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"Garnishing" the photosynthetic reaction center for bioelectronics

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The photosynthetic reaction center is an extraordinarily efficient natural photoconverter, which can be ideally used in combination with conducting or semiconducting interfaces to produce electric signals in response to absorption of photons. The actual applicability of this protein in bioelectronic devices critically depends on the finding of (a) suitable deposition methods enabling controlled addressing and precise orientation of the protein on electrode interfaces and (b) chemical manipulation protocols able to tune and enhance the protein light absorption in specific or broader spectral regions. Literature reports several examples of approaches to fulfill these requirements, which have faced in different ways the fundamental issues of assembling the biological component and non-natural systems, such as electrode surfaces and artificial light harvesting components. Here we present a short overview of the main methods reported to accomplish both the objectives by properly "garnishing" the photosynthetic reaction center (RC) via chemical modifications.

Introduction

Photosynthetic organisms, *i.e.* plants, algae and some kinds of bacteria are widespread both on land and water, where they efficiently use sunlight to generate biomass: an estimated energy value of about 4.0×10^{21} J is converted in one year by these organisms into valuable biomolecules and molecular oxygen.[1] All life forms on Earth strictly depend on photosynthesis and today we have a quite deep knowledge of this natural process at a molecular level. Many research efforts have been inspired by photosynthesis, aiming to develop artificial machineries for efficient energy conversion.[2-5]

The photochemical core of photosynthesis is represented by a specific transmembrane multi-subunit protein, known as the reaction center, whose acronym, RC, comes from the initials of Roderick Clayton who reported, for the first time in 1968, its isolation from the purple *Rhodopseudomonas* (later reclassified as *Rhodobacter*) *sphaeroides* bacterium.[6] The RCs of photosynthetic bacteria are generally simpler and less prone to photo-oxidative degradation with respect to their counterparts in more evolved photosystems.[7, 8]

The RCs are the sole photoenzymes in the biosphere employing solar energy to generate charge separated states with almost unitary conversion efficiency.[9]

The possibility of taking advantage of this unmatched photoconversion efficiency has suggested to assemble bio-hybrid devices. In fact, implementing the complex functions of the RC into optoelectronic devices opens the way to intriguing applications ranging from electronic biosensing to alternative approaches for transducing sunlight into electrical signals or chemical energy.[10] In addition, a number of genetically engineered RCs have been expressed in photosynthetic bacteria, paving the way to their use in a variety of applications[11] and, even more interestingly, opening the fascinating possibility of massive biotechnological production of active materials for electronic devices. Chasing a robust and reliable strategy for implementing the biological photoconverters in bioelectronics is worthwhile, as the chances of realizing successful devices are real. Efforts are continuously made to improve the "connectivity" between the enzymatic (the RCs), and the non-enzymatic components of the device, as witnessed by the growing number of publications on this topic in the last ten years, which have been also recently reviewed.[12-16]

This account paper offers a rather different perspective, focusing on the key role of the RC chemical manipulation to optimize implementation of this biological photoconverter in electronic devices. In particular we will focus on selective chemical modification of the RC protein as a tool to: 1) address and orient the protein on the electrodes surfaces to optimize charge injection; 2) improve the overall photoconversion by

increasing the optical cross-section of the reaction center and synthesize hybrids outperforming the pristine RC in non-physiological environment.

An insight into the bacterial RC activity

The key processes of the bacterial photosynthesis are sketched in Figure 1, together with the role of the RC. These processes take place in the photosynthetic cell membrane, where photon energy is captured by light harvesting antenna complexes (cyan) and then transferred to the RC (green). Here a cascade of electron transfer reactions leads to the production of a fully reduced and doubly protonated ubiquinol (QH_2, purple) which diffuses in the membrane and passes its electrons to the cytochrome bc_1 complex (orange). Electrons are lastly returned to the RC by cytochrome Cyt c_2 (purple). A transmembrane proton gradient is established since quinone takes protons from the cytoplasm upon reduction and releases them in the periplasm upon oxidation, thanks to the vectorial orientation of RC and bc_1 within the membrane. This gradient is used to drive the ATP synthesis by ATPase, which fuels the biosynthetic part of the photosynthetic process (red).[17]

As shown in Figure 2, the *Rhodobacter sphaeroides* reaction center is composed of a protein portion composed by three subunits, named M, L and H (cyan, magenta and green in Figure 2a, respectively) and a series of non-covalently bound cofactors. The hydrophobic L and M subunits are structurally very similar and they are composed of five transmembrane α-helices whereas the hydrophilic H subunit is located on the cytoplasmic side of the membrane and contains one transmembrane α-helix.

The nine cofactors (Figure 2b) located within the protein scaffolding are spatially organized in two branches (A and B) and are directly responsible for the electron transfer reactions. In particular, the cofactors are: four bacteriochlorophylls a (BChl), two of which are strongly coupled to form a BChl dimer (D) located on the periplasmic side, two bacteriopheophytins (BPh) consisting of a free chlorinic ring, two ubiquinone-10 molecules (Q) and one non-heme Fe^{2+}ion.

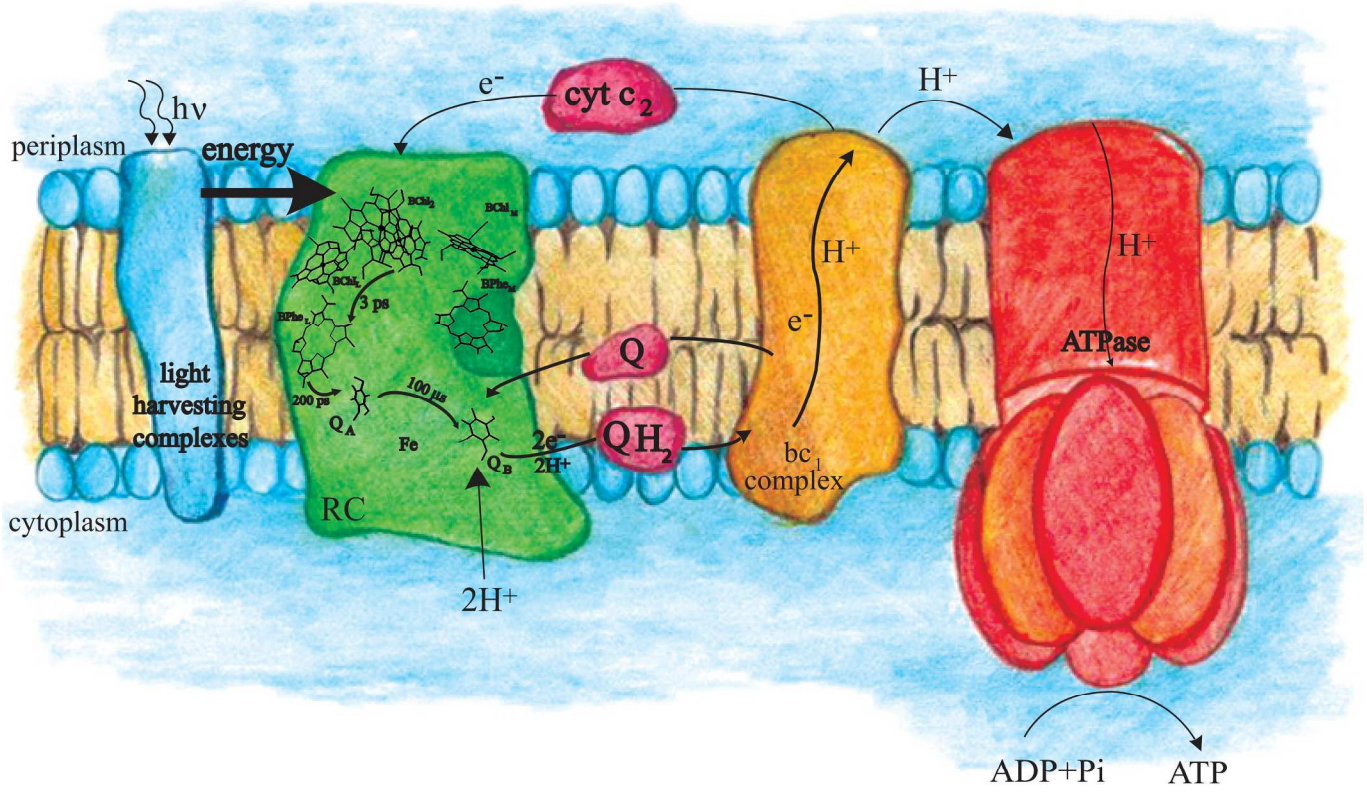

Figure 1. Representation of the membrane proteins involved in the light promoted reactions of purple bacterial photosynthesis.

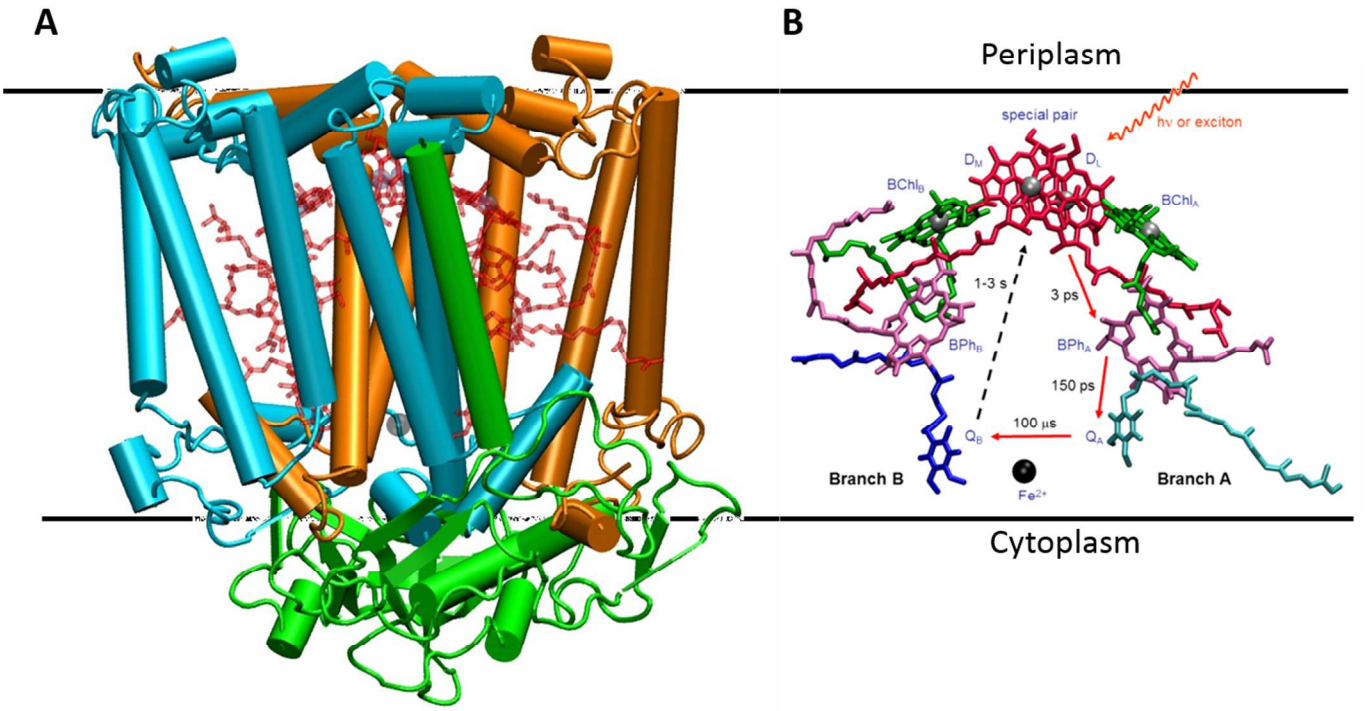

Figure 2. (a) Schematic drawing of the *R. sphaeroides* R26 reaction center. Subunits: H green, M cyan, and L brown. (b) Organization of cofactors in the *R. sphaeroides* RC, with indication of photoinduced electron transfer reactions and relevant time constants. The subscripts A and B are used to indicate cofactors located in the corresponding branches.

Light induced electron transfer within the RC consists of a cascade of reactions: upon photon absorption, the primary electron donor D is excited and very fast (3 ps) electron transfer from D* to BPh_A occurs. Then, an electron is transferred, within 150 ps, to the primary ubiquinone acceptor Q_A and, in 100 µs, to the secondary electron acceptor Q_B leading to the final $D^+Q_B^-$ charge separated state.[18, 19] This state has an intrinsic long lifetime of 1-3 s. This is a very important feature that makes the RC an extremely attractive structure for energy conversion applications.[20]

In addition, it is worth noting that RC can be easily isolated from the bacterial cultures by dissolving the cytoplasmic membranes with proper detergents. Detergent molecules confer high stability to the purified protein in aqueous solution by forming a toroid around its L and M hydrophobic subunits. The resulting surfactant protected protein in water can be easily handled, chemically modified or processed for possible applications in bioelectronic devices.

RC integration in electronic devices

Application of the photosynthetic RC protein in electronics discloses very fascinating possibilities, ranging from biosensing to elegant ways of sunlight transduction into electric signals or chemical energy[10]. So far, RCs extracted from *R. sphaeroides* have been mainly applied in the fabrication of photoelectrochemical cells, devices converting sunlight into electrical energy. In principle, the simplest photoconverter setup consists of a layer of photoactive protein molecules which, under illumination, generate electron-hole couples and inject charges into a (possibly transparent) electrode (Figure 3). This device also includes a redox couple (mediator) which ensures the neutral state regeneration and an external circuit for current collection.

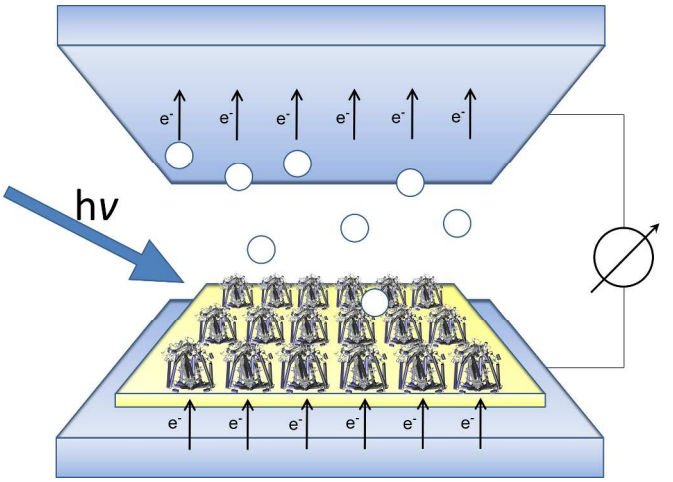

Figure 3. Ideal simplest photoconverter device formed by two electrodes (the grey platforms), one platform designed to ensure electrical conduction from the ordered RC array and the electrode (yellow platform). White circles represent the redox mediators ensuring circuit closure. Illumination generates a flow of electrons in the external circuit.

The main advantage of such biohybrid setup with respect to completely artificial devices is that the photoconverter has an efficiency close to unit, an extraordinary achievement obtained

by evolution and hardly affordable by artificial photoconverters. On the other hand, a major issue is represented by the conjugation of a biological structure with an electronic device in non-physiological environment. This requires effective strategies to stably immobilize intact functional photosystems on electrodes surfaces. In particular, the extent of photocurrent generated in photoenzyme-based devices critically depends on the quality of their immobilization, on the number of proteins anchored (whether in a uniform monolayer or multilayers), on the stability of the assembly, on proteins orientation with respect to the surface and on the effectiveness of the charge extraction process.

Stable assemblies of photosynthetic proteins on electrode surfaces can be created in different ways. Approaches driven by electrostatic interactions, physisorption and adsorption are aspecific. Chemical modifications, on the contrary, enable selective immobilization of the RC scaffold in an oriented way by, for instance, covalent linkages, affinity tags on genetically modified proteins and molecular plugging.

RC physisorption on active electrodes can be obtained by simple contact between the electrode and a protein solution. Yaghoubi *et al.* demonstrated that the protein layer proposed in this study is permeable to redox mediators such as Cyt c and ubiquinone (UQ_2). Improvement of the photoelectrode performances was observed by illumination in presence of these mediators.[21] However, this approach offers very little or no control over protein orientation on the surface.

Electrospray deposition of RC on a HOPG (highly ordered pyrolytic graphite) electrode[22] is more convenient than physisorption achieved by dip coating. In the electrospray technique (Figure 4a), a high voltage power supply is connected to a conductive needle which is attached to a syringe pump containing the protein solution. The electrode substrate is grounded and the protein solution is forced through the needle. The solution exiting from the needle is dispersed into small droplets in air, due to a high coulombic droplets mutual repulsion in the electric field imposed between the needle and the substrate. As a result, the charged droplets hit the substrate at a high velocity and are electrostatically attached to the grounded electrode. After deposition, AFM analysis of the HOPG surface revealed a grainy-textured surface coating corresponding to RCs globular aggregates, which could be distinguished from the HOPG. Remarkably, the RCs not only survived exposure to the high electric fields but also yielded peak photocurrent densities of up to 7 μA cm^{-2}, with action spectra revealing the absorption profile of RC (Figure 4d). The high currents attained from films produced by electrospray technique is a considerable advantage even if this technique does not offer any control over protein orientation.

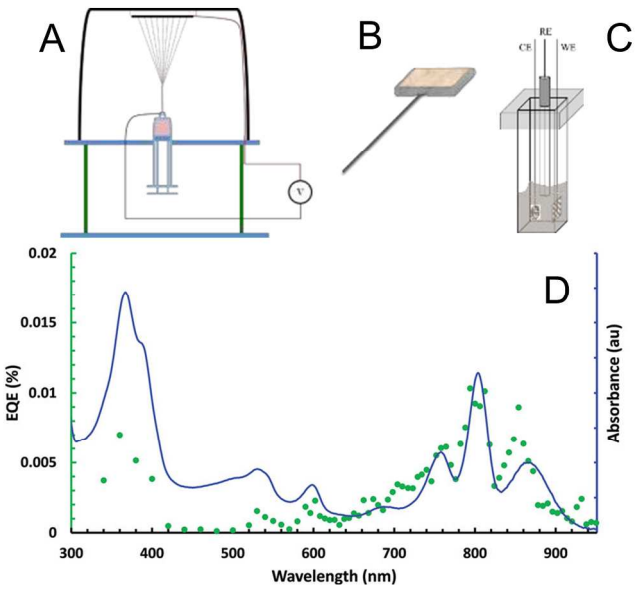

Figure 4. a) Schematic representation of the electrospray apparatus, with a voltage applied between the syringe needle and the HOPG substrate; b) HOPG working electrode, c) electrochemical cell used to measure the light response of the RC treated HOPG electrodes and d) action spectrum of the electrosprayed photoconverter. Adapted with permission.[22] Copyright 2014, Wiley-VCH.

RCs were also directly chemisorbed on gold.[23] Direct contact of RC with gold did not compromise protein integrity and functionality (Figure 5a). The RCs would likely attach to the gold surface via cysteine tags (the H156 cysteine on the H subunit from *R. sphaeroides* RCs), forming an RC layer permeable to the mediators. The resulting electrode was compared to multilayered electrodes, where the formation of a self-assembled monolayer (SAMs) of appropriate organic molecules on gold can function as effective interlayer between the metal and proteins. Irradiation at 850 nm wavelength produced similar photocurrents from RCs on both bare gold and on C2 SAM modified gold. The cathodic current indicates electron flow from gold electrode to the adhered RCs. Electrons are then transferred to the counter electrode via the redox mediators. An action spectrum confirmed that the photoconverter produced electrons at the same wavelengths absorbed by RCs (Figure 5a and 5b). Increasing SAM thickness produced a drop in the photocurrent, showing that immobilization via SAMs potentially slows electron tunneling rates leading to a quantum yield drop as a consequence of the increased distance between the electrode surface and the redox centers of the RC.

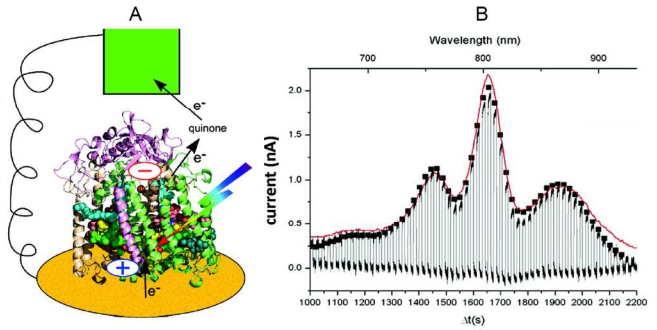

Figure 5. a) Direct assembly of *R. sphaeroides* RC on gold; b) photoinduced currents action spectrum of the biohybrid photoconverter. Reprinted with permission.[23] Copyright 2011, American Chemical Society.

An elegant approach exploits the specific docking interaction of RC with Cyt c by covalently anchoring the latter protein on a SAM modified gold electrode: this offers control on the orientation of RCs, since it selectively interacts with Cyt c close to the dimer site.[21, 24-26] In a recent work[26] the structure was assembled by layer-by-layer deposition: first a 6-mercaptoesanoic acid self-assembled monolayer was formed on gold, and layers of Cyt c and RC were deposited on top (Figure 6). The Au|SAM|cyt c|RC working electrode was applied in a three-probe electrochemical cell where a peak cathodic photocurrent density of 0.5 $\mu A \cdot cm^{-2}$ was achieved. The electrode has 70% coverage of fully active RCs, but it was found that its work function and the large barrier of the SAM are accountable for the low conductance in the devised linker structure.

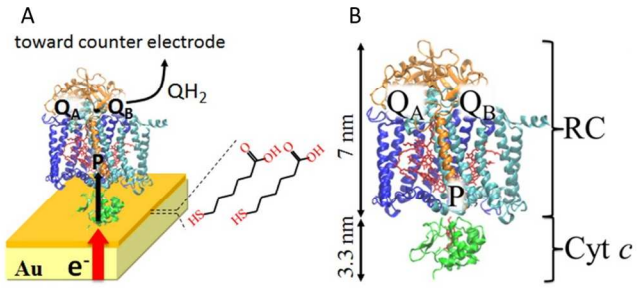

Figure 6. (a) Representation of the Au|SAM|cyt|RC photoelectrode. Arrows evidence the electrons pathway from the 6-mercaptohexanoic acid-modified Au electrode, Cyt c, and the RC. The photon-generated electrons reach the final electron acceptor Q_B and are eventually transferred to the counter electrode via diffusion of QH_2 behaving as mediator. (b) Dimension of the two proteins involved in the docking. Reprinted with permission.[26] Copyright 2014, American Chemical Society.

A genetically engineered RC from *R. sphaeroides* having a polyhistidine (polyHis) tag at the C-terminal of its M subunit offers a more elaborated protocol for stable immobilization with the special pair oriented towards the electrode, since it possesses a unique recognition group protruding from a specific position of the protein structure. This tag would interact with Ni-NTA (Ni-nitrilotriacetic acid) with micromolar affinity. In the first examples of immobilization of RCs on gold electrodes via His-tag technology, Ni-NTA-terminated linker molecules of different lengths were explored. Photocurrents in the range of nA were recorded after light excitation, mediated by Cyt c and UQ_2. Charge-recombination represents the limiting factor preventing better performances.[24, 27] As a further elaboration of this strategy, polyHis tagged RCs were attached to gold *via* Ni-NTA terminated SAM and reconstituted into a lipid bilayer by *in situ* dialysis (Figure 7). The resulting film is a protein-tethered lipid bilayer membrane (ptBLM) with an optimized packing density.[28] The new electrode offers the advantage of an unmediated electron transfer. Photocurrents of around 10 μA cm^{-2} were attained with this setup. Under continuous illumination, D^+ and UQH_2 were generated in equal amounts. This was a fascinating and surprising observation since RCs usually yield ubiquinol only if D^+ is reduced back to D by an external electron donor. A possible explanation lies in intraprotein redox reactions[29] responsible for the boost in photocurrent versus RCs simply bound to the electrode *via* NTA linkers.

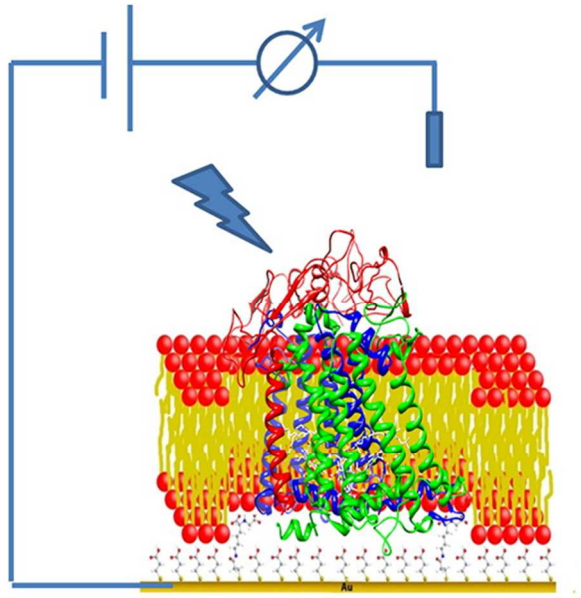

Figure 7. Affinity tag of RC-tethered lipid bilayer membrane on gold electrode *via* Ni-NTA linker. Reprinted with permission.[28] Copyright 2015, American Chemical Society.

Increasing RC photoconversion activity by artificial light harvesting units.

Optimization of the RC light harvesting function represents an effective way to improve the overall efficiency of this enzyme for optoelectronic applications. Combination of RC with properly tailored antennas can in fact extend its physiological light absorption in the visible spectral region where it does not efficiently absorb. In fact, as shown in Figure 8, the sturdy RC of *R. sphaeroides* strain R26 has very limited absorption cross section in the visible (450-700 nm) range where solar irradiance is maximum, whereas it captures light mainly in the ultraviolet

(UV) and near infrared (NIR) spectral regions. This is not surprising since in Nature the RC is always associated with Light Harvesting complexes.

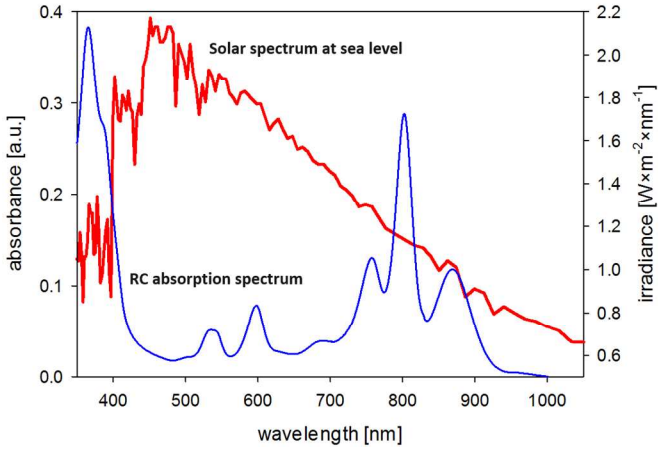

Figure 8. RC absorption spectrum (in blue) superimposed with solar irradiance at sea level (in red).

Hence, building hybrids which combine the isolated RC with artificial light harvesting antennas in the visible range represents a possible approach to endow the natural photoconverter with increased photoconversion efficiency.

Artificial antennas conceived to replace the natural LH complexes must have efficient light absorption in the visible range and high emission quantum yield (QY) in correspondence of an absorption peak of the protein. High Stokes shift between antenna absorption and emission spectra is also desirable to avoid the antenna self-absorption and to excite the RC in a wavelength range where the protein does not efficiently absorb. Additionally, the antenna should be chemically functionalized to be directed and firmly affixed as close as possible to the RC absorbing BChl pigments.

These requirements can be fulfilled using various approaches, each of them having strong and weak points.

The first strategy proposed by Nabiev *et al* consists in using CdTe quantum dot nanoparticles (QDs) as RC light harvesting antennas (Figure 9a).[30]

QDs have broadband absorption spectrum, an intrinsically variable and high Stokes shift, excellent resistance to photobleaching and high emission quantum yield in the visible range.[31] QDs emission wavelengths are strictly dependent on their size. Thus, control of nanoparticle growth during synthesis is a tool to tune their emission in correspondence of an RC absorption peak: the bigger the QD size, the longer the emission wavelength. Unfortunately, the hydrophobic character of QDs limits, in principle, their use in aqueous surfactant solution of RC. An additional issue is represented by their size, comparable to that of the protein, that can prevent intimate contact with the RC.

Nabiev *et al.* used CdTe nanoparticles with 2.7 nm diameter and 0.3 QY, whose emission is peaked at 570 nm next to the RC absorption maximum at 600 nm (Figure 9b).

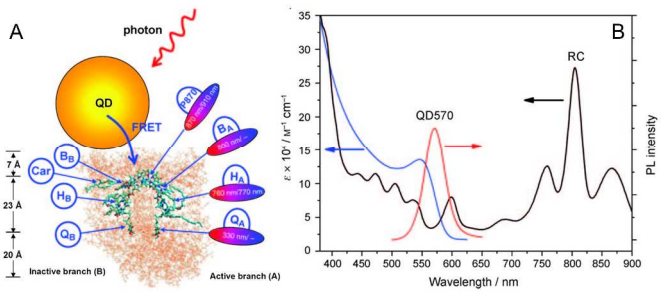

Figure 9. (A) RC structure with highlighted cofactors and QD depicted in scale as an orange sphere. (B) Absorption and emission spectra of QD570 (in blue and red respectively) superimposed with RC absorption spectrum (black). Reprinted with permission.[30] Copyright 2010, Wiley-VCH.

The QDs' surface was decorated with the hydrophilic capping agent thioglycolic acid, and electrostatic interactions determined assembly of the hybrid QD/RC system. Efficient energy transfer was demonstrated by this approach: upon excitation at 450 nm, both antenna emission quenching at 570 nm and RC photoluminescence enhancement at 910 nm (directly related to the charge separated state concentration) were observed.

The major issue of this approach is the fact that electrostatic interactions between QD and RC do not allow an accurate control of the antenna position with respect to the protein and the QD sterical hindrance may affect the activity of the hybrid system.

More profitably, the RC absorption cross section can be enhanced by regioselective covalent binding molecular dyes to the protein scaffold. An important advantage of organic molecular fluorophores is the possibility to finely tune, by proper chemical design, their absorption and emission properties as well as their reactivity towards specific RC aminoacids. This enables to fulfill both spectral and chemical requirements of highly efficient light harvesting antennas for RC. Therefore, despite the synthesis of tailored organic fluorophores often requires greater efforts than QD preparation, this approach affords unhindered molecules that (a) can be selectively bound, without jeopardizing the protein function, in exactly controlled proximity of the RC dimer region where charge separation occurs and (b) can be endowed with a tailored π-conjugated skeleton fulfilling spectral requirements for light harvesting and energy transfer to RC.

This has been recently proven in our group[32] by efficient functionalization of the RC from *R. sphaeroides* with an *ad hoc* synthesized aryleneethynylene (AE) fluorophore with a bis(thiophene)-benzothiadiazole central core bearing a succinimidyl ester moiety used to selectively covalently bind the AE to the amino groups of the RC lysines (Figure 10a). The AE has maximum absorption at 450 nm, where the RC cross section is very low, and maximum emission at 602 nm, in correspondence of an RC absorption peak (Figure 10b).

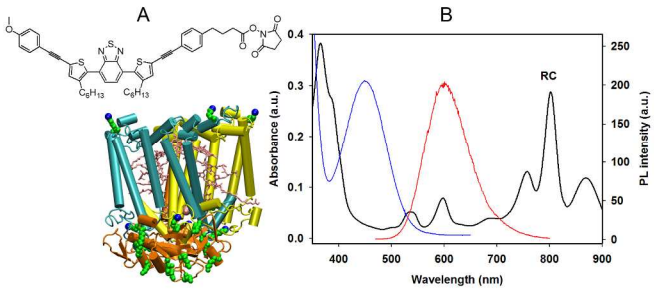

Figure 10. (A) RC structure with target lysines highlighted in green and AE structure. (B) Absorption and emission spectra of AE (in blue and red respectively) superimposed to the RC absorption spectrum (black). Adapted with permission.[32] Copyright 2012, Wiley-VCH.

Moreover, the almost linear structure of AE promotes its intercalation with the surfactant molecules surrounding the protein in aqueous medium and allows the organic dye to reach the reactive lysine residues, which are the most abundant functionalization sites present in the natural photoenzyme. An average number of four AE molecules was estimated to bind to each RC and efficient energy transfer was observed by steady state and time resolved emission spectroscopies. The ability of AE to boost the RC activity was demonstrated observing that the concentration of charge separated state generated exciting the hybrid at 450 nm is five fold higher than that recorded for the native protein at the same excitation wavelength. Finally, the enzymatic activity of the AE-RC was found to outperform that of the pristine RC, by a three fold increase of the rate of the cytochrome C reduction during the protein photocycle.

An alternative RC bioconjugation approach, reported by Dutta *et al.*[33], consists in mutating the natural protein by genetic engineering, which introduces cysteine moieties in proximity of the chlorophyll dimer site (Figure 11a). These moieties were used to covalently bind three antenna molecules of commercially available Alexa Fluor dyes (AF647, AF660 and AF750) functionalized with maleimide groups.

This work provides a smart example of the structural flexibility of the reaction center that can be genetically modified *in vivo* inserting, in proper positions, non native aminoacid reactive sites suitable for further chemistry on the isolated protein. However, the Alexa Fluor dyes have low Stokes shift (20-30 nm), which limits their ability to transfer energy to the modified RC. In the case of AF660, 2.2-fold increase of generation of the charge separated state has been observed exciting the hybrid at the single excitation wavelength of 650 nm (Figure 11b).

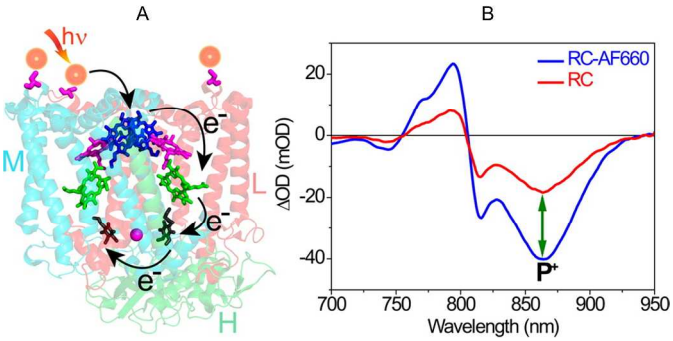

Figure 11. (a) Reaction center from *R. sphaeroides 2.4.1* with the three genetically engineered cysteines residues highlighted. (b) Light-minus-dark spectra of the pristine and bioconjugated RC. Reprinted with permission.[33] Copyright 2014, American Chemical Society.

The same research group reported covalent functionalization of the genetically modified RC with a well-defined light harvesting antenna complex obtained attaching two different pairs of dyes (Cy3 and Cy5, or AF660 and AF750) to two strands (Strand-2 and -3, respectively) of a 3arm-DNA nanostructure (Figure 13).[34] The third strand (Strand-1) of this multiple antenna system was conjugated to a cysteine residue of the modified RC by a *N*-succinimidyl-3-(2-pyridyldithio)propionate cross-linker.

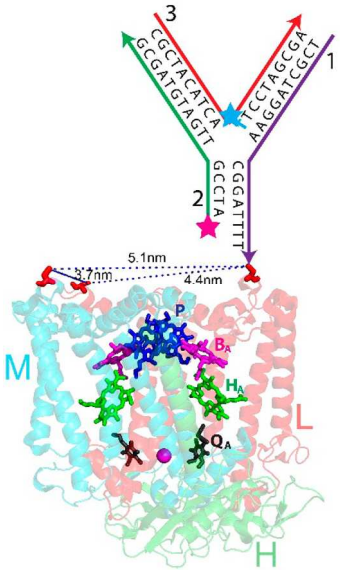

Figure 12. Modified structure of RC from *R. sphaeroides* 2.4.1 (PDB 2J8C 10) with sequences of the 3arm-DNA construct shown. Stars represent the positions of the two dye molecules (the cyan star corresponds to either Cy3 or AF660, and the pink star corresponds to either Cy5 or AF750). Due to the presence of three Cys residues, up to three 3arm DNA junctions (and three pairs of dyes) can be conjugated to the RC. Reprinted with permission.[34] Copyright 2014, American Chemical Society.

This work paves the way to more sophisticated hybrid machineries that rely on the combined effect of multiple antenna dyes and mimic in a very efficient way the function of a natural photosynthetic apparatus. In this case, spatial placement, spectral properties and excited state kinetics of organic dyes play a key role in determining the light harvesting

ability of the supramolecular antenna complex. In particular, the rate of photon capture is proportional to the number of dye molecules and increasing the number of DNA-dye constructs attached to the reaction center leads to an increase of the protein functional cross section.

Conclusions

Implementation of the photosynthetic reaction center in electronic devices represents a perfect case of study of the emerging field of organic bioelectronics. It shows the enormous potentialities arising from the combination of highly specialized and efficient protein natural structures with organic electronic devices. Especially, it is possible to envisage the exciting perspective of combining biotechnological production of active materials with the electronic technologies. On the other hand, studies carried out on photoelectrodes based on the reaction center clearly illustrate the main issues relevant to the manipulation of the complex and delicate biological macromolecules for implementation in electronic devices. This small review has offered a special perspective on this topic, focusing on the role of highly selective chemical modification to optimize the performances of functional proteins and to selectively address these systems at the interfaces with electronic devices. We consider that the chemical tools of organic synthesis and self-assembly will play a key role in developing organic bioelectronics, bridging the gap between biotechnological production of the materials and engineering of the devices.

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Notes

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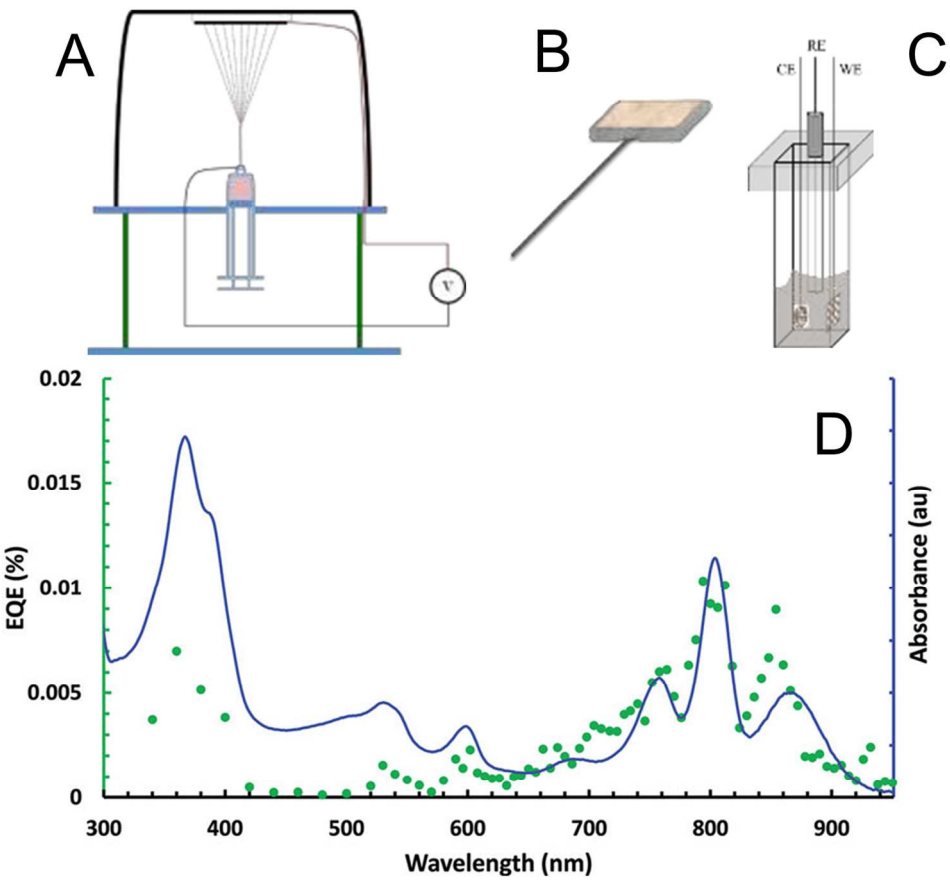

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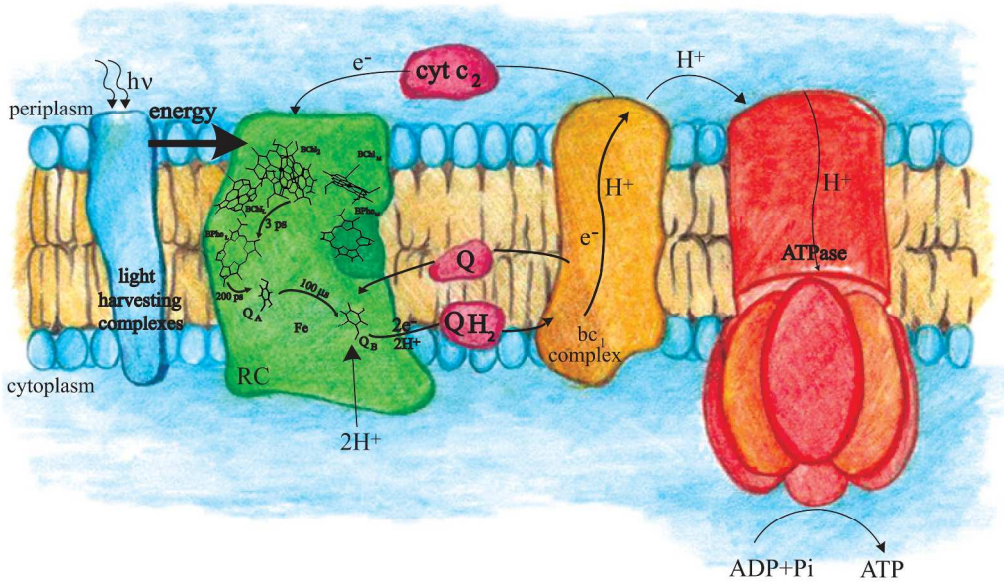

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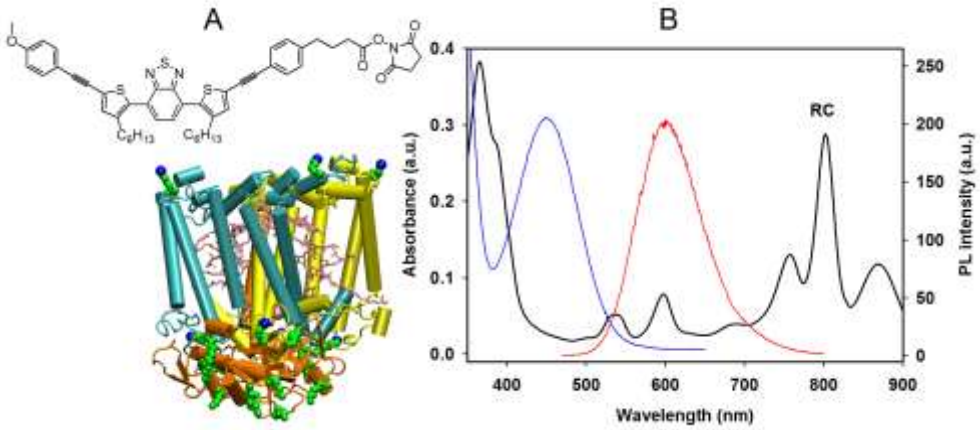

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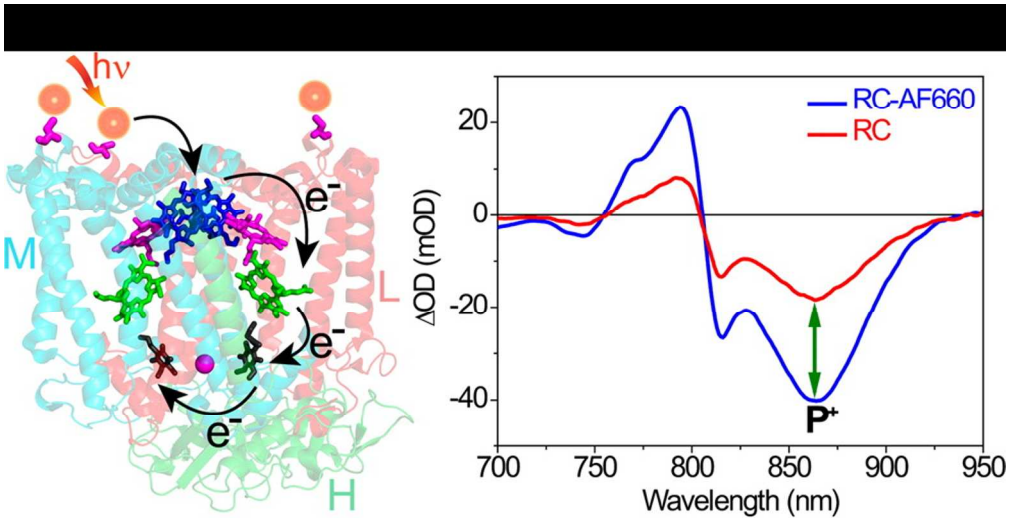

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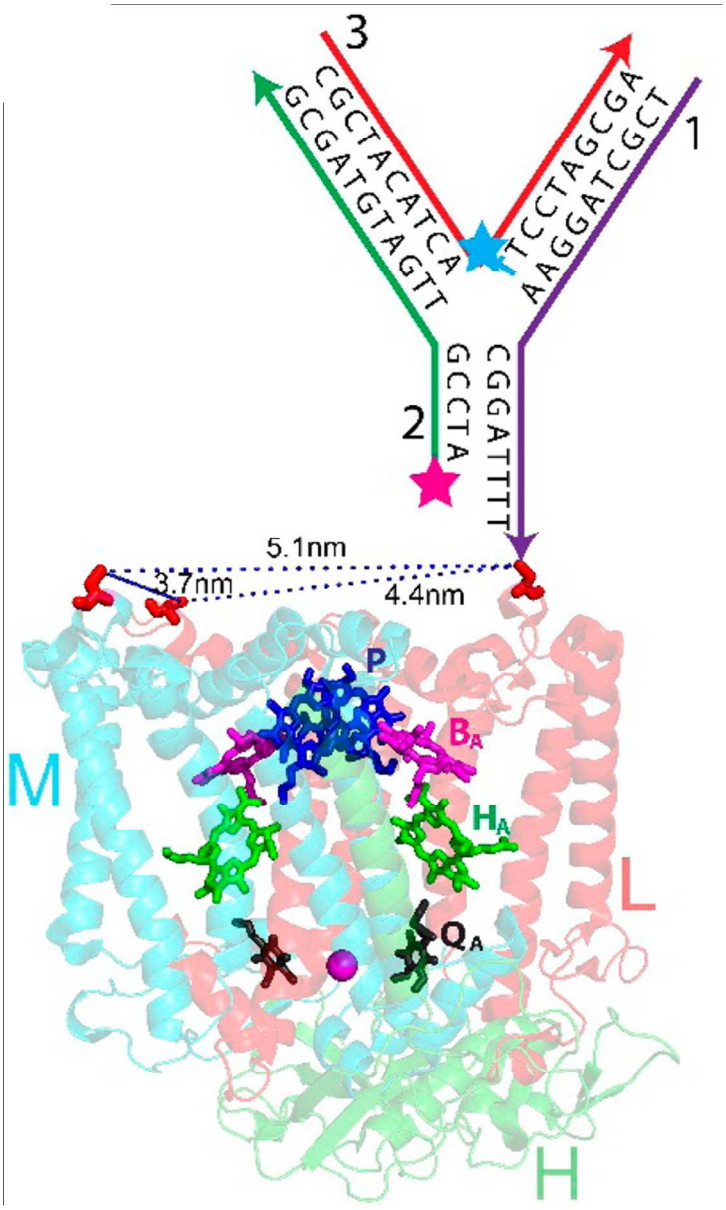

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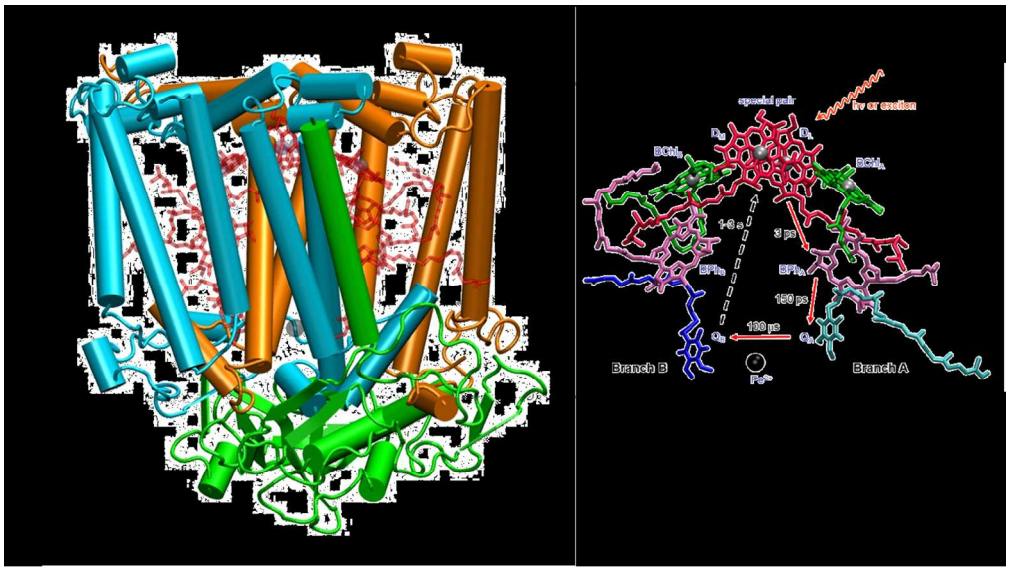

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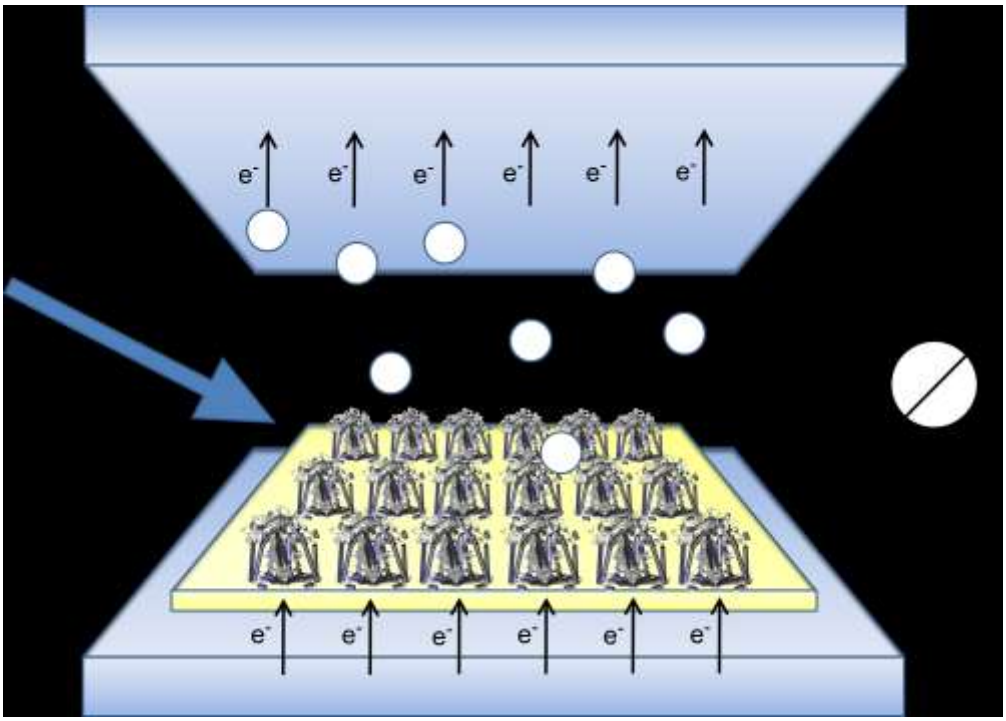

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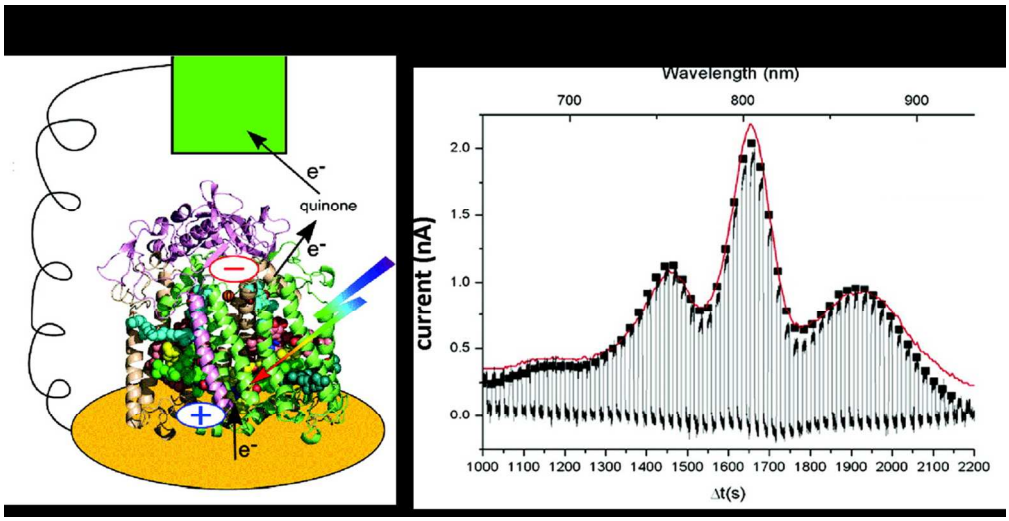

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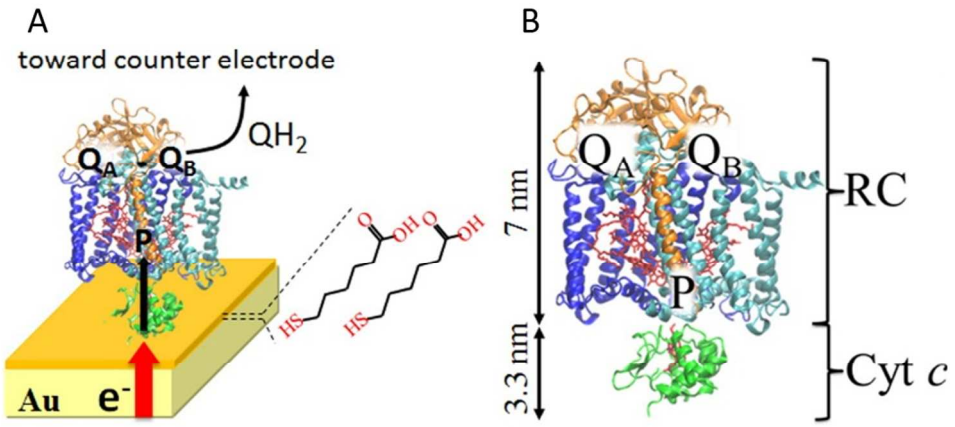

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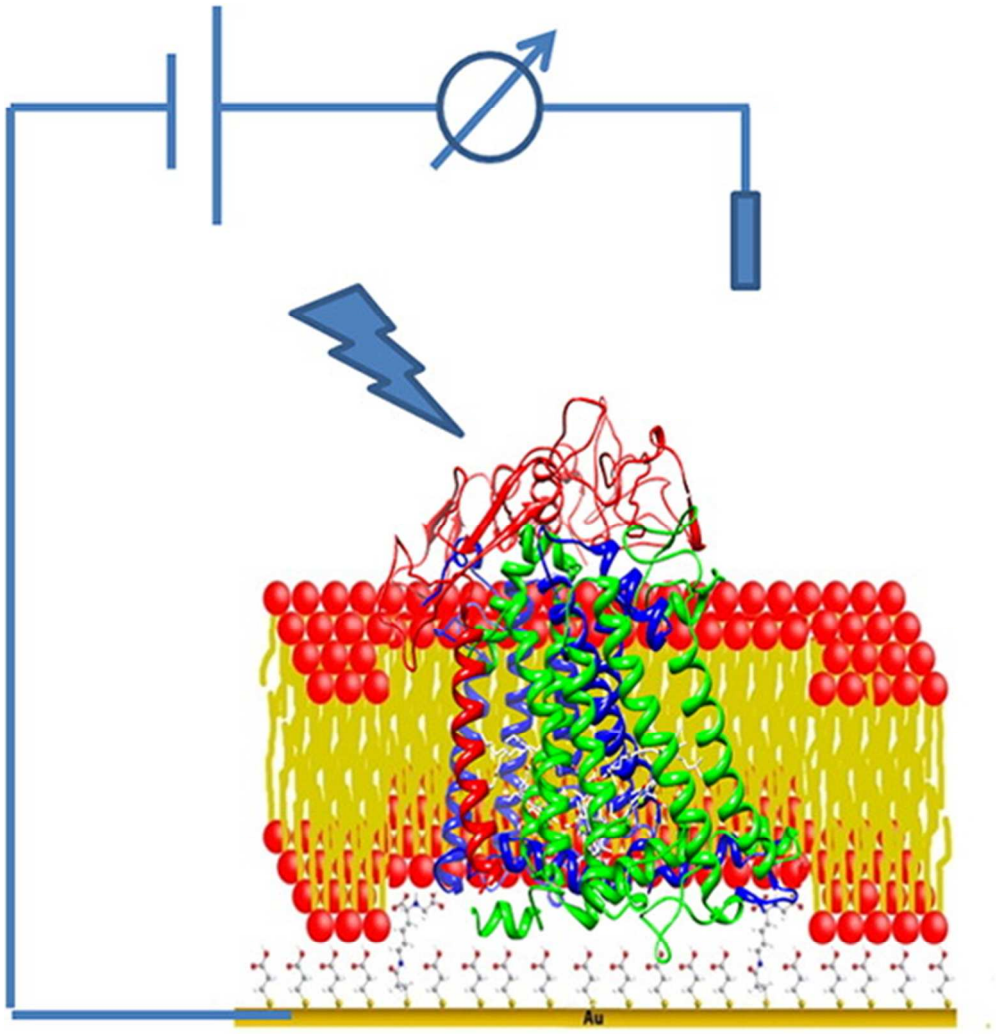

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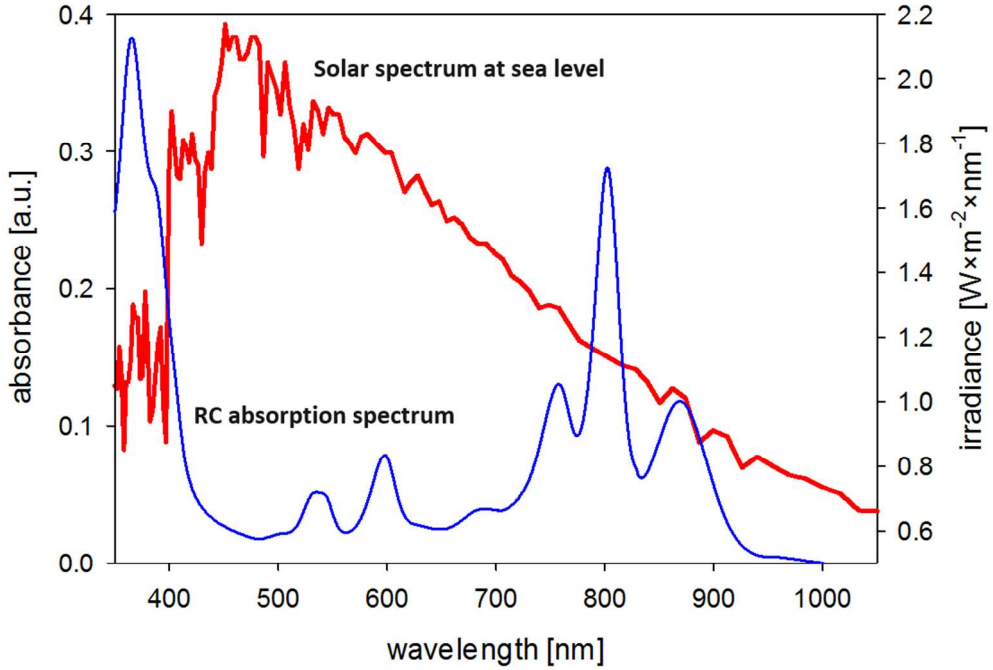

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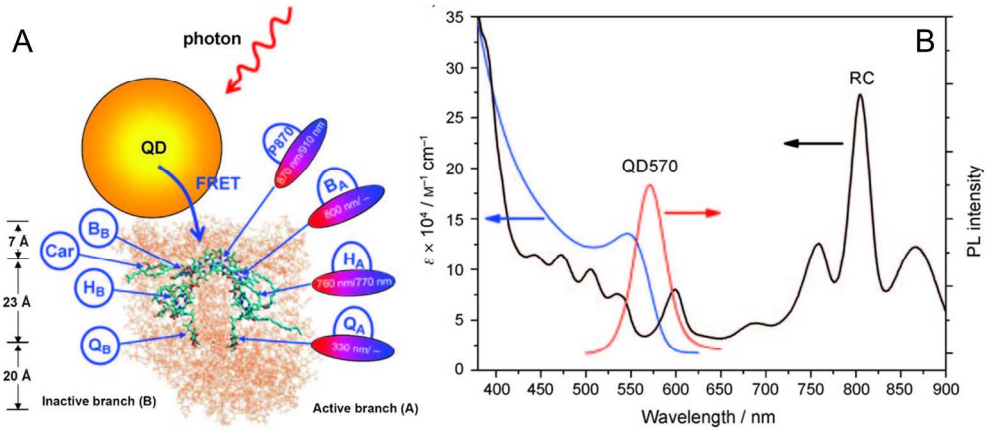

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