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Research Article

# ssDNA degradation along capillary electrophoresis process using a Tris buffer

Tris-Acetate buffer is currently used in the selection and the characterization of ssDNA by capillary electrophoresis (CE). By applying high voltage, the migration of ionic species into the capillary generates a current that induces water electrolysis. This phenomenon is followed by the modification of the pH and the production of Tris derivatives. By injecting ten times by capillary electrophoresis ssDNA (50 nM), the whole oligonucleotide was degraded. In this paper, we will show that the Tris buffer in the running vials is modified along the electrophoretic process by electrochemical reactions. We also observed that the composition of the metal ions changes in the running buffer vials. This phenomenon, never described in CE, is important for fluorescent ssDNA analysis using Tris buffer. The oligonucleotides are degraded by electrochemically synthesized species (present in the running Tris vials) until it disappears, even if the separation buffer in the capillary is clean. To address these issues, we propose to use a sodium phosphate buffer that we demonstrate to be electrochemically inactive.

**Keywords:**
Biomolecules / Capillary electrophoresis / Oligonucleotides / ssDNA degradation / Tris-Acetate buffer
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## 1 Introduction

It is well established that electrochemical phenomena happen beside electromigration and electroosmosis during capillary electrophoresis (CE). As the CE separation requires high voltage, to induce the migration of ionic species, an electric current is generated. On the electrodes, immersed in the buffer, electrolysis of water happens. In consequence, the pH in the buffer vials changes. This phenomenon is called buffer depletion and has been described by many authors [1–4]. The modification of the buffer pH is mainly due to the poor buffering capacity of the background electrolyte and also the small volumes of the vials. This can be overcome by a frequent refreshing of the buffer vials or, when possible, by changing the buffer composition. Among the commonly used buffers 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) is very popular and can be employed for many CE experiments run at pH around 8.5. The main application of this buffer concerns slab gels and CE applications in nucleic acids separations. Actually, the sentence "for reasons not fully evident today, Tris became established as the favored cation for DNA electrophoresis" opens the "Tris reigned" paragraph of an article concerning the history of DNA separations [5]. Today, most of DNA separations by gel and capillary electrophoresis use Tris based buffers. It is still commonly employed for ssDNA studies [6], PCR using CE [7], or on-line preconcentration studies of DNA fragments [8]. Twenty years ago, Ray et al [9, 10] have shown that during gel electrophoresis the DNA of two *Streptomyces* species undergo a Tris-dependent strand scission. The authors indicated that the nucleolytic activity acts at the modification sites and implies a peracid derivative of Tris. Formed at the anode, it can be chemically mimicked by addition of peracetic acid to Tris buffers. The aim of this "ancient" paper (only cited 46 times) was to prevent the electrophoretic community to neither use the Tris buffer nor amine containing buffers, but to prefer buffers using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). This recommendation was poorly followed by the analysts using CE. Indeed,

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**Abbreviations: CRB**, CE running buffer; **ECGS**, electrochemical generated species; **HRMS**, high resolution mass spectrometry; **NCRB**, non-CE running buffer; **Tris**, 2-amino-2-(hydroxymethyl)-1,3-propanediol

**Colour Online:** See the article online to view Fig. 3 in colour.

they claimed that the capillary diameter is small compared to the buffer vial volume, and that the electro-synthesized species of the back-ground electrolyte cannot interfere with the injected DNA.

In this paper, we will show that the same ssDNA degradation phenomenon can be observed and becomes very important for fluorescent ssDNA like aptamers analysis in CE using Tris buffer. We will show that this buffer is modified along the electrophoretic process by electrochemical reactions, as it was observed that the composition of the metal ion changes in the buffer vials. The electrochemically synthesized molecules are produced and pollute the sample even if the separation buffer in the capillary is clean.

## 2 Materials and methods

### 2.1 Chemicals

Tris (two different lots were studied), acetic acid, sodium phosphate dibasic anhydrous, sodium chloride, hydrochloric acid, magnesium chloride, sodium hydroxide, ammonium persulfate, xylene cyanol, and bromophenol blue were purchased from Sigma-Aldrich - Fluka (St Quentin Fallavier, France). Urea was from Euromedex (Souffelweyersheim, France), TEMED (N,N,N',N' Tetramethyl-1-, 2-diaminoethane) from Eurobio (Courtaboeuf, France), acrylamide (Fischer Scientific Illkirch, France). The Sequence of ssDNA was the following: 5′-AGTCCGTGGT AGGGCAGGTTGGGGTGACT-3′. ssDNA was labeled with a fluorophore, 6-carboxyfluorescein: FAM in 5′ end (Sigma-Aldrich—Fluka).

### 2.2 Instrumentation

An Agilent Technologies CE 7100 instrument was equipped with a 50 μm id fused silica capillary (Polymicro Technologies, Phoenix, Arizona) or a PVA coated capillary (Agilent, Walbronn, Germany) effective length 69 cm, total length 90 cm. Zetalif LED detector from Picometrics Technologies (Toulouse, France) was equipped with a LED at 480 nm. For some experiments, the electrodes were cleaned using ultrasonic bath in ethanol/water (50/50, v/v). CE experiments were performed with 50 mM Tris-Acetate pH 8.2 as BGE, the volume of the vials at anode and cathode was 1.5 mL (the volume of buffer that can be contained in a vial is 1.5 mL maximum). +30 kV for an uncoated capillary (current intensity: 30 μA) or −30 kV (current intensity: −30 μA) for a coated capillary. The 50 mM Tris-Acetate buffer at the inlet and outlet was formerly described for ssDNA analysis by CE [11].

Samples of ssDNA were prepared in 50 mM Tris-HCl with 100 mM NaCl, 1 mM $MgCl_2$. The volume of ssDNA sample is 20 μL. The ten injections of ssDNA (50 nM) were performed by pressure (50 mbar during 30 s). The duration of the entire process (injections and separations) was 300 min.

Polyacrylamide gel was prepared by weighing 10.5 g of urea diluted in 5 mL of Tris-Borate EDTA (TBE) 5×. A 6.25 mL of acrylamide 40% was added in the mixture. The reaction of polymerization was then initiated by the formation of free radicals by the persulfate ammonium (150 μL of 10% solution) and catalyzed by the addition of TEMED (15 μL). A 20 μL of ssDNA sample (50 nM) was hydrodynamically injected six times in CE and then dropped in the polyacrylamide gel. A fresh ssDNA sample is used as the reference. Samples of ssDNA were diluted in a buffer consisting of 95% of formamide, 20 mM of EDTA, 0.05% of xylene cyanol, 0.05%, bromophenol blue, and glycerol. A 20 μL of ssDNA at 50 nM was dropped on the polyacrylamide gel. Samples of ssDNA migrated in a TBE - 7M urea, buffer with 100 V. After migration, ssDNA samples were revealed with ethidium bromide under UV illumination or by fluorescent detection (λexc: 480 nm).

LC/MS experiments: UPLC Acquity Xevo G2 QTOF Waters, a column BEH C18 (1.7 μm; 2.1 × 15 mm) and a gradient ACN 3–30% in water with 0.1% formic acid in 4 min with a flow rate of 0.3 mL/min, bearing of 1 min at the beginning, were used at T = 35°C. Mass spectrometry calculations were done using ChemCalc, [12].

A Thermo-Finnigan Neptune MCICP-MS was employed to determine the relative concentrations of elements in the Tris solutions. These solutions were diluted with precision between 4.5 and 5.1 times in 2% $HNO_3$.

## 3 Results and discussion

### 3.1 ssDNA degradation in Tris buffer during CE

During our work, we washed the uncoated or coated capillary with a Tris buffer which was never submitted to the high voltage (HV). The anode and cathode vials during the separation (with the HV) were changed every ten runs. When we injected several times (around ten injections) a sample containing 50 nM solution of a FAM labeled ssDNA, we observed a decrease of the ssDNA peak until its extinction was raised. A concomitant increase of two peaks was observed (Fig. 1A). Meanwhile, the pH of the anodic vial decreased slightly (from 8.7 to 8.3) after ten separations of 30 min each. The ssDNA degradation phenomenon was also observed using the PVA coated capillary. In these analyses, the electroosmotic flow is negligible and doesn't change the migration time of the ssDNA. These analyses confirm that the ssDNA is degraded.

We decided to load ssDNA samples on a denaturing urea polyacrylamide gel, to control its size after one injection and after six injections by CE. Two samples of ssDNA diluted in Tris-Acetate buffer were analyzed: the first one is the control ssDNA CE sample (50 μL) with the non-CE run buffer (NCRB) (Fig. 1B - lane a): this sample was injected

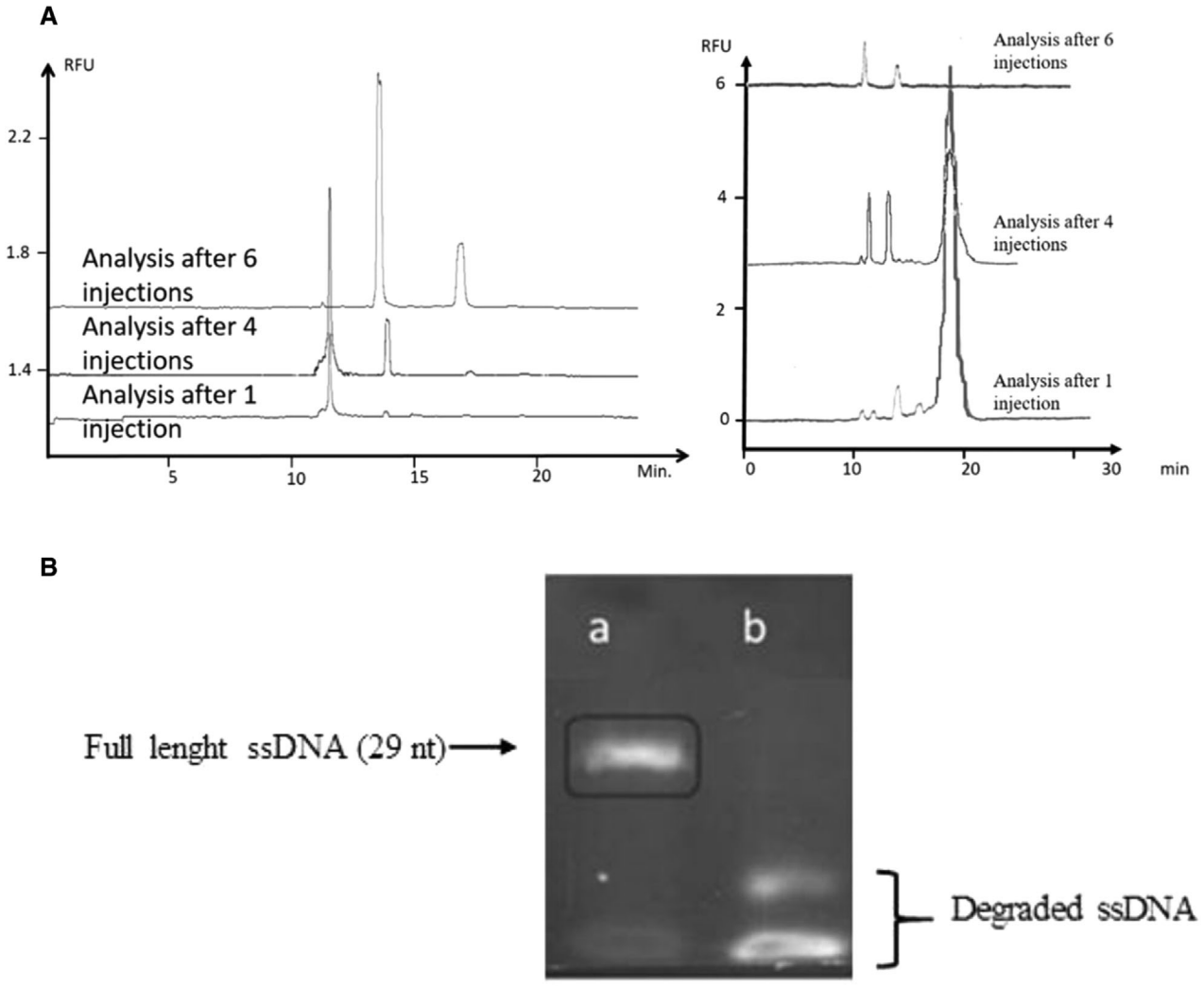



**Figure 1. (A)** Analysis of ssDNA in Tris-Acetate migration buffer (50 mM pH 8.2) during six injections with a 50 μm id, PVA coated capillary (Fig. 1A on the left). Identification of degradation peaks at 14 and 17 min. The same experience was realized with an uncoated capillary with a 50 μm id (Fig. 1A on the right) and ssDNA injected six times. Identification of degradation peaks at 11 and 13 min. **(B)** polyacrylamide gel 7 M urea of (**a)** the ssDNA sample injected six times in CE. The revelation of the fluorophore (FAM) is performed with UV at 480 nm. Two fluorescence spots are observed at low size, but no smear of ssDNA is observed which indicates that the ssDNA is affected by electrochemical radicals and degraded. (**b)** ssDNA-FAM not injected in CE.

one time and the electropherogram showed any degradation. The second one is the same ssDNA CE sample but injected six times in CE, with the same CE run buffer (CRB) at anode and cathode: the electropherogram confirmed its degradation and the gel-run with the residual sample in the vial (injected 6 times) demonstrated a complete alteration (Fig. 1B - lane b). We could only detect, by fluorescence at 480 nm two fluorescent signals with low DNA size. These two fluorescent signals corresponded to free fluorophore and to very small DNA fragments. When ethidium bromide was used to detect the ssDNA after 6 CE injections, no band could be identified, while the control ssDNA can be observed (data not shown) indicating the smallness of DNA fragments. This observation highlights that the ssDNA sample was affected by the CE buffer and degraded into the vial. We assumed that electrochemical generated species (ECGS) were produced from the Tris inside the electrode vials and proceed on ssDNA in the vial. Furthermore, we have shown that the ssDNA degradation was associated with its concentration. When the concentration of ssDNA was increased (from 50 nM to 1 μM), the degradation process was lengthened. This was probably due to the fact that we used the same final concentration of Tris and therefore the amounts of ECGS were the same. Thus, with a larger amount of ssDNA (1 μM injected), the ECGS mediated degradation occurs but slower: the ssDNA peak is constant during 13 injections and then decreases rapidly (data not shown).

To better identify the electrochemical processes in the buffer vials, we measured the influence of the electrode material in the buffer submitted to electrophoresis.

### 3.2 Analysis of Tris buffer before and after CE separation by ICP/MS

What is the behavior of the electrode along the CE run? The electrodes of the CE instruments are made of platinum/iridium. Nothing is published about how the Pt/Ir electrode can act during the electrophoresis. Therefore, we wanted to know how the electrode is affected during the run. We measured the different amounts of sodium, potassium, platinum, and iridium in Tris vials (cathode and anode) after 200 min of the high voltage CRB, compare to the same vials before the CE running NCRB. Compared to the initial value (NCRB), we observed an increase of 55% $Na^+$ (70 μM) in the anode CRB due to traces of NaOH which was used to wash the capillary. As washes were done with an empty vial at the cathode, we did not have any increase of $Na^+$ in the cathode CRB compare to the NCRB. An increase of 33% of potassium (35 μM) at the anode compared to the cathode was more surprising, because we used potassium buffer several weeks ago (no increase at the cathode). In consequence, the behavior of sodium and potassium on the platinum/iridium electrode showed a retentive effect. It seems that these ions are adsorbed on the electrode and are only slowly released.

We also noticed an increase of 5 and 6-fold of the Si at the anode and cathode respectively, compared to NCRB. The huge increase of the concentration of Pt (estimated concentration at the anode 25 nM, more than 2220- and 1200-fold at the anode and the cathode respectively) and Ir (8 nM, 2000, 800) showed that an oxidation was realized from the surface of each electrode. It was known that when the Pt was used for electrochemical reactions, it was first oxidized. Then the PtO surface electro-reacted with the electrolyte [13]. In our case the electro-osmotic flow and electromobility help the metallic species to move to the cathode.

We conclude that during the CE process, even if the current was very low (30 μA), we observed some amounts of Pt

**Table 1.** List of the principal chemical formulas of molecules (ECGS) identified, analyzed in $ESI^{+/-}$HRMS at the anode and cathode vials of Tris. DB, number of double bonds

| Experimental mass | Absolute intensity | Theoretical mass | Proposed chemical formula | DB | Δ (mDalton) |
|---|---|---|---|---|---|
| **Anode ESI+** | | | | | |
| 103.0623[c] | 1.3 E4 | 103.0633 | C4H9NO2 | 1 | −1.0 |
| 113.0851[b] | 4.0E3 | 113.0840 | C6H11NO | 2 | 1.1 |
| 121.0749[a] | 2.11 E4 | 121.0739 | C4H11NO3 | 0 | 1.0 |
| 124.0644 | 3.3 E4 | 124.0636 | C6H8N20 | 4 | 0.8 |
| 131.0593 | 1.10 E4 | 131.0582 | C5H9NO3 | 2 | 1.10 |
| 139.0630 | 2.7E3 | 139.0633 | C7H9NO2 | 4 | −0.30 |
| 145.0752 | 5.2E3 | 145.0738 | C6H11NO3 | 2 | 1.40 |
| 157.0751 | 2.4 E4 | 157.0739 | C7H11NO3 | 3 | 1.21 |
| 173.0684 | 3.8E3 | 173.0688 | C7H11NO4 | 3 | −0.40 |
| 175.0861 | 9.8E3 | 175.0844 | C7H13NO4 | 2 | 1.7 |
| 205.0285 | 1.7E4 | 205.0276 | C9H5N2O4 | 8.5 | 0.9 |
| 210.1026 | 2.8E3 | 210.1004 | C10H14N2O3 | 5 | 2.2 |
| 228.1132 | 3.2E3 | 228.1110 | C10H16N2O4 | 4 | 2.2 |
| 244.1064 | 2.2E3 | 244.1059 | C10H16N2O5 | 4 | 0.5 |
| 294.147 | 2.2 E4 | 294.1440 | C12H18N6O3 | 8.5 | 3.0 |
| a: Tris ; b : impurity in Tris ; c: this molecule corresponds to an ion after the loss of $H_20$ of Tris, in the ESI+ source. | | | | | |
| **Cathode ESI+** | | | | | |
| 117.0588 | 0.9 E3 | 117.0578 | C8H7N | 6 | 1.0 |
| 131.0596 | 3.5 E3 | 131.0582 | C5H9NO3 | 2 | 1.4 |
| 133.0749 | 2.6 E3 | 133.07389 | C5H11NO3 | 1 | 1.0 |
| 157.0753 | 6.7 E3 | 157.0738 | C7H11NO3 | 3 | 1.4 |
| 159.0225 | 3.2 E3 | 159.01945 | C8H3N2O2 | 8.5 | 3.0 |
| 196.0843 | 5.0 E3 | 196.0848 | C9H12N2O3 | 5 | 0.5 |
| 206.0146 | 1.7 E3 | 206.0175 | C5H6N2O7 | 4 | 2.9 |
| 214.0955 | 4.2 E3 | 214.0953 | C9H14N2O4 | 4 | 0.1 |
| 244.1064 | 2.5 E3 | 244.1059 | C10H16N2O5 | 4 | 0.5 |
| **Anode ESI-** | | | | | |
| 113.9928[b] | 4.0 E4 | 113.9953 | C4H2O4 | 4 | 2.5 |
| 121.0753[a] | 1.9 E4 | 121.0749 | C4H11NO3 | 0 | 0.4 |
| 135.0554 | 4.9 E4 | 135.0531 | C4H9NO4 | 1 | −2.2 |
| 149.0698 | 4.3 E4 | 149.0688 | C5H11NO4 | 1 | −1.0 |
| 258.0978 | 3.1 E5 | 258.0978 | C11H16NO6 | 4.5 | −0.0 |
| 260.0951 | 1.0 E5 | 260.2652 | C14H14NO4 | 8.5 | −2.8 |
| 268.1265 | 2.1 E5 | 268.1271 | C9H20N2O7 | 1 | 0.5 |

a: Tris; b: impurity in the Tris, greyed lined are identical chemical formulas in anodes and cathode

(or PtO) and Ir which were displaced into the buffer. This highlights that the electrochemical processes can be important on the electrode during CE.

### 3.3 Analysis of ECGS by HRMS

Using UPLC/ESI$^+$HRMS or UPLC/ESI$^-$HRMS we identified different compounds in the Tris buffer. The major one was the Tris ($C_4H_{11}NO_3$) itself.

After 10 runs of the same Tris CRB from the anode and cathode vial, were analyzed in ESI$^+$/HRMS and MS/MS. Its composition was slightly changed. We identified at least 24 new compounds. Table 1 lists the different chemical formulas of the molecules which were identified, while we proposed some structures considering the reaction between different Tris molecules, in Fig. 2. We noticed that we obtained unsaturated molecules. The UV spectrum at an absorption wavelength of 280 nm confirmed the formation of aromatic molecules.

Using ESI$^-$ ionization, new compounds were identified, and presented in Table 1. The $C_4H_2O_4$ acid corresponded to the oxidation of a Tris molecule and the two other compounds resulted in the condensation of at least three Tris molecules. One corresponded to the mass of a radical which can be stabilized thanks to electron conjugation. None peracidic species could be identified.

In the literature, to our best knowledge, we did not identify a work reporting on the electrochemical modification of the Tris molecules. It was well known that phenolic compounds can be oxydized at the platinum anode. Actually, when phenol is adsorbed on the electrode it becomes conductive and its oxidation involves ring cleavage, leading to simple organic acids. It can also be oxidized in hydroxiquinone, benzoquinone, phenolic ether, biphenol, and polymers, induced by phenoxy radicals [14]. Diols were also oxidized and resulted in lactones [15]. Likewise, another well-known electrochemical oxidation was the Shono oxidation. Shono's protocol generates N-acyliminium ions from the oxidation of amides [16]. Polyaniline was reported to be a very good conducting

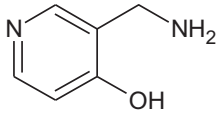
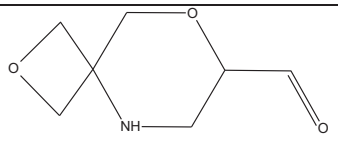
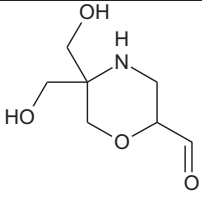
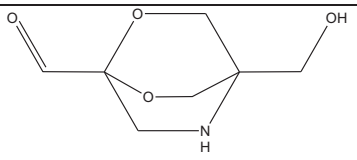
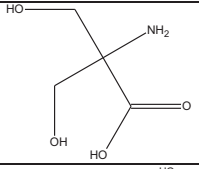
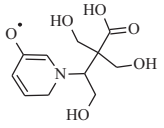
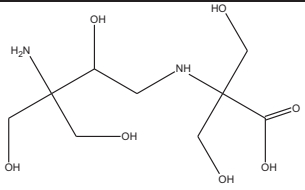
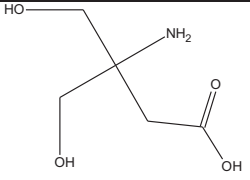
| Chemical structure | $M_{exp}$ | $M_{exp}$ | Chemical structure |
|---|---|---|---|
| **Cathode** | | | |
|  | 124.0644 | 157.0751 |  |
|  | 175.0861 | 173.0684 |  |
| **Anode** | | | |
|  | 135.0531 | 258.0978 |  |
|  | 268.1265 | 149.0688 |  |

**Figure 2.** Proposal of some chemical structures of molecules identified in ESI$^+$ and ESI$^-$.

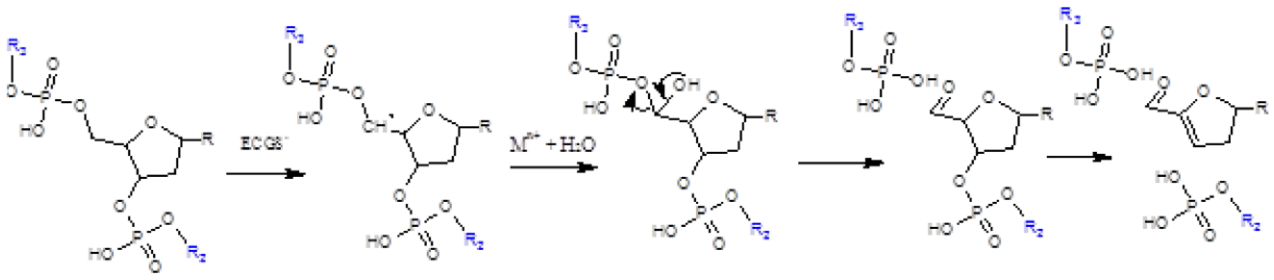



**Figure 3.** Proposal of chemical reactions of degradation of ssDNA following the scheme proposed by [21]. R = nucleic base, R2 and R3 are the following nucleotides.

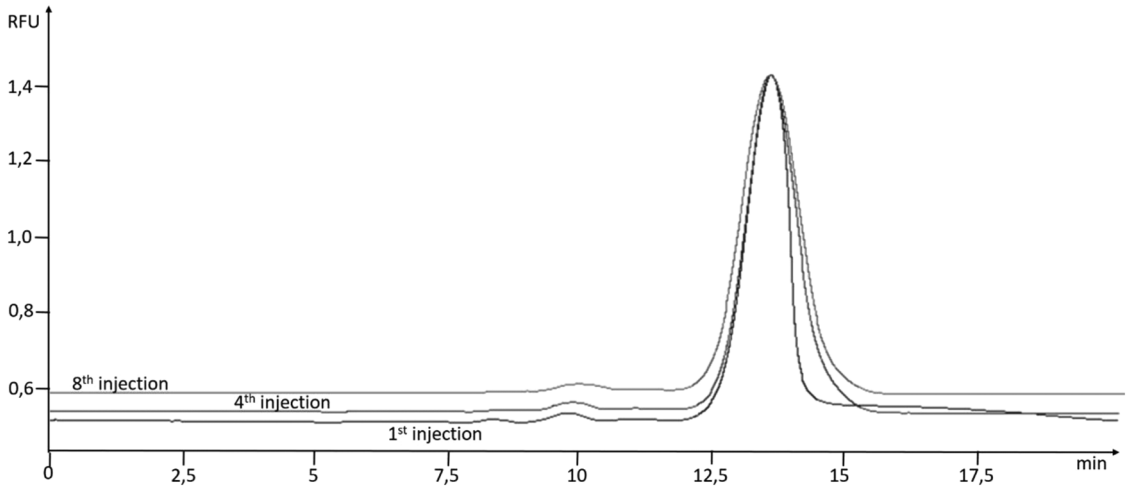



**Figure 4.** Electropherogram of ssDNA in 50 mM phosphate buffer (pH 8.6) after one, four, and eight injections.

organic material, and can be synthesized on the Pt electrode. It has been shown that with amine, the reactivity of radicals can also have induced such polymerization reactions to obtain polyaniline [17]. Such electrochemical reactions were also observed with pyrrole [18] or 1, 5diaminonaphtalene [19]. In consequence, many compounds can be synthetized using electrophoresis in the buffer. All these studies highlighted that more or less complex electrochemical reactions can occur on aromatic compounds. From our results, even if Tris was not an aromatic molecule, we showed that we had also a very high number of different electrochemical reactions.

Among the molecules which were electrochemically synthesized, two radicals can be identified at the anode while we had only one at the cathode. We can imagine that the radicals presented in Fig. 2 could initiate diverse reactions on the ssDNA.

We supposed that we can have the following reactions at the anode:

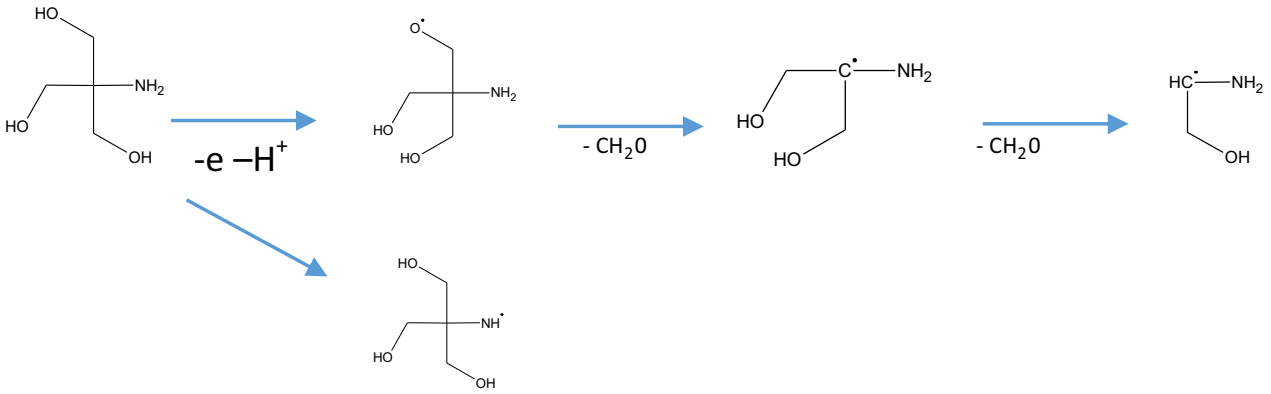
**Reactions of Tris at the platinum anode**

The four radicals can react on Tris and form different adducts able to lose $H_2O$, CO, $CO_2$, $NH_3$ and other simple molecules. We expected that these radicals reacted also with Tris molecules and result in products which can evolve in the different C5 to C11 compounds of Table 1. These molecules can as well lose electrons and undergo other electrochemical reactions.

The cathode CRB composition also differed from the initial Tris sample. It contained low quantities of the compounds identified at the anode vial, certainly thanks to the electroosmotic flow. We found molecules ranging from C5 to C10, and an aromatic molecule ($C_8H_7N$). In the literature, the cathodic reduction of quinones was well documented [20, 21], it showed that ECGS can also be synthetized at the cathode.

Finally, we remarked that some impurities were present in the commercial Tris. Low amounts of $C_6H_{11}NO$ (an amine which can be seen in $ESI^+$) and $C_4H_2O_4$ (an acid visible in $ESI^-$) were present even before electrophoresis. These molecules could also have some importance in the described electrochemical synthesis.

Because radicals were produced as well in the anodic as in the cathodic vials, several side reactions will be undergone in presence of substrate. These radicals can react on ssDNA in the presence of metals (Pt, Ir) and degrade it totally, as mentioned in many papers [22]. As reported elsewhere, we present in Fig. 3, a proposal of the chemical reactions which explains the total degradation of the 50 nM ssDNA sample.




A question remains: how long can the ECGS radicals, produced in the running buffer, react on ssDNA?

### 3.4 How stable are the actives ECGS molecules?

To investigate the stability of the ECGS molecules, a Tris-Acetate buffer was run with the CE during 400 min. A first separation of the ssDNA was run with this 400 min buffer by injecting a 50 nM ssDNA sample. The second separation was run 24h later with this same Tris buffer (run during 400 min, 24 h sooner) and a novel ssDNA (50 nM). In the first run, we only observed the degradation peak, the ssDNA peak disappeared. In the second run, we observed the degradation peak at the same migration time but the ssDNA peak decreased only of a factor 2 compared to the undegraded ssDNA.

This experience showed that ECGS were quite stable molecules because the half of ssDNA was still degraded when the buffer was rested.

### 3.5 The use of phosphate buffer

Our results obtained with Tris-Acetate buffer showed that this buffer was not appropriate for separating low concentrations of ssDNA. So, we decided to use a buffer without nitrogen atoms. We chose a phosphate buffer (pH 8.6) as propose by Kanoatov and Krylov [23]. To prove the efficiency of phosphate buffer, we prepared different concentrations (50, 25, 10, and 5 mM). As the different concentrations of phosphate buffer did not induce the ssDNA degradation (Fig. 4), we preferred to use 50 mM concentration because the buffer capacity is better. In these conditions, we did not observe the degradation of ssDNA after eight injections in phosphate buffer corresponding to 160 min of CE runs while it was observed after six injections in Tris buffer (corresponding to 180 min of CE runs).

## 4 Concluding remarks

Tris buffer is degraded during the CE experiments; many different compounds are electrochemically synthetized. Among them we were able to identify radical species. These compounds can react on the ssDNA and induce its total degradation. When the ssDNA from the sample vial is observed using slab gel separation after 10 runs, no ssDNA can be observed; solely the fluorescein is detected. This shows that the degradation of the ssDNA is induced in the vial. Using a phosphate buffer, no ssDNA degradation is observed.

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