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Photoactive film by covalent immobilization of a bacterial photosynthetic protein on reduced graphene oxide surface

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ABSTRACT
Covalent functionalization of reduced graphene oxide (rGO) with the photosynthetic reaction center (RC) from *Rhodobacter sphaeroides* R26 *via* click chemistry reaction has been performed. The hybrid system was characterized by flash photolysis and infrared spectroscopy and the RC was found to retain its photoactivity and structural integrity. The strategy is applicable for the fabrication of hybrid bio-electronic devices capable of absorbing and converting solar energy.

INTRODUCTION
Photosynthesis can be considered as one of the most important biological reactions in the biosphere, since all life on Earth, directly or indirectly, depends on it as a source of energy. Nature performs the photosynthetic process using specialized protein pigments complexes organized to ensure up to 98% conversion of the absorbed photons in stable, long-living charge-separated states [1]. A proper combination of the photosynthetic core protein, the reaction center (RC), and engineered materials, i.e. metals or inorganic semiconductor electrodes, attracts great attention to build hybrid devices for solar energy conversion [2, 3]. The class of carbon-based electrodes, composed by bi-dimensional layers of sp^2-hybridized carbon atoms, has intriguing properties: high charge-carrier mobility, high elasticity, large thermal conductivity (~3000 $Wm^{-1}K^{-1}$ in plane) and elevated transparency over the entire visible spectrum. Nanostructured carbon materials, such as carbon nanotubes (CNTs) or graphene films have been employed in association with the RCs, looking for potential devices for efficient solar energy conversion [4-6].
A tight and efficient association of a biological macromolecule to an exogenous material requires a functional controlled combination of the two components of the system which is hard to attain and may eventually result detrimental even for a sturdy membrane protein like RCs. A possible strategy is exploitation of the hydrophobic interaction to drive the RCs on the surface of a graphene electrode by simple physisorption [5]. This approach has two main disadvantages: the loose interaction between RCs and graphene and the absence of control of the protein

orientation. Covalent binding of the protein to the electrode surfaces would ensure a more stable and a correct orientation of the protein. Lysine or cysteine aminoacids represent the proper sites for anchoring the protein *via* suitable linker molecules, such as carboxylic N-succinimidyl ester (NHS) for lysines and maleimide compounds for cysteines. This strategy has been recently pursued by Milano et al. to bind an *ad hoc* designed and properly functionalized organic fluorophore to the lysine residues of *Rb. sphaeroides* RC [7].
Herein we propose a bioconjugation strategy to covalently bind the photosynthetic reaction center of *Rb. sphaeroides* R26 to reduced graphene oxide (rGO) [8, 9] bearing reactive groups selective for the amino groups of lysine aminoacids (Figure 1 a-b). The possibility of chemically modifying rGO surfaces makes it an attractive substrate for a wide range of applications, including bio-interfacing.
A heterogeneous-phase strategy for RC bioconjugation onto rGO is illustrated and discussed. Results show that the covalent binding is more efficient than the simple physisorption.

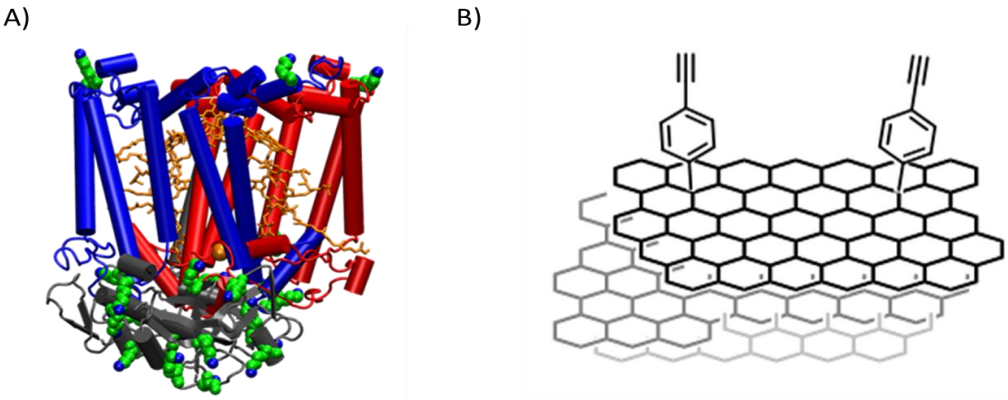

Figure 1. (A) Three-dimensional structure of the photosynthetic RC from the *Rb. sphaeroides* R26 (PDB entry: 2J8C[10]). The RC is a membrane-spanning protein composed of three subunits L, M, and H (in red, blue and black) non-covalently allocating nine cofactors (not shown): two ubiquinone-10 molecules (Q), one iron ion, two bacteriopheophitins (BPhe) and four bacteriochlorophylls (Bchl), two of which forming a functional dimer (D). The 22 lysine residues are represented as van der Waals green spheres. The terminal N_Z lysine targets of bioconjugation are in blue. (B) Reduced GO bearing phenyl ethynyl groups prepared through aryl diazonium chemistry [8, 9].

EXPERIMENTAL

Reduced GO (rGO) - ITO substrate preparation

Chemical functionalization of rGO flakes (SLG, ACS Materials) with the diazonium tetrafluoborate salt of 4-ethynylaniline was carried out according to a previous reported procedure [11, 12].
The deposition of the flakes onto ITO was obtained by spin-coating, distributing 60 μL of a 0.5 mg/mL suspension of rGO in DMF onto 1x0.8 cm substrates. To complete the removal of the organic solvent, the deposited film was subjected to an annealing procedure at 70°C for about 10 minutes.

RC isolation and purification

RC isolation from *Rhodobacter sphaeroides* strain R26 was performed following a standard procedure [13]. The reaction centre purity and integrity were checked by the absorbance ratio A_{280}/A_{802} and A_{760}/A_{865}, which were kept below 1.3 and 1 respectively.

Click chemistry strategy

To bioconjugate RC to functionalized rGO deposited on ITO, a two-step strategy was performed (Figure 2). Firstly, RC was derivatized with NHS-PEG_4-azide (Thermo Scientific™) by selectively binding the succinimidyl moiety to lysine residues. In the second step, the azide functionalization was exploited to anchor the RC to ethynyl modified graphene sheets by a 1,3 dipolar addition reaction [14].

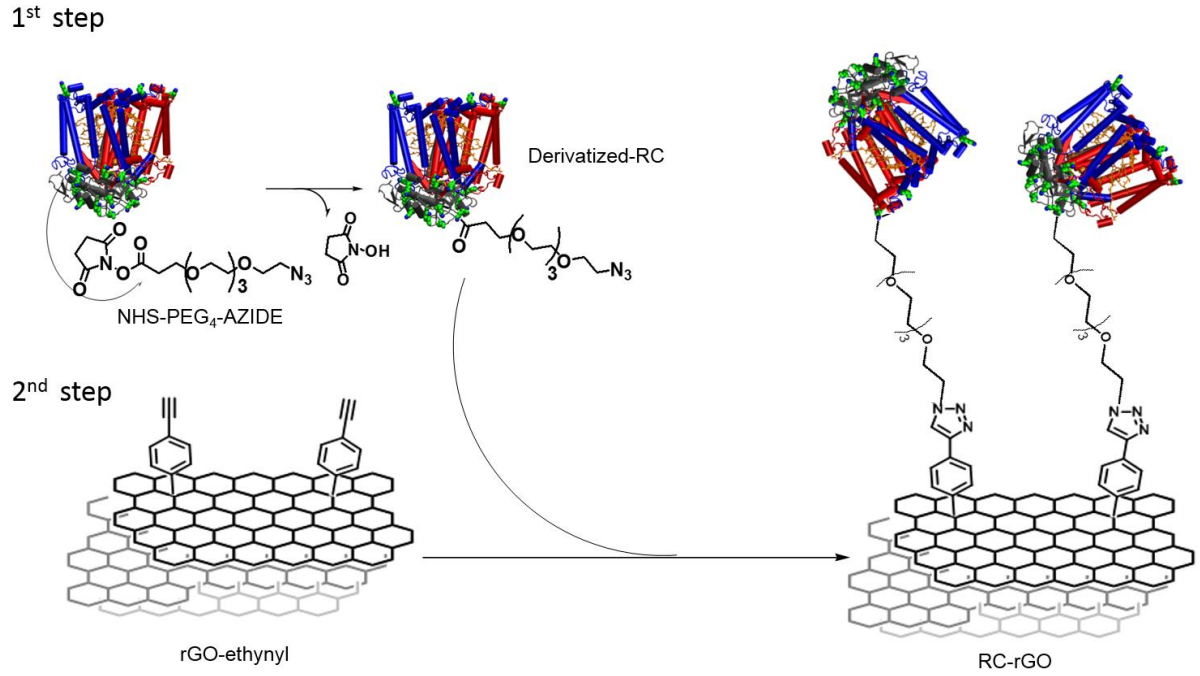

Figure 2. Two-step bioconjugation strategy of RC to functionalised rGO deposited onto ITO substrates. Lysine residues as bioconjugated targets are higlighted in green van der Waals spheres.

RC functionalization with NHS-PEG_4-azide linker

Bioconjugation of NHS-PEG_4-azide to RC was carried out by adding to a 2 µM RC solution in buffer phosphate 50 mM, KCl 100 mM and LDAO 0.025% at pH 7.2, a 10-fold molar excess of linker from a stock solution (10 mM in dry DMSO). The mixture was incubated for 1 hour in the dark. The excess of unreacted linker was removed by ultracentrifugation with 100 kDa cut-off centrifuge tubes with ultracell regenerated cellulose membrane (Millipore). Size exclusion chromatography (Sephadex G-50 – Sigma Ald.) was used to remove unreacted precursors.

RC coupling onto rGO-ITO substrates procedure

rGO/ITO substrate was immersed for 4 hours under stirring in 800 µL of 10 µM PEG_4-azide derivatized RC, 30 µM $CuSO_4 \cdot 5H_2O$ and 156 µM sodium ascorbate, followed by rinsing with deionized water.

Bioconjugated RC-rGO system characterization

The morphology of the resulting surface and structural integrity of the RC-rGO films were investigated. Retention of the protein photoactivity after the bioconjugation process was also verified.

AFM images of ITO and rGO-ITO were obtained in air in tapping mode by using a PSIA XE-100 atomic force microscope equipped with a standard 125 µm long rectangular cantilever with a force constant of 42 N/m and a nominal resonant frequency of 320 kHz. Topographic images were analyzed using the software XEI (PSIA Corp., version 1.7.3) and the cross section profiles were obtained.

Mid-infrared spectra were acquired with a Perkin Elmer Spectrum One FTIR spectrometer, equipped with a DTGS detector, with a spectral resolution of 4 cm^{-1}. A variable specular reflection device designed specifically for thin film analyses (Amplif-IR, Smiths Detection) was employed for IR measurements on rGO and RC-rGO films onto ITO substrates [15, 16]. ITO substrates were chosen for the deposition of rGO since it is a suitable IR reflecting material.

Charge recombination reactions were recorded by a kinetic spectrophotometer of local design [17]. Data were collected as 128 averages onto a Digital Oscilloscope (Tektronics TDS-3200) and trace deconvolution was performed using a locally developed C-code.

DISCUSSION

ITO substrate was chosen for the deposition of rGO as a suitable flat and reflecting material in order to perform characterization both via AFM and Amplif-IR.

ITO and rGO-ITO substrates were preliminarily characterized by atomic force microscopy in order to evaluate the morphology of the obtained graphene film.

Figure 3 shows micrographs of the ITO substrate and the spin-coated rGO film on ITO. Functionalized graphene flakes with lateral dimensions in the micrometric range are not homogeneously distributed on surface, displaying heights up to 40 nm. Despite the non-uniformity of rGOs distribution on ITO substrate, the obtained films are suitable for our purposes.

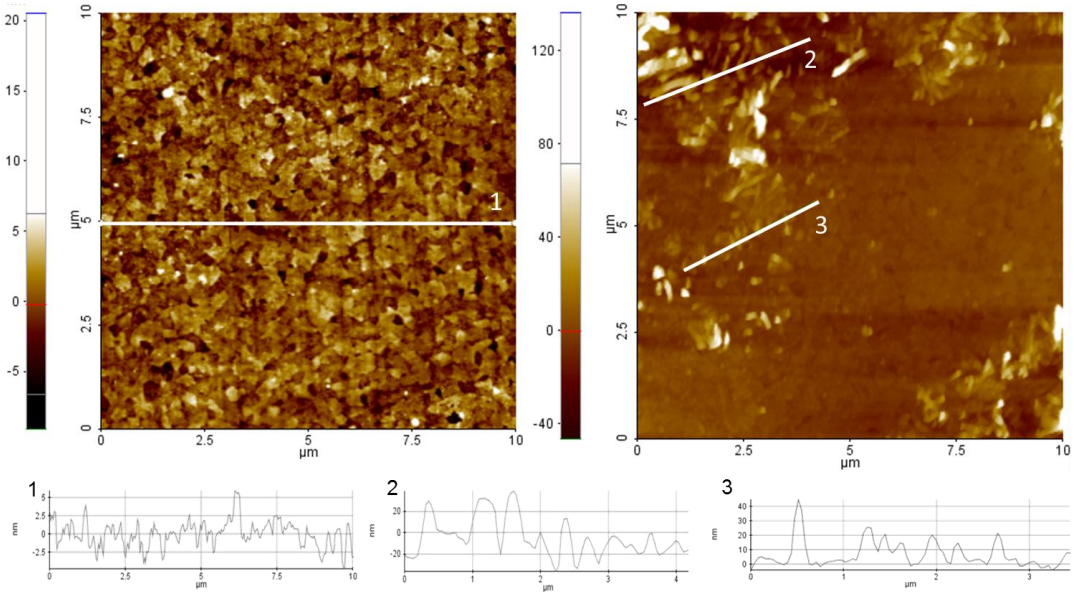

Figure 3. AFM images of ITO substrate (left) and rGO film onto ITO (right) and corresponding height-profiles.

In the two-step procedure RC is covalently bound via the lysine a.a. to a heterobifunctional linker containing a NHS moiety and an azide reacting groups at the two opposite ends of a tetraethylene glycol chain. The derivatized protein is anchored to ethynyl functionalized rGO film onto ITO substrates. The functionalization reaction is a 1,3-dipolar reaction between the azide and the ethynyl group, by a biocompatible strategy employing as catalyst Cu (I) salt reduced *in situ* by sodium ascorbate (Figure 2). Infrared spectroscopy confirmed the effective RC bioconjugation to rGO and the protein integrity, while photoinduced charge recombination kinetic was used to assess the integrity of RC photochemical activity.
Figure 4 left panel compares the IR spectra of rGO and RC-rGO adducts deposited onto ITO. In the case of the RC-rGO film, the IR spectrum reveals the typical protein fingerprint, i.e. peaks at ~1650 and ~1550 cm^{-1}, due to the amide I and amide II bands respectively [18] with the underlying contribution of rGO. For comparison, native RCs were also bound to rGO by simple physisorption. In the right panel of figure 4 the IR spectra of bioconjugated (B) and physisorbed (B) RC minus rGO are compared with the native RC casted onto ITO (A). Both spectra B and C are very similar to A, indicating that the protein is in its native state, but bioconjugated rGO-RC shows twice the intensity of physisorbed RC revealing that the proposed bioconjugation strategy allows effective immobilization of RC.

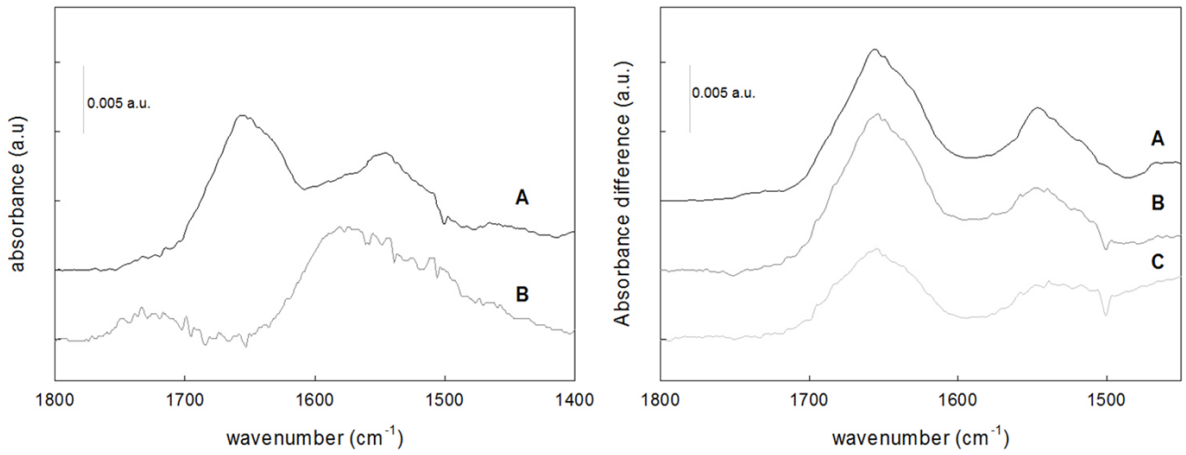

Figure 4. Left: IR spectra of RC bioconjugated to rGO on ITO (A), rGO onto ITO (B). Amide I and II characteristic RC peaks at 1655 and 1549 cm^{-1} respectively are present in spectrum A. Peak attribution in rGO (B): 1732 cm^{-1} C=O stretching; 1580 cm^{-1} skeleton aromatic ring stretching. Right: IR spectra of native RC (A), bioconjugated RC-rGO minus rGO/ITO (B) and physisorbed RC minus rGO/ITO (C). All spectra are translated in abscissa to facilitate their display.

The ability of the bioconjugated rGO-RCs to generate photoinduced charge separate states was tested by recording the formation of the electron-hole pair induced by a white light flash and its dark decay spectrophotometrically followed at 865 nm, as shown in figure 5. The charge recombination time constant in the RCs covalently anchored to rGO substrates is identical to the typical value obtained in native RCs, indicating that nor the click-chemistry reaction, nor the bioconjugation, nor the close proximity of the protein to the rGO actually jeopardizes the enzymatic activity of the protein.

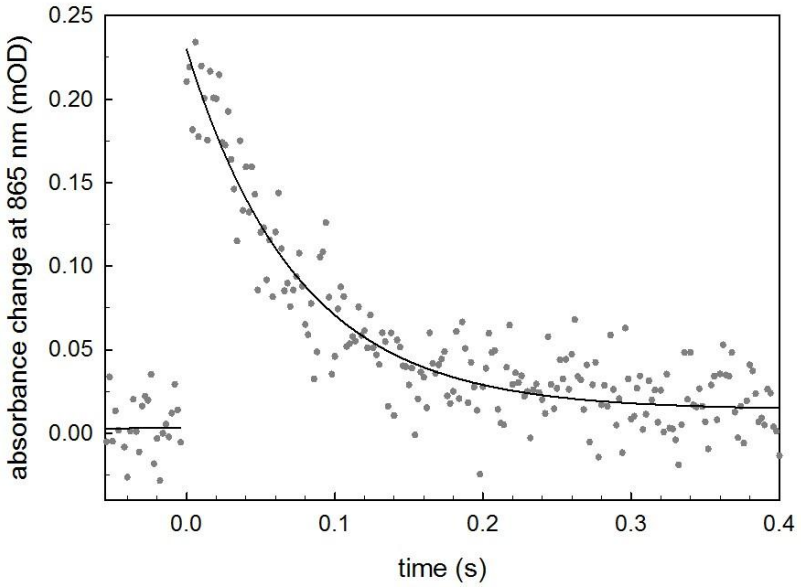

Figure 5. Time course of the charge separated state of the bioconjugated reaction centre to rGO deposited on ITO surface. The monoexponential interpolation is shown in black trace.

The fitting of the experimental data with a monoexponential curve ($A(t)=A_0e^{-kt}$) allowed to determine a kinetic constant of 10 s^{-1} in accordance with the literature values associated with the recombination kinetic from Q_A, which is one of the two possible electron-acceptor ubiquinone molecules of the RC [19]. Moreover, from the absorbance change at t_0 (A_0), using the differential molar extinction coefficient $\Delta\varepsilon_{865}$ = 105 $mM^{-1}cm^{-1}$, it is also possible to calculate the photoactivated RC concentration. This leads to determine an average surface photoactive RC concentration of $2.4 \cdot 10^{-12}$ mol/cm^2, an amount of the same order of magnitude as the surface concentration calculated for the densely packed RC monolayer ($5\ 10^{-12}$ mol/cm^2) [20].

CONCLUSIONS

A heterogeneous bioconjugation strategy for covalently anchoring bacterial RCs from *Rb. sphaeroides* R26 to suitably functionalized graphene nanoplatelets deposited onto ITO substrates is illustrated. Experimental data show that this procedure successfully binds twice as much reaction centers to reduced graphene oxide as compared to the simple physisorption technique and that can be further pursued for more efficient protein anchoring. Moreover, the procedure is mild enough to maintain functional integrity of the protein.

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