Porous silicon pillar structures/photosynthetic reaction centre protein hybrid for bioelectronic applications

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Abstract

Photosynthetic biomaterials have attracted considerable attention at different levels of the biological organisation, from molecules to the biosphere, due to a variety of artificial application possibilities. During photosynthesis, the first steps of the conversion of light energy into chemical energy take place in a pigment–protein complex, called reaction centre (RC). In our experiments photosynthetic reaction centre protein, purified from *Rhodobacter sphaeroides* R-26 purple bacteria, was bound to porous silicon pillars (PSiP) after the electropolymerisation of aniline onto the surface. This new type of biohybrid material showed remarkable photoactivity in terms of measured photocurrent under light excitation in an electrochemical cell. The photocurrent was found to increase considerably after the addition of ubiquinone (UQ-0), an e^- -acceptor mediator of the RC. The photoactivity of the complex was found to decrease by the addition of terbutryn, the chemical which inhibits the e^- -transport on the acceptor side of the RC. In addition to the generation of sizeable light-induced photocurrents, using the PSiP/RC photoactive hybrid nanocomposite material, the system was found to be sensitive towards RC inhibitors and herbicides. This highly ordered patterned 3D structure opens new solution for designing low-power (bio-)optoelectronic, biophotonic and biosensing devices.

Graphical abstract

Keywords Porous silicon · Photosynthetic reaction centre · Biocomposite

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1 Introduction

Design and fabrication of photoactive biohybrid materials, biocomposites and investigation of their physical/chemical characteristics is an emerging field in academic and industrial laboratories. One of the most promising materials in this field is porous silicon (PSi), which is a widely used semiconductor with exceptional photonic [1], structural and electronic properties. Due to its unique properties and diverse structural design, PSi is an ideal candidate not only for optoelectronic devices [2, 3] and novel drug delivery systems but also for chemical and biosensing applications [4–12].

Silicon has been applied in biosensors since the very beginning of the development of biosensing technologies [13–15]. After the discovery of PSi and recognising its unique optical properties [16, 17], new prospects were revealed in the area of sensing [18] and biosensing [19–21]. In the simplest case, the light interference effect of the PSi photonic structure provides a characteristic reflectance spectrum with a specific reflection mode, which is sensitive to any surface modification [8, 22]. Depending on the architecture of the PSi structures, the refractive indices of both the silicon skeleton and the material inside the pores (air, water, ethanol, etc.) enable qualitative and quantitative detection of analytes. Besides having a modulable reflectance spectra, PSi is also photoactive, which allows the generation of different signals (photoluminescence, plasmonic resonance, photoacoustics) [17, 23–26] and enables an efficient detection of various molecular species [23, 27, 28].

Even if the PSi surface pattern can be modulated during the fabrication process, its use in sensing is limited due to the constrained transport of the diffusing medium (water or organic solvents) inside the pores [29]. Apart from enhancing the diffusion inside the pores, the 3D PSi structures, such as nanowires or a periodic array of micropillars, increase the actual useful surface area utilized for the detection of any analyte or biomolecule. This could be particularly advantageous, specially, if the detection requires the presence of a continuous layer of any functional material (e.g. metallic oxides) [30]. The 3D PSi structures can be readily fabricated by metal-assisted chemical etching (MACE), a chemical etching reaction that dissolves silicon using an oxidising agent and a metal as catalyst [31]. In addition to a controllable and large exposed surface area that might be decorated with a functional material [32], PSi pillar structures have been demonstrated to be useful in the optical detection of chemical analytes [33]. Highly ordered patterned 3D structures provide wide number of sensitive, selective and stable fingerprint-sensing devices [33, 34].

Enhanced yield of immobilisation of biomolecules can provide electrochemical sensing with extreme sensitivity

and specificity. Conductive polymers are suitable for amperometric and potentiometric applications by chemically modifying the surface, stimulating the redox reactions on it. Polyaniline (PANI) is one of the well-studied conductive polymers that has already been successfully combined with PSi [5, 35–37]. PANI deposition might assure proper functional interface for biomolecules, facilitating the physical/chemical sorption of the desired biological entity.

Various biological systems—at any level of the organisation (e.g. cells, membrane fragments, molecular complexes)—have been used to create different approaches based on light excitation [38–45]. Photosynthetic reaction centre proteins (RCs) are of special interest due to their exceptionally high quantum yield of energy conversion [46]. The first steps of the photosynthetic energy conversion take place in the RCs. These pigment—protein complexes are embedded in the photosynthetic membranes of oxygenic and anoxygenic bacterial photosynthetic organisms. In the RC, the energy of an absorbed photon induces the separation of positive and negative charges within the protein complex, i.e. $P^+BPheo^-Q_A$ state, which is followed by the $P^+BPheoQ_A^-$ state, where P^+ is the oxidised primary electron donor, a specialized bacteriochlorophyll dimer, $(BChl)_2$, $BPheo$ is the first electron acceptor, a monomer bacteriopheophytin, and Q_A^- is the reduced quinone-type primary electron acceptor. The separated charges are then further stabilized in the form of the $P^+Q_B^-$ state, where Q_B^- is the reduced secondary quinone acceptor [47, 48]. In living organisms secondary e^- -donors to P^+ and e^- -acceptors of Q_B^- (more precisely from the doubly reduced and protonated form Q_BH_2 , see details in Allen and Williams [47]) reset the RC, making it ready to perform successive charge separation and to participate in the vectorial electron transport driving the metabolic pathways of the cells.

RCs purified from *Rhodobacter (Rb.) sphaeroides* purple bacteria are widely used components in photoactive biocomposites. Combining RCs with conductive or semiconductive carriers, direct redox interactions can proceed in the hybrid composites. After light excitation, photocurrent is generated by the RC which is sensitive to the added redox-active components and different quinone-site inhibitors [49–56].

It has already been shown that the donor and acceptor sides of the RC remain accessible for the externally added horse heart cytochrome c donor and UQ-0 (2,3-methoxy-5-methyl-1,4-ubiquinone) acceptor in photo-electrochemical cell [22, 57] after binding RC to PSi. Hence, it facilitates the detection of active components—like specific inhibitors—for which RC shows selectivity and sensitivity [58]. These results provided the basis for further applications of the composite as photoactive electrode. Since bionanocomposite materials are considered as future materials [59, 60], one can envision that such composites may contribute to

the development of new-generation low-power photonic, optoelectronic, photovoltaic, electrochemical and biosensing technologies, as reviewed, e.g. by Nagy et al. [61, 62].

In this work it is demonstrated that PSiP, used as carrier matrix for the RC, offers new applications in the field of biohybrid systems in nanobionics. Photocurrent can be generated by the PSiP/RC complex, and the donor and acceptor sides of the RC, after binding the protein to PSi, remain accessible for redox turnover either through carrier surfaces or externally added redox mediator molecules.

2 Materials and methods

2.1 Sample preparation

Rb. sphaeroides R-26 cells were grown photoheterotrophically [63]. RCs were prepared by solubilizing the intracytoplasmic membrane fragments of the cells by LDAO (N,N-dimethyldodecylamine-N-oxide, Fluka) and purified by ammonium sulphate precipitation, followed by DEAE Sephacel (Sigma) anion-exchange chromatography [64].

Porous silicon pillar structures (PSiP) were fabricated by pre-patterning the silicon surface with colloidal lithography and then with MACE. First, a continuous hexagonal array of self-assembled poly(N-isopropylacrylamide) (polyNIPAM) hydrogel microspheres was formed over monocrystalline silicon (p-type, specific resistivity is 0.001–0.005 Ωcm) substrate. Gold nanoparticles were deposited over the pre-patterned surface followed by the removal of the polyNIPAM microspheres. Gold nanoparticles were synthesised by the Turkevich method [65]. Heterodisperse gold solution was used with particle diameters between 10 and 40 nm.

The thickness of the remaining layer of gold nanoparticles was increased by electroless gold deposition. The gold patterned silicon substrate was submitted to MACE with a solution of ethanol, HF (48%) and H_2O_2 (36%) in a volumetric ratio of 1:1:1 for 5 min. Finally, the gold layer was removed with Aqua Regia. Detailed information of this process has been described elsewhere [66].

PSiP/RC composites were fabricated by immobilisation of the RCs on the PSiP surface. First, electrochemical polymerisation of aniline was carried out in an electrochemical cell with three-electrode arrangement, where the working, counter and reference electrode were PSiP, platinum and Ag/AgCl, respectively. 0.25 M of aniline was dissolved in 0.5 M HCl solution. Voltage was swept between 0 V and 1.7 V and the number of stop-crossings was 4 [24]. Finally, the cyclic voltammogram of PSiP before and after deposition of PANI by electrochemical polymerisation was measured in the range of – 1 to 1.5 V. Glutaraldehyde (GTA) in 2.5 m/v% concentration served as an amine-targeted crosslinker between the PANI-covered silicon surface and

the RC, as described earlier [7, 22, 57]. Briefly, the surface was dipped in the GTA solution for 15 min, then washed with phosphate buffer and dried under the stream of nitrogen. After the primary functionalisation, the samples were incubated in 11 μM of RC for 2 h at 4 $^\circ\text{C}$, followed by the rinsing with phosphate buffer (10 mM, pH: 7.0) and drying under the stream of nitrogen.

2.2 Fabrication of the working electrode

PSiP/RC working electrodes were prepared by a laminating procedure. A copper wire, as an electrical contact, was fixed on the non-porous part of the PSiP plate with the use of conductive carbon paste (SUPELCO). The electrodes were covered with a laminating foil (Swardfish). As a result of the lamination, only a circular patch of the porous surface was in contact with the electrolyte solution (10 mM phosphate buffer, pH: 7.0). The approximate diameter of the available porous silicon surface was 6 mm that corresponds to 28.5 mm^2 of the illuminated circular surface area. Although the pillar structure (diameter, length and distribution of pillars) can be well characterised (see below), at the present state of our investigations we do not know the degree of the surface coverage by the RC protein. It is generally accepted in the literature to use the net illuminated surface area in similar cases.

2.3 Scanning electron microscopy

Scanning electron microscopy (SEM) was performed with a Hitachi S-4700 type II FE-SEM equipped with a cold-field emission gun operating in the range of 5–15 kV. The hybrid PSiP surfaces were mounted on a conductive carbon tape and sputter coated with a thin Au/Pd layer in an Ar atmosphere prior to the measurement.

2.4 Electrochemical measurements

An electrochemical cell with three-electrode arrangement was used for the measurements [67]. The working, counter and reference electrodes, i.e. PSiP/RC composite, platinum and Ag/AgCl, respectively, were immersed in the reaction mixture containing 10 mM phosphate buffer (pH: 7.0) and UQ-0 in different concentrations. After the equilibration, the open-circuit potential was fixed to – 60 mV and the photocurrent was measured under white light illumination of a 150 W halogen lamp. For inhibitor treatment, the required terbutryn concentration was adjusted by adding calculated amount of the chemical from the 60 mM ethanolic stock solution. In order to avoid the possible effect of the solvent on the stability of the RC, the final concentration of ethanol never exceeded 0.2% in the reaction mixture. The

Fig. 1 Electrochemical arrangement of the system measuring the photocurrent of the PSiP/RC working electrode

Fig. 2 Cyclic voltammogram of PSiP before (blue line) and after deposition (red line) of PANI by electrochemical polymerisation. Inset shows the I/V characteristics of the subsequent cycles of the electropolymerisation process carried out in the range of 0–1.7 V. The reaction mixture contained 0.5 M HCl with the addition of 0.25 M aniline or without this monomer for the polymerisation and for the comparing the samples before and after deposition, respectively. Figure shows results of typical experiments

arrangement of the electrochemical measurement is depicted in Fig. 1.

3 Results and discussion

3.1 Electropolymerisation

Porous silicon pillar substrates were covered with precisely controlled electrochemical polymerisation of aniline to form thin and homogenous polymer coverage and to preserve the available surface area of the PSiP structure (cf. Materials and methods). Cyclic voltammograms indicate successful

PANI deposition on the PSiP surface. Figure 2 shows the result of anodic cyclic voltammetry (CV) performed on PSiP before and after PANI deposition, complemented with the electropolymerisation process.

During the electropolymerisation, typically a decrease in the current indicates the decreasing amount of reacting monomers in the electrolyte (ref. to Inset Fig. 2). Small change in the current during the cycles suggests the deposition of relatively few aniline molecules on the surface, which is favourable for the formation of a thin polymer layer.

After the polymerisation process, the usual negative shift [68] of the oxidising current peak in the cyclic voltammograms is revealed in the –1 to 1.5 V range, which shows the formation of a thin dielectric layer on the surface that changes the charge capacitance of the working electrode PSiP (Fig. 2). These measurements indicate that the coverage of the Si surface with conducting organic polymer layer together with the modified layer structure of the electrode area may play important role in the photocurrent characteristics of the PSiP_PANI_RC composite electrode (explained in later paragraphs).

Scanning electron micrographs of the PSiP structure were taken before and after the PANI deposition process to observe the morphological changes due to the surface modification. Figure 3 shows a successful homogeneous polymer coverage achieved without considerable damage to the PSiP matrix. The figure shows a slight increase in the width of the column, which in turn reduces the inter columnar space due to the polymer coverage.

Figure 3A and B shows the structure of the PSiP pillars, which appear highly homogeneous in length (~2800 nm) and in thickness (~900 nm). Figure 3C and D shows the results after PANI deposition. The external walls of the pillars are well covered with the polymer without significant alteration of the hexagonal pattern of the silicon skeleton supporting the polymer (Fig. 3). Also, a small increment change in the length and diameter of the pillars due to the thickness of the polymeric layer was estimated by visual investigation (final length and diameter are approximately 3000 nm and 1000 nm, respectively). We expect that the change in the pillar structure geometry (i.e. the changes in the roughness of the pillars' surface and the reduction of the pillar-to-pillar spacing) is due to the polymer deposition. The bottom part of the pillars has been used to calculate the thickness of the coverage, which came out to be 91.29 ± 4.23 nm.

3.2 Photocurrent generated by the PSiP/RC composite

Our aim was to create functional PSiP/RC composite material which is capable of generating photocurrent in an electrochemical cell under light excitation. In order to check

Fig. 3 SEM images of PSiP structures. **A** Transversal and **B** top view before, and **C** transversal and **D** top view, after PANI deposition

Fig. 4 Time course of light-induced photocurrents measured on bare PSiP, PSiP covered with PANI (PSiP_PANI) and RC bound to PSiP electrode (PSiP_PANI_RC) in the absence of quinone. The measurements were performed in detergent-free phosphate buffer (10 mM, pH: 7.0). The time instants when the light was switched on (↓) and off (↑) are indicated. Curves represent the results corresponding to typical measurements

the photochemical/photophysical activity of the composites, light-induced photocurrent was measured on PSiP before and after PANI deposition as well as after RC immobilisation. The applied potential was fixed to -60 mV with respect to the Ag/AgCl electrode in all samples [53] (Fig. 4). As reference, bare and PANI-covered PSiP samples were also tested before immobilising RC, and only a low intensity steady photocurrent was found after light excitation in the absence of quinones. The low intensity of the photocurrent

in the absence of the RC can be explained by the quantum confinement phenomenon which might be shown occasionally by the oxidised porous silicon [69]. However, when the PSiP_PANI electrode was functionalised with RC, a noticeable photocurrent could be observed even without quinones. The presence of the continuous steady-state photocurrent indicates the continuous turnover of the RC protein bound to the PSiP. Since the acceptor side was reconstituted only by the added UQ-0, the turnover is indicative of the continuous electron flow from the functionalised PSiP to UQ-0 through the RC photochemistry/-physics.

Quinones play essential role as electron acceptors of the RC in native systems. Water-soluble ubiquinone (UQ-0)—an artificial electron acceptor at the RC's docking site—was used as electron transport mediator at different concentrations (Fig. 5). There is a clear correlation between the steady-state photocurrent and the increasing concentration of the added mediator with tendency to saturate the signal at higher concentrations. Coverage of PSiP by PANI improves the conductivity considerably, since the saturation value is approximately the double of the one measured before the polymerisation. After binding the RC to the PANI-decorated PSiP surface, a noticeable photocurrent could be measured even without the addition of quinone, which then saturates at high UQ-0 concentration. Detailed explanation is given in similar (quinone less) conditions for the origin of anodic and cathodic current in Chatzipetrou et al. [51]. Positive and negative charges are formed after the absorption of light that leads to redox interaction between the peptide complex and the silicon wafer. Also, change in the surface charge

Fig. 5 Light-induced photocurrents measured as a function of UQ-0 concentration before (PSiP) and after (PSiP_PANI) polymerisation, and after RC immobilisation (PSiP_PANI_RC). Photocurrents were measured in detergent-free phosphate buffer (10 mM, pH: 7.0). For better viewing, the magnitude of the photocurrents is demonstrated regardless of their direction. The potential was fixed to -60 mV with respect to the Ag/AgCl electrode in all samples in all measurements. All data points represent an average of three measurements. Error bars indicate maximum errors

arrangement can induce the polarization of the working electrode and, subsequently, an ion transport (an apparent current) in the electrochemical cell (Note that 10 mM concentration of the phosphate buffer is relatively high for this measurement). These data are in accordance with the experimental evidences provided by RCs (isolated from *Rb. sphaeroides* purple bacteria) interfaced with gold electrodes in a photoelectrochemical cell, generating photocurrent under a wide range of conditions of illumination, applied potential and mediator concentration [49, 50], even in the absence of quinone mediators [51].

3.3 The effect of terbutryn on PSiP_PANI_RC composite electrode

In further experiments terbutryn was also added to the reaction mixture. This chemical belongs to the group of the so-called triazine-type compounds, which are widely used for specific blocking of the RC photochemistry. Although terbutryn can undergo redox transition in special oxidative or reductive conditions (like photo [70]- or electro [71]-chemical decompositions), there are a large number of indications that this chemical is not redox-active when bound to the RC. Consequently, after competitive binding to the acceptor side of the protein, it eliminates the redox transition between Q_A and Q_B . Hence, it was possible to check whether the measured photocurrent was associated with the light-induced charge pair or not. Figure 6 demonstrates the effect of terbutryn on the light-induced photocurrent generation. UQ-0 concentration was optimized at 100 μ M, based on the previous measurements shown in Fig. 5. The resulting

Fig. 6 Time course of the light-induced photocurrents measured on RC/PSiP sample with different terbutryn concentrations, as indicated. The electrolyte consisted of 100 μ M UQ-0 and 10 mM phosphate buffer (pH: 7.0). The time instants when the light was switched on ($\downarrow$) and off ($\uparrow$) are indicated. Curves represent results of typical measurements

photocurrent decreases with increasing terbutryn concentrations (Fig. 6).

To check whether the effect of terbutryn on the photocurrent is specific to the RC coverage, measurements were also performed on PSiP in the absence of RC. After the addition of terbutryn at three different concentrations (100 nM, 1 μ M and 2 μ M), no significant change was found in the photocurrent (data not shown).

It is already demonstrated in Fig. 5 that PANI coating increases the photocurrent measured on PSiP electrodes in the presence of UQ-0. However, PANI/PSiP electrode can be sensitised to the added herbicides by immobilisation of RC onto the electrode. There are two effects of the sensitisation by the RC. Apart from an increase in the generated photocurrent due to RC coverage, it facilitates the detection of e^- -transport inhibitors in a very specific and sensitive way. In addition, the RC/PSiP composite was tested as an electrochemical biosensor for the detection of terbutryn in the concentration range from 200 nM to 100 μ M. The specific and effective reaction of the inhibitor is important for testing the goodness of the sample for other type of RC-based applications. For this measurement the electrolyte consisted of 100 μ M UQ-0 and 10 mM phosphate buffer (pH: 7.0). The percentage of inhibition was calculated and plotted against the terbutryn concentration (Fig. 7).

The photocurrent plateau intensity was fitted with logistic function (Eq. 1):

$$Y(\%) = 100 \times \frac{Y_{\text{Max}} - Y_{\text{Min}}}{1 + \frac{[I_{50}]}{[I]}} \quad (1)$$

where Y_{Min} and Y_{Max} are the photocurrents measured with and without the inhibitor treatment, respectively, I_{50} is the

Fig. 7 Level of inhibition, given in percentage, as a function of the terbutryn concentration in logarithmic representation. The electrolyte consisted of 100 μM UQ-0 and 10 mM phosphate buffer (pH: 7.0). Solid line was calculated by the fitting of $I_{50}=1.19\ \mu\text{M}$ ($\text{SD}=\pm 0.7\ \mu\text{M}$). The curve was completed by titrating three independent samples with overlapping concentrations of terbutryn; SD was calculated for the completed curve. I_{50} values for the individual three samples were in the range of 0.95–1.33 μM . Error bars indicate maximum errors. The goodness of the fitting is indicated by $r^2=0.93$ (for individual experiments r^2 was in the range of 0.88–0.99)

fitted apparent inhibitor constant (i.e. the concentration that causes half of the maximum inhibition), and $[I]$ is the inhibitor concentration [53, 72]. By fitting the measured steady-state photocurrent data $I_{50}=1.19\pm 0.7\ \mu\text{M}$ ($r^2=0.93$) was determined. This obtained value of I_{50} is somewhat higher than that reported in Ref. [51], where, in a similar arrangement, the half maximal inhibitor concentration was found to be at 0.208 μM . However, in this latter experiment the RC was adhered to the surface of gold electrode in an electrochemical cell. Similarly, $0.38\pm 0.14\ \mu\text{M}$ was determined by Szabó et al. [53] who used silanised ITO decorated with RC/MWCNT (multiwalled carbon nanotube) as a working electrode in a microfluidic cell.

Note, that the inhibitor constant K_I value, which corresponds to the concentration of half maximum of the inhibition, agrees well with the values found in the literature, which are in the range of 0.7–3 μM [58, 72–77]. These values (for RCs purified from different species of purple bacteria) were determined by flash-induced absorption change measurements in detergent solutions.

4 Conclusion

Successful immobilisation of photosynthetic bacterial reaction centre (RC) was performed on PANI covered porous silicon pillar structure (PSiP). After the controlled electrodeposition of the conducting polymer, with respect to the reference (bare PSiP), the magnitude of the light-induced photocurrent significantly increased. Further

enhancement of the photocurrent was achieved by the immobilisation of RCs. The enhanced current density revealed a redox interaction between the RC and the PSiP after light excitation, and hence causing a change in the charge state of the electrode surface and inducing current flow in the electrochemical cell.

Due to the presence of specific redox cofactors and binding sites of the RCs, the photocurrent of the biocomposite device was found to be sensitive to terbutryn, as a result of its interaction with the RC's acceptor side. After the required optimization, the RC/PSiP biocomposite device can potentially operate as an electrochemical biosensor for the detection of e^- -transport inhibitors, like herbicides or some other endocrine disruptor compounds. Such sensitive and selective systems can be further developed by reducing the measured sample quantity, e.g. in a microfluidic cell [53]. The unique pillar structure of the PSi provides possibility for specific (bio) photonic applications.

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Data availability All data generated or analysed are included in this published article.

Declarations

Conflict of interest The authors have no conflicts of interest.

Dedication The authors would like to dedicate this publication to Professor László Szalay, former member of the executive committee of the European Society for Photobiology (ESP), the founder and head of the Department of Biophysics at the József Attila University and the founder of the Institute of Biophysics of the Biological Research Center at Szeged, Hungary. He was awarded the precious prize of the ESP for his pioneering activity in photobiology.

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