**Supplementary information**

# Development of immobilised enzymes and heterogeneous cascade systems with recoverable and recyclable catalysts for green applications

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## Materials and Methods

### 1 Chemicals and Materials

Iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) (98-102%), ethylene glycol (≥99%), urea (≥98%), polyvinylpyrrolidone (PVP) (MW 40,000), acetophenone (≥99%), sodium pyruvate (≥99%), (s)-methylbenzylamine (s-MBA) (≥98%), pyridoxal 5' phosphate hydrate (PLP, ≥98%), Nicotineamide adenine dinucleotide phosphate disodium salt ($NADP^+$) (≥98%), Nicotineamide adenine dinucleotide phosphate, tetrasodium salt (NADPH) (≥98%), Nitrotetrazolium Blue chloride (NBT), Phenazine methosulfate (PMS) (90%), Alcohol dehydrogenase from saccharomyces cerevisiae (ADH), D-Glucose (≥99.5%), Iron (III) nitrate, nonahydrate ($Fe(NO_3)_3.9H_2O$) (≥98%) all were purchased from Sigma Aldrich.

Nickel (II) chloride hexahydrate ($NiCl_2.6H_2O$) (97%) potassium dibasic monohydrogen phosphate (99%+) were purchased from Acros Organics. Potassium monobasic dihydrogen phosphate anhydrous, acetonitrile (HPLC gradient grade, ≥99.9%), hydrochloric acid (approx. 37%) were purchased from Fischer. 10% $NiO/SiO_2$ was a gift from Johnson Matthey Catalysts (Billingham, UK). His-tagged enzymes (*Halomonas elongata* ω-transaminase, HeωT) and *Bacillus* species glucose dehydrogenase (*Bs*-GDH)) were cultured by the Campoiano group at the University of Edinburgh. Characterisation and activities of these free enzymes are available in a recent publication (Lau et al., 2023). All solutions were made using distilled water unless stated otherwise.

### 2. Synthesis and characterisation of $NiFe_2O_4$ MNPs

The production of nickel ferrite magnetic nanoparticles ($NiFe_2O_4$ MNPs) was carried out via a solvothermal synthesis route (Atacan et al., 2019). Firstly, 2.38g of $NiCl_2.6H_2O$ and 5.41g $FeCl_3.6H_2O$ was dissolved in 20ml of ethylene glycol. Then 2.5g of urea and 0.2g of PVP were dissolved in a separate solution using 20ml of ethylene glycol, this was then added to the first mixture of nickel and iron precursors and stirred at room temperature until fully dissolved. The reaction mixture was then transferred to a Teflon lined stainless steel autoclave (Parr, 45 mL) and heated in an oven at 180 °C for 20 hours. After 20h, the autoclave was cooled to room temperature and the $NiFe_2O_4$ MNPs were harvested with a magnet, then washed with distilled water 20 times (10 mL) followed by ethanol a further 10 times (5 mL) to remove any unreacted chemicals and residual solvent. The MNPs were then dried at under vacuum at 65 °C for 24 hours.

The synthesised MNPs were characterised by TEM, XRD, and SQUID magnetomotry, these techniques provided information on the morphology of the nanoparticles. TEM was mainly used to study the size and shape of the MNPs. This was carried out using a Technai T20 Transmission Electron Microscope (FEI) operating at 120kV. The .tif images were captured using Olympus Scandium software and analysed through imageJ software. X-ray diffraction (XRD) patterns were obtained on a Panalytical diffractometer (Cu K $\alpha$ radiation, $\lambda$ = 1.5406 nm) for 2 $\theta$ from 20° - 80° at a scan rate of 2° per minute. Fourier-transform infrared (FTIR)

spectra were acquired using a ThermoScientific Nicolet iS5 Fourier-transform infrared spectrometer with an ATR diamond with 16 scans between 500 - 4000 $cm^{-1}$. Thermogravimetric analysis was carried out using TA instruments SDT Q600 Thermal Balance, where the sample was heated up at a heating rate of 10 °C /min from 20 to 800 °C under flowing air (75 mL/min). To study the magnetic properties of the $NiFe_2O_4$ MNPs, measurements of hysteresis loops were performed on a Quantum Design MPMS3 SQUID magnetometer equipped with a 7 T DC magnet. In a typical experiment, a sample was weighed into a gel capsule. A small drop of Eicosene (Sigma-Aldrich) preheated to 60°C was added to the capsule to prevent the sample from moving within strong magnetic fields. The capsule then was weighed again to account for the mass of Eicosene. The hysteresis loops of the magnetic moment (M) versus field (H) for the samples were measured at room temperature with a maximum field of about ±15 kOe.

3. Immobilisation of HeωT

Once the MNPs were washed and dried, a solution of 1 mg/mL of MNPs was prepared in potassium phosphate reaction buffer (containing, 0.25% DMSO and 0.1 mM PLP at 50 mM, pH8). Then 1 mL (5.13 mg/mL) of HeωT was added to the MNP solution and shaken for 4 hours at 4°C. After immobilisation the supernatant was removed and the immobilised MNPs were washed with 3 portions reaction buffer (5 mL) and redispersed in reaction buffer ready for reactions.

4. Immobilisation of GDH on $NiO/SiO_2$

GDH was immobilised on to non-magnetic $NiO/SiO_2$ particles using the his-tag present on GDH molecule to chelate with the free nickel sites on the particles. In a typical experiment 10 mg of $NiO/SiO_2$ particles were suspended in 1 mL of potassium phosphate buffer (50 mM, pH 8) and sonicated for 15 mins until particles were fully dispersed. Then 1 mL of purified his-tagged GDH (2.03 mg/mL) was added to the particles and rotated for 4 hours at 4 °C. After the GDH was immobilised, the mixture was centrifuged using the Eppendorf mini spin at 12000 rpm for 7 minutes, for separation between the immobilised enzymes and supernatant. The supernatant was pipetted out and underwent Bradford assay to determine the concentration of GDH unbound. The particles were washed with 5 volumes of 2 mL buffer. The immobilised GDH was then dispersed in 500 µL of buffer ready for reactions.

5. Study on the separation of $NiFe_2O_4$ and $NiO/SiO_2$ from a mixed suspension

To investigate the efficiency of the separation between magnetic and non-magnetic particles in the cascade system, a typical experiment was set up with 10 mg of $NiFe_2O_4$ and 10 mg of $NiO/SiO_2$ dispersed in 1 mL of potassium phosphate buffer and homogenised for 10 mins to ensure full mixing. The mixture then was put under a NdFeB magnet for 2 mins to allow for magnetic separation between $NiFe_2O_4$ and $NiO/SiO_2$. The $NiO/SiO_2$ suspension was then pipetted out and digested with 10 mL of concentrated HCl acid for 72 hours. Atomic absorption spectrometry (ThermoScientific S series AA Spectrometer) was used to analyse the digested

$NiO/SiO_2$ solution and quantify any iron from $NiFe_2O_4$ being left in the in the remainder of the sample.

6. Cascade reaction from combined free enzymes

Figure S1. shows the reaction scheme of the cascade reaction system from the combined free enzymes, involving the three enzymes working in tandem. The first step of the cascade involves the enzyme HeωT to convert the substrate s-methylbenzyl amine to acetophenone. The second step uses ADH to convert the acetophenone to 1-phenylethanol by consuming the cofactor NADPH to produce $NADP^+$, which is then regenerated to form NADPH using GDH, using glucose as the sacrificial hydrogen donor. Reaction conditions were at 37 °C and pH 8, this is the optimal operational conditions for all 3 enzymes. **Table S1** shows the components and concentration of the 1 mL reaction carried out for the free enzyme cascade reaction.

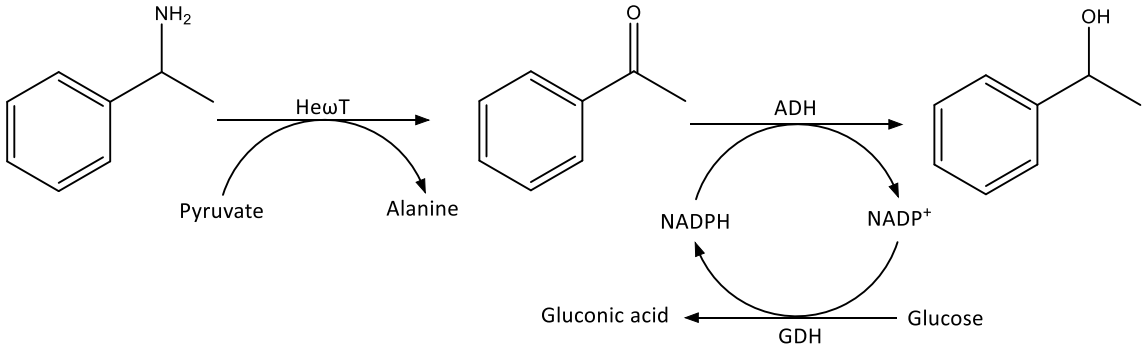


**Figure S1.** Reaction scheme of the cascade reaction of the combined enzyme system

**Table S1.** Reaction components and their concentrations in a 1 mL volume for the combined system using free enzymes.

| Component | Concentration in cuvette |
|---|---|
| HeωT | 0.072 mg/mL |
| ADH | 1.025 mg/mL |
| *Bs*-GDH | 0.4 mg/mL |
| s-methylbenzyl amine | 100 mM |
| Sodium pyruvate | 100 mM |
| D-glucose | 70 mM |
| NADPH | 1 mM |
| Buffer | 50 mM |

7. Cascade reaction from immobilised enzymes

The cascade reaction using combined immobilised enzymes, with HeωT immobilised on $NiFe_2O_4$ MNPs (MNP-HeωT), GDH immobilised on $NiO/SiO_2$ ($NiO/SiO_2$-GDH) and ADH being kept as a free enzyme. Reaction conditions were at 37 °C and pH 8 (same as the free enzyme cascade). **Table S2** shows the components and

concentration of the 1 mL reaction carried out for the cascade reaction using immobilised enzymes.

**Table S2.** Reaction components and their concentrations in a 1 mL volume for the combined system using immobilised enzymes and free ADH.

| Component | Concentration in cuvette |
|---|---|
| MNP-HeωT | 0.072 mg/mL |
| ADH | 1.025 mg/mL |
| $NiO/SiO_2$-*Bs*-GDH | 0.4 mg/mL |
| s-methylbenzyl amine | 100 mM |
| Sodium pyruvate | 100 mM |
| D-glucose | 70 mM |
| NADPH | 1 mM |
| PLP | 0.1 mM |
| Buffer | 50 mM |

8. HPLC analysis for ADH activity, free and immobilised combined enzyme cascade

S-methylbenzyl amine, acetophenone and 1-phenylethanol in ADH, and free and immobilised cascades were analysed using the Agilent 1100 Series with a Zorbax Eclipse Plus C18 (4.6 x 100mm, 3.5µm) with an isocratic elution of acetonitrile/water (35:65 v/v) at a flowrate of 1 mL $min^{-1}$. Sample volume of 10 µL was injected to the column at a temperature of 25 °C and the UV detector set to 211 nm.

Reference:

Atacan K, Güy N, Çakar S, and Özacar M, Efficiency of glucose oxidase immobilized on tannin modified $NiFe_2O_4$ nanoparticles on decolorization of dye in the Fenton and photo-biocatalytic processes, J Photochem Photobiol A Chem 382:111935 (2019). doi: https://doi.org/10.1016/j.jphotochem.2019.111935.

Lau ECHT, Dodds KC, McKenna C, Cowan RM, Ganin AY, Campopiano DJ and Yiu HHP. Direct purification and immobilization of his-tagged enzymes using unmodified nickel ferrite NiFe2O4 magnetic nanoparticles. Sci Rep 13:21549 (2023). https://doi.org/10.1038/s41598-023-48795-x