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**Integrated microfluidic device for the separation,
decomposition and detection of low molecular weight
S-nitrosothiols**

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Abstract

S-nitrosothiols (RSNOs) are considered as biological circulating stocks of nitric oxide (NO) that have many roles *in-vivo*. The variation of RSNOs proportion occurs in several diseases, which makes them potent biomarkers. The identification and quantitation of each RSNO is therefore important for biomedical studies. For now, miniaturized devices have been used to detect RSNOs, based on their total quantitation without a preceding separation step. This study reports on an original and integrated microdevice allowing for the successive separation of low molecular weight RSNOs, their decomposition under metal catalysis, and the quantitation by amperometric detection of the produced nitrite, leading to their quantitation in a single run. For this purpose, a commercial SU-8/Pyrex microfluidic system was coupled to a portable and wireless potentiostat. Different operating and running parameters were optimized to achieve the best analytical performance allowing for LODs of 20 μM . The simultaneous separation of S-nitrosoglutathione and S-nitrosocysteine was successfully obtained within 75 s.

Introduction

S-Nitrosothiols (RSNOs) are nitric oxide (NO) carrier molecules that play important roles in several physiological functions (vasodilatation and relaxation^{1, 2}, antiplatelet aggregation^{3, 4}, antimicrobial⁵, regulation and signaling protein function⁶...) and pathological events (neurodegenerative diseases such as Parkinson and Alzheimer⁷, apoptosis⁸, chronic obstructive pulmonary disease⁹, preeclampsia¹⁰, diabetes¹¹...). RSNOs can be divided into low molecular weight (LMW) and high molecular weight (HMW) RSNOs. Although there is no defined border in terms of molecular mass, it is common to use the term “low molecular weight” for peptides and aminoacid S-nitrosothiols (such as S-nitrosoglutathione (GSNO) and S-nitrosocysteine (CySNO)) and “high molecular weight” for s-nitrosylated proteins (such as S-nitrosoalbumin (AlbSNO) and S-nitrosohemoglobin (HbSNO)). RSNOs store, transport and release NO. They can also interchange NO through transnitrosation reaction¹³. The variation of RSNOs concentration has been shown to occur in many diseases¹². For all these reasons, the development of powerful methodologies for the simultaneous quantitation of all RSNOs in a biological sample is crucial.

Numerous methods have been developed for RSNOs analysis, based on direct or indirect detection. **Examples of direct detection** consist in the

separation of RSNOs from other species by capillary electrophoresis (CE) or liquid chromatography followed by mass spectrometric or spectrophotometric detection¹⁴. For example, the simultaneous separation of S-nitrosogluthathione (GSNO) and S-nitrosocysteine (CySNO) was performed by our group using conventional CE equipped with capacitively coupled contactless conductivity detection but in a conventional system¹⁵. In another work, the simultaneous separation of GSNO, GSH, glutathione sulfonic and sulfinic acid by CE coupled to mass spectrometry was obtained¹⁴. Most standard methods developed for RSNOs quantitation reported in the literature remain indirect. They are based on the detection of their decomposition products¹⁶, through homolytic or heterolytic cleavage of S-NO bond generating NO or NO⁺ leading finally to NO₂⁻. These decomposition products are then detected by spectrophotometry, fluorimetry, electrochemistry or chemiluminescence^{16, 17}. Various reagents have been used to decompose RSNOs, such as metal cations¹⁸ (Hg²⁺, Cu⁺), light¹⁹ and heat²⁰, leading to different decomposition products: NO is generated¹⁸ if Cu⁺, light or heat are employed, whereas nitrite is directly generated^{18, 21} when Hg²⁺ or Ag⁺ are used.

Nowadays, miniaturization in chemical analysis has become a powerful tool contributing to reduce the samples/reagents amount, analysis time and

77 waste generation. Such an approach can be beneficial for the quantitation
78 of RSNOs. Indeed, our group has recently reported on the colorimetric
79 analysis of RSNOs in a microfluidic paper-based analytical device²². This
80 system allowed to perform analysis of total RSNOs in plasma samples
81 without any separation step. Other approaches were proposed by Hunter et
82 al. for NO²³ and total RSNO detection²⁴ (after light decomposition) using a
83 single PDMS microfluidic channel with amperometric detection. In all cases
84 no separation of RSNOs occurred before detection in these miniaturized
85 devices. Also, Gunasekara et al²⁵ used microchip capillary electrophoresis
86 (MCE) with amperometric detection to separate a NO donor
87 (DEA-NONOate or Proli-NONOate) from NO and nitrite in less than one
88 minute. Tu et al.²⁶ used MCE with fluorescence detection to separate and
89 detect NO, reduced glutathione (GSH) and cysteine (Cys). Herein we report
90 the design and optimization of a single-run MCE analytical strategy allowing
91 for the first time the simultaneous quantitation of two low molecular weight
92 RSNOs (S-nitrosoglutathione and S-nitrosocysteine), thanks to the
93 integration of successive electrokinetic separation, RSNO decomposition by
94 Hg²⁺ to nitrite and nitrite quantitation by amperometry.

Experimental

Chemicals

All reagents were of analytical grade and used as received. L-arginine (ARG), 2-(N-morpholino) ethanesulfonic acid (MES), acetic acid (HAc), L-histidine (His), sodium tetraborate, Sodium Nitrite, N-acetyl-p-aminophenol (Paracetamol), Mercury(II) Chloride, EDTA, hydrochloric acid, sodium phosphate monobasic, sodium phosphate dibasic, L-cysteine (Cys) and reduced glutathione (GSH), were purchased from Sigma Aldrich (St. Louis, MO, USA). All aqueous solutions were made using ultra-pure water with a resistivity of 18.2 MΩ.cm from a Pure Lab Flex system (ELGA Labwater, France).

Synthesis of S-nitrosothiols

GSNO was synthesized as described elsewhere²⁷. Briefly, an equimolar amount of nitrite was added to the equimolar amount of GSH and HCl. The resulting pure solid was rinsed once with 80% acetone, twice with 100% acetone and three times with diethyl ether and then stocked in the dark at 20 °C.

S-Nitrosocysteine (CySNO) was daily synthesized using the method described by Peterson and coworkers²⁸. Briefly, solutions of 5 mM CysNO

were prepared by reacting cysteine with an equimolar concentration of nitrite in acidic medium (0.1 M HCl) in a dark flask to avoid light decomposition. After 5 min, more than 90% of cysteine was converted into CysNO. The solution was neutralized by 0.1 M PBS buffer (pH 7.4) containing 0.5 mM EDTA to prevent decomposition by trace metal cation contaminants.

Final concentrations of RSNOs were determined spectrophotometrically in aqueous solution at 335 nm ($\epsilon = 586$ and $503 \text{ M}^{-1} \text{ cm}^{-1}$ for GSNO and CysNO, respectively)²⁹.

Instrumentation

Electrophoretic experiments were performed using a SU-8/Pyrex microchips with integrated micro band platinum electrodes at the outlet end of the separation channel from Micrux Technologies (Oviedo, Spain) (MCE-SU8-Pt001T) (**Figure 1**). Only working (WE) and reference (RE) electrodes were used, with widths of 50 μm and 250 μm , respectively. The separation and injection channel length were 35 mm and 10 mm, respectively. The microchannels width and depth were 50 μm and 20 μm , respectively. A microfluidic platform (Oviedo, Spain) (MCE-HOLDER-DC02) was used to interface the microchip with the amperometric detector and the high voltage

source. The high voltage source was a programmable HVS448-3000V 8-channels high-voltage supply (LabSmith Inc., CA, USA) controlled by Sequence software v.1.165. Amperometric detection was performed by a modified model 9051, 2-channel, wireless, portable and electrically isolated potentiostat (Pinnacle Technology, Lawrence, KS. USA) operating in a two-electrode format at a 5 Hz sampling rate (gain=5 000 000 V/A, resolution= 30 fA). This potentiostat is isolated which eliminates interferences from the high voltage power supply system used for the separation. This potentiostat was controlled by Sirenia Acquisition Software v.1.7.6. The WE and RE were connected to the corresponding electrodes using the commercial chip holder.

Figure 1 - Schematic presentation of A) dimensions and B) design of Micrux MCE-SU8-Pt001T chip. Adapted from ³⁰

C⁴D detection was performed using a commercial detector model ER815 acquired from eDAQ Pty (Denistone East, Australia). A microfluidic

platform EDAQ ET121 containing external electrodes was used to interface a commercial PMMA microchip (model 02-0750-0082-01, ChipShop, Jena, Netherlands) with the detection system. This microchip layout comprised separation and injection channels (50 μm wide/deep) with 87 and 10 mm long, respectively.

Electrophoresis and Decomposition procedure

Prior to analysis, microchannels were conditioned with 0.1 M NaOH, deionized water and running buffer. Samples of RSNOs and paracetamol (1 mM each) were electrokinetically injected by gated mode³¹ by applying potentials of 800 V and 1000 V to sample and buffer reservoirs, respectively, while both waste reservoirs were grounded for the loading step. The injection was performed by floating the potential at the buffer reservoir for 3 s, giving the start of the separation step. The same procedure but under reversed polarity was performed for nitrite (1 mM) quantitation. For the decomposition step, HgCl_2 (10 mM) was added in the detection reservoir. Before RSNOs reach the buffer waste reservoir the polarity was inverted allowing detection of nitrite generated from decomposition. For amperometric detection of nitrite and paracetamol, potentials from 0.7 to 1.2 V vs. Pt were applied. Analysis using C^4D detection was realized under the same electrophoretic conditions and

170 detection was performed applying a sinusidal signal of 600 kHz and
171 90 V_{peak-to-peak}.

Results and discussion

Herein a method for successive separation, decomposition, and detection of RSNOs is proposed by integrating all the three steps in a microfluidic device. In order to achieve this goal, the decomposition should be (i) much faster than the separation process (otherwise the peaks originating from different RSNOs will overlap) (ii) quantitable and (iii) the decomposition product should be stable through the analysis time and operating conditions. It is well known that the decomposition product of RSNOs depends on the decomposition agent. As indicated above, the use of Cu^+ or light leads to homolytic cleavage and to the formation of NO, while the use of Hg^{2+} leads to heterolytic cleavage and to the production of NO^+ that transforms immediately to NO_2^- . Decomposition of RSNOs by light is slow (tenth of minutes are needed to decompose the sample¹⁵) and only partial decomposition of RSNOs have been obtained by Hunter et al²⁴ in a microfluidic device using 530 nm LED. Cu^+ decomposition is faster than light decomposition but still insufficient ($>2 \text{ min}^{32}$) in comparison with the separation time scale. Moreover Cu^+ is poorly soluble and stable in aqueous solution and it is usually produced by reduction of Cu^{2+} with reducing agent such as GSH. Decomposition using Cu^{2+} is affected by the variation of GSH concentration in the sample, which is difficult to control³². Decomposition

using mercuric (II) is instantaneous leading to NO_2^- which is stable and electroactive³³. Consequently, Hg^{2+} was chosen as the decomposition agent.

BGE plays an important role in the migration and electrochemical detection steps in micro chip electrophoresis (MCE)^{34, 35}. As the objective was the detection of nitrite generated from RSNO decomposition, the BGE optimization was focused on nitrite signal/noise (S/N) ratio during detection. Several BGEs usually used for biological samples during MCE were tested : 20 mM MES / His (pH 6.0), 20 mM MES / Arg (pH 7.5), 20 mM Arg / Acetic Acid (pH 5.8). Nitrite (1 mM) was injected in the gated mode (see experimental section), separated and detected by amperometry using these various BGEs. For each BGE, the detection potential applied between WE and RE was varied from +0.5 V to +1.5 V keeping constant same electrophoretic injection and separation conditions. The optimal potential for nitrite detection in all BGE was 0.7 V. **Figure 2** shows electropherograms for the separation of nitrite in various BGE. 20 mM MES/His (pH. 6.0) provided the highest amperometric detection signal. However, it was not selected for this design as Hg^{2+} , that will be used as the decomposition reagent for RSNO, reacts with histidine to form a precipitate. Although MES/Arg leads to the highest signal intensity, it however results in the

lowest signal to noise ratio. Therefore, the selected BGE, leading to the highest S/N ratio without interference with other molecules in the solution, was 20 mM Arg adjusted to pH 5.8 with HAc.

Figure 2 – Electrophoretic separation of 1 mM nitrite in SU-8/Pyrex microchip. BGE: MES (20 mM)/His (20 mM) pH 6.0 in blue, MES (20 mM)/ Arg (20 mM) pH 7.5 in red, Arg (20 mM) pH 5.8 adjusted with Acetic Acid in green. Gated injection V1=-800V, V2= -1000V, injection time 3s, successive injections: 70s, detection 0.7 V vs Pt.

For an optimized on-chip integration of the three steps involved in RSNOs characterization (separation, decomposition and detection) in the microchip, the apparent mobilities of each of the compounds (different RSNOs, nitrite, mercury) were determined. The overall procedure was first optimized for GSNO, as it is the most abundant low molecular weight RSNO. **Control experiments by direct addition of nitrite into the BW reservoir, with or without application of electrophoretic voltage resulted in an amperometric**

228 signal, proving the efficiency of the electrochemical detection step. A
229 neutral electroactive marker (paracetamol) allowed determining the
230 electroosmotic mobility as $1.85 \pm 0.07 \times 10^{-4} \text{ cm}^2.\text{V}^{-1}.\text{s}^{-1}$ and GSNO
231 electrophoretic mobility was determined as $-0.64 \pm 0.06 \times 10^{-4} \text{ cm}^2.\text{V}^{-1}.\text{s}^{-1}$,
232 employing a C⁴D detector, as GSNO is not electroactive (results not
233 shown).

234 Under these experimental conditions, GSNO migrates towards the detector
235 under a positive polarity. The device was primarily developed as follows:
236 GSNO was electrokinetically injected from the sample reservoir (S) in the
237 gated mode in positive polarity (see experimental section). Hg²⁺ was
238 introduced in the waste reservoir (connected to the cathode) where it
239 should decompose GSNO into nitrite upon reaching the buffer waste
240 reservoir (BW). However, no amperometric signal was observed (results not
241 shown). One hypothesis is based on the fact that Hg²⁺ undergoes diffusion
242 from the BW within the separation channel, inducing the GSNO
243 decomposition within the separation channel. As nitrite electrophoretic
244 mobility under these experimental conditions ($-4.25 \times 10^{-4} \text{ cm}^2.\text{V}^{-1}.\text{s}^{-1}$) is
245 higher in absolute value than the electroosmotic mobility, nitrite moves back
246 to the sample reservoir (S) instead of the BW reservoir where it should be
247 detected.

A new design was then developed, including an additional step allowing for voltage inversion just before GSNO decomposition (**Figure 3A**). In this new design, the loading, injection and separation steps were performed under positive polarity (step I, II and III, respectively), Hg^{2+} is added before GSNO reaches the channel end in step III, and polarity is inverted (step IV). This inversion of polarity leads to the migration of Hg^{2+} and GSNO to the sample reservoir (S). As Hg^{2+} migrates faster than GSNO, the migration zone of Hg^{2+} enters that of GSNO, allowing for GSNO decomposition. The produced nitrite migrates towards the BW reservoir and is detected (**see detail in step IV, Figure 3A**). A typical electropherogram obtained characterizing all the analytical steps is presented in **Figure. 3B**. Control experiments (without Hg^{2+} or without GSNO) did not show any signal (data not shown).

Figure 3 - (A) Scheme of the main steps for RSNOs quantitation. Loading step (I): Voltages of 800 and 1000 V are applied during 45 s to the sample (S) and buffer (B) reservoirs, respectively, grounding both waste reservoirs (SW and BW). Injection step (II): samples are injected into separation channel by floating the voltage applied to B reservoir during 3 s. Migration step (III): The potentials were then re-established to step I condition allowing the migration of RSNO sample towards separation channel. 15 s before the end of this step, Hg^{2+} was added to BW reservoir. Inversion and detection step (IV): In this

268 step the potential polarity is inverted which leads to the migration of Hg^{2+} into the separation channel
269 faster than RSNO leading to RSNO decomposition. This is followed by nascent nitrite (in green) opposite
270 migration towards the electrodes and detection by applying a potential of 1.2 V vs *Pt*. (B) Typical
271 electropherogram obtained for GSNO (1 mmol/L) analysis characterising all the steps of the process.

272

273 Therefore, the overall integrated protocol includes (1) a separation of the
274 RSNOs under positive polarity, (2) an inversion of the separation polarity,
275 (3) a decomposition of RSNO thanks to the on-line crossing and mixing of
276 RSNO and Hg^{2+} zones, respectively, due to different migration velocities,
277 (4) the migration of the produced nitrite to the detector. So as to further
278 optimize the experimental conditions to improve the limit of detection for
279 nitrite, three parameters were studied; the BGE ionic strength (from 10 to
280 50 mM), the detection voltage (from 0.5 to 1.5 V vs *Pt*) and the time of
281 polarity inversion. Considering the two first parameters, best signal
282 intensities and S/N ratios for nitrite detection were obtained for a detection
283 potential of 1.2 V (**Figure 4**).

Figure 4 - Histogram of peak current (in nA) and signal-to-noise ratio (S/N) of nitrite (1 mM) injection in SU-8/Pyrex microchip obtained by variation of the detection potential from 0.5 to 1.5 V vs Pt. BGÉ: 20 mM arginine solution adjusted at pH 5.6 with acetic acid.

The time of polarity inversion is a crucial parameter as it should allow for the best separation of the RSNOs, their total decomposition and their optimal detection. For each studied compound, the electroosmotic and apparent mobilities must therefore be determined. For RSNOs of positive apparent mobility, the time of inversion should be chosen between the migration time of the neutral marker and the one of the RNSOs. This parameter was optimized for GSNO and CysNO. CysNO, another important nitrosothiol, as it is smaller than GSNO and with similar charge at this pH, presents a higher apparent mobility than GSNO. The best signal intensities were obtained for an inversion time of 90 s and 75 s, for GSNO and CysNO respectively (**Figure 5**). These results indicate the versatility of the

procedure for all types of RSNO. In these experimental conditions, the analytical performances of this methodology were determined for GSNO. The linearity was verified in the 100-700 μM concentration range ($y=0.0485x - 5.0485$, $R^2=0.9936$) with a LOD of 20 μM

Figure 5 - Electropherograms for detection of 1 mM GSNO (Black) and 1 mM CysNO (Red and Blue) to determine the time of polarity inversion. In black and red 90 s was used for inversion while in blue 75 s. BGE: 20 mM arginine solution adjusted at pH 5.6 with acetic acid.

The final objective of such a micro-total analysis system is to allow for the simultaneous quantitation of various RSNOs. Therefore three main challenges have to be addressed : (i) the efficient separation of the different RSNOs, (ii) the choice of a unique time of polarity inversion in the process, and (iii) the efficient detection of the successive nitrite zones produced from each RSNOs decomposition.

The simultaneous separation of GSNO and CysNO was performed so as to prove the versatility of the system. For this purpose, GSNO and CysNO were first separated and detected individually. The crucial parameter being the inversion time, different values were applied from 75 to 90 s. In a second step, an equimolar mixture of CysNO and GSNO (1 mM each) was separated and detected. **Figure 6** presents the resulting electropherograms for an optimized inversion time of 75 s that corresponds to the migration time of CysNO. As expected the signal arising from GSNO appears after the one of CysNO, as CysNO has a higher apparent mobility. These results indicate a powerful simultaneous separation and indirect detection of GSNO and CysNO. The similar intensities for GSNO and CysNO, injected at the same concentration indicate that decomposition efficiency is similar in both cases. This method is therefore applicable for the quantitation of pharmaceutical RSNOs, future drug candidates. Some work is in progress for decreasing the LODs to reach biological concentrations (less than 16 μM^{22}).

332

333 **Figure 6** - Electropherograms corresponding to the electrophoretic profile of CysNO (1 mM), GSNO (1
 334 mM) and mixture GSNO (1 mM) / CysNO (1 mM) in blue, red and black, respectively. Experiments were
 335 performed in Su-8/pyrex microchip with gated injection. Procedure : (1) 3s injection, (12) application of
 336 V1=800V, V2=1000V , during 3 minutes (not visible on the graph as it occurs before running the
 337 electropherogram), (3) addition of Hg²⁺ at t=0s of the electropherogram, and inversion of polarity V1=-
 338 800V, V2= -1000V for t=100s. Detection 1V vs Pt. BGE: ARG 20 mM adjusted to pH 5.8 with HAC

Conclusions

An original method to simultaneously quantify two low molecular RSNOs in a mixture using MCE was developed. A commercial microchip of SU-8/Pyrex microchip and a wireless isolated potentiostat were used. After the electrokinetic separation step, an inversion of electrokinetic polarity was necessary to mix the RSNOs with the decomposition agent within the separation channel and detect the produced nitrite by amperometric detection at the buffer waste reservoir. Optimization of BGE composition and detection potential were performed in order to obtain the best signal intensity and S/N ratio. The LODs were 20 μM for GSNO and CysNO. This methodology can be applied for the quantitation of pharmaceutical RSNOs, future drug candidates. Using a more environmental friendly decomposition reagent such as immobilized gold nanoparticles is envisaged. The method developed herein has shown to be versatile, opening the way to the quantitation of complex mixtures of RSNOs.

Conflicts of interest

The author declares no conflicts of interest.

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References

1. B. T. Mellion, L. J. Ignarro, C. B. Myers, E. H. Ohlstein, B. A. Ballot, A. L. Hyman and P. J. Kadowitz, *Mol. Pharmacol.*, 1983, **23**, 653-664.
2. J. S. Stamler, D. I. Simon, J. A. Osborne, M. E. Mullins, O. Jaraki, T. Michel, D. J. Singel and J. Loscalzo, *Proc. Natl. Acad. Sci. U.S.A.*, 1992, **89**, 444-448.
3. E. J. Langford, A. S. Brown, R. J. Wainwright, A. J. Debelder, M. R. Thomas, R. E. A. Smith, M. W. Radomski, J. F. Martin and S. Moncada, *Lancet*, 1994, **344**, 1458-1460.
4. M. W. Radomski, D. D. Rees, A. Dutra and S. Moncada, *Br. J. Pharmacol.*, 1992, **107**, 745-749.
5. G. F. P. de Souza, J. K. U. Yokoyama-Yasunaka, A. B. Seabra, D. C. Miguel, M. G. de Oliveira and S. R. B. Uliana, *Nitric Oxide-Biol. Chem.*, 2006, **15**, 209-216.
6. C. G. Kevil and R. P. Patel, *Curr. Opin. Investig. Drugs*, 2010, **11**, 1127-1134.
7. M. Ito, *Annu. Rev. Neurosci.*, 1989, **12**, 85-102.
8. A. R. Butler and P. Rhodes, *Anal. Biochem.*, 1997, **249**, 1-9.
9. K. M. Beeh, J. Beier, N. Koppenhoefer and R. Buhl, *Chest*, 2004, **126**, 1116-1122.
10. V. A. Tyurin, S. X. Liu, Y. Y. Tyurina, N. B. Sussman, C. A. Hubel, J. M. Roberts, R. Taylor and V. E. Kagan, *Circ. Res.*, 2001, **88**, 1210-1215.
11. A. B. Milsom, C. J. H. Jones, J. Goodfellow, M. P. Frenneaux, J. R. Peters and P. E. James, *Diabetologia*, 2002, **45**, 1515-1522.
12. M. W. Foster, T. J. McMahon and J. S. Stamler, *Trends Mol. Med.*, 2003, **9**, 160.
13. B. C. Smith and M. A. Marletta, *Curr. Opin. Chem. Biol.*, 2012, **16**, 498-506.
14. A. Ismail, F. d'Orlyé, S. Griveau, J. A. F. Da Silva, F. Bedioui and A. Varenne, *Anal. bioanal. chem.*, 2015, **407**, 6221-6226.
15. A. Ismail, F. d'Orlye, S. Griveau, F. Bedioui, A. Varenne and J. A. F. da Silva, *Electrophoresis*, 2015, **36**, 1982-1988.
16. S. Griveau and F. Bedioui, *Analyst*, 2013, **138**, 5173-5181.
17. D. Giustarini, A. Milzani, I. Dalle-Donne and R. Rossi, *J. Chromatogr. B*, 2007, **851**, 124-139.
18. D. L. H. Williams, *Acc. Chem. Res.*, 1999, **32**, 869-876.
19. M. M. Veleparampil, U. K. Aravind and C. T. Aravindakumar, *adv. phys. chem.*, 2009, **2009**.
20. M. G. de Oliveira, S. M. Shishido, A. B. Seabra and N. H. Morgon, *J. Phys. Chem. A*, 2002, **106**, 8963-8970.

21. H. R. Swift and D. L. H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1997, 1933-1935.
22. A. Ismail, M. O. Araujo, C. L. S. Chagas, S. Griveau, F. D'Orlye, A. Varenne, F. Bedioui and W. K. T. Coltro, *Analyst*, 2016, **141**, 6314-6320.
23. R. A. Hunter, B. J. Privett, W. H. Henley, E. R. Breed, Z. Liang, R. Mittal, B. P. Yoseph, J. E. McDunn, E. M. Burd, C. M. Coopersmith, J. M. Ramsey and M. H. Schoenfish, *Anal. Chem.*, 2013, **85**, 6066-6072.
24. R. A. Hunter and M. H. Schoenfish, *Anal. Chem.*, 2015, **87**, 3171-3176.
25. D. B. Gunasekara, M. K. Hulvey, S. M. Lunte and J. A. F. da Silva, *Anal. Bioanal. Chem.*, 2012, **403**, 2377-2384.
26. F. Q. Tu, L. Y. Zhang, X. F. Guo, Z. X. Zhang, H. Wang and H. S. Zhang, *J. Chromatogr. A*, 2014, **1359**, 309-316.
27. J.-W. Yoo, G. Acharya and C. H. Lee, *Biomaterials*, 2009, **30**, 3978-3985.
28. L. A. Peterson, T. Wagener, H. Sies and W. Stahl, *Chem. Res. Toxicol.*, 2007, **20**, 721-723.
29. R. A. Hunter and M. H. Schoenfish, *Anal. Chem.*, 2015, **87**, 3171-3176.
30. <https://www.micruxfluidic.com/en/> Accessed on 04/12/2018
31. S. C. Jacobson, S. V. Ermakov and J. M. Ramsey, *Anal. Chem.*, 1999, **71**, 3273-3276.
32. A. Ismail, S. Griveau, F. d'Orlyé, A. Varenne and F. Bedioui, *Electroanalysis*, 2015, **27**, 2857-2863.
33. B. Thirumalraj, S. Palanisamy, S.-M. Chen and D.-H. Zhao, *J. Colloid Interface Sci.*, 2016, **478**, 413-420.
34. P. Kubáň and P. C. Hauser, *Electrophoresis*, 2009, **30**, 3305-3314.
35. J. L. Beckers and P. Boček, *Electrophoresis*, 2003, **24**, 518-535.