**Electronic Supporting Information (ESI)**

Multilayer enzyme-coupled magnetic nanoparticles as efficient, reusable biocatalysts and biosensors

*Josep Garcia,* a *Yue Zhang,* a *Hannah Taylor,* a *Oscar Cespedes,* b *Michael E. Webb* a and *Dejian Zhou* *a*

a School of Chemistry and Astbury Centre for Structural Molecular Biology, University of Leeds, Woodhouse Lane, Leeds LS2 9JT, United Kingdom. Email: d.zhou@leeds.ac.uk

b School of Physics and Astronomy, University of Leeds, Leeds LS2 9JT, United Kingdom.

**Estimation of enzyme coupling efficiency**

All UV-vis absorption spectra were determined on a quartz cuvette with a 1 cm path length. To estimate the percentage of protein being coupled to the magnetic nanoparticles, we used the following equation:

\[
\left(1 - \frac{[A_{280(\text{sup})} - A_{280(\text{blank})}][A_{800(\text{std})} - A_{800(\text{blank})}]}{[A_{280(\text{std})} - A_{280(\text{blank})}][A_{800(\text{std})} - A_{800(\text{blank})}]}ight) \times 100
\]

Where \( A \) is the optical absorbance at the indicate wavelength, \( \text{sup} \) is the supernatant, the \( \text{blank} \) used is PBS and \( \text{std} \) is standard solution. The absorption spectrum of the protein was done in a scan on a 1 cm path length quartz cuvette.

**Protein biotinylation (BSA and GOX)**

The protein and enzyme biotinylation was carried out following the standard protocols given by the biotinylation kit provided by Pierce Biotechnology.

**Preparation of biotinylation solution (20 mM)**

A micropipette tip was used to puncture the foil top of a biotin reagent microtube (containing 2 mg NHS-PEO₄-biotin, MW = 589, and the NHS and biotin groups were linked by a hydrophilic tetra(ethylene glycol), PEO₄ linker ) and into which was added 170 µl of pure water. The resulting solution was thoroughly mixed by pipetting up and down to make a 20 mM solution of NHS-PEO₄-Biotin. The solution was freshly prepared and used immediately.

**Determination of biotin incorporation.**

This was done by following the procedures of the biotinylation kit. 5 mg Avidin was dissolved in 300 µl HABA (10 mM) and 9.7 ml PBS was added to give a HABA/Avidin solution. A background absorption (at 500 nm) of 1 ml PBS in 1 cm path length quartz cuvettes was measured and the spectrometer zeroed. The absorbance of HABA/Avidin solution was measured at 500 nm and if the solution was prepared correctly, the absorbance at 500 nm must be between 0.9 and 1.3. 900 µl HABA/Avidin (H/A) solution was put in a cuvette and \( A_{500} \) was measured. 100 µl biotinylated protein...
was added to the cuvette and the sample was mixed well. Finally the HABA/Avidin/b-protein (H/A/B) absorbance was measured at 500 nm and the level of biotin incorporated can be estimated.

Preparation of Biotin-GOX
50 mg of glucose oxidase (GOX, containing ~20% of protein) was weighted and dissolved in 1.2 ml of dH₂O. Into which was added NHS-PEO₄-Biotin (63 µl, 20 mM, biotin to GOX molar ratio is ~20) and the resulting solution was kept in a fridge at 4 °C overnight to achieve good coupling between biotin and glucose oxidase. After that, the biotinylated GOX was purified on a spin column. The resulting biotin-GOX concentration was calculated as 18.9 µM using an extinction coefficient of 37,800 cm⁻¹M⁻¹ for GOX at 450 nm using the Beer-Lambert law. The level of biotinylation for GOX was determined as 8.0 by the HABA assay using the equations provided by the biotin labelling kit.

Table S1. Summary of the total enzyme weight and molar loading rates for the HRPₓ-MNP systems.

<table>
<thead>
<tr>
<th>HRPₓ-MNP system</th>
<th>Total enzyme weight loading (mg/g (enzyme/MNP))</th>
<th>Total enzyme mole loading rate (µmol enzyme/g MNP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP₁-MNP</td>
<td>17</td>
<td>0.15</td>
</tr>
<tr>
<td>HRP₂-MNP</td>
<td>32</td>
<td>0.49</td>
</tr>
<tr>
<td>HRP₃-MNP</td>
<td>62</td>
<td>0.76</td>
</tr>
<tr>
<td>HRP₄-MNP</td>
<td>69</td>
<td>0.91</td>
</tr>
<tr>
<td>HRP₅-MNP</td>
<td>130</td>
<td>1.47</td>
</tr>
</tbody>
</table>

Figure S1. Schematic structure of the MNP-HRP₁ system where the HRP-Av conjugate is attached to the MNP surface via a biocompatible BSA linker biotin groups through its avidin conjugate, not the HRP enzyme part. This reduces the possible steric blocking to the HRP enzyme, allowing it to maintain high activity.
Scheme S1: Schematic of the HRP activity assay: in the presence of H₂O₂, Amplex Red (colourless) is turned over into a strongly colored product, resorufin, which absorbs strongly at 571 nm catalysed by HRP.

Figure S2. Three kinetic assay traces (A₅₇₁ nm versus incubation time plot) for the (HRP)₁-MNP (left) and the same amount of free HRP-Av (right) in PBS buffer with 10 μM Amplex red and 10 μM H₂O₂ where both assay samples contained 0.75 pmol of HRP-Av. The average initial enzyme activities for the (HRP-Av)₁-MNP and HRP-Av are 0.00427 ± 0.00024 and 0.00396 ± 0.00005 (A₅₇₁.S⁻¹) respectively, confirming the HRP-Av-MNP has the same or slightly higher level of activity as the corresponding free enzyme in solution.

To convert the above activity to per enzyme turnover rate, we used the following calculation:

1. To convert A₅₇₁ rate change (optical path length: 1 cm) to concentration rate change:
   \[ \text{rate change} = \text{rate change (optical path length: 1 cm)} / (7.3 \times 10^4 \text{ M}^{-1} \text{cm}^{-1}) = 5.85 \times 10^{-8} \text{ M}^{-1} \text{s}^{-1} \]

2. This corresponds to per enzyme turnover rate (for the HRP-MNP system):
   \[ 5.85 \times 10^{-8} \text{ M}^{-1} \text{s}^{-1} \times 10^{-3} \text{ L (volume)} / 0.75 \times 10^{-12} \text{ mol (enzyme amount)} = 78 \text{ s}^{-1} \]

Similarly, the free HRP-MNP (0.00396 A₅₇₁S⁻¹) turnover rate can be calculated as 72 s⁻¹.
Figure S3. Representative assay curves (A$_{571}$ v.s. time) for the same amount of HRP-biotin (black) and HRP-Av (red line) in PBS with 10 μM H$_2$O$_2$, 10 μM Amplex red and 1 mg/mL BSA. The initial enzyme activity rates are 0.0132 and 0.00568 s$^{-1}$ (A$_{571}$) for HRP-Av and HRP-biotin respectively. The relative activity of HRP-biotin is ~43% (0.00568/0.0132) that of the same amount of HRP-Av.

Figure S4. Kinetic assay traces (A$_{571}$ nm versus time) for assessing possible enzyme leaking from the MNP-HRP$_x$ system. The assay curves for the MNP only (black, 5 μg), MNP-HRP$_3$ (red, 5 μg), and supernatants obtained from a series samples containing the same amounts of the MNP-HRP$_3$ (5 μg) after complete removal of the MNP-HRP$_3$ at storage time of 0, 1, 3, 5 days are shown. The supernatants were obtained on a series of MNP-HRP$_3$ samples (each containing the same amount of the MNP-HRP$_3$ as that used in the MNP-HRP$_3$ assay shown in the red line) after being stored for a specific period followed by the complete removal of all MNPs by magnetic separation and centrifugation. All assays were carried out under identical conditions in PBS with 10 μM Amplex red and 10 μM H$_2$O$_2$. The table below shows the free (leaked) enzyme activity in the surpernatant is less than 1% that of the MNP-HRP$_3$, suggesting almost no enzyme leaking over 5 days.

The resulting fitted enzyme kinetic data for the enzyme leaking assays.

<table>
<thead>
<tr>
<th>MNP-HRP$_x$ system</th>
<th>Activity (A$_{571}.S^{-1}$)</th>
<th>Storage time (day)</th>
<th>Supernatant activity (A$_{571}.S^{-1}$)</th>
<th>Supernatant/MNP-HRP$_3$ activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNP-HRP$_3$</td>
<td>$7.6 \times 10^{-3}$</td>
<td>Day 0</td>
<td>$3.6 \times 10^{-5}$</td>
<td>0.47%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 1</td>
<td>$4.1 \times 10^{-5}$</td>
<td>0.54%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 3</td>
<td>$3.8 \times 10^{-5}$</td>
<td>0.50%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Day 5</td>
<td>$4.7 \times 10^{-5}$</td>
<td>0.62%</td>
</tr>
</tbody>
</table>
Figure S5. (A) A photograph of the MNP (~1.3 mL) in water in an eppendorf tube showing that the MNPs are well-dispersed as a uniform solution. (B) The same eppendorf tube after being placed on a standard biomag separation device for 1 mins, almost all MNPs have now been pulled to the side of the tube and the solution becomes clear, confirming that the MNPs are easily retrieved by applying a magnetic field for recycling and reuse.

Figure S6. Control assay curves (A₅₇₁ v.s. time) for the MNP only samples at 5 μg (black line) and 100 μg (red line) in PBS with 10 μM H₂O₂, 10 μM Amplex red. No changes of A₅₇₁ was observed for both MNP only samples, suggesting that the MNPs used here is not active for the HRP catalyzing reaction, i.e. all the observed enzyme activity comes from the HRP, not the MNP carrier. The higher A₅₇₁ absorbance for the 100 μg MNP sample is due to the absorption and light scattering of the MNP itself.
**Figure S7.** Typical assay curves ($A_{571}$ vs. time) for MNP-HRP$_5$-GOX (red) and MNP-HRP$_5$-GOX-HRP (both contains 25 μg of equivalent dry MNP) under identical conditions. The specific activities (change of $A_{571}$ s$^{-1}$) are $2.3 \times 10^{-3}$ and $5.6 \times 10^{-4}$ s$^{-1}$ for MNP-HRP$_5$-GOX and MNP-HRP$_5$-GOX-HRP, respectively. Thus incorporation of a further HRP layer (HRP-Av) on top of the MNP-HRP$_5$-GOX system decreased its coupled enzyme activity by 76%.