Designing Electrochemical Biosensing Platforms Using Layered Carbon-Stabilized Porous Silicon Nanostructures

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ABSTRACT: Porous silicon (pSi) is an established porous material that offers ample opportunities for biosensor design thanks to its tunable structure, versatile surface chemistry, and large surface area. Nonetheless, its potential for electrochemical sensing is relatively unexplored. This study investigates layered carbon-stabilized pSi nanostructures with site-specific functionalities as an electrochemical biosensor. A double-layer nanostructure combining a top hydrophilic layer of thermally carbonized pSi (THCpSi) and a bottom hydrophobic layer of thermally hydrocarbonized pSi (THCcPSi) is prepared. The modified layers are formed in a stepwise process, involving first an electrochemical anodization step to generate a porous layer with precisely defined pore morphological features, followed by deposition of a thin thermally carbonized coating on the pore walls via temperature-controlled acetylene decomposition. The second layer is then generated beneath the first by following the same two-step process, but the acetylene decomposition conditions are adjusted to deposit a thermally hydrocarbonized coating. The double-layer platform features excellent electrochemical properties such as fast electron-transfer kinetics, which underpin the performance of a TCpSi-THCcPSi voltammetric DNA sensor. The biosensor targets a 28-nucleotide single-stranded DNA sequence with a detection limit of 0.4 pM, two orders of magnitude lower than the values reported to date by any other pSi-based electrochemical DNA sensor.

KEYWORDS: porous silicon, layered nanostructures, controllable surface chemistry, dual-surface functionality, electrochemical biosensor

INTRODUCTION

Porous silicon (pSi) has demonstrated its advantages and versatility in various biomedical applications, such as biosensing and drug delivery.1−3 The ease of controlling pore morphology has implications in its sensing capabilities and has engendered advances in the detection of a large range of chemical and biological species.5−6 Other features of pSi, such as the large available internal surface area and excellent biocompatibility, have also been harnessed to develop high-performance biosensing platforms.7−8 Of particular interest for biosensing purposes is the versatile surface chemistry of pSi, allowing a broad range of functionalization routes (e.g., hydrosilylation,9−11 silanization,12 carbon-based thin layers13,14) to introduce functional groups further used to covalently immobilize diverse biomolecules as bioreceptors (e.g., antibodies,15,16 oligonucleotides,17,18 enzymes19,20), and simultaneously protect from surface degradation even under oxidizing conditions. The advantages of using layered pSi nanostructures as optical sensing platforms have been well demonstrated.21,22 Interferometric biosensors based on pSi double layers have been used not only to separate biomolecules based on size exclusion but also to effectively monitor biomolecule penetration into specific layers and improve the sensitivity of the label-free output signal.19,22 Control of surface functionalization for each porous layer separately enables discrimination on the basis of molecular affinity via the specific capture probes grafted onto selected layers.

Nonetheless, to the best of our knowledge, no report has yet described the use of layered pSi nanostructures as electrochemical biosensing platforms. This is likely due to the low stability of porous silicon nanostructures when subjected to aqueous electrochemical conditions and the tendency of silicon to form an electrically insulating oxide (SiO2). Moreover, the distribution of the space-charge layer within the silicon walls around pore voids favors the restriction of charge transfer to the pore tips. To overcome these limitations and thus allow charge-transfer reactions proceeding over the entire pSi contour, researchers have demonstrated the possibility to prevent oxidation by chemically derivatizing pSi with nonpolar Si−C=C−R linkages11 or introducing a protective thin layer of graphene oxide.23 These approaches have demonstrated the possibility of pSi to operate in aqueous environments under...
oxidizing conditions. Similarly, our research group recently explored the use of a carbon-rich coating for the inner pore walls of a single-layer pSi structure, finding that the layer imparted substantial improvements in chemical stability and electrical conductivity, which resulted in an excellent performance as an electrochemical transducer.14 Here we combine this carbonization chemistry with the precise control that electrochemical anodization provides in the silicon system, generating a porous double-layer structure that substantially improves the selectivity of the electrochemical system. The double-layer structure combines a top hydrophilic layer of thermally carbonized pSi (TCpSi) and a bottom hydrophobic layer of thermally hydrocarbonized pSi (THCpSi), which is fabricated via a tandem electrochemical anodization/carbonization process (Figure 1a–f). The resulting double layer displays different average pore sizes and surface chemistries in each layer, which enables the discrimination of analyte on the basis of both size and chemical properties. Most importantly for the intended voltammetric DNA sensor, the carbonization chemistry imparts improved stability to the electrochemical biosensor. We report the specific detection of a 28-nucleotide single-stranded DNA (ssDNA) sequence, with a limit of detection (LOD) of 0.4 pM, which is two orders of magnitude lower than the best LOD (50 pM)23 achieved for previously reported pSi-based electrochemical DNA sensors.

# EXPERIMENTAL SECTION

**Materials.** p-type Si wafers with 0.00055–0.001 Ω cm resistivity, (100)-oriented were purchased from Siltronix (France). Hydrofluoric acid (HF) (48%, AR grade) was purchased from Scharlau (Australia). Potassium ferrocyanide (K₄[Fe(CN)₆]), potassium ferricyanide (K₃[Fe(CN)₆]), undecylenic acid, N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)N-ethylcarbodiimide hydrochloride (EDC), phosphate-buffered saline (PBS) tablets, 2-(N-morpholino)ethanesulfonic acid (MES), (3-amino propyl) triethoxysilane (APTES), (3-glycidylxypropyl) trimethoxysilane (GPTMS), sodium chloride, tris(hydroxymethyl)aminomethane, hydrochloric acid (37%), and fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich (Australia). Cyanine5 amine (Cy5-NH₂) was purchased from Luminoprobe (USA). The acetylene gas cylinder (1 m³ industrial grade, dissolved) was purchased from BOC (Australia). All the DNA strands were purchased from Integrated DNA Technologies, Inc. The sequence of the amino-modified ssDNA capture probe was 5′-5AmMC₆/GTC CAC GCC GTA AAC GAT GTC GAC TTG G-3′. The amino-modified nonspecific ssDNA capture probe was 5′-5AmMC₆/CAC AAA TTC GGT TCT ACA GGG TA-3′. The sequence of the target ssDNA was 5′-CCA AGT CGA CAT CGT TTA CGG CGT GGA C-3′.

**Apparatus.** Scanning electron microscope (SEM) images were obtained with an FEI NovaNano SEM 430 at an accelerating voltage of 10 kV. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy was performed with a Thermo Scientific Nicolet 6700 FTIR spectrometer. Raman spectra were acquired using a Renishaw inVia Raman microscope with a 100 mW 532 nm laser excitation source. A 10% excitation power density was applied to avoid damage to the surface. Fluorescence microscopy images were

![Figure 1. Synthesis of the TCpSi-THCpSi double-layer nanostructure. (a) Bulk silicon wafer. (b) A single layer of porous silicon (pSi) is prepared via electrochemical anodization; this layer is referred to in this work as the top layer. (c) Decomposition of acetylene gas on the pSi surface at 525 °C (thermal hydrocarbonization) followed by (d) annealing at 800 °C (thermal carbonization) generates a TCpSi surface coating on the top pSi layer. (e) A second pSi layer is generated directly underneath the TCpSi top layer via a second anodization step; this layer is referred to as the bottom layer. (f) A second thermal hydrocarbonization at 525 °C of the sample generates a THCpSi surface on the freshly etched bottom layer. Cross-sectional scanning electron microscope (SEM) images of the resulting TCpSi-THCpSi double layer at (g) low magnification and (h) high magnification.](https://doi.org/10.1021/acsami.2c02113)
collected with a laser scanning confocal microscope (Nikon Instrument TIRF with Ti-U system).

**Fabrication of pSi Single Layer.** A whole 6-in. p-type Si wafer was anodically etched in an electrolyte solution containing 1:1 (v/v) aqueous 48% HF: absolute ethanol (caution: HF is highly toxic and corrosive, and proper care should be exerted to avoid contact with skin, eyes or lungs) to produce a first pSi layer, using a wet etching system (A.M.M.T. GmbH, Germany), which can load a 6-in. Si wafer with an exposed etching area of 132 cm². Firstly, a sacrificial layer was produced at an anodic current density of 60.6 mA cm⁻² for 30 s. This was removed with 1 M sodium hydroxide. The etching cell was rinsed with water followed by absolute ethanol and dried with N₂ gas. Nexan was thoroughly washed with PBS and transferred to a 2 mM Tris buﬀer, pH 5.5, at room temperature and then vacuum filtered from the solution and stored at −20 °C overnight. To assess the performance of the developed DNA sensors, ssDNA target solutions were prepared at various concentrations (from 1 to 1000 pM) in 10 mM Tris buﬀer, pH 5.5, at room temperature for 30 min to produce succinimidyl ester groups. Subsequently, a 10 μg mL⁻¹ Cy5-NH₂ solution in 10 mM PBS was incubated on the surface for 30 min. The Cy5-modified sample was ﬁnally rinsed with PBS and absolute ethanol. To modify the NH₂-terminated TCpSi bottom layer, a 10 μg mL⁻¹ FITC solution in 10 mM PBS was incubated onto the surface for 30 min. The FITC-modiﬁed sample was rinsed with PBS and then absolute ethanol. Control samples exclusively modiﬁed either with FITC or Cy5 were simultaneously prepared.

**Optical Characterization of pSi Double Layer.** Interferometric reﬂectance spectra were collected using a tungsten lamp (Ocean Optics) and a CCD spectrometer (Ocean Optics S-2000). White light was fed through one end of a bifurcated optic cable and focused through a lens onto the pSi surface with a spot size of approximately 1 mm in diameter. Light reﬂected from the pSi layer was collected through the same optical lens and transferred to the CCD spectrometer via the second arm of the bifurcated optic cable. A fast Fourier transform using algorithm from the WaveMetrics Inc. Igor program library was applied to the resulting spectra.

**Electrochemical Characterization.** Electrochemical measurements were carried out on an electrochemical analyzer (CH Instruments, model 600D series, USA) using a three-electrode conﬁguration in a Teflon cell containing the pSi nanostructure on an aluminum ﬁlm as the working electrode, a Ag/AgCl reference electrode, and a platinum wire as a counter electrode.

**DNA Detection Using a TcPSc-ThcPSc Double Layer-Based Sensor. Fabrication and Modiﬁcation of DNA Sensor.** In order to fine-tune the pore size to enable DNA hybridization, the pSi top layer was electrochemically etched in a 1:1 (v:v) aqueous 48% HF: absolute ethanol solution, by applying a current density of 49.9 mA cm⁻² for 80 s. The pSi bottom layer was etched in a 3:1 (v:v) aqueous 48% HF: absolute ethanol solution, using a Teflon etching cell and applying a current density of 21.4 mA cm⁻² for 60, 150, and 300 s to obtain samples with different bottom layer thickness. First, in order to form hydroxyl groups on the TcPSc layer, the TcPSc-ThcPSc double layer was treated in 1:1 (v:v) aqueous 48% HF: absolute ethanol solution for 15 min and dried at 65 °C for 3 h. Second, the OH-terminated TcPSc top layer was subsequently modiﬁed using 10 mL 10% GPTMS dissolved in anhydrous toluene for 30 min at room temperature. After reaction, the samples were thoroughly rinsed with 1:1 (v:v) anhydrous toluene/methanol, pure methanol, and then pure ethanol. Third, 100 μL of either 10 μM of NH₂-ssDNA capture probe or nonspeciﬁc NH₂-ssDNA capture probe, both prepared in 0.1 M MES buﬀer, pH 5.5, were incubated on the double-layer nanostructures for 2 h at room temperature. Samples were subsequently rinsed thoroughly with PBS and ready to proceed with sensing experiments. DNA detection protocol. To assess the performance of the developed DNA sensors, ssDNA target solutions were prepared at various concentrations (from 1 to 1000 pM) in 10 mM Tris buﬀer with 75 mM NaCl, pH 7.5, were incubated on the sensor surface for 15 min. After each incubation step, the biosensor surfaces were thoroughly washed with PBS and transferred to a 2 mM absolute ethanol. The TCpSi top layer is natively covered with a thin oxide layer, preventing from potential introduction of −COOH groups as a result of the alkene grafting process. Prior to further reaction, the COOH-modified sample was immersed into 1:1 (v:v) aqueous 48% HF: absolute ethanol solution for 15 min at room temperature and then vacuum filtered from the solution and ﬁnally dried at 65 °C for 3 h. The COOH-terminated THCpSc bottom layer resisted HF attack and was stable until further modiﬁcation. However, exposure to HF enables hydroxylation of the TcPSc surface. The OH-terminated TcPSc top layer was subsequently modiﬁed in 10 mL 0.5% APTES in anhydrous toluene for 30 min at room temperature. The silane solution was then removed and replaced by anhydrous toluene. Then, the sample was sonicated for 3 min in successive washing steps with fresh toluene, 1:1 (v:v) toluene/methanol, methanol, and ethanol, to remove any traces of loosely bound APTES. The sample was dried at 105 °C overnight.
FTIR) spectroscopy. The as-etched pSi layer displayed bands characteristic of Si–H and Si–H2 stretching vibrations at 2087 and 2114 cm\(^{-1}\), respectively, and a band assigned to the Si–H deformation mode at 905 cm\(^{-1}\) (Figure 2a). Compared to the as-etched pSi, TCpSi (Figure S3) did not show the bands characteristic of Si–H; instead new bands associated with the carbon layer were observed: the stretching vibration of saturated C–H at 3047 cm\(^{-1}\), the unsaturated carbon double-bond stretching at 1600 cm\(^{-1}\), and the CH\(_2\) symmetric deformation mode of Si–CH\(_2\) at 1250–1260 cm\(^{-1}\). Figure 2b shows the ATR-FTIR spectrum of the TCpSi-pSi double layer, which was derived from an initial TCpSi single layer that was subjected to a second electrochemical anodization process to produce a pSi bottom layer (Figure 1). After the second anodization step, a new band associated with Si–H, at ∼2100 cm\(^{-1}\) was observed in the TCpSi-pSi double layer. All the bands associated with the original TCpSi layer (e.g., C–H, C=CH, C=CH\(_2\)) persisted in the TCpSi-pSi double layer, suggesting that the carbon-coated layer is stable to the HF-containing electrolyte, and it is not removed by the anodization process. After the second thermal hydrocarbonization step, the resulting TCpSi-THCpSi double layer no longer displayed bands associated with Si–H, while the peaks associated with carbon layers such as C–H, C=CH, and CH\(_3\) were still apparent (Figure 2c).

Raman spectroscopy was used to characterize the carbon layers introduced on the pSi surface. Both the TCpSi single layer (Figure 2e) and the TCpSi-THCpSi double layer (Figure 2f) displayed a characteristic Si lattice mode at 515 cm\(^{-1}\) demonstrating that the crystallinity of Si was preserved after carbonization. Unlike as-etched pSi (Figure 2d), the TCpSi single layer displayed the D and G bands (at 1350 and 1580 cm\(^{-1}\), respectively), which are the Raman signatures of carbon materials. The D band is due to the breathing mode of sp\(^2\) atoms in rings, and it is linked to defects in the structure. The first-order D band is not usually observed in pristine graphene due to its crystal symmetries. Hence, the presence of the D band in the Raman spectra of both the TCpSi single layer and the TCpSi-THCpSi double layer suggests the presence of a
disordered and defective carbon layer. In contrast, the G band, primarily an in-plane vibrational mode of sp2 atoms in hydrocarbon chains and rings, is indicative of high crystallinity of the carbon layer. The intensity ratio of the D and G bands \( \frac{I_D}{I_G} \) is typically used to evaluate the level of disorder in carbon coatings. In the present case, \( \frac{I_D}{I_G} \) values were obtained by Lorentzian fitting of the bands after baseline subtraction. The TCpSi-THCpSi double layer showed a value of \( \frac{I_D}{I_G} \) (0.83) lower than that of the TCpSi single layer (0.95, Figure 2e) suggesting that the deposition and annealing steps (Figure 1) reduced defect density within the carbon coating of the double-layered nanostructure. To further investigate this characteristic, we calculated \( \frac{I_D}{I_G} \) for a TCpSi-pSi double layer that was prepared from a TCpSi single layer via second anodization but prior to the second carbon deposition step. This sample showed a slightly lower \( \frac{I_D}{I_G} \) value (0.92, Figure S4b) compared with that of the TCpSi single layer precursor (0.95, Figure S4e). This decrease in the level of disordered carbon suggests that the HF-containing electrolyte and the anodization conditions more readily remove the more highly disordered portions of the carbon coating. Thus, the more highly disordered carbon is likely associated with surface SiO2, as most of the oxide layer formed on the TCpSi single layer is removed by HF during the second electrochemical etching step. We hypothesize that the greatly reduced defect level in the final TCpSi-THCpSi double-layer structure (evidenced by a lower \( \frac{I_D}{I_G} \) of 0.83, Figure 2f) is due to a combination of increased crystallinity in the bottom layer of THCpSi and the removal of SiO2 and disordered carbon from the top layer of TCpSi during the second etching step. The Raman spectra comparing THCpSi and TCpSi single layers with THCpSi-pSi and TCpSi-pSi double layers (Figure S4) confirmed that the carbon coating in both THCpSi and TCpSi is resistant to HF, which is essential to allow the fabrication of multilayered nanostructures that display different degrees of carbonization in one layer relative to the other.

We next exploited the controlled surface chemistry of TCpSi-THCpSi to prepare biointerfaces containing different functional groups in the different layers. Amine and carboxylic acid moieties were grafted to the TCpSi and THCpSi surfaces of the double-layer nanostructure, respectively (Figure 3). The site-specific chemistries used to prepare the TCpSi-THCpSi double layer are described in Figure 3b. We first prepared a 7.7 μm-thick TCpSi-THCpSi double layer with the microstructure depicted in Figure 3a. The THCpSi bottom layer was then functionalized with COOH groups via thermal grafting with undecylenic acid. Next, the TCpSi-THCpSi double layer was treated with aqueous ethanolic HF and dried at 65 °C. While the COOH-terminated THCpSi bottom layer was stable to HF, the TCpSi top layer reacted with the HF-containing solution to introduce surface –OH groups. This reactivity was confirmed by performing the same HF treatment on a
TCpSi single layer; the ATR-FTIR data are shown in Figure S5, which is consistent with previous reports. A broadening of the OH band over 3000 cm$^{-1}$ and a clear shoulder structure around 3650 cm$^{-1}$ is indicative of the formation of OH groups (Figure S5b). The TCpSi surface, while primarily composed of silicon and carbon species, also contained some surface oxide species (primarily Si oxide that is back-bonded to carbon atoms); exposure of this surface to HF generated the surface hydroxyls. The hydroxylated TCpSi top layer was then functionalized with 3-aminopropyl triethoxysilane (APTES), a common linker used to graft amines to silanol or hydroxyl surfaces. This resulted in an amine-terminated TCpSi top layer.

The two layers were then differentially modified with fluorescent dyes in order to validate the differential grafting chemistries. The COOH species on the THCpSi bottom layer was attached to the free NH$_2$ group on a Cy5 fluorescent dye via carbodiimide coupling chemistry, and the fluorescent dye fluorescein was grafted to the NH$_2$-terminated TCpSi top layer by reaction with fluorescein isothiocyanate (FITC). The site-specific labeling of the TCpSi-THCpSi double layer was confirmed by confocal fluorescence microscopy. As presented in Figure 3c, the green and red layers correspond to the FITC-labeled TCpSi top layer and Cy5-labeled THCpSi bottom layer, respectively. Control confocal microscope images were acquired on TCpSi-THCpSi double-layer samples that were functionalized exclusively with either FITC or Cy5. Functionalized TCpSi-THCpSi double-layer samples labeled with FITC showed characteristic green fluorescence only at the TCpSi top layer (Figure 3d), while samples labeled with Cy5 displayed red fluorescence only at the THCpSi bottom layer (Figure 3e). The thickness and location of each dye-labeled layer were consistent with the physical thickness and location of the layers as measured by SEM (Figure S6).

The electrochemical properties of the TCpSi-THCpSi double-layer nanostructure were investigated by cyclic voltammetry (CV) in the presence of [Fe(CN)$_6$]$^{3-/4-}$ in 10 mM phosphate-buffered saline solution (PBS), at pH 7.4. The [Fe(CN)$_6$]$^{3-/4-}$ redox system, extensively used in the electrochemical characterization of sp$^2$ carbon materials (e.g., graphite and glassy carbon), was chosen because it displays quasi-reversible redox electrochemistry on carbon electrodes. First, to demonstrate the ability of the whole double-layer nanostructure to work as an electrochemical transducer, the effective surface area ($A_{\text{eff}}$) of both TCpSi single layer and TCpSi-THCpSi double-layer, the latter with a top TCpSi

![Figure 4](https://doi.org/10.1021/acsami.2c02113)
standard deviation, properties such as the double-layer nanostructure possessed surface and electronic relaxation that the stabilized TCpSi-THCpSi double-layer structure showed reasonable performance as an electrode for redox events in the range from −0.2 to 0.8 V vs Ag/AgCl, as shown by the high oxidation and reduction peak currents and small peak-to-peak potential difference ΔEp (Figure 4a). The ΔEp value of 123 mV is larger than the expected theoretical value of 59 mV at 25 °C for a fully reversible one-electron transfer reaction, representing a quasi-reversible electron-transfer system. The ratio of peak currents Ip(Ox)/Ip(Re) was approximately 1 (Table S2), indicating that the stabilized TCpSi-THCpSi double-layer nanostructure possessed surface and electronic properties sufficient to support relatively rapid electron transfer. The fast electron transfer these nanostructures elicit was confirmed by the estimated value of heterogeneous standard rate constant k0 of electron transfer. We calculated k0 using a modified version of the Nicholson method reported by Lavagnini and colleagues41 that introduces an empirical equation to simplify the calculation of the dimensionless parameter, Ψ:

Ψ = (−0.6288 + 0.0021X)/(1 − 0.017X)

where X is ΔEp in mV (ΔEp values extracted from Figure 4c are included in Table S2). The relationship between Ψ and k0 is given by the following equation:

Ψ = k0 [DnF/(RT)]^{1/2}

Therefore, the slope of the linear fitting of the plot Ψ vs [πDnF/(RT)]^{1/2} ν^{1/2} can be assigned to the kinetic parameter k0, which corresponded to 0.16 cm s⁻¹ for the carbon-stabilized TCpSi-THCpSi double-layer nanostructure. This value exceeds the k0 value of 3.6 × 10⁻⁸ cm s⁻¹, reported by Lavagnini et al.41 for a carbon paste electrode under the same working conditions. It also supports fast electron transfer when compared with a glassy carbon electrode, based on the k0 values estimated using other methods of calculation: 8.9 × 10⁻⁹ cm s⁻¹ determined by digital simulation42 or 1.3 × 10⁻⁹ cm s⁻¹ estimated from EIS data.43

The stability of the electrode was evaluated by recording Ip(Ox)/Ip(Re) values through many cycles, over a time period of 90 h. The response was stable over this period, with average peak current values of 0.36 ± 0.01 and 0.35 ± 0.01 mA, respectively (RSD < 3%) (Figure 4b). Moreover, Ip(Ox) and Ip(Re) increased linearly with the square root of scan rate ν^{1/2} (Figure 4c,d), suggesting that even in the restricted dimensions of the porous nanostructure, the reaction is controlled by semi-infinite linear diffusion typical of planar electrodes at the timescales measured.44

To demonstrate the suitability of the TCpSi-THCpSi double-layer structures for electrochemical biosensing, a label-free voltammetric DNA sensor was constructed. The TCpSi top layer, possessing relatively large pores of 27 ± 9 nm diameter, was selectively functionalized with an ssDNA probe while the THCpSi bottom layer with smaller pores (<5 nm diameter) acted as an electrochemical transducer to convert the hybridization event confined in the top layer into an output current signal via differential pulse voltammetry (DPV) measurements. Successful immobilization of the ssDNA...
The sensing mechanism of the label-free voltammetric DNA sensor developed here relies on the hypothesis that DNA hybridization will induce partial blockage of the nanochannels as shown in Figure 5a. When the target ssDNA hybridizes with the ssDNA capture probe immobilized at the TCPsi top layer, partial blockage of the top layer nanochannels can be expected. This partial nanochannel blockage hinders the diffusion of redox species such as $[\text{Fe(CN)}_6]^{3-}/[\text{Fe(CN)}_6]^{4-}$ into the electrochemically active bottom layer (the electrochemical transducer), resulting in a decrease in the intensity of peak current monitored by DPV. Changes in peak current obtained from differential pulse voltammograms acquired prior to and after hybridization were normalized via the relationship:

$$\Delta i = \frac{(i^b - i)}{i^b}$$  \hspace{1cm} (1)

where $\Delta i$ is the normalized current change, $i^b$ is the peak current value measured after 15 min incubation in buffer blank, and $i$ is the peak current value measured after 15 min incubation in the ssDNA target solution at a given concentration. Both $i^b$ and $i$ were extracted from the differential pulse voltammograms obtained in a 2 mM $[\text{Fe(CN)}_6]^{3-}/[\text{Fe(CN)}_6]^{4-}$ solution. Control samples were prepared using a random sequence of ssDNA as a capture probe.

Three double-layer structure types were tested: all three were prepared with a top layer of similar thickness and porosity, and the thickness of the bottom layer (the transducer layer) was systematically varied, and the analytical performance of each DNA sensor was assessed. The normalized $\Delta i$ values plotted as a function of Log[ssDNA] (Figure 5) exhibited a linear relationship to the target ssDNA concentration in the range from 1 to 1000 pM. Control experiments using a noncomplementary capture probe (hollow symbols) showed nearly no electrochemical response compared with the corresponding specific DNA sensors (solid symbols). As shown in Figure 5, the transducer thickness (i.e., THCpSi bottom layer) had a pronounced effect on the sensitivity of the designed DNA sensor. The structure with the thinnest THCpSi bottom layer tested (0.8 μm) showed the highest sensitivity.

The LOD values, calculated using the equation $y_b + 3 \times SD$, where $y_b$ is the DPV current value for the blank (normalized $\Delta i$ in buffer) and SD is the associated standard deviation ($n = 3$), were 0.4, 1.6, and 2.8 pM for TCPsi-THCpSi double-layer nanostructured DNA sensors featuring 0.8, 2.4, and 4.8 μm transducer thickness at the bottom layer, respectively. The developed pSi double layer-based DNA sensor allowed the detection of a 28-nucleotide DNA sequence, with a LOD as low as 0.4 pM, showing two orders of magnitude enhancement compared with the best LOD (50 pM) \(^{23}\) achieved to date by a pSi-based electrochemical DNA sensor reported by Lugo et al., which exploited the semiconductor characteristics of pSi. That DNA sensor used oxidized pSi as the immobilization platform of a DNA capture probe complementary to the target DNA, and DNA hybridization was monitored by measuring the oxidation of guanine using a ruthenium bipyridine [Ru(bpy)_2]\(^{2+}\) redox indicator.\(^{25}\) The present approach takes advantage of the electrical conductivity of the carbonized pSi matrix, allowing for a more stable and more sensitive detection modality.

For electrochemical sensing, the double-layer structure shows a key advantage over a single-layer structure because it provides a robust means to incorporate an electrochemical transducer into the nanostructured sensing element. For example, when a single layer-based TCPsi transducer is used, the biomolecular coating acts as a highly resistive series element, inhibiting electron transfer from solution species into the electrode and thus degrading its performance as an electrochemical transducer. The electrochemical characterization data obtained on such a control single-layer structure is given in Table S3. Covalent immobilization of the capture probe (ssDNA) to a TCPsi single layer caused a sharp decrease in the measured anodic and cathodic peak currents, and an increase in $\Delta E_p$ in the cyclic voltammograms. The inclusion of an unmodified THCpSi layer featuring small (<5 nm) pores directly underneath the DNA-modified TCPsi layer overcomes this limitation; indeed, the electrochemical performance exceeded the sensitivity achieved on conventional flat electrodes.\(^{45-46}\) Because the pore size and thickness of THCpSi bottom layer are readily adjustable in the fabrication process, the double-layer system provides a means to optimize surface area of the transducer, as well as its wettability. The small dimension of the micropores in this electrochemically active layer also acts to exclude larger species (such as proteins) that might interfere with the electrochemical measurement. Compared to single layer-based transducers, TCPsi-THCpSi double layers also offer the possibility to tune the electrochemical performance by varying pore size and depth in each layer, providing a means to tune the sensitivity level required for a specific biosensing purpose.

### CONCLUSIONS

In summary, this work demonstrates for the first time the design and fabrication of a carbon-stabilized pSi double-layer nanostructure featuring fine-tuned wettability and layer-specific functionalization to facilitate further fit-for-purpose modification. Along with retaining the unique physical features derived from pSi, the carbon-stabilized TCPsi-THCpSi double-layer nanostructures possess controllable surface functionalities and fast electron transfer kinetics suitable to harness them as both a biointerface and an electrochemical transducer. Critical to designing high-performance electrochemical biosensors is the demonstrated capacity of the carbon-stabilized TCPsi-THCpSi double-layer nanostructures to tune their sensitivity to suit to a specific application. Our results support the feasibility of increasing sensitivity by adjusting the morphological features of the transducer layer to maximize the electrode active area and those of the biorecognition layer to maximize pore blockage upon analyte binding. Their potential for use as electrochemical biosensors is demonstrated by the developed TCPsi-THCpSi double-layer-based voltammetric DNA sensor, showing an LOD of 0.4 pM in buffer, two orders of magnitude lower than the best performing previously reported pSi-based electrochemical DNA sensor. While not as sensitive as the best label-free electrochemical biosensors\(^{50}\) or PCR methods that can detect DNA in the low fM range,\(^{50}\) the controllability of the nanostructural morphology and surface chemistry of this combination of carbon and pSi provides a means to incorporate features, such as selective filtration, that can enable more complex (bio)sensing functions. Future research will unlock the potential of these double-layer structures as sensing platforms for sequential detection of various analytes. Sequential sensing is envisaged by combining discrimination...
based on molecular affinity, provided by the site-specifically immobilized bioreceptors and the differential penetration of molecules into specific layers.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.2c02113.

- SEM images of pSi, THCP$i$, and TCp$i$.
- Interferometric reflectance spectra of pSi, TCp$i$, and TCp$i$-THCP$i$.
- Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectrum of TCp$i$.
- Raman spectra of THCP$i$-pSi, TCp$i$-pSi, pSi, THCP$i$, and TCp$i$.
- ATR-FTIR spectra of TCp$i$ before and after HF immersion.
- Cross-sectional SEM image of the pSi double-layer sample used for confocal microscopy characterization after selective dye labeling.
- ATR-FTIR spectra of TCp$i$-THCP$i$ after HF-dipping and drying at 65 °C for 3 h, epoxy silane modification, and NH$_2$-ssDNA probe immobilization.
- Ellipsometry analysis to calculate the thickness of carbon layer, summary of peak current and $\Delta E_p$ extracted from the cyclic voltammograms obtained using TCp$i$-THCP$i$; summary of peak currents and $\Delta E_p$ extracted from the cyclic voltammograms obtained during functionalization and DNA immobilization, using TCp$i$ and TCp$i$-THCP$i$.

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Notes

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