DOCTORAL THESIS

REDOX PROTEINS IN BIONANOCOMPOSITES

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Introduction

The most abundant energy source of humanity is solar energy. Photosynthesis is the process by which it is converted into stored energy in the form of reduced carbon by plants, algae, and cyanobacteria. The energy demands of earth has been supported by this process since the beginning of life and through the use of fossil fuels derived from ancient plant material continues to fuel the increasing consumption. In recent years the over-exploitation of this stored energy has resulted in numerous problems such as climate change, environmental pollution, political conflicts, and inadequate division of the supply. Huge research efforts have been focused on providing new routes for converting and storing of alternative energy. One of the most promising avenues is solar energy because of its abundance, availability, and safety. Since the discovery of the photoelectric effect researchers have worked on methods which help efficiently capture and convert sunlight into usable and storable energy.

On the basis of their efficiency, sensitivity and specificity biological systems are today in the focus of emerging research technologies, although, these work with great efficiency only in their natural environment. Thus, the main challenge is to fabricate an artificial environment for them where they can preserve their activity. The main advantage when nano- and bio-materials are associated is that, by combining their advantageous properties, the new (bio-nano) material exhibit exceptionally new characteristics with (perhaps predictable) designable parameters. Promising technical applications, such as in nano(molecular)electronics as integrated optical devices, diodes, biosensors, and memory bits have opened up new directions for devices based on bio-matter (bio-nanocomposites).

Photosynthetic reaction centre (RC) is a pigment protein complex, a redox-active enzyme, which uses sunlight to power a chain of intraprotein electron transport events that leads to the separation of electrical charges. Although its size is only a few nanometers, and the energy converted by the protein is also on nanoscale, the present form of life in Earth depends on this protein complex. The quantum efficiency of the primer charge separation in the RC is almost 100% and it owns characteristic light-absorption in the near-infrared.

During my research work I have bound photosynthetic reaction centre to carbon nanotubes (functionalized, non-functionalized, single-walled, multiwalled) and conductive indium tin oxide (ITO) by different chemical and physical methods. These carrier matrices own exceptional physical characteristics, like biocompatibility, specific surface area, electric and mechanical properties, etc. The photochemical/-physical processes and the stability of the complexes were investigated by optical spectroscopy measurements.

Based on the experiments gained from the research of RC complexes, I have also worked with other redox protein, like horseradish peroxidase. Peroxidases can oxidize hydrogen atoms and for example xenobiotics in the presence of H_2O_2. It is the most commonly used enzyme in understanding the biological behaviour of catalysed oxidation of H_2O_2. I have bound the enzyme to multiwalled carbon nanotubes and ITO to create a biosensor which allows real-time sensing and the limit of detection of H_2O_2 is few picomoles. I investigated the fluorescence and absorption kinetics and electrochemical properties of the complex.

Aims

1. Investigation and optimization of conditions (temperature, pH applied during preparation, incubation time) affecting the stability of SWCNT/RC complexes made by physical binding.
2. Working out such chemical binding methods which are adaptable to our laboratory circumstances.
    - 2.1. Chemical binding of photosynthetic reaction centre to amino- and carboxyl functionalized multiwalled carbon nanotubes. Morphological (EM) and functional (flash photolysis) characterization of the prepared MWCNT/RC complexes.
    - 2.2. Chemical binding of MWCNT/RC complex to the surface of ITO, structural characterization (SEM) of the complexes and determination of the photochemical/-physical activity (flash photolysis) of the RC after the different binding methods.

3. Determination of the enzyme activity and limit of detection of H_2O_2 of horseradish peroxidase with absorption and fluorescence measurements after the addition of guaiacol and amplex red hydrogen donors.
    3.1. Chemical binding of horseradish peroxidase to amino- and carboxyl functionalized multiwalled carbon nanotubes. Detecting the activity of the complexes by fluorescence measurements.
    3.2. Morphological characterization (AFM) of MWCNT/HRP complex and determination of the enzyme activity and limit of detection of H_2O_2 with fluorescence measurements.
    3.3. Chemical binding of MWCNT/HRP complex to the surface of ITO to create an enzyme electrode. Morphological characterization (SEM) of the electrode, determination of the enzyme activity and limit of detection of H_2O_2 with fluorescence measurements, detection of the activity by cyclic voltammetry.

Materials and methods

Preparation of biological samples and biocomposites

<u>Photosynthetic reaction centre preparation</u>

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

<u>Peroxidase enzyme preparation</u>

1 µM solution was made by dissolving horseradish peroxidase (salt free, activity 350 Unit/mg, Reanal) in distilled water.

<u>Single-walled carbon nanotube preparation</u>

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 7.0. Finally they were dried at 120 °C for 30 minutes.

Multiwalled carbon nanotube preparation

MWCNTs were synthesized in the catalytic decomposition (CCVD) of acetylene at 720 °C on catalysts supported by $CaCO_3$. For purification, raw MWCNT samples were stirred in 2 M HCl overnight, filtered and washed with distilled water acid-free.

Composite preparation

SWCNT/RC composite preparation by physical binding

500 µL RC (c ≈ 100 µM) was added to 25 µL SWCNT suspension (0.1 mg/mL) and dialyzed in PBS (0.1 M; pH 7.0) for 3 days at 4 °C, to remove the detergent. The unbound RCs were removed by series of washing and centrifugation in phosphate buffer (0.1 M; pH 7.0). The suspension was dried on a glass surface.

MWCNT/RC composite preparation by chemical binding with sulfo-SMCC crosslinker

500 µL NH_2-functionalized MWCNT were mixed with sulfo-SMCC (4.5 mM) and stirred for 2 h, then the MWCNT and RC (c ≈ 65 µM) were dialyzed in PBS (MWCNT: 0.1 M; pH 7.2; 150 mM NaCl; 0.006% LDAO; RC: 0.1 M; pH 7.0; 0.006% LDAO) for 2 h. Then the MWCNT and RC was mixed and stirred overnight at 4 °C. The unbound RCs were removed by series of washing and centrifugation in phosphate buffer (0.1 M; pH 7.2; 0.006% LDAO).

MWCNT/RC composite preparation by chemical binding with carbodiimide crosslinker

Two possible cases: 1. Carboxyl functional group of the MWCNT is activated; 2. RC's carboxyl group is activated. 1. case: 500 µL carboxyl-MWCNT (0.14 mg/mL) and 100-100 µL EDC/NHS crosslinker (0.125 M) was mixed and stirred for 2 h. The unbound EDC/NHS was removed by dialysis (2 h, PBS: 0.1 M; pH 7.0; 0.006% LDAO). Then the MWCNT and RC was mixed and stirred overnight at 4 °C. The unbound RCs were removed by series of washing and centrifugation in phosphate buffer (0.1 M; pH 7.0; 0.006% LDAO). 2. case: crosslinkers were added to the RC solution and it was bound to amino functionalized MWCNT.

MWCNT/RC composite preparation by chemical binding through nickel complex

NTA·Ni^{2+} complex were bound to carboxyl-MWCNT by EDC/NHS crosslinkers and by glutaraldehyde to amino-MWCNT. 500 µL MWCNT and the crosslinkers were mixed (EDC/NHS: 100-100 µL; 0,125 M, 2 h; GTA: 100 µl; 50%, 10 min). The unbound crosslinkers were removed by dialysis (4 h, PBS: 0.1 M; pH 8.0; 0.006% LDAO). 200 µL NTA (5 mM) and 200 µL $NiCl_2$ (10 mM) were mixed for 1 h, then added to the MWCNT suspensions and stirred for 2 h. The unbound NTA·Ni^{2+} was removed by dialysis. Then the MWCNT and RC was mixed and stirred for 1 h at 4 °C. The unbound RCs were removed by series of washing and centrifugation.

Chemical binding of MWCNT/RC composite to the surface of ITO

ITO covered glass was cleaned by acetone and ethanol, then functionalized by silane monolayer (3-Mercaptopropyl)trimethoxy-silane and (3-Aminopropyl) triethoxysilane to create thiol and amino groups on it, respectively. The amino- and carboxyl functional groups of MWCNTs were activated with sulfo-SMCC or EDC/NHS. MWCNTs were deposited on the surface of the ITO for 2 h and washed intensively. The RCs were fixed on the MWCNTs with EDC/NHS by depositing them on the surface for 2 h, at 4°C. After this immobilization procedure the electrode was washed intensively with phosphate buffer (0.1 M; pH 7.0; 0.006% LDAO) and distilled water several times.

Chemical binding of MWCNT/RC composite to the surface of ITO through conducting polymer

Amino functionalized MWCNTs (0.14 mg/mL) were mixed with PTAA solution (1 mg/mL in 0.1 M PBS, pH 8.0) and incubated for 2 h at room temperature. PTAA–MWCNT complexes were separated by ultracentrifuge, then washed with PBS (0.1 M; pH 8.0). RC was added to the PTAA/MWNT complex at a final concentration of 65 µM and the detergent was dialyzed out. Finally the RC/PTAA/MWCNT complex was separated by ultracentrifuge.

ITO covered glass was cleaned by acetone and ethanol, then it was immersed into phosphate buffer (0.1M, pH 8.0) for 5min and into PDDA solution (1.9 mM; pH 1.0 HCl) for 10 min. Following this pre-treatment the electrode was immersed into PTAA/MWCNT/RC solution for 15 min.

MWCNT/HRP composite preparation

500 μL carboxyl-MWCNT were mixed with 100-100 μL EDC/NHS (0,125 M) and stirred for 2 h. The unbound EDC/NHS was removed by dialysis (2 h, PBS: 0.1 M; pH 6.0; 0.006% LDAO). Then the MWCNT and HRP (1 mg/mL) was mixed and stirred overnight at 4 °C. The unbound HRP was removed by series of washing and centrifugation in phosphate buffer (0.1 M; pH 7.0).

ITO electrode functionalized by MWCNT/HRP complex -

ITO covered glass was cleaned by acetone and ethanol, then functionalized by silane monolayer (3-Aminopropyl)triethoxysilane to create amino groups on it. The carboxyl functional groups of MWCNTs were activated with EDC/NHS. MWCNTs were deposited on the surface of the ITO for 2 h and washed intensively. HRP was fixed on the MWCNTs with EDC/NHS by depositing them on the surface for 2 h, at 4°C. After this immobilization procedure the electrode was washed intensively with phosphate buffer (0.1 M; pH 7.0) and distilled water several times.

New Results

1. I bound photosynthetic reaction centre protein purified from *Rhodobacter sphaeroides* purple bacteria to non-functionalized single walled carbon nanotube by physical binding, then I investigated the conditions (temperature, pH applied during preparation, incubation time) affecting the stability of SWCNT/RC complexes. [**Magyar et al., Phys. Status Solidi B, 2011**]

   I determined that

   1.1 according to the flash-photolysis measurements the immobilized RCs keep their photoactivity even after the binding procedure for several months;

   1.2 the contribution and lifetime of the slow component of the RC's light-induced absorption change show faster decay as a function of time than in the case of SWCNT/RC complexes;

   1.3 at 4 °C, both the contribution and the lifetime of the slow phase is more stable as compared to the case of room temperature.

2. I adapted different chemical binding strategies to our laboratory circumstances for the binding of RCs to functionalized carbon nanotubes.

Conclusions:

2.1 I bound photosynthetic reaction centre chemically to amino-functionalized multiwalled carbon nanotubes using EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) and NHS (N-hydroxysuccinimide) crosslinker molecules. [**Hajdu et al., Phys. Status Solidi B, 2011**]

a) Morphological characterization (TEM) showed that there is a multilayer RC coverage on the MWCNT surface.

b) Steady state absorption spectra proved that there is a dramatic change in the environment of the donor within the protein.

c) The change in the environment of the donor indicates that there is a dramatic change in the electrostatic interactions around the bacteriochlorophyll monomer and dimer within the protein after the binding to the MWCNTs.

d) According to the flash-photolysis measurements the immobilized RCs keep their photoactivity even after the binding procedure, but the lifetime of the slow component of the charge recombination shows a considerable decrease (τ_{slow} = 368 ms) compared to the case in detergent solution (τ_{slow} = 1200 ms).

2.2 I prepared bio-nanocomposites by the chemical binding of RCs to amino- and carboxyl-functionalized multiwalled carbon nanotubes using different crosslinkers (EDC, NHS, sulfo-SMCC). According to the flash-photolysis measurements the immobilized RCs keep their photoactivity even after the binding procedures. [**Nagy et al., Current Protein and Peptide Science, 2014**]

2.3 I prepared MWCNT/RC composites through nickel complex, in which I labelled the amino- and carboxyl-functionalized multiwalled carbon nanotubes with the complex of nitrilotriacetic acid and nickel ($NTA{\cdot}Ni^{2+}$), then I bound the polyhistidine labelled RC to it. [**Nagy et al., Current Protein and Peptide Science, 2014**]

3 I created electrodes by the binding of MWCNT/RC complexes onto the surface of ITO carriers, which can be then applied in electrochemical cells.

3.1 I bound photosynthetic reaction centre chemically by EDC/NHS crosslinkers to the amino-functionalized MWCNT immobilized on the surface of the silanized ITO by sulfo-SMCC crosslinker. [**Szabó et al., Phys. Status Solidi B, 2015**]

a) According to the electrochemical cell measurements, I established that the RC bound to the ITO/MWCNT electrode keeps its redox activity even after the binding procedure.

3.2 I bound RCs to the amino-functionalized MWCNTs through PTAA conductive polymer, then I immobilized this complex on the surface of the PDDA electrolyte covered ITO. [**Szabó et al., Phys. Status Solidi B, 2012**]

Conclusions:

a) According to the flash-photolysis measurements the immobilized RCs keep their photoactivity even after the binding to the ITO.
b) The efficiency of the binding was highly affected by the detergent concentration. The absence of detergent indicates homogenous, the presence of it indicates heterogeneous binding.
c) Light-induced photocurrent could be measured in electrochemical cell.

4 I determined the enzyme activity and the limit of detection of H_2O_2 of horseradish peroxidase enzyme by absorption kinetic and fluorescence measurements, using guaiacol hydrogen donor. [**Magyar et al., Phys. Status Solidi B, 2013]**

Conclusions:

4.1 According to the calibration made from the absorption kinetic and fluorescence measurements belonging to the same enzyme concentrations, the limit of detection of H_2O_2 of horseradish peroxidase is 124 nM H_2O_2 s^{-1}.

4.2 According to the calibration made from the absorption kinetic and fluorescence measurements belonging to the same enzyme concentrations, the enzyme activity of horseradish peroxidase is 7 M [H_2O_2]/(M [HRP]· sec).

5 I bound horseradish peroxidase chemically to carboxyl-functionalized multiwalled carbon nanotubes using EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) and NHS (N-hydroxysuccinimide) crosslinker molecules. [**Magyar et al., Phys. Status Solidi B, 2013**]

Conclusions:

5.1 According to fluorescence measurements made by the addition of guaiacol the immobilized HRP keeps its enzyme activity even after the binding and its active centre stays accessible to the substrate.

5.2 According to the calibrations made by the HRP solution, the limit of detection of H_2O_2 of the MWCNT/HRP complex is 9,6 pM H_2O_2 s^{-1}.

6 I bound horseradish peroxidase chemically by EDC/NHS crosslinkers to the carboxyl-functionalized MWCNT immobilized on the surface of the silanized ITO by the same crosslinkers. [**Magyar et al., Phys. Status Solidi B, 2013**]

6.1 I determined that in electrochemical cell measuring cyclic voltammograms, the ITO/MWCNT/HRP enzyme electrode shows direct electron transfer at -350mV which is characteristic to H_2O_2 reduction. The results show that electron transfer can be facilitated between the active sites of the enzyme and the surface of the electrode and HRP keeps its enzyme activity.

Publication list

a, Full papers that the thesis is based on

1. **M. Magyar**, K. Hajdu, T. Szabó, K. Hernádi, A. Dombi, E. Horvath, L. Forró, L. Nagy (2011) Long term stabilization of reaction center protein photochemistry by carbon nanotubes, Phys. Status Solidi B, 248, No.11, 2454–2457.
   IF= 1.316
2. K. Hajdu, T. Szabó, **M. Magyar**, G. Bencsik, Z. Németh, K. Nagy, A. Magrez, L. Forró, Gy. Váró, K. Hernádi, L. Nagy (2011) Photosynthetic reaction center protein in nanostructures, Phys. Status Solidi B, 249, No.12, 2700–2703.
   IF= 1.316
3. T. Szabó, **M. Magyar**, Z. Németh, K. Hernádi, B. Endrődi, G. Bencsik, Cs. Visy, E. Horváth, A. Magrez, L. Forró, L. Nagy (2012) Charge stabilization by reaction center protein immobilized to carbon nanotubes functionalized by amine groups and poly(3-thiophene acetic acid) conducting polymer, Phys. Status Solidi B, 248, No.10, 2386–2389.
   IF= 1.489
4. **M. Magyar**, K. Hajdu, T. Szabó, B. Endrődi, K. Hernádi, E. Horváth, A. Magrez, L.Forró, Cs. Visy, L Nagy (2013) Sensing hydrogen peroxide by carbon nanotube/horse radish peroxidase bio-nanocomposite, Phys. Status Solidi B, 250, No.12, 2559–2563.
   IF= 1.605
5. L. Nagy, **M. Magyar**, T. Szabó, K. Hajdu, M. Dorogi, L.Giotta, F. Milano (2014) Photosynthetic Machineries in Nano-Systems, Special Issue: “Sensors and transducers in the landscape of photosynthesis”, Current Protein & Peptide Science, 15, No. 4, 363-373.
   IF= 2.328
6. T. Szabó, E. Nyerki, T. Tóth, R. Csekő, **M. Magyar**, E. Horváth, K. Hernádi, B. Endrődi, Cs. Visy, L. Forró, L Nagy (2015) Generating photocurrent by nanocomposites based on photosynthetic reaction centre protein, Phys. Status Solidi B (accepted)
   IF= 1.61

b, Other full papers

1. T. Szabó, G. Bencsik, **M. Magyar**, Cs. Visy, Z. Gingl, K. Nagy, Gy. Váró, K. Hajdu, G. Kozák, L. Nagy (2013) Photosynthetic reaction centre/ITO hybrid nanostructure, Materials Science and Engineering C, 33, 769-774.
   IF= 2.736
2. P. Boldog, K. Hajdu, **M. Magyar**, É. Hideg, K. Hernádi, E. Horváth, A. Magrez, K. Nagy, Gy. Váró, L. Forró, L. Nagy (2013) Carbon nanotubes quench singlet oxygen generated by photosynthetic reaction centers, Phys. Status Solidi B, 250, No.12, 2539–2543.
   IF= 1.605
3. L. Nagy, K. Hajdu, Sz. Torma, S. Csikós, T. Szabó, **M. Magyar**, D. Fejes, K. Hernádi, M. Kellermayer, E. Horváth, A. Magrez, L. Forró (2014) Photosynthetic reaction centre/carbon nanotube bundle composites, Phys. Status Solidi B, 251, No.12, 2366–2371.
   IF= 1.605
4. I. Husu, **M. Magyar**, T. Szabó, B. Fiser, E. Gómez-Bengoa, L. Nagy (2015) Structure and binding efficiency relations of QB site inhibitors of photosynthetic reaction centres, Gen. Physiol. Biophys. 34, 119–133.
   IF=0.88
5. L. Nagy, V. Kiss, V. Brumfeld, K. Osvay, Á. Börzsönyi, **M. Magyar**, T. Szabó, M. Dorogi, S. Malkin (2015) Thermal effects and structural changes of photosynthetic reaction centres characterized by wide frequency band hydrophone: Effect of carotenoids and terbutryne, Photochemistry and Photobiology (in press)
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6. G.P. Szekeres, K. Németh, A. Kinka, **M. Magyar**, B. Réti, E. Varga, Zs. Szegletes, A. Erdőhelyi, L. Nagy, K. Hernádi (2015) Controlled nitrogen doping and carboxyl functionalization of multi-walled carbon nanotubes, Phys. Status Solidi B (in press)
   IF=1.61
7. T. Szabó, **M. Magyar**, K. Hajdu, M. Dorogi, E. Nyerki, T. Tóth, M. Lingvay, Gy. Garab, K. Hernádi, L. Nagy (2015) Structural and functional hierarchy in

photosynthetic energy conversion - from molecules to nanostructures, Nanoscale Research Letters (accepted)
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c, Published conference abstracts

1. **M. Magyar**, K. Hajdu, K. Hernádi, E. Horváth, A. Magrez, K. Nagy, Gy. Váró, L. Forró, L. Nagy (2011) Photosynthetic reaction center/carbon nanotube hybrid nanostructures, 2011 Eur Biophys J., 40 (1):35–241, 526.
   IF=2.139
2. K. Hajdu, T. Szabó, D. Fejes, **M. Magyar**, Zs. Szegletes, Gy. Váró, E. Horváth, A. Magrez, K. Hernádi, L. Forró, L. Nagy (2013) Carbon nanotube as functional matrix for bacterial photosynthetic reaction centers, Eur. Biophys J., 42 (1):S1-236, 408.
   IF= 2.474
3. **M. Magyar**, T. Szabó, B. Endrődi, K. Hajdu, Cs. Visy, Zs. Szegletes, Gy. Váró, E. Horváth, A. Magrez, K. Hernádi, L. Forró, L. Nagy (2013) Photocurrent generated by photosynthetic reaction centers/carbon nanotube/ITO bio-nanocomposite, Eur. Biophys J., 42. (1):S1-236, 411.
   IF= 2.474

d, Invited lectures

1. **Melinda Magyar -** “Photosynthetic reaction center/carbon nanotube hybrid nanostructures”
   Swiss Contribution 7/2, Annual Progress Report, SZAB Székház, Szeged, 28 September 2011.
2. **Melinda Magyar** - “Strategies to bind photosynthetic reaction centers to nano-systems”
   PHOTOTECH: Photosynthetic proteins for technological applications: biosensors and biochips – First Plenary Workshop COST Action TD1102, Antwerpen, Belgium, 10-12, June, 2012.
3. **Melinda Magyar** - “Strategies to bind redox proteins to nanosystem”
   SNSF Swiss National Science Foundation Valorization Meeting, Szeged, 5-8, June, 2013.

4. **Melinda Magyar** - “Photocurrent generated by photosynthetic reaction centers/carbon nanotube/ITO bio-nanocomposite”
   Bionanotechnology - Recent Advances, Satellite meeting to the 9th European Biophysics Congress EBSA2013, Sesimbra, Portugal, 10-13, July, 2013.
5. **Melinda Magyar** - “Strategies to bind photosynthetic reaction centres to carbon nanotubes” (Hungarian)
   XXIV. Congress of the Hungarian Biophysical Society, Veszprém, Hungary, 27-30, August, 2013.
6. **Melinda Magyar -** “Redox proteins in carbon nanosystems”
   Swiss Contribution 7/2, Final Report, Lausanne, Switzerland, 29 March – 1 April, 2015.
7. **Melinda Magyar** - “Photosynthetic reaction center protein optoelectronics in nano-hybrid systems” (Hungarian)
   Hungarian Chemical Society 2. National conference, Hajdúszoboszló, Hungary, 31 August – 2 September, 2015.