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# Selective sampling of multiply phosphorylated peptides by capillary electrophoresis for electrospray ionization mass spectrometry analysis

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**Abstract**

The ionization of phosphorylated peptides in positive ion mode mass spectrometry is generally less efficient compared with the ionization of their non-phosphorylated counterparts. This can make phosphopeptides much more difficult to detect. One way to enhance the detection of phosphorylated proteins and peptides is by selectively isolating these species. Current approaches of phosphopeptide isolation are based on the favorable interactions of phosphate groups with immobilized metals. While these methods can be effective in the extraction, they can lead to incomplete sample recovery, particularly for the most strongly bound multiply phosphorylated components. A non-sorptive method of phosphopeptide isolation using capillary electrophoresis (CE) was recently reported [Zhang et al., Anal. Chem. 77 (2005) 6078]. The relatively low isoelectric points of phosphopeptides cause them to remain anionic at acidic sample pH. Hence, they can be selectively injected into the capillary by an applied field after the electroosmotic flow (EOF) is suppressed. The technique was previously coupled with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). In this work, the exploitation of selective sampling in conjugation with electrospray ionization mass spectrometry (ESI-MS) is presented. The transition was not immediately straightforward. A number of major alterations were necessary for ESI interfacing. These adaptations include the choice of a suitable capillary coating for EOF control and the incorporation of organic solvent for efficient ESI. As expected, selective injection of phosphopeptides greatly enhanced the sensitivity of their detection in ESI-MS, particularly for the multiply phosphorylated species that were traditionally most problematic. Furthermore, an electrophoretic separation subsequent to the selective injection of the phosphopeptides was performed prior to analysis by ESI-MS. This allowed us to resolve the multiply phosphorylated peptides present in the samples, predominantly based on the number of phosphorylation sites on the peptides.


*Keywords:* Phosphorylation; Multiply phosphorylated peptides; Separation; EOF suppression; Polymer capillary coatings; Non-aqueous CE

## 1. Introduction

Protein phosphorylation is an extremely important cellular event that governs the actions of numerous processes, including gene expression, which is mediated through the phosphorylation of transcription factors by serine and threonine kinases in the nucleus [1]. Phosphorylation is carried out by a family of enzymes known as protein kinases, which are encoded by over 500 genes [2]. It is believed that one-third of all proteins present in a mammalian cell are regulated through reversible phosphorylation [3]. Given its importance, great effort has gone into better understanding phosphorylation and its connection to disease. Mass spectrometry (MS) is widely used in proteomics and is the current method of choice to detect protein phosphorylation for high sample throughput and sensitivity [4].

Unfortunately, there are several disadvantages facing the MS-based detection of phosphopeptides. Firstly, phosphorylated proteins are generally present at very low stoichiometic ratios relative to their non-phosphorylated counterparts. Secondly, acidic phosphopeptides are inherently more difficult to ionize in positive ion mode MS than less acidic, non-phosphorylated, components. As a result, phosphopeptides are subject to considerable ionization suppression, making detection difficult without first isolating them from the non-phosphorylated peptides. There are numerous techniques available to isolate phosphopeptides, many of which are chromatographic or sorptive methods based on the distinctive interactions of phosphate-containing peptides

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with solid phases. Two of the more recent popular methods are immobilized metal ion affinity chromatography (IMAC) [5] and titanium dioxide ($TiO_2$) chromatography [6], in which phosphopeptides are selectively isolated based on their interactions with iron or titanium, respectively. Unfortunately, the retentive characteristic of these columns can lead to sample loss particularly for the most strongly retained multiply phosphorylated peptides [4,6]. Alternatively, the relatively polar character of phosphopeptides has been used for its isolation in reversed phase chromatography, which is biased against peptides with low isoelectric points (p*I*) [7]. Hence, phosphopeptides generally elute earlier than their non-phosphorylated forms [8]; nevertheless, in a complex mixture, they can co-elute with other hydrophilic non-phosphorylated peptides. Likewise, the non-retentive behavior of phosphopeptides allows their isolation in the flow-through fractions from a reversed phase column [9]. However, this can contain non-volatile salts, buffers, and additives that can be deleterious to MS-based analyses. Therefore, on-target cleanup [9] or further chromatographic resolution with porous graphitic carbon chromatography [10] was necessary.

A more effective, non-retentive, approach of phosphopeptide isolation has recently been developed in our laboratory using capillary electrophoresis (CE) [11]. This technique takes advantage of the acidic characteristics of phosphopeptides, which remain negatively charged even in low pH environments when most non-phosphorylated peptides have a net positive charge. When electrokinetic injection is performed without a significant electroosmotic flow (EOF), the migration of sample ions into the capillary is predominantly determined by their net charges. This allows for the selective sampling of anionic peptides, such as the phosphopeptides from a tryptic digest of phosphorylated proteins [11]. A similar approach was applied to analyze selectively sialylated glycoproteins by CE [12]. Compared with the conventional injections used in CE, selective sampling permits focusing on a sub-set of the sample and greatly reduces the complexity for subsequent separation and detection.

Selective sampling of phosphorylated peptides by CE is particularly effective for isolation of multiply phosphorylated peptides. Since these species have extremely low p*I*s (p$I < 3$), they can be better distinguished from the acidic non-phosphorylated peptides, such as those rich in Asp and Glu residues. In addition, multiply phosphorylated peptides generally have higher net negative charges than singly phosphorylated peptides and are consequently injected to a greater extent during selective sampling [11]. The sample bias towards multiply phosphorylated peptides is particularly advantageous as it is complementary to the commonly used IMAC and $TiO_2$, both of which tend to give poor recovery of multiply phosphorylated peptides [4]. Other advantages realized by using CE include improved sample recovery since there is no binding to a stationary phase, and CE's ability to handle extremely small sample volumes.

Our previous reports of selective phosphopeptide sampling focused on the interfacing with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [11,13]. In the current report, we are interested in adapting the selective sampling of phosphopeptides for detection by electrospray ionization mass spectrometry (ESI-MS). The transition from MALDI-MS to ESI-MS for the selective injection of phosphopeptides involves a number of non-trivial alterations. It begins with the re-assessment of capillary coatings for compatibility with ESI-MS. Previously, a phospholipid coating was employed to generate the required near-zero EOF for selective sampling at acidic pH conditions. While this coating appeared to be compatible with MALDI-MS, it was nevertheless a non-covalently adsorbed coating. Desorption of coating could impose issues with ESI-MS. Instead, permanent, covalently attached polymeric coatings may be more suitable for this work.

Over the years, a large collection of capillary coatings, both covalently bound and physically adsorbed, have been reported in the literature for the prevention of peptide or protein adsorption during their separations in CE. This area of development was last reviewed by Doherty et al. in 2003 [14] and briefly summarized by Huang et al. in 2006 [15]. Examples of covalently attached coatings include polyacrylamide (PA) [16], poly(vinyl alcohol) (PVA) [17], poly(vinylpyrrolidone) (PVP) [18], poly(ethylene oxide) (PEO) [18] and poly(ethylene glycol) (PEG) [19]. Some of these polymers, namely PVA [17] and PEO [20], and other neutral polymers such as hydroxypropylmethyl cellulose [21], were also used as dynamic adsorbed coatings. Other dynamic coatings included charged polymers (polybrene [22], polyarginine [23], polyethyleneimine [24], chitosan [25]), amines [26] and surfactants [27–29] including the phospholipids used in our previous work [11,13,30].

Capillary coatings previously employed for CE–MS were summarized in a review by Simpson and Smith [31]. Many of the aforementioned coatings were included. While no one technique appeared dominant, preference was given to charged coatings that could generate a significant EOF, since such a solution flow during electrophoresis is beneficial in maintaining a stable electrospray. This is illustrated by developments in CE–MS coatings within the last year, which predominantly focus on charged coatings: polyamine [32], polybrene-poly(vinyl sulfonate) bilayer [33], and ionene [34]. Unfortunately, in contrast to the traditional CE separations, the selective injection of phosphopeptides cannot be performed with a fast EOF. We will, therefore, focus on the more conventional uncharged polymeric coatings for this work. This brings us to the second focus of this work, which is to determine the best conditions to deliver the selectively injected phosphopeptides from the capillary to the ESI-MS under a near-zero EOF.

The selection of phosphopeptides based on their electromigration dictates the use of capillaries with surface modifications that result in near-zero EOFs. While the injection step can be performed offline, decoupled from the detection step by ESI-MS, it is unfortunately not possible to transfer the analytes from one capillary to another without introducing significant bandbroadening. Hence, ESI-MS can only be performed in the absence of an EOF in this case. To create a flow, a make-up solution can be added near the ESI emitter using either a T-junction connector or a sheath-flow interface [35]. Unfortunately, the introduction of makeup flow can dilute the analytes and lower the sensitivity. In addition, the pressure used to deliver the make-up solution can potentially drive the capillary contents