Published in final edited form as:

Nanotechnology. 2012 September 28; 23(38): 385101. doi:10.1088/0957-4484/23/38/385101.

Selective Filling of Nanowells in Nanowell Arrays Fabricated Using Polystyrene Nanosphere Lithography with Cytochrome P450 Enzymes

Lance A. Wollenberg^{1,†}, John E. Jett^{1,†}, Yueting Wu², Darcy R. Flora³, Nianqiang Wu², Timothy S. Tracy⁴, and Peter M. Gannett^{1,*}

¹West Virginia University, Basic Pharmaceutical Sciences, P.O. Box 9530, Morgantown, WV 26506

²West Virginia University, Department of Chemical Engineering, P.O. Box 6102, Morgantown, WV 26506

³College of Pharmacy, University of Minnesota, Minneapolis, MN, 55455

⁴University of Kentucky, College of Pharmacy, 789 S. Limestone, Lexington, KY 40536

Abstract

This work describes an original and simple technique for protein immobilization into nanowells, fabricated using nanopatterned-array fabrication methods, while ensuring the protein retains the normal biological activity. Nanosphere-lithography was used to fabricate a nanowell array with nanowells that were 100 nm in diameter and a periodicity of 500 nm. The base of the nanowells was gold and the surrounding material was silicon dioxide. The different surface chemistries of these materials were used to attach two different self-assembled monolayers (SAM) with different affinities for the protein used here, cytochrome P450 (P450). The nanowell SAM, a methyl terminated thiol, had high affinity for the P450. The surrounding SAM, a polyethylene glycol silane, displayed very little affinity toward the P450 isozyme CYP2C9, as demonstrated by x-ray photoelectron spectroscopy and surface plasmon resonance. The regularity of the nanopatterned array was examined by scanning electron microscopy and atomic force microscopy. P450-mediated metabolism experiments of known substrates demonstrated that the nanowell bound P450 enzyme exceeded its normal activity, as compared to P450 solutions, when bound to the methyl terminated self-assembled monolayer. The nanopatterned array chips bearing P450 display long term stability and give reproducible results making them potentially useful for high throughput screening assays or as nanoelectrode arrays.

Keywords

Nanowell array; Self-assembled monolayer; cytochrome P450

I. Introduction

Nanoscale protein patterns have a number of applications such as biosensing [1;2], control of cell adhesion and growth [3-6], and the fabrication of biochips [7-10]. Reduction of the feature size is beneficial as it often results in the reduction of the amount of analyte or

*Please address correspondence to: Peter Gannett, 1 Medical Center Drive, P.O. Box 9530 Morgantown, WV 26505, USA, 304-293-1480 (ofc), 304-293-2576 (fac), pgannett@hsc.wvu.edu.

[†]Authors contributed equally to this work

reagent required, permits an increased density of sensor and chip elements, and faster reaction or response times [11;12]. At the micrometer level, protein patterning has been achieved by the application of techniques such as photolithography [13;14] and microcontact printing [15;16]. These approaches prepare surfaces such that discrete regions of reactive terminal groups (e.g., self-assembled monolayers (SAMs)), organic thin films, or polymers, are created for subsequent protein adsorption [17]. It should be noted that these approaches often rely on the availability of chemistries that provide differential surface reactivities for immobilizing proteins onto surfaces, such as the photoactivation of SAM surfaces [18].

These techniques provide a high throughput means for assembling proteins at a size scale of hundreds of nanometers or larger [18]. Atomic force microscopy (AFM) based lithography has been developed that can pattern SAMs at the dimension of nanometers [19;20], allowing further miniaturization. To produce large arrays of nanometer-sized structures, particle or nanosphere lithography has been developed [21;22]. In this approach, monodisperse particles are closely packed into 2D periodic structures and then these structures are used as a template where the void space can be filled with the material of interest. The particles are then removed [23;24] leaving raised features that can be subsequently elaborated.

Patterned arrays can also be constructed to create micro or nanowells for use as small scale reactors, high throughput technologies, and nanoelectrode arrays. Several approaches have been used to create micro/nanoscale reactors [25] including liposomes [26;27], reverse-micelles [28], oil-dispersed droplets [29], biomolecules [30], chip-based [31] and other strategies [32]. Reducing the volume and constraining the reactants to small volumes has both basic science and practical applications [33]. At the same time, this creates the problem of how reactants are deposited in the nanowells prior to the reaction, constrained to the nanowells during the reaction, released following the reaction, and identification and/or quantitation of the resulting products.

In this work, we have created a nanowell array by a nanosphere lithography technique. In this approach, polystyrene beads were coated on a gold surface and then reduced in size by plasma ion etching. The exposed surface was backfilled with silicon dioxide and then the polystyrene spheres were dissolved away, leading to nanowells with gold on the bottom and silicon dioxide on the walls and in the intervening space. These nanowells were then used as nanoincubators to conduct cytochrome P450 (P450) mediated metabolism of model substrates. Protein confinement to the nanowells was achieved by using selective surface chemistries to attach different SAMs to the floor of the nanowells and to the surrounding walls and surface. The characteristics of these SAMs were such that the P450 bound to the SAM bonded to the floor of the nanowell and not to the surrounding surface. Moreover, the approach used during enzyme immobilization resulted in an active enzyme, fully capable of metabolizing typical substrates, remaining active through the course of the metabolic reaction. Finally, the SAM layer and bound P450 can be removed and reapplied such that the nanowell chip can be reused. The procedures used have been optimized to produce low-defect periodic arrays bearing active and isolated protein nanostructures. Nanowells are an attractive format for conducting metabolism studies as the wells will minimize protein aggregation, lead to improved reproducibility, and, importantly, are amenable to high throughput screening.

II. Experimental Section

1. General

Unless otherwise noted, potassium phosphate buffer (KPi) was made using 40mM solutions of mono and dibasic potassium phosphate to provide solution pH=7.4. Running buffer for

SPR experiments was comprised of sodium chloride (150mM) in KPi buffer (phosphate buffered saline (PBS)). CYP2C9 adsorption to the nanowells was conducted in the presence of flurbiprofen and dapsone (1:1, 40 μ M each) in KPi. Cryobuffer solution was made from KPi and glycerol (80:20, v/v). Diclofenac sodium, dapsone, flurbiprofen, beta-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), 8-octanethiol (OT), and N-phenylanthranilic acid were purchased from Sigma-Aldrich (Milwaukee, WI). 4'-OH-Diclofenac was purchased from Toronto Research Chemicals (Toronto, Canada). Cytochrome P450 reductase (CPR) was purchased from BD Biosciences (San Jose, CA). Purified CYP2C9 was prepared as previously described [34], 2-[Methoxy(polyethyleneoxy)propyl]trimethoxysilane was purchased from Gelest (Morrisville, PA). Microscope cover slides were purchased from VWR (Radnor, PA). SPR substrate holders were purchased as part of an SIA Au kit from GE Healthcare (Piscataway, NJ). Gold coated glass microscope slides were purchased from Evaporated Metal Films (Ithaca, NY). All metals were purchased from the Kurt J. Lesker Company (Clariton, PA). Polystyrene spheres (500 nm diameter) were purchased from Thermo-Fisher Scientific (Waltham, MA). A Barnstead NANOpure water system (Thermo Scientific, Waltham, MA) was the source of water for all aqueous solutions, water washes, etc.

2. Nanopatterned array chip fabrication

A large-area close-packed polystyrene sphere monolayer template was prepared as previously reported [35]. Briefly, a silicon chip (15 mm $\times$ 15 mm) with a resistivity of $> 2000 \Omega \text{ cm}^{-1}$ (p-type, 111° , undoped) was cleaned in piranha solution ($\text{H}_2\text{O}_2:\text{H}_2\text{SO}_4$, 3:7, v/v) for 1 h (Caution: piranha solution reacts violently with organic chemicals) and was soaked, with sonication, in concentrated ammonia, hydrogen peroxide and water (1:1:5, v/v) for 1 h to produce a hydrophilic surface. A mixture (5 μ L) of polystyrene spheres (PS) ($d=500 \text{ nm}$), made up in water (10% w/v) and diluted with 100% ethanol (1:1 v/v) prior to use, was dispensed onto the pretreated silicon chip. After the PS covered the silicon chip, it was slowly immersed into pure water in a petri dish (10 cm diameter, water depth $> 1 \text{ cm}$) to release the PS spheres from the silicon surface, forming a self-assembled, close-packed PS microsphere monolayer on the water surface. A gold coated glass microscope slide (15 mm $\times$ 15 mm), cleaned with piranha solution for 10 min, was dipped into the water bearing the surface PS monolayer to cover 10 mm (total area 10 mm $\times$ 15 mm) and then slowly pulled out, thereby transferring the PS monolayer onto the gold substrate.

After solvent removal, the PS microsphere monolayer present on the gold surface, was first baked in an oven (50°C) for 16 hr then placed in a March PX-250 Plasma Asher (Nordson Corporation, Westlake, OH) and ashed at a low power of 30 watts and a high oxygen pressure of 110 mtorr for 5-30 min to reduce the PS to the size desired (monitoring by SEM at 1-5 min intervals). A thin film (10 nm) of SiO_2 was then deposited by e-beam evaporation with a Temescal BJD-2000 (Edwards Vacuum, Phoenix, AZ) system with an Inficon XTC/2 deposition controller (Inficon, East Syracuse, NY) at a rate of 5-10 $\text{\AA/s}$. Finally, samples were soaked in ethanol and heavily sonicated for 30 min to remove the PS spheres and nanowell arrays were fabricated. The morphology of the nanoarrays was examined by a scanning electron microscope (SEM) and an atomic force microscope (AFM).

3. Surface characterization

SEM micrographs were obtained using a JEOL JSM-7600F field emission analytical scanning electron microscope (Tokyo, Japan) using a low beam energy (5.0 kV). Chips with nanowell arrays were mounted on a sample stage and the chip edges were grounded using silver paint to minimize charging effect.

AFM imaging was performed using a multimode scanning probe microscope (SPM) with a Nanoscope IIIA controller (Veeco Instruments, Woodbury, NY). AFM images were obtained by first mounting the samples on a magnetic AFM sample disk and placing the disk on the AFM scanner (E-head, Veeco Instruments). A silicon AFM cantilever (NCS12, Micromass) was used with an oscillation frequency between 273-310 KHz and oscillation amplitude of 0.5-2 nm. The tip of the cantilever used to scan the surface had a radius of curvature between 4 nm and 12 nm. The AFM was operated in tapping mode with a scan rate of 1-1.5 Hz

XPS analysis was performed with a Physical Electronics VersaProbe 5000 XPS (Chanhassen, MN), equipped with an EA125 energy analyzer. Photoemission was stimulated by monochromated Al K α X-ray (1486.6 eV). Nitrogen 1s (N1s) core level scans were scanned from 411-391 eV and carbon 1s (C1s) core level from 299-279 eV. Electron counts were analyzed every 0.1 eV for high resolution core-level scans. Spectra (C1s, N1s) were acquired for the protein-adsorbed and nano-array samples using a pass energy of 58.7 eV. Peak binding energies were referenced to the Au 4f peak at 84.0 eV.

4. SPR Film Fabrication

Microscope cover slides (40 mm $\times$ 24 mm, No. 2) (VWR, Radnor, PA) were cut into 12 mm $\times$ 10 mm rectangles and were loaded into a Temescal BJD-2000 (Edwards Vacuum, Phoenix, AZ) system with an Inficon XTC/2 deposition controller (Inficon, East Syracuse, NY) for metal evaporation at a pressure 1.0×10^{-5} Torr and a system voltage of 10.0 kV. Typical currents were approximately 40 mA for titanium and 80 mA for gold. Samples were rotated 1-2 RPM in the chamber to ensure uniform coverage and a crystal monitor with gold 6 MHz piezoelectric crystals (Kurt J. Lesker Co.) was used to monitor metal thickness during evaporation.

The emission current was first allowed to stabilize at 12-13 mA, and then the current was slowly ramped up in 5 mA increments until the metal began to melt at which time the shutter was opened and evaporation onto the SPR substrates would begin. The deposition rate onto the samples was controlled by adjusting the emission current and was typically maintained at 0.3-0.5 Å/s. The Inficon XTC/2 deposition controller automatically closed the shutter when the desired final thickness had been reached. Target thickness for SPR substrates was 50 nm of gold on a 2 nm thick titanium adhesion layer. Upon completion of deposition, the chamber was returned to atmospheric pressure and samples were allowed to cool for 30-60 minutes before removal. Deposition of silicon dioxide was conducted as described in the nanopatterned array fabrication section (Section 2), above.

5. Self-assembled monolayer (SAM) formation

Preparation of octanethiol (OT) SAM on gold—Thin gold films on glass microscope slides (XPS analysis) or gold coated microscope cover slides (SPR analysis) were prepared by first rinsing with distilled water and ethanol (3X each) and then submerging in an ethanolic solution of octanethiol (OT) (10 mM) (16 h). After removal from the thiol-containing solution they were again rinsed with ethanol and distilled water (3X each) and then dried under a gentle stream of nitrogen.

Preparation of PEG-Silane SAM on silicon dioxide—Thin films of silicon dioxide (10 nm) on gold coated microscope slides (XPS analysis) or gold coated microscope cover slides (SPR analysis) were first rinsed with distilled water, ethanol and anhydrous toluene (3X each) before being submerged 2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane (6 mM) in anhydrous toluene containing triethylamine (1%, v/v) (16 h). The silane-SAM films were then rinsed with anhydrous toluene, ethanol, and distilled water (3X each) and dried

under a gentle stream of nitrogen. Regardless of the substrate (microscope slide, cover slip, silicon), a SAM made from OT on gold is referred to as Au-OT. Likewise, for 2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane derived SAM on silicon dioxide are referred to as SiO₂-PEG-Silane.

Preparation of mixed SAM on nanowell substrates—Self-assembled monolayers were prepared in two steps. The nanowell substrate was first washed with distilled water and ethanol (3X each), then submerged in an ethanolic solution of octanethiol (10 mM) (16 h), removed from the thiol-containing solution, rinsed with distilled water and ethanol, and anhydrous toluene (3X each), and submerged 2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane (6 mM) in anhydrous toluene containing triethylamine (1%, v/v) (16 h). The nanowell was then rinsed with anhydrous toluene, ethanol, and distilled water (3X each) and dried under a gentle stream of nitrogen.

6. SPR Binding Analysis

Au-OT or SiO₂-PEG-Silane coated chips were affixed with double sided tape to SPR substrate holders. Assembled SPR cartridges were loaded into a Biacore X100 SPR (GE Healthcare, Piscataway, NJ) for analysis. SPR experiments were conducted with a continuous flow of (PBS) at 10 uL/min. Running buffer was flowed over the sensor surface for a total of 90 sec, then a solution of CYP2C9 in PBS (100 nM, 10 uL/min) for 480 sec, and then running buffer for an additional 480 sec. Quantitation of approximate surface coverage was determined as the difference in response values (Δ RU) before and after CYP2C9 injection. Values were selected at time points before and after injection that allowed sufficient stabilization of the SPR system. The approximate coverage was then calculated from the Δ RU as one RU corresponds to approximately 1pg/mm² of protein [36]. Each sensorgram trace is an average of three separate injections and each injection measurement was conducted on a freshly made SPR sensor film.

7. CYP2C9-SAM immobilization

Either Au-OT or SiO₂-PEG-Silane bearing substrates or nanowell chips, prepared as described above, were treated with CYP2C9 (100 nM) dissolved in KPi (16 h) containing flurbiprofen (40 μ M) and dapsone (40 μ M). [37] Inclusion of flurbiprofen and dapsone is essential to retain enzymatic activity. They are removed in the subsequent rinsing procedure. The protein treated SAM surfaces were rinsed in KPi (3X) and distilled water (3X) and then dried under a gentle stream of nitrogen and then stored in cryobuffer (KPi:Glycerol, 1:4) at -80°C at least for 4 h prior to use

8. CYP2C9 Incubation Conditions

Nanopatterned array chips bearing SAM (OT in the wells, PEG-Silane elsewhere) and CYP2C9 were immersed in solutions containing cytochrome P450 reductase (200 nM) and diclofenac (250 μ M) in KPi buffer in 1.5mL Eppendorf tubes. Incubations were initiated by the addition of NADPH (1 mM, final concentration) in KPi (1 mL, final volume). The mixtures were placed in a water bath and incubated at 37°C (16 h). Positive control experiments used a solution of CYP2C9 (2.5 nM) containing dilaurophosphatidyl choline (50uL, 1 mg/mL), CPR (200 nM) and diclofenac (250 μ M) in KPi (1 mL total volume) and were initiated by the addition of NADPH (1 mM final concentration). Incubations were quenched by the addition of glacial acetic acid (250 μ L) containing N-phenylanthranilic acid (internal standard) in acetonitrile (250 μ L, 5 μ g/mL HPLC grade), centrifuged (13,000 x g) for 10 min to precipitate protein, the supernatant isolated, and dried down (Savant SC110 Speedvac (Thermo-Fisher, Pittsburgh, PA)). Once dry, samples were dissolved in (200 μ L)

sodium acetate buffer (75 mM, pH 5) and acetonitrile (60:40, v/v), and loaded into HPLC vials for analysis [38]. Incubations were conducted in triplicate and on three different chips.

9. Chromatographic Detection of CYP2C9 metabolites

Separation of metabolite and parent compound was conducted using a Waters Alliance 2965 separations module (Milford, MA). [39] Injection volume was 10 μ L, the column was an Agilent Zorbax SB C-18 (150 mm $\times$ 4.6 mm, 5 μ m particle size (Agilent Technologies, Santa Rosa, CA)), flow rate=0.5 ml/min, the mobile phase was a 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 at 280 nm. Data were processed using the Waters Empower V2.0 software (Waters Corporation, Milford, MA). The peak corresponding to 4'-hydroxydiclofenac was quantitated by comparison to a 4'-hydroxydiclofenac standard curve. Approximate retention times for diclofenac, 4'-hydroxydiclofenac and N-phenylanthranilic acid were 23.4 min, 10.2 min, and 20.5 min, respectively.

III. Results and Discussion

Nanopattern array fabrication

The process for fabrication of the nanopatterned array bearing the nanowells is shown in Figure 1 [35]. The steps for the fabrication of the nanowells are described in the Experimental section. Similar approaches have been used to produce arrays of nanowells though the nanowells that result from these alternative approaches typically have diameters that are 2-3 times greater than those fabricated here [4;10;40;41]. Using the approach described, we have been able to obtain nanowell arrays with nanowell diameters down to 50 nm, though below 100 nm it becomes more difficult. Finally, we note that, using the method described here, nanowell arrays can be made over relatively large areas (cm dimensions).

A SEM image of the nanowell surface, prior to SAM formation, is shown in Figure 2. Under low magnification (Figure 2a) the regularity of the pattern over a large area can be seen. Measurement of the nanowell (diameter 100 nm) and periodicity between (500 nm) were made from higher magnifications (Figure 2b). An AFM image of the same chip is shown in Figure 3 and is in agreement with the SEM images with respect to nanowell diameter and periodicity. In addition, the image shows that the silicon dioxide surface is rough with variations in height of several nanometers. While this is not a problem with regard to the functionality of the chip, it does make it impossible to determine, by AFM, whether and where the P450 enzyme binds after SAM formation (surface, nanowells, or both) as the surface roughness is of the same magnitude as the enzyme (roughly 6 nm in diameter [42]). Consequently, indirect methods were utilized to determine P450 binding and location.

XPS Characterization of the Nanopatterned Array Fabrication Process

The desired goal is to utilize the nanowells as nanoreactors. To achieve this result we utilized selective protein binding to nanowell floors by utilizing SAMs with varying terminal moieties and, therefore, different P450 affinities. The nanowell array was examined by XPS at each step of the fabrication process. Once the nanowell chip had been made, a SAM of octane thiol (OT) was created in the nanowells utilizing the gold layer on the nanowell floor. While there is some concern that this layer might not be confined to the gold surface, it is known that SAMs of thiols do not form on silicon dioxide unless the thiol is applied neat [43]. XPS, discussed below, confirms this. After formation of the OT SAM in the nanowells, a second SAM, comprised of 2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane (PEG-Silane), was formed on the silicon dioxide surface. Finally, the enzyme, CYP2C9, was adsorbed to the chip. The XPS data (O1s, Si2p, C1s and N1s core) following the creation of the OT self-assembled

monolayer, after the addition of the PEG-Silane SAM, and after enzyme binding are presented in the plots shown in Figure 4. The silicon and oxygen plots are consistent with the surface changes but the data is not very sensitive to SAM variation or the CYP2C9. Both the C1s (Figure 4c) and N1s (Figure 4d) are more sensitive to the changes in the surface composition. Figure 4c (1) displays XPS data that is consistent with the presence of OT on the chip surface as the observed peak is consistent with the C-C bonds of OT [44]. Spectrum 4c (2) was obtained following the addition of the PEG-silane. This spectrum exhibits a characteristic double peak (indicated by the arrow) consistent with the presence of a C-O bond [45]. Therefore, this suggests that the PEG-Silane SAM is present on the surface. Finally, the XPS spectrum shown in Figure 4c (3) was obtained following binding of CYP2C9 to the chip. The corresponding N1s core spectra are shown in Figure 4d and demonstrate that after addition of CYP2C9 (Figure 4d (3)), C-N can be detected, consistent with the binding of the enzyme to the surface, but that prior to treatment with the enzyme, species with a C-N bond were not present (Figure 4d (1) & (2)).

To assess the likely location of CYP2C9 binding on the surface, two separate samples were prepared on glass microscope slides: 1) a gold under layer with an OT SAM to mimic the floor of the wells, and 2) a silicon dioxide under layer with a PEG-Silane SAM to mimic the walls and intervening spaces. Both samples were then treated with CYP2C9 and the XPS data obtained (Figure 5). The intensity of the N1s signal obtained from the OT SAM treated sample with CYP2C9 was roughly 20 times that of the PEG-Silane SAM treated sample. The difference may be greater under conditions where both OT and PEG-Silane SAMs are present, as in the case of the nanowell chip, as CYP2C9 may preferentially bind to the OT SAM. Nevertheless, the result indicates that, on a nanowell chip with OT lining the floor of the wells and PEG-Silane elsewhere, the CYP2C9 will preferentially bind in the well. Furthermore, it has been well demonstrated in the literature that hydrophobic proteins tend to strongly bind to alkyl groups but not to PEG terminated SAMs [46;47].

SPR Characterization of CYP2C9 Adsorption to OT and PEG-Silane SAMs

A second approach to demonstrating selective binding to the OT SAM, and to better quantitate the process, utilized surface plasmon resonance (SPR). Figure 6 displays the SPR sensorgrams that were obtained from either the adsorption of CYP2C9 to Au-OT SAM sample or to the SiO₂-PEG-Silane SAM sample. For each experiment, sensor surfaces were equilibrated with running buffer for 90 sec. CYP2C9, in running buffer, was then passed over the surface of the sensor (Figure 6, arrow 1) for 480 sec and then switched back to running buffer alone (Figure 6, arrow 2). During the last phase (550 sec), loosely bound CYP2C9 is washed from the surface or from surface bound CYP2C9. The relative response of the SPR to CYP2C9 binding to the Au-OT SAM sample was more than 50 times greater than for the SiO₂-PEG-Silane SAM sample. Thus, binding of CYP2C9 to Au-OT SAMs is predicted to be greatly favored over SiO₂-PEG-Silane SAMs.

The change in response (Δ RU), determined by the difference of the response value taken between $t < 90$ sec and at $t = 1120$ sec, corresponds to the mass of CYP2C9 that is bound to the surface. Previous studies conducted with radioactive proteins [36] have determined that 1 RU corresponds to a coverage of protein equivalent to 1 pg/mm². This allows the calculation of the amount of enzyme on the surface. For the Au-OT SAM sample, 5.853 ± 0.15 ng/mm² (Δ RU = 5853 ± 150) was bound to the surface while for the SiO₂-PEG-Silane SAM sample 0.109 ± 0.10 ng/mm² (Δ RU = 109 ± 100) was bound. These values represent a 53-fold increase in CYP2C9 binding to the Au-OT-SAM sample relative to the SiO₂-PEG-Silane SAM sample. In the context of the nanowell chip, then, since the nanowells have an OT SAM in the bottom of the well while the surrounding area (nanowell wall and the intervening space between wells) bears a PEG-Silane SAM, CYP2C9 is expected to bind to

the OT SAM and be located in the nanowells. Effectively, this approach constitutes a method for filling nanowells.

CYP2C9 activity

As noted, the goal here was to selectively place CYP2C9 in the nanowells by binding them to the SAM that is attached on the floor of the nanowell, while preventing non-specific binding to the remaining SAM. In addition, we required that the bound enzyme retain the endogenous activity associated with the enzyme. If this can be achieved then the CYP2C9 will be solely located in the nano-array well and can be used in a variety of applications such as high throughput screening for binding. The nanopatterned array of wells bearing SAMs was prepared as described above and then treated with a solution of CYP2C9 to bind the enzyme in the nanowells. This chip was then evaluated for whether the bound CYP2C9 retained metabolic activity toward a known molecule utilizing the endogenous co-factors and co-enzymes (NADPH and cytochrome P450 reductase) as the electron source. The results of this and several reference incubations are shown in Table 1 for CYP2C9 mediated metabolism of diclofenac to 4'-hydroxydiclofenac.

The reference incubations with CYP2C9 included a solution incubation, CYP2C9 bound to a OT SAM on a planar gold on silicon chip, and CYP2C9 bound to SiO₂-PEG-Silane. The solution incubation yielded the greatest amount of metabolite (12.56 ng), the OT SAM on gold approximately 75% of the solution incubation, and the PEG-Silane only a trace of metabolite. In the case of the nanowell chip, 7.41 ng of metabolite was formed or approximately 60% of that observed from the solution incubation. Metabolite production, in the latter two cases, must have been formed from surface bound enzyme as these incubations do not contain phospholipid, necessary for metabolite production in solution-based incubations [37].

The results suggest increased activity of the CYP2C9 when confined to the nanowells relative to the other conditions based on the amount of CYP2C9 present in each system and may be due to differences in aggregation, known to affect enzyme activity. [48] The amount of enzyme present in the solution-based incubations contained 2.5 picomole (M.W. ~ 55000, 138 ng) of enzyme. The amount of enzyme present on the nanowell chip is difficult to determine though an upper limit can be calculated from the size of the chip and the SPR data. The size of the chip used in the incubation was 10 mm × 15 mm (area with nanowells) and would bear 6×10^8 nanowells, each with an area of 7.85×10^3 nm² (total area of 4.71 mm²). The SPR binding gave a response corresponding to a coverage (on a OT SAM on gold) of 5.85 ng/mm². Combining the two, a total 27.6 ng is calculated to be bound to the nanowells. Thus, even though there is less CYP2C9 on the chip metabolite, metabolite production per ng of enzyme is greater in the nanowells by a factor of ~8 relative to the solution incubation.

Note that to observe metabolite production from a P450 in solution requires the presence of phospholipids in the case of CYP2C9 and diclofenac [38]. Thus, if the nanowell immobilized CYP2C9 desorbs into solution, it will not be able to metabolize diclofenac. We also note that the SAM and enzyme may be removed from the chip by plasma ashing, the two SAMs reapplied, enzyme re-immobilized, and a metabolically competent chip obtained. Therefore, the nanowell chip can be reused and we have done so here (three cycles with no loss in activity). While it is likely that there is a limit to the number of repetitions of this process that can be performed before the metabolic activity falls off to an unacceptable value, to date we have not determined this limit.

IV. Conclusions

Here we describe a novel approach to creating large, periodic arrays of nanowells and for selectively filling them with an enzyme. Given their small size (zeptoliter range), selectively loading the nanowells can be a challenge. To accomplish this we fabricated the array such that the floor of the well was gold and the surrounding substrate, silicon dioxide, and then took advantage of selective surface chemistries to create SAMs with different affinities toward CYP2C9. In this form, the nanobiochip has potential applications for high throughput screening of pharmaceutical agents that may bind or be metabolized by the P450 captured in the nanowell. Whether a drug candidate binds to a P450 is very important and can determine if further development is warranted. Alternatively, by replacing the OT SAM with one made from mercaptoundecanoic acid (MUA), the P450 enzymes can be bonded to the MUA thereby placing them in electrical contact with the underlying gold surface as we have shown on planar gold surfaces [49]. In this configuration, the nanobiochip can be used for metabolism studies where the electrons required for enzyme activity can be supplied electrochemically. In this mode, the array could be used for enzyme kinetics and for the generation of metabolites (i.e., a biochemical reactor) [49]. It may also be possible to further reduce the diameter of the nanowells such that the wells will contain a single enzyme. This control will permit study of protein-protein interactions on enzyme activity, not otherwise achievable in bulk solution where aggregation effects produce multimers and modulate activity. Finally, it is of particular interest to note that the enzyme activity is retained after fabrication. Normally, immobilized P450 enzymes require electrochemical sources of electrons for activity while here, *in vivo* activity is retained [37] and the normal co-factors and co-enzymes serve as the electron source.

Acknowledgments

The authors thank NIH (GM086891, NSF (EPS-1003907), and fellowships to JEJ and LAW (HEPC.dsr.09013) for support of this research.

V. References

- [1]. Morrison, DWG.; Dokmeci, MR.; Demirci, U.; Khademhosseini, A. Clinical Applications of Micro- and Nanoscale Biosensors. In: Gonsalves, KE.; Halberstadt, CR.; Laurencin, CT.; Nair, CT., editors. Biomedical Nanostructures. John Wiley & Sons; 2008. p. 433-454.
- [2]. Gaubert HE, Frey W. Nanotechnology. 2007; 18:135101. [PubMed: 21730372]
- [3]. Lan S, Veisoh M, Zhang M. Biosensors and Bioelectronics. 2005; 20:1697. [PubMed: 15681184]
- [4]. Kuo CW, Chien F-C, Shiu J-Y, Tsai S-M, Chuch D-Y, Hsiao Y-S, et al. Nanotechnology. 2011; 22:265302. [PubMed: 21576808]
- [5]. Veisoh M, Zhang M. Journal of the American Chemical Society. 2006; 128:1197. [PubMed: 16433536]
- [6]. Pollheimer PD, Kastner M, Ebner A, Blaas D, Hinterdorfer P, Gruber HJ, et al. Bioconjugate Chemistry. 2009; 20:466. [PubMed: 19196022]
- [7]. Blawas AS, Reichert WM. Biomaterials. 1998; 19:595. [PubMed: 9663732]
- [8]. Scouten WH, Luong JHT, Brown RS. Trends Biotechnol. 1995; 13:178.
- [9]. Zhang S, Yan L, Altman M, Lassle M, Nugent H, Frankel F, et al. Biomaterials. 1999; 20:1213. [PubMed: 10395390]
- [10]. Ruiz A, Valsesia A, Bretagnol F, Colpo P, Rossi F. Nanotechnology. 2007; 8:505306.
- [11]. Bergveld P. Sens Actuators. 1996; 56:65.
- [12]. O'Brien JC, Jones VW, Porter MD. Analytical Chemistry. 2000; 72:703. [PubMed: 10701253]
- [13]. Arshak K, Mihov M, Arshak A, McDonagh D, Sutton D. Microelectron Eng. 2004; 73-74:144.
- [14]. Donthu S, Pan ZX, Myers B, Shekhawat G, Dravid VA. Nano Letters. 2005; 5:1710. [PubMed: 16159210]

- [15]. Salaita K, Wang Y, Fragala J, Vega RA, Liu C, Mirkin CA. *Angew Chem , Int Ed.* 2006; 45 A
- [16]. Fresco ZM, Suez I, Backer SA, Fréchet JMJ. *Journal of the American Chemical Society.* 2004; 126:8374. [PubMed: 15237983]
- [17]. Love JC, Estroff LA, Kriebel JK, Nuzzo RG, Whitesides GM. *Chemical Reviews.* 2005; 105:1103. [PubMed: 15826011]
- [18]. Ta T, McDermott MT. *Analytical Chemistry.* 2000; 72:2627. [PubMed: 10857646]
- [19]. Lee K-B, Park S-J, Mirkin CA, Smith JC, Mrksich M. *Science.* 2002; 295:1702. [PubMed: 11834780]
- [20]. Kenseth JR, Harnisch JA, Jones VW, Porter MD. *Langmuir.* 2001; 17:4105.
- [21]. Xia Y, Gates B, Yin Y, Lu Y. *Adv Mater.* 2000; 12:693.
- [22]. Wu M-H, Whitesides GM. *Appl Phys Lett.* 2001; 78:2273.
- [23]. Haynes CL, Van Duyne RP. *J Phy Chem.* 2001; B 105:5599.
- [24]. Tessier PM, Velev OD, Kalambur AT, Lenhoff AM, Rabolt JF, Kaler EW. *Adv Mater.* 2001; 13:396.
- [25]. Rondelez Y, Tresset G, Tabata K, Arata H, Fujita H, Takeuchi S, et al. *Nature Biotechnology.* 2005; 23:361.
- [26]. Karlsson M, Davidson M, Karlsson R, Bergenholtz J, Konkoli Z, Jesorka A, et al. *Annu Rev Phys Chem.* 2004; 55:613. [PubMed: 15117264]
- [27]. Nicolau DV, Taguchi T, Taniguchi H, Yoshikawa S. *Langmuir.* 1998; 14:1927.
- [28]. Uskokovic V, Drofenik M. *Adv Colloid Interface Sci.* 2007; 133:23. [PubMed: 17400166]
- [29]. Jones VW, Kenseth JR, Porter MD. *Analytical Chemistry.* 1998; 70:1233. [PubMed: 9553488]
- [30]. Comellas-Aragones M, Engelkamp H, Claessen VI, Sommerdijk NAJM, Rowan AE, Christianen PCM, et al. *Nature Nanotechnology.* 2012; 2:635.
- [31]. Dontha N, Nowall WB, Kuhr WG. *Analytical Chemistry.* 1997; 69:2619. [PubMed: 9230677]
- [32]. Palivan CG, Fischer-Onaca O, Delcea M, Itel F, Meier W. *Chemical Society Reviews.* 2012
- [33]. Hong J, Edel JB, deMello AJ. *Drug Discovery Today.* 2009; 14:134. [PubMed: 18983933]
- [34]. Haining RL, Hunter AP, Veronese ME, Trager WF, Rettie AE. *Archives of Biochemistry and Biophysics.* 1996; 333:447. [PubMed: 8809086]
- [35]. Li H, Wu NQ. *Nanotechnology.* 2008; 19:275301. [PubMed: 21828697]
- [36]. Stenberg E, Persson B, Roos H, Urbaniczky C. *J Colloid and Interface Science.* 1991; 143:513.
- [37]. Gannett PM, Kabulski J, Perez FA, Liu Z, Lederman DL, Locuson CW, et al. *Journal of the American Chemical Society.* 2006; 128:8374. [PubMed: 16802783]
- [38]. Kumar V, Rock DA, Warren CJ, Tracy TS, Wahlstrom JL. *Drug Metabolism and Disposition.* 2006; 34:1903. [PubMed: 16928789]
- [39]. Kaphalia L, Kaphalia BS, Kumar S, Kanz MF, Treinen-Moslen M. *J Chromatog B.* 2006; 830:231.
- [40]. Ctistis G, Papaioannou E, Patoka P, Gutek J, Fumagalli P, Giersig M. *Nano Lett.* 2009; 9:1. [PubMed: 19072720]
- [41]. Zheng YB, Wang SJ, Huan AC, Wang YH. *J Appl Phys.* 2006; 99:034308.
- [42]. Wester MR, Yano JK, Schoch GA, Yang C, Griffin KJ, Stout CD, et al. *Journal of Biological Chemistry.* 2004; 279:35630. [PubMed: 15181000]
- [43]. Yan C, Zharnikov M, Golzhauser A, Grunze M. *Langmuir.* 2000; 16:6208.
- [44]. XPS, AES, UPS and ESCA. 2011. <http://www.lasurface.com/accueil/index.php>
- [45]. Li L, Chen S, Zheng J, Ratner BD, Jiang S. *J Phy Chem.* 2005; B 109:2934.
- [46]. Mrksich M, Sigal GB, Whitesides GM. *Langmuir.* 1995; 11:4383.
- [47]. Ostuni E, Chapman RG, Liang MN, Meluleni G, Pier G, Ingber DE. *Langmuir.* 2001; 17:6336.
- [48]. Backes WL, Kelley RW. *Pharmacol Ther.* 2003; 98:221. [PubMed: 12725870]
- [49]. Yang M, Kabulski JL, Wollenberg L, Chen X, Lederman D, Tracy TS, et al. *Drug Metabolism and Disposition.* 2009; 37:892. [PubMed: 19171677]

Figure 1.

The five-step process used to create a gold nanowell array using nanosphere lithography. a) deposition of gold (yellow) on silicon (gray), b) formation of monolayer of polystyrene spheres (black), c) reduction in sphere diameter by oxygen plasma ashing, d) deposition of silicon dioxide layer (purple), and e) removal of the polystyrene spheres by dissolution to form nanowells.

Figure 2.
(a) Low magnification scanning electron micrograph (SEM) of the nanowell array following removal of the polystyrene spheres and prior to SAM formation and b) high magnification SEM image of the nanowell array.

Figure 3. Atomic force microscopic image of the nanowell array structure. The image was obtained on the same chip as the SEM data.

Figure 4.

XPS spectra of the a) O1s core level spectra, b) Si 2p level spectra, c) C1s core level spectra and d) N1s core level spectra obtained during fabrication of the nanowell chip bearing OT, PEG-Silane, and CYP2C9. Successive spectra (from bottom) in each panel are of the step-by-step fabrication of the nanowell array and are 1) nanowell array with OT SAM, 2) nanowell array, OT SAM, and PEG-Silane SAM, and 3) nanowell array, OT and PEG-Silane SAMs, and CYP2C9.

Figure 5. X-ray photoelectron spectra comparing N1s core level scans of CYP2C9 adsorbed to self-assembled monolayers comprised of OT (black) or PEG-Silane on silicon dioxide. Each spectrum is the sum of 15 scans.

Figure 6. SPR sensorgram comparing the binding of CYP2C9 to self-assembled monolayers comprised of either Au-OT (black) or SiO₂-PEG-Silane (gray). Each sensorgram trace represents an average of 3 separate experiments.

Table I. Metabolite Production from CYP2C9 and Diclofenac.^a

Number	Sample	4'OH-Diclofenac (ng/mL) ^b
1	Solution CYP2C9 (+NADPH)	12.56 ± 0.56
2	Solution CYP2C9 (-NADPH)	ND
3	Au-OT-CYP2C9 (+NADPH)	9.9 ± 0.33
4	Au-OT-CYP2C9 (-NADPH)	ND
5	SiO ₂ -PEG-Silane (+NADPH)	Trace
6	SiO ₂ -PEG-Silane (-NADPH)	ND
7	Nanowell CYP2C9 (+NADPH)	7.41 ± 0.32
8	Nanowell CYP2C9 (-NADPH)	ND

^aIncubation conditions: Solution incubations contained CYP2C9 (2.5 pmol), CPR (10 pmol), phospholipid (1 mg/ml), NADPH (1 mM), and diclofenac (250 uM). Negative controls omitted NADPH. Nanowell-CYP2C9 incubations contained CPR (10 pmol), NADPH (1 mM), and diclofenac (250 uM).

^bMetabolite formation determined by HPLC.