CZE of peptides using a multi-pixel detector array

ABSTRACT

Capillary electrophoresis has been used in the analysis of peptides for decades, however traditional methods show excessive variability due to composition and sequence of the peptide, and variation in the interaction of the peptide with the separation medium. Multi-pixel detection along with the associated data analysis software improves reproducibility and sensitivity beyond current methods using UV absorption detection. The reproducibility of the data and the sensitivity of this 'no-label' multi-pixel detection approach are investigated using a mixture of known peptides. In the near future this will enable peptide mass finger printing based on the ability to assign molecular weight values to the peptides separated by charge.

INTRODUCTION

Capillary Zone Electrophoresis (CZE) is a common technique in peptide analysis (1). It offers high resolution and greater separation efficiency while using smaller sample volumes and reduced separation times. Peptides are short amino acid polymers linked together in a specific sequence via a peptide bond. The electro migration of peptides through a capillary relies on several intrinsic properties of the peptide such as the number, composition and sequence of its amino acid residues. Variation of these properties changes the overall peptide electric charge, size, shape and hydrophobicity. These factors, in combination with the properties of the separation medium, (pH, ionic strength, viscosity, permittivity etc.) and the interaction of the peptide with the components of the separation medium, determine the effective mobility of the peptide (2, 3).

There are several models, which seek to explain and thus predict peptide electrophoretic mobility. The first, and most commonly used model was described by Offord (3), where the effective mobility of the peptide is inversely proportional to its molecular mass raised to a fractional power. Consequently the separation conditions must be kept constant to limit any variation in mobility measurements. Multi-pixel detection capillary electrophoresis was performed on a compact bench-top device designed around a universal constituent layout. The light source (a Deuterium lamp HEREAUS Noblelamp DS 225/05J, optical parts (UV lenses-Newport, UV filters-Andover Optic), separation phase (Capillaries-Composite Metal Services Ltd) and detector (HAMAMATSU PDA 3904 S3904-512Q) are arrayed on a common rail. Light from the low-noise deuterium lamp passes through a filter wheel allowing the selection of detection wavelength, in this case through a 20 nm band pass filter centred at 220 nm for peptide bond detection at 214 nm.

Peptide	Number of amino acid residues	Molecular Weight	Sequence
Leucine Enkephalin	5	555	YGGFL
Bradykinin fragment	5	572	RPPGF
Methionine Enkephalin	5	573	YGGFM
Bradykinin	9	1059	RPPGFSPFR
Oxytocin	9	1009	CYIQNCPLG
[Arg8] – Vasopressin	9	1087	CYFQNCPRG
Luteinising Hormone releasing hormone	10	1207	EHWSYGLRPG
Substance P	11	1347	RPKPQQFFGLM
Bombesin	14	1638	EQRLGNQWAVGHLM

Table 1. Information on peptides present in the peptide mixture.

The light is then focused on a fused silica capillary, with an internal diameter of 50 μ m, outside diameter 375 μ m. As a bio molecule passes the light beam it absorbs energy dependent on its spectral characteristics. The light beam is then focused on to the photo diode array detector where the drop in signal due to the energy absorbed by the bio molecule is measured. The photodiode array data are preaveraged to a 20 Hz rate, and then transferred to a standard computer via a USB cable for further analysis by two signal-processing algorithms.

signal-processing algorithms.
The Generalised Separation Transform (GST) is designed to retain maximum information on temporal shape as well as quantification. The GST algorithm maximizes signal-to-noise, averaging the signal from the 512 pixels but retains the shape of the analyte peak. GST is less sophisticated than the EVA approach, discussed later, but provides the familiar electropherogram output with superior signal-to-noise ratio.

electropherogram output with superior signal-to-noise ratio. The Equiphase Vertexing Algorithm (EVA) takes a complementary approach to the analysis of the 512 electropherograms by analyzing the electropherogram from each pixel and reducing it to a set of space-time points corresponding to detected absorption peaks. In this multi-pixel signal analysis, absorption data from the entire detector array are combined to track the analyte bands as they migrate across the wide (12.7 mm) detector window. The bands migration speeds are then extrapolated to find their space-time origin (the vertex), which corresponds to the position and time at which the electrophoretic separation began. The vertex constraint imposed by the larger bands permits unparalleled resolution of significantly smaller absorption features.

Further advantages to multi-pixel array detection include artefact detection, direct measurement of thermal diffusion rates, and use of multiple injections into the same capillary, with each injection being identified by their vertex. In this paper we report the separation of a peptide mixture using CZE on a multi-pixel detector CE instrument along with GST and EVA data analysis. No labels were used and improvements in signal-to-noise between the single pixel and the processed data is demonstrated. The reproducibility of the data and the sensitivity of this 'no-label' detection approach are also investigated. The 'vertexing' capability and label free detection allows for molecular imaging that exceeds canonical absorption imaging techniques.

POLYMICRO TECHNOLOGIES

Separations were performed on a multi-pixel detector Capillary Electrophoresis platform. All data was analysed using the proprietary software obtained with the instrument. Samples were

separated on a bared fused silica capillary of total length 32 cm, effective length 20 cm and internal diameter of 50 µm obtained from Polymicron Technologies USA. The capillary was initially prepared by flushing with methanol, 0.1 M NaOH and water for 3 minutes each at 30 p.s.i. This was followed by a rinse with 1 M HCl for 10 minutes at 30 p.s.i. Between separations the capillary was rinsed with 0.1 M HCl for 3 minutes at 30 p.s.i., then filled with separation buffer. A 0.1 M phosphate buffer, pH 2.5, was used as the separation buffer. The peptide mixture (see Table 1) and individual peptides used in these studies were obtained from Sigma (UK). The individual peptide samples were prepared by diluting them to the appropriate concentration in the peptide separation buffer while the mixture was prepared as detailed in

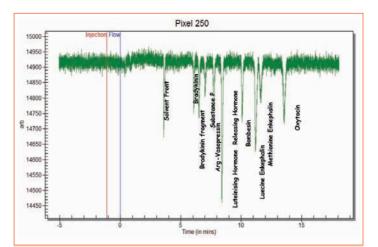
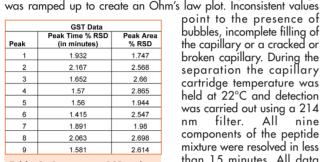


Figure 1. Single pixel (250) trace for a peptide mixture. The sample was injected at 2 p.s.i. for 4 seconds and separated under an electric field of 294.11 V/cm. Individual peaks are labelled.

shown by the highly reproducible separation of the peptide mixture over ten consecutive runs. For each peptide peak, the respective peak times and areas were recorded over the ten separations and the percentage Relative Standard Deviation (RSD) values determined from them. Figure 4 details the overlay of the GST processed data for the ten

repeats of the peptide separation and Table 2 lists the peak time and peak area percentage RSD values obtained for each peak from these separations.

All the peak time values from Table 2 show less than 2 percent RSD except for Peaks 2 and 8, which are slightly higher. This substantiates the ability of CE to perform high-resolution separations based on charge with exceptional reproducibility. Deviations are more frequent in the case of peak areas. Most of the peak amplitudes still have a percent RSD that is less than 3 percent. The inconsistencies in peak areas could possibly be due to slight variations in the EOF, which leads to variable sample injection.



the

V/cm

Sigma

Samples were loaded on to the capillary via a

hydrodynamic injection at

2 p.s.i. for 4 seconds or

an electrokinetic injection

at 8 kV for 10 seconds.

The separation was

conducted under an

electric field of 294.11

polarity. Prior to every

separation a conductivity

at

information sheet.

product

standard

Table 2. Percentage RSD values for peak time and peak area for all nine peaks of the peptide standard mixture (N=10).

point to the presence of bubbles, incomplete filling of the capillary or a cracked or broken capillary. During the separation the capillary cartridge temperature was held at 22°C and detection was carried out using a 214 All filter. components of the peptide mixture were resolved in less than 15 minutes. All data was analysed using both the Equiphase Vertexing Algorithm (EVA) and Generalised Separation

Transform (GST) algorithms. GST is a method of combining the data from the 512 pixels in a natural way which preserves the peak shape information of the electropherograms while at the same time maximising the signal to noise ratio. A 10-fold increase in signal to noise using GST as compared to single electropherograms is typically observed. The EVA signalprocessing algorithm uses a space-time constraint to significantly increase the signal-to-noise of the data. It also improves the resolving power of the system and allows associations of a given band in the electrophoretic window with a given injection, allowing (for example) large increases in throughput through multiple injections. The results quoted in this report are based upon the GST and EVA analysed traces.

check was performed by tracing current readings as the voltage

RESULTS AND DISCUSSIONS

To demonstrate the capabilities of multi-pixel detection, we have investigated the charge separation of peptides, the reproducibility of the separation in terms of peak area and peak time and the sensitivity of detection at 214 nm.

Charge separation and reproducibility

The peptide mixture used for separation was composed of Bradykinin, Bradykinin fragment, Substance P, Arg-Vasopressin, Luteinising Hormone Releasing Hormone, Bombesin, Luecine Enkephalin, Methionine Enkephalin and Oxytocin. Figures 1, 2 and 3 show the single pixel, GST and EVA traces of the aforementioned nine peptides, separated as a mixture.

The separation parameters were optimised to allow the best resolution between the sample peaks in the shortest time. The separation is performed in a low pH buffer; consequently most of the silanol groups on the surface of the capillary are protonated leading to negligible electro-osmotic flow (EOF). The sample is also prepared in the same low pH buffer resulting in an overall positive charge for all the sample peptides. This allows their separation under standard polarity. Sample absorption to the capillary surface due to hydrophobic or electrostatic interaction is possible, however the effects of adsorption were not observed as

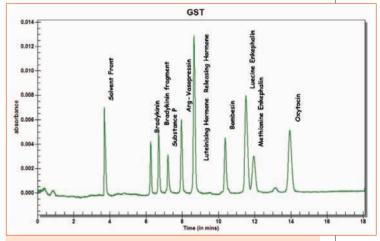


Figure 2. GST processed data for a peptide mixture. Separation conditions are detailed in the experimental section of this paper. Individual peaks are labelled.

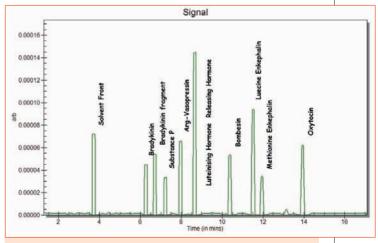


Figure 3. EVA processed data for peptide mixture.

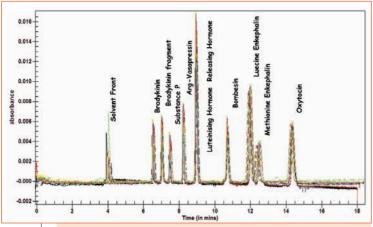


Figure 4. Overlay of the GST processed data for ten consecutive runs of the peptide standard separated under identical separation parameters.

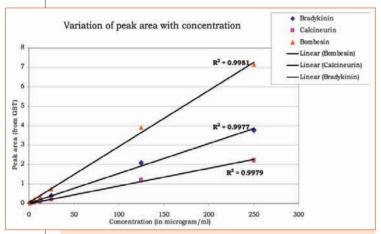


Figure 5. Plot of sample concentration versus peak area.

Another reason may be that due to the bias to charged species during electrokinetic injection sample depletion may occur. This bias is insignificant when separating higher concentration samples. Electrokinetic injections are typically less reproducible when compared to pressure injections, but when large sample volumes and a pre-coated capillary are used the reproducibility improves. The large surface to volume ratios of low volume glass inserts, typically 250 µl and below, can also lead to greater adsorption of peptides on to the glass surface compared to the larger buffer vials causing drops in concentration of sample and signal acquired. The adhesion of peptides to silica in the vials or in the capillary may be reduced or eliminated using specialised coatings, for example the low volume glass vial may be silanized and the capillary may be coated dynamically or permanently. These capillary coatings function by ion pairing with the negatively charged silanolic groups at the surface of the capillary or are covalently bound to them (4). This improves the efficiency and resolution obtained by decreasing the EOF and preventing peptide absorption.

Sensitivity

A mixture of three peptides including Bradykinin fragment, Calcineurin and Bombesin were prepared at a stock

FIONA PEREIRA¹, STUART HASSARD², ANTHONY CASS3, JUDIT M. NAGY3*

- *Corresponding author
- 1. Imperial College London Dept Chemistry and deltaDOT Ltd
- deltaDOT Ltd, Bessemer Building London, SW7 2BP, United Kingdom
- 3. Imperial College London Institute of Biomedical Engineering **Bessemer Building**
 - London, SW7 2AZ, United Kingdom

Concentration (µg/ml)	Peak Area Bradykinin	Peak Area Calcineurin	Peak Area Bombesin
250	3.77193	2.22354	7.14418
125	2.08041	1.20705	3.91269
25	0.390426	0.201989	0.734045
12.5	0.169431	0.0865758	0.365962
6.25	0.10854	0.0579064	0.22311
2.5	0.0520427	0.0298304	0.0886316
1.25	0.024003	n/a	0.0383524

Table 3. GST processed peak area values obtained from the dilution series for the three test peptides.

concentration of 2 mg/ml and a dilutions series was set up from 250 µg/ml to 1.25 µg/ml in peptide separation buffer. Figure 5 shows the plot of sample concentration versus peak area for each peptide, the peak area values obtained for the three peptides at each concentration is detailed in Table 3. Molecular weights and sequences are summarised in Table 4.

As the molecules are not labelled, detection is dependent on their intrinsic UV absorption. At 214 nm the peptide bond is the main absorbing moiety, with some contribution from R groups, particularly those of the aromatic amino acids. Therefore the peptide with the longest chain length and highest number of aromatic groups should show the lowest limit of detection. (5, 6)

The absorbances show direct correlation to the primary sequence and the absorption is not a direct function of the molecular weight, lower the the molecular weight the more relevant this is. Good linearity was

Peptide	Molecular Weight (in Dalton)	Sequence
Bombesin	1591.83	EQKLGNQWAVGHLM
Bradykinin	1060.21	RPPGFSPFR
Calcineurin	2112.35	DLDVPIPGRFDRRVSVAAE

Table 4. Molecular weights and sequences of the three peptides.

observed for all three peptides over the sample concentration range. The trend lines for all three peptides showed R² values above 0.997. The limit of detection observed for Bradykinin, Calcineurin and Bombesin was 1.25 µg/ml, 2.5 µg/ml and 1.25 µg/ml respectively. These results were obtained without any sample pre-concentration.

CONCLUSION

The data shows the utility of the label free, multi-pixel approach due to its ability to map the intrinsic absorption with an accuracy and precision impossible in labelled systems. It removes the error inherent with label systems due to the chemistry of the label/molecular interaction and enables a direct measurement of the physiochemical properties of the peptide themselves. Direct measurement of the physiochemical properties of the molecular enables the significance of both primary sequence and peptide side chain absorbances to be analysed. This study demonstrated the advantages of a multi-pixel detector combined with advanced data processing in terms of reproducibility and sensitivity.

Future work will include investigation of the correlation between peptide sequence and measured absorbance/peak amplitudes. This will lead to the ability to assign molecular weight to the peptides separated by charge, enabling accurate peptide mass finger printing to be achieved in a CZE system. This will offer significant cost savings over canonical mass spectrometers.

REFERENCES AND NOTES

- K. Vaclav, Electrophoresis 20, pp. 3084-3105 (1999).
 M.B. Peirotti, M.V. Piaggio et al., J. Sep. Sci. 31, pp. 548-554 (2008).
 M. B. Peirotti, M.V. Piaggio et al., Electrophoresis 27, pp. 4631-4647
- B. Verzola, C. Gelfi et al., J. Chromatogr. A **874**, pp. 293-303 (2000).
- R. Goldfarb, J. Biol. Chem. 197, pp. 317-320 (1952)
- B.J.H. Kupiers, H. Gruppen, J. Agric. Food Chem. (2007)