Electrochemiluminescence of 2′,6′-difluorophenyl 10-methyl-9,10-dihydroacridine-9-carboxylate

Robert Wilson,*[a] Hashem Akhavan-Tafti,[b] Renuka DeSilva[b] and A. Paul Schaap[b]

[a] *Robert Wilson, Department of Chemistry, Liverpool University, Liverpool, UK L69 7ZD. E-mail: R.Wilson@liv.ac.uk*
[b] *Lumigen, Inc., 24485 W. Ten Mile Rd., Southfield, MI 48034, USA. E-mail: aps@lumigen.com*

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Electrochemical oxidation of the acridine ester 2′,6′-difluorophenyl 10-methyl-9,10-dihydroacridine-9-carboxylate yields the corresponding acridinium ester which reacts with H_2O_2 to generate intense chemiluminescence.

Electrochemiluminescence (ECL) has received widespread attention during the previous decade, especially in the field of chemical analysis.[1] It combines the well known sensitivity of chemiluminescence (CL) with the precise control over the time and position of light emitting reactions afforded by electrochemistry. As an alternative approach for conducting immunoassays and nucleotide assays it offers advantages such as increased sensitivity and precision, reduction in time and labor, and the elimination of radioisotopes.[2] In order to exploit the full potential of this technology there is a requirement for new CL compounds which can be initiated electrochemically. In this communication we show for the first time how acridinium ester CL can be triggered by electrochemical oxidation of 2′,6′-difluorophenyl 10-methyl-9,10-dihydroacridine-9-carboxylate (DMC).

Prior to the present communication ruthenium chelates[3] and luminol† derivatives[4] are the only compounds that have been used in a significant number of analytical applications involving ECL. Ruthenium chelates have been used for enzyme assays,[5] but their most significant impact has been as labels for immunoassays[6] and nucleotide assays.[7] Luminol has also been used for enzyme assays[8] and immunoassays.[9] Light is emitted when electrochemically oxidized luminol reacts with H_2O_2, which allows the reaction to be coupled to oxidase enzymes such as glucose oxidase.[10] The latter enzyme has been used as an antibody label in ECL enzyme immunoassays which constitute a further consolidation of existing technology.[9] The chemiluminescence reaction of luminol is also catalyzed by electrochemically oxidized ferrocenes[11] suggesting that these compounds could be used as labels in an ECL system resembling the one based on ruthenium chelates.

Acridinium esters have been used as labels in chemiluminescence immunoassays for many years.[12] The CL reaction of these compounds does not require a catalyst and is usually triggered by addition of an acidic solution of H_2O_2 followed by a NaOH solution. The acidic solution ensures that inactive pseudo-base is converted to the active acridinium ester and the alkali triggers the CL oxidation of the ester by a H_2O_2.[13] Two advantages of these compounds which do not appear to have been fully exploited are freedom from interference in matrices such as whole blood[14] and their ability to intercalate into the double helix of polynucleotides.[12] Intercalation of an acridinium ester into an oligonucleotide duplex shields it from attack by hydroxide and forms the basis of homogenous hybridization protection assays for RNA.[15]

Recently a large number of esters derived from 9,10-dihydroacridines (reduced acridinium esters and thioesters) based on the *N*-alkyldihydroacridinecarboxylate nucleus, including DMC, have been made at Lumigen.[16] These dihydroacridine compounds are stable in the presence of H_2O_2 and do not form an inactive pseudo-base. Light emission can be triggered by oxidising the acridan with horseradish peroxidase (HRP) in the presence of H_2O_2 and an enhancer such as *p*-iodophenol. HRP oxidizes the 9,10-dihydroacridine to the corresponding acridinium ester, which is immediately subject to nucleophillic attack by the peroxide anion (OOH^-) at the 9 position of the acridinium nucleus; the possibility of pseudo-base formation does not arise because peroxide is several orders of magnitude more nucleophillic than hydroxide.[13] Nucleophillic attack results in the formation of a dioxetanone which decomposes to form the singlet excited state of *N*-methylacridone. This relaxes to the ground state accompanied by the emission of intense blue light with a maximum wavelength of 430 nm. By using these compounds as a substrate for HRP it has been possible to detect as little as 0.1 amol of this enzyme in a 15 min assay.[16]

Although there have been several reports of ECL in aqueous solutions involving acridinium esters[17] and the related compound lucigenin[18] they are all based on the reduction of dissolved oxygen to H_2O_2; this has two disadvantages: reliance on dissolved oxygen concentration and the susceptibility of the acridinium ester to pseudo-base formation.[13] These drawbacks are avoided when DMC is used because the acridinium ester is produced *in situ* from a passive precursor.

Cyclic voltammetry and linear sweep voltammetry with luminometric detection were carried out using an experimental arrangement described previously;[11] all potentials *vs.* Ag/AgCl. The concentrations of DMC and the corresponding acridinium ester were 50 and 5 μM respectively. The second cyclic voltammogram (CV) of DMC shown in Fig. 1 has three peaks located at 0.76 V (peak A), −0.25 (peak B) and −0.11 V (peak C). A plot of light intensity at 430 nm against applied potential for DMC in the presence of H_2O_2 (Fig. 2) has a single peak at 0.75 V corresponding closely to the position of peak A in Fig. 1. This suggests that peak A represents the two electron oxidation of DMC to the corresponding acridinium ester as shown in Scheme 1a, which then reacts with H_2O_2 to generate chemiluminescence as shown in Scheme 1b. Peaks B and C in

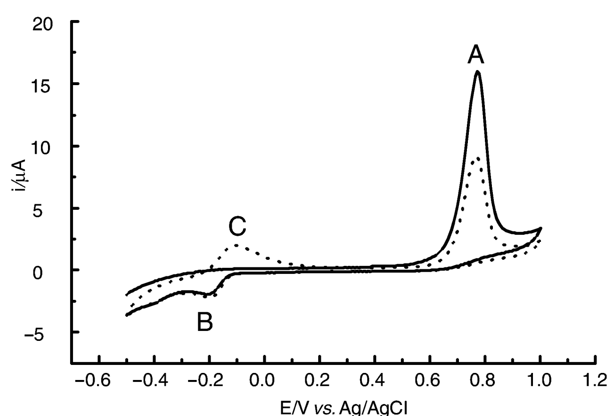

Fig. 1 Two successive cyclic voltammograms of 50 μM DMC in 10 mM Tris buffer with 0.1 M NaCl, 10 mM H_2O_2, and 0.025% Tween-20. Scan rate 100 mV s^{-1}, 1st scan (solid line); 2nd scan (dashed line).

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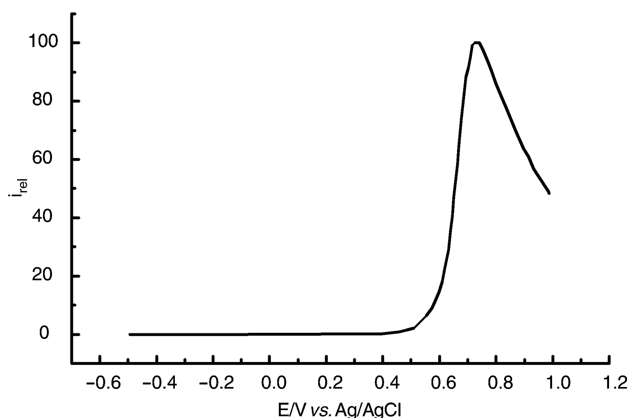
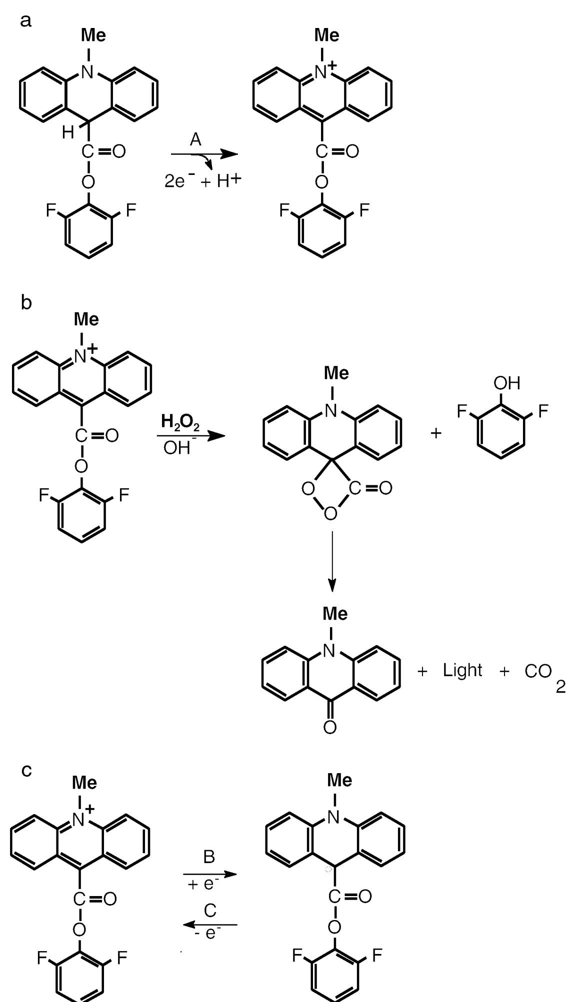


Fig. 2 Dependence of electrochemiluminescence on potential for 50 μM DMC at pH 8.0, in 10 mM Tris buffer with 0.1 M NaCl, 10 mM H_2O_2, 1 mM EDTA and 0.025% Tween-20. Scan rate 10 mV s^{-1}.



Scheme 1 Large capital letters refer to peaks in CV. (a) Two-electron oxidation of DMC to acridinium ester; (b) CL reaction of acridinium ester with H_2O_2; (c) one-electron redox reactions of acridinium ester/radical couple.

Fig. 1 only appear in the CV after oxidation of DMC and therefore peak B must represent the reduction of an oxidation product. Light emission in the presence of H_2O_2 indicates that an acridinium ester is produced when DMC is oxidized and the simplest explanation for peak B is that it represents reduction of this product as shown in Scheme 1c. This is supported by the observation that peak B is almost absent when a CV is obtained in the presence of H_2O_2, as would be expected if the compound responsible reacts with peroxide. Further investigation of this hypothesis with an authentic sample of the acridinium ester corresponding to DMC[16] gave a CV with two peaks identical to B and C in Fig. 1.

Enzymatic oxidation of DMC by HRP has been reported to occur in two one-electron oxidation steps separated by a non-enzymatic deprotonation.[16] The corresponding electrochemical pathway would be a classic ECE mechanism[19] in which the two enzymatic steps are replaced by electrochemical oxidations. Previous work on acridans, however, has suggested an alternative mechanism in which the second oxidation step occurs in solution as a result of disproportionation between protonated and unprotonated radical intermediates.[20] Further work is required to reveal which mechanism applies to DMC.

We have shown for the first time that CL of the dihydroacridine ester DMC can be triggered by electrochemical oxidation in the presence of H_2O_2. Presently work is in progress to extend this investigation to other 9,10-dihydroacridine esters in order to build up a detailed picture of the mechanism, and the relationship between electrochemiluminescence and molecular structure. ECL of 9,10-dihydroacridines in matrices where light emission from compounds such as luminol is quenched[14] will also be investigated as will the effect of nucleotide duplexes on ECL. The latter investigation is based on the assumption that if intercalation of an acridinium ester into a nucleotide double helix can shield it from hydrolysis it may also shield a 9,10-dihydroacridine ester from oxidation by an electrode.

Notes and references

† The IUPAC name for luminol is 5-amino-2,3-dihydrophthalazine-1,4-dione.

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