Communications

Sub-microliter Electrochemiluminescence Detector—A Model for Small Volume Analysis Systems

Arun Arora, Andrew J. de Mello and Andreas Manz*

Zeneca/SmithKline Beecham Centre for Analytical Sciences, Department of Chemistry, Imperial College of Science, Technology & Medicine, Exhibition Road, South Kensington, London, UK SW7 2AY

A small volume, electrochemical cell for the generation and detection of electrochemiluminescence from tris(2,2′-bipyridyl) ruthenium(II) has been fabricated. The flowcell is a poly(methyl methacrylate) (PMMA)–acetate–PMMA sandwich construct, containing two platinum, thin-film electrodes. Operation of the microchip establishes sub-microliter detection of tris(2,2′-bipyridyl) ruthenium(II) electrochemiluminescence in continuous flow. Initial experiments demonstrate a detection limit of 5×10^{-13} M at an effective cell volume of 100 nl. This corresponds to the detection of only 30 000 molecules.

Electrogenerated chemiluminescence (ECL) is a highly specific and sensitive detection protocol used in a diversity of analytical applications. These include bioassays in clinical diagnostics and high-throughput screening for drug discovery.[1,2] ECL methods have significant advantages over more conventional chemiluminescent techniques. In particular, the necessary reactants are produced *in situ* at a given electrode, enabling the reaction to be controlled through small changes in the applied potential. Furthermore, since light emission is located only in the immediate vicinity of the electrode surface, light collection is both efficient and facile.

ECL resulting from the reaction between tri-propylamine (TPA) and tris(2,2′-bipyridyl) ruthenium(II) (TBR) has been shown to be very successful in the detection of a range of analytes.[1,3] TBR labels are cheap, non-radioactive and stable. In addition, the high efficiency and low oxidation potential of the reaction, affords the possibility of specificity at low analyte concentrations (10^{-14} M).[4] The documented mechanism is illustrated in Fig. 1.[4] Briefly, both TBR and TPA are oxidized in aqueous solution at the anode. The TPA cation is unstable and becomes deprotonated almost immediately to form TPA′. Subsequent electron transfer between TPA′ and TBR$^+$ causes the formation of an excited state TBR molecule (TBR*), which then relaxes radiatively back to the ground state ($\lambda_{emission} \approx 610$ nm). It is noted that although TPA is consumed during the reaction, the TBR is recycled.

Smith and co-workers recently reported a microfabricated electrochemical cell for the generation and detection of ECL of TBR.[5,6] More contemporary studies demonstrated the detection and quantitation of single-stranded DNA attached to paramagnetic beads.[7] Using a 1 cm^2 Pt anode and a sample volume of 150 µl, a detection limit of 4×10^{-14} moles was realised.

As previously noted TBR is recycled during reaction. Consequently, if a flowing reactant solution (with TPA in excess) is manoeuvred over the surface of the anode, reactant concentrations will remain constant, and luminescence intensity becomes a function of TBR concentration only. This paper reports the fabrication of a poly(methyl methacrylate) (PMMA)–acetate (cellulose acetate) hybrid chip for ECL detection on nanoliter volumes. Detection limits for ECL in continuous flow are investigated for the reaction between TBR and TPA in aqueous solution.

Experimental

A 5 mM solution of TPA (pH 6.8) was prepared by diluting a 180 mM stock solution (Procell Assay, Boehringer, Mannheim, Germany) with high resistivity deionised water (Maxima Ultrapure Elga, UK). To this was added TBR hexahydrated chloride (Fluka, Buchs, Switzerland) to create a TBR–TPA (1 mM–5 mM) stock solution. Test solutions (5×10^{-14}–5×10^{-12} M) were made through serial dilution with the 5 mM TPA solution.

The hybrid chip consists of an acetate film sandwiched between two blocks of PMMA (ICI, Cheshire, UK). A flow channel is fabricated by removing a rectangular section (30 mm × 1 mm) from the acetate sheet with a sharp scalpel blade. In addition, two platinum foil electrodes (1 mm wide) are positioned across the channel. The assembly is bonded using epoxy-based glue (Alraldite, Ciba-Geigy, Duxford, UK), and two drilled conduits in the upper PMMA block facilitate access to the enclosed flow channel. A schematic of the chip is shown in Fig. 2(*a*). ECL is only generated where the test solution contacts the working electrode. Consequently, only 100 nl (100 µm × 1000 µm × 1000 µm) of solution is probed at any given time. Continuous flow is achieved by connecting the conduits, *via* silicone tubing (Cole Parmer, Hanwell, London, UK), to a peristaltic pump (Werner Meyer, Zürich, Switzerland). A Ag/AgCl reference electrode (World Precision Instruments, USA) is connected to the output conduit through use of a 'T' connector. A scanning potentiostat (Sycopel Scientific, Boldon, UK) is used to control electrode potentials in all cases. Emitted light is detected by placing an R1477 photomultiplier tube (Hamamatsu, Hamamatsu City, Japan) operating at 999 V directly (2 mm) below the chip. The PMT output is digitally
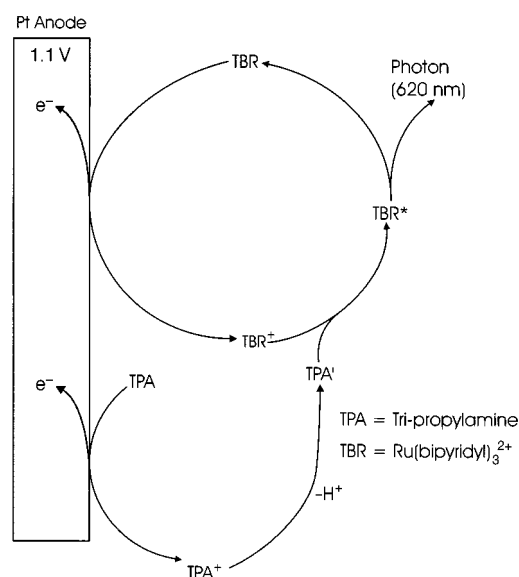

Fig. 1 Electrochemiluminescence reaction mechanism of TBR in aqueous solution, according to Leland.[4]

recorded using a multifunction digital I/O board (NB-MIO-16X, National Instruments, USA) and a PC (XPS200n, Dell, UK). A schematic of the complete experiment is shown in Fig. 2(*b*).

Results

Electrochemical characterization was performed to determine the optimum oxidation potential for the Ru^{2+} ion in the current system. The cyclic voltammogram illustrated in Fig. 3 was obtained using the 1 mM TBR solution (not flowing), and the Ag/AgCl reference electrode. The applied voltage is scanned between +0.3 and +1.5 V at a speed of 100 mV s^{-1}. The peak in the anodic sweep is at +1.1 V. This indicates the optimum oxidation potential for the Ru^{2+} ion. This potential was used for all subsequent measurements.
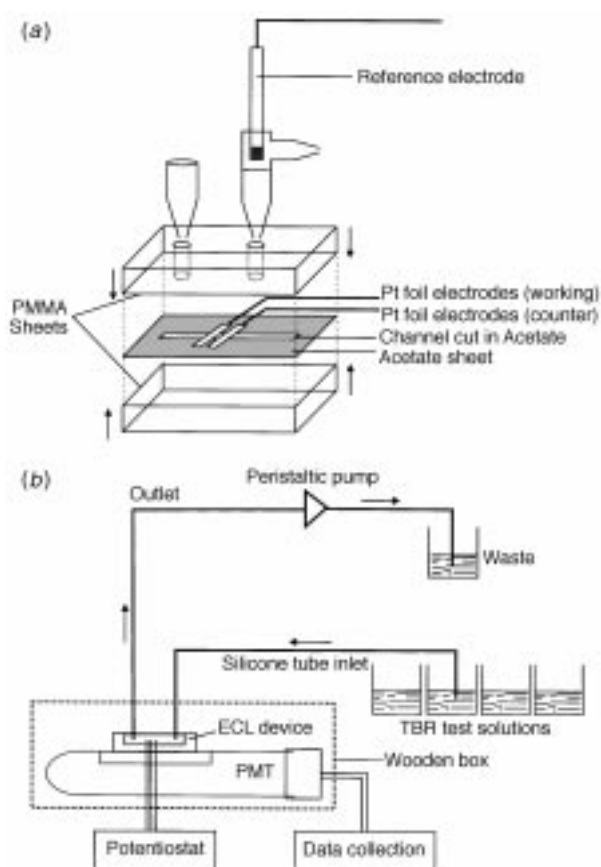

Fig. 2 (*a*) PMMA–acetate hybrid microchip for ECL detection, (*b*) schematic of ECL detector instrumentation.
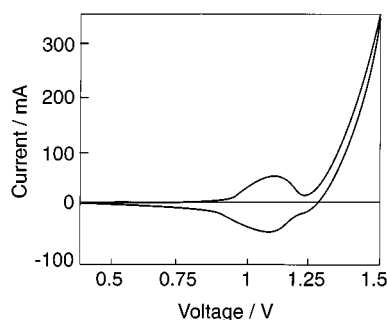

Fig. 3 Cyclic voltammogram of 1 mM TBR solution using a saturated Ag/AgCl reference electrode.

TBR–TPA test solutions were then sequentially pumped through the hybrid chip at a constant flow rate (170 µl s^{-1}). ECL initially increases as a function of time, but plateaus soon after at a limiting value (within 30 s of sample injection). An average light intensity is calculated from each limiting value and tabulated in Table 1. It is noted, that subsequent to each intensity measurement, the chip is thoroughly flushed with 5 mM TPA solution to ensure the removal of all previous sample. Repeat measurements on identical solutions yield consistent results, demonstrating electrode continuity. A calibration plot of emission intensity *versus* TBR concentration is shown in Fig. 4. Within the concentration range studied (5×10^{-13}–5×10^{-12} M) the plot is approximately linear (correlation coefficient ≈ 0.998). The lowest measurable concentration (S/N = 3) is 5×10^{-13} M, which compares favorably with the best-reported literature value.[4] This concentration corresponds to only 3×10^4 molecules in the probe volume of 100 nl. It should be realized that since ECL occurs only for molecules in the immediate vicinity of the working electrode, the actual number of molecules contributing to the measured signal could be considerably less.

Discussion and Future Work

The use of ECL as an effective detection protocol for separation techniques such as capillary electrophoresis (CE) and liquid chromatography (LC) is yet to be established. However, it is clear that the unique characteristics of ECL make it a desirable alternative to more conventional optical schemes such as absorption and fluorescence spectroscopies.[8] The success of any detection protocol is, in part, determined by its applicability to small volume measurement. To date, little work has focussed on the application of ECL to measurement in small volumes. This first generation, hybrid microchip has been successful in detecting ECL from sub-microliter volumes. In this case, the probe volume (defined by the dimensions of the acetate channel and working electrode) is at most 100 nl. Additionally, it is clear that measurement from this small volume is not at the expense of sensitivity (lowest measurable concentration). The use of

Table 1 ECL emission intensity resulting from various TBR test solutions

Concentration/ mol dm^{-3}	Number of molecules*	Light intensity (arbitrary units)
0	0	0
5×10^{-13}	30 100	2.1 ± 0.5
1×10^{-12}	60 200	5.2 ± 0.4
2×10^{-12}	120 400	11.1 ± 0.7
4×10^{-12}	240 800	28.0 ± 0.6
5×10^{-12}	301 000	34.1 ± 0.4

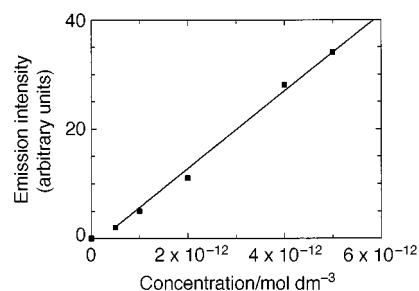* Calculated assuming a probe volume of 100 nl.

Fig. 4 Plot of emission intensity *versus* TBR solution concentration.

continuous flow has also proved to be useful in optimizing signal stability. Electrode 'fouling' is precluded by continually replacing reaction by-products (such as di-propylamine and propyl aldehyde) with fresh reactants. Consequently, emission intensity becomes solely a function of TBR solution concentration.

The planar microchip construct affords the possibility of performing 'on-line' ECL detection when combined with an electrophoretic or chromatographic separation. Consequently, work is currently addressing the fabrication of glass microchip systems with integrated ECL detectors. The detection of TBR in small volumes (≈ 100 nl) establishes the feasibility of analyzing labelled protein and nucleic acid moieties on a similar scale using ECL. The eventual aim being to perform ultra-sensitive detection of clinically relevant samples in real time and at low cost. Minor modifications in electrode construction and light collection should enable the required improvements in performance.

References

1 Knight, A. W., and Greenway, G. M., *Analyst*, 1994, **119**, 879.
2 Motmans, K., Raus, J., and Vandevyver, C., *J. Immunol. Methods*., 1996, **190**, 107.
3 Forbes, G. A., Nieman, T. A., and Sweedler, J. V., *Anal. Chim. Acta,* 1997, **347**, 289.
4 Leland, J. K., and Powell, M. J., *J. Electrochem. Soc.,* 1990, **137**, 3127.
5 Hsueh, Y. T., Smith, R. L., and Nothrup, M. A., *Conference Proceedings of Transducers '95–Eurosensors IX, Stockholm, Sweden, June 25–29*, 1995, pp. 768–771.
6 Hsueh, Y. T., Smith, R. L., and Nothrup, M. A., *Sen. Actuators, B,* 1996, **33**, 110.
7 Hsueh, Y. T., Collins S. D., and Smith, R. L., *Conference Proceedings of Transducers '97, Chicago, Illinois, USA, June 16–19*, 1997, **1**, pp. 175–178.
8 Pentoney, S. L., and Sweedler, J. V., in *Handbook of Capillary Electrophoresis*, ed. Landers, J. P., CRC Press, 2nd edn., 1997, 409.