



# Highly efficient solid-phase derivatization of sugar phosphates with titanium-immobilized hydrophilic polydopamine-coated silica



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## ABSTRACT

Sugar phosphates are a type of key metabolic intermediates of glycolysis, gluconeogenesis and pentose phosphate pathway, which can regulate tumor energetic metabolism. Due to their low endogenous concentrations, poor chromatographic retention properties as well as ionization suppression from complex matrix interference, the determination of sugar phosphates in biological samples is very difficult. In this study, titanium-immobilized hydrophilic polydopamine-coated silica microspheres ( $\text{SiO}_2@\text{PD-Ti}^{4+}$ ) were synthesized for highly efficient solid-phase derivatization of sugar phosphates. Sugar phosphates were selectively captured onto the surface of the  $\text{SiO}_2@\text{PD-Ti}^{4+}$  microspheres by chelating with phosphate groups, and then reacted with 3-amino-9-ethylcarbazole via reductive amination based on solid-phase derivatization, which could not only increase the retention and resolution of sugar phosphates on reversed-phase liquid chromatography (RPLC), but also improve the mass spectrometry (MS) sensitivity of sugar phosphates. The adsorption capacity of  $\text{SiO}_2@\text{PD-Ti}^{4+}$  microspheres towards glucose-6-phosphate is 0.76 mg/g, which is much larger than that of commercial  $\text{TiO}_2$ . Compared with the traditional liquid-phase derivatization, the solid-phase derivatization based on the  $\text{SiO}_2@\text{PD-Ti}^{4+}$  microspheres displayed several superiorities including shorter derivatization time (within 10 min), higher product purity and much lower limit of detection (up to 38 pmol/L). In addition, good linearity ( $R^2 \geq 0.99$ ), excellent recovery (80.6–118%) and high precision (RSDs with 2.8–7.8%) were obtained when the developed method was used for quantitative analysis of sugar phosphates. Finally, the  $\text{SiO}_2@\text{PD-Ti}^{4+}$  microspheres combined with RPLC–MS were successfully applied to the determination of sugar phosphates from hepatocarcinoma cell lines and could even detect the trace sugar phosphates in thousands of cells.

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## 1. Introduction

As a kind of important metabolic intermediates, sugar phosphates such as glucose-6-phosphate (G6P), ribose-5-phosphate (R5P), erythrose-4-phosphate (E4P) and glyceraldehyde-3-phosphate (G3P) play vital roles in glycolysis, gluconeogenesis and pentose phosphate pathway (PPP), which can conduct as signaling molecules in gene transcription and regulate tumor energetic metabolism [1,2].

In the past, many analytical methods based on gas chromatography (GC) [3], liquid chromatography (LC) [4–9], capillary

electrophoresis (CE) [10,11] coupled with mass spectrometry (MS) detection were employed for the determination of sugar phosphates. Among them, LC–MS has become the most widely used technique for its high sensitivity and resolution [12]. However, the determination of sugar phosphates is still a challengeable task because of their low endogenous concentrations, poor chromatographic retention on reversed-phase liquid chromatography (RPLC) as well as the ionization suppression from complex matrix interference of biological samples. Ion pairing reagents, like tributylamine were often employed as the counterion to improve chromatographic retention on RPLC [7,9] or hydrophilic interaction liquid chromatography (HILIC) was employed for determination of the sugar phosphates [13,14]. However, the extra ionization suppression resulting from the ion pairing reagents in RPLC or the volatile salts in HILIC could seriously reduce the detection sensitivity of sugar phosphates [12].

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In recent years, chemical derivatization has drawn great attention in the analysis of carbohydrates [15–18]. Meanwhile, the derivatization approach based on reductive amination has been employed for the LC–MS analysis of sugar phosphates in biological samples by using aniline or 3-amino-9-ethylcarbazole (AEC) as derivative reagents [12,19]. However, the derivatization was often carried out in the liquid phase, which had inherent disadvantages including time-consuming, residual derivative reagent and low efficiency of derivatization. Recently, a highly efficient solid-phase derivatization approach based on boronic acid functionalized mesoporous silica nanoparticles was designed for the analysis of saccharides, which could remarkably overcome these drawbacks [20]. Nevertheless, no reports about solid-phase derivatization of sugar phosphates have been published so far.

From their chemical structure, sugar phosphates contain two different functional groups including phosphate group and aldehyde group. Immobilized metal ion affinity chromatography (IMAC) has been widely used to capture the molecules containing phosphate group such as phosphopeptides via the affinity of the phosphate groups to metal ions [21–26]. Recently, it has been reported that a thin polydopamine (PD) layer can adhere to the surface of IMAC substrates by the polymerization of dopamine, which not only increases the hydrophilicity of IMAC materials but also provides numerous adjacent hydroxide groups as the binding sites for metal ions [27–29]. Moreover, the PD coated IMAC materials have some unique properties with good environmental stability, biocompatibility and excellent dispersibility in water [30], which are favorable for the enrichment of compounds with strong polarity such as sugar phosphates.

In this work, an IMAC material called  $\text{SiO}_2@\text{PD-Ti}^{4+}$  was synthesized via a facile two-step approach and then a solid-phase approach based on the  $\text{SiO}_2@\text{PD-Ti}^{4+}$  microspheres for the highly efficient enrichment and derivatization of sugar phosphates was proposed for the first time. Initially, sugar phosphates in a biological sample were selectively captured onto the surface of the microspheres ( $\text{SiO}_2@\text{PD-Ti}^{4+}$ ) while the other interferents were excluded out of the microspheres. Then, a solid-phase derivatization with high efficiency was carried out by introducing the derivative reagent of AEC via reductive amination. Next, a liquid chromatography tandem mass spectrometry approach was used to analyze the derivatives of the sugar phosphates. Finally, the linearity, limit of detection (LOD), limit of quantitation (LOQ), recovery, precision and application of the developed method were further investigated.

## 2. Experimental

### 2.1. Materials

Silica microspheres (3  $\mu\text{m}$ , 100  $\text{\AA}$ ) were purchased from Fuji Silysia Chemical (Kasugai, Japan). Commercial  $\text{TiO}_2$  beads were purchased from GL Sciences (Tokyo, Japan). Dopamine hydrochloride was acquired from Aladdin Chemistry Co., Ltd. Titanium sulfate ( $\text{Ti}(\text{SO}_4)_2$ ) was purchased from Sinopharm Chemical Reagent Co., Ltd. 3-Amino-9-ethylcarbazole (AEC) and glutamic acid were obtained from J&K Scientific Co., Ltd. D-(+)-glucose-6-phosphate (G6P), D-fructose-6-phosphate disodium salt (F6P), D-ribose-5-phosphate (R5P), D-Ribulose-5-phosphate sodium salt (Rb5P), D-erythrose-4-phosphate (E4P), D/L-glyceraldehyde-3-phosphate (G3P), D-fructose 1,6-bisphosphate trisodium salt (F1,6-P), dihydroxyacetone phosphate dilithium salt (DHAP), D-ribulose 1,5-bisphosphate sodium salt hydrate (RbDP), D-glucosamine-6-phosphate ( $\text{NH}_2\text{-G6P}$ ) and 5-phospho-D-ribose-1-diphosphate sodium salt (R5P-DP) were acquired from Sigma-Aldrich (St. Louis, MO). D-mannose-6-phosphate disodium salt hydrate (M6P) and N-

Acetyl-D-glucosamine-6-phosphate disodium salt (N-Acetyl-G6P) were purchased from J&K (Shanghai, China). Sodium cyanoborohydride ( $\text{NaBH}_3\text{CN}$ ) was acquired from Sahn chemical technology Co., Ltd (Shanghai, China). Methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Tris-HCl, acetic acid and formic acid (FA) were of analytical reagent grade. Ultra pure water was produced by a Milli-Q system (Millipore, Milford, MA, USA).

Stock solutions of G6P (3.8 mM), F6P (3.3 mM), R5P (4.3 mM), Rb5P (3.6 mM), E4P (5.0 mM), G3P (5.9 mM), F1,6-P (2.5 mM), DHAP (5.5 mM), RbDP (3.2 mM), M6P (3.3 mM), N-Acetyl-G6P (2.9 mM),  $\text{NH}_2\text{-G6P}$  (3.9 mM) and R5P-DP (2.1 mM) were respectively prepared by dissolving each standard substance in methanol/water (3/1, v/v) solution. Working solutions were prepared via diluting stock solutions with ACN/ $\text{H}_2\text{O}$  (65/35, v/v) solution containing 2% FA and saturated glutamic acid (loading buffer). All of the above solutions were kept at  $-20^\circ\text{C}$  before use.

### 2.2. Synthesis of $\text{SiO}_2@\text{PD-Ti}^{4+}$ microspheres

The synthetic procedure of  $\text{SiO}_2@\text{PD-Ti}^{4+}$  microspheres was illustrated in Fig. 1a. Firstly, polydopamine (PD) coated  $\text{SiO}_2$  microspheres were prepared via the oxidative self-polymerization of dopamine. Briefly, 200 mg of  $\text{SiO}_2$  microspheres were dispersed in 40 mL of dopamine hydrochloride solution at a concentration of 10 mM, and followed by the addition of 40 mL tris-HCl buffer solution (10 mM, pH 8.5). The above mixture was stirred for 10 h at  $40^\circ\text{C}$ . The generated  $\text{SiO}_2@\text{PD}$  microspheres were washed with water and collected by centrifugation. The second step was the immobilization with titanium ions on the surface of the  $\text{SiO}_2@\text{PD}$  microspheres. The prepared  $\text{SiO}_2@\text{PD}$  microspheres were incubated in 80 mL of 100 mM  $\text{Ti}(\text{SO}_4)_2$  solution for 3 h at  $40^\circ\text{C}$ . The synthesized  $\text{SiO}_2@\text{PD-Ti}^{4+}$  microspheres were rinsed with water for several times, and then stored in water for further use.

### 2.3. Measurements and characterizations of $\text{SiO}_2@\text{PD-Ti}^{4+}$ microspheres

The morphology of the microspheres was observed by transmission electron microscopic (TEM) (JEM-2100, Jeol, Japan) and scanning electron microscopy (SEM) (JSM-7800F, Jeol, Japan). The energy-dispersive X-ray analysis (EDX) was measured by scanning electron microscopy (SEM) (JSM-7800F, Jeol, Japan). The group changes on the surface of the microspheres were measured by Fourier-transform infrared spectroscopy (FT-IR) (TENSOR27, Bruker, Germany).

### 2.4. Optimization of enrichment and solid-phase derivatization conditions

The equilibration times of extraction and desorption processes were investigated by using G6P as a test compound. 200  $\mu\text{L}$  of G6P solution (0.04 mM) was incubated with 5 mg of  $\text{SiO}_2@\text{PD-Ti}^{4+}$  microspheres, the mixture was vortex shaken for various extraction times from 0.5 to 60 min. Next, the supernatant was removed by centrifugation and the microspheres were rinsed with loading buffer for three times. Then the captured G6P was derivatized with AEC at different molar ratios of G6P to AEC to optimize the amount of derivative reagent. The above mixtures were reacted at different temperatures from 25 to  $70^\circ\text{C}$  in a thermomixer (Eppendorf, Germany) at a shaking frequency of 1000 rpm for 2, 5, 10, 20, 30 and 40 min, respectively, to optimize the reaction temperature and time for solid-phase derivatization. After the microspheres were washed with methanol/ $\text{H}_2\text{O}$  (3/1, v/v) (wash solution) for three times, 200  $\mu\text{L}$  of 5% ammonium hydroxide was used to elute the derivatives of G6P from the  $\text{SiO}_2@\text{PD-Ti}^{4+}$  microspheres. The equi-

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