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Signal enhancement and low oxidation potentials for miniaturized ECL biosensors *via* *N*-butyldiethanolamine

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We present studies on ruthenium-based electrochemiluminescence (ECL) focusing on conditions supporting signal enhancement and low oxidation potentials. Low oxidation potentials (LOPs) are especially attractive for miniaturized ECL biosensors, as microfabricated electrodes tend to detach from their support when used with high currents and operated at high potentials. Furthermore, high potentials or current densities can lead to damage of typical biosensor surface coatings and biological probes. The possibility of generating LOP ECL signals at a potential below 900 mV was therefore studied for $Ru(bpy)_3^{2+}$ with two typical coreactants, *i.e.* 2-(dibutylamino)ethanol (DBAE) and tripropylamine (TPA), as well as with the tertiary amine *N*-butyldiethanolamine (NBEA). Furthermore, the effect of buffer components and pH values on ECL signal generation was investigated. We could show a significant LOP ECL signal for NBEA. We found that Tris buffer, with its ability to form complexes with transition metal ions, has a positive influence on this ECL signal in terms of signal strength and LOP capabilities. Specifically, at basic pH values significant increases in ECL signals were observed at 900 mV and at 1.2 V. In fact, the ECL signal at 1.2 V was three times higher than the signal observed in phosphate buffer at a pH of 7, and it was thirty times higher than the ECL signal for TPA under these conditions. The LOP signal for NBEA in Tris buffer at pH 8.5 was similar to the signal obtained for TPA in phosphate buffer at pH 8.5 but three times higher than for TPA at pH 7.0. Interestingly, the coreactant DBAE was neither significantly influenced by the buffer system or pH nor did it present a valuable LOP ECL signal. Finally, it was found that high peak currents in cyclic voltammograms are not the indicators for high ECL signals, which should be obvious because the ECL mechanism requires more complex electron transfers. Overall, the standard TPA ECL at 1.2 V in phosphate buffer at pH 7.0 can successfully be replaced by NBEA ECL at 900 mV in Tris at pH 8.5 providing significantly higher signals accompanied by more gentle electrochemical conditions.

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Introduction

Electrochemiluminescence (ECL) is an analytical technique which was first introduced for $Ru(bpy)_3^{2+}$ by Allen Bard in 1972 and has since been applied in numerous sensor systems.[1] It has found a vast number of applications in bio- and chemosensing, as well as in immunoassays. ECL allows for spatial- and time-controlled assays through its generation mechanism, which simultaneously leads to a "zero" background signal.[2–4] For commercial analytical ECL instruments, a coreactant-based ECL mechanism is typically employed.[4] Tripropylamine (TPA) is the most commonly represented coreactant since its discovery for $Ru(bpy)_3^{2+}$ ECL in 1990.[5] For coreactant-based ECL, one of the coreactant's intermediate states, produced upon electron transfer reaction at an electrode, can react with the ECL molecule, *e.g.* $Ru(bpy)_3^{2+}$, to form an excited state. The underlying mechanism for $Ru(bpy)_3^{2+}$ ECL can be either direct oxidation, catalytic oxidation, or reactions between the $Ru(bpy)_3^{2+}$ molecules and the amine cation radicals.[6–18]

Another important aspect, if not the most important one in ECL research, is to increase the sensitivity of ECL-based sensors. Even though TPA is the standard coreactant, other coreactants have been reported to enhance the ECL signal of $Ru(bpy)_3^{2+}$. 2-(Dibutylamino)ethanol (DBAE)[9] and *N*-butyldiethanolamine (NBEA)[19] have been shown to lead to stronger $Ru(bpy)_3^{2+}$ ECL signals than TPA. In general, it was demonstrated that monoamines enable higher ECL signals than diamines. The higher promotion of the amine oxidation

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by two hydroxyethyl groups instead of one is thought to increase ECL signals. Furthermore, the ECL process is directly related to the oxidation of NBEA.[19]

The voltage required to initiate $Ru(bpy)_3^{2+}$ ECL is typically 1.2 V *vs.* Ag/AgCl reference electrodes: a high voltage, close to the potential difference of water. This can cause the formation of bubbles on the electrode surface. While disc or macro electrodes are stable against such high potentials, it is often a true challenge for thin microfabricated electrodes, especially when combined with high currents and gas formation in solution. Electrode damage, for example, due to electrode fouling and detachment of the electrode from its substrate can occur. Furthermore, high potentials can interfere with DNA stability, as oligonucleotide sequences get damaged at +1.0 V *vs.* SCE.[20,21]

An approach to overcome this issue is the use of low oxidation potential (LOP) ECL which is an ideal scenario for microfluidic systems but has not been reported yet for miniaturized analytical systems. The existence of a LOP ECL signal for $Ru(bpy)_3^{2+}$ with TPA and the nonionic fluorosurfactant Zonyl FSN was demonstrated by Li *et al.* in 2004[8] on gold and platinum electrodes. The hydrophobic conditions on the electrode surface and, most importantly, the inhibiting effect on oxide layer growth on the electrodes were thought to facilitate the direct oxidation of TPA. The same group published a second paper characterizing in greater detail the LOP of the $Ru(bpy)_3^{2+}$/TPA system on gold electrodes in the presence of Zonyl FSN-100.[22] In the search for coreactants leading to a higher LOP ECL signal, multiple tertiary amine coreactants for $Ru(bpy)_3^{2+}$ were compared to TPA, in terms of their LOP ECL capability.[23] Tri-*n*-ethylamine (TEtA), TPA, and tri-*n*-butylamine (TBuA) were investigated at a Zonyl-FSO-100-modified gold electrode. The changes due to the electrode hydrophobicity showed that more hydrophobic surfaces increased the LOP ECL signal.

In general, the addition of a surfactant increases the ECL signal.[8,11] Triton X-100 is frequently added to the $Ru(bpy)_3^{2+}$/TPA system. An 8-fold increase in ECL efficiency in the presence of Triton X-100 was demonstrated for TPA as the coreactant.[11] Zonyl FSN was introduced as an alternative to Triton X-100.[8] In addition to the occurrence of LOP ECL, the recorded ECL signal, at 1.25 V *vs.* SCE, was higher than the ones observed for Triton X-100. The increase in the electrode hydrophobicity and retardation of oxide formation on the electrode are assumed to cause the increased ECL signal. However, LOP ECL is also possible without the addition of actual surfactants. Jiang *et al.*[24] showed the existence of a LOP for the $Ru(bpy)_3^{2+}$/TPA ECL system at gold electrodes while introducing pyridine and its analogues, quinoline or 4,4′-dipyridine, to the system. The proposed assumption was that pyridine and its analogues could act as surfactants and facilitate direct TPA oxidation.

More recent studies demonstrated that LOP ECL is not limited to the $Ru(bpy)_3^{2+}$/TPA system. For ECL quantum dot (QD) applications, Zou *et al.*[25] showed that, at low oxidation potentials on glass carbon electrodes, anodic near-infrared (NIR) ECL from CdTe QDs could be achieved. Furthermore, a strategy for greatly enhanced band-gap NIR ECL at low oxidation potentials with the dual-stabilizer-capped CdTe QDs was presented. The CdTe QDs were used as ECL emitters, while TPA was employed as the coreactant, leading to two distinct ECL processes at 0.63 and 0.88 V. The two identified ECL potentials are much lower than those for other reported NIR-emitting QDs[26,27] and visible-emitting QDs.[28,29]

Interestingly, the ECL signal produced at the low oxidation potential can furthermore be used to measure quenching, as the quenching effects on the ECL pathway of $Ru(bpy)_3^{2+}$ with TPA can be more significantly and sensitively observed under LOP ECL conditions. This phenomenon and detection approach has been, for example, used for the detection of oxygen,[30] uric acid[31] and phenolic compounds.[32]

Because of the potentially wide applicability, especially in microfabricated electrode systems, we investigated LOP ECL and present here a new LOP ECL strategy using NBEA as the coreactant instead of TPA. We paired these studies with investigations on optimal conditions for ECL at potentials of 1.2 V and below 1 V for one to directly compare the signal under otherwise identical conditions but also to identify conditions that make LOP ECL attractive as an analytical method.

Experimental

Reagents

Tris(2,2′-bipyridyl)dichlororuthenium(II) hexahydrate, tripropylamine, *N*-butyldiethanolamine, 2-(dibutylamino)ethanol, Triton™ X-100 and Zonyl® FSN fluorosurfactant were purchased from Sigma Aldrich (Taufkirchen, Germany). Potassium phosphate dibasic, potassium phosphate monobasic and Tris were purchased from Fisher (Schwerte, Germany), and glycine from Merck Millipore (Darmstadt, Germany).

Data recording and analysis

The concentration of $Ru(bpy)_3^{2+}$ was at 1 μM, 50 mM for NBEA and DBAE, 200 mM for TPA, and 0.05 wt% for Zonyl FSN. Tris and potassium phosphate (PB) buffers were prepared at a concentration of 0.1 M each in Millipore® water. 0.1 M PB was prepared with 0.1 M potassium phosphate monobasic and 0.1 M potassium phosphate dibasic to reach pH 7 and Tris was prepared by dissolving it in powdered form at a concentration of 0.1 M and then adjusting the pH to 8.5 with HCl. Glycine buffer was prepared by dissolving the powder in Millipore® water at a concentration of 0.1 M and the pH was adjusted with NaOH. The three-electrode setup consisted of a gold disc WE (diameter of 1.6 mm), a Pt wire CE, and a Ag/AgCl RE (all electrodes were ordered from BASi, West Lafayette, IN, USA). An Autolab potentiostat (Metrohm Autolab B.V., Utrecht, The Netherlands) was used to apply 1.2 V for 30 s to a sample volume of 4 mL placed in a transparent glass beaker. The electrodes were placed in a metal holder, securing exact repositioning of the electrodes for every measurement. The WE was aligned, *via* the metal holder, to be positioned atop an optical fiber connected to the luminescence spectrometer

(AMINCO-Bowman Series 2, Thermo Electron Scientific Instruments Corporation, Madison, WI, USA). The signal itself was recorded *via* the luminescence spectrometer (PMT voltage set to 1000 V, interval time of 1 s) and the over time integrated signal was determined directly *via* AMINCO-Bowman Series 2 software. Each condition was repeated in triplicate and the integrated signal over time, determined *via* AMINCO-Bowman Series 2 software, was averaged and errors are given as standard deviation.

Results and discussion

NBEA was investigated as a relevant new alternative to the commonly used coreactant TPA, as it has been demonstrated to lead to higher ECL signals.[19] In order to identify enhancing ECL conditions, the buffer and pH composition were investigated in greater detail. NBEA was previously studied in a phosphate based buffer.[19] However, other buffer systems, mainly Tris buffer, have also been employed for ECL. Furthermore it is known that the pH value strongly influences the ECL intensity.[4] Therefore, we chose to investigate the influence of pH of Tris and glycine buffers compared to phosphate buffer in their respective buffering ranges. It is obvious that the ECL signal with NBEA as the coreactant increases with more basic pH (Fig. 1).

At the same time, when switching the buffer system itself, the ECL signal dropped. This implies that both pH and the buffer system influence the ECL reaction. We assume that the higher pH values enable the formation of a larger number of intermediate and deprotonized NBEA molecules and radicals, as it was also presented for TPA pH dependency.[4,5] The effect of the nature of the buffer species itself is more complex. It can be assumed that the buffer media itself either stabilizes or enhances the formation of the excited ruthenium complex itself or the coreactant radical state, participating in the electron transfer reactions.

One possibility might be the presence of the amine group of Tris and glycine buffers and its influence on the coreactant radical formation. Here, further studies are needed that elucidate the complex ECL pathway similar to the electrochemical (EC) and ECL research done on introduction of TPA by several groups.[5,10,12,17,18,33]

Furthermore, the studies on the influence of Tris buffer on the $Ru(bpy)_3^{2+}$ ECL system were also used to investigate the strong ECL signals at potentials well below 1.0 V. As an example, solutions of 1 μM $Ru(bpy)_3^{2+}$ with 50 mM NBEA and 0.05 wt% Zonyl FSN in Tris buffer at pH 8.5 were investigated at various potentials (Fig. 2). At potentials as low as 800 mV, a significant ECL signal was detectable, which increased in strength until a plateau was reached between 880 mV and 910 mV *vs.* Ag/AgCl. A potential of 890 mV was chosen for further studies.

The existence of LOP ECL has been shown for TPA, involving ECL pathways that do not include the oxidation of $Ru(bpy)_3^{2+}$, but instead require a direct oxidation route of the coreactant.[22] This is likely the same mechanism for NBEA, substantiated by the fact that the LOP occurs at the potential for the direct oxidation of NBEA, which was shown to be around 900 mV.[19] Since a LOP ECL signal with NBEA as the coreactant has not been reported yet, a range of studies was initiated to further understand this interesting phenomenon: (a) comparison between Tris and phosphate buffers, (b) influence of pH, (c) influence of the surfactant Zonyl FSN and finally (d) comparison of DBAE and TPA for their LOP ECL capabilities.

When recording the electrochemical response in cyclic voltammograms for NBEA in combinations of PB and Tris buffer at pH values of 7 and 8.5, strong current peaks were observed at voltages well below 1 V for all systems except for Tris buffer at a pH of 7 (Fig. 3).

Subsequently, the ECL signal was recorded at the voltages at which the current peaks were found. Indeed, 1 μM $Ru(bpy)_3^{2+}$ with the coreactant NBEA in Tris buffer at a pH of 7

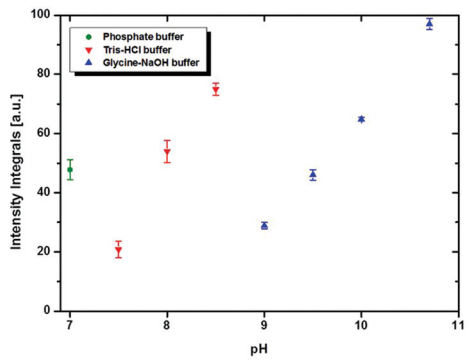

Fig. 1 pH dependence in PB, Tris and glycine buffers for 1 μM Ru $(bpy)_3^{2+}$ with 50 mM NBEA and 0.05 wt% Zonyl FSN. The ECL signal was integrated over 30 s at 1.2 V *vs.* Ag pseudo-RE under different buffer conditions: phosphate buffer (●), Tris buffer (▼) and glycine buffer (▲); $n = 3$.

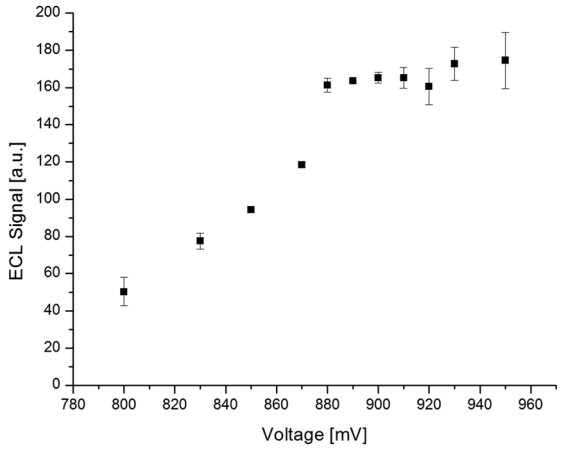

Fig. 2 ECL signal for 1 μM $Ru(bpy)_3^{2+}$ with 50 mM NBEA and 0.05 wt% Zonyl FSN in Tris buffer pH 8.5 according to the applied potential *vs.* Ag/AgCl. The ECL signal was generated on an Au disc electrode *vs.* Ag/AgCl, and integrated over 30 s; $n = 3$.

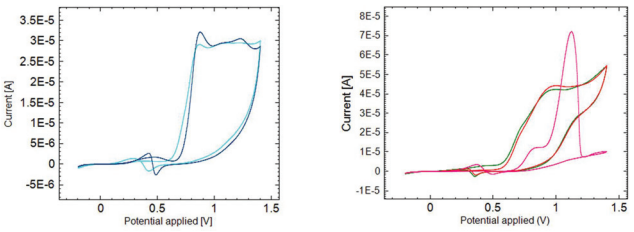

Fig. 3 CVs for 1 μM $Ru(bpy)_3^{2+}$ with the coreactant NBEA. Left: PB at a pH of 7 (blue) and at a pH of 8.5 (cyan). Right: Tris buffer at a pH of 7 (pink), at a pH of 8.5 (red) and at a pH of 8.5 in the absence of Zonyl FSN (green). Recorded at a scan rate of 10 mV s^{-1} *vs.* Ag/AgCl.

did not lead to an LOP ECL signal, as could be expected from the CV data (Fig. 3 and 4). The LOP ECL signal for PB was minimal, and did not correlate with the strong current peaks observed in the CV.

This means that no sufficient intermolecular electron transfer can be obtained under those conditions which would have led to the formation of the excited state and luminescence signals. Therefore, EC data can be used as an indicator for ECL performance only, while signal intensities don't have to translate into the corresponding ECL luminescence signals. Too many interactions can occur between the different components that lead to electron transfer but do not support the formation of the excited photon-emitting state of $Ru(bpy)_3^{2+}$. This fact is worth mentioning because in too many ECL-related studies only EC data are reported without the correlating reporting of luminescence analysis. Hence, wrong conclusions can be drawn.

In the case of NBEA, a clear LOP ECL signal was detectable only in Tris buffer at pH 8.5 (Fig. 4). Interestingly, the same LOP ECL intensity was observed in the presence and absence of the surfactant Zonyl FSN.

TPA affords an ECL LOP signal in PB at pH 8.5, but only minor signals in Tris. The optimal TPA LOP ECL signal has the same intensity as the optimal NBEA signal. For DBAE, a significantly smaller LOP ECL signal was observable under all tested conditions (7–20 times lower than the optimal NBEA signals), except for LOP ECL in Tris buffer at pH 7, where DBAE was in fact the coreactant with the highest averaged signal.

We can conclude that the LOP ECL signal is not only dependent on the buffer system but also on the pH. The same proved to be true for the ECL intensity at 1.2 V *vs.* Ag/AgCl (Fig. 1 and 5). It is therefore assumed that Tris interacts with the $Ru(bpy)_3^{2+}$ ECL pathway and, consequently enhances the production of the excited state. The effect caused by pH alone should be the same for each buffer system, and is supported by the data. In fact, highest signals are obtained under the same conditions (Tris, pH 8.5) with NBEA as the coreactant for both LOP ECL and normal ECL (1.2 V *vs.* Ag/AgCl). While LOP ECL with TPA as the coreactant showed almost equivalent intensities in PB buffer at pH 8.5, as did NBEA in Tris buffer, NBEA still was shown to be a more reliable coreactant, due to the smaller standard deviations, and the overall stronger ECL signal at 1.2 V.

It is known that Tris can form complexes with transition metal ions.[34] These complexes are mostly formed through the deprotonation of one of the OH-groups of Tris.[34] This means that at high pH values, Tris can promote the ECL pathway better than at lower pH values due to an enhanced ratio of coreactant radical formation or more likely a stabilization of the excited $Ru(bpy)_3^{2+*}$ state, due to the possible interaction of Tris with metal complexes. More studies are needed to confirm this influence, as no information has been found in the literature regarding this so far. Even though Tris can inter-

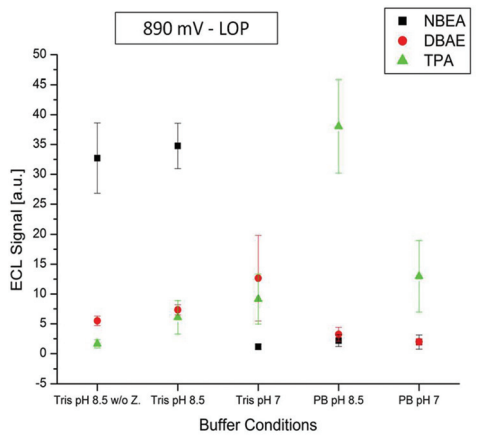

Fig. 4 LOP ECL signal for 1 μM $Ru(bpy)_3^{2+}$ with the coreactants NBEA (■), DBAE (●) and TPA (▲) and the surfactant Zonyl FSN under different buffer conditions. The investigated buffers were, from left to right: Tris buffer pH 8.5 without Zonyl (w/o z.), Tris buffer pH 8.5, Tris buffer pH 7, PB pH 8.5 and PB pH 7. The ECL signal was recorded on a gold disc electrode at a potential of 890 mV *vs.* Ag/AgCl; $n = 3$.

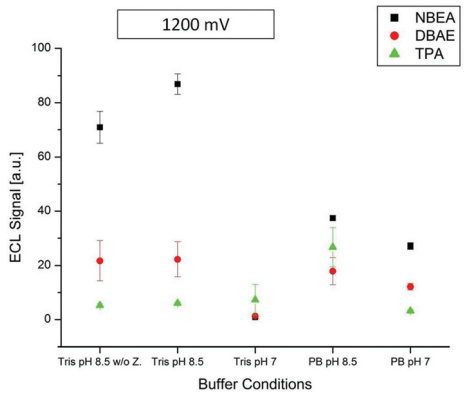

Fig. 5 LOP ECL signal for 1 μM $Ru(bpy)_3^{2+}$ with the coreactants NBEA (■), DBAE (●) and TPA (▲) and Zonyl FSN as the surfactant under different buffer conditions. The investigated buffers were, from left to right: Tris buffer pH 8.5 without Zonyl (w/o z.), Tris buffer pH 8.5, Tris buffer pH 7, PB pH 8.5 and PB pH 7. The ECL signal was recorded on a gold disc electrode at a potential of 1.2 V *vs.* Ag/AgCl; $n = 3$.

fere with metals, and has been studied in the presence of metal ions such as Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+},[35] we are not aware of any studies with $Ru(bpy)_3^{2+}$ nor of possible influences on ECL generation.

As can be seen from data shown in Fig. 4 and 5, the surfactant Zonyl FSN has no influence on the LOP ECL, yet, its presence increases the ECL signal intensity by a factor of 22.5% for normal ECL at 1.2 V. We assume that this is due to two effects. One being that the surfactant Zonyl FSN enables a closer distance between $Ru(bpy)_3^{2+}$ and the coreactant NBEA. More precisely NBEA with its two OH-groups that have a negative partial charge will be able to approach the positively charged $Ru(bpy)_3^{2+}$ complex in closer proximity in the more surfactant enriched environment at the electrodes. The second effect could be a shift from the radiative to non-radiative relaxation to the ground state, favoring the route *via* photon emission.

Further studies are needed to better understand these mechanisms in the future. Overall, it is clear that significant signal enhancements can be obtained when optimizing the buffer component, pH and surfactant conditions, as expected. Here, it is shown that for normal ECL conditions with NBEA, the signal is increased by a factor of 2, and for LOP ECL by a factor of 17 when switching from PB at pH 7 to Tris at pH 8.5.

Finally, it was determined that the optimal LOP ECL signal of the $Ru(bpy)_3^{2+}$/NBEA/Zonyl system is 40% lower than the optimal ECL signal at 1.2 V. However, it is still 2.7 times higher than the commonly used TPA/Triton X-100 system at pH 7 (ECL signal of 13 ± 6 A.U.) in a phosphate buffer (Fig. 4 and 5), which thus makes it a viable option for all (bio)assay applications and very attractive for miniaturized systems.

Conclusion

ECL is a known powerful tool for a large variety of analytical procedures, yet its use in analytical devices, especially in microfluidic systems, continues to be limited in comparison with other luminescence or electrochemical detection strategies. By investigating new co-reactants and ECL reaction conditions, we have demonstrated here that LOP-ECL can provide high signal intensities under mild electrochemical conditions, which will favor their application in microfabricated electrodes.

Specifically, the coreactant NBEA, especially when combined with the surfactant Zonyl FSN and when used in Tris buffer at pH 8.5, increases the ECL signal by almost 30 times at 1.2 V compared to the standard coreactant TPA in phosphate-based buffer at pH 7. Furthermore, even the LOP ECL signal is 2.7 times higher than the standard TPA ECL. We assume that deprotonation of NBEA's radical cation at higher pH values causes a more efficient ECL reaction similar to a mechanism described for TPA. However, it remains a subject of further studies, to better understand the enhancing factor of Tris *vs.* phosphate buffers. Here, further research on metal complexation will be useful, *e.g.* determining the effect of Tris buffer in iridium complex formation. This has not been explored thoroughly and will provide new opportunities and insights into ECL for sensing applications.

We propose as a key step towards improved ECL performance that NBEA be used as a co-reactant instead of TPA and even DBAE. We demonstrated that TPA, even though commonly used in the literature, is by far not the optimal coreactant for $Ru(bpy)_3^{2+}$-based ECL and should be replaced by NBEA. The replacement would not require any additional assay work steps, nor more expensive chemicals and is therefore easily applicable to any existing and future analytical devices employing $Ru(bpy)_3^{2+}$-based ECL. Through increased signals and broader voltage ranges NBEA usage can be a step towards establishing ECL as a high sensitivity standard in (microfabricated) sensing devices.

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