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Márquez, Augusto; Santiago, S.; Dominguez, Carlos; [et al.]. «Photoelectroenzymatic glucose reusable biosensor by using dithienylethene mediators». Chemistry, Vol. 26, Issue 40 (July 2020), p. 8714-8719. DOI 10.1002/chem.202000865

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Photo-Electro-Enzymatic Glucose Reusable Biosensor using dithienylethene mediators

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Keywords

Optical biosensor • photoelectrochromism • enzymatic mediator • glucose sensing • regenerable biosensor

Abstract

In the development of colorimetric biosensors, the use of electrochromic mediators has been accepted and widely used during decades. The main drawback of these types of enzymatic substrates is the difficult recovery of the initial redox state of the molecule, which can be done electrochemically or by antioxidants addition, complicating the initially simple structure of the biosensor. Actually, those strategies are rarely followed; being the disposable biosensor configuration the most extended for this detection mechanisms. Alternatively, we propose the first reported use of a diacid dithienylethene 1,2-bis(5-carboxy-2-methylthien-3-yl)cyclopentene (DTE) photoelectrochromic compound as a substrate of the horseradish peroxidase (HRP). The photoisomerization between the open (DTEo) and closed (DTEc) forms of the molecule and the respective shift in the redox potential allowed the light-induced enzymatic detection of glucose in the glucose oxidase (GOx) – HRP cascade system. This fast and easy control over the enzymatic substrate availability by light pulses permits a gradually consumption and the light-regeneration of the biosensor for a number of cycles. We consider the presented results transcendent in the development of reusable and light-controlled photonic biosensing systems.

Introduction

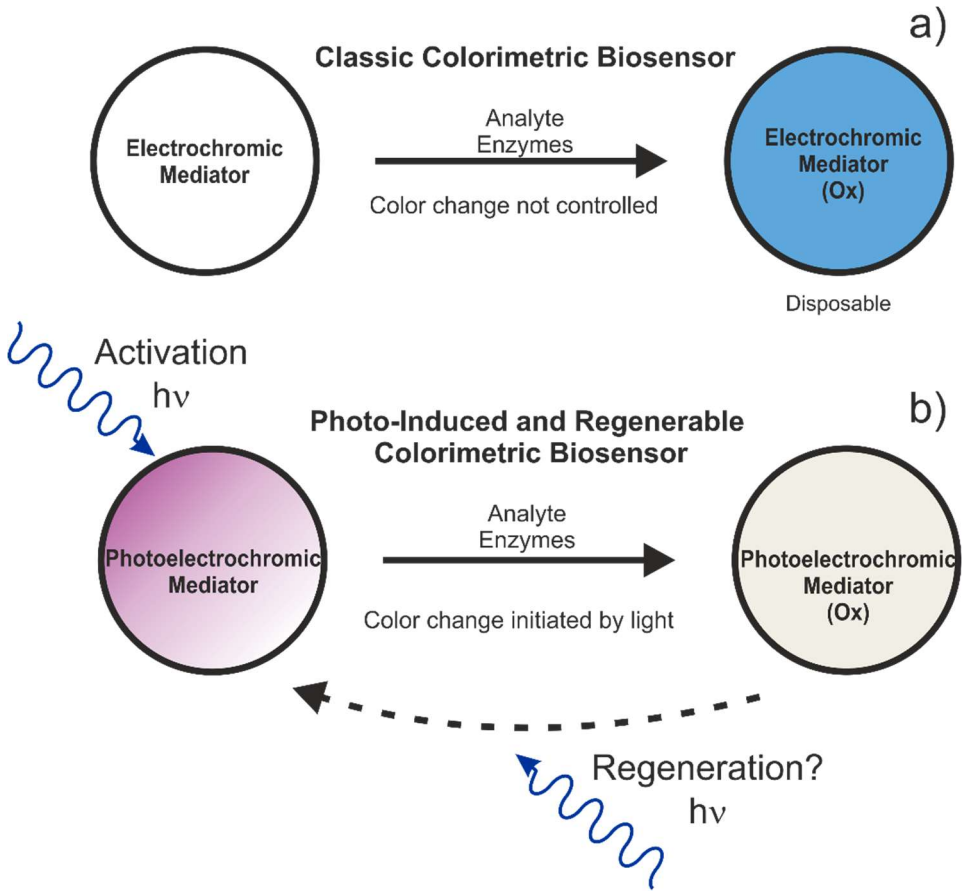
Photoelectrochromic compounds are characteristic for undergo reversible isomerization, ruled photonically (at a certain wavelengths) and/or electrochemically (at a certain potentials), what changes the optical material properties (e.g. UV-Vis-absorbance or refractive index) but also the structural conformation.[1,2] This photoelectrochemical modulation of the intrinsic properties of the material enhances the application of such compounds in a wide variety of fields like smart windows and glasses,[3] color-changing displays,[4] smart cards,[5] e-paper,[6] computing technology (data storage),[7] optics (active filters),[8] etc...

These properties of photoelectrochromic compounds have been also applied in sensing. For example, recently Zou et al. have reported the use of a polyoxometalate in low cost ultraviolet radiations sensors for real-time solar UV dosimeters.[9] Furthermore, photochromic spiropyrans, integrated in polydimethylsiloxane (PDMS), have been applied in HCl gas sensing[10] and also inorganic composites such as Au-WO_3 or Ag-TiO_2 have been employed in H_2[11] and acetone gas [12] detection, respectively.

Among different families of organic photochromic molecules (i.e. phenoxyquinones, spiropyrans, azobenzenes), in the present work we discuss the possibility of using a dithienylethene dye (DTE) as enzymatic mediator for glucose detection. Diarylethenes are a family of thermally irreversible photoelectrochromic molecules (P-type) composed by two basic entities: aromatic thienyl groups and a hexatriene subunit.[13] DTE experiments reversible cyclization of the hexatriene skeleton by UV (closure) and visible (opening) irradiation, translated in the coloration and discoloration of the molecule, respectively. Furthermore, DTE structure is also interesting since its highly symmetry, highly fatigue resistance and, the most important, its electrochromic behaviour, what makes the molecule both photo- and electro-chromic.[14] Despite DTE has been used as GOx redox relay for electrochemical glucose biosensing,[15] it has never been reported in optical biosensing applications where it could play a role as an enzymatic colorimetric mediator itself. Besides glucose, the detection of many biomarkers of interest in healthcare or professional sports (e.g. ethanol, lactate, cholesterol…) can be achieved, among others strategies, by an oxidase-peroxidase cascade system in which firstly the oxidase coproduces H_2O_2 after the oxidation of

the target analyte and secondly the peroxidase uses the produced H_2O_2 to oxidase the mediator. Afterwards, the mediator can be detected either electrochemically or optically (if the oxidation is accompanied by a color change).

It is the objective of this work to investigate an alternative to classical electrochromic enzymatic mediators by the use of photoelectrochromic molecules, such as DTE, with the aim of develop a photo-inducible and regenerable enzymatic biosensor (**Scheme 1**). In order to study this possibility, the case of glucose detection is taken as an example, based on the oxidation by the GOx enzyme with O_2 coupled to the peroxidase activity.



Scheme 1. Scheme of the classic colorimetric disposable biosensor (a) and the pursued photo-inducible and regenerable one (b), using photoelectrochromic molecules as enzymatic mediators. The activation of the molecule is due to the UV-induced isomerization that changes the redox potential, being accessible for peroxidase catalysis. The system (not the single molecules) can be restored for a number of cycles if the activation of the initial amount of molecules is partial.

Results and Discussion

Enzymatic coupling to DTE bleaching

The isolated DTE was first deprotonated prior to enzymatic essays, increasing 4 times the solubility in water (**Figure S1**). Then, the catalysis of the DTE first only by the HRP in presence of H_2O_2 was studied for both isomers revealing that only the closed one could be catalysed (**Figure S2**). Remarkably, there is no DTE bleaching in presence of H_2O_2 and in absence of HRP, what revealed that the DTE is not sensitive to the presence of the oxidant if the reaction is not catalysed by the peroxidase. This fact is corroborated in terms of redox potential of both isomers, being only the closed one accessible for the HRP oxidation. Secondly, the oxidation of glucose by the GOx was coupled to the HRP reaction, ending in the detection of the analyte following the enzyme induced bleaching of the DTEc.

The capability of the HRP to catalyse the oxidation of the DTE was evaluated spectroscopically by consecutive cycles of UV-induced coloration and enzyme-induced bleaching of the molecule. First, a PBS solution of DTE in presence of HRP and GOx was irradiated with 300 nm UV light to close the molecule and the absorbance spectrum was measured respect to the same solution before irradiation (**Figure 1a**). Secondly, a PBS glucose solution was mixed with the prior volume and, instantly, a spectrum was taken every 30 s to follow the absorbance decay. The same cycle was repeated 21 times (**Figure 1b**) with a full molecule recovering in terms of absorbance at 515 nm up to cycle 16^{th}. As the glucose was in excess respect to the DTE ($2\ 10^{-3}$ M : $7.5\ 10^{-5}$ M) and not all the DTE was closed upon UV irradiation in each cycle, extra additions of glucose were not needed. Although in principle recovered, by increasing the number of cycles the DTE signal decreased gradually until disappearance (cycles 16^{th} to 21^{st}), dismissing a possible reversibility of the DTE to the initial state. Furthermore, three more absorbance signals were detected: one transitory at 760 nm and other two at 345 nm and 370 nm that increased its value with the consecutive cycles (**Figure S3**). Actually, the increment of these two signals ceased simultaneously to the breakage of the 515 nm signal recovery, pointing to a fully catalysis of DTE.

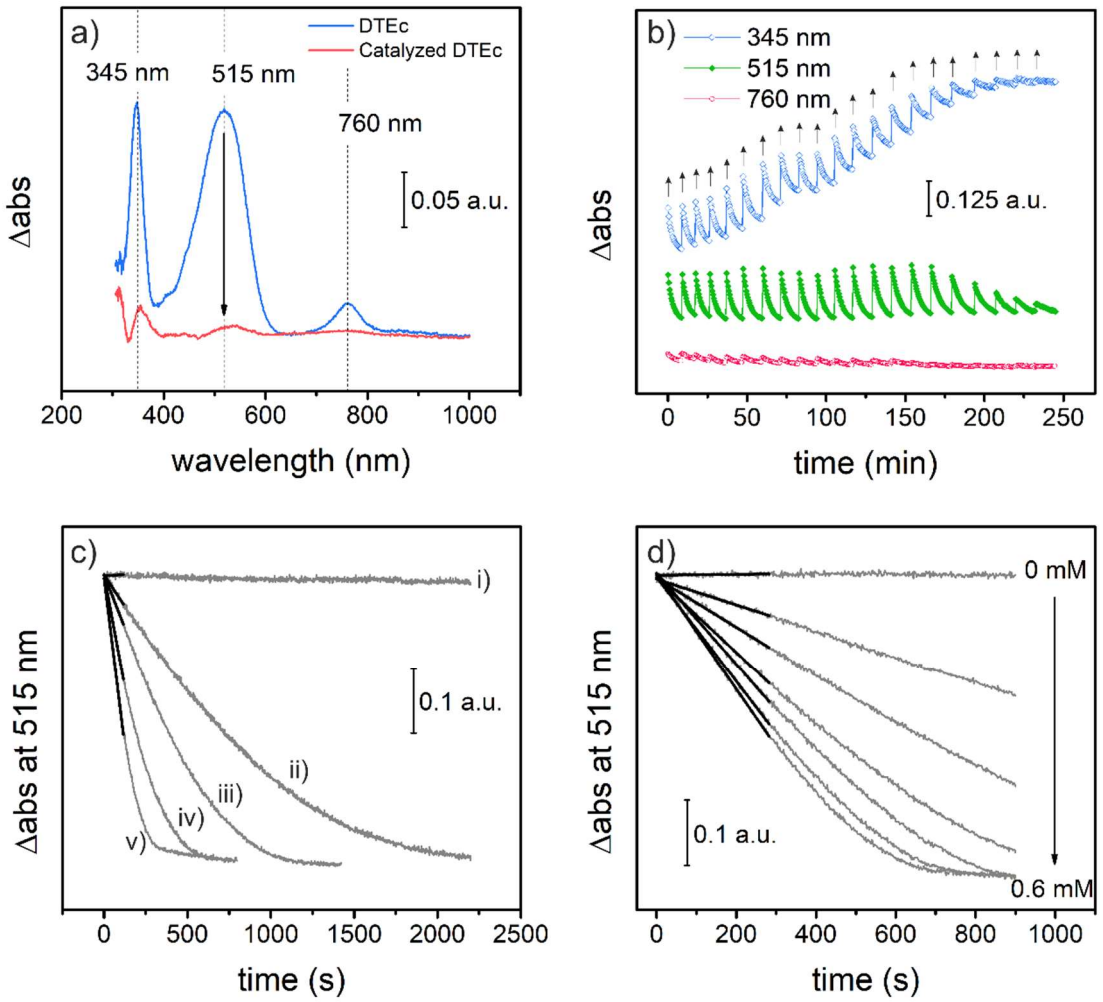


Figure 1. a) Absorbance spectra evolution of DTEc in PBS solution after the addition of HRP-GOx and glucose. b) Absorbance values at different wavelengths within subsequent cycles of DTE coloration upon UV irradiation (arrows) and bleaching by HRP enzymatic activity. Solution: 7.5 10^{-5} M DTE (B^{2-}), 0.01 M PBS, 2 10^{-3} glucose, 1 u ml^{-1} HRP and 0.67 u ml^{-1} GOx. c) DTEc (B^{2-}) bleaching by the enzymatic activity of i) 0 u ml^{-1}, ii) 0.25 u ml^{-1}, iii) 0.5 u ml^{-1}, iv) 1 u ml^{-1} and v) 2 u ml^{-1} of GOx in combination of HRP (2:3 relation) in the presence of 2 10^{-3} M glucose 0.01 M PBS solution. d) DTEc (B^{2-}) bleaching by GOx-HRP system (1 and 1.5 u ml^{-1} respectively) in different glucose concentrated 0.1 M PBS solutions (from 0 to 6 10^{-4} M, increments of 1 10^{-4} M).

Varying the reaction parameters, the bleaching rate of DTE changed. For instance, the increment of GOx-HRP concentration in the reaction volume accelerated the discoloration of DTE (**Figure 1c**) for the same concentration of 2 10^{-3} M glucose in PBS solution, denoting a clear dependence on the enzymatic activity and the possibility to couple the DTE bleaching to the oxidase-peroxidase system. The DTE is then potentially useful to detect any analyte that can be catalysed by an oxidase enzyme (e.g. glucose, ethanol, lactate, cholesterol, monoamines, tryptophan, etc…) that coproduces H_2O_2.

The system also demonstrated to be dependent on the analyte concentration (**Figure 1d**) and showed clear different bleaching rates between 0 and 6 10^{-4} M glucose maintaining the same amount of enzymes (1 and 1.5 u ml^{-1} of GOx and HRP respectively), what means that the system is useful not only for the detection but also for the analyte quantification. With this linear response, it would be possible to analyze the glucose presence in samples such as sweat[16] or tears,[17] where the concentration values oscillates between 2 and 6 10^{-4} M. However, for such type of analysis further studies must be carried out, as the biosensor optimization and system immobilization in a solid-state platform.

Regarding the additional signals that appeared in the visible spectrum during the reaction (at 760 nm) and in the UV (at 345 and 370 nm) (**Figure 1a and S3**), they provide useful information to figure out the reaction pathway of the system. Spectra of the 21 cycles at two different moments are depicted: just after the UV irradiation and before the next irradiation, when the spectrum was stable. After the UV irradiation, a sudden increment at 515 nm is appreciated because of the DTE closure. When the UV irradiation was stopped, a second signal appeared at 760 nm, with some seconds of delay. Finally, both signals decreased while the signals in the UV remained and even increased in the subsequent cycles (**Figure S3**). This signal was associated to a final reaction product of the catalysis of the DTEc by the HRP in presence of H_2O_2.

DTEc catalysed study by ATR-FTIR.

To seek more information about the product of the enzymatic reaction, IR measurements were done when all the DTE present had been catalysed (**Figure 2**). In a first comparison between the open and closed forms of DTE, it can be appreciated small changes at 1660 (O – H vibration) and

1345 cm^{-1}, having a higher absorption the DTEc compound in both cases. While the first one is attributed to a higher moisture retention from the less hydrophobic DTEc compound, the second is attributed to $–CH_3$ bending changes related to ring closure. The IR spectrum of catalysed DTE shows remarkable differences in the C = O (1800 – 1500 cm^{-1}) region, while the intense fingerprint peaks[18] of the cyclic structure in the 1500 – 1300 cm^{-1} remain unchanged.

As the fingerprint signals of the cyclic structure remains unchanged, the authors suggest that IR changes are related to protonation changes in the carboxylic moieties of the DTE. In this sense, the most notable changes between the non-catalysed DTE (both open and closed) and catalysed DTEc are the peak shift from 1630 cm^{-1} to 1670 cm^{-1}, and the new contribution at 1720 cm^{-1}. While the first two signals are related to the asymmetric stretch (ν_{as}) of the carboxylate groups, the signal at 1720 cm^{-1} is associated to the carbonyl stretch $\nu_{C=O}$. As the initial DTE is initially deprotonated to increase the solubility in aqueous medium (Figure S1 and 2a,b), both changes can be associated to a protonation process of the carboxylic groups[19] from degradation products of DTE (see proposed mechanism in **Figure 3**).

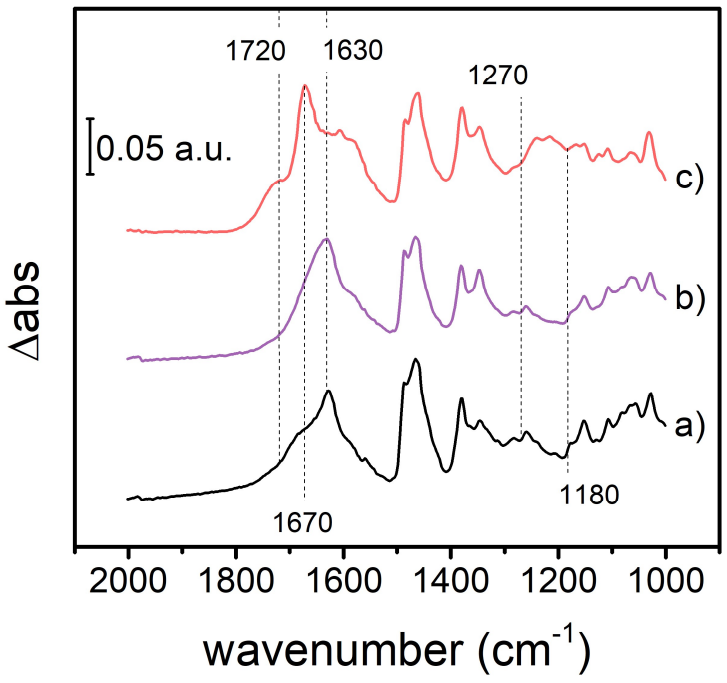


Figure 2. IR absorbance of the a) DTEo, b) DTEc and c) DTEc after complete catalysis by HRP in presence of H_2O_2. Medium: 0.01 M PBS, 6 u ml^{-1} HRP, 2.5 10^{-3} M DTE and, in c), 0.01 M H_2O_2. The sample was deposited over an ATR prism and dried with a N_2 flow prior to data acquisition.

Proposed Mechanism

In presence of H_2O_2, there is a well described oxidation transition of Fe III to Fe IV in the heme group of the enzyme[20] (named Compound I) by direct bonding of one oxygen atom from the H_2O_2 to the Fe, liberating H_2O (**Figure 3**). Sequentially, the oxygen is reduced first to hydroxide (Compound II) (II) and finally to H_2O (III), by the uptake of $2H^+$ and $2e^-$ from a donor (i.e. DTEc). Regarding the obtained results, the fast initial interaction of HRP with the DTEc in presence of H_2O_2 provokes the appearance of a transitory signal at 760 nm wavelength, which may be related to the initial interaction of Compounds I and II with the substrate, and a final, and remaining signal at 345 nm wavelength, related to the radical DTEo$^\bullet$ (spontaneously opened from DTEc$^\bullet$). Previous studies revealed a similar behaviour when Cu II was added to a DTE solution.[21] Finally, DTEo$^\bullet$ is restored to DTEo by proton recover from the buffer (i.e. PBS) which is finally degraded).The role of PBS is demonstrated in **Figure S4**, where the absence of the buffer lead to an only one cycle of DTEc decoloration. After the first (and fast) decoloration, it was not possible to recover the initial DTEc by UV irradiation: the intensity of the 515 nm absorbance signal was negligible. On the other hand, the signal at 760 nm belonging to the HRP catalytic center presents the same cycles and intensity as in the case of PBS presence.

Figure 4a shows a cyclic voltammogran of DTE_o, an irreversible oxidation wave is detected at 1.04 V (E_{pa} , anodic peak potential) vs. SCE. After UV irradiation the DTEo isomerizes leading to the DTEc, Figure 4b shows the cyclic voltammogram of DTEc sample obtained after reaching the photostationary state (PSS) of a DTEo 0.01 M PBS solution after UV irradiation. The amount of DTEc can be easily monitored and controlled by terms of cyclic voltammetry and irradiation time, since DTEc showed a pseudo-reversible oxidation peak at 0.41 V vs. SCE. A controlled-potential electrolysis of a 0.01 M PBS DTEc solution at 0.7 V vs. SCE revealed the decoloration of the sample as well as the isomerization of DTEc to DTEo. Note that the same electrochemical response was observed for a DTEc solution in presence of glucose, GOx and HRP (or simply H_2O_2 and HRP). Hence, the DTEc is oxidized leading to the DTEo$^\bullet$, which eventually evolves to DTEo (Epa= 1.04 V vs. SCE, Figure 4c). These electrochemical results confirms that the HRP

(E^0 = 0.59 V)[22] can catalyze only the DTEc (Eox = 0.41 V) as the DTEo (E = 1.04 V) is not accessible in terms of potential[21], as it can be in **Figure 4**.

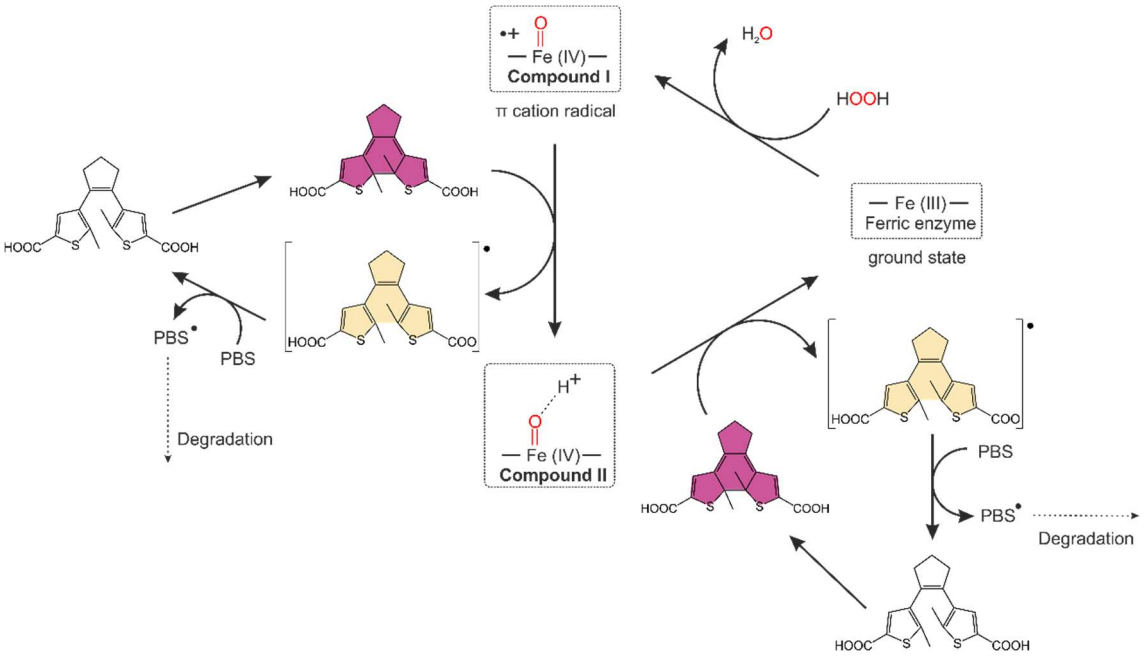


Figure 3. Proposed catalytic pathway**.** HRP catalytic center, in ground state, becomes cation radical (named Compound I) by H_2O_2 reduction to H_2O. Then, it oxidizes consecutively two molecules of DTEc, which become DTEo radicals after the ring opening. These radicals recover the initial DTEo state by proton uptake from PBS. The compound I passes to compound II and again to ground state by the successive DTEc oxidations.

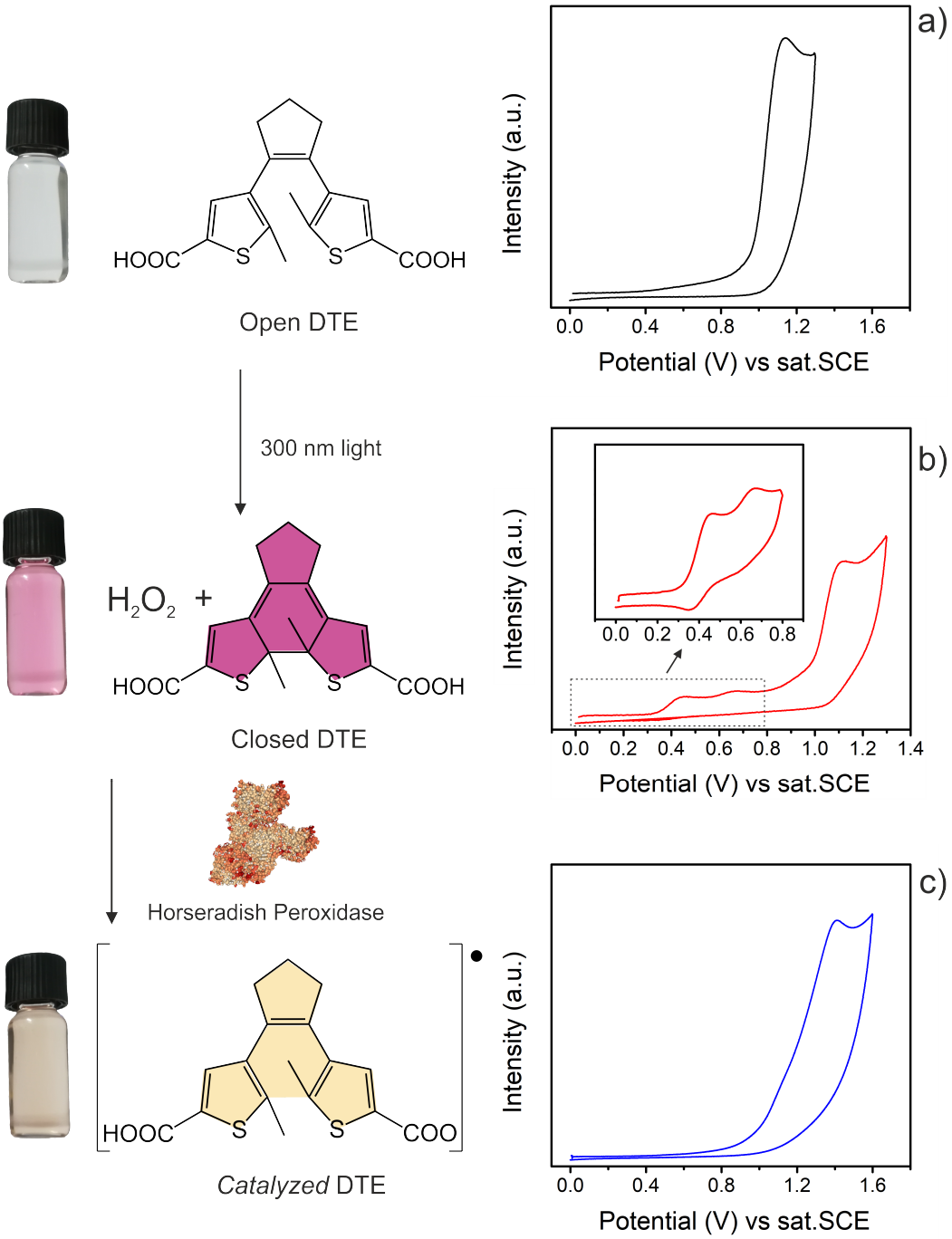


Figure 4. Proposed reaction scheme for DTE. a) The DTEo is closed by UV irradiation at 300 nm. b) In presence of glucose, GOx and HRP (or simply H_2O_2 and HRP), the closed molecule is oxidized by the last, ending in a yellowish product that absorbs mainly in the UV and slightly 400-450 nm. The cyclic voltammetries of 2.5 10^{-3} M DTE, measured in 0.01 M PBS, revealed that only the DTEc is accessible for HRP catalysis. Scan rate: 0.5 V s^{-1}, Working electrode: Glassy carbon, Reference electrode: Saturated Calomel, Counter Electrode: Pt.

The possible interference of common substances present in patients' samples was also studied in order to prevent additional contributions. Some of them did not affect the bleaching rate of DTE (uric acid, creatinine, salicylic acid). However, in case of ascorbic acid and acetaminophen there was a significant change, retaining the color loss or accelerating it, respectively (**Figure S5**). This fact must be taken in account in the future for the development of the matrix-based biosensor for the analysis of subsequent samples.

Conclusions

In summary, the photoelectrochromic DTE molecule can be used as a colorimetric mediator for the HRP enzyme, which catalyse the molecule oxidation once closed by UV irradiation due to the redox potential fall respect to the open ring isomer. We demonstrate the first enzymatic reaction for biosensing that employs a photoelectrochromic molecule as colorimetric mediator. The described system is very interesting as the amount of available mediator for the reaction can be modulated by UV irradiation (DTEc) regardless the total amount of mediator present in the essay (DTEc and DTEo). This fact is interesting because permits a controlled mediator consumption in the reaction, enabling consecutive analyte detections using the same enzymes and restarting the system only by an UV pulse. Additionally, there ison-going work to fabricate 2D solid structures based on silk fibroin with immobilized DTE using the same precursor but irradiating differentially adjoining areas. The availability of mediator for the detection would be different in those areas and would be given only by the irradiation time. The decoloration of more or less previously UV colored areas could be therefore associated to an analyte concentration in the sample in a semi-quantitative essay. The possibility of HRP enzymatic activity coupling to the molecule color change prospects the adaptation to any oxidase-peroxidase cascade system for further biomolecules detection, as it has been demonstrated for glucose.

Experimental Section

Reagents

H_2O_2 (30% in H_2O), NaCl (ACS reagent, ≥99 %), D-(+)-Glucose (≥ 99.5%), tetramethylammonium hydroxide solution (TBAOH, 1 M in methanol), peroxidase from horseradish (HRP; Type VI-A, essentially salt-free lyophilized powder, 250-330 units mg^{-1}) and glucose oxidase (GOx) from *Aspergillus niger* (Type X-S, lyophilized powder, 100–250 units mg^{-1}) were purchased from Sigma-Aldrich, Na_2HPO_4 (ACS reagent, ≥99 %) from Panreac and KCl (≥99 %) and KH_2PO_4 (ACS reagent, ≥99.5 %) from Fluka. All chemicals were used as received and aqueous solutions were prepared using de-ionized water with a resistivity of 2 M Ω cm. Used PBS contains NaCl (8 g L^{-1}), KCl (0.2 g L^{-1}), Na_2HPO_4 (1.42 g L^{-1}) and KH_2PO_4 (0.24 g L^{-1}). Diacid dithienylethene 1,2-bis(5-carboxy-2-methylthien-3-yl)cyclopentene (DTE) was synthesized following a procedure previously described in the literature.[12]

UV-Vis measurements

For the UV-Vis measurements, a cuvette setup from Ocean Optics was used. One extreme of an optical fiber of 1 mm core was connected to the light source and the other to the cuvette light input. At the cuvette light output, another optical fiber of 600 µm guided the light to the Qe65 Pro Spectrometer controlled by OceanView software (both from Ocean Optics). The used light source, depending on the experiment was a deuterium-halogen (DH-2000-BAL, Mikropack) or halogen (HL-2000-FHSA, Ocean Optics) lamp. To close the DTE molecule, a 300 nm LED (M300F2, Thorlabs) was situated on the top of the cuvette. During the experiment, the setup was maintained at every moment in darkness to prevent any interference from environmental light over the compound. For the absorbance spectra acquisition, the cuvette was only irradiated at the time of the measurement.

IR measurements

IR measurements were performed using FTIR Tensor 27 Bruker spectrophotometer, and a Specac Golden Gate accessory for the attenuated total reflectance measurements (ATR). OPUS 5.5 software was used for monitoring the IR spectra. The IR spectra were registered measurements in solid state for better resolution, hence the solvent of the samples were

evaporated using a N_2 flow at room temperature. The results are represented as –log (R/R_0) where R and R_0 are the reflectance values corresponding to the sample and reference, respectively.

Electrochemical measurements

Cyclic voltammetry (CV) experiments were performed using a BioLogic potentiostat (VSP100) controlled with EC-Lab V9.51 software. The measurements were performed in a conical electrochemical cell using a glassy carbon disk (ø=0.5 cm) as a working electrode, Pt wire as a counter electrode and Saturated Calomel Electrode as reference electrode. For CV experiments, the working electrode is polished using a 1 µm diamond paste after each measurement. 2.5 10^{-3} M DTE solutions were prepared in 0.01 M PBS aqueous solution (pH 7).

Acknowledgements

This work was supported by the Spanish R & D National Program (MEC Project TEC2014-54449-C3-1-R and CTQ2015-65439-R). Dr. X. M.-B. also acknowledge the “Ramón y Cajal” program from the Spanish Government. A.M. is also grateful to the MICINN for the award of a research studentship from FPI program (BES-2015-072946).

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