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Biomimetic silication of surfaces and its application to preventing leaching of electrostatically immobilized enzymes

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ABSTRACT

Biomimetic condensation of silicic acid onto a polyethyleneimine (PEI) coated surface was investigated using quartz crystal microbalance measurements (QCM). It was shown that precipitation of silicic acid proceeds in two sequential stages. Very fast PEI-assisted silica formation is followed by slow non-specific gel deposition. Electrostatic adsorption of enzyme and precipitation of silicic acid on the surface of PEI-modified screen-printed carbon electrodes (SPCE) were found to be competing and therefore silica formation does not increase enzyme load. However, incubation of the PEI-modified surface in silicic after immobilization of enzyme can be used to solidify and reinforce the PEI backbone via non-covalent concreting by silica. This simple and quick procedure ultimately stabilizes electrostatically immobilized enzymes against washing-off in low pH and/or ionic strength environments.

1. Introduction

Inspired by natural silica formation in diatoms, biomimetic synthesis of silica particles *in vitro* was carried out using several polyamines and proteins, e.g. frustulines [1], pleuralines [2], and silaffins [3–7]. The latter proteins, isolated from *Cylindrotheca fusiformis*, have been investigated most intensively. Silaffin-1A is a short polypeptide having phosphorylated serine units and methylated or polyamine-grafted lysine units. Phosphorylation of serine is not essential for the formation of silica if multivalent anions are present in the solution. Also, not much difference was found between polyamines bound to the polypeptide backbone of silaffin-1 and freely diffusing polyamines [8]. Consequently, synthetic polyamines have been demonstrated to cause condensation of silicic acid into silica particles. For example, poly-(L-lysine) [9–11], poly-(L-arginine) [12–14], amine-terminated dendrimers [15], poly(ethyleneimine) [16–18], poly(propyleneimine) [17] and poly(allylamine) [19,20] have all been used to obtain silica particles of different sizes ranging from several nm to μm. Several papers have reported on the formation of silica on surfaces modified by various polyamines, e.g. poly(L-lysine) [21], poly(2-(dimethylamino)ethyl methacrylate) [22] and poly(ethyleneimine) [23], after their incubation in silicic acid solutions.

Despite entrapment of enzymes inside growing silica particles being shown to be a robust and effective immobilization technique [24,25] only a few studies have been carried out to evaluate the applicability of biomimetic surface silication for immobilization of enzymes for biosensor applications [26]. In the latter example, surface specific condensation of silicic acid from a glucose oxidase-containing solution was achieved using a carbon electrode with non-specifically adsorbed lysozyme. Successful immobilization of the enzyme was demonstrated by various methods, including the amperometric response of the resulting biosensor to sequential injections of glucose. We were interested to explore if a similar approach could be extended to inexpensive and readily available polyamines, such as polyethyleneimines (PEI), attached to the surface of screen-printed carbon electrodes (SCPE) and whether this brings advantages over other techniques of enzyme immobilization.

2. Materials and methods

2.1. Materials

Cobalt phthalocyanine (CoPc), polyethyleneimines (PEI) Mw 25,000 and 750,000, diethylene glycol butyl ether, polygalacturonic acid (PGA) Mw 25,000–50,000, 3-mercapto-1-propanesulfonic acid sodium salt, acetylthiocholine iodine, 30% H_2O_2, tetramethyl orthosilicate (TMOS) and acetylcholinesterase (AChE) from *Electrophorus electricus* Type VI-S were purchased from Sigma–Aldrich. Glucose oxidase from *Aspergillus niger* (GOx) was bought from

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Biozyme in the form of lyophilized powder. D-Glucose, D-sucrose, KH_2PO_4, KOH and KCl were supplied by BDH.

Conducting carbon inks type Electrodag PF-407A and dielectric inks type Electrodag PF-455 were obtained from Acheson (Plymouth, UK), whilst ceramic tiles (96% Al_2O_3 Coorstek Grade ADS96R) were sourced from Laser Cutting Ceramics Ltd. (Sheffield, UK). Water was deionised using a Milli-Q reagent water system (Millipore, USA). 25 mM or 50 mM KH_2PO_4 buffer solution (PBS) was used in most experiments to maintain the pH value.

2.2. Fabrication of SPCE

SPCE were produced as described previously [27]. Briefly, two carbon layers (Electrodag PF-407A) were sequentially printed onto the ceramic support and cured for 1 h at 240 °C in air. Finally, ceramic tiles were covered with an insulating layer of Electrodag PF-455 to restrict a round working area of 7 mm^2 (geometric area) per electrode. The insulating layer was dried in a vacuum oven for 1 h at 200 °C at less than 1020 mbar pressure. After fabrication, the electrodes were stored at room temperature in a closed box. A DEK-248 screen printer (DEK Northern Europe, Weymouth, UK) was used for all printing procedures.

2.3. Modification of SPCE

To provide SPCE with sensitivity to thiols and hydrogen peroxide, the carbon surface was modified with a CoPc redox mediator. Derivatisation of the CoPc-doped surface with PEI was made to support electrostatic enzyme adsorption as well as biomimetic formation of silica. The whole modification route was performed in a single step procedure where CoPc and PEI were simultaneously adsorbed onto the surface of the SPCE using 1 h incubation at 50 °C in the saturated CoPc solution in diethylene glycol butyl ether containing 10 mg mL^{-1} PEI Mw 750,000 and 10 mg mL^{-1} water. Adsorption of components was accomplished by washing at room temperature in diethylene glycol butyl ether for 3 min followed by another 3 min washing in ethanol. Electrodes were then dried for 15 min under vacuum (<1 mbar) and stored in a closed dark box before use.

To prepare a saturated CoPc solution in diethylene glycol butyl ether, a suspension of CoPc powder in the 50 °C preheated solvent (containing 10 mg mL^{-1} PEI Mw 750,000 and 10 mg mL^{-1} water) was ultrasonicated for 15 min. After this, the mixture was thoroughly shaken and quickly distributed into 1.5 mL microcentrifuge tubes. The tubes were centrifuged for 15 min at 14,000 rpm (Eppendorf Centrifuge 5418) to give a blue solution of CoPc equilibrated with its precipitant. The SPCE were incubated directly in these tubes.

2.4. Immobilization of enzymes

Enzymes were adsorbed onto the modified SPCE via electrostatic interactions with the surface-bound PEI. AChE was adsorbed for 5 min from a 0.1 mg mL^{-1} solution in 50 mM PBS pH 7.0. GOx was immobilized via 5 min incubation in 10–0.01 mg mL^{-1} enzyme solution in 50 mM PBS pH 7.0 containing 50 mM silicic acid (unless stated otherwise). Freshly prepared silicic acid was obtained from a 10 min hydrolysis of 150 μL TMOS in 850 μL of 1 mM HCl.

2.5. Electrochemical measurement of biosensor response

A three-electrode cell equipped with the saturated calomel electrode as a reference electrode and a platinum rod as an auxiliary electrode was used in all experiments. Electrochemical measurements were performed using a μAutolab-III FRA12 system and the general purpose electrochemical software operating system GPES4 from Eco Chemie B.V. (Utrecht, Netherlands). Home-made SPCEs were employed as working electrodes after appropriate modifications. All measurements were carried out at room temperature in intensively stirred solutions. The baseline current was allowed to settle before the injections of substrates were started. The response was calculated by subtraction of this baseline value from the actual readings.

Activity of immobilized GOx was observed by the H_2O_2 release in response to the addition of glucose. This was achieved by injections of 10–50 μL aliquots of 1 M glucose into 5 mL of 25 mM PBS, 100 mM KCl, pH 7.0. Oxidation of H_2O_2 was monitored at +600 mV unless specially stated.

AChE activity was measured using the injection of 5–100 μL aliquots of 20 mM or 100 mM acetylthiocholine iodine into 25 mM PBS, 100 mM KCl, pH 8.0. Thiocholine, a hydrolytic product of AChE, was assayed by electrochemical oxidation at +100 mV.

2.6. Synthesis of silica nanoparticles in solution

Silica nanoparticles were obtained by dissolving an appropriate amount of TMOS (usually 5–15 μL) in 1 mL of 100 μM PEI Mw 25,000, 25 mM PBS at pH 7.5. When the stoichiometry of the silica formation was determined, the concentration of PEI was varied. A mixture of PEI and PBS was prepared using stock solutions of 25 mM PBS pH 7.5 and 200 μM PEI Mw 25,000 pH 7.5 (pH adjusted with HCl).

2.7. Quartz crystal microbalance measurements

Mass deposition on the surface of a sensor crystal over time was monitored using a Maxtek RQCM instrument at a flow rate of 500 μL min^{-1} in a 100 μL flow chamber. Sensor crystals were 5 MHz 2.54 cm diameter piezoelectric Au on Cr. Before any experiments were carried out, the sensor crystals were cleaned by 3 min incubation in "piranha" solution containing 30% H_2O_2:98% H_2SO_4 = 3:7 (v/v). Note that piranha solution is extremely corrosive and should be handled with care. The electrodes were then thoroughly rinsed with de-ionised water, ethanol and dried with a nitrogen flow.

To investigate PEI-assisted condensation of silicic acid, sensor crystals were modified with negatively charged sulfonic groups via 2 h incubation in 100 mM 3-mercapto-1-propanesulfonic acid, 100 mM Tris–HCl buffer pH 8.0, followed by rinsing with water, ethanol and drying. 50 mM silicic acid in 50 mM PBS pH 7.0 was prepared by appropriate dilution of 1 M freshly prepared silicic acid. The latter was obtained by 10 min hydrolysis of 150 μL TMOS in 850 μL of 1 mM HCl.

3. Results and discussion

3.1. PEI-assisted condensation of silicic acid in homogeneous solution

When silicic acid produced by the hydrolysis of TMOS in 1 mM HCl was added to the mixture of PEI and phosphate buffer (pH 7), a white cloudy precipitate of silica was formed almost instantly. Such a rapid condensation of silicic acid prevents equal distribution of reagents in the mixture prior to the formation of silica particulates which resulted in fusing together of silica particulates which quickly settled down as large aggregates. In order to obtain relatively small discrete particles essential for the formation of a stable suspension, silicic acid should be slowly added to the PEI solution whilst employing mixing [28,29].

We have found that a stable, highly dispersed suspension of silica particles can also be obtained if the hydrolysis of TMOS was