Journal of Fluorescence, Vol. 16, No. 1, January 2006 (© 2006)
DOI: 10.1007/s10895-005-0023-2

Time-Resolved Detection of Hot Electron-Induced Electrochemiluminescence of Fluorescein in Aqueous Solution

Tiina Ylinen,[1] Johanna Suomi,[1,3] Mika Helin,[1] Timo Ala-Kleme,[2] and Sakari Kulmala[1]

Received July 1, 2005; accepted October 14, 2005
Published online: February 23, 2006

Strong electrogenerated chemiluminescence (ECL) of fluorescein is generated during cathodic pulse polarization of oxide-covered aluminum electrodes and the resulting decay of emission is so sluggish that time-resolved detection of fluorescein is feasible. The present ECL in aqueous solution is based on the tunnel emission of hot electrons into the aqueous electrolyte solution, which probably results in the generation of hydrated electrons and hydroxyl radicals acting as redox mediators. The successive one-electron redox steps with the primary radicals result in fluorescein in its lowest excited singlet state. The method allows the detection of fluorescein (or its derivatives containing usable linking groups to biomolecules) over several orders of magnitude of concentration with detection limits well below nanomolar concentration level. The detection limits can still be lowered, e.g., by addition of azide or bromide ions as coreactants. The results suggest that the derivatives of fluorescein, such as fluorescein isothiocyanate (FITC), can be detected by time-resolved measurements and thus be efficiently used as electrochemiluminescent labels in bioaffinity assays.

KEY WORDS: electrogenerated chemiluminescence; ECL; time-resolved detection; fluorescein; FITC; hot electron; oxide-covered aluminum electrode.

INTRODUCTION

We have previously shown that time-resolved detection of cathodic electrochemiluminescence (ECL) of Tb(III) labels at thin insulating film-coated electrodes can be efficiently used in electrochemiluminoimmunoassays (ECLIA) [1–3]. It has also been shown that some metalloporphyrins [4] are usable as electrochemiluminescent labels displaying relatively long-lived cathodic ECL. The feasibility of Tb(III) chelate labels is much better than that of metalloporphyrins mainly because most of the Tb(III) chelates are practically insensitive towards the presence of molecular (triplet) oxygen [1–3,5]. Different types of multiparametric bioaffinity assays would need labels emitting at different wavelength ranges and/or time domains. For instance, fluorescein has been observed to emit ECL at about 515 nm, and Tb(III) chelates are not emitting ECL at that wavelength [6].

The fluorescence quantum yield of fluorescein (2-(6-hydroxy-3-oxo-xanthen-9-yl)benzoic acid) is relatively good (about 0.91 in alkaline aqueous solution [7]). The derivatives of fluorescein are amongst the oldest and best-studied photoluminescent labels usable in bioaffinity assays and their use is widely distributed [8]. In addition to fluorescent label derivative forms linkable with biomolecules, such as fluorescein isothiocyanate (FITC), fluorescein is also used e.g. as fluorescent probe in detection of singlet oxygen and oxidizing radicals [9–11].

Fluorescein is also in use in several chemiluminescent systems as a light emitter. However, fluorescein often behaves only as an energy acceptor and secondary emitter which receives energy, e.g., from reaction products of luminol, [12], singlet oxygen [13–15], or sulfide [16].

Fluorescein is known to show radiochemiluminescence (RCL) induced by hydrated electrons and

[1] Laboratory of Analytical and Inorganic Chemistry, Helsinki University of Technology, P.O. Box 6100, FIN-02015 HUT, Finland.
[2] Laboratory of Analytical Chemistry, Department of Chemistry, University of Turku, FIN-20014, Finland.
[3] To whom correspondence should be addressed. E-mail: johanna.suomi@hut.fi

hydroxyl radicals [17,18]. These are the same primary species we have suggested to be the primary reactive species generated at our tunnel emission electrodes inducing chemiluminescence from various luminophores at the vicinity of our electrode surfaces [19–22]. Actually our excitation method, cathodic hot electron injection into aqueous electrolyte solutions, seems to have more common with pulse radiolysis than with traditional electrochemistry [8,23].

We have earlier shown that $Ru(bpy)_3^{2+}$-based labels [24], luminol derivatives [25], and coumarins [26] can be excited by cathodic pulse-polarization of conductor/insulator/electrolyte (C/I/E) tunnel junction electrodes, e.g., at oxide-coated aluminum, silicon and magnesium electrodes [1,19–22]. These thin insulating film-coated electrodes are known to tunnel-emit hot electrons (e^-_{hot}) into aqueous solutions in an analogous manner to that occurring in solid-state devices [1,19–22,27–29].

If the energy of tunnel-emitted hot electrons is above the conduction band edge of water, they may enter the conduction band of water and are likely to become hydrated electrons (e^-_{aq}) after thermalization and solvation [1,19–22]. Meanwhile, strongly oxidizing species such as sulfate and hydroxyl radicals can be cathodically generated from added coreactants or from dissolved oxygen [1,19–22,24,25]. In addition, F^+-centers existing in oxide film of C/I/E electrodes may act as oxidants [1,21,22]. Hence, highly reducing and oxidizing conditions are simultaneously achieved in the vicinity of the electrode surface. Thus, our hot electron electrochemistry at thin insulating film-coated cathodes resembles more pulse radiolysis of water than traditional electrochemistry utilizing active metal electrodes or semiconductor electrodes [1,19–22].

We have shown with several luminophores that typical ECL excitation routes based on our cathodic pulse polarization method are (i) reduction-initiated oxidative (red-ox) and (ii) oxidation-initiated reductive (ox-red) excitation pathways, in which the luminophores are excited by the cathodically generated species at the oxide film/electrolyte interface by successive one-electron transfer steps [1,19–22,28]. Normally, this results in the luminophore in its original oxidation state but in singlet or triplet excited state [19–22,24]. However, sometimes the ECL mechanism is based on the decomposition of the luminophore [25].

The present work was carried out to study the rise and decay of cathodic ECL of fluorescein during pulsed excitation as well as the possible feasibility of time discrimination in detection of fluorescein.

EXPERIMENTAL

The ECL intensities of fluorescein were detected through an interference filter with a center wavelength at 515 nm and transmission band half-width of 10 nm, using single photon counting and previously described apparatus [20,30]. The ECL measurements were normally made in 0.2 M boric acid buffer at pH 9.2 containing 0.1 M sodium sulphate. Either a coulostatic pulse generator [2] or Pine Instrument RD4 potentiostat was used in the measurements, and the working electrodes were Al-cup [30] electrodes with a Pt wire counter electrode. The effective surface area of the oxide-covered working electrodes was 2.1 cm^2 in the case of Al-cup electrodes (350 μL samples). The disposable Al electrodes were cut from a nominally 99.9% pure aluminum band (Merck Art. 1057, batch 721 K4164557) and the electrodes were covered with the 2–3-nm thick natural oxide film [31,32].

The ECL spectra were measured in a 1-cm polystyrene cuvette, in which an Al strip working electrode and a Pt wire counter electrode were set between two PTFE support pieces. ECL and fluorescence spectra were measured using Perkin-Elmer LS5 luminescence spectrometer.

Most of the measurements were made in 0.05 M sodium tetraborate buffer at pH 9.2. This buffer is known to be quite unreactive with hydrated electrons, hydroxyl radicals and sulfate radicals [23]. Fluorescein, NaI, NaBr, $Na_2B_4O_7{\cdot}10H_2O$, NaN_3, $K_2S_2O_8$, $NaNO_3$, and H_2O_2 were pro analysi grade reagents from Merck. Potassium peroxodiphosphate was supplied by Polysciences, Inc., Warrington, PA 18976.

RESULTS AND DISCUSSION

Basic Features of the ECL

During cathodic excitation pulses at oxide-covered aluminum electrodes, fluorescein emits an ECL emission spectrum similar to its fluorescence emission spectrum peaking at about 515 nm (Fig. 1). In addition, fluorescein isothiocyanate (FITC) has been previously observed to be simultaneously excitable under similar conditions with Eu(III) labels [6].

The decay of fluorescein ECL emission is much slower than that of, e.g., coumarins which we have studied quite recently [26] (Fig. 2). The present decay is multi-exponential and can be quite nicely fitted with two exponential functions, the first giving luminescence lifetime of 17 μs and the second of about 60 μs (Fig. 2). We assume that the slow decay is actually based on the

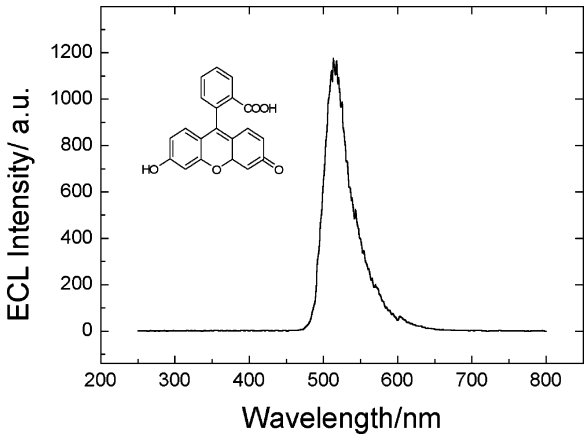

Fig. 1. ECL spectrum and structure of fluorescein. Conditions: 1×10^{-6} M fluorescein in 0.05 M $Na_2B_4O_7$ buffer at pH 9.2. Coulostatic pulse generator: pulse voltage –40 V, pulse frequency 90 Hz, pulse charge 120 μC, aluminum strip cathode, platinum wire anode. Spectrum was measured with Perkin-Elmer LS5 spectroluminometer, emission slit 20 nm.

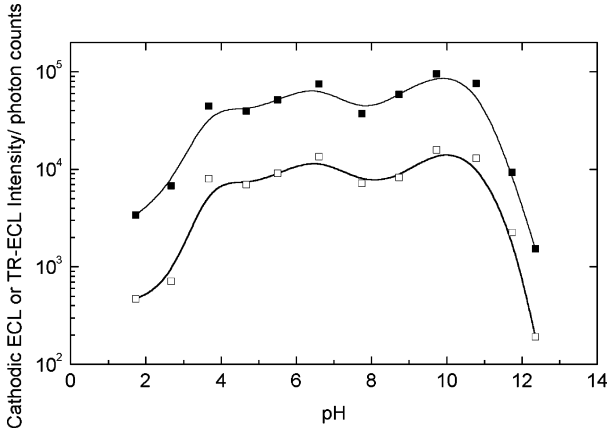

Fig. 3. Effect of pH on the cathodic ECL (solid squares) and TR-ECL (open squares) of fluorescein. Conditions: 1.0×10^{-7} M fluorescein in 0.1 M Na_2SO_4 supporting electrolyte solution. Solutions were adjusted to the desired pH with sulfuric acid or sodium hydroxide. Pulse voltage –10 V, pulse time 200 μs, pulse frequency 50 Hz. ECL was measured through 515-nm interference filter and the total sum of photons was integrated over 1000 excitation cycles. In the TR-ECL measurements the delay time was 5.0 μs and gate time 2.00 ms.

excitation reactions still progressing after the cathodic pulse and that the radiative decay of the excited state is not any slower than in case of fluorescence induced by photoexcitation. Interestingly, the rise time of fluorescein ECL is much faster than those of coumarins and the rather long lag time of, e.g., 7-hydroxy-4-methyl coumarin is missing (Fig. 2). We believe that this is based on the slower reaction rates of 7-hydroxy-4-methyl coumarin with the primary radicals of the present excitation system.

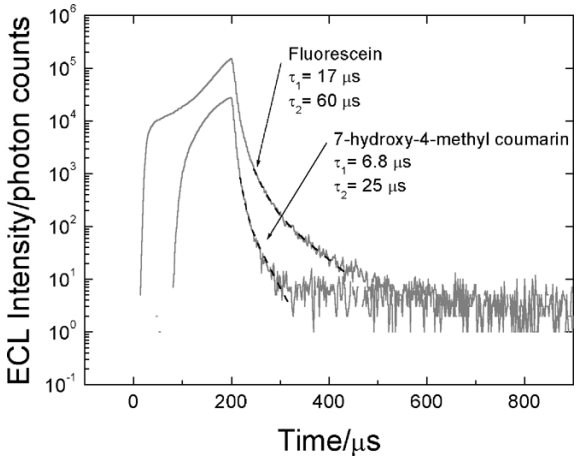

Fig. 2. The rise and decay of the cathodic ECL pulse of fluorescein and 7-hydroxy-4-methyl coumarin. Conditions: 0.05 M $Na_2B_4O_7$ buffer at pH 9.2 containing 0.1 M Na_2SO_4, pulse time 200 μs, pulse voltage –10 V, pulse frequency 50 Hz, Pine Instrument RD4 potentiostat.

The dependences of the cathodic ECL and TR-ECL of fluorescein on pH in air-saturated solution are presented in Fig. 3. The ECL intensity is relatively constant within pH range from 4 to 11, and the behavior of cathodic ECL and TR-ECL appears to be similar. Fluorescein is efficiently excited over the whole pH range of the stability of Al_2O_3 film. The pK_a values of fluorescein have been reported to be 2.2, 4.4, and 6 [33]. Thus, fluorescein exists as a dianion in alkaline solutions and seems to be exited best below pH 10 at oxide-covered aluminum electrodes. It is believed that a wider usable pH range would be obtained at oxide-coated silicon electrodes [3,34].

Effect of Free-Radical Scavengers on the ECL and the Mechanism of the ECL

If the common luminophore excitation routes based on our cathodic pulse polarization method were valid also in the present study, hot or hydrated electrons would be the primary reducing species and hydroxyl radical the primary oxidizing species inducing the present ECL. Therefore, free-radical scavengers should have a significant effect on the present ECL.

The effects of several free-radical scavengers on TR-ECL of fluorescein are shown in Fig. 4. Peroxodisulfate, peroxodiphosphate and hydrogen peroxide are fast hydrated electron scavengers producing strongly oxidizing

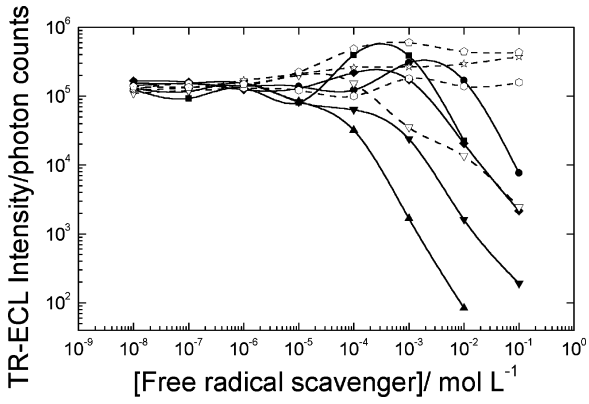

Fig. 4. Effect of several free-sradical scavengers on the TR-ECL intensity of fluorescein. Hydrated electron scavengers: $K_2S_2O_8$ (*solid squares*), H_2O_2 (*solid diamonds*), $K_4P_2O_8$ (*solid circles*), $NaNO_3$ (down triangles), Benzophenon-4-carboxylate (*up triangles*). Hydroxyl radical scavengers: NaCl (*open hexagons*), NaBr (*open stars*), NaI (*open triangles*), NaN_3 (*open pentagons*). Conditions: Fluorescein concentration 1×10^{-6} M, 0.05 M $Na_2B_4O_7$ buffer at pH 9.2 containing 0.1 M Na_2SO_4, pulse time 200 μs, pulse voltage –10 V, pulse frequency 50 Hz, Pine Instrument RD4 potentiostat, 1000 excitation cycles, 515-nm intereference filter, delay time 5.0 μs, gate time 2.00 ms.

radical by one-electron reduction:

$$e_{aq}^- + S_2O_8^{2-} \rightarrow SO_4^{\bullet -} + SO_4^{2-} \quad (1)$$

$$e_{aq}^- + P_2O_8^{4-} \rightarrow PO_4^{\bullet -} + PO_4^{2-} \quad (2)$$

$$e_{aq}^- + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- \quad (3)$$

with second-order reaction rate constants $k_1 = 1.2 \times 10^{10}$ L mol^{-1} s^{-1} and $k_2 = 1.8 \times 10^8$ L mol^{-1} s^{-1} and $k_3 = 1.2 \times 10^{10}$ L mol^{-1} s^{-1}, respectively [23]. Of these oxidizing radicals, sulfate radical seems to have the strongest enhancing effect, while phosphate and hydroxyl radical cannot much enhance the ECL intensity beyond the intensity obtained due to the F^+ centers [1] and to the presence of dissolved molecular oxygen:

$$O_2 + e_{aq}^- \rightarrow O_2^{\bullet -} \quad (4a)$$

$$O_2^{\bullet -} + 2H^+ + e_{aq}^- \rightarrow H_2O_2 \quad (4b)$$

with the second-order rate constants $k_{4a} = 1.9 \times 10^{10}$ L mol^{-1} s^{-1} and $k_{4b} = 1.3 \times 10^{10}$ L mol^{-1} s^{-1}. Oxygen concentration of aerated electrolyte solutions is reported to be about 2×10^{-4} M [35]. When the concentration of these added coreactants is too high, the ECL intensity is strongly deceased due to the spoiling of the balance between the reducing and oxidizing equivalents (Fig. 4).

Nitrate ion and benzophenone-4-carboxylate are fast-hydrated electron scavengers [23] not producing useful secondary radicals. Therefore, they efficiently prevent the ECL generation (Fig. 4).

Azide ion has an equal enhancing effect to that of peroxodisulfate (Fig. 4). It is known to react at near diffusion-controlled rate with hydroxyl radical and to produce azide radical with second-order rate constant 1.2×10^{10} L mol^{-1} s^{-1} [23]:

$$OH^{\bullet} + N_3^- \rightarrow OH^- + N_3^{\bullet} \quad (5)$$

Also bromide at its highest concentration had an enhancing effect. This is due to the much smaller rate constant ($k(OH^{\bullet} + Br^-) = 2.2 \times 10^8$ L mol^{-1} s^{-1} at pH 7, and must even be smaller at the present pH). Chloride ($E^{\circ}(Cl^{\bullet}/Cl^-) = 2.41$ V vs. SHE [36] did not have any effect because at the present pH it is not oxidized by hydroxyl radical [23,37,38]. The formal reduction potential of hydroxyl radical is about 2.3 V (at pH 7), 2.1 V (at pH 9), and 2.0 V vs. SHE (at pH 12) [37]. Iodide had an ECL enhancing effect only at its low concentrations and it quenched TR-ECL strongly at its higher concentrations.

Iodide is also rapidly oxidized by hydroxyl radical ($k_6 = 1.1 \times 10^{10}$ L mol^{-1} s^{-1}):

$$OH^{\bullet} + I^- \rightarrow OH^- + I^{\bullet} \quad (6)$$

The reduction potential of iodine atom is closer to that of azide radical (1.33 V vs. SHE) [36], but iodine atom, unlike azide radical, is rapidly [38] associated with its parent ions to form a series of complexes which are much weaker oxidants:

$$I^{\cdot} + I^- \rightarrow I_2^{\bullet -} \quad (7)$$

$$I^{\bullet} + I^{\bullet} \rightarrow I_2 \quad (8)$$

$$I_2 + I^- \rightarrow I_3^- \quad (9)$$

$I_2^{\bullet -}$ is already a very poor oxidant in the series ($E^{\circ}(I_2^{\bullet -}/I_2) = 0.21$ V vs. SHE [36]). Thus, 1.3 V vs. SHE seems to be sufficient oxidizing power for the oxidant of the present ECL system.

A solvated fluorescein dianion is known to be one-electron oxidized by both $Br_2^{\bullet -}$ ($E^{\circ}(Br_2^{\bullet -}/Br_2) = 1.62$ V vs. SHE [36]) and $N_3^{\bullet}$ at pH 7.4 according to the experiments with pulse radiolysis by Brooke *et al.* [39]. This

means that the formal oxidation potential of solvated fluorescein must be below that of azide ion, i.e., clearly less than 1.33 V vs. SHE [36].

Hydroxyl radical is known to react with fluorescein dianion (Fl^{2-}) at near diffusion-controlled rate ($k = 1.2 \times 10^{10}$ L mol^{-1} s^{-1}, pH = 10). However, the reaction results only partly in one-electron oxidation (10) as hydroxyl radical also forms an adduct radical with fluorescein (11) [33].

$$Fl^{2-} + OH^{\bullet} \rightarrow Fl^{\bullet -} + OH^{-} \quad (10)$$

$$Fl^{2-} + OH^{\bullet} \rightarrow OH - Fl^{2-\bullet} \quad (11)$$

The second step of the ox-red pathway has been observed to be efficiently carried out by hydrated electron [17,18].

$$Fl^{\cdot -} + e^{-}_{aq} \rightarrow Fl^{2-\bullet} \quad (12)$$

Due to the very negative reduction potential of hydrated electron (−2.9 V vs. SHE [23]) this step is easily energy-sufficient even if the formal one-electron oxidation potential of fluorescein would be somewhat on the negative side of the zero point of the SHE scale.

In our case, addition of azide blocks the side reaction (11) and therefore enhances the TR-ECL intensity because azide radical almost exclusively reacts as a one-electron oxidant and does not have tendency for association reactions such as reactions (7)–(9) [37,38].

Fluorescein dianion is also known to be rapidly reduced by hydrated electron ($k_{13} = 1.4 \times 10^{10}$ L mol^{-1} s^{-1}, pH = 9.2) [40]:

$$Fl^{2-} + e^{-}_{aq} \rightarrow Fl^{3\bullet -} \quad (13)$$

Therefore, the red-ox excitation pathway can be thought to be completed by hydroxyl radical:

$$Fl^{3\bullet -} + OH^{\bullet} \rightarrow Fl^{2-\bullet} + OH^{-} \quad (14)$$

The formal one-electron reduction potential of solvated fluorescein at pH 7 has been reported to be of –0.57 V vs. SHE [41]. As mentioned above, the reduction potential of hydroxyl radical at pH 7 is about 2.3 V vs. SHE. Thus, the Gibbs free-energy change of reaction (14) is ca. −2.9 eV and the enthalpy change a bit less negative. According to our fluorescence measurements, the 0–0 transition of fluorescein is at about 2.47 eV, which means that the excitation step (14) is energetically possible. However, when hydroxyl radical is replaced by azide radical in Eq. (14) the free-energy change is only about –1.9 eV which makes the red-ox excitation pathway energy-deficient.

On the other hand, a comproportionation excitation pathway between $Fl^{\bullet -}$ and $Fl^{3\bullet -}$ yields Gibbs free-energy change of only about –1.6 eV (assuming 1.0 V vs. SHE for the one-electron oxidation potential of Fl^{2-}). Thus, it cannot be the source of the present ECL.

In conclusion, we suggest that in the present case fluorescein was excited mainly by ox-red excitation pathway proposed by Pruetz and Land a long time ago in case of RCL [18]. In addition, it is well known that the hydrated electron does not follow Marcus electron transfer theory but is showing Rehm–Weller type of behavior [23]. Thus, it is capable of completing the ox-red excitation pathway by an extremely rapid reduction step (12) although the Gibbs free-energy change in this excitation step is highly negative. On the other hand, the Gibbs free-energy change of reaction (12) in case of hydroxyl, and even more clearly in the case of sulfate radical, is likely to be in the Marcus inverted region because these radicals are likely to follow Marcus electron transfer theory. Therefore excitation reaction (12) cannot be fast and efficient.

Analytical Applicability of ECL

The calibration plots of TR-ECL of fluorescein were measured under several conditions. The best results were obtained in plain borate buffer and especially with azide ion as a coreactant (Fig. 5). About one order of magnitude lower detection limit is obtained in the presence of 0.1 M azide ion. When the enhancing effect of peroxodisulfate or peroxodiphosphate was utilized, the linearity of the calibration plot was lost at low concentrations. The results obtained in the presence of 1 mM peroxodiphosphate are shown as an example in Fig. 5. Thus, the best detectability of fluorescein, and probably also its

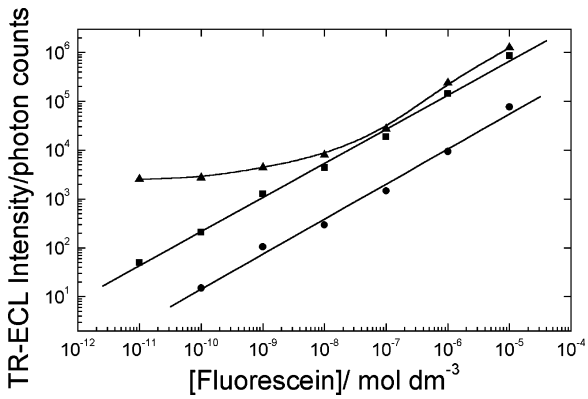

Fig. 5. Calibration plots of fluorescein using disposable oxide-covered aluminum electrodes. TR-ECL in the absence of azide ions (*circles*), TR-ECL in the 0.1 M NaN_3 solution (*squares*), TR-ECL in the presence of 1 mM $K_4P_2O_8$. Conditions: as in Fig. 4 except delay time was 50.0 μs.

derivatives, is achieved using azide ion as a coreactant. When the oxidizing power of azide radical is sufficient, it is an excellent one-electron oxidant with less side reactions than hydroxyl radical tends to have [42] (e.g. addition to aromatic ring, forming $X_2^{\bullet -}$ type of radical complex with the parent ion, etc.).

The analytical performance of the present ECL system allows us to suggest that many commercially available antibodies and oligonucleotides labeled with different derivatives of fluorescein could be detectable on the basis of TR-ECL. We have earlier demonstrated that, e.g., isothiocyanate derivatives of certain aromatic Tb(III) chelates can be efficiently and sensitively used as labels in immunoassays carried out at oxide-covered aluminum electrodes on the basis of TR-ECL detection [1,2,43]. Thus, at least FITC should also be well suited to similar purposes.

CONCLUSIONS

Fluorescein could be sensitively detected on the basis of TR-ECL measurements at oxide-covered aluminum electrodes. The results obtained suggest that the hydrated electrons and hydroxyl radicals are primary species in aerated solutions for the generation of the present ECL. The predominating excitation mechanism seems to be ox-red pathway. In this pathway, cathodically produced oxidizing radicals first one-electron oxidize fluorescein, which is then reduced by hot or hydrated electrons back to its original oxidation state but now in its lowest excited singlet state.

Linear calibration curves of fluorescein spanning several decades of concentration were obtained with detection limits well below nanomolar concentration levels. The presence of azide ions strongly enhances the ECL of fluorescein, and azide ion was found to be the best coreactant for the present ECL system studied in this work. The present method is suggested to be useful in detecting fluorescein-based labels in various types of bioaffinity assays.

REFERENCES

1. S. Kulmala, A. Kulmala, T. Ala-Kleme, and J. Pihlaja (1998). Primary cathodic steps of electrogenerated chemiluminescence of lanthanide(III) chelates at oxide-covered aluminum electrodes in aqueous solution. *Anal. Chim. Acta* **367**(1–3), 17–31.
2. S. Kulmala, M. Hakansson, A.-M. Spehar, A. Nyman, J. Kankare, K. Loikas, T. Ala-Kleme, and J. Eskola (2002). Heterogeneous and homogeneous electrochemiluminoimmunoassays of hTSH at disposable oxide-covered aluminum electrodes, *Anal. Chim. Acta* **458**(2), 271–280.
3. M. Helin, L. Väre, M. Håkansson, P. Canty, H.-P. Hedman, L. Heikkilä, T. Ala-Kleme, J. Kankare, and S. Kulmala (2002). Electrochemiluminoimmunoassay of hTSH at disposable oxide-coated n-silicon electrodes, *J. Electroanal. Chem.* **524/525**, 176–183.
4. P. Canty, L. Väre, M. Håkansson, A.-M. Spehar, D. Papkovsky, T. Ala-Kleme, J. Kankare, and S. Kulmala (2002). Time-resolved electrochemiluminescence of platinum(II) coproporphyrin, *Anal. Chim. Acta* **453**(2), 269–279.
5. S. Kulmala, T. Ala-Kleme, M. Latva, K. Loikas, and H. Takalo (1998). Hot electron-induced electrogenerated chemiluminescence of rare earth(III) chelates at oxide-covered aluminum electrodes, *J. Fluoresc.* **8**(1), 59–65.
6. Q. Jiang, M. Håkansson, A.-M. Spehar, J. Ahonen, T. Ala-Kleme, and S. Kulmala (in press). Hot electron-induced time-resolved electrogenerated chemiluminescence of a europium(III) label in fully aqueous solutions. *Anal. Chim. Acta*
7. J. S. Poole, C. M. Hadad, M. S. Platz, Z. P. Fredin, L. Pickard, E. L. Guerrero, M. Kessler, G. Chowdhury, D. Kotandeniya, and K. S. Gates (2002). Photochemical electron transfer reactions of tirapazamine. *Photochem. Photobiol.* **75**(4), 339–345.
8. S. Kulmala and J. Suomi (2003). Current status of modern analytical luminescence methods. *Anal. Chim. Acta* **500**(1–2), 21–69.
9. K. Tanaka, T. Miura, N. Umezawa, Y. Urano, K. Kikuchi, T. Higuchi, and T. Nagano (2001). Rational design of fluorescein-based fluorescent probes. Mechanism-based design of a maximumfluorescence probe for singlet oxygen. *J. Am. Chem. Soc.* **123**(11), 2530–2536.
10. B. Ou, M. Hampsch-Woodill, and R. L. Prior (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **49**(10), 4619–4626.
11. B. Ou, M. Hampsch-Woodill, J. Flanagan, E. K. Deemer, R. L. Prior, and D. Huang (2002). Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. *J Agric. Food Chem.* **50**(10), 2772–2777.
12. J. Nikokavouras, C. Papadopoulos, A. Perry, and G. Vassilopoulos (1976). Chemiluminescence of a luminol-fluorescein amide. *Chim. Chron.* **5**(3), 223–229.
13. A. Kahn and M. Kasha (1966). Physical theory of chemiluminescence in systems evolving molecular oxygen. *J. Am. Chem. Soc.* **88**(7), 1574–1576.
14. J. Hadjianestis and J. Nikokavouras (1992). Luminol chemiluminescence in micellar media: Energy transfer to fluorescein, *J. Photochem. Photobiol. A* **69**(3), 337–343.
15. M. Voicescu, M. Vasilescu, and A. Meghea (2000). Energy transfer from the aminophtalate dianion to fluorescein. *J. Fluoresc.* **10**(3), 229–236.
16. J. Burguera and A. Townshend (1980). Determination of ng/ml levels of sulphide by a chemiluminescent reaction. *Talanta* **27**(4), 309–314.
17. W. Pruetz, K. Sommermeyer, and E. Land (1966). Light emission after pulse radiolysis of aqueous solution of dyes. *Nature* **212**(5066), 1043–1044.
18. W. Pruetz and E. Land (1967). Phosphorescence of aqueous dye solutions after irradiation with electron pulses. *Biophysik* **3**(4), 349–360.
19. S. Kulmala, T. Ala-Kleme, L. Heikkilä, and L. Väre (1997). Energetic electrochemiluminescence of (9-fluorenyl)methanol induced by injection of hot electrons into aqueous electrolyte solution. *J. Chem. Soc. Faraday Trans.* **93**(17), 3107–3113.
20. T. Ala-Kleme, S. Kulmala, and M. Latva (1997). Generation of free radicals and electrochemiluminescence at pulse-polarized oxide-covered silicon electrodes in aqueous solutions, *Acta. Chem. Scand.* **51**(5), 541–546.
21. S. Kulmala, T. Ala-Kleme, H. Joela, and A. Kulmala (1998). Hot electron injection into aqueous electrolyte solution from thin insulating film-coated electrodes. *J. Radioanal. Nucl. Chem.* **232**(1/2), 91–95.
22. M. Håkansson, Q. Jiang, M. Helin, M. Putkonen, A. J. Niskanen, S. Pahlberg, T. Ala-Kleme, L. Heikkilä, J. Suomi, and

S. Kulmala (2005). Cathodic Tb(III) chelate electrochemiluminescence at oxide-covered magnesium and n-ZnO:Al/MgO composite electrodes, *Electrochim. Acta*. **51**(2), 289–296.
23. G. V. Buxton, C. L. Greenstock, W. P. Helman, and A. B. Ross (1988). Critical Review of rate constants for reactions of hydrated electrons. Chemical Kinetic Data Base for Combustion Chemistry. Part 3: Propane. *J. Phys. Chem. Ref. Data* **17**(2), 513–886.
24. T. Ala-Kleme, S. Kulmala, L. Väre, P. Juhala, and M. Helin (1999). Hot Electron-Induced Electrogenerated Chemiluminescence of $Ru(bpy)_3^{2+}$ Chelate at Oxide-Covered Aluminum Electrodes, *Anal. Chem.* **71**(24), 5538–5543.
25. S. Kulmala, T. Ala-Kleme, A. Kulmala, D. Papkovsky, and K. Loikas (1998). Cathodic electrogenerated chemiluminescence of luminol at disposable oxide-covered aluminum electrodes, *Anal. Chem.* **70**(6), 1112–1118.
26. M. Helin, Q. Jiang, H. Ketamo, M. Håkansson, A.-M. Spehar, S. Kulmala, and T. Ala-Kleme (2005). Electrochemiluminescence of coumarin derivatives induced by injection of hot electrons into aqueous electrolyte solution, *Electrochim. Acta*. **51**(4), 725–730.
27. D. Arnold, E. Cartier, and D. DiMaria (1994). Theory of high-field electron transport and impact ionization in silicon dioxide, *Phys. Rev. B* **49**(15), 10278–10297.
28. D. DiMaria and M. Fischetti (1988). Vacuum emission of hot electrons from silicon dioxide at low temperatures, *J. Appl. Phys.* **64**(9), 4683–4691.
29. D. DiMaria and E. Cartier (1995). Mechanism for stress-induced leakage currents in thin silicon dioxide films, *J. Appl. Phys.* **78**(6), 3883–3894.
30. J. Kankare, K. Fälden, S. Kulmala, and K. Haapakka (1992). Cathodically induced time-resolved lanthanide(III) electroluminescence at stationary aluminum disk electrodes, *Anal. Chim. Acta* **256**(1), 17–28.
31. S. Tajima (1977). Luminescence, breakdown and colouring of anodic oxide films on aluminum, *Electrochim. Acta* **22**(9), 995–1011.
32. A. Despic and V. Parkhutik (1989). In J. Bockris, R. White, and B. Conway (Eds.). *Modern Aspects of Electrochemistry,* Vol. 20, Plenum, New York, pp. 400–503, and the references cited therein.
33. W. A. Pruetz and E. J. Land (1974), Chemiluminescent reactions after pulse radiolysis of aqueous dye solutions. Absolute yields. *J. Phys. Chem.* **78**(13). 1251–1253.
34. Q. Jiang, M. Kotiranta, K. Langel, J. Suomi, M. Håkansson, A.-M. Spehar, T. Ala-Kleme, J. Eskola, and S. Kulmala (2005), Ruthenium(II) tris(2,2′-bipyridine) chelate as a chemiluminophore in extrinsic lyoluminescences of aluminum and magnesium in aqueous solution. *Anal. Chim. Acta* **541**(1–2), 177–184.
35. W. Koppenol and J. Butler (1985). Energetics of interconversion reactions of oxyradicals, *Adv. Free Rad. Biol. Med.* **1**(1), 91–131.
36. D. Stanbury (1989). Reduction potentials involving inorganic free radicals in aqueous solution. *Adv. Inorg. Chem.* **33**, 69–138.
37. W. Koppenol (1987), Thermodynamics of reactions involving oxyradicals and hydrogen peroxide, *Bioelectrochem. Bioenerg* **18**(1–3). 3–11.
38. P. Neta (1976), Application of radiation techniques to the study of organic radicals, *Adv. Phys. Org. Chem.* **12**, 223–297.
39. R. Brooke, R. Bisby, and F. Ismail (2004). Characterisation and quantification of phenolic antioxidants using the "Oxygen Radical Absorbance Capacity" (ORAC) assay for antioxidants. Free Radicals and Excited States in Aqueous and Non-Aqueous Solutions, 27th–29th October 2004, p. 4., CCLRC Daresbury Laboratory.
40. P. Cordier and L. I. Grossweiner (1968). Pulse radiolysis of aqueous fluorescein, *J. Phys. Chem.* **72**(6), 2018–2026.
41. P. S. Rao and E. J. Hayon (1973). Reduction of dyes by free radicals in solution. Correlation between reaction rate constants and redox potentials. *J. Phys. Chem.* **77**(23), 2753–2756.
42. P. Neta, R. E. Huie, and A. B. Ross (1988). Rate constants for reactions of inorganic radicals in aqueous solution. *J. Phys Chem. Ref. Data* **17**(3), 1027–1284.
43. J. Kankare, K. Haapala, S. Kulmala, V. Näntö, J. Eskola, and H. Takalo (1992). Immunoassay by time-resolved electrogenerated luminescence, *Anal. Chim. Acta* **266**(2), 205–212.