Programmable assembly of a metabolic pathway enzyme in a pre-packaged reusable bioMEMS device†

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We report a biofunctionalization strategy for the assembly of catalytically active enzymes within a completely packaged bioMEMS device, through the programmed generation of electrical signals at spatially and temporally defined sites. The enzyme of a bacterial metabolic pathway, S-adenosylhomocysteine nucleosidase (Pfs), is genetically fused with a pentatyrrosine “pro-tag” at its C-terminus. Signal responsive assembly is based on covalent conjugation of Pfs to the aminopolysaccharide, chitosan, upon biochemical activation of the pro-tag, followed by electrodeposition of the enzyme–chitosan conjugate onto readily addressable sites in microfluidic channels. Compared to traditional physical entrapment and surface immobilization approaches in microfluidic environments, our signal-guided electrochemical assembly is unique in that the enzymes are assembled under mild aqueous conditions with spatial and temporal programmability and orientational control. Significantly, the chitosan-mediated enzyme assembly can be reversed, making the bioMEMS reusable for repeated assembly and catalytic activity. Additionally, the assembled enzymes retain catalytic activity over multiple days, demonstrating enhanced enzyme stability. We envision that this assembly strategy can be applied to rebuild metabolic pathways in microfluidic environments for antimicrobial drug discovery.

Introduction

Microelectromechanical systems (MEMS) based on semiconductors convey electrical and optical signals and exhibit high throughput analysis. Biological components, such as nucleic acid, enzymes and antibodies, exhibit molecular recognition capabilities for biosensing functions. The integration of biology with MEMS (bioMEMS), especially with microfluidic systems, offers major advances in our ability to manipulate biomolecular systems.1-10 The microfluidic environment of bioMEMS devices provides unprecedented advantages for enzyme analysis because of the ability to work with smaller reagent volumes, shorter reaction times, and the possibility of parallel operation.1,2,11,12 Recently, researchers have explored enzyme assembly approaches in microfabricated devices using either entrapment approaches, such as packed beads,11,13-15 or surface immobilization approaches.15,16-25 However, robust and reproducible enzyme assembly within microfluidic devices remains challenging due to the labile nature of these biological molecules that is incompatible with lengthy and dry processing conditions often occurring in the device fabrication.

Additionally, semiconductor-based devices are expensive due to the need of costly photolithographic equipments and the access of clean rooms, and the microfabrication processes are time-consuming. Therefore, tremendous research has been directed to fabricate cheap disposable devices for biological applications by using alternative polymeric materials, such as polydimethylsiloxane (PDMS),26-30 and soft lithographic techniques, including microcontact printing.30,26-28,31 However, this substitution of polymers for semiconductors as the basis for bioMEMS poses new challenges in (a) integrating multiple electrical and optical functions together with microfluidics, and (b) simultaneously facilitating the incorporation of biological species in the bioMEMS.

In this paper, we report a chitosan-mediated biofunctionalization strategy for the assembly of catalytically active enzymes onto spatially and temporally programmed sites within completely packaged and systematically controlled bioMEMS devices. Specifically, we report assembly of a bacterial metabolic pathway enzyme, S-adenosylhomocysteine nucleosidase (Pfs), and demonstrate that it retains catalytic activity for small
molecule biosynthesis. Pfs catalyzes the irreversible cleavage of S-adenosylhomocysteine (SAH) into S-ribosylhomocysteine (SRH) and adenine, and is a member of the autoinducer-2 (AI-2) biosynthesis pathway, a metabolic pathway found in many bacterial species. AI-2 is a small signaling molecule that mediates interspecies bacterial cell–cell communication termed “quorum sensing” (QS), a process in which the entire population coordinates behavior in response to environmental cues. QS is involved in regulating many of the genes associated with bacterial pathogenesis. Inhibition or knock-down of enzymes in this pathway represents new opportunities for antimicrobial drugs that target population phenotype as opposed to essential biological functions. Therefore, the enzyme assembly strategy reported here provides a template toward rebuilding metabolic pathways in microfluidic environments for novel antimicrobial drug discovery.

Different from conventional approaches, which have immobilized enzymes onto packed beads or on entire surfaces of microchannel walls, our biofunctionalization strategy assembles enzymes at a specific address within a microchannel through the programmed generation of electrical signals at spatially and temporally defined sites. First, the bioMEMS device in Fig. 1(a) is prefabricated for multiple uses. Second, Pfs–chitosan conjugate solution is prepared by covalently conjugating Pfs to chitosan in solution upon tyrosinase activation of the pro-tag genetically fused at the enzyme’s C-terminus, as shown in Fig. 1(b). This conjugation step confers the pH-responsive properties of chitosan to the enzyme Pfs for one-step electro-assembly onto the readily addressable sites within the microfluidic channels. Third, the Pfs–chitosan conjugate is electrodeposited onto an assembly site inside a microfluidic channel by applying negatively biased electrical signals, as shown in Fig. 1(c). With biofunctionalization complete, Fig. 1(d) shows that the Pfs-catalyzed enzymatic reaction is performed by introducing the enzyme substrate SAH into the microchannel, which is then catalytically converted by the assembled Pfs into products SRH and adenine. After the reaction, Fig. 1(e) indicates that a mild acid wash removes the assembled Pfs–chitosan conjugate for reuse of the bioMEMS.

The unique features of this work are that we employ localized electrical signals to guide the assembly of a biocatalytically-active enzyme at a specific electrode address within a completely packaged microfluidic channel. This assembly approach is important because it allows device fabrication to be separated from biofunctionalization and enables the prefabricated bioMEMS to be repeatedly biofunctionalized for multiple uses.

Materials and methods

Materials

S-adenosylhomocysteine (SAH), bovine serum albumin (BSA), chitosan (minimum 85% deacetylated chitin; molecular weight 200 000 g mol$^{-1}$) from crab shells, imidazole, isopropyl-$\beta$-D-thiogalactopyranoside (IPTG), nickel sulfate, phosphate buffered saline (PBS) (2.7 mM KCl, 137 mM NaCl, 1.5 mM KH$_2$PO$_4$, 8.1 mM Na$_2$HPO$_4$, pH 7.5), sodium cyanoborohydride, and tyrosinase from mushroom were purchased from Sigma (St. Louis, MO, USA). Tyrosinase was reported by the manufacturer to have an activity of 1530 Units mg$^{-1}$ solid. LB (Luria broth) medium was purchased from Becton Dickinson (Cockeysville, MD, USA). Acetonitrile (HPLC grade), ampicillin sodium salt, chloroform, glycerol, sodium phosphate (monobasic), sodium phosphate (dibasic), and water (HPLC grade) were purchased from Fisher Chemical (Fair Lawn, NJ, USA). Hydrochloric acid and sodium chloride were purchased from J. T. Baker (Phillipsburg, NJ, USA). Non-fat dry milk was purchased from BioRad (Hercules, CA, USA). Bleach was

![Fig. 1 Schematic flow of programmable enzyme assembly in a pre-packaged reusable bioMEMS device. (a) Prefabricated device, (b) enzyme–chitosan conjugation, (c) electrically programmed assembly of Pfs–chitosan conjugate, (d) enzymatic small-molecule reaction, (e) mild acid wash to remove biofunctionalization and reuse bioMEMS device.](image-url)
purchased from James Austin Co. (Mars, PA, USA). De-ionized water (ddH₂O, 18 MΩ·cm, Milli-Q) and PBS (dissolved in de-ionized water) were autoclaved before use.

**Plasmid construction**

pTrcHis-Pfs-Tyr plasmid construction was reported elsewhere. Briefly, the plasmid was constructed by PCR amplification of Pfs from *E. coli* wild type strain W3110. Following digestion, the PCR products were extracted by gel purification and inserted into pTrcHisC (Invitrogen). DNA sequencing was performed to verify construct integrity. The plasmid was transformed into *E. coli* DH5α (defective luxS strain).

**Purification of (His)₆-Pfs-(Tyr)₅**

*E. coli* DH5α containing pTrcHis-Pfs-Tyr was cultured at 37 °C and 250 rpm in LB medium supplemented with ampicillin at 50 μg mL⁻¹. When the OD₆₀₀ nm reached 0.5–0.6, IPTG was added to induce enzyme production at a final concentration of 1 mM IPTG. After an additional 5 h, the culture was centrifuged for 10 min at 10 000 g and the cell pellet stored at −20 °C. The thawed pellet was resuspended in PBS + 10 mM imidazole, pH 7.5, placed in an ice-water bath, and the cells lysed by sonication (Fisher Scientific Sonic Dismembrator 550). The lysed cells were centrifuged for 10 min 16 000 g to remove insoluble cell debris, and the supernatant filtered though 0.22 μm PES filter. The enzyme was purified from the filtered soluble cell extract by immobilized metal-ion affinity chromatography (IMAC) using a 5 mL HisTrap chelating column (Amersham Biosciences). Prior to loading the filtered extract, the column was charged with Ni²⁺ ions using 0.1 M NiSO₄, washed with de-ionized water, and equilibrated with 3 column volumes (CVs) of 20 mM sodium phosphate, 250 mM NaCl, 10 mM imidazole, pH 7.4. After loading the filtered extract, the column was washed with 3 CVs of the previous buffer, washed again with 3 CVs of 20 mM sodium phosphate, 250 mM NaCl, 50 mM imidazole, pH 7.4, and the protein was eluted using 1.5 CVs of 20 mM sodium phosphate, 250 mM NaCl, 350 mM imidazole, pH 7.4. All steps were performed at 2 mL min⁻¹ (1 cm min⁻¹ linear velocity). The eluted sample was dialyzed overnight (16 h) at 4 °C into PBS. Purified protein concentration was determined with a UV/vis spectrophotometer (DU 640, Beckman, Fullerton, CA, USA) using UV light at 280 nm wavelength. The protein solution was mixed 2 : 1 with glycerol, aliquoted, and stored at −80 °C.

**Chitosan and Pfs–chitosan conjugate preparation**

Chitosan solution was prepared by adding chitosan flakes in de-ionized water, with HCl added dropwise to maintain pH ∼ 2, and mixing overnight. The pH was then adjusted to pH 4.8 by adding 1 M NaOH dropwise, and the chitosan solution was then filtered and stored at 4 °C.

The conjugate was prepared by incubating (His)₆-Pfs-(Tyr)₅ (0.2 mg mL⁻¹), tyrosinase (0.1 mg mL⁻¹ or 166 Units mL⁻¹), and chitosan (0.5% (w/w)) in 50 mM sodium phosphate buffer (final pH of mixture 6.0) for 2 h at room temperature (23–24 °C) and 250 rpm, followed by incubation in sodium cyanoborohydride (0.2 mg mL⁻¹) for 30 min at 250 rpm to stabilize Pfs–chitosan binding.

**BioMEMS device fabrication and packaging**

The fabrication process of our bioMEMS device with packaging was reported previously. Briefly, our bioMEMS device (Fig. 2)
features six identical microchannels evenly distributed on a 4" Pyrex wafer with two rectangular gold electrodes underneath each microchannel. A Cr adhesion layer (90 Å) and then a gold layer (2000 Å) were deposited onto a 4" Pyrex wafer, and rectangular gold electrode patterns (1 mm × 0.5 mm) were created by photolithography. SU8–50 (MicroChem, MA, USA) was patterned on the top of substrate and electrode surfaces to form structures which serve a dual function, namely (1) sidewalls for a microfluidic channel, and (2) sharp "knife-edge" structures for reliable leak-tight sealing to a PDMS layer above. The microfluidic channels were thus sealed by a 300 μm-thick top sealing PDMS layer spun on a Plexiglas plate, and the SU8–50–PDMS junction was compressed by two package-level Plexiglas plates with six pressure-adjustable compression bolts (1/4"-28) hexagonally spaced on the ring and six force tunable socket screws (4-40) between every two microchannels. The microchannels thus formed were 500 μm wide by 150 μm high. Fluidic connectors (Nanoport™) and electric Pogo pins (Interconnect Devices, Inc.) were assembled through punched holes on the sealing PDMS and drilled-holes through the top sealing and packaging Plexiglas plates, and then connected to external pressure-driven aqueous transport and electrical signal, correspondingly.

**BioMEMS system control technology**

A bioMEMS control system was built to systematically control the selections from multiple solution sources and the pumping into microfluidic channels. The control system shown in Fig. 2 mainly consists of a peristaltic micropump (Masterflex1 pump drive, Cole-Palmer Instrument Co) with two cartridges in alternative directions on an 8-roller cartridge pump head (Masterflex pump head, Cole-Palmer Instrument Co) to achieve near pulseless combined output flow; a 6-to-1 solenoid valve (Bio-Chem Valve/Omnifit, NJ, USA) with separate tubing near pulseless combined output flow; a 6-to-1 solenoid valve (Masterflex pump head, Cole-Palmer Instrument Co) to achieve pump drive, Cole-Palmer Instrument Co) with two cartridges for controlling the microfluidic flow rate in a range of 2.8–280 μL min⁻¹. Another LabView program was used to control electrodeposition process and to monitor the voltage of the applied electrical signal (2–3 V measured during the electrodeposition process). A network simulation (VisSim™ 3.2, Design Science Inc.) showed that at 5 μL min⁻¹ flow rate, the velocity inside the channel (cross section: 500 μm × 150 μm) is 1.1 mm s⁻¹. If the pump is restarted after the channel is filled with static fluid, the response time of the flow in the channel is 30 s.

**One-step electrodeposition of enzyme–chitosan conjugate and sequential enzymatic reactions**

As shown in Table 1, the microchannel and all the connecting tubing were first rinsed with DI water at 50 μL min⁻¹ flow rate for 30 min. Then, bovine serum albumin (BSA) solution (1% (w/v) in PBS buffer) was pumped into the microchannel at 3 μL min⁻¹ for 2 h to block non-specific binding. After PBS buffer rinsing at 3 μL min⁻¹ for 30 min, Pfs–chitosan conjugate solution was pumped into the microchannel at 5 μL min⁻¹. After the microchannel was completely filled, the pump was stopped and an electrical signal of constant current density 3 A m⁻² was applied to maintain negative bias voltage on the gold (working) electrode for 240 s, while a second gold electrode served as the anode (counter). The Pfs–chitosan conjugate solution was then drained from the system, and the electrodeposited Pfs–chitosan conjugate was washed with PBS buffer at 5 μL min⁻¹ and then at 20 μL min⁻¹, each for 15 min. Next, enzymatic reactions were performed by continuously pumping the SAH substrate solution (1 mM SAH in 50 mM sodium phosphate buffer pH 7.2) for 2 h at each flow rate. During the second hour at each flow rate, samples were collected every 10 min for 3 min each. They were then extracted with chloroform and stored at −20 °C before analyzing via HPLC.

**Table 1** Experimental procedure to demonstrate programmable assembly of a catalytically active enzyme and its reproducibility

<table>
<thead>
<tr>
<th>Step #</th>
<th>Procedure</th>
<th>Flow rate/μL min⁻¹</th>
<th>Time/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>1</td>
<td>DI water cleaning</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>BSA</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>PBS buffer</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Pfs–chitosan assembly</td>
<td>Static</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>PBS buffer</td>
<td>5, 20</td>
</tr>
<tr>
<td>Day 2</td>
<td>6</td>
<td>Enzymatic reaction (SAH)</td>
<td>3, 22</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>HCl wash</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>DI water cleaning</td>
<td>50</td>
</tr>
<tr>
<td>Day 3</td>
<td>9</td>
<td>Enzymatic reaction (SAH)</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>DI water cleaning</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>BSA</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>PBS buffer</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>Pfs–chitosan re-assembly</td>
<td>Static</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>PBS buffer</td>
<td>5, 20</td>
</tr>
<tr>
<td>Day 4</td>
<td>15</td>
<td>Enzymatic reaction (SAH)</td>
<td>3, 22</td>
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<tr>
<td>Day 5–7</td>
<td>16</td>
<td>In PBS buffer</td>
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<tr>
<td>Day 8</td>
<td>17</td>
<td>Enzymatic reaction (SAH)</td>
<td>3, 22</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>Cleaning</td>
<td>50</td>
</tr>
</tbody>
</table>
Negative controls

Several control experiments were run to evaluate the signal-directed assembly of the conjugate and free enzyme within the MEMS. For control Set #1, the microchannel was incubated for 240 s with free Pfs solution (0.2 mg mL\(^{-1}\)) without chitosan (therefore no need of electrical bias) and without tyrosinase. For control Set #2, an electrical signal of constant current density (therefore no need of electrical bias) and without tyrosinase. For control Set #3, the process was similar to that shown in Table 1, except there was no electrical signal applied when incubating the microchannel with the same batch of Pfs–chitosan conjugate solution for 240 s. Finally, enzymatic reactions were performed for all sets of controls and the corresponding experiments, and the downstream reaction products were collected and analyzed via HPLC.

Analysis of enzymatic reaction products

A Waters Spherisorb Silica column (250 × 4.6 mm) with 5 μm beads (80 Å pore) was used in reversed-phase mode with 5 μL sample injection size and a mobile phase of 70 : 30 acetonitrile : water at 0.5 mL min\(^{-1}\). Conversion was calculated from elution data at 210 nm. The HPLC system consisted of two Dynamax model SD-200 pumps (with 10 mL pump heads and mixing valve) and a Dynamax Absorbance Detector model UV-D II, and data was analyzed using Star 5.5 Chromatography Software (Rainin).

Results

One-step electrodeposition of enzyme–chitosan conjugate and sequential enzymatic reactions

We demonstrate the assembly of Pfs enzyme onto a readily addressable site in a prefabricated and packaged microfluidic channel by one-step electrodeposition of Pfs–chitosan conjugate. Additionally, we demonstrate retention of catalytic activity of the assembled Pfs, and robustness and stability of the assembled Pfs throughout repeated flow cycles over extended time. For this, we first prepared the Pfs–chitosan conjugate solution by incubating chitosan (buffered to pH 6.0 by the addition of sodium phosphate buffer), tyrosinase, and the pro-tagged Pfs (Fig. 1(b)). Tyrosinase activates the pro-tag to covalently conjugate Pfs to the chitosan in solution. Next, we electrodeposited Pfs–chitosan conjugate onto the patterned assembly site inside a microfluidic channel by filling the microchannel with Pfs–chitosan conjugate solution and then applying negatively biased electrical signals to the patterned electrode (Fig. 1(c)). This microchannel was previously incubated in BSA solution to block non-specific binding of Pfs to the channel surfaces. Next, we continually transported through the microchannel a solution containing the substrate SAH, which was catalytically converted into reaction products SRH and adenine by the assembled Pfs (Fig. 1(d)). Finally, we performed HPLC analysis of the enzymatic reaction mixtures collected downstream.

As shown in Fig. 3(a), Pfs–chitosan conjugate was first assembled onto the assembly site in the microchannel by applying negatively-biased electrical signals (day 1), and enzymatic reactions were performed by continuously pumping SAH solution through the channel in a cyclic manner between 3 μL min\(^{-1}\) and 22 μL min\(^{-1}\), as shown in Fig. 3(b) (day 2). Reaction mixtures collected downstream were analyzed by HPLC. Next, Pfs enzyme was disassembled by mild acid solution (day 2), SAH was continuously pumped through the channel (day 3), and HPLC analysis was performed on the solutions collected downstream to demonstrate that Pfs enzyme was in fact completely disassembled and the device was cleaned for reuse. With the acid wash completed, Pfs–chitosan conjugate from the same solution batch was re-assembled (day 3), and enzymatic reactions were performed at the same flow rates, as shown in Fig. 3(b) (day 4), to demonstrate reproducible enzyme assembly and catalytic activity. Finally, the assembled enzyme was left in PBS buffer for 4 days at room temperature (days 4–7) before the final cycle of enzymatic reactions were performed (day 8) to demonstrate the stability of the assembled enzyme with extended time.

The HPLC analysis results in Fig. 3(c) show the following behavior. (1) By varying the flow rate in a cyclic manner between 3 μL min\(^{-1}\) (low flow rate) and 22 μL min\(^{-1}\) (high flow rate), the SAH conversion correspondingly cycled between 46 ± 4% at the low flow rate and 12 ± 1% at the high flow rate (day 2). (2) After cleaning the channel with mild acid solution, there is no conversion (day 3), demonstrating that the assembled enzyme was in fact completely removed, and thus allowing for repeated biofunctionalization and reuse of the bioMEMS device. (3) After re-assembly of Pfs enzyme, the SAH conversion recovered back to the cyclic behavior, alternating between 46 ± 4% and 12 ± 1% (day 4), demonstrating reproducible enzyme assembly and catalytic activity. (4) After leaving the enzyme in the microfluidic environment at room temperature for 4 days (days 4–7), the conversion cycled between 33 ± 2% and 9% (day 8). Specifically, the 33 ± 2% conversion represents >70% of the original conversion (46 ± 4%), demonstrating the stability of the enzyme with extended time. These results show that our model enzyme Pfs was successfully and robustly assembled onto readily addressable sites within prefabricated and packaged microfluidic channels by one-step electrodeposition of the Pfs–chitosan conjugate. Importantly, the assembled enzyme retained reproducible activity throughout the repeated flow cycles.

Combined, these results demonstrate the reproducibility of enzyme catalytic activity, the spatial and temporal programmability of our enzyme assembly process, the robustness and stability of the assembled enzyme with time, and the reusability of the devices when using our electrochemical enzyme assembly process.
Fig. 3  Programmable enzyme assembly, reproducibility after removal and robustness over time. (a) Schematic of programmable enzyme assembly, disassembly and re-assembly and the corresponding enzymatic reactions. (a1) Pfs enzyme was assembled in a microchannel (day 1), and enzymatic reaction was performed by introducing substrate SAH in cyclic flow rates between 3 μL min⁻¹ and 22 μL min⁻¹ (day 2). Reaction products downstream were collected and analyzed by HPLC. (a2) Enzyme was then disassembled by acid (day 2) and enzymatic reaction was performed (day 3) to demonstrate complete enzyme disassembly. (a3) Next, enzyme was re-assembled (day 3) and enzymatic reaction was performed in cyclic flow rates (day 4) to demonstrate reproducibility of enzyme assembly. (a4) Finally, enzyme was left in PBS buffer for 4 days (day 4–7) before final cycle of enzymatic reaction was performed (day 8) to demonstrate enhanced stability with extended time upon immobilization. (b) Flow rates. (c) Reproducible catalytic activity after enzyme assembly, disassembly and re-assembly, and stability of assembled enzyme after 4 days. The background colours of each step in (a) correspond to the background colours in (b) and in Table 1.

Negative controls to examine non-specific binding and dead volume

We next examined what portion, if any, of the SAH conversion in Fig. 3(c) was a result of Pfs non-specifically assembled onto the microchannel surfaces and/or of solution-phase Pfs retained in the dead volume of the bioMEMS. For this, we designed the following three sets of experiments as shown in Fig. 4(a).

The most simple experiment Set #1 was designed to test the non-specific binding of free, unconjugated, solution-phase Pfs to the bioMEMS surfaces. In the experiment, Pfs-chitosan conjugate was electrodeposited, as shown in Fig. 3 (day 2), while the negative control was performed in the same microchannel after rinsing with acid solution. The negative control only differed from the experiment in that the microchannel was incubated with Pfs solution without chitosan and without tyrosinase. The HPLC analysis results in Fig. 4(d) show that at 3 μL min⁻¹ flow rate, there was 16 ± 3% conversion of SAH to SRH and adenine for the negative control, while there was 48 ± 2% conversion for the experiment on day 2 in Fig. 3(c).

Experiment Set #2 was designed to compare the non-specific binding versus covalent binding of free Pfs to an already electrodeposited chitosan scaffold in the bioMEMS. For this, we first applied electrical signals to electrodeposit chitosan scaffold onto the assembly electrode, then incubated the microchannel in milk solution to block non-specific binding of Pfs to chitosan and the channel surfaces. Next, we incubated the microchannel with a mixed solution of Pfs and tyrosinase to in situ activate the pro-tag of Pfs for its covalent assembly onto the electrodeposited chitosan scaffold, and finally we performed enzymatic reactions. Following the same procedure, the negative control was performed in the same microchannel after rinsing with acid solution, except that no tyrosinase was added to the free Pfs solution. The HPLC results in Fig. 4(d) shows that at 3 μL min⁻¹ flow rate, there was 17 ± 1% conversion of SAH to SRH and adenine for the negative control, while there was 97 ± 2% conversion for the experiment. The higher conversion in the experiment of Set #2 (97 ± 2%) compared to that of Set #1 (48 ± 2%) might be due to the fact that the conjugate electrodeposition process in Set #1 might (1) partially bury the Pfs molecules in the electrodeposited chitosan matrix, making them less accessible to SAH substrates and (2) shortly expose Pfs molecules to high pH. However, the conjugate assembly in Set #1 is advantageous than in situ activation and assembly in Set #2 in that this strategy allows for sequential assembly of multiple enzymes representing a metabolic pathway onto different assembly sites within the same microfluidic channel. That is, each enzyme of the pathway can be conjugated to chitosan separately, and then
each conjugate can be electrodeposited onto its own assembly site within a microchannel. We believe the most stringent control was in experiment Set #3, which was designed to test the non-specific binding of Pfs–chitosan conjugate in bioMEMS without biasing the assembly electrode. This was performed in a new microchannel and with a new batch of Pfs–chitosan conjugate solution. Following the similar experimental procedure shown in Table 1, we first incubated the microchannel in BSA solution to block non-specific binding of Pfs to the channel surfaces. Next, we performed the experiment by electrodepositing Pfs–chitosan conjugate onto the patterned assembly site, and conducting enzymatic reactions by cycling the SAH flow rate between 3 μL min⁻¹ and 22 μL min⁻¹ and analyzed the reaction products via HPLC. Following the same procedure, the negative control was performed in the same microchannel after rinsing with acid solution, except that no electrical signal was applied during Pfs–chitosan conjugate incubation. The HPLC analysis of the enzymatic reaction products in Fig. 4(d) shows that at 3 μL min⁻¹ flow rate, there was 25 ± 3% conversion of SAH into SRH and adenine in the negative control, while there was 79 ± 4% conversion in the experiment. At 22 μL min⁻¹ flow rate, there was 3 ± 1% conversion in the negative control and 19 ± 1% conversion in the experiment. The conversion difference at 3 μL min⁻¹ flow rate in experiment Set #1 (48 ± 2%) versus Set #3 (79 ± 4%) might be because the experiment Set #3 was performed within a different microchannel and with a new batch of Pfs–chitosan conjugate solution.

**Performance of enzyme assembled on electrode**

In all three experimental sets shown in Fig. 4, the results consistently yield 3–6 times more conversion than the corresponding negative controls, which demonstrates that the majority of conversion resulted from catalytic reactions at the target electrode sites. We confirmed that there was a significant statistical difference between the enzymatic conversion of the negative control and that of the experiment in each set by analyzing via single-factor ANOVA tests and multiple comparison tests ($p < 0.01$). For Set #3, this analysis was performed on conversions at both 3 and 22 μL min⁻¹ flow rates. This is particularly striking because (1) the electrode site comprises only 0.2% of the total surface area within the bioMEMS device available for non-specific binding of the enzyme, and (2) the fluid volume above the electrode site comprises only 0.3% of the total fluid volume in the bioMEMS available for trapping free, unattached, solution-phase enzyme. In other words, these results demonstrated that the enzyme intentionally assembled on the electrode is far more ($> 10^3 = 3–6/0.2\%$) efficient than the enzyme at non-specific binding sites in the entire microchannel.

We are currently optimizing our bioMEMS design to further minimize system dead volume, and are investigating alternative methods for blocking non-specific enzyme attachment. Nonetheless, non-specific protein attachment and system dead volume are issues common in biofunctionalized bioMEMS systems, due to the structural characteristics of enzymes and other proteins, the high surface area to volume ratio of the...


system, and the minimal mixing due to the laminar flow characteristics of the system.\textsuperscript{1,13,21,44}

**Enzyme stability and activity**

The experimental results in Fig. 3(c) show that the assembled enzyme on the selected site in the microfluidic channel retained substantial catalytic activity for at least four days. We further examined Pfs stability by comparing specific catalytic activities over 4 days of the surface-assembled Pfs–chitosan conjugate at the electrode in a microchannel to (1) unassembled Pfs–chitosan conjugate solution and to (2) free and unconjugated Pfs solution (Table 2). For this, we reacted the Pfs solutions (1) and (2) with SAH on day 1 and then re-reacted the same Pfs solutions with fresh SAH on day 5. The free Pfs–chitosan conjugate formed a suspension when it was mixed with SAH substrate (due to the higher pH of SAH solution). All reaction mixtures were analyzed via HPLC. Shown in Table 2 are the specific activities of the experiments on day 1 and on day 5 after remaining 4 days at room temperature in PBS buffer, calculated as $\mu$mol SAH converted per min per mg Pfs ($\mu$mol SAH min$^{-1}$ mg Pfs$^{-1}$). For the case of the unassembled conjugate, we assumed that all of the initially available Pfs had conjugated to chitosan during the conjugation reaction (as chitosan was added in excess of Pfs). For the case of the assembled conjugate, the mg Pfs assembled was estimated by electrodepositing Pfs–chitosan conjugate onto a microfabricated chip under the same conditions, and then resolubilizing the conjugate with dilute hydrochloric acid and performing Western blot analysis. Further information can be found in the electronic supplementary information.\textsuperscript{†}

Table 2 shows that on the first day the specific activity of the assembled Pfs–chitosan conjugate is 1.8 $\mu$mol SAH min$^{-1}$ mg Pfs$^{-1}$, while the specific activities of the unassembled conjugate suspension and the free unconjugated Pfs solution are 12 $\mu$mol SAH min$^{-1}$ mg Pfs$^{-1}$: both values are within the range of reported Pfs specific activities, which vary over 3 orders of magnitude.\textsuperscript{12,45,46} The decrease in activity upon assembly is not surprising, given the steric hindrance effects of immobilized enzymes, and the diffusional limitations due to the minimal mixing associated with laminar flow in bioMEMS systems.

However, Table 2 demonstrates that the activity of the assembled Pfs–chitosan conjugate is better retained with time, as shown by the % activities remaining after 4 days incubation in PBS buffer at room temperature: 70% remaining for the assembled conjugate, only 26% remaining for the conjugate suspension, and only 13% remaining for the free unconjugated enzyme. We conclude that the assembled enzymes in our bioMEMS are more stable and resistant to environmental changes for better retention of catalytic activities with time than are the bulk free or chitosan-conjugated enzyme solutions; thus, our approach allows for repeated use of the bioMEMS in continuous or intermittent processes. This stability advantage for surface-assembled enzyme over those in bulk solution is consistent to what has been observed in literature.\textsuperscript{47}

**Transient response**

Using our current bioMEMS packaging system, we observed a time delay between changing the flow rate over the reaction site (assembled with Pfs enzyme) and measuring the corresponding change in SAH concentration at the sample collection point downstream. To further understand the transient system response, we performed numerical modeling of the bioMEMS by simulating the mixing purely due to mass diffusion and laminar transport (Reynolds number = 0.1). The simulation result in Fig. 5 shows that in the low flow rate case (3 $\mu$L min$^{-1}$) it takes 10 min for a concentration change at the reaction site to travel downstream and arrive at the sample collection site, and it takes 25 min for the concentration response at the sample collection site to reach 95% of the concentration change at the reaction site. This is mainly due to dead volume (~20 $\mu$L) in the packaging between the microchannel and the external

<table>
<thead>
<tr>
<th>Enzyme conditions</th>
<th>Specific activity day 1/$\mu$mol SAH min$^{-1}$ mg Pfs$^{-1}$</th>
<th>Specific activity day 5/$\mu$mol SAH min$^{-1}$ mg Pfs$^{-1}$</th>
<th>% activity after 4 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heterogenous:</strong> Assembled Pfs–chitosan conjugate in bioMEMS</td>
<td>3.7$^\ast$</td>
<td>2.6$^\ast$</td>
<td>70.4</td>
</tr>
<tr>
<td><strong>Homogenous:</strong> Unassembled enzyme</td>
<td>Pfs–chitosan conjugate$^b$ in batch reactors</td>
<td>12.1</td>
<td>26.4</td>
</tr>
<tr>
<td>Unconjugate free Pfs</td>
<td>12.0</td>
<td>1.5</td>
<td>12.8</td>
</tr>
</tbody>
</table>

$^\ast$ The enzymatic reaction by the assembled Pfs–chitosan conjugate on spatially selected sites in a bioMEMS channel is heterogeneous. Quantification of the specific activities is more difficult than that in homogeneous reactions and is the focus of ongoing research. Assumptions were made to estimate the specific activities shown in the table, and more details are available in the ESI.\textsuperscript{†} $^b$ Free Pfs–chitosan conjugate forms a suspension when it was mixed with SAH due to the higher pH of SAH solution.
Enzyme assembly and activity in bioMEMS

Here we report an enzyme assembly strategy and demonstrate corresponding enzymatic activity based on electrodeposition of the enzyme-chitosan conjugate onto readily addressable electrode sites into a microchannel of a pre-fabricated and packaged bioMEMS device. Specifically, we report assembly of Pfs enzyme, a member of the AI-2 biosynthesis pathway, which catalyzes the cleavage of SAH into SRH and adenine. The significance of this result is that enzymes can be programmably assembled within a bioMEMS, while maintaining their catalytic activity over time. This provides the underpinnings for a viable bioMEMS technology platform to support metabolic engineering research and development for applications from elucidating biochemical reaction kinetics to discovering new drugs.

Our enzyme assembly strategy described here offers several unique advantages over conventional techniques, such as microcontact printing and self assembly layers. First, the enzymes conjugated to chitosan are covalently bonded, and the assembly of the enzyme-chitosan conjugate onto the patterned electrode in the microchannel can be programmed conveniently by electrical signals. Second, the enzyme assembly is performed in mild aqueous conditions inside prefabricated and completely packaged bioMEMS devices, thus avoiding direct contact and complex facilities. Third, we achieve temporal programmability, since we are able to assemble the enzymes just prior to using the enzyme for small molecule biocatalysis. This is advantageous for biological components that have limited shelf life.

Quantification

While the purpose of this paper was to demonstrate the assembly and activity of enzymes within the bioMEMS, quantification of the activity and comparison to alternatives is an important and natural question. Such quantification is challenging and is the subject of ongoing research. In the meantime, it is possible here to identify or estimate several semi-quantitative results of note. Figure 4(d) shows that some parasitic or background reaction occurs, presumably through non-specific enzyme assembly onto the microchannel surfaces or through any free, unattached, solution-phase enzyme. Figure 4(d) also demonstrates that such conversion is significantly less and statistically different compared to the conversion achieved by the electrodeposited enzyme–chitosan conjugate. Additionally, the electrode, with only 0.5 mm² area, represents only 0.2% of the total microenvironment surface (221 mm²), and the volume above the electrode site (0.075 µL) represents only 0.3% of the total microenvironment volume (23.9 µL). Therefore, the enzyme-activated electrode is >3 orders of magnitude more efficient in executing the catalytic reaction than other areas of the microfluidic environment.

In a meaningful sense, these quantitative results are already encouraging. An overriding concern with microfluidics technology and applications is that the vastly enhanced surface-volume ratio compared to conventional chemical reactors may dramatically alter pathways and kinetics, rendering microfluidic environments not viable. For localized reaction sites in a bioMEMS, the concern takes two somewhat different but equally important forms: (1) nonspecific binding at the large area of channel surface dominates over the small area of an active electrode; and (2), will parasitic reaction of enzyme in the aqueous phase of the entire channel volume dominate over that at the electrode? Results here show that the assembled enzyme on the small electrode can control the catalytic conversion of small molecules in the bioMEMS.

Optimization

There is ample opportunity to optimize conversion rates in our bioMEMS environments. One means is through process parameters, such as flow rates, process time, concentrations, surface passivation, and pH. Another is through device design, such as channel dimensions and geometry, reduction of dead volumes, such as reservoirs, and new network designs which minimize cross-talk between enzyme assembly and subsequent catalytic conversion. With present conversion rates of 46%, there is room to improve the efficiency of enzyme conversion at the electrode, and reduction of parasitic conversion pathways (non-specific binding at channel surfaces and/or reaction in aqueous volumes), control and specificity of reaction at the active electrode sites can be improved as well.

Conclusions

This work demonstrates a chitosan-mediated biofunctionalization strategy for the assembly of catalytically active enzymes onto spatially and temporally programmed sites within a completely packaged and systematically controlled bioMEMS device. The HPLC analysis of downstream reaction mixtures...
demonstrates that the assembled enzymes are catalytically active, robust, and stable with time, and that our strategy is reproducible, allowing for multiple uses of bioMEMS devices. While further quantification is needed, the assembled enzyme at the small active electrode is much more effective overall in catalytic conversion of the SAH substrate than are parasitic channels associated with non-specific enzyme attachment to the channel surfaces or with solution-phase enzyme. In any case, we report here for the first time the signal-guided assembly of catalytically active enzymes at localized sites, which can be programmed both spatially and temporally within a pre-packaged bioMEMS. The demonstration of their catalytic activity represents a key step in progress toward a bioMEMS technology to support metabolic engineering research and development, where multi-step biochemical reactions are common and separation of these steps is highly desirable for understanding reaction details and modifying pathways and kinetics for various applications (e.g. drug discovery).

This novel strategy of enzyme assembly was achieved through two unique techniques: (1) the covalent conjugation of the enzyme to chitosan in solution upon biochemical activation of a pro-tag and (2) the electrodeposition of the resulting enzyme–chitosan conjugate. Because the assembly of biological elements is signal-guided through the electrodeposition process, the active biology (enzyme–chitosan conjugate) can be introduced into prefabrication bioMEMS devices upon demand. We anticipate that the methodology can be extended to multiple sites and with different enzymes to accommodate multi-step metabolic pathways as would be valuable for replicating specific bacterial pathways and seeking new antimicrobial drugs that modify or suppress those pathways.

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References