DEVELOPMENT OF REDOXPROTEIN-BASED BIOHYBRID SENSOR DEVICE

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Abstract
We have successfully developed an enzyme electrode based on photosynthetic reaction centre (RC) protein of *Rhodobacter sphaeroides* purple bacteria. We bound the protein chemically to the electrode surface through a layer of multi-walled carbon nanotubes (MWCNTs). The surface was borosilicate glass covered with indium-tin-oxide (ITO) for its transparency and well-known qualities as an electric semiconductor. We achieved a bio-nanocomposite with good features to support the protein's photosynthetic activity. To test it, we used the nanocomposite as a working electrode in a traditional electrochemical cell with electrolytes containing only mediators or mediators and inhibitors (herbicides that block the protein's activity specifically). Our measurements provided great results in terms of electric current ("photocurrent") generated by the photon-excited RC, regeneration of the electrode surface and stability of the composite. For further optimization of the system, we decided to replace the RC/MWCNT part with a two-component protein complex of chemically bound RC and cytochrome c, which is a natural electron donor of the RC protein. We have successfully prepared the complex and made several measurements to determine the quantity and quality of the product, with good results.

Introduction
The unique attributes of the RC protein [1] offers a great number of possible applications in photoelectric devices [2-4]. It also has to be emphasized that in the presence of mediator molecules and light, the RC completes its photosynthetic cycle even *in vitro*. In an electrochemical cell as a working electrode it produces an easily measureable amount of electric current [5]. It has been proven that the RC protein, bound to a gold electrode in a photoelectrochemical cell produces notable activity under various conditions [6]. This photocurrent can be blocked by several herbicides or quinone-side inhibitors [7]. There are numerous "triazine-type", RC specific, environmentally hazardous herbicides long forbidden in the EU but still used in USA, China, etc. Monitoring their concentration in the soil is an important goal of environmental protection.
The RC has a natural electron donor protein, c type cytochrome. There are many experiments noted in the literature [8-10] to bind cytochrome and RC together in a complex formula. This would open ways to control the orientation of the RC bound on the surface. A nanocomposite with an oriented dual protein complex layer immobilized chemically and used as a working electrode of an electrochemical cell would give further opportunities to examine and apply the RC's photosynthetic activity in the future.

Experimental
Preparation of the electrodes We created thiol groups on the ITO surface by a functionalization procedure with (3-mercapto-propyl)trimethoxy-silane, and used these groups to bind MWCNTs through their amine groups, which we previously activated with sulfo-

SMCC. After 2 hours of reaction time the excess nanotubes were washed down from the surface. As crosslink agents we used NHS and EDC, solved in distilled water, to immobilize the RCs deposited on the surface for 2 hours at 4°C. After the fixation of the proteins, we washed the electrode surface intensively with 0.1M pH 7 phosphate buffer.

Electrochemistry We used the prepared nanocomposite as a working electrode in an electrochemical cell with three electrodes. The counter and the reference electrode was Pt and Ag\AgCl, respectively. As an electrolyte, we used UQ-0 and ferrocene solved in 20 mM TRIS buffer, pH 8.00. The measurements were carried out using a Metrohm PGSTAT204 type potentiostat/galvanostat at ambient temperature. The light source was a 150W halogen lamp. The light intensity was 78 $mW \cdot cm^{-2}$.

Sensory measurements We used orto-phenantroline (o-Phe) and terbutryn as inhibitors of the protein activity. Both are herbicides that bind specifically to the RC's secondary quinone binding site, thus preventing it from binding its electron acceptors and completing its charge separation cycle. In the absence of the herbicides, we measured current densities of a few $\mu A\ cm^{-2}$ after 100 s dark incubation. The measured photocurrent was successfully inhibited with o-Phe gradually increased from zero to 600 µM. The same experiment was completed with terbutryn as well (data not shown). We have determined the fitted inhibitor constants of both terbutryn and o-Phe from the figure of the photocurrent inhibition as a function of herbicide concentration. We stored the prepared composites in dark environment and 4°C, and gained evidence that the RCs bound to the electrode surface stayed active (produced measurable photocurrent) even after weeks. Dipping the electrode into the solution of the RC's substrate ubiquinone-0 caused the herbicides to dissociate from the protein thus led to the regeneration of the electrode surface. The speed and efficiency of the regeneration increased with greater substrate concentrations from 100 µM UQ-0 to 1 mM.

Preparation of the RC/cytochrome protein complex We have prepared protein complexes from RC and horse heart cytochrome c by chemically binding them together in their natural state of redox interaction. We followed the steps described by Drepper et al. [10] and Rosen et al. [9] for the preparation of the complex. The proportion of cytochrome to RC was 3:1, and both proteins were in a solution of 10 mM pH 7.5 HEPES buffer in the presence of 0.025% LDAO to prevent the RC from aggregation. The proper redox environment for the reaction was granted by 1 mM of ascorbate and 60 µM of 1,4-naphtoquinone. After the addition of 12 mM NHS and 6 mM EDC crosslinking agents, the reaction was carried out in 3 hours of dark incubation at room temperature. We stopped the reaction with the addition of 50 mM ammonium acetate. After 10 minutes, we dialyzed the solution at 4°C overnight against 10 mM TRIS-HCl, pH 8 with 0,025% LDAO.

Purification and measuring of the complex The dialyzed sample was eluated through a Sephadex G-75 column (1x15 cm) with pH 8.00 TRIS-HCl buffer to separate the complex from the excess RC and cytochrome molecules. We took 12 fractions of 0.5 cm^3 and measured them one by one with UV-Vis spectroscopy. The fractions providing the best results of RC and cytochrome presence were passed through a Sephadex G-50 column again for better resolution. The second set of 0.5 cm^3 fractions was measured with UV-Vis spectroscopy as well, and 3 fractions (4th, 5th and 6th) contained the full amount of the product. They were put together in a filtered Falcon tube and concentrated by centrifuge at 5000 RPM. We studied the concentrated sample with absorption kinetics spectroscopy at 860 nm wavelenght to search for the characteristic absorption change of the light excitation and charge separation in the RC.

Results and discussion

Electrochemistry Figure 1 shows the unmodified photocurrent generated by the RC/MWCNT/ITO electrode in the electrochemical cell, and the steps of increasing inhibition by the successive addition of o-Phe up to a concentration of 600 μM. The electrolyte contained 100 mM UQ-0, 20 μM ferrocene as electron donor and 20 mM TRIS-HCl buffer. The results clearly show the capability of sensory application of the system.

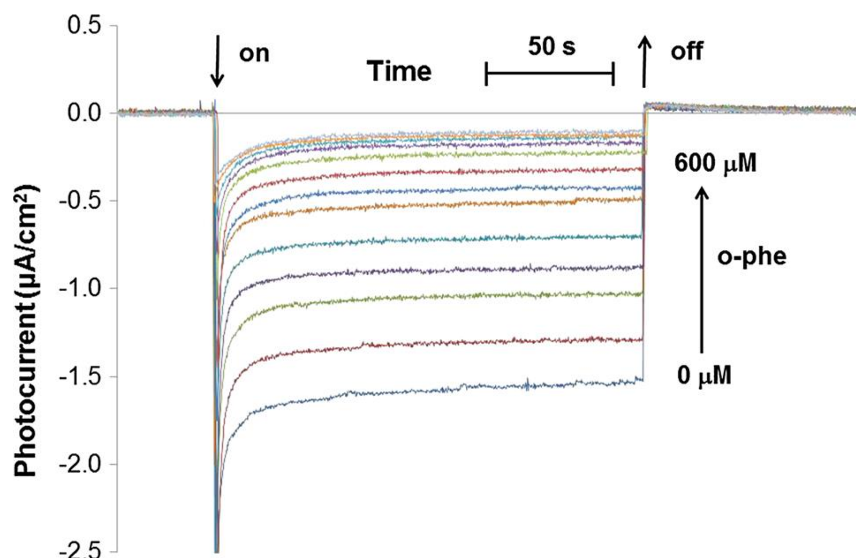

Figure 1. Generated photocurrent in the absence and in the presence of herbicide.

Based on this measurement we fitted a logistic function (Figure 2) to determine the K_I, the fitted inhibitor constant (the concentration that causes half of the maximum inhibition). The fitting was done with the function: $Y = \frac{Ymax - Ymin}{1 + \frac{[KI]}{[I]}}$

Y_{Max} and Y_{Min} are the signals in the absence and presence of the inhibitor, respectively; K_I is the fitted inhibitor constant, $[I]$ is the actual inhibitor concentration.

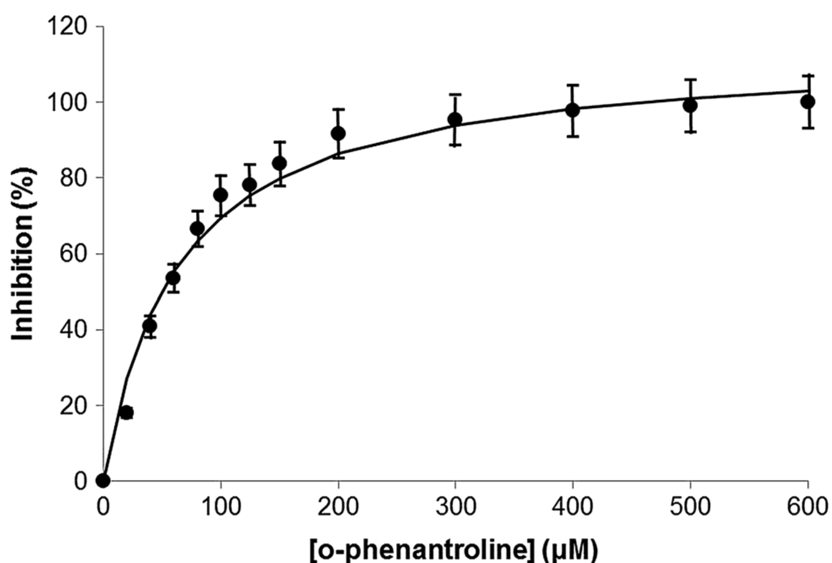

Figure 2. Fitted logistic function of the photocurrent inhibition.

Measuring the RC/cytochrome complex After cleaning the complex from the excess RC and cytochrome we took measurements with an UNICAM UV4 type spectroscope. We collected data through the 325-900 nm wavelength interval, but figures show only the part of the spectra that has information value. Figure 3 shows the spectra of the fractions containing the protein complex, with two samples from the start and end of the set as references.

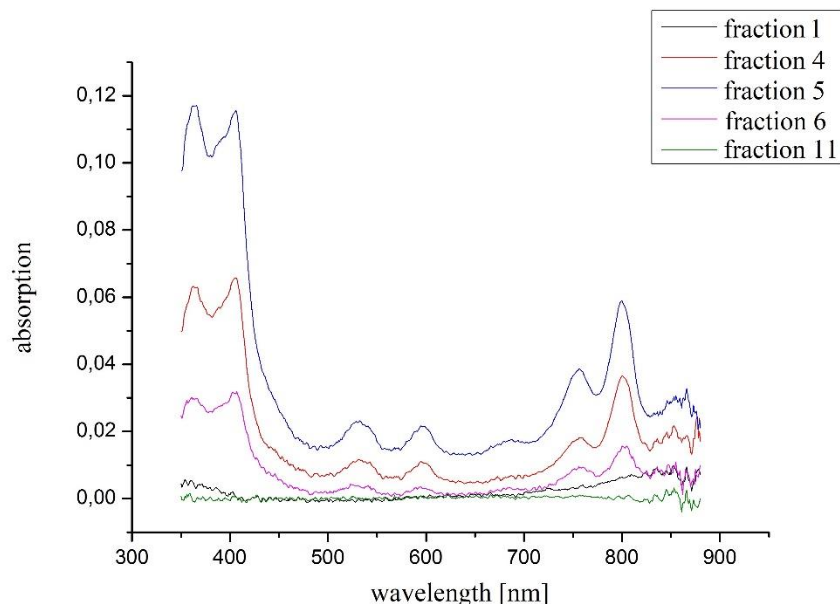

Figure 3. Only three of the 12 fractions contained the product.

After we concentrated these fractions with a centrifuge, we tested the complex to see if the cytochrome was still intact and active. We measured the sample in both oxidized and reduced state to see the characteristic reduced double peak at 530 nm. Figure 4 shows the result of the measurement.

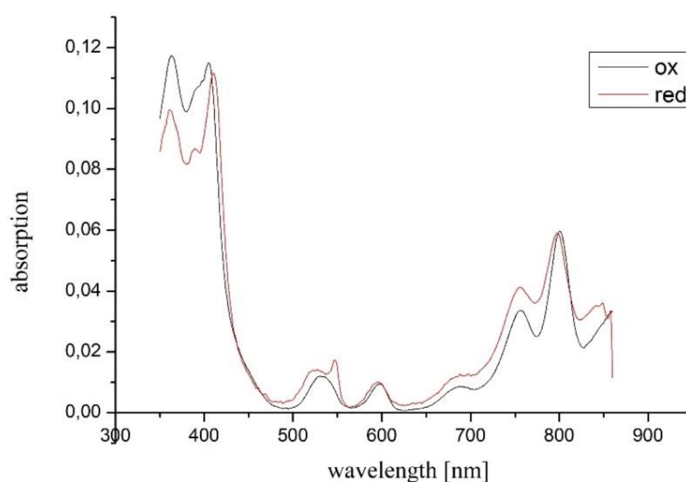

Figure 4. The spectroscopy proved we had an intact and working cytochrome bound to the RC

This was followed by a measurement of absorption kinetics for real time monitoring of the redox activity of the complex. Background signal was complemented using offset voltage to set the base line to zero, then flash kinetics were measured on 860 nm where the P*/P+ shift can be seen upon the light excitation and charge separation of the RC protein. Figure 5 shows the result of the absorption kinetics experiment.

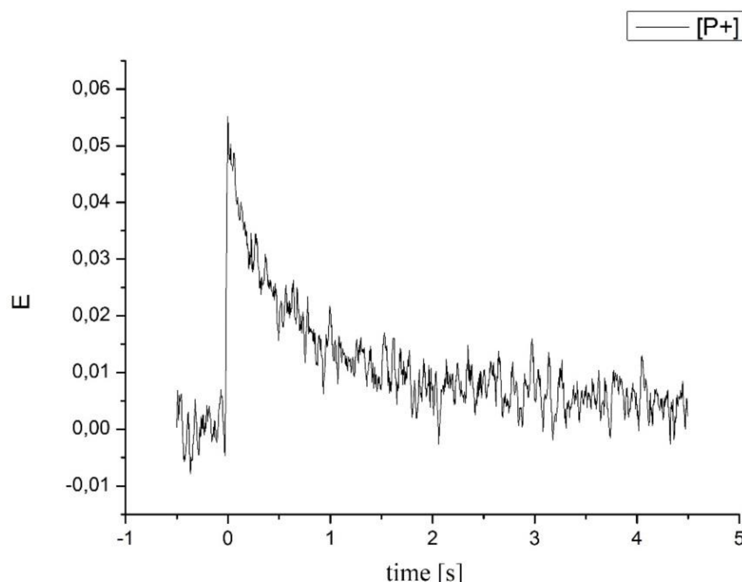

Figure 5. Flash absorption kinetics of the protein complex at 860 nm.

Conclusion

It is clear that the RC/MWCNT/ITO nanocomposite produced good results of photoelectric activity and as a sensor for herbicides. The system can be further optimized by utilizing a RC/cytochrome complex to control the orientation of the RC protein. Preparation of this complex has been successfully done at our lab. Results of the RC/cytochrome complex measurements indicate good chances for its application in the electrochemical biosensor.

Acknowledgements

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