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Facile fabrication of recyclable and active nanobiocatalyst: purification and immobilization of enzyme in one pot with Ni-NTA functionalized magnetic nanoparticle

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Materials: Ferric chloride hexahydrate (FeCl₃·6H₂O, 97%), ferrous chloride tetrahydrate (FeCl₂·4H₂O, 99%), potassium oleate (40 wt. % in H₂O), ammonium hydroxide (~28% NH₃ in H₂O), ammonium persulfate ((NH₄)₂S₂O₈, \geq 98.0%), glycidyl methacrylate (97%), ethylenediamine (NH₂CH₂CH₂NH₂, 99%), glutaraldehyde solution (25% in H₂O), N α ,N α -Bis(carboxymethyl)-L-lysine hydrate (\geq 97.0%), nickel chloride (NiCl₂, 98%), 2-(4-chlorophenyl)oxirane (96%), potassium phosphate dibasic anhydrous (\geq 99%) were purchased from Sigma-Aldrich. Potassium dihydrogen phosphate (99%) was supplied by Merck. 1-(4-chlorophenyl)ethane-1,2-diol (~98%) was purchased from Spectra Group. De-ionized (DI) water was purified through Millipore Elix water purification systems. Acetonitrile (HPLC), hexane (HPLC), ethyl acetate (HPLC) and magnesium sulfate (anhydrous) were supplied by Fisher. Cell-free extract of *Pichia pastoris* containing his-tagged stEH1 was obtained from VTU Technology GmbH, Austria.

Characterization of the materials: Field emission transmission electron microscope (FETEM) was performed on JEOL JEM-2010F model. Field emission scanning electron microscope (FESEM) was performed on JEOL JSM-6700F model. Energy-dispersive X-ray spectroscopy (EDX) was performed on Oxford instrument INCAx-act. The absorption was analyzed by UV-vis equipment: Hitachi U-1900. High-performance liquid chromatography (HPLC) was performed on Shimadzu HPLC system. Fourier transform infrared spectroscopy (FTIR) was performed on Bio-Rad Excalibur Series. Dynamic light scattering (DLS) was conducted by zetasizer nano-ZS from Malvern. Magnetic analysis was accomplished by using vibrating sample magnetometer (VSM) ADE magnetic EV-7.

Synthesis of oleic acid coated iron oxide magnetic nanoparticles (OA-MNPs): A solution of 0.01 mol ferric chloride hexahydrate and 0.005 mol ferrous chloride tetrahydrate in 100 mL of DI water in a 3-neck round bottom flask was stirred by mechanical stirrer at 80 °C with argon bubbling for 30 min. 0.01 mol of potassium oleate was added, and the mixture was stirred for another 30 min. 5 mL of 28% ammonium hydroxide was rapidly added to the mixture to form iron oxide supersaturating solution.¹ The reaction system turned to black immediately. The reaction continued at 80 °C under argon protection for 30 min. The black liquid was centrifuged

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at 16700 g for 10 min at 25 °C to separate unreacted oleic acid from magnetic nanoparticles solution. Excess oleic acid was further removed by using high gradient magnetic separator (Frantz isodynamic model L-1) to operate at 150 V with 2.2 A with a steel wool packed column. The obtained nanoparticle has a concentration at 4.6 mg/mL.

Synthesis of core-shell magnetic nanoparticles (PGMA-MNPs): 0.025 g ammonium persulfate and 0.316 mL glycidyl methacrylate (GMA) monomer were added into a 30 mL water solution containing 0.023 g OA-MNPs. The reaction was continued for 1 hour at 80 °C under magnetic stirring. The resultant mixture was centrifuged at 16700 g for 10 min and washed by DI water for several times. The solid was resuspended into DI water with concentration at 10.3 mg/mL of PGMA-MNPs.

Synthesis of amino-functionalized core-shell magnetic nanoparticles (EDA-MNPs): 3mL ethylenediamine (EDA) was added into 30 mL aqueous suspension containing 0.308 g PGMA-MNPs. This mixture was incubated at 80 °C for 12 hours. Afterwards, the particles were controlled by magnetic field and washed by DI water for several times. The EDA-MNPs were suspended into 30 mL DI water at concentration of 10.0 mg/mL for further application.

Synthesis of aldehyde-functionalized core-shell magnetic nanoparticles (GA-MNPs): 30mL of glutaraldehyde solution was heated to 80 °C and vigorously stirred. 30mL EDA-MNPs solution was slowly added into the above-mentioned solution to avoid any unwanted aggregation. This reaction mixture was incubated at 80 °C for 3 hours. Afterwards, the particles were centrifuged at 12000 g for 10 min and washed by DI water for several times. The particle was resuspended into 30 ml DI water and was named as GA-MNPs at concentration of 9.8 mg/mL.

Synthesis of NTA-functionalized core-shell magnetic nanoparticles (NTA-MNPs): 2 mL of 0.1 M Nα,Nα-Bis(carboxymethyl)-L-lysine hydrate solution at pH 8 was added into 20 ml GA-MNPs solutions. This mixture was incubated at 25 °C for 12 hours. Afterwards, the particles were

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centrifuged at 12000 g for 10 min and washed by DI water for several times. The particle was redisperesed into 20 mL DI water and was named as NTA-MNPs at concentration of 9.5 mg/mL.

Synthesis of Ni-NTA-functionalized core-shell magnetic nanoparticles (Ni-NTA-MNPs): 1 mL of 1 M nickel chloride solution at pH 8 was added into 10 mL NTA-MNPs. The reaction mixture was incubated for 12 hours before collection. The particle could be magnetically separated by permanent magnet, and washed by DI water for several times. The final concentration is around 9 mg/mL, which is corresponding to 90% of the yield from beginning.

Characterization of magnetic nanoparticles by DLS:



Figure S1. Size distribution (by number) of (a) OA-MNPs, (b) PGMA-MNPs, (c) EDA-MNPs, (d) GA-MNPs

Figure S1 shows the result from DLS. The hydrodynamic size of OA-MNPs is 24.2±9.7nm. After coating with GMA, the size increases to 90.2±22.5nm. The size of EDA-MNPs is 105.5±26.7nm, while the size of GA-MNPs is 114.2±25.5nm. By functionalization of the MNPs with different groups, the hydrodynamic size increases a little bit, which indicates successful attachment of those functions.

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Characterization of magnetic nanoparticles by FESEM/TEM:







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Figure S2. FESEM of (a) GMA-MNPs, (b) EDA-MNPs, (c) GA-MNPs, (d) Ni-NTA-MNPs; TEM of (e) OA-MNPs, (f) GMA-MNPs, (g) EDA-MNPs, (h) NTA-MNPs

Characterization of magnetic nanoparticles by FTIR:





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Figure S3. FTIR of (a) OA-MNPs, (b) GMA-MNPs, (c) EDA-MNPs, (d) GA-MNPs, (e) Ni-NTA-MNPs Table S1. Summary of functions from FTIR

symbol	wavenumber (cm ⁻¹)	functional group	nanoparticles
а	1701	C=O stretch in oleic acid	OA-MNPs
b	3000	O-H stretch in oleic acid	OA-MNPs
с	2850-2916	Fe-O-Fe stretch	OA-MNPs
d	1732	C=O stretch in GMA	GMA-MNPs
e	979	epoxy group ²	GMA-MNPs
f	1651	N-H	EDA-MNPs
g	1716	C=O stretch in aldehyde	GA-MNPs
h	1716	C=O stretch in carboxylic acids	Ni-NTA-MNPs
i	3375	O-H stretch in carboxylic acids	Ni-NTA-MNPs

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Characterization of magnetic nanoparticles by EDX: The loading of Ni could be verified by using EDX. From Figure S4, it could be noticed that characteristic peaks of Ni element can be observed in the sample of Ni-NTA-MNPs. Besides, there is no such peak in the EDX profile of NTA-MNPs. These results proved the existence of Ni element in the sample of Ni-NTA-MNPs.



Figure S5. EDX of (a) NTA-MNPs, (b) Ni-NTA-MNPs

Purification and immobilization of his-tagged epoxide hydrolase from cell-free extract in one pot with Ni-NTA-MNPs to prepare stEH1-Ni-NTA-MNPs in 1 mL scale: 1 mL cell-free extract of *Pichia pastoris* producing his-tagged stEH1 and other enzymes at a total protein amount of 0.5 mg was incubated with 2.0 mg Ni-NTA-MNPs at pH 8, 0.1 M phosphate buffer at 4 °C and 30 rpm for 12 h. The supernatant was separated from particles by using magnetic field. The protein concentration in the supernatant was analyzed by using Bradford reagent with 1mg/mL BSA as

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standard. The particle was further washed by using 0.1 M phosphate buffer at pH 8 for two times, and then named as stEH1-Ni-NTA-MNPs.

Purification and immobilization of his-tagged epoxide hydrolase from cell-free extract in one pot with Ni-NTA-MNPs to prepare stEH1-Ni-NTA-MNPs in 25 mL scale: 25mL of immobilization was conducted under similar conditions as mentioned previously with same concentration of stEH1 and MNPs. The specific loading capacity was 137 μg protein/mg particles after 12 hours at 4 °C.

Theoretical calculation for immobilization of stEH1 on MNPs Ni-NTA-MNPs: A theoretical calculation was made for estimating the total weight of epoxide hydrolase loaded on single Ni-NTA-MNPs nanoparticle. From TEM result of MNPs, we could hypothesize that the core-shell structured particle possesses a core size at 20nm and the total size of 60 nm. From X-ray results, the size of stEH1 is 55.9×98.5×122.0 Å.³ In this model, enzyme molecule is considered as spherical with a diameter at 6 nm, which is perfectly packed on the particle surface with monolayer distribution. The footprint of each enzyme is supposed as rectangular footprint. Figure S6 describes that hypothesis virtually.

Several important parameters are calculated and estimated from the model, including the specific surface area, total enzyme number on single MNPs, weight of single MNPs, and specific Enzyme loading amount, which is similar as previous published paper.⁴ Equations S1-S4 show the formula of these parameters.

$$A = \frac{4\pi (R+1)^2}{m_{3B}} (\text{Equation S1.}) \qquad m_{3p} = \frac{4\pi}{3} (\rho_0 r^2 + \rho_3 R^2 - \rho_5 r^2) (\text{Equation S2.})$$
$$N = \frac{4\pi (R+1)^2}{d^2} (\text{Equation S3.}) \qquad S = \frac{3 (R+1)^2 M_w}{N_A d^2 (\rho_0 r^2 + \rho_3 R^3 - \rho_5 r^3)} (\text{Equation S4.})$$

In order to get specific number of those parameters in the model, simple estimation was made. $\rho_c = 5.18 \text{ g/cm}^3$ (density of Fe₃O₄), while $\rho_s = 1.14 \text{ g/cm}^3$ (density of poly (GMA)). The M_w of stEH1 is around 37.1 kDa.⁵ The length of linker is estimated as 3nm. After simple calculation, the

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specific surface area is $104 \text{ m}^2 / \text{ g}$. Total stEH1 number on single MNPs is around 380. The weight of single MNPs is $1.31* 10^{-16}$ g. Finally, the maximum specific enzyme loading amount is 178 mg stEH1 / g MNPs, which is the ideal case when considering spatial hindrance with monolayer loading.

Based on the experimental results, the immobilization of stEH1 is 146 mg stEH1 /g support. From the value of single particle weight and stEH1 molecular weight, about 311 stEH1 molecules were bonded on single EDA-MNPs, which covers 82% surface area of the nanoparticles.



Figure S6. Model of immobilization of enzymes on MNPs

Verification of binding selectivity between Ni-NTA-MNPs and his-tagged EH: Several samples of EH-Ni-NTA-MNPs were incubated with different concentration of imidazole solution (0 mM-250 mM) at 4 °C for 1 h. The elute was obtained afterwards by separating particles out from the

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system. CFE and supernatant during immobilization, together with the above-mentioned eluted solution were further analyzed by using SDS-PAGE.

Hydrolysis of 2-(4-chlorophenyl)oxirane with stEH1-Ni-NTA-MNPs or purified free EH in 1 mL scale: stEH1-Ni-NTA-MNPs or purified free enzyme with 0.342 mg protein was added into a 990 μ L of 0.1 M phosphate buffer at pH 8. This reaction was started by adding a 10 μ L acetonitrile solution containing 1 M 2-(4-chlorophenyl)oxirane. The reaction mixture was incubated at 30 °C for 2 h. Several samples were taken along the reaction at different time intervals. The supernatant was separated by centrifugation at 5000 g and then under magnetic field. For preparing samples for reverse phase HPLC, the solution was mixed with equal amount of acetonitrile containing 2 mM of propiophenone as internal standard. Figure S7 presented the ee of 2-(4-chlorophenyl)oxirane for free and immobilized stEH1 at different time intervals. It can be seen that after 40min, the ee for both of the reaction system reached 99%. There was a slightly decreasing for free enzyme, while no decrease was observed for immobilized enzyme.

Detection methods of concentration and ee: Reverse phase HPLC was used to quantitatively analyze the product formation: C18 column (Agilent Eclipse Plus, 4.6*155 mm, 3.5 μ m); mobile phase consisted of acetonitrile (55% v/v) and water (45% v/v); flow rate of 1.0 mL/min; detection at 210 nm; retention time: 2.06 min for 1-(4-chlorophenyl)ethane-1,2-diol, 5.50 min for 2-(4-chlorophenyl)oxirane, 6.49 min for n-butyrophenone (internal standard). Normal phase HPLC was used to analyze the ee of diol and epoxide (Figure S8): AS-H column (Diacel, 250 * 4.6 mM, 5 μ m); mobile phase consisted of hexane (90% v/v) iso-propanol (10% v/v); flow rate of 1.0 mL/min; detection at 210nm; retention time: 6.00 min for (*R*)-2-(4-chlorophenyl)oxirane, 6.76 min for (*S*)-2-(4-chlorophenyl)oxirane, 10.83 min for (*S*)-1-(4-chlorophenyl)ethane-1,2-diol, 12.36min for (*R*)-1-(4-chlorophenyl)ethane-1,2-diol.

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Figure S7. ee of 2-(4-chlorophenyl)oxirane for free and immobilized stEH1 at different time intervals



Figure S8. Normal phase HPLC results for immobilized enzyme after 40min's reaction

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Hydrolysis of 2-(4-chlorophenyl)oxirane with stEH1-Ni-NTA-MNPs in 20 mL and preparation of (R)-2-(4-chlorophenyl)oxirane: stEH1-Ni-NTA-MNPs with 0.342 mg/mL protein was added into a 19.8 mL of 0.1 M phosphate buffer at pH 8. This reaction was started by adding a 200 μ L acetonitrile solution containing 1 M 2-(4-chlorophenyl)oxirane. The reaction mixture was incubated at 30 °C for 1 h. The conversion and ee of 2-(4-chlorophenyl)oxirane was analyzed at different time. Figure S8 presented the results. The resulted solution containing 2-(4chlorophenyl)oxirane and 1-(4-chlorophenyl)ethane-1,2-diol was further extracted by hexane for three times (1:1 ratio). Hexane was evaporated by vacuum evaporator with bath temperature at 60°C. The final purified product (*R*)-2-(4-chlorophenyl)oxirane had a yield of 33% (theoretical maximum at 50% for racemic resolution) and ee > 99%.



Figure S7. ee and conversion of 2-(4-chlorophenyl)oxirane in 20 mL reaction

Theoretical Calculation of Released Enzyme of stEH1-Ni-NTA-MNPs in Each Equilibrium Cycle: The relationship of the release kinetics could be presented in Scheme, where [P], [L] and [C] represent molar concentrations of the protein, ligand and complex, respectively. The definition of K_d could be presented in Equation S7. The dissociation constant (K_d) between Ni-NTA functional group and 6×his-tagged protein was reported with the value of 10⁻¹³ M.⁶ In current biotransformation system presented in this paper, initial concentration of immobilized enzyme complex [I] is 9.21×10⁻⁶ M. If in each cycle, the system could reach equilibrium, the

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released free protein amount [X] could be calculated by Equation S8, which is 1.91×10^{-9} M. This means that the release of free enzyme is only 0.02% of the total immobilized enzyme after one equilibrium cycle, which could be negligible.

 $C \longrightarrow P + L \text{ (Scheme S1.)}$ $K_{d} = \frac{[L][P]}{[C]} \text{ (Equation S7.)}$ $K_{d} = \frac{[X][X]}{[1] - [X]} \text{ (Equation S8.)}$

Recycle and Reuse of stEH1-Ni-NTA-MNPs: 990 μ L of 0.1M phosphate buffer at pH 8 was added into stEH1-Ni-NTA-MNPs containing 0.342 mg protein. This reaction was started by adding a 10ul acetonitrile solution containing 1M 2-(4-chlorophenyl)oxirane. The reaction mixture was incubated at 30 °C for 40 min in each cycle. The particles were separated by magnetic control. The supernatant was analyzed by HPLC. Afterwards, fresh buffer and substrate were added into particle precipitate for the next cycle of catalysis.

References:

- 1. B. Olle, S. Bucak, T. Holmes, L. Bromberg, T. Hatton and D. Wang, *Ind. Eng. Chem. Res*, 2006, **45**, 4355-4363.
- M. Sánchez-Soto, P. Pagés, T. Lacorte, K. Briceño and F. Carrasco, Compos. Sci. Technol., 2007, 67, 1974-1985.
- S. L. Mowbray, L. T. Elfström, K. M. Ahlgren, C. E. Andersson and M. Widersten, *Protein Sci.*, 2006, 15, 1628-1637.
- 4. W. Wang, Y. Xu, D. I. C. Wang and Z. Li, J. Am. Chem. Soc., 2009, 131, 12892–12893.
- 5. L. Elfström and M. Widersten, *Biochem. J*, 2005, **390**, 633.
- 6. J. F. Hainfeld, W. Liu, C. M. R. Halsey, P. Freimuth and R. D. Powell, *J. Struct. Biol.*, 1999, **127**, 185-198.