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Micro sequential injection: automated insulin derivatization and separation using a lab-on-valve capillary electrophoresis system

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Automated sampling and fluorogenic derivatization of islet proteins (insulin, proinsulin, c-peptide) are separated and analyzed by a novel lab-on-valve capillary electrophoresis (LOV-CE) system. This fully integrated device is based on a micro sequential injection instrument that uses a lab-on-valve manifold to integrate capillary electrophoresis. The lab-on-valve manifold is used to perform all microfluidic tasks such as sampling, fluorogenic labeling, and CE capillary rejuvenation providing a very reliable system for reproducible CE separations. Fluorescence detection was coupled to an epiluminescence fluorescence microscope using a customized capillary positioning plate. This customized plate incorporated two fused-silica fiber optic probes that allow for simultaneous absorbance and fluorescence detection, extending the utility of this device. Derivatization conditions with respect to the sequence of addition, timing, injection position, and volumes were optimized through iterative series of experiments that are executed automatically by software control. Reproducibility in fluorogenic labeling was tested with repetitive injections of 3.45 mM insulin, yielding 1.3% RSD for peak area, 0.5% RSD for electromigration time, and 2.8% RSD for peak height. Fluorescence detection demonstrated a linear dynamic range of 3.43 to 6.87 μM for insulin (r^2 = 0.99999), 0.39 to 1.96 pM for proinsulin (r^2 = 0.99195) and 260 to 781 nM for c-peptide (r^2 = 0.99983). By including hydrodynamic flushing immediately after the detection of the last analyte, the sampling frequency for islet protein analysis was increased. Finally, an *in vitro* insulin assay using rat pancreatic islet excretions was tested using this lab-on-valve capillary electrophoresis system.

Introduction

Monitoring insulin, proinsulin and c-peptide levels has clinical significances in hypoglycemia investigations, pathogenesis studies, and treatment of diabetes mellitus. Within the pancreatic β cell of the islets, the proinsulin is converted into insulin and c-peptide by a series of enzymatic cleavages.[1] Most proinsulin (~85%) is converted to insulin and c-peptide, which are then secreted in equimolar amounts into the serum. Unconverted proinsulin can be excreted intact or as a partial hydrolyzed product of equal molecular weight. Quantitation of c-peptide in the serum can reflect the effectiveness of the conversion of proinsulin to insulin in the β cells since its only source is from endogenous pancreatic β cells, thus, distinguishing endogenous from exogenous insulin sources. The functionality of the islet cells can also be determined by the concentration ratio of insulin and proinsulin in the serum. Since the circulating concentration ranges of these peptides are very low, normally in the pico-molar to nano-molar range, it is a true analytical challenge to assay these peptides in a complex serum matrix containing relative high concentration of other biomolecules.

Chromatographic technique, along with bioassay and immunoassay, are three main methods for the determinations of insulin, proinsulin, and c-peptide.[2] High performance liquid chromatography (HPLC) has been widely used for the determination of these peptides in academic research and in pharmaceutical industry. Most of these assays are single event measurements that require manual preparation prior to analysis. However, in real-time studies of the cellular functional response, full automation in sample gathering, preparation, and analysis are required in a rapid and repeatable fashion. None of the above mentioned techniques provide this feature readily. The goal of this work is focused on the development of a system for evaluating the functional response of islet cells through time and under repeated stimulations as a means of evaluating there relative viability and health.

Previous work with LOV manifold[3] coupled with CE system[4] confirmed the feasibility and versatility of this novel technique for real-time islet stimulation studies, characterized by high separation efficiency and short assay time. However, low concentration sensitivity is the major disadvantage of CE due to its nature of the instrumental limitations such as curvature capillary surface and short detection path length. Use of LOV allows low concentration samples to be automatically derivatized thus increasing the CE detectability prior to the CE injection. The CE system can also be automatically flushed and regenerated after the completion of the separation with the computer controlled operating protocols thus enhancing the performance of the CE system.

There are several fluorescence labeling reagents that can be used for enhancing peptide and protein detection, such as 6-AQC (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate),[5] NDA (naphthalene-2,3-dicarboxaldehyde), OPA (*o*-phthaldialdehyde),[6] and CBQ (3-(*p*-carboxybenzoyl)quinoline-2-carboxaldehyde)[7,8] *etc*. Most of the derivatization processes need extended reaction time and auxiliary reagents to complete the derivatization. Of these we choose fluorescamine[9] being a one-step-reagent derivatization and also because it provides a sensitive method for determining the concentration of small, low molecular weight peptides.[10,11] The fluorescamine has been proven useful to derivatize small peptides of less than 20 amino acids in length.[12] It reacts with the primary aliphatic amine groups present in the peptide samples and produces a stable compound that upon excitation at 390 nm emits strong fluorescence from 460 to 490 nm.[13,14]

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The maximum fluorescence intensity of the derivatized product depends on the reaction conditions such as pH and buffer selection.[15] The reaction product has maximum fluorescence intensity at pH 9 using phosphate buffer if the fluorescamine was prepared with acetonitrile or at pH 8 when acetone is used.[14] The fluorescamine reacts readily with any primary amino groups on the peptide and the reaction products are stable during the assay time.[16] The insulin molecule has three primary amine sites (glycine at A1, phenylalanine at B1, and lysine at B29) available for the fluorescamine reagent to react.[17] It is expected that multiple-site labeling of the insulin will also generate several peaks that reflect a distribution of the fluorogenic addition. Since fluorescamine is a fluorogenic reagent, excess fluorescamine molecules are deactivated *via* hydrolyzation[18] producing a non-fluorescent compound that is invisible to the fluorescence detector but visible to the absorbance detector. Optimal conditions for maximizing fluorogenic labeling are identified and well maintained by means of the programmable fluidic control offered by the μSI-LOV system. The fluid driven mechanics of sequential injection allows automated optimization and assay to be performed without the need of manual intervention.

Experimental

System configurations

LOV-CE fluorescence UV-VIS detection system was constructed using an epiluminescence fluorescence microscope equipped with a photomultiplier tube (PMT, Model P10232, Electron Tubes Inc., Rockaway, NJ, http://www.electrontubes.com), and two spectrophotometers (USB2000, Ocean Optics Inc., Dunedin, FL, http://www.oceanoptics.com) for absorbance spectroscopy (Fig. 1a). The whole system was served by a stepper motor driven syringe pump (XL 3000, 24000 step full range with a 500 μL syringe, Cavro Scientific Instruments, Inc., San Jose, CA, http://www.cavro.com). The LOV manifold was mounted on the top of a 6 way selector valve[4] (Model C22, Valco Instruments Co. Inc., Houston, TX, http://www.valco.com). A two way normally open isolation valve (HP225T022, NResearch Inc., West Caldwell, NJ, http://www.nresearch.com) was used for hydrodynamic sample injection, refreshing the CE buffer, and automated capillary flushing/reconditioning. FEP Teflon tubing (Cat. 1520, 1.588 mm od, 0.762 mm id) was used for connecting all fluid channels. A smaller id FEP Teflon tubing (Cat. 1548, 1.588 mm od, 0.508 mm id) was used for the sample flow-through port (#6 in Fig. 1a). PEEK tubing (Cat. 1532, 1.588 mm od, 0.508 mm id) was used for reagent channel to prevent light exposure of the reagent. Optical fibers (240 μm od, 200 μm id, core type UVMI, H_2 loaded, modified, Polymicro Technologies LLC, Phoenix, AZ, http://www.polymicro.com) enclosed in short PEEK tubes (Cat. 1531B, 1.588 mm od, 254 μM id) were inserted in the built-in LOV detection channels. These two fibers were facing each other and were 1.5 mm apart from each other. μSI protocols were executed through FIAlab for Windows software (Version 5.9.54, FIAlab Instruments, Inc., Bellevue, WA, http://www.flowinjection.com) installed in a personal computer (Intel Pentium II, 300MHz, 64MB RAM) loaded with Microsoft Windows XP Professional Edition operating system (Microsoft Inc., Edmond, WA, http://www.microsoft.com).

For the CE separation system (Fig. 1a, left), a bare platinum (Pt) wire was used as the cathode placed in a container filled with CE buffer (~ 25 mL). The anode was housed in a PEEK tubing (Cat. 1533, 1.588 mm od, 0.762 mm id) and was connected away from the LOV manifold.[4] A polyimide coated fused silica capillary (Cat. TSP050150, 50 mm id, 150 mm od, Polymicro Technologies LLC, Phoenix, AZ) with total length (Lt) of 50.0 cm and effective length (Ld) of 30.0 cm was used for CE separation. The capillary was enclosed in a PEEK tubing (Cat. 1536, 1.588 mm od, 178 μm id) and the capillary opening was adjusted to the light path of the two optical fibers in the LOV manifold. Capillary detection window was prepared by removing the polyimide coating using heated sulfuric acid (96.5%, J.T. Baker Chemical Co., Phillipsburg, NJ, http://www.jtbaker.com). CE separation was driven by a high voltage power supply (CZE1000R, Spellman Inc., Hauppauge, NY, http://www.spellmanhv.com) working under forward polarity mode at a constant voltage of 15.0 kV with the cathode (−) side away from the LOV manifold and controlled by the personal computer that controls the μSI-LOV system.

An epiluminescence fluorescence microscope (ZEISS, Germany) capable of epi-illumination excitation was used for CE detection (Fig. 1b). The excitation radiation source was from a 75 W Xenon arc lamp equipped with an external adjustable power source. Use of the xenon arc lamp has an advantage of strong broadband emission and thus flexibility in the choice of the excitation wavelength to match the fluorophore. An UV excitation band-pass filter (A46–095, center wavelength λm = 330 nm, Edmund Industrial Optics, Barrington, NJ, http://www.edmundoptics.com) was used for fluorescence excitation and a VIS long pass filter (A46–422, limit of the stopband λs = 370 nm, cut-off position λc = 400 nm, limit of the passband λp = 475 nm, Edmund Industrial Optics, Barrington, NJ) was used for the collection of analyte fluorescence emission. The fluorescence emission was collected by a PMT (280 nm to 630 nm spectral response range) mounted on the top of the microscope. Sampling frequency of the PMT was set to 10 Hz. Factory installed photon counting program was used for data collection with a DELL™ notebook computer (Intel Pentium III, 1.2GHz, 256MB RAM) loaded with Microsoft Windows XP Home Edition operating system (Microsoft Inc., Edmond, WA).

Supplementary UV absorbance detection systems were incorporated within both LOV manifold and CE separation system. A deuterium lamp (Model D-1000, Analytical Instrument Systems, Inc., Flemington, NJ, http://www.aishome.com) was used as the UV source and an USB-interfaced UV-VIS spectrophotometer (USB2000, Ocean Optics Inc., Dunedin, FL) was used as the detector. Under the sample objective of the microscope, the CE capillary and two UVMI optical fibers were glued on the glass specimen plate (Fig. 1b). These two optical fibers were facing each other and were about 300 μm apart. The capillary detection widow was then positioned in between and so the absorbance was measured at point A in Fig. 1b. The sample objective of the fluorescence microscope was subsequently focused at 1 mm upper stream from the absorption point B in Fig. 1b. The difference in height of the capillary and the optical fibers was compensated by using 50 μm thick 3M™ tape to elevate the capillary to a proper position. Listed tubing, unless specified, was obtained from Upchurch Scientific, Oak Harbor, WA, http://www.upchurch.com.

Reagents

A 20 mM phosphate buffer stock solution was made by dissolving 0.1647 g potassium phosphate monobasic (KH_2PO_4, Cat. 60219) and 3.2732 g potassium phosphate dibasic (K_2HPO_4, Cat. 60353) in 1.0 L of deionized (DI) water. The pH was adjusted using 3.0 M sodium hydroxide solution to pH 8.4. A working CE buffer solution containing 20 mM phosphate buffer solution and 20 mM TAPS and was made by dissolving 3.0413 g TAPS ([(2-hydroxy-1,1-bis(hydroxymethyl) ethyl)-amino]-1-propanesulfonic acid, Cat. T9659) using the prepared 500 mL phosphate buffer solution mentioned above. The sample buffer solution containing 20 mM phosphate and 200 μM EDTA was made by dissolving 0.0292 g ethylenediamine-

tetraacetic acid (EDTA, Cat. EDS) into the remaining 500 mL phosphate buffer solution.

Borate CE buffer used for demonstrating simultaneous absorbance and fluorescence detections was prepared by dissolving 1.9068 g sodium tetraborate 10-hydrate ($Na_2B_4O_7 \cdot 10H_2O$, Cat. 3568, J.T. Baker Chemical Co., Phillipsburg, NJ) in 250 mL DI water to a final concentration of 20 mM and pH of 9.1. Sample dilution buffer used to prepare insulin standard used in this demonstration was made by dissolving 1.1915 g 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, Cat. H-7523) and 0.0146 g ethylenediaminetetraacetic acid (EDTA, Cat. EDS) in 250 mL DI water to final concentrations of 20 mM HEPES and 20 μM EDTA following by the pH adjustment to 7.5 using 1.0 M sodium hydroxide standard solution (VW3222-1, VWR International, West Chester, PA, http://www.vwrsp.com).

Insulin and c-peptide standard stock solutions were made by dissolving bovine pancreatic insulin and human c-peptide (I-8405 and C-5051) in the sample buffer. Proinsulin standards were made by adding proinsulin standard solution kit (8015-K, Linco Research Inc., http://www.lincoresearch.com) containing 2, 5, 10, 20, 50, and 100 pM pre-dissolved proinsulin standard solutions in a 0.05 M phosphate buffer with 0.08% sodium azide, 0.15 M NaCl, 0.025 M EDTA, and 1% BSA with a pH of 7.4. An islet cell perfusion sample solution was also diluted using the sample buffer. A 3.60 mM (or 1000 ppm) fluorescemamine (F-2332, Molecular Probes Inc., Eugene, OR, http://www.probes.com) protein staining reagent was made by

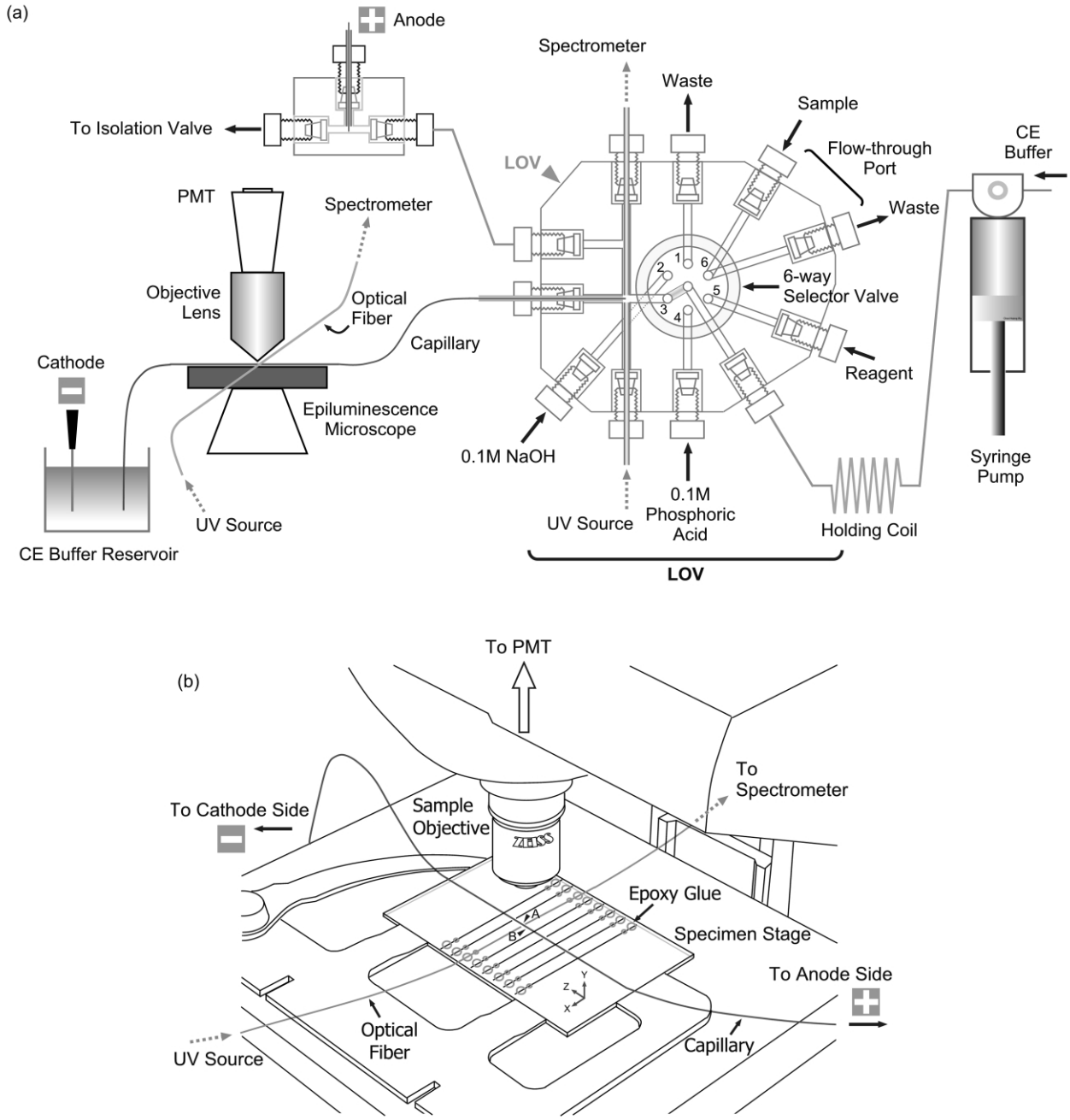

Fig. 1 Configuration of LOV-CE peptide derivatization and detection system. (a) Experimental setup shows the LOV system (right) and the CE detector (left). The CE buffer solution was used as the carrier for the μSI system. The 0.1 M sodium hydroxide solution was used to prime the new capillary and a 0.1 M phosphoric acid bolus was used to flush the capillary after each sample injection. The capillary was re-equilibrated in the CE buffer thereafter. (b) The geometry of the fluorescence/absorbance detection system mounted on the fluorescence microscope. The CE capillary was positioned between two optical fibers and the absorbance signal was collected at point A where the optical fibers are facing each other, while fluorescence was collected at point B. The purpose of offsetting both point A and B was to prevent the background reflection from the capillary surface by the radiation source fiber.

dissolving 100 mg fluorescamine in 100 mL acetonitrile reagent (Cat. 27100–4) in an amber glass bottle and stored at 2 ~ 5 °C when not in use. A 0.167 mM fluorescein solution (F-7505) was used for simulating sample and reagent volumetric gradients and for positioning the capillary under the microscope. All DI water used was generated by NANOpure II water purification system (at 18.0 MΩ cm, Barnstead/Thermolyne, Dubuque, Iowa, http://www.barnstead.com). Listed chemicals and reagents, unless specified, were obtained from Sigma-Aldrich Chemical Co. Inc., Milwaukee, WI, http://www.sigmaaldrich-.com.

Results and discussions

The flow reversal volume and the selection of the concentration gradient

The concept of the flow reversal volume is explained in Fig. 2. A multi-position valve (MPV) serves as the central hub for the sample (S) and reagent (R). Solutions connected to the MPV are aspirated into the holding coil (HC) by aspiration action of the syringe pump. The aspirated sample and reagent, with the order of the sample first followed by the reagent into the HC is called the "sequenced solution profile." Since the CE capillary is located downstream from the HC, the sequenced solution profile needs to be transported to the capillary input orifice by a flow-reversal stroke of the syringe pump. The volume that the syringe pump delivered is the "flow reversal volume." As the flow reversal volume increases, the dispersion of the sequenced solution profile increases as well. The dispersion in the radial direction assists the mixing of the sample and the reagent. Product (P) formed from the reaction of the sample and the reagent and its concentration profile along the channel inside the LOV can be visualized as shown in Fig. 3.

The concentration gradient of the reaction product, denoted as the shaded trace in the "Concentration *vs*. Channel Position" plot in the Fig. 3, is due to product formation at the dispersion zone. As a flow reversal volume shown in the Fig. 3a, the capillary is sampling the product zone at its highest concentration. Shown in the Fig. 3b is an increased flow reversal volume resulting in a higher degree of product dispersion at the capillary

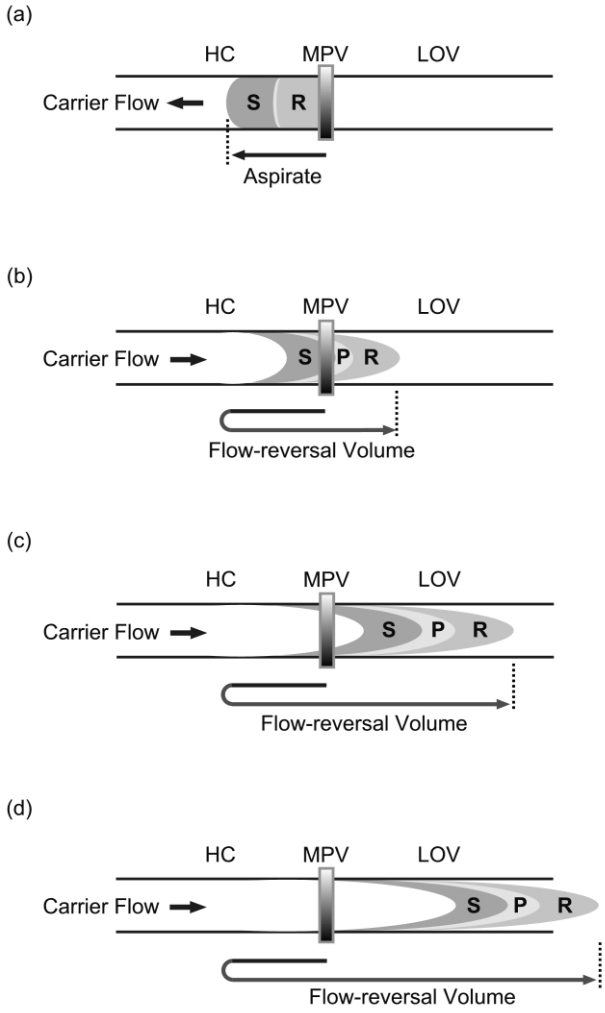

Fig. 2 The flow reversal volume. (a) The solution profile containing the sample (S) and the reagent (R) was aspirated into the holding coil (HC) through the multi-position valve (MPV) in the LOV-CE system. (b) The sequenced solution profile was then delivered to the detector (or the CE capillary orifice in this assay) downstream from the HC by the flow reversal action of the syringe pump. (c) Zone dispersion increases with the increase of the flow reversal volume. (d) The larger the flow reversal volume, the greater the dispersion of the sequenced solution profile will be.

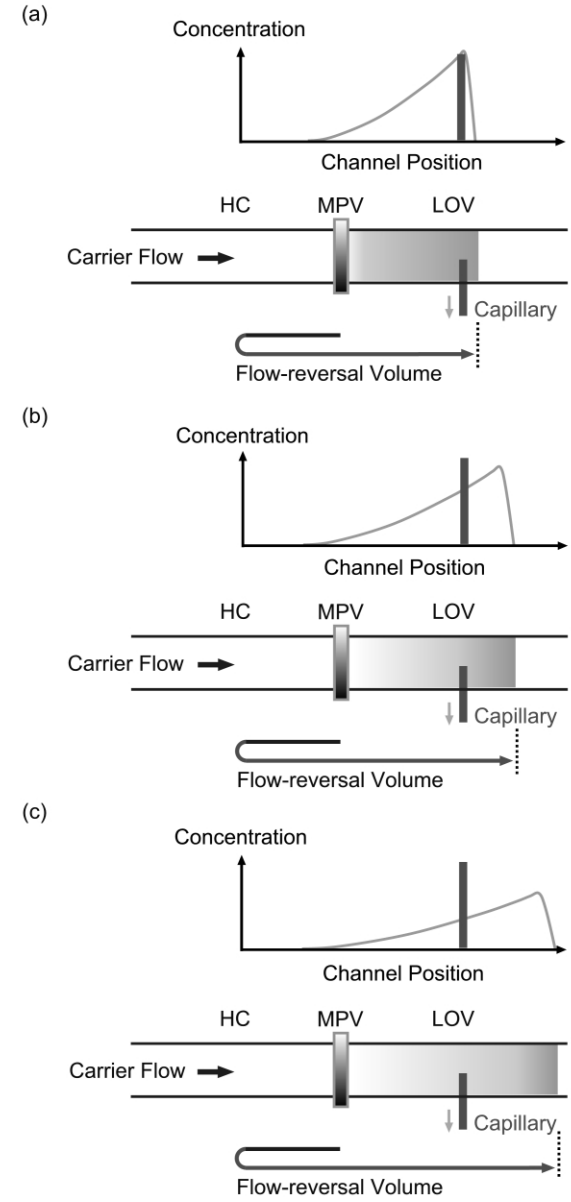

Fig. 3 The selection of the concentration gradient by the flow reversal volume. The concentration of the product zone (as seen in the Fig. 2) is visualized in this figure. (a) The CE capillary samples at the highest dispersed product concentration with the optimized flow reversal volume. (b) Increased flow reversal volume results in a decreased product concentration at the CE capillary orifice. (c) Sampling at an even higher degree of the dispersed product zone can be achieved by further increasing the flow reversal volume.

orifice. Further advancement of the product zone with even a larger flow reversal volume (Fig. 3c) results in even greater dispersion and a decreased product concentration at the CE capillary orifice. Once the optimized condition of the sample-to-reagent volumetric ratio is identified, a selected and fixed flow reversal volume is used for the assays to ensure that exactly the same volume fraction of each component in each assay run will be electrokinetically injected into the capillary. It should be noted that the flow reversal volume is not the amount that was physically introduced into the capillary, instead, the flow reversal volume represents the location (or the position of the dispersed concentration gradient) where the CE capillary will be sampled.

Optimizations of assay protocols for peptide derivatization

Manual fluorogenic derivatizations lack reproducibility due to poor kinetic control of the reaction conditions. The use of a LOV module as the central sample processing unit for sample pretreatment addresses this problem through a precise control of the derivatization condition with respect to the degree of mixing, reaction time, and product delivery in an automated and timely manner. Sequential injection of sample and reaction zones into the holding coil results in the formation of a complex concentration gradient that offers a continuous variation of sample/reagent concentrations and volumetric ratios.[3] By injecting a sample zone followed by a reagent zone (Fig. 4a, left) upstream into the holding coil, the central streamline of the reagent volume will penetrate into the sample zone that has already acquired a parabolic profile at its upstream end. Since the center streamline travels twice the mean flow velocity, the injection of 150 μL reagent into 350 μL of sample will telescope the reagent further upstream into the holding coil. Following by the flow reversal (provided by the syringe pump), the sample and reagent zones are delivered to the CE injection site. During the flow reversal, the radial mixing also takes place being assisted by the geometry of the flow channel and the holding coil, and by the effort of the accelerated flow.

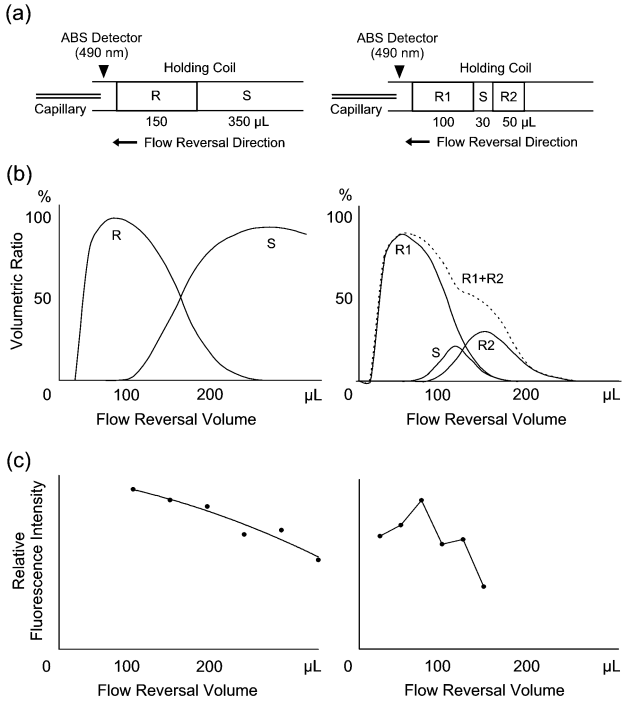

Fig. 4 Optimization of the derivatization condition. (a) The two sequencing methods for insulin derivatization are demonstrated using the two zone mixing method (left) and the sandwich mixing method (right). (b) The volumetric ratio of each component in the sequenced solution profile. (c) The relative fluorescence intensity plotted against the flow reversal volume for the selection of best derivatization condition.

Two approaches were engaged to investigate two zone mixing (Fig. 4a, left) and sandwich mixing (Fig. 4b, right). The method is designed to monitor excreted insulin from pancreatic cells, where the concentration of insulin or peptides may be extremely low while the availability of the sample volume is not a limiting factor. For this purpose, the two zone mixing method was selected. On the other hand, it was also desired to find the condition for the assay where the analyte concentration is high while sample volume may be limited and thus the sandwich method would be more appropriate. In order to map the resulting volumetric ratio and concentration gradient during and after the flow reversal, a 0.167 mM fluorescein solution was injected in separate runs through the sample port and through the reagent port. Optical fibers were inserted close to capillary inlet to measure absorbance of fluorescein tracer at 490 nm. The peaks of the tracer were separately recorded and overlaid in Fig. 4b to provide the image of the volumetric ratios of sample and reagent within CE injection site (Fig. 1a, in the LOV manifold). Since the absorbance detector was in close vicinity to the capillary inlet, the graphs provide an estimation of the volumetric ratios sampled by the capillary during the electro-kinetic injection. For actual assays, the same protocol was applied; however, the reagent was then replaced with 3.60 mM (1000 ppm) fluorescamine and the sample port charged with 50 μM insulin sample. Electropherograms were recorded for variable flow reversal volumes in a range of 100 to 350 μL for two zone mixing and in a range of 25 to 150 μL for the sandwich mixing (Fig. 4b). Series of electropherograms for the two zone mixing is shown in Fig. 5. In order to compare these two mixing methods, peak marked “a” in Fig. 5 was selected as marked for the degree of the derivatization for the selection of optimized conditions. Relative fluorescence intensity (RFI), corresponding to a given flow reversal volume, was plotted in Fig. 4c. It was concluded that the flow reversal volume in the range of 100 to 200 μL was suitable for the two zone mixing method while the flow reversal volume in the range of 40 to 100 μL was appropriate for the sandwich mixing technique.

Another variable in this investigation was the injected sample concentration. For the two zone mixing the insulin sample zone concentration was 5.15 μM, while for the sandwich mixing the insulin sample zone concentration was 51.5 μM. In the case of the two zone mixing using 5.15 μM insulin concentration, the flow reversal volume of 150 μL was chosen corresponded to approximately 65% v/v (or 2.34 mM) fluorescamine reagent and 40% v/v (or 2.06 μM) insulin sample at the CE injection site. In the case of the sandwich mixing using the 51.5 μM insulin sample, a 75 μL flow reversal volume was appropriate, corresponding to approximately 85% v/v (or 3.06 mM) fluorescamine reagent and 2% v/v (or 1.03 μM) insulin sample at the CE injection site.

The duration of derivatization was also explored by varying the reaction time (Table 1). It was found that the maximum signal response for peaks a, b, and c (Fig. 5) was observed at a short derivatization time of 10 s. Therefore, the flow reversal volume of 150 μL and 10 s for derivatization were chosen. Overall system performance was then examined by repetitive injections and separations of 3.45 μM insulin sample using the optimized protocol. The system performance in terms of the reproducibilities of peak areas, the electromigration time, and the peak heights is listed in Table 2.

Simultaneous absorbance and fluorescence detection

The fluorescamine reagent is fluorogenic so it doesn’t have any fluorescence property until it reacts with a primary amine or peptide or protein. Combination of absorbance and fluorescence measurements can be used to examine the derivatization process

by simultaneous monitoring at 280 nm for absorbance and at 490 nm for fluorescence at the microscope region (Fig. 6a). Excess reagent molecules are unable to be detected by fluorescence (*i.e.* PMT) but can be detected by the absorbance detector (Fig. 6b). Moreover, simultaneous absorbance and fluorescence measurement of protein can be useful when the protein samples are at high concentrations.

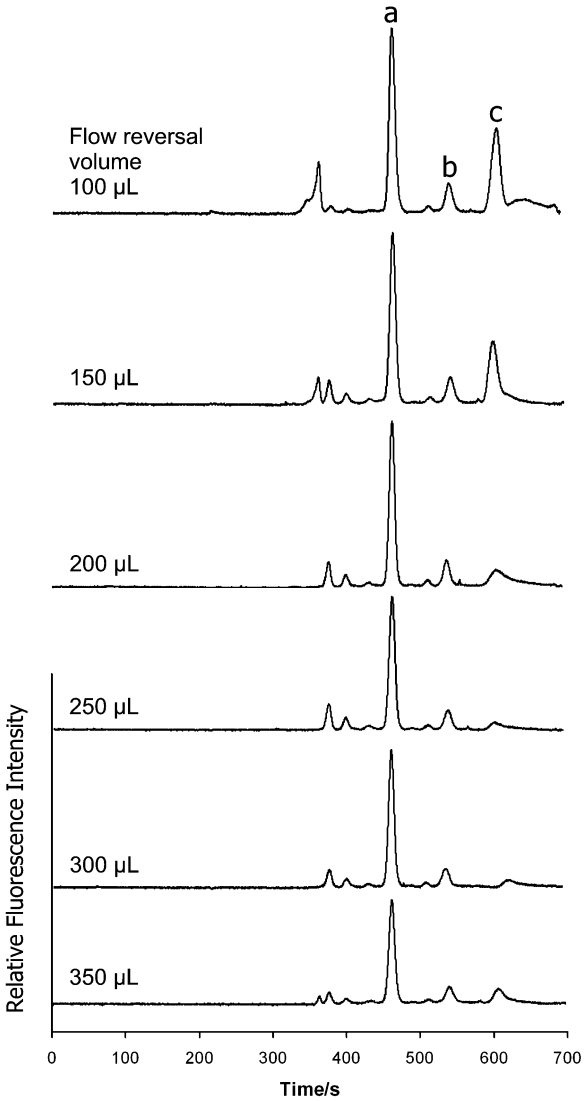

Fig. 5 Selection of the CE injection position. The selection of the zone injection position will determine the best condition for optimized derivatization. The sequenced solution profile in the holding coil (150 μL fluorescamine reagent and 350 μL diluted insulin sample) was delivered at a flow rate of 50 μL s^{-1} downstream to the CE injection site (Fig. 1a, port #3) with the flow reversal volumes of 100 to 350 μL. The sample containing 5.15 μM bovine insulin was derivatized in this way and yielded three major peaks (a, b, and c) in the electropherogram. The flow reversal volume of 150 μL was chosen and it corresponded to approximately 65% v/v (Fig. 4b, left) of the reagent in the derivatized sample. (CE parameters: capillary Lt = 50 cm, Ld = 30 cm, 15 kV, 28 μA, EK injection = 1 s at 15kV.)

Table 1 Selection of the derivatization time

Derivatization time of 5.15 μM insulin sample	Peak area[a]/arbitrary unit		
	Peak a	Peak b	Peak c
10 s[b]	64174	1454162	245842
5 min	54096	664634	121585
10 min	48509	614295	107350

[a] Peak areas were calculated based on valley-to-valley baseline subtraction for each peak. [b] The derivatization time excludes the sample and reagent aspirations time and valves turning and the solution delivery time for total 15 s per sample injection.

Assay of insulin, proinsulin, and c-peptide samples

Insulin, proinsulin, and c-peptide standard solutions were diluted ranging from 50 to 300 fold by the sample buffer. The reason for such extensive dilution is that the method will be ultimately used for on-line assay of perfusion liquid from live pancreatic cells. The standard solutions were stored in the freezer when not in use. The fluorescamine reagent can be prepared in acetonitrile, acetone, or ethanol. Since the LOV was fabricated from rigid polyvinylchloride (PVC), it has sufficient chemical resistance for ethanol and acetonitrile. Acetonitrile was chosen instead of ethanol since it reportedly produces more

Table 2 System performance

Sample: 3.45 μM insulin (n = 5)	RSD%		
	Peak a	Peak b	Peak c
Peak area	0.7	2.0	0.4
Electromigration time	0.2	0.4	0.8
Peak height	2.1	2.3	4.7

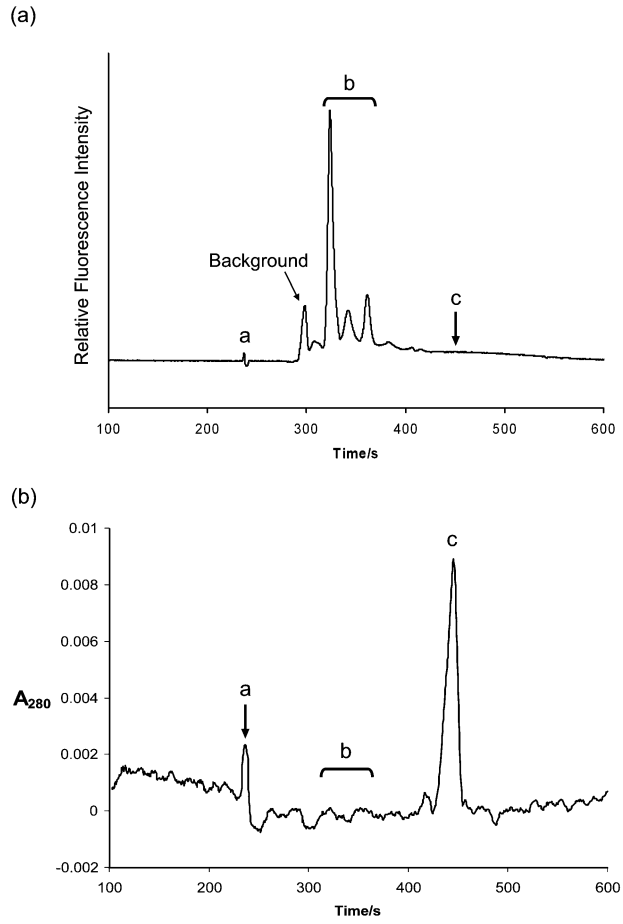

Fig. 6 Simultaneous fluorescence and absorbance detections. (a) 5.15 μM bovine insulin standard was derivatized and separated for the demonstration. Peak a was the solvent, b was the insulin peaks, and c was where the fluorescamine should be eluted. Since unreacted fluorescamine molecules don't have any fluorescence property it is undetectable under the fluorescence detector. (b) However, unreacted fluorescamine can be detected by the supplementary absorbance detector yielding the peak c. Capillary: 50 cm × 50 μM inner diameter, 30 cm to detector; CE buffer: 20 mM pH 9.1 borate buffer; sample was diluted in 20 mM pH 7.2 HEPES buffer with 20 μM EDTA; field strength: 300 V cm^{-1}.

intense fluorescence emission.[14] In order to reduce protein adhesion to conduit walls, TAPS was used as a zwitterionic additive to the CE buffer solution. The zwitterionic molecules can have low electrical conductivity[19] when the buffer pH is adjusted to its pI allowing the pH of the carrier buffer to be adjusted to pH 8.4. The target analyte was identified by first injecting the sample buffer as the blank followed by doping the blank with the analytes (Fig. 7a for c-peptide and Fig. 7b for proinsulin). With the optimized experimental parameters stated above, linear concentration dynamic ranges of 3.43 to 6.87 μM for insulin, 0.39 to 1.96 pM for proinsulin, and 260.4 to 781.1 nM for c-peptide samples were obtained with the regression responses of r^2 = 0.99999 for insulin, r^2 = 0.99195 for proinsulin, and r^2 = 0.99983 for c-peptide samples were found. As a precaution, BSA (bovine serum albumin) is routinely added to the proinsulin samples in order to avoid losses by adsorption onto the walls of the plastic containers. However, at high concentrations (> 0.02% w/v) of BSA, electrophoretic separation can be hindered and thus must be avoided in the sample preparation. Therefore, the proinsulin samples were extensively diluted. Other unidentified peaks from the acetonitrile reagent were observed. A phosphoric acid flush was performed after each assay[20] and the CE capillary was regenerated by sodium hydroxide daily.

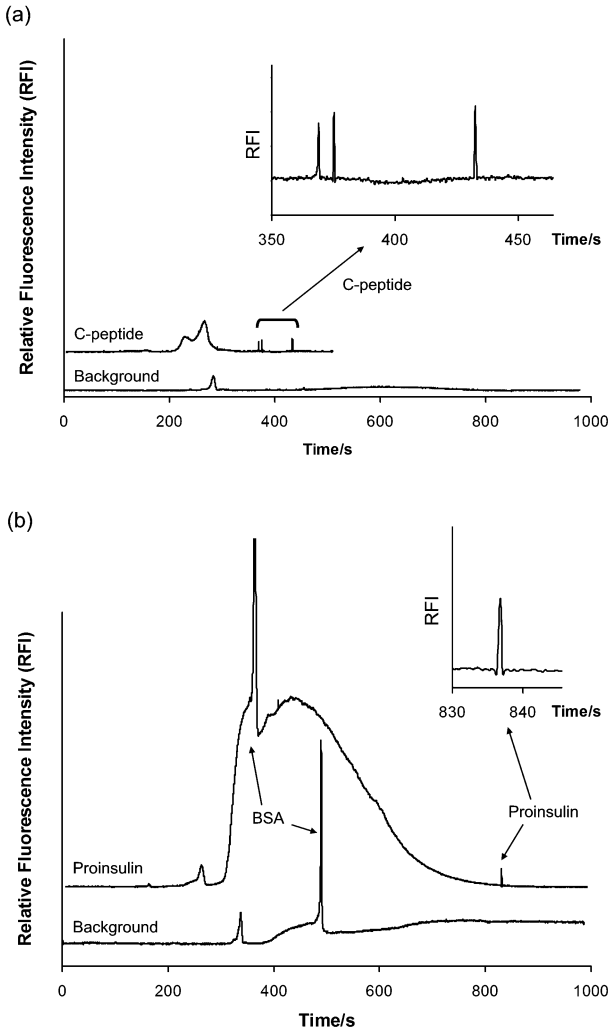

Fig. 7 Identifications of c-peptide and proinsulin peaks. Identification of (a) 781.1 nM c-peptide with the sample buffer as the background, (b) 1.96 pM proinsulin with 0.01% BSA in the sample buffer as the background. Capillary: 50 cm × 50 μM inner diameter, 30 cm to detector; CE buffer: 20 mM pH 8.4 phosphate buffer with 20 mM TAPS; sample was diluted in 20 mM pH 8.4 phosphate buffer with 200 μM EDTA; field strength: 300 V cm^{-1}.

Insulin assay in the cellular perfusion liquid

Due to the complexity of the sample matrix, insulin assay in the cellular perfusion liquid was based on standard addition method (Fig. 8). The tallest peak was chosen to identify the presence of insulin in the sample. Each doped sample (3.43, 5.15, and 6.87 μM final insulin concentrations) was repeatedly injected six times (n = 6) yielding a linear response of 0.99999 (RSD% of 4.51%, 4.18%, and 6.88% respectively). The calculated concentration in the perfusion liquid was 1.54 μM insulin.

At this point it should be reiterated that the peptide standards were initially diluted 50 to 300 times to approximate the expected concentration levels in cell perfusion. Consequently a very large sample volume, 350 μL, was injected into the LOV since cellular perfusions will be available in such amounts. However, considering that LOV-CE methodology is suitable for limited sample volumes, it should be pointed out that a less diluted insulin sample was easily analyzed while injecting only 30 μL of sample into the LOV manifold (Fig. 4a, right).

Conclusions

The previously introduced LOV-CE system was used for the first time for the assay of biomolecules and peptide derivatization *in situ* in this work. The use of the LOV as the "front end" for CE allows for a versatile solution handling and a fast sample pretreatment such as derivatization by fluorogenic reagent. It should be noted that an alternative CE technique know as EMMA[21] (electrophoretically mediated microanalysis) in principal differs from our approach in that sample and reagent addition takes place in the capillary, promoted by electromobility differences between them. In the presented LOV-CE system, the CE capillary was used for separation only, while the LOV manifold was used for the sample derivatization. The

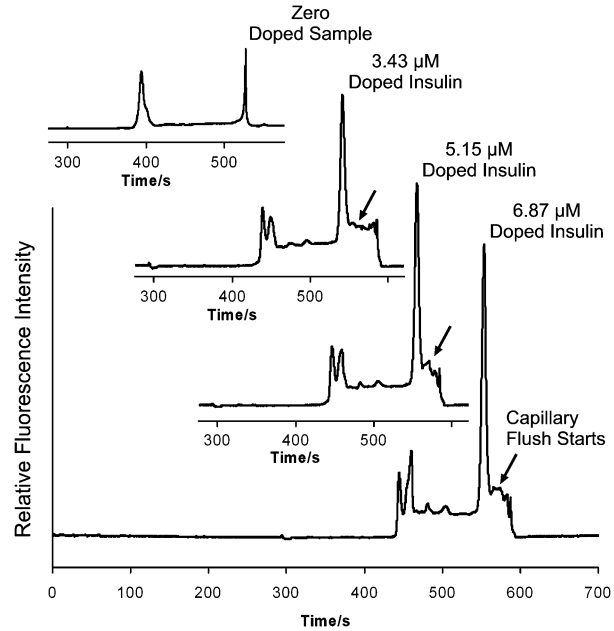

Fig. 8 Determination of insulin from the cellular perfusion liquid. The diluted cellular perfusion samples (50 times dilution of the perfusion solution from 50 islet cells) were doped with insulin standard to a final doped concentrations of 3.43, 5.15, and 6.87 μM insulin. Peak a (as seen in Fig. 5) in each electropherogram was taken for the standard addition calculation. After the appearance of the target peak (the peak a, t_m = 553 s, in this assay), the remaining sample materials was hydrodynamically flushed out of the CE capillary. Capillary: 50 cm × 50 μM inner diameter, 30 cm to detector; CE buffer: 20 mM pH 8.4 phosphate buffer with 20 mM TAPS; sample was diluted in 20 mM pH 8.4 phosphate buffer with 200 μM EDTA; field strength: 300 V cm^{-1}.

LOV manifold acts as the sample pretreatment "front end" allowing sample and reagent to be physically mixed to any desirable degree, providing the ability to optimize the condition of a chemical reaction. This approach is more versatile since it does not limit reactions or selection of reagents due to electromobility constraints.

In order to increase sampling frequency, the LOV was used to hydrodynamically flush the remaining sample material out of the capillary as soon as the target peak was recorded. Also automated reconditioning of the capillary after completion of the assay (by pumping a conditioning solution) resulted in an increase capillary life and improved reproducibility. Simultaneous detection of fluorescence and absorbance, described in this work, allows for the monitoring of the reagent and the reaction products as well as the selection and the optimization of the assay protocol. It is believed that the novel approach described in this work will find a wide application for functional assay measurements on living cells/tissues. Our aim is to apply this novel LOV-CE technique to the real-time monitoring of insulin secretion of living pancreatic cells to test their functional response with respect to repeated stimulations and to evaluate their overall health.

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References

1 K. I. J. Shennan, N. A. Taylor, J. L. Jermany, G. Matthews and K. Docherty, *J. Biol. Chem.*, 1995, **270**, 1402.
2 P. M. Clark, *Ann. Clin. Biochem*, 1999, **36**, 541.
3 J. Ruzicka, *Analyst*, 2000, **125**, 1053.
4 C.-H. Wu, L. Scampavia and J. Ruzicka, *Analyst*, 2002, **127**, 898.
5 H. Liu, B.-Y. Cho, R. Strong, I. S. Krull, S. Cohen, K. C. Chan and H. J. Issaq, *Anal. Chim. Acta.*, 1999, **400**, 181.
6 E. L. Gump and C. A. Monnig, *J. Chromatogr. A*, 1995, **715**, 167.
7 E. A. Arriaga, Y. Zhang and N. J. Dovichi, *Anal. Chim. Acta*, 1995, **299**, 319.
8 J. Liu, Y.-Z. Hsieh, D. Wiesler and M. Novotny, *Anal. Chem.*, 1991, **63**, 408.
9 M. Weigele, S. L. De Bernardo, J. P. Tengi and W. Leimgruber, *J. Am. Chem. Soc.*, 1972, **94**, 5827.
10 M. A. Bridges, K. M. McErlane, E. Kwong, S. Katz and D. A. Applegarth, *Clin. Chim. Acta*, 1986, **157**, 73.
11 G. M. Funk, C. E. Hunt, D. E. Epps and P. K. Brown, *J. Lipid Res.*, 1986, **27**, 792.
12 D. S. Brown and D. R. Jenke, *J. Chromatogr.*, 1987, **410**, 157.
13 T. K. Narayanan and L. M. Greenbaum, *J. Chromatogr.*, 1984, **306**, 109.
14 R. Zhu and W. T. Kok, *J. Chromatogr.*, 1998, **814**, 213.
15 V. K. Boppana, C. Miller-Stein, J. F. Politowski and G. R. Rhodes, *J. Chromatogr.*, 1991, **548**, 319.
16 M. E. Szulc and I. S. Krull, *J. Chromatogr., A*, 1994, **659**, 231.
17 N. G. Hentz, J. M. Richardson, J. R. Sportsman, J. Daijo and G. S. Sittampalam, *Anal. Chem.*, 1997, **69**, 4994.
18 S. Stein, P. Bohlen and S. Udenfriend, *Arch. Biochem. Biophys.*, 1974, **163**, 400.
19 A. V. Stoyanov and P. G. Righetti, *J. Chromatogr., A*, 1997, **790**, 169.
20 M. Zhu, R. Rodriguez, D. Hansen and T. Wehr, *J. Chromatogr.*, 1990, **516**, 123.
21 J. Bao and F. Regnier, *J. Chromatogr.*, 1992, **608**, 217.