Electronic Supplementary Material (ESI) for ChemComm.

Characterizing Protein Modifications by Reactive Metabolites using Magnetic Bead Bioreactors and LC-MS/MS

Supporting Information

Chemicals and materials

Human liver supersomes 2E1 (Cyt P450 2E1OR with p450 reductase and Cytochrome b5) were purchased from CORNING (Woburn, MA, USA). Carboxylated magnetic particles were from Polysciences (Warrington, PA; 1 μm diameter; concentration 20 mg mL^{-1}). Acetaminophen, Authentic N-acetyl-p-benzoquinone imine (NAPQI), urea, reduced glutathione (GSH), Glutathione S-transferase from human placenta, NADPH regenerating system: glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PDH), and nicotinamide adenine dinucleotide phosphate (NADP), were from Sigma-Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT), iodoacetamide (IAA) were obtained from Thermo Scientific (Rockford, IL, USA). Sequencing grade modified trypsin was from Promega (Madison, MI, USA). All of the solvents were of high-performance liquid chromatography (HPLC) grade from Sigma-Aldrich (St. Louis, MO, USA).

Experimental procedures:

Fig. S1 Film fabrication on magnetic beads (MB). 200μl of negatively charged magnetic particles (2mg ml^{-1} in tris buffer, pH 7.4) was mixed with 200μl of polycation poly(diallyldimethylammoniumchloride) (PDDA) (2mg/ml in water, containing 50 mM NaCl). The mixture was kept in ice for 20-min until PDDA adsorption saturated on the particles. Then the PDDA-coated particles were first separated from the solution using magnets, followed by washing and redispersing in 10 mM tris buffer (pH 7.4). The same assembly and washing steps were repeated for polyanion poly(styrene sulfonate) (PSS) and CYP2E1 supersomes film growth. Supersomes required 30 min to reach steady state adsorption.[1] The final architecture on the magnetic bioreactors was MP/PDDA/PSS/PDDA/2E1.

Protocols for magnetic biocolloid reactors incubation (Fig. 1)
After film fabrication, magnetic biocolloid reactor particles were dispersed in 400 μl 0.1 M phosphate buffer (pH 7.4) containing NADPH-regenerating system (10 mM glucose-6-phosphate, 4 units of glucose-6-phosphate dehydrogenase, 10 mM $MgCl_2$, 0.80 mM β-$NADP^+$), 2mM DTT and 1mM acetaminophen. For metabolites study, 1mM GSH was added to the solution to trap the reactive metabolites NAPQI. For protein adducts study, 50 μg hGSTP was used as the target protein for covalent modification of acetaminophen metabolites. Negative control experiments were conducted in the absence of NADPH-regenerating system or acetaminophen substrate. After incubation for 30 min at 37°C, the reaction was terminated by magnetic separation. Positive control experiment was performed by using 100 μM authentic NAPQI directly react with 50 μg hGSTP in phosphate buffer solution.

Tryptic digestion of hGSTP
Buffer exchange. After magnetic separation the supernatant was transferred to Amicon® Ultra centrifugal filters (Millipore, Billerica, MA, USA) with 3,000 molecular weight cutoff to desalt and exchange buffer. After loading sample solution to inner tube with mass cutoff filtering membrane, it was centrifuged at 16,000×g for 30 min to remove majority of phosphate buffer into the outer tube. Nevertheless, about 40 μl of protein containing buffer stay in the inner tube. Then 500 μl of 50 mM ammonium bicarbonate buffer (pH 8.0) was added into the inner tube, and centrifuge again for 30 min at the same speed to filter out the rest of phosphate salt. The step for removing phosphate salt was repeated twice. Finally, the concentrated sample was reconstituted with 100 μl ammonium bicarbonate buffer and subjected to tryptic digestion.
Tryptic digestion. hGSTP reduction was carried out by adding 4 μl of 500 mM DTT to the sample solution for one hour at room temperature. The free thiol group on cysteine residues were then alkylated by addition of 8 μl of 500 mM IAA and incubated in dark for one hour at room temperature. Excess IAA was then neutralized by 8 μl of DTT for 15

min. The resulting samples were digested by sequencing grade modified trypsin in a trypsin/protein (w/w) ratio of 1:25 for 15 hours. The reaction was stopped by adding 2 µl of formic acid.

LC-MS/MS analysis

NAPQI-GSH metabolites analysis. A capillary LC (Waters, Capillary LC-XE, Milford, MA) was used as previously described.[1] 10 µL of sample was injected to a Luna C18 trap column (0.5mm × 20mm, 5µm, Phenomenex) and flushed at a flow rate of 10 µL min^{-1} with water (with 0.1% formic acid) to eliminate the phosphate buffer salt. After 3 min, the analytes were flushed to the analytical column, Luna C18 column (0.5mm × 150mm, 5µm, Phenomenex) with A, water (with 0.1% formic acid) and B, acetonitrile (with 0.1% formic acid). The gradient was 5% B for 5 min, 5-90% B for 20 min, 90% B for 2 min, 90-5% B for 2 min, 5% B for 1 min. A 4000 QTRAP® (AB Sciex, Foster City, CA) mass spectrometer with Analyst 1.5 software was operated in the positive mode with a Turbo IonSpray ionization source. Multiple reactions monitoring (MRM) was conducted at 5300 V ion spray voltage, 275 °C, 50 V declustering potential, 30 eV collision energy, and 0.15 s dwell time for different mass transitions: internal standard (136→94), NAPQI-GSH metabolites (457→328). Enhanced product ion scanning (EPI) was performed using collision-induced dissociation (CID) with 30 eV collision energy for NAPQI-GSH parent ion m/z 457.

NAPQI-hGSTP analysis. 2 µL of tryptic peptide mixtures were loaded to a Jupiter C18 column (0.5 mm × 150mm, 5µm, Phenomenex). The gradient for the analytes separation in the analytical column is: 5% B for 5 min, 5-50% B for 65 min; 50-95% B for 5 min, 95% B for 5min, 95-5% B for 3 min and 5% B for 2 min at a flow rate of 15 µL min^{-1}. Information-dependent acquisition (IDA) was performed on a QSTAR® Elite mass spectrometer (AB Sciex, Foster City, CA) equipped with a TurboIonSpray source in positive mode with a spray voltage of 5500 V and desolvation temperature of 300 °C. The method ran a full ion scan survey in the *m/z* range 300 to 1500, followed by MS/MS acquisition on the two most prominent precursor ions from the survey scan. After an ion was selected once it would be excluded from MS/MS acquisition in the following 30 seconds.[2] The mass-to-charge value and retention time from negative control sample IDA scan was listed in a rejected mass list for the analysis of enzymatic incubation samples.

Mascot search

The LC–MS⁄MS data were submitted to a local mascot server for an MS⁄MS protein identification search using Mascot Daemon (version 2.3) against NCBInr database with the following parameter settings, Taxonomy: Homo sapiens; Fixed modifications: none; Variable modifications: oxidized methionine (M), NAPQI (C); Enzyme: trypsin; Number of allowed missed cleavages: 2; Peptide mass tolerance: 100 ppm; MS/MS mass tolerance: 0.5 Da. Charge state: 2, 3 and 4. The NAPQI modified hGSTP was identified with peptides that exhibit ion score > 30 ($P < 0.05$).

Characterization of films on magnetic bioreactors

Table S1. Estimated amount of metabolic enzymes deposited on the magnetic particles (MP) using Bradford assay. Magnetic particles modified with triple-layer composites was observed about 15% increase in 2E1 adsorption comparing to magnetic particles with single polyion layers.

Composition (per mg particles)	Metabolic enzymes (µg of protein)
PDDA/2E1	487 ± 11
PDDA/PSS/PDDA/2E1	558 ± 45

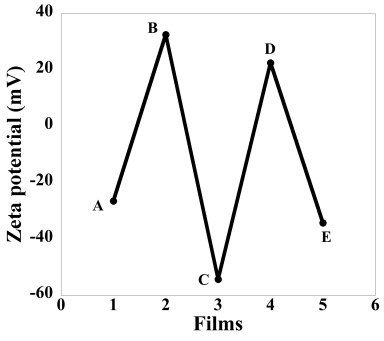

Fig. S2. Zeta potential was measured to determine the electrophoretic mobility of each surface layer in the colloidal system. (A) MP, (B) MP/PDDA, (C) MP/PDDA/PSS, (D) MP/PDDA/PSS/PDDA, (E) MP/PDDA/PSS/PDDA/2E1. Surface charge sign changed with each successive layer. The zeta potential of the final MP/PDDA/PSS/PDDA/2E1 bioconjugate particles appeared negatively charged.

References

1 Zhao, L.; Schenkman, J. B.; Rusling, J. F. *Anal. Chem.* 2010, **82**, 10172–10178.

2 Yukinaga, H.; Iwabuchi, H.; Okazaki, O.; Izumi, T. *J. Pharm. Biomed. Anal.* 2012, **67-68**, 186–192.