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Focusing Review

In situ Photopolymerization of Functionalized Polyacrylamide-Based Preconcentrators for Highly Sensitive Specific Detection of Various Analytes by Microchip Electrophoresis

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Abstract

Microchip electrophoresis (ME) has emerged as a promising tool for rapid analysis of various sample types and it is one of the most important modules of miniaturized total analysis systems. Electrokinetic effects provide both efficient transport and separation of sample components in microfluidic channels. Moreover, miniaturization of devices drastically reduces reagent consumption, significantly decreases analysis time, and allows for easier parallel screening and automation. However, most microfluidic systems are insensitive, which is a serious problem. In order to increase the sensitivity of ME, several on-line pretreatment applications such as specific and non-specific preconcentration, extraction, and derivatization have been proposed. Here, we review various specific on-line preconcentration methods utilizing in situ photopolymerized polyacrylamide-based preconcentrator gels. These techniques allowed for highly sensitive specific detection of various sample types by ME.

Keywords: Microchip electrophoresis; On-line derivatization; On-line concentration; Specific concentration

1. Introduction

ME is one of the most important methods in miniaturized total analysis systems [1,2]. Electrokinetic effects provide both efficient transport and separation of sample components in microfluidic channels. Translation of the separation modes developed for traditional capillary electrophoresis to a microchip platform enables analysis of various types of sample components [3-5]. Moreover, miniaturization of device reduces reagent consumption and analysis time. It also allows for parallel screening and ease of automation [6].

However, most microfluidic systems are insensitive, which is a serious problem. Since 2000, several approaches designed to improve the detection limits by exploiting various physical and chemical phenomena have been developed and reviewed [7-12]. Several reports proposed numerous methods for sample preconcentration in micro/nanofluidic devices, including stacking [13,14], isotachophoresis, [15,16] and multiple preconcentration techniques [17,18]. Of these various techniques, electrokinetic trapping based on nanoporous charge-selective membranes [19,20] or nanofluidic channels [21-23] is one of the most commonly used methods for the detection of charged analytes. Electrokinetic trapping has several important advantages for the preconcentration of charged samples. For example, the buffer requires no temporal or partial changes and the electrokinetic driving mechanism is readily compatible with other analytical techniques. The present article provides a comprehensive review of various techniques and other hydrogel materials for sample preconcentration utilizing ion concentration polarization (ICP) effects and affinity ME.

2. The mechanism of ICP

When performing ME, sample preconcentration is often required to increase the detection limit. One of the most commonly used methods for sample preconcentration utilizes ICP [24-29]. The method requires only a single homogeneous analyte phase. A nano-junction region between microchannels and nano-passages (nanochannel or nanoporous membrane) is also required to achieve preconcentration.

Figure 1 shows a schematic illustration of ICP induced by an external electric field for the case of a microchannel with negatively charged perm-selective nanochannels. When an external electric field is applied, only cations can pass through the ion-selective nanochannels, which are depicted on the left side of Figure 1. In contrast, the anions...
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in this region cannot move through the nanochannels to the right side. In order to satisfy the electrical neutrality of the original homogeneous analyte, a depletion region (that forces anions to move to the left boundary of the region) is formed in the neighborhood of the nano-microchannel junction. The depletion region is nearly devoid of charged ions and has a very low local electrical conductivity value. As a result, a very large electric field is generated across this region. Therefore, electrophoretic migration is dominant in the depletion zone and pushes the anions toward the anodic side.

3. Electrokinetic trapping in front of charged narrow gaps fabricated using various techniques

Most of the recent advancements in the field of on-line preconcentration have been driven by the needs of existing ICP-based technologies namely, to increase their applicability and scale. For example, the cost and difficulty of device fabrication has been reduced through the expansion of ICP-based focusing of charged analytes to paper microfluidic platforms [32-34]. In another application area that benefits populations living in settings with limited resources, desalination by ICP is an alternative to reverse osmosis and requires a simpler infrastructure [35].

The recent incorporation of constrictions and valves into microfluidic ICP devices has enabled the recovery of focused analyte plugs after enrichment [36]. These methods are commonly based on the electrokinetic trapping mechanism. In this mechanism, the sample components are moved continuously and trapped in a boundary space immediately in front of charged narrow gaps fabricated using an etching technique. The device enables extremely robust concentration of proteins by $10^6$-$10^8$-fold. However, the ionic nanofluidic filter is fabricated on a silicon wafer with a specially designed channel, which is expensive and therefore hinders general use.

We developed a novel method for fabricating an ionic preconcentrator on a commercial poly(methyl methacrylate) (PMMA) cross-channel chip based on the photopolymerization of ionic acrylamide [24-27]. An ionic polyacrylamide gel was fabricated by introducing the acrylamide solution into a microchip channel. Next, polymerization of the acrylamide solution was conducted by irradiation for a few minutes with an argon ion laser.
beam near the channel cross. This fabricated ionic gel preconcentrator had sufficient mechanical strength. The preconcentrator efficiently trapped ionic analytes possessing an opposite charge in the front of the nanofilter by application of hundreds of volts for a few minutes. These methods enabled greater than 10^4-fold concentration. Figure 2 shows the sample preconcentration images of an acidic fluorescent reagent at the channel cross of a PMMA microchip comprised of sulfonate-type polyacrylamide. The sulfonate-type polyacrylamide gel was prepared as a perm-selective preconcentrator to bank up anionic samples at the outlet channel near the intersection of the microchip. After exchanging the channel solution with 10 mM acetate (pH 4.2) as the background electrolyte, the sample reservoir was filled with a 10^-5 M solution of 8-aminopyrene-1,3,6-trisulfonic acid (APTS), which emits fluorescence after irradiation with an argon ion laser. Voltage (100 V) was applied across the preconcentration gel. The APTS immediately moved to the channel cross and did not penetrate the gel. The concentration of APTS began after 10 s and increased over 1.5-2 min then finally reached a plateau. This method enabled greater than 10^4-fold concentration of APTS [24]. However, this method has some limitations; Sample anions as well as all anionic components present in the sample lane are concentrated just before the gel, which occasionally results in insufficient sample injection. Moreover, since most biological samples are weak acids or bases, the presence of inorganic salts, such as sodium chloride, may hinder the effective preconcentration of the biological ionic species.

In an attempt to address the limitations of our sulfonate-type preconcentrator gel, we prepared a carboxylate-functionalized polyacrylamide gel near the cross of a microchip channel. This gel was then used to preconcentrate a sample containing weak anions. An acrylamide solution containing acrylamidoglycolic acid was introduced at a sample outlet channel near the cross and was subsequently polymerized by irradiation with a 100 μm diameter argon ion laser beam. The preconcentrator gel had sufficient mechanical strength. It specifically entrapped weak anionic analytes in front of the gel. The concentrated weak anions were introduced into a separation channel, using the pinched injection method. Next, they were separated and detected in the separation channel. This method enabled greater than 10^5-fold concentration of fluorescein isothiocyanate (FITC)-labeled amino acids [25].

We also utilized this carboxylic acid-type gel and 5-(4,6-dichlorotriazinyl) aminofluorescein (DTAF) for continuous on-line preconcentration and fluorescent derivatization of Asp. This system was chosen because of the rapid reaction between Asp and the DTAF reactivity toward both primary and secondary amines [37]. The carboxylic acid-type gel continuously captured Asp as well as DTAF. Therefore, the fluorimetric labeling reaction was also catalyzed in front of the gel. The fluorescently labeled Asp were resolved and detected near the outlet of the channel. This method enabled greater than 10^4-fold concentration of Asp with derivatization in as little as 5 min [26].

The majority of previous ICP on-line concentration studies have been based on an anionic nanochannel or polymer, hence they are limited to anionic samples [38, 39]. Many experimental studies, however, require the analysis of cationic samples. A cation-selective preconcentration system can significantly enhance the detection sensitivity for cationic analytes; this can be used to detect biological toxins in bodily fluids. However, only one study reported the use of a cation-selective preconcentration technique using a fluorescent reagent [40]. Thus, we developed a method for the fabrication of a cationic preconcentrator utilizing an acrylamide solution containing (3-acrylamidopropyl) trimethylammonium. This method enabled greater than 10^4-fold preconcentration of rhodamine. We utilized this method to analyze both Rho 110 derivatives of oligosaccharides (Fig. 3) and a FITC-labeled protein.

4. Affinity ME for the analysis of post-translational modifications (PTMs)

PTMs, which comprise the covalent attachment of chemical entities to the side chains of modifiable residues on proteins, act as molecular switches to allow cells to respond to diverse conditions [41]. PTMs play a vital role in the control of protein activity, stability, and subcellular localization, thereby contributing to intracellular regulation.
developed a specific medium borate buffer (pH 11.0), and the concentration and time development of PTMs spectrometry at substoichiometric levels, considerably impeding mass postphosphorylation.[42,43]

Over the past decade, the demand for in-depth PTM-based proteomic studies has spawned the development of a variety of selective affinity materials capable of capturing trace amounts of PTM-containing peptides from highly complex biosamples. However, novel strategies are urgently required for fulfilling the increasingly complex and accurate requirements of PTMs proteomics analysis, which can hardly be met using conventional enrichment materials [44,45]. Thus, we used an in situ photopolymerized acrylamide gel for affinity matrix.

4.1. Affinity ME of oligosaccharides utilizing lectin-immobilized gels

Oligosaccharides prepared from biological samples often contain various components and analytes at concentrations below the detection threshold. Consequently, these samples often require preliminary fractionation and concentration. Lectins are proteins that interact specifically with carbohydrates without modifying them [46]. Most lectins primarily interact with monosaccharides with a higher affinity compared to the monosaccharide binding affinity. The high specificity of lectin binding to oligosaccharides is helpful for glycoproteomic profiling analysis. Lectin affinity capillary electrophoresis [48-50] has been utilized for this purpose.

We developed a specific on-line preconcentration technique by fabrication of a lectin-impregnated gel at the channel crossing point using in situ photopolymerization [51]. This method included the following four steps: (1) affinity matrix fabrication, (2) saccharide concentration, (3) elution, and (4) separation and detection. The lectin that was chosen for gel impregnation was concanavalin A (Con A). Con A binds to α-glucoside and α-mannoside with a $K_a$ of $10^3$ M$^{-1}$ and to high-mannose-type oligosaccharides with a $K_a$ of $10^5$ to $10^7$ M$^{-1}$ [52]. A Con A-containing acrylamide solution was delivered to the channels from R2 under pressure. A round Con A-impregnated acrylamide gel was fabricated at the intersection of the channels in the same manner as the ionic acrylamide gel described above. This step is referred to as the affinity matrix fabrication step. After washing the channels with 25 mM Tris/acetate buffer (pH 7.0, neutral buffer), R1 was filled with a solution of APTS-labeled mannobiose, R2 was filled with the neutral buffer, R3 with an acidic buffer (25 mM phosphate, pH 2.0), and R4 with a basic buffer (200 mM sodium borate buffer, pH 11.0). Figure 4 shows the time-course of the concentration and release of APTS derivatives of mannobiose at the channel crossing point in the Con A-impregnated polyacrylamide gel. Dim fluorescence from riboflavin was observed after the washing and preconditioning processes at the lectin-immobilized plug (interior of the broken circle in Fig. 4(a)). After 150 V was applied across R1 and R2, with R2 as the anode, the fluorescence intensity at the lectin plug gradually increased, spread over the gel, and finally reached saturation (Fig. 4(b)–(d)). This indicated that the negatively

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**Fig. 4.** Time-course of the concentration and release of APTS derivatives of mannobiose at the channel crossing point in the Con A-impregnated polyacrylamide gel. All of the channels were filled with 25 mM Tris/acetate buffer (pH 7.0). Next, the left chamber (R3) was filled with 25 mM Tris/phosphate buffer (pH 2.0), the right chamber (R4) was filled with 200 mM sodium borate buffer (pH 11.0), and the lower chamber (R1) was filled with 10$^{-6}$ M APTS-mannobiose. Next, 150 V was applied across R1 and R2 for 0 s (a), 30 s (b), 90 s (c), and 180 s (d). The voltage settings were then changed to 200, 200, 0, and 800 V for R1, R2, R3, and R4, respectively. Images (e) and (f) show the decrease in the fluorescence of APTS-mannobiose after 10 and 15 s. Reproduced from [51] with permission from the Royal Society of Chemistry.

[42,43]. Among these modifications, protein phosphorylation and glycosylation are two of the most well-studied PTMs. However, in living organisms, native post-translationally modified proteins are typically present at substoichiometric levels, considerably impeding mass spectrometry (MS)-based analyses and identification.

Over the past decade, the demand for in-depth PTM-based proteomic studies has spawned the development of a variety of selective affinity materials capable of capturing trace amounts of PTM-containing
charged APTS-labeled mannobiose was trapped in the Con A plug. This step is referred to as the saccharide concentration step. The maximum fluorescence intensity of APTS-mannobiose at this stage may be limited by the amount of Con A immobilized in the gel. Following the saccharide concentration step, an electric field (800 V, 5 min) was applied across R3 (acidic buffer) and R4 (basic buffer). After 10 s, the concentrated APTS-mannobiose was released from the gel to the separation channel by delivering phosphate ions to the gel from R3. These phosphate ions induced the denaturation of Con A. As shown in Fig. 4(e) and (f), trapped APTS-mannobiose was released as a relatively broad band from the gel. This step is referred to as the elution step. The sample components were finally stacked at the boundary of the phosphate-acetate ions in a pH gradient generated by a large amount of sodium ions delivered from the anode. The stacked sample was separated and detected at the end of the separation channel. This step is referred to as the separation and detection step.

This method may be used for highly sensitive analysis of glycoprotein-derived oligosaccharides. Since numerous lectins with various specificities are available, this method could be applied to screening and profiling of oligosaccharides in glycan mixtures. In addition, these results indicated that Con A protein activity was maintained in an acrylamide gel. Therefore, we developed a method for the fabrication of an enzyme-impregnated gel in a commercial pipette tip that was photopolymerized in situ by irradiation with a LED laser. We chose the enzymes trypsin and PNGaseF to impregnate the acrylamide gels for fast digestion of glycoprotein. The deglycosylation of glycoprotein samples was performed by a two-step digestion in trypsin- and PNGaseF-impregnated gels, which was completed in 16 min. The cost of preparing a single sample including fluorescent labeling was only 1 dollar. Thus, this method provided an inexpensive, rapid, and convenient analysis of glycoprotein glycans [53].

### 4.2. Affinity ME of phosphorylated compounds utilizing Phos-tag gels

Protein phosphorylation is one of the most well-studied PTMs and greater than 30% of eukaryotic proteins are phosphorylated [54]. The addition of a phosphate group to an amino acid residue of a substrate protein may disrupt the existing electrostatic interactions and create new hydrogen bonds in the substrate protein. Therefore, the protein structure may be significantly altered, which may further affect the stability, activity, and subcellular localization of the protein. These phosphorylation events specifically regulate multiple cellular processes [55-58].

MS is a promising tool to analyze protein phosphorylation and has gained a great deal of attention due to its ability to profile thousands of proteins in a single analysis [59, 60]. The analysis of protein phosphorylation is not straightforward because the abundance of most phosphorylated proteins is not only low but also dynamically changes depending on physiological states and cellular activities. Therefore, one of the major challenges in phosphoproteome analysis is to develop a specific method to capture low-abundant phosphorylated proteins or peptides [61]. We developed a phosphate-specific on-line concentration method utilizing the affinity ligand 1,3-bis[bis(pyridin-2-ylmethyl)amino]propan-2-olate referred to as Phos-tag [62]. This method included the four steps described above (section 4.1) for the affinity ME of
generated Phos-tag-impregnated acrylamide gels formed at the channel crossing point of microchip by irradiation with a UV LED laser. Figure 5 shows a time-course of the specific concentration of FITC-labeled phosphoserine in a Phos-tag-impregnated polyacrylamide gel at the channel crossing point. After washing and preconditioning the Phos-tag polyacrylamide gel, no fluorescence was detected from the gel (interior of the square in Fig. 5(a)). After applying voltage, the fluorescence intensity of the Phos-tag polyacrylamide gel gradually increased (Fig. 5(b)), expanded, and finally reached a maximum (Fig. 5(c)). This indicated that the FITC-labeled phosphoserines were specifically trapped in the Phos-tag acrylamide gel. Next, an electric field (800 V, 2 min) was applied across R3 and R4. After 10 s, the entrapped FITC-labeled phosphoserines were released from the gel and delivered to the separation channel by the administration of high concentrations of phosphate ions and EDTA to the gel. As shown in Figures 5(d) and (e), the trapped phosphoserines were completely released from the gel.

In order to expand the applicability of our Phos-tag ME method, we tested whether phosphorylated peptides could be concentrated and separated with this system. The on-line concentration and separation fluorescent profiles of $5.0 \times 10^{-9}$ g mL$^{-1}$ of FITC-labeled ovalbumin peptides subjected to Phos-tag acrylamide gel ME is shown in Figure 6(a). The fluorescence intensities of the pinched injection analysis of $1.0 \times 10^{-6}$ g mL$^{-1}$ of FITC-labeled ovalbumin tryptic peptides (without Phos-tag gel ME) is depicted in Figure 6(b). The pinched injection analysis of the ovalbumin peptides exhibited a complex separation profile and peaks appeared within 2 min. In contrast, Phos-tag acrylamide gel ME produced two large peaks at 1.1 and 1.5 min. When the sample was pre-treated with alkaline phosphatase no peaks were detected. Therefore, the peaks observed at 1.1 and 1.5 min were tentatively assigned to monophosphorylated peptides [63].

5. Conclusions

This review summarizes our recent studies of sample preconcentration utilizing ICP in conjunction with ME. An ionic polyacrylamide gel preconcentrator was fabricated and exhibited sufficient mechanical strength. The ionic analytes were trapped in the front of the nanofilter upon application of voltage ($\sim 100$ V between the sample and sample waste reservoirs) for a few minutes. Subsequently, the analyte was introduced into the separation channel using the gated injection method. This technique enabled greater than 10-fold concentration of ionic samples. A lectin-immobilized gel and a Phos-tag gel were formed by in situ polymerization of an acrylamide solution. Upon application of voltage for a few minutes, the lectin-immobilized gel and Phos-tag gel trapped specific oligosaccharides and phosphorylated compounds, respectively, with high sensitivity. The total analysis time for each type of ME was approximately 10 min. The choice of the electrophoretic buffer system for transient isotachophoresis stacking was carefully considered and enabled specific detection without the loss of resolution. By combining these methods, most analytes can be effectively concentrated, which may enhance the applicability of ME for various purposes.

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References

[1] Manz, A.; Fettinger, J. C.; Verpoorte, E.; Lüdi, H.; Widmer, H. M.; Harrison, D. J. Trends Anal. Chem. 1991, 10, 144-149.
[2] Manz, A.; Graber, N.; Widmer, H. M. Sens. Actuators B Chem. 1990, 1, 244-248.
[3] Suzuki, S. Chromatography 2014, 35, 1-22.
[4] Baba, Y. Chromatography 2015, 36, 73-79.
[5] Yamamoto, S.; Nagai, E.; Asada, Y.; Kinoshita, M.;
Chromatography

Chromatogr. A 1988, 438, 73-84.

[53] Yamamoto, S.; Ueda, M.; Kasai, M.; Ueda, Y.; Kinoshita, M.; Suzuki, S. J. Pharm. Biomed. Anal. 2020, 179, 112995.

[54] Olsen, J. V.; Blagoev, B.; Gnad, F.; Macek, B.; Kumar, C.; Mortensen, P.; Mann, M. Cell 2006, 127, 635-648.

[55] Ha, J.; Kang, E.; Seo, J.; Cho, S. Int. J. Mol. Sci. 2019, 20, 6157.

[56] García-Blanco, N.; Vázquez-Bolado, A.; Moreno, S. Int. J. Mol. Sci. 2019, 20, 6228.

[57] Kumar, A.; Gopalswamy, M.; Wolf, A.; Brockwell, D. J.; Hatzfeld, M.; Balbach, J. Proc. Natl. Acad. Sci. 2018, 115, 3344-3349.

[58] Bah, A.; Vernon, R. M.; Siddiqui, Z.; Krzeminski, M.; Muhandiram, R.; Zhao, C.; Sonenberg, N.; Kay, L. E.; Forman-Kay J. D. Nature 2015, 519, 106-109.

[59] Maes, E.; Tirez, K.; Baggerman, G.; Valkenborg, D.; Schoofs, L.; Encinar, J. R.; Mertens, I. Mass Spectrom. Rev. 2016, 35, 350-360.

[60] Potel, C. M.; Lemeer, S.; Heck, A. J. R. Anal. Chem. 2019, 91, 126-141.

[61] Ishihama, Y. Chromatography 2019, 40, 89-97.

[62] Kinoshita, E.; Takahashi, M.; Takeda, H.; Shiro, M.; Koike, T. Dalton Trans. 2004, 8, 1189-1193.

[63] Yamamoto, S.; Himeno, M.; Kobayashi, M.; Akamatsu, M.; Satoh, R.; Kinoshita, M.; Sugiura, R.; Suzuki, S. Analyst 2017, 142, 3416-3423.