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Electrochemiluminescence of coumarin derivatives induced by injection of hot electrons into aqueous electrolyte solution

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Abstract

Hot electrons can be injected from conductor/insulator/electrolyte (C/I/E) junctions into an aqueous electrolyte solution by cathodic pulse-polarization of the electrode. Injected hot electrons induce electrogenerated chemiluminescence of various luminophores including coumarins in fully aqueous solutions. This is based on the tunnel emission of hot electrons into aqueous electrolyte solution, which can result in the generation of hydrated electrons as reducing mediators. These tunnel-emitted electrons allow also the production of highly oxidizing radicals from added precursors. This work shows that coumarin derivatives are suitable candidates as ECL labels for bioaffinity assays or other analytical applications in which detection is based on the ECL of pulse-polarized C/I/E tunnel-emission electrodes in fully aqueous solutions. The mechanisms of the ECL of coumarins are discussed and the analytical applicability of the ECL of three coumarin derivatives is studied.

Keywords: Electrochemiluminescence (ECL); Hot electron; Tunnel junction conductor/insulator/electrolyte-electrodes; Coumarin derivatives

1. Introduction

General reviews on electrogenerated chemiluminescence, i.e., electrochemiluminescence (ECL) are published very frequently and detailed information on the mechanisms and applicability of different ECL systems is easily found from the literature [1–5]. Presently, ECL methods can be divided into following subclasses on the basis of the mechanistic principles: (i) anodic ECL methods normally using $Ru(bpy)_3^{2+}$ labels [1,2] and (ii) the newest available ECL technology, hot electron-induced cathodic ECL [3–5].

Many kinds of luminophores can be excited by cathodic pulse-polarization at conductor/insulator/electrolyte tunnel junction (C/I/E) electrodes, e.g. oxide-covered aluminium, silicon and magnesium electrodes. These thin insulating film-coated electrodes are known to act as cold-cathodes and tunnel-emit hot electrons (e^-_{hot}) into aqueous solutions with quite an analogous manner as solid state devices are known to operate [6–9]. The cathodic pulse-polarization of thin insulating film-coated electrodes, i.e. conductor/insulator/electrolyte-junctions (C/I/E-junction), induces a tunnel emission of hot electrons into the aqueous electrolyte solution as a primary step of ECL. During high amplitude cathodic pulse-polarization of an insulating film-coated electrode in dilute aqueous solutions it is possible that not all of the hot electrons are reacting at the insulator/solution interface (e.g. Al_2O_3, MgO or SiO_2/aqueous electrolyte solution interface) with the solute species. If the energy of tunnel-emitted hot electrons is above the conduction band edge of water it is likely that electrons become hydrated electrons (e^-_{aq}) after thermalization and solvation via pathways known from photoemission and photoionization studies. Therefore, cathodic reductions can probably be produced simultaneously by heterogeneously transferred, presolvated hot and hydrated electrons.

These hot or hydrated electrons can react with compounds that are hard to reduce, and therefore, cathodic reductions

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usually not possible to carry out in aqueous solutions can be made. Also according to previous studies strongly oxidizing species such as sulphate and hydroxyl radicals can be cathodically generated from added coreactants at this kind of C/I/E-electrodes in fully aqueous solutions. Hence highly reducing and oxidizing conditions are simultaneously achieved in the vicinity of the electrode surface by appropriate selection of the concentration of the cathodic coreactant allowing the redox excitation of a wide variety of luminophores [10,11]. In the absence of added coreactants F^+-centres of the oxide film can act as strong one-electron oxidants, as described elsewhere [12–15].

These above mentioned transient species cannot be produced electrochemically in fully aqueous solutions at any active metal electrodes. That is why the ECL of aromatic compounds at conventional active metal electrodes in aqueous electrolyte solutions is generally impossible for energetic reasons [16]. The mechanism of tunnel emission of hot electrons into aqueous electrolytes for different type of electrode materials has been described in detail with schematic energetic diagrams in references [10,11,14,15]. So the special feature of ECL induced by hot electron injection into aqueous solution is that luminophores having very different optical and redox properties can be simultaneously excited and constructionally simple detection system can be used with disposable or non-disposable C/I/E-working electrodes and quite freely selectable counter electrode in many analytical applications.

The present work was carried out to study the mechanism of hot electron-induced ECL of three differently substituted coumarins 7-hydroxy-4-methylcoumarin (HMC), 6,7-dihydroxy-4-methylcoumarin (DHMC) and 7-amino-4-methylcoumarin (AMC) at thin insulating film-coated aluminium electrodes. This contribution will also show that this new promising group of ECL compounds are potential labels for immunoassay applications based on the ECL detection methods. Compounds of coumarins have been known for a long time ago [17] and are nowadays used in the wide fields of biology, medicine, polymer science and industry in general [17]. In the analytical point of view, derivatives of coumarins have been used in various types of immunoassays: as labels for antigens in homogeneous and heterogeneous fluoroimmunoassays [18], as blue emitting luminophores in immunofluorometry and flow cytometry, and as polymeric multi-coumarin labels for immunofluorometric assays. Coumarin derivatives has also been used as fluorogenic substrates for enzyme determinations used in fluorometric enzyme immunoassays and as fluorogenic substrates coupled to antigens in substrate labelled fluorescence immunoassays [19–25].

2. Experimental

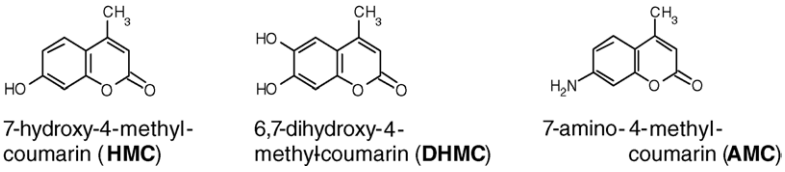
A schematic diagram of the principal components of the apparatus and electrochemical cell used in these ECL measurements has been presented in detail earlier [26]. In a two-electrode cell, the disposable aluminium cup working electrodes (area 2.1 cm^2) were used as cathode electrodes and a thin Pt-wire counter electrode (diameter 0.9 mm, area 2.6 mm^2) was used as anode. Briefly, ECL measurements were conducted using either a coulostatic pulse generator (made in our laboratory) with 120 μC, 20 Hz, −50 V cathodic pulses or a potentiostatic pulse generator (Pine Instruments RD4) with 0.2 ms, −10.0 V, 100 Hz cathodic pulses with a 10 ms intermittent zero level. The ECL intensities of coumarins were detected through interference filters of 420 and 450 nm (transmittance 50–70% and half width about 10 nm) with a photomultiplier tube combined with single photon counting (Stanford Research Systems SR400). ECL spectra were recorded with Perkin-Elmer LS-5 luminescence spectrometer with excitation shutter closed. OriginLab program Origin was used to acquisition of ASCII-data collected from measurements. Also photoluminescence measurements were made with Perkin-Elmer LS-5 spectrofluorometer (quartz cuvettes). Aluminium cup electrodes were made from a nominally 99.9% pure aluminium band (Merck) and were covered with natural about 2–3 nm thick oxide film. All used coumarins HMC, DHMC and AMC were purchased from Aldrich. $Na_2B_4O_7$, $K_2S_2O_8$, $NaNO_3$, $NaNO_2$, NaBr, HCOONa, NaI, NaSCN, H_2SO_4, NaOH, were pro analysi products and Na_2SO_4, NaCl were suprapur products of Merck. H_2O_2, $Co(NH_3)_6^{3+}$, $K_4P_2O_8$ were purchased from J.T. Baker, Ventron and Polysciences Inc., respectively. Boric acid-borate buffer pH 9.2 was used as measuring buffer, because it is known to be unreactive with hydrated electrons and hydroxyl radicals [27]. Quartz distilled water was used for the preparation of all solutions.

3. Results and discussion

3.1. Photoluminescence of coumarin derivatives

The structures of the three studied coumarins are presented in the Scheme 1.

The photoluminescence excitation and emission spectra of HMC, DHMC and AMC (Fig. 1) show that the excitation and emission maxima of these three coumarins are in a same green-blue area, the intensity of fluorescence of HMC at wavelength of 442 nm being the strongest, DHMC at wavelength of 451 nm being the next strongest and AMC at wavelength of 439 nm being the weakest. All three studied coumarins are substituted with donor groups of –OH or $–NH_2$. This donor group in the coumarin molecule is known to be responsible for charge-transfer nature of the emissive excited state and causing the observed relatively large Stokes' shifts between absorption and emission maxima. It has been reported that the substitutions of the coumarin structure shifts the fluorescence band, for example adding a methyl group to the 4-position of 7-hydroxy- or 7-methoxycoumarin red shifts, i.e. shifts to the longer wavelengths. The addition of electron-repelling groups in the 4-, 6- or 7-position or



Scheme 1. The structures of coumarins used in ECL measurement.

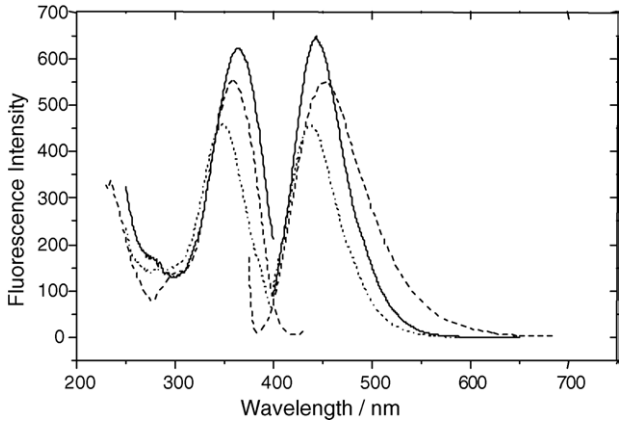


Fig. 1. Fluorescence excitation and emission spectra of HMC (solid line; λ_{ex} = 365, λ_{em} = 442), DHMC (dashed line; λ_{ex} = 356, λ_{em} = 451) and AMC (dotted line; λ_{ex} = 348, λ_{em} = 439). Conditions: Spectrofluorometer Perkin-Elmer LS-5 excitation and emission slits 15 and 10 nm, respectively, scan speed 120 nm/min. Aqueous solutions of 1.0×10^{-7} M coumarins in 0.2 M boric acid-borate buffer pH 9.2.

electron-attracting groups in the 3-position has been reported to shift the fluorescence band to longer wavelengths [28,29]. The same effect was confirmed by our fluorescence measurements (Fig. 1).

3.2. Electrochemiluminescence of coumarin derivatives

The general scheme of tunnel emission and Fowler-Nordheim tunnelling of hot electrons into aqueous electrolyte solutions has been described in detail elsewhere [11]. Briefly, the cathodic reductions at C/I/E-junctions could be due to direct action of hot dry electrons ($e^-_{quasifree}$), e^-_{aq}, or heterogeneously transferred electrons from the bottom of the insulator conduction band or somewhere above it ($e^-_{CB\ of\ the\ insulator}$) and less energetic electrons via the surface states ($e^-_{SS\ of\ the\ insulator}$) (reactions (1)–(3)).

$$e^-_{hot}\,(\text{electrode}) \rightarrow e^-_{quasifree}\,(\text{in the conduction band of water}) \quad (1)$$

$$e^-_{quasifree} \rightarrow e^-_{aq} \quad (2)$$

$$e^-_{hot}\,(\text{electrode}) \rightarrow e^-_{CB\ or\ SS\ of\ the\ insulator} \quad (3)$$

However, strong ECL of luminophores at C/I/E-junctions has been observed only in the direct tunnel emission regime [11] (insulating oxide film thickness between 2 and 6 nm), which implies that $e^-_{quasifree}$ or e^-_{aq} have an important role in the ECL pathways. Under air-saturated solutions, and due to oxygen evolution at the counter electrode, oxyradicals and hydrogen peroxide are formed, if hydrated electrons are produced at the working electrode.

$$O_2 + e^-_{aq} \rightarrow O_2^{\bullet -} \quad (4)$$

$$O_2^{\bullet -} + e^-_{aq} + 2H_2O \rightarrow H_2O_2 + 2OH^- \quad (5)$$

$$H_2O_2 + e^-_{aq} \rightarrow {}^{\bullet}OH + OH^- \quad (6)$$

The second-order rate constants of the above mentioned reactions are $k_4 = 1.9 \times 10^{10}$ L mol^{-1} s^{-1}, $k_5 = 1.3 \times 10^{10}$ L mol^{-1} s^{-1}, and $k_6 = 1.2 \times 10^{10}$ L mol^{-1} s^{-1}, respectively [27]. The oxygen concentration of air-saturated electrolyte solutions is about 2×10^{-4} M [30]. If coreactants such as hydrogen peroxide (6) or peroxydisulphate ions (7) are added, strongly oxidizing radicals are directly cathodically generated, $k_7 = 1.2 \times 10^{10}$ L mol^{-1} s^{-1} [27]:

$$e^-_{aq} + S_2O_8^{2-} \rightarrow SO_4^{\bullet -} + SO_4^{2-} \quad (7)$$

Peroxydisulphate ion produces by one-electron reduction a strongly oxidizing sulphate radical (7), which can rapidly oxidize aromatic compounds even benzene [31]. The simultaneous presence of hydrated electrons ($E^o = -2.9$ V) [27] and sulphate radicals ($E^o = 3.4$ V) [32] is one of the harshest conditions in aqueous solution that can exist, capable of generating chemiluminescence from a wide variety of compounds, including coumarins, that otherwise are hard to reduce or hard to oxidize [33–35].

In all cases, the ECL of coumarins was measured during the electrical cathodic excitation pulse because they show strong singlet state emission and the time-resolved delayed long-lived ECL luminescence from triplet states was so short that it has no analytical meaning. Fig. 2 displays the uncorrected ECL spectra of HMC, DHMC, AMC and background as present of peroxodisulphate. As it can be seen all the investigated coumarins emits ECL at same 450 nm wavelength, which is also the same as the photoemission wavelength of DHMC. AMC was noticed to be very poor electrochemiluminophore, in comparison to HMC and DHMC, which two were used as model coumarins in the mechanisms studies.

As already above mentioned, certain radical scavengers have a strong effect on the ECL of studied coumarins. With the help of the effect of these hydrated electron scavengers ($Co(NH_3)_6^{3+}$, H_2O_2, $S_2O_8^{2-}$, $P_2O_8^{4-}$, NO_2^-, NO_3^-, benzophenon-4-carboxylate) and sulphate radical scavengers (N_3^-, Br^-, Cl^-, SCN^-, $HCOO^-$, I^-, CH_3CH_2OH) it is also possible to get valuable information of the reaction

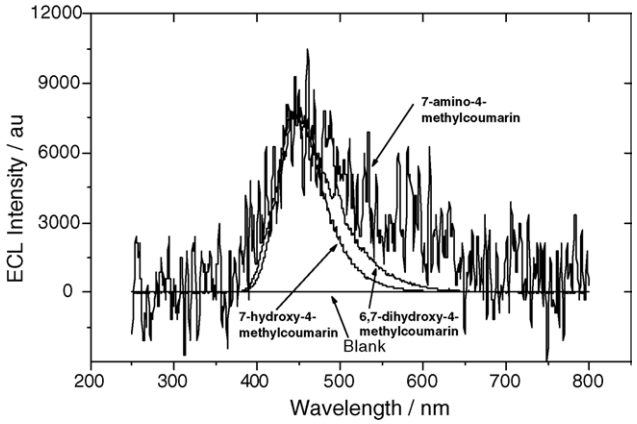


Fig. 2. Uncorrected ECL spectra of coumarins at the oxide-covered aluminium electrode. Spectra are normalized and thus the signal measured from 1.0×10^{-5} M solutions of HMC, DHMC, AMC and background (blank, measuring buffer) multiplied by factor 1.0, 8.0, 600 and 1.0, respectively. Conditions: 0.2 M boric acid-borate buffer pH 9.2, 1×10^{-3} M $S_2O_8^{2-}$, Perkin-Elmer LS-5 spectrofluorometer excitation slit closed and emission slit 20 nm, scan speed 240 nm/min, coulostatic pulse generator excitation pulse charge 120 μC, frequency 80 Hz and voltage −50 V.

mechanisms of the examined ECL of coumarins. Fig. 3 shows the effect of the hydrated electron and Fig. 4 the effect of the sulphate radical scavengers on the ECL of HMC. The adding of special coreactants, free radical scavengers especially peroxodisulphate $S_2O_8^{2-}$ and azide N_3^-, to the solution enhanced the ECL intensity of the examined coumarins about six times. This enhancement is important in the view of analytical applications.

On the basis of these scavenger measurements (Figs. 3 and 4), it can be said that peroxodisulphate was a strong ECL enhancer with all coumarins in the range of 10^{-5}–10^{-3} M of scavenger concentration. When the concentration of peroxodisulphate exceeds the limit of

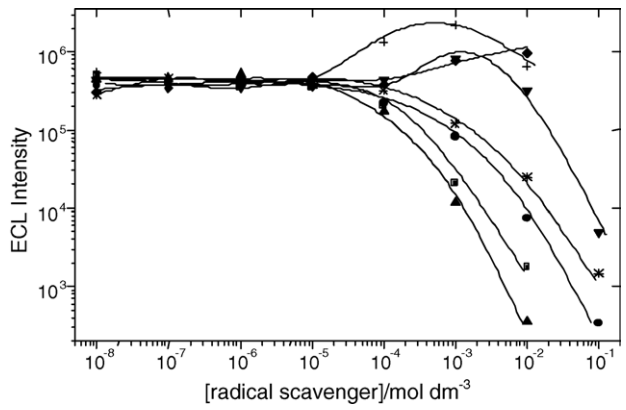


Fig. 3. Effect of hydrated electron scavengers on the ECL of HMC (1.0×10^{-7} M) at the oxide-covered aluminium electrode. Conditions: 0.2 M boric acid-borate buffer pH 9.2, Potentiostatic pulse generator with 0.2 ms, −10 V and 100 Hz cathodic excitation pulses. Interference filter 450 nm and presented ECL signals integrated as a sum of 1000 excitation pulses. $S_2O_8^{2-}$ (+), H_2O_2 (▼), $P_2O_8^{4-}$ (♦), NO_2^- (*), NO_3^- (●), $Co(NH_3)_6^{3+}$ (■), benzophenon-4-carboxylate (▲).

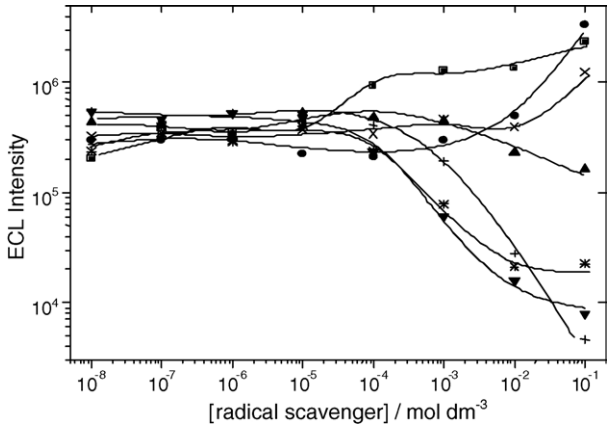


Fig. 4. Effect of sulphate radical scavengers on the ECL of HMC (1.0×10^{-7} M) at the oxide-covered aluminium electrode. Conditions: as in Fig. 3. Br^- (●), Cl^- (×), SCN^- (▲), $HCOO^-$ (*), I^- (▼), CH_3CH_2OH (+), NaN_3 (■).

5×10^{-3} M, the ECL intensity starts to fall down, for the reason for that the production of oxidizing radicals is so effective that they use all reducing radicals from the aqueous solution and the ECL is quenched. The enhancing effect of peroxodiphosphate and hydrogenperoxide was clearly smaller. The most effective luminescence quenchers like $Co(NH_3)_6^{3+}$, benzophenon-4-carboxylate, nitrite and nitrate was noticed to quench the ECL intensity in the order of the reaction rate constant with hydrated electron (Fig. 3). This is again one fact that emphasizes the vital importance of the existence of hot and hydrated electrons in the ECL process at C/I/E-tunnel junction electrodes in aqueous solutions [10].

In the same manner also the effects of the sulphate radical scavengers (Fig. 4) were quite similar for all three coumarin derivatives. Azide enhances the ECL intensity of luminophores as a function of its concentration because the azide radical $N_3^{\bullet -}$ is almost purely a one-electron oxidant and it has a very weak tendency to the association reactions. The enhancing effect of the azide is greater for the reason that it does not enhance the blank background signal like other enhancers usually do, e.g. the often used peroxodisulphate.

Ethanol, formiate and iodide, which are capable of producing reductive secondary radicals, are the most effective ECL quenchers from the group of sulphate radical scavengers. The halide and pseudohalide ions are unreactive with hydrated electrons and produce oxidising radicals in a reaction with sulphate radicals. These ions were observed to quench the ECL more strongly the faster the scavenging reaction and the weaker the secondary radicals produced was as an oxidant. Iodide is the most effective ECL quencher of these halides and pseudohalides because it reacts fastest with the sulphate radical and the formed secondary radical $I_2^{\bullet -}$ is the weakest oxidising agent.

The effect of chloride and bromide to the ECL intensity is based on the fact that the redox potential of hydroxyl radical is 2.2 V versus SHE (pH 9.0) and it cannot oxidise chloride ion (E^o (Cl/Cl^-) = 2.4 V versus SHE). This causes

that chloride has no the special effect to ECL of coumarins. Instead bromide (E^o (Br/Br^-) = 1.9 V versus SHE) is possible to be oxidised by hydroxyl radical and this effect could be even observed with the enhancement of coumarins ECL intensity as the function of bromide concentration. Bromide atom formed as a result of the bromide ion becoming oxidised is more effective oxidant than hydroxyl radical. When the bromide concentration is high, the primary oxidising product is dibromide radical ion ($Br_2^{\bullet -}$) that is weaker oxidant than hydroxyl radical and in this case the ECL intensity is not increased. The decreasing effect of thiocyanate ion is weaker than the quenching effect of iodide, because the reaction velocity of the reaction between thiocyanate and sulphate radical is half of that of iodide and sulphate radical and also because the radical generated from thiocyanate is stronger oxidant radical than the radical formed from iodide.

The most obvious ECL excitation routes for coumarins are the luminophore-reduction-initiated oxidative-excitation pathway (red-ox pathway) and the luminophore-oxidation-initiated reductive-excitation pathway (ox-red pathway) (reactions (8)–(12), where HMC is used as model coumarin and Ox(is someone of the strong oxidants, e.g. sulphate or hydroxyl radical).

$$HMC + e_{aq}^- \text{ (or } e_{hot}^-) \rightarrow HMC^{\bullet -} \quad (8)$$

$$HMC^{\bullet -} + Ox^{\bullet -} \rightarrow HMC^* + Ox^- \quad (9)$$

$$HMC + Ox^{\bullet -} \rightarrow HMC^{\bullet +} + Ox^- \quad (10)$$

$$HMC^{\bullet +} + e_{aq}^- \text{ (or } e_{hot}^-) \rightarrow HMC^* \quad (11)$$

$$HMC^* \rightarrow HMC + h\nu\,(450\,\text{nm}) \quad (12)$$

It is impossible to say which pathway is exactly predominant, until the rate constants of coumarins are unknown because it is necessary to take into account the stability of the radical ions of coumarin derivatives together with thermodynamic and kinetic issues. Anyway it seems that the ox-red pathway (reactions (10)–(12)) could be the usual reaction rate, because of the strong oxidants like F-centre, hydroxyl and sulphate radicals formed in cathodically pulse-polarized thin insulating oxide film-covered aluminium electrodes are capable of being oxidants strong enough to oxidize these coumarins as their cation radicals, which further can react with hydrated electrons to form the coumarin molecule as in its excited state.

First, we interpreted the ECL spectra measurements so that the final ECL emitting molecule should be the same in all cases and that particularly DHMC molecule would be the active emitter in all performed ECL experiments. Consequently, HMC and AMC molecules would be converted to DHMC during electrochemical excitation. However, this hypothetical assumption, that AMC^+ and HMC^+ radical cations would undergo nucleophilic substitution with recovering of DHMC, needs further studies which will be carried out later as comparative studies at oxide-coated n-Si and magnesium electrodes with the present measurements. However, the weak ECL signal of DHMC and especially

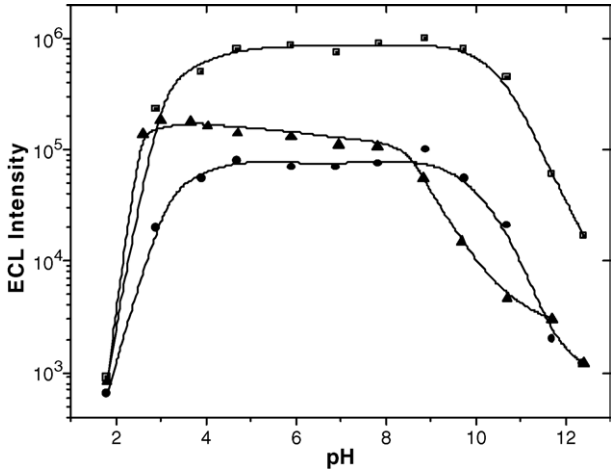


Fig. 5. ECL intensity of HMC (■), DHMC (●), AMC (▲) as a function of solution initial pH at the oxide-covered aluminium electrode. Conditions: 1.0×10^{-6} M coumarins in 0.1 M Na_2SO_4 supporting electrolyte (solution was adjusted to the desired pH with 0.1 M H_2SO_4 or NaOH). Potentiostatic pulse generator with 0.2 ms, −10 V and 100 Hz cathodic excitation pulses. Interference filter 450 nm and presented ECL signals integrated as a sum of 1000 excitation pulses.

of AMC and the use of Perkin-Elmer LS5 for ECL spectra measurements (it is only designed to be useful for PL measurements) produces quite much uncertainity to the emission maxima results. Thus, it can be equally well assumed that in each cases the ECL and fluorescence emission spectra were actually similar (Figs. 1 and 2) and therefore in all cases the emissive species was most probably the original coumarin compound emitting its singlet state emission.

In the literature, it has been shown that the solvent or the solution pH can affect the fluorescence spectra of coumarins and the fluorescence intensity of coumarins being strongly dependent on the pH. As a rule of thumb, it seems that increasing of the solution pH raised the fluorescence intensity [36]. The pH dependence of the group of coumarins in the ECL system was measured using Na_2SO_4 as an inert electrolyte (Na_2SO_4 was separately tested and noticed to be usable in the whole pH area used) in the solutions and adjusting the appropriate pH with the help of 0.1 M H_2SO_4 and 0.1 M NaOH (Fig. 5). In the case of HMC and DHMC, the ECL intensity as the function of pH is at similar level in the pH region 4–10 and drops dramatically outside of this pH region. The pH dependence of the third studied coumarin derivative AMC was noticed to be a little more complicated. It seems to have a small ECL intensity maximum at the pH 3–4 after which the intensity slowly crawls down about quarter of decade until after the pH of 8.5 falls rapidly down. The reason for this quite unordinary behaviour comparing for the others might be the effect due to the amino group in the structure of the molecule. The ECL intensity of AMC would be a little higher (about quarter of the decade) if the buffer pH used would be between 3 and 8.5 but anyway all measurements were made in borate buffer pH 9.2 for the reason that all the results would be comparable to each other owing to the measurement conditions.

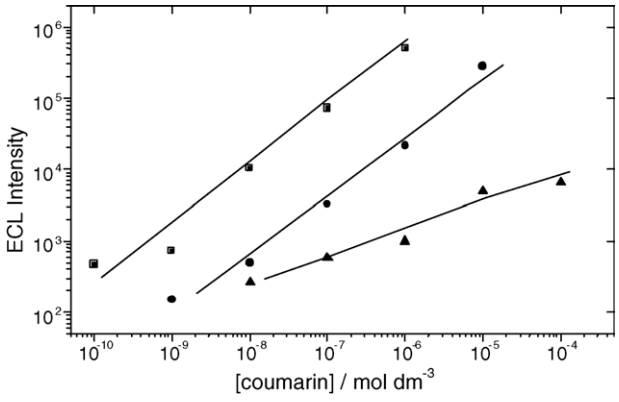


Fig. 6. ECL calibration curves of HMC (■), DHMC (●) and AMC (▲) at the oxide-covered aluminium electrode. Conditions: 0.2 M boric acid-borate buffer pH 9.2, 1×10^{-3} M $S_2O_8^{2-}$. Potentiostatic pulse generator with 0.2 ms, −10 V and 100 Hz cathodic excitation pulses. Interference filter 450 nm and presented ECL signals integrated as a sum of 1000 excitation pulses.

The other reason for use of the borate buffer was that it is known to be unreactive towards e_{aq}^- and hydroxyl radicals, as well as towards the sulphate radicals and dichlorine radical ions [27].

The calibration graphs of these three coumarins were all observed to cover over four orders of magnitude of concentration and the lowest detection limit for HMC (strongest ECL signal) must be close to 10^{-10} M (Fig. 6). The linear slopes for HMC and DHMC were close to unity, but the slope of AMC (about half) differs totally from the others, which might be due to undergoing nucleophilic substitution of AMC^+ radical cation or due to a different kind of side reaction and really would need more detailed studies.

4. Conclusions

Coumarins show strong singlet state ECL emission but unfortunately the long-lasting triplet state emission is extremely weak and has no analytical applicabilty. Therefore, the time-resolved ECL detection method is not applicable and, thus, coumarins do not belong to the group of the most sensitive ECL luminophores. Among the coumarins studied so far, HMC has the highest ECL yield and the detection limit is approximately 10^{-10} M under appropriate conditions. Thus, it can be said that some coumarin derivatives can be regarded as potential ECL labels for bioaffinity assays.

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