

Electrochemiluminescence Enhanced by a Non-Emissive Dual Redox Mediator

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Abstract: A sulfonated tris(1-phenylpyrazolato)iridium(III) complex ($[\text{Ir}(\text{sppz})_3]^{3-}$) serves as a proof-of-concept non-emissive enhancer of the widely used ECL detection system of tris(2,2'-bipyridine)ruthenium(II) ($[\text{Ru}(\text{bpy})_3]^{2+}$) with tri-*n*-propylamine (TPrA) co-reactant, acting through electrocatalysis of TPrA oxidation and efficient chemi-excitation of the luminophore. Using self-interference ECL spectroscopy, we show that the enhancer extends diffusion of the required electrogenerated precursors from the electrode surface. Previously reported enhancement through these pathways has been confounded by the inherent ECL of the enhancer, but the increase in $[\text{Ru}(\text{bpy})_3]^{2+}$ ECL intensity using $[\text{Ir}(\text{sppz})_3]^{3-}$ was obtained without its concomitant emission. The most prominent enhancement (11-fold) occurred at low potentials associated with the 'indirect' co-reactant ECL pathway, which translated to between 2- and 6-fold enhancement when the luminophore was immobilised on microbeads as a general model for enhanced ECL assays.

Introduced over three decades ago, the co-reactant electrochemiluminescence (ECL) of tris(2,2'-bipyridine)-ruthenium(II) ($[\text{Ru}(\text{bpy})_3]^{2+}$) with tri-*n*-propylamine (TPrA) (Schemes 1a–1c) has become a fundamental detection platform for in vitro diagnostics and remains the benchmark for ECL research.^[1] Together with advances in instrument

automation and affinity assays, optimising ECL detection efficiency is critical for the fastest and most sensitive quantification of biomarkers.^[2]

Extensive research has therefore focused on enhancing the ECL signal. In commercialised ECL systems, the TPrA is delivered in 'ProCell' buffer solution containing a surfactant that enables dissolution of higher concentrations of this co-reactant. The introduction of compounds such as propanamide and boric acid to the ProCell solution has been found to improve the limit of detection by 30–40%.^[2] Recent proposals to enhance the ECL of the $[\text{Ru}(\text{bpy})_3]^{2+}$ -TPrA system involve either modification of the reaction microenvironment or electrogeneration of additional intermediates that increase the efficiency at which the excited state luminophore can be formed.^[3] One of the most promising of these approaches exploits a redox mediator that can act as both an electrocatalyst for TPrA oxidation (Scheme 1d) and a more stable alternative to TPrA^{•+} for the chemi-excitation of the luminophore (Scheme 1e).^[3e,4] To date, the most effective mediator reported is tris(2-(2-pyridinyl-*κ*N)-4-sulfonatophenyl-*κ*C)iridium(III) ($[\text{Ir}(\text{sppy})_3]^{3-}$, Figure 1), due to its combination of (i) high solubility in aqueous solution, (ii) a chemically and electrochemically reversible oxidation (E_{ox}) at a potential close to that of the irreversible oxidation of TPrA, (iii) an E_{red} beyond the reduction strength of TPrA^{•+}, and (iv) a greater excited state energy than $[\text{Ru}(\text{bpy})_3]^{2+}$.^[4c]

All of the iridium(III) redox mediator enhancers explored to date^[3e,4] also proceed through the direct ECL

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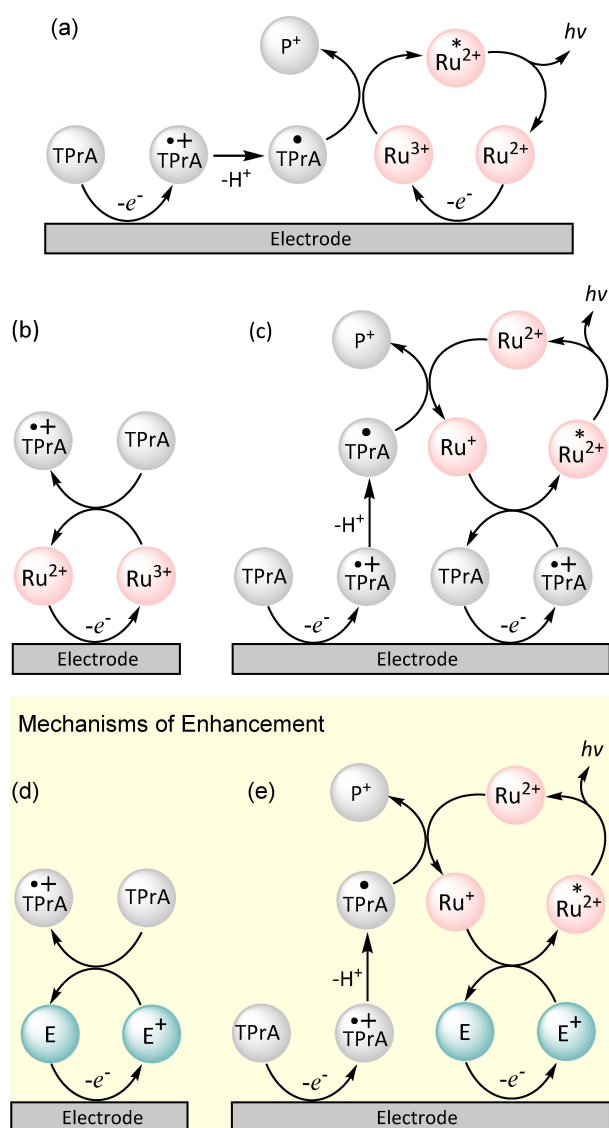
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Scheme 1. Reaction pathways for the co-reactant ECL of $[Ru(bpy)_3]^{2+}$ and TPrA, where $Ru^{+2/+3+} = [Ru(bpy)_3]^{+2/+3+}$, $TPrA^{•+} = Pr_3N^{•+}$, $TPrA^+ = Pr_2NC^+HCH_2CH_3$, P^+ is an iminium cation ($Pr_2N^+ = CHCH_2$) that hydrolyses in aqueous solution,^[1b,c,3e,4a] and E is an enhancer (e.g., $[Ir(sppy)_3]^{3-}$).^[1b,4c] (a) The 'direct' or 'oxidative-reduction' route, requiring electrooxidation of the metal complex. (b) The 'catalytic' route, in which TPrA is homogeneously oxidised. (c) The 'indirect' (also referred to as the 'remote' or 'low-oxidation potential') pathway, in which $[Ru(bpy)_3]^{2+}$ is not oxidised. (d) Electrocatalysis of TPrA oxidation by the enhancer. (e) The 'redox-mediator' route,^[3e,4a] in which the oxidised enhancer (E^+) reacts with $[Ru(bpy)_3]^{2+}$ to generate the emissive species.

excitation pathway (analogous to Scheme 1a) to simultaneously emit light. Under certain circumstances, this can be exploited for internal standardisation,^[3e] but the need to distinguish the emission from each luminophore limits the analytical sensitivity. Moreover, it is incompatible with conventional ECL instrumentation, and the greater instrumental complexity is not ideal for the development of portable analytical devices based on ECL detection. We therefore sought a species that would enhance the ECL of $[Ru(bpy)_3]^{2+}$ with TPrA co-reactant through the same

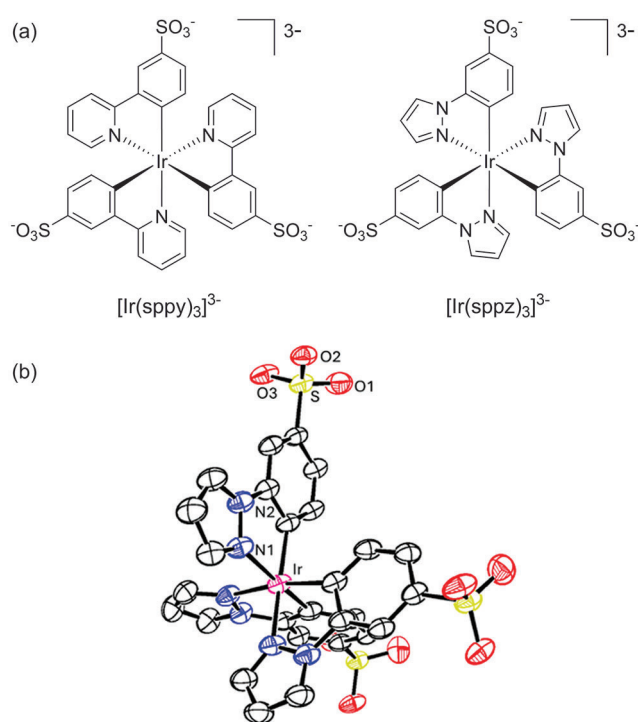


Figure 1. (a) Structures of the emissive enhancer $[Ir(sppy)_3]^{3-}$ and the novel non-emissive enhancer $[Ir(sppz)_3]^{3-}$. (b) ORTEP representation of $Na_3[Ir(sppz)_3]$.^[6] Ellipsoids drawn at 50% probability and hydrogen atoms, counterions and solvent molecules omitted for clarity.

mechanisms (Schemes 1d and 1e) without concomitant emission.

As proof-of-concept, we prepared a mediator satisfying the above four criteria by sulfonating tris(1-phenylpyrazolato)iridium(III) ($[Ir(sppz)_3]^{3-}$, Figure 1). The parent $Ir(ppz)_3$ has a low quantum yield (<0.01 in 2-methyltetrahydrofuran) due to efficient population of a metal centred (3MC) excited state.^[5] Moreover, the reaction of the oxidised $[Ir(ppz)_3]^+$ with TPrA $^{\bullet}$ is not sufficiently energetic to generate $Ir(ppz)_3^*$ (i.e., a pathway analogous to Scheme 1a is not feasible). The sulfonated $[Ir(sppz)_3]^{3-}$ complex exhibits similar spectroscopic and electrochemical properties to those of $Ir(ppz)_3$ (Table S2; Figures S2–S5), where oxidation is observed at marginally higher potential than that of the phenylpyridine analogue and reduction does not occur within the electrochemical window of the solvent.

The relative intensity and spectral distribution of ECL from $[Ru(bpy)_3]^{2+}$ and TPrA with: (i) no enhancer; (ii) $[Ir(sppy)_3]^{3-}$; or (iii) $[Ir(sppz)_3]^{3-}$, upon application of 0.95 V vs Ag/AgCl, are shown in Figures 2a, 2b and 2c, respectively. Both Ir(III) complexes greatly increased the co-reactant ECL of $[Ru(bpy)_3]^{2+}$, but concomitant ECL was observed only from $[Ir(sppy)_3]^{3-}$. At the same concentration, the enhancement by $[Ir(sppy)_3]^{3-}$ was greater (Figure S6–S9), but without inherent ECL, the novel $[Ir(sppz)_3]^{3-}$ complex can be used at higher concentrations providing superior enhancement of $[Ru(bpy)_3]^{2+}$ ECL.

For a comprehensive assessment of the enhancement over a wide range of applied potentials, we used the

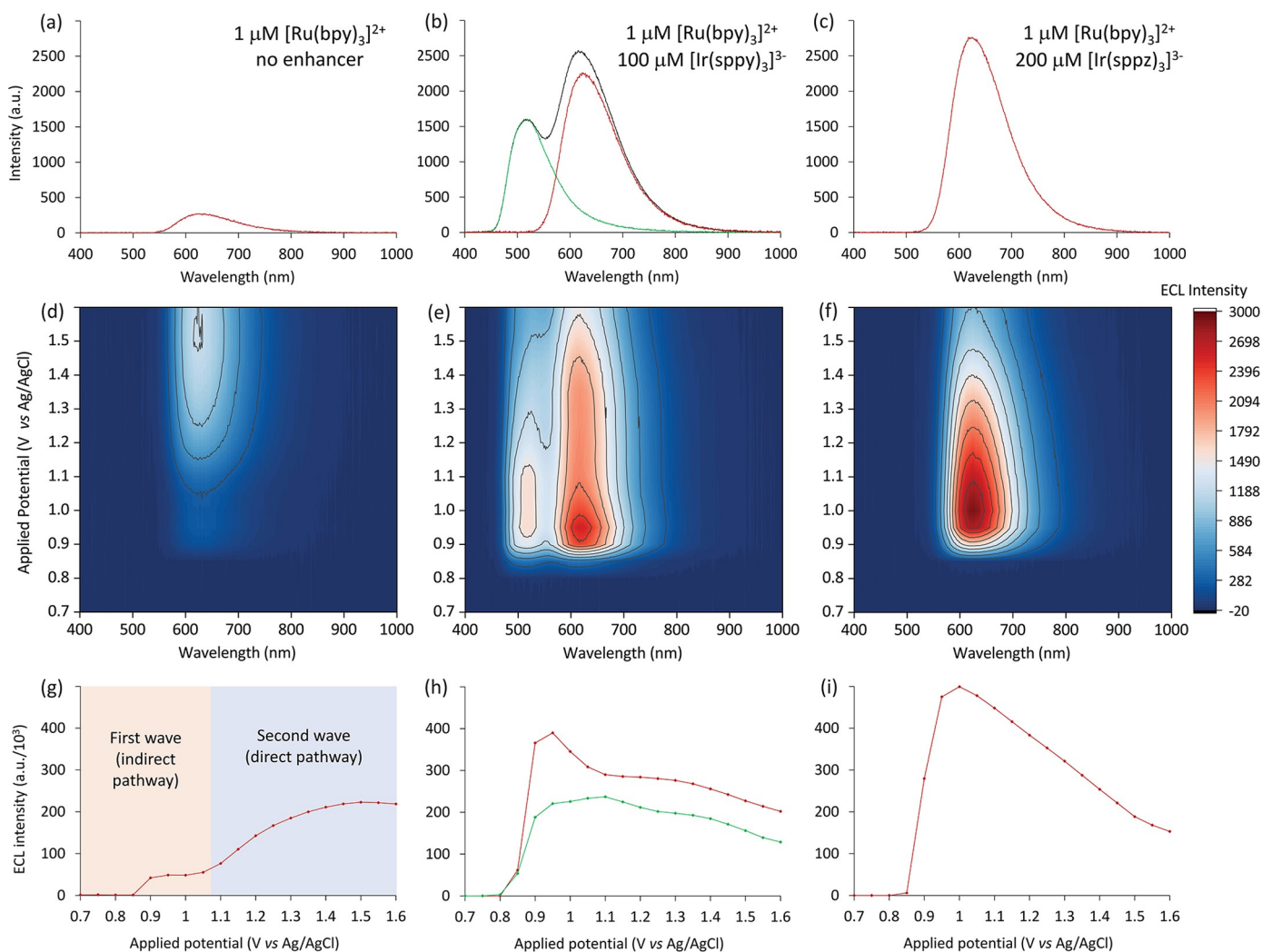


Figure 2. (a–c) ECL spectra of $1 \mu\text{M}$ $[\text{Ru}(\text{bpy})_3]^{2+}$ in ProCell with (a) no enhancer, (b) $100 \mu\text{M}$ $[\text{Ir}(\text{sppy})_3]^{3-}$, or (c) $200 \mu\text{M}$ $[\text{Ir}(\text{sppz})_3]^{3-}$, when applying a potential of 0.95 V vs Ag/AgCl. In Figure 2b, the overall emission (black plot) has been deconvoluted into the emissions from $[\text{Ru}(\text{bpy})_3]^{2+}$ (red plot) and $[\text{Ir}(\text{sppy})_3]^{3-}$ (green plot). (d–f) ‘3D ECL’ contour plots of the same reaction mixtures, showing the ECL spectral distribution (10 s acquisition time) over a series of applied potentials (50 mV intervals), interspersed with 10 s wait times at 0 V vs Ag/AgCl. (g–i) Comparisons of relative ECL intensity (integrated spectral distribution) over the applied potential range, extracted from the data shown in Figure 2d–2f, respectively. In Figure 2h, the overall emission (not shown) has been deconvoluted into the contributions from $[\text{Ru}(\text{bpy})_3]^{2+}$ (red plot) and $[\text{Ir}(\text{sppy})_3]^{3-}$ (green plot). Electrodes: working: GC, counter: Pt wire, reference: Ag/AgCl.

‘spooling’ (‘3D’) ECL approach⁷¹ to create plots of ECL spectra versus the increasing potential of a series of chronoamperometric pulses (Figure 2d–2f). From these data, the ECL intensities of $[\text{Ru}(\text{bpy})_3]^{2+}$, without and with an enhancer, over the applied potential range were extracted (Figures 2g–2i). The unenhanced reaction exhibits the characteristic ‘two waves’ of ECL^{1b,8j} in this system (Figure 2d and 2g): the first at potentials that oxidise only the co-reactant, arising from the indirect pathway (Scheme 1c) and the second at potentials that oxidise both the co-reactant and luminophore, from the direct pathway (Scheme 1a). The addition of $100 \mu\text{M}$ $[\text{Ir}(\text{sppy})_3]^{3-}$ results in both a new peak arising from its ECL and a significant enhancement of $[\text{Ru}(\text{bpy})_3]^{2+}$ ECL, particularly in the ‘first wave’ (Figure 2e). Deconvolution of these emissions (Figure 2h) reveals a 9-fold enhancement at 0.90 V vs Ag/AgCl (close to

the oxidation potential of the enhancer: 0.79 V vs Ag/AgCl; Figure S5). Alternatively, adding $[\text{Ir}(\text{sppz})_3]^{3-}$ resulted in enhanced ECL from only $[\text{Ru}(\text{bpy})_3]^{2+}$, with the greatest enhancement (11-fold using $200 \mu\text{M}$ $[\text{Ir}(\text{sppz})_3]^{3-}$) at 1.0 V vs Ag/AgCl, corresponding to the slightly higher potential required to oxidise this Ir(III) complex (Table S2). Similarly, for the detection of the free $[\text{Ru}(\text{bpy})_3]^{2+}$ luminophore in ProCell solution, the addition of $200 \mu\text{M}$ $[\text{Ir}(\text{sppz})_3]^{3-}$ increased the sensitivity (calibration gradient) by 10-fold or 4-fold when applying a squarewave potential sequence (1 Hz) to 1.0 V (insufficient to oxidise $[\text{Ru}(\text{bpy})_3]^{2+}$) or 1.3 V (sufficient to oxidise $[\text{Ru}(\text{bpy})_3]^{2+}$), respectively (Figure S10).

For comparison, we also examined the influence of the $[\text{Fe}(\text{bpy})_3]^{2+}$ complex, which meets some but not all the proposed criteria for an effective enhancer of the ECL of

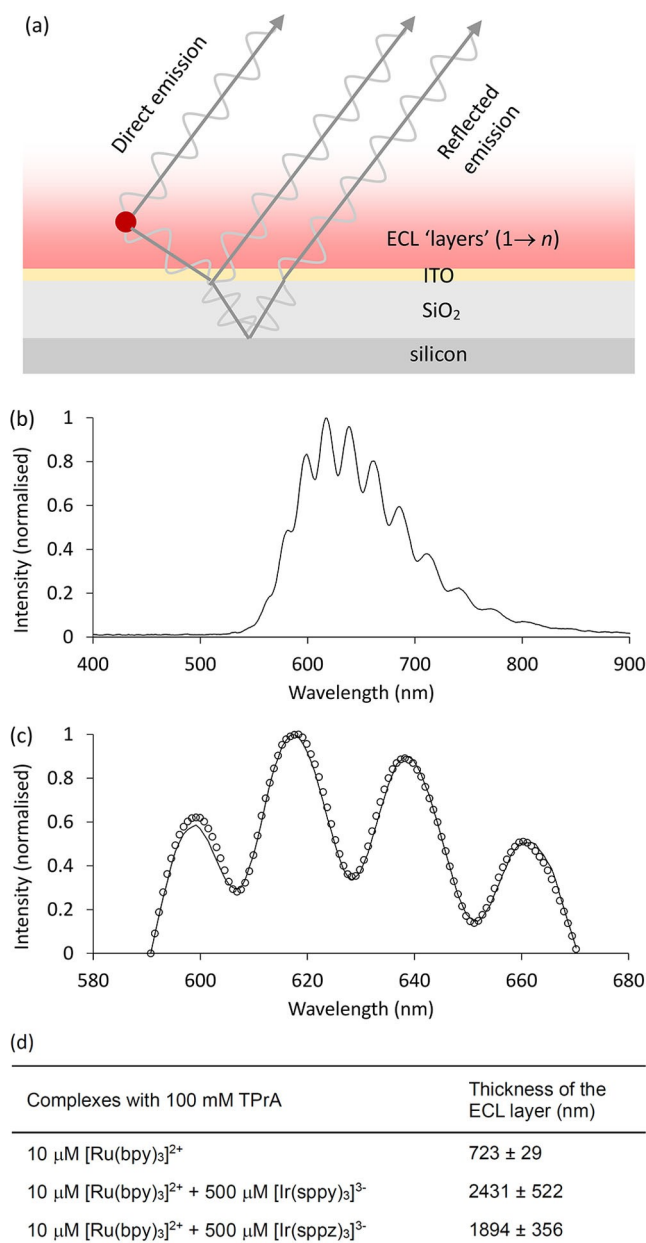


Figure 3. (a) Schematic of ECL self-interference spectroscopy using an ITO/SiO₂/Si working electrode. (b) Normalised ECL self-interference spectrum (1 s integration time at 1.3 V vs Ag/AgCl) from a solution of 10 μM $[\text{Ru}(\text{bpy})_3]^{2+}$, 500 μM $[\text{Ir}(\text{sppz})_3]^{3-}$ and 100 mM TPrA in 0.1 M phosphate buffer (pH 7.4). (c) Simulation (open circles) of the experimental data (solid line) from Figure 3b. (d) Thickness of the $[\text{Ru}(\text{bpy})_3]^{2+}$ ECL layer without and with the enhancers in solution. The complete set of experiments used to obtain these data is shown in Figure S14–S18. Other electrodes: counter: Pt wire, reference: Ag/AgCl.

$[\text{Ru}(\text{bpy})_3]^{2+}$,^[11a] and the thickness of the ECL layer generated using different co-reactants.^[11b] Analysis of the interference spectra for the unenhanced and enhanced systems (Figures 3b, 3c and S14–S18) showed an increase in the thickness of the $[\text{Ru}(\text{bpy})_3]^{2+}$ ECL layer when using either enhancer (Figure 3d).^[12] In the case of $[\text{Ir}(\text{sppz})_3]^{3-}$, despite its much greater concentration, the thickness of the ECL layer corresponding to its inherent emission (with or

without $[\text{Ru}(\text{bpy})_3]^{2+}$ in the reaction mixture) was thinner than even the unenhanced ECL layer of the Ru(II) complex (Table S3).

In some of the above experiments, the indirect route (Scheme 1c) was isolated from the direct route (Scheme 1a) by applying potentials at which TPrA, but not $[\text{Ru}(\text{bpy})_3]^{2+}$, is oxidised. It is under these conditions (~ 1.0 V vs Ag/AgCl) that the greatest enhancement was observed (Figure 2) via Schemes 1d and 1e. The indirect route is critical for magnetic-microbead supported assays (such as those used in commercial ECL instruments for immunodiagnostics), where the vast majority of luminophores are not close enough to the electrode to undergo direct oxidation.^[2,13] The redox mediators were therefore anticipated to enhance the co-reactant ECL of $[\text{Ru}(\text{bpy})_3]^{2+}$ -based luminophores immobilised on microbeads. Under these conditions, the ECL mechanism is limited to the indirect route irrespective of the applied potential, but enhancement via Scheme 1d should be less prominent when higher overpotentials are applied. On the other hand, the immobilisation of luminophores away from the electrode places a much greater dependence on the diffusion of reactive intermediates away from the electrode surface. As demonstrated by ECL self-interference spectroscopy (Figure 3 and Table S3), the redox mediators can indeed be used to manipulate the distance at which the reactive intermediates diffuse from the electrode surface.

We employed two approaches to examine the influence of the non-emissive $[\text{Ir}(\text{sppz})_3]^{3-}$ redox mediator on the co-reactant ECL of the $[\text{Ru}(\text{bpy})_3]^{2+}$ luminophore when immobilised on beads, as general models for bead-based ECL assays. In the first, the luminophore was covalently immobilised on 12 μm polystyrene beads that were drop-casted onto the working electrode (Figure S19–S20). Using microscopy,^[14] an increase in the ECL from the $[\text{Ru}(\text{bpy})_3]^{2+}$ luminophore by 4.8-fold at 1.0 V and 3.4-fold at 1.2 V vs Ag/AgCl was detected when adding $[\text{Ir}(\text{sppz})_3]^{3-}$ into the ProCell solution (Figure 4a). Analogous experiments using $[\text{Fe}(\text{bpy})_3]^{2+}$ as the redox mediator resulted in quenching of the ECL (Figure S21). In the second approach, the $[\text{Ru}(\text{bpy})_3]^{2+}$ luminophore was covalently bound to 1 μm magnetic beads, that were attracted to the working electrode surface with a magnet, and a photomultiplier tube was used to measure the ECL intensity when the potential was applied. Under these conditions, the addition of $[\text{Ir}(\text{sppz})_3]^{3-}$ increased the intensity of the $[\text{Ru}(\text{bpy})_3]^{2+}$ ECL by 6.2-fold at 1.0 V and 2.3-fold at 1.3 V vs Ag/AgCl (Figure 4b). Both approaches demonstrated the amplification of the ECL signals under bead-based assay conditions using the non-emissive redox-mediator.

The $[\text{Ir}(\text{sppz})_3]^{3-}$ complex is an effective enhancer of the ECL reaction of $[\text{Ru}(\text{bpy})_3]^{2+}$ and TPrA in aqueous solution, without an inherent interfering emission. When the $[\text{Ru}(\text{bpy})_3]^{2+}$ luminophore is free in solution, the electron-transfer mechanisms of enhancement elicit greatest influence at low potentials that are sufficient to initiate the indirect ECL pathway (oxidation of co-reactant and enhancer). When the luminophore was immobilised on microbeads, the vast majority of the observed ECL occurs through the indirect pathway irrespective of the applied

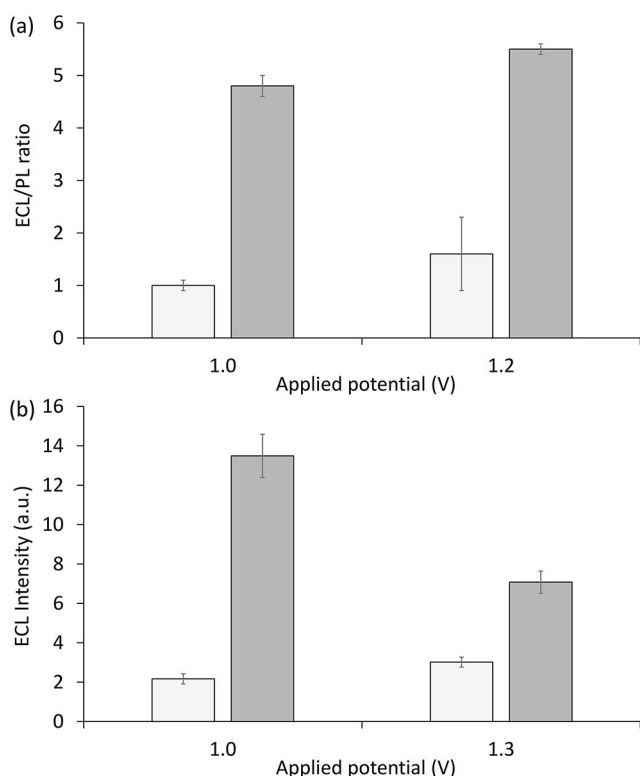


Figure 4. (a) ECL intensity (after background subtraction, and normalisation to photoluminescence) of [Ru(bpy)₃]²⁺ covalently bound on 12 μm polystyrene beads in ProCell solution without (light columns) and with (dark columns) 200 μM [Ir(sppz)₃]³⁺, upon application of 1.0 V (2 s) or 1.2 V (1 s) vs Ag/AgCl, measured by ECL microscopy. (b) ECL intensity of [Ru(bpy)₃]²⁺ covalently bound on 1 μm magnetic beads in ProCell solution without (light columns) and with (dark columns) 200 μM [Ir(sppz)₃]³⁺, upon application of 1.0 V (10 s) or 1.3 V (10 s) vs Ag/AgCl, measured using an electrochemical microcell with a photomultiplier tube. ECL intensities were integrated over the period of time that the oxidative potential was applied. Error bars represent ±1 standard deviation ($n=4$). Electrodes: working: GC, counter: Pt wire, reference: Ag/AgCl.

potential. Under these conditions, greater enhancement was still observed at low potentials, which can be ascribed to the greater rate of heterogeneous TPrA oxidation with increasing overpotentials, and thus the diminishing influence of electrocatalysis. The redox mediators also enable manipulation of the distance that the reactive intermediates can diffuse from the working electrode surface, measured in this study through the thickness of the emissive layer by ECL self-interference spectroscopy. This aspect, in conjunction with the electron transfer pathways of enhancement, could also be exploited for greater ECL intensity and detection sensitivity in not only bead-supported assays, but also modified electrode and other assay formats. We anticipate the redox-mediator approach to enhance co-reactant ECL will be beneficial for a wide range of ECL-based applications in bioanalysis and microscopy.

Supporting Information

The authors have cited additional references within the Supporting Information.^[15]

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: luminescence · electrochemistry · transition metal complexes · electrogenerated chemiluminescence

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