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

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Abstract

We report here label-free metabolite-protein adduct detection and identification employing magnetic beads coated with metabolic enzymes as bioreactors to generate metabolites and possible metabolite-protein adducts for analysis by liquid chromatography-tandem mass spectrometry.

Pollutant and drug toxicity in humans is very often caused by reactive metabolites that damage proteins, DNA, and other biomolecules.^{1, 2} Covalent binding between a carcinogenic molecule and tissue proteins was first reported over 70 years ago.³ In the early 1970s, pathways of liver toxicity induced by drug-protein reactions were revealed using radiolabeled toxicants in rodents.⁴ Metabolite-protein adduct studies still rely on expensive radiolabeled reactants or affinity capture to determine the adducted proteins. Sample fractionation and protein digestion are often necessary before mass spectrometry analyses.^{5, 6}

Label-free strategies for metabolite-protein adduct determinations have been investigated. Yukinaga et al.⁷ incubated human glutathione S-transferase pi (hGSTP), microsomal enzymes and drugs, then fractionated the sample by LC and collected adducted and unadducted hGSTP fractions. The hGSTP fractions were digested and analyzed by subsequent LC-MS/MS and a database search. This method advanced metabolite-protein adduct detection, but sample fractionation steps are cumbersome and target modified protein is difficult to separate from the many microsomal proteins. A major challenge for enzymatic protein adduct characterization in complex samples is that binding levels are generally low so that differentiating tiny amounts of adducted protein from complex proteomes is difficult. In this communication, we report a label-free, cell-free *in vitro* assay suitable for screening using magnetic microsomal bioreactor beads to generate metabolites, and subsequently protein adducts, for detection of covalent protein modification (Fig. 1). The magnetic bead-enzyme bioconjugates, used for the first time to generate metabolite-protein damage, greatly simplify separation of damaged target proteins from the microsomal enzymes.

We chose acetaminophen as the test compound for proof-of-concept since it is responsible for ~80% of drug-related liver failures,⁸ even though it has been used as an analgesic agent for 100 years.⁹ Acetaminophen is bioactivated by microsomal enzymes and transformed into reactive metabolite N-acetyl-*p*-benzoquinone imine (NAPQI).¹⁰ Limited amounts of NAPQI can be detoxified by reduced glutathione (GSH). However, in the case of acute overdoses, GSH depletion saturates the liver's detoxifying capacity. NAPQI covalently binds to microsomal proteins, which can lead to hepatic necrosis.¹¹ Liver microsomal glutathione S-transferase can catalyze GSH conjugation with electrophiles.¹² With depletion of GSH in the liver, GST itself becomes a covalent binding target for NAPQI.¹³ Human glutathione S-transferase pi (hGSTP) was used as a model target protein of NAPQI. It is easily expressed in *E. coli* and well characterized.^{14, 15} This relatively small protein features four cysteine residues (Cys-14, 47, 101 and 169), among which Cys-47 has the highest reactivity due to its relatively low pKa (3.5–4.2) and accessibility.¹⁶

We previously developed magnetic and silica beads coated with microsomal enzymes as bioreactor beads for metabolic profiling, as well as with enzyme/DNA coatings for investigating DNA damage by reactive metabolites.^{17,18} Microsomal enzyme layers were formed by alternate electrostatic layer-by-layer (LbL) adsorption of oppositely charge layers on these beads. This simple but universal method produces stable, active enzyme-magnetic beads without the need for chemical reactions.¹⁹ Human cytochrome P450 (CYP) CYP2E1 is the main cyt P450 enzyme isoform responsible for bioactivation of acetaminophen.²⁰ In the present work, we used supersomes of CYP2E1, which are recombinant microsomes containing only this single cyt P450 and its reductase. The negatively charged supersomes were deposited onto magnetic beads with underlying polycation/polyanion/polycation layers via the LbL method to make metabolic enzyme bioreactor beads. (SI, Fig. S1, S2) The optimized bioreactor beads included sequential layers of poly(diallyldimethylamine) chloride (PDDA), polystyrene sulfonate (PSS), and PDDA before supersomal enzyme adsorption. The final architecture PDDA/PSS/PDDA/CYP2E1 provided ~15% more CYP2E1 enzyme than a simpler architecture PDDA/CYP2E1. (SI, Table S1)

An acetaminophen metabolite study was done by dispersing CYP2E1-bioreactor beads into phosphate buffer pH 7.4 that contained GSH, acetaminophen and NADPH cofactors to initiate the reaction. GSH in the incubation system trapped the reactive metabolite NAPQI after its generation, and the bioreactor beads were removed with a magnet. Detection of the NAPQI-GSH conjugates provided confirmation that acetaminophen was metabolized by CYP2E1-bioreactor beads.²¹ The enhanced product ion (EPI) scanning spectra showed typical fragmented ion patterns m/z 382, m/z 328, m/z 311, m/z 208, m/z 182, m/z 140, which are characteristic of NAPQI-GSH.²¹ (Fig. 2A)

The amount of NAPQI formed was measured by LC-MRM using acetanilide (m/z 136>94) as an internal standard. In 30 min reactions, the formation rate of NAPQI-GSH was $0.34 \pm 0.03 \text{ nmol min}^{-1} (\text{nmol CYP2E1})^{-1} (\text{mM acetaminophen})^{-1}$. (Fig. 2B) These results indicate that the CYP2E1 on magnetic bioreactor beads is enzymatically active and efficiently converts acetaminophen into the expected reactive metabolite NAPQI.

To investigate formation of metabolite-protein adducts, CYP2E1-bioreactor beads were reacted with acetaminophen and hGSTP in the NADPH/pH 7.4 buffer in the presence of hGSTP protein dissolved in the solution (Fig. 1) The reaction was terminated by magnetically removing the CYP2E1-bioreactor beads from the buffer, leaving NAPQI-adducted and non-adducted hGSTP in the solution. Due to low extent of reaction between NAPQI and hGSTP, the disulfide bonds at cysteine residues in unreacted hGSTP were subsequently reduced by dithiothreitol and following by alkylation using iodoacetamide before tryptic digestion. The purpose of alkylation is to protect the free thiol group from undesirable reactions that may lead to failure to detect NAPQI-modified peptides. After tryptic digestion, LC-MS/MS analysis for the drug-protein adduct sample employed information dependent acquisition (IDA) using a reject mass list, which excludes the most abundant unwanted ions and gives better opportunity to select the target modified peptide ions for MS/MS.⁷

The resulting peptide MS/MS spectra were searched for peptide sequences match against human NCBItr peptide database using the MASCOT search engine to identify proteins and modified fragment ions.²² NAPQI with monoisotopic mass of 149.048 Da was added to the database for a NAPQI-adducted peptide search. As a result, NAPQI-adducted peptide ASCLYGQLPK at protein hGSTP position 45 to 54 was successfully identified. The m/z of the NAPQI-modified peptide 45–54 parent ion $[M+2H]^{2+}$ was 614.800 (Fig. 3A&B). Comparing with the theoretical value 614.805, the mass accuracy was 8.7 ppm. In the negative control experiment without metabolic reaction, the unmodified peptide 45–54 with m/z 568.811 was found. (Fig. 3C) Note that the thiol groups from the cysteine residue in unmodified peptide 45–54 have been alkylated by iodoacetamide before tryptic digestion. Therefore, the observed parent ion $[M+2H]^{2+}$ m/z 568.811 contains an additional 57.022 Da from S-carbamidomethylation of Cys, and the real m/z for unmodified peptide should be 540.300. The MS/MS spectrum of NAPQI-modified peptide 45–54 is shown in Fig. 3B. The NAPQI-modified residue was determined to be Cys-47, since the fragment ion of b_3^{1+} m/z 411.156 and y_8^{1+} m/z 1070.530 showed a mass shift of 149.0 Da comparing to unmodified hGSTP peptide control. Meanwhile, m/z of all of the detected fragment ions that involve Cys-47 residues, b ions from b_3 to b_8 and y ions from y_8 to y_9 , showed the addition of 149.0 Da from NAPQI (Fig. 3B&C). No other cysteine-containing peptides were found to be modified by NAPQI from our search.

Characterizing the ability of metabolites to covalently modify proteins and determining the adducted protein structure is important for drug and chemical safety assessment. Modified protein conjugates may reduce or destroy the protein activity or function,^{23,24} alter protein-protein interactions,²⁵ and/or induce responses from element-mediated gene transcription.²⁶ However, not all covalent binding leads to toxicity. Some non-hepatotoxic drugs may show a higher covalent binding level than hepatotoxins. The relationship between protein covalent binding and hepatotoxicity is not completely clear. Nevertheless, the assay we describe here represents a simple new screening tool screen for the possibility that covalent binding of a metabolite to proteins can occur, and provides chemical information that can be combined with other toxicity tests for informed decisions to predict potential human toxicity.

In summary, we have demonstrated a novel metabolite-protein adduct generation and characterization assay using magnetic bioreactor beads and LC-MS/MS analysis. The model drug acetaminophen was enzymatic activated by CYP2E1 supersomes on the bioreactor beads, and the resulting reactive metabolite NAPQI subsequently reacted with target protein hGSTP in the buffer. Reacted and unreacted hGSTP are isolated for LC-MS/MS analysis by using a magnet to trap the bioreactor beads, and removing the supernatant liquid. This approach provides a simple, label-free way to generate and purify reactive metabolite-protein conjugates. Advantages over previous methodology include the lack of reactant labelling or complex fractionation schemes. This relatively rapid, chemically informative *in vitro* methodology could be adapted for virtually any drug metabolites and microsomal enzyme combination for protein damage screening at early stages of drug or environmental chemical development. It could also be easily adapted to multiple target proteins, and to the 96-well plate format of our previously developed high throughput biocolloid bead DNA damage assays.¹⁸

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Work flow for protein adduct generation and characterization using magnetic bioreactor beads.

Fig. 2. LC-MS/MS results for GSH-trapped acetaminophen metabolites. (A) EPI spectrum of NAPQI-GSH; (B) TIC (Total Ion Current) chromatogram of LC-MS/MS with MRM mass transition pair m/z 457 to m/z 328.

Fig. 3.

Quadrupole-TOF MS analysis of peptide ASCLYGQLPK from hGSTP: (A) MS¹ spectrum of NAPQI-modified peptide from sample after incubation of acetaminophen and hGSTP with CYP2E1 bioreactor beads: (B) representative MS/MS spectrum of NAPQI-modified peptide ASCLYGQLPK from sample after incubation with acetaminophen. Parent ion [M+2H]²⁺ is m/z 614.800 The b or y fragment ions that reflect the NAPQI mass shift are in red. (C) MS/MS spectrum of standard unmodified peptide ASCLYGQLPK from hGSTP after S-carbamidomethylation at Cys 47. The parent ion [M+2H]²⁺ was 568.810.