Anal. Chem. **2002,** *74,* 2257−2260

Ultratrace Analysis Based on Hadamard Transform Capillary Electrophoresis

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Hadamard transform capillary electrophoresis, which is based on a multiple sample injection technique, was combined with laser-induced fluorometry and utilized in the determination of analytes at subpicomolar levels. The sensitivity was substantially improved by increasing the order, i.e., the number of elements, of the Hadamard matrix. In fact, the signal-to-noise ratio was enhanced 18-fold by the use of a matrix of order 2047. A feasibility study was carried out by computer simulation to study the detection of an average of less than a single molecule in a single injection volume. The signal peak was clearly observable even under conditions at which only 0.3 molecule is present in the volume. Thus, this approach is potentially useful for ultratrace analysis, in which conventional single-injection capillary electrophoresis cannot be applied.

Sensitivity is one of the most important issues in analysis. Several research groups have already reported on the detection of a single molecule using laser-induced fluorescence.[1−3] However, these studies constitute demonstrations of ultratrace analysis under ideal conditions and a considerable number of issues need to be resolved for the practical application of such techniques. For example, numerous interfering substances are present in an authentic sample, and a sophisticated separation technique must be used prior to spectrometric detection. Capillary electrophoresis has been used successfully because of its high separation resolution. The concentration detection limit is, however, rather poor, due to the short path length (∼10 μm) of the detector. Recently, several mathematical approaches, which provide a multiplex advantage, were proposed for sensitivity enhancement. For example, a Fourier transform technique has been widely used in spectroscopies, e.g., infrared absorption spectrometry, nuclear magnetic resonance spectrometry, and mass spectrometry. We previously reported on the application of a Hadamard transform technique to capillary electrophoresis (HTCE), which substantially improves the signal-to-noise ratio, as would be expected from theory.[4,5] In this investigation, an optically gated sample injection technique was employed for multiple sample injection according to a Hadamard code. The electropherogram was constructed by decoding the observed signal by calculating the product with an inverse Hadamard matrix. Techniques using other mathematical calculations involve Shah convolution Fourier transform[6,7] and cross-correlation[8,9] techniques. However, the Hadamard transform technique is simpler and requires a shorter time for calculation.

In this study, we demonstrate ultratrace analysis based on HTCE combined with sensitive laser fluorometry. The signal-to-noise ratio was substantially improved, and detection of analytes at subpicomolar levels was achieved using an injection code of order 2047. In addition, a computer simulation was performed, to evaluate the potential advantage of HTCE, in which a feasibility study of ultratrace analysis allowing the detection of an average of less than a single molecule in a single injection volume was demonstrated.

EXPERIMENTAL SECTION

Instrumentation. The experimental setup is essentially the same as that reported previously,[4,5] except for a few minor changes; a disposable syringe was used as a reservoir at the sample injection side; the total and effective lengths of the capillary (25-μm i.d., 375-μm o.d., GL Sciences Inc.) were 14 and 4.5 cm, respectively. An argon ion laser emitting at 488.0 nm (GLG3200, Nippon Electric Co., Tokyo, Japan) is split into two parts for use as gating and probe beams, respectively. The gating and probe beams were focused by objective lenses onto the capillary, positioned 4.5 cm from the focal point of the gating beam to that of the probe beam. The gating beam is passed through or blocked by an optical shutter (F77-4, Suruga Seiki, Shizuoka, Japan) which is modulated by a controller (F77-6, Suruga Seiki), interfaced with a personal computer (PC9801 RX, Nippon Electric Co.). When the shutter is opened, the light-absorbing analyte is photodegraded by strong irradiation by a visible light. Thus, the sample is injected only when the shutter is closed. The sample was injected according to a pseudorandom sequence based on the cyclic *S*-matrix consisting of "1" or "0" in the Hadamard transformation. The time for data sampling was adjusted to be equal to the injection time (0.5 s) in this study. Four different orders of

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10.1021/ac011149b CCC: $22.00
Published on Web 04/12/2002

matrices, i.e., 255, 511, 1023, and 2047, were made by employing a method using maximal length shift-register sequences.[10]

Materials. All reagents used in this study were analytical grade. Glycine, sodium hydroxide, and pyridine were purchased from Kishida Chemical Co. (Osaka, Japan). All other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Deionized water was prepared using an Elix purified water system (Millipore Co. Ltd., Molsheim, France). A carbonate buffer solution was prepared by dissolving sodium hydrogencarbonate in deionized water and by adjusting the pH with sodium hydroxide solution. A sodium fluorescein solution was prepared by dissolving 0.037 g of sodium fluorescein in 100 mL of water (1.0 mM). This solution is further diluted by mixing with 30 mM carbonate buffer (pH 9.2). Amino acid solutions (3 mM, 3 mL) were dissolved in 30 mM $KHCO_3$ (pH 9.2) and were derivatized with 6 mM fluorescein isothiocyanate (FITC) isomer I dissolved in 1 mL of acetone. The derivatization reaction was catalyzed by addition of 4 μL of pyridine. The reaction was carried out in the dark at 25 °C for 3 h, and the resulting solutions of FITC-labeled amino acids were stored at 4 °C. The mixture samples of FITC-labeled amino acids (glycine, arginine) were prepared by mixing the appropriate amount of each FITC-amino acid solution, prior to the measurements. The concentrations were adjusted to be 0.5 nM for glycine and 10 nM for arginine (1:20) or 0.5 nM for glycine and 50 nM for arginine (1:100).

Simulation. A computer simulation was performed to demonstrate the potential advantage of HTCE. The aim of this computer simulation is to demonstrate a capability of HTCE, which may allow the detection of the analyte molecule at concentration levels of an average of less than a single molecule in a single injection volume. In the computer simulation, the data for the calculation of an electropherogram were generated by adding a single molecule in each injected segment and by assuming the detection of these molecules with detectabilities of 100%. To demonstrate a capability for analyte detection at lower concentrations, the molecules were randomly removed from the segments and then the electropherogram was reconstructed by HT.

A fundamental equation of HTCE is represented by,

$$[\eta] = [S] \times [E] \tag{1}$$

where η is a series of data encoded by S which is the $n \times n$ matrix consisting of "zero" or "one" element and E is a series of data representing an electropherogram. The encoded electropherogram, η, is decoded to the electropherogram, E, by multiplying an inverse cyclic S matrix of S, S^{-1}, as follows,

$$[E] = [S]^{-1} \times [\eta] \tag{2}$$

Detail of the procedure for HTCE are reported in the previous paper and article.[4,5]

RESULTS AND DISCUSSION

Sensitivity of HTCE. In the previous study, we described some preliminary work and reported an 8-fold improvement in the S/N ratio using a 255 × 255 Hadamard matrix, relative to the value obtained by a single-injection method. In this study, an analytical curve for fluorescein was constructed using a laser operating at a total output power of 150 mW (gating beam 120 mW, probe beam 9 mW). A straight line extending from 2.0×10^{-12} to 1.5×10^{-7} M with a correlation coefficient of 0.997 was obtained. The detection limit (S/N = 3) was 2.0×10^{-12} M under the conditions used here. Further improvement in the detection limit was achieved by increasing the laser power. Panels A and B of Figure 1 show the electropherograms for a sample of fluorescein at 2 pM and 500 fM, which were obtained using laser powers of 150 and 250 mW, respectively. The S/N ratio in the latter was 3 with a detection limit of 5×10^{-13} M. The S/N ratio was improved by a factor of 4 by increasing the laser power from 150 to 250 mW. Thus, an improvement may be achieved by an increase in both the output power of the probe and the gating beams. In the case of optically gated injection, a high-power gating beam is desirable in order to achieve a more efficient destruction of the analyte molecule by photolysis, resulting in a deeper modulation of the analyte concentration. Because of this, a high-power laser is preferred for improving sensitivity. On the other hand, the intensity of the probe beam (15 mW) may be below the saturated level. Therefore, room exists for further improving the detection limit. The injection time for a single segment of the code was 0.5 s, and the migration velocity of the fluorescein ion was determined to be 2.2 mm s^{-1}. The injection volume for a segment was calculated to be 0.55 nL. The amount of fluorescein in a single injection volume was then calculated to be 280 ymol, which corresponds to ~170 molecules.

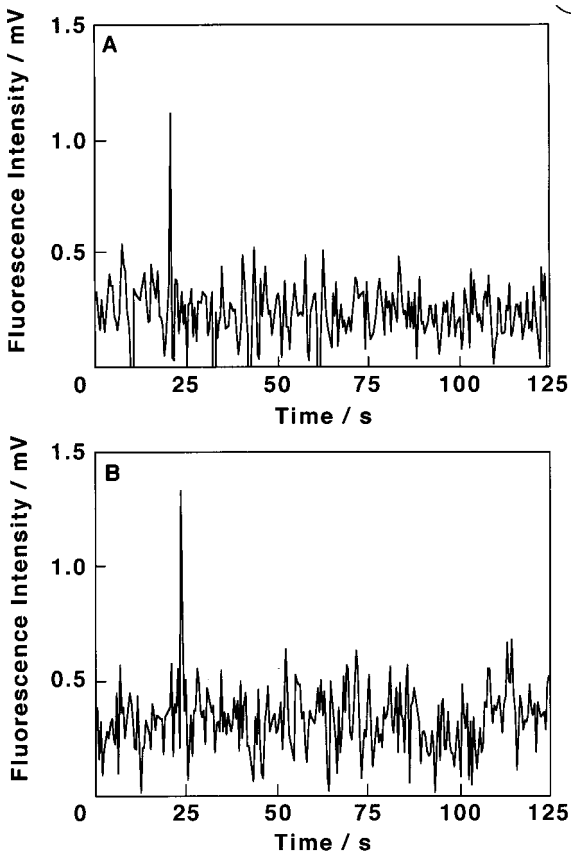

Figure 1. Electropherograms of fluorescein: (A) laser power, 150 mW; sample concentration, 2 pM (B) laser power, 250 mW; sample concentration, 500 fM. Buffer, 30 mM carbonate buffer (pH 9.3); laser wavelength, 488 nm; migration voltage, 10 kV; matrix order, 255.

Table 1. Relationship between the Order of Matrix, the Enhancement of S/N Ratio, and the Time for Analysis[a]

	enhancement of S/N ratio		
matrix order	observed	theoretical	time for analysis (s)
255	7.1	8.02	254.5
511	9.6	11.32	510.5
1023	12	16.01	1022.5
2047	18	22.03	2046.5

[a] The enhancement of the S/N ratio was calculated as the ratio of the S/N values obtained in the electropherograms measured by HTCE and a single-injection method. The sample concentration was adjusted to 50 pM. The injection time for a single segment was 0.5 s.

In HTCE, the S/N ratio is increased by a factor of $(n + 1)/2n^{1/2}$, where n is the order of the Hadamard matrix.[10] Thus, the S/N ratio can be increased with an increase in the matrix order. Table 1 shows the relationship between the order of the Hadamard matrix and the enhancement in the S/N ratio. The observed values are in reasonably good agreement with the theoretical values, although the observed values are slightly smaller than those expected from theory. This small discrepancy may arise from errors induced in the process of numerical calculation by a computer. For example, when the fluorescence signal is digitized by a computer, the signal may possibly be overestimated or underestimated in the digitizing process. In HTCE, such errors are more serious than in conventional CE, since the signal intensity is obtained by addition or subtraction of large signals in HT. It should be noted that substantial enhancement of the S/N ratio is observable in Table 1, especially in the case of a matrix of order 2047, but at the expense of the time required for analysis.

Separation. Numerous interferences are often present in an authentic sample. In a multiple-injection method, e.g., in a Hadamard transformation or a cross-correlation technique, a major component in the sample is known to disturb the detection of minor species. To confirm this limitation in multicomponent analysis using HTCE, several amino acids labeled with FITC were measured at different concentration ratios. Figure 2 shows a series of electropherograms for a sample containing glycine and arginine at different concentrations. Panels A and B of Figure 2 represent data for a sample containing 0.5 nM glycine and 10 nM arginine (molar ratio, 1:20), as measured by conventional (single-injection) CE and HTCE, respectively. The S/N ratio obtained by HTCE is superior to that obtained by conventional CE. The electropherogram obtained by HTCE for a sample containing 0.5 nM glycine and 50 nM arginine (1:100) is shown in Figure 2C. The peaks arising from FITC and glycine are no longer clearly observable because of a larger background signal. This is, unfortunately, unavoidable in HTCE, since a small peak is calculated as a result of the difference of large modulated signals. Because of this, the signal must be measured precisely for detection of minor species, and the measurement should typically be performed using an intense and stable light source, a sensitive detector, and a high-precision analog-to-digital converter/computer. However, the present result indicates that a minor species can be measured even in the presence of a 20-fold higher level of interferences.

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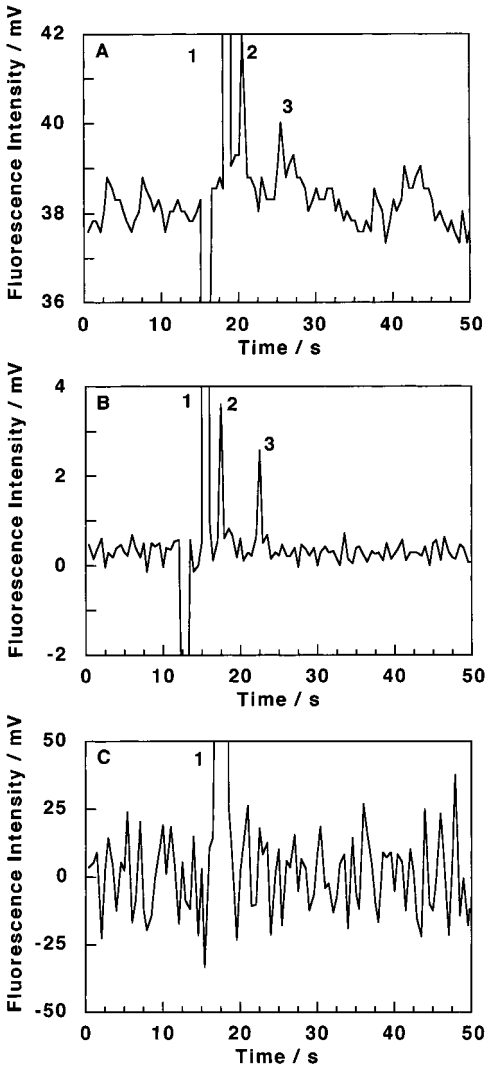

Figure 2. Electropherograms for multiple samples: (A) 0.5 nM glycine and 10 nM arginine (1:20), single injection; (B) 0.5 nM glycine and 10 nM arginine (1:20), multiplex injection; (C) 0.5 nM glycine and 50 nM arginine (1:100), multiplex injection. Peak identification: 1, arginine; 2, FITC; 3, glycine. The other conditions are the same as those described in Figure 1.

Detection of an Average of Less Than a Single Molecule in a Single Injection Volume. The detection of a single molecule has already been demonstrated using laser fluorometry. The detector in the present study is, unfortunately, not so sensitive because of poor isolation of fluorescence using the filters and the low quantum yield of the photomultiplier. It is known that a sheath-flow detector is effective in reducing background emission. A laser with a higher output power is also useful for deeper modulation of the concentration and for the more efficient excitation of the analyte molecule. It is known that an avalanche photodiode is more sensitive because of its higher quantum yield.[11] Therefore, it may be possible to further improve the sensitivity and to combine a single-molecule detector with HTCE. In conventional CE, it is essentially difficult to measure an electropherogram under conditions at which less than an average of a single molecule is present in a single injection volume. However, the measurement of analytes at such concentration levels is possible in HTCE, since

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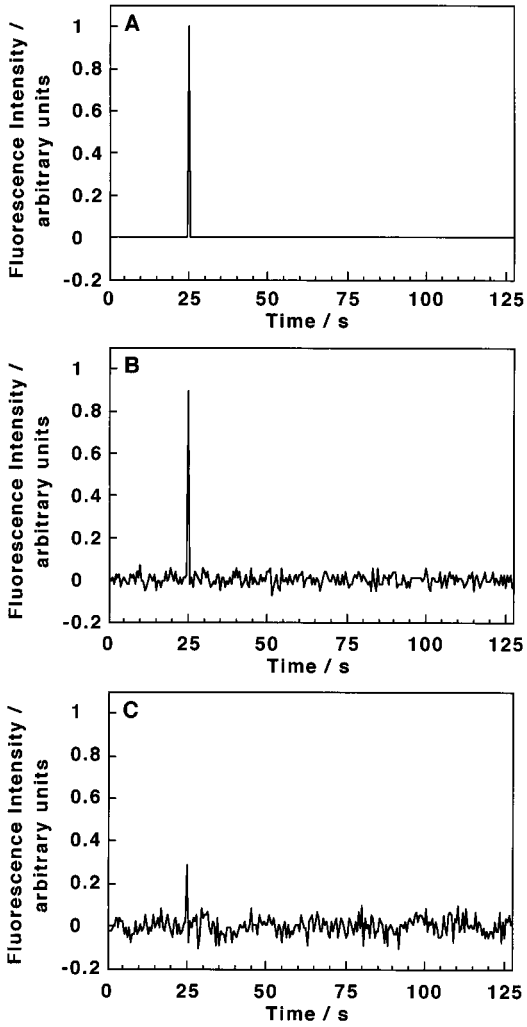

Figure 3. Computer simulation of HTCE at different analyte concentrations. Concentration: (A) 100%; (B) 90%; (C) 30%. In computer simulation, a single molecule is assumed to be present in a single injection volume. This condition is referred to as a "concentration of 100%" in this study. If the concentration is lower than 100%, some of the peaks in the observed data (encoded matrix of η) are missing. The data were artificially generated by randomly converting specified percentages of the signals into zero from the data of 100%.

numerous molecules are injected in a series of segments. Figure 3 shows the electropherograms obtained at various analyte concentrations. The S/N ratio decreases with decreasing analyte concentration. However, the peak remains clearly observable even at 30%. The dependences of the sample concentration on the peak height and the S/N ratio are shown in Figure 4. In a study of single-molecule detection, a detectability of 97% was reported.[12] Therefore, the assumption of 100% detectability in this study is not necessarily invalid. It should be noted that the peak height is proportional to the concentration of the sample and the S/N ratio becomes 3 at 10%. Thus, quantitative analysis at sub-single-molecule levels is possible using HTCE. On the other hand, in conventional CE, at least nine molecules are required for the determination (S/N = 3) since the signal intensity is 9 and the

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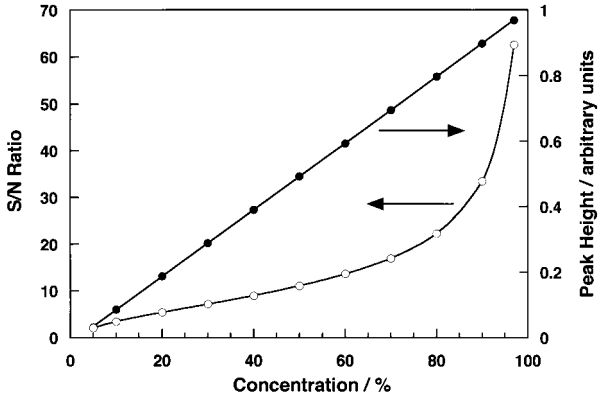

Figure 4. Dependences of the sample concentration on the peak height (solid circle) and the S/N ratio (open circle). The peak height is proportional to the sample concentration, and the S/N ratio becomes 3 at a concentration of 10%. This suggests that an electropherogram can be obtained even when only 13 molecules are present in the average in a total injection volume of 70 nL, providing a concentration detection limit of 310 aM, which cannot be achieved using conventional CE.

noise (statistical variation in the shot-noise-limit case) is $\sqrt{9} = 3$, even at detectabilities of 100% for single molecules. It should be noted that the total number of molecules detected in HTCE is much larger than those in conventional CE by sequential injections. Therefore, the detection limit determined by the shot noise can be substantially decreased in HTCE.

CONCLUSION

The concentration detection limit in capillary electrophoresis can be improved by using a Hadamard transformation technique. An analysis of samples at subpicomolar levels was demonstrated in this study. The S/N ratio was enhanced by increasing the order of the matrix at the expense of the time required for analysis. A further increase in sensitivity may be possible by improving the detection system. Thus, the detection of analytes at less than an average of a single molecule in a single injection volume might be possible using the present approach based on HTCE.

An optically gated sample injection technique was employed in this study. This approach is simple but somewhat expensive. Moreover, the modulation depth was insufficient due to a low output power of the laser used. As has already been demonstrated in other reported studies, microchip technology is useful not only for the miniaturization of the experimental setup but also for simple electrokinetic injection of the sample. Therefore, HTCE combined with microchip technology has the potential for widespread use in various applications, including environmental and biological analyses.

ACKNOWLEDGMENT

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan.

Received for review November 5, 2001. Accepted February 24, 2002.

AC011149B