

Lighting up Electrochemiluminescent Inactive Dyes by Intramolecular Resonance Energy Transfer

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Abstract:

By virtue of near-zero optical background and photobleaching, electrochemiluminescence (ECL), an optical phenomenon excited by electrochemical reactions, has drawn extensive attention in both fundamental studies and wide applications especially of ultrasensitive bioassay. Developing diverse ECL emitters is crucial to unlock their multiformity and performances, but remains a formidable challenge, due to the rigorous requirements for ECL. Herein, we report a general intramolecular ECL resonance energy transfer (iECL-RET) strategy to light up ECL-inactive dyes in aqueous solutions using an existing high-performance ECL initiators. As a proof-of-concept, a series of luminol donor-dye acceptor based ECL emitters with near unity RET efficiency and coarse/fine tunable emission wavelengths were demonstrated. Different to previous exploitation of new molecule single-handedly to address all the prerequisites of ECL, each unit in the proposed ECL ensemble performed maximally its own functions. The iECL-RET strategy would greatly expand the family members of ECL emitters for more demanding future applications.

Keywords: electrochemiluminescence, electrochemiluminescent emitters,
fluorescence dyes, resonance energy transfer

Introduction

Due to near-zero background optical noises and photobleaching, electrochemiluminescence (ECL) is widely used to pursue the ultimate analytical limit in diverse fields ranging from clinical diagnosis, food analysis, environmental monitoring to bioimaging since the pioneering works in the mid-1960s.¹⁻⁵ As one of the most essential criteria, ECL emitter directly determines the strategies and performances of application methods.^{6, 7} For this, developing new emitters has been one of the forefronts of ECL-based research, but remains a formidable challenge, even by using dyes with strong fluorescence (FL). In principle, for the common co-reactant mechanism-based ECL, there are three essential prerequisites for an efficient emitter (**Figure 1a**).^{8,9} Firstly, ECL emitters should be ready to exchange high-energy electrons with working electrodes. Secondly, ECL emitters in high energy state could react with a co-reagent radical and generate excitation state, during which self-annihilation or quenching by ambient molecules in aqueous solution are not evident. Lastly, the radiative transition should dominate the final recombination of excitons. Generally, it is hard to meet all these requirements (**Figure 1b**), making the type of ECL active molecules be limited.

Current available ECL emitters mainly include metal complexes,¹⁰⁻¹² semiconducting nanoparticles/nanoclusters,¹³⁻¹⁶ conjugated organic molecules¹⁷⁻¹⁹. Great efforts of modulating electron transfer among electrode, emitters and/or co-reactants by potential control,²⁰⁻²⁴ aggregation-induction,²⁵⁻²⁹ self-enhancement,³⁰⁻³⁴ crystallization,³⁵⁻³⁸ as well as other strategies,³⁹⁻⁴² have been devoted to improving ECL efficiency and adapting to different application schemes. Despite these unprecedented success in mechanism studies and some applications,⁴³⁻⁴⁶ developing ECL emitters with reasonable ECL efficiency, good water-solubility/-dispersibility, and facile being labeled is still in infancy. For instance, only tris(2,2'-bipyridine)ruthenium(II) ($[\text{Ru}(\text{bpy})_3]^{2+}$) has been used in commercialized ECL system, which significantly hampers their prospective applications. Therefore, there is an increasing demand for

developing diverse ECL emitters to unlock their multiformity and performances.

Rather than rigorous designing a totally new molecule that addressed all the prerequisites for ECL in previous reports,^{47, 48} we reasoned that if it was possible to create ECL ensemble, in which each unit performed its own functions. For instance, resonance energy transfer (RET) is a mature strategy to give materials new functions and innovating applications, mediated by electronic dipole-dipole coupling to induce non-radiative transfer of electronic excitations from donor to acceptor.⁴⁹⁻⁵¹ Lighting up inactive ECL dyes by RET from an existing high-performance ECL initiators would be an intriguing general way to prepare new ECL emitters with variable properties. Despite there have been biosensing investigations regarding RET between molecular recognition-triggered ECL donor-acceptor pairs for sensitivity enhancement,⁵²⁻⁵⁴ development of new ECL emitters using RET has not been reported.

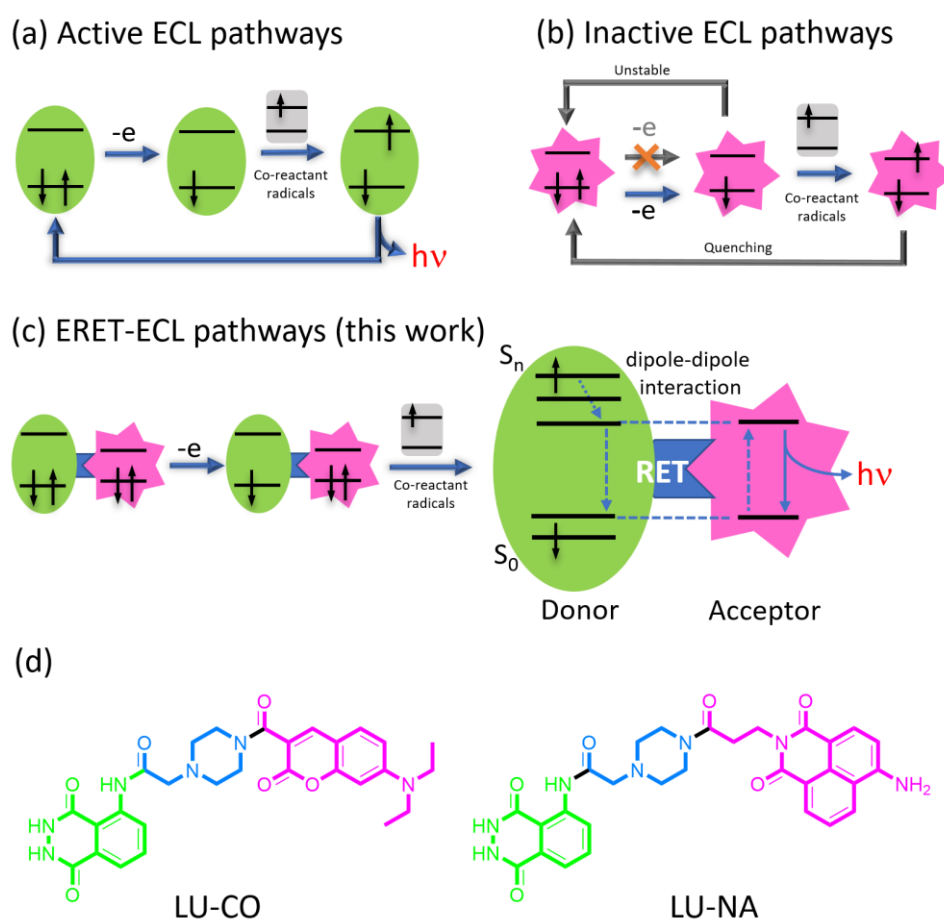


Figure 1 Design of lighting up inactive ECL emitters by iECL-RET. Brief mechanism of ECL active (a) and inactive (b) emitters, and (c) the proposed iECL-RET activation of ECL-inactive emitters. For clarity, only anodic ECL processes are shown. (d) Two examples of iECL-RET-based ECL emitters.

Here, we report a general intramolecular ECL resonance energy transfer (iECL-RET) strategy to light up ECL-inactive dyes in aqueous solutions (**Figure 1c**). As a proof of concept, two classical dyes, i.e., (7-(Diethylamino) coumarin-3-carboxylic acid (**CO**) and 3-(4-amino-1,8-naphthalimido) propanoic acid (**NA**) that are ECL inactive, were demonstrated to be lighted up using covalently linked luminol as an ECL initiator with a RET efficiency of near-unity (**Figure 1d**). Moreover, modification of electron-withdrawing substituents on dyes further fine-tuned the ECL emission wavelength.

Results and Discussion

For a highly efficient iECL-RET, the choosing of the donor, acceptor, and linker is crucial. As a proof of principle, luminol (**LU**) with high ECL activity was used as the donor. Considering the ECL emission wavelength of luminol and the absorption wavelength of FL luminophores, **CO** and **NA** were selected as the two-model acceptors. As shown in **Figure 2a** and **2b**, the overlap between the emission of luminol and the absorption of **CO** and **NA** were profound. The quantitative analysis indicated the spectral overlap integrals (J) was up to $6.46 \times 10^{14} \text{ nm}^4 \text{ M}^{-1} \text{ cm}^{-1}$ and $6.83 \times 10^{14} \text{ nm}^4 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. Complementarily, the frontier orbitals energies of HOMOs and LUMOs of **LU**, **CO**, and **NA** were also calculated by density functional theory (DFT). As shown in **Figure 2c** and **S1**, the HOMO-LUMO energy levels were adequately aligned, allowing energy transfer between **LU** and **CO** or **NA**. Therefore, the experimental spectra and theoretical calculation suggested that **CO** and **NA** are two ideal energy acceptors for the luminol donor.

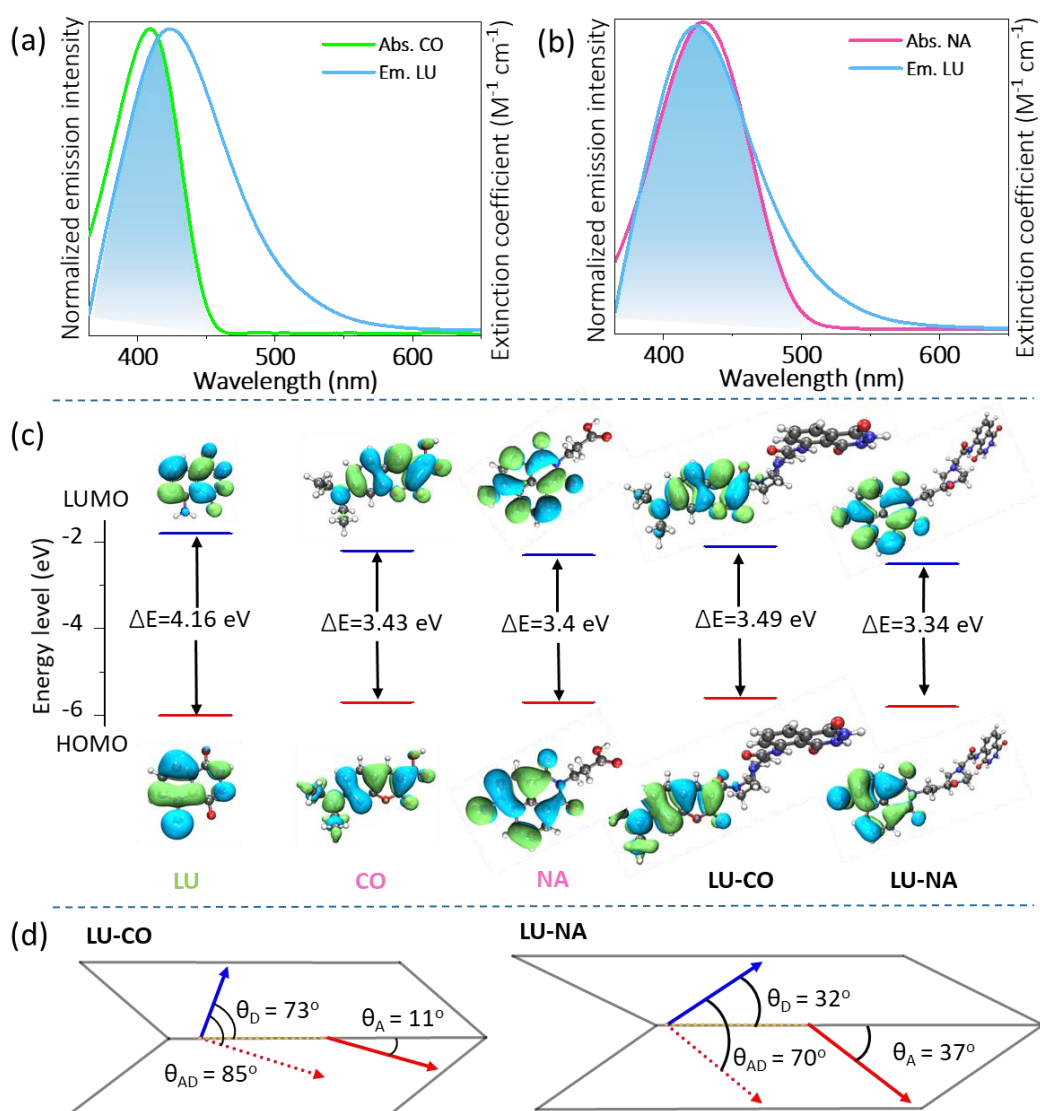


Figure 2. Principle validation of RET between LU and CO or NA. Absorption spectra (Abs.) of CO (a, green line), NA (b, pink line) and normalized ECL emission spectra (Em.) of luminol (a, b, blue line) in PBS (pH = 8.0, 0.01 M). Blue area: the spectral overlap integrand. Electronic isodensity plots and frontier orbitals energies (c) of HOMOs and LUMOs of LU, CO, NA, LU-CO, and LU-NA based on the DFT/b3lyp/6-311+g(d). Chart of dipole angles with the center-to-center vector of the LU-CO and LU-NA (d). Blue: transition dipole moments of donor emission. Red: the acceptor absorption. Yellow: the connecting vector. θ_{DA} : the angle between donor emission and acceptor absorption dipole moments. θ_D and θ_A : the angles between the respective dipole moments.

Appropriate distance and angle between LU and CO or NA are another critical factor

for effective iECL-RET. For this, a piperazine derivative was chosen as the linker to conjugate **LU** and **CO/NA** because piperazine has an advantage in self-adjustable conformations.^{55, 56} Along this line, the structures of the targeted **LU-CO** and **LU-NA** molecules were firstly optimized by DFT calculation (**Figure S1**). The electronic isodensity plots and frontier orbitals energies of **LU-CO** and **LU-NA** were obtained by DFT and time dependent DFT (TD-DFT). As shown in **Figure 2c** and **Table S1**, the HOMO-LUMO gap of **LU-CO** and **LU-NA** were much smaller than the **LU** monomer and close to the corresponding dye monomer. In addition, the electrons were mainly distributed in the dye unit, indicative of effective donor-acceptor interaction with **LU** and the potential domain in charge of the final ECL emission. The core-to-core distance (*R*) between **LU** and the dyes in **LU-CO** or **LU-NA** were calculated as 11 Å and 16 Å, respectively (**Figure S2**), which is in line with the distance rule of RET.^{57, 58} Moreover, the dipole transitions moments and the angles with the center-to-center vector of **LU-CO** and **LU-NA** were also calculated. As shown in **Figure 2d & S3**, the orientation factor (κ^2) of **LU-CO** and **LU-NA** were calculated as 0.59 and 2.89, respectively, which were also within the reasonable range ($0 \leq \kappa^2 \leq 4$).⁵⁸ Accordingly, the critical Förster radius (*R*₀) of **LU-CO** and **LU-NA** was evaluated to be 27 Å and 35 Å, respectively, indicative of a very promising RET efficiency (Φ_{RET}) by taking the practical lower *R* into account.^{57, 58} Therefore, principally, the proposed **LU-CO** and **LU-NA** meet the requirements of RET.

Guided by the above theoretical prediction, **LU-CO** and **LU-NA** were synthesized from luminol and dyes (**Figure 3a**) using piperazine derivative as the bridge. Since carboxyl group is a typical structure in most organic dyes, in order to make the synthetic routes general, the intermediate product, i.e., amino group-terminated luminol (**LU-NH**), was first synthesized. Consequently, **LU-CO** and **LU-NA** were obtained by a facile amidation reaction between dyes and **LU-NH** with a total yield of ca. 37%. The structures of all compounds were confirmed by ¹H-NMR, ¹³C-NMR, and high-resolution mass spectrum (**Figure S4-S12**).

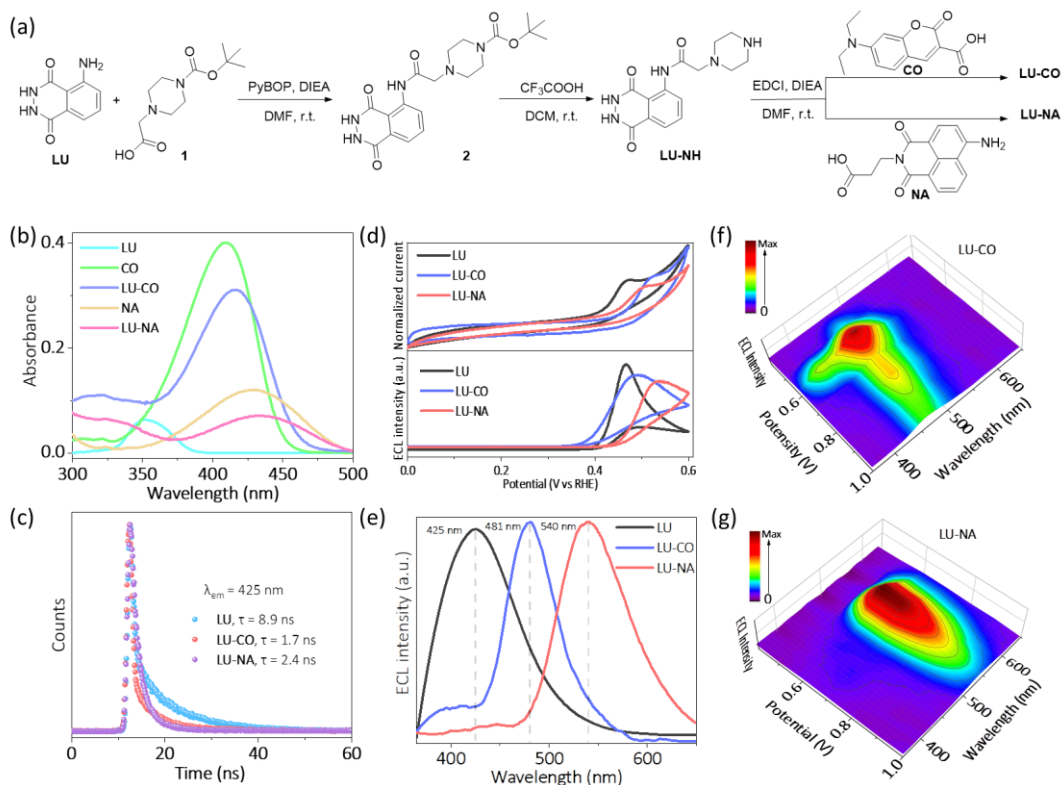


Figure 3. Synthesis, photophysical and ECL properties of LU-CO and LU-NA. (a) Brief synthetic routes of LU-CO and LU-NA. (b) UV-Vis absorption of LU-CO, LU-NA, and the controls. (c) Time-resolved decay curves of LU, LU-CO, and LU-NA under 350 nm excitation in PBS (0.01M, pH = 8.0). CV curves (top) and corresponding ECL intensity ($\lambda_{em} = 425$ nm) of LU, LU-CO, and LU-NA (d). ECL emission spectra of LU, LU-CO and LU-NA (e). Normalized ECL emission spectra changes of LU-CO (f) and LU-NA (g) at different potentials. Electrolytes: PBS (0.01M, pH = 8.0) with H₂O₂ (10 mM) as co-reactant.

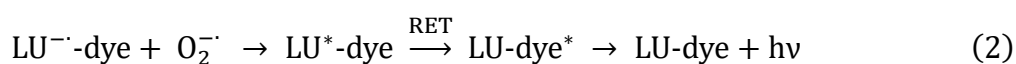
The photophysical properties of LU-CO and LU-NA were investigated in phosphate buffer saline under ambient conditions. As shown in the UV-Vis absorption spectra (Figure 3b), since the amino group (electron-donating group) of LU was turned into amide group (electro-withdraw group), the absorption bands of the LU part in the LU-CO and LU-NA were blue-shifted, while that of dye unit was slightly red-shifted due to the conversion of carboxyl group into amide group. The molar absorption coefficient of LU-CO and LU-NA was found to be different from that of the monomers (Table S7), consistent with the covalent conjugation between luminol and the dyes. The FL

spectra in **Figure S13** further showed that the maximum emission of **LU-NA** was almost identical to that of **NA**; while that of **LU-CO** was slightly red-shifted, compared with **CO**. It may attribute to the different electron-withdrawing abilities of carboxyl and amide groups. Interestingly, when excited at 350 nm (the maximum exciting wavelength of **LU**), the fluorescence intensity of **LU-CO** and **LU-NA** was much higher than that of **CO** and **NA** (**Figure S14**), strongly suggesting the critical role of **LU** in photon absorption for the following intermolecular RET. More importantly, the FL lifetime (τ) of **LU** after conjugating with the **CO** and **NA** acceptors remarkably decreased (**Figure 3c**), with an associated RET efficiency (E_{RET}) of 81 % and 73 %, respectively. All these experimental results confirmed that the effective RET from the **LU** donor unit to the dye acceptor units in **LU-CO** and **LU-NA** could occur under light irradiation.⁵⁸

We next exploited if RET in **LU-CO** and **LU-NA** could be driven and lighted up under electrogenic excitation. As shown in **Figure 4a**, **LU**, **LU-CO**, and **LU-NA** oxidized at almost the same peak potential of ca. 0.50 V under cyclic voltammetry in PBS with H_2O_2 as the co-reagent. Such oxidation could be assigned to the electron loss of the **LU** donor,^{59, 60} as it was not observed for the single dyes (**CO** and **NA**) in the same condition (**Figure S16**). Simultaneously, **LU-CO** and **LU-NA** exhibited ECL signals from the starting of the electrochemical oxidation, and reached the maximum intensity at ca. 0.5 V; while the dye monomers did not show any ECL signals (**Figure S16**). The ECL emission spectra of **LU-CO** and **LU-NA** were recorded to explore the ECL properties further. As shown in **Figure 4b**, except for the minor ECL emission of luminol at 425 nm, **LU-CO** and **LU-NA** showed a new ECL emission at 481 nm and 540 nm, respectively, the profile of which was almost identical to that of the FL spectra (**Figure S13**). In contrast, no peaks at 481 nm or 540 nm were found under the same condition for the physically mixed dye (**CO** or **NA**) and **LU** (**Figure S17**), further verifying the occurrence of RET in the covalent linkage form. The efficiency of iECL-RET in **LU-CO** and **LU-NA**, evaluated by ECL intensities (I) of the donor in the absence (I_D) and in the presence (I_{DA}) of the acceptor like that of FL (**Figure S18**),⁵⁸

was approximated both as near-unity. These results evidently proved that the ECL-inactive dyes were successfully lighted up by the efficient iECL-RET pathway.

Notably, typical excellent ECL properties of **LU** were well inherited in **LU-CO** and **LU-NA**. For instance, the ECL intensity of **LU-CO** and **LU-NA** could be modulated by the electrogenic excitation conditions, including the co-reagent concentration (**Figure S20**), the pH of aqueous solutions (**Figure S21**), and the electrochemical scanning rate (**Figure S22**). The ECL signals of **LU-CO** and **LU-NA** also exhibited high stability and reproducibility (**Figure S23**). Moreover, the 3D dynamic ECL emission plots of **LU-CO** and **LU-NA** at different potentials also strongly depended on **LU** (**Figure 4c, 4d & S19**), e.g., the detectable ECL emission from ca. 0.4-0.5 V, and reached the maximum at ca. 0.6 V. Therefore, considering the theoretical calculation, photophysical characterization, and the ECL properties, the ECL mechanism of **LU-CO** and **LU-NA** associated with iECL-RET was briefly described in **Equation 1 & 2** (see the detailed mechanism in **Figure S24**):⁶¹⁻⁶³



To prove the universality of our approach for lighting up ECL-inactive molecules, except for the blue **LU-CO** and yellow **LU-NA**, a series of ECL emitters with more refined color-tuning, was exploited by engineering electron-withdrawing substituents (**Figure 4a**). For this purpose, **LU-NA-NO₂** was firstly synthesized (see characterization in **Figure S25-28**). Such -NO₂ terminal group has a strong electron-withdrawing ability, making the intramolecular charge transfer (ICT) in the acceptor unit interdict (**Figure 4a and S29**).^{64, 65} Consequently, it led to the interruption of iECL-RET, and the ECL emission was mainly contributed by the **LU** donor. Interestingly, the nitro-group could be quantitatively reduced to amino-group with a much lower electron-withdrawing capacity by a facile enzymatic reaction using nitroreductase (NTR) and NADH.⁶⁶⁻⁶⁸ As a result, the ICT for the acceptor was improved, the iECL-RET was boosted, and the ECL emission from the acceptor unit was enhanced. Along

this line, the emission wavelength of **LU-NA-X** could be modulated by varying the concentration of NTR. As shown in **Figure 4b**, with the concentration of NTR increased from 25 to 200 $\mu\text{g/mL}$, the maximum ECL emission wavelength was shifted significantly from 425 nm to 540 nm. Therefore, based on the iECL-RET strategy, the color of ECL emitters could be modulated not only in a wide range by using different task-specific dyes but also in a refined manner by further harnessing substituents of different electronegativity (**Figure 4c**).

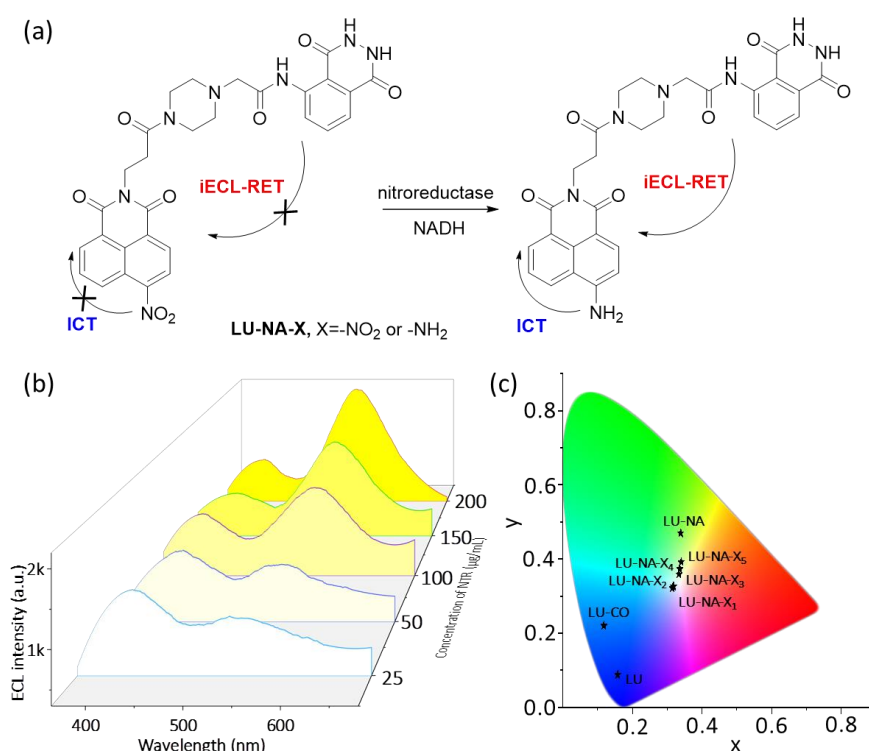


Figure 4. Generality of iECL-RET activation for a series of ECL emitters with fine-tuned properties. Enzymatic reduction conversion (a) and ECL emission spectra (b) of **LU-NA-X** with different amount of electron-withdrawing substituent. Color modulation emissions of **LU**, **LU-CO**, **LU-NA**, and a series of **LU-NA-X** in the CIE coordinate diagram (c).

It should be noted that the number of FL molecules with excellent photophysical properties is huge, but most of them are ECL inactive. The strategy of generating ECL signals from FL molecules through RET induced by an ECL initiator would provide an intriguing facile way to greatly expand the family members of ECL emitters. It would

be highly envisioned that the well-developed sensing mechanism specifically for FL system could also be inherited by the proposed iECL-RET platform. For instance, upon the delicate correlation between **LU-NA-X** molecular structures and the unique ECL emission, a proposed example for sensitive and selective detection of NTR activities could also be constructed in turn. As shown in Figure S30, after the reaction between **LU-NA-X** and the analyte, the chromaticity coordinate showed a highly linear relationship to the concentration of NTR, while the other species were not included in the given range. Moreover, the proposed excited-state manipulation by RET to endow FL molecules with crossover luminescence is not only limited to ECL, but also promising for other type of luminescence, such as phosphorescence and X-ray fluorescence, which deserves future more fundamental and application studies.

Conclusions

In summary, a general iECL-RET strategy was proposed to facilitate turning ECL inactive dyes into ECL activity emitters in aqueous phase. By screening the donors, acceptors, linkers and substituents according to the spectrum properties and theoretical prediction, a series of ECL emitters (**LU-CO**, **LU-NA** and **LU-NA-X**) were synthesized with a near unity RET efficiency and coarse/fine tunable emission wavelengths up to 115 nm. Different to previous exploitation of new molecule single-handedly to address all the prerequisites of ECL, each unit in the proposed ECL ensemble performed maximally its own functions. Thanks to the generality of iECL-RET and huge number of dyes with diverse photophysical properties, this study would open a new era for developing ECL emitters and advance their multifunctionality and performances in more demanding prospective applications. Nonetheless, although the RET efficiency was close to unity, the overall ECL efficiency of **LU-CO** and **LU-NA** relative to **LU** was low (ca. 3%) and need a future substantial improvement.

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