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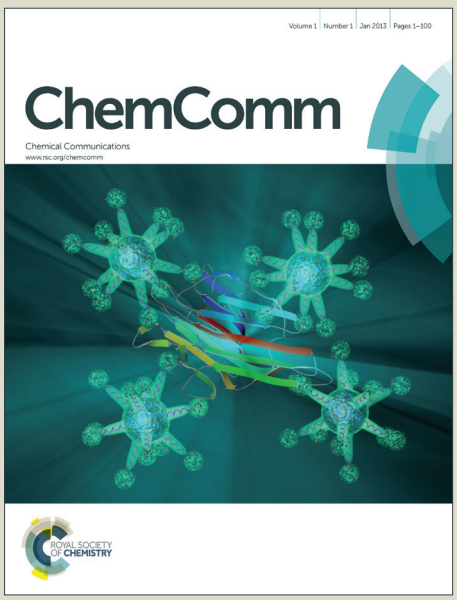

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Synthesis and Enzymatic photo-activity of O_2 tolerant hydrogenase/CdSe@CdS quantum rod bioconjugate

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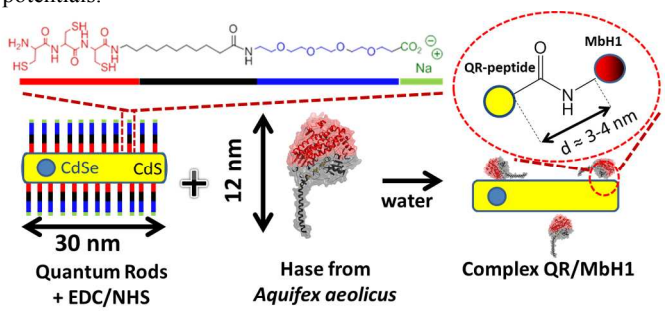
This communication reports on the preparation of stable and photo-active nano-heterostructures composed of O_2 tolerant [NiFe] hydrogenase from *Aquifex aeolicus* bacterium grafted on hydrophilic CdSe/CdS quantum rods in view of the development of H_2/O_2 biofuel cells. The resulting complex is efficient towards H_2 oxidation, displays a good stability and new photosensitive properties.

The same length scale of biomolecules and inorganic nanocrystals allows the preparation of nanotools where the unique, photonic, electronic and catalytic properties of nanoparticles are combined with the properties of biomolecules. Among the nanoparticles, anisotropic semi-conductor nanocrystals, namely quantum rods, have emerged as a promising class of materials for a broad range of applications such as energy conversion.[1] In particular core@shell quantum rods made of CdSe@CdS are known to efficiently convert light in order to perform redox reactions in comparison to their spherical counterpart.[2] Indeed the life time of the photo-induced charge separation (i.e. exciton) is enough longer to favor electron transfer at the surface of the quantum rods due to their anisotropic shape.

In a natural photosynthesis, plants absorb solar radiation using pigments that efficiently separate the charge carrier and deliver it to catalysts, such as hydrogenase (Hase) in order to perform the reversible reaction of hydrogen decomposition/generation. Previously complexes composed of an O_2-sensitive FeFe Hase and either a CdS nanorod[3] or CdTe quantum dot[4] have been obtained through electrostatic interaction for hydrogen generation. In the present work, our artificial system consists in an O_2-tolerant and hyperthermophilic Hase (MbH1) covalently grafted on a hydrophilic peptide-coated quantum rod where the latter acts as a photosensibilizer. To the best of our knowledge the covalent attachment of such an enzyme on an inorganic nanoparticle, with the preservation of its catalytic activity, has never been reported in colloidal suspension. MbH1 contains a NiFe active center and presents a bias toward the catalytic hydrogen splitting. Based on these properties, MbH1 has been integrated in a H_2/O_2 biofuel cell on an anode modified by carbon nanotubes.[5] Furthermore, we previously demonstrated a light-induced reactivation under turnover of an inhibited state of the NiFe MbH1 (Ni-B) formed at high redox potentials.[6]



Scheme 1: a) Cartoon showing the strategy adopted to graft covalently MbH1 on the QR surface (30*5 nm) *via* a peptidic coupling.

Herein we report on an efficient and simple strategy to covalently graft MbH1 from the hyperthermophilic bacterium *Aquifex aeolicus* to quantum rods made of CdSe@CdS in water. The resulting biohybrid complex has been characterized by gel electrophoresis and by electrochemistry. We showed that the enzyme retains its activity on the quantum rod surface. We have next investigated the photosensitive properties of this complex in electrochemistry. This work would be a further step towards the design of an efficient biofuel cell.

Quantum rods CdSe@CdS were dispersed in water thanks to a robust peptidic exchange strategy according to a previously published procedure on quantum dots CdSe@ZnS.[7] The interaction between the tricystein sequence of the hydrophilic ligands (see scheme 1) is strong enough to allow purifying the suspension of the free ligands while limiting aggregation process. Besides those advantages, the peptidic ligand used in this work is terminated by a carboxylic acid that can react to form an ester via (EDC/NHS) coupling agents. The QR suspension is then activated and can further react with a primary amine at the surface of MbH1 in order to form a QR/Hase complex by a covalent bond (see Scheme 1).

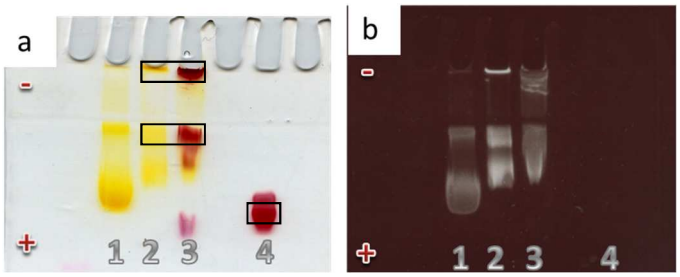


Figure 1: Migration of the different components by nativePAGE: (a) detection of MbH1 activity in gel assay (b) QR (30*5 nm) detection by UV illumination. **1**: QRs alone (20 mg/mL), **2**: QRs after activation of the carboxylic acid *via* a mixture of (400/100 mM; EDC/NHS) coupling agents (Initial concentration 20 mg/mL), **3**: MbH1 (28 µM) grafted on QR. **4**: MbH1 alone. The concentration of QRs is fixed at 20 mg/mL for all lanes containing QRs (1, 2, 3). The framed bands were analyzed by MS.

In order to investigate the putative binding of MbH1 to QR, native PAGE has been performed (Figure 1). First the colloidal stability and the monodispersity of the quantum rods grafted with peptidic derivatives were evidenced by a single spot in agarose gel electrophoresis corresponding to the migration of the nanoparticles (see the gel Figure S1). In the case of nativePAGE, one has to notice that the migration of the quantum rods in the gel (lanes 1, Figure 1) is non-uniform even if the colloidal suspension is homogeneous and the nanorods are monodisperse in size (see TEM Figure S2). The heterogeneous migration of the quantum rods is attributed to the higher concentration of polyacrylamide gel (5% m/v polyacrylamide gel versus 0.5 % m/v for agarose gel electrophoresis). A decrease in the quantum rods migration is observed when 400 mM/100 mM EDC/NHS are added in the quantum rods suspension (lanes 2, Figure 1) corresponding to an overall decrease of the particle charge due to the formation of ester on the terminal function of the peptidic ligands or/and to the crosslinking between QRs (see Figure S3). Then, the activity of –MbH1 in one hand and its grafting on QR surface in the other hand were detected and visualized in gel. No visible aggregation of the QR/Hase suspension was observed before deposition on the gel even if one can not exclude that protein coupling induces partially the formation of some aggregates.The enzymatic activity for H_2 oxidation is revealed with a gel assay displaying a red color induced by the reduced triphenyltetrazolium chloride (see protocols in supporting information). Colocalization of red-active MbH1 with the fluorescent quantum rods clearly appears on the gel (lane 3, Figure 1a and 1b).[8] This last observation assesses the formation of the QR-MbH1 complex and that the MbH1 retains its activity on the QR surface. MbH1 is composed of two subunits a large one (72 kD) and a small one (39 kD). The formation of the QR-MbH1 complex has been confirmed in Peptide Mass Fingerprint. The MALDI-ToF and LC-Q-ToF mass spectrometry evidenced the grafting of the MbH1 protein (see supporting informations, Figure S4). No protein was detached from the surface of the particle for the duration of the electrophoresis experiment (about 2 hours after the coupling) therefore non-covalent adsorption of the protein appears not to be significant.

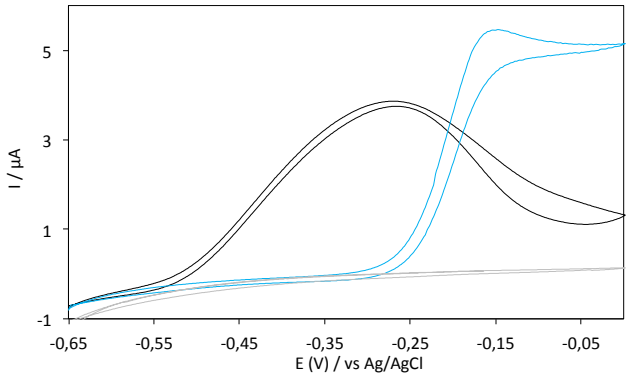


Figure 2: Cyclic voltammetry (CV) for adsorbed MbH1 (2.8 µM, black curve), QR/MbH1 (2 mg.mL^{-1}/2.8 µM) complex before (grey), and after 10µM methylene blue (MB) (blue) addition in solution. The deposition on the pyrolitic graphite PG electrode was immobilized through a dialysis membrane. 50 mM HEPES buffer solution, pH 6.8, 60°C (v = 5 mV.s^{-1}). The experiments are performed under H_2 atmosphere.

When MbH1 is immobilized at the membrane graphite electrode, under H_2 at 60°C, a direct current for H_2 oxidation is observed (Figure 2, black curve). This is rationalized by the fact that some MbH1 molecules are oriented at the rough surface of the PG electrode with the last electronic relay (the distal FeS cluster) at a tunnel distance of the PG electrode.[9] A decrease in the current is observed for potentials higher than -0.2 V. This reversible inhibition process has been widely studied, and is linked to the formation of a hydroxide bridge between the Ni and Fe atoms (called Ni-B state). By lowering the potential, electron injection allows the reduction of this bridging ligand and consequently the formation of an active state of the NiFe site.

In the same electrode configuration, the complex QR/MbH1 was then studied at 60°C under H_2 using cyclic voltammetry (Figure 2, grey curve). No oxidative current can be detected until MB was added into the solution (Figure 2, blue curve). Three important conclusions can be derived from these measurements. First, MbH1 engagement in a complex with QR is confirmed since the direct electron transfer (DET) process obtained for H_2 oxidation with MbH1 at the same concentration in the absence of QR disappears thus no free MbH1 is present in the QR/Hase suspension at the concentration used. Second, the length of the derivative for MbH1 immobilization, and/or the site of fixation of MbH1 on the QR, as suggested by mass spectrometry, precluded approach of the distal FeS cluster to the electrode so that no DET process can be achieved *via* the QR/MbH1 complex. However, MbH1 is still efficient for mediated H_2 oxidation. After addition of MB into the buffer solution, a mediated process for H_2 oxidation appears at the potential for MB oxidation. A second order rate constant of $8x10^7$ $M^{-1}.s^{-1}$ was calculated. The mediated catalytic process is not plateau-shaped, and a decrease in the current density is observed above -0.15 V, which

may be linked to MbH1 inhibition. Moreover, the complex showed great a long-term stability as the H_2 oxidation occurs even one week after the peptidic coupling.

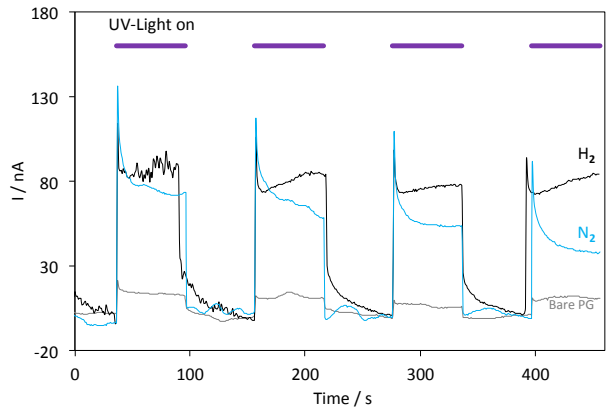


Figure 3: Chronoamperometry at -0.1 V vs Ag/AgCl for the complex QR/MbH1 immobilized at the membrane PG electrode in the presence of 10 µM MB under N_2 or H_2 with light pulse applied every 60 s. 50 mM HEPES buffer solution, pH 6.8, 60°C.

The QR/MbH1 complex has been next studied by chronoamperometry at an oxidizing potential (-0.1 V) where the NiFe active site bears a hydroxo ligand (Ni-B state). Short light pulses using a LED (λ = 405 nm) were applied to the working electrode and the photoinduced charge transfer between the complex and the electrode, named hereafter photocurrent, was recorded in the presence of MB. The wavelength at 405 nm was selected to have a strong absorption of QR while having low absorption of the protein. The intensity of illumination applied on the working electrode was fixed to 700 lux. The bare PG electrode shows weak photocurrents under H_2 (Figure 3, grey curve). Conversely, the QR/MbH1 complex displays significant higher photocurrent (Figure 3, black line). The contribution of the individual constituents of the bioelectrode on the total photocurrent was established by working either under H_2 or N_2 atm. We have shown recently that the enzymatic activity of MbH1 can be triggered by light at those oxidizing potentials.[6] Indeed, under N_2 only the photocurrent induced by the QR and MB must be recorded. In this latter case, the photocurrent profile is similar to the one obtained in the absence of MbH1 on the QR (Figure S5). It is characterized by a sharp increase corresponding to an oxidation photocurrent that sharply decreases when the light is switched off (Figure 3, blue curve). This behavior has been described on quantum dots and was assigned to the alignment of the Fermi level at the electrode interface which is potential dependent.[10] The photocurrent link to the QR is thus strongly related to the potential applied on the electrode (see Figure S6). In addition, the decay of the photocurrent observed under illumination increases with the number of pulses. This latter observation is attributed to a partial photooxidation of the nanocrystals in absence of any sacrificial hole scavenger (see Figure 3).[11] Under H_2, (Figure 3, black line) the photocurrent evolution with time is notably different. A continuous increase is recorded when the light is on, and the photocurrent slowly decreases when the light is switched off. Those results suggest a synergistic effect between the QR and MbH1 as the observed photocurrent differs from the individual constituents on the bioelectrode. The increase in photocurrent under turnover at an oxidizing potential where the enzyme is in an inactive state can be attributed to a reactivation of the enzyme by a light induced process.

In conclusion, a complex composed of quantum rods and an enzyme has been obtained through a covalent peptidic coupling. The techniques employed to characterize the complex demonstrate that the enzyme immobilized on the nanorod surface presents a long-term stability. We demonstrate also an original light triggered reactivation process of the active site of MbH1 at oxidizing potential. We are currently working on the surface chemistry of the quantum rods in order to avoid the use of the electron mediator that limits the integration of such system in biofuel cells. The aim is to improve the life time duration of biofuel cell technology thanks to light reactivation of the hydrogenase.

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Notes and references

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Electronic Supplementary Information (ESI) available: Figure S1: native PAGE gels of QRs with various aspect ratios; Figure S2: corresponding TEM images of the QRs; Figure S3: Native PAGE gels of QRs with various amounts of coupling agents; Figure S4: Native PAGE gels of MbH1 in the presence or not of coupling agents; Figure S5: Chronoamperogram of QRs immobilized at the electrode in presence of MB with light pulses; Figure S6: linear sweep voltammogram of the QRs immobilized at the electrode with light pulses. See DOI: 10.1039/c000000x/

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