Supporting information on

Magnetic enzyme nanogel (MENG): a universal synthetic route of biocatalysts

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1. **Synthesis**

Bare magnetic nanoparticles (MNPs) were prepared based on a modification of Massart’s method.\textsuperscript{[1, 2]} For amination, bare MNPs (50 mg) were dispersed in 10\% (3-aminopropyl)triethoxysilane (APTES) solution (20 mL, containing the appropriate amount of APTES dissolved in 1:1 water/ethanol), and the suspension was purged with N\textsubscript{2} for 20 min and then refluxed at 50 °C for 5 h. The black precipitate (MNP-I) was collected using a neodymium-iron-boron magnet, and washed repeatedly with doubly distilled (dd) H\textsubscript{2}O to ensure complete removal of ethanol. \textit{N}Acryloylsuccinimide (NAS) (2.5 mg, dissolved in dimethyl sulfoxide 100 µL) was slowly added to a suspension of MNP-I (20 mg, dispersed in borate buffer 50 mM, pH 9.0 or Tris-HCl buffer 50 mM, pH 8.0, 50 mL). The reaction mixture was then incubated at 30 °C for 2 h, and the black product MNP-II was collected and washed by magnetodecantation. To convert the remaining amine groups into carboxylate groups, MNP-II was reacted with succinimide (0.5 g) in tetrahydrofuran (5 mL) by stirring at room temperature for 6 h. The final product MNP-III was collected and washed by magnetodecantation.

\textit{Fabrication of MENGs:} MNP-II or MNP-III (1 mg/mL) was mixed with respective enzymes (0.5 mg/mL), and the reaction mixtures were topped up with appropriate buffers to give a total volume of 4 mL. For CRL-MNPs and HRP–MNPs, 50 mM Tris-HCl pH 8.0 buffer was used for both assembly and polymerization. For CyC-MNPs and Tr-MNPs, phosphate buffer pH 6.0 was used for both assembly and polymerization. The reaction mixture was stirred at room temperature for 2 h and then the unabsorbed proteins were removed by magnetodecantation and washed three times with buffer. The supernatant and washes were collected and dialyzed against the respective buffer at 4 °C prior to further BCA analysis to calculate the enzyme
loading efficiency. The enzyme–particle ensembles were re-dispersed in pre-polymerization solution (30 mg/mL AM, 0.15 mg/mL MBA crosslinker in buffer), ammonium persulfate (8 mg, dissolved in 100 µL water) and TEMED (8 µL) were added to initiate the polymerization, and the reaction was carried out at 30 °C for 4 h. The resulting MENGs were collected magnetically and washed three times with buffer to remove the unbound polymer. The supernatant and washes were collected magnetically, and dialyzed against water. The dialyzed supernatant was then subjected to BCA assay to determine the protein concentration. No protein was detected, indicating that all of the protein (0.5 mg/mL) had been adsorbed onto the surface of the particles or encapsulated within the MENGs. The MENGs were finally dispersed in buffer for the respective enzyme assays and kept at 4 °C.
2. Characterizations

AFM
Atomic force microscopy (AFM) measurement of samples was carried out using NanoFirst-3100 SPM (Suzhou Haizisi). A drop of diluted samples was added to mica slide and air-dried for a few hours, prior to examination.

S Figure 1. AFM images of (a) bare MNPs and (b) CRL-MENGs. (scale bar 1 µm)
FT-IR
FT-IR studies were conducted using a Thermo Nicolet Nexus spectrometer. The samples were dried in air for two days and grinded using a motor.

S. Figure 2. FTIR results of bare MNPs, MNP-I (APTES-MNPs), MNP-II (acryloylated MNPs); and MNP-III (carboxylated MNPs).

The surface chemical composition of MNPs was confirmed by FTIR. For bare MNPs, the strong absorption peak at 570 cm⁻¹ was due to Fe-O bond. For MNP-I (APTES-MNPs), the presence of silane polymers on the surface of MNPs was evidenced by the appearance of Si-O vibration peak at 993 cm⁻¹. Two new peaks at 1621 and 1526 cm⁻¹ were assigned to amide I and amide II of MNP-I and MNP-II (acyryloylated MNPs). There was a strong absorption peak at 1700 cm⁻¹ due to C-O vibration, indicating successful carboxylation of MNP-III (carboxylated MNPs).
Zeta-potential measurements of bare MNPs, MNP-I, -II and –III were carried out in

\[\text{Zeta-potential (mV)}\]

\[\text{pH}\]

Bare MNPs
MNP-I
MNP-II
MNP-III
MENs

7.4
11.4
10.6
3.5

S. Figure 3. Zeta-potential of bare MNPs, MNP-I, MNP-II, MNP-III and CRL-MENs, as a function of pH from pH 3- pH 12, isoelectric point (pI) was indicated with an arrow.

The pI value of bare MNPs was determined 7.4, consistent with the published results. After amination with APTES, the pI of MNP-I (APTES-MNPs) was shifted to 11.4, due to the abundance of amine groups. The pI of MNP-II (after acryloylation), was determined to be 10.6 (slightly decreased compared to pI of MNP-I), and the trend of the zeta-potential against pH was similar to MNP-I, indicating that very small amount of amine groups were converted to acryloyl groups. The pI value of MNP-III (carboxylated MNPs) was decreased to 3.5 (corresponding to the pK_a value of carboxylic group), suggesting that the remaining amine groups were converted to carboxylate groups. The CRL-MENs curve showed a relatively constant zeta-potential (around zero) regardless of environmental pH, hence no pI value was observed, which could be attributed to the non-charged polyacrylamide shell. Unlike bare MNPs and MNP-I, -II and –III, CRL-MENs were quite stable at the whole range of pH, because they were stabilized by the steric repulsion of the polymeric shell instead of surface charges.
Quantification of functional groups of MNP-I and MNP-II

Determination of Fe content in MNP suspensions
Fe content was determined colorimetrically according to Mykhaylyk's report.\textsuperscript{[5]} 20 µL MNP suspensions were dissolved in 180 µL concentrated HCl to yield a dark yellow solution and diluted to 4 mL ddH\textsubscript{2}O. Reaction buffer was prepared by mixing 200 µL concentrated HCl, 200 µL 10 % hydroxylamine solution, 2 mL ammonium acetate solution, 800 µL 1 % 1,10-phenolanaline solution and 7.3 mL ddH\textsubscript{2}O. 150 µL sample solution or standard solution was mixed to 1000 µL reaction buffer and incubated at room temperature for 20 min and absorbance at 510 nm of the mixtures were recorded in a quartz cuvette by a UV-VIS spectrophotometer model UV-2450 (Shimadzu, Japan). FeCl\textsubscript{3}/FeCl\textsubscript{2} (molar ratio of 2:1) solution total Fe concentration up to 0.6 mM was used a Fe standard solution.

Determination of surface amine groups in functionalized MNP suspensions
Amine group determination was carried out based on a modified fluorescamine method developed by Udenfrie \textit{et al.}\textsuperscript{[6]} 1.5 mL of fluorescamine solution (in acetone, 0.1 mg/mL) was firstly added to 0.5 mL of functionalized MNPs dissolved in pH 7.0, 50 mM phosphate buffer. After 10 min reaction at room temperature, the fluorescence intensity was determined at an excitation wavelength of 390 nm and an emission wavelength of 483 nm using a Shimadzu spectrofluorometer. Bare MNPs was used as a control to eliminate the effect of MNPs. APTES solution up to 0.1 mM was used as standard amine solutions.
S. Table 1. a) Schematic illustration of the hydrolysis and deposition of APTES on the surface of MNPs and b) reaction conditions of APTES functionalization and degree of amination determined by ICP.

(a)

(b) Reaction temperature (°C) Acid/Base catalysis Solvent Initial APTES amount (w/v) Degree of amination (amine/NP)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Acid/Base</th>
<th>Solvent</th>
<th>Amount</th>
<th>Degree of amination</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>HAc pH3.0</td>
<td>ddH2O</td>
<td>15%</td>
<td>25.1</td>
</tr>
<tr>
<td>90</td>
<td>HAc pH3.0</td>
<td>Water/ethanol</td>
<td>15%</td>
<td>125.4</td>
</tr>
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<td>50</td>
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<td>ddH2O</td>
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<td>20.2</td>
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<td>ddH2O</td>
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</tr>
<tr>
<td>70</td>
<td>NaOH pH11.0</td>
<td>ddH2O</td>
<td>15%</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>NaOH pH11.0</td>
<td>Water/ethanol</td>
<td>3%</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>NaOH pH11.0</td>
<td>Water/ethanol</td>
<td>10%</td>
<td>38.8</td>
</tr>
<tr>
<td>50</td>
<td>NaOH pH11.0</td>
<td>Water/ethanol</td>
<td>15%</td>
<td>75.5</td>
</tr>
<tr>
<td>70</td>
<td>NaOH pH11.0</td>
<td>Water/ethanol</td>
<td>3%</td>
<td>11.2</td>
</tr>
<tr>
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<td>Water/ethanol</td>
<td>10%</td>
<td>129.1</td>
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<tr>
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<td>Water/ethanol</td>
<td>15%</td>
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<tr>
<td>90</td>
<td>NaOH pH11.0</td>
<td>ddH2O</td>
<td>15%</td>
<td>45.4</td>
</tr>
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</table>

In order to optimize the reaction condition of amination, several batches of MNP-I (APTES-MNPs) were prepared at different temperature, using acid or base as catalysts, using different solvent. The degree of amination was determined as mM of amine / mM of MNPs. NaOH works better as catalysts compared to acetic acid, because at basic condition, the hydrolyzed saline molecules tend to condensate on the surface of iron oxide particles, instead of self-polymerization. [7]
S. Table 2. a) Generation of vinyl groups on the surface of MNPs via NAS reaction with amine groups on the surface of MNPs; and b) reaction condition of NAS acryloylation of MNPs and acryloylation degree.

(a)

(b) Reaction temperature (°C) | Buffer | Initial NAS and MNP molar ratios | Degree of amination (amine/NP) | Calculated acryloylation (acryloyl/MNP) |
---|---|---|---|---|
90 | borate buffer pH 9.0 | 5/1 | 170 | 25.1 |
90 | borate buffer pH 9.0 | 10/1 | 160 | 125.4 |
50 | borate buffer pH 9.0 | 20/1 | 155 | 20/ |
50 | borate buffer pH 9.0 | 50/1 | 82 | 82.7 |
70 | borate buffer pH 9.0 | 100/1 | 21 | |
70 | borate buffer pH 9.0 | 200/1 | 0 | 0 |
50 | Tris-HCl buffer pH 8.0 | 5/1 | 172 | 0 |
50 | Tris-HCl buffer pH 8.0 | 10/1 | 170 | 38.8 |
50 | Tris-HCl buffer pH 8.0 | 20/1 | 152 | 75.5 |
70 | Tris-HCl buffer pH 8.0 | 50/1 | 109 | 11.2 |
70 | Tris-HCl buffer pH 8.0 | 100/1 | 29 | 29.1 |
70 | Tris-HCl buffer pH 8.0 | 200/1 | 0 | 338.1 |
90 | ddH2O | 200/1 | 0 | 45.4 |

The reaction condition of NAS modification was optimized. At slightly basic environmental pH, NAS reacted with the surface amine to yield an amide bond; two buffers were used in the experiments. MNP-I dispersed very well in Tris-HCl buffer pH 8.0 and the acryloylation degree could be controlled by adjusting the NAS amount, hence Tris-HCl buffer pH 8.0 was chosen for NAS modification. In order to control the ratio of surface vinyl groups and surface amine groups, NAS amount was strictly limited.
Protein BCA assays

The protein concentration was colorimetrically determined by BCA protein assay kit (Biyuntian, Beijing), according to the supplier’s instructions. Briefly, 20 µL reagent A was mixed with 1 mL reagent A to prepare green reaction solution. In a 96-well plate, 20 µL sample solution was mixed with 200 µL reaction solution in each well and incubated at 37 °C for 45 min for the purple colour to develop. The absorbance at 560 nm of the reaction mixture was measured by a Sunrise plate reader (Tecan). Bovine serum albumin (BSA) standard solution with protein content up to 0.5 mg/mL was used as a protein standard solution.

The loading efficiency was determined indirectly by protein BCA assays. The enzyme-particle hybrids were subjected to magnetodecantation and washed with buffers for 3 times, which were collected and dialyzed against phosphate buffer pH 7.0. The dialyzed supernatant was subjected to BCA assay to determine the protein concentration.

\[
E_{\text{loading}} = \frac{n_{\text{total}} - n_{\text{supernatant}}}{n_{\text{NP}}}
\]

Where \( n_{\text{total}} \) and \( n_{\text{supernatant}} \) are the molar amount of total proteins and protein content in the supernatant measured by BCA assays, and \( n_{\text{NP}} \) is the molar amount of MNPs.

It is well established that proteins can be adsorbed onto the carrier surfaces through a variety of interactions, including hydrophobic, van der Waals, electrostatics, and hydrogen bonding. In this work the main driving force of formation of enzyme-MNPs hybrids was electrostatic interaction, for example, acidic enzyme CRL was assembled under conditions where it was negatively charged in buffered solution since it has an isoelectric point (pI) of 4.8 (Lip1, 89%) and 5.8 (Lip3, 11%), and functionalized MNP-1 (pI of 10.2) was positively charged. (S Figure 5.) Whereas basic enzymes, such as cytochrome C (pI of 10.7) and trypsin (pI of 10.0) were also assembled onto the negatively charged MNP-2 (pI of 4.0, S Figure 5.) at acidic pH.
Optimization of assembly-polymerization conditions

S Figure 4. Enzyme loading efficiencies onto the MNPs (µM/µM)\textsuperscript{[a]} and relative activities\textsuperscript{[b]} of (a) CRL-MNPs, (b) HRP-MNPs, (c) Tr-MNPs, and (d) CyC-MNPs.

\textsuperscript{[a]} Proteins were adsorbed onto the surface of functionalized MNPs at a concentration of 0.5 mg/mL, with an MNPs concentration of 1.0 mg/mL, at 25 °C. The experiments were conducted in triplicate with a standard error of <10%. The loading efficiency is expressed in terms of the molar ratio of enzyme molecules and MNPs.

\textsuperscript{[b]} The enzymatic activities of the MENGs were measured and compared with those of their free enzyme counterparts at the optimal pH using the respective substrates. Substrates: \(p\)-NPP for CRL at pH 7; ABTS for CyC at pH 8; TMB for HRP at pH 5.5; and BAPNA for trypsin at pH 8.
2. Enzyme assays

**Determination of CRL hydrolytic activity**

The hydrolytic activity of CRL was determined by using p-nitrophenyl palmitate (p-NPP) as substrates.[8] p-NPP substrate solution was prepared by dissolving 9.6 mg p-NPP in acetone and slowly added the to 50 mL 1.25 % (w/v) Triton X-100 solution (1.25 g Triton X-100 dissolved in 100 mL 50 mM phosphate buffer, pH 7.0). 50 µL sample solution (containing CRL, free form or in MENs) was added to 950 µL substrate solution and quickly mixed and the hydrolytic activity of CRL was measured by recording the initial velocity of absorbance changes at 348 nm in a quartz cuvette by a UV-VIS spectrophotometer model UV-2450 (Shimdzu, Japan), using molar absorption coefficient of n-nitrophenol (12,800 M⁻¹·cm⁻¹).[8] The initial reaction rate and the hydrolytic activity for each sample were then obtained and normalized against the concentration of CRL for comparison.

![Graph](image-url)

S Figure 5. (a) Hydrolytic activity of free CRL and CRL-MENs at varied p-NPP concentration, and (b) kinetic parameters interpretation by a Lineweaver–Burk plot.
Determination of activity of HRP

During a run, 0.9 ml of pH 5.5, 100mM phosphate citrate containing 1.1mM \( \text{H}_2\text{O}_2 \), 0.05 ml of 0.02 M \( \text{H}_2\text{O}_2 \), and 10uL of 0.2 \( \mu \)g/mL HRP was added into a test tube. The reaction was initiated by adding 0.05 ml of DMSO containing 0.02 M 3,3’5,5’-tetramethylbenzidine (TMB) and monitored at 655 nm. The oxidation rate of TMB were interpreted from slope of the initial linear parts of the adsorption curve at 655 nm using a molar absorption coefficient (39,000 M\(^{-1}\)cm\(^{-1}\)) for the oxidation product of TMB.\(^9\) The Michaelis-Menten equation was applied to the free enzymes and MENs with TMB concentration from 0 to 1mM. The kinetic parameters were interpreted using a Lineweaver–Burk plot.

S Figure 6. (a) Peroxidative activity of free HRP and HRP-MENs at varied TMB concentration in the fixed amount of \( \text{H}_2\text{O}_2 \), and (b) kinetic parameters interpretation by a Lineweaver–Burk plot.
Determination of protease activity of trypsin

1 mL of 0.2 mg/mL N-a-benzoyl-DL-arginine-p-nitroanilide (BAPNA) solution (BAPNA dissolved in DMSO firstly followed by dilution with 50 mM HAc/NaAc buffer pH 4) was mixed with 200 µL of 0.1 mg/mL trypsin solution and incubated at room temperature for 10 min. After incubation, 200 µL 30% acetic acid was added to the reaction mixture to stop reaction. The absorbance of 405 nm using a molar absorption coefficient (9920 M⁻¹cm⁻¹) was measured in a quartz cuvette by a UV-VIS spectrophotometer model UV-2450 (Shimadzu, Japan). [10] The Michaelis-Menten equation was applied to the free enzymes and MENs with BAPNA concentration from 0 to 5 mM. The kinetic parameters were interpreted using a Lineweaver–Burk plot.

S Figure 7. (a) Hydrolytic activity of free trypsin and Tr-MENs at varied p-NPP concentration, and (c) kinetic parameters interpretation by a Lineweaver–Burk plot.
Determination of peroxidative activity of cytochrome C

Cyc and CyC-MENGs was assayed for peroxidative activity chromogenic substrate 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonate) in the presence of H$_2$O$_2$. Ferri-Cyc mediates the oxidation of ABTS in the presence of H$_2$O$_2$, with one mole of H$_2$O$_2$ reacting with two moles of ABTS. Fresh ABTS solution was added to the Cyc samples. Hydrogen peroxide was added to give a final assay mixture (contained 1.5mM CyC, 25mM ABTS, and 120 mM hydrogen peroxide) for free CyC and Cyc MENs. After adding H$_2$O$_2$, samples were immediately transferred to a UV-vis spectrophotometer and the absorbance increase at 415 nm using a molar absorption coefficient (36,000 M$^{-1}$cm$^{-1}$) was recorded for 5 min. The initial reaction rate and the peroxidase activity for each sample were then obtained.$^{[11]}$ The Michaelis-Menten equation was applied to the free enzymes and MENs with ABTS concentration from 0 to 0.08 mM. The kinetic parameters were interpreted using a Lineweaver–Burk plot.

S Figure 8. (a) Peroxidative activity of free CyC and CyC-MENGs at varied ABTS concentration in the fixed amount of H$_2$O$_2$, and (b) kinetic parameters interpretation by a Lineweaver–Burk plot.
S Table 3. Sample notation, preparation condition, Km, Vmax values and half-life under 50 °C and 60 °C of native CRL, CRL-MNPs complexes and CRL-MENs prepared under various conditions were listed.

<table>
<thead>
<tr>
<th>Sample notation</th>
<th>AM (mg/mL)</th>
<th>MBA (mg/mL)</th>
<th>Km (mM)</th>
<th>Kcat (S⁻¹)</th>
<th>Half-life at 50 °C (min)</th>
<th>Half-life at 60 °C (min)</th>
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</thead>
<tbody>
<tr>
<td>Native</td>
<td>/</td>
<td>/</td>
<td>0.23</td>
<td>3.44</td>
<td>240.7</td>
<td>30.3</td>
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<tr>
<td>CRL-MNPs</td>
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<td>0</td>
<td>0.19</td>
<td>1.52</td>
<td>259.9</td>
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<td>CRL-MENGs-1</td>
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<td>1.14</td>
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<td>89.8</td>
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<tr>
<td>CRL-MENGs-2</td>
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<td>0.26</td>
<td>1.2</td>
<td>&gt;480</td>
<td>94.3</td>
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<tr>
<td>CRL-MENGs-3</td>
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<td>0</td>
<td>0.29</td>
<td>1.2</td>
<td>&gt;480</td>
<td>211.4</td>
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<tr>
<td>CRL-MENGs-4</td>
<td>50</td>
<td>0</td>
<td>0.30</td>
<td>0.8</td>
<td>&gt;480</td>
<td>93.2</td>
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<td>1.25</td>
<td>&gt;480</td>
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<tr>
<td>CRL-MENGs-6</td>
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<td>0.28</td>
<td>1.51</td>
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<td>CRL-MENGs-7</td>
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<td>0.29</td>
<td>1.4</td>
<td>&gt;480</td>
<td>66.8</td>
</tr>
</tbody>
</table>

To investigate the effect of in situ polymerization, several batches of CRL-MENs with various amounts of monomers and crosslinkers were prepared. The Km and Kcat values were determined using increasing amount of p-NPP as substrates, and the thermal stability were assessed by incubating at 50 °C and 60 °C. Clearly, without polymerization, the thermal stability of CRL-MNPs hybrids was not improved. When increasing the initial monomer amount, the optimal monomer amount (30 mg/mL) was observed.
S. Table 4. CRL content of CRL-MENGs as prepared, determined by BCA assay and hydrolytic activity, in the supernatant by magnetic separation.

<table>
<thead>
<tr>
<th>Sample notation</th>
<th>MBA (mg/mL)\textsuperscript{a}</th>
<th>Protein content in supernatant\textsuperscript{b}</th>
<th>Hydrolytic activity in supernatant</th>
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<tr>
<td>CRL-MENGs 2</td>
<td>0</td>
<td>15%</td>
<td>10%</td>
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<tr>
<td>CRL-MENGs 7</td>
<td>0.075</td>
<td>0.20%</td>
<td>0</td>
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<tr>
<td>CRL-MENGs 6</td>
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<td>0.10%</td>
<td>0</td>
</tr>
<tr>
<td>CRL-MENGs 5</td>
<td>0.15</td>
<td>0.20%</td>
<td>0</td>
</tr>
<tr>
<td>CRL-MENGs 8</td>
<td>0.3</td>
<td>Not suitable for magnetic separation</td>
<td>/</td>
</tr>
</tbody>
</table>

\textsuperscript{a} All samples were prepared at monomer acrylamide concentration of 30 mg/mL, with varied crosslinker concentration.

\textsuperscript{b} the prepared CRL-MENGs were magnetically separated and dispersed in 50 mM phosphate buffer (pH = 6.0) and incubated at R.T. for 2 hrs. The re-dispersed CRL-MENGs were then magnetically collected and the supernatant were subjected to BCA assay to determine the protein content and p-NPP hydrolytic activity assay to determine the residual enzymatic activity.

In order to investigate the enzyme leakage problems of CRL-MENGs, the protein content and hydrolytic activity in the supernatant was measured. For batch 2, (no crosslinker), after incubation for 2 hrs, significant amount of CRL and hydrolytic activity were detected in the supernatant, hence batch 2 is not suitable for biocatalytic applications. When applying crosslinker during in situ polymerization, no traceable hydrolytic activity can be detected in the supernatant, and very small amount of protein content was detected in the supernatant in BCA, indicating the polymeric network prevent the leakage of proteins. Overuse of the crosslinker should not be recommended, as indicated in table, when MBA amount reaches 0.3 mg/mL, the product become too viscous and not suitable for magnetic separation.
References