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Nanostructured Porous Si Optical Biosensors: Effect of Thermal Oxidation on Their Performance and Properties

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ABSTRACT

The influence of thermal oxidation conditions on the performance of porous Si optical biosensors used for label-free and real-time monitoring of enzymatic activity is studied. We compare three oxidation temperatures (400, 600, and 800°C) and their effect on the enzyme immobilization efficiency and the intrinsic stability of the resulting oxidized porous Si (PSiO₂), Fabry-Pérot thin films. Importantly, we show that the thermal oxidation profoundly affects the biosensing

performance in terms of greater optical sensitivity, by monitoring the catalytic activity of horseradish peroxidase and trypsin-immobilized PSiO_2 . Despite the significant decrease in porous volume and specific surface area (confirmed by nitrogen gas adsorption-desorption studies) with elevating the oxidation temperature, higher content and surface coverage of the immobilized enzymes is attained. This in turn leads to greater optical stability and sensitivity of PSiO_2 nanostructures. Specifically, films produced at 800°C exhibit stable optical readout in aqueous buffers combined with superior biosensing performance. Thus, by proper control of the oxide layer formation, we can eliminate the aging effect; thus achieving efficient immobilization of different biomolecules, optical signal stability and sensitivity.

INTRODUCTION

Nanostructured porous Si (PSi) has emerged as an attractive and versatile material for optical biosensing applications due to its large internal surface area and tunable optical properties.¹⁻⁵ Various biosensing schemes have shown numerous advantages of these nanosystems over conventional bio-analytical techniques, such as enhanced detection sensitivity, real-time and label-free fast analysis.^{1, 6-24} However, a critical challenge in designing PSi-based biosensors is the relative chemical instability of the Si scaffold in biologically relevant environments.²⁵⁻²⁸ Specifically, PSi can be oxidized and/or dissolved in aqueous environments, which can lead to significant variations in the optical and electrical properties, e.g., luminescence, refractive index and absorption coefficient.^{26, 29-31} For example, PSi oxidation can significantly affect the refractive index of the material ($n=3.5$ and $n=1.4$ for Si and SiO_2 , respectively) and thus it can interfere with the signal transduction in PSi optical biosensors. Moreover, PSi dissolution in aqueous media causes even higher change in refractive index, which may ultimately result in the structural collapse of the porous matrix.^{14, 31} Several chemical routes are used to enhance PSi

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3 stability in biological media, including thermal oxidation, hydrosilylation, electrochemical
4 alkylation and thermal hydrocarbonization.^{2, 31-33} Thermal oxidation is frequently utilized for PSi
5 biosensors passivation, owing to the wide repertoire of chemical modifications available for Si
6 oxide surfaces. The high temperature treatment completely converts the reactive SiH_x species
7 into relatively inert Si-O bonds, resulting in a greater optical stability, potentially eliminating
8 zero-point drift, and protection against aqueous dissolution.^{19,21}
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18 Herewith, we study the effect of thermal oxidation conditions on the stability and sensitivity of
19 PSi-based optical biosensors, which are designed for monitoring enzymatic activity. The effect
20 of different oxidation conditions (temperature) on the chemical stabilization of the biosensor
21 surface i.e., PSi Fabry-Pérot thin film is investigated. We demonstrate that by proper control of
22 the oxide layer formation, the aging effect can be eliminated; thus leading to an efficient
23 bioconjugation of different molecules, optical signal stability and sensitivity.
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32 EXPERIMENTAL SECTION

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36 **Materials.** Silicon wafers (highly-doped p-type, 0.8 mΩ-cm resistivity, <100> oriented, Boron-
37 doped) are supplied from Siltronix Corp. Aqueous HF (48%) and absolute ethanol are obtained
38 from Merck. 3-Aminopropyl(triethoxyl)silane (APTES), diisopropylethylamine (DIEA), Bis (N-
39 succinimidyl)carbonate (DSC), Horseradish peroxidase (HRP) type VI, trypsin from bovine
40 pancreas (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone, TPCK), acetonitrile,
41 hydrogen peroxide (H₂O₂), Ampliflu Red, 4-chloro-1-naphthol (4CN) and analytical grade
42 buffers are supplied from Sigma Aldrich Chemicals. Fluorescein thiocarbamoyl-labeled casein
43 (FTC-casein) and Fluorescein-5-EX, succinimidyl ester are obtained from Thermo Fisher
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Preparation of porous SiO₂ nanostructures. PSi thin films are prepared by anodization at a current density 385 mA cm⁻² as previously described by Massad-Ivanir *et al.*⁶ Then, the PSi samples are thermally oxidized at three temperatures: 400, 600, and 800°C for 1 hr, resulting in porous SiO₂ (PSiO₂) layers.⁶

The following sections describe the biofunctionalization procedures and the consecutive biosensing experiments, which are carried out for the PSiO₂ layers, formed at 400, 600, and 800°C.

Biofunctionalization of PSiO₂.

Preparation of APTES-modified and DSC-modified surfaces: The PSiO₂ samples are prepared as previously described by Massad-Ivanir *et al.*⁶

Preparation of enzyme-immobilized surfaces: The DSC-modified surfaces are incubated with 30 µL of enzyme solution (2.5 µM and 4.6 µM, HRP and trypsin, respectively) in 0.1 M HEPES buffer (pH 8) for 1 hr at room temperature. After the removal of solution, the surfaces are washed with HEPES buffer, soaked for 20 min in buffer, and washed with HEPES to exclude the non-specifically bound enzymes from the surfaces.

Nitrogen adsorption-desorption measurements. The different oxidized (400, 600, and 800°C) PSiO₂ nanostructures are analyzed as previously described.³⁴ Note: Before conducting the adsorption experiment, the samples are degassed *in situ* at 200°C for 10 h.

Gravimetric determination of porosity and infrared spectroscopy. Detailed analysis is performed as previously described.⁶

Thermal gravimetric analysis (TGA). Thermogravimetric analysis is conducted using a TA Instruments Q5000-0486 TGA. The freshly-etched PSi samples are heated between 25°C and the

designated oxidation temperature (400, 600, and 800°C), under nitrogen atmosphere, at a constant heating rate of 20°C min⁻¹.

Energy-dispersive X-ray spectroscopy (EDS). A Carl Zeiss Ultra Plus high-resolution scanning electron microscope (HRSEM) coupled with EDS detector is used to determine the oxidation degree of the different porous nanostructures. EDS elemental mapping is performed at an acceleration voltage of 10 kV using Espirit 1.8 software.

Confocal laser scanning microscopy (CLSM). HRP and trypsin are modified with Fluorescein-5-EX, succinimidyl ester according to the manufacturer's protocol. The labeled enzyme solution in HEPES buffer (at concentration of 2.5 μM fluorescein-HRP and 4.6 μM fluorescein-trypsin, respectively) is incubated with the DSC-modified PSiO₂ for 1 hr at room temperature. After the solution is removed, the surfaces are thoroughly washed with HEPES buffer, soaked for 1 hr in buffer, and washed again with buffer to exclude any non-specifically bound enzymes from the surfaces. Following the conjugation, the samples are scanned with a LSM 700 confocal laser scanning microscope (Carl Zeiss, Inc.) connected to a Zeiss inverted microscope equipped with a Zeiss X63 oil immersion objective. Combinations of 405 nm and 488 nm laser lines are used for the excitation of PSiO₂ structure and FTC-modified enzymes, respectively. For three-dimensional (3D) image projection of the enzyme-immobilized PSiO₂, z-scans in 0.35 μm increments are taken over a depth of ~ 6.6 μm and projected by using standard Carl Zeiss software (ZEN 2009). Image analysis is performed by Imaris Bitplane scientific software.

Enzymatic activity. *HRP activity:* Ampliflu Red substrate solutions and HRP activity assay are performed as previously described.³⁵

Trypsin activity: FTC-casein solution (200 μL of 0.38 μM) in a HEPES buffer is introduced onto trypsin-immobilized PSiO_2 samples. The catalytic reaction progress is monitored at room temperature by recording the fluorescence values at 538 nm (excitation wavelength of 485 nm).

Quantification of the immobilized enzyme. The amount of immobilized enzyme is determined by comparing the specific activity rates of various concentrations of the free enzyme in a solution (calibration curve: trypsin and HRP, 2-650 nM and 20-160 nM, respectively) to the immobilized enzyme activity.

Optical measurements. Detailed optical characteristics of PSiO_2 film, system setup and data analysis are described elsewhere.⁶ Briefly, the effective optical thickness (EOT) refers to the $2nL$ term in the Fabry-Pérot formula (where n is the average refractive index and L is the porous layer thickness).⁶ In the present work, the data is presented as relative EOT and defined as:

$$EOT/EOT_0 = \left(\frac{EOT_{\text{Chemical modification}}}{EOT_{\text{PSiO}_2}} \right)_T \quad (1)$$

where $EOT_{\text{Chemical modification}}$ is the value of nL obtained after different biofunctionalization steps (e.g., APTES, DSC, HRP immobilization); EOT_{PSiO_2} is the nL_0 value of the neat PSiO_2 ; and T refers to the thermal oxidation temperature.

Optical readout stability of PSiO_2 based transducers. Neat PSiO_2 , APTES-modified PSiO_2 , DSC-modified PSiO_2 , Trypsin and HRP-immobilized PSiO_2 are washed with HEPES buffer at a constant flow of 0.5 mL min^{-1} for 260 min. The reflectivity spectra are collected throughout the experiments every 30 s.

Optical biosensing of HRP activity. HRP-immobilized PSiO_2 surfaces are washed with HEPES buffer for 30 min. Then, 0.8 mM of 4CN in HEPES buffer is cycled through a custom-made flow cell for 20 min. Lastly, 0.16 M H_2O_2 is introduced into the cycled solution for enzyme activation. The reflectivity spectra are recorded throughout the experiments every 30 s.³⁶

RESULTS AND DISCUSSION

Characterization of PSiO₂ films. The PSiO₂ surfaces are prepared using anodic electrochemical etching of heavily-doped Si, followed by thermal oxidation in ambient air at three different temperatures (400, 600, and 800°C) to generate a hydrophilic scaffold.⁶ Previous work by Pap *et al.*²⁹ has established that thermal oxidation of PSi at 800°C for a duration of 1 hr results in a high conversion (>80%) of the hydrogen-terminated Si to SiO₂. At lower temperatures (400°C and 600°C) extending the oxidation time has a limited effect on the oxidation degree. These results are explained by taking into account the thickness and porosity of the porous nanostructure and the rate of diffusion through the layer. The structural properties of the obtained thermally oxidized porous nanostructures correspond to previously described by Segal *et al.*³ The porous film is 6,830±150 nm thick, characterized by cylindrically shaped pores with a diameter of 60 to 100 nm. EDS-coupled to HRSEM and TGA are employed in order to characterize the resulting oxide layer and the PSiO₂ films. Figure 1a summarizes the elemental analysis results, expressed in terms of relative atomic percentage of oxygen to Si, for the different PSiO₂ layers.³⁷ The oxygen to Si ratio is observed to significantly increase with oxidation temperature, indicative of a thicker and more dense oxide layer,³³ in agreement with TGA data (see Fig. S1, Supporting Information section). It should be noted, that the porous film is highly irregular and rough at the nano-scale and a thicker and denser oxide layer would have a profound effect on the surface homogeneity. Figure 1b-d depicts EDS-HRSEM results for the PSiO₂ films oxidized at 800°C.

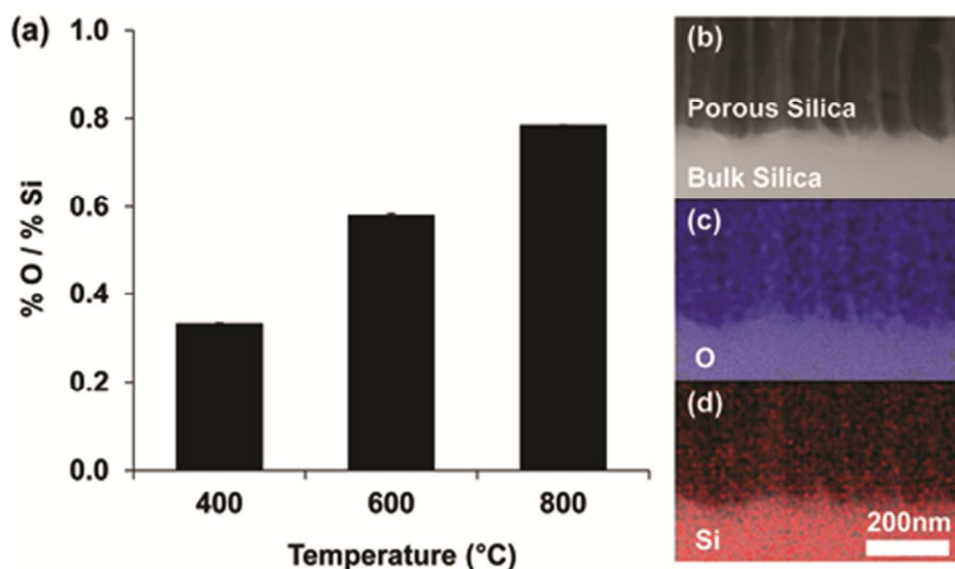


Figure 1. Elemental analysis of the PSi nanostructure oxidized at different temperatures. **(a)** The ratio of atomic percentages, obtained by EDS mapping, of oxide to Si across the porous layer, for films oxidized at different temperatures (400, 600, 800°C). The error is less than ± 0.004 . **(b)** Cross-section HRSEM micrograph of the PSiO₂ oxidized at 800°C, focusing at the interface between the bulk Si support and porous layer. **(c)** Oxygen and **(d)** Si elemental mapping of the corresponding layer (blue and red, respectively).³⁷

Furthermore, ATR-FTIR spectroscopy is used as a complementary tool for surface characterization. Figure S2 depicts the ATR-FTIR spectra of the different oxidation temperatures (400, 600, 800°C). A significant increase in absorbance of Si-O-Si stretching mode and –(O_ySiH_x) vibration mode (observed at 1039 and 801 cm⁻¹, respectively) with elevation of the oxidation temperature is shown in Figure S2a. These results correlate well with the disappearance of the Si-H_x peak (observed at 878 cm⁻¹) at 600 and 800°C, due to the oxidation of all hydrogen-terminated Si groups. Figure S2b shows a similar increase in the absorbance of Si-OH vibration mode at 3747 cm⁻¹, resulting from a thicker oxide layer.³⁸

Nitrogen adsorption-desorption experiments followed by BET and BJH analyses are used to further characterize the different oxidized P*Si* films, in terms of pore size distribution, specific surface area and the porous volume. Table 1 summarizes the physical characteristics of the different oxidized films. The average pore diameter and the total porosity (obtained by gravimetric analysis) decrease with the increase of the oxidation temperature. The resulting pore clogging by oxygen layer obtained at elevated temperatures lowers the porous volume and accordingly the surface area.³³ It should be noted that the pore diameter obtained by this method is significantly lower than the visual estimates by HRSEM (Table 1), as previously reported.³

Table 1. Physical characteristics of P*Si* films oxidized at different temperatures (400, 600, 800°C) measured by nitrogen adsorption-desorption and gravimetry.

Oxidation temperature (°C)	Average pore diameter (nm) ^a	Total Porosity (%) ^b	Surface area (m ² gr ⁻¹) ^c	Porous volume (cm ³ gr ⁻¹) ^c
400	16.1	83±1	268.8±0.6	1.13±0.02
600	15.8	80±1	257.7±1.0	1.08±0.02
800	11.8	75±1	247.7±0.6	0.81±0.02

^a Values calculated from nitrogen desorption isotherms using BJH model.

^b measured by gravimetric analysis.

^c Values calculated based on the BET model.

Biofunctionalization of P*SiO*₂ with enzymes. The synthetic approach for grafting enzymes onto the three different oxidized surfaces is based on a well-described silanization methodology.³⁹⁻⁴⁰ First, APTES in combination with organic base DIEA are used to introduce amino groups to the thermally oxidized nanostructures and obtain an activated surface for grafting of homobifunctional cross-linker, DSC. The cross-linker reacts with primary amines through nucleophilic attack of the amine on C=O succinimidyl ester, eliminating one NHS group.

Finally, primary amines stemming from the surface Lys groups of the HRP enzyme are used for immobilization onto the surface via the second reactive group of the DSC,³⁹ as schematically illustrated in Figure 2. ATR-FTIR spectroscopy is used to confirm the modification of the porous scaffold. The ATR-FTIR spectra of the different surfaces oxidized at 400°C (neat P_{SiO}₂, APTES, DSC and HRP) are shown in Fig. 2. The neat P_{SiO}₂ surface spectrum (Fig. 2, trace a) shows a typical $-(O_ySiH_x)$ vibration mode at 801 cm^{-1} and a peak at 1039 cm^{-1} that is related to the Si-O-Si stretching mode. The APTES-modified surface spectrum (Fig. 2, traces b and b1) depicts two additional peaks; the 1641 cm^{-1} is ascribed to the bending of primary amine and the 1555 cm^{-1} to the bending of protonated amines. The spectra taken after following synthetic step (DSC modification) is displayed in Figure 2, traces c and c1. Amide bands appear at 1635 cm^{-1} and 1531 cm^{-1} (amide I and amide II, respectively), exhibiting a red shift compared to bands of the APTES-modified surface. Moreover, two substantial peaks appear at 1734 cm^{-1} and 1774 cm^{-1} (Fig. 2, trace c1), are ascribed to the asymmetric and symmetric stretching bands of succinimidyl ester, respectively.³⁹ Finally, for the HRP-immobilized surface (Fig. 2, traces d and d1), amide bands appear at 1645 cm^{-1} and 1558 cm^{-1} (amide I and amide II, respectively), showing a blue shift compared to the DSC-modified surface. These results verify that the HRP macromolecules are conjugated to the P_{SiO}₂ surface. ATR-FTIR spectra for the biofunctionalization of the other two surfaces (oxidized at 600 and 800°C) show identical spectra (data not shown).

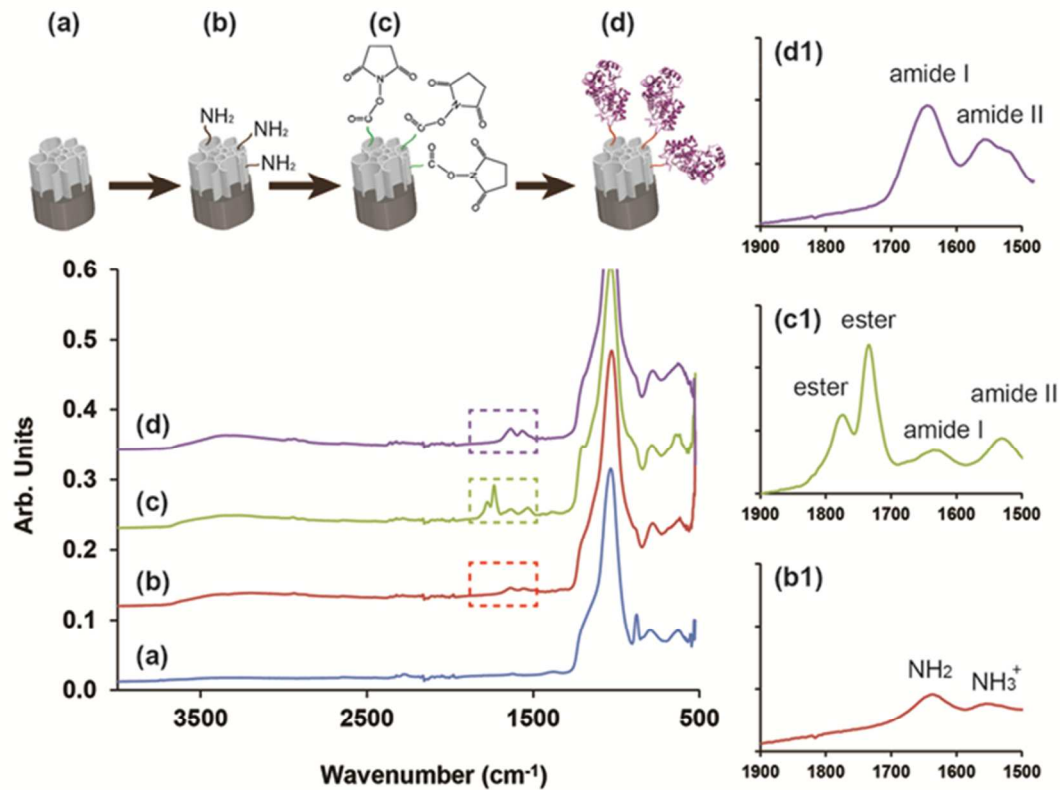


Figure 2. Top: A schematic illustration of the synthetic steps followed for enzyme immobilization onto P_{SiO}₂ surface: **(a)** neat P_{SiO}₂ surface oxidized at 400°C; **(b)** APTES-modified surface; **(c)** DSC-modified surface and **(d)** HRP-immobilized surface. **Bottom:** The corresponding ATR-FTIR spectra. **Right:** Insets b1, c1, d1 show the corresponding spectra for clarity.

Reflective interferometric Fourier transform spectroscopy. To monitor the optical changes following different biofunctionalization steps on the P_{SiO}₂ surfaces, reflective interferometric Fourier transform spectroscopy (RIFTS) is used as an additional tool. This method is characterized by high sensitivity to minor alterations in the average refractive index of the porous nanostructure and thus allows for direct and real-time monitoring of the binding of different species onto the pore walls.^{1-2, 7-9, 41} It is expected that chemical modification of the P_{SiO}₂

surfaces (as illustrated in Fig. 2) will induce a red shift in the EOT due to the increase in the value of n upon immobilization of the different biomolecules. It should be noted that the P SiO_2 nanostructure is designed to allow a proper infiltration of the desired biomolecules into the pores. Indeed, substantial relative EOT changes are perceived after each of the described immobilization steps for the three different oxidized surfaces (400, 600, and 800°C), see Figure 3. Additionally, the EOT values after each modification are observed to increase, as the oxidation temperature rises (e.g., after silanization, an increase of 1.017 ± 0.002 , 1.020 ± 0.001 , 1.0257 ± 0.003 in the relative EOT or 245 ± 28 , 285 ± 9 , 338 ± 54 nm shifts are observed for oxidation temperatures of 400, 600, 800°C, respectively). This phenomenon can be ascribed to the increase of the amount of Si-OH present on the surface at elevated temperatures (see Fig. S2 for details), which serves as an anchor for the following biofunctionalization steps.²⁹⁻³⁰

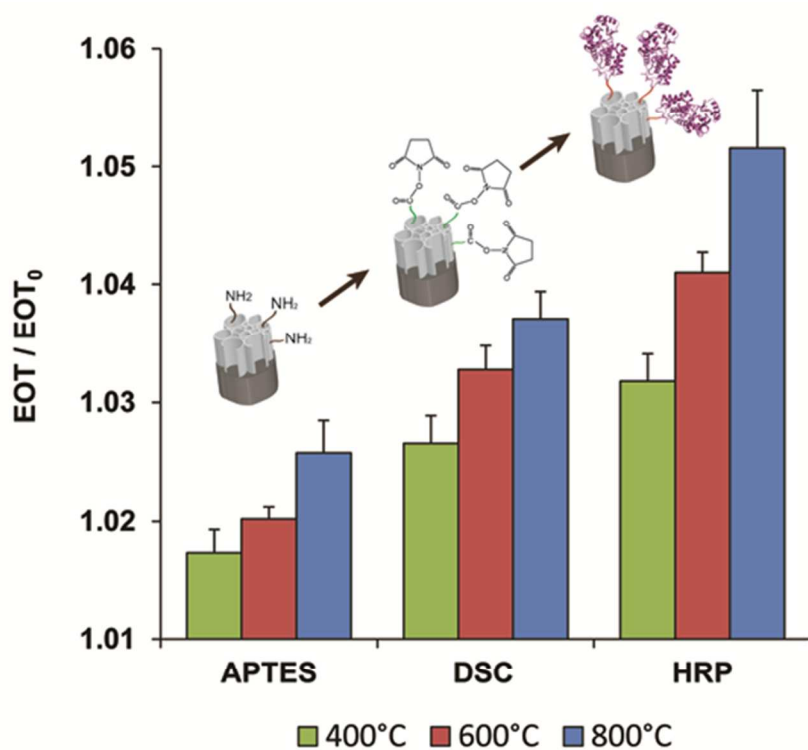


Figure 3. Relative EOT changes upon HRP biofunctionalization onto P SiO_2 scaffolds oxidized at different temperatures. The synthetic steps followed for enzyme immobilization onto the P SiO_2 surface are: (i) APTES modification, (ii) DSC modification, and (iii) HRP conjugation. Note: the EOT values obtained after each modification step are normalized with respect to the EOT value of the neat oxidized P Si scaffold (termed as EOT_0) at a specific temperature.

Quantification of the immobilized enzyme. Two different classes of enzymes are investigated in this study. First, HRP, one of the most powerful peroxidases with a molecular weight of 44 kDa and dimensions of $6.2 \times 4.3 \times 1.2 \text{ nm}^3$ (Fig. 4), is used.⁴²⁻⁴³ The second is trypsin, an extensively used protease for peptide mapping, characterized by a molecular weight of 24 kDa and typical dimensions of $3.8 \times 3.8 \times 3.8 \text{ nm}^3$ (Fig. 4).⁴⁴ To quantify the amount of enzymes anchored onto the different P SiO_2 scaffolds, the substrate oxidization capability of HRP and proteolytic activity of trypsin are studied using colorimetric tests. Enzyme-immobilized P SiO_2 surfaces are introduced with the corresponding substrates (Ampliflu Red and FTC-casein for HRP and trypsin, respectively), allowed to react, while the specific activity parameters are spectrophotometrically evaluated. By comparing the obtained results to the respective activity values of the neat enzymes in solution, we estimate the amount of enzyme immobilized onto the different oxide layers,³⁵ see Figure 4. For both HRP and trypsin, a clear trend of a higher immobilized enzyme amount is obtained as the oxidation temperature is raised, values as high as 1.2 ± 0.2 and $2.3 \pm 0.3 \text{ pmol}$ at 800°C are calculated for HRP and trypsin, respectively. These results are in agreement with the RIFTS experiments results, presented in the previous section. It should be noted that the quantification of the enzymes is performed by comparing the specific activity of the immobilized enzymes to that of the free enzymes in a solution, assuming that all immobilized enzymes are fully active.^{35, 45} This assumption underestimates the extent of enzyme

immobilization as it does not consider important parameters such as enzyme-support interactions, diffusion limits, structural changes in the enzyme induced by the local surface geometry and nano-confinement effects. All these parameters may impair the catalytic performances of immobilized enzyme.⁴⁶⁻⁴⁷ The obtained results agree well with steric crowding effects, which significantly affect the immobilization efficiency of biomolecules onto the porous scaffold.^{14, 25, 48} Thus, as the gyration radius of trypsin is smaller than that of HRP (approximately twofold smaller) than that of HRP, its physical accessibility into the porous nanostructure is expected to be higher, resulting in a greater immobilization efficiency. Indeed, significantly higher values (>2x) of immobilized trypsin are observed at all oxidation conditions, as shown in Figure 4. Moreover, these results show, that despite the decrease in porous volume upon thermal oxidation at elevated temperatures (Table 1), the pore clogging by thicker oxide layer has no negative effect on enzyme immobilization. On the contrary, the substantial oxide layer offers means for effective surface coverage and successful biofunctionalization (e.g., higher coverage of Si-OH groups at elevated temperatures).

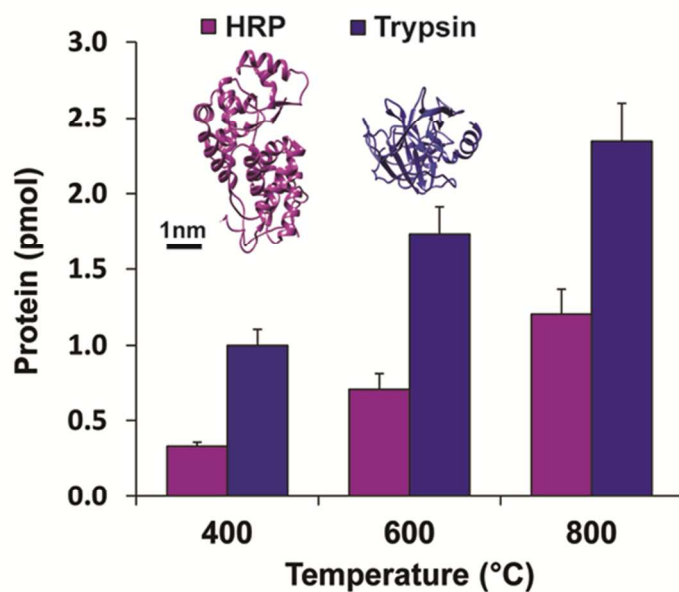


Figure 4. Quantification of immobilized enzymes (HRP and trypsin) onto the P SiO_2 at three different oxidation temperatures (400, 600, 800°C). Inset: 3D-structure of the immobilized enzymes analyzed by Chimera program.⁴⁹ Note that enzymes quantification is performed by comparing the specific activity of the immobilized enzyme to that of the unrestricted enzyme in a solution.

To further characterize the extent of the enzymes immobilization within the different P SiO_2 scaffolds, fluorescein-labeled HRP (FTC-HRP) and trypsin are immobilized as previously discussed, followed by observation using a confocal laser scanning microscope (CLSM). Figure 5 presents CLSM 3D projection images of P SiO_2 nanostructures following the immobilization of (FTC-HRP). The fluorescence intensity is observed to significantly increase with oxidation temperature (see Fig. 5, top-row images), indicative of higher enzyme immobilization efficiency. The fluorescence signal from the labeled HRP is observed throughout the entire volume of the porous scaffold, for P SiO_2 processed at higher temperatures, see Figure S3 for details. A similar trend is obtained when immobilizing FTC-trypsin onto the different P SiO_2 layers (data not shown). Figure 5 (middle row) and Figure S3 depict the photoluminescence (PL) of the P SiO_2 skeleton. The PL intensity increases as the oxide layer is grown at elevated temperatures.^{33, 50-53} These results further confirm that the thicker and more uniform oxide layer, obtained at elevated temperatures, has a profound impact on the extent of enzyme immobilization.³³ This is clearly observed in Figure 5 (bottom row), which combines the PL (originates from the P SiO_2 scaffold) and the fluorescence (from the labeled HRP) signals, where the labeled enzyme densely decorates the porous scaffold throughout.

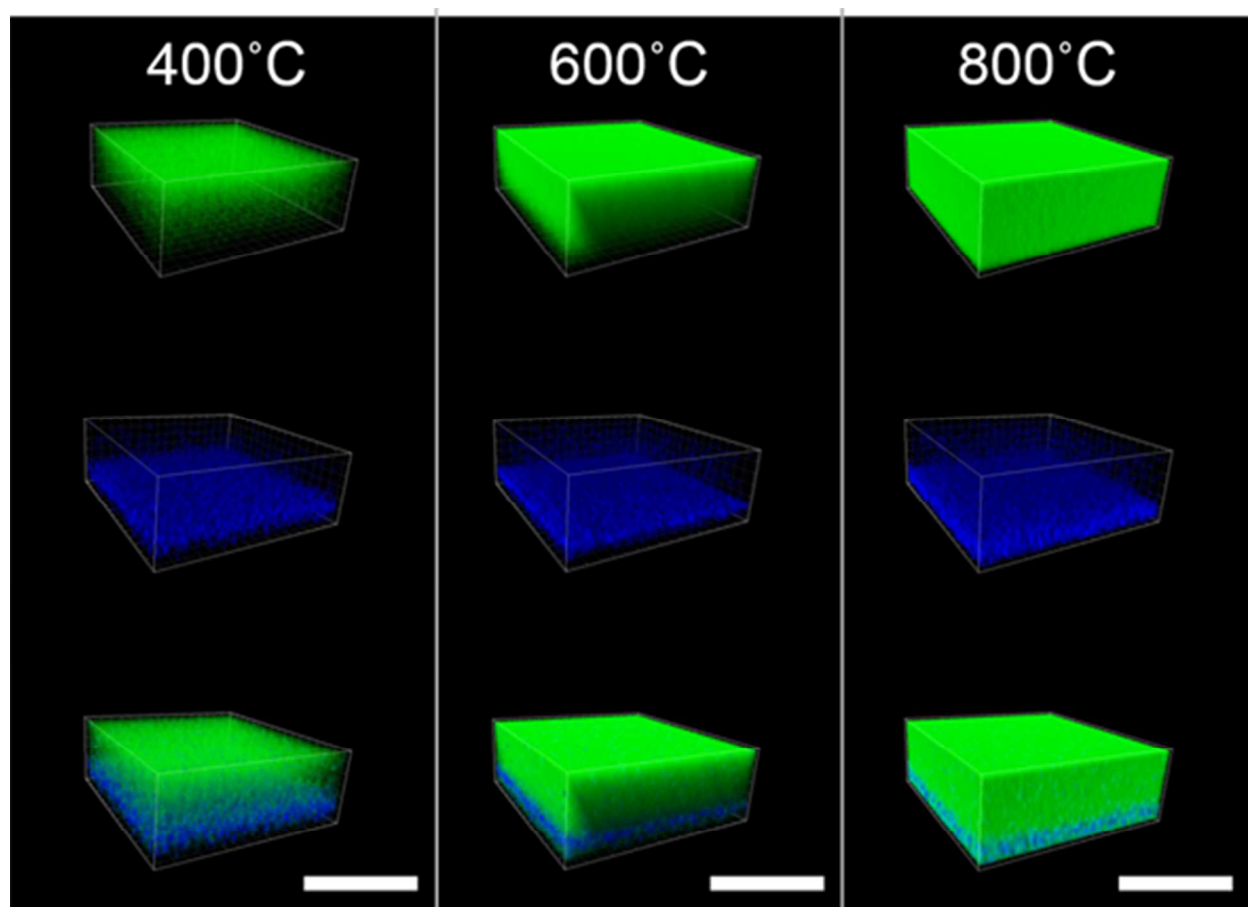


Figure 5. CLSM 3D projection images of fluorescently-labeled HRP immobilized onto PSiO₂ nanostructures oxidized at three temperatures (400, 600, 800°C). **Top:** FTC-HRP immobilized surfaces. **Middle:** photoluminescence (PL) of the different oxidized porous scaffolds. **Bottom:** combined view of top and middle row. The scale bar is 10 μ m.

Optical signal stability in aqueous media. Before we investigate the potential of the enzyme-immobilized PSiO₂ platforms to act as optical biosensor for label-free monitoring of enzyme activity, their optical readout stability in biologically relevant media should be assessed.^{4, 10, 25, 35,}

³⁹ Thus, two transducers (neat PSiO₂ and enzyme-immobilized PSiO₂) are fixed in a flow cell and HEPES buffer is circulated, while their optical properties are monitored in real time. Figure 6 depicts the changes in the relative EOT as a function of time for neat PSiO₂ and the

corresponding HRP-immobilized P SiO_2 layers. During 4 hr buffer flow experiments, negligible baseline drift in EOT values ($4.0 \times 10^{-7} \text{ min}^{-1}$, see Fig. S4) are observed for HRP-immobilized P SiO_2 , which is oxidized at 800°C . On the contrary, HRP-immobilized P SiO_2 oxidized at lower temperatures of 400°C and 600°C , show significant and rapid decrease in their relative EOT values (9.0×10^{-6} and $2.9 \times 10^{-6} \text{ min}^{-1}$, respectively, see Fig. S4). These profound changes in the EOT signals are attributed to time-dependent degradation and dissolution of the porous scaffold.^{4, 25, 27} A similar trend is observed for neat P SiO_2 (no enzyme), APTES-modified P SiO_2 , DSC-modified P SiO_2 and the trypsin-immobilized P SiO_2 films, shown in Figure 6b, S5, S6 and S7, respectively. Thus, these results clearly demonstrate that the optical readout of the neat P SiO_2 , APTES-modified P SiO_2 , DSC-modified P SiO_2 and the enzyme-immobilized P SiO_2 are highly stable at elevated temperatures (800°C) under biologically relevant aqueous conditions. It should be noted the surface modification and the enzyme immobilization process facilitate the optical signal stability at lower oxidation temperatures due to surface passivation (Fig. S4).

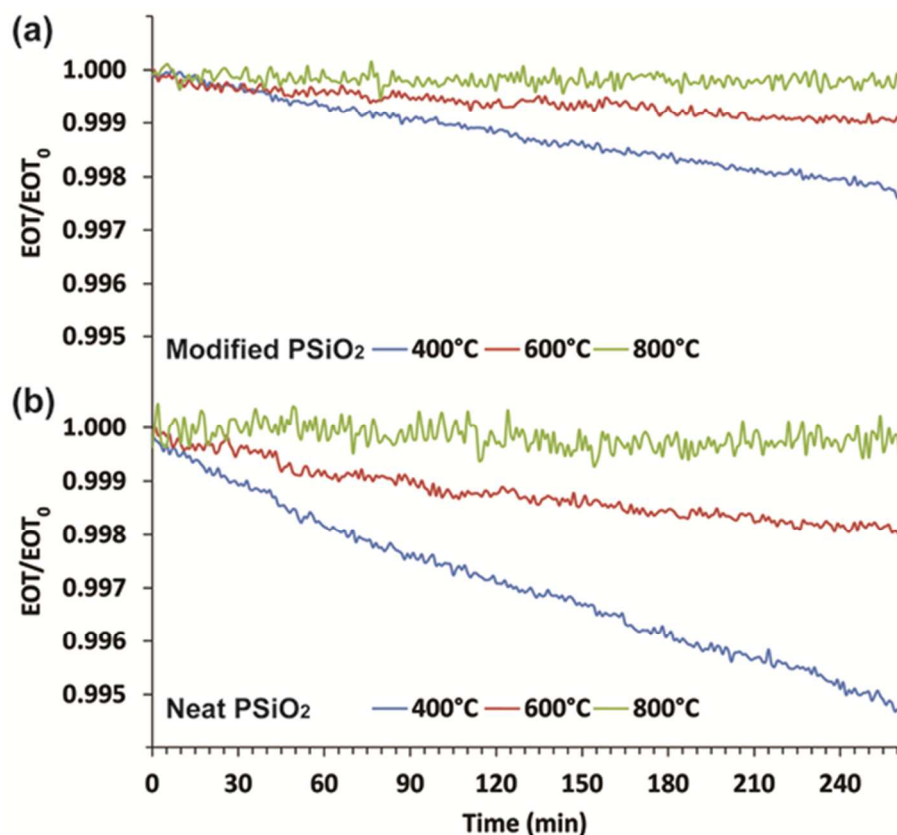


Figure 6. Optical response, expressed in terms of the relative EOT, of enzyme-immobilized and neat PSiO₂ films vs. exposure time to aqueous solution flow conditions. The nanostructures are continuously washed with HEPES buffer at 0.5 mL min⁻¹. **(a)** HRP-immobilized PSiO₂ oxidized at three different temperatures (400, 600, 800°C). **(b)** Neat PSiO₂ (no enzyme) oxidized under similar conditions.

Optical monitoring of enzymatic activity. Previous sections have confirmed that oxidation temperature of the porous nanostructure has a profound effect on enzyme immobilization efficiency and the optical signal stability. Next, we investigate the potential of HRP-immobilized PSiO₂ scaffolds to serve as optical biosensor for monitoring sensitive biochemical reaction through RFTS experiments and the influence of oxide layer on the biosensors performance. Figure 7 shows the relative EOT change of HRP-immobilized PSiO₂ after introduction of the

substrate, 4-chloro-1-naphthol (4CN), which is oxidized by HRP into an insoluble product, 4-chloro-1-naphthol (4CNn).^{36, 54} A rapid increase in the relative EOT values is observed after addition of 0.16 M H₂O₂ to the cycled solution for all oxidized surfaces (Fig. 7II). This increase is ascribed to the precipitation and accumulation of the enzymatic reaction product within the pores⁵⁵, as schematically illustrated in Fig. 7. As the oxidation temperatures are increased from 400 to 800°C, more significant EOT changes rapidly introduced to the system are observed. This can be attributed to the to higher enzyme content within the different PSiO₂ scaffolds, which results in the higher substrate oxidation efficiency and rapid pore blockage by insoluble product.

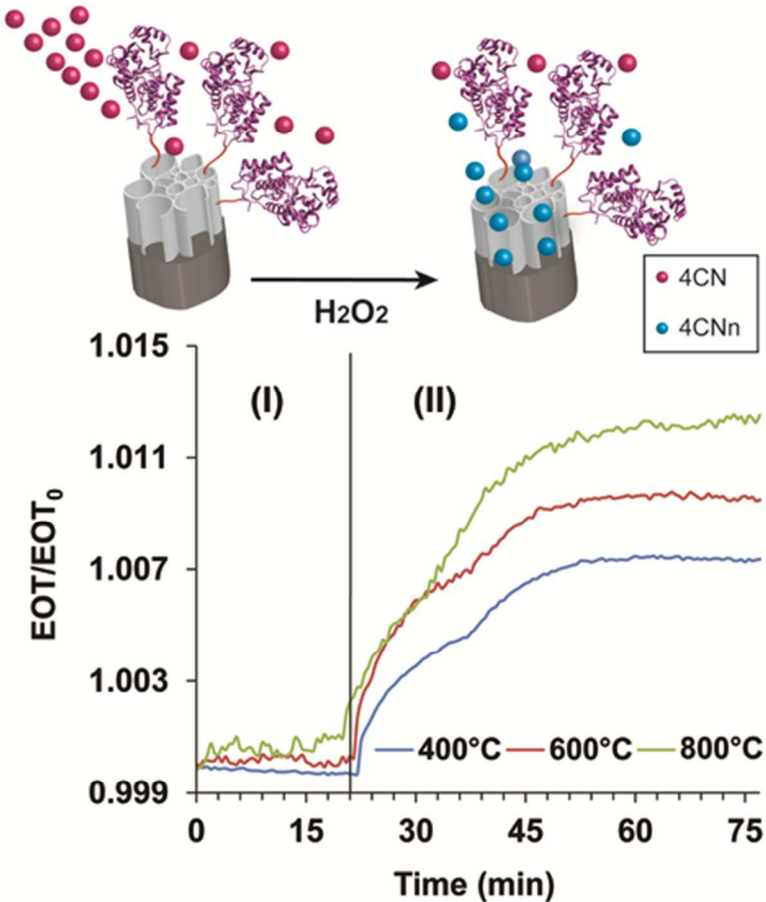


Figure 7. A schematic illustration of the optical response of HRP-immobilized PSiO₂ to enzymatic reaction products infiltrating the nanostructure (red dots – 4CN, blue dots – 4CNn

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3 molecules). The HRP-immobilized P SiO_2 (oxidized at 400, 600, 800°C) are pretreated with
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5 HEPES buffer to reduce nonspecific adsorption of enzymes. **(I)** Wash with 0.8 mM 4CN in
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7 HEPES buffer (pH 8). **(II)** Introduction of 0.16 M H_2O_2 to the cycled solution.
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10 11 CONCLUSIONS 12 13

14
15 We demonstrate the effect of oxidation temperature on the chemical stability of P SiO_2 optical
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17 transducers. Substantial oxide layer formed at different temperatures stabilizes the highly porous
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19 scaffold and thus preserving the characteristic optical properties in biologically-relevant media.
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21 Importantly, by monitoring the catalytic activity of HRP-immobilized P SiO_2 we show that the
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23 thermal oxidation profoundly affects the biosensing performance in terms of greater optical
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25 sensitivity. Thus, despite the significant decrease in specific surface area upon thermal oxidation
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27 at elevated temperatures, higher enzyme content and surface coverage are achieved, leading to
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29 enhancement of biosensor's sensitivity. Therefore, surface passivation by thermal oxidation
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31 enhances the P Si versatility and applicability as a fundamental platform for the development of
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33 optical sensing systems.
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39 ASSOCIATED CONTENT 40 41

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43 **Supporting Information.** TGA, ATR-FTIR, image analysis and optical stability experiments.
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45 This information is available free of charge via the Internet at <http://pubs.acs.org/>.
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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

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