Supporting Information

Site-specific Covalent Immobilization of Lipase on Natural Polyphenol Modified Magnetic Nanoparticles for Effective Biodiesel Production

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Supporting information contents:

Number of pages: 15
Number of Figures: 10
Number of Tables: 1
Experiments:

BCL random immobilization procedure

For preparation of BCL random immobilization through covalent bond formation between amine groups on commercial Fe$_3$O$_4$-NH$_2$ nanoparticles (Xi'an Ruixi Biological Technology Co., Ltd) and carboxyl groups (-COOH) on BCL. Briefly, a certain amount of BCL was added in 9.0 mL of phosphate-buffered solution (pH 7.0, 0.05 M) containing 5 mg of N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide (EDC) and 6.0 mg of N-hydroxysuccinimide (NHS) were stirring for 1 h to active carboxyl of BCL. Then, a suitable amount of commercial Fe$_3$O$_4$-NH$_2$ nanoparticles was added to the BCL mixed solution for stirring at 4 ℃ for another 10 h. After that, the product was carefully collected by magnetic separation thoroughly and rinsed with fresh buffer to remove unbound BCL.

For preparation of BCL random immobilization through covalent bond formation between blank Fe$_3$O$_4$ nanoparticles and hydroxyl groups (-OH) on the BCL. Briefly, carbonyldiimidazole (CDI) as a chemical crosslinking agent to reveal the hydroxyl groups (-OH) of BCL conjugated on the surface of Fe$_3$O$_4$ nanoparticles according to previous study$^1$. The condition of immobilization was the same as BCL site-specific immobilization procedure.

Characterization

The TEM images of Fe$_3$O$_4$, Fe$_3$O$_4$-EGCG NPs and Fe$_3$O$_4$-EGCG-BCL were obtained
using JEM-1400 (JEOL, Japan) transmission electron microscope (TEM) at an accelerating voltage of 120 kV. The Fourier Transform infrared spectroscopy (FTIR spectra) were recorded on an IFS 55 FT-IR spectrometer (Bruker, Switzerland) with a resolution of 2 cm⁻¹ for each spectrum to detect the chemical structures of samples. Thermogravimetric analysis (TGA) was performed by a TGA-50 Thermogravimetric analyzer (Shimadzu, Japan) in the temperature range of 30-800°C at heating rate of 10°C/min in a N₂ atmosphere. The magnetic properties of synthesized nanoparticles were analyzed on a PPMS DynaCool magnetometer (Quantum Design, CA). X-ray powder diffraction (XRD) of as-synthesized samples was measured by RINT2000 vertical goniometer (Rigaku, Japan) with Cu Kα irradiation. The surface composition of Fe₃O₄-EGCG NPs was identified by X-ray photoelectron spectrometry (XPS) on an ESCALAB 250 X-ray photoelectron spectrometer (Thermo Electron Corporation, USA) with monochromatized Al Kα X-ray source (1486.6 eV photons). CD spectra measurements were recorded through a Chirascan-plus system (Applied Photophysics, England) in the wavelength rang of 190-260 nm.

**Leakage experiment**

In order to evaluate the stability of Fe₃O₄-EGCG-BCL, the leakage experiment was also conducted. Specifically, Fe₃O₄-EGCG-BCL was dispersed in PBS solution at 200 rpm, the leaked enzyme concentration in reaction mixture was periodically detected by Bradford’s method.
**Figure S1.** The distribution of residues (in orange) with hydroxyl (-OH) groups on BCL after 180° rotation of Figure 1B.
Figure S2. The distribution of residues (in cyan) with carboxyl (-COOH) groups on BCL after 180° rotation of Figure 1C.
**Figure S3.** The CLSM image of Fe₃O₄-EGCG-BCL nanoparticles, and the BCL protein were labeled by FITC. Scale bar: 50 μm.
Figure S4. FTIR partial enlarged detail of Fe$_3$O$_4$ NPs and Fe$_3$O$_4$-EGCG NPs.
Figure S5. High resolution XPS spectra of the O 1s peak for Fe$_3$O$_4$-EGCG NPs.
As shown in Figure S6, the solvent-accessible surface (SAS) area was used to describe the medium exposition degree of seven Lys and N-terminal Ala residues as immobilization sites. Among them, Lys22 (K22), Lys70 (K70), Lys165 (K165), Lys283 (K283) and Ala1 (A1) have better medium exposition degree than other Lys residues, allowing a greater effective contact with Fe$_3$O$_4$-EGCG NPs through Schiff-base/Michael addition reaction. This result indicated that Lys and N-terminal Ala residues as immobilization sites could effectively reduce the excessive BCL conformation distortion caused by the formation of excess covalent bonds during immobilization, leading to proper balance between the rigidity and flexibility of BCL molecules.
Figure S7. The effect of molar ratio of oil to methanol on biodiesel yield.

Methanol plays critical roles on the transesterification reaction. Specifically, methanol tends to push the reaction process in the direction of transesterification, but excessive methanol is harmful to proteins. Therefore, the amounts of methanol added to the reaction mixture was optimized first. Figure S7 showed that as the molar ratio increased from 1:1 to 1:4, the biodiesel yield gradually increased caused by methanol driven. When the molar ratio was further increased, the biodiesel yield began to decrease obviously. This result might be explained by the reason that the excessive amount of methanol removes the essential water layer on lipase surface and consequently deactivates the biocatalyst. Thus, the optimal molar ratio of oil to methanol was set at 1:4.
The effect of methanol addition steps on biodiesel yield. The methanol was added in one step (Method I), in two steps at an interval of 5 h (Method II), in three steps at intervals of 4 h (Method III), in four steps at intervals of 3 h (Method IV) and in five steps at intervals of 2 h (Method V).

The effects of methanol addition method on biodiesel yield was shown in Figure S8. The methanol was added in one step (Method I), in two steps at an interval of 5 h (Method II), in three steps at intervals of 4 h (Method III), in four steps at intervals of 3 h (Method IV) and in five steps at intervals of 2 h (Method V). The results revealed that the biodiesel yield increased along with the increase of methanol addition steps. However, there was no obvious difference between the Method II, IV and V, indicating that the methanol concentration in the reaction medium was not harmful on BCL. Therefore, the three addition steps method (Method III) was selected as the optimal approach for biodiesel production.
Water plays an important role in transesterification reactions and has a great influence on the stability and activity of lipase. A certain amount of water is usually required to maintain the catalytic activity and native conformation of lipase. Figure S9 revealed that with the water content increased, biodiesel yield was increased gradually. However, when water content beyond 3 wt.%, the biodiesel yield decreased continuously. This result could be explained by the fact that less water is necessary to activate the lipase, while excessive water content may increase the flexibility of lipase and then leading to side reactions such as hydrolysis reaction. Thus, the optimal water content was 3 wt.%. 

Figure S9. The effect of water content on biodiesel yield.
Temperature is an important factor to be considered in the biodiesel preparation. As depicted in Figure S10, a rise in the biodiesel yield catalyzed by immobilized BCL was observed when the reaction temperature is gradually increased from 30 to 40 °C. However, higher reaction temperatures may inactivate the immobilized BCL. Thus, when temperature further rised to 55 °C, biodiesel yield decreased. Thus, the optimum reaction temperature for immobilized BCL-medicated transesterification reaction was found to be 40 °C.
**Table S1** The immobilization efficiency of supports and activity of immobilized BCL by different immobilization strategy.

<table>
<thead>
<tr>
<th>Immobilization Strategy</th>
<th>Added enzyme (mg/g)</th>
<th>Bound enzyme (mg/g)</th>
<th>Activity recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site-specific immobilization</td>
<td>250</td>
<td>154.5</td>
<td>71.3</td>
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<td>(through -NH₂ groups)</td>
<td></td>
<td></td>
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<tr>
<td>Random immobilization</td>
<td>250</td>
<td>169.3</td>
<td>42.5</td>
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<tr>
<td>(through -OH groups)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Random immobilization</td>
<td>250</td>
<td>164.8</td>
<td>55.9</td>
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<td>(through -COOH groups)</td>
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References