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Microchip electrophoresis with hydrodynamic injection and waste-removing function for quantitative analysis

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Abstract

Quantitative analysis is problematic for microchip electrophoresis for several reasons including chip-to-chip variation, discontinuous sample re-loading, channel reconditioning, and electrokinetic injection bias. In this study, the capability for quantitative analysis on a flow-through based microchip electrophoresis, which provides continuous sample re-loading, channel washing, reconditioning and hydrodynamic injection as well as waste removing is demonstrated to be more quantifiable and more reproducible compared to manual electrokinetic injection method. Using the flow-through microchip with waste-removing function, FITC-labeled estrogen or Rhodamine B could be continuously analyzed without significant changes (R.S.D. < 6.6%) in signal intensity for over 3 h, which is sufficient for a complete set of quantitative analysis. With the use of a phosphorylated kinase substrate as the model, a calibration curve for quantitative analysis of phosphopeptides were constructed and results indicate that both R^2 value of the linearity and R.S.D. values of the peak intensity were around 0.9961 and 3.16%, respectively, without the use of an internal standard. These values were slightly improved to be around 0.9986 and 2.27%, respectively, with the use of a non-phosphopeptide counterpart as the internal standard. The potential of this flow-through device for the development of a kinase phosphorylation assay based on the quantitative method was also briefly discussed.

Keywords: Microchip electrophoresis; Quantitative analysis; Hydrodynamic injection; Kinase assay

1. Introduction

Microchip electrophoresis has been one of the most powerful analytical tools in recent years because of its short analysis time and feasibility for multichannel or high throughput analysis [1–8]. The simplest electrophoresis-based microchip contains a simple cross channel and four reservoirs for sample, sample waste, electrophoretic buffer and buffer waste. Repetitive injections and analyses can be performed simply by voltage switching among these reservoirs [2,5]. With conventional microchips, however, extensive time and labor for channel washing, reconditioning, and sample re-loading are required. These processes are usually incompatible with the efficiency of the microchip, especially when lots of samples are to be analyzed. Although some microchips, such as those made on PMMA or PDMS substrate, are fairly cheap and can be disposable, the speed of analysis is still limited because of the interruption between two analyses. Moreover, variations caused by chip-to-chip or channel-to-channel may lead to large deviations, particularly for quantitative analysis.

There are several methods developed to facilitate efficient sample introduction for microchip electrophoresis. Fang et al. [9] have developed an interface for continuous sample introduction, which allows 80 samples to be analyzed in one hour. Harrison and co-workers have reported a chip design enabling the sample to be directly and continuously introduced into the microchip without perturbing the liquid within the microfluidic device [10]. The injection of these methods, however, is still under electrokinetic mode, which is likely to suffer from injection bias, leading to large deviations for quantitative analysis. In our previous work [11], a commercial autosampler, which allows as few as 60 nL of sample to be injected, was connected to a novel microchip electrophoresis system [12,13] for continuous and automatic analysis. Results indicated that automation of the whole process including

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sample loading, injection and analysis as well as channel reconditioning and sample re-loading could be realized in such design without noticeable sample contaminations caused by the absorption of sample onto the surface. Moreover, because the injection is by hydrodynamic force rather than electrokinetic force, such design should lead to more quantifiable and more reproducible results compared to manual electrokinetic injection method.

Since the development of a robust quantitative method is highly dependent on the reproducibility of the measurement, several factors that could influence the reproducibility would need to be characterized. First, the cross contamination due to insufficient washing had been investigated before [11] and was considered to be minor for a relatively "pure" sample investigated here. Sufficient washing will remove molecules absorbed on the channel surface from previous analyses. Second, the variation of optical re-focusing, due to the removal of the microchip from the microscope stage for sample re-loading, channel washing, and reconditioning by manual method, would also lead to deviations. The flow-through method, however, would not suffer from this defect since the microchip was not removed throughout the experiment but the manual method will suffer from this variation. Third, as mentioned previously, the variation caused by the injection bias is common to capillary electrophoresis using electrokinetic injection and it may not be compensated even by the use of an internal standard. Lastly, although the chip-to-chip or channel-to-channel variation can be prevented by using the same chip, the lifetime of one disposable chip would need to be sufficiently long in order to conduct a complete set of quantitative analysis. The first factor had been excluded based on previous studies. This study will focus on characterizing the last two factors and further applying the flow-through chip for the quantitative analysis of phosphopeptides, which holds a great potential for the development of a simple kinase assay.

2. Experiment

2.1. Chemicals and reagents

Sodium phosphate, sodium tetraborate and Rhodamine B were obtained from Fluka (Buchs, Switzerland). Both the FITC-labeled phospho and non-phosphopeptides with the sequence of AEEEIYGVLFAKKKK were synthesized from Sigma–Genosys (Woodland, TX, USA). FITC-labeled estrogen was purchased from Panavera (Madison, WI, USA). Tris-acid and glycerol were purchased from J.T. Baker (Phillipsburg, NJ, USA) and DDT was purchased from Sigma (St. Louis, MO, USA).

For quantitative analysis, phosphopeptides were dissolved in 5 mM sodium phosphate buffer that contains 0.2 mM sodium dodecyl sulfate (SDS) (pH 7.68) at the concentration ranging from 25 to 250 ppm. Non-phosphopeptides, which were used as the internal standard, were added into each phosphopeptide standard at a fixed concentration of 25 ppm. All reagents were of the highest grade available. Finally, the CE water was deionized distilled water filtered through a Barnstead E-pure system, and had a resistance over 18.0 MΩ/cm.

2.2. Instrumental setup

A schematic diagram of the microchip system is displayed in Fig. 1. Briefly, a syringe pump (Series 74900, Cole Parmer, Vernon Hills, IL, USA) was used to drive the buffer through the injector, the connection capillaries, into the microchip. We will use "loading" and "injection" to refer the sample introduction by the injector and by voltage switching, respectively. A high-voltage power supply (Series 230, Bertan High Voltage, Hicksville, NY, USA) was employed to furnish the gating and separation voltages. Fig. 1B depicts the whole voltage switching process for the flow-through injection method: A high positive voltage, so-called the gating voltage (2.0 kV), was first applied to reservoir C for 13 s, while reservoirs B and D were grounded (Fig. 1B, gating). Under this voltage scheme, the inlet flow was directed to reservoir B. For sample injection, reservoirs B and D were set to float for 5 s to allow the analyzed sample to be pumped into the separation channel (Fig. 1B, injection). For sample analysis (Fig. 1B, separation), reservoirs B and D were immediately grounded and a positive high voltage, so-called the separation voltage (2.0 kV), was applied to reservoir C to start the sample analysis for 13 s. The above voltage switching was controlled by a program written with LabVIEW (National Instruments, Austin, TX, USA) and the cycle was continuously repeated throughout the experiment even during the interval time between two samples. Channel washing was achieved by continuously pumping the electrophoresis buffer into the microchannel between loaded samples. Occasionally, the microchip was removed from the microscope stage for complete vacuum washing between different sets of runs.

For manual sampling, the capillary to sample inlet A was disconnected. The sample was pipetted into reservoir A and then injected for electrophoretic separation using a voltage switching scheme shown in Fig. 1C: a high positive voltage HV1 (1.5 kV) and HV2 (2.0 kV) was first applied to reservoir A and C for 12 s, respectively, while reservoirs B and D were grounded. In this manner, the sample was driven to the cross-section for injection by electrokinetic force. For sample injection, HV2 was turned off for 5 s to allow the sample to flow into the separation channel. For sample analysis, HV2 was re-applied to reservoirs C to start the separation. Consecutive injections for the same sample such as FITC-estrogen and Rhodamine B were conducted by repeating the above voltage switching scheme. For different samples, the applied voltage was turned off and the microchip was removed from the detection microscope for washing and reconditioning by vacuum, and then, the next sample was pipetted into reservoir A to re-start the above cycle for separation and detection.

Signals were detected on-chip via fluorescence detection, with the detection system being constructed by modifying

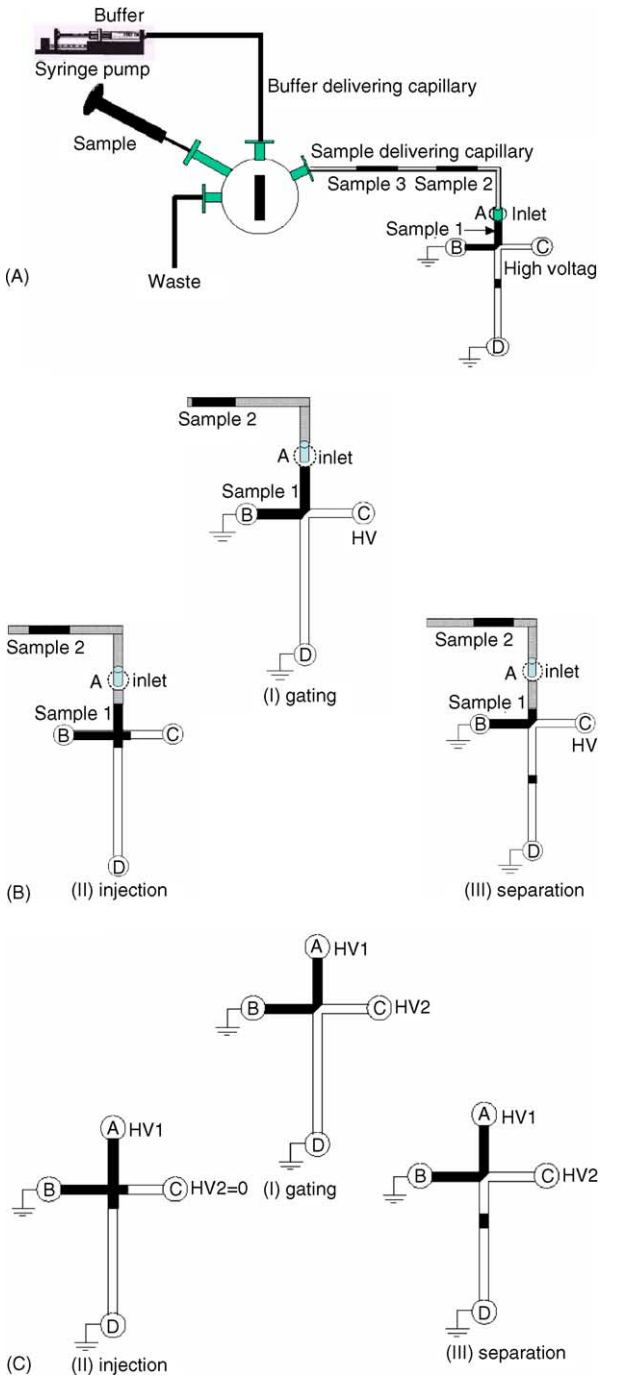

Fig. 1. (A) Schematic diagram of the microchip system. (B) Voltage gating scheme for sample injection using flow-through injection method. (C) Voltage gating scheme for sample injection using conventional manual injection method. See the text for detailed information.

a commercial reflection microscope (model BX40, Olympus, Tokyo, Japan) [2]. The light source radiated from a mercury lamp was filtered by a band-pass filter (510–550 nm for Rhodamine B and 470–490 nm for FITC-labeled peptides and estrogen) and was focused on the microchannel downstream 2.0 cm from the intersection by a 50× (NA = 0.5) long working distance objective. Fluorescence was collected by the same objective lens and passed through a dichroic cube (570 nm for Rhodamine B and 500 nm for FITC-labeled peptides and estrogen) with a long-pass filter (590 nm for Rhodamine B and 515 nm for FITC-labeled peptides and estrogen), followed by spatial filtering and finally detected by a photomultiplier tube operated at −600 V (R928, Hamamatsu, Tokyo, Japan). Amplified photoelectron signals were converted to the digital signal and processed by a computer using a commercial interface (model 9524, SISC, Taipei, Taiwan) running on a computer.

2.3. Fabrication of the microchip

The cross channels on the microchip are 1.2 cm and 4 cm in length, respectively, and both have a width of 90 μm at the top, 20 μm at the bottom and a depth of 35 μm. The microchip was fabricated on a soda-lime glass substrate using standard photolithography techniques in Micro-Nanotechnology Center at National Cheng Kung University [14]. Before bonding, the through holes A–D with a diameter of 1.5 mm were mechanically drilled on the cover plate to bond with four reservoirs by epoxy adhesive, and hole A was used as the sample inlet. A 13.5 cm fused silica capillary (I.D. 50 μm, O.D. 375 μm) was glued to hole A through a PTFE fitting. Detailed description regarding the fitting was described in [11]. Before experiments, the microchannel was sequentially rinsed with 1 M NaOH, DI water, and the running buffer for 10 min, respectively.

For waste-removing function, several modifications were added to the microchip as depicted in Fig. 2. A PMMA plate was fixed to the bottom of the microchip, reservoir C was replaced by a larger plastic reservoir with a volume of 1000 μL, two sub-reservoirs E and F fabricating by PMMA plate were fixed to the bottom plate and then connected to reservoir B and D, respectively, through the joint glass tubes.

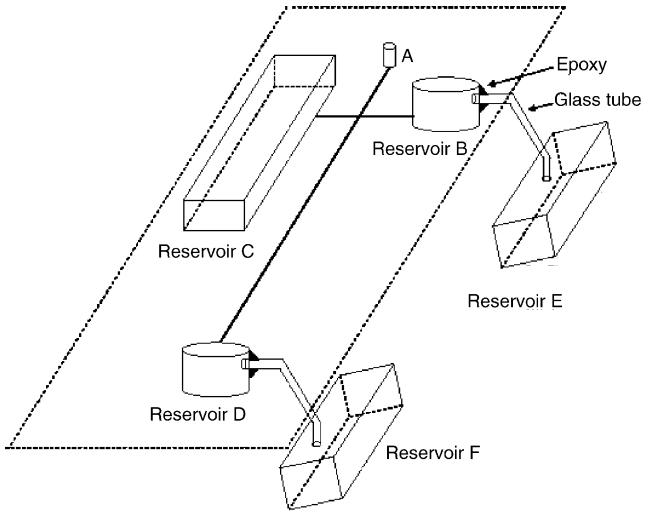

Fig. 2. Layout of microchip with waste-removing function.

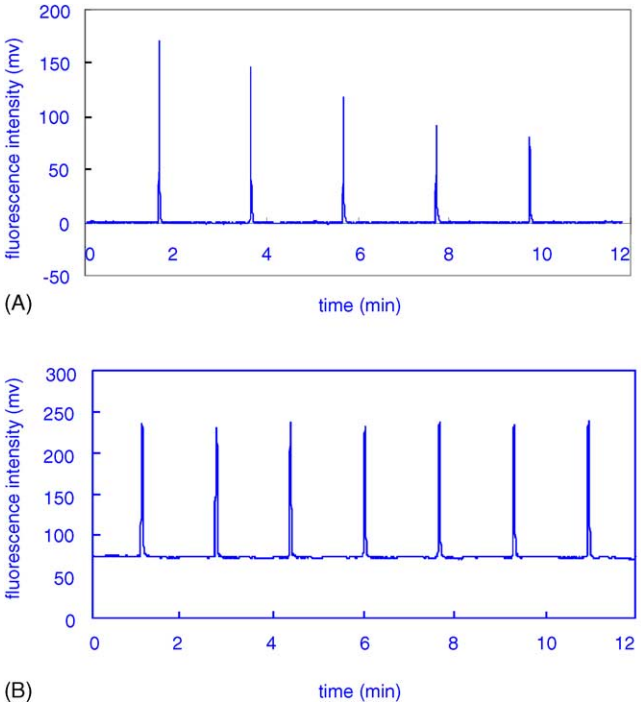

Fig. 3. Electropherograms of FITC-labeled estrogen using (A) manual injection method and (B) flow-through injection method. FITC-labeled estrogen has a concentration of 40 nM in a buffer containing 10 mM of Tris-acid, 1% glycerol and 0.2 mM of DDT (pH 7.4). The separation buffer was 1.5 mM boric acid (pH 7.4) containing 16 mM of sodium cholate and 5 mM of β-CD.

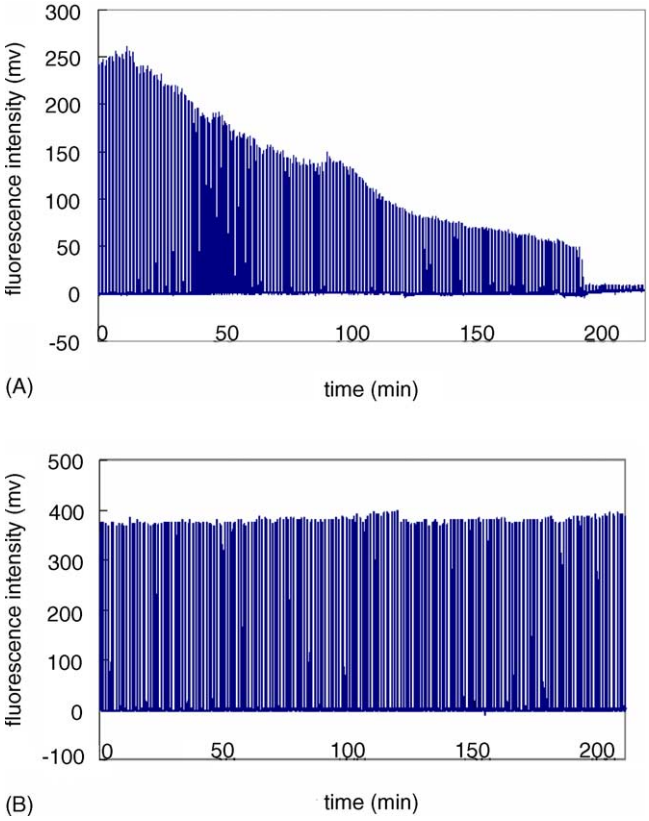

Fig. 4. (A) Electrokinetic injection of 10−5 M Rhodamine B dissolved in electrophoretic buffer containing 1 mM sodium tetraborate (pH 9.21). (B) Flow-through injection of 10−5 M Rhodamine B dissolved 1 mM sodium tetraborate (pH 9.21).

3. Results and discussions

3.1. Hydrodynamic versus electrokinetic injection for quantitative analysis

Compared to the manual cross injection method commonly used for microchip device, the flow-through method is free of injection bias [13] since the sample was driven to the cross-section by the hydrodynamic rather than electrokinetic force. We had further investigated this effect by analyzing a relatively neutral compound, FITC-labeled estrogen (40 nM). The injection bias was expected to be more severe when a neutral compound is injected since background electrolytes will dominate the injected sample. In order to eliminate the focusing problem, consecutive injections without removing the microchip was performed using both flow-through and manual injection methods. As seen in Fig. 3A, by electrokinetic method, a great reduction in peak intensity was observed and the signal completely disappeared after several injections. By flow-through method as shown Fig. 3B, the peak intensity was kept almost constant.

3.2. Waste-removing function for quantitative analysis

Because of a relatively large flow-through rate (1–2 μL/min), one flow-through microchip can only hold several analyses due to the overflow from the waste reservoir (100 μL), which has limited the analysis time up to 40 min. This time period, however, may not be sufficient to conduct an experiment to complete a quantitative analysis without changing the microchip. Moreover, the gravity force may arise to perturb the flow in the microchannel due to the unequal liquid level in the reservoir [15]. As shown in Fig. 4A, with manual electrokinetic injection, the signal of Rhodamine B degraded substantially with the time. It was also noticed that the color of Rhodamine B in reservoir A became much faded after 3 h of operation (data not shown) due to the selective injection of the charged analytes under electrokinetic injection mode. With the waste-removing function implemented in the microchip, Fig. 4B shows that Rhodamine B could be continuously analyzed without significant change (R.S.D. < 6.6%) in signal intensity for more than 3 h, which is generally sufficient to conduct a complete set of quantitative analysis.

3.3. Quantitation of phosphopeptides for the development of kinase assays

In this study, we attempted to construct a calibration curve for quantitative analysis of phosphopeptides using a phosphorylated peptide substrate for Lck, a nonreceptor cytoplasmic phosphotyrosine kinase (PTK), as the model. Lck

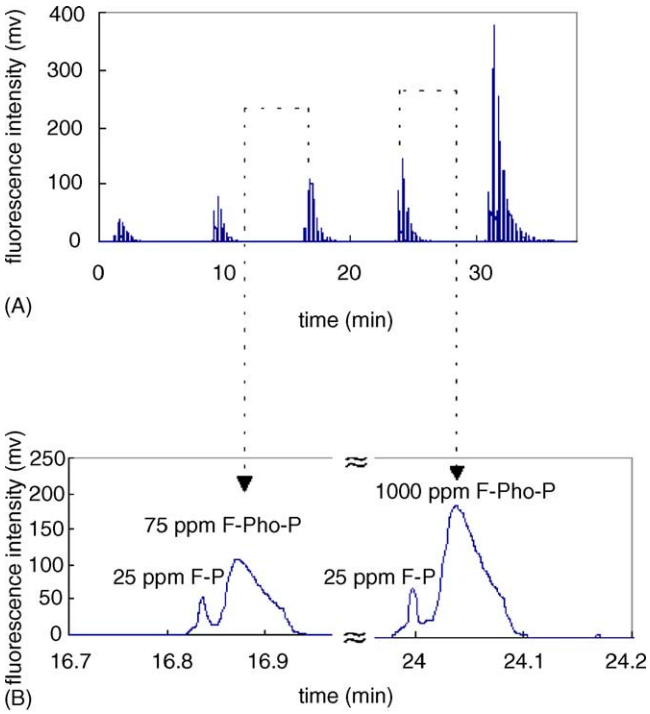

Fig. 5. (A) Electropherograms obtained from the introduction of a series of mixtures containing FITC-labeled phosphopeptides with a concentration ranging from 25 to 250 ppm and FITC-labeled non-phosphopeptides at a concentration of 25 ppm in sodium phosphate (5 mM, pH 7.68). The pump flowrate was 1.0 μL/min, and other conditions were described in the experimental section. (B) The enlarged electropherograms. The phospho and non-phospho pepetide pair could be separated as indicated.

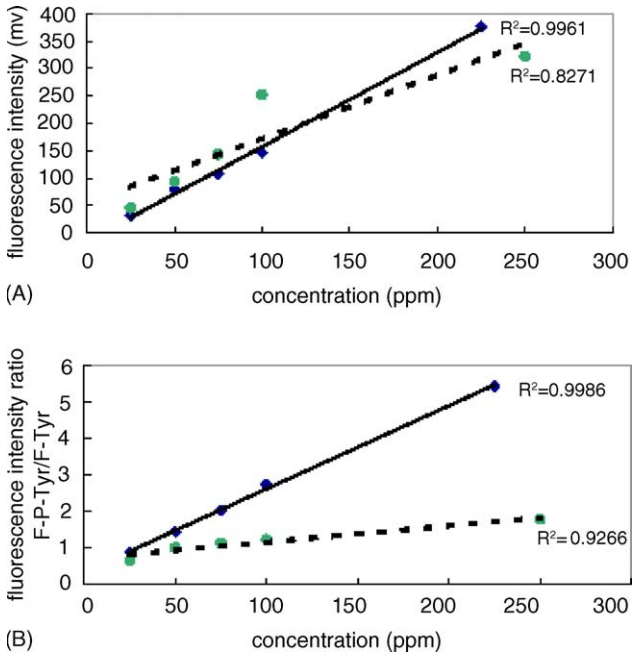

Fig. 6. Calibration curves constructed from the electropherograms shown in Fig. 5. The R.S.D. values of these data were listed in Table 1. The sampling methods include continuous flow-through (♦) and manual electrokinetic method (●) and the data were treated (A) without the use of internal standard and (B) with the use of internal standard. For the flow-through method, the greatest peak intensity among consecutive injections from one loaded sample (Gaussian plug) was used.

belongs to Src-family PTKs and plays important roles in the regulation of growth, proliferation, and differentiation of many types of cells [16]. Hence, developing a simple, reliable PTK assay based on chip-based quantitative method to screen compound collections for inhibitors holds a great potential in developing drugs for various diseases. For this study, a non-phospho counterpart of this peptide substrate was also synthesized as the internal standard for the quantitation. Alternatively, the detection of non-phospho counterpart would be valuable to quantify the extent of protein phosphorylation. Moreover, each sample was introduced into the hydrodynamic flow by a Valco valve to minimize the sample consumption. As described in previous section, a 3-h period would allow about 30 samples to be continuously analyzed when the injection valve is connected, which should be sufficient for this investigation. All analyses were completed by the same microchip to minimize the chip-to-chip or channel-to-channel variations.

The electropherogram acquired from consecutive loadings and injections of five standards (25–250 ppm) of phosphopeptides was depicted in Fig. 5A, which shows a proportional increase of peak intensity with the concentration. As shown in the profile for each concentration, the sample plug (60 nL) introduced by Valco valve was virtually a Gaussian shape [11] and each plug was subsequently fractionated and injected into the electrophoretic microchannel for separation by the voltage switching scheme described above. The washing time between two samples takes about 1–5 min, which is generally longer than the separation time. We are currently investigating other substrate materials to reduce the absorption effect and therefore reducing the required washing time. The enlarged electropherogram shown in Fig. 5B indicates that the phospho and non-phospho pepetide pair could be separated along a 2-cm microchannel. The calibration curves were constructed with flow-through and manual sampling methods as well as without (Fig. 6A) and with (Fig. 6B) the use of the internal standard. For flow-through method, only the greatest peak among a Gaussian profile (one loaded plug) was used for the calculation. With the use of the internal standard, Fig. 6B shows that continuous flow-through method has the best linearity (R^2 = 0.9986) compared to that obtained by manual injection method (0.9266). As indicated in Table 1, without the use of the internal standard, the R.S.D. value of the peak height associated with four replicate loadings ranges from 2.72 to 4.04% by flow-through method within the investigated concentration range compared to the range of 10.39–23.8% by manual injection method. With the use of the non-phospho-peptide as the internal standard, the R.S.D. value obtained by manual injection method was dramatically decreased to a range from 4.84 to 20.02% but the reproducibility was still much poorer than those obtained by flow-through method without the use of the internal standard. The use of the internal standard should compensate any system-

Table 1
The R.S.D. values calculated from a series of phosphopeptide standards analyzed with and without the use of the non-phosphopeptide as the internal standard based on both manual and flow-through sampling methods

Concentration of phosphopeptides (ppm)	Without/with internal standard	Injection methods	
		Continuous flow-through	Manual electrokinetic
25	Without	2.72	23.80
	With	2.69	10.84
50	Without	2.99	17.74
	With	1.46	4.84
75	Without	3.39	19.27
	With	3.02	8.63
100	Without	4.04	10.39
	With	2.60	5.43
250	Without	2.64	30.44
	With	1.58	20.02
Average	Without	3.16	20.33
	With	2.27	10.35

The R.S.D. values were deduced from three or four replicates.

atic error, such as the optical alignment discussed previously. With the use of the internal standard, the R.S.D. valves for manual sampling method (Table 1) were greatly improved compared to those without the internal standard. For flow-through sampling method, however, the R.S.D. values were just slightly decreased from 3.16 to 2.27%, indicating that the flow-through injection method has fewer systematic errors than manual injection method.

Based on the results obtained, the injection bias could not be compensated by the use of the non-phosphopeptide as the internal standard for manual electrokinetic injection. This is due to the fact that the non-phosphorpeptide used in this study as the internal standard has a different charge to mass ratio compared to the standard phosphopeptides. However, the simultaneous detection of the phospho/non-phosphopeptide pair would enable the development of a comparative assay for quantifying the extent of kinase phosphorylation, which is important in characterizing kinase activity.

4. Conclusions

From this study, the hydrodynamic injection-based microchip electrophoresis shows superior capabilities for quantitative analysis compared to conventional manual method due to its capability in continuous and reproducible sampling up to 3 h and inherent nature of injection-bias free. Besides, the required amount of samples, analysis time and labor for handling are greatly reduced compared to the manual method. In view of the increasingly importance of quantitative analysis in many analytical fields, the usefulness of the device will be many.

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