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Overcoming the Pitfalls of Cytochrome P450 Immobilization Through the Use of Fusogenic Liposomes

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This work describes a new nanotechnology-based immobilization strategy for cytochrome P450s (CYPs), the major class of drug metabolizing enzymes. Immobilization of CYPs on solid supports provides a significant leap forward compared with soluble enzyme assays by enabling the implementation of through-flow microreactors for, e.g. determination of time-dependent inhibition. Immobilization of the complex CYP membrane-protein system is however particularly challenging as the preservation of the authentic enzyme kinetic parameters requires the full complexity of the lipid environment. The developed strategy is based on the spontaneous fusion of biotinylated fusogenic liposomes (b-FL) with lipid

bilayers to facilitate the gentle biotinylation of human liver microsomes (HLM) that incorporate all main natural CYP isoforms. The same process was also feasible for the biotinylation of recombinant CYPs expressed in insect cells, same as any membrane-bound enzymes in principle. As a result, CYPs could be immobilized on streptavidin-functionalized surfaces, both those of commercial magnetic beads and customized microfluidic arrays, so that the enzyme kinetic parameters remained unchanged, unlike in previously reported immobilization approaches that often suffer from restricted substrate diffusion to the enzyme's active site and steric hindrances. The specificity and robustness of the functionalization method of customized microfluidic CYP assays was also carefully examined.

Liposomes are widely utilized as nanocarriers in e.g. gene transfection^[1] and drug delivery,^[2] where cellular uptake is mainly governed by endocytosis. Certain, tailored, lipid compositions have been found to enable spontaneous bilayer fusion, the propensity of which is mainly promoted by the negative curvature^[3] of the monolayer as well as by close contact, small size and unilamellarity of the liposomes.^[4] Liposome fusion with cell membranes has been demonstrated using customized fusogenic liposomes (FL) comprising of neutral and positively charged lipids,^[5] and a variety of cell-labeling approaches have been introduced for functionalization of mammalian cells with, e.g., fluorescent^[6] or biotinylated lipids^[7] or immune cell activating lipopolysaccharides.^[8] In this work, we introduce a novel use for FLs, which facilitates a universal approach to immobilization of any membrane proteins on solid surfaces *via* the well-characterized avidin-biotin chemistry (**Figure 1a**). Here, we focused on the cytochrome P450 (CYP) system, the complexity of which places special demands on immobilization and has puzzled researchers for decades.^[9] Mammalian CYPs are complex microsomal enzymatic systems consisting of both CYPs themselves as well as several electron-supplying redox partner enzymes, such as cytochrome P450 reductase,^[10] embedded in a lipid membrane environment which is also essential for their correct function.^[11] CYPs

1 constitute the majority of drug detoxification pathways,^[12] and drug-drug interactions
2 mediated by CYPs are thus the primary target of preclinical drug characterization. Human
3 liver microsomes (HLM) are the preferred *in vitro* matrix, because they show CYP activities
4 much higher than those of intact hepatocytes^[13] and thus allow for high-throughput drug
5 screening *in vitro*. By making use of the spontaneous fusion of customized biotinylated FL (b-
6 FL) with subcellular HLM fractions, we were able to biotinylate the HLMs in a natural and
7 gentle manner and thus preserve the authentic enzyme kinetic functions of the membrane-
8 bound CYPs upon immobilization of the biotinylated HLMs on streptavidin-functionalized
9 surfaces, both those of commercial microbeads and of customized microfluidic pillar arrays.

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24 CYP immobilization on solid support structures has raised considerable interest, since it
25 would facilitate certain mechanistic and relative activity assays, such as time-dependent
26 inhibition under continuous flow conditions, as well as more straightforward purification of
27 the reaction products for both production and analytical purposes. The ability to manipulate
28 substrate and inhibitor concentrations using flow chemistry additionally speeds up the
29 determination of enzyme/inhibitor kinetics.^[14] When further combined with the integration
30 and parallelism possibilities of the emerging microarray technology, immobilized enzyme
31 reactors (IMERs) would offer a compelling tool for high-throughput drug metabolism assays.
32 However, the commonly applied enzyme immobilization strategies tend to result in diffusion-
33 limited mass transfer (e.g. physical entrapment of enzymes/microsomes^[15]) or steric
34 hindrance due to uncontrolled enzyme orientation (e.g. nonselective covalent bonding *via*
35 amino acid residues^[16]), which may result in flawed enzyme kinetic determinations and thus
36 impair the *in vitro-in vivo* correlation.^[17] Covalent immobilization may also alter the protein
37 surface charge and thus reduce the enzyme activity and/or stability.^[18]

The b-FL composition used in this study was modified from previous works^[7,19] and contained both a (spacer-)biotinylated lipid (biotin-cap-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 0.1 m-%) and a fluorescent labeled lipid (Lissamine Rhodamine B-DOPE), 0.05 m-%) to facilitate not only immobilization of the biotinylated-HLM (b-HLM) but also monitoring of the immobilization efficiency based on fluorescence. Prior to fusion with HLMs (37°C, 15 min), multilamellar liposomes of the given composition and average hydrodynamic radius of ca. 493 ±87 nm (**Figure S1a**) were prepared and extruded through a 100 nm membrane to yield unilamellar b-FLs with an average hydrodynamic radius of 220 ±25 nm (n=4 batches) (Figure S1b). Upon mixing, the opposite net charges of HLM (-44.3±4.9 mV, **Figure S2**) and b-FL (69.3±1.8 mV) promoted close contact *via* electrostatic inter-actions, facilitating membrane fusion. As expected, the biotinylation process did not significantly affect the activity of soluble CYP (**Figure S3** and **Figure S4**), since the membrane fusion is a gentle process and the biotin label is small and locates in the lipid membrane considerably far from the enzyme's active site. Also upon immobilization on streptavidin-functionalized magnetic beads, the enzyme affinities (K_M) of four main CYP isoforms were similar to those of the soluble enzymes (Figure 1b and **Figure S5**) and the enzyme activities of the immobilized enzymes were preserved over at least 15 days (Figure 1c). These results confirmed that by embedding the biotin in the lipid membrane for anchoring the HLMs on avidin-functionalized surfaces leaves the CYPs' active sites accessible better than by covalent bonding of the CYP enzyme itself (as in, e.g. ^[16a]). Our approach also allows unhampered mass transfer to and from the enzyme's active site (unlike in, e.g. ^[15a]) providing clear advantages over other previously reported immobilization approaches based on enzyme entrapment or covalent bonding. Moreover, no significant loss of CYP activity was observed with immobilized b-HLMs even after two weeks of storing at 4°C, whereas negative controls prepared by solubilizing nonbiotinylated HLMs on lipid-coated magnetic beads lost half of their activity already in 6 days (Figure 1c). Upon repeated

1 use of the same batch of beads, the CYP activity was typically halved in all cases (Figure 1c),
2 which likely resulted from incomplete recovery of the beads in subsequent incubations.

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4 Similar activity loss of CYPs immobilized on magnetic particles was also reported by
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7 Schejbal et al.^[16b]
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11 To address the limitations related to handling and packing of magnetic beads, we further
12 developed the concept toward implementation of microfluidic through-flow IMERs. The
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14 microfluidic IMERs were fabricated using a custom composition of thiol-enes, which are an
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16 emerging class of polymers facilitating low-cost microfabrication *via* non-cleanroom replica-
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18 molding (**Figure S6**) based on UV-induced thiol-ene click chemistry.^[20] By mixing the thiol
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20 or allyl monomers in off-stoichiometric ratios, the thiol-ene surfaces can be readily
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22 (bio)functionalized to incorporate excess of either free thiol or allyl functional groups.^[21] In
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24 this work, thiol-rich surfaces were prepared and biotinylated with biotin-PEG₄-alkyne *via* yet
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26 another UV induced thiol-yne reaction, followed by functionalization with fluorescent StrA
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28 (ex 488 nm) and eventually with b-HLM. As a preliminary characterization, we determined
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30 the water contact angles at each step of the functionalization protocol and observed that the
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32 functionalization resulted in clear changes in the surface wettability (**Figure 2a**). To maximize
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34 the surface area, and thus, the amount of immobilized CYP, we implemented a dense
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36 micropillar array^[22] at the reaction zone (Figure 2a). Compared with porous polymer monolith
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38 packings, commonly used for enzyme immobilization in capillaries,^[23] the micropillar arrays
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40 are less prone to clogging and reproducibility issues, and thus provide a convenient approach
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42 to implementation of well-ordered IMERs by microfabrication means. The robustness of the
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44 functionalization protocol was determined with respect to the effects of the bulk monomer
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46 (thiol/allyl) ratio and biotin and StrA concentrations on the amounts of immobilized StrA and
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48 b-HLM, which were quantitated based on their fluorescence tags (Figure 2b-d). As expected,
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50 the amount of bound StrA increased along with increasing amount of biotin, which could be
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1 altered by adjusting either the number of free surface thiols (Figure 2b) or the biotin
2 concentration (Figure 2c). The amount of immobilized b-HLM was, however, inversely
3
4 dependent on the amount of bound StrA (Figure 2d). This likely resulted from more efficient
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6 biotin-streptavidin interactions in the absence of steric hindrances caused by excess
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8 streptavidin. For further assays, we used 0.1 mM biotin and 0.5 mg mL⁻¹ StrA concentrations
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10 and a 25 mol-% excess of thiol functional groups in the bulk.
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16 The CYP activities of the IMERs were determined at a flow rate of 5 μ L min⁻¹ using
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18 temperature-insensitive Luciferin-H (200 μ M in PBS) hydroxylation *via* CYP2C9 (**Figure 3a**)
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20 and temperature-sensitive coumarin (50 μ M in 0.1 M Tris buffer) 7-hydroxylation *via*
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22 CYP2A6 (Figure 3b) as the model activities. In both cases, the IMERs maintained their CYP
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24 activity for at least 60 min, while the reaction temperature was shown to have an effect on the
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26 efficiency of the latter reaction, as expected. The developed method was also shown to be
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28 feasible for immobilization of recombinant CYPs (rCYPs) expressed in baculovirus-
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30 transfected insect cells (Figure 3a), which highlights the universal nature of the developed
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32 enzyme immobilization strategy. Since our approach targets the lipid membrane, not the
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34 protein itself, it is universally applicable to biotinylation and immobilization of any
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36 membrane-bound protein on any avidin-coated surface. Finally, the specificity of the
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38 functionalization protocol was determined by omitting each of the functionalization steps at a
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40 time and determining the cumulative (60 min) amount of produced metabolite using three
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42 parallel IMERs in each condition. Although some traces of nonspecific binding of both StrA
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44 and b-HLM was observed in the fluorescence measurements, the activities of the CYP-IMERs
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46 prepared using the optimized functionalization protocol were significantly higher than those
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48 of any of the negative controls (Figure 3c), suggesting that the nonspecifically adsorbed lipid
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50 vesicles leaked out of the IMER upon application of through-flow.
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On the basis of these results, the developed immobilization method, which is based on natural membrane fusion between biotinylated fusogenic liposomes and the desired lipid membrane incorporating the enzymes of interest, provides a universal tool for studying membrane-bound enzymes on solid surfaces. Furthermore, the thiol-ene click chemistry -based microchip fabrication and functionalization protocol, specifically used in this study, further provides a robust tool for implementation of through-flow IMERs for drug metabolism research. Efficient immobilization of the CYP system combined with the advantages of the microarray technology pave the way to novel methodologies, such as mechanistic drug inhibition studies. The concept developed herein is particularly suitable for applications where unaltered enzyme kinetics are of paramount importance, such as the preclinical drug screening and prediction of the *in vivo* drug response on the basis of *in vitro* enzyme kinetic results.

Experimental Section

Liposome preparation and fusion with human liver microsomes or recombinant cytochrome P450: Biotin-containing fusogenic liposomes (b-FL) were prepared using a lipid composition modified from Hersch et al.^[7] and Csiszár et al.^[9] by mixing stock solutions (in chloroform) of DOPE (10 mg mL⁻¹), DOTAP (10 mg mL⁻¹), biotin-cap-DOPE (10 mg mL⁻¹) and Lissamine Rhodamine B-DOPE (1 mg mL⁻¹) in a lipid mass ratio of 1:1:0.1:0.05, respectively. After mixing, the bulk solvent was evaporated under a stream of nitrogen. To remove any solvent residues, the lipid mixture was kept in vacuum for 2 h. Next, the dry lipid film was solvated in PBS to yield a total lipid concentration of 2 mg mL⁻¹ and vortexed for 1 h at room temperature. To prepare unilamellar vesicles, the multilamellar liposome mixture was passed through a polycarbonate membrane (pore size 100 nm) 51 times using a benchtop extruder (Avanti Polar Lipids, Alabaster, AL). The size distribution of multilamellar and unilamellar b-FLs (Figure S1) was determined by dynamic light scattering (DLS) using Zetasizer APS and the size distribution of HLM and the zeta potential of b-FLs and HLM (Figure S2) was

determined using Zetasizer Nano ZS (both devices from Malvern, Worcestershire, UK). Before measurements, the HLM stock (20 mg mL⁻¹ total protein) was diluted to a concentration of 0.125 mg mL⁻¹ and the b-FL stock to a concentration of 0.002 mg mL⁻¹ with de-ionized water. The b-FL dispersion was stored at ≤4°C until use. For HLM immobilization, equal volumes of the b-FL dispersion (2 mg mL⁻¹ total lipid) and the HLM stock solution (20 mg mL⁻¹ total protein) were mixed and incubated at 37°C for 15 min before applying the biotinylated HLM (b-HLM) vesicles onto the streptavidin functionalized magnetic beads or microchips. The recombinant cytochrome P450 (rCYP) isoenzymes were biotinylated in a similar manner by mixing the b-FL stock with an equal volume of the rCYP3A4, rCYP2D6 or rCYP2C9 stock (each 1 000 nmol L⁻¹) followed by incubation at 37°C for 15 min.

Enzyme immobilization on streptavidin functionalized magnetic beads: Desired amount of streptavidin prefunctionalized, superparamagnetic beads (Dynabeads™ M-280, 10 mg mL⁻¹, Life Technologies) was pre-treated according to the supplier's protocol.^[24] Next, the beads were divided in 25 µL aliquots (corresponding to 0.25 mg of beads) of which the supernatant was separated and discarded. The b-HLM vesicles (15 µL) were immediately added onto the beads and the suspension was incubated on a tube rotator at room temperature for 30 min, after which the supernatant was discarded and the beads were washed 4–5 times with 50 µL of PBS using magnetic separation. After functionalization, the beads were stored in 50 µL of PBS at ≤4°C until use. In addition to b-HLM vesicles, the pretreated beads were also functionalized with biotinylated DPPE lipids and then with bare HLM vesicles (negative control), as described in the Supporting Information.

Microchip fabrication and biofunctionalization: The immobilized enzyme microreactors (IMERs) were implemented on micropillar arrays that were fabricated in-house and sequentially functionalized with biotin and streptavidin. Briefly, the micropillar arrays were

1 fabricated from thiol-ene polymers following a previously developed protocol.^[22] The
2 microreactor design comprised a 30-mm-long and 4-mm-wide microchannel featuring an
3 array of ca. 13 600 micropillars ($\varnothing$ 60 μ m) (Figure 2a). The channel height was 200 μ m and
4 the total chip volume ca. 25 μ L (including the connecting channels between inlet/outlet and
5 excluding the space occupied by the micropillars). The chip fabrication protocol included four
6 steps: (i) SU-8 master fabrication in cleanroom conditions, (ii) casting of a PDMS mold using
7 the SU-8 master as a template, (iii) UV replica-molding of the microchannel and the cover
8 layers out of thiol-ene polymers using the PDMS mold, and (iv) bonding of the two thiol-ene
9 layers by lamination (Figure S6). The cleanroom fabrication of the SU-8 masters (step i) is
10 described in the Supporting Information. The PDMS molds (step ii) were prepared by mixing
11 the base elastomer and the curing agent in a ratio of 10:1 or 9:1 (w/w), degassing in vacuum
12 for 30 min, and pouring the mixture onto the SU-8 master prior to curing by heat (80°C for 3h
13 or 65°C overnight). The thiol-ene layers (step iii) were prepared by mixing tetrathiol and
14 triallyl monomers in a molar ratio of 3:2 (with respect to free thiol and allyl functional
15 groups), which resulted in thiol-rich (25 mol-% excess of thiols) bulk composition. In
16 addition, stoichiometric compositions (tetrathiol and triallyl monomers mixed in a molar ratio
17 resulting in equal amounts free thiol and allyl functional groups) were used as controls during
18 the validation of the biofunctionalization process. Next, the monomer mixture was poured
19 onto the PDMS mold, degassed in vacuum, and cured under UV for 5 min (Dymax 5000-EC
20 Series UV flood exposure lamp, nominal power 225 mW cm⁻², Dymax Corporation,
21 Torrington, CT). Both microchannel and cover layers (with inlet/outlet holes) were prepared
22 in the same manner. The two, preheated (70°C) layers were laminated together and exposed to
23 UV for an additional 2 min (step iv). The average amount of free surface thiols was
24 determined by titration with Ellman's reagent and was, for instance, 133 $\pm$ 13 nm⁻² when 50
25 mol-% excess of thiol functional groups was used in the bulk composition. Finally, the thiol-
26 ene surface was functionalized with streptavidin according to a protocol adopted from Lafleur
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et al.^[21a] Briefly, free surface thiols were functionalized with biotinylated PEG₄-alkyne (0.1 mM in ethylene glycol with 1% Irgacure TPO-L as the photoinitiator) using photopolymerization under UV ($\lambda = 365$ nm) for 1–2 min (Figure 2a). Next, the microreactor was thoroughly rinsed sequentially with methanol and deionized water (≥ 5 mL each) and filled with 0.5 mg mL⁻¹ Alexa Fluor® Streptavidin in PBS. After incubation at room temperature for 30–45 min, the microreactor was again thoroughly rinsed with PBS (≥ 5 mL) and filled with the b-HLM vesicles or biotinylated rCYPs. Finally, after incubation at $\leq 4^\circ\text{C}$ overnight (b-HLM) or at room temperature for 30 min (biotinylated rCYP), the microreactor was thoroughly rinsed with PBS (≥ 5 mL) and used immediately. For validation of the surface biofunctionalization process, controls were prepared using varying biotin (0.01–1 mM) and streptavidin (0.005–0.5 mg mL⁻¹) concentrations and characterized in terms of the efficiency of streptavidin and b-HLM immobilization by fluorescence (for details, see Supporting Information). The success of the surface modifications were also monitored based on changes of the water contact angle (for details, see Supporting Information) compared to that of native thiol-ene surface (Figure 2a).

CYP incubations with enzymes immobilized on magnetic beads: The CYPs immobilized on magnetic beads were incubated in Eppendorf tubes similar to soluble enzymes (see Supporting Information) with the following exceptions: Instead of the specified amount of HLM (see Table S1), 0.25 mg of magnetic beads incorporating the immobilized CYPs were added to each reaction and preincubated with the substrate prior to addition of NADPH. The reactions were stopped by separating the beads from the supernatant using an external magnet. After reaction, the beads were washed twice with PBS buffer (50 μL) and stored (in PBS buffer) at $\leq 4^\circ\text{C}$ until further use. The supernatants (100 μL) were either acidified for fluorescence detection by adding 10 μL of 4 M perchloric acid (CYP2A6) or allowed to react

with the Luciferin detection reagent (100 μ L) for luminescence detection (all other CYP isoforms).

CYP incubations on immobilized enzyme microreactors: The CYP activity of the IMERs was assessed by infusing the substrate solution containing NADPH (0.1 mM, in the specified buffer) through the IMER at a flow rate of 5 μ L min⁻¹ and collecting fractions of 50 or 100 μ L of the outcome for further (off-line) analysis. The collected fractions were either acidified or allowed to react with the luminescence detection reagent as previously specified. For reactions conducted at physiological temperatures, the IMERs were heated using a resistive heater ($R=0.5 \Omega$, $P=0.6-0.8$ W) connected to an external power supply. The temperature on the top surface of the IMER was measured with a thermocouple and adjusted to 35°C (corresponding to roughly two degrees higher temperature inside the microchannel on the basis of a prior publication^[25]) using a PID-controller connected to the circuit.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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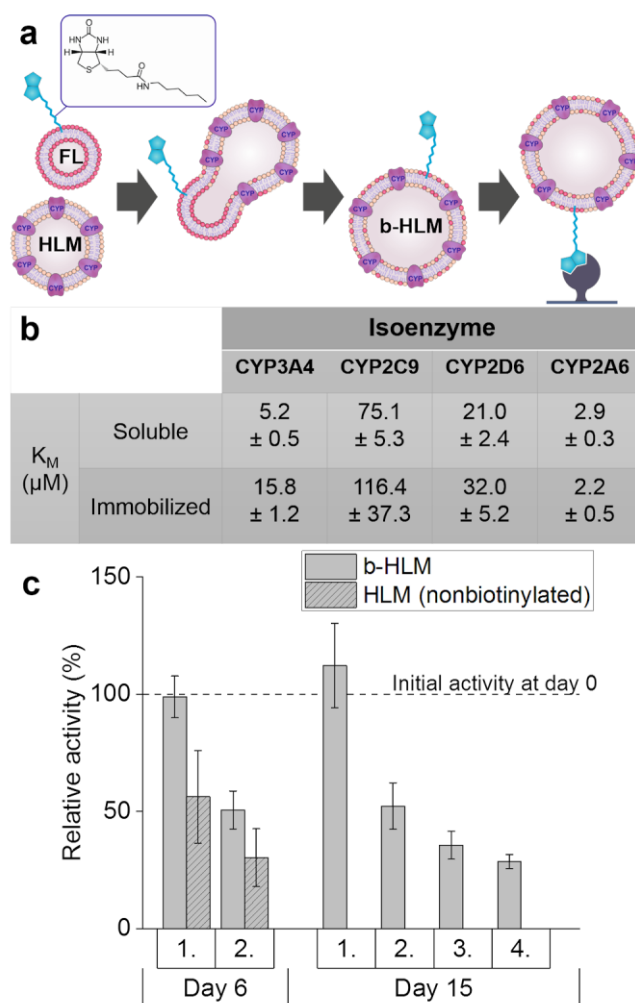


Figure 1. Immobilization of human liver microsomes (HLM) on streptavidin-functionalized magnetic particles using fusogenic liposomes. a) A schematic describing the immobilization protocol. b) Isoenzyme-specific K_M values of four main CYPs, when immobilized on magnetic beads, compared to values determined for the same isoenzymes incorporated in soluble HLM. c) Relative activity (percentage of initial activity on day 0) of b-HLM immobilized on magnetic particles ($n=4$ batches each) on consecutive incubation cycles (1.–4.) of the same batch of beads after 6 or 15 days of storing at 4°C.

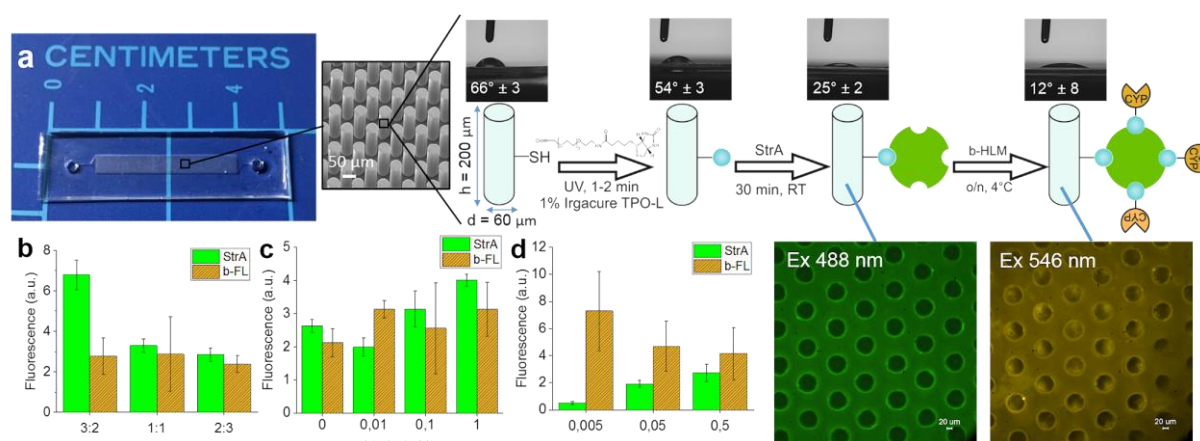


Figure 2. Development of CYP-IMERs utilizing immobilization *via* fusogenic liposomes. a) Photograph of the thiol-ene micropillar chip, scanning electron microscope (SEM) image of the micropillar array and a schematic of the biotin-streptavidin functionalization of the thiol-ene pillars with inlays of fluorescence microscopy pictures and water contact angle measurements of thiol-ene surfaces (25% molar excess of thiol monomer in the bulk) after each step of the functionalization protocol. The effect of b) thiol-ene molar ratio, c) biotin and d) streptavidin concentration on the binding efficiency of streptavidin (StrA) and biotinylated fusogenic liposomes (b-FL) on thiol-ene micropillar surfaces as quantified ($n=3$ IMERs) by fluorescence microscopy (streptavidin: ex 488/em 500–700 nm; b-FL ex 546/em 575–640 nm).

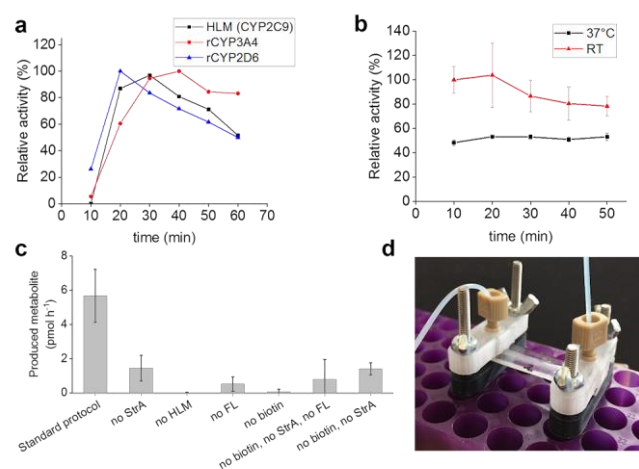


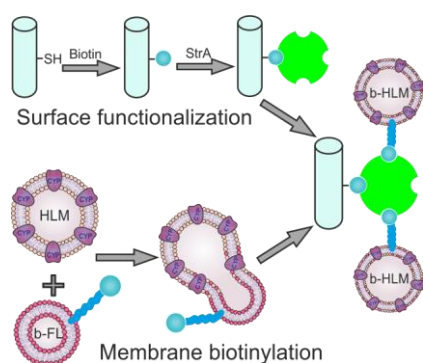
Figure 3. Figure 3. Characterization of CYP-IMER performance. a) Relative activity of CYP-IMERs immobilized with HLM ($n=3$) and two recombinant CYP enzymes ($n=2$ each) as a function of time. b) Relative CYP2A6 activity of CYP-IMERs as a function of time in room temperature and 37°C as percentage of the initial activity at 37°C. c) Cumulative metabolite production (60 min) of CYP-IMERs ($n=3$) prepared by sequentially omitting all the steps in the functionalization protocol. d) A photograph of the complete chip setup with fluidic connectors.

Immobilization of membrane-bound enzymes, such as the drug-metabolizing cytochromes P450 (CYPs), is facilitated by a novel, nanotechnology inspired immobilization method utilizing membrane biotinylation *via* customized fusogenic liposomes. Unlike in previously reported immobilization approaches, the kinetic characteristics of CYPs remain unchanged using the developed method.

Keywords: enzyme immobilization, liposomes, cytochrome P450, drug metabolism, immobilized enzyme reactors

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Overcoming the Pitfalls of Cytochrome P450 Immobilization Through the Use of Fusogenic Liposomes



Supporting Information

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Materials and methods

Materials and reagents: 7-hydroxycoumarin, β -nicotinamide adenine dinucleotide 2'-phosphate (NADPH) reduced tetrasodium salt hydrate, biotin-PEG₄-alkyne, coumarin, Trizma® base and phosphate buffered saline (PBS, pH 7.4) were purchased from Sigma Aldrich, St. Louis, MO. Perchloric acid (HClO₄) and magnesium chloride (MgCl₂) were from Riedel-de-Haën, Seelze, Germany. Irgacure® TPO-L (Ethyl phenyl(2,4,6-trimethylbenzoyl)phosphinate (84434-11-7) photoinitiator was from BASF, Ludwigshafen, Germany. Streptavidin (Alexa Fluor® 488 conjugate) was from Life Technologies, Eugene, OR. All chemicals were of analytical grade unless otherwise stated. Deionized water was purified with a Milli-Q water purification system (Millipore, Molsheim, France). The luminescent cytochrome P450 (CYP) substrates (P450-Glo™ assays), Luciferin-IPA (CYP3A4), Luciferin-ME EGE (CYP2D6), Luciferin-H (CYP2C9), and the Luciferin detection reagent were purchased from Promega, Madison, WI. The human liver microsome (HLM) preparation used (as the source of CYP activity) was Corning® Gentest 20-Donor Pool (BD Biosciences, Woburn, MA). Recombinant isoenzymes used (CYP3A4, CYP2D6 and CYP2C9) were of Corning® Supersomes™ series (BD Biosciences, Woburn, MA). All lipids used in the study were from Avanti Polar Lipids, Alabaster, AL: 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-

(Cap biotiny) (sodium salt) (biotin-cap-DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Lissamine Rhodamine B-DOPE). The magnetic particles used in this study were commercial paramagnetic beads (Dynabeads® M-280, $\varnothing = 2.8 \mu\text{m}$, $\sim 6\text{--}7 \times 10^8$ beads per mL) manufactured by Life Technologies, Oslo, Norway. The monomers used for microchip fabrication, pentaerythritol tetrakis(3-mercaptopropionate) ('tetrathiol') ($\geq 95.0\%$) and 1,3,5-triallyl-1,3,5-triazine-2,4,6(1H,3H,5H)-trione ('triene') ($\geq 98.0\%$), were purchased from Sigma-Aldrich (Saint Louis, MO). Poly(dimethyl siloxane) (PDMS) was prepared by mixing Sylgard 184 base elastomer and curing agent in a 10:1 or 9:1 (w/w) ratio (Down Corning Corporation, Midland, MI).

Fabrication of SU-8 masters: For fabrication of the SU-8 masters (step i of the chip fabrication protocol), silicon wafers were first dipped into hydrofluoric acid to improve photoresist adhesion. SU-8 100 (Micro Resist Technology, Germany) was spin coated (1700 rpm, 30 s) and soft baked on a hotplate (65°C for 25 min followed by 95°C for 3.5 h) to yield a 200- μm -thick SU-8 layer, which was then UV exposed with a dose of 0.63 J cm⁻² on the MA-6 mask aligner (Süss Microtec, Garching, Germany). Post exposure bake was done on a hotplate (65°C for 1 h followed by a slow 4-hour ramp back to room temperature) and the development in propylene glycol methyl ether acetate for 75 min. After development, the master was hard baked on the hotplate at 150°C for 3 h. Finally, the master was coated by a nominally 40-nm-thick fluoropolymer layer for anti-adhesion. The fluoropolymer was deposited by plasma-enhanced chemical vapor deposition (PECVD) (Plasmalab 80+, Oxford Instruments, UK), out of the trifluoromethane (CHF₃) precursor.

Control functionalizations of magnetic particles: For functionalization with biotinylated DPPE lipids, 400 μL (4 mg) of the pretreated beads in PBS were added to the evaporated biotin-DPPE to yield a lipid concentration of 2.5 mM, and incubated at 65°C for 1h. Next, the

lipid-functionalized beads were divided in 25 μL aliquots and 15 μL of bare HLM vesicles (10 mg mL^{-1} total protein in 0.1 M Tris, pH 7.5, containing 3.3 mM MgCl_2 and 1% Pluronic F127) was added to each sample. After incubation at room temperature for 30 min, the supernatant was discarded and the beads were washed 4–5 times with 50 μL of PBS using magnetic separation and stored in 50 μL of PBS at $\leq 4^\circ\text{C}$ until use.

CYP model activities and enzyme incubation conditions: The CYP model activities (probe substrates and their metabolites) are listed in Table S1 along with the isoenzyme-specific incubation conditions (selection of buffer, amount of HLM, incubation time).

CYP incubations with soluble enzymes: The CYP (control) incubations were conducted in Eppendorf tubes at 37°C at a total volume of 100 μL using NADPH (1 mM) as the cofactor. The selected CYP model activities were determined using isoenzyme-specific probe substrates as described in Table S1. The incubation buffer, the amount of HLM, and the incubation time were adjusted isoenzyme specifically so that the model activities followed Michaelis-Menten kinetics (see Table S1). Prior to initiating the reaction by the addition of NADPH, the HLM and the substrate were mixed and preincubated at 37°C for 5 min. The CYP2A6 reactions were stopped by the addition of 10 μL of ice-cold perchloric acid (4 M), after which the samples were kept on ice for 20 min, centrifuged at 16 000 g for 10 min, and the supernatants were transferred for fluorescence analysis (ex 325 nm, em 470 nm). All other CYP reactions were stopped by the addition of the Luciferin detection reagent (100 μL), incubated at room temperature for 20 min and analyzed for luminescence. The fluorescence and luminescence measurements were carried out on a Varioskan Flash or a Varioskan LUX microplate reader (both from Thermo Scientific, Vantaa, Finland). To ensure that the enzymatic activity of HLM is not degraded during the incubation steps of the immobilization procedure, the enzyme activity of HLM (untreated) was determined following

storing/incubation at 4°C, room temperature and 37°C for given periods of time (Figure S3).

Based on the results, CYP activity is not compromised during the immobilization process.

Fluorescence measurements: The reaction mixtures from CYP-IMERs or magnetic bead CYP assays were analyzed with a Varioskan Flash or Varioskan LUX well plate reader using either fluorescence (CYP2A6, ex 325/em 470 nm, bandwidth 12 nm, measurement time 100 ms) or luminescence (CYP2C9, CYP2D6 and CYP3A4) spectroscopy. The binding of streptavidin and the immobilization efficiency of b-HLM vesicles and biotinylated rCYPs on thiol-ene microchannels was monitored on a Zeiss AxioScope A1 upright epifluorescence microscope (Zeiss Finland, Espoo, Finland) equipped with a Plan-Neofluar 20X/0.4 Corr objective and a HAL100 halogen lamp (100W). The fluorescence signal of streptavidin and lissamine rhodamine B (ex 488/em 500–700 nm and ex 546±6 nm, em 575–640 nm, respectively) was monitored using a Moticam 3500 color CCD camera (Motic, Xiamen, China) or quantified using a Hamamatsu R5929 photomultiplier tube equipped with a signal amplifier module (Cairn Research, Faversham, UK) and a PicoScope 2203 AD converter (Pico Technology, St. Neots, UK).

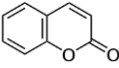
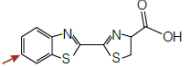
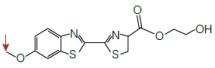
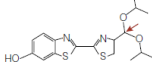
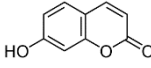
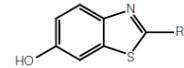
Water contact angle measurements: The wetting properties of thiol-ene surfaces after different functionalization steps were characterized by a sessile water drop contact angle method.^[1] Water drop profiles were recorded with an optical contact angle and surface tension meter (KSV Instruments Ltd., Helsinki, Finland) equipped with a camera (CAM 200, 30 fps). To determine the water contact angle (WCA), three drops of Milli-Q water were placed randomly over the surface and the contact angle was determined by processing the images with Attension Theta software (Version 4.1.0) and averaged.

Scanning electron microscope: Scanning electron microscope (SEM, FEI Quanta™ FEG, Hillsboro, OR) was used for characterization of the micropillar arrays. Samples were attached onto the sample stage with a carbon-coated double-sided tape and sputtered with platinum

(Quorum Q150TS, turbomolecular-pumped high resolution coater, Quorum Technologies,
UK) for 25 seconds (30 mA) resulting approximately in a 5 nm thick coating.

Supplementary figures and tables

Table S1. CYP model activities and incubation conditions used in the study.

Isoenzyme	CYP2A6	CYP2C9	CYP2D6 ^(a)	CYP3A4
Substrate	Coumarin 	Luciferin-H 	Luciferin-ME EGE 	Luciferin-IPA 
[S] used for Michaelis constant (K _M) determination	1-2-4-8-16-32-64 μM	25-50-75-100-150-200-400 μM	7.5-15-22.5-30-45-60-120 μM	2-4-6-8-12-16-32 μM
Metabolite	7-hydroxycoumarin 	Luciferin ^(b) 		
Buffer	Tris (0.1M, pH 7.5) + 3.3 mM MgCl ₂	Potassium phosphate (0.1M, pH 7.4)		
HLM (total protein)	0.4 mg mL ⁻¹	0.4 mg mL ⁻¹	0.4 mg mL ⁻¹	0.2 mg mL ⁻¹
Incubation time (@ 37°C)	15 min	30 min	30 min	10 min
Stop reagent	4M perchloric acid (+10 μL per reaction)	Luciferin detection reagent (+100 μL per reaction)		
Detection	fluorescence ex 325 nm/em 470 nm	luminescence ^(b)		

^{a)}The CYP2D6 incubations were carried out in the presence of 5 μM furafylline (inhibitor of CYP1A1 and CYP1A2 isoenzyme activity) due to limited specificity of Luciferin-ME EGE to CYP2D6.

^{b)}CYP enzymes act on a luminogenic proluciferin to produce a luciferin product that generates light upon reaction with luciferase, which is added after the CYP reaction has been completed. The CYP substrate selectivity depends on the specific structure of the proluciferin substrate.

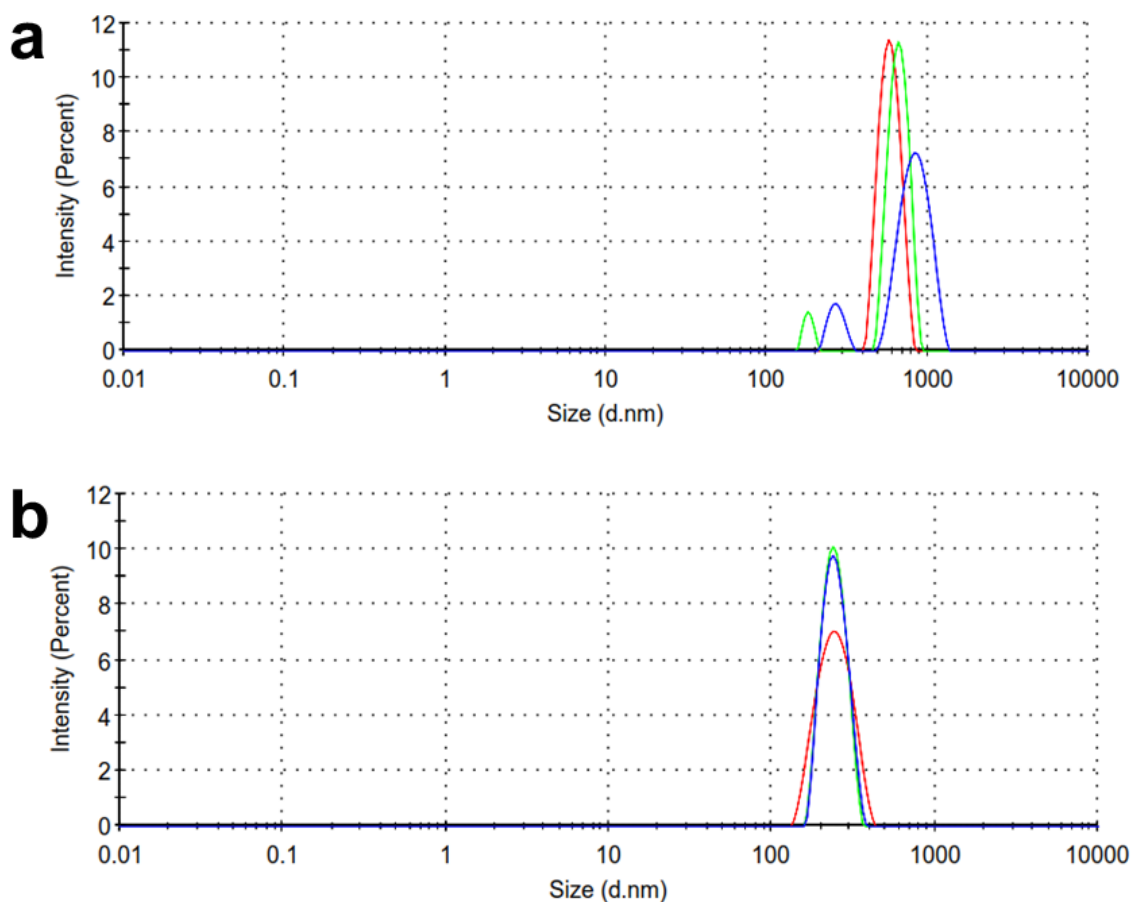


Figure S1. Size distribution of fusogenic liposome dispersion by dynamic light scattering (DLS) a) before extrusion b) after extrusion (51x through a 100 nm membrane). Extrusion of the liposome dispersion resulted in a significant decrease in both average liposome size (Z-average size of 662 nm vs 241 nm) and polydispersity (polydispersity index, PDI) of 0.176 vs 0.094). The results shown are means from three repeated runs of 13 measurements on the same sample. It should be noted that because the z-average gives only a single average value for the whole particle population, it does not accurately describe the size distribution of the polydisperse nonextruded sample. The zeta potential of the fusogenic liposomes was measured to be 69.3 ± 1.8 mV.

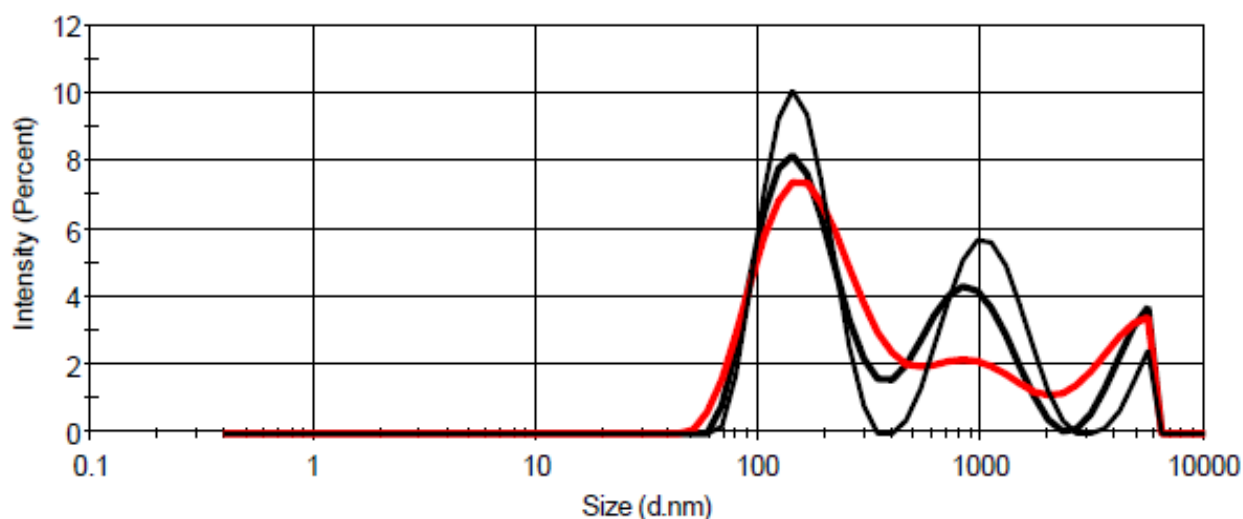


Figure S2. Vesicle size distribution of HLM (0.125 mg mL^{-1} in deionized water) by DLS. The vesicle size distribution showed three distinct fractions with average vesicle diameters of 150 nm, 1000 nm, and 5000 nm. As the scattering intensity is proportional to the sixth power of the particle diameter, scattering from large particles may easily suppress signals from smaller particles.^[2] Taking this into account, the quantity of the smallest vesicles (size fraction in the range of 100–200 nm) is much more than what can be directly inferred from the data. The zeta potential of the HLM preparation used was measured to be $-44.3 \pm 4.9 \text{ mV}$.

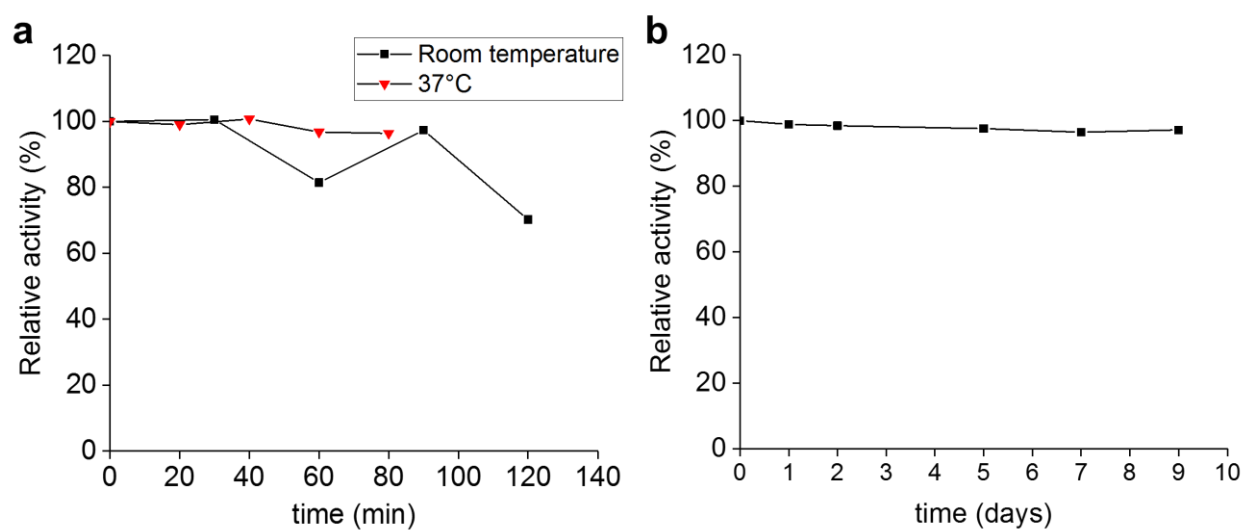


Figure S3. Relative activity of HLM stored for given periods of time at a) room temperature and 37°C and b) 4°C. Data points are means of two independent samples.

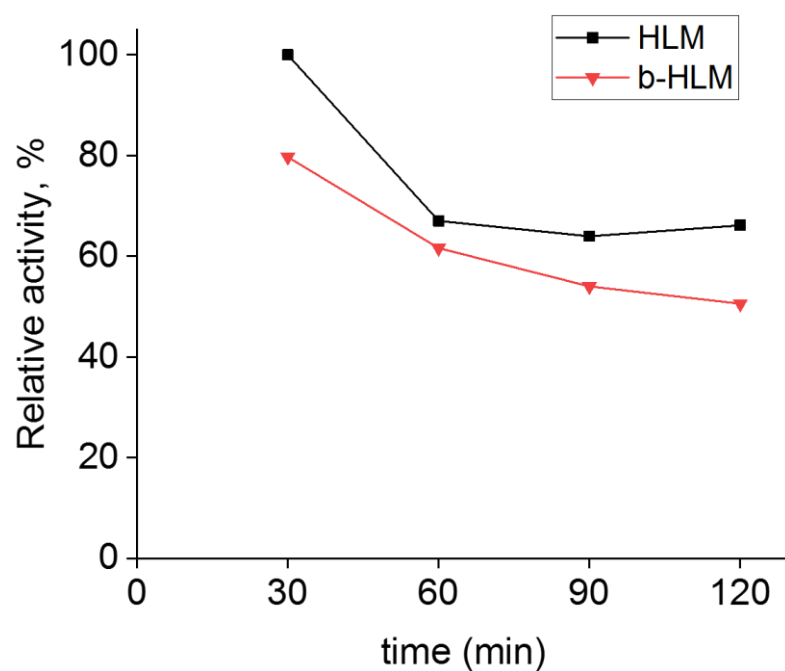


Figure S4. The relative activity of HLM and biotinylated HLM (both in soluble form, nonimmobilized) as a function of time. The initial activity of nonbiotinylated HLM is used as the reference point for the relative activities of both HLM and biotinylated HLM. Data points are means of two independent samples.

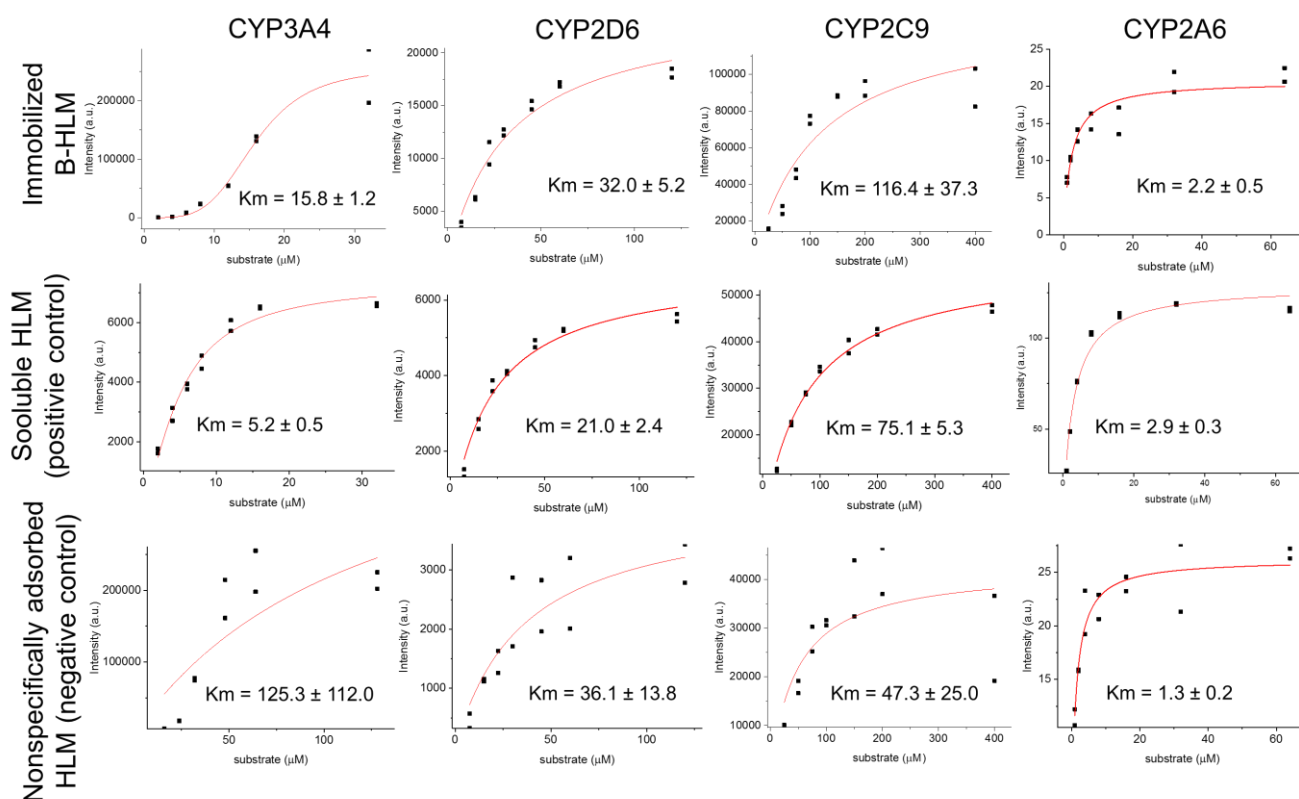


Figure S5. Michaelis-Menten curves for four CYP isoforms determined with soluble HLM, b-HLM immobilized on streptavidin-coated magnetic particles and HLM solubilized on lipid-functionalized magnetic particles (control). For CYP3A4, a sigmoidal fit was chosen based on a comparison of Akaike information criterion values.

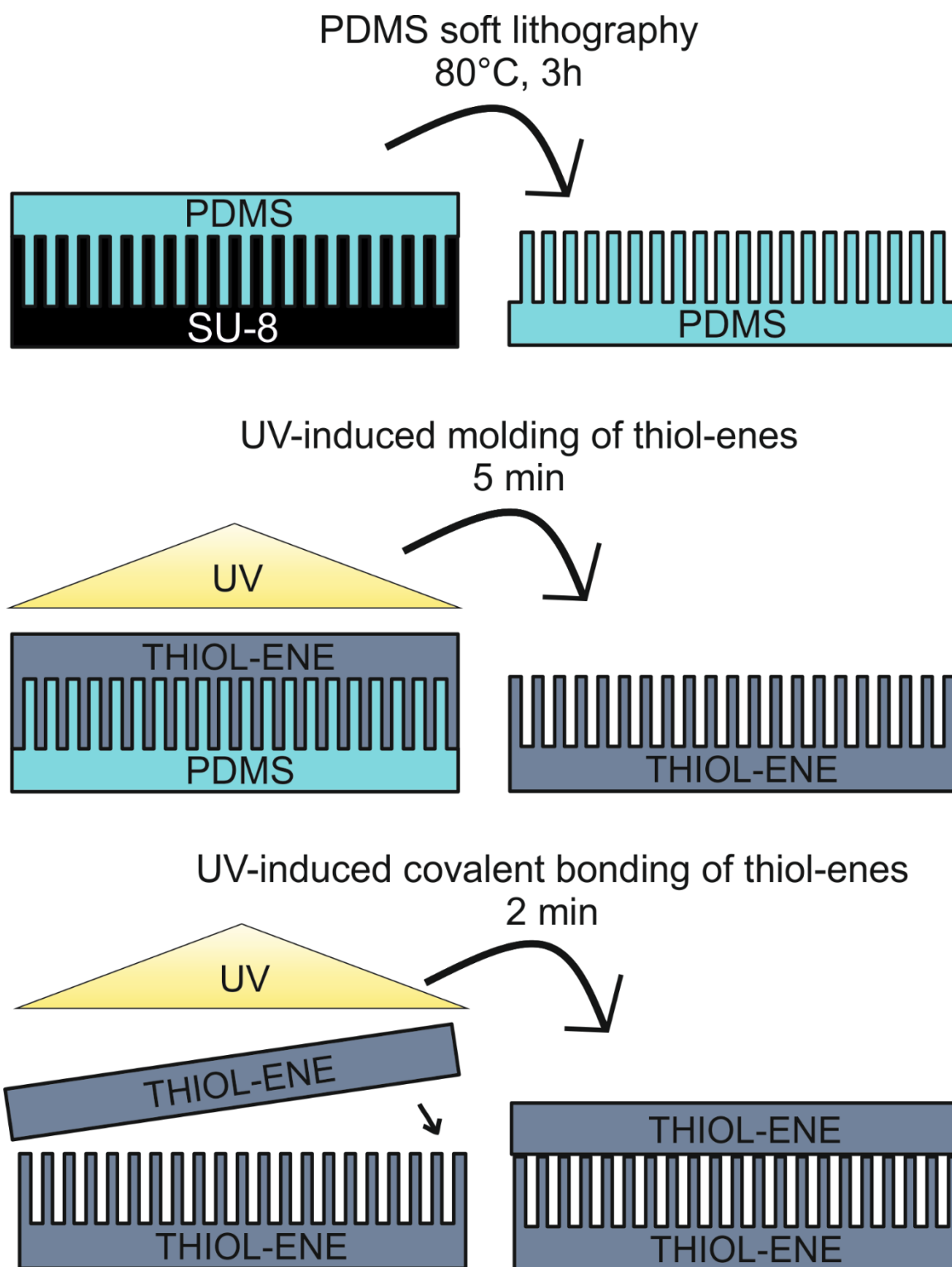


Figure S6. Schematic of the thiol-ene chip fabrication protocol.

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