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**Development of a new in-line coupling of a miniaturized boronate affinity monolithic column with
reversed-phase silica monolithic capillary column for analysis of cis-diol-containing nucleoside
compounds**

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Abstract

In-line coupling of capillary columns is an effective means for achieving miniaturized and automated separation methods. The use of multimodal column designed to allow the direct integration of a sample preparation step to the separation column is one example. Herein we propose a novel in-line coupling at the capillary scale between a boronate affinity capillary column (μ BAMC unit) and a reversed-phase separation column. This has been made possible due to the elaboration of a new and efficient μ BAMC unit. A thiol-activated silica monolithic capillary column was functionalized through thiol-ene photoclick reaction. This simple and fast reaction allows to prepare stable μ BAMC units having grafting densities of 1.93 ± 0.17 nmol cm⁻¹. This grafting strategy increases the surface density by a factor 4 compared to our previous strategies and opens the frame to in-line coupling with reversed-phase capillary column. Proof of concept of the in-line coupling was done by coupling a 1-cm length μ BAMC unit to a 7-cm length reversed phase capillary column. The conditions of loading, elution and separation were optimized for cis-diol nucleosides analysis (uridine, cytidine, adenosine, guanosine). A loading volume (at pH 8.5) of up to 21 hold volume (i.e 1 μ l) of the μ BAMC unit can be loaded without sample breakthrough. For the least retained nucleoside (uridine) a limit of detection of 50 ng mL⁻¹ was estimated. Elution and full separation of the four nucleosides was triggered by flushing the multimodal column with an acetic acid (50mM) / methanol (98/2, v/v) mobile phase.

Keywords: multimodal chromatography, photoclick chemistry, in-line coupling, boronate affinity, capillary chromatography.

1. Introduction

Miniaturized analytical methods are attractive in the context of waste and cost reduction, automation, in-situ / handheld analysis and personalized medicines. This non-exhaustive list shows the great diversity of applications that are in demand. In such frame, separation sciences are also part of this process. To go beyond the straightforward column reduction, in-line coupling of the sample preparation step to the separation column is proposed. In-line coupling of a sample preparation step allows not only to purify but also to preconcentrate analytes. This is key feature regarding sensibility problem associated to miniaturization. In addition, in-line couplings are “automated by-design”.

However, relevance of such in-line coupling depends on (i) the binding capacity of the purification/preconcentration zone (ii) the ability to elute the captured fraction in a small plug of solution whose composition has also to be adapted to the following separation step (mechanism). Finding appropriate conditions to meet these requirements is challenging and may account for the relatively limited number of reported in-line coupling combinations. Classically, miniaturized in-line couplings implement purification steps based on strong interactions mechanisms (i.e. ion exchange, immunoaffinity) coupled to reversed-phase analyses. Indeed, the purification and elution steps are done under aqueous media and the elution plug directly transferred to the reversed-phase column.

Herein we investigated a novel in-line coupling of a boronate affinity purification/preconcentration step to a reversed-phase separation. Boronate affinity chromatography (BAC) has unique affinity towards cis-diol containing compounds that cover a wide range of molecules of interest in pharmaceutical and biomedical research (catecholamines, glycoproteins, RNA, etc) [1,2] and supports the use of salt and organic solvent [3]. This reversible reaction allows the capture of cis-diol compounds under basic conditions and their rapid release under acidic media. Miniaturized boronate affinity monolithic unit (μ BAMC) were in-line coupled to electrokinetic separations[4,5]. While only on-line coupling of boronate affinity column to reversed-phase liquid chromatography has already been reported [6–10] highlighting the pertinence of such coupling, no chromatographic in-line couplings were developed. Analysis of complex mixtures at trace levels has pushed forward the development of integrated and miniaturized separation systems, coupling the preconcentration and separation steps. Besides the “off-line” classical approach, two different coupling approaches, so-called “on-line” or “in-line” couplings, have been proposed in the literature.

The on-line approach is referred as the physical coupling of the two distinct preconcentration and separation columns, through specific interfaces like 10-port valves. Although this approach has benefits in terms of automation, the instrumentation set-up is usually complex and band broadening consecutive to the physical coupling is critical in miniaturized systems [11]. The in-line mode represents the highest level of integration and automation and is well adapted for nano-separations. The μ -SPE unit, located at the inlet of the capillary column, is an integral part of it [12]. In such systems, adapted to microliter sample volumes, the elution of the μ -SPE unit is achieved with a very low volume of solution (few tens of nanoliters) that is sharply directed to the separation zone, without dilution or band broadening effects. Our objective is to demonstrate the feasibility of a miniaturized in-line coupling of monolithic boronate affinity column to reversed-phase monolithic capillary column. We describe the preparation of μ BAMC units using a thiol-ene photoclick reaction, which allows a 4-fold increase in the number of phenylboronate sites compared to photopolymerization reaction. This is of paramount importance to balance the relatively low affinity of cis-diol compounds for boronate affinity column. Conditions of in-line coupling of such preconcentration units were investigated. Finally, a proof of feasibility of the in-line coupling was done by coupling a 1-cm length μ BAMC unit with a 7-cm length reversed-phase monolithic column.

2. Materials and methods

2.1 Reagents and chemicals

All the reagents used were of analytical grade. Tetramethoxysilane (TMOS), methytrimethoxysilane (MTMS), urea, polyethylene glycol (PEG, MW =10,000), 3-(acrylamido)phenylboronic acid (AAPBA), triethylamine (TEA), methanol (MeOH, HPLC grade), 1,2-dihydroxybenzene (catechol), 1,4-Benzenediol (hydroquinone), adenosine, 2'-deoxyadenosine, uridine, cytidine, guanosine, thiourea, 3-(mercaptopropyl)trimethoxysilane (MPTMS), benzophenone, Na_2HPO_4 , NaH_2PO_4 were purchased from Sigma-Aldrich (Sigma-Aldrich, Saint-Quentin-Fallavier, France). n-Octadecyl dimethyl(dimethylamino)silane was from Gelest inc. (Germany). Acetonitrile (ACN) (HPLC grade) was purchased from VWR (VWR, Fontenay-sous-bois, France). Acetic acid (AcOH) was from Riedel-de Haënn (Riedel-de Haënn, Seelze, Germany). All aqueous solutions were prepared using $>18 \text{ M}\Omega\text{-cm}$ water (Millipore, Molsheim, France). PTFE-coated fused-silica capillaries (TSU deep-UV

Transparent Coating 75 μm i.d.) were purchased from Polymicro Technologies (Polymicro Technologies, Arizona, USA).

2.2 Instrumentation

Nano-LC experiments were carried out with an Agilent HP3D CE system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector and exclusively used in the pressurization mode with an external pressure device allowing to work up to 12 bars (no voltage applied). System control and data acquisition were carried out using the Chemstation software (Agilent). In-line coupling was realized by physically coupling μBAMC unit to RP-monolithic column using fluorinated ethylene-propylene tubing (FEP tubing 330 μm i.d. x 1.55 mm o.d. (Cluzeau info labo, Sainte-Foy-La-Grande, France) (see Figure S1).

2.3 Preparation of functionalized silica monoliths

2.3.1. Synthesis of silica monoliths

The silica monolith preparation followed our reported procedure [13]. Briefly, a 18 mL mixture of TMOS/MTMS (85/15 v/v) was added to 40 mL of 0.01 M acetic acid solution containing 1.9 g PEG and 4.05 g urea. The sol mixture was stirred at 0°C for 30 min. Then, the temperature was raised to 40°C and the mixture was loaded into the 75- μm fused-silica capillary and kept overnight at 40°C for gelification completion. Mesopores were formed by urea decomposition gently raising the capillary temperature at 0.5°C min⁻¹ up to 120°C maintained for 4 hours. After cooling, the monolith was washed with methanol and the capillary was cut to desired length.

2.3.2. C18-functionalization of silica monoliths

10-cm length monolithic capillary columns were filled with pure n-Octadecyl dimethyl(dimethylamino)silane and heated at 110°C for 2 hours (adapted from [14]). Capillary columns were then thoroughly rinsed with THF.

2.3.3. AABPA-functionalization of silica monoliths

- Thiol pre-functionalization

Prior to surface modification by photo-click chemistry, silica monoliths were prefunctionalized with 3-(mercaptopropyl)trimethoxysilane (MPTMS) to anchor thiol moieties onto the surface by covalent bonding. The silanization solution was composed of

MPTMS (5% v/v) in toluene. Silanization was performed at 37°C during 3 hours under hydrodynamic flow and then cured at 100°C overnight. After silanization, monoliths were thoroughly rinsed with THF and MeOH.

- “Thiol-ene” photo-click functionalization of monoliths

The thiol pre-functionalized silica support was filled with a solution containing a photoinitiator (12.5 mM benzophenone) and the 3-(acrylamido)phenylboronic acid monomer (100 mM AAPBA) in an aqueous TEA (pH 9)/acetonitrile mixture (50/50, v/v). Then, the ends of the capillary were plugged with rubber and the capillary irradiated for 10 min at 365 nm. The illumination system used for photo-click reaction was a BioLink cross linker (VWR International, Strasbourg, France) equipped with five 8 W UV tubes emitting at 365 nm. The capillary was then washed extensively with a water / acetonitrile (50/50 v/v) mixture and then with methanol. In some cases, several successive graftings (from 3 to 10 as indicated in the text) were implemented after refreshing the grafting solution inside the capillary in-between each irradiation step. A schematic representation of the AAPBA-functionalization is presented in Figure S2.

2.3 Determination of the number of actives sites and cis-diol affinity (K_d) by in-capillary frontal analysis [15]:

A 1-3-cm length silica monolithic segment at the inlet of a 10-cm length fused silica capillary was used to determine the total number of actives sites (B_{act}) on boronate affinity monolithic columns by frontal analysis. Catechol was used as cis-diol model molecule to probe the photografting reaction. Indeed, catechol is widely used in boronate affinity materials to characterize the number of actives sites due to its relatively high affinity with phenylboronate ($K_d \sim 300 \mu\text{M}$). Catechol interacts with phenylboronate groups through specific cis-diol interactions to form 5-membered ester rings under basic conditions. Hydroquinone was used to evaluate the specificity of the μBAMC . Indeed, hydroquinone is a positional isomer (non cis-diol) with comparable non-specific interaction than catechol with the solid support. Breakthrough curves (detection at 270 nm) with catechol as model solute at 100, 300 and 500 $\mu\text{g mL}^{-1}$ in phosphate buffer (15 mM Na_2HPO_4 , pH 8.5)/MeOH (80/20, v/v) were used to determine the amounts of catechol (q) captured at different catechol concentrations [L] according to equation 1:

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$$q = \Delta t \times F \times [L] \quad (\text{Eq. 1})$$

F being the flow rate and Δt being the time difference between dead time, t_0 , and saturation time, t_{plateau} . The total number of active sites on the affinity column, B_{act} , and the dissociation equilibrium constant (K_d) were determined according to equation (2) after plotting $1/q$ versus $1/[L]$ (linear plot):

$$\frac{1}{q} = \frac{K_d}{B_{\text{act}}} \times \frac{1}{[L]} + \frac{1}{B_{\text{act}}} \quad (\text{Eq. 2})$$

2.4 Determination of the loading capacity and LOD

After preconditioning the column with phosphate buffer at pH 8.5, an injection volume of 50 nL of uridine solution (corresponding to 1.2 volume of the μBAMC unit) was loaded on the multimodal nano-column. The interstitial liquid was then removed from the μBAMC unit by percolating 42 nL of phosphate buffer pH 8.5 (corresponding to 1 volume of μBAMC unit). This step allowed to remove unretained compounds from the μBAMC unit before the final elution step. The μBAMC was eluted by flushing the multimodal column with a 50 mM acetic acid solution. This experiment was repeated with increasing concentration of uridine (from 1 to 100 $\mu\text{g mL}^{-1}$). All steps were conducted at a constant pressure of 12 bars (i.e. a linear velocity of 0.16 mm s^{-1}).

The limit of detection (LOD) was determined by using a capillary column composed of a 1-cm μBAMC monolith coupled to a 7-cm length reversed-phase column. Solutions of uridine solubilized in the loading buffer (15 mM phosphate solution (pH 8.75)) were loaded through the column at a constant pressure of 12 bars (i.e. a linear velocity of 0.16 mm s^{-1}) for increasing duration depending on the volume to be loaded. The interstitial liquid was then removed from the μBAMC unit by percolating 42 nL of phosphate buffer pH 8.5 (corresponding to 1 volume of μBAMC unit). The elution and separation steps were performed using a 50 mM acetic acid mobile phase.

3. Results and discussion

3.1 Thiol-ene versus Ene-thiol photoclick grafting of AAPBA

We previously detailed the *in-situ* preparation of two phenylboronate affinity capillary columns (75 μm i.d.) : a porous polymer monolith used to work under harsh pH conditions [5] and a silica-based monolith [4], characterized (using catechol) by a number of phenylboronate sites of 0.16 nmol cm^{-1} (3.6 $\text{nmol } \mu\text{L}^{-1}$) and 0.43 nmol cm^{-1} (9.7 $\text{nmol } \mu\text{L}^{-1}$), respectively. In order to further increase the boronate affinity capacity of silica monoliths i.e to increase the preconcentration capacity (and the loading volume), we investigated an alternative grafting approach on silica based monolith. Herein, a photografting process based on the thiol-ene photoclick reaction is proposed to prepare monolayer-like functionalized silica monolithic columns [12,16]. In first instance, we tried the “ene-thiol” version (referred as vinyl functionalized silica and thiol-modified PBA in solution) as described by Chen et al [17]. But we failed to produce stable PBA material. The grafts were leached-out during experiment revealing adsorption rather than covalent grafting of the PBA derivatives on the support (data not shown). The alternative “thiol-ene” version was then implemented on thiol-terminated silica surface using vinyl-functionalized PBA derivative (AAPBA). After optimization of the overall process (silane derivatization and photoclick grafting reactions) this chemical pathway gives rise to silica monolith having 0.93 nmol cm^{-1} (i.e 21 $\mu\text{mol } \mu\text{L}^{-1}$) of active sites, which is 2 fold higher than our previous AAPBA functionalized monolith [4]. It was shown that high concentration of reagents (up to 1M) is useful to prepare dense layers of grafted molecules [16] but AAPBA monomer was not soluble up to 0.1 M. To increase the AAPBA grafting density, it was intended to proceed to successive illumination steps (from 3 to 10), after refreshing the solution inside the capillary in-between each illumination. The grafting density increased up to $1.93 \pm 0.17 \text{ nmol cm}^{-1}$ for 3 successive grafting steps and then remained constant.

This thiol-ene grafting pathway on silica monolith produces stable and dense phenylboronate layers with a loss of active phenylboronate site inferior at 5% after ten affinity / elution cycles.

3.2 Nucleosides affinity

Determination of affinity (K_d) for nucleosides were also determined using frontal affinity breakthrough curves. Hydroquinone elutes at the column hold-time (not shown), whereas the breakthrough curve plateaus are delayed for catechol (Figure S3). This result clearly indicates that no non-specific interactions occur and that the retention of catechol is solely due to boronate affinity. Table 1 gives the K_d values at pH 8.5 in a 15 mM phosphate buffer mobile phase :

Table 1

The retention factor (k) for cis-diol compounds can be estimated through the K_d value according to equation (2) [18]

$$k = \frac{1}{K_d} \frac{B_{act}}{V_0} \quad (\text{Eq 3})$$

For example, a retention factor of about 18 is estimated for uridine with the experimental K_d value of 2830 μM , a total number of active sites (B_{act}) of 2 nmol cm^{-1} and a hold-up volume (V_0) of 40 nL for a 1-cm length monolithic column. The migration velocity of uridine in the μBAMC (u') can be estimated by equation 3,

$$u' = \frac{u}{1+k} \quad (\text{Eq 4})$$

where u is the mobile phase velocity. In other words, the maximum loading volume before elution of a fraction of uridine should be about 18 times the column hold-up volume.

3.3 Multimodal chromatography: in-line coupling μBAMC -RP nano-LC

A capillary composed of a 1-cm length μBAMC unit coupled to a 7-cm length RP column was used to evaluate the effectiveness of such in-line coupling strategy.

3.3.1 Determination of the loading capacity

The loading capacity of the 1-cm length photoclicked AAPBA monolith (μBAMC) was evaluated by injecting small volumes of increasing uridine concentration (1 – 100 $\mu\text{g mL}^{-1}$). Uridine was chosen as model solute due to its lowest affinity on the AAPBA silica monolith.

Figure 1

The peak area (Figure 1A) increases linearly with the concentration of uridine in the sample solution up to approximately $50 \mu\text{g mL}^{-1}$ afterwards it can be noticed a deviation from the linearity for the last point at $100 \mu\text{g mL}^{-1}$. According to figure 1, up to $50 \mu\text{g mL}^{-1}$ the recovery is complete (the peak area increases proportionally to the amount injected). The amount injected for a $50 \mu\text{g mL}^{-1}$ concentration and a 50 nL injection volume is 2.5 ng. Since the length of the μBAMC is 1cm, the loading capacity is 2.5 ng cm^{-1} .

This capacity increases for nucleosides exhibiting higher boronate affinity. However, regarding in-line coupling the preconcentration factor is a critical parameter. The maximum loading volume was thus also investigated.

3.3.2 Determination of the maximum loading volume and LOD for uridine:

As previously, after preconditioning, various injection volumes (from 50 to 1500 nL) of uridine solution ($1 \mu\text{g mL}^{-1}$ in phosphate buffer pH 8.5) were loaded on the multimodal column. After washing the μBAMC unit with a phosphate buffer pH 8.5, the peak area of uridine was determined during the elution step. The plot of the recovered peak area versus loading volume is presented Figure 1B. Experimentally, the breakthrough of uridine during the loading step is observed after percolation of a sample volume of approximately 21 μBAMC hold-up volumes that is in good agreement with the previously estimated value (i.e 18 V_m). In order to demonstrate the usefulness of the presented in-line coupling to preconcentrate cis-diol compounds, low concentration of uridine solutions solubilized in phosphate buffer (pH 8.5) and in acetic acid (50 mM) were analyzed on the multimodal column (Figure 2 (A)). Injection of uridine solubilized in phosphate buffer at pH 8.5 gives a peak at 5.5 min (with a large S/N ratio) while no peak is detected when uridine is solubilized in acetic acid (50 mM). The μBAMC unit is “activated” when the sample is loaded at pH 8.5. Under acidic medium (i.e 50 mM acetic acid) the boronate affinity is turned off and no preconcentration effect was observed (Figure 2A).

The quantity of uridine loaded (2.1 ng cm^{-1}) was lower than the maximum loading capacity estimated previously. A loading volume of 1 μL (corresponding to 21 μBAMC hold-up volume) was thus fixed as the maximum volume of sample that can be loaded on the 1-cm length μBAMC unit of the multimodal column. Estimation of the limit of detection for uridine was done by measuring the peak height in function of the concentration of uridine using a

loading volume of 1 μ L. LODs (S/N= 3) were estimated at 57 ng mL⁻¹ for uridine in such experimental set-up. In addition, the linearity of the overall method is demonstrated by plotting the corrected peak areas versus the analyte concentration Figure 2 (B).

Figure 2

3.4 Separation of nucleosides using multimodal chromatography (μ BAMC-RP nano-LC)

A proof of feasibility of this novel in-line coupling strategy was done for the preconcentration and separation of 4 nucleosides (uridine, cytidine, adenosine and guanosine). 1 μ L of sample (at a nucleoside concentration of 0.5 μ g mL⁻¹) was loaded on a preconditioned multimodal column. The μ BAMC unit was washed with the same phosphate buffer (pH 8.5) before elution and separation of nucleosides using acetic acid mobile phase. The elution and separation of the first two peaks were done by continuously flushing the column with acetic acid (50 mM). The mobile phase was then changed to acetic acid (50 mM)/MeOH (98/2, v/v) (Figure 3). The 4 nucleosides were preconcentrated during the loading step. Elution is strong enough to elute the captured nucleosides in a short injection plug in order to avoid detrimental band broadening effect. This is of paramount importance to successful in-line coupling. Indeed, performances of all in-line coupling strategies rely on the specificity to preconcentrate efficiently and to transfer the captured target molecules to the separation area in the lowest “injection” volume. The base-line separation of the four nucleosides on the reversed-phase monolithic column demonstrates the power of the presented in-line coupling.

4- Conclusion :

The elaboration of miniaturized boronate affinity monolithic column through thiol-ene photoclick chemistry was shown to greatly improve the surface density of active sites on silica monolith. The thiol-ene version of such photo-initiated reactions allows to produce stable and dense layer-like phenylboronate surface of 1.93 ± 0.17 nmol cm⁻¹ for 3 successive grafting steps. With regards to the previously photopolymerization pathway, this method allows to increase the surface density by a factor 4. High binding capacity is a prerequisite to in-line coupling of such μ BAMC for preconcentration and purification purpose. We evaluated

the retention behavior of such AAPBA photoclicked columns with nucleosides and determined the optimal capture conditions. The coupling with a reversed-phase capillary column was then investigated. Determination of the capacity and loading volume on a composite capillary composed of 1-cm length μ BAMC unit with 7-cm length reversed-phase monolithic column demonstrated the potentiality of such coupling at the capillary scale. The evaluation of this novel in-line coupling was done on a capillary electrophoresis system under pressurization mode. NanoLC experiments with such experimental set-up allows to control the injection volume and to use a large diversity of mobile phases owing to absence of pump and injection valve. Using the least retained nucleoside (uridine) it was shown that a loading volume as large as 20 μ BAMC hold-up volume can be loaded without breakthrough of sample from the preconcentration unit. This preconcentration factor allows to greatly decrease the nucleosides concentration that can be detected. The in-line coupling was successfully applied to the preconcentration and separation of 4 nucleosides.

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388 **Figure captions**

389 Figure 1. Plots of the peak area in function of the concentration of uridine at a constant
390 loading volume of 50 nl (1.2 μ BAMC volume) (A) and for increasing loading volume at a
391 constant uridine concentration of 1 μ g mL⁻¹ (B). The multimodal capillary column is
392 composed of a 1-cm length μ BAMC unit connected to a 7-cm length reversed-phase
393 monolithic column. The percolation volume was increased from 50 to 1420 nL and uridines
394 standard solutions at 1, 5, 20, 50 and 100 μ g mL⁻¹ in 15mM phosphate (pH 8.5) were
395 percolated at 0.01 cm min⁻¹.

396 Figure 2. Capture and elution of uridines solubilized in phosphate buffer pH 8.5 (black curve)
397 and in acetic acid (grey curve) on a μ BMAC-RP multimodal capillary column (A) and plot of
398 the evolution of the peak area versus concentration of uridine in binding buffer (B). Length of
399 the μ BAMC segment: 1 cm; length of the reversed-phase segment: 7 cm; mobile phase 50
400 mM acetic acid. The multimodal capillary was preconditioned with binding buffer (15 mM
401 phosphate buffer at pH 8.5) prior to injection of 1 μ l (corresponding to 21 volumes of the
402 μ BAMC unit) of uridine. Before elution and separation, the μ BAMC unit was washed with 50
403 nl of binding buffer.

404 Figure 3. Preconcentration and separation of the 4 nucleosides on multimodal capillary. The
405 multimodal capillary (1-cm length μ BAMC – 7 cm-length RP-capillary column) was
406 preconditioned with binding buffer (15 mM phosphate buffer at pH 8.5) prior to injection of 1
407 μ l (corresponding to 21 volumes of the μ BAMC unit) of the 4 nucleosides at a concentration
408 of 0.5 μ g mL⁻¹ each. Before elution and separation, the μ BAMC unit was washed with 50 nl
409 of binding buffer. Elution and separation of the 4 nucleosides was done using an acetic
410 acid/MeOH (92/8, v/v) mobile phase.