Supplementary Information

Rapid protein immobilization for thin film continuous flow bioreactors

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**Experiment details**

All immobilizations and substrate transformations experiments were performed at 8 krpm rotational speed with a tilt angle of 45° relative to the horizontal position. To test the immobilization efficiency, the confined mode of operation was used with β-glucosidase as the model enzyme. For immobilization efficiency experiments, the following analysis was performed: first, β-D-glucopyranoside (1.50 mL, 0.01 M) was added to the sample tube by pipette, the sample tube was then capped and rotated for 5 min. Thereafter, the substrate solution was removed and added to an Eppendorf tube (2 mL) containing a quenching solution (NaOH-glycine buffer, 0.7 M glycine, pH 10.8, 200 µL). To analyze substrate conversion levels, 100 µL of this solution was then transferred to a UV transparent, 96-well microtiter plate (Costar), and the absorption at 405 nm recorded. Each sample tube was tested six consecutive times, with each set of reaction conditions tested on two individual sample tubes. The error reported indicates the standard deviation around the mean (n=12).

**Preparation of the APTES coated sample tube**

A 20 mm external diameter sample tube was loaded into the VFD and then fresh piranha solution (3 mL) was added. The sample tube was capped by a B19 Suba Seal and then rotated at 8 krpm for 1 min. **Note:** the piranha solution is highly dangerous and corrosive. This step should only be performed by an experienced, well-trained technician expert in appropriate safety measures. After rotation, the sample tube was emptied before washing with diH₂O (10 x 10 mL) and then oven-dried for 2 h at 160 °C. Next, the sample tube was removed from the oven and immediately capped. After cooling to ambient temperature, MeOH (3 mL) was added to the sample tube along with APTES (60 µL). The sample tube was then capped, loaded into the VFD, and rotated at 8 krpm for 30 min. Next, the sample tube was rinsed with MeOH (6 x 5 mL), and heated (1 h, 160 °C) in an oven. The sample tube was then cooled to ambient temperature before usage. The reaction shown above is a simplified representation of the APTES-treated surface.¹,²

**Non-covalent immobilization**

The enzyme to be immobilized was solubilized (3 mL, 0.30 mg/ mL in PBS (150 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) pH 8.0) and then added to a freshly prepared APTES-coated sample tube. The sample tube was then capped, loaded into the VFD, and rotated at 8 krpm for 30 min. Next, the sample tube is removed from the
VFD and washed with PBS (5 x 5 mL, pH 8.0) and then sodium acetate (50 mM, pH 5.0) to create a non-covalent immobilized enzyme reactor. The sample tube surface must not be allowed to dry completely (especially in vacuo) as such conditions could denature the protein. Immediate usage of the enzyme immobilized sample tubes is recommended, as storage decreases activity, as described in Fig. 3d.

Imine-glutaraldehyde cross-linker immobilization

Glutaraldehyde solution (90 µL in 3.00 mL PBS) was added to a freshly coated APTES sample tube. The sample tube was then capped, and inserted into a VFD. The sample tube was rotated at 8 krpm for 30 min to introduce the APTES-glutaraldehyde cross-linker. After rotation, the sample tube is then rinsed with PBS (6 x 5 mL) and inverted to remove the majority of the buffer solution. After 10 min, enzyme immobilization was carried out as described above for the non-covalent immobilization. We suggest immediate usage of the enzyme-immobilized, sample tubes as storage decreases activity as described in Fig. 3d.

Amine-glutaraldehyde cross-linker immobilization

Glutaraldehyde solution (90 µL in 3.00 mL PBS) was added to a freshly coated sample tube. The sample tube was then capped, and inserted into a VFD. The sample tube was rotated at 8 krpm for 30 min to create the APTES-glutaraldehyde cross-linker. After rotation, the sample tube was then rinsed with PBS (6 x 5 mL) and inverted to remove the majority of the buffer solution. The enzyme immobilization step was then carried out as described above. Following
this, the sample tube was removed from the VFD and washed with PBS (5 x 5 mL, pH 8.0), and inverted to remove the majority of the buffer solution. The sample tube was then loaded back into the VFD before addition of NaBH₃CN (18.8 mg) in sodium acetate buffer (50 mM, pH 5.0, 3.00 mL). The sample tube was then capped and rotated at 8 krpm for 30 min. Next, the sample tube was rinsed with sodium acetate buffer (2 x 5 mL) and then inverted for 10 min to remove excess buffer, and to afford the amine-glutaraldehyde cross linker. Immediate usage of the enzyme immobilized sample tubes is recommended as storage decreases activity as described in Fig. 3d.

**Enzymes, Buffers and Assays**

**Enzymes** Alkaline phosphatase was purchased from Life Technologies (Fast thermosensitive alkaline phosphatase, 1 U/µL, 0.11 mM, 4.4 mg/mL). β-glucosidase was purchased from Sigma and Aldrich (Lyophilized powder, 2 U/mg). These enzymes were used without further purification. Phosphodiesterase (2 mg/mL) was prepared using bacteria expression as previously described and purified using immobilized metal affinity chromatography to 95% purity. Quantitates and concentrations for the enzymes and substrates used in the continuous flow experiments as detailed in Fig 3b are described below. The flow rate used for this experiment was 1.0 mL/min with the continuous flow set up detailed previously.

**Alkaline phosphatase conditions**

![Alkaline phosphatase reaction](image)

The p-nitrophenol phosphate substrate solution (0.01 M in 1.0 M diethanolamine) was prepared as follows: 140 g of diethanolamine was added to 1.0 L of H₂O, then the pH of the solution was adjusted to pH 9.8 using 5 M HCl. This buffer was further diluted to 1 M diethanolamine, and then 500 µL of 1 M MgCl₂ was added. The resulting buffer was filtered-sterilized through a 0.22 µm filter (Corning), and stored wrapped in aluminum foil at 4 °C. In creating the active substrate solution, p-nitrophenol phosphate (3.714 g, 0.01 M) was added to this buffer and this solution was then immediately used. The enzyme solution (0.25 mg/mL) was formulated by adding alkaline phosphatase (170 µL) to PBS (3.00 mL, pH 8.0) and then used immediately for immobilization. Sampling occurred at the times indicated in Fig. 3b. For this sampling method, a 1.6 mL aliquot was collected from the continuous flow exit, and was immediately quenched with a NaOH solution (4.0 M, 150 µL). The aliquot was then analyzed via absorption spectroscopy as described above. The molar absorption coefficient of p-nitrophenol after the quench described above was 15644 M⁻¹ cm⁻¹.

**β-Glucosidase conditions**

![β-Glucosidase reaction](image)

The 4-nitrophenyl β-D-glucopyranoside substrate solution (0.01 M in 50 mM sodium acetate) was prepared as follows: 4.37 g sodium acetate was dissolved in 1.0 L diH₂O and ≈1.1 mL of glacial acetic acid to generate a buffer of pH 5.0. The buffer was then filtered-sterilized through a 0.22 µm filter and stored at 23±2 °C. To formulate the active substrate solution, 4-nitrophenyl β-D-glucopyranoside (3.125 g, 0.01 M) was added to this buffer, and was used
immediately. The enzyme solution (0.30 mg/mL) composed of β-glucosidase (3 mg) added to 10 mL of PBS (3.00 mL) was immediately used for immobilization. Sampling occurred at the times indicated in Fig. 3b. For this sampling method, a 1.6 mL aliquot was collected from the continuous flow exit and was immediately quenched with glycine-NaOH solution (0.7 M glycine, pH 10.8, 200 µL). The aliquot was then analyzed via absorption spectroscopy as described above. The molar absorption coefficient of p-nitrophenol after the quench described above was 9413 M⁻¹ cm⁻¹.

Phosphodiesterase conditions

![Phosphodiesterase reaction](image)

The bis(p-nitrophenyl)phosphate substrate solution (0.7 mM in 50 mM diethanolamine) was prepared as follows: diethanolamine (5.257 g, 50 mM) and NaCl (2.320 g, 40 mM) were added to 1.0 L of H₂O. The pH of the solution was adjusted to pH 9.8 with 5 M HCl. Following this, NiCl·6H₂O (237 mg, 1.0 mM) was added to the solution and the resulting buffer was then filtered-sterilized through a 0.22 µm filter (Corning), and stored wrapped in aluminum foil at 4 °C. To create the active substrate solution, sodium bis(p-nitrophenyl)phosphate (237 mg, 0.7 mM) was added to this buffer, and the solution was used immediately. The enzyme solution (0.30 mg/mL) was formulated by adding phosphodiasterase (450 µL) to PBS (3.00 mL), and the solution used immediately for immobilization. Sampling occurred at the times indicated in Fig. 3b as follows. A 1.6 mL aliquot was collected from the continuous flow exit and was immediately quenched with NaOH (4.0 M, 300 µL). The aliquot was then analyzed via absorption spectroscopy as described above. The molar absorption coefficient of p-nitrophenol after the quench described above was 4242 M⁻¹ cm⁻¹.

Determination of the amount of β-glucosidase attached to the reactor surface

To determine the quantity of β-glucosidase on the surface of the reactor, two complimentary methods were used. The first method quantified the enzyme removed during the wash steps; thus, estimation of quantities of β-glucosidase adhering to the surface was simply the difference between enzyme levels in the initial and the wash solutions. The second method determines the amount of β-glucosidase present by monitoring substrate conversion rates for the modified surface. A calibration plot with substrate conversion rates allowed determination of quantities of enzyme. Both methods provided similar and complementary results.

Method 1: Comparing initial and wash solutions of enzyme concentrations

This method quantifies the amount of β-glucosidase washed out of the tube after immobilization with a simple difference calculation used to determine enzyme immobilization on the reactor surface. This method assumes that no enzyme is lost to unfolding during the half hour for this experiment.

The calibration curve required enzyme solutions at three different concentrations, which were prepared as follows. β-glucosidase at the indicated quantities (0.90, 0.60 or 0.3 mg) was added to PBS (3 mL, pH 8.0). Each solution was then added to a separate 500 mL volumetric flask. The 500 mL volumetric flasks were then filled with PBS (50 mL, pH 8.0) and then sodium acetate buffer (447 mL, 50 mM, pH 5.0).

The amount of β-glucosidase washed out of the tube after immobilization by non-covalent and imine-glutaraldehyde cross-linking was determined with the following procedure. First, the sample tube was rinsed with PBS (50 mL, pH 8.0)
and the wash solutions added to a 500 mL volumetric flask. The contents of the volumetric flask were then diluted to 500 mL with sodium acetate buffer (50 mM, pH 5.0). The pH for the wash steps can maximize enzyme immobilization during this processing (pH 8.0), and switching to pH 5.0 allows the enzyme catalysis assay to proceed at its optimal pH.

The following protocol quantified enzyme activities and therefore enzyme concentration. Each enzyme solution (500 µL) was added to 4-nitrophenyl β-D-glucopyranoside solution (500 µL, 0.01 M). The reactions were incubated at RT for 10 min. Thereafter, NaOH-glycine quenching solution (200 µL) as described above was added to halt the reaction. The solutions were then analyzed using absorption spectroscopy as detailed above. The error is reported as standard deviation around the mean (n=3, Fig. S1).

![Absorbance vs Amount of Protein Graph](image)

**Fig. S1** Determining the amount of enzyme removed from the stock solution during non-covalent and covalent immobilization. The results demonstrate that very low quantities of β-glucosidase are removed from the stock solution for both the non-covalent and the imine-glutaraldehyde cross-linking immobilizations (<1% in each case). To ascertain the amount of protein for the tested solution, the quadratic equation shown was solved for each absorption value. The error is reported as standard deviation around the mean (n=3).
Method 2. Determination of \( \beta \)-glucosidase quantity by enzyme activity

This method looks at the amount of \( \beta \)-glucosidase present on the VFD reactor surface by direct monitoring of the catalytic rates for enzyme attached to the VFD reactor. Such rates were compared to identical control solutions with specified amounts of enzyme, and not subjected to VFD processing. This method assumes that the enzyme rate remains unchanged by attachment to the VFD reactor.

For this experiment, the protocol above was used with the following changes. The concentration of enzyme was varied as indicated in Fig. S2. The reaction time was five minutes.

![Fig. S2 Determining the amount of enzyme by substrate transformation levels.](image)

The results demonstrate that very low quantities of \( \beta \)-glucosidase are attached to the reactor, agreeing with the results in Fig. S1. For this analysis, the imine-glutaraldehyde cross linker immobilization method was used. The quadratic equation (shown) was solved for the minimum and maximum absorption values obtained in the experiments reported in this manuscript. The minimum value yielded a mass of 15.4 ng, and the maximum value obtained yielded 69.8 ng \( \beta \)-glucosidase on the reactor surface. The error is reported as a standard deviation around the mean (n=3).

References