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In-capillary non-covalent labeling of insulin and one gastrointestinal peptide for their analyses by capillary electrophoresis with laser-induced fluorescence detection

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Abstract

The potential of the commercially available dye sypro orange for in-capillary derivatization was evaluated for the detection of insulin and one gastrointestinal peptide (Arg-Arg-gastrin) by capillary electrophoresis with laser induced fluorescence (CE–LIF). The fluorescent emission intensity (λ_{ex} = 488 nm, λ_{em} = 610 nm) of this probe is very low in aqueous medium, and increases strongly in less polar solvent, e.g. methanol. The hydrophobic character of the two analyzed peptides is too low to induce sufficient interaction with the fluorescent probe for good sensitivity when the latter is alone in the background electrolyte. Thus, the potential of several neutral, zwitterionic, cationic and anionic surfactants to favor probe/peptide interactions has been evaluated. It was demonstrated that a borate buffer (pH 8.5) containing tetradecyltrimethylammonium bromide (TTAB) in sub-micellar conditions can be considered as the most suitable buffer for insulin CE–LIF analysis. In addition, the method showed a good linearity between insulin concentration and the peak area of the labeled insulin, allowing quantitative measurements. The sensitivity achieved so far is comparable with that achieved with UV absorption detection, but even at this level it is interesting for microchip analysis, in which fluorescence detection is much more commonly available than UV absorption detection.

Keywords: Capillary electrophoresis; Laser induced fluorescence (LIF); In-capillary labeling; Fluorescent probe; Sypro orange; Peptide

1. Introduction

Capillary electrophoresis (CE) is a powerful separation technique for peptides due to its high efficiency, good resolution and fast separation speed [1,2]. In this context, labeling with a fluorophore is an attractive method to enhance detection sensitivity for the CE analysis of peptides or proteins, due to the low detection limits that can be attained using laser induced fluorescence (LIF) detection and to the wide variety of derivatizing reagents available. Therefore, peptides and proteins are usually covalently labeled with a fluorescence dye for LIF detection in the UV or visible region (see [3–5] for reviews). The main problem encountered with covalent labeling involving, for example, amino groups of peptides and proteins is the production of multiply tagged derivatives arising from the coexistence of several functionalities in such molecules [6]. Alternatively, non-covalent labeling of proteins has been explored. In this method, the labeling dye, weakly fluorescent in water, interacts with the protein to form a strongly fluorescent protein-dye complex. Application of non-covalent labeling of proteins for CE–LIF analyses was first described by Swaile and Sepaniak using in-capillary derivatization of proteins with the non-covalent labeling reagents 1-anilinenaphtalene-8-sulfonate (ANS) and 2-*p*-toluidininaphtalene-6-sulfonate (TNS) [7]. The improvement in LOD with this method relative to on-column UV absorbance remained low since protein concentrations below 10^{-7} M could not be detected. In 1999, Benito et al. [8] also reported the in-capillary derivatization by ANS

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and TNS for CE–LIF analysis of bovine whey proteins. The limit of detection obtained for bovine serum albumin (BSA), β-lactoglobulin A (β-LGA) and β-lactoglobulin B (β-LGB) were lower using LIF compared to UV-absorption detection, whereas LOD for α-lactalbumin (α-LA) was better with UV-absorption detection. Horka and Slais [9] also reported very low LOD for the analysis of a protein mixture by adding pyrenebutanoate as fluorescent additive in the background electrolyte. More recently, new types of non-covalently labeling dyes for proteins, such as nano orange, sypro red and sypro orange have appeared. These dyes, initially developed for staining of proteins following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [10], have also been exploited in electrophoretic separations of proteins performed either on conventional CE and on microchip. The labeling of proteins with non-covalent probes has been successfully applied to various CE separation modes. As an example, Sze et al. [11] showed the potential of sypro orange and sypro red for LIF detection of BSA and hemoglobin in capillary isoelectric focusing. They demonstrated that non-covalently labeled proteins focused to well characterized peaks, and that the related calculated p*I* values did not change significantly with LIF detection as compared to UV detection. Moreover, nano orange has been investigated as a potential clinical diagnostic tool due to its low cost, ease of use and ability to detect nanomolar concentrations of human serum albumin with CZE–LIF analysis [12]. Use of sypro red has been reported for the size separation of proteins by SDS–CGE method [13,14]. Sypro red labeling of SDS–BSA complexes at nanomolar protein concentrations has been demonstrated, leading to detection limits surpassing those of silver staining [13]. These last years, electrophoretic separations of non-covalently labeled proteins on microchip have also been described [15–18]. As an application, Bousse et al. [16] have developed a microchip device that performs a protein sizing assay with sizing accuracy better than 5% and high sensitivity (30 nM for carbonic anhydrase). In 2000, Liu et al. [17], have described the electrophoretic separation of α-lactalbumin, β-lactoglobulin A and B separated and labeled on-chip by post-column addition of nano orange dye. The authors have shown that no band broadening is generated by the post-column labeling step.

In the present study the potential of sypro orange dye to perform in-capillary labeling of peptides is examined. This dye has already been used for protein labeling but never for non-covalent in-capillary labeling of smaller molecules such as peptides. Labeling of peptides and proteins for CE–LIF analyses can be accomplished by pre-, post- or in-capillary procedures. In-capillary derivatization can be accomplished by different strategies which may be classified into three groups: zone-passing, at-inlet and throughout-capillary technique [3,5]. In the present work, we have investigated the throughout-capillary procedure to non-covalently label peptide. In this approach, sample solution is introduced at the inlet of the capillary previously filled with a running buffer which contains the labeling reagent. When the electric field is applied, the analyte migrates, mixes with the reagent, allowing the binding to occur.

We have first investigated the fluorescence properties of the sypro orange dye in various aqueous (water, acidic and alkaline buffers) or organic media (methanol). Moreover, as the chemical structure of sypro orange is not available, and in order to determine the possible ionic character of the dye, the electrophoretic behaviour of the probe has been studied by CE using UV detection. Then, the potential of sypro orange dye for in-capillary non-covalent labeling has been investigated using carbonic anhydrase and insulin selected as small protein and large peptide models, respectively. Preliminary studies have shown that the fluorescence emission of the dye–peptide complex requires the presence of surfactant in the background electrolyte, thus we investigated the influence of the nature (neutral, ionic) and molar concentrations of several surfactants (see Table 1) on the fluorescence peak intensity. The effect of the sypro orange concentration in the background electrolyte on the sensitivity was also studied. The non-covalent in-capillary labeling method has been validated in terms of linearity and sensitivity. Finally, the method was applied to the CE–LIF analysis of a mixture of two peptides, insulin and Arg-Arg-gastrin peptide.

2. Experimental

2.1. Capillary electrophoresis

CE experiments were performed using a Beckman P/ACE 2100 system equipped with either a UV detector (Beckman Instruments, Fullerton, CA, USA) or a LIF detector. The excitation was performed by an argon ion laser source at 488 nm. Data acquisition and instrument control were carried out using Beckman P/ACE 2100 system software.

Separations were carried out with fused-silica capillaries (Beckman Coulter, Fullerton, CA, USA), 75 μm I.D., effective length 37 cm, total length 47 cm. Samples were

Table 1
Surfactant characteristics

Surfactant	Abbreviation	Formula	CMC (mM)	M_r
Sodium dodecyl sulfate	SDS	$C_{12}H_{25}O_4SNa$	8	288.4
Polyoxyethylene (23) dodecanol	Brij 35	$C_{58}H_{118}O_{24}$	0.1	1198.0
N-Hexadecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate	SB-16	$C_{21}H_{45}NO_3S$	0.06	391.0
Tetradecyltrimethylammonium bromide	TTAB	$C_{17}H_{38}NBr$	3.5	336.4

CMC: critical micelle concentration; M_r: relative molecular mass.

introduced in the hydrodynamic mode (3.5 kPa/10 s). Separations were performed under a negative voltage of 25 kV and the temperature in the capillary cartridge was set at 30 °C.

The background electrolytes consisted of boric acid, sodium hydroxide, sypro orange and surfactant. Peptide and protein samples were prepared in a tetraborate sodium solution (2.5 mM) and diluted at the appropriate concentration in the background electrolyte. The background electrolytes were sonicated 3 min prior each analysis. New capillaries were conditioned by successive flush with 1 and 0.1 M NaOH and then with water for 10, 5 and 10 min, respectively, under a pressure of 138 kPa. Prior to each sample injection, the capillary was rinsed with the separation electrolyte for 2 min.

2.2. Spectrofluorimetric measurements

Fluorescence measurements were performed at room temperature (23 ± 1 °C) using a Perkin-Elmer LS50B spectrofluorimeter, equipped with a red sensitive R6872 photomultiplier. The excitation wavelength was set at 488 nm and emission spectra were recorded between 510 and 800 nm. The excitation slit was set at 5 nm.

2.3. Chemicals

Buffers and standard solutions were prepared with Milli-Q water (Millipore, Bedford, MA, USA). Sodium hydroxide was obtained from Prolabo (Paris, France). Sodium tetraborate was from Fisher scientific (Pittsburgh, USA). Sodium dodecyl sulfate (SDS), tetradecyltrimethylammonium bromide (TTAB) and *N*-hexadecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (SB-16) were obtained from Sigma (St. Louis, MO, USA). Polyoxyethylene (23) dodecanol was from Fluka (Brij 35) (Buchs, Germany). Insulin (human), Arg-Arg-gastrin and carbonic anhydrase were purchased from Sigma. Sypro orange 5000*x* concentrate in DMSO was from Molecular Probe (Eugene, OR, USA).

3. Results and discussion

3.1. Preliminary study

3.1.1. Sypro orange characterization

As the chemical structure of the studied dye is not provided by the supplier, we first examined the spectral characteristics of sypro orange in the absence of peptide. The emission spectra of sypro orange at a 5*x* concentration in water and in methanol are presented in Fig. 1A. These spectra show that sypro orange does not fluoresce in aqueous medium, whereas in less hydrophilic medium (e.g. methanolic medium) a significant enhancement in fluorescence is observed. Consequently, the fluorescence emission of sypro orange can be considered as solvent polarity dependent and this property will be exploited for peptide detection. Thereafter, two hydro-organic buffers were chosen for evaluating the

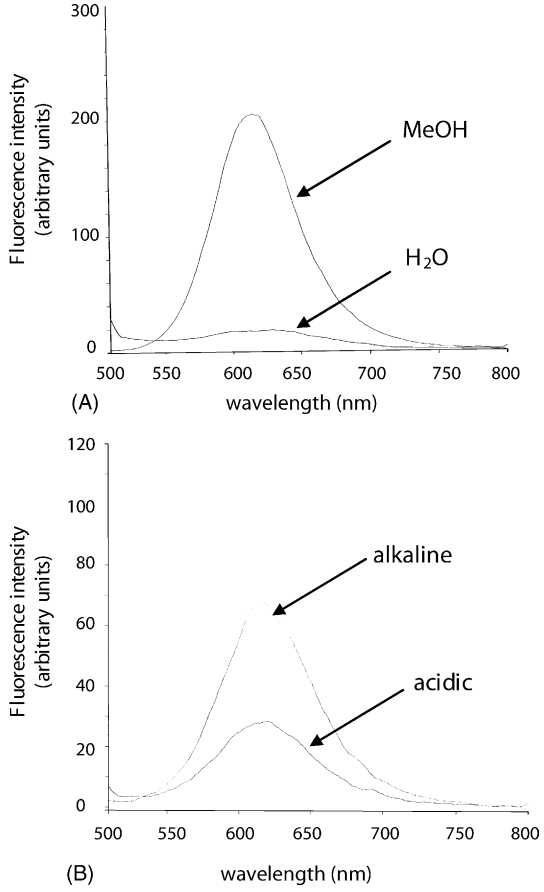

Fig. 1. (A) Emission spectra of sypro orange in water and in MeOH λ_{ex} = 488 nm; sypro orange: 5*x*. (B) Emission spectra of sypro orange under acidic and alkaline conditions λ_{ex} = 488 nm; sypro orange: 5*x*. Acidic conditions: MeOH–aqueous buffer (phosphate, 50 mM, pH 3.1) (25/75: v/v). Alkaline conditions: MeOH–aqueous buffer (borate, 50 mM, pH 8.5) (25/75: v/v).

fluorescent quantum yield of sypro orange under acidic and alkaline conditions. As shown in Fig. 1B, the fluorescence of sypro orange is enhanced under alkaline conditions.

Then, the electrophoretic behavior of the probe was investigated using CE with direct UV detection using a background electrolyte adjusted at different pH values. The probe co-migrates with the electroosmotic flow marker over the whole pH range comprised between 2.0 and 10.3. It can be concluded that the sypro orange is neutral over the wide range of pH studied and, consequently, that no ionic interaction can occur between the probe and the peptides. From these results we have selected alkaline conditions for the further studies.

3.1.2. Effect of surfactant nature on fluorescence intensity

Numerous peptides of interest, and in particular insulin and Arg-Arg-gastrin are rather hydrophilic, and do not present sufficient interactions with polarity-sensitive fluorescent probes for sensitive detection.

In order to compensate for this low hydrophobicity, a surfactant can be introduced in the background electrolyte

Table 2
Physicochemical parameters of protein and peptides

Compound	M_r	p*I*	Hydrophobicity
Carbonic anhydrase	29000	5.9	−0.550
Insulin	5807	5.3	−0.159
Arg-Arg-gastrin	1380	4.1	−2.055

M_r: relative molecular mass; p*I*: isoelectric point. Hydrophobicity was estimated on http://www.expasy.org with protscale, using parameters of Kyte and Doolittle [21].

to enhance probe/peptide interactions and thereby fluorescence detection, a strategy that proved extremely efficient with proteins. The potential of four surfactants (Table 1) to assist peptide-probe interaction for the analysis of peptides by CE–LIF has been investigated. Anionic (SDS), cationic (TTAB), zwitterionic (SB-16) and neutral (Brij 35) surfactants have been tested to evaluate the role of electrostatic and hydrophobic interactions in enhancing the affinity of the probe for the peptides.

In a first approach, a large peptide (insulin) and a small protein (carbonic anhydrase) were chosen as models to investigate their affinity toward the fluorescent probe. Some of their physico-chemical properties are listed in Table 2. The surfactant was added to the background electrolyte and insulin and carbonic anhydrase were analysed by CE–LIF. The concentration of each surfactant in the background electrolyte was chosen below their respective critical micelle concentration to limit the background noise.

As highlighted in Fig. 2, addition of surfactant is necessary to allow measurable emission of the fluorophore. These results show that, in the absence of surfactant, no peak is detected neither for the peptide nor for the protein. As reported in Table 2, the analytes present a low hydrophobic character which could explain the lack of fluorescent signal

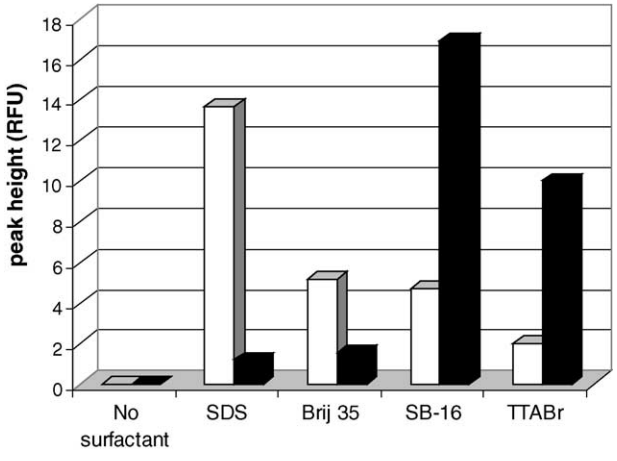

Fig. 2. Influence of the presence and nature of surfactant in BGE on the fluorescent signal of insulin obtained by CE–LIF. Conditions: BGE, boric acid 50 mM, sodium hydroxide, pH 8.5, sypro orange 5*x*; surfactant concentration: SDS 0.01 mM, Brij 35 0.01 mM, SB-16 0.05 mM, TTAB 2.5 mM; applied voltage ±25 kV, LIF detection (λ_{ex} = 488 nm); temperature 30 °C; sample: insulin 1 mg/mL (black), carbonic anhydrase 1 mg/mL (white); hydrodynamic injection: 10 s/3.5 kPa.

observed in absence of surfactant. Non-covalent labeling studies reported earlier in the literature have been carried out with proteins exhibiting higher molecular masses and thereby more hydrophobic regions (e.g. β-lactoglobulin, bovin serum albumin).

Among the surfactants tested, the anionic surfactant (SDS) routinely employed for 2D-gel electrophoresis gives clearly the most intense fluorescent signal for the protein. In contrast, for the peptide analysis, the higher fluorescent signal has been observed in presence of the zwitterionic (SB-16) or the cationic (TTAB) surfactant in the running buffer. The affinity of a non covalently bound dye for an analyte can involve various interactions: hydrophobic, electrostatic interactions and/or hydrogen bonding. As in alkaline conditions, insulin is negatively charged, electrostatic interactions could occur between insulin and the cationic group of the surfactant enhancing the probe/peptide interactions. This explains the higher fluorescent signal obtained in presence of surfactant exhibiting cationic groups.

Further CE–LIF analyses have been performed in presence of TTAB or SB-16. The electropherograms obtained in presence of SB-16 have shown that insulin peak migrates very close to the peak of electroosmosis, whatever the concentration of zwitterionic surfactant and/or analyte employed. This indicates that the complex formed between insulin and SB-16 probably has a too low charge/friction ratio, leading to an electrophoretic mobility close to zero and preventive accurate quantitative analysis. Thus, we selected the cationic surfactant TTAB for the continuation of this study.

3.2. Optimisation of non-covalent labeling

In this section, we investigated by CE–LIF the influence of both the surfactant and probe concentrations on the fluorescent detection of insulin.

3.2.1. CMC of TTAB determination

We first determined the TTAB critical micelle concentration (CMC) in presence of sypro orange and in our experimental conditions (pH, ionic strength and temperature). Cifuentes et al. [19] have demonstrated the usefulness of capillary electrophoresis instrumentation for determining CMC values. This method is based on the concept that the conductivity of ionic surfactants in an electrolyte solution depends on its aggregation state. By plotting the electric current measured at a given voltage against the surfactant concentration, experimental points fit into two straight lines exhibiting, as expected, markedly different slopes corresponding to the monomeric and micellar aggregation states (Fig. 3). The intercept of these straight lines was estimated at 3 mM. This concentration can be considered as the CMC value of TTAB in the presence of probe. It is very close to the CMC reported in the literature for pure TTAB in water (3.5 mM). Thus, the presence of sypro orange in the background electrolyte does not lead to a major alteration of the TTAB aggregation process.

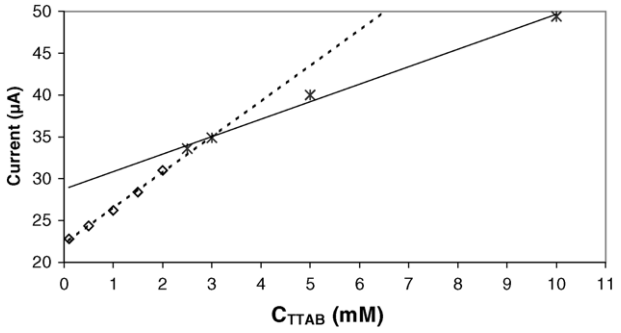

Fig. 3. Plots of electric current vs. concentration of TTAB added to the electrolyte. Conditions: BGE, boric acid 50 mM, sodium hydroxide, pH 8.5, sypro orange 5*x*, applied voltage +25 kV, temperature 30 °C. C_{TTAB}: concentration of TTAB.

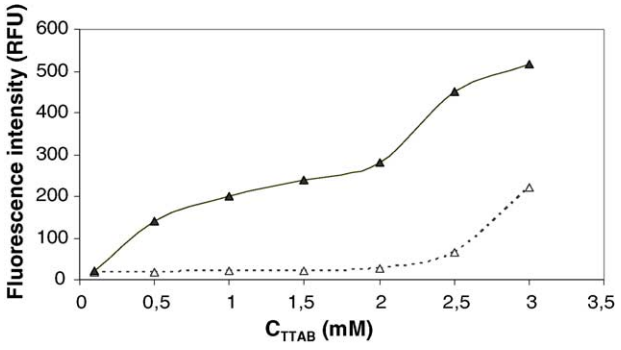

Fig. 4. Influence of TTAB concentration on the emission fluorescent signal in absence (dotted line) and in presence (solid line) of insulin. The lines are included only to guide the eyes. Sample: boric acid 50 mM, sodium hydroxide, pH 8.5, sypro orange 5*x*, various concentration of TTAB, ±4.7 μg/mL insulin. Excitation at 488 nm. C_{TTAB}: concentration of TTAB.

3.2.2. Influence of TTAB concentration

The CMC value has been used for delimiting the range of surfactant concentration studied, with the goal of optimizing detection sensitivity. The influence of the cationic surfactant concentration has been studied by spectrofluorimetry between 0.1 and 5 mM. The emission spectra of the different electrolytes were carried out in the absence and in the presence of insulin (Fig. 4). At low concentrations of surfactant and in the absence of insulin, the fluorescent background, due to the running buffer itself, can be considered as negligible. Above 2.5 mM of TTAB, the fluorescence emission of the running buffer increases markedly. The results obtained for TTAB concentrations superior to the CMC are not shown because of saturation of the spectrofluorimeter. These observations confirm that at concentrations of TTAB above the CMC, the baseline noise of the running buffer is too high, due to the presence of a significant amount of dye in the hydrophobic core of the micelles. In presence of insulin, as a general trend, the fluorescence signal increases with the concentration of surfactant. However, for the lowest concentrations studied the signals in the presence or in the absence of insulin can be considered as equal. These results confirm that a minimal concentration of surfactant is necessary to favour the interaction between the probe and the peptide, and show that it is also essential to keep the system under submicellar concentrations in order to obtain a good sensitivity. As illustrated by Fig. 4, the higher signal-to-noise ratio is obtained for a surfactant concentration comprised between 1 and 2.5 mM of TTAB. Then, similar experiments have been carried out with CE–LIF in order to take into account the specific noise due to the CE equipment. This study has shown that the best sensitivity was obtained for a concentration of TTAB of 2.5 mM.

In order to interpret the results obtained during the in-capillary non-covalent labeling, we have proposed a mechanism which is illustrated in Fig. 5. In-capillary labeling can be achieved by using an electrolyte containing both the probe and the cationic surfactant. In this case, TTAB plays a double role: reversing the electroosmotic flow and enhancing the probe/peptide interactions. Indeed, in the presence of TTAB, the direction of electroosmotic flow (EOF) was reversed (i.e. from cathode to anode) by creating a cationic layer on the inner surface of the fused-silica capillaries. It is likely that in presence of insulin, the cationic surfactant leads to a synergic effect which permits the formation of an aggregate containing simultaneously the probe, the peptide and the cationic surfactant. In this hydrophobic environment, the sypro orange generates a fluorescent signal. However, it is also probable that the fluorescent probe interacts with the hydrophobic domains of the monomeric surfactants leading to a noisy baseline and then to a sensitivity of detection not as high as expected. A second hypothesis can be proposed to explain the previous results, indeed we cannot exclude the possibility that under our conditions, insulin monomer could interact with other monomers leading to an insulin hexamer as previously described [20]. This hexamer would

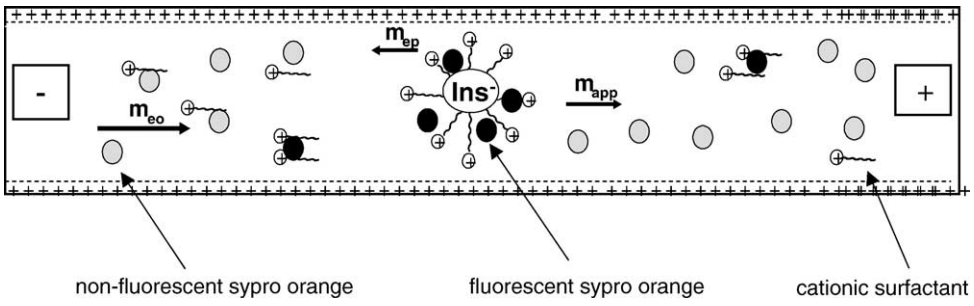

Fig. 5. Schematic representation of the in-capillary non-covalent labeling method.

present hydrophobic domain allowing interaction with sypro orange, leading to a fluorescent signal. This hypothesis will be discussed in the next section.

3.2.3. Influence of sypro orange concentration

The influence of the probe concentration on the sensitivity was also investigated. Insulin was analysed by CE–LIF under previously described conditions and with sypro orange added to the background electrolyte at concentrations ranging from 1*x* to 20*x*. This study showed that for a concentration of sypro orange lower than 5*x*, no significant signal was detected whereas for concentrations over 10*x* the signal-to-noise ratio decreased (results not shown). The higher S/N ratio was obtained for sypro orange concentration at 5*x*. Since sypro orange is provided as a 5000*x* concentrate without any information of molarity, it is not possible to express binding stoichiometry in terms of mole fraction.

3.2.4. Quantitative aspect

In the optimised conditions, the corrected area of the insulin peak correlates with the concentration of injected insulin (Fig. 6). The linearity of the non-covalent in-capillary labeling method was investigated using insulin solutions with concentrations ranging from 6×10^{-7} to 5×10^{-5} M and using the optimised conditions. Linear regression analysis of the corrected area of labeled insulin peak plotted against the molar concentration gave the following equation: $y = 2.24 \times 10^{13}x + 1.16 \times 10^{8}$ with a satisfactory determination coefficient ($R^2 = 0.996$). The sensitivity of the method was studied by calculating the limit of detection (LOD) corresponding to a signal-to-noise ratio of 3. The LOD of labeled insulin by CE–LIF method was 6.5×10^{-7} M whereas the LOD of insulin by CE–UV (200 nm) was estimated at 7×10^{-7} M. The LOD value obtained by CE–LIF is of the same order of magnitude than that obtained by CE-UV. In spite of this low sensitivity, the non-covalent in-capillary labeling method developed is particularly interesting for transposition to microsystems. Generally speaking, LIF is more amenable to miniaturization, since decrease of the illuminated volume (e.g. by a change in focusing objectives) does not change the number of emitted photons in a comfortable range of analyte concentrations, whereas UV absorption detection is proportional to the optical path.

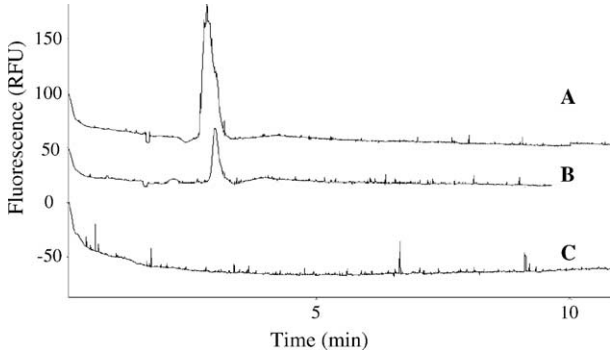

Fig. 6. Electropherograms illustrating the effect of an increasing concentration of insulin in the sample. Conditions: BGE, boric acid 10 mM, sodium hydroxide, pH 8.5, sypro orange 5*x*, TTAB 2.5 mM; applied voltage −25 kV, LIF detection (λ_{ex} = 488 nm); temperature 30 °C; hydrodynamic injection: 10 s/3.5 kPa. Insulin: (A) 2.4×10^{-5} M, (B) 6.0×10^{-6} M, (C) 0 M.

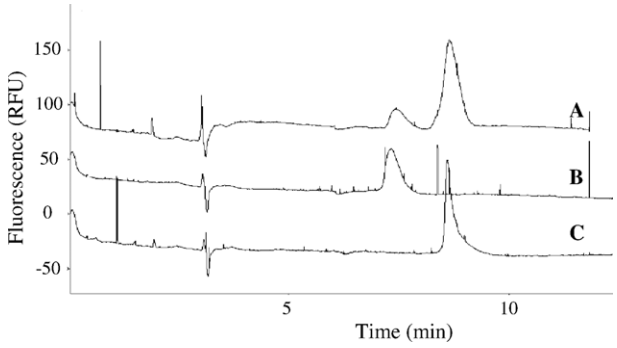

Fig. 7. In-capillary non-covalent labeling and separation of two peptides by CE–LIF. Conditions as in Fig. 6 except: capillary: total length L = 57 cm, effective length l = 47 cm. (A) Mixture of insulin at 0.1 mg/mL and Arg-Arg-gastrin at 0.1 mg/mL, (B) insulin at 0.1 mg/mL, (C) Arg-Arg-gastrin at 0.1 mg/mL.

3.2.5. Application of the non-covalent in-capillary labeling method

Finally, the method previously described was applied to the CE–LIF analysis of a mixture of two peptides which do not exhibit fluorescent properties (Fig. 7). In order to identify the peaks obtained, insulin and Arg-Arg-gastrin peptide were also analysed separately. The results showed that this in-capillary non-covalent labeling method is not restricted to insulin and can be applied to a mixture of two peptides exhibiting different hydrophobicities, isoelectric points and relative molecular masses (Table 2). Then, we can conclude that this labeling method is also suitable for peptides smaller than insulin. We can also infer from these last results, that the sensitivity obtained with insulin is not a consequence of a specific hexameric association (see Section 3.2.2), and that this new labeling approach will probably be applicable to many other peptides. Finally, it is also interesting to note in Fig. 7, that the peptide migration times are not modified by the simultaneous presence of the two peptides in the sample and that these peptides are resolved under these conditions.

4. Conclusions

In this work, we demonstrated the potential of sypro orange dye to perform in-capillary non-covalent labeling of peptides. To efficiently label peptide, this in-capillary procedure requires, in the background electrolyte, the presence of surfactant exhibiting cationic groups. We showed that sensitivity is greatly dependent on the surfactant and probe concentration. Furthermore, the method showed a good linearity between the derivative area and insulin concentration, allowing quantitative measurements. Although the sensitivity attained is not as high as expected with a LIF detection,

the ability to perform a rapid on-line labeling of peptides, without the complications and limitations of multiple labeled species encountered with covalent labeling, was demonstrated for the first time. Compared to a pre-capillary labeling, this in-capillary method is superior in terms of simplicity and ease of automation. Finally, this in-capillary approach should be useful for the development of micro total analysis systems (μTAS) dedicated to the separation of non-fluorescent molecules.

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