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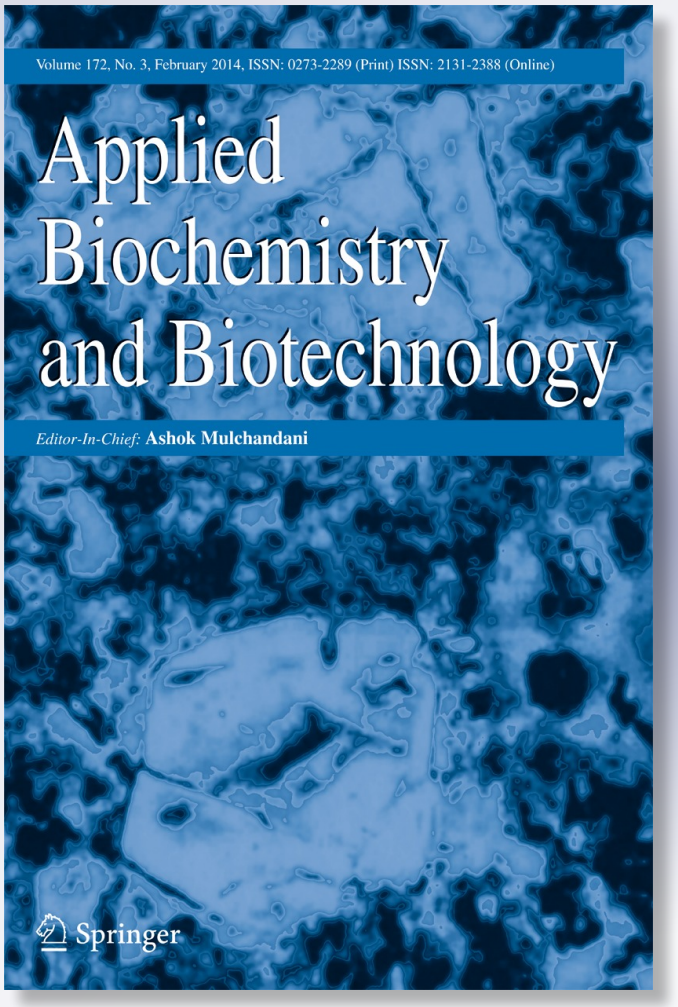

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The Use of Immobilized Cytochrome P4502C9 in PMMA-Based Plug Flow Bioreactors for the Production of Drug Metabolites

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Abstract Cytochrome P450 enzymes play a key role in the metabolism of pharmaceutical agents. To determine metabolite toxicity, it is necessary to obtain P450 metabolites from various pharmaceutical agents. Here, we describe a bioreactor that is made by immobilizing cytochrome P450 2C9 (CYP2C9) to a poly(methyl methacrylate) surface and, as an alternative to traditional chemical synthesis, can be used to biosynthesize P450 metabolites in a plug flow bioreactor. As part of the development of the CYP2C9 bioreactor, we have studied two different methods of attachment: (1) coupling via the N-terminus using *N*-hydroxysulfosuccinimide 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and (2) using the Ni(II) chelator 1-acetato-4-benzyl-triazacyclononane to coordinate the enzyme to the surface using a C-terminal histidine tag. Additionally, the propensity for metabolite production of the CYP2C9 proof-of-concept bioreactors as a function of enzyme attachment conditions (e.g., time and enzyme concentration) was examined. Our results show that the immobilization of CYP2C9 enzymes to a PMMA surface represents a viable and alternative approach to the preparation of CYP2C9 metabolites for toxicity testing. Furthermore, the basic approach can be adapted to any cytochrome P450 enzyme and in a high-throughput, automated process.

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Introduction

Cytochrome P450 (P450) enzymes play a major role in the metabolism of pharmaceutical compounds [1] and, consequently, new chemical entities are screened with human P450 enzymes for metabolism [2, 3]. When metabolites are formed, it becomes necessary to assess toxicity or whether other undesirable effects are produced by the metabolites formed [4–10]. Prior to testing, the structure of the metabolite must be determined [11] and then synthetic routes must be devised and traditional organic chemistry techniques applied to prepare the metabolite(s).

The metabolism of substrates by P450 enzymes is known for producing compounds in a regiospecific and/or stereoselective manner [12–14] and may occur at locations that can be difficult to achieve chemically. Synthetic challenges may be problematic since they can cause delays in drug development, yet metabolite toxicity must be assessed prior to moving the drug development process forward. Solutions for this potential bottle neck will expedite the drug development process and, thus, there is a need for approaches to rapidly produce small quantities of metabolites [11].

P450 enzymes have been used to synthesize compounds that are otherwise difficult to prepare [15, 16]. However, to be able to produce sufficient quantities for characterization or other purposes, some type of bioreactor will likely be required [17]. One approach is to incorporate the P450 enzyme into an electrode and by controlled potential electrolysis electrochemically generate the desired metabolite(s) [18–21]. There are several advantages to this approach. The stability of the P450 enzymes in the electrode matrix is generally good which allows multiple uses. The electrodes are usually easily prepared and the approach does not require the uses of coenzymes or cofactors. At the same time, there are some concerns. In some cases, the metabolite(s) may not be stable and undergo additional enzyme-mediated transformations to compounds that are not biologically relevant [22]. Further, there is no guarantee that the metabolites that the full set of metabolites will be produced as are observed when the endogenous cofactors and coenzymes are used.

An alternative approach is to use a bioreactor. The physical design of bioreactors [23] falls into two general types: batch [24, 25] and continuous flow bioreactors [26, 27]. Batch bioreactors are generally inexpensive to make but do not allow for the addition of biochemically necessary agents or the removal of products that might undergo further transformation. In contrast, continuous flow bioreactors pass the reactants and other required materials into the reactor and remove products from the reactor allowing for a variety of configurations. Thus, it may be possible to use a bioreactor to prepare small quantities of materials needed for metabolite toxicity studies.

Recent advances for the production of recombinant P450 enzymes [28] has resulted in methods that can efficiently produce P450 enzymes in high concentrations facilitating the use of P450 enzymes in bioreactor applications [29]. Current strategies for bioreactor-based metabolite generation include the use of bacterial [30], insect [31, 32], and hepatocyte [33] cell-based systems. Other strategies include the coupling of human liver microsomes (HLM)-based systems to high-performance liquid chromatography (HPLC) instrumentation [34–36] in a plug flow setup. In the case of the latter system, these constructs provide a tool for structural determination of metabolites from pharmaceutical compounds though they are not intended for the generation of sufficient quantities of metabolite necessary for toxicity testing or other

research and development activities. However, with modification, it may be possible to scale up this system to produce a sufficient quantity of metabolite to assess toxicity.

Here, we present a plug flow bioreactor design, where the P450 CYP2C9 is bonded to a poly(methyl methacrylate) (PMMA) surface. Amine-directed coupling of recombinant CYP2C9 to UV-activated PMMA is easily achieved. Key for the success of this bioreactor is to bond CYP2C9 in the presence of the substrates flurbiprofen and dapsone; otherwise, normal biological activity is lost [37]. Furthermore, we have tested several different conditions, such as varying the concentration of the enzyme used during attachment and variation of the time used for enzyme attachment, to maximize metabolite production. Finally, we demonstrate the ability of the resulting bioreactor to generate metabolites of interest from the CYP2C9 probe substrate, diclofenac.

Experimental

Materials

Diclofenac sodium, flurbiprofen, *N*-hydroxysulfosuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*′-ethyl-carbodiimide hydrochloride (EDC), beta-nicotinamide adenine dinucleotide phosphate, reduced form (nicotinamide adenine dinucleotide phosphate (NADPH)), nickel chloride, dapsone, and *N*-phenylanthranilic acid were purchased from Sigma-Aldrich (Milwaukee, WI). 4′-Hydroxydiclofenac was purchased from Toronto Research Chemicals (Toronto, Canada). 1-Aceto-4-benzyltriazacyclononane (AcBzTacn) was a gift from L.L. Martin (Monash University, Australia). Cytochrome P450 reductase (CPR) was purchased from BD Biosciences (San Jose, CA). Purified CYP2C9 was prepared as previously reported [38]. Potassium phosphate buffer (KPi) was made by mixing 40 mM solutions of dibasic and monobasic potassium phosphate such that the resulting solution of mixed components had a pH of 7.4. Mixtures of EDC and NHS used for enzyme immobilization consisted of 3 mM EDC and 5 mM NHS in KPi. Solutions were combined in a 1:1 ratio (*v*/*v*) and substrates were immersed. Solutions used for immobilization of CYP2C9 contained flurbiprofen (40 μM) and dapsone (40 μM). Cryobuffer solution was comprised of KPi and glycerol (80:20, *v*/*v*).

PMMA Chip Fabrication

For initial, proof-of-concept experiments, small (4×6 mm) PMMA chips were cut from a PMMA sheet (Goodfellow, Oakdale, PA) using an Epilog Legend EXT carbon dioxide laser (Golden, CO). Each side of the PMMA chips was irradiated with ultraviolet light (280 nm) (Newport Corporation, Irvine, CA) for 15 min prior to use.

PMMA Bioreactor Fabrication

The PMMA-based bioreactor was fabricated by micromilling the channel using a Sherline Model 2010 milling station (Vista, CA) from PMMA blanks (McMaster Carr, Elmhurst, IL). The milling of the channel was conducted by Protea Biosciences (Morgantown, WV). Access holes and threads were all drilled with a standard drill press and were designed to fit standard HPLC fittings (Upchurch Scientific, Oak Harbor, WA). The milling schematic and dimensions for the plug flow PMMA bioreactor are shown in Fig. 1. Both the top cover and milled channel were irradiated with 280 nm (Newport Corporation, Irvine, CA) for 15 min prior to fusing the two pieces of the PMMA bioreactor together.

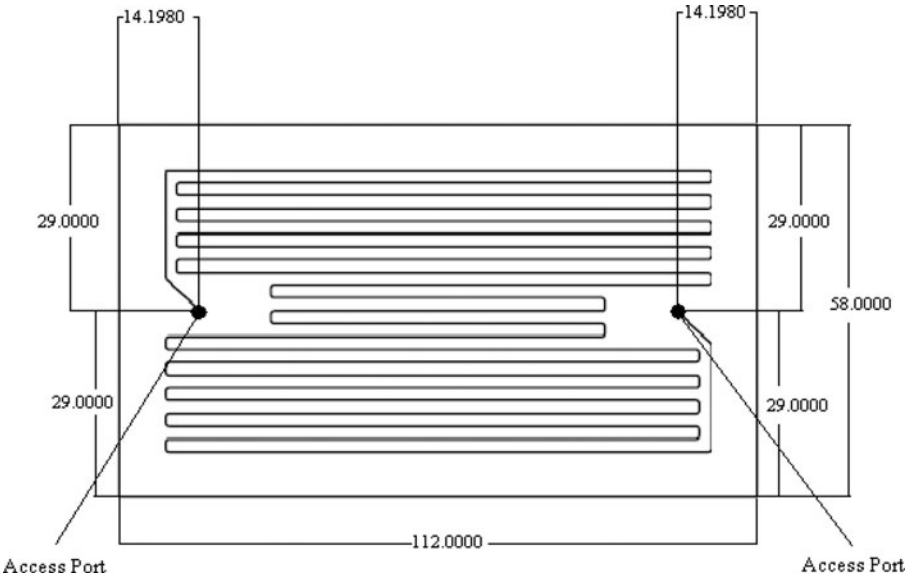

Fig. 1 Schematic drawing of the plug flow reactor fabricate and used for generation of metabolites. Substrates were injected into one of the two access ports and collected at the other. All dimensions given are in millimeters

PMMA-CYP2C9 Chip Preparation

The bonding of CYP2C9 to PMMA is based upon our previous work in which CYP2C9 was bonded to gold-coated silicon chips [37]. It is essential to conduct the bonding in the presence of both flurbiprofen and dapsone. If these compounds are not present, CYP2C9 activity will not be realized. The flurbiprofen and dapsone are removed from the enzyme after bonding by flushing with substrate free buffer. PMMA chips were sonicated in KPi for 5 min and then placed in a solution containing EDC/NHS in KPi (10 mL) for 2 h. After activation of the surface, chips were rinsed with KPi to remove excess EDC and NHS and then were immersed in a solution of CYP2C9 (100 nM) in KPi containing flurbiprofen and dapsone (40 μM, each) and allowed to stand overnight at room temperature for a minimum of 2 h to bond CYP2C9 to the PMMA. Longer periods, of up to 24 h, resulted in modest improvement in metabolite formation (10–20 %) and, consequently, 24 h attachment times were typically used. Finally, the PMMA-CYP2C9 chips were rinsed in KPi before being stored in cryobuffer at −80 °C prior to use. While storage at −80 °C is not essential, it has been found to significantly improve the activity of the CYP2C9 (~2×) albeit for unknown reasons. The fabricated chips could be stored for at least 2 months without loss in activity. A schematic of the fabrication process is shown in Fig. 2.

PMMA-AcBzTacn-CYP2C9 Chip Fabrication

PMMA chips were sonicated in KPi for a total of 5 min. After sonication, chips were immersed in a solution of EDC/NHS in KPi (10 mL) for 2 h, rinsed with KPi, and treated with AcBzTacn (2 mL, 20 mM, NaOH, pH 10) for 30 h [39]. The PMMA surface was then treated with a solution of $NiCl_2$ (10 mL, 100 mM) for 1 h. Finally, the PMMA chips were treated with a solution of CYP2C9 (2 mL, 100 nM) in KPi containing flurbiprofen and dapsone (40 μM, each) and allowed to stand overnight to allow for Ni(II)-coordinated immobilization of CYP2C9 to the PMMA surface. The chips were then rinsed in KPi and stored in cryobuffer at −80 °C to

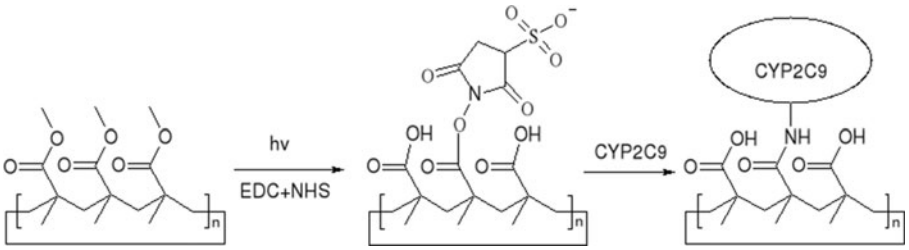

Fig. 2 Scheme for N-terminus attachment of CYP2C9 which requires UV-mediated cleavage of the PMMA methyl esters and EDC/NHS activation (*left*) to yield 'activated' esters (*center*) and then treatment with CYP2C9 to yield the enzyme bonded to the PMMA (*right*)

activate the enzyme for at least 4 h before use. All processes were performed under argon and at room temperature. A schematic of this fabrication process is shown in Fig. 3.

PMMA-CYP2C9 Chip-Mediated Metabolic Incubation

PMMA-CYP2C9 chips were immersed in a solution containing CPR (200 nM, final concentration) and diclofenac (250 μM, final concentration) in KPi buffer. Incubations were initiated by the addition of NADPH (250 μM, final concentration) in KPi (200 μL, final volume). The mixtures were incubated in a water bath for 16 h at 37 °C. Positive controls were solution-based incubations (200 μL, final volume) and contained CYP2C9 (2.5 nM), CPR (5.0 nM), diclofenac (250 μM), and 10 μL of dilaurophosphatidyl choline (1 mg/mL). Note that chip incubations did not contain dilaurophosphatidyl choline, which improves metabolite production in solution, but eliminates it when CYP2C9 is bonded to a solid surface [37].

Attachment of CYP2C9 to Bioreactor Channels

Three approaches were used to attach CYP2C9 to PMMA. Two utilized N-terminus attachment and the third C-terminus attachment. These three approaches are described here.

N-Terminus Attachment Under Flow Conditions After fabrication of the PMMA bioreactor and its exposure to ultraviolet radiation, the plug flow channel was cleaned by flowing KPi (10 mL, 40 mM) through the PMMA block at a rate of 500 μL/h using a syringe pump (Harvard Apparatus Pump II, Holliston, MA). The channel was then filled with EDC/NHS (2 mL) in KPi and allowed to stand for 2 h, rinsed with KPi (2 mL) for 2 h at a flow rate of 1 mL/h, and then a mixture of CYP2C9 (10 mL, 100 nM) containing flurbiprofen and dapsone

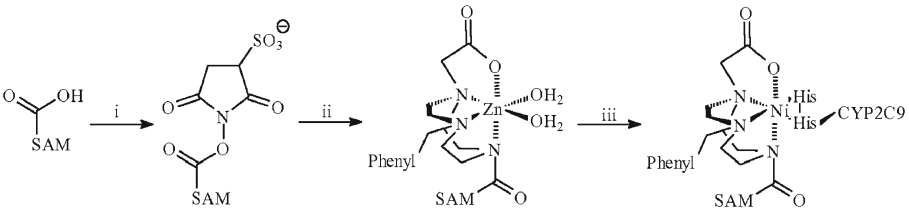

Fig. 3 Scheme for C-terminus attachment of CYP2C9 to PMMA. (*i*) UV-mediated cleavage of ester moieties present within the PMMA polymer and EDC/NHS activation of the resulting carboxylic acids followed by (*ii*) bonding to AcBzTacn and chelation with Ni(II) and the (*iii*) complexation to the C-terminus of CYP2C9 via the His-tag

(40 μM, each) in KPi was delivered through the channel continuously for a total of 20 h at 500 μL/h. After CYP2C9 immobilization, the channel was rinsed with KPi (4 h at 500 μL/h).

N-Terminus Attachment Under Static Conditions The plug flow bioreactor channel was fabricated as described up to the point of enzyme attachment. Then, the plug flow channel was filled with a CYP2C9 solution (500 nM) containing flurbiprofen (40 μM) and dapsone (40 μM) in KPi. The CYP2C9 solution was allowed to stand in the channel for 4 h, and then, the channel was rinsed by flowing KPi (500 μL/h) through the channel for 4 h.

C-Terminus Attachment Under Flow Conditions To attach CYP2C9 by the C-terminus AcBzTacn was used [39]. The bioreactor channel was first cleaned by flowing KPi (40 mM) through the PMMA block (500 μL/h, 20 h) using a syringe pump (Harvard Apparatus Pump II). The bioreactor was then filled with EDC/NHS in KPi, allowed to react for 2 h, and then rinsed with KPi (1 mL/h) for 2 h. The bioreactor was then filled with AcBzTacn (20 mM) in aqueous potassium hydroxide (pH 10), allowed to react for 30 h, and then flushed with KPi (1 mL/h) for 2 h [39]. Next, the bioreactor was filled with aqueous $NiCl_2$ (100 mM) and allowed to stand for 1 h, rinsed free of excess $NiCl_2$, with KPi (2 mL, 1 mL/h), and the enzyme was then bound to the PMMA channel by flowing CYP2C9 (100 nM, 500 μL/h) containing flurbiprofen (40 μM) and dapsone (40 μM) for 20 h. Finally, the channel was flushed with KPi (500 μL/h, 4 h) to remove enzyme that was not bound to the AcBzTacn-nickel complex. Regardless of the method of attachment, the complete bioreactors maintained their activity for a minimum of 2 months when stored at −80 °C.

PMMA-CYP2C9 Bioreactor-Mediated Metabolite Generation

A syringe and syringe pump were coupled via HPLC style fittings and polyetheretherketone (PEEK) tubing to the PMMA-CYP2C9 bioreactor in a water-jacketed beaker which was coupled to a circulating water bath to maintain the internal temperature of the jacketed beaker at 37 °C. Metabolite generation was initiated by pumping (500 μL/h) a solution (10 mL) containing diclofenac sodium (250 μM, final concentration), NADPH (1 mM, final concentration), and CPR (200 nM, final concentration) in KPi through the bioreactor. The bioreactor eluent was coupled to a fraction collector via PEEK tubing and fractions collected at 1-h intervals (500 μL) over a 20-h period. The syringe was then refilled with KPi and pumped through the bioreactor (2 mL, 500 μL/h, 4 h) to remove any remaining metabolite from the channel. Fractions were collected as described above, resulting in a total of 24 fractions. Immediately upon collection, fractions were centrifuged to dryness in vacuo using a Speedvac (Savant SC110, Thermo-Fisher, Pittsburgh, PA) and redissolved in aqueous acetonitrile (5 % *v*/*v*, 200 μL).

CYP2C9-Mediated Solution Incubations of Diclofenac

Reconstituted CYP2C9 enzyme incubations with diclofenac were conducted with solutions containing diclofenac (50 μM), CYP2C9 (125 nM), dilaurophosphatidyl choline (1.6 mM, final concentration), and CPR (250 nM, final concentration) in a total volume of 200 μL of KPi. Samples were pre-incubated for 3 min at 37 °C before the addition of NADPH (1 mM, final concentration). Incubations were conducted for 20 min at 37 °C, quenched with acetic acid (50 μL), an internal standard, *N*-phenylanthranilic acid (5 μg/mL) in acetonitrile (50 μL), was added, samples were centrifuged (13,000×*g*) for 10 min to precipitate protein, and the supernatant (200 μL) was transferred into vials for chromatographic analysis [40].

Chromatographic Determination 4′-Hydroxydiclofenac

Metabolic reactions with PMMA chips and bioreactor fractions were quenched by adding acetic acid (50 μL) to either PMMA chip incubations or bioreactor fractions and an internal standard *N*-phenylanthranilic acid (5 μg/mL) in acetonitrile (50 μL) was also added, samples were centrifuged (13,000×*g*) for 10 min to precipitate protein, and the supernatant (200 μL) was transferred into vials for chromatographic analysis. Separation of metabolite, parent compound, and internal standard was conducted using a Waters Alliance 2965 separations module (Milford, MA). Approximately 10 μL of the analyte solution was injected into an Agilent Zorbax SB C-18 column (150×4.6 mm, 5 μm particle size) (Agilent Technologies, Santa Rosa, CA) and was eluted at 0.5 mL/min using a mobile phase mixture of aqueous sodium acetate (75 mM, pH 5) and acetonitrile (60:40, *v*/*v*). Eluted compounds were detected with a Waters 2487 Dual Absorbance Detector (280 nm). Data was processed by Waters Empower ver. 2.0 software. The peak corresponding to 4′-hydroxydiclofenac was determined by comparison to an authentic sample of 4′-hydroxydiclofenac.

Results

Introduction

To optimize metabolite production by the plug flow bioreactor, several different parameters were examined using PMMA chips as a model system of the plug flow reactor. These parameters include (a) the concentration of CYP2C9 used during coupling, (b) the time allowed for attachment of CYP2C9 to PMMA, and (c) two different attachment methods. Parameters which gave the maximal metabolite output were then used for the fabrication of the plug flow bioreactor and tested for metabolite production.

Variation of CYP2C9 Immobilization Concentration

The optimal concentration of CYP2C9 used during coupling is a tradeoff between producing a high CYP2C9 surface concentration and, at the same time, not overcrowding the surface and thereby inhibiting CPR access to the immobilized CYP2C9. To this end, UV-activated PMMA chips (4×6 mm) were treated with EDC/NHS and reacted with 50, 100, 200, and 400 nM CYP2C9 solutions for 24 h to bond the CYP2C9 to the chips. These chips were then exposed to diclofenac sodium, CPR, and NADPH and the resulting amount of metabolite formed was quantitated. Figure 4 shows the metabolite formation data for solution controls (white) and PMMA chips (gray bars). The positive control experiment (solution) contained 0.5 pmol CYP2C9, dilaurophosphatidyl choline (typically present in solution incubations but not in chip-based incubations [39]), and NADPH. Negative control experiments omitted NADPH and metabolite formation (4′-hydroxydiclofenac) was not detected in the negative controls (data not shown). Maximal metabolite formation was observed with PMMA-CYP2C9 chips prepared with a 100-nM CYP2C9 solution.

CYP2C9 Attachment Time

In addition to determining the optimal enzyme concentration to treat the PMMA surface with, and thus maximize metabolite output, a series of experiments were run to determine the time of exposure of the PMMA chip to CYP2C9 necessary to maximize metabolite

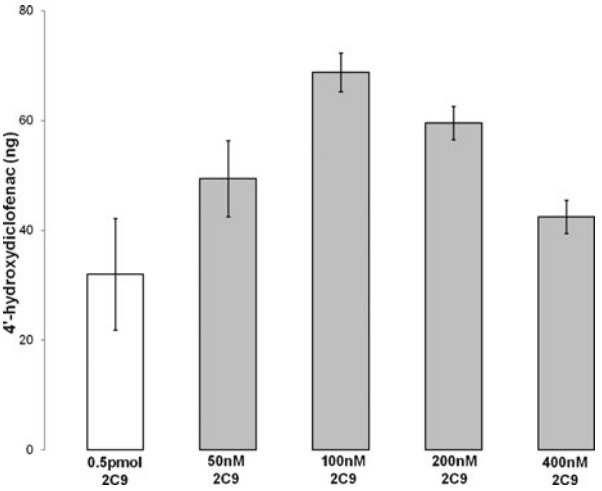

Fig. 4 Diclofenac metabolite formation from PMMA chips prepared with different concentrations (50, 100, 200, and 400 nM) of CYP2C9 applied to EDC/NHS-activated PMMA chips (*gray*). Solution incubation contains 0.5 pmol CYP2C9 and dilaurophosphatidyl choline (*white*). All incubations contain diclofenac, CPR, and NADPH

formation. This experiment used metabolite formation from UV-activated PMMA chips (4× 6 mm) that had been treated with CYP2C9 for different time periods (2, 4, 6, 8, and 24 h). Negative control experiments omitted NADPH. The results of this experiment are shown in Fig. 5 and are displayed as the fold change from the first measured time point (2 h attachment time). Metabolite formation (4′-hydroxydiclofenac) was not detected in the negative controls (data not shown), and no significant difference ($p<0.05$) was detected

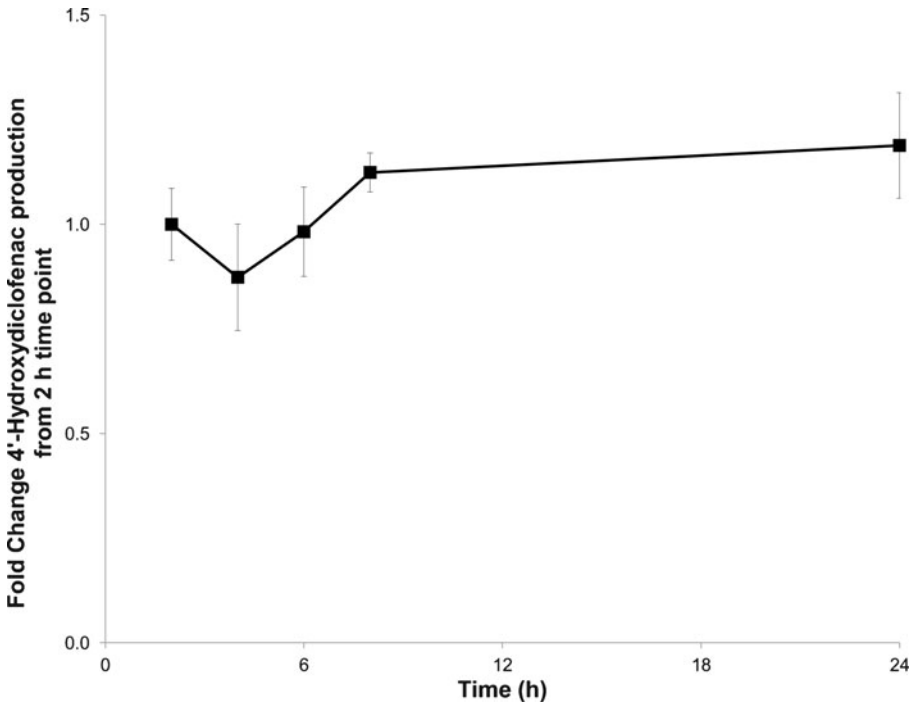

Fig. 5 Time course study (2, 4, 6, 8, and 24 h) to optimize CYP2C9 attachment time to an EDC/NHS-activated PMMA chip

between any of the different time intervals. Thus, attachment times of longer than 2 h do not lead to improved performance.

N- versus C-terminus Attachment of CYP2C9

Two different attachment chemistries were studied to determine the effect on metabolite production. The first involves using EDC/NHS activation of the surface carboxylic acid groups present on the PMMA surface toward amide bond formation with the N-terminus of the protein [22, 37]. The second used AcBzTacn, a molecule capable of chelating divalent cations such as Ni(II), which could, in turn, be used to chelate to the His-tag located on the C-terminus of the CYP2C9 [39]. Figure 6 shows the amount of metabolite formed in solution incubations (white bar) and with PMMA chips (gray bars) that used EDC/NHS or AcBzTacn to immobilize the CYP2C9. Negative control experiments omitted NADPH and no metabolite was observed regardless of the immobilization method (data not shown). The data suggest that the AcBzTacn (C-terminus) may be the better bonding modality.

Bioreactor Metabolite Production

In the first approach, CYP2C9 was immobilized in plug flow bioreactor blanks by flowing the enzyme through the bioreactor following activation with either EDC/NHS (N-terminus attachment) or AcBzTacn (C-terminus attachment). Solutions containing diclofenac, CPR, and NADPH were then flowed through the bioreactor (500 μL/h), and equal volume samples (500 μL) of the eluent were collected over a 24-h period and followed by quantitation of metabolite in each fraction by HPLC (Fig. 7).

Analysis of two different coupling chemistries, N-terminus via EDC/NHS (filled squares) or C-terminus via AcBzTacn chelation (filled circles), was compared in the flowed-through mode (500 μL/h) for a total of 24 h. Total metabolite production from the C-terminus CYP2C9 chelated reactor was 114 ng. The N-terminus CYP2C9-bonded reactor prepared using continuous flow yielded 296 ng.

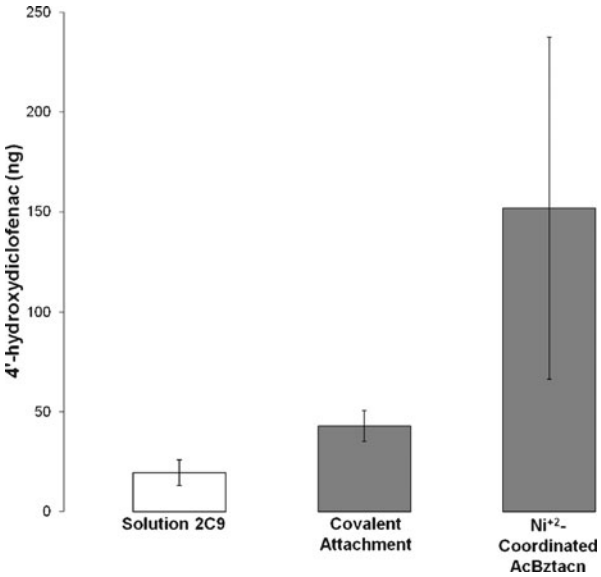

Fig. 6 Comparison of diclofenac metabolite production based on Ni(II)-directed coordination attachment (C-terminus) and covalent attachment (N-terminus). Solution incubation (*white*) contains 0.5 pmol CYP2C9 and dilaurophosphatidyl choline. All incubations contain diclofenac, CPR, and NADPH

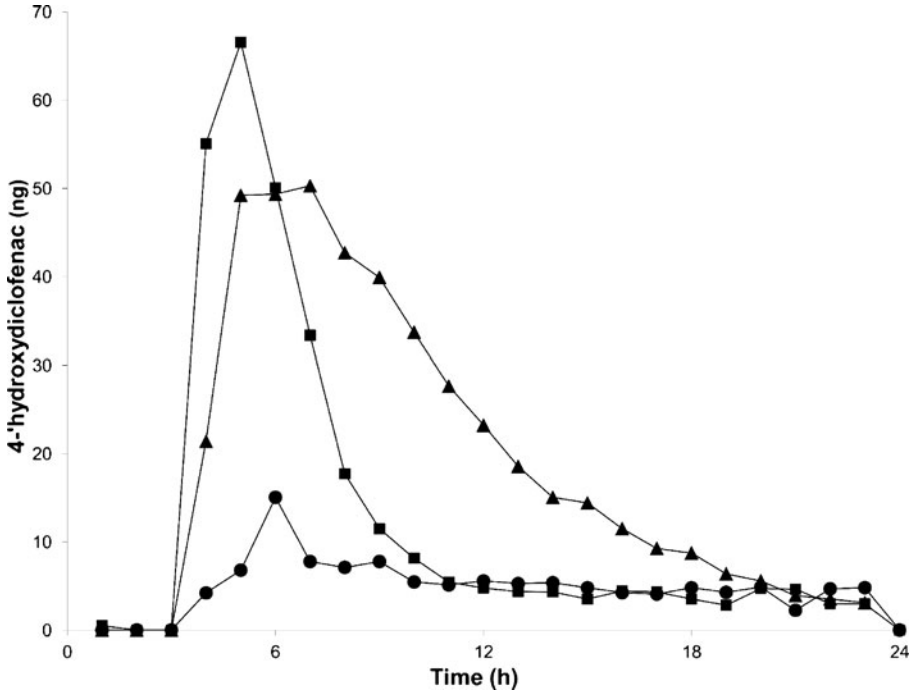

Fig. 7 Time course analysis of metabolite production by a CYP2C9-immobilized plug flow PMMA bioreactor. His-tag-directed coordination with AcBzTacn (*circles*) is compared to plug and flow channels fabricated using EDC/NHS covalent immobilization. Further comparison of EDC/NHS immobilization techniques is shown by continuous injection (10 mL, continuously injected at 500 μL/h for 20 h total) of CYP2C9 (100 nM) (*squares*) and static injection (2 mL, injected in one bolus and over a 20-h period) of CYP2C9 (500 nM) (*triangles*)

In the alternative approach, CYP2C9 was attached after EDC/NHS activation (N-terminus attachment) by filling the channel with a solution (2 mL) of CYP2C9 (500 nM). Attachment was allowed to occur for 2 h, followed by rinsing and then flowing a solution of diclofenac sodium, CPR, and NADPH through the bioreactor. Under these conditions, total metabolite production was 437 ng. Thus, the third approach was the method of choice.

Discussion

In the course of drug development, new potential drugs are routinely screened against P450 enzymes to determine whether they bind and, if so, whether metabolites are formed. In the event metabolites are produced, it is then necessary to determine whether they are toxic. The primary method that has been used to prepare metabolites for toxicity testing is synthetic organic chemistry. This requires the development and execution of a synthetic route. This approach can be expensive, time consuming, and labor intensive. An alternative approach is to prepare the metabolite directly from the drug and the specific P450 isoform.

There are key advantages to this approach. First, it is certain that the desired metabolite(s) will be produced. Second, when there is a specific enantiomer, the issues surrounding stereochemistry are eliminated. Enzyme-mediated synthesis will result in the formation of the desired enantiomer. To achieve sufficient quantities needed for preliminary toxicology screens,

however, requires the use of some form of a bioreactor as simple incubations will not yield sufficient quantities.

There are two general types of bioreactors commonly used in industrial applications. These include batch and continuous flow bioreactors [23]. Batch reactors, generally, are inexpensive to build. However, they have no flow of materials into or out of the bioreactor and this leads to the build-up of waste products, which may decrease the overall efficiency of the bioreactor. The alternative is a continuous flow bioreactor that has a continuous flow into and out. There are a variety of configurations possible, including bioreactors that are made of a simple tube or channel to which reagents are supplied and are commonly referred to as plug flow bioreactors [26, 27]. Generally, these types of devices utilize a tube-shaped structure that contains a catalyst on the tube walls, an inlet and an outlet. Compared to batch bioreactors, they are more expensive to build, tend to have poorer mixing, particularly in the axial direction, and temperature regulation can be more difficult to control. Nevertheless, we selected to use a plug flow reactor because product build up is less problematic, a concern since it is possible that metabolites may be further metabolized, especially at high concentrations.

To maximize the production of metabolite, we sought to optimize several aspects of the bioreactor construction and use. Time allotted for enzyme immobilization, the concentration of the enzyme during attachment, the concentration of the coenzyme, and how the substrate was exposed to the enzyme were all investigated. Most, but not all, of these factors were found to affect metabolite production.

Several experiments were conducted to optimize the coverage of enzyme on the bioreactor surface by varying the concentration of CYP2C9 present during the immobilization. The optimal concentration of CYP2C9 during immobilization was found to be approximately 100 nM. Metabolite production fell off if either higher or lower concentrations of enzyme were used. We hypothesize that a lower concentration of CYP2C9 during immobilization results in a surface too sparsely populated with enzyme [37]. In contrast, when concentrations of CYP2C9 above 100 nm were employed, the enzyme may have become too densely packed, reducing the access of CPR to the surface-bonded CYP2C9, and therefore resulting in reduced metabolite production.

A second parameter examined was the time allowed for bonding of the CYP2C9 to the PMMA. The time for bonding was varied between 2 and 24 h. Statistically, there was no variation in the production of metabolite. Shorter periods of time could be explored but it is unlikely that the use of shorter bonding times would increase metabolite production and, more likely, would result in a decrease of metabolite production.

A third factor that significantly affected metabolite production was whether the enzyme was attached to the bioreactor under flow or static conditions. In either case, the bioreactor was treated with the same amount of enzyme, at the same concentration, and for the same period of time. However, metabolite formation was approximately 50 % greater when the enzyme was attached under static conditions than under flow conditions. Under flow conditions, the concentration of CYP2C9 may not be uniform throughout the channel as the solution passes through. At the inlet, the CYP2C9 concentration would be at its highest and potentially may lead to overcrowding of the enzyme such that CPR access is prevented. Nearing the outlet, enzyme concentration would be lower and so would the amount of attached enzyme. Either of these situations would lead to a reduction in metabolite production. In contrast, under static conditions, the concentration of the CYP2C9 is uniform throughout the channel.

Fourth, we examined whether there was a difference between N- and C-terminus attachment of the CYP2C9. We obtained contradictory results between PMMA chips and the bioreactor. C-terminus coupling leads to improved activity on PMMA chips, but N-terminus coupling

appears to favor metabolite production when employed in the bioreactor. A potential reason is that N-terminus bonding of CYP2C9 in the bioreactor may be more efficient and results in more enzyme on the surface than when the C-terminus method is used. The time period for coupling of the CYP2C9 to the PMMA surface was also examined for its effect on metabolite production. Over the period examined (2–24 h), no significant effect on metabolite production was observed suggesting that coupling to the PMMA surface is a relatively fast process.

Three aspects of the overall fabrication were not examined but are nevertheless important to note. First, the attachment of CYP2C9 was always conducted in the presence of flurbiprofen and dapsone. As we have previously described [37], when CYP2C9 is bonded to a surface via amide bond formation, it must be done in the presence of these two compounds to maintain metabolic activity with CPR/NADPH. We believe that both flurbiprofen and dapsone are then in the active site during bonding and stabilize it. Similar effects have been noted for other CYP enzymes. For example, CYP1A2 can only be isolated and purified if 7,8-benzoflavone is bound to the active site [41]. Second, following attachment, the bioreactor (or chips) is cooled to −80 °C in cryobuffer for at least 4 h. While it is unknown why, chips that are not refrigerated prior to use are less efficient at producing metabolite [42]. At the same time, complete bioreactors or chips with bonded enzyme maintain their activity for at least 2 months after preparation if they are stored in cryobuffer and at −80 °C.

Interestingly, the plug flow bioreactor was found to generate metabolite without having the large excess concentration of CPR. In our previous studies, we found that high concentrations of CPR were required with the gold chip incubations to optimize metabolite formation [37]. Previously, on gold chips, we found that metabolite production was greatest when the CPR/CYP2C9 ratio was 10:1. This is ten times greater than the concentration of CPR used for solution incubations where a 2:1 ratio is common. However, in the case of the plug flow bioreactor, a ratio of 2:1 maximized metabolite production, a fivefold reduction in the optimal CPR/CYP2C9 ratio. This difference may be due to the difference in surface area/volume ratio between the two systems. The cross section of the plug flow channel is 1×1 mm and the channel is 2.1 m in length. Thus, the internal volume is approximately 2,100 μL and the surface area is 8,400 mm^2 or a ratio of 4 mm^2:1 μL. In comparison, the immobilized chip experiments are conducted in 200 μL of solution and the surface area of the chip is approximately 48 mm^2 or 0.24 mm^2:1 μL, approximately one eighth of the plug flow reactor. Though it may be coincidental, the factor roughly corresponds to the reduction in the CPR/CYP2C9 ratio.

The reduction in CPR/CYP2C9 ratio was not expected. Plug flow reactors have reduced mixing in the channel, particularly in the axial direction. As a result, a less favorable interaction between CPR and the P450-PMMA surface is expected. Moreover, the decreased mixing may also hinder mixing of other components necessary for P450-mediated metabolism (NADPH, O_2, and substrate). Regardless, the significant reduction in the amount of CPR required to run the bioreactor also significantly reduces the cost to run the bioreactor.

A problem that has made it difficult to use P450 enzymes in a bioreactor, regardless of the type, is that their activity is quickly lost. This is particularly true with solution-based incubations, which are typically conducted for 30 min or less. However, immobilizing P450 enzymes appears to extend the period of time they remain active. We show here that CYP2C9 activity lasts at least 24 h. Others have observed a similar result upon immobilization (e.g., CYP2D6 and CYP3A4 [36]). The reasons for the improved lifetime of the enzyme relative to solution are unknown but, nevertheless, appear to be a general result.

Similar approaches, as described here, have been pursued for generating drug metabolites using plug flow bioreactors with HLM preparations [22, 29, 43]. HLM preparations contain multiple P450 enzymes in physiologically relevant ratios. Consequently, for any given substrate, multiple metabolites may be formed and require separation, though this approach is useful for

screening purposes. In contrast, plug flow bioreactors that utilize recombinant P450 enzymes are comprised of only one isoform and therefore only the metabolite(s) of interest will be formed. In many instances, only one metabolite will be formed, though if more are formed, it is likely that separating the metabolites will be easier than when HLM-based reactors are used.

Systems do exist that will generate large quantities of metabolite; in particular insect cell-based systems designed by Rushmore et al. [31]. These systems are very efficient for metabolite production and can be tailored by transfection of P450 baculosomes into insect cell lines to produce specific metabolites of interest. However, bioreactors containing living cells have several problems associated with their use that can render them undesirable for use in bioreactors. For example, temperature control can be a serious challenge and is a problem well known to plague batch and fed-batch bioreactors, which often require complex temperature and waste control systems.

The use of a plug flow bioreactor based on PMMA-conjugated P450 enzymes can likely be easily converted into a system for commercial use. For example, commercially available PMMA tubing is inexpensive and would eliminate the need to mill PMMA blocks and the associated costs (time, labor, and equipment). Coupling the PMMA tubing with HPLC-like fittings readily allows for interfacing to, for example, a mass spectrometer and/or an HPLC that would allow for separation and isolation of metabolites. Further, the use of PMMA tubing would result in a very inexpensive system and, upon loss of enzyme activity, could be replaced with new tubing and the desired immobilized P450. Finally, several PMMA tubes, each functionalized with a different P450 isoform, could be bundled and utilized in hollow fiber bioreactor constructs [43], thus serving to further increase the metabolite output of PMMA in the immobilized P450 bioreactor.

Conclusion

In summary, we have shown the ability of immobilized CYP2C9 in a plug flow bioreactor to biosynthesize drug metabolites of interest. The method described herein provides advantages over traditional organic synthesis in that metabolites can be produced in a rapid and high-throughput fashion, with a high level of regio- and stereochemical control. We propose this as a novel system to produce metabolites for pharmaceutical companies to conduct toxicity testing. This approach to metabolite production could reduce the time for and the cost of development associated with drug discovery by eliminating the need to synthesize compounds for testing by traditional medicinal chemistry methods.

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