Supporting Information

Co-immobilization of Three Cellulases on Au-Doped Magnetic Silica Nanoparticles for the Degradation of Cellulose

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Experimental Section

Materials.

Mercaptopropyltriethoxysilane were purchased from Aldrich. All other materials were of analytical grade and commercially available, including ferric chloride hexahydrate (FeCl₃•6H₂O), ferrous chloride tetrahydrate (FeCl₂•4H₂O), ammonium hydroxide (25% [w/w]), tetraethyl orthosilicate (TEOS), cupper chloride, imidazole, and ethylenediaminetetracetic acid (EDTA). Bio-Rad reagent for protein assay was obtained from Bio-Rad Laboratories (Hercules, CA). The water used throughout this study was de-ionized and filtered using a U.S. Filter purification system.
**Instruments.**

IR spectra were obtained for KBr pellets, in the range 400-4000 cm\(^{-1}\), with a Shimadzu FT-IR 8400S instrument. Field Emission Transmission Electron Microscope (FE-TEM) was measured with a Tecnai F20 and JEOL JEM-1400. Field Emission Scanning Electron Microscope (FE-SEM) was measured with a JEOL JSM-7500F(EDS; Oxford). X-ray Photoelectron Spectroscopy (XPS) was measured with a VG Multilab 2000. High Resolution X-Ray Diffractometer (HR-XRD) was measured with a X'Pert PRO Multi Purpose X-Ray Diffractometer. Magnetic properties were measured with a vibrating sample magnetometer (VSM, model-7404, Lakeshore) at room temperature. High performance liquid chromatography (HPLC) was measured with an RI detector (Waters 2414, USA) using a Rezex RPM column (4.6 x 300 mm; Phenomenex, USA). HPLC-grade water was supplied at a flow rate of 0.6 mL/min as a mobile phase, and temperature was controlled at 80 °C.

**Preparation of Magnetic Nanoparticles (MNP).**

FeCl\(_3\)•6H\(_2\)O (1.0 M) and FeCl\(_2\)•4H\(_2\)O (0.5 M) were dissolved in aqueous hydrochloric acid (0.4 M, 10 mL) at room temperature under sonication. After the salts were completely dissolved in solution, the mixture was degassed using a pump. Aqueous sodium hydroxide (0.5 M, 100 mL) was slowly added under nitrogen with stirring at room temperature. The mixture was left to react, heated in an oil bath at 80°C for 30 min. After cooling to room temperature, the magnetic nanoparticles were rinsed with aqueous hydrochloric acid (0.1 M) and ethanol to remove any unreacted impurities followed by resuspension in deionized water (40 mL) under sonication for 30 min. The supernatant was collected after centrifugation at 13500 rpm for 30 min at 4°C.

**Preparation of Magnetic Silica Nanoparticles (MSNP).**

Magnetite nanoparticles in suspension were added to a freshly prepared solution of tetraethyl orthosilicate (TEOS, 98%) in ethanol. The aqueous ammonia solution was dropped until the pH of the mixture raised to 12. Then, the mixture was refluxed at 100°C for 24 h and dense silica layer onto magnetite surfaces. Finally, the product was washed with ethanol for 3 times and dried at 60°C for 12 h in vacuum oven.
Preparation of MSNP with Immobilized Mercaptopropyltriethoxysilane.

Mercaptopropyltriethoxysilane (125 mg) was dissolved in anhydrous toluene (5 mL). The mixture was stirred under nitrogen for 5 min. The MSNP (40 mg) was added as solid. The reaction mixture was refluxed and stirred for 24 h. The collected solid was washed copiously with THF to rinse away any surplus mercaptopropyltriethoxysilane and then dried under vacuum.

Preparation of Au Nanoparticles.

Gold nanoparticle seeds were prepared according to the literature. Typically, this involves preparation of a 20 mL aqueous solution containing. To this solution was added 0.6 mL of ice cold 0.1 M NaBH₄ with stirring. The solution immediately turned orange-red, indicating formation of gold nanoparticles. The average particle size measured from transmission electron microscopy (TEM) was ~2.0 nm. Citrate serves as a capping agent in this case, and gold particles are stable for a couple of weeks. To the vial was added 9.0 mL of growth solution containing a mixture of 2.5 × 10⁻⁴ M HAuCl₄ and 0.10 M CTAB solutions. To this solution was added 50 μL of 0.10 M freshly prepared ascorbic acid, and the resulting solutions were stirred gently. Next, 1.0 mL of the seed solution was mixed with vial solution. After 10 s, 1.0 mL was drawn from vial solution.

Preparation of Au-MSNP.

To immobilize gold nanoparticles onto the surface of MSNP, the MSNP was added to Au nanoparticle solution (2 mL) for 24 h at room temperature. The collected solid was washed copiously with water (20 mL) three times to rinse away any surplus Au nanoparticles and then dried under vacuum.

Immobilization of Cysteine-Containing Cellulases with Au-MSNP or AuNP.

The phosphate buffer solution (PBS/NaCl, KCl, Na₂HPO₄, KH₂PO₄, 200 μL) contains cysteine-containing proteins (50 μg) with added Au-MSNP (5 mg) or AuNP (1 mL). The mixture was stirred at 0 °C in a darkroom for 1 day. The collected solid was washed
copiously with water (10 mL) to rinse away any surplus cysteine-containing proteins.

**Characterization**

The size and morphology of iron oxide nanoparticles before and after enzyme immobilization were determined by TEM using a JEOL 100-CX electron microscope. The binding of cellulase to the nanoparticle surface was determined using the Bradford protein assay. The protein found in the removed supernatant was measured by a colorimetric method involving the binding of Coomassie Brilliant Blue G-250 to the protein and then measuring the concentration across a wavelength of 595 nm. Bio-Rad dye reagent was used for the protein assay and bovine serum albumin as the standard. The binding of the enzyme was confirmed by Fourier transform infrared spectroscopy using a Thermo Nicolet Nexus 670 FTIR model and by X-ray photoelectron spectroscopy (XPS) using a Kratos Axis 165 XPS/Auger.

3,5-Dinitrosalicylic acid (DNS) is an aromatic compound which reacts with reducing sugars and other reducing compounds to form 3-amino-5-nitrosalicylic acid, which absorbs light strongly at 540 nm. The DNS method was used in this study to determine the amount of glucose formed as reducing sugars resulting from a reaction with dinitrosalicylic acid reagent. Sugars that are contained in polysaccharides, such as cellulose and starch, or other complex organic compounds, are not accounted for by this method. d-Glucose was used as the standard.

**Properties of the Free and Immobilized Cellulase.**

1. **Protein assay**

   Protein levels were determined using the Bradford assay.[1]

2. **The binding efficiency of enzyme**

   The binding efficiency of enzyme to magnetic particles was determined by first evaluating the saturation of cellulase enzyme on the surface of the precipitates. Subsequently, the ideal weight ratio (weight enzyme:weight of nanoparticles) was then determined in order to find
the optimum condition allowing for maximum activity. Weight ratio was determined by measuring the mass of immobilized enzyme as indicated by Bradford and associating this value with the mass of corresponding nanoparticles. The mass of magnetic silica nanoparticles was kept constant at 2 mg.

2 mg of Au-MSNP (or 1mL of AuNP) was added to 200 μL of phosphate buffer (PBS/NaCl, KCl, Na₂HPO₄, KH₂PO₄). The mixture was sonicated for 15 min and added cysteine-containing proteins from 20 μg to 500 μg. The mixture was stirred at 0 °C in a darkroom for 1 day. The mixture was centrifuged at 14000 rpm for 10 min. The amount of protein in the supernatant was determined by a colorimetric method at 595 nm using the Biorad Protein Assay Reagent Concentrate with bovine serum albumin (BSA) as the protein standard.

3. Effect of pH on cellulases activity

The activity assays were carried out over the pH range 3.0-7.0 to determine the pH profiles for the free and immobilized cellulases. 0.1 M citrate–phosphate buffer was used. Hydrolysis reactions were performed using 10 mg of cellulase-bound nanoparticles, 50 μg of microcrystalline cellulose, and 1 mL buffer. The results of pH are presented in a normalized form, with the highest value of each set being assigned the value of 100% activity. The pH stability of the free and immobilized cellulases was ascertained by the residual activity of the enzyme exposed to 3.0-7.0 under optimal conditions. For pH study, an equal amount of free cellulase enzyme (50 μg), as was immobilized on the AuNP and Au-MSNP, was tested for activity comparisons.

4. Effect of thermal stability

The thermal stability of the free and immobilized cellulases was measured at pH 4.5 and 80 °C. The enzymes were preincubated in citrate-phosphate buffer under optimal conditions for 0-48 h, and the residual activity was determined using pNPG as substrate.

Activity measurements

1. BglB assay
BglB activity was determined by measuring p-nitrophenyl release from p-nitrophenyl β-D-glucopyranoside (pNPG). The assay mixture containing 10 mM pNPG in citrate–phosphate buffer (pH 4.5) was incubated with the enzyme for 15 min at 80 °C in a total volume of 1 mL. The reaction was stopped by adding 1 M Na₂CO₃, and absorbance was measured at 405 nm.

2. CMC assay

CBHII activity was ascertained by measuring the amount of reducing sugars released from a 1% carboxymethyl cellulose (CMC) solution in 100 mM sodium acetate buffer (pH 5.0) for 1 h at 50 °C. One unit of activity was defined as the amount of enzyme that produced 1 M of glucose-reducing sugars per minute. The amount of reducing sugars was measured according to the dinitrosalicylic acid (DNS) method.[2]

3. Filter Paper assay

The filter paper assay was carried out by the method of Mandels' using 2 mg Whatman No. 1 filter paper strips as a substrate. The enzymes were diluted in 0.05M citrate buffer, pH 5.0, with a final volume in the assay of 0.5 mL. All assays were carried out in 18 mm test tubes at 50 °C for 1 h. The amount of reducing sugars was measured according to the DNS method.

Reusability assay

Cellulase enzyme complex (each 50 μg) co-immobilized on 5 mg of Au-MSNP were subjected to a hydrolysis reaction with microcrystalline cellulose under optimal conditions. After the specified reaction time, the enzyme-bound nanoparticles were magnetically separated and introduced into solution containing fresh substrate. Activity was determined following each recycle until the activity had fallen below 50%.

Additionally, a series of controls were measured in order to compensate for any interference, one containing enzyme alone and another containing substrate alone. Any responses measured by the spectrophotometer from these controls were subtracted from the primary hydrolysis reaction to aide in increasing accuracy among measurements.
Preparation of cystein-tagged β-glucosidase.

The β-glucosidase gene (bg1B) of Thermotoga maritima encodes 725 amino acids with a predicted molecular weight of 81kDa, and belongs to family 3 glycosyl hydrolases. The recombinant plasmid DNA, pRsetA vector harboring bg1B, was transferred to E.coli BL21 for recombinant protein expression. The protein was purified using affinity chromatography and confirmed by SDS-PAGE and obtains 81kDa single protein band.

Amino acid sequence of BglB protein (Thermotoga maritima):

1  TCATGGTTTG AATCTCTTCT CTCCCTCAAC CAGAAAAATA
41  TCTCTCAACC TTATATCCCT CGAAGATGCA CCGACCCTGA
81  CCTCGTATTC TCCTGACTCG ACAACCCATT CTTTCCCATC
121 GAAACTCGCA AGATCTCTGA GAGGAATTTC CAAGGAGATT
161 TCTTCTGATT CACCCCGGTT CAAAAGTTTT GTTTTGTGAA
201 ACGCTTTTCG CTCTGGAAG GTTTGTCTTA TTTTCCCTTT
241 TGGAGCTTTTG ATGTAGACCT GTGAGACTTC TTTTCCAGCT
281 CTGTCCCCAG TGTGTTGTGAT CGTGTACGAC ACTCTGAGCG
321 TCTCACCCTG TATAGCGATT TTAAATCTTT TGTATTCAAA
361 CTTTGTGAA GAGAGGCGTG AGCCGAATTTC GTAGGCAGGT
401 TCCACACCAG AGGTGTCGTA GTACCCTGTAT CCCACGTAGA
441 TGTCTTATCT GTACCACACT CTTTCCGGAT TGTCTTCTTG
481 CTCTTCTGGG AACGTCCAGG ATGGAACGTC CGAGTAATCC
521 TTCGAGGAAGG TCGGTGGAAG TTTTCCGGAG GGATTAATCT
561 TTCCCACAAG AACATCGGCC ACTATTCTTC CCATCTCCTG
601 TCCCGCCCTGC CAGACGAGAA GAATTCCATC CACAAGTCT
641 TCCACGCTTG CGACTTCGAT GGGACTTCGG ATGTTCAGAA
681 GAACCACAAC TTTCCTTACC CGTCGCTGGGA ATCTTCTCAG
721 GACGGTTTTT ATGAGTTTCA GCTCGTCATC GGAGAGGTAG
761 AAGTCCACTT CCACCAGGTT TCTGCGTAAT CCTCACCAGG
801 AGATCTCTACT GATCACAACA ACTGCAACAT CGTTTCTCTT
841 TGCAGCTTTT CTTATCTCCT TTCGTGAGAG AAGATTCTCT
881 GGGAGTTCTG GTTTATGAC CGTTCCCCAA GAGTCGGTTC
921 TGGGTTTATA TTCCTCTGTT TCTCTCATCT TTTTTATGTA
961 CTCCTCATATA GTGGAAGCGA GTTCTTCTGTC GAACCTCATG
1001 TTTCTTTCTT TTATGCTTTC AAGGATAGAG ATCGTGTATC
1041 TCGGATGGGT GTCTCCACTT CCGGTTTCTC CTTTTATGT
1081 TTGGATTTGA CCAGGTGCCAA AGACGCGCAC ATGGGTAATT
1121 TCATCGAACG GAAGAACACC GTTGTCTTCA AGAAGGAACA
1161 CACCCCTCGC ACCTGCTTCA AGGCGACTT CCGGATGAGA
1201 TTGTGATGCC GCCTTGTTTG AGTACCTGTA CCGGGTTGAAG
1241 GAAGGCGGCT TCACAAGAAC TTTGAGAATG TTTTTCACAC
1281 ACTCATCGAG AACCTCCTCA CTCAAATTTG CTTCCCTCAG
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1521 TTCCATTCTG ATTTTCTGAA GCGCTCATCAG GCGCTCCAGG
1561 TCTTGCTTTT TGGACAGCAG AAGCTAAGAC TTTTTCAGAT
1601 AATTTCAATG CCGATGTTAC CAGTCATGCA GTGTCGACAA
1641 CACCGCTTGT CTTTTTCTTG TGTGTCGAGCA CAAATGTGTT
1681 TATACATGCT CCCACCCCTT GGATGAGATC TCCCTTGCAC
1721 AGAGCCTGAA CCATTCTGAG GAAAGGGAAC GAGATCTTTCTG
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1801 AATGGTCTAC GCAGGATTGA AAAGCAGCAT GCAGCCGAT
1841 CCACTTACCTG CTTTCATCTC GGGCTCGGAG AAGCTTCCCAG
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1921 GGAAATGCT GGGTGTGATG AAGTGTTTTC ATCGTCTTCC
1961 CTGGGCTGAT TTATTCGAG TCTCGGGGA CCATCGGCAA
2001 GAGACTCAGC AAGATTTTGA AGTCTTGGGA GGGATGTGT
2041 TTGCTCATGC GCACCCCGGA CTCTGGAATG GGGTTCTCAG
2081 AAGATCTGAC GAAAGCAACCCCCACGAC AGCTTCACCT
2121 TTACGCTCTGT AGTAACTGCA GAGGAATTT CATCGATCCT
2161 TTCCATTGCTGCTG
Amino acid sequence of CBHII protein (Neurospora crassa):

1   ATGGCTGCCA AGAAGCTCCT TCTCGCTGCC GCCTTGACGG
41  CCTCTGCCCT CGCCGCTCCC GTCCTCGAGG ATCGTCAGAA
81  CTGCGGCTCT GCCTGGAGCC AGTGTGGTGG CATTTGGCTGG
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Amino acid sequence of EGIVCBDII protein (Ruminococcus albus gene for endo-1,4-beta-glucanase):

1 ATGTTGGATA AACTAAAGT AATAAATGGA AAACCTGACAG
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Figure S1. TEM images of MSNP (a) before and (b) after functionalization with mercaptopropyltriethoxysilane. The images show nanoparticles with dark Fe₃O₄ cores (a single-crystalline cubic spinel structure, about 20 nm in diameter) surrounded by lighter amorphous silica shells about 3–4 nm thick. The diameter of MSNP also increased by another 2–3 nm after coating with MPTES.
Figure S2. EDX spectrums of MSNP (a) before and (b) after functionalization with mercaptopropyltriethoxysilane. EDX measurements confirmed the presence of sulfur atoms.
Figure S3. XRD patterns of MSNP (a) before and (b) after functionalization with mercaptopropyltriethoxysilane. XRD diffractograms of the MSNP exhibited patterns consistent with those of spinel ferrites described in the literature. The diffraction peaks at (220), (311), (400), (422), (511), and (440) are characteristic of magnetite. The same peaks were observed in the diffractograms of MSNP with immobilized MPTES.
Figure S4. FT-IR spectra of MSNP (a) before and (b) after functionalization with mercaptopropyltriethoxysilane: (KBr pellet). Following the attachment of MPTES to MSNP, a CH$_2$ stretching peak appeared at 2920 cm$^{-1}$ in the FT-IR spectrum, strongly indicating covalent attachment of the MPTES group to the surface of the MSNP.$^4$
Figure S5. Magnetization curve of (a) magnetic silica nanoparticles (MSNP) (saturation magnetization value: 91.1 emu/g), (b) MSNP functionalized with mercaptopropyltriethoxysilane (65.6 emu/g), and (c) the gold-doped MSNP (Au-MSNP) (33.3 emu/g), obtained by VSM at room temperature.
Figure S6. UV-Vis spectrum of (a) colloidal gold solution and (b) after stirring with SH-functionalized MSNP. The surface plasmon resonance (SPR) of particles in the colloidal gold solution can be seen at 530 nm.
Figure S7. TEM images of (a) AuNP@cellulases and (b) Au-MSNP@cellulases.
Figure S8. EDX spectrums of (a) AuNP, (b) AuNP@cellulases, and (c) Au-MSNP@Cellulases.
Figure S9. EDX mapping of the cellulases co-immobilized Au-MSNP. (a) Zero-loss image, (b) iron component, (c) oxygen component, (d) silicon component, (e) gold component, (f) carbon component and (f) nitrogen component.
Figure S10. XPS analysis of (a) Au-MSNP, and (b) Au-MSNP@cellulases. (c) C1s XPS spectra.
Table S1. Elemental analysis of **Au-MSNP** and **Au-MSNP@cellulases** from evaluation by XPS.

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Figure S11. Binding efficiency for varying amounts of cellulases added to 2 mg of Au-MSNP.
Figure S12. Effect of pH on free and co-immobilized cellulases activity using (a) CMC (at 50 °C) and (b) filter paper assays (at 50 °C).
Scheme S1. Illustration of the mechanism of enzymatic cellulose degradation.
Figure S13. Activity loss over 7 reuses of cellulase enzymes immobilized on AuNP and Au-MSNP.; (a) Enzymatic activity on CMC, (b) Enzymatic activity on filter paper.
References.


