Supporting Information

In Situ Synthesis of Peptide Nucleic Acids in Porous Silicon for Drug Delivery and Biosensing

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Table of Contents

I. PSi surface functionalization

All porous silicon films were thermally oxidized at 800°C for 30 min in ambient air. Films were then loaded with PNA using the three following methods.

In situ PNA synthesis

Oxidized PSi films were functionalized with 3-aminopropyltriethoxysilane (3-APTES) by drop-casting a solution of 1% 3-APTES in anhydrous toluene onto the porous surface and incubating the sample at room temperature for 10 min. Following 3-APTES attachment, films were rinsed 3x with ethanol to remove excess silane. The silanized films were thermally annealed at 150ºC for 15 min to promote stable 3-APTES monolayer formation, and the films were then soaked at room temperature in deionized water for 3 h to remove any unwanted silane multilayers.

In situ PNA syntheses of anti-miR122 and a 16-mer biosensing PNA (NH_2-TAG CTA TGG TCC TCG T-COOH) were conducted directly on APTES-functionalized PSi within a custom reaction vessel. The synthesis process is presented in Figure S1. After synthesis, Bhoc protecting groups were removed from nucleobases by 10 min incubation with 5% (v/v) anisole in trifluoroacetic acid (TFA), followed by 5 min washing with dichloromethane (DCM), 2x, rinsing with methanol, 3x, drying under nitrogen gas, and storage in a vacuum desiccator.

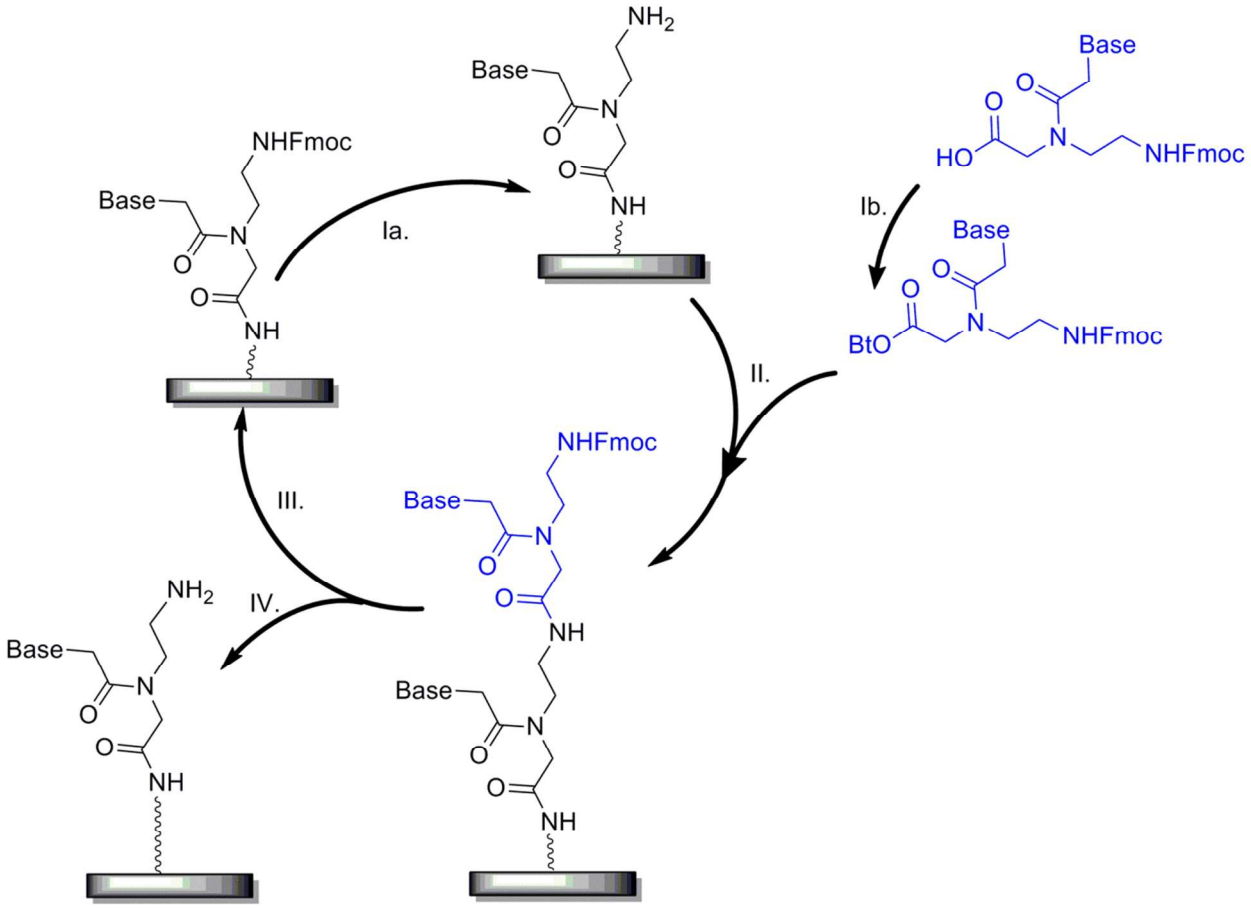

Figure S1. *In situ* synthesis of PNA from a PSi film solid support (shaded box). Each base addition requires **(Ia)** Fmoc deprotection of growing PNA chains on the PSi surface, 2x, **(Ib)** activation of PNA monomer C-termini and **(II)** coupling of preactivated monomers to growing PNA oligos, 3x, followed by either **(III)** automated repetition of the cycle to further elongate PNA oligos, or **(IV)** final deprotection of N-termini and exit from the cycle.

Pre-synthesized PNA conjugation

Pre-synthesized cys-PNA were conjugated to APTES-functionalized PSi via an amine-to-sulfhydryl heterobifunctional crosslinker, *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP). SPDP was first reacted with the freshly-silanized surface in ethanol at room temperature for 1 h using a 20x molar excess of SPDP. The number of successfully attached SPDP groups was then quantified with a control sample by reducing conjugated SPDP with TCEP and measuring the absorbance of released pyridine-2-thione at 343 nm.

Prior to attachment, pre-synthesized cys-PNA dissolved in deionized water was reduced on immobilized TCEP resin. Immediately afterwards, the SPDP-functionalized PSi film was incubated with the reduced cys-PNA at a PNA:SPDP molar ratio of 4:1. The reaction was allowed to proceed at room temperature on a shaker for 2 h, after which the reaction supernatant was removed and retained for subsequent characterization. The PNA-conjugated film was rinsed 3x with water, 2x with ethanol and 2x with methanol, then dried with nitrogen gas and stored in a desiccator under vacuum. Reaction efficiency was quantified by comparing moles of pyrdine-2-thione released from PNA attachment to moles of SPDP conjugated to the surface.

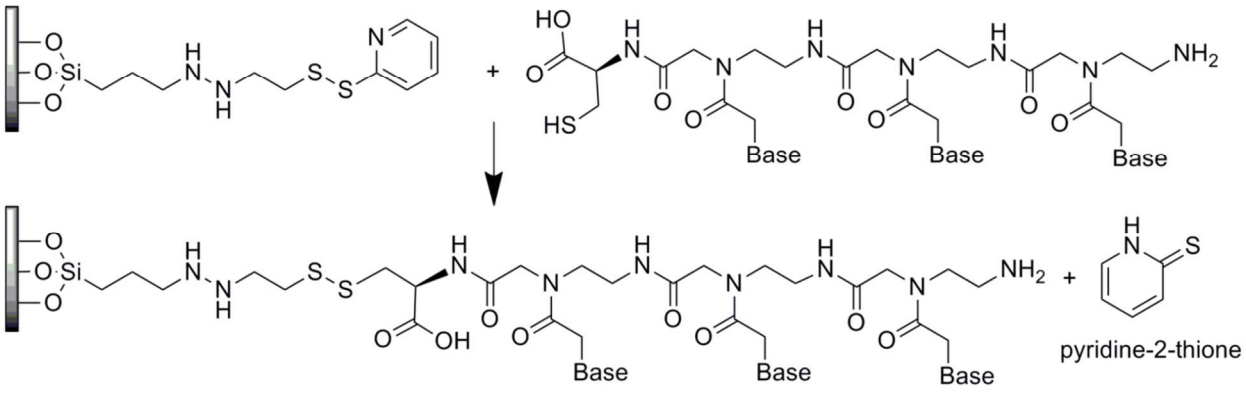

Figure S2. Direct conjugation of pre-synthesized PNA to a PSi film (shaded box).

Physical adsorption

Physical adsorption of PNA was accomplished using by incubating oxidized PSi films with PNA in water at an equivalent concentration to that used to conjugate pre-synthesized PNA to PSi (1 μmole PNA/2.6 mg PSi) for 2 hours at room temperature. Following incubation, films were washed 3x with water, 2x with ethanol and 2x with methanol. In these films, PNA are either adsorbed to the PSi internal surface, or absorbed within the open pore volume.

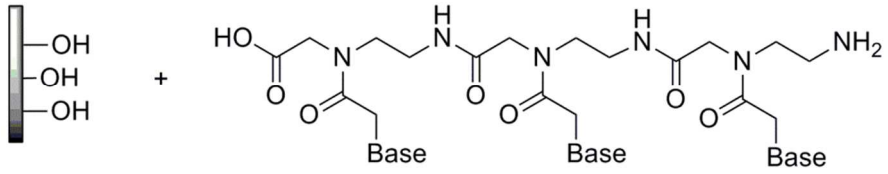

Figure S3. Physical adsorption of pre-synthesized PNA to an oxidized PSi film (shaded box).

II. Mass spectrometry experimental methods

Matrix assisted laser desorption-ionization mass spectrometry (MALDI-MS) was utilized to confirm the expected mass of the synthesized PNA.[1] MALDI spectra were acquired using a Voyager DE-STR mass spectrometer in linear, positive mode with an external Nd:YAG laser ionization source (frequency tripled, 355 nm). The matrix employed for the MALDI measurement consisted of either 2.5 mg of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) or 1 mg of α-cyano-4-hydroxycinnamic acid (CHCA), dissolved into 100 µL of a 7:3 v/v solution of acetonitrile:water, with 2 µL of 10% TFA. In order to liberate PNA from the PSi interior, an area of PNA-loaded porous silicon film (approximately 6 mm^2) was scraped into 40 µL of deionized water, sonicated for several minutes and allowed to dissolve for approximately 1 h. This solution-suspension was then added in equal volume to the matrix solution and agitated. The matrix was drop-cast onto a conventional stainless steel MALDI plate and allowed to dry prior to measurement.

III. *In situ* PNA synthesis in pore-widened PSi

To investigate whether pore widening improves monomer coupling efficiency for longer oligos, changes in optical thickness were monitored during synthesis of anti-miR122 PNA from a KOH-exposed PSi film (Figure S4). Following electrochemical etching, PSi films were exposed to a 9 mM pore-widening solution of KOH in ethanol at room temperature for 20 min. Pore size distributions were quantified from SEM micrographs using ImageJ analysis software.[2] Pore widening increased shifts in optical thickness, and therefore increased monomer coupling efficiency, for oligos greater than five nucleotides in length.

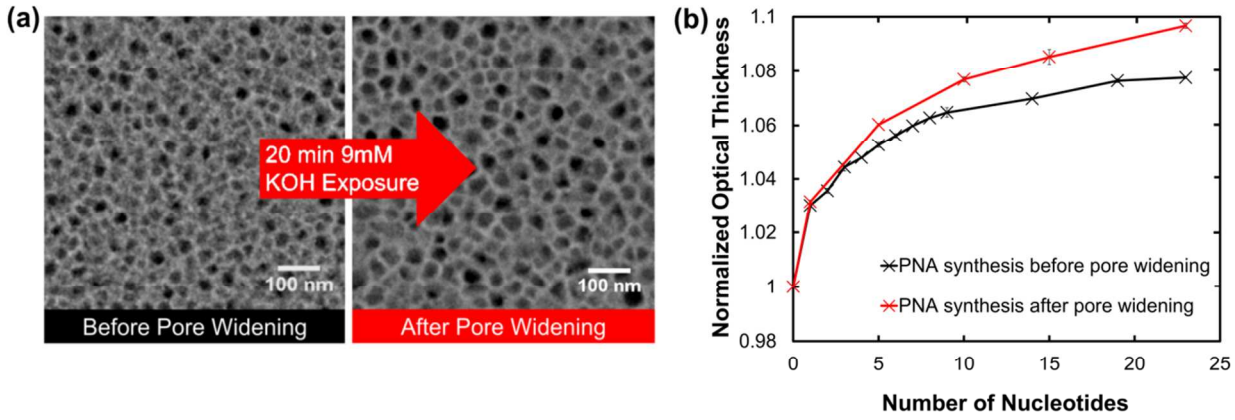

Figure S4. **(a)** Top-view of PSi films before and after KOH pore-widening (SEM; scale bars=100nm). **(b)** Characterization of optical thickness profile during PNA synthesis to illustrate changes in monomer coupling efficiency and yield of longer oligos following pore widening. Results are normalized to the optical thickness of each film's corresponding optical thickness following APTES functionalization.

IV. *In vitro* PNA release experimental methods

Release studies were performed by incubating PNA-loaded PSi films in 1x PBS at 37ºC. Releasate was collected at pre-determined times and analyzed for PNA concentration by monitoring the absorbance of the nucleic acid at 260 nm. Collected releasate was replaced with fresh PBS to maintain infinite sink conditions over the course of the study.

V. PSi nanoparticle fabrication and functionalization

Multilayer films used for PSi nanoparticle (PSNP) generation were fabricated by multiple repetitions of two anodization steps: 50 mA/cm^2 for 8 s to yield "particle" layers with 70% porosity, and 60 mA/cm^2 for 2 s to form mechanically fragile "lift-off" layers (Figure S5). Following *in situ* PNA synthesis from multilayer PSi, films were ultrasonicated in isopropanol (IPA) for 2 h. Deionized water was used to reduce the concentration of IPA in the sonicated particle suspension to 65%. PSNPs were then filtered through a 2.6 μm syringe filter and immediately isolated using a 10kDa molecular weight cutoff centrifugal filter to separate free PNA from PNA-conjugated PSNPs. Free PNA was collected from the filtrate, and PNA-functionalized particles (PNA-PSNPs) were collected from the concentrate. Aliquots of both the free PNA and PNA-PSNPs solutions were taken for analysis by UV-Vis spectroscopy and nanoparticle tracking analysis (NTA).[3] In order to quantify the amount of PNA conjugated per g PSi, a known concentration of PNA-PSNPs was dissolved in 5x PBS overnight, and PNA concentration was determined by measuring PNA absorbance at 260 nm.

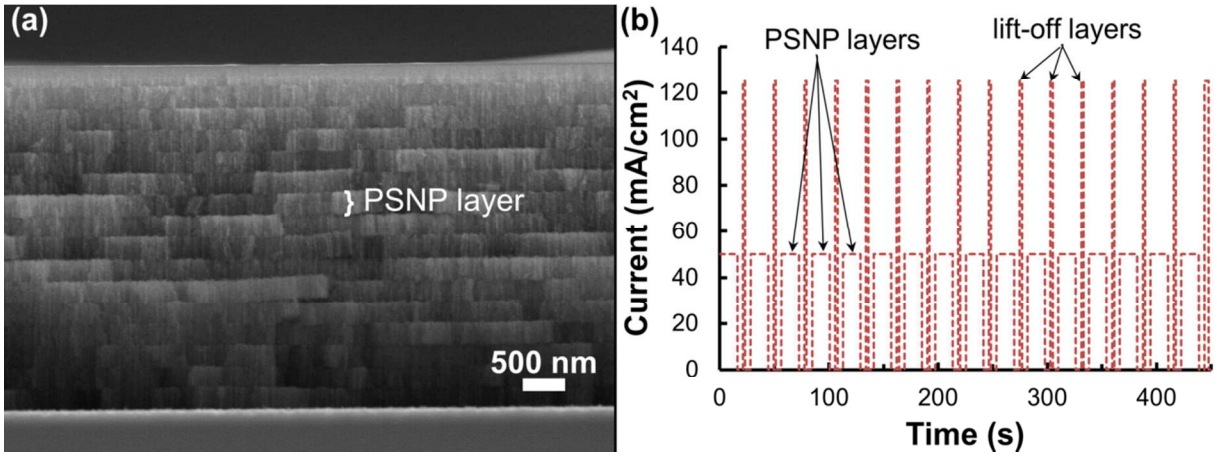

Figure S5. (a) Side-profile SEM of a 16-layer PSi film used to generate PSNPs by ultrasonic fracture. **(b)** Current profile used to generate the multilayer film shown in (a).

VI. Cell culture and biological assays

All Huh 7 human hepatic carcinoma cells were maintained in Dubelco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 2% penicillin-streptomycin antibiotics, and 2μg/mL ciprofloxacin at 37ºC/5% CO2.

Intracellular uptake

To enable visualization of intracellular PNA delivery, N-termini of free and PSi-bound PNA were labeled with NHS-Alexa-488 at a molar ratio of 1:2 (Alexa:PNA). Excess NHS-Alexa and reaction byproducts were removed by sequential centrifugal filtration (3 kDa cutoff centrifugal filters) and resuspension in deionized water, 4x. Alexa-labeled free PNA and PNA-PSNPs were then lyophilized for storage until used.

Cells were seeded onto 8-well chamber slides (Thermo Fisher Scientific Inc., Waltham, MA) at a density of 12,500 cells/well and incubated at 37ºC for 8 h. Cells were then treated for either 1 or 24 h with free PNA or PNA-PSNPs at a 2 μM dose of PNA (~3.7 μg/mL PSNPs for PNA-PSNP treatments). Subsequently, the cells were washed with PBS and stained for 5 min with Hoechst nuclear dye prior to imaging with confocal microscopy (Zeiss LSM 710Meta, Oberkochen, Germany).

Cytotoxicity

PSNP cytotoxicity was determined using a lactate dehydrogenase CytoTox-ONE™ homogenous membrane activity assay (Promega Corporation, Madison, WI). Huh7-psiCHECK-miR122[4] cells were seeded at 6,000 cells per well in black, clear-bottom, 96-well plates (BD Falcon, San Jose, CA) and incubated at 37ºC for 8 h. Cells were then treated with free PNA, empty PSNPs, or PNA-PSNPs at a 2 μM dose of PNA (~3.7 μg/mL PSNPs for PSNP and PNA-PSNP treatments)

in DMEM containing 1% FBS for 24 h at 37ºC/5% CO_2. Note that all PSNPs in this study were fabricated from pore-widened PSi. After 24 h, the cells were washed with PBS and the media was replaced with DMEM containing 10% FBS, 2% penicillin-streptomycin, and 2μg/mL ciprofloxacin. Cells treated with a miR-122 antagomir (AMO) (2′-OMe PS modified oligonucleotide 5′ ACAAACACCAUUGUCACACUCCA 3′; IDT DNA)[4-5] were transfected using Fugene 6 (3μL reagent: 1μg AMO) (Promega) in Opti-Mem media. Cells treated with AMO were incubated at 37 °C for 4 h followed by the replacement of transfection media with standard DMEM growth media. The cell viability was determined after 44 h after treatment according to the published proliferation assay protocol.[6]

Luciferase anti-miR122 activity

Huh7-psiCHECK-miR122[4] cells were seeded at 6,000 cells per well in black, clear-bottom, 96-well plates (BD Falcon, San Jose, CA) and incubated at 37ºC for 8 h. Cells were then treated with free PNA, empty PSNPs, or PNA-PSNPs at a 2 μM dose of PNA (~3.7 μg/mL PSNPs for PSNP and PNA-PSNP treatments) in DMEM containing 1% FBS for 24 h at 37ºC/5% CO_2. Note that the PSNPs and PNA-PSNPs in this study were fabricated from pore-widened PSi. After 24 h, the cells were washed with PBS and the media was replaced with DMEM containing 10% FBS, 2% penicillin-streptomycin, and 2μg/mL ciprofloxacin. As a positive control, an optimized AMO (2′-OMe PS modified oligonucleotide 5′ ACAAACACCAUUGUCACACUCCA 3′; IDT DNA)[4-5] was transfected using Fugene 6 (3μL reagent: 1μg AMO) (Promega) in Opti-Mem media. Cells treated with AMO were incubated at 37 °C for 4 h followed by the replacement of transfection media with standard DMEM growth media. Luciferase activity was measured based on bioluminescence using an in vivo imaging system (IVIS; Perkin Elmer, Waltham, MA) after addition of the luciferase substrate d-Luciferin.

VII. Adsorbed PNA surface stability study

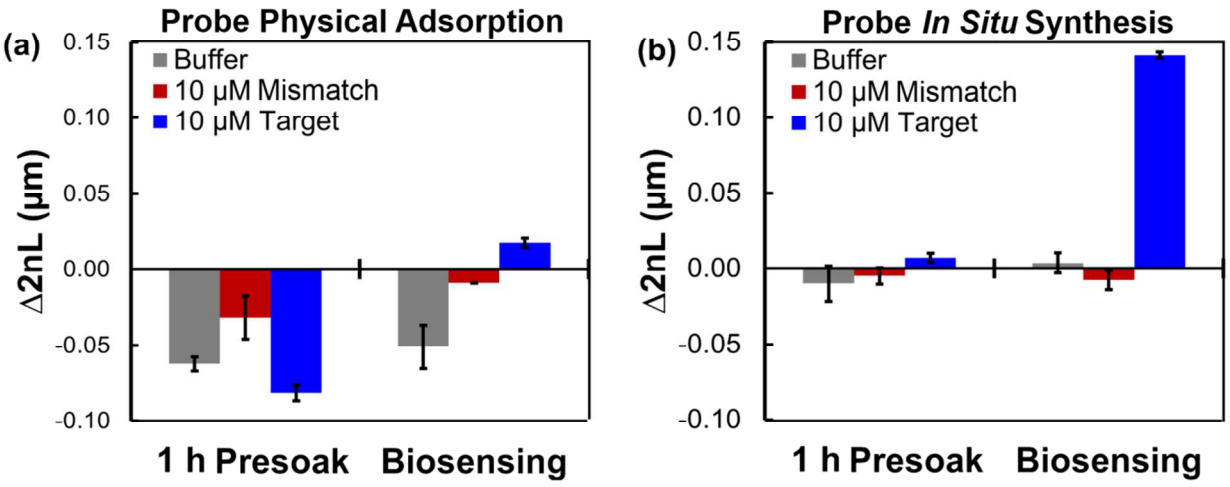

Figure S6. Hybridization assay following either **(a)** physical adsorption, or **(b)** *in situ* synthesis of a 16-mer PNA on a single-layer PSi biosensor. Prior to characterizing sensor selectivity, biosensor stabilities were evaluated by pre-soaking in deionized water at room temperature for 1 h. A decrease in optical thickness following the 1 h presoak indicates instability of the sensor surface, which was apparent for the physically adsorbed PNA samples. Following the 1 h water presoak, biosensing was demonstrated by incubating samples with HEPES buffer (pH 7.4) alone, or HEPES buffer containing either 10 μM mismatch or 10μM target DNA. Hybridization with a complementary target sequence during biosensing experiments is indicated by a positive shift in optical thickness. These data demonstrate selective sensing of a complementary probe sequence via the in situ synthesized PNA samples.

References

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