Hydrolysis of acetylcholinesterase inhibitors – organophosphorus acid anhydrolase enzyme immobilization on photoluminescent porous silicon platforms†

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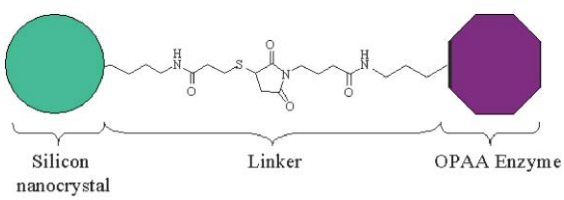
We report on the immobilization of an OPAA enzyme on luminescent porous silicon devices, and on the utilization of this new platform to hydrolyze *p*-nitrophenyl-soman.

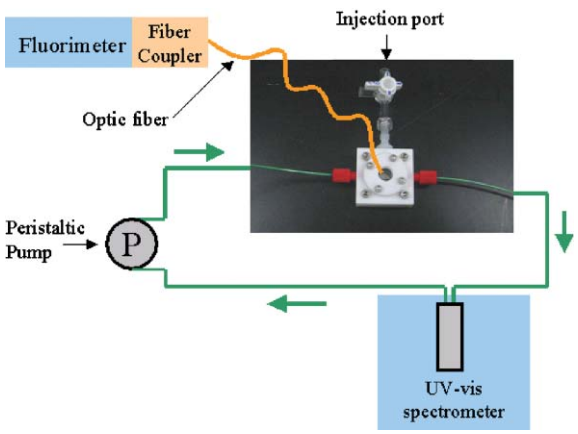
We recently developed a technique to covalently anchor molecules onto porous silicon (PSi) platforms through a direct Si–C bond on the silicon surface.[1,2] This new method is now part of a series of techniques used to immobilize molecules on silicon surfaces.[3–9] We demonstrated its flexibility by anchoring various types of molecules including dansyl cadaverine, which retained its fluorescent properties, biotin, which remained accessible for streptavidin binding,[1] and glucuronidase, which retained its enzymatic activity when anchored on the silicon surface.[2] A mechanism of charge transfer between the silicon quantum dots and the product of the enzymatic breakdown was postulated as responsible for the reversible decrease in luminescence observed during glucuronidase action. To further demonstrate the relevance of this technique, we present in this communication, the attachment of the Organophosphorus Acid Anhydrolase (OPAA) enzyme, EC 3.1.8.2 from *Alteromonas* sp. JD6.5, which has been demonstrated to have high catalytic activity towards the hydrolysis of acetylcholinesterase inhibitors.[10–13] We report on the activity of the bound enzyme tested with the nerve agent surrogate *p*-nitrophenyl-soman as well as on the transduction of this activity by the PSi platform.

Luminescent PSi samples were prepared by electrochemical etching of n-type silicon in hydrofluoric acid. The porous devices were then rinsed in de-ionized water and the hydride-terminated silicon surface was functionalized with a chemical linker (shown in Scheme 1) by hydrosilylation followed by protein cross-linking techniques (details of the functionalization procedure can be found in the ESI as well as in previous publications).[1,2] OPAA enzyme was then covalently immobilized on the functionalized PSi platform by incubating the samples at room temperature for 4 h in 1 mL of enzyme solution (1 mg/mL in bis-trispropane buffer at pH = 7.5). The attachment point of the OPAA has not been determined but is thought to be through lysine residues. The samples were washed extensively in order to eliminate non-covalently bound enzyme (twice in 1-mL aliquots of buffer, once in a 1-mL aliquot of 1 M aqueous solution of sodium chloride, and again twice in 1-mL aliquots of buffer). The protein content of the incubation and wash solutions was analyzed by micro bicinchoninic acid (BCA) assay (Pierce Scientific), which has a detection limit of 0.5 μg/mL. The results showed that more than 99% of the enzyme present in the incubation solution was immobilized on the device, which represents a total mass of 0.99 mg for a geometric surface of 0.8 cm^2. No detectable amount of enzyme was found in any of the wash solutions, indicating the absence of non-covalent binding. It should be stressed that the optical properties of the silicon platform were retained after functionalization. Also, the red luminescence remained bright enough to be observed with the naked eye.[1,2]

In order to assay the activity of the immobilized OPAA enzyme, the functionalized silicon samples were tested in a flow cell set-up (shown in Scheme 2) allowing for real-time measurement of enzyme activity with concurrent examination of PSi photoluminescence (PL). The functionalized PSi was mounted into the cell that was connected to a peristaltic pump, and exposed to the substrate *p*-nitrophenyl-soman (synthesized at Edgewood Chemical and Biological Center, Aberdeen, MD). The total volume contained in



Scheme 1



Scheme 2

† Electronic supplementary information (ESI) available: porous silicon synthesis, device functionalization, enzyme preparation and immobilization, enzymatic activity assays and luminescence measurements. See http://www.rsc.org/suppdata/cc/b4/b412215a/

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the system was 2.5 mL and the liquid was re-circulated through a UV-vis spectrophotometer (see ESI for details). It should be pointed out that, to our knowledge, there are no published data for the human toxicity of the substrate *p*-nitrophenyl-soman. It therefore has to be assumed to be comparable to the available data for soman (the LD_{50} values for a 70 kg person are 350 mg for skin exposure and 30 mg for ingestion).[14] Safety measures include preparing all the substrate dilutions in a fume hood while wearing proper personal protective equipment and using a perfectly sealed flow cell (see ESI for details).

Fig. 1 shows the activity assay for an OPAA-functionalized PSi device in the flow cell upon injection of various concentrations of the substrate *p*-nitrophenyl-soman ranging from 400 to 25 μM. An increase of the absorption of the solution at 405 nm was observed, which is the absorption maximum for *p*-nitrophenol, the breakdown product of *p*-nitrophenyl-soman by the OPAA enzyme. There is a linear correlation between the amount of *p*-nitrophenol produced and the initial substrate concentration (see ESI for details). The platform was shown to be remarkably stable. Activity of the samples upon storage at 5 °C in a 1 : 1 (v : v) solution of bis-trispropane buffer and glycerol was measured to be constant for at least six months.

The transduction of the enzymatic breakdown of the substrate *p*-nitrophenyl-soman by our hybrid platform was also investigated in the flow cell. Fig. 2 shows two sets of experiments for substrate concentrations of 50 and 25 μM. The black lines depict the PL signal of a functionalized sample in bis-trispropane buffer solution before and after the injection of substrate and the red and green lines show the PL signal recorded 5 minutes after the injection of the substrate. A significant decrease in the PL associated with the injection of substrate was observed, as seen in our previous work with β-glucuronidase enzyme.[2] There is an inverse linear correlation between the amount of decrease in PL and the initial substrate concentration (see ESI for details). The transduction is fast (less than 2 min), sensitive (10% decrease for 25 μM) and reversible. Moreover, the samples are stable and can therefore be reused.

We have demonstrated that the OPAA enzyme, which has specific catalytic activity toward the hydrolysis of acetylcholinesterase inhibitors, including G-type nerve agents, can be immobilized on a luminescent PSi platform and that both the luminescence properties of the silicon device and the enzymatic activity are

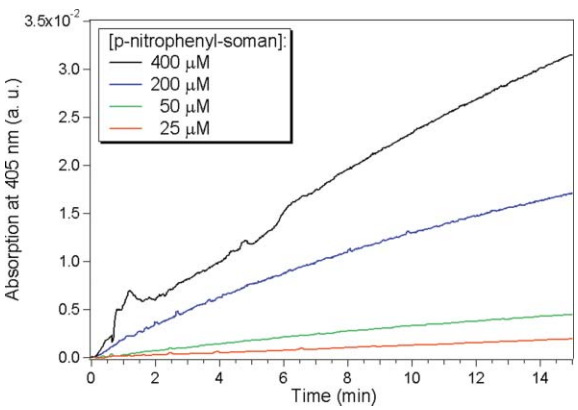

Fig. 1 Detection of *p*-nitrophenol production as a function of time by OPAA-functionalized PSi device recorded in the flow cell in real time for various concentrations of substrate *p*-nitrophenyl-soman.

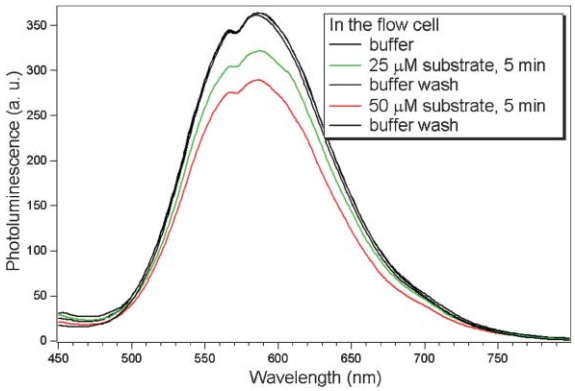

Fig. 2 Emission spectra (λ_{ex} = 290 nm) of OPAA-functionalized PSi before exposure to *p*-nitrophenyl-soman, during exposure to 25 μM and 50 μM substrate concentrations, and following PBS buffer washes.

retained. This platform retains activity for at least six months and is able to transduce its exposure to the substrate *p*-nitrophenyl-soman as a decrease in its PL output. Agent concentrations as low as 25 μM, which corresponds to a mass concentration of 7.5 mg/L, were easily detected in less than 2 minutes of exposure and the transduction mechanism was shown to be reversible.

Further work will be required to ultimately develop our hybrid system into a platform for nerve agent detection. The quenching of luminescence recorded in the present study is due to the interaction of the aromatic product of the enzymatic decomposition of *p*-nitrophenyl-soman with the platform. In real field cases, the breakdown of the G-type nerve agents by the OPAA enzyme will yield HF. Although the effect of high concentrations of HF on the luminescence of PSi has been studied for a few different surface terminations,[15,16] the effect of low concentrations of HF on enzyme-functionalized platforms remains to be studied.

Since silicon processing is already well established, the large-scale production of OPAA constitutes the key step toward the development of this new generation of enzyme-based sensors. With the availability of high expression clones of *Alteromonas* sp. JD6.5 OPAA, the enzyme is now in the process of transitioning to an industrial scale production.[12]

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