Doctoral Thesis

PHOTOSYNTHETIC ORGANISMS IN BIOCOMPOSITES

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Introduction

Biological materials developed by nature are extremely efficient and complex systems. It is a real challenge to use them in biohybrid composites and harness all their advantageous properties. This way it is possible to build environmentally friendly biodegradable items like drug delivery systems, biosensors. In addition, it is also probable to use them for special (opto)electronic devices, photoelectric energy conversion or different imaging systems. Biomaterials are sensitive when they are isolated out of their natural environment, so these applications usually require their integration into biocompatible abiotic matrices. Combining different materials several new possibilities and new advantageous properties have been upcoming.

The photosynthetic conversion of solar energy into chemical energy is one of the most important processes on Earth. Photosynthetic biomaterials have always had special interest in every level of organization of being, from photoactive molecules to whole plant cells. Numerous studies have already been carried out about the application of whole plant cells in artificial systems to achieve different special tasks like biosensors or bioreactors. In these research works one of the most popular materials is *Arabidopsis thaliana* as the viability of these plant cells are excellent even after immobilization. So, I used *Arabidopsis thaliana* cell culture and encapsulated it in a silica matrix with sol-gel method. The main challenge was to fabricate an artificial environment for the cells where they can preserve their cell viability.

Working with photosynthetic organisms the efficiency of our system can be much better if we use only a part of the whole cells, as there are a lot of mechanisms that can cause energy loss during the light harvesting. The first steps of energy conversion in photosynthesis take place in a pigment-protein system called reaction centre (RC). It is really thought-provoking that although its size is only a few nanometers, the present form of life in Earth depends on this protein complex. After photoexcitation a charge pair is formed inside the protein. The charge separation and stabilization is connected to protonation and deprotonation of specific amino acids. The efficiency of this system is near to 100% as every

absorbed photon creates a charge pair which is further stabilized within the protein.

During my research work I have bound photosynthetic reaction centre to conducting and semiconducting materials by chemical and physical methods. Two different carrier matrices (carbon nanotubes and porous silicon) were used in my experiments because of their exceptional physical characteristics, like biocompatibilitiy, specific surface area, electric and mechanical properties, etc.

For any possible practical application it must be taken into account that some of the excitation energy is captured in triplet states of chlorophylls and transferred easily to triplet oxygen. On one hand, this process can be useful because of the longer life time of the excited state. On the other hand, unuseful due to the damaging effect of the produced singlet oxygen. The role of carbon nanotubes in quenching the singlet oxygen after photoexcitation of RC-bionanocomposit was also investigated.

Aims

1. Immobilization of *Arabidopsis thaliana* whole plant cells in silica gel matrix using sol-gel process, finding the best properties for this method and characterization of photochemical/-physical properties of the composite.

2. Preparation of bionanocomposites by the chemical or physical binding of bacterial photosynthetic reaction centre purified from *Rhodobacter sphaeroides* purple bacteria, to the surface of conducting or semiconducting matrix.

1.1. Chemical binding of photosynthetic reaction centre on the surface of amine-functionalized single walled carbon nanotube (SWCNT). Morphological (AFM, SEM) and functional (flash photolysis) characterization of the prepared RC/SWCNT complexes.

2.2. Chemical and physical binding of photosynthetic reaction centre on the surface of porous silicon (PSi), morphological (SEM, EDX) characterization

of the composites, detection of the different binding methods' efficiency (Fourier Transfrom Infrared Spectroscopy) and the measurement of the photoactivity of the complexes after different binding methods (flash-photolysis measurements).

Materials and methods

Preparation of biological samples and biocomposites

Plant Cell Culture

Suspension-cultured *Arabidopsis thali*ana cells derived from leaves of *A. thaliana* strain L-MM1 ecotype *Landberg erecta* were cultivated in Murashige and Skoog medium (4.43 g/l, pH 5.7) supplemented with sucrose (30 g/l), 0.05 μg/ml of kinetin, and 0.5 μg/ml of NAA (naphtalene-acetate).

Photosynthetic Reaction Centre Preparation

Rb. sphaeroides R-26 cells were grown photoheterotrophically. RCs were prepared by LDAO (N,Ndimethyldodecylamine-N-oxide, Fluka) solubilization and purified by ammonium sulfate precipitation, followed by DEAE Sephacell (Sigma) anion-exchange chromatography.

Carbon Nanotube (CNT) Preparation

The purification of the carbon nanotubes was done by wet oxidation method: 100 g pure HiPco single walled carbon nanotubes (SWCNT) was oxidized in the mixture of 60 ml 30% H_2O_2 and 110 ml 22% HCl. The solution was under continuous reflux for 9 h at 70 °C. After that the temperature was adjust to 25 °C and the SWCNTs were filtered and washed with distilled water to reach pH 7. Finally they were dried at 120 °C for 30 minutes.

Porous silicon (PSi) Preparation

PSi wafers were prepared by a wet electrochemical etching using highly boron-doped p-type silicon wafers (thickness 500−550 μm) with a 0.002−0.004 ohm/cm specific resistivity and with a crystallographic orientation of (100). The microcavity structures were fabricated with the configuration of (HL)x5HH(LH)x5 where H and L represent high and low porosity layers, respectively. As the optical thickness of each layer was kept at λ/4 (with λ = 700

nm), the presence of two successive layers of high porosity in the middle creates a cavity mode with an optical thickness of λ/2. To ensure good macromolecular penetration, the multilayers were prepared by assuring higher porosity for the first layer.

Composite preparation

Plant Cell Encapsulation

Anaqueous sol-gel route was used to encapsulate fragile plant cells into pure and organically modified silica matrices. MTGS (methyl triglicerol silane) was employed to synthetise the organically modified hybrid material. This compound is synthetised by mixing MTMS (100 mM, Fluka, >98%) and Glycerol (300 mM, Fluka, 99.5%). Sodium silicate solution was made from Na_2Sio_3 (Mercks). Resin (Acros) was rinsed with acid water pH 0.5-1 (1 M HCl Sigma) and after freezing, it was mixed with the sodium silicate solution and then a clear sol was recovered after filtration. Organically modified silane stock solution was obtained by mixing the solution described above, different concentrations of MTGS, NaCl and KOH solution and plant cell solution (20 V%). During the gel preparation the pH is the determining factor. First the pH is acidic so before adding the plant cell it has to be adjusted to 5.7.

Reaction Centre/Carbon Nanotube Composite Preparation

RC was bound to the amine functionalized SWCNTs using glutaraldehyde (GTA) homobifunctional crosslinker molecule. 50 µl GTA was mixed with 800 µl SWCNT suspension and mixed for 10 minutes at 400 rpm. The unbound GTA was removed by dialysis (20 minutes, phosphate puffer, pH 6.5). 100 µl RC (c=64 µM) was put into this solution and dialised, again, for 2 h in phosphate buffer (pH 7.0). The unbound RCs were removed by series of washing and centrifugation in phoshpate buffer (pH 7.0, 0.03% LDAO). The suspension was dried on a glass surface.

Reaction Centre/Porous Silicon Preparation

RC was bound to PSi in two different ways. The first method involved a three-step conjugation protocol. The PSi surface was first functionalized using the amine group of 3-aminopropyl-triethoxysilane (APTES), followed by the binding of glutaraldehyde (GTA) as an amine-targeted homobifunctional cross-linker.

Finally, the RC was bound to GTA through its surface-accessible lysine side chains.

In the second method, the RC was bound to PSi through a 12-mer peptide (SPGLSLVSHMQT) previously elaborated as a specific linker for the used p-type PSi surfaces. Briefly, the deoxidized (by HCl), freshly etched PSi sample was exposed to the peptide solution (20 μM in PBST, (PBS buffer, 0.1% Tween 20)) for 2h. The unbound peptide was washed away with PBST. To bind the RC, drops of the protein solution (in 10 mM TRIS, 0.03% LDAO, pH 8.0) were placed on the sample surface for 90 min, followed by washing with PBS buffer.

Investigation methods

Biological Activity and Viability of Plant Cells

The physiological functions of entrapped cells were assessed by monitoring the oxygen consumption in a Clark cell vessel (Oxy-lab manufactured by Hansatech Instruments). Typically, 500 mg of monolithic gel pieces were added to 1 ml of Murashige and Skoog medium (MS medium). The activity of the plant cell suspension was measured just before immobilization and was taken as the reference (100%). Cell viability was determined by gently crushing hybrid gels on the surface of a MS agar medium. The ability of encapsulated cells to grow and form so-called callus tissues (unorganized plant tissues) was used as an indicator of cell viability.

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 samples were mounted on a conductive carbon tape and sputter coated with a thin Au/Pd layer in an Ar atmosphere prior to the measurement. To characterize the element distribution within the PSi structure, energy-dispersive X-ray analyses (EDX) were also performed using a RÖNTEC XFlash Detector 3001 with a silicon drift detector (SDD).

Specular Reflectometry

Reflectivity spectra were recorded with a Bruker 66 V Fourier Transform Infrared spectrometer using the Bruker A 510, 11° specular reflection unit. The PSi samples were illuminated with the tungsten source, and the reflected beam was

detected with the silicon diode detector. The resulting spectrum was captured in the range of 25 000−9000 cm^{-1} after each modification step of the porous silicon. It was the average of 100 scans and had a spectral resolution of 2 cm^{-1}.

Optical Spectroscopy

Flash-induced absorption changes were measured at 430 and 860 nm by the single-beam kinetic spectrophotometer of local design. In the case of the PSi samples there was a geometrical modification to allow its operation in reflection mode instead of transmission mode. In this case the measuring light beam was incident on the surface of the RC functionalized PSi sample at 45° and reflected toward the photomultiplier tube detector (Hamamatsu R928). For activation, the beam of a Xe flash lamp (EG&G FX200, $t_{1/2}$= 8.5 μs) was projected perpendicularly on the sample surface through an optical fiber. At 860 nm, the redox state of the P/P^+ couple can be monitored without a substantial contribution by the other cofactors of the RC. The oxidation of horse heart cytochrome c (Sigma) by RCs bound to PSi was measured at 550 nm in transmission mode. The PSi sample containing the bound RCs was placed in a 1 cm × 1 cm spectroscopic cuvette containing buffer solution (10 mM TRIS, 100 mM NaCl, 0.03% LDAO, pH 8.0) next to its rear wall facing the Xe flash beam, thereby avoiding the perpendicular measuring light beam. A series of actinic Xe flashes were applied with a repetition rate of 1 Hz. The amount of cytochrome c oxidized by the RCs was determined using the difference in the extinction coefficient $\varepsilon_{red} - \varepsilon_{ox} = 21.1 \pm 0.4$ mM^{-1} cm^{-1}. Cytochrome c was reduced by ascorbate before the experiments. For the reconstitution of the quinone electron-acceptor side, water-soluble UQ_0 (2,3-methoxy-5-methyl-1,4-ubiquinone, Sigma) was used.

New Results

1. I managed to immobilize *Arabidopsis thaliana* plant whole cells in silica gel matrix using sol-gel method. The cells preserved their activity even after immobilization and they were able to produce oxygen. (Meunier et al., Langmuir, 2010)

2. I prepared bio-nanocomposites by the chemical or physical immobilization of photosynthetic reaction centre (RC) purified from *Rhodobacter sphaeroides* purple bacteria. The carrier matrices were conducting or semiconducting materials.

2.1. I bound photosynthetic reaction centre chemically to amine-functionalized single walled carbon nanotubes (SWCNT) using glutaraldehyde crosslinker molecule. (Boldog et al., Physica Status Solidi B, 2013)

a) Morphological characterization (AFM, SEM) showed that there is a monolayer RC coverage on the SWCNT surface.

b) According to the flash-photolysis measurements the immobilized RCs keep their photoactivity even after the binding procedure.

c) After photoexcitation the life time of the uprising oxidized chlorophyll dimer (P^+) increases significantly in the complex (τ=1200 ms and 3100 ms in solution, detergent micelle and bound to single walled carbon nanotube).

d) The increase of the life time can be explained by the effect of the carbon nanotubes. SWCNT can have effect on the stabilization of the charge pair inside the RC.

2.2. I bound photosynthetic reaction centre to porous silicon wafers chemically – with glutaraldehyde crosslinker molecule, and supported with a specific peptide molecule as well. (Hajdu et al., Langmuir, 2012; Hajdu et al., Nanoscale Research Letters, 2012)

a) Morphological characterization (AFM, SEM) and optical reflection measurements showed that the RCs were bound successfully into the PSi's porous and preserved their photoactivity after both binding procedure.

b) RC binding had a saturation kinetics that could be characterized by the shift of the specific modus in the reflection spectra.

c) In both case, the half of the saturation maximum was reached at about 3 μM RC concentration, however the shift of the reflection spectra was always bigger with the "peptide method" ($\Delta\lambda max$ = 59(±5) and 38(±8) nm using the "peptide" and "GTA method")

d) Flash photolysis measurements showed that the RC remained active after the immobilization on the PSi surface. After photoexitation the relaxation time of the oxidized chlorophyll dimer (P^+) had one component in the case of the "peptide method" (τ=27 ms), since using the "GTA method" there were two components (τ_1 =14 ms, τ_2 =230 ms).

e) After series of saturating flash excitation cytochrome oxidation can be measured. It was possible to block this mechanism by using terbutrin indicating that the donor and acceptor sides of the RC are accessible after immobilization.

Publication list

a, Full papers that the thesis is based on

1, Meunier, C. F., Rooke, J. C., Hajdu, K., Cutsem, P. V., Alexandre, P. C., Léonard, A., Su, B. L. (2010) Insight into cellular response of plant cells confined within silica-based matrices, Langmuir, 26, 6568–6575. IF: 4,19

2, Hajdu, K., Gergely, Cs., Martin, M., Cloitre, T., Zimányi, L., Tenger, K., Khoroshyy, P., Palestino, G., Agarwal, V., Hernádi, K., Németh, Z., Nagy, L. (2012) Porous silicon/photosynthetic reaction center hybrid nanostructure, Langmuir, 28, 32, 11866–11873. IF: 4,19

3, Hajdu, K., Gergely, Cs., Martin, M., Zimányi, L., Agarwal, V., Palestino, G., Hernádi, K., Németh, Z., Nagy, L. (2012) Light-harvesting bio-nanomaterial using porous silicon and photosynthetic reaction center, Nanoscale Research Letters, 7:400. IF: 2,52

4, Boldog, P., Hajdu, K., Magyar, M., Hideg, É., Hernádi, K., Horváth, E., Magrez, A., Nagy, K., Váró, Gy., Forró, L., Nagy, L. (2013) Carbon nanotubes quench singlet oxygen generated by photosynthetic reaction centers, Physica Status Solidi B, 12, 2539–2543. IF: 1,49

b, Other full papers

1, Hajdu, K., Szabó, T., Magyar, M., Bencsik G., Németh, Z., Nagy, K., Magrez, A., Forró, L., Váró, Gy., Hernádi, K., Nagy, L. (2011) Photosynthetic reaction center protein in nano structures, Phys. Status Solidi B, 1–4, / DOI 10.1002/pssb.201100046. IF: 1,49

2, Magyar, M., Hajdu, K., Szabó, T., Hernádi, K.,. Dombi, A., Horváth, E., Forró, L. Nagy, L. (2011) Long term stabilization of reaction center protein photochemistry by carbon nanotubes, Phys. Status Solidi B, 248, No.11, 2454–2457, DOI 10.1002/pssb.201100051. IF: 1,49

3, Szabó, T., Bencsik, G., Magyar, M., Visy, Cs., Gingl, Z., Nagy, K., Váró, Gy., Hajdu, K., Kozák., G., Nagy, L. (2013) Photosynthetic reaction centre/ITO hybrid nanostructure, Materials Science and Engineering C, 33, 769-773. IF: 2,59

4, Magyar, M., Hajdu, K., Szabó, T., Endrődi, B., Hernádi, K., Horváth, E., Magrez, A., Forró, L., Visy, Cs., Nagy, L. (2013) Sensing hydrogen peroxide by carbon nanotube/horseradish peroxidase bio-nanocomposite, Phys. Status Solidi B, 12, 2559–2563. IF: 1,49

c, Published conference abstracts

1, Hajdu, K., Gergely, Cs., Martin, M., Zimányi, L., Agarwal, V., Palestino, G., Leza, D. S., Hernádi, K., Németh, Z., Nagy, L. (2011) Porous silicon/photosynthetic reaction center hybrid nanostructure, 2011. Eur Biophys J., 40(1), 35–241, 518.

2, Magyar, M., Hajdu, K., Hernádi, K., Horváth, E., Magrez, A., Nagy, K., Váró, Gy., Forró, L., Nagy, L. (2011) Photosynthetic reaction center/carbon nanotube hybrid nanostructures, 2011 Eur Biophys J., 40 (1), 35–241, 526.

d, Invited lectures

1. Hajdu K. (2011) Photosynthetic reaction center/porous silicon hybrid nanostructure, SH_7_2_20 workshop, Szeged, Hungary, 28-30. September 2011.
2. Hajdu K. (2012) Porous silicon-photosynthetic reaction centers nanostructure, Photosynthetic proteins for technological applications: biosensors and biochips (PHOTOTECH), Joint Working Groups Meeting, Bukarest, Romania, 11-12. May 2012.
3. Hajdu K. (2013) Detection of singlet oxygen generated by reaction centre, (PHOTOTECH) First Plenary Workshop COST Action TD1102, Antwerpen, Belgium, 10-12. June 2012.

4. Hajdu K. (2013) Detection of singlet oxygen generated by reaction center, SNSF Swiss National Science Foundation Valorization Meeting, Szeged, Hungary, 5-8. June 2013.
5. Hajdu K. (2013) Nanotube as functional matrix for bacterial photosynthetic reaction centers, Bionanotechnology - Recent Advances, Satellite meeting to the 9th European Biophysics Congress EBSA2013, Sesimbra, Portugal, 10-13. July 2013.
6. Hajdu K., Márquez León J., Cloitre T., Martin M., Agarwal V., Nagy L., Palestino G., Gergely C., Zimányi L. (2013) Optical and electronic properties of biomolecule-functionalized porous silicon photonic structures, IMRC International Materials Research Congress, Cancun, Mexico, 11-15. August 2013.
7. Ramarkrishnan S. K., Estephan E., Martin M., Cloitre T., Hajdu K., Nagy L., Zimányi L., Palestino G., Agarwal V., Gergely C. (2013) Comprehensive study on porous silicon microcavities' functionalization with peptides for biosensing, IMRC International Materials Research Congress, Cancun, Mexico, 11-15. August 2013.