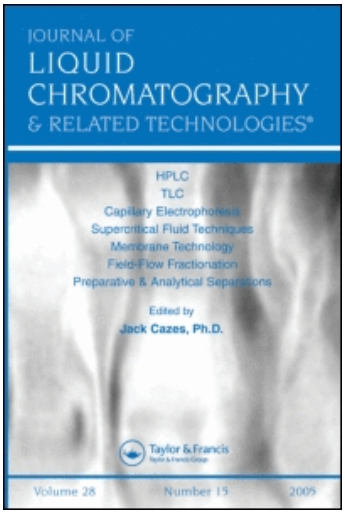


**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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**To cite this Article** Feng, Hua-tao , Li, Sam Fong-Yau , Chia, Pei-shan and Loke, Weng-keong(2006) 'Detection of Proteins by On-Column, Non-Covalent Labeling with NanoOrange During Capillary Zone Electrophoresis', Journal of Liquid Chromatography & Related Technologies, 29: 10, 1447 — 1455
**To link to this Article: DOI:** 10.1080/10826070600674844
**URL:** http://dx.doi.org/10.1080/10826070600674844

*Journal of Liquid Chromatography & Related Technologies*®, 29: 1447–1455, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070600674844

# Detection of Proteins by On-Column, Non-Covalent Labeling with NanoOrange During Capillary Zone Electrophoresis

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**Abstract:** To avoid prior derivatization of proteins with fluorescent reagents, on-column labeling of non-covalent dye NanoOrange on proteins was evaluated with sodium dodecyl sulfate (SDS) as an additive using laser induced fluorescence (LIF) monitoring in capillary electrophoresis (CE). Performance of NanoOrange in buffer solutions of various pH values was tested. Acidic buffer solution was found to be unsuitable for on-column NanoOrange labeling. SDS enhanced the fluorescence intensities of basic proteins with high *pI* values. The fluorescence intensities of protein solutions with or without SDS additives were compared using a fluorescence spectrophotometer. The applicability of on-column labeling and detection of staphylococcal enterotoxin B was also demonstrated. This on-column labeling method provides a rapid, direct, widely applicable technique for protein studies.

**Keywords:** Capillary electrophoresis, Proteins, Laser induced fluorescence, NanoOrange, Non-covalent labeling

## INTRODUCTION

Many studies have been carried out for separation and determination of proteins using CE methods.[1–3] Though LIF detection can provide

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sensitivities several orders higher than that of UV detection, the pre- or post-column covalent labeling of proteins with fluorescent dyes is time consuming and often requires special skills to achieve acceptable results. The multiple labeling sites on proteins can lead to incomplete labeling, which may cause a single protein to be separated into several peaks. Such difficulties can be avoided by applying fluorescent dye into the separation buffer and establishing a dynamic interaction between dye and proteins. This on-column labeling or dynamic labeling technique is similar to the ubiquitous method of on-column DNA labeling, allowing one of the main advantages of CE, namely speedy determination, to be exploited readily.

Non-covalent dyes are possible choices for on-column labeling. Swaile and Sepaniak presented the first report on on-column labeling of proteins in CE using the non-covalent dyes 1-anilinonaphthalene-8-sulfonate (ANS) and 2-p-toluidinonaphthalene-6-sulfonate (TNS).[4] Since then, several methods for non-covalent labeling of proteins for their subsequent analysis by CE-LIF were developed.[5–9]



NanoOrange is a merocyanine dye that binds to protein surfaces or hydrophobic domains of nondenatured proteins. The dye shows little background fluorescence in free solution and undergoes significant fluorescence enhancement due to conformational changes induced by binding.[10,11] Recently, NanoOrange has been used with CE postcolumn labeling for protein determination.[10] In addition, dynamic labeling of proteins with NanoOrange in sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CGE) was reported.[11,12] Non-covalently on-column labeling of serum albumin by NanoOrange was studied using both capillary isoelectric focusing (CIEF)[13] and capillary zone electrophoresis (CZE).[14] However, no other proteins were tested in these two reports.

In this paper, the feasibility and stability of on-column protein labeling by NanoOrange without heating were tested in CZE mode with different proteins, though NanoOrange labeling was designed to work after protein thermal denaturing.[15,16] The performance of NanoOrange in various pH value buffer solutions was evaluated. SDS was found to be a good additive, enhancing the fluorescence of basic proteins with high *pI* values. The method was developed and successfully applied to the rapid detection of a toxin and biowarfare agent, staphylococcal enterotoxin B (SEB).

## EXPERIMENTAL

### Materials

Lysozyme (*pI*: 10.7 $\sim$ 11.0, MW 14,400) from egg white, myoglobin (*pI*: 8.1, MW 16,700) from horse heart, bovine serum albumin (BSA) (*pI*: 4.8, MW 67,000), and SEB were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ribonuclease A (*pI*: 9.45, MW 13,700) from bovine pancreas was

the product of Fluka (Buchs, Switzerland). NanoOrange stock solution (500×) was purchased from Molecular Probes, (Eugene, OR, USA). Other reagents were of analytical-reagent grade. Water (≥18 MΩ) used throughout the experiments was generated by a NANOpure ultrapure water system (Barnstead, IA, USA).

## Apparatus

Proteins were dissolved in buffer solutions of different pH values. NanoOrange stock solution was added to protein solutions at 1× concentration without heating. A SPECTRAFluor Plus (TECAN, Switzerland) was used to study the fluorescence of the solutions in microplate format. Since the reactions between proteins and NanoOrange occurred rapidly, fluorescence was measured immediately after addition of NanoOrange using the fluorometer equipped with filters allowing excitation at 485 ± 20 nm and measurement of emission at 595 ± 35 nm. The duration of fluorescence measurement was kept as short as possible to minimize photobleaching effects.

The CE separations were performed on a CE-P2 system (CE-Resources, Singapore) coupled with a homemade LIF detector. The excitation wavelength was 488 nm. A photomultiplier tube was used for the fluorescence detection with a 580 nm band-pass filter (Full Width-Half Maximum: 10 nm, Edmund, NJ, USA). An alternative UV-VIS detector–LCD 2083.2 (ECOM, Praha, Czech) was used when UV detection was applied. Except where otherwise noted, total length of untreated fused silica capillary (Polymicro Technologies, AZ, USA) was 50 cm (35 cm to detection window). The inner diameter was 75.0 μm, and the outer diameter 365.0 μm. Hydrodynamic injections were carried out by applying 0.30 PSI for 15 s. When a capillary was first used, it was rinsed with 0.1 M NaOH and water for 10 min each. A pressure of 30 PSI was applied for the rinse procedure. Subsequently, the capillary was flushed by a separation buffer for 10 min. Between two runs, the capillary was rinsed for 2.0 min with a running buffer. The running buffer was changed after each five runs. Electrophoresis was performed at +20 kV and a temperature of 25 ± 1°C.

## Labeling of Proteins with NanoOrange

Three buffer solutions with different pH values were tested, 20 mM phosphate buffers with pH values at 7.1 and 3.0 and 20 mM sodium tetraborate buffer at pH 9.1. To stabilize the complexes formed between proteins and NanoOrange, especially for basic proteins with high *pI* values, 0.01% SDS was added into the buffers, subsequently. These buffers were used in both microplate fluorescence measurements, as well as in CE experiments. For CE experiments, protein sample solutions did not contain NanoOrange, and the buffers with

1× NanoOrange were prepared fresh daily. Because of the toxicities of NanoOrange and SEB, the researchers were wearing personal safety devices, and all experiments were carried out in a fume hood, except for the microplate fluorescence measurements, which hadn't been used to test the SEB sample. Sample and buffer solutions which were used were immersed in bleach solution first and collected by DSO National Laboratories waste treatment department.

## RESULTS AND DISCUSSION

### Direct Labeling of NanoOrange and Proteins on CE

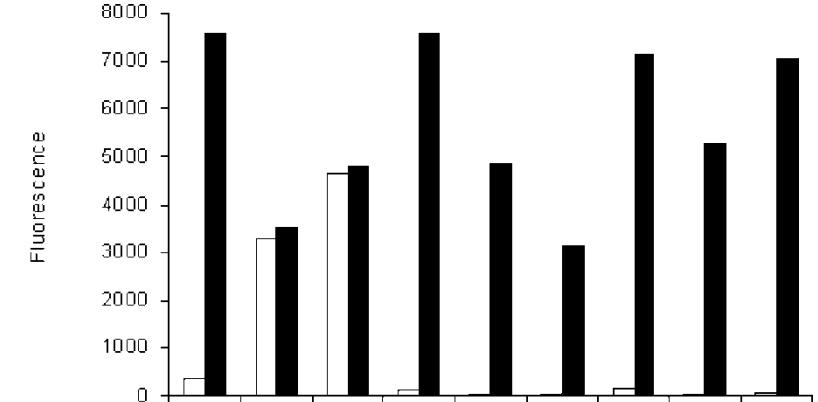
To evaluate the use of NanoOrange for on-column labeling, 7.30 μM ribonuclease A, 6.94 μM lysozyme, 5.99 μM Myoglobin, and 1.49 μM BSA (100 mg/L each) proteins were tested by three different pH value separation buffers with 1× NanoOrange. No obvious association between NanoOrange and proteins was found under low pH condition, as in pH 3.1 buffer. Also, the peaks of ribonuclease A and lysozyme could not be observed with neutral and basic buffers. In pH 7.1 and 9.1 buffers, BSA could be labeled effectively on column and the peak area under pH 9.1 was 2.5 times larger than that under 7.1. Myoglobin could show a quite small peak only with pH 9.1 buffer.

As mentioned above, NanoOrange can bind to protein surfaces and hydrophobic domains of non-denatured proteins. BSA is a transporter molecule for lipids and has more hydrophobic domains. That is one possible explanation why only BSA could show strong fluorescence.

### Fluorescence Measurement

Detergents may have helped the binding of NanoOrange on proteins.[15,17] A powerful detergent, such as SDS, can disrupt both hydrophobic and hydrogen bonds and dissolves even hydrophobic proteins effectively. Existence of SDS can convert proteins from "native" forms into more linear shape forms and may lead to larger surface area and more hydrophobic domains. Moreover, fluorescence can be enhanced by SDS via another mechanism, as SDS itself can associate with NanoOrange and produce fluorescence. Therefore, SDS bound to protein molecules will subsequently produce fluorescence when labeled with NanoOrange and upon excitation.

To confirm the effect of SDS, both 1× concentration NanoOrange and 0.01% SDS, which is the maximum tolerance level for SDS contamination advised by the manufacturer, were added into protein sample solutions. The average fluorescent values of five measurements, with or without SDS, are shown in Figure 1. After subtracting fluorescence value of blanks, which




| | BSA | | | Ribonuclease A | | | Lysozyme | | |
|---|---|---|---|---|---|---|---|---|---|
| □ Without SDS | 352 | 3287 | 4650 | 119 | 37 | 33 | 154 | 45 | 52 |
| ■ 0.01% SDS | 7581 | 3530 | 4806 | 7588 | 4855 | 3122 | 7154 | 5286 | 7037 |

***Figure 1.*** Effect of SDS on fluorescence of proteins labeled with NanoOrange. Fluorescence measurements of proteins in three different pH buffers were performed with 1× concentration NanoOrange. The excitation wavelength is 485 ± 20 nm and emission wavelength is 595 ± 35 nm. Proteins: 7.30 μM ribonuclease A, 6.94 μM lysozyme, and 1.49 μM BSA (100 mg/L each).



are the fluorescence intensities of solutions without proteins, only BSA displayed high fluorescence value without SDS addition at a pH 7.1 and 9.1 environment. With addition of SDS in the same pH environment, both ribonuclease A and lysozyme exhibited strong fluorescence, and the fluorescence of BSA increased slightly too. Moreover, all proteins showed obvious fluorescence enhancements after addition of SDS in pH 3.0 buffer. This result can explain why on-column labeling with different proteins was reported under SDS-CGE mode[11,12] and only serum albumin was labeled successfully under CZE mode so far.

## On-Column Labeling with SDS Addition

On-column labeling of proteins in CE was carried out further using all three pH value buffers with 1× concentration NanoOrange and 0.01% SDS. However, the proteins always displayed low, undetectable fluorescence with the pH 3.0 buffer. In contrast, the proteins in the pH 9.1 solutions could give stable peaks. Strong fluorescence from BSA, ribonuclease A, and lysozyme were observed. Proteins could be detected in pH 7.1 buffer, but adsorptions of proteins on the capillary wall might be the reason of bad peak shapes. SDS was added into separation buffer in this experiment, and it reacted dynamically to proteins;

only one main peak could be acquired from each protein rested. This had been confirmed using the UV detector. However, similar to the results in a recent paper,[18] myoglobin showed exceptional peak shape at pH 9.1, which may be triggered by loss of Heme groups and formation of intermediates with the presence of SDS.

Six different SDS concentrations were investigated ranging from 0.001% to 0.05%, in the pH 9.1 buffer. Higher SDS concentrations usually could lead to higher peak heights. However, higher SDS concentration resulted in higher background noise at the same time. The signal/noise ratio of 6.94 μM lysozyme sample acquired from different SDS concentration buffers was studied. The protein remained undetected with 0.001% SDS. When 0.05% SDS was added into the buffer, the background signal was so large that the irregular negative peaks were acquired. The addition of 0.01% SDS showed the highest signal/noise value and was selected for further experiments. The addition of SDS could enhance the fluorescence intensities. However, at the same time SDS may decrease protein resolution, since it brings negative charges to all proteins. The separation of 1.19 μM BSA, 5.84 μM ribonuclease A, and 5.55 μM lysozyme (80 mg/L each) was preformed with a longer capillary (total/effective length: 75/60 cm). With the SDS additive, these three proteins could still be separated within ten minutes (Figure 2), though 9.1 is not an ideal buffer pH value for separation of basic proteins.



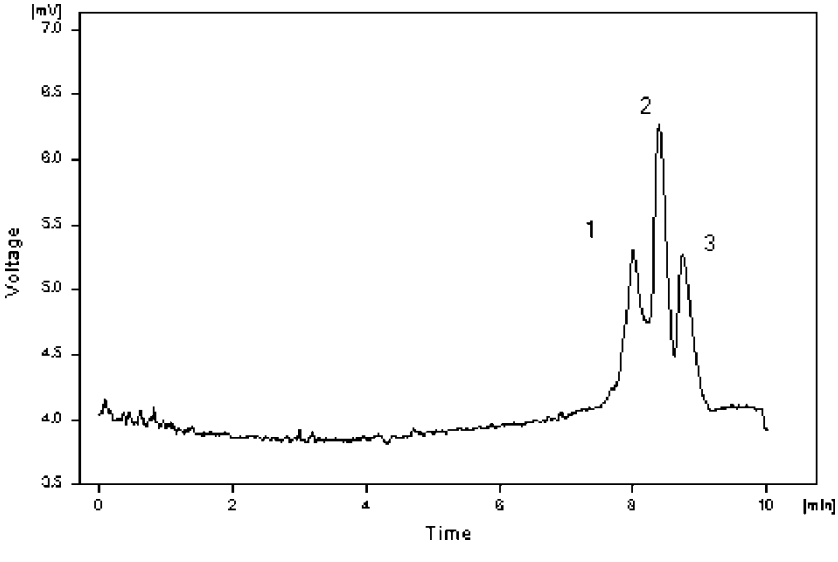



***Figure 2.*** Separation of BSA, ribonuclease A, and lysozyme. Separation buffer: 20 mM sodium tetraborate, pH 9.1, 0.01% SDS and 1× concentration NanoOrange. Total capillary length: 75 cm, effective length: 60 cm. The inner diameter was 75.0 μm. Injection: 0.30 PSI for 15 s. Separation voltage: 20 kV. Proteins: 1, BSA; 2, lysozyme; 3, ribonuclease A.

## Application to SEB Detection

The toxin staphylococcal enterotoxin B monomer has a molecular weight of 28366 Da.[19,20] Previous studies had applied capillary electrophoresis with LIF detection to the determination of staphylococcal enterotoxins (SEs).[21–23] However, SEs had to be prelabeled, or labeled carefully, by dye in the lab before detection. As a toxin, SEB can contaminate the lab environment, so the labeling procedure is a threat to analysts. Also, as a potential biological warfare agent, it should be detected as fast as possible in some cases.

The method developed in this study was used to the online detection of SEB, which has a *pI* value of 8.6 and is slightly negatively charged in pH 9.1 solution. Without the SDS additive, no clear peak of SEB could be detected. Figure 3 shows the electrophoregram of on-column labeled SEB with an SDS additive. Detection limit was about: 0.061 μM. Though the LIF method was established on a home made detector, its sensitivity was still ~10 times better than that achieved from the UV detector with 20 mM sodium tetraborate buffer at pH 9.1.

Five successive runs were preformed to test the reproducibility. The R.S.D. (n = 5) of peak areas was 3.03% and the R.S.D. of migration times was 3.10%. The correlation coefficient ($R^2$) of five concentration levels (from 0.1 to 2.0 μM) was 0.9969. Note, that this detection was obtained via

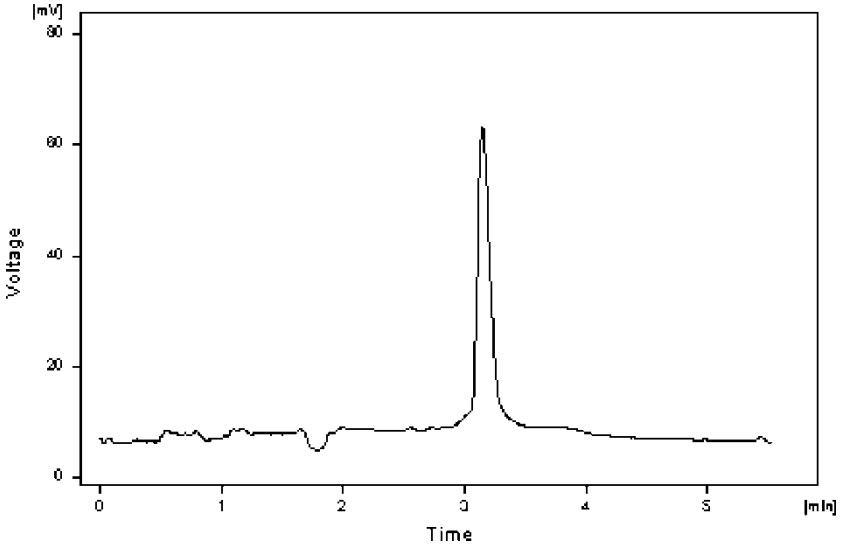



***Figure 3.*** Detection of SEB with CE-LIF by on-column labeling with NanoOrange. Separation buffer: 20 mM sodium tetraborate, pH 9.1, 0.01% SDS and 1× concentration NanoOrange. Total length of capillary: 50 cm; effective length: 35 cm. SEB concentration: 3.53 μM. Other conditions as in Fig. 2.

online labeling with NanoOrange in buffer, and the migration time was less than 4 min. The direct and rapid detection of SEB with an LIF detector became possible.

## CONCLUSION

In most cases, prior derivatization of proteins with a fluorescent label molecule is necessary to LIF detection. On-column labeling of proteins can offer a rapid, direct, and broad application range and satisfactory sensitivity relative to other detection methods. A method using a non-covalent dye, NanoOrange, was developed for on-column labeling. With the presence of SDS in the separation buffer, proteins with different *pI* values, including the toxin SEB, were non-covalently labeled and, subsequently, analyzed by CE-LIF.



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Received November 30, 2005
Accepted December 26, 2005
Manuscript 6776