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Redox switchable rhodamine-ferrocene dyad : exploring imaging possibilities in cells

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Abstract

An original redox-responsive fluorescent probe combining a rhodamine derivative and a ferrocenyl moiety used as the fluorescence modulator was designed, synthesized and characterized. The fluorescence of this new dyad could be tuned from the redox state of ferrocene, a feature observed both electrochemically and on cancer cells incubated with this probe.

Keywords

Molecular electrochemistry;

Spectroelectrochemistry;

Rhodamine;

Ferrocene;

Electrofluorochromism.

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1. Introduction

The design and development of molecules whose fluorescence can be switched as a function of their redox state is a very attractive approach in analytical and bioanalytical chemistry since many analytes are redox active and be detected and mapped with high sensitivity through fluorescence [1-5]. Within this context, the most effective strategy to tune fluorescence is based on the development of dyads in which an internal electron transfer or an energy transfer between a redox-active system and a fluorescent core can be enabled or suppressed. Within the past decade, increasing attention has been paid to the development of dyads made of a fluorophore linked through a spacer to a redox moiety which switches fluorescence through photoinduced electron transfer or energy transfer between the excited state of fluorophore and the redox functionality.

Redox moieties are generally either organic or metal-containing electroactive groups where ferrocene is a very attractive redox quencher due to its stability and versatility in terms of chemical functionalization. Accordingly, ferrocene has been combined to various common fluorophores such as pyrene [6], perylene diimide [7,8], BODIPY [9,10] as well as non-established fluorophores such as zinc porphyrin [11,12] or europium [13]. All these investigations clearly demonstrated that ferrocene, under its reduced form, can be advantageously used to quench fluorescence whereas its oxidized form leaves fluorescence unaffected. Ferrocene was also combined with anthracene with a double bond as a linker. In this case, fluorescence was quenched via an intramolecular charge transfer process whereas fluorescence enhancement was due to the specific reaction of the double bond with hypochlorous acid [14].

Ferrocene has also been grafted to rhodamine derivatives in view of developing chemosensors. In these cases, restoration of fluorescence was not obtained through oxidation of the ferrocene moiety, but was triggered by ion sensing [15-21]. Surprisingly, no work was reported so far on the use of ferrocene-rhodamine dyes from which fluorescence was triggered through ferrocene oxidation. Based on recent investigations on rhodamine 101 (Rh101) [22], we designed an original rhodamine-ferrocene dyad (Rh-Fc, **2** in Figure 1) in which the Fc moiety can be used as the fluorescence modulator depending on its redox state.

2. Experimental

2.1. Chemicals

Compounds for the synthesis of compound **2** were used as received: acetic acid, CF_3COOH , CH_2Cl_2 , cyclohexane, DMF, EtOAc, EtOH (Carlo Erba), CuSO_4 , EDTA (Alfa Aesar), ferrocenemethanol and sodium ascorbate (Sigma-Aldrich), H_2O (distilled with Aquatron® model A4000), MgSO_4 and NaHCO_3 (VWR), silica gel (Macherey-Nagel), sodium azide (TCI S0489). Rhodamine with an alkyne moiety was previously prepared in our laboratory [23]. Compounds for electrochemical and spectroelectrochemical experiments were also used without further purification: anhydrous MeCN (Sigma-Aldrich), ferrocene (Sigma-Aldrich). Tetrabutylammonium tetrafluoroborate (TBA.BF_4) was synthesized from tetrabutylammonium hydrogen sulfate by anion metathesis with sodium tetrafluoroborate.

2.2. Synthesis of the ferrocene-rhodamine Rh-Fc derivative (2)

A solution of CuSO₄ (6.0 mg, 24.0 μmol) and sodium ascorbate (7.0 mg, 35.3 μmol) in H₂O (1 mL) was added to a solution of rhodamine **1** (30.0 mg, 48.8 μmol) [24] and azido-ferrocene (23.0 mg, 95.4 μmol – prepared as described in [24]) in DMF (9 mL). The mixture was stirred at room temperature overnight and the solvents were evaporated. The residue was dissolved in CH₂Cl₂ (50 mL) and washed with 0.1 M EDTA aqueous solution (2 x 20 mL). The organic layer was dried over MgSO₄ and the solvent was removed on a rotary evaporator. The resultant residue was purified by column chromatography on silica gel (CH₂Cl₂ /EtOH/CF₃COOH 900:100:1). Compound **2** was isolated as purple oil in 31 % yield (13 mg, 15 μmol).

¹H NMR (300 MHz, CDCl₃): δ (ppm) = 1.90 (m, 4H, Hb or Hb'); 1.99 (m, 4H, Hb or Hb'); 2.59 (m, 4H, Ha); 2.89 (t, J = 6.5 Hz, 4H, Ha'); 3.41 (m, 8H, Hc, Hc'); 3.90 - 4.08 (m, 9H, H Cp ring); 5.07 (m, 4H, O-CH₂-Triaz and Triaz-CH₂-Cp); 6.64 (s, 2H, H1); 7.00 (dd, J = 1.8, 7.5 Hz, 1H, Har); 7.09 (m, 1H, Har); 7.30 (d, J = 8.5 Hz, 1H, Har); 7.45 (s, 1H, HTriaz); 7.51 (m, 1H, Har). ¹³C NMR (75 MHz, CDCl₃): δ (ppm) = 20.0 (Cb or Cb'), 20.2 (Ca'), 20.9 (Cb or Cb'), 27.8 (Ca), 50.8 (Cc or Cc'), 51.3 (Cc or Cc'), 68.9 – 69.3 (C Cp ring), 105.2, 113.3 (CH Triaz), 121.3 (C ar), 121.7 (Cq), 123.6 (Cq), 126.8 (C1), 131.0 (C ar), 131.6 (C ar), 151.2 (Cq), 152.3 (Cq), 152.9 (Cq), 155.5 (Cq). HRMS (ESI) m/z: [M⁺] (C₄₅H₄₄FeN₅O₂) calc.: 742.2839, found: 742.2852.

2.3. Instrumentation

Cyclic voltammetry was performed at room temperature under argon atmosphere in a three-electrode cell using an Autolab potentiostat (PGSTAT 20). The reference electrode was an SCE (Radiometer), separated from the solution by a bridge compartment filled with the same solvent/supporting electrolyte solution as used in the cell. The counter electrode was a 1 cm platinum wire (Goodfellow). The glassy carbon working electrode was homemade (1 mm diameter; Goodfellow). Ferrocene was used as an internal standard. The half-wave potential of the ferrocene/ferrocenium redox couple (Fc/Fc⁺) was 0.420 V/SCE [25].

Spectroelectrochemical experiments were carried out on Autolab PGSTAT 20 potentiostat in a quartz glass spectroelectrochemical cell with 0.5 mm optical path length (Biologic) using platinum mesh as a working electrode, non-aqueous Ag/Ag⁺ (Biologic instruments; silver wire soaking in a non-aqueous electrolyte Ag⁺/MeCN/TBAP (tetrabutyl ammonium perchlorate)) as a reference electrode, and a platinum wire as a counter electrode. Perkin Elmer Lambda 45 spectrometer was used in absorption spectroelectrochemical experiments and JASCO FP-8300 spectrofluorometer in fluorescence spectroelectrochemical experiments. Experiments were performed under argon purging, at room temperature, with ferrocene as an internal standard. The half-wave potential of the ferrocene/ferrocenium redox couple (Fc/Fc⁺) was 0.095 V with the non-aqueous Ag/Ag⁺ reference electrode. All potentials were recalculated to SCE by adding the difference of 0.325V to all measured values.

2.4. Experiments on PC-12 cells in the presence of the Rh-Fc (2) compound

PC-12 cells were cultured according to the procedure recommended by ATCC (CRL-1721). For their observation under fluorescence microscopy with a set filter 74 HE from Zeiss, PC-12 cells were studied in Petri dishes (P50G-1.5-14-F, MatTekCultureware, Ashland, MA) previously treated with a collagen IV solution (0.1 mg / mL) as described elsewhere [26]. After the subculture stage, PC-12 cells were re-suspended in their complete growth medium and diluted to reach a concentration of 10^5 cells /mL. Samples were maintained in the incubator (at 37 °C and under an atmosphere of 5% CO₂), for 24 hours, before experiments. Then media were renewed with complete growth media supplemented with Rh-Fc (**2**). Samples were stored in the incubator for different time durations (10, 20, 30 and 60 minutes). Afterwards, Petri dishes were rinsed three times with filtered Phosphate Buffered Saline (PBS - 7.4) and cells were observed in this solution.

3. Results

3.1. Electrochemical behavior of Rh-Fc (**2**)

The oxidation of the ferrocene (Fc) unit appeared fully reversible (Fig. 1(a)) and occurred at a more positive potential value compared to unsubstituted Fc (+0.59 vs. +0.46 /SCE) accounting for the electron withdrawing effect of the rhodamine-triazole structure linked to the Fc group. On the other hand, the reduction of the rhodamine moiety was observed at the expected potential value for rhodamine 101 derivatives though less reversible (Fig. 1(b)) [22].

< Figure 1 >

3.2. Photophysical properties of **2**

The normalized absorption and fluorescence spectra of **2** were similar, in terms of shape and maximum bands, in MeCN, H₂O, and PBS (Fig. 2). The absorption and fluorescence emission maxima of the Rh-Fc complex **2** were also similar to that obtained for other rhodamine 101 derivatives previously investigated in both methanol and acetonitrile [22] showing no effect of the Fc moiety on the photophysical properties of **2**.

However, in terms of intensity, only very weak fluorescence was obtained for the Rh-Fc complex suggesting an intramolecular charge transfer between the Fc unit and the Rh moiety. The fluorescence quenching observed in the presence of the Fc unit was quantified through the determination of quantum yields using Rh101 as a standard [27]. As shown in Fig. 2(C), the Rh-Fc complex possesses significantly reduced relative emission quantum yields.

Actually, the emission yield for Rh-Fc is only 5% that of Rh101. Importantly, similar fluorescence quenching were also obtained in water and PBS (Fig.2(D)).

The origin of the fluorescence quenching in dyad **2** was ascribed to a photoinduced electron transfer between the ferrocene unit and the rhodamine moiety, an assessment based on several considerations: First of all, the electronic absorption spectra of both the Rh101 and Rh-Fc were similar whatever the solvent nature. Therefore, in case an intramolecular charge transfer (ICT) took place between the donor Fc and the acceptor Rh, an additional absorption

band would have been observed [9]. Then, an estimation of ΔG for the photoinduced electron transfer from the Fc to the Rh moiety would be -0.55eV, accounting for a thermodynamically favorable reaction. This was calculated from the Rehm-Weller equation: $\Delta G_{\text{PET}} = [E^\circ(\text{ox}) - E^\circ(\text{red})] - E(\text{ex}) - e^2/\epsilon d$, with $E^\circ(\text{ox}) = 0.55 \text{ V}$, $E^\circ(\text{red}) = -0.97$, $\lambda(\text{ex}) = 600 \text{ nm}$. Note that the coulombic attraction term ($e^2/\epsilon d$) could be neglected in our case since the global charge remained the same after the electron transfer. Finally, there is no spectral overlap between the absorption spectrum of the ferrocene moiety and the fluorescence spectrum of the Rh unit, ruling out the possibility of an energy transfer between Fc and Rh.

< Figure 2 >

3.3. Fluorescence modulation of **2**

3.3.1. Electrochemical oxidation of the Fc moiety

Fluorescence modulation of **2** was first tested through the electrochemical oxidation of its Fc group by fluorescence spectroelectrochemical measurements. At open circuit potential, the emission spectra of **2** displayed no fluorescence (peak expected at 608 nm - $\lambda_{\text{exc}} = 580 \text{ nm}$) due to the PET process. However, during electrolysis at a potential value corresponding to the oxidation of the Fc moiety (+0.7 V), the fluorescence emission of **2** appeared and increased as a function of time (Fig.3(a)) clearly indicating that PET no longer happened. Importantly, after 5 minutes, switching the electrolysis potential back to a value allowing the ferricinium moiety could be reduced led to the disappearance of the fluorescence emission intensity (Fig.3(b)). Under these conditions a full ON/OFF switching could be achieved between the non-fluorescent reduced Fc state and its fluorescent oxidized state.

3.3.2. Incubation of **2** with cancer cells

The fluorescence modulation of **2** was tested in a practical application for living-cell imaging. Since physiological as well as pathological processes are closely associated with alterations of cellular redox status (which itself is a consequence of the precise balance between reactive oxygen species and reducing species) [28, 29], we decided to explore the incubation of cancer PC-12 cells in the presence of our original Rh-Fc dyad. Indeed, it is now well-documented that ferrocene, which is a prosthetic group present in a growing number of bio-organometallic compounds with potent antitumoral properties, can be easily oxidized *in vitro* or *in vivo* [30-32]. On the other hand, PC12 cells were considered due to their possible ability to oxidize the ferrocene probe inside the intracellular medium. This cell model should indeed contain basal sources of oxidants, like Reactive Oxygen Species due to the intracellular autoxidation of dopamine [33].

Fluorescence of the PBS solution as well as that of complex **2** dissolved in PBS were first tested as control analyses. As expected, the results showed the absence of fluorescence in both cases indicating that compound **2** remained in its reduced form in PBS solution.

Samples containing PC-12 cells in their complete growth medium supplemented with 20 μM of Rh-Fc, were then stored in the incubator for different durations (10, 20 and 60 minutes). After each incubation time, Petri dishes were removed from the incubator and rinsed three times with filtered PBS. Cells were therefore observed in PBS free of growth medium. In order to check the intrinsic fluorescence of cells, one sample containing PC-12 cells, incubated only with their complete growth medium (without any Rh-Fc molecule inside), was also prepared and analyzed under the same conditions. PC-12 cells exhibited fluorescence when incubated with Rh-Fc whereas no fluorescence was detected upon incubation with growth medium only (Fig.3(B)).

This observation demonstrated that Rh-Fc molecule has been internalized by PC-12 cells and oxidized in the intracellular medium. According to the pictures taken for different incubation periods, the fluorescence intensity reached a plateau after 20 min of incubation.

< Figure 3 >

4. Conclusion

An original ferrocene-appended rhodamine 101 complex possessing interesting electrofluorochromic properties was designed and synthesized. The fluorescence quantum yield of the Rh101 derivative dropped drastically after clicking the ferrocenyl unit onto the Rh moiety. However, emission of the Rh-Fc complex could be recovered upon oxidation of the Fc part. This fluorescence modulation was established from the electrochemical behavior, and allowed the observation of specific light emission from PC-12 cancer cells, opening new perspectives to image alterations of the cellular redox status.

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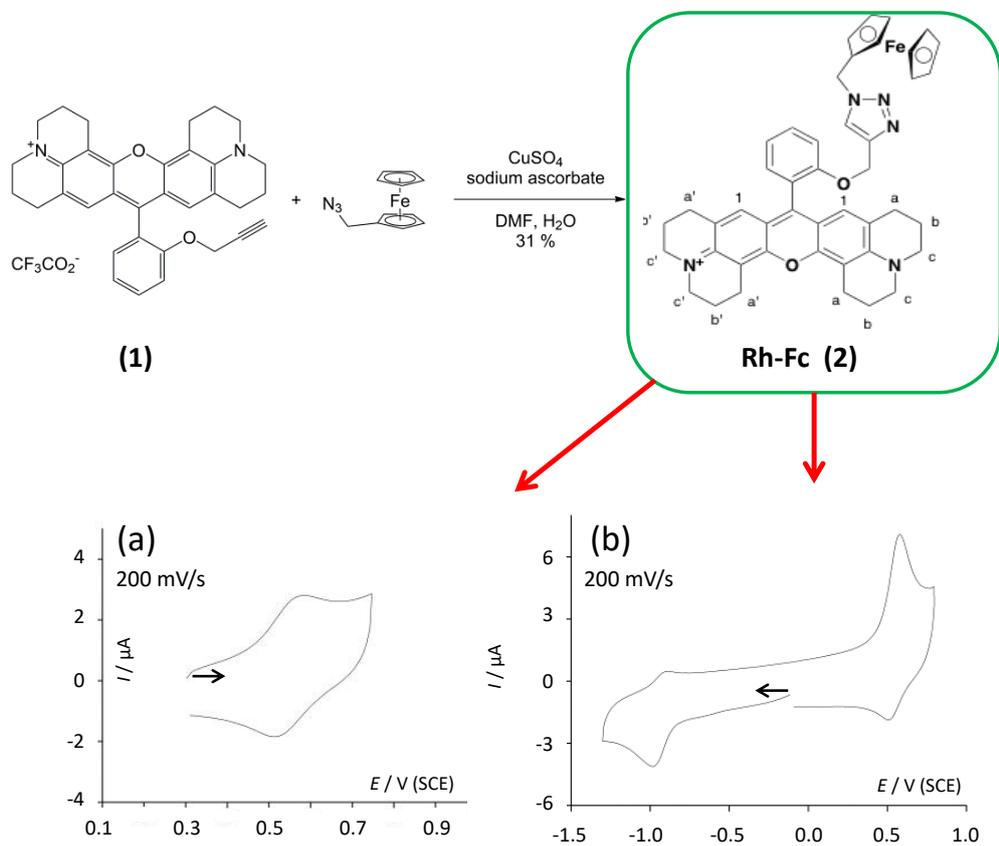
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Figure Captions

Fig. 1. Synthesis of the Rh-Fc (**2**) complex and its corresponding cyclic voltammograms (0.5 mM) in MeCN/[TBA][BF₄] (0.1 M). Scans performed towards (a) positive and (b) negative potential values.



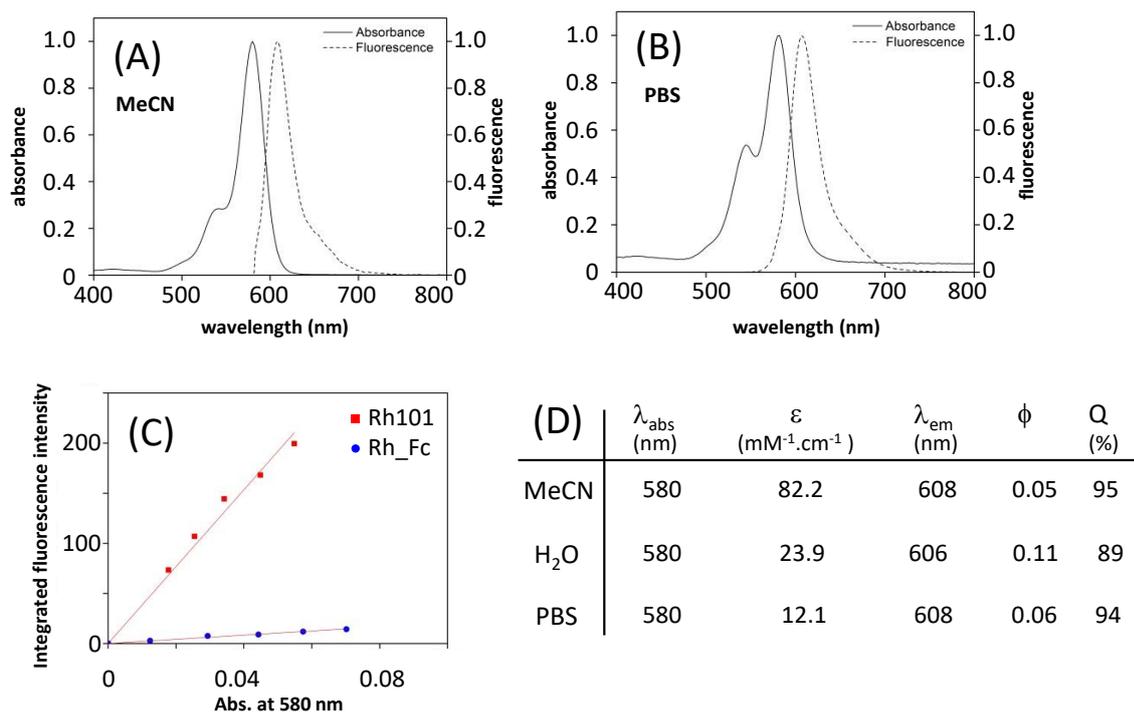


Fig. 2. Absorption and fluorescence normalized spectra of **(2)** in (A) MeCN (20 μM) and in (B) PBS (28 μM). (C) Slope comparison of **(2)** and Rh 101 used as standard. (D) Summary of photophysical properties of **(2)**; Q – quenching efficiency, $Q = (\Phi_{\text{DYE}} - \Phi_{\text{DYAD}}) / \Phi_{\text{DYE}} \times 100\%$.

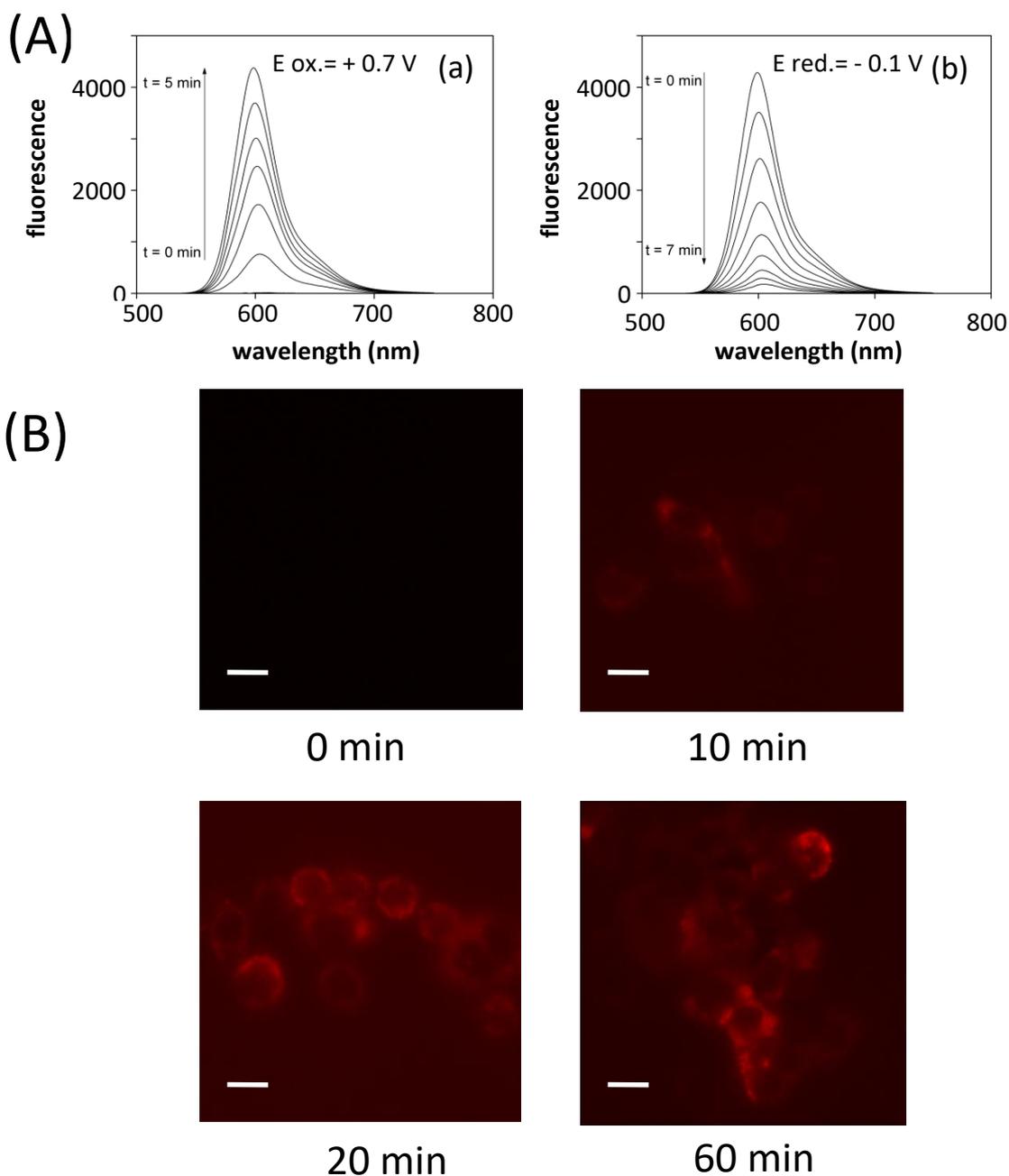


Fig. 3. (A) Fluorescence emission spectra recorded in MeCN/[TBA][BF₄] (0.1 M) upon (a) oxidation of **2** (5 μ M, λ_{exc} = 580 nm, spectra recorded every 50s) at +0.7 V and upon (b) reduction of the electrogenerated ferricinium species at -0.1 V (λ_{exc} = 580 nm, spectra recorded every 50s). Note that time evolution of fluorescence emission spectra shown in (b) have been performed just after (a) (same experiment). (B) Incubation of PC-12 cells in the presence of the compound **2** (20 μ M) as a function of time. Fluorescence maximum level (100%) is stable for incubation times between 20 min and 60 min; fluorescence intensity with incubation time 10 min: 30%. The white scale bar represents 10 μ m. Evolution of fluorescence in cells with time was measured with the Image J software.