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Detection of Nonderivatized Peptides in Capillary Electrophoresis Using Quenched Phosphorescence

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A capillary electrophoresis detection technique for (small) peptides is presented, i.e. quenched phosphorescence, a method that is generally applicable and does not require chemical derivatization. For this purpose, a novel phosphorophore, 1-bromo-4-naphthalenesulfonic acid (BrNS), was synthesized. BrNS has sufficient water solubility and provides strong phosphorescence at room temperature over a wide pH range. The detection is based on the dynamic quenching of the BrNS phosphorescence background signal by electron transfer from the amino group of the peptides at pH 9.5−10. For the di- and tripeptides Val-Tyr-Val, Val-Gly-Gly, Ala-Ser, Gly-Asn, Gly-Ala, and Gly-Tyr, detection limits in the range of 5−20 μg/L were obtained. The novel technique is even a good alternative for the (limited) group of peptides containing tyrosine and, thus, exhibiting native fluorescence as well as strong UV absorption: using Gly-Tyr, Val-Tyr-Val, methionine enkephalin, and human angiotensin II as test compounds, quenched phosphorescence detection was found to compare favorably with absorption detection at 190- and 266-nm laser-induced fluorescence detection, as performed with a recently developed, small-size, quadrupled Nd:YAG laser.

The separation and detection of peptides is one of the most challenging tasks in capillary electrophoresis (CE).[1,2] It is rather difficult to detect nonderivatized peptides sensitively as well as selectively. Absorption detection can be applied at 185−220 nm with the peptide bond as the chromophoric group.[3] Detection limits (LODs) in the low 10^{-6} M range can thus be achieved at 190 nm, but there is a marked lack of selectivity because of the deep-UV wavelength applied. Of course, the combination of CE with mass spectrometry (using an electrospray interface) is much more selective, but also for CE−MS generally the LODs are relatively high.[4−7] Laser-induced fluorescence (LIF) is considered to be the most sensitive detection mode in CE.[8] However, detection of peptides often requires derivatization with a fluorogenic compound, unless natively fluorescent amino acids such as tryptophan or tyrosine are present in the molecule. In the latter case, LIF detection can be applied directly, but in practice, this is not often done since expensive long-frame gas lasers are required for excitation in the deep UV. Here, a recent breakthrough should be noted: a small-size quadrupled Nd:YAG laser, emitting 266-nm pulses at a high repetition rate of ∼10 kHz, was found to be well-suited for CE-LIF.[9,10] Finally, detection of peptides in CE is also possible by using direct or indirect amperometric detection,[11] as well as indirect fluorescence detection.[12,13] These latter techniques are very well suited for achieving low absolute LODs (low-to-mid attomole range) in small-inner diameter capillaries; however, the concentration LODs are moderate.

The separation of peptides in CE is usually performed at low pH (2.5−3.0), but a high pH has also been used.[14] Under both conditions, the interactions of the peptides with the (uncoated) capillary wall are reduced. At low pH, dissociation of the silanol groups is inhibited and the electrostatic forces are small; at high pH, both the peptides and the capillary wall are negatively charged and repulsion occurs.

In this work, the quenched phosphorescence detection method recently introduced in CE[15,16] was modified and applied to the separation of small, nonderivatized peptides. The method is shown to be generally applicable to (small) peptides, including those that are difficult to detect sensitively by other techniques such as LIF and UV absorption.

The quenched phosphorescence technique is based on the bimolecular quenching interaction of the analytes with the

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(1) Grossman, P. D., Colburn J. C., Eds. *Capillary Electrophoresis, Theory and Practice*; Academic Press: San Diego, CA, 1992; Chapter 9.
(2) Kasicka, V. *Electrophoresis* **1999**, *20*, 3084−3105.
(3) Lim, B. C.; Sim, M. K. *J. Chromatogr., B* **1994**, *655*, 127−131.
(4) Johansson, I. M.; Huang, E. C.; Henion, J. D.; Zweigenbaum, J. *J. Chromatogr.* **1991**, *554*, 311−327.
(5) Mück, W. M.; Henion, J. D. *J. Chromatogr.* **1989**, *495*, 41−59.
(6) Wahl, J. D.; Gale, D. C.; Smith, R. D. *J. Chromatogr., A* **1994**, *659*, 217−222.
(7) von Brocke, A.; Nicholson, G.; Bayer, E. *Electrophoresis* **2001**, *22*, 1251−1266.
(8) Swinney, K.; Bornhop, D. J. *Electrophoresis* **2000**, *21*, 1239−1250.
(9) Chan, K. C.; Muschik, G. M.; Issaq, H. J. *Electrophoresis* **2000**, *21*, 2062−2066.
(10) Kuijt, J.; García-Ruiz, C.; Stroomberg, G. J.; Marina, M. L.; Ariese, F.; Brinkman, U. A. Th.; Gooijer, C. *J. Chromatogr., A* **2001**, *907*, 293−301.
(11) Olefirowicz, T. M.; Ewing, A. G. *J. Chromatogr.* **1990**, *499*, 713−719.
(12) Gross, L.; Yeung, E. S. *Anal. Chem.* **1990**, *62*, 427−431.
(13) Yeung, E. S.; Kuhr, W. G. *Anal. Chem.* **1991**, *63*, 275A−282A.
(14) Lauer, H. H.; McManigill, D. *Anal. Chem.* **1986**, *58*, 166−170.
(15) Kuijt, J.; Brinkman, U. A. Th.; Gooijer, C. *Anal. Chem.* **1999**, *71*, 1384−1390.
(16) Kuijt, J.; Brinkman, U. A. Th.; Gooijer, C. *Electrophoresis* **2000**, *21*, 305−1311.

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phosphorophore present in the buffer solution—either via energy transfer or via electron transfer—which leads to negative peaks in the electropherogram. Peptides are expected to induce efficient quenching via electron transfer, provided that the amino group is not protonated. In other words, to enable quenched phosphorescence detection of peptides, a phosphorophore should be involved that can be used at high pH. Unfortunately, the phosphorophore used until now, biacetyl, cannot be used at pH >9: enolization occurs at high pH and phosphorescence is inhibited.[15,16] Therefore, a new phosphorophore, 1-bromo-4-naphthalenesulfonic acid (BrNS), was synthesized in order to meet the following requirements: sufficient water solubility, strong phosphorescence in aqueous solutions at room temperature, pH-independent phosphorescence, and efficient quenching by relevant analytes.

EXPERIMENTAL SECTION

Chemicals and Materials. Boric acid and methanol were obtained from Riedel-de Haën (Seelze, Germany). All peptides were received from Sigma (St. Louis, MO). 4-Amino-1-naphthalenesulfonic acid (sodium salt hydrate) was purchased from Aldrich (Steinheim, Germany). Sodium nitrite (p.a.) and cuprous bromide (98%) were obtained from Acros (Geel, Belgium) and HBr (48%) from Fluka (Buchs, Switzerland). All chemicals were used as received. All buffers and samples were filtered over 0.2-μm syringe filters (Schleicher & Schuell, Dassel, Germany) before use.

Synthesis of 1-Bromo-4-naphthalenesulfonic Acid. The phosphorophore, 1-bromo-4-naphthalenesulfonic acid was synthesized from 4-amino-1-naphthalenesulfonic acid by substitution of the amino group. 4-Amino-1-naphthalenesulfonic acid (193.0 g, 760 mmol) and sodium nitrite (55.1 g, 798 mmol) were dissolved in 1.5 L of water using a 6-L flask equipped with a mechanical stirrer. This yielded a purple suspension. Upon the addition of HBr (210 mL mixed with 750 g of crushed ice), the color turned to light brown. The suspension was cooled in an ice bath after which 120 g (836 mmol) of cuprous bromide, dissolved in 1.1 L of HBr, was slowly added (to prevent excessive foaming) under rapid stirring. The suspension was removed from the ice bath and stirred for 2 days. Next, the resulting dark purple suspension was boiled for 30 min to achieve dissolution. Upon cooling to room temperature, pink crystals were obtained which were rinsed twice with 250 mL of an aqueous 5% HCl solution and once with brine using a Buchner funnel. After drying, the crystals (218 g) were extracted for 2 days with 750 mL of methanol in a Soxhlet apparatus. Filtration of the extract over a Buchner funnel and rinsing the filtrate twice with 100 mL of cold methanol yielded 130 g (60%) of pink crystals. These were boiled for 1 h in 2.5 L of an aqueous solution of 1 M NaOH. The dark brown suspension was left standing for 2 days at 5 °C and decanted. The precipitate was washed twice with 100 mL of cold water and dried at 90 °C to yield 116 g (49%) of 1-bromo-4-naphthalenesulfonic acid (light brown powder). The proton NMR spectrum consisted of four doublets centered at 7.81 (H2), 7.91 (H3), 8.61 (H5), and 8.24 ppm (H8) and a multiplet from 7.58 to 7.75 ppm (H6, H7). Chemical shifts and coupling constants agreed with the literature.[17]

Capillary Electrophoresis. CE was performed using a Prince CE system (Prince Technologies, Emmen, The Netherlands). In all experiments, uncoated fused-silica capillaries of 75-μm i.d. (BGB Analytik, Anwil, Switzerland) were used. The capillaries were flushed for 15 min (1000 mbar) with 0.1 M NaOH at the beginning of each day. For quenched phosphorescence detection, capillaries of 110-cm length (l_{eff} = 55 cm) were used and separations were performed at −15 kV (a negative separation voltage was used because sample injection has to be performed at the "outlet" side in this setup). For absorption and 266-nm LIF detection, a 105-cm capillary with an effective length of 60.5 cm was used and separations were performed at +20 kV.

Detection. Phosphorescence excitation and emission spectra were recorded in a 1-cm cuvette on an LS-50B luminescence spectrometer (Perkin-Elmer, Beaconsfield, U.K.). For absorption detection at 190 nm, an SP 8480 XR detector (SpectraPhysics, Mountain View, CA) was used with a capillary detection window of 3 mm. For 266-nm LIF detection, a home-built LIF setup, equipped with a NanoUV quadrupled Nd:YAG laser (Uniphase, Meylan, France), was used. The system has been described before,[10] but in this work, a lens with a focal distance of 2.5 cm was used to produce a spot of ~100 μm in diameter. For quenched phosphorescence detection, a pulsed LS-40 luminescence detector (Perkin-Elmer) was used (delay time, 0.05 ms; gating time, 5.00 ms). Excitation was performed at 294 nm, and the phosphorescence emission was collected using the total emission mirror (TEM) and the 390-nm cutoff filter provided with the instrument. To obtain phosphorescence, oxygen levels in the separation buffer should be low, because molecular oxygen is an efficient quencher. Therefore, an adapted CE setup was used, which has been described in detail before.[15] Before use, the buffer was deoxygenated using overnight purging with nitrogen, while the buffer was being circulated by the LC pump. To start electrophoresis, the exit of the pump was connected with the automated six-way valve of the system and the capillary was filled in the normal fashion.[15,16]

RESULTS AND DISCUSSION

Phosphorophore. It is well known that bromine-substituted naphthalenes can produce strong phosphorescence signals in deoxygenated solutions at room temperature.[18] Unfortunately, bromine-substituted naphthalenes lack adequate solubility in aqueous buffers (millimolar concentrations are required to generate a strong phosphorescence background signal). It was expected that BrNS would be sufficiently water soluble, while providing a strong phosphorescence background signal even at high pH. Indeed, the phosphorescence signals of BrNS recorded in distilled and demineralized water were large and showed only minor differences in intensity over the pH range from 7 to 12. The phosphorescence excitation and emission spectra obtained for a 1 μM solution at neutral pH, after 10 min of purging with nitrogen gas, are shown in Figure 1: the excitation maximums are at 228 and 294 nm, and the emission maximums are at 512 and 535 nm. It should be noted that no fluorescence is observed in Figure 1 because of the applied delay time.

For a further characterization of the phosphorophore, the triplet lifetime of 1 mM BrNS dissolved in a 50 mM borate buffer

(17) Cerfontain, H.; Zou, Y.; Bakker, B. H.; van de Griendt, F. *Can. J. Chem.* **1994**, *72*, 1966−1971.

(18) Donkerbroek, J. J.; Elzas, J. J.; Gooijer, C.; Frei, R. W.; Velthorst, N. H. *Talanta* **1981**, *28*, 717−723.

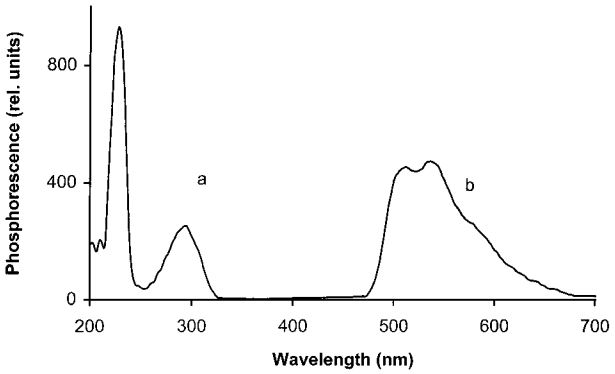

Figure 1. Phosphorescence excitation (a) and emission (b) spectrum of 1 μM BrNS recorded in demineralized and distilled water (pH 7). Spectral bandwidths (excitation/emission) 10/10 nm, gating time 3.00 ms. Excitation spectrum: λ_{em} 535 nm, delay time 1.00 ms. Emission spectrum: λ_{ex} 294 nm, delay time 2.50 ms.

Table 1. Triplet Lifetimes of 1 mM 1-Bromo-4-naphthalenesulfonic Acid in 50 mM Borate Buffer, as a Function of pH and CE Conditions

	triplet lifetime (ms) (current, μA)		
CE conditions	pH 9.0	pH 9.5	pH 10.0
−180 mbar	0.58	0.44	0.45
−15 kV	0.40 (−9)	0.29 (−15)	0.23 (−20)
−30 kV	0.31 (−21)	0.22 (−34)	0.18 (−48)

was determined at various pH values. To this end, a capillary was filled with the buffer solution and pressure (−180 mbar) or voltage (−15/−30 kV) was applied; the experimental results are presented in Table 1. The triplet lifetime is an important parameter: together with the quenching rate constant, k_q, and the noise on the baseline, it determines the sensitivity that can be reached in quenched phosphorescence detection.[15] Because the amount of oxygen produced at the anode depends on the current through the capillary,[15] shorter lifetimes are obtained at higher voltage and pH (both lead to a higher current). Nonetheless, as indicated by the data of Table 1, the lifetime at pH 10 (−48 μA) is still as large as 0.18 ms.

Finally, it is interesting to compare the triplet lifetime of BrNS with that of biacetyl under pH conditions where also the latter phosphorophore can be applied. In borate buffers at pH 8.5−9.0, the lifetime of BrNS was typically 3-fold longer than that of biacetyl. This should lead to 3-fold lower LODs.[15,16] It should be realized, however, that BrNS is negatively charged, so that the quenching interaction may be reduced if negatively charged analytes are dealt with. It was observed that these effects combined lead to similar or slightly improved LODs for nitrophenols and naphthalenesulfonates ($(1-3) \times 10^{-8}$ M) compared to those obtained using biacetyl ($(1-6) \times 10^{-8}$ M).

Capillary Electrophoresis. As outlined above, quenched phosphorescence detection of peptides requires a separation buffer pH of 9 or higher, because the amino groups should be deprotonated. Because the deprotonated peptides are negatively charged, electrophoretic mobility is induced at the same time. In Figure 2, the observed electrophoretic mobilities, μ_{el}, of three di- and two tripeptides are plotted as a function of the buffer pH; in the pH range tested, they are much smaller than the electroosmotic flow

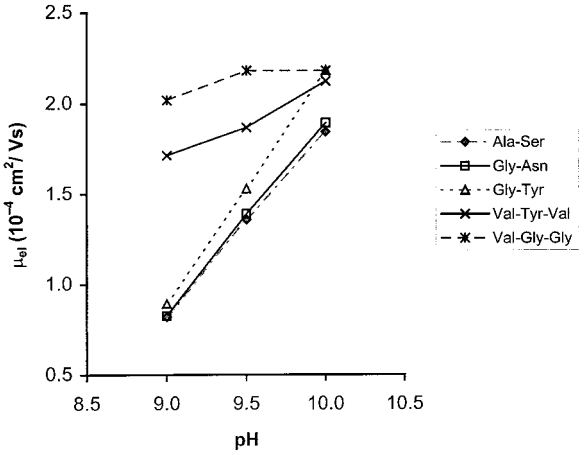

Figure 2. Electrophoretic mobilities of di- and tripeptides as a function of buffer pH. Buffer: 50 mM borate, 1 mM BrNS.

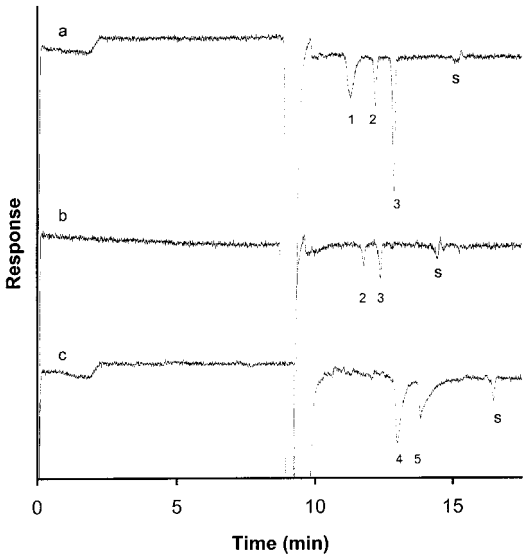

Figure 3. Electropherograms of different mixtures of di- and tripeptides recorded using quenched phosphorescence detection. Buffer: 50 mM borate, 1 mM BrNS, voltage: −15 kV (−9 μA). Peaks: (1) Ala-Ser, (2) Val-Tyr-Val, (3) Val-Gly-Gly, (4) Gly-Asn, (5) Gly-Tyr, and (s) system peak. Trace a: pH 9.5; peptide concentrations, 100 μg/L; injection, $-30 \times 9 = -270$ mbar·s. Trace b: pH 9.5; peptide concentrations, Val-Tyr-Val 20 μg/L; Val-Gly-Gly, 10 μg/L; injection, $-70 \times 9 = -630$ mbar·s. Trace c: pH 10; peptide concentrations, 200 μg/L; injection, $-30 \times 9 = 270$ mbar·s.

(EOF), which is $\sim(7-9) \times 10^{-4}$ cm^2/Vs. This leads to a rather short time window in which all analytes migrate. In other words, the peak capacity is rather small. This problem was partly solved by using a relatively low voltage (−15 kV). Probably, the situation can be improved further by reducing the EOF either by adding an organic modifier to the buffer or by using coated capillaries. In the present study, no such attempts were made, because the emphasis was on detection rather than on separation.

Quenched Phosphorescence Detection. The potential of quenched phosphorescence for the detection of peptides was examined with the nonderivatized di- and tripeptides Val-Tyr-Val, Val-Gly-Gly, Ala-Ser, Gly-Asn, Gly-Ala, and Gly-Tyr. Figure 3 shows electropherograms obtained with a 50 mM borate buffer containing 1 mM BrNS, at pH 9.5 (traces a and b) and pH 10.0 (trace c). It should be recalled here that quenched phosphorescence

Table 2. Detection Limits and Theoretical Plate Numbers Obtained for Di- and Tripeptides, Using CE with Quenched Phosphorescence Detection[a]

peptide	LOD μg/L	LOD M	N ($\times$ 1000)
Val-Tyr-Val	10	2×10^{-8}	150
Val-Gly-Gly	5	3×10^{-8}	145
Ala-Ser	20	8×10^{-8}	14
Gly-Asn	20	1×10^{-7}	13
Gly-Tyr	20	1×10^{-7}	16
Gly-Ala	15	1×10^{-7}	7.5

[a] Buffer: 50 mM borate, 1 mM BrNS, pH 9.5. Voltage: -15 kV (-9 μA). Injection: $-30 \times 9 = 270$ mbar·s.

Table 3. Detection Limits (μg/L) for Tyrosine-Containing Peptides, Using CE with 190-nm Absorption, 266-nm LIF, and Quenched Phosphorescence Detection

peptide	absorption	266-nm LIF	quenched phosphorescence
Gly-Tyr	50	10	20
Val-Tyr-Val	75	15	10
Met- enkephalin	75	50	65
angiotensin II	180	200	80

[a] Experimental conditions: see text.

detection is nonlinear, since the quenching interaction is governed by the Stern−Volmer equation.[15,16] This is obvious from trace b where the concentrations of Val-Tyr-Val and Val-Gly-Gly are respectively 5 and 10 times lower than in trace a; it also serves to illustrate the LODs that can be obtained using higher injection volumes. In terms of peak efficiency, the pH range 9.0−9.5 provides the best results. Mutual separation of Gly-Asn and Gly-Tyr is not achieved at pH 9.5 but requires pH 10.0, obviously with the disadvantage of more peak broadening (trace c).

The LODs (expressed in micrograms per liter as well as in molar) and the theoretical plate numbers, N, for the individual peptides at a buffer pH of 9.5, are given in Table 2. Obviously, LODs in the 5−20 μg/L range can be obtained for all analytes. It is interesting to look at the LODs in terms of molarity; for the tripeptides, LODs of 2×10^{-8} and 3×10^{-8} M were obtained, which is close to the best results achieved with quenched phosphorescence in CE thus far.[16] For the dipeptides, LODs of $\sim 10^{-7}$ M were obtained, because the peaks of these peptides were ~4 times broader than those of the tripeptides. Taking this into account, it is clear that the quenching efficiencies of all peptides are approximately equal.

Finally, the results obtained using CE with quenched phosphorescence detection were compared with those provided by UV absorption and 266-nm LIF detection. For this purpose, Gly-Tyr, Val-Tyr-Val, the pentapeptide methionine enkephalin, and the octapeptide angiotensin II—all of them peptides containing one tyrosine residue—were used as test analytes. It should be noted that not only their native fluorescence is exceptional: the LODs obtained for this class of peptides using UV absorption are also not representative for the group of peptides as such. This is because the tyrosine residue contributes significantly to the overall absorption (in addition to the peptide bonds). For quenched phosphorescence, CE was performed using a 50 mM borate, 1 mM BrNS buffer at pH 9.5. For the electropherograms obtained with UV and LIF detection, a buffer at pH 9.0 and a separation voltage of + 20 kV were adequate to obtain a baseline separation of the analytes. For tryptophan-containing peptides, which emit ~100-fold stronger fluorescence signals than tyrosine-containing peptides, LODs are reported to be in the nanomolar range.[9] Consequently, for the tyrosine-containing peptides discussed here, LODs in the 10−100 μg/L range should be expected. As regards absorption detection, the best detectability can be expected at a short wavelength, i.e., 190 nm.

The LODs for the three detection methods involved, as presented in Table 3, show some interesting features. With LIF detection they vary from 10 to 200 μg/L as expected. Interestingly, quenched phosphorescence detection shows the same performance, with LODs in the 10−80 μg/L range. This underlines its relevance since—in contrast to LIF—it is generally applicable to the detection of peptides. In comparison to 190-nm absorption detection—a technique that is also generally applicable—the LODs obtained using quenched phosphorescence are somewhat better. It should be noted that the advantage of quenched phosphorescence detection will be more pronounced for (small) peptides that do not contain tyrosine, because the peptide bonds are the only chromophores present in that case. Furthermore, the difference in selectivity should be stressed: while an overwhelming number of compounds absorb radiation at 190 nm and, therefore, may interfere with UV-based peptide detection, such problems will be far less serious in quenched phosphorescence since the analyte characteristics required to induce phosphorescence quenching are quite stringent.[15,16] Admittedly, this selectivity will have to be demonstrated in the analysis of real-life samples and will be the subject of future research.

CONCLUSIONS

This preliminary study shows that quenched phosphorescence provides good perspectives for the detection of (small) peptides in CE and deserves further attention, especially as regards the selectivity and the CE separation conditions. Quenched phosphorescence does not require chemical derivatization and provides LODs similar to those of LIF as applied to natively fluorescent, tyrosine-containing peptides. Because detection is directed at the terminal amino group and, if present, also at side-chain amino groups, quenched phosphorescence will be most advantageous for small peptides. Future research will be dealing with the application of quenched phosphorescence detection to peptides in real-life samples.

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