Journal of Chromatography A, 1232 (2012) 93–100

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Preparation and full characterization of a micro-immunoaffinity monolithic column and its in-line coupling with capillary zone electrophoresis with Ochratoxin A as model solute

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ARTICLE INFO

Article history:
Available online 28 October 2011

Keywords:
Immunoaffinity
Monolith
Antibody
Ochratoxin A
Solid phase extraction
CZE
Laser induced fluorescence

ABSTRACT

A micro-immunoaffinity monolithic column (μIAC) was developed and in-line coupled with capillary zone electrophoresis in a fully automated way with Ochratoxin A as test solute. The in-line micro-immunoaffinity columns based on monolithic methacrylate polymers (EDMA-GMA) were prepared in situ at the inlet end of a PTFE coated fused silica capillary by UV initiated polymerization and subsequently grafted with antibodies. These μIACs were thoroughly characterized. The synthesis of the polymeric support was first demonstrated to be reproducible in terms of permeability, surface properties and efficiency. The antibodies immobilization was then studied by a new original hydrodynamic method (ADECA) allowing the in situ quantitative determination (at a miniaturized scale) of the total amount of immobilized antibodies. The combination of this measurement with the binding capacity of the μIAC allowed, for the first time, the in situ determination of immobilized antibody activity. A total of 260 ± 15 ng (1.6 ± 0.1 pmol) of IgG antibodies/cm in 75 μm i.d. monolithic column (i.e. 18 μg mg^{-1}) was obtained with (anti-Ochratoxin A/Ochratoxin A) as antibody/antigen model. 40% of the immobilized antibodies remain active corresponding to a binding capacity of 1.2 ± 0.2 pmol antigen/cm (i.e. 600 pg/cm of our test solute OTA), a very high capacity when dealing with trace analysis and with regard to the detection limits (30 pg and 0.5 pg with UV and LIF detection, respectively). The recovery yields were quantitative with negligible non-specific adsorption and allow analysis of diluted samples (1 ng mL^{-1}) for a percolated volume of 10 μL. It was also demonstrated that despite the progressive denaturation of antibodies consecutive to the elution step, the binding capacity of the μIAC remained high enough to implement at least 15 consecutive analyses with the same column and in a fully automated way.

1. Introduction

The relentless rise in commercial trade in the world hustles border control services and control of some potential sources of toxic, hazardous and pathogen agents' dissemination becomes a public health issue. The frame of controls and analyses covers wide domains of applications ranging for example from fight against terrorism to drug compliance, each domain being characterized by its peculiar specificities. However, whereas the analysis of complex mixtures at trace levels becomes widespread, it relies yet on the use of cumbersome methods and sophisticated analytical tools. Therefore, demand of rapid, high throughput and robust/simple analytical tools is spreading and is revealed challenging from an analytical point of view. For complex mixtures at trace levels, sample treatment turns into key parameter and is usually used in combination with a separation step in order to increase specificity and sensibility of analysis. In addition, reducing the sample volume is one of today's preoccupations especially when it comes to work with biological samples. The implementation of miniaturized analytical tools (e.g. μTAS [1–3], in-capillary techniques [4–7]) integrating the different analysis steps can meet to the aforementioned specifications. For this miniaturization purpose, electrokinetic techniques are interesting since separations are driven by application of an electric field between the extremities of the separation column (capillary, microchannel) thus avoiding sophisticated hydrodynamic pumping systems. Among electrokinetic techniques, capillary zone electrophoresis (CZE) offers high selectivity and efficiencies with fast separations of charged solutes and low sample and solvent consumption.

The hyphenation of a CZE separation step with a sample pretreatment step was reported in recent reviews [8–12]. Direct adaptation of existing methodology concerns the miniaturization

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doi:10.1016/j.chroma.2011.10.061

of the sample treatment step using micro-SPE tools and the handling of minute volume of extracted sample in CZE. This straightforward *off-line* approach allows purifying and preconcentrating large volumes of crude sample but analyses are time consuming and are potentially sources of sample loss or contamination [13,14]. Coupling of the purification/preconcentration steps is crucial and efforts are now focused to develop automated tools for high throughput analyses adapted to non-skilled people. Physical coupling, also called on-line coupling, mainly relies on elaborated instrumental approaches allowing simplification of analyses compared to off-line approach [5]. However, only a part of the purified sample is injected in the separation system [1,13,15]. Gain in sensibility implies the analysis of the total amount of the purified sample and can be only attained by *in-line* integration of the two separation dimensions. The purification/preconcentration step should be inserted inside the separation column thus allowing the elution of the total fraction of analyte in a short plug of liquid (relatively high concentration of purified analytes) which is directly sent in the separation column. Purification and preconcentration of analytes are classically performed via solid phase extraction that allows treating relatively large volumes of crude sample. If SPE in-line coupling is straightforwardly realized in functionalized open-tubular capillaries [16–19] it suffers from a lack of capacity. In order to address these issues of detection limits with respect to the low volume of treated sample, extractions can be performed on porous supports inserted inside the capillary. Different strategies for entrapment of functionalized particles have been implemented inside miniaturized systems (microsystems, capillaries) such as the use of magnetic field to immobilize magnetic particles [20,21] or the use of retaining frits [4,22–26] or even the use of membranes [27,28]. A more convenient way to insert a solid phase inside microchannels relies on the in situ synthesis of functionalized porous materials. The photopolymerization of organic monoliths fulfills these requirements [29–32]. The diversity of monomers (acrylate and methacrylate monomers) involved in the UV-initiated polymerization allows to cover all interaction mechanisms, ranging from ion exchange to popular reversed phase mode [33].

We report here on the development of a simple, automated and robust in-capillary analytical system composed of a micro immunopreconcentration column (μIAC) coupled *in-line* to a capillary zone electrophoresis (CZE) separation and a diode array or LIF detection system. We investigated and thoroughly characterized the synthesis and biofunctionalization of short length poly(glycidyl-methacrylate-co-ethylene dimethacrylate) monoliths in situ synthesized at one extremity of a 75 μm inner-diameter capillary. The repeatability and reproducibility of the monolith synthesis was followed by reversed phase chromatography experiments whereas the antibodies immobilization was assessed through an innovative in situ colorimetric technique (ADECA method [34]), based on the reversible and specific interactions of the Coomassie Brilliant Blue (CBB) dye with amino groups of antibodies. This simple, rapid, and robust method allowed demonstrating the repeatability and reproducibility of the biofunctionalization step for each synthesis batch. Moreover, the possibility to implement several successive cycles of antibodies quantification via ADECA on the same monolith was used to validate the stability of the grafting. The developed μIACs were then successfully in-line coupled with capillary zone electrophoresis in a fully automated way (from sample loading to the quantification of the antigen) with anti-OTA/OTA as antibody/antigenmodel. The ratio between the total binding capacity (representative of the active antibody amount) and the total immobilized antibody amount was used to determine, the yield of active immobilized antibodies. Recovery yields were then determined and non-specific adsorption evaluated.

2. Materials and methods

2.1. Instrumentation

Capillary electrophoresis experiments were carried out with an Agilent HP^{3D} CE system (Waldbronn, Germany) equipped with a diode array detector (DAD) and an external pressure device allowing to work up to 12 bar. The control of the electrochromatographic system and the data acquisition were carried out by the Chemstation system (Agilent Technologies, Waldbronn, Germany). Analyses of dilute samples (1 ng mL^{-1}) were realized in LIF mode detection using a Picometrics LIF detector (LIF UV-01, Picometrics, France) equipped with a helium–cadmium ion laser with an excitation wavelength of 325 nm. The acquisition of the LIF's data was carried out by Azur from Datalys (France). Bio-link UV cross-linker (VWR International, Strasbourg, France) was used for photopolymerization reactions; it was equipped with five 8 W UV tubes emitting at 365 nm. A UVX-radiometer (Fisher Scientific, Lyon, France) equipped with a 365-nm sensor (1 cm^2) was used to record the total UV-light energy supplied during each polymerization. An LC pump (Shimadzu LC 10AD, Tokyo, Japan) was used to rinse the capillaries after their fabrication. Morphological characterization of the monoliths was done using a JEOL nanoscope JCM 5000 (Japan) scanning electron microscope after a gold coating of the samples.

2.2. Reagents

Ethylene dimethacrylate (EDMA, 97% pure), glycidyl methacrylate (GMA, 98% pure), 1-propanol, 1,4-butanediol, (3-methacryloxypropyl)-trimethoxysilane, acetonitrile (HPLC grade), methanol (HPLC grade) sodium nitrate, pentylbenzene, octylbenzene and ochratoxin A (OTA; MW = 403 g mol^{-1}) were purchased from Sigma–Aldrich (France). 2,2-Azobisisobutyronitrile (AIBN) was from Acros (France). Ammonia (25%) was purchased from Carlo Erba (Italy). Coomassie Brilliant Blue G-250 (CBB) from Acros (France) and was purified using counter current chromatography [35]. Borate buffer (20 mM, pH 9.3) was purchased from Agilent (Germany). Phosphate buffered saline (PBS) 10 mM, pH 7.4 was prepared using the adequate proportion of KH_2PO_4 and Na_2HPO_4 in water with the addition of 0.15 M of NaCl (all reagents were from Sigma–Aldrich, France). Monoclonal antibodies anti-ochratoxin A (anti-OTA; MW = 160,000 g mol^{-1}) from mouse were purchased from Covalab (France). All aqueous solutions were prepared using >18 MΩ DI water.

2.3. Capillary monolith preparation

Poly(tetrafluoroethylene) (PTFE) coated fused silica capillaries (75 μm i.d.) were purchased from Polymicro Technologies (USA). Pre-treatment and filling steps of capillary columns were carried under nitrogen (N_2) pressure using a Nanobaume System (CIL-Cluzeau Info Labo SA). Capillary pre-treatment procedure was carried out in order to ensure covalent attachment of the monolith to the capillary walls. The inner wall of the fused-silica capillary was rinsed with 1 M NaOH (1 h, 4 bar, 25 °C) to eliminate any impurities present in the capillary, then rinsed with a mixture of ethanol/water (95/5) (1 h, 4 bar, 25 °C) and finally flushed with a 1% (v/v) solution of 3-(trimethoxysilyl) propyl methacrylate in 95/5 ethanol/water mixture (1 h, 4 bar, 25 °C). Thereafter, the capillary was rinsed with ethanol (30 min, 4 bar, 25 °C) and dried under nitrogen stream. The pre-treated capillary was then filled with the polymerization mixture under gas pressure (4 bar). In order to localize the monolith inside the capillary, a PEEK tubing (300 μm i.d.) was used as a mask for non irradiated areas. Finally, the capillary ends were sealed with silicone.

Polymerization mixture adapted from [36,37] was obtained by mixing 0.3 mL of EDMA, 0.9 mL of GMA, 1.05 mL of 1-propanol, 0.6 mL of 1,4-butanediol and 0.15 mL of ultra pure water. 12 mg of 2,2-azobisisobutyronitrile were added under magnetic stirring. Prior to irradiation, dissolved oxygen in solution was degassed by sonication for 15 min. The photopolymerization reaction was then initiated by a 365 nm UV light using the UV crosslinker for a total energy supply of 3 J/cm^2. After polymerization, the monolithic column was rinsed with methanol (1 h) using a LC pump to remove any remaining reagents.

2.4. Chromatographic characterization of the monolithic capillary columns

In order to evaluate the reproducibility and the efficiency of the prepared capillaries, the latter were applied in nano-LC in reversed phase mode using a 70:30 (v:v) acetonitrile:water mixture as mobile phase for the separation of three test molecules, sodium nitrate (unretained analyte), pentylbenzene and octylbenzene. In this case the monolith's length was of 8.5 cm. This evaluation was performed using the CE system on the shorter end. The system was operated with an in situ hydrodynamic injection, and in situ UV detection (214 nm) through the capillary. The injection was performed by applying a 50 mbar pressure at the outlet for 45 s. The efficiency (N) was calculated at half peak height with the Chemstation software, while the hydrodynamic permeability was calculated using Darcy's law:

$$K = \frac{u_0 \eta L}{\Delta P}$$

where K is the permeability (m^2), u_0 the linear velocity (m s^{-1}), η the mobile phase viscosity (Pa s), L the column length (m) and ΔP the pressure drop (Pa).

2.5. Monolithic capillary column biofunctionalization

The antibodies immobilization conditions, previously optimized on batch macromonoliths in static mode [38] were adapted to micro-format in a hydrodynamic mode.

After drying of the μIAC (monolith's length = 5 cm) under nitrogen stream, the antibody solution (750 μg mL^{-1}) in PBS was hydrodynamically flushed under a 3 bar pressure (u = 2 cm min^{-1}) for 5 h and then left to react overnight. In previously published results [38] it was demonstrated that the antibodies are first rapidly adsorbed and then covalently bound to the monolith's surface moieties. The capillary was later washed with PBS to remove the non-covalent grafted antibodies (3 bar, 1 h).

2.6. Hydrodynamic ADECA method for the in-capillary determination of the total immobilized antibodies

The ADECA method was adapted to micro-format and hydrodynamic mode from previously published results obtained in a static mode on macro monoliths synthesized in batch [38]. The experiments were conducted using the CE system in the short end injection mode and using the external pressuring device. The injection-to-detection length was 8.5 cm and the monolith's length was varied between 1 and 5 cm (n > 8).

Staining, washing and elution reagents were prepared as follow. Coomassie Brilliant Blue (CBB) staining solution was prepared at 50 mg/L in 10% (v/v) methanol and 5% (v/v) acetic acid in ultrapure water (18.2 MΩ). The washing solution was made of 10% methanol and 5% acetic acid in ultrapure water. Two elution buffers were tested: the first one consisted in 50% carbonate–bicarbonate buffer 0.25 M at pH 11.2 with 50% methanol [34,38] and the second one consisted in 50% of 1 M ammoniacal buffer at pH 11.2 with 50% of methanol.

Briefly, after the grafting step, the monolith was successively washed with ultrapure water to remove the PBS and then conditioned with the washing solution to protonate the amino groups (6 bar, 20 min). Staining step was performed by flushing the CBB solution under a 6 bar pressure until saturation of the monolith, the saturation being monitored at 595 nm with the DAD detector. The excess of CBB that did not interact with protonated amine groups was removed by flushing the monolith with the washing solution until the baseline at 595 nm reaches stability, followed by a washing with ultrapure water (6 bar, 20 min). The destaining or elution step was performed by applying a pressure of 6 bar to the elution buffer and the CBB was detected at 595 nm or at 320 nm in case of saturation of the detector at 595 nm.

2.7. Determination of the maximal loading capacity and recovery yields of the μ-IAC

Anti-OTA/OTA was used as antibody/antigen model for this study. After saturation of the μIAC with an antigen solution (OTA), the quantification of the recognized antigen (OTA) was used to evaluate its maximal loading capacity and subsequently to calculate the amount of active antigen binding sites. The experiments were conducted using the CE system, the injection-to-detection length was 26 cm and the monolith's length was 5 cm (n = 4 from three different batches).

The saturation of the μ-IAC was done by flushing it (10 min at 10 bar) with an excess of antigen (10 μg mL^{-1} OTA in PBS) followed by a washing step with PBS (20 min, 10 bar) to remove the excess of antigen, then with a 10 mM borate buffer pH 9.3 (the separation buffer; 20 min, 10 bar) (Fig. 1b). The recognized antigen was then hydrodynamically eluted with a 1 cm methanol plug at a linear velocity of 2 cm min^{-1} (Fig. 1c) which was later pushed with the borate buffer far from the monolith at the same speed (Fig. 1d). The antigen was eluted in methanol since methanol is able to disrupt the antigen–antibodies interactions. Then an electric field of 0.32 kV/cm was applied for 3 min while maintaining the pressure (Fig. 1e). Since the antigen is negatively charged at the pH of separation buffer that surrounds the small methanol elution plug, the application of an electric field drives the antigen out from the methanol plug allowing its stacking and its separation from methanol and from potential pollutants non-specifically adsorbed onto the monolithic support in the case of real samples. After the electrokinetic step, thiourea (a non-retained and UV-absorbing solute) was injected (50 mbar, 0.1 s) to control the linear velocity during the elution step and ascertain the robustness of the method (as described in the quantification section the knowledge of the linear velocity during the elution step is required for the quantification). Finally, the capillary column was flushed with borate buffer at the same speed to elute the methanol plug, then the antigen with detection at 380 nm a specific wavelength for OTA quantification (Fig. 1f) and finally the thiourea used as flow velocity marker.

For analyses of dilute samples (OTA at 1 ng mL^{-1}) and recovery experiments, a LIF detector was used. The injection-to-detection length increased to 32 cm. The OTA solutions were prepared from a certified standard solution (10 μg mL^{-1} in acetonitrile). First, 10 μL of dilute solution of OTA were hydrodynamically percolated through the μ-IAC (4 bar, 120 min or 8 bar, 60 min), followed by 2 washing steps with PBS (20 min, 10 bar) then with Borate buffer (20 min, 10 bar). The elution was realized with a 1 cm methanol plug at a linear velocity of 2 cm min^{-1}, and this plug was later pushed away from the monolith (5 cm). The electrokinetic separation step was achieved by applying an electric field of 0.32 kV/cm for 5 min while maintaining the pressure. After the electric field was shut down, a very small plug of methanol (a non retained solute leading to a detectable signal with the LIF detector) was injected (4 bar,

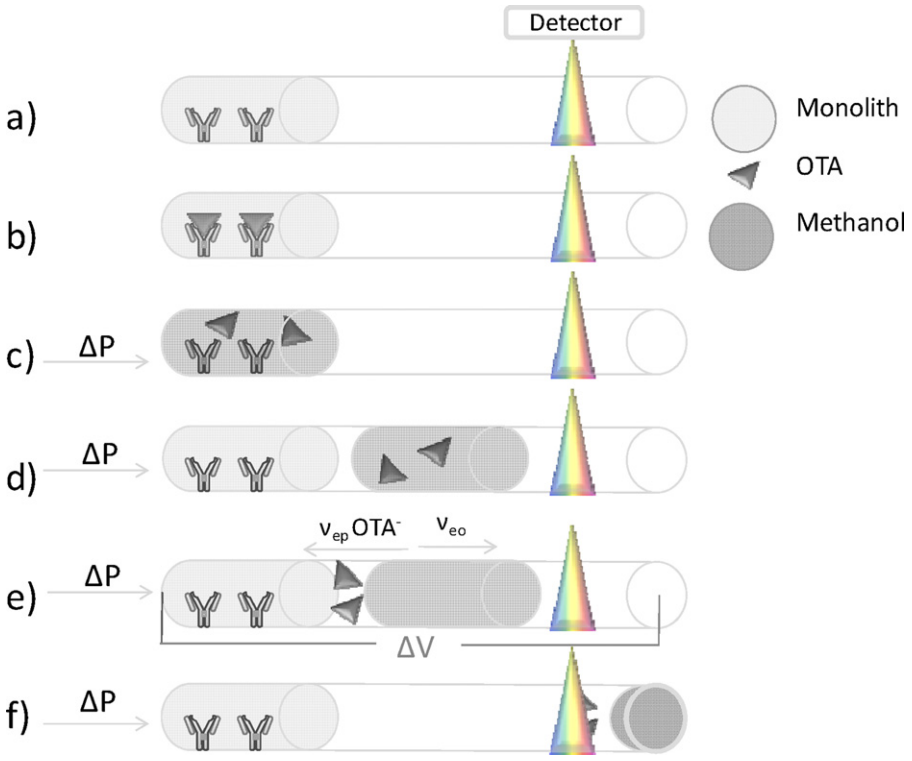

Fig. 1. Schematic representation of the developed in-line method, (a) immobilization of the antibodies; (b) binding of the OTA; (c) hydrodynamic elution with a methanol plug; (d) flushing of the methanol plug with the borate buffer; (e) electrokinetic separation step; (f) final hydrodynamic elution with the borate buffer.

6 s) to control the linear velocity during the final elution step. Then, the capillary was flushed with borate buffer to elute the methanol and the OTA. The electrokinetic step allows to separate OTA from the methanol plug and to detect it in borate buffer in which its fluorescence yield is better thus enhancing the sensitivity.

2.8. Antigen and CBB in situ quantification

After the elution step of CBB or of the antigen, the quantity of eluted CBB or antigen can be determined with the corresponding peak surface area and according to the following equation:

$$\text{Quantity} = \int CdV = \int \frac{\text{Signal}}{K}\,\text{section} \times \text{velocity} \times dt$$

$$= \frac{\text{section} \times \text{velocity}}{K} \int \text{Signal} \times dt = \frac{\ell \pi d_{\text{cap}}^2}{4Kt_0}\,\text{peak area}$$

where ℓ is the effective length in cm, d_{cap} the capillary inner diameter in cm, t_0 the dead time in s and K the response factor.

Frontal analysis of antigen (OTA) or CBB solutions prepared at different concentrations were conducted to determine their respective response factors, the height of the concentration step being directly proportional to the solution concentration (height of the concentration step = KC). Depending on the detection mode, K is expressed in mAU mL μg^{-1} in UV mode and in mV mL ng^{-1} in LIF mode while C the concentration is in $\mu g\,mL^{-1}$ in UV mode and in $ng\,mL^{-1}$ in LIF mode.

2.8.1. CBB quantification

For the quantification of CBB in UV mode, CBB solutions with concentrations ranging from 10 to 100 $\mu g\,mL^{-1}$ in the elution buffer were prepared for frontal analysis. The signal was recorded at two wavelengths 595 nm and 320 nm. The latter was used when the signal of the eluted CBB saturated the detector at 595 nm.

The calculated response factors in 75 μm i.d. capillaries were $K_{(595\,nm)} = 0.56$ mAU mL μg^{-1} and $K_{(320\,nm)} = 0.09$ mAU mL μg^{-1}.

2.8.2. OTA quantification

For the quantification of OTA in UV mode, solutions having concentrations ranging from 50 to 200 $\mu g\,mL^{-1}$ of OTA in borate buffer were prepared. For the LIF analysis, solutions of OTA were also prepared in Borate buffer but with lower concentrations ranging from 50 to 500 $ng\,mL^{-1}$. The frontal analysis of these solutions allowed the determination of the response factors in each detection mode. Calculated response factors in UV (380 nm) and LIF detection modes were respectively 0.18 mAU mL μg^{-1} and 0.17 mV mL ng^{-1} (in 75 μm i.d. capillary).

3. Results and discussion

3.1. Characterization of the acrylate monolithic support

Poly(GMA-co-EDMA) monoliths were used in this study since these porous materials allow a direct and straigthforward immobilization of biomolecules through the reaction of amino groups with the surface epoxy moieties. Their polymerization was directed by UV-irradiation allowing their in situ synthesis and easy integration inside miniaturized microchannels. In this work, monoliths were synthesized inside 75 μm i.d. capillaries. Since these monoliths were to be used in the hydrodynamic mode, their regular and satisfactory anchoring to the capillary walls was a prerequisite to avoid any preferential pathway. Moreover, since methacrylate monoliths have been reported to be irreproducible [39], their preparation procedure had to be validated before any immunoaffinity application.

Therefore, the in situ synthesized monoliths (inside a 75 μm i.d. capillary) were first characterized by SEM. The SEM image (Fig. 2a) illustrates the radial homogeneity of the monolith and the absence of voids between the monolith and the capillary wall. The interface between the prepared monolith and the void in the masked section

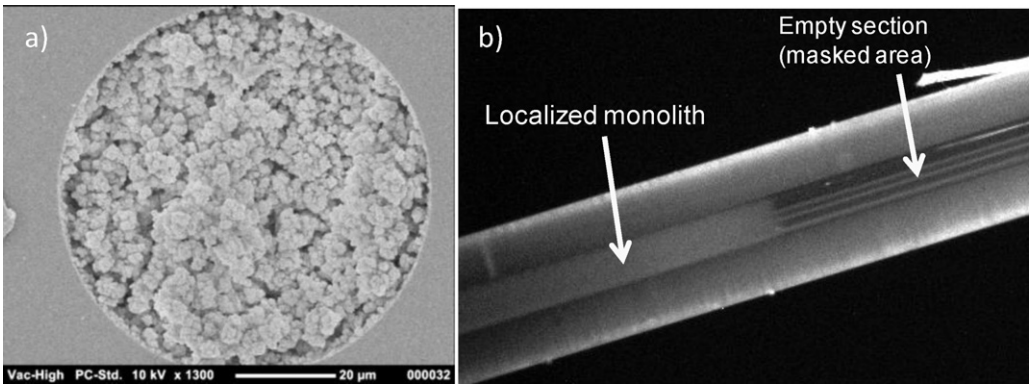

Fig. 2. (a) SEM image of 75 μm i.d. monolithic capillary; (b) interface between the monolith and the empty "masked" area observed with optical microscopy.

of the capillary was observed with optical microscopy. As can be seen in Fig. 2b, the interface is very clear showing the efficiency of the masking.

The monoliths were later characterized in reversed mode chromatography for the separation of three test molecules. The aim of this characterization was to determine the reproducibility of the preparation and the efficiency of the monolithic support. Three parameters were chosen for the monolithic capillary validation, (i) the permeability, a prerequisite to be able to percolate liquids through the monolith, (ii) the retention factor, as an indicator of the surface properties and (iii) the efficiency as an indicator of the homogeneity of the monolithic structure. Fig. 3 shows a chromatogam of the separation of the test molecules obtained on a 8.5 cm monolith. The results obtained on three monoliths from different batches indicate that the preparation of the monoliths and their structure can be considered reproducible and homogeneous since the relative standard deviations are <10% and high efficiencies (100,000 ± 5000 plates/m) are observed.

3.2. A new method for the in situ determination of the grafting step efficiency

Antibodies were further grafted in the hydrodynamic mode as mentionned in the materials and methods section. The amount of grafted antibodies has to be quantified to evaluate the grafting efficiency. Since the classical method usually used in batch (μ-BCA assay) may not be implemented at the miniaturized scale inside the capillary in an hydrodynamic mode, this quantification was realized using a colorimetric method (ADECA) based on the reversible

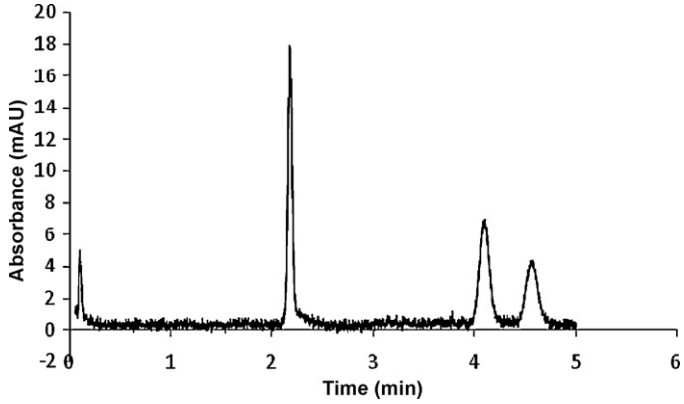

Fig. 3. Separation of nitrates, pentylbenzene and octylbenzene with a poly(GMA-co-EMDA) monolithic capillary; monolith's length = 8.5 cm; distance to the detector = 8.5 cm; u = 3.8 cm min^{-1}; mobile phase: 70:30; acetonitrile:water (v:v); UV detection at 214 nm.

interactions between the Coomassie Brilliant Blue dye (CBB) and the protonated amine groups of immobilized antibodies [34,38]. Briefly, this method consists in three consecutive steps; a staining step with a CBB solution, followed by a washing step to remove non-interacted CBB, before the final elution and quantification of the eluted CBB. In order to implement the ADECA method inside the capillary column in hydrodynamic mode, several adaptations of the initial static protocol were necessary.

3.2.1. Modification of the elution buffer

In the original method (in batch mode), the elution of CBB is realized with a carbonate buffer (i.e. at a basic pH to deprotonate the amino moieties of the antibodies and thus disrupt its electrostatic interactions with CBB), and the carbonate elution solution is acidified with 3 M HCl just before the absorbance measurement in order to increase the molar absorption coefficient of CBB. Since the post-acidification step is not possible in the in-line hydrodynamic mode, the carbonate buffer was replaced with an ammoniacal one (at the same pH), in which CBB exhibits a high molar absorption coefficient equivalent to that in the carbonate acidified solution at 595 nm (around 80,000 cm^{-1} M^{-1} in the ammoniacal buffer and in the acidified carbonate buffer while it is around 36,000 cm^{-1} M^{-1} in the non acidified carbonate buffer). The elution of CBB with this ammoniacal buffer was compared to the elution with the initial carbonate buffer in batch experiments. The amount of eluted CBB was identical with both elution buffers. This adaptation, thus simplifies the elution and allows the quantification of the CBB with a high sensitivity for in-capillary determination.

3.2.2. Optimization of the washing step

The washing step was optimized on ungrafted monolithic capillary columns in order to reduce the quantity of non-specifically adsorbed CBB in the elution step. All other things being equal, the surface area of the CBB elution peak for an ungrafted monolith represented less than 1% of the surface area measured for a μ-IAC. This result shows that the efficiency of the washing step is improved by the convective mass transfer when compared with the static protocol [38] and that non specific adsorption of CBB can be negleted in the hydrodynamic mode.

3.2.3. Staining step

When compared to the original ADECA static protocol, the concentration of the CBB staining solution was decreased from 500 mg/L down to 50 mg/L in order to shorten the washing step. It was verified that such a decrease does not impair the staining efficiency since the same results were obtained with both CBB concentrations.

As shown in Fig. 4, monitoring of the UV signal at 595 nm allows following each step of the ADECA in-line hydrodynamic method.

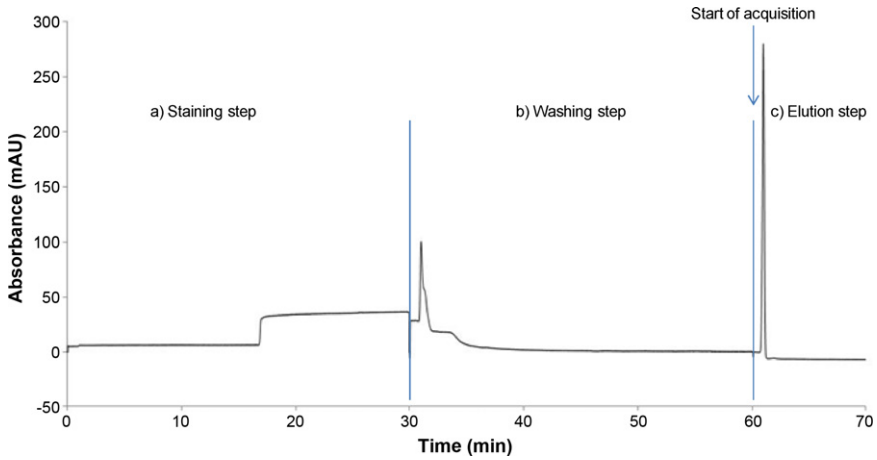

Fig. 4. Chromatogram illustrating the UV signal recorded during the different steps of the ADECA hydrodynamic method; detection at 595 nm. (a) Staining of the grafted monolith with a 50 mg/L CBB solution; (b) washing of the stained monolith with the acidic buffer; (c) elution of the fixed CBB with the ammoniacal elution buffer.

The surface area of the obtained elution peak (Fig. 4c) was used for the quantification of the CBB bound to antibodies, which can be correlated to the total antibody amount using a predetermined ratio of 122 moles of CBB per mol of antibody [38].

It was calculated that 260 ± 15 ng of antibodies (1.6 ± 0.1 pmol) are immobilized per cm of 75 μm i.d. μ-IAC ($n = 5$ from 3 different batches) which is equivalent to 18 μg of antibodies per mg of monolith (assuming a density of the monolith of 0.32 g cm^{-3}). This value is in the same order of magnitude than the values reported by Hage [40] and two fold higher than that we obtained with batch monoliths with the same antibodies [38]. As already mentioned in the literature [40], the higher grafting efficiency in the hydrodynamic mode may be due to convective mass transfer that allows delivery of antibodies to the monolith's surface in addition to diffusion that only occurs in the static mode. Furthermore, the in-line hydrodynamic ADECA method was repeated several times ($n \geq 3$) on each μ-IAC. The same quantity of CBB was obtained with a RSD < 5% reflecting the stability of the grafting: non-covalently immobilized antibodies (if any) should have been released during the first cycle owing to the harsh conditions used.

This simple and sensitive in-line hydrodynamic ADECA method is revealed to be useful to measure directly the amino surface density at the miniaturized scale and to quantify total amount of immobilized antibodies inside capillary columns provided that the number of CBB molecules that bind one antibody is known. As described previously [41], this method should be implemented for the in situ determination of any molecule bearing amine group.

3.3. Full characterization of the μ-IAC in-line coupled to capillary zone electrophoresis

Affinity chromatography columns are usually characterized through the determination of their loading capacity, binding capacity and recovery yields of a target antigen. Their loading capacity corresponds to the quantity of antigen that can be extracted after saturation of the column. The binding capacity of the μ-IAC, that represents the maximum quantity of specifically immunoextracted antigens, is then obtained by subtracting the amount of non-specifically extracted antigens to the loading capacity. The knowledge of this binding capacity allows the subsequent determination of the activity of immobilized antibodies; Monitoring of the binding capacity through multiple uses of the same μ-IAC may also allow to estimate the loss of biological activity during the elution step and to evaluate the possibility to implement successive analysis on the same μIAC. The characterization of the μ-IAC performances implies its coupling to a separation method and the selection of an antibody/antigen model. The μIAC was thus in-line coupled to a capillary zone electrophoresis step to perform the analysis of OTA used as antigen model.

3.3.1. Automated in-line coupling of the μ-IAC with CZE

The in-line coupling of the μ-IAC to CZE involves several successive steps. The affinity column is first loaded by percolating the antigen solution. Non-adsorbed solutes are then washed from the μIAC prior conditioning the whole capillary with the separation background electrolyte. Then, the antigens are eluted from the μ-IAC with a short plug of organic solvent. Indeed, the highly specific antigen/antibody interactions are disrupted in organic media like methanol. Finally, an electrokinetic separation of the antigen from the methanol plug and from potential pollutants non-specifically retained onto the μIAC allows its identification through the measurement of its migration time, and its quantification through the measurement of the peak area. All steps until the separation step are performed under hydrodynamic mode. In the case of OTA, the electrokinetic separation step in borate buffer also allows to increase the sensitivity of the detection (the fluorescence quantum yield of OTA is higher in borate buffer than in methanol). Fig. 5 illustrates the chromatogram recorded after the elution with a 1 cm length methanol plug of a μ-IAC saturated with OTA. When a successive second plug of methanol was injected, no further elution was observed showing that such a short plug (~44 nL) is sufficient

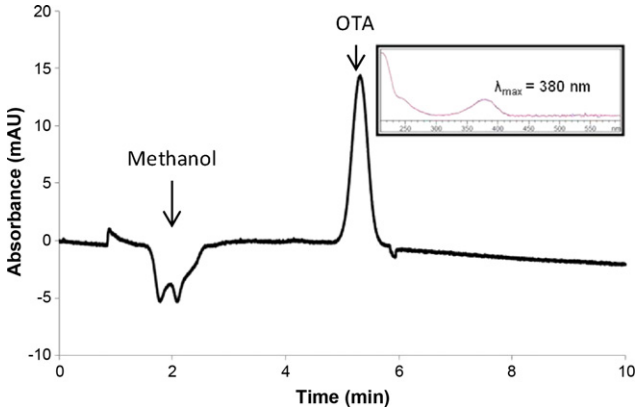

Fig. 5. Chromatogram obtained during the elution step of OTA; monolith's length 5 cm; injection-detection length = 23 cm; capillary length = 31.5 cm; $u = 2$ cm min^{-1}; UV detection 380 nm; elution buffer: borate 10 mM pH 9.3; the insert represents the UV spectrum of the eluted peak showing the maximum wavelength of the OTA.

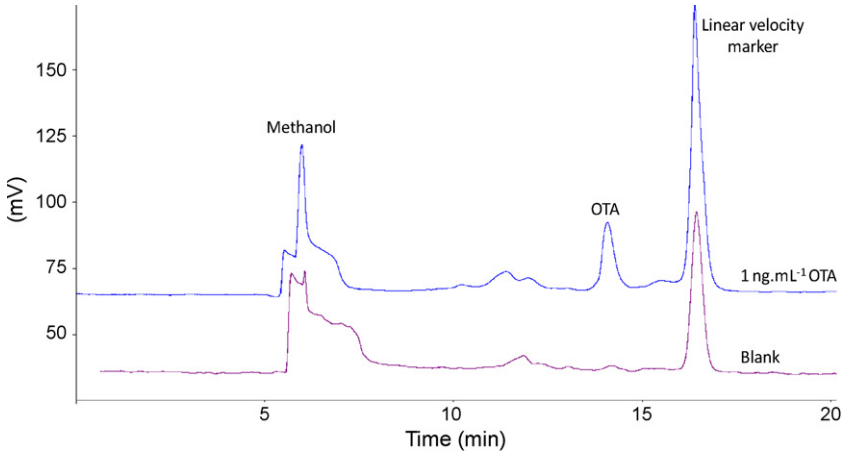

Fig. 6. Chromatograms recorded during the elution step (a) after preconcentration of OTA on the μ-IAC, (b) blank run obtained without OTA during the loading step; monolith's length = 5 cm; injection-to-detection length = 33.5 cm; capillary length = 47.5 cm; $u = 2\,\text{cm}\,\text{min}^{-1}$; elution buffer: borate 10 mM pH 9.3; LIF detection.

for the complete elution of the antigen, thus allowing its preconcentration in a very small volume. The detection may not be improved by smaller plugs of methanol since the antigen is electrokinetically removed from the methanol plug, and then stacked at the borate/methanol solutions boundary.

The whole process (from percolation to antigen quantification) is fully automated and allows the analysis of samples in a total analysis time of about 2 h with the ability to implement automated sequence analysis; this analysis time should be further reduced by decreasing the monolith's length in the capillary.

3.3.2. Determination of the antigen loading and binding capacities

Since the grafting of antibody onto glycidyl functionalized solid support is non oriented, antibodies are randomly immobilized. Some immobilization configurations could reduce their biological activity [42,43] that influences the maximum binding capacity of the μ-IAC (the amount of Ag specifically recognized by antibodies).

The determination of the total extracted antigen quantity was realized as described in Section 2 by saturating the μIAC with the antigen. The mean amount of antigen eluted from different μIACs ($n = 4$, from three different batches) was 1.2 ± 0.2 pmol antigen/cm representing the maximal antigen loading capacity.

In order to determine the contribution of non-specifically adsorbed antigen to the maximal loading capacity, the same experiments were carried out on a denaturated μ-IAC. An elution peak corresponding to approximately 1 fmol antigen/cm was detected with the more sensitive LIF detection mode which accounts for less than 1% of the maximal loading capacity of the μIAC. Hence, the 1.2 ± 0.2 pmol of antigen/cm previously determined represents the binding capacity.

Considering the test solute solute used, this binding capacity is high enough (i.e. 1000 fold higher than that required) with regard to the LOD in LIF (1.2 fmol) to allow analysis of samples in a wide range of concentrations, i.e. from 0.05 μg/L to 250 μg/L (considering an arbitrary percolated volume of 10 μL).

3.3.3. Activity of the grafted antibodies

The binding capacity corresponds to the number of accessible and active antigen binding sites (1.2 ± 0.2 pmol/cm of monolith) and hence to twice the number of active immobilized antibodies (0.6 pmol/cm), assuming 2 antigen binding sites per active antibody. The ratio between active and total antibodies ($39 \pm 8\%$) reflects the remaining activity after the antibody immobilization step. To our knowledge, this information has not been previously reported in the litterature for μ-IAC mainly due to difficulties encountered for in situ determination of the total immobilized antibodies inside microchannels. This is the first time that an in-line hydrodynamic method for the determination of the antibodies activity is reported in the litterature. The activity obtained here is comparable to the 57% of activity reported by Hage [40] for anti-FITC and significantly lower than the 70% reported by Faye et al. [38] for anti-HRP in batch mode. The different nature of the antibodies and/or of the immobilization processes may account for such discrepancies. Indeed, the activity determined with anti-OTA in batch monolith was about 30% lower than the activity obtained with anti-HRP (results not shown). Finally, the satisfactory remaining activity obtained in this work, shows that this non-oriented and straightforward immobilization process is well-adapted to the in situ grafting of miniaturized columns since it does not impair the activity in a great extent.

3.3.4. Reusability of the μ-IAC column and recovery yields

Commercially available immuno-SPE cartridges are sold by their suppliers as single-use cartridges owing to the denaturation of the antibodies by the high volume of methanol used during the elution step (i.e. the high duration of the elution step). In our fully automated process, the methanol volume used to disrupt the antigen–antibodies interactions is reduced as well as its contact time with the antibodies. Thus, one may consider that the antibodies are not fully denaturated at the end of a cycle and that the μIAC cartridge may be multiple-use. We evaluated the global remaining capacity of our μIAC columns after several successive cycles with an arbitrary (non optimized) regeneration duration of 2 h in PBS between each cycle. Despite the unavoidable partial denaturation of the antibodies, the μIAC column retained a non negligible activity. It was determined that after 5 successive cycles, the binding capacity of a column is decreased by about a 10 fold factor (i.e. down to 750 fmol for a 5 cm lengh μ-IAC) but remains, e.g. 600 times higher than the LOD for OTA. The complete automatisation of the process allowed to perform several successive immunopreconcentration/elution cycles on the same μIAC column to determine the recovery yield and reusability.

The recovery yields of the μ-IAC were determined by measuring the ratio between the amounts of loaded (known amount) and eluted antigen. For this set of experiments, 10 μL of a 1 ng mL^{-1} OTA solution (25 fmol of OTA) were percolated through the μ-IAC at two different percolation speeds (2 and 4 $\text{cm}\,\text{min}^{-1}$). Fig. 6 shows the electrophoregram recorded during the elution of the μ-IAC treated with 2.5 fmol of OTA.

Recovery yields of about 100% ($114 \pm 11\%$) were obtained for up to 15 successive analyses on the same μ-IAC (whatever the

percolation speed). These results show that the whole process is quantitative and that the μ-IACs are multiple use (with a non-optimized regeneration time of about 2 h in PBS between each analysis).

4. Conclusion

This work exhibits the synthesis and the full characterization of a μIAC and the ability to couple it in-line and in a fully automated way (from sample loading to antigen quantification) to capillary zone electrophoresis with anti-OTA/OTA antibody/antigen model.

The in situ synthesized immunopreconcentration monolithic supports have been thorougly characterized with various analytical tools including of a new, robust and very sensitive in-line-hydrodynamic method for the determination of the total immobilized antibodies directly inside the capillary at a miniaturized scale. Owing to its basic principle, simplicity and great sensitivity, this method should be implemented in other applications for the quantification of other amino containing molecules grafted inside a capillary or a microchannel. This original method also opens the door to the comparison of grafting methods either for the evaluation of their immobilization yields and/or for their influence on the antibodies' activity.

It is also demonstrated that the non-oriented but straigthforword immobilization method used thoughout this work allows to retain at least at 40% of the antibodies activity (this ratio being antibodies dependant). Despite the progressive (and unavoidable) partial denaturation of the antibodies during successive recognition cycles, the activity of the μIAC remains high enough to implement successive cycles. As an example, it is demonstrated that the remaining activity allows the full recovery and quantification of OTA at a 1 μg/L concentration level for at least 15 recovery-elution cycles, i.e. that the μIAC are multiple use owing to their great initial binding capacity. It is demonstrated that the in line-coupling of this immunoaffinity capillary column with capillary zone electrophoresis allows the quantitative recovery of OTA in very diluted samples, with a preconcentration factor of about 250 fold considering a percolated volume of 10 μL and an elution one of about 40 nL. Higher preconcentration factors may be reached by percolating larger sample volumes. The non-specific adsorption is demonstrated to be negligible. Further work is now underway to study very complexe matrices such as coffee and cocoa extracts and to compare the results obtained with our in-line method to the classical off-line one.

As already discussed by Valcárcel et al. [26] such an in-line coupling (vs off-line and on-line counterparts) minimizes sample handling, avoids potential losses and facilitates the control of the all analytical process. A supplementary advantage lies in the very low consumption of sample and solvents relative to the off-line method.

Acknowledgements

The authors would like to thank the ANR PCV for financial support (No. ANR-07-PCVI-0036; D-aminochip). The authors would also like to thank Pr. Alain Berthod and Nazim Mekaoui for the CCC purification.

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