Talanta 116 (2013) 308–314

Contents lists available at ScienceDirect

Talanta

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

Discovered triethylamine as impurity in synthetic DNAs for and by electrochemiluminescence techniques

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ARTICLE INFO

Article history:
Received 21 May 2012
Received in revised form
4 July 2012
Accepted 14 March 2013
Available online 24 May 2013

Keywords:
Impurity
Triethylamine
Synthetic DNAs
Electrochemiluminescence

ABSTRACT

The purity of the synthetic oligonucleotides is very important because it is crucial for the accuracy of the established biological assays. Herein, it was discovered that one impurity in synthetic DNAs might affect the experiment results of electrochemiluminescence (ECL) detection techniques, which was never reported before. According to a series of experiments using ECL detection methods combined with capillary electrophoresis (CE) (CE–ECL), the impurity was identified as triethylamine (TEA), which came from incomplete removal after HPLC purification of synthetic DNAs. Moreover, CE–ECL technique was for the first time to be proposed for discovering, identifying and sensitive determining the possible impurity such as TEA in various DNA samples, which was usually neglected by other detection techniques for purification quality control of synthetic oligonucleotides. A detection range from 5.00×10^{-10} to 2.00×10^{-5} M with a detection limit as low as 50 nM ($S/N=3$) was reached for TEA. Through further designed ECL methods and data analysis, situations which would be really affected by the impurity of TEA were studied. To avoid or eliminate the impact of the TEA impurity on ECL applications, judgment basis for choosing purification ways was discussed according to individual requirements.

1. Introduction

Synthetic oligonucleotides are important in a wide variety of applications such as hybridization probes, DNA sequencing, polymerase chain reaction, microarrays, etc. [1]. And their annual production has increased sharply in recent years in order to meet the considerable demand in the biology field [2]. Apparently, the quality of the synthetic oligonucleotides is very important because they are crucial for the accuracy of the established biological assays. Even low levels of impurities in the synthetic oligonucleotides can potentially lead to unwanted and possibly detrimental experiment results. Thus, it is important to determine whether the purity of the synthetic oligonucleotides is enough or whether further purification is still needed [1,2]. Currently, the most common techniques for synthetic oligonucleotides purification are polyacrylamide gel electrophoresis (PAGE) [3] and high performance liquid chromatography (HPLC) [1,2]. In addition, the purification is application-dependent, for example, PAGE is the most efficient means of purification for unmodified oligonucleotides, while HPLC is usually applicable for oligonucleotides that have been modified with an additional linker or spacer, such as primary amines (–NH_2), sulfhydryls (–SH), etc. [4,5]. To achieve efficient purification, both the mobile and stationary phases of HPLC have to be optimized. In last century, buffers typically containing triethylamine (TEA) as the ion-pairing agent have been proposed as the mobile phase for oligonucleotide separations with good results [2,6]. Theoretically, the eluent buffer is volatile and can be easily removed from column effluents by freeze-drying. Until now, TEA contained buffer is still the most traditionally employed ion-pairing mobile phases in HPLC for oligonucleotide separations [7,8].

During the purification process, not only the by-products and short failure sequences, but also the inorganic salts, traces of organic compounds, low molecular weight impurities, etc. have to be removed. Although the scientists tried their best to be considerate to the purification strategies, no one single method is perfect enough to meet all the requirements for eliminating such comprehensive and complex impurities. Thus, to detect, identify, and quantify these impurities is an ongoing urgent task for the bioanalyst. Usually UV–visible absorbance and mass spectrometry (MS) such as matrix assisted laser desorption ionization-time of flight and electrospray ionization (ESI) are applied to characterize the purification quality [9]. Capillary electrophoresis (CE) is also one way to be used due to its superiorities such as minimal sample

Abbreviations: CE, capillary electrophoresis; CE–ECL, ECL combining with CE technique; CV, cyclic voltammetry; ECL, electrochemiluminescence; ESI, mass spectrometry; HAc, glacial acetic acid; HFIP, hexafluoroisopropanol; HPLC, high performance liquid chromatography; MS, mass spectrometry; –NH_2, primary amines; NH_2-, NH_2-labeled; OTA, ochratoxin A; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffer; RSDs, relative standard derivations; -SH, sulfhydryls; TC, tetracycline; TEA, triethylamine; Tris, tris(hydroxymethyl)aminomethane.

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http://dx.doi.org/10.1016/j.talanta.2013.03.089

preparation, rapidness, high resolution and automatic data handling, etc. [9,10]. The most traditional detection techniques combined with CE are UV–visible absorbance and laser induced fluorescence methods [10,11]. Similar to the purification techniques, each method by itself is insufficient for purification quality characterization, thus more techniques have to be developed for providing more reliable oligonucleotide impurity analysis. Electrochemiluminescence (ECL) is with advantages of highly sensitive and selective, cost-effective, etc. [12]. Except for its individual biochemical applications, they can also be combined with CE for detection and analysis of inorganic ions, drugs, amino acids [11]. However, as far as we know, that ECL combining with CE technique (CE–ECL) used for quality control of synthetic oligonucleotides was seldom reported.

In addition, the intrinsic high sensitivity of the ECL detection technique not only endows it with sensitive ability to detect oligonucleotides with ECL active functional groups, but also make their experiment results more easily be influenced by even very low levels of ECL-active impurity substances in the synthetic oligonucleotides. Because the most frequently used detection techniques during the process of purification and impurities identification are UV and MS, impurities that have influences on ECL methods could be probably neglected, even these impurities are often present.

In the present work, it was discovered that one impurity in synthetic DNAs probably had great interferences with the DNA-based bio-assay results using ECL detection techniques. The impurity was recognized as TEA, which was from incomplete removal after HPLC purification of synthetic oligonucleotides. Moreover, taking TEA as an example, CE–ECL technique was proposed to discover, identify and sensitively determine possible impurity in synthetic oligonucleotides, which was usually neglected by other detection techniques for purification quality control of synthetic oligonucleotides. According to the further designed methods and data analysis, situations which will be really affected by the impurity of TEA were studied and discussed. Thus, by determining the amount of certain impurity, either rejecting or accepting the oligonucleotide samples for further experiments will be determined. It also provided with judgment basis for choosing purification ways or avoiding certain buffer components in HPLC purification according to individual requirements.

2. Experimental

2.1. Chemicals and materials

Tris(2,2′-bipyridyl)ruthenium(II) chloride hexahydrate was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Synthetic oligonucleotides were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) and Takara Biotechnology (Dalian) Co., Ltd. (Dalian, China). TEA, hexafluoroisopropanol (HFIP), $K_3[Fe(CN)_6]$, $K_4Fe(CN)6 \cdot 3H_2O$, $Na_2HPO_4 \cdot 12H_2O$, $NaH_2PO_4 \cdot 2H_2O$, tris(hydroxymethyl) aminomethane (Tris), KCl, NaCl $CaCl_2$, $MgCl_2$, glycerol, acetonitrile, glacial acetic acid (HAc) were obtained from Beijing Chemicals Inc (Beijing, China). All chemicals and reagents were of analytical grade and used without further purification. All solutions were prepared with deionized water processed with Milli-Q ultra-high purity water system (Millipore, Bedford, MA, USA). They were stored in the refrigerator at 4 °C and filtered through 0.45 μm pore disposable filter membranes before use.

2.2. Methods and equipments

All ECL experiments were carried out with a computer controlled CE–ECL system (Xi'an Remex Electronics Co. Ltd. Xi'an, China), including a high voltage power supply for electrophoretic separation and electrokinetic injection, an electrochemical potentiostat, a multifunctional chemiluminescence detector and a multichannel data processor. A three-electrode configuration was used in the detection cell consisting of a 500 μm Pt disk as a working electrode, Ag/AgCl as a reference electrode and Pt wire as a counter electrode. ECL detection reservoir used herein is the same as the one reported previously [13]. All separations were performed in a 45-cm-long fused-silica capillary with 50 μm i.d. and 360 μm o.d. (Yongnian Optical Conductive Fiber Plant, Hebei, China). The capillary was rinsed with 1.0 M NaOH overnight, washed for 5 min with 0.1 M NaOH, followed by double-distilled water and equilibrated with the running buffer for 5 min before use so as to maintain an active and reproducible inner surface. The voltage of photomultiplier tube for collecting the ECL signal was set at 800 V in the process of detection. Electrokinetic injections were performed at 13 kV for 8 s. The inlet end of the capillary was held at a positive potential and the outlet end was maintained at ground.

For adsorption studies, the Au working electrode was firstly polished with 0.3 μm alumina slurry to obtain a mirror surface, sonicated and thoroughly rinsed with Milli-Q water; And then they were subjected to repeated cycling between −0.2 V and 1.5 V (vs. Ag/AgCl) at a scan rate of 100 mV s^{-1} in 0.1 M H_2SO_4 until a reproducible voltammogram was obtained; Finally it was separately dipped into solutions of 10 μM TEA or 1.0 μM SH-Random DNA-2 containing 10 μM TEA for 2 h. The modified electrodes were rinsed gently with H_2O for 1 min to remove any nonadsorbed species. Before and after being modified, the electrodes were characterized by the cyclic voltammetry (CV) ranging from 0 to 0.6 V in a solution of 1.0 mM $Fe(CN)_6^{3-/4-}$ containing 100 mM KCl, respectively. Meanwhile, ECL signals were also recorded for the electrodes before and after modification in the potential range of 0–1.3 V in 1.0 μM $Ru(bpy)_3^{2+}$ solution containing 100 mM PBS (pH 7.4), respectively.

To characterize the discovered impurity in the DNA samples, MS analysis was carried out on a Quattro Premier XE (Waters, Milford, MA, USA.). The MS conditions were as follows: ionization mode: ESI; polarity: positive; function type: full scan; capillary voltage: 3.0 kV; cone voltage: 20 V; source temperature: 110 °C; cone gas flow: N_2, 80 L/h; desolvation temperature, desolvation gas flow: N_2, 480 °C, 600 L/h; mass range: 50–500 Da.

2.3. Preparation of standard solutions

All ECL measurements were performed in phosphate buffer (PBS), which was prepared from 100 mM NaH_2PO_4 with the pH adjusted to 7.4 by 100 mM NaOH. This avoided the introduction of unnecessary ions into the electrolyte which might affect the ECL results. $Ru(bpy)_3^{2+}$ (5 mM) with 50 mM PBS was added in the detection cell. All the synthetic DNA samples were prepared from 50 mM stock solutions, which were quantitated by 260 nm UV absorbance and the corresponding extinction coefficient. SH-labeled Random DNA-2 was prepared in the buffer of 100 mM tris–HCl (pH 7.4) containing 233 mM NaCl, 8.5 mM KCl, 1.7 mM $CaCl_2$, 1.7 mM $MgCl_2$ and 8.5% glycerol; All the sequences of the used synthetic DNAs herein were listed in the Table S1 in the Supporting information.

3. Results and discussion

3.1. Discovering and recognizing the impurity in synthetic DNAs

As can be seen in Fig. 1A-b, significant ECL signal were unexpectedly obtained when NH_2-labeled (NH_2-) cocaine aptamer was used as injection sample for CE–ECL experiments. As no work has been reported about ECL properties of the cocaine aptamer,

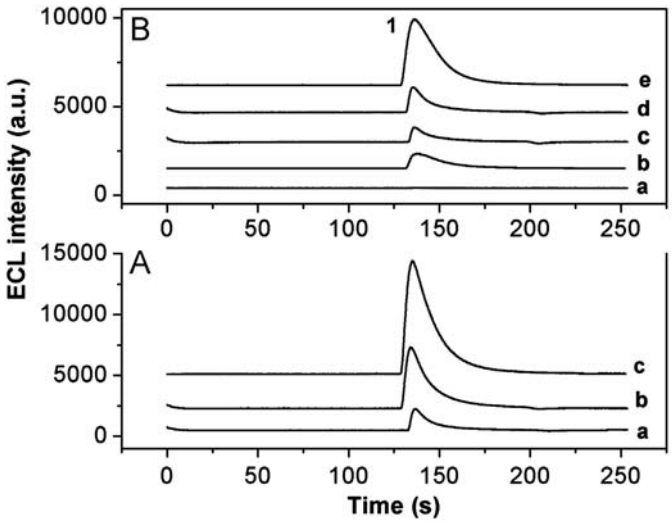

Fig. 1. (A) Electropherograms of (a) 5 μM TEA, (b) 20 μM NH_2-cocaine aptamer, (c) 20 μM NH_2-cocaine aptamer spiked with 5 μM TEA. (B) Electropherograms of 2 μM Random DNA-1 purified by (a) PAGE combining with MS qualification (ULTRAPAGE) or (b) HPLC; as well as of (c) 2 μM NH_2-Random DNA-1, (d) 5 μM NH_2-OTA aptamer, (e) 15 μM NH_2-TC aptamer purified by HPLC, respectively. Peak 1: ECL signal of TEA. Conditions: detection potential, 1.20 V; running buffer, 20 mM PBS (pH 7.4); 5 mM $Ru(bpy)_3^{2+}$ with 50 mM PBS (pH 7.4) in the detection reservoir; electrokinetic injection, 8 s at 13 kV; separation voltage, 18 kV.

this inexplicable phenomenon motivated us to carry out further experiments to characterize the unknown compound or functional group in this DNA sample.

First, different synthetic DNAs labeled with or without NH_2 groups were tested by CE–ECL techniques. As aptamers are nowadays popularly used, other aptamers such as ochratoxin A (OTA) aptamer and tetracycline (TC) aptamer were selected to study. To make the experiment results with extensive adaptability, other DNAs with random sequences named Random DNA-1 and Random DNA-2 were also applied for further tests. As shown in Fig. 1B-c-e and Table 1, DNAs labeled with NH_2 as injection samples could result in obvious ECL signals, while there were no such ECL peaks when DNAs without NH_2 groups were analyzed (Fig. 1B-a). These results excluded the possibility that the ECL signals were derived from the intrinsic ECL properties of the synthetic oligonucleotides constituents such as G and A nucleobases [14]. Generally, for the alkylamines as the coreactants of $Ru(bpy)_3^{2+}$, the ECL intensity follows the order of tertiary > secondary > primary. Analytes containing primary amines have very low ECL intensity [15]. Thus, the significant ECL signal should not be from the NH_2 group as it belongs to one primary amine. This conclusion was again confirmed by the experiments as follows: assumed that the ECL signals were from the NH_2 group, different conditions such as different components and pHs of running buffers, different separation voltages, etc. were tested trying to separate the NH_2 labeled DNAs by CE–ECL techniques. The tested DNAs were with 9, 36, 30, 76 bases for Random DNA-1, cocaine aptamer, OTA aptamer and TC aptamer, respectively. Their sizes and charge-to-mass ratios were with great difference from each other. Theoretically, they could be separated easily in common CE conditions and four ECL peaks should be obtained [16]. However, only one signal was obtained in the ECL electropherograms at the explored conditions. It was because even they were separated successfully by CE, they could not be represented by ECL detection techniques because they were not with ECL active groups by themselves. Thus the above assumption was false and it indicated that the ECL signals were not from the NH_2-DNAs themselves. In addition, as shown in Table 1, when the cocaine aptamer or Random DNA-2 was labeled with SH labels and used as samples for CE–ECL detection, there was also obvious ECL signal for these two DNAs. All these experiment results indicated that the ECL signals were not from the DNAs or related labels.

The purity should be a process-related impurity resulted from the synthesis or purification procedure. Compared these DNAs with and without NH_2 or SH groups, all the preparation procedures were the same except their purification methods. For DNAs without NH_2 or SH groups, they were in ULTRAPAGE degree, which were purified by PAGE combining with MS qualification to obtain synthetic oligonucleotides with much higher purity (the explanation of ULTRPAGE was obtained on the website of Sangon Biotech (Shanghai) Co., Ltd. (http://www.sangon.com/sangon_detail.aspx?newsID=501). While for DNAs with NH_2 or SH groups, commonly they were purified by HPLC to remove the short failure sequences to achieve high quality of DNA products [4,5]. Thus this ECL signals should probably come from the process of HPLC. This assumption was confirmed by the result that significant ECL signal was observed when unmodified Random DNA-1 was obtained through HPLC purifying process (see Fig. 1B-b and Table 1). The most traditional and sophisticated mobile phase in HPLC for synthetic oligonucleotides purification contains HAc, acetonitrile, TEA and sometimes HFIP [7,8]. Thereinto, TEA is one kind of tertiary amine, which is volatile and can be theoretically obviated from the synthetic oligonucleotides. However, even trace residual quantity of TEA could cause significant ECL by co-reacting with $Ru(bpy)_3^{2+}$ [17]. HAc, acetonitrile, HFIP and TEA have been tested as injection samples for CE–ECL techniques, respectively. It can be seen in Table 1, there were not any ECL signal for HAc, acetonitrile and HFIP. While for TEA, not only were ECL signals obtained, but also the migration time in the CE–ECL electropherogram (see Fig. 1A-a) was the same as the ones of impurity in NH_2-DNA samples (see Fig. 1A-b). Moreover, by adding different concentrations of TEA to the NH_2-DNA samples, the ECL intensities of the samples increased with increasing concentration of the added TEA (Fig. 1A-c).

To further confirm the impurity is TEA, several conditions were studied. First, NH_2-random DNA-1 was obtained only through desalination for purification process, avoiding HPLC purification. As supposed, the ECL peaks disappeared at the corresponding migration time of TEA in the CE–ECL electropherogram (Fig. 2c). Same result was also observed when the NH_2-random DNA-1 was obtained by PAGE purification process (see Fig. 2d). In another way, ethanol precipitation was additionally carried out to further eliminate the TEA in the product of synthetic DNAs after HPLC purification [18]. As a result, the ECL peaks for these TEA-further-eliminated DNA products were obviously decreased at the time of TEA in electropherograms (Fig. 2b) comparing with the previous NH_2 labeled DNA samples (Fig. 2a).

Subsequently, ways of choosing DNA purification to avoid or eliminate the influence effects on ECL applications can be summarized as follows:

(1) If no labels such as –NH_2 or –SH are needed to be modified on the DNAs, the purity of ULTRAPAGE degree is enough for further experiments and HPLC purification can be avoided.
(2) If labels are needed to be modified on the DNAs and TEA in DNA samples will not influence the experiment results, HPLC is the better way to guarantee the quality of the synthetic DNAs. However, if the TEA will affect the ECL applications significantly, there are several ways to be considered: (1) if the experiment will not be affected by the short failure DNA sequences, only desalination for synthetic DNAs is suggested; (2) PAGE is also one way to obtain the labeled DNAs while avoiding HPLC, but it is not commonly recommended or even scarcely applied in many DNA Synthesis and Biotechnology Research Industries due to its lower success efficiency, accuracy and purify than HPLC for labeled DNAs. (3) If HPLC

Table 1
ECL signals of different DNA products or substances used as injection samples for CE–ECL techniques.

Samples	Labels	Purification methods	ECL signal
Random DNA-1	N/A[a]	ULTRAPAGE	N[b]
		HPLC	Y[c]
	5′ NH_2-$(CH_2)_6$	HPLC	Y
		HPLC plus with ethanol precipitation	Y but much weaker
		Only desalination	N
		PAGE	N
Random DNA-2	5′SH-$(CH_2)_6$	HPLC	Y
Cocaine aptamer	N/A	ULTRAPAGE	N
	5′ NH_2-$(CH_2)_6$	HPLC	Y
		HPLC plus with ethanol precipitation	Y but much weaker
	5′SH-$(CH_2)_6$	HPLC	Y
OTA aptamer	N/A	ULTRAPAGE	N
	5′ NH_2-$(CH_2)_6$	HPLC	Y
		HPLC plus with ethanol precipitation	Y but much weaker
TC aptamer	N/A	ULTRAPAGE	N
	5′ NH_2-$(CH_2)_6$	HPLC	Y
		HPLC plus with ethanol precipitation	Y but much weaker
5% (V/V) HAc	N/A	N/A	N
5%(V/V) acetonitrile	N/A	N/A	N
5%(V/V) HFIP	N/A	N/A	N

[a] N/A, not applicable.
[b] N represents no ECL signals.
[c] Y represents strong ECL signals.

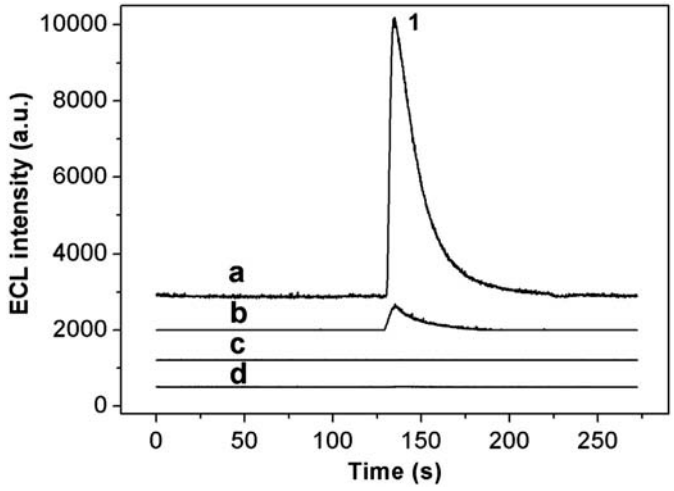

Fig. 2. Electropherograms of 20 μM NH_2-Random DNA-1 purified by (a) HPLC, (b) HPLC plus with ethanol precipitation, (c) only desalination; and (d) PAGE. Peak1: ECL signal of TEA. Conditions are the same as Fig. 1.

purification has to be applied, further step of ethanol precipitation is needed to eliminate the TEA as little as possible. However, as long as the mobile phase of HPLC purification process contained TEA, the TEA could only be reduced from the DNA products and could not be eliminated completely until now. At least, it was still be detectable by the CE–ECL technique. (4) The fundamental solution to resolve the influence of TEA impurity on ECL applications is to look for other alternative to TEA as efficient ion-pairing agent for HPLC purification of synthetic oligonucleotides in further investigations.

Furthermore, 10 μM NH_2–cocaine aptamer was characterized and checked for purity by MS determination, which was performed using ESI ionization mode scanning in the molecular range from 50 to 500 Da. As can be seen in Fig. 3A, it clearly indicated the existence of TEA (molecular weight 101.9) in the NH_2–cocaine aptamer sample. However, as can be seen in the Fig. S1, usually molecular weight range around the ones of the required DNAs were set to characterize the purity of required DNAs after the DNAs were synthesized. As the molecular weight of TEA was much less than the DNAs', the TEA was usually neglected by ESI MS spectra. Another technique used to quantify the DNAs is UV–visible absorbance. As shown in Fig. 3B, except the 260 nm UV absorbance peak, there was no additional peak for DNAs with or without NH_2 labels in the range of 200–800 nm. Moreover, there was not so much difference of the UV absorbance as long as the concentrations of DNA were the same. Thus, TEA in DNA sample couldn't be discovered by UV absorbance method.

That is to say, to characterize the impurity TEA in synthetic DNAs, MS can be applied; however, molecular weight range should not be limited to around the ones of the required DNAs, lower scanning range around the molecular weight of TEA should be set. Meanwhile, it still needs to explore more efficient techniques such as CE–ECL for qualifying and quantifying impurities in synthetic DNAs.

3.2. Quantification of the impurity in synthetic DNAs by CE–ECL technique

Subsequently, using TEA as a representative, CE–ECL technique was developed for quantifying the impurity in synthetic DNAs. To obtain the highest sensitivity of TEA in synthetic DNAs, the detection conditions were optimized according to the procedures in previous reports [13] and were as follows: ECL detection at 1.20 V, separation voltage at 18 kV, 20 mM PBS (pH 7.4) running buffer, 5 mM $Ru(bpy)_3^{2+}$ with 50 mM PBS (pH 7.40) in the detection reservoir, electrokinetical injection for 8 s at 13 kV. Under the optimized conditions, a standard solution of 2 μM TEA was injected consecutively six times to determine the repeatability of ECL intensity based on peak height and migration time. Relative standard derivations (RSDs) of the ECL intensity and the migration time were 4.53% and 0.79%, respectively. The high reproducibility was indicative of the high accuracy for detecting TEA by the proposed CE–ECL technique. To evaluate the linearity of the established method, standard curves were prepared by analyzing different concentrations of TEA between 1 pM and 1 mM. The standard curves were linear in the range of 5.00×10^{-10} to 2.00×10^{-5} M. The calibration equations and regression coefficients were $y = 0.38684 \times +40.19941$ and $R = 0.9911$ ($n = 20$),

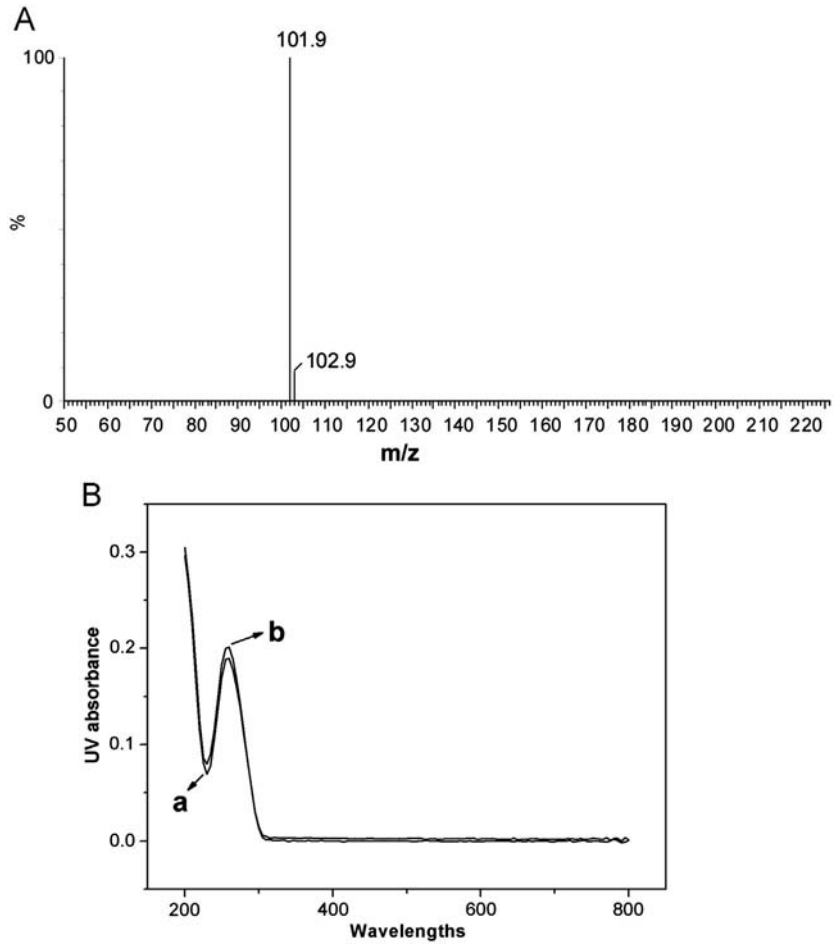

Fig. 3. (A) Mass spectrum of 10 μM NH_2-cocaine aptamer using ESI ionization mode in the mass range of 50–500 Da; (B) UV–vis absorbance spectra of (a) 0.540 μM NH_2–cocaine aptamer and (b) 0.575 μM cocaine aptamer scanning from 200 to 800 nm.

respectively, in terms of peak height response as a function of TEA concentration (nM). Detection limit of 0.1 nM was achieved ($S/N=3$).

The optimized CE–ECL methods were adopted to the determination of TEA in different NH_2 labeled DNA samples. The amount of TEA was quantified by the calibration curve. In addition, to determine the validity of the proposed method, four groups of NH_2 labeled DNAs were prepared. Each group contained three different concentrations of corresponding DNA and was spiked with three different concentrations of TEA at 10, 1.0, 0.1 or 10, 3.0, 0.5 μM, respectively. The detection results and recovery rates were obtained by replicate determinations ($n=3$) of the original or spiked samples of NH_2 labeled DNAs. The recovery rates were calculated with the following equation: Recovery rates (%)=(total found−found)/Spiked. All the results were shown in Table 2. Recovery rates of range from 95.80 to 106.60% were obtained.

These results indicated that the analytical method proposed was sensitive and accurate enough for the determination of the impurity TEA in NH_2 labeled DNA samples. Due to fast separation, lower detection limits, good selectivity, high sensitivity and effective resolution of CE–ECL technique [12], it has the great potential to be a powerful tool for analyzing other ECL-active impurities in synthetic oligonucleotides. Meanwhile, it also indicated that the high sensitive ECL related analytical methods were vulnerable to the ECL active interference in the DNA samples. Thus, attention should be paid on the impurity of DNA samples for the ECL biological assays.

Table 2
The results for CE–ECL analysis of TEA in different DNA samples and spiked with different concentrations of standard TEA.

DNA samples	Concentrations of DNA	Found[a] (μM)	Spiked (μm)	Total found[b] (μM)	Recovery (%)
NH_2-Random	20 μM	19.315	10	N/A[c]	N/A
DNA-1	2	2.108	1	3.174	106.6
	0.2	0.234	0.1	0.337	103
NH_2–Cocaine	20 μM	12.893	10	N/A	N/A
aptamer	2	1.273	1	2.226	95.8
	0.2	0.129	0.1	0.23	101
NH_2-OTA	15	10.334	10	N/A	N/A
aptamer	5	3.615	3	6.509	99.17
	1	0.666	0.5	1.192	105.2
NH_2-TC	15	9.538	10	19.267	97.29
aptamer	5	3.07	3	6.096	100.87
	1	0.63	0.5	1.143	102.6

[a] The average of three repeated measurements.
[b] The average of three repeated measurements.
[c] It is not applicable because the ECL value is out the linearity range.

3.3. Discussion of situations affected by the impurity

As mentioned above, the impurity TEA in the DNA samples was with significant ECL signals and even at trace amount may interfere with the results of normal ECL related experiments. However, it must also be pointed out that the influence degree of TEA would be different depending on the different experiment

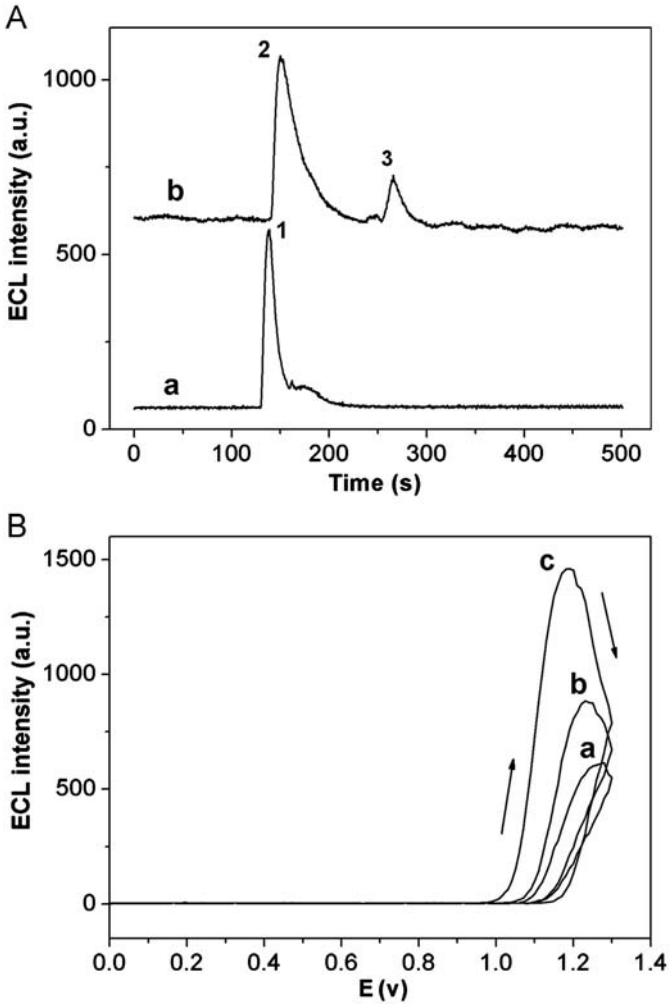

Fig. 4. (A) Electropherograms of 2 μM NH_2–cocaine aptamer with 10 μM cocaine using (a) 10 mM PBS (pH 7.4) as running buffer and separation voltage 18 kV; and (b) 20 mM PBS as running buffers and separation voltage 15 kV. ECL signals of (1) unseparated TEA and cocaine, (2) TEA and (3) cocaine, respectively. Other conditions were the same as in Fig. 1. (B) ECL intensity–potential curves of 10 μM Ru $(bpy)_3^{2+}$ solution containing (a) 0, (b) 10 μM cocaine, (c) 10 μM cocaine and 10 μM NH_2-cocaine aptamer, respectively, at Pt electrode in 100 mM PBS (pH 7.4).

situations. Circumstances that would be or not be affected by the impurity TEA were studied and discussed according to further designed experiments as follows:

1) In assays using CE–ECL techniques, if the TEA in synthetic DNAs can be distinguished from the ECL-active analytes by CE techniques, the influence can be avoided; otherwise, it will result in inaccurate results. For example, in establishing aptamer-based assays, cocaine was usually used as a model analyte. Moreover, cocaine is with one tertiary functional group [13], which can be detected by ECL techniques [13]. Thus, taking it as an example, the influences of impurity TEA on the DNA related experiments were analyzed. As can be seen in Fig. 4A, when the DNA products containing TEA impurity were used for injection sample for CE–ECL techniques, if the TEA and cocaine could be separated successfully (Fig. 4A-b), no influence will be exerted on the experiment results. Oppositely, the results will be affected significantly if successful separation between TEA and cocaine (Fig. 4A-a). That is to say, misleading appearance will be possibly obtained if the impurity was not discovered or considered.
2) When the DNAs with TEA impurity was directly scanned in the solution, the impact on ECL results will be great. For example, 10 μM $Ru(bpy)_3^{2+}$ solution containing 10 μM cocaine or 10 μM cocaine and 10 μM NH_2-cocaine aptamer, were prepared, respectively. Scanning range of 0–1.3 V was set to obtain their ECL signals, as can be seen in Fig. 4B, the ECL intensity of cocaine (Fig. 4B-b) was significantly increased and affected by addition of the added NH_2–cocaine aptamer due to the existence of TEA (Fig. 4B-c). Unfortunately, as all the analytes were in the solution and no separating strategies were carried out, the influence will be very significant and can't be avoided.
3) If the DNAs are immobilized or adsorbed on the electrodes, the influence of TEA impurity can be avoided. For example, adsorption studies were designed to see whether the TEA could adsorb on the electrode or not. Detailed procedure was described in Section 2.2. As shown in Fig. 5A, before being immersed in the DNA or TEA solutions, no obvious ECL signal was obtained for 1.0 μM $Ru(bpy)_3^{2+}$ being scanned between 0 and 1.3 V (Fig. 5A-a). Upon immersion of the electrode in the SH-DNA containing TEA solution for two hours, ECL signal was observed for scanning in the same conditions. This was because SH-DNA could bind on the electrode surface through Au–S specific interaction and accordingly resulted in the ECL signal due to the inherent ECL properties of DNA [20] (see Fig. 5A-c and inset of Fig. 5A). In the subsequent cycles, however, the ECL intensity dropped significantly, even disappeared. Because the oxidation of the DNA was irreversible and inherent ECL properties of the adsorbed DNA on the Au electrode was weak, therefore, after the first voltammetric cycle, the electrode behaved just liked a bare one and nearly no DNA was adsorbed on the electrode. Meanwhile, it still presented no ECL signal after the electrode being immersed in the pure TEA solution for 2 h (Fig. 5A-b). According to the comparison results, it clearly indicated the non-absorption of TEA on the electrode surface. This conclusion was again confirmed by the typical EC experiments: upon immersion in the SH-DNA containing TEA solution for two hours, the current response of the Au electrode in 1.0 mM $Fe(CN)_6^{3-/4-}$ containing 100 mM KCl was significantly decreased (Fig. 5B-c). This indicated the changing surface structure of the electrode, on which SH-DNAs self-assembled on the electrode through the Au–S interaction [19]. Meanwhile, no obvious current response change was observed before and after being immersed in pure TEA solution (Fig. 5B-a and B-b), indicating no surface structure change of the Au electrode. This meant no TEA was adsorbed on the electrode surface during the process of preparing this modified electrode. Thus, when the DNAs with TEA impurity were applied for electrode immobilization or modification, for example, ECL biosensors using Au electrode, because TEA will not adsorb on the electrode surfaces, TEA will not affect the result of biosensor preparation and further experiment results.

All in all, influence or not will depend on the actual situation. When ECL experiments were carried out, the impurity of DNA should be paid more attention to.

4. Conclusions

In the present work, one impurity in synthetic DNAs that might influence the experiment results of ECL detection techniques was discovered. According to a series of experiments using ECL detection methods as well as combined with CE, the impurity was identified as TEA, which was from incomplete removal after HPLC purification of synthetic DNAs. In addition, the CE–ECL technique was proposed to discover, identify and sensitively determine

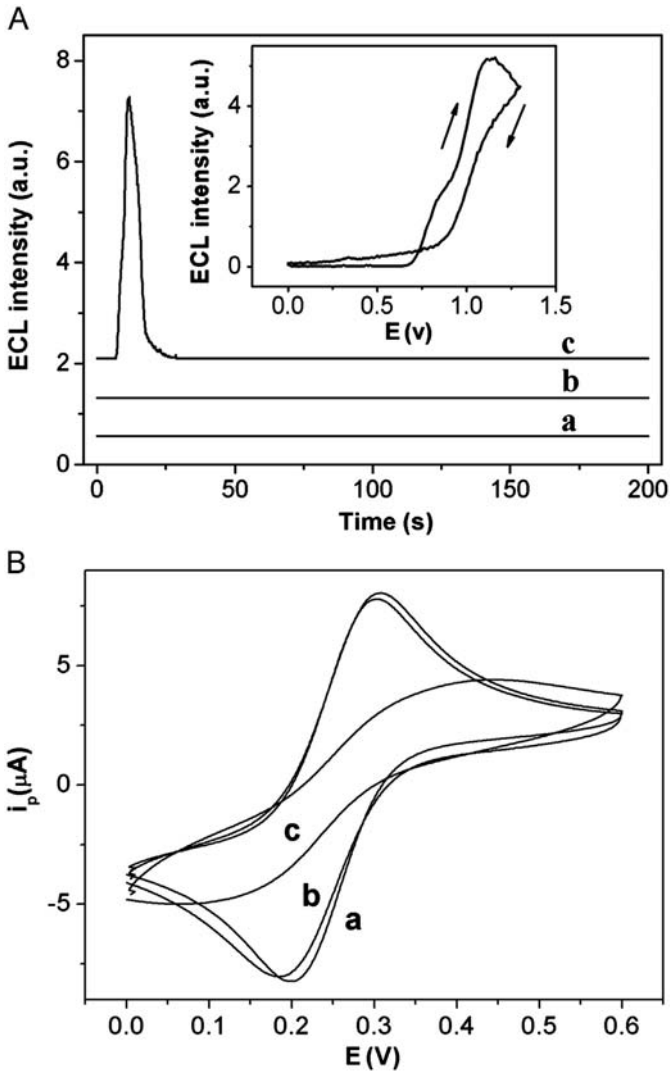

Fig. 5. (A) ECL signals for 1.0 μM $Ru(bpy)_3^{2+}$ solution containing 100 mM PBS (pH 7.4) with scanning range of 0–1.3 V and (B) Cyclic voltammograms of 1.0 mM $Fe(CN)_6^{3-}/^{4-}$ containing 100 mM KCl with scanning range of 0–0.6 V at Au electrode (a) before and after being modified with (b) 10 μM TEA or (c) 1.0 μM SH-Random DNA-2 containing 10 μM TEA, respectively. Scan rates: 100 mV s^{-1}. Inset of (A): ECL intensity-potential curve of the first cycle for 1.0 μM $Ru(bpy)_3^{2+}$ solution containing 100 mM PBS (pH 7.4) at Au electrode after being modified with 1.0 mM SH-Random DNA-2 containing 10 μM TEA.

possible impurity such as TEA in synthetic DNAs, which was usually neglected by other detection techniques for purification quality control of synthetic oligonucleotides. Due to fast separation, lower detection limits, good selectivity, high sensitivity and effective resolution of CE–ECL technique [12], it has the great potential to be a powerful tool for analyzing other ECL active impurities in synthetic oligonucleotides. According to the designed ECL and EC methods and data analysis, situations which will be really affected by the impurity of TEA were studied and discussed. Thus, by determining the amount of certain impurity, either rejecting or accepting the oligonucleotide samples for further experiments will be decided. It also provided with judgment basis for choosing purification ways or avoiding certain buffer components of HPLC for DNA purification according to individual requirements. Moreover, it will motivate the scientist to explore new alternative to TEA in the HPLC purification methods for synthetic oligonucleotides.

It is well known that a little neglect may breed great mischief. Thus, this work reminds the researchers that to obtain accurate experiment results, attention should be paid to throughout all the synthetic oligonucleotides-related processes including synthesizing, purifying, quality controlling and applying to obtain accurate results in the biological assays.

Acknowledgements

This work is supported by the National Natural Science Foundation of China with Grant Nos. 21190040 and 21075120.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.03.089.

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