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COMMUNICATION

Switching of fluorescence mediated by a peroxynitrite–glutathione redox reaction in a porous silicon nanoreactor†

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A nanostructured porous silicon chip functionalized with dichlorofluorescein is employed as a nanoreactor to respond to Reactive Oxygen Species (ROS) and to real-time studying redox reactions.

Reactive Oxygen Species (ROS) cause oxidative stress, and are co-factors in cell damage during inflammation, ischemia, cancer and aging. Regardless of how and where they are generated, a rise in intracellular oxidant levels has potentially two important effects: damaging of biological molecules and activation of specific signaling pathways.[1,2] Peroxynitrite is an endogenous oxidizing and nitrating agent, formed by the very fast reaction of nitrogen monoxide with superoxide radicals.[3–5] During the last few years peroxynitrite has been widely studied as a potential pathogenic mediator in human diseases and as a cellular toxin in host defense mechanisms against invading microorganisms.[6] One of the most employed methods is based on the measurement of the oxidative behaviour of nonfluorescent forms (chemically reduced) of highly fluorescent dyes such as fluorescein and rhodamine.[7,8] The nonfluorescent reduced form of such dyes is oxidized to the luminescent one by the reaction with oxidizing species.[8] Systems to monitor the presence of ROS are indeed desirable, and in this context we propose the employment of a hybrid material based on porous silicon (pSi) modified with dichlorofluorescein (DCF), where the fluorescence is activated by the presence of ROS.

Porous silicon (pSi) is widely used as a host matrix for drug delivery[9] and biosensing applications[10,11] because of its large surface area,[12] as a photonic crystal,[13] and the relatively easy ways to modify the chemical properties of its surface introducing specific functional groups.[14–16] Surface modification has been employed to impart selectivity towards specific molecules for application such as the detection of immunoglobulin in whole blood[17] or to increase sensing performances.[18,19] In a previous study[18] chitosan has been successfully employed to improve the sensitivity towards negatively charged molecules, taking advantage of the large number of protonated amino groups present on its surface. The advantages of both a porous matrix (in terms of large surface area compared to a flat substrate) and of chitosan (in terms of binding sites, antibacterial properties, biocompatibility and cost) stand out from our previous study; the deep characterizations carried out on the substrate showed that the range of the pore size (~20–30 nm) did not cause relevant diffusion issues through the hybrid matrix. Taking advantage of these results, here we immobilized DCF on a pSi surface covered by chitosan oligomers that provide a large number of binding sites for DCF conjugation through amide bonds. The behavior of such a hybrid system to monitor redox reactions is then followed using peroxynitrite and glutathione as oxidizing and reducing agents, respectively.

The pSi layer was fabricated by electrochemical etching of a highly boron-doped monocrystalline (100) silicon substrate, at room temperature, performing a two-step chemical treatment of the silicon wafer before the electrochemical etching to prevent the formation of a parasitic surface layer.[18] From our previous characterisation data and on the basis of well known literature data, the produced pSi film is estimated to have pore size diameters ranging between 20 and 30 nm. Surface modification of nanostructured pSi (Scheme 1) is achieved by thermal hydrosilylation with undecylenic acid, followed by a common cross-linking reaction to covalently bind chitosan oligomers (M_n = 5000 g mol^{-1}). Chitosan has been chosen because of the large amount of amine groups which provides very good sites for covalently binding[18] carboxylated molecules, such as DCF.

Reduced 2′,7′-dichlorofluorescein (DCFH) was synthesized following the procedure reported by Low *et al.*,[20] starting from 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma Aldrich). The solution was kept in the dark to slow down its spontaneous oxidation to the fluorescent compound 2′,7′-dichlorofluorescein (DCF).

Crosslinking of the carboxylic acid function present on DCF with amine groups of chitosan was performed using EDC and NHS in a two-step reaction (please see ESI† for more details). First, a solution (2 ml) of DCFH made previously, which contained also DCF because of its spontaneous oxidation to DCF,[21] was reacted with 3 mg of EDC and 3 mg of NHS for 15 minutes; then, the solution was added to the chitosan-terminated pSi sample and reacted overnight. However, in order to bind the dye to chitosan, the two forms (DCF *vs.* DCFH) are chemically equivalent (at least in the proximity of the reacting group), because they both have the free carboxylic group that reacts with amino

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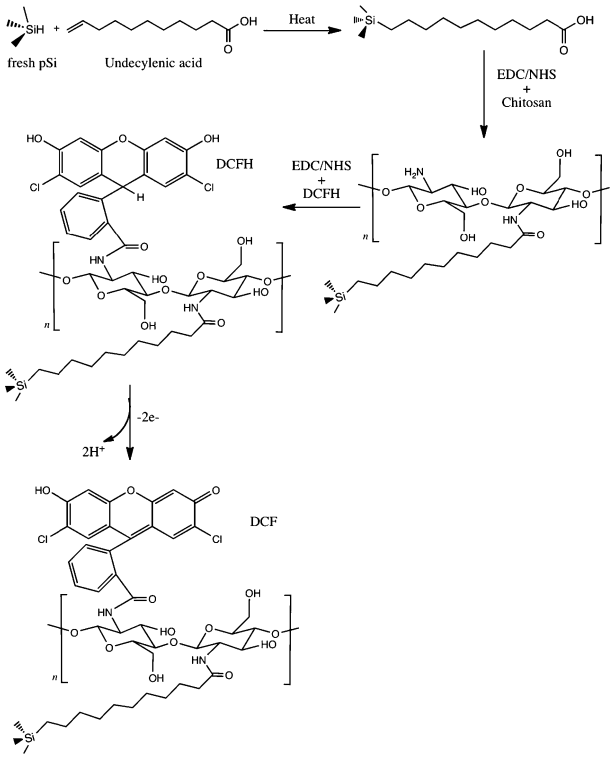


Scheme 1 Reaction pathway.

groups in the presence of EDC/NHS. After reaction, the pSi sample was copiously rinsed with deionized water and incubated overnight in water. Then it was again rinsed with deionized water and ethanol and dried under a stream of nitrogen. After this step any DCFH bound to the surface is oxidized to DCF. We expect DCF to retain its fluorescence properties after being immobilized on the pSi surface, as the chitosan acts as a spacer, avoiding any energy transfer between the dye and the semiconducting pSi surface.[22] The absence of any photoluminescence from the pSi matrix in the fluorescence spectra (see ESI†) bears this out.

Peroxynitrite was synthesized following the method reported by Robinson *et al.*[23] The concentration of the obtained peroxynitrite was 20 mM, measured by absorbance measurement with UV-visible spectroscopy, using an extinction molar coefficient of 1670 M^{-1} cm^{-1}.[23] The obtained solution was diluted 1 : 100 in PBS right before its introduction in the flow cell, as peroxynitrite degrades quickly at that pH.[4]

The characterizations of the functionalized pSi samples were performed by FTIR spectroscopy in attenuated total reflectance (ATR) mode at every functionalization step (Fig. 1). Curve A shows the infrared spectrum of the pSi chip functionalization with undecylenic acid. The functionalization is confirmed by the vibration bands at 1710 cm^{-1} and at 1463, 2855 and 2925 cm^{-1} that are assigned to the $\nu_{C=O}$ stretching vibration mode of the carboxylic acid and deformation and stretching (symmetric and asymmetric) vibration modes of the aliphatic C–H_2 groups respectively.[18] However, the surface is not entirely modified, because part of silicon hydride remains unreacted, as demonstrated by the presence of bands at 905 and 2100 cm^{-1}, assigned to vibrational modes of Si–H_x groups.[18]

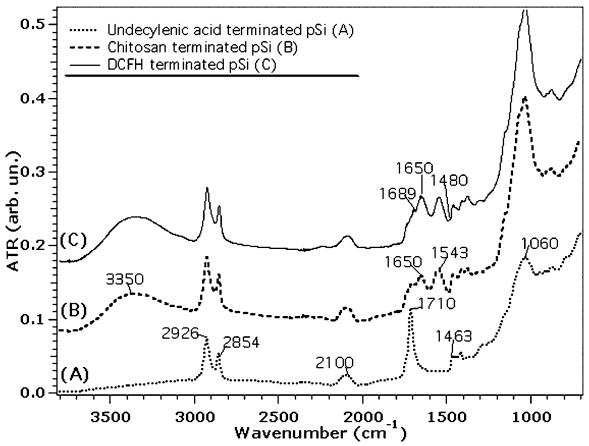
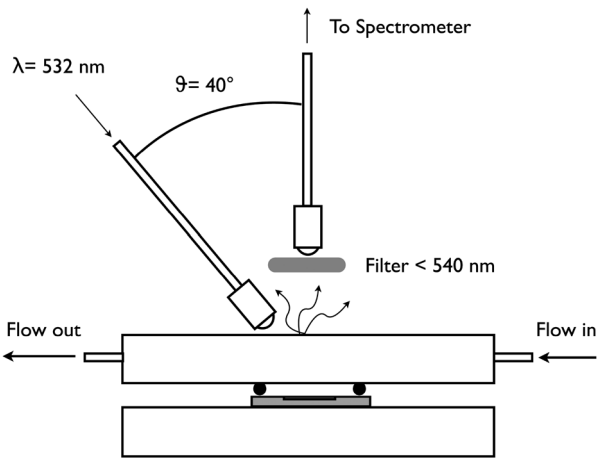


Fig. 1 ATR spectra of porous silicon samples obtained at key points in the transformation of freshly etched pSi to a surface suitable for the detection of ROS species.

Curve B (Fig. 1) shows the IR spectrum after the crosslinking reaction with the amine groups of the chitosan oligomers in the presence of EDC and NHS.[18] The amide I, II bands at 1650 and 1543 cm^{-1} confirm the presence of chitosan, covalently bound to the carboxylated surface. The broad band around 3350 cm^{-1} is associated with the stretching modes of hydroxyl and amino groups both present on chitosan and probably engaged in hydrogen bonding interactions. The large amount of free amine functions on the immobilized chitosan plays a key role in further conjugation with carboxylic groups present on DCF (fluorescent in its oxidized form). Curve C in Fig. 1 reports the IR spectrum after covalent bonding of DCF with chitosan. The increase of the band at 1650 cm^{-1} (amide) and the appearance of new bands at 1689 cm^{-1} (conjugated $\nu_{C=O}$ stretching) and 1480 cm^{-1} ($\nu_{C=C}$ aromatic stretching) confirm that DCF was successfully immobilized on the functionalized pSi chip.

The performance of the DFC functionalized nanostructured pSi in the presence of ROS species was monitored in a flow cell set-up (shown in Scheme 2) allowing for real-time measurement of fluorescence. In such a configuration, the optical fibers were mounted on coaxial goniometrical stages, allowing for angle resolved measurements (not reported). The reactivity of the DCF



Scheme 2 Setup employed to measure the fluorescence.

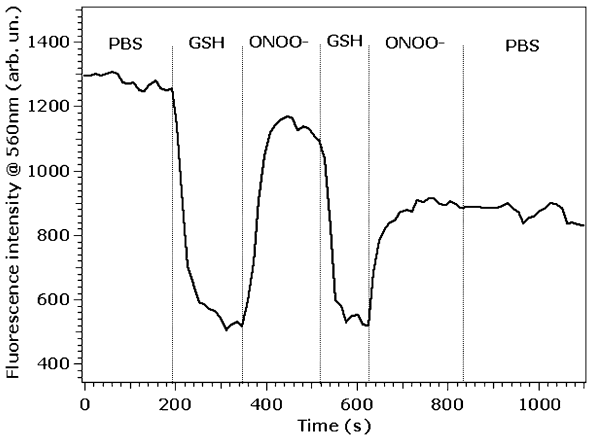


Fig. 2 Intensity of the fluorescence emission at 560 nm with respect to the background over Redox species dosing.

functionalized pSi nanoreactor towards reducing and oxidizing agents was evaluated by exposing the sample, respectively, to freshly prepared solutions of glutathione (12 mg ml^{-1}, Sigma Aldrich) and peroxynitrite (200 μM, freshly prepared; see ESI†) in PBS, under laser irradiation (λ = 532 nm), and measuring the fluorescence emitted by the pSi chip in real-time. A long pass filter was faced to the collecting fiber to reject the laser light scattered by the nanoporous structure.

Fig. 2 shows the real-time measurements of the emission intensity at 560 nm upon the exposure of the pSi nanoreactor to the solutions. PBS at pH 7.4 is first flowed to have a reference for the fluorescence intensity, which is mainly caused by the presence of DCF, as the photoluminescence of nanostructured pSi synthesized under these experimental conditions occurs at longer wavelength.[24] A strong decrease of fluorescence is observed when the chip is exposed to the GSH solution, indicating the reduction of DFC to DFCH,[7] which is very weakly fluorescent.[8] A weak emission is still observed, likely due to the poor fluorescence of DCFH or some unreacted DCF molecules.

When the chip is exposed to the peroxynitrite solution, an increase in fluorescence rapidly occurs, indicating the oxidation of DCFH to the fluorescent DCF. Both the reduction and oxidation processes are very quick, of the order of some tens of seconds, as evidenced by Fig. 2, in accord with data available in the literature for the range of pH used here.[4] A further exposure of the functionalized pSi chip to the GSH solution leads again to a decrease in fluorescence intensity, to the minimum value, confirming the reversibility of the process. However, the fluorescence measure after a further exposure to the peroxynitrite solution increases but does not reach the maximum value. The possible degradation of the peroxynitrite solution before it diffuses within the pores could explain such a phenomenon. In fact, the peroxynitrite solution employed is freshly made, and it is not diluted in the buffer solution until the last moment before flowing into the flow cell, because of its short half-life at pH 7.4. Therefore, the time before the solution is actually in contact with the surface could be slightly different at every cycle and so will be the action of the peroxynitrite.

In previous results Estevez *et al.*[25] have shown that a 1 mM bolus addition of peroxynitrite is equivalent to generating a submicromolar concentration over 1 h, because of the quick decomposition of peroxynitrite at pH 7.4. Considering this result, we can assume that the actual concentration of the peroxynitrite inside the porous matrix is comparable to physiologically relevant concentrations, which is in the micromolar range and known to be toxic for cells.[25] The described behavior to respond quickly and reversibly to redox reactions is a proof of concept that makes this functionalized pSi nanoreactor appealing for the detection of peroxynitrite, a ROS that takes part in the development of oxidative damage in various pathologies.[6] Moreover, the versatility demonstrated to optically detect the presence of reducing as well as oxidizing agents indicates possible applications as a nanoreactor for studying and monitoring luminescent redox reactions occurring in biological systems. In fact, pSi could be employed as a substrate for cell reactions (occurring on the top of its surface) and monitoring by-products such as ROS, diffusing underneath within the dye functionalized nanoporous matrix.

This work further confirms also how the surface functionalization can play an extremely important role in sensing applications, and the benefits of using chitosan as an intermediate molecule for conjugation, as the large number of amino groups can easily bind a large number of carboxylic groups.

The combination of the surface functionalization described here, with the design of more sophisticated optical structures such as microcavities that have proved efficient on their own to improve sensitivity of pSi based sensors, will be part of future work.

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