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# PAMAM dendrimers: a multifunctional nanomaterial for ECL biosensors

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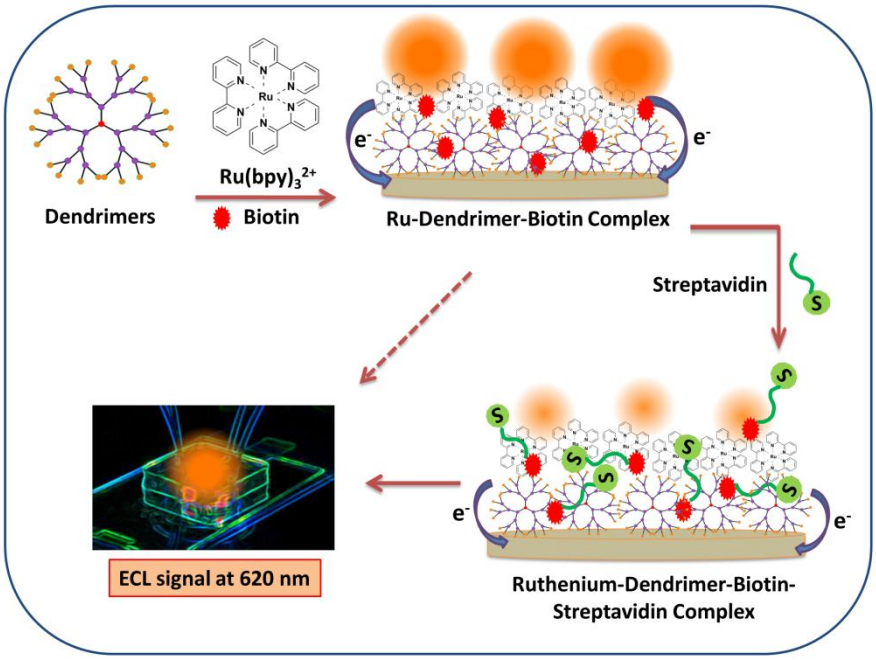
**Abstract**

Polyamido amine (PAMAM) dendrimers have been shown to function as electrochemiluminescence (ECL) co-reactant and have the inherent capability of improving immobilization of molecules on surfaces due to their dendritic structure. Here, we investigated the combination of both of these properties as the basis for biosensor development. Dendrimers with 5, 8, 10 and 16 terminal amine groups, respectively, were used. These were covalently coupled to biotin as model recognition site, and tagged with $Ru(bpy)_3^{2+}$ via adsorption. Due to their hydrophilicity, Ru-dendrimers showed significantly improved electrochemical activity in comparison to the standard tripropylamine (TPA) assisted ECL and similar luminescence yields even though 10 fold less dendrimer concentration was required in comparison to TPA. Best signals were obtained for D8 and D10 dendrimers. These Ru-dendrimers were subsequently used for the quantification of streptavidin, as its binding to the biotin-tag caused a proportional decrease in ECL signal with a dynamic range of 5 nM to 1μM. These preliminary studies demonstrate that PAMAM dendrimers can function as responsive signal generators in solution-based ECL-bioassays with an assumed even higher impact when being immobilized directly on the electrode-surface.

Graphical abstract




Schematic representation of the dendrimer-ECL platform for the biotin-streptavidin assay

Keywords: PAMAM dendrimer; ECL; biosensor



## 1. Introduction

Electrogenerated chemiluminescence (ECL) involves the generation of electroactive species which can undergo highly-energetic electron transfer (redox or enzymatic) reactions that emit light upon relaxation to the ground state. This technique has attracted attention due to its application in medical diagnostics, pharmacy, food and environmental analysis [1]. Commonly used ECL reagents (luminophores) are luminol, quantum dots and tris(2,2'-bipyridyl) ruthenium(II) ($Ru(bpy)_3^{2+}$) and their analogues, among which $Ru(bpy)_3^{2+}$ is most frequently used due to its available low oxidation potential, high emission yield, and low cost [2]. Commonly, a co-reactant (mostly tripropylamine, TPA) is used to enhance the sensitivity of the Ru-based ECL system [3]. However, TPA is toxic, volatile, and is required in high concentrations (~100 mM), which leads to an increase in background noise. Further, TPA exhibits slow electrochemical oxidation that limits the ECL efficiency. Moreover, TPA is basic in nature and surfactants are needed to bring it close to the electrode surface to enable

an efficient electron transfer. All these drawbacks prompted researchers to find alternative co-reactants. Here, tertiary amines are interesting as they show higher ECL efficiencies than primary and secondary amines [4]. In fact, several parameters contribute to the overall performance of an ECL assay such as hydrophilicity, luminescence lifetime, wavelength of emission, the location of the excited state within the complex, and the redox potential of ground and excited states. The ability to control these parameters depends on the luminophore and the co-reactant, which ultimately enhances the sensitivity and specificity of the assay either by providing longer excited-state lifetime or by reducing the redox potential, respectively [5, 6]. Of late, nanoparticles are found to enhance the ECL signal because of their ability to diffuse into the solution layer near the electrode, thereby enhancing the ECL signals. As they possess a large surface area they also provide many binding sites for attaching biomolecules of interest [7, 8]. Among the studied nanomaterials, the hyper-branched, three-dimensional polyamidoamine (PAMAM) dendrimers received considerable interest as they bear a substantial number of primary, secondary and most importantly tertiary amine groups. Furthermore, it is anticipated that PAMAM dendrimers can not only serve as co-reactant to the ECL luminophores [9], but can simultaneously serve as a multifunctional label loaded with luminophores and biomolecules [10]. Kim et al. [11] reported an enhanced ECL of $Ru(bpy)_3^{2+}$ with TPA co-reactant on ITO electrodes that were modified with dendrimers encapsulating nanoparticles. Using these modified electrodes, nicotine was detected with a ~210-fold increase of the ECL signals in comparison to signals obtained from bare ITO. Similarly, Lin et al. [12] constructed an ECL platform by functionalizing a PAMAM dendrimer with titanate nanotubes. In this work, we have explored the ECL co-reactant pathway of $Ru(bpy)_3^{2+}$ using plain PAMAM dendrimers as postulated by Xiong et al. [13] (Equations 1-5). It is proposed that PAMAM in solution is oxidized along with the luminophore ($Ru(bpy)_3^{2+}$) at the same electrode potential to form $Ru(bpy)_3^{3+}$ and excited PAMAM$^{*+}$. Then, $Ru(bpy)_3^{3+}$ is reduced by PAMAM* to produce $Ru(bpy)_3^{2+}$*, ensuing emission of light. We studied this mechanism by varying the dendrimer core molecules (ethylenediamine and triethylenetriamine) and using different numbers of peripheral amine groups.

## 2. Assay Principles

Based on the terminal amine groups, the PAMAM dendrimers were classified as D5, D8, D10 and D16 having 5, 8, 10 and 16 -$NH_2$ groups, respectively (Figure S1). To trigger an ECL signal, the dendrimers were oxidized at the electrode surface generating a radical. At the

same time, Ru(II) is oxidized to Ru (III) which in turn is reduced and excited by the deprotonated dendrimer radical. Luminescence occurs consequently by radiative relaxation of the excited Ru(II).

$$PAMAM - e^{-} \rightarrow PAMAM^{\bullet +} \quad \text{..... (Eq. 1)}$$

$$Ru(bpy)_3^{2+} - e^{-} \rightarrow Ru(bpy)_3^{3+} \quad \text{..... (Eq. 2)}$$

$$PAMAM^{\bullet +} \rightarrow PAMAM^{\bullet} + H^{+} \quad \text{..... (Eq. 3)}$$

$$Ru(bpy)_3^{3+} + PAMAM^{\bullet} \rightarrow Ru(bpy)_3^{2+*} \quad \text{.....(Eq. 4)}$$

$$Ru(bpy)_3^{2+*} \rightarrow Ru(bpy)_3^{2+} + h\nu \quad \text{..... (Eq. 5)}$$

The ECL platform based on Ru-dendrimers (Ru-D5, Ru-D8, Ru-D10 and Ru-D16) was then used to study the binding of biotin to streptavidin as straightforward affinity binding model as depicted in Figure 1.

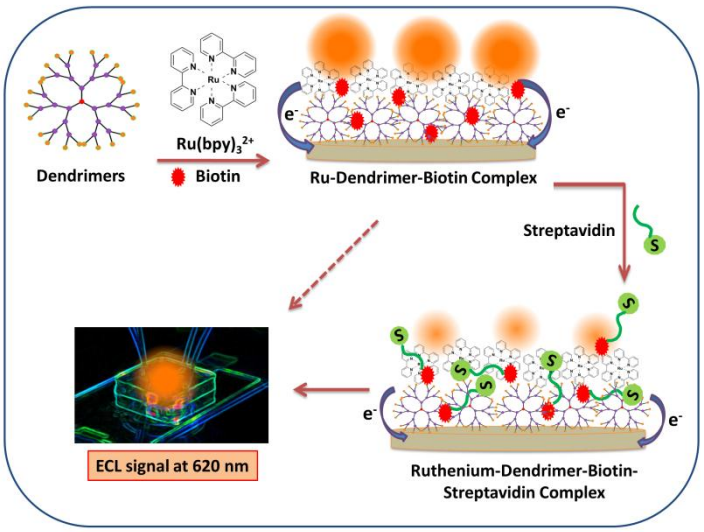



Figure 1: Schematic representation of the dendrimer-ECL platform for the biotin-streptavidin assay

## 3. Materials and Methods

An indium tin oxide (ITO) electrode coated on PET (polyethylene terephalate) foil, Ag wire and Pt wire were used as working, pseudo reference and counter electrodes, respectively. An Autolab potentiostat was used for the ECL measurements. The PMT voltage was set to 620 V and the set potential was maintained at +1.5V. Luminescence spectra were measured using a 620 nm emission wavelength. For CV measurements with $Ru(bpy)_3^{2+}$ and its associated analysis, the potential was scanned from +0.5 to +1.7 V with a step potential of 0.01 V and scan rate of 50 mV/sec.

The dendrimers (D5 and D10) and (D8 and D16) were synthesized from initiator cores diethylenetriamine and ethylenediamine, respectively with branching monomers (methyl acrylate and ethylenediamine) by Michael addition-amidation reaction [14, 15].

Typically, 10 mM dendrimers were incubated with $Ru(bpy)_3^{2+}$ (0.1 mM) for 5 hours in a buffer solution of pH 9.0 with glycine-NaOH buffer. KCl (0.1 M) was used as supporting electrolyte. No surfactant was used for the ECL measurements. For experiments employing streptavidin, the ITO cells were blocked with 0.1% BSA solution for 30 minutes and washed prior to use. For biofunctionalization of the dendrimers, they were tagged with biotin using NHS-biotin (0.1 mg in 30 µL total volume of glycine buffer pH 9.0) by incubation under magnetic stirring for 3 hours. In the case of experiments with streptavidin, an additional incubation for 30 minutes was used. Here, varying concentrations of streptavidin (SA) were made from the stock solution (200µM) by serial dilution in the same glycine buffer.

## 4. Results and Discussion

Based on previous knowledge [10, 11] it can be predicted that dendrimers with their layered architecture and structural homogeneity have the potential to serve multiple purposes in an ECL reaction. They offer a large reactive surface area for both, the immobilization of proteins/nucleic acids and simultaneously function as co-reactant for the ECL luminophore. Furthermore, the residual terminal amines render stable aqueous dendrimer dispersions. The primary and tertiary amine groups of PAMAM dendrimers are mostly responsible for ECL reactions, whereas the latter are more efficient as co-reactants than the former since the electron-donating alkyl side chains have a tendency to enhance ECL [16]. Further, it is known that the enhancement of ECL intensity is also correlated with the amine structure wherein the amines with an α-hydrogen atom are easily oxidized to form a cation radical that

in turn can deprotonate easily to form strongly reducing free-radical species [16]. Thus, the ability of all dendrimers studied here to act as co-reactant was expected.

Initial experiments in which dendrimers were adsorbed to the ITO surface demonstrated, that a layer of dendrimers does effectively inhibit the ruthenium-based ECL reaction. At the same time, it became clear that the dendrimers could function as co-reactants in solution. As the primary and tertiary amines of the G1.0-G3.0 PAMAM dendrimers are fully deprotonated at pH 10, we assume that both participate in the ECL mechanism. Based on these results the assay was changed. As the dendrimers are highly hydrophilic, they could also be used in solution. In addition, Yuan et al. [2] had already shown that the ECL reaction time, the electron transfer efficiency and consequently the ECL signal were enhanced when adding luminophore and coreactant simultaneously to the electrode surface. Therefore, dendrimers and the luminophore were incubated for 30 min prior to the ECL measurements, also no immobilization such as simple absorption was performed. The preliminary studies showed best ECL performances of the D8 (ethylenediamine core) and D10 (triethylenetriamine core) dendrimers with respect to the reliability of the data obtained and signal height, so that these two systems were further characterized with respect to their electrochemical performances (Figure S2).

Figure 2 demonstrates the electroactivity of the PAMAM dendrimers at 10 mM (Ru-D8 and Ru-D10) in comparison to the conventional co-reactant TPA (100 mM). The cyclic voltammograms show that direct oxidation of the $Ru(bpy)_3^{2+}$ started at the same electrode potential (~+0.9 V vs pseudo Ag/AgCl). Maximum ECL emission can be observed at +1.5V similar to TPA-assisted ECL. Further, an apparent increase in the oxidation current suggested that the dendrimers exhibited a very good electrocatalytic effect on the oxidation of $Ru(bpy)_3^{2+}$ even though they are present at a 10-fold lower concentration than TPA. We assume that this enhancement is due to the hydrophilic nature of the dendrimers in contrast to the hydrophobicity of TPA. Ruthenium can more easily access the electrode surface under hydrophilic conditions which consequently leads to an enhanced electron transfer (Fig. 2) and also luminescence signal (data not shown). Further, the presence of multiple amine groups in the dendrimers unlike TPA, is directly correlated with the enhancement of the ECL intensity since the α-H atom of the amine is easily oxidized and the newly formed cation radical can deprotonate easily to form reducing free radical species [16].

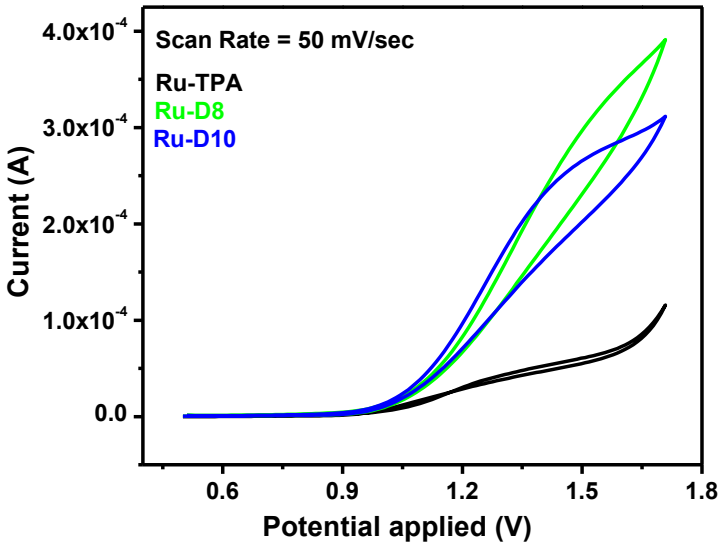



Figure 2: Comparison of the electroactivity of Ru-D8 and Ru-D10 with Ru-TPA

Based on these findings, the dendrimer ruthenium concept was explored to simultaneously function as co-reactant and as biorecognition element in a bioassay, using biotin-streptavidin as affinity binding model. The dendrimers were chemically modified with an abundance of biotin, while keeping at least one free primary amine group per dendrimer statistically to serve as co-reactant. (This resulted in lower ECL activity as described further below.) In the assay, biotinylated dendrimers, tris(2,2'-bypyridine)ruthenium(II) and streptavidin were incubated for 30 minutes prior to addition to the ITO electrodes. The expected decrease in the luminescence intensity of Ru-D8 (Figure S3) and Ru-D10 (data not shown) upon specific streptavidin binding was clearly detectable.

To further investigate this finding, the influence of the binding interaction of biotin and streptavidin on the ECL reaction was also monitored by the change in the luminescence intensity with time. Figures 3 demonstrate the ECL decay of Ru-D10in presence of biotin and streptavidin. While the biotinylation did not alter the ECL signal trace of the Ru-D10 significantly, the reaction with streptavidin causes a two-fold drop in signal. We assume that the streptavidin binding causes steric hindrance for the ECL mechanism or affects the diffusion process to the electrode surface. In the case of the Ru-D8 complexes also the biotinylation led to a decrease in signal. We assume this is due to fewer primary amine groups available after the biotinylation reaction and further optimization of the biotinylation reaction in the future is warranted.

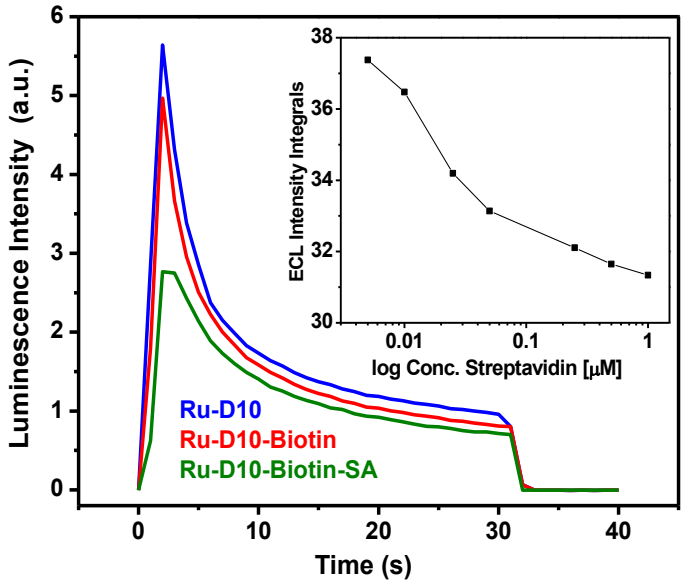



Figure 3 Changes in the ECL intensity of Ru-D10 over time on immobilization with biotin and streptavidin. Inset: Dose-response ECL intensity curve showing decrease in luminescence with increase in the concentration of streptavidin.

Finally, the detection principle was assessed by subjecting the Ru-Dendrimer-Biotin solution to different concentrations of streptavidin (Figure S4). The ECL intensity decreased with an increase in the streptavidin concentration as depicted in the dose responsive curve which is due to specific binding between the analyte streptavidin to the dendrimer-coupled biotin. The calibration curve of ECL versus logarithm concentration of SA showed an exponential relationship in a dynamic range from 0.005 to 1μM. The regression equation was found to be $I = 31.7 (\log C_{SA}) - 1.74$ with a correlation coefficient of R = 0.97523 where $I$ is the ECL intensity and $C$ is the concentration of streptavidin.

Table 1 Changes in Zeta potential values of Ru-D8 and Ru-D10 upon immobilization with biotin and streptavidin

| Sl. No. | Samples | Zeta Potential (mV) ± S.D |
|---|---|---|
| 1 | D8 | -19.1 ± 1.3 |
| 2 | D8-Ru | -7.5 ± 2.7 |
| 3 | D8-Ru-Biotin | -12.8 ± 4.0 |
| 4 | D8-Ru-Biotin-Streptavidin | -3.99 ± 0.3 |
| 5 | D10 | -34.9 ± 2.4 |
| 6 | D10-Ru | -26.2 ± 4.4 |

| | | |
|---|---|---|
| 7 | D10-Ru-Biotin | -32.4 ± 7.4 |
| 8 | D10-Ru-Biotin-Streptavidin | -9.89 ± 3.6 |

Finally, the potential stability of the dendrimer complex in buffer solution (0.1 M glycine-NaOH buffer, pH 9.0; 25°C) was assessed using zeta potential measurements. (Table 1). The refractive index of the dendrimers was experimentally found to be 1.334. Upon modification with Tris(2,2'-bipyridine)ruthenium(II) and biotin, the zeta potential changes as expected. Interestingly, the addition of streptavidin indicates that the complexes become less colloidally stable due to the positive charge of the protein (+20 mV at pH ≤ 8) [17]. A schematic representation of the above is shown in Figure S4. Further experiments need to be carried out to determine, how this contributes to the sensitive signal response upon streptavidin binding.

## 5. Conclusion

The dendrimer-$Ru(bpy)_3^{2+}$ approach shows promise as analytical platform for the direct detection of bioaffinity events. The dendrimers function as efficient immobilization matrix due to their tethered structures and primary amine groups. Simultaneously they function as highly efficient coreactant due to their primary and tertiary amine groups. In fact, Newkome et al [18] postulated in a similar mechanism that Ru-species promote and stabilize the inter-dendrimer assembly on electrode surfaces by joining the individual dendrimers and interacting the (bipyridine) ligand with the exterior amine groups of the dendrimers. The presence of primary and tertiary amines in the PAMAM dendrimers therefore provides for specific coupling reactions and for ECL enhancement. The proposed assay here is based on a homogeneous format allowing full distribution of the biorecognition element (here biotin) within the sample. Preliminary studies with immobilized dendrimers indicate increased sensitivity, which will be studied in more detail in the future.

**Acknowledgements**

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Highlights

- Four different PAMAM dendrimers are compared towards their ECL capabilities
- The simultaneous function of PAMAM dendrimers as ECL coreactants and biorecognition elements is investigated
- This bivalent role was successfully tested with the model system biotin/streptavidin