Food Chemistry 366 (2022) 130590

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Paper-based electrochemiluminescence device for the rapid estimation of trimethylamine in fish via the quenching effect of thioglycolic acid-capped cadmium selenide quantum dots

Nisachon Praoboon [a], Suphawuth Siriket [a], Narin Taokaenchan [b], Surasak Kuimalee [c], Sirirat Phaisansuthichol [a], Pusit Pookmanee [a], Sakchai Satienperakul [a,*]

[a] Department of Chemistry, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand
[b] Faculty of Agricultural Production, Maejo University, Chiang Mai 50290, Thailand
[c] Department of Industrial Chemistry and Textile Chemistry, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand

ARTICLE INFO

Keywords:
Microfluidic paper-based analytical device
Electrochemiluminescence
Trimethylamine
TGA–capped CdSe quantum dots

ABSTRACT

A paper-based electrochemiluminescence device (μPAD-ECL) for the estimation of trimethylamine (TMA) concentration in fish was developed using tris(2,2'-bipyridyl)ruthenium(II) complex coupled with water soluble thioglycolic acid–capped CdSe quantum dots on the inkjet-printed paper-based device. The quenching effect of tertiary amines on the ECL intensity was found to be sensitive and concentration dependent. This effect allows the measurement of TMA at low concentrations. Under the optimal conditions, the linear concentration range was exhibited from 1×10^{-12} to 1×10^{-7} M and a detection limit of 2.09×10^{-13} M, with relative standard deviation of 1.97 %. The applicability of μPAD-ECL is demonstrated by the rapid estimation of trimethylamine concentration in fish tissue, and could be used as a method for screening the total amount of tertiary amines in fishery products in remote communities. The results obtained using the paper-based devices agreed well with those obtained applying high performance liquid chromatography with benzoyl derivatization, at a confidence level of 95%.

1. Introduction

Odour is one of the conditions used to assess fish freshness and considered as a major benefaction to the quality of fish (Olafsdóttir et al., 1997). Tertiary amines are based on the volatile compounds contributing to fish odour, and the measurement of volatile amines can indicate the freshness or spoilage level of fish, thus assess and predict their quality (Malle & Tao, 1987). The microbial decomposition process produces trimethylamine (TMA) from trimethylamine *N*-oxide (TMAO) in spoilage of most fish. The microbial growth and the spoilage of fish related to TMA formation with the fish quality deterioration depend on fish species, temperature and time of storage. Therefore, the growth of TMA content due to freshness reduction becomes an important standard and an indicator of freshness of fish products during transportation and storage. Several instrumentation methods for the analysis of trimethylamine have been developed for the evaluation of fish freshness such as gas chromatography (GC) (Fiori, Turroni, Candela, Brigidi & Gotti, 2018), liquid chromatography (LC) (Monser & Greenway, 1996), high-performance liquid chromatography (HPLC) (Anderson, 2008), flow injection analysis (FIA) with photometric detection (Sadok, Uglow & Haswell, 1996; García-Garrido & Luque de Castro, 1997), chemiluminescence detection (Cobo, Silva & Pérez-Bendito, 1997) or capillary electrophoresis (CE) (Li & Lee, 2007). The modern instruments come with a high selectivity, high sensitivity, suitability for TMA determination, and are commercially available for most of the laboratories. However, the complicated and expensive equipment barricaded these methods from end-users in developing or developed countries, due to the tedious sample preparations and fairly well-trained scientists that are often required for the operation.

The development of simple, inexpensive and affordable sensors for quantitative determination of TMA plays an important role in the early evolution of fish freshness evaluation. Among these techniques, electrochemiluminescence or electrogenerated chemiluminescence (ECL) detection has a great potential because of its high performance (Sojic, 2020; Hao & Wang, 2016). ECL is a phenomenon of light emission from an energetic electron transfer reaction of electrogenerated species at an

* Corresponding author.
E-mail address: sakchais@mju.ac.th (S. Satienperakul).

https://doi.org/10.1016/j.foodchem.2021.130590
Received 21 September 2020; Received in revised form 30 May 2021; Accepted 11 July 2021
Available online 14 July 2021

electrode surface. The combination of electrochemistry and chemiluminescence have associated benefits such as selectivity, ease of use, wide dynamic range, rapidity, high sensitivity and low background noise (Tang, Li, Guo & Nie, 2019; Li et al., 2019; Li, Han, Hu & Xu, 2009; Zhao, Thuo & Liu, 2013). Moreover, ECL can miniaturize the size of the electrode without affecting the sensitivity and selectivity. The electrode can then be considered as a "green" analytical method when combined with a microfluidic technique since the fabrication of the disposable sensor is aimed for a mass-manufacture at low-cost, easy to use, low consumption of reagent and minimum waste release.

The microfluidic paper-based analytical device (μPAD) has widely evolved as an analytical tool because it is inexpensive, portable and simple to operate (Martinez, Phillips, Whitesides & Carrilho, 2010; Xia, Si & Li, 2016, Liu, Zhang & Liu, 2015). In 2011, the μPAD was successfully applied with ECL for the determination of 2-(dibutylamino)-ethanol (DBAE) and nicotinamide adenine dinucleotide (NADH) (Delaney, Hogan, Tian & Shen, 2011). The μPAD, consisting of a network of hydrophilic channel from printing alkenyl ketene dimer, was used as cellulose hydrophilization agent. Using a commercial digital inject printer and a hydrophobic boundary that acts as a detection zone separating in micro-scale, the μPAD is able to transport fluids via capillary action without external pump (Dou et al., 2015; Rossini et al. 2018; Almeida, Jayawardane, Kolev & McKelvie, 2018). In addition, their lightweight, end-users friendly, disposability, and biodegradability characteristics make the μPAD a desirable analytical device which leaves low harm to the environment (Nechaeva, Shishov, Ermakov, & Bulatov, 2018; Akyazi, Basabe-Desmonts & Benito-Lopez, 2018).

Since the ECL is generated by redox reactions, with suitable reaction conditions and certain reagents, the chemiluminescence light occurs from intermediate radicals in the relaxation process. The ECL can be replaced by direct oxidation of goal molecules which would establish the enhancement or inhibitory effect of ECL signal. However, the electrochemical cell resistance may occur in μPAD due to the resistance from the paper fiber. Hence, the signal amplification is essential to enhance the ECL emission for paper-based analytical applications by using sensitizers or reaction strategies, such as nanomaterial transition-elements, catalytic processes and resonance energy transfer processes with high quantum efficiency. Quantum dots (QDs) are a type of luminescent semiconductor nanomaterial, and some investigations have illustrated that the use of quantum dots in fluorescence-visualized paper-based sensors has provided new avenue to enhance the inherent sensitivity of the paper-based sensitizing platform for food and agricultural applications (Chen, et al., 2020; Wang, et al., 2019). However, the enhancing effect of QDs on ECL-μPAD systems has not been reported.

In this paper, water soluble thioglycolic acid (TGA)-capped CdSe QDs was synthesized by a facile colloid aqueous phase route. The improvement of the tris(2,2'-bipyridyl)ruthenium(II) electrochemiluminescence reaction via the microfluidic paper-based analytical device was presented using water soluble thioglycolic acid (TGA)-capped CdSe QDs for the determination of the tertiary amines total values in freshwater and ocean fish samples via an ECL quenching effect. The fabrication of a low cost, disposable, low chemical consumption and easy to use μPAD-ECL was proposed. By using a conventional photodetector principle, the device can be favorably used as a new sensing platform for a rapid on-site detection of TMA to estimate the degree of freshness of the product in fishery industries, and food safety monitoring during the storage or transportation from remote areas.

2. Experimental

2.1. Chemicals and equipment

All the chemicals are analytical reagent (AR) grade. The experiments were prepared in purified water using a compact ultrapure water system (18.2 MΩ, Milli-pore, France). The tris (2,2′-bipyridyl) ruthenium (II) chloride ($Ru(bpy)_3Cl_2$) was synthesized in our laboratory by a modification of the method reported by Broomhead (Broomhead, Young & Hood, 2007). Selenium powder, 2,2′-bipyridyl, ruthenium (III) chloride, sodium borohydride ($NaBH_4$), cadmium chloride, thioglycolic acid were purchased from Sigma-Aldrich (USA). Tertiary amines, including trimethylamine (TMA), triethylamine (TEA) and trimethylamine *N*-oxide were purchased from Sigma-Aldrich (USA) and N,N,N'-trimethyl ethylene diamine (N,N,N'-TMEDA), N,N,N',N'-tetramethyl ethylene diamine (N,N,N',N'-TMEDA) were purchased from Fluka (UK). Biogenic amines, such as histamine, putrescine and cadaverine were purchased from Sigma-Aldrich (USA). Tertiary and biogenic amines standard solutions were prepared in 0.05 M phosphate buffer solution pH 7.5 and stored at 4 °C. The heavy metal standard solutions used for the selectivity studies were prepared by the appropriate dilution of certified stock solutions of 1000 $mg{\cdot}L^{-1}$ of each heavy metal (Pb(II), Ni(II), Cu(II) and Zn(II)) purchased from Merck (Germany). Ammonia, chloroform and ethanol purchased from Fluka (UK) were also used for interference studies.

The ECL measurements were performed via a cyclic voltammetric (CV) technique on the Autolab PGSTAT101 (Autolab, Switzerland). A standard three-electrode configuration was employed, together with a carbon screen printed electrode (Dropsens, Spain) as the working electrode. The ECL signal was monitored in a sealed black box, the paper based μPAD was put flush against a red-sensitive PMT (Thorn-EMI 9828 SB, Electron Tubes Ltd., UK). The operational potential for the PMT was provided by a high voltage power supply (Thorn-EMI model PM 20, Electron Tubes Ltd., UK) at a constant voltage of 850 V. The output of the PMT, proportional to the ECL intensity, was monitored continuously and displayed on a personal computer via a digital multimeter USB/RS-232 (UT60F, Hong Kong) interfaced with the voltage divider (C637BFN2, Electron Tubes, UK). UNI-Ts UT60F AC/DC software was used to determine the maximum peak.

2.2. Synthesis of TGA-capped CdSe nanoparticles

The synthesis of water-soluble CdSe-QDs followed a procedure developed by Li et al. (2016) with some modifications. Briefly, 0.0496 g of $CdCl_2$ 2.5 H_2O was dissolved in 50 mL of ultrapure deionized water in a three-necked round bottom flask. Then, 37.5 μL of thioglycolic acid (TAG), used as stabilizer agent, was injected in the solution and adjusted to a pH of 10 using 2 M NaOH. After the mixture solution was bubbled with pure N_2 gas for 30 min, 2 mL of 50 mM NaHSe (0.0100 g Se powder mixed with 3 mL aqueous solution of $NaBH_4$) was carefully added to the mixture to obtain a clear yellow solution. The solution mixture was refluxed for 8 h at 100 °C until a clear orange solution was formed. The TGA-capped CdSe QDs solution was kept refrigerated at 4 °C.

The size and morphology of the TGA-capped CdSe-QDs were characterized by a JOEL 2100 transmission electron microscope (TEM) (JEOL Ltd., Japan), operated at 200 keV. Bright field TEM (BF-TEM) images and selected area electron diffraction pattern (SADP) were used for morphology and crystallinity identification. The CdSe-QDs were dispersed in ethanol using ultrasonic vibration for approximately 5 min. The mixture was dropped onto a 200-mesh copper grid coated with continuous carbon films and allowed to dry at room temperature before TEM evaluation.

In the meantime, the fluorescence spectra of TGA–capped CdSe-QDs was analyzed using a luminescence spectrometer LS50B (PerkinElmer Corporation, USA). The TGA–capped CdSe-QDs solution, at an appropriate dilution, was put in a quartz fluorescence cuvette with 10-mm optical path length, while the excitation and emission slits were set at 5 nm. The emission spectra were observed when the excited wavelength was set at 270 nm and recorded over the wavelength range of 200–800 nm.

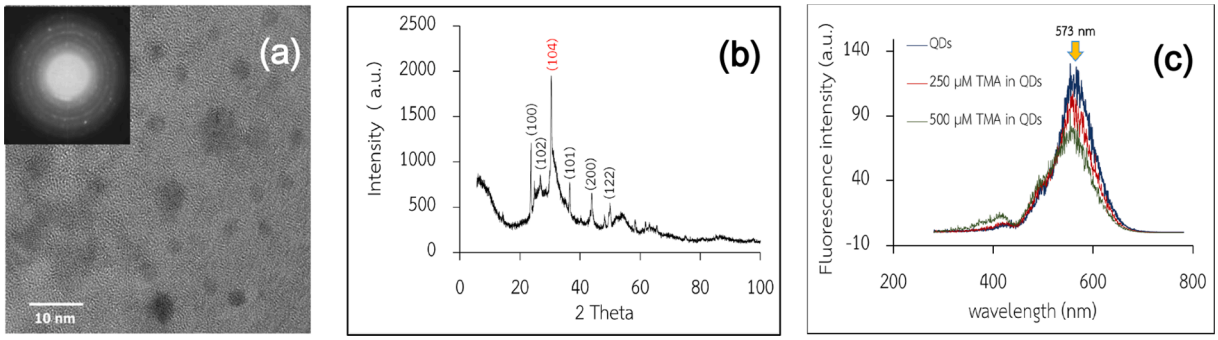

Fig. 1. (a) Bright-field -TEM image of the TGA-capped CdSe-QDs indicated in dark particles. The inset shows the selected area diffraction pattern (SADP) corresponding to the polycrystalline structure and (b) XRD pattern of TGA-capped CdSe-QDs. (c) The emission spectra of thioglycolic acid (TGA)–capped CdSe quantum dots (TGA-capped CdSe-QDs) at different concentrations of trimethylamine (TMA) (excitation at 270 nm).

2.3. Fabrication of microfluidic paper-based devices for electrochemiluminescence sensors

The preparation of the microfluidic paper-based analytical devices, µPAD, (Fig. S1) were similar to Delaney et al. (2011). The spoon shape of the hydrophobic barrier on paper was designed using Adobe illustrator. The channel was 1.5 cm long and 0.7 cm wide, and the detection zone had a diameter of 0.5 cm. The alkenyl ketene dimer (AKD) was dissolved in analytical grade *n*-heptane, representing the cellulose hydrophobilizing agent. The AKD-heptane solution was put into a black cartridge in a commercial digital inkjet printer (Canon ip 2770, Japan). The Whatman chromatography paper (No.4) was cut into an A4-size to be used as paper substrate. The microfluidic patterns were printed onto the A4-size chromatography paper. This printed chromatography paper was then heated in an oven at 100 °C for 6 min to cure AKD into the cellulose fibers and to create a hydrophobic barrier. The printed microfluidics paper was then loaded with 12 µL of 10 mM $Ru(bpy)_3^{2+}$ or $Ru(bpy)_3^{2+}$/TGA-capped CdSe-QDs solutions and left to air-dry. The printed microfluidic paper was cut to a size of 1.0 × 3.0 cm and each piece was placed onto a carbon screen printed electrode (SPE) where they were laminated with transparent cellophane tape (shown in Fig. S1a) and was ready for ECL testing.

2.4. Operation and assay procedure of the electrochemiluminescence sensor

A piece of µPAD was loaded with the mixed solution of $Ru(bpy)_3^{2+}$/TGA–capped CdSe-QDs sensitizer follow by either tertiary amine (e.g. TMA, TEA, N,N,N'-TMEDA and N,N,N',N'-TMEDA) on a little incision at the base of µPAD (shown in Fig. S1b). The fully wetted detection zone was then put in contact with the carbon SPE. For optimal reproducibility, a paper clip was used to apply pressure and keep the aperture of µPAD in even contact with the screen-printed electrode during the ECL measurement. The ECL-µPAD was placed close to the window of a photomultiplier tube (PMT) where the ECL reaction was initiated by scanning or stepping the potential to a value more positive than the oxidation potential of the tris(2,2'-bipyridyl)ruthenium(II) complex. The applied potential was controlled in the range of 0.0 to 1.6 V by a scan rate of 0.20 $V.s^{-1}$. The output of the PMT, which was proportional to the ECL intensity, was monitored continuously. The quenched ECL intensity of the $Ru(bpy)_3^{2+}$/CdSe-QDs system corresponding to the peak heights was plotted versus various concentration of each compound, where $\Delta I = I_0 - I_{CL}$; I_0 is the background ECL intensity of the $Ru(bpy)_3^{2+}$/CdSe-QDs system in the absence of TMA ; and I_{CL} is the intensity in the presence of a TMA standard or sample.

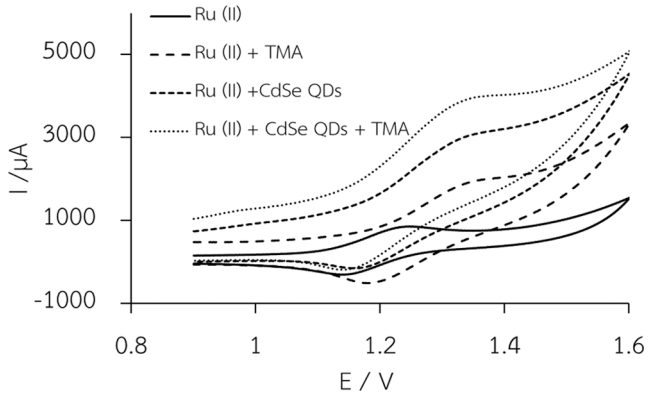

Fig. 2. Cyclic voltammograms of 10 mM $Ru(bpy)_3^{2+}$ at carbon screen-printed electrode in 0.1 M phosphate buffer solution pH 7.9, with and without the presence of TMA and/or TGA-capped CdSe-QDs. Scan rate of 50 mVs^{-1}; temperature of 25.0 °C.

3. Results and discussion

3.1. Characterization of the thioglycolic acid-capped cadmium selenide quantum dots

The morphology of TGA–capped CdSe-QDs was studied through TEM measurement as indicated in Fig. 1a, which shows a bright field-TEM image of CdSe-QDs. The shapes of TGA–capped CdSe-QDs nanoparticles are quasi-spherical and less congregated, with an average size of 3–10 nm and a narrow size distribution. The nanoparticles aligned well with the orange luminescence obtained by ultraviolet irradiation at 366 nm. The inset in Fig. 1a presents the selected area electron diffraction pattern (SADP) of these particles which corresponds to the polycrystalline structures.

The XRD spectra of TGA-capped CdSe-QDs is shown in Fig. 1b The distinct diffraction peaks were observed at 2θ values of 23.8°, 26.9°, 35.0°, 41.8° and 49.5°, corresponding to the (1 0 0), (1 0 2), (1 0 1), (2 0 0) and (1 2 2) crystalline planes of multiphase of hexagonal CdSe and rhombohedral $CdCO_3$, respectively, The diffraction peaks were in agreement with the joint committee powder diffraction standard (JCPDS) file no. 002–0330 and 001–0907. The dominant peak at 30.4° was representative of $CdCO_3$ crystalline plane at (1 0 4). Thus, the XRD pattern of multiphase was represented by the polycrystalline nanocomposites, which agrees well with the TEM results.

The fluorescence spectra of TGA-capped CdSe-QDs showed the maximum absorption and emission peaks at 573 nm. It is shown in Fig. 1c that the fluorescence intensity was significantly quenched by the addition of different concentrations of TMA at 250 and 500 µM. It can be observed that the fluorescence quenching effect of QDs by TMA was

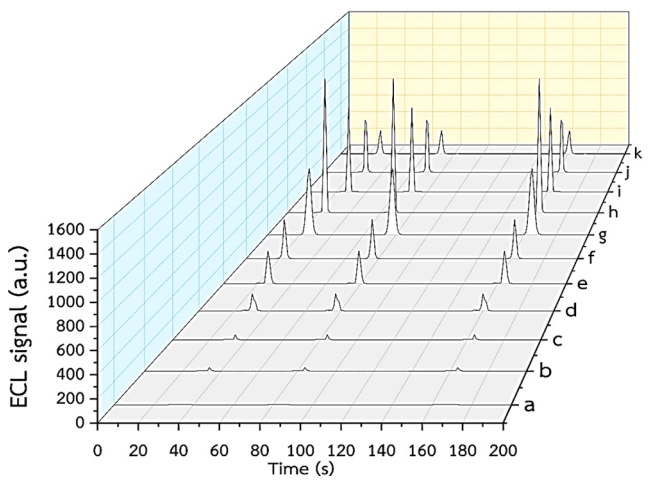

Fig. 3. The ECL signals of the electrochemiluminescence reaction in paper: (a) TMA concentration at 100 μM in PBS pH 7.5 (b) TGA-capped CdSe-QDs concentration at 1 mM ; (c) 100 μM TMA + TGA-capped CdSe-QDs; (d) 10 mM of $Ru(bpy)_3^{2+}$; (e–g) 10 mM of $Ru(bpy)_3^{2+}$ with 100, 250 and 500 mM of TMA , respectively; (h) 10 mM of $Ru(bpy)_3^{2+}$/1 mM TGA-capped CdSe-QDs; (i–k) 10 mM of $Ru(bpy)_3^{2+}$/1 mM TGA-capped CdSe-QDs with 1, 5 and 50 μM of TMA, respectively. The ECL was initiated in each case by applying the potential sensor from 0.8 to 1.6 V following addition of a drop of 12 μL of various solutions in 0.1 M PBS pH 7.9.

found to be concentration dependent. This system can be hence applied for the development of a TMA sensor.

3.2. ECL behavior of the $Ru(bpy)_3^{2+}$/TGA-capped CdSe-QDs in paper

In order to prove the μPAD-ECL feasibility, the cyclic voltammogram behavior was observed when the different solutions were directly introduced to μPAD previously filled with 12 μL of 10 mM $Ru(bpy)_3^{2+}$. The cyclic voltammograms of $Ru(bpy)_3^{2+}$, in presence of TMA and/or TGA-capped CdSe-QDs (Fig. 2), presented a quasi- reversible redox process at 1.30 V, which is associated with the oxidation of the amine group. The $Ru(bpy)_3^{2+}$ is reversibly oxidized to $Ru(bpy)_3^{3+}$ at 1.20 V under the same experimental condition. Hence, a potential range of 0.8–1.6 V was applied to the screen-printed electrode to oxidize the Ru $(bpy)_3^{2+}$ complex and the amine at a pH of 7.9. Under these conditions the amines would be deprotonated.

As shown in Fig. 3, the ECL emission behavior of $Ru(bpy)_3^{2+}$/TGA-capped CdSe-QDs on μPAD was thoroughly investigated. Weak ECL signals were obtained from TMA or TGA-capped CdSe-QDs without Ru $(bpy)_3^{2+}$ solution in paper (curves a, b) or even with the mixture of TMA and TGA-capped CdSe-QDs (curve c). The significant higher ECL signal obtained by only 10 mM $Ru(bpy)_3^{2+}$ solution in paper is shown in curve d. The increasing of ECL signals (curves e-g) were obtained by adding the relatively high concentrations of TMA at 100, 250 and 500 mM into the μPAD, filled with 10 mM of $Ru(bpy)_3^{2+}$ solution, since TMA (as all tertiary amines) could act as an efficient ECL co-reactant at quite high concentration (Egashira, Kumasako, Uda & Ohga 2002).

The mechanism for the production of ECL using $Ru(bpy)_3^{2+}$ and TMA is similar to that of other well-known co-reactants, such as tripropylamine (TPA) or 2-(dibutylamino) ethanol (DBAE), according to the following mechanism:

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^- \quad (1)$$

$$TMA \rightarrow TMA^{\bullet+} \quad (2)$$

$$TMA^{\bullet+} \rightarrow TMA^{\bullet} + H^+ \quad (3)$$

$$Ru(bpy)_3^{3+} + TMA^{\bullet} \rightarrow Ru(bpy)_3^{2+*} \quad (4)$$

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

The increase in the chemiluminescence intensity is due to the oxidation products of the amine group, which forms a radical cation $TMA^{\bullet+}$ in a short lifetime, and then is deprotonated, generating the reduced $TMA^{\bullet}$ radical. The radical then reduces the $Ru(bpy)_3^{3+}$to the excited state $Ru(bpy)_3^{2+*}$ which emits the chemiluminescence light when it relaxes back to ground state. The oxidation and deprotonation of TMA to form an intermediate radical reducer were critical steps of the reaction, which occurs favorably in weak alkaline solutions since the amines intermediates are the crucial source of chemical energy to produce the excited $Ru(bpy)_3^{2+*}$. The medium solution with pH less than 7.0 may diminish the ECL signals (Lindino & Bulhões, 2007).

The strong ECL signal was enhanced greatly when TGA-capped CdSe-QDs mixed with $Ru(bpy)_3^{2+}$ solution was introduced to the μPAD (Fig. 3, curve h). The preliminary test result indicates that the $Ru(bpy)_3^{2+}$ ECL emissions enhancement resulted from TGA-capped CdSe-QDs. This effect is likely due to the electron-transfer processes during the ECL reaction which correlates to the excellent optical and electronic properties of QD. Moreover, the great CL intensity could be generated from the excitement of QDs, which is formed by electron- and hole-injection after direct oxidation (Chen, Lin, Haifeng & Lin, 2014).

On the other hand, the quenched ECL intensity of the $Ru(bpy)_3^{2+}$/TGA-capped CdSe-QDs was observed even when very low concentrations of TMA (1, 5 and 50 μM) were added to the ECL-μPAD system (Fig. 3, curves i-k). The electron–transfer competition between CdSe-QDs in excited state and oxidation products of trimethylamine might be responsible for the ECL quenching effect, where an addition of TMA tends to reduce the ECL intensity (Taokaenchan et al., 2015). The ECL intensity decreased gradually by increasing the different concentrations of TMA in the solution mixture and was found to be concentration dependent. Based on this quenching principle, it is possible to apply the proposed ECL-μPAD system on the determination of TMA in aqueous samples.

3.3. Optimization of the electrochemiluminescence condition

It was notoriously observed that TMA quenches the ECL intensity of $Ru(bpy)_3^{2+}$/TGA-capped CdSe-QDs in a concentration dependent manner. Therefore, a series of experiments were conducted to evaluate the optimal experimental parameters that provides the highest ECL sensitivity, which was then used in the quantitation of TMA in real samples. Optimization of the experimental conditions was carried out based on the so-called univariate method. The chemical and ECL system variables affecting the microfluidic detection of TMA, such as pH and the concentration of phosphate buffer solution, reagent volumes, reagent concentrations and CV scan rate of the respective measurements were investigated.

3.3.1. Effect of pH values and phosphate buffer solution concentration

It is well known that the characteristics of most ECL signals are pH-dependent and are sensitive to its changes. Therefore, pH was the first parameter to be evaluated for its effect on the analytical response. The effect of the pH was studied using 0.1 M phosphate buffer solution adjusted to various pH values in the range of 6.0–9.0. The maximum quenching occurred at a pH where an amine was deprotonated. The maximum ECL signal of $Ru(bpy)_3^{2+}$/TGA-capped CdSe-QDs system occurred when the pH was at 7.5, and beyond this point the ECL signal gradually decreased associated with a significantly unstable baseline (Fig. S2a); hence, a phosphate buffer solution at pH 7.5 was used as solvent for posterior work. Additionally, the influence of buffer concentration on the ECL response was studied using different concentrations of the phosphate buffer solution in the range of 2.5–500 mM at pH 7.5. From Fig. S2b, the increase the buffer concentration enhances the ECL response from 2.5 to 50 mM. It reaches a maximum peak at 50 mM and then decreases. Therefore, 50 mM phosphate buffer solution was

Table 1
Optimized system parameters.

Parameter	Range studied	Optimal value
pH of phosphate buffer solution	6.0–9.0	7.5
Phosphate buffer solution concentration (mM)	2.5–500	50
$Ru(bpy)_3^{2+}$ concentration (mM)	2–20	10
TGA-capped CdSe-QDs concentration (mM)	0.5–3.0	2.0
CV scan rate ($V{\cdot}s^{-1}$)	0.02–1.00	0.20
Volume of $Ru(bpy)_3^{2+}$/TGA-capped CdSe-QDs in paper (μL)	9–16	12

Table 2
Maximum tolerable concentration ratios of interference on the ECL determination of 0.1 μM of TMA.

Foreign species	Tolerable concentration ratios
Formaldehyde, Triethylamine, N,N,N'-TMEDA, N,N,N',N'-TMEDA	1
Histamine, heavy metals (Pb(II), Ni(II), Cu(II), Zn(II))	5
Trimethylamine *N*-oxide, chloroform, putrescine, cadaverine	10
Ammonia	100
Ethanol	1000

selected for subsequent experiments.

3.3.2. Effect of the volume of $Ru(bpy)_3^{2+}$/TGA-capped CdSe-QDs mixture in paper

Different volumes of 10 mM $Ru(bpy)_3^{2+}$/1 mM TGA-capped CdSe-QDs mixed solution were introduced into the microfluidic channel and tested. The ECL intensity only changed slightly when a volume from 11 to 12 μL was filled in the channel. The maximum ECL signal was obtained at 12 μL (Fig. S2c), which was considered suitable for the following experiments.

3.3.3. Effect of the concentration of $Ru(bpy)_3^{2+}$ and TGA-capped CdSe-QDs

The effect of the concentration of $Ru(bpy)_3^{2+}$ on the ECL intensity was subsequently studied with $Ru(bpy)_3^{2+}$ solutions at different concentrations (5–50 mM). The ECL signal reached a maximum at a concentration of 10 mM (Fig. S2d) and a gradually decrease of ECL signal was observed with higher concentrations. Consequently, the $Ru(bpy)_3^{2+}$ concentration at 10 mM was chosen for further studies. Furthermore, the effect of TGA-capped CdSe-QDs concentration was studied in the range of 0.5 mM-3.0 mM. It was also found that the highest ECL signal was observed when 2 mM TGA-capped CdSe-QDs was added in the ECL μPAD system (Fig. S2e).

3.3.4. Effect of the CV scan rate on the ECL response

The scan rate could affect the ECL signal based on the rate of generation/ annihilation of $Ru(bpy)_3^{2+}$ in paper. The scan rates of CV were investigated at various levels from 0.02 to 1.00 $V{\cdot}s^{-1}$. With the increase of the scan rate, the ECL intensity also increased and reached a maximum at the scan rate of 0.20 $V{\cdot}s^{-1}$, demonstrating that the ECL intensity reached a high point (Fig. S2f). Therefore, a scan rate of 0.20 $V{\cdot}s^{-1}$ was chosen for the following studies.

Based on these findings, all the optimal values are shown in Table 1. These parameters would ensure a high sensitivity and potentially minimize the cost of operation.

3.4. Interference studies

To assess the selectivity of the proposed method, the interference effect of some coexisting substances present in fish samples on the determination of TMA was examined. A solution containing a fixed amount of TMA at 0.1 μM and different weight ratios of interference compound, analyte ranging from 1 to 1000, were applied to the ECL-μPAD and investigated under the optimal analytical conditions following the proposed method. Selected potential interfering compounds that are occasionally found in raw fish and deteriorated fish such as NH_3, trimethylamine *N*-oxide (TMAO), ethanol, formaldehyde chloroform, some heavy metals, biogenic and tertiary amines were investigated. The maximum tolerable concentrations for each interference are shown in Table 2. A substance was considered not to interfere if it caused a relative error of less than 10% for 0.1 μM of TMA.

The interfering results show that the ECL method is not as selective as the chromatographic method (Yen & Hsieh, 1991; Anderson, 2008) since the determination of TMA is greatly affected when certain biogenic or tertiary amines, formaldehyde and some heavy metals are present in the sample up to 10-times the weight ratio of TMA. However, the method can be used as a screening method to predict the freshness level of fish by monitoring the biogenic and total tertiary amines concentration (Biji, Ravishankar, Venkateswarlu, Mohan & Srinivasa Gopal, 2016), since the interference caused by heavy metals can be eluted by adding metal masking agent. In addition, chloroform contamination can be removed from the sample during a liquid–liquid extraction process.

Compared with the recommended method (AOAC, 1990), the method proposed on this work is much simpler and faster for the determination of fish freshness through the quantification of trimethylamine. Hence the method can be used as a screening method to predict the freshness level of fish by monitoring the biogenic and tertiary amines concentration before the determination with an *in-situ* instrument.

3.5. Analytical performance on ECL-μPAD

Under the optimal conditions for the determination of TMA, the ECL intensity on the ECL-μPAD was studied for each selected tertiary amines tested, namely trimethylamine, triethylamine (TEA), N,N,N'-trimethyl ethylenediamine (N,N,N'-TMEDA) and N,N,N',N'-tetramethyl ethylenediamine (N,N,N',N'-TMEDA). The ECL intensity was proportional to the concentration of tertiary amines in the range from 1×10^{-12} to 1×10^{-6} M. The analytical characteristics of each tertiary amines were determined. The regression equations, the correlation coefficients (r^2), limits of detection (LOD) and relative standard deviation (RSD) were calculated. The precision of the method was assessed in 12 repeated measurements of 1×10^{-7} M of each tertiary amine. The results shown in Table S1 indicates that the method is acceptable for the determination of tertiary amines at low concentration levels in fish.

3.6. Application of the ECL-μPAD method in the analysis of fish samples

In order to examine the applicability and accuracy of this method, five fresh fish were analyzed by the proposed ECL-μPAD under the optimal experimental conditions. Three freshwater and two ocean fish of different types, obtained from a local market in Chang Mai, Thailand, were pre-treated following the recommendations of the commission of analytical methods for analysis of fish and derivatives for direct analysis of fish tissue muscle (Li & Lee, 2007). The method of the extraction was modified by Anderson (2008). Briefly, around 5 g of ground tissue of each fresh fish was homogenized with 20 mL of 6% (w/v) trichloroacetic acid (TCA) and then allowed to stand for 1 h at room temperature. The mixture was transferred into a polypropylene tube and centrifuged for 10 min at 3000 rpm. Afterwards, the supernatant was filtered, and the solid residue was washed with 6% TCA. The washing product was added to the extraction aliquot to give a final volume of 25 mL, then adjusted to pH 7.5 and finally diluted to 50 mL using 50 mM phosphate buffer. Lastly, aliquots of 3 μL were taken for the ECL-μPAD determination of trimethylamine under the optimized conditions described above.

The HPLC procedure with photodiode-array detection after benzoyl chloride derivatization (Yen & Hsieh, 1991) was used as a reference

Table 3
Comparative determination of TMA residues in fish samples by the proposed ECL-μPAD method and the reference HPLC method.

Feed sample	Amount found (mg/100 g)	
	ECL-μPAD method[a]	HPLC method[b]
Red Tilapia	0.52 ± 0.02*	NA
Yellow Tail	2.18 ± 0.03	2.24 ± 0.02
Salmon	2.47 ± 0.02	2.53 ± 0.03
Tuna	0.91 ± 0.01	NA
Walking catfish	2.58 ± 0.02	2.56 ± 0.02

* Units in ng/100 mg.
[a,b] Mean ± standard deviation, n = 3 for the proposed method and the reference method.

method to identify each tertiary amine in fish samples prior to compare with the ECL-μPAD method. The HPLC results showed that every fresh fish sample contained only TMA.

Since the proposed ECL-μPAD method shows equal ECL response for most of the tertiary amines found in spoiled fish, the determination of the freshness and the spoilage level of fresh fish could be easily achieved by calculating the mean of the total tertiary amines measurements. The TMA contents from ECL-μPAD were compared with the data provided by HPLC procedure in all instances (shown in Table 3). The comparison showed statistically no significant difference between the two methods at a 95% confidence level by the Student *t*-test (*t*-value 0.417) with regard to accuracy and precision. Therefore, the proposed ECL-μPAD method was proved to be a simple approach with a potentially lower cost instrumentation setup compared to current methods, where a reliable disposable paper-based sensor could be very useful in a context where a rapid and cost-effective method is required. In addition, the capability of this disposable sensor allows the monitoring of fish freshness in rural areas or during transportation where scientific equipment and trained personnel are in short supply.

4. Conclusion

An ECL detection combined with μPADs has been successfully developed as a simple, inexpensive and sensitive sensing platform for the rapid estimation for TMA without any process derivatization. The ECL signal from the $Ru(bpy)_3^{2+}$/thioglycolic acid–capped CdSe-QDs system assessed the total amount of tertiary amines through a quenching effect. This significant effort demonstrates the utility of a portable device in the field as a rapid screening method to improve the capability of fish freshness quality control under aerobic storage. Compared with the HPLC method, similar results were obtained using the ECL-μPAD method in a shorter time (2 min versus 25 min for the HPLC method). In addition, the ECL-μPAD provides the possibility of miniaturization of an analytical device which could become a great tool for food quality monitoring in remote regions and has also the potential to be used during the transportation from developing or developed countries. Complementary qualitative confirmation may be required using a specific instrumental method for exported food products.

CRediT authorship contribution statement

Nisachon Praoboon: Formal analysis, Investigation, Methodology, Writing - original draft. **Suphawuth Siriket:** Software. **Narin Taokaenchan:** Data curation. **Surasak Kuimalee:** Visualization. **Sirirat Phaisansuthichol:** Validation. **Pusit Pookmanee:** Visualization. **Sakchai Satienperakul:** Conceptualization, Funding acquisition, Project administration, Resources, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

N. Praoboon gratefully acknowledges financial support from the Royal Golden Jubilee (RGJ) Ph.D. Program of the Thailand Research Fund for this work. Additionally, S. Satienperakul would like to thank the Commission on Higher Education, Ministry of Education for their very kind partial support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130590.

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