which makes it a better choice compared with other methods [39]. In the present study, two types of nano-BECs consisting of two different enzymes with distinct catalytic properties conjugated within a single universal polymer scaffold were constructed using a general and modular approach. This provides a new method for the effective synthesis of R-HPBE.

MATERIALS AND METHODS

1. Materials and Equipment

PAA (Mv=450,000 g mol^{-1}, where Mv is the viscosity average molecular weight), EDC, GDH (*Bacillus*), dibutyl phthalate, n-hexane, n-heptane, benzene, and ethyl acetate were purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. CBR was separated and purified from *Saccharomyces cerevisiae*, CGMCC No. 3361. Oxidized coenzyme II/$NADP^+$ (>98%) was purchased from Nanjing Odofoni Biotechnology Co., Ltd. Ethyl 2-oxo-4-phenylbutyrate (98%) was purchased from Shanghai Merrill Chemical Technology Co., Ltd. R-HPBE and S-HPBE standards were purchased from Saen Chemical Technology (Shanghai) Co., Ltd. All reagents used in the electrophoresis experiment were purchased from Shenggong Bioengineering (Shanghai) Co., Ltd. Electrophoresis (DYCZ-24DN) used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was purchased from Beijing Liuyi Biotechnology Co., Ltd.

2. Analytical Method

The conversion and enantiomeric excess of R-HPBE were analyzed using a Shimadzu GC-2014 gas chromatograph. The conversion was defined as the ratio of the converted substrate concentration to the initial substrate concentration. The sample was analyzed by GC equipped with an Agilent CP7502 J&W CP-Chirasil-Dex CB chiral column (Machery-Nagel; 25 m×0.25 mm×0.25 mm). The injector, column, and FID temperatures were 250, 130, and 250 °C, respectively. The split ratio was 1 : 15. The flow rate was

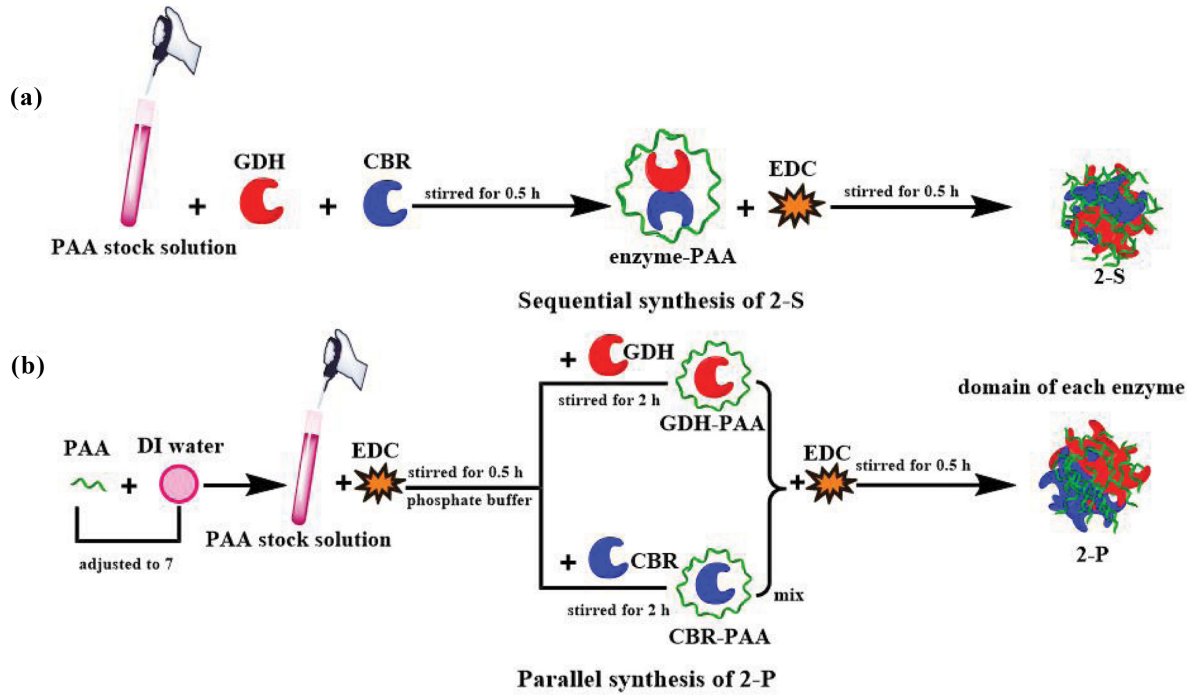

Fig. 1. Two different routes to synthesize nano-BECs. (a) Sequential synthesis of 2-S. (b) Parallel synthesis of 2-P.

2 mL/min. The retention times of OPBE, R-HPBE, and S-HPBE were 20.898, 27.948, and 28.923 min, respectively. Eq. (1) and (2) define the conversion (X) and enantiomeric excess (ee) of HPBE.

$$X\,(\%) = \frac{P \times Ms}{Q \times Mp} \times 100\% \quad (1)$$

Ms and Mp are the molecular weights of the substrate and the product, respectively. P and Q represent the mass of the product at the end of the reaction and the initial mass of the substrate, respectively.

$$ee_P = \frac{C_R - C_S}{C_R + C_S} \times 100\% \quad (2)$$

C_R and C_S represent the concentrations of R-HPBE and S-HPBE, respectively.

3. Preparation of Nano Bienzyme Conjugates

Nano-BECs were synthesized using two different enzymes (CBR and GDH) and PAA via two different synthesis routes, which include sequential and parallel synthesis routes. The preparation process is shown in Fig. 1.

3-1. Sequential Synthesis of 2-S

Synthesis of nano-BECs was in one step. The synthetic method was reasonably improved based on the current literature [19]. A 2% stock solution of PAA (40 μL) was prepared by dissolving PAA in distilled water, and the pH was adjusted to 7. The PAA stock solution was mixed with 40 μL 10 mM phosphate buffer (PB; pH 7.4). 0.124 mg and 0.089 mg of CBR and GDH dissolved in PB (100 μL) were added drop wise, and the mixture was stirred for 0.5 h. EDC was added to this mixture with a mass of 2.585 mg and the total solution volume was adjusted to 1 mL by adding PB solution. The mixture was stirred for 0.5 h to prepare the nano-BECs. The sample was dialyzed using a 25 kDa dialysis membrane at pH 7.4 PB for 3 cycles of 5 h to remove unreacted EDC and EDC-urea byproducts. The total mass of 2-S was 3 mg and the final enzyme activity of each enzyme in 2-S is 2.5 U (1 U is defined as the amount of enzyme that can convert 1 μmol substrate in 1 minute).

3-2. Parallel Synthesis of 2-P

Nano-BECs were synthesized using a two-step parallel process. In step 1, CBR-PAA and GDH-PAA conjugates were synthesized using EDC. Twenty microliters of PAA stock solution (2%) was mixed with an equal volume of 10 mM phosphate buffer (pH 7.4) and activated by 1.292 mg of EDC. The mixture was stirred for 0.5 h, and then 50 μL CBR stock solution was prepared by dissolving 0.124 mg CBR in PB solution and added drop wise, and stirred for 2 h. Finally, covalent conjugation was achieved through carboxyl groups on PAA and lysine amino groups on the enzymes, and CBR-PAA conjugates were prepared. In a similar manner, another GDH-PAA conjugate was synthesized with 0.089 mg of GDH. Enzyme-PAA conjugates including CBR-PAA and GDH-PAA were purified by dialysis using a 25 kDa dialysis membrane at pH 7.4 PB for 3 cycles of 5 h each. In step 2, CBR-PAA and GDH-PAA were mixed in equal volume and cross-linked with 2.585 mg of EDC. The mixture volume was 1 mL and stirred for 0.5 h to prepare the nano-BECs (2-P). The sample was dialyzed to remove unreacted EDC and EDC-urea byproducts. The total mass of 2-P was 5.5 mg and the final enzyme activity of each enzyme in 2-P is 2.5 U, similar to 2-S.

4. Characterization of the Enzyme, Enzyme-PAA, and Nano-BECs (2-S and 2-P)

Successful conjugation of enzymes to PAA and formation of nano-BECs (2-S and 2-P) was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The structural characterization of nano-BECs was by using circular dichroism (CD) studies (J-815; JASCO, Japan). Morphology, structure, and particle size distribution of BECs, single enzyme-PAA, and unmodified native enzymes were analyzed using a 120 kV transmission electron microscope (TEM). Samples 2-S, 2-P, CBR-PAA, GDH-PAA, CBR, and GDH were imaged using TEM (HT 7700; Hitachi, Japan).

5. Reduction Reaction Catalyzed by Nano-BECs in the Single Aqueous Phase

5-1. Influence of Different Substrate Concentrations on the Reduction Reaction

The two types of nano-BECs with each enzyme activity of 2.5 U prepared in Section 2.3 were added to the reaction mixture containing 0.05 mM $NADP^+$, co-substrate d-glucose (1.5 equiv.), and OPBE at different concentrations of 388 mM, 436 mM, 485 mM, 533 mM, 582 mM, and 630 mM, and the total volume of the reaction system was adjusted to 12 mL using PB (10 mM, pH 7.4). The reactions were performed as batch processes in a rotary shaker at 180 rpm and 35 °C for 40 h. The conversion of substrate and e.e.% of R-HPBE was determined as described in Section 2.2.

5-2. Influence of Shaker Speed on the Reduction Reaction

OPBE (485 mM), co-substrate d-glucose (1.5 equiv.), coenzyme $NADP^+$ (0.05 mM), and nano-BECs (3 mg 2-S or 5.5 mg 2-P) were added to the reaction flask containing PB (10 mM, pH 7.4) to maintain the total volume of the reaction system at 12 mL. The reaction systems were placed in a constant temperature (35 °C) shaker at different speeds (150, 160, 170, 180, and 190 rpm) for 40 h. After the reaction, the reaction solution was extracted with an equal volume of ethyl acetate. The conversion and e.e.% of R-HPBE were determined.

6. Reduction Reaction Catalyzed by Nano-BECs in the Organic-aqueous System

6-1. Influence of Organic Solvent Type on the Reduction Reaction

Nano-BECs (3 mg 2-S or 5.5 mg 2-P) were dispersed in PB (10 mM, pH 7.4) and then an equal volume (6 mL) of different types of organic solvents (n-hexane, dibutyl phthalate, benzene, toluene, and n-heptane) was added to the mixture. The 12 mL reaction mixture additionally contained 533 mM OPBE, 0.05 mM $NADP^+$, and 799.5 mM d-glucose (1.5 equiv.). After the reduction was carried out at 35 °C in a 120 r/min shaker for 40 h, an equal volume of ethyl acetate was added. The mixture was thoroughly mixed and the organic phase was isolated by centrifugation at 8,000 rpm for 10 min. The organic phase was dried over anhydrous Na_2SO_4 and analyzed by GC to determine the conversion and e.e.% of R-HPBE.

6-2. Influence of Different Phase Volume Ratios on the Reduction Reaction

Nano-BECs (3 mg 2-S or 5.5 mg 2-P) were dispersed in PB (10 mM, pH 7.4). Subsequently, different volumes of dibutyl phthalate solution were added to bring the volume ratio of the organic

and aqueous phases (Vo/Va) to 1 : 3, 2 : 3, 3 : 3, 4 : 3, and 5 : 3. Five hundred and thirty-three millimolar of OPBE, 0.05 mM $NADP^+$, and 799.5 mM d-glucose (1.5 equiv.) were added to the biphasic system. The total solution volume was maintained at 12 mL. The reaction flasks with different phase volume ratios were placed in a shaker (35 °C, 120 rpm) for 40 h, and then extractions were carried out using an equal volume of ethyl acetate. The organic phase was dried over anhydrous Na_2SO_4 and analyzed by GC to determine the conversion and e.e.% of R-HPBE.

6-3. Influence of Temperature on the Reduction Reaction

To change only the temperature while other conditions remained the same (Vo/Va 1 : 1, 533 mM OPBE, 0.05 mM $NADP^+$ and 799.5 mM d-glucose), the reaction flasks were placed in a constant temperature shaker (120 r/min) at 20 °C, 25 °C, 30 °C, 35 °C, and 40 °C for 40 h. At the end of the reduction, the reaction solution was extracted with an equal volume of ethyl acetate. The reaction solution was centrifuged (8,000 r/min, 10 min) to obtain a layered aqueous phase and an organic phase. The organic phase was dried over anhydrous Na_2SO_4 and analyzed by GC to determine the conversion and e.e.% of R-HPBE.

6-4. Influence of Substrate Concentration on the Reduction Reaction

An equal volume of dibutyl phthalate solution was added to 6 mL PB solution (10 mM, pH 7.4) containing nano-BECs (3 mg 2-S or 5.5 mg 2-P). Three hundred and eighty-eight millimolar, 388 mM, 436 mM, 485 mM, 533 mM, 582 mM, and 630 mM OPBE was added separately to the biphasic system. After addition of 0.05 mM $NADP^+$ and 1.5 equivalents of d-glucose, the reaction flasks were placed in a shaker (35 °C, 120 rpm) for 40 h. At the end of the reduction, the reaction solution was extracted using an equal volume of ethyl acetate. The samples were analyzed by GC to determine the conversion and e.e.% of R-HPBE.

6-5. Influence of Shaker Speed on the Reduction Reaction

533 mM OPBE, 799.5 mM d-glucose, and 0.05 mM $NADP^+$ were added to 12 mL of the dibutyl phthalate-PB (1 : 1) biphasic system dispersed with nano-BECs (3 mg 2-S or 5.5 mg 2-P). The reaction systems were placed in a constant temperature (35 °C) shaker at different speeds (80 rpm, 100 rpm, 120 rpm, 150 rpm, and 180 rpm) for 40 h. At the end of the reduction, the effect of shaker speed on the reduction was investigated by determining the conversion and e.e.% of R-HPBE.

7. Study on the Coenzyme Regeneration Ability of Nano-BECs

Different amounts of CBR (0.124 mg), GDH (0.089 mg), CBR-PAA (1.8 mg), GDH-PAA (1.7 mg), unconjugated enzymes (0.213 mg) and nano-BECs (3 mg 2-S or 5.5 mg 2-P) were used as catalysts and dispersed in 6 mL PB solution (10 mM, pH 7.4), respectively. An equal volume of dibutyl phthalate solution was added to the above four solutions. The 12 mL reaction mixture additionally contained 533 mM OPBE, 0.05 mM $NADP^+$, and 799.5 mM d-glucose. After the reduction was carried out in a 120 r/min shaker at 35 °C for 40 h, the sample was analyzed by GC to determine the conversion and e.e.% of R-HPBE.

8. Study on the Stability of Nano-BECs

A series of OPBE with different concentrations (0.25-8 mM) were configured as substrates. The enzyme activity at different substrate concentrations was measured, and the corresponding reaction rate was calculated. Double reciprocal mapping method (Lineweaver-Burk) was used to obtain Km. In order to determine the pH stability, the enzymes (CBR, GDH, unconjugated enzymes CBR/GDH, and nano-BECs) were placed in different buffers with pH 5.0-9.0. After being placed at 25 °C for 16 h, the remaining enzyme activity was determined. In the temperature stability experiment of the immobilized enzyme and the free enzyme, the residual enzyme activity was measured after placing them at different temperatures (30, 37, 40, 45, 50, 55 °C) in their respective optimal pH buffers for 30 min.

9. Reuse of Nano-BECs

Refer to the experimental method in Section 2.7, using immobilized enzyme nano-BECs as a catalyst to catalyze the reduction reaction. After the first batch of reaction, the two-phase interface was washed five times with the corresponding organic solvent dibutyl phthalate and PB buffer solution to remove free enzymes and reaction products. The free enzymes in the eluate were collected and added to the reaction system to perform reaction under optimized conditions to ensure that the recovered enzymes were nano-BECs. After that, the nano-BECs were collected for the second batch of reactions, a total of six batches.

RESULTS AND DISCUSSION

1. Characterization of the Enzyme, Enzyme-PAA, and Nano-BECs

A general and modular strategy to synthesize novel, synthetic, bienzyme polymer chemical conjugates using different enzymes (CBR, GDH) and PAA as the polymer scaffold is presented. This strategy was developed mainly based on the need for coenzyme regeneration and improvement of enzyme activity at the organic-aqueous phase interface. Successful syntheses of 2-S, 2-P, and single enzyme-PAA conjugates were confirmed using sodium SDS-PAGE, TEM, and CD studies.

1-1. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Successful conjugation of enzymes to PAA and formation of nano-BECs (2-S and 2-P) were confirmed by SDS-PAGE. Since

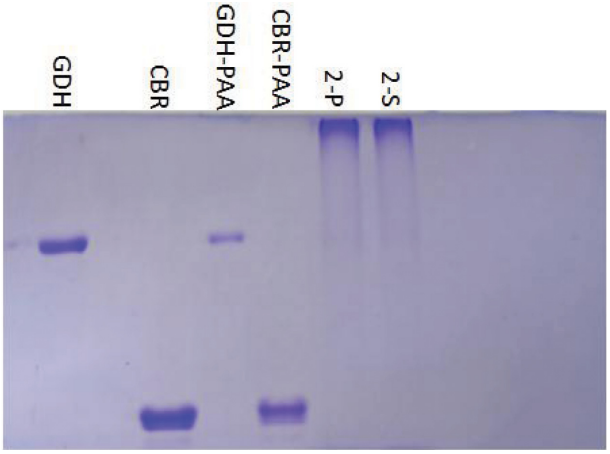

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of enzymes (GDH, CBR, GDH-PAA, CBR-PAA, 2-P, 2-S).

the anionic surfactant (SDS) has a large amount of negative charge, the amount of charge carried by the protein can be ignored after the protein molecule is fully combined with the SDS. The relative molecular mass of the protein determines the mobility of the SDS-protein complex during gel electrophoresis. The smaller the molecular weight of the protein, the faster it moves in the electric field; conversely, the slower it moves. The electrophoresis results of GDH, CBR, GDH-PAA, CBR-PAA, 2-P, and 2-S, were recorded and are presented in Fig. 2. From left to right, the protein electropherograms of six samples, GDH, CBR, GDH-PAA, CBR-PAA, 2-P, and 2-S are shown. As the size of nano-BECs increased, the nano-BECs (2-S and 2-P) were stuck in the well of the polyacrylamide gel with some streaking. The absence of any other bands pertinent to free enzymes and single enzyme-PAA conjugates in 2-S and 2-P under electrophoresis confirmed the formation of nano-BECs using PAA as a scaffold.

1-2. Transmission Electron Microscopy

TEM micrographs clearly show the nanogel morphologies of

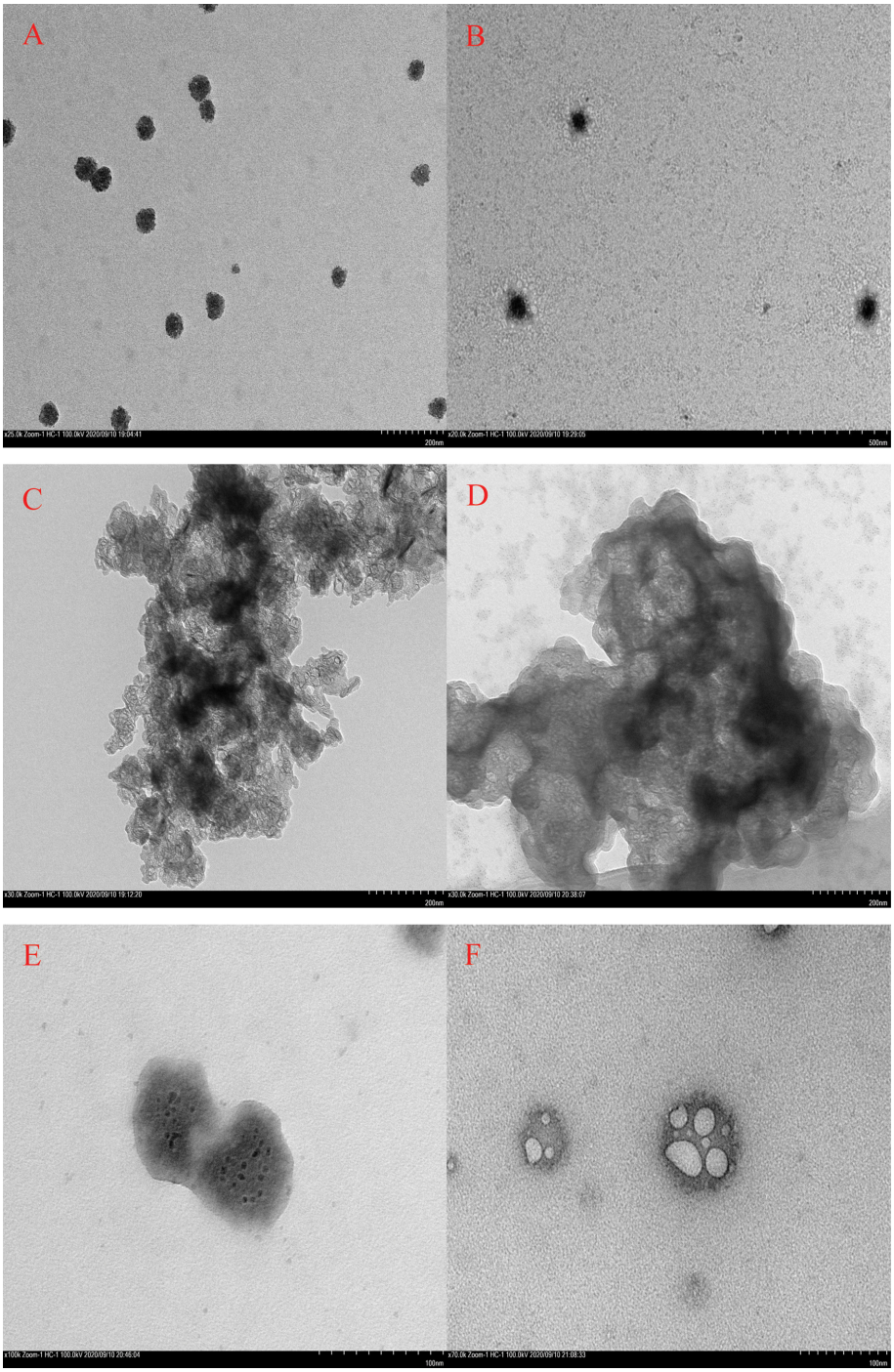

Fig. 3. Transmission electron micrographs (A) GDH, (B) CBR, (C) GDH-PAA, (D) CBR-PAA, (E) 2-P, (F) 2-S.

GDH-PAA (Fig. 3C) and CBR-PAA (Fig. 3D), similar to reported single enzyme-PAA conjugates [19,40], whereas the native enzyme shows discrete aggregated particles (Fig. 3A and 3B). Due to the polyvalence of the PAA as well as the proteins, chemical crosslinking of these units can result in conjugates in a distribution of sizes. The distribution and average size of the cross-linked conjugates depend on the polymer concentration, protein concentration, EDC concentration, and reaction time. The reaction conditions were carefully optimized to obtain nano-BECs of 50-70 nm (Fig. 3E and 3F). It can be observed in Fig. 3 that the appearance of 2-P and 2-S was almost spherical. In addition, obvious pore-like structures can be observed on the surface of their spherical structure. This illustrates that the two types of nano-BECs are similar in size and structure, but the complete diameter of 2-S is slightly larger. This difference may have been caused by the different preparation methods.

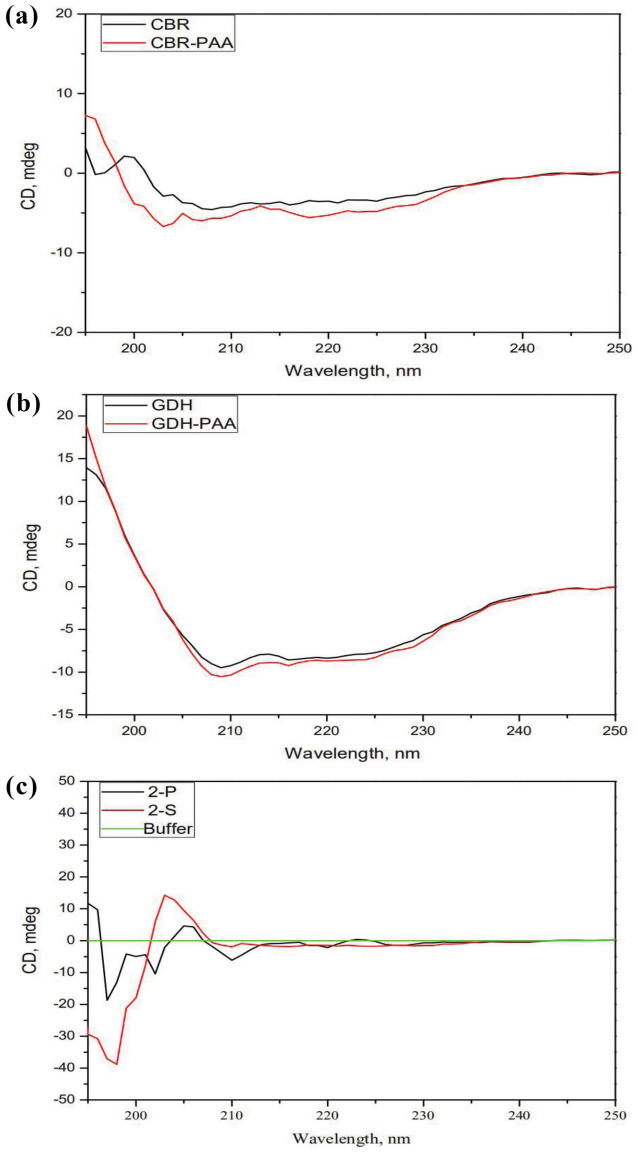

Fig. 4. Circular dichroism (CD) spectra of (a) CBR (black), CBR-PAA (red), (b) GDH (black), GDH-PAA (red) and (c) 2-S (red), 2-P (black), buffer (blue).

1-3. CD Studies

Retention of the secondary structure of BECs is important for biocatalytic activity, which is established using far-UV CD studies to compare the CD spectra of free enzymes and enzyme-PAA conjugates (Fig. 4). CD spectra were monitored from to 190-250 nm in pH 7.4 PB at room temperature (25 °C). For GDH-PAA, the spectra are reasonably similar in intensity as well as peak positions, while CBR-PAA is dissimilar. Thus, some structural changes occur upon conjugation with the polymer. The secondary structure of each enzyme within 2-S and 2-P could not be compared with the single enzyme conjugates or native enzymes because of the difficulty in resolving and deconvoluting the spectra of each enzyme in the BECs. It can be observed that the spectral shapes of 2-S and 2-P are slightly similar to the single enzyme-PAA conjugates or free enzymes, which may indicate at least partial retention of secondary structure. In the absence of concrete structural retention data from UV and CD studies, the enzyme catalytic ability of 2-S and 2-P was investigated and compared with single enzyme-PAA conjugates, and is an indirect form of structural retention investigation.

2. Study of the Asymmetric Reduction Ability of Nano-BECs

2-S or 2-P Nano-BECs were applied in the asymmetric reduction of OPBE in a single aqueous phase and a biphasic system. By comparing the transformation results in the two systems after exploring the optimal conditions of biotransformation, the optimal system was obtained.

2-1. Reduction Reaction Catalyzed by Nano-BECs in the Single Aqueous Phase

2-1-1. Influence of Substrate Concentration on Reduction in the Aqueous Phase

In this experiment, the influence of the concentration of OPBE (388-630 mM) on the reduction was investigated in 10 mM PB (pH 7.4). Fig. 5(a) shows that a high concentration of OPBE may cause changes in the molecular structure of the enzyme and inhibit the full play of enzyme activity, but too low an OPBE concentration reduces the reaction efficiency. When the concentration of OPBE increased from 388 mM to 485 mM, all the substrates could still be completely converted to HPBE. When the concentration was higher than 485 mM, the conversion clearly showed a downward trend. The conversion reached 94.17% and 87.08% by 2-S and 2-P with 533 mM of OPBE, respectively. The e.e. of R-HPBE decreased slightly with 2-P as a catalyst when the OPBE concentration exceeded 485 mM, while the e.e. of R-HPBE remained at the highest value (99.9%) with 2-S as the catalyst. The different tolerance to substrate concentration may be due to differences in the structure and preparation process of the two types of nano-BECs. It is obvious that the most suitable OPBE concentration is 485 mM for both 2-S and 2-P.

2-1-2. Influence of Shaker Speed on Reduction in the Aqueous Phase

Shaker speed affects the diffusion and distribution of the substrate and product in the reaction system. This ultimately affects the molar conversion and configuration of the product. Fig. 5(b) shows that the conversion increases with an increase in shaker speed, when the shaker speed is less than 180 rpm. The rapid increase in molar conversion indicates that mass transfer is the main

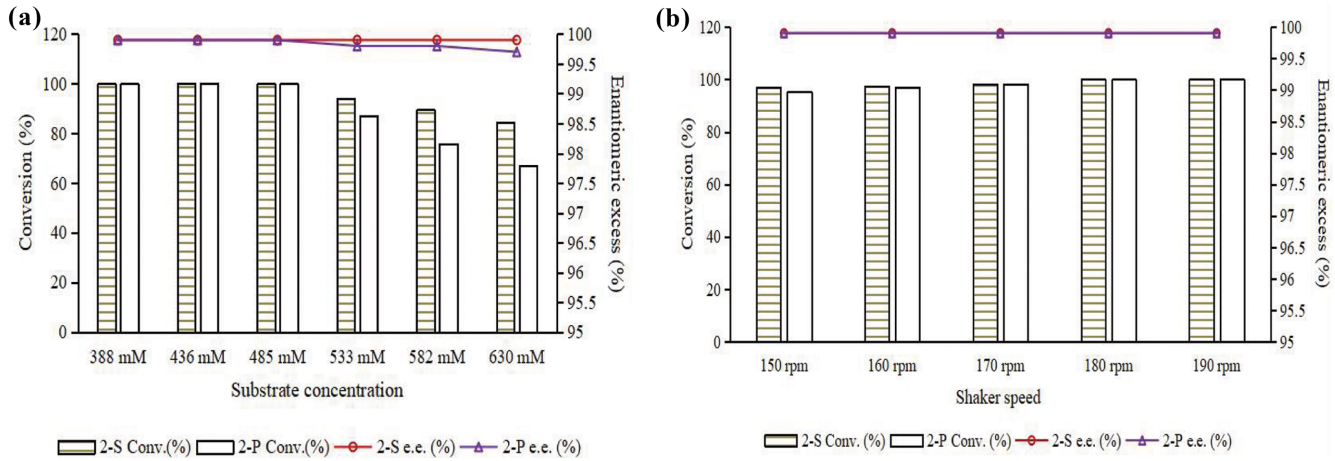

Fig. 5. (a) The influence of different substrate concentration on reduction reaction in single aqueous phase. Studies carried out at 180 rpm, 35 °C for 40 h. (b) The influence of different shaker speed on reduction reaction in single aqueous phase. Studies carried out at 35 °C for 40 h with substrate concentration of 485 mM OPBE.

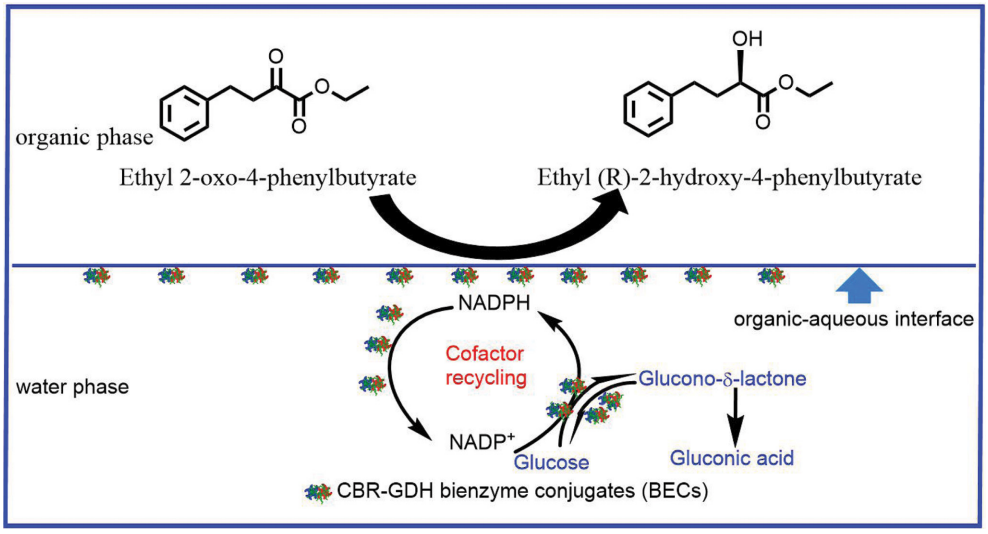

Fig. 6. Reaction mechanism of asymmetric reduction of ethyl 2-oxo-4-phenylbutyrate (OPBE) by nano-BECs in the organic-aqueous biphasic system.

determinant of the reaction in this case. If the shaker speed is further increased above 180 rpm, the conversion and the e.e. of R-HPBE will remain unchanged. Therefore, 180 rpm is considered to be the most appropriate shaking speed for nano-BECs (2-P and 2-S).

2-2. Reduction Reaction Catalyzed by Nano-BECs in the Organic-aqueous System

Targeting the low solubility of the substrate and the toxic effects of the organic substrate and product on the activity of the enzyme in a single aqueous phase, this study aimed to construct an organic-aqueous biphasic reaction system to overcome these problems. The reaction mechanism of the asymmetric reduction of OPBE by nano-BECs in the organic-aqueous biphasic system is presented in Fig. 6.

2-2-1. Influence of Organic Solvent Type on Reduction in the Organic-aqueous System

The selection of an appropriate organic solvent *in situ* extraction agent is highly important for an organic-aqueous biphasic system. This study explored the biocatalytic effect in a biphasic system composed of different organic solvents and PB, and the results are shown in Fig. 7(a). The logP (hydrophobic constant) of an organic solvent is an important constant for evaluating its influence on enzyme catalysts, being the logarithmic value of the solvent distribution constant in the n-butanol/water system. It was found that when dibutyl phthalate (1ogP>5) was used as the organic phase, the molar conversion and the e.e. of the R-HPBE were higher. In the biocatalysis of a biphasic system, alkanes (n-hexane, n-heptane) with logP values of 3.5 and 4, respectively, have higher conversion as organic phases than aromatic hydrocarbons (benzene, toluene) with logP values of 2 and 2.5. The results show that as the number of carbon atoms in the solvent increases, the conversion is higher for the same type of organic solvent. The reason why organic solvents (n-hexane, benzene, toluene, n-heptane) are not highly converted when used as the organic phase may be that they lose most

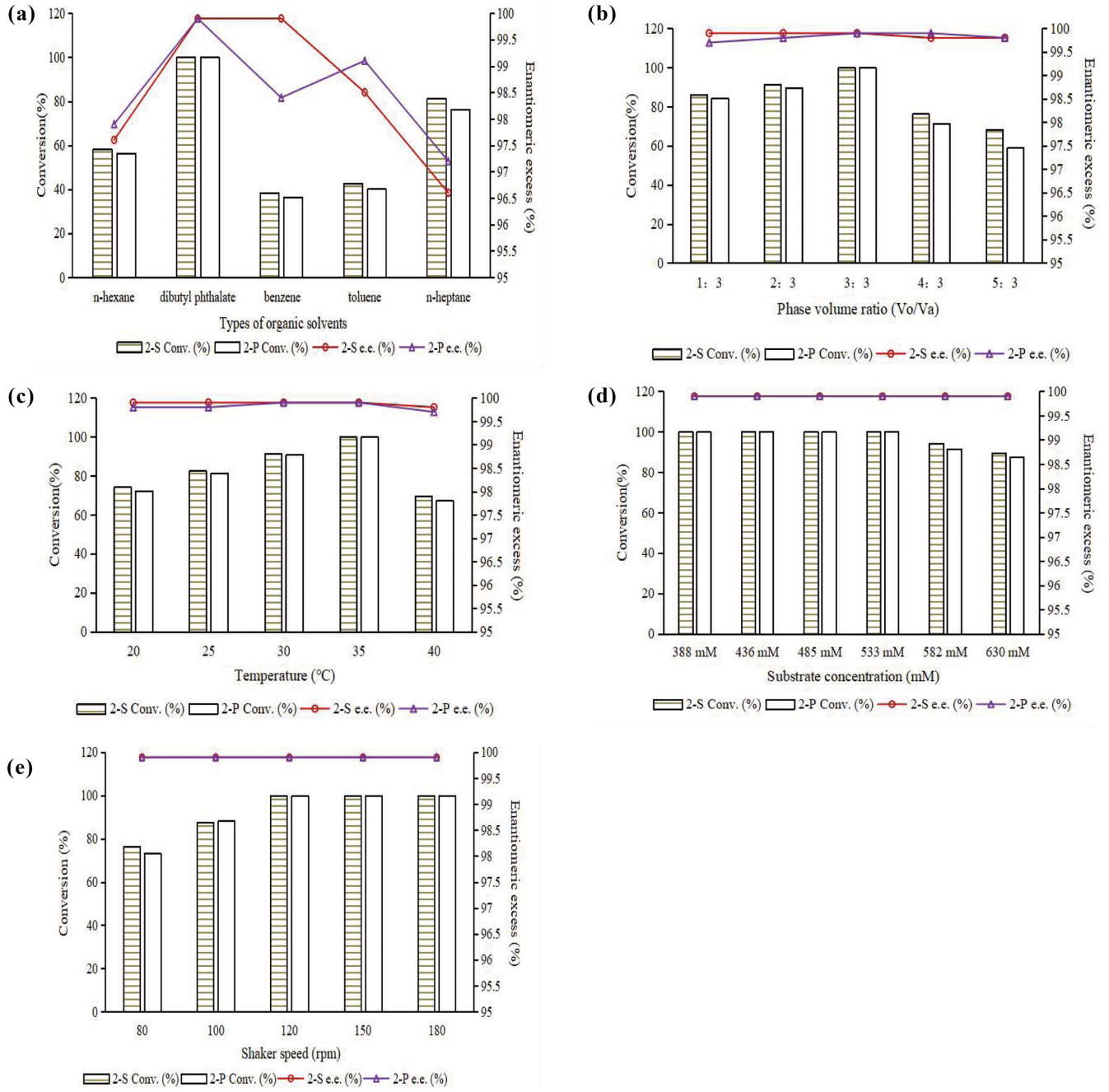

Fig. 7. (a) The influence of different organic solvents on reduction reaction in organic-aqueous biphasic reaction system. (b) The influence of phase volume ratio of dibutyl phthalate and PB on reduction in dibutyl phthalate-PB biphasic reaction system. (c) The influence of different temperature on reduction reaction in dibutyl phthalate-PB (Vo/Va 1 : 1) biphasic reaction system. (d) The influence of different substrate concentration on reduction reaction in dibutyl phthalate-PB (Vo/Va 1 : 1) biphasic reaction system. (e) The influence of different shaker speed on reduction reaction in dibutyl phthalate-PB (Vo/Va 1 : 1) biphasic reaction system.

of the enzyme activity, affecting the catalytic performance of the enzyme. It was found that when dibutyl phthalate was used as the organic phase, the molar conversion and the e.e. of R-HPBE were the highest. This may be attributed to the fact that dibutyl phthalate not only has good biocompatibility but also has suitable substrate and product distribution. According to the experimental results, dibutyl phthalate, which has a good biocompatibility and extraction performance, was selected as the optimal organic phase to carry out the following research.

2-2-2. Influence of Phase Volume Ratio on Reduction in the Organic-aqueous System

Usually, the volume ratio of organic solvent and aqueous solution (Vo/Va) has a greater impact on enzyme-catalyzed reactions. Excessive ratios will cause serious enzyme inactivation. However, when it is too low, the aim of increasing the substrate treatment capacity and reducing the inhibition of substrate and product on

enzyme-catalyzed reactions will not be achieved. This study investigated the influence of the volume ratio of organic solvent (0-62.5%) on the molar conversion and e.e. of R-HPBE in the dibutyl phthalate-PB biphasic reaction system. It can be seen from Fig. 7(b) that the phase volume ratio has a significant effect on the substrate treatment capacity, but has little effect on the e.e. of R-HPBE. With the increase of Vo/Va within a certain range (≤1), the molar conversion showed a slow increasing trend and the conversion was at a relatively high level. The conversion decreased significantly when the proportion of organic solvents continued to increase. Therefore, a 50% organic solvent ratio (Vo/Va of 1 : 1) was selected as the optimal volume ratio for further study.

2-2-3. Influence of Temperature on Reduction in the Organic-aqueous System

In the enzymatic reaction, temperature has varying degrees of influence on the activity and stereoselectivity of the biocatalyst. At the optimal reaction temperature, the biocatalyst exhibits high catalytic activity. This experiment investigated the changes in the conversion of OPBE and the e.e. of R-HPBE within the range of 20-40 °C. Fig. 7(c) shows that the conversion increased significantly to a stable maximum (100%) when the temperature increased from 20 °C to 35 °C. As the temperature further increased, the conversion began to decrease. This rule that the conversion is affected by temperature is applicable to reduction reactions catalyzed by both 2-S and 2-P. This may be due to the destruction of the spatial structure of the enzyme because of the excessively high temperature. The decrease in enzyme activity results in a decrease in the conversion. Moreover, the e.e. of R-HPBE does not change significantly with the change in temperature. Fig. 7(c) shows that when the reaction temperature was 35 °C, the molar conversion and e.e. of R-HPBE reached 100% and 99.9%, respectively. Therefore, 35 °C is regarded as the optimum temperature for reduction with 2-S or 2-P as the catalyst.

2-2-4. Influence of OPBE Concentration on Reduction in the Organic-aqueous System

Substrate concentration affects the catalytic efficiency of the co-immobilized bienzymes at the interface between the organic phase and aqueous phase. Without the use of substrate feeding, this study investigated the influence of initial substrate concentrations (388-630 mM) on the asymmetric reduction in the biphasic system with 2-S and 2-P as the biocatalyst. Fig. 7(d) shows that the conversion remained stable at the highest value (100%) when the substrate concentration increased from 388 mM to 533 mM. However, there was a sharp downward trend in conversion when the substrate concentration was further increased. It is probable that a high substrate concentration (>533 mM) leads to an excessive accumulation of substrate in the aqueous phase, which in turn inhibits the reaction. It was found that the e.e. of R-HPBE was minimally affected by the substrate concentration. Therefore, the most suitable substrate concentration is 533 mM for the reduction catalyzed by 2-P and 2-S in the dibutyl phthalate-PB (1 : 1) biphasic system.

2-2-5. Influence of Shaker Speed on Reduction in the Organic-aqueous System

An appropriate rotating speed of the shaker can cause the substrate and the product to more fully dissolve in the dibutyl phthalate and increase the material transfer efficiency. In this experiment, the influence of shaker speed in the range of 80-180 rpm on the conversion and e.e. of R-HPBE was explored when other conditions remained unchanged (Fig. 7(e)). In the range of 80-120 rpm, the conversion continued to increase until it reached the maximum (100%) and then remained stable with further increases in shaker speed. It is probable that the contact between the nano-BECs (2-S and 2-P) accumulated at the oil-water interface and the organic substrate was insufficient when the shaker speed was lower than 120 rpm. The optimal shaker speed was 120 rpm using 2-P or 2-S as the catalyst. 2-P or 2-S can achieve maximum catalytic activity in the reaction system at 120 rpm.

3. Comparison of Reduction between the Organic-aqueous Biphasic System and the Single Aqueous Phase

The optimal reduction conditions obtained in the organic-aqueous system were compared with those obtained in the single aqueous phase. The conversion and e.e. of R-HPBE obtained in the organic-aqueous system and the aqueous phase system were constructed as comparison parameters. The results are shown in Table 1. OPBE (533 mM) could be converted completely when the shaker speed was only 120 rpm in the organic-aqueous biphasic system with nano-BECs (2-S or 2-P) as the catalyst. However, 485 mM could be converted completely when the shaker speed was 180 rpm in a single aqueous phase with nano-BECs (2-S or 2-P) as the catalyst. The catalytic performance of 2-S and 2-P was compared in a systematic investigation of the influence of factors such as organic solvent type, phase volume ratio, reaction temperature, substrate concentration, and shaking speed on the molar conversion and optical purity of the product. Under optimal reaction conditions, both 2-S and 2-P showed excellent catalytic performance, but under the same conditions (except for the optimal conditions), the conversion of the reduction reaction catalyzed by 2-S was always slightly higher than for 2-P. These trends in catalysis may be due to the microenvironment in nano-BECs produced due to the synthetic strategy and may allow the substrate better access to active sites in 2-S compared to 2-P, leading to enhanced activity in 2-S. In the interface catalysis of organic-aqueous solutions, the three-dimensional

Table 1. Comparison of reduction reaction process between organic-aqueous biphasic system and single aqueous phase

	Catalyst	Substrate concentration (mM)	Shaker speed (rpm)	Conversion (%)	Optical purity (% e.e.)
Organic-aqueous biphasic system	2-S	533	120	100	>99.9
	2-P	533	120	100	>99.9
Single aqueous phase	2-S	485	180	100	>99.9
	2-P	485	180	100	>99.9

structure of the enzyme molecule is different from that in the aqueous phase. Therefore, the catalytic activity of the enzyme in the organic-aqueous biphasic system is usually lower than that in the aqueous phase. At the same time, the contact of organic solvents and substrates with enzyme molecules will also cause changes in the structure of the enzyme molecules, inhibiting full enzyme activity. In this study, the enzyme protein molecular modification method, in which PAA is the backbone to immobilize CBR and GDH to form nano-BECs simultaneously, was used to improve the catalytic activity of the enzyme in the organic-aqueous biphasic system. It can be seen from Table 1 that dibutyl phthalate-PB as the reaction medium for the two-phase system increases the utilization rate of the substrate OPBE and increases its conversion.

4. Study on the Coenzyme Regeneration Ability of Nano-BECs

CBR, GDH, CBR-PAA, GDH-PAA, unconjugated enzymes CBR/GDH and nano-BECs (2-S and 2-P) were used as catalysts in the dibutyl phthalate-PB (1 : 1) biphasic system to investigate coenzyme regeneration ability. R-HPBE could not be detected in the reaction system with GDH or GDH-PAA as the catalyst. In addition, the amount of R-HPBE (349 mM) obtained in the reaction with CBR-PAA as the catalyst was significantly lower than the amount of R-HPBE (533 mM) obtained in the reaction with bienzyme-PAA conjugate (2-S or 2-P) as the catalyst. The co-immobilization of CBR and GDH on PAA resulted in a 34.52% increase in the overall conversion compared with CBR-PAA (Table 2). Combined with the analysis of the reaction mechanism (Fig. 6), the nano-BECs (2-S or 2-P) have coenzyme regeneration ability, which helps to increase the conversion. The amount of the product R-HPBE with single enzyme CBR as the catalyst was 391 mM in a dibutyl phthalate-PB biphasic system, while the amount of R-HPBE was 349 mM using immobilized single enzyme CBR-PAA as the catalyst, indicating that the enzyme activity was lost during the conjugation process. From the experimental results (Table 2), it can be seen that whether the two free enzymes or their conjugated enzymes (2-S or 2-P) are used to catalyze the reduction reaction, the substrate can be completely converted. Therefore, it can be deduced that the effect of co-immobilization of dual enzymes to increase the local reaction concentration may eliminate the effect of enzyme activity loss caused by the change of enzyme structure during the immobilization process.

5. Comparison of Immobilized Enzyme Nano-BECs and Free Enzyme

In the multi-enzyme system, the effective contact between the catalytic enzyme CBR, the coenzyme regenerating enzyme GDH, the coenzyme NADPH and the substrate OPBE is an important factor that determines the effective application of the extracellular multi-enzyme catalytic system. Due to the proximity of the two enzymes, the immobilized enzyme nano-BECs limit the diffusion of intermediates. Multi-step reactions are completed quickly in the same reactor, which can increase the local concentration of the reaction and improve the catalytic efficiency. The pH of the reaction medium is a key factor affecting enzyme activity. Different pH can significantly affect the dissociation state of key amino acids in the active center of the enzyme, which in turn affects the catalytic activity of the enzyme. Enzyme thermal stability is another property that plays an important role in the catalytic function of enzymes. Through the research on the stability of immobilized enzyme and free enzyme, it is found that co-immobilization technology not only maintain the better conversion of the enzyme but also improves the stability including pH and temperature (Table 3).

6. Reuse of Nano-BECs

In the process of using the enzyme coupling system to catalyze the reaction, the repeated utilization of the catalyst can increase the final yield of the reaction. Based on the yield of the first batch of free enzyme coupling reaction, the relative yield of each batch of reaction catalyzed by immobilized enzyme is shown in Fig. 8. For

Table 2. Validation experiment results of coenzyme regeneration ability of nano-BECs

	Catalyst	Initial substrate concentration (mM)	R-HPBE concentration (mM)	Conversion (%)	Optical purity (% e.e.)
Single-enzyme	GDH	533	0	0	0
	CBR	533	391	73.36	>99.9 (R)
Unconjugated enzymes	CBR and GDH	533	533	100	>99.9 (R)
Single enzyme-PAA	GDH-PAA	533	0	0	0
	CBR-PAA	533	349	65.48	>99.9 (R)
Nano-BECs	2-S	533	533	100	>99.9 (R)
	2-P	533	533	100	>99.9 (R)

Table 3. Comparison of immobilized enzyme nano-BECs and free enzyme

		Stable range of pH	Optimal pH	Stable range of temperature (°C)	Optimal temperature (°C)	Km (mM)	Optimal shaker speed (rpm)
Single enzyme	CBR	6-8	7	30-40	30	0.6	100
	GDH	5.5-8	7	30-55	30	/	100
Unconjugated enzymes	CBR/GDH	6-8	7	30-40	30	0.6	100
Nano-BECs	2-S	5.5-8.5	7.4	30-45	35	1.6	120
	2-P	5.5-8.5	7.4	30-45	35	1.7	120

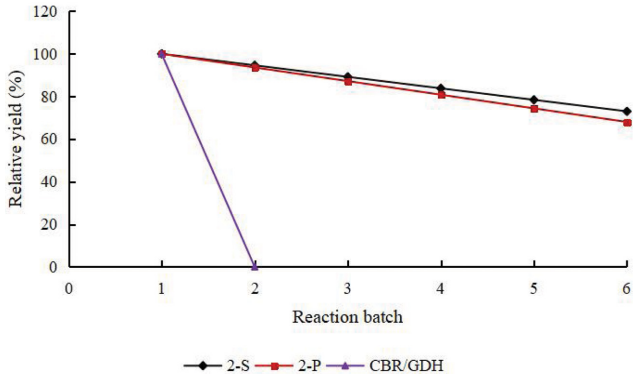

Fig. 8. Relative yield of multi-batch reaction of free and immobilized CBR/GDH coupling systems.

the two nano-BECs, the catalytic performance and reuse of 2-S are slightly better than 2-P. It can be seen from Fig. 8 that the nano-BECs coupled catalysis can maintain a high conversion in several batches of reactions, and the relative yield in the sixth batch is still close to 70%. In multi-batch reactions, the cumulative conversion of immobilized enzymes is much higher than that of free enzymes, so this immobilization technology is conducive to the realization of high-efficiency multi-batch catalytic reactions in a multi-enzyme catalytic system.

CONCLUSIONS

In this study, PAA was used as the backbone to immobilize CBR and GDH to form nanopore-bienzyme conjugates (nano-BECs) using two distinct strategies. The nano-BECs (2-S and 2-P) were used for the asymmetric conversion of OPBE in an organic-aqueous biphasic system to prepare R-HPBE. The method can realize the *in situ* regeneration of a coenzyme and increase conversion, with good prospects for application in the field of chiral alcohol preparation by asymmetric reduction of carbonyl compounds.

ACKNOWLEDGEMENTS

We thank the National Nature Science Foundation of China (21978267) and the Natural Science Foundation of Zhejiang Province (LY15B060005) for the financial support.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

HUMAN AND ANIMAL RIGHTS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

REFERENCES

1. Z. Q. Zhao, H. L. Wang, Y. P. Zhang, L. F. Chen, K. Wu and D. Z. Wei, *Biotechnol. Lett.*, **38**, 1799 (2016).
2. J. Deng, Z. Q. Yao, K. L. Chen, Y. A. Yuan, J. P. Lina and D. Z. Wei, *J. Biotechnol.*, **217**, 31 (2016).
3. K. Javidnia, E. Faghih-Mirzaei, R. Miri, M. Attarroshan and K. Zomorodian, *Indian J. Pharm. Sci.*, **78**, 73 (2016).
4. H. L. Zhang, C. Zhang, C. H. Pei, M. N. Han and W. Li, *J. Appl. Microbiol.*, **126**, 127 (2019).
5. M. Hibi, K. Takahashi, J. Kako, Y. Wakita, T. Kodera, S. Shimizu, K. Yokozeki and J. Ogawa, *Bioorg. Med. Chem.*, **26**, 1327 (2018).
6. G. C. Xu, Y. P. Zhang, Y. Wang and Y. Ni, *Bioresour. Technol.*, **247**, 553 (2018).
7. Q. Xu, W. Y. Tao, H. Huang and S. Li, *Biochem. Eng. J.*, **106**, 61 (2016).
8. K. Chen, X. Y. Huang, S. B. J. Kan, R. K. Zhang and F. H. Arnold, *Science*, **360**, 71 (2018).
9. Y. G. Zheng, H. H. Yin, D. F. Yu, X. Chen, X. L. Tang, X. J. Zhang, Y. P. Xue, Y. J. Wang and Z. Q. Liu, *Appl. Microbiol. Biot.*, **101**, 987 (2017).
10. S. Oksuz, E. Şahin and E. Dertli, *Chem. Biodivers.*, **15**, e1800028 (2018).
11. K. Honda, M. Inoue, T. Ono, K. Okano, Y. Dekishima and H. Kawabata, *J. Biosci. Bioeng.*, **123**, 673 (2017).
12. L. H. Zheng, X. Y. Zhang, Y. P. Bai and J. H. Fan, *Algal. Res.*, **35**, 432 (2018).
13. S. Shah, R. Agera, P. Sharma, A. V. Sunder, H. Bajwa, H. M. James, R. P. Gaikaiwari and P. P. Wangikar, *Process Biochem.*, **70**, 71 (2018).
14. X. Chen, Z. Q. Liu, C. P. Lin and Y. G. Zheng, *Bmc. Biotechnol.*, **16**, 70 (2016).
15. Z. Q. Liu, L. M. Zhou, P. Liu, P. J. Baker, S. S. Liu, Y. P. Xue, M. Xu and Y. G. Zheng, *Appl. Microbiol. Biot.*, **99**, 8891 (2015).
16. A. Scomparin, H. F. Florindo, G. Tiram, E. L. Ferhuson and R. Satchi-Fainaro, *Adv. Drug Deliv. Rev.*, **118**, 52 (2017).
17. Z. Y. Sun, U. Glebe, H. Charan, A. Boker and C. Z. Wu, *Angew. Chem. Int. Edit.*, **57**, 13810 (2018).
18. X. L. Wu, J. Ge, J. Y. Zhu, Y. F. Zhang, Y. Yong and Z. Liu, *Chem. Commun.*, **51**, 9674 (2015).
19. O. V. Zore, P. Pande, O. Okifo, A. K. Basu, R. M. Kasi and C. V. Kumar, *RSC Adv.*, **7**, 29563 (2017).
20. X. Huang, M. Li, D. C. Green, D. S. Williams, A. J. Patil and S. Mann, *Nat. Commun.*, **4**, 1 (2013).
21. G. Premaratne, L. Coats and S. Krishnan, *Method. Enzymol.*, **590**, 225 (2017).
22. P. van Rijn, *Polymers*, **5**, 576 (2013).
23. N. Suthiwangcharoen and R. Nagarajan, *Biomacromolecules*, **15**, 1142 (2014).
24. P. Bosiger, G. Tegl, I. M. T. Richard, L. Le Gat, L. Huber, V. Stagl, A. Mensah, G. M. Guebitz, R. M. Rossi and G. Fortunato, *Carbohydr. Polym.*, **181**, 551 (2018).
25. Y. Zhao, Y. Wang, X. B. Zhang, R. M. Kong, L. Xia and F. L. Qu, *Talanta*, **155**, 265 (2016).
26. S. D. Gur, N. Idil and N. Aksoz, *Appl. Biochem. Biotechnol.*, **184**, 538 (2018).
27. N. S. Rios, C. Mendez-Sanchez, S. Arana-Pea, N. Rueda, C. Ortiz, L. R. B. Goncalves and R. Fernandez-Lafuente, *Biochim. Biophys. Acta.*, **1867**, 741 (2019).
28. Y. Ido, A. L. B. Macon, M. Iguchi, Y. Ozeki, S. Koeda, A. Obata, T. Kasuga and T. Mizuno, *Polymer*, **132**, 342 (2017).

29. D. Skoulas, V. Stuettgen, R. Gaul, S. A. Cryan, D. J. Brayden and A. Heise, *Biomacromolecules*, **21**, 2455 (2020).
30. C. M. Riccardi, D. Mistri, O. Hart, M. Anuganti, Y. Lin, R. M. Kasi and C. V. Kumar, *Chem. Commun*., **52**, 2593 (2016).
31. B. Ozbek and A. Unal, *Korean J. Chem. Eng*., **34**, 1992 (2017).
32. V. K. Mudhivarthi, K. S. Cole, M. J. Novak, W. Kipphut, I. K. Deshapriya, Y. X. Zhou, R. M. Kasi and C. V. Kumar, *J. Mater. Chem*., **22**, 20423 (2012).
33. R. Chen, J. Deng, J. P. Lin, X. P. Yin, T. Xie, S. L. Yang and D. Z. Wei, *Biotechnol. Appl. Bioc*., **63**, 465 (2016).
34. X. L. Qian, J. Pan, N. D. Shen, X. Ju, J. Zhang and J. H. Xu, *Biochem. Eng. J*., **91**, 72 (2014).
35. J. Deng, Z. Q. Yao, K. L. Chen, Y. A. Yuan, J. P. Lin and D. Z. Wei, *J. Biotechnol*., **217**, 31 (2016).
36. Z. G. Wang, S. Zhou, S. L. Zhang, S. Zhang, F. M. Zhu, X. L. Jin, Z. M. Chen and X. L. Xu, *Sci. Rep*., **7**, 4007 (2017).
37. S. H. Huang and S. W. Tsai, *J. Mol. Catal. B: Enzym*., **28**, 65 (2004).
38. P. D'Arrigo, G. Pedrocchi-Fantoni and S. Servi, *Tetrahedron: Asymmetry*, **21**, 914 (2010).
39. Y. X. Yang, Y. G. Shi, L. F. Feng and S. Y. Tian, *Molecules*, **25**, 2056 (2020).
40. H. A. Klok, *J. Polym. Sci. Part A: Polym. Chem*., **43**, 1 (2005).