Analytica Chimica Acta 1193 (2022) 339394

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

Crosslinked zwitterionic polymeric ionic liquid-functionalized nitinol wires for *fiber-in-tube* solid-phase microextraction and UHPLC-MS/MS as an amyloid beta peptide binding protein assay in biological fluids

Israel D. Souza [a], Jared L. Anderson [b], Maria Eugênia C. Queiroz [a, *]

[a] Departamento de Química, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil
[b] Department of Chemistry, Iowa State University, Ames, IA, 50011, United States

HIGHLIGHTS

- Zwitterionic PIL sorbent was synthetized by subsequently *in-situ* polymerization.
- PEEK capillary was packed with crosslinked zwitterionic PIL-coated nitinol wires.
- *Fiber-in-tube* SPME and UHPLC-MS/MS method was developed and validated.
- The method was successfully applied to evaluate the binding of HSA to Aβ in biological fluids.
- Zwitterionic PIL selectively preconcentrates Aβ (ion-exchange and dispersive interactions).

ARTICLE INFO

Article history:
Received 20 September 2021
Received in revised form
18 December 2021
Accepted 22 December 2021
Available online 25 December 2021

Keywords:
Amyloid beta peptide
Zwitterionic ionic liquids
Fiber-in-tube SPME
UHPLC-MS/MS
Protein binding

ABSTRACT

Alzheimer disease (AD) is a neurodegenerative disorder characterized by extracellular accumulation of amyloid-β peptide (Aβ) in the brain interstitium. Human serum albumin (HSA) highly binds to Aβ in blood plasma and is thought to inhibit plaque formation in peripheral tissue. Thus, the evaluation of albumin binding to Aβ is an important key to understand the dynamics of these molecules in the biological system of patients with AD. In this work, a *fiber-in-tube* solid-phase microextraction (*fiber-in-tube SPME*) and ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method was developed to estimate Aβ fraction binding to HSA in cerebrospinal fluid (CSF) and plasma samples. Crosslinked zwitterionic polymeric ionic liquid (zwitterionic PIL)-coated nitinol wires were developed and packed into a polyether ether ketone (PEEK) capillary for a *fiber-in-tube* SPME and UHPLC-MS/MS method. Zwitterionic PIL sorbent was synthetized from 1-vinyl-3-(butanesulfonate)imidazolium ([$VIm^+C_4SO_3^-$]) and 1,12-di(3-vinylimidazolium)dodecane dibromide ([$(VIm)_2C_{12}$]2[Br]) monomers by *in-situ* thermally-initiated polymerization. Morphological characterization by scanning electron microscopy (SEM) and atomic force microscopy (AFM) revealed a decrease in the surface roughness of the nitinol wires from ~17 nm to 1 nm after the *in-situ* polymerization. The zwitterionic PIL sorbent selectively preconcentrates Aβ through a two-pronged interaction mechanism. The *fiber-in-tube* SPME and UHPLC-MS/MS method presented lower limits of quantification (LLOQ) of 0.4 ng mL^{-1} for Aβ38 and 0.3 ng mL^{-1} for Aβ40 and Aβ42, a linear range from LLOQ values to 15 ng mL^{-1} with coefficients of determination higher than 0.99, precision with coefficient of variation (CV) values ranging from 2.1 to 7.3% and accuracy with relative standard deviation (RSD) values from −0.3 to 7.4. This method was successfully applied to evaluate the binding of HSA to Aβ in cerebrospinal fluid (CSF) and plasma samples.

1. Introduction

Amyloid-β peptide (Aβ) is produced through the proteolytic cleavage of a transmembrane protein, amyloid precursor protein (APP). The peptides constituted of 42 (Aβ42), 40 (Aβ40), and 38 (Aβ38) amino acid residues are the main fragments produced in

* Corresponding author. Av. Bandeirantes, 3900, CEP 14040-901, Ribeirão Preto, SP, Brazil.
E-mail address: mariaeqn@ffclrp.usp.br (M.E.C. Queiroz).

https://doi.org/10.1016/j.aca.2021.339394
0003-2670/

this process [1]. Aβ accumulation in the brain is proposed to be an early event in the pathogenesis of Alzheimer's disease (AD), which is the most common form of dementia associated with plaques and tangles in the brain [2]. Human serum albumin (HSA) binds highly to Aβ in blood plasma and is thought to inhibit plaque formation in peripheral tissue. However, the evaluation of albumin binding to Aβ is an important key to understand the dynamics of these molecules in the biological system of AD patients [3]. Aβ−protein binding phenomena can be studied by different techniques, which provide different information. Spectroscopic techniques can provide details on protein structural changes [4] whereas immunoassay provides high sensitivity for quantitative results of Aβ-protein binding fraction [5]. Solid-phase microextraction (SPME)-based approaches allow the uptake of a negligible amount of free analytes in the sample, thereby not disturbing the equilibrium between the free analytes and the analytes bound to proteins [6]. Furthermore, these approaches offer advantages such as low sample-handling volume, and the ability to minimize matrix effects in MS/MS analysis by using biocompatible extraction sorbents. For example, C18-coated solid-phase microextraction 96-pin devices [6] and fiber SPME [7] have been applied in the determination of plasma−protein binding properties of pharmaceutical drugs in plasma and *in vivo* studies, respectively.

Fiber-in-tube SPME is a sample preparation technique combining fiber SPME and *in-tube* SPME features [8]. In this technique, the analytes are extracted in a short capillary that is longitudinally packed with fine fibers (fine solvent-resistant synthetic polymer filaments), as the extraction medium. *Fiber-in-tube* SPME offers reduced pressure drop during extraction and desorption as compared to *in-tube* SPME with sorbent-packed capillary [9]. Recently, some advancements include not only the development of selective coatings but also high-pressure resistant capillaries [9]. Special features including high flexibility and excellent shape memory make nitinol wires as a new generation of SPME supports [10,11]. Crosslinked polymeric ionic liquids (PIL) chemically bonded onto nitinol wires have also demonstrated superior advantages [11].

Zwitterionic ionic liquids, a subclass of ionic liquids, are composed of both cations and anions that are covalently linked. The presence of both charges lead to high dipole moments while maintaining charge neutrality [12]. Similar to some biopolymers [13,14], the physical and chemical properties of zwitterionic ionic liquids can be influenced by external stimuli including pH [15]. This interesting property has motivated a wide range of applications including their use as enhanced solvents for cellulose dissolution [16] and as gas chromatographic (GC) stationary phases with high selectivity, strong retention, and excellent peak symmetry in the analysis of volatile carboxylic acids [17]. Zwitterionic ILs that contains polymerizable moieties can produce zwitterionic PILs through polymerization. This procedure is attractive for zwitterionic PIL applications as the sorbent because thermal and chemical stability are improved without sacrificing the polymer structure. For example, compared with commercial polyacrylate fibers SPME, the crosslinked zwitterionic PIL sorbent exhibited better extraction efficiency (headspace mode) in the extraction of short chain free fatty acids in red wine samples followed by GC-mass spectrometry detection [12]. These examples highlight the potential of zwitterionic PILs and has inspired their use in different SPME techniques.

In this work, an innovative *fibers-in-tube* SPME capillary was developed by combining the tensile strength of nitinol supports and selectivity/and robustness of crosslinked zwitterionic PIL. Zwitterionic PIL monomers as well as crosslinker were synthesized and characterized. Modification of the nitinol wires with a crosslinked zwitterionic PIL coating was obtained through an *in-situ* thermal-initiated polymerization process. *Fiber-in-tube* SPME extractions (in *offline* mode) exploit the capability of the crosslinked zwitterionic PIL coating in establishing ion-exchange and dispersive interactions to successfully preconcentrate Aβ peptides. Finally, the *fiber-in-tube* SPME and UHPLC-MS/MS method was applied to estimate the fraction of protein binding to HSA in CSF and plasma samples. The novelties of this study rely not only in the development of a new biocompatible coating but also applying it to the innovative *fiber-in-tube* SPME technique and UHPLC-MS/MS to determine Aβ peptides in biological fluids.

2. Experimental

2.1. Reagents and analytical standards

Human amyloid beta synthetic peptides (Aβ38, Aβ40 and Aβ42) and nitrogen-15 stable-isotope labeled amyloid beta peptides ($^{15}N_{51}$-Aβ38, $^{15}N_{53}$-Aβ40 and $^{15}N_{55}$-Aβ42) were all purchased from rPeptide (Athens, USA). Polypropylene Protein LoBind® tubes and LoRetention pipette tips were acquired from Eppendorf (Hamburg, Germany). 1,4-butane sultone (≥99%), vinyltrimethoxysilane (VTMS, 98%), 1,12-dibromododecane (98%), toluene, formic acid (≥98%), ammonium hydroxide (ACS reagent 28−30% NH_3 basis), chloroform, and HSA were purchased from Sigma-Aldrich (São Paulo, SP, Brazil). 2,2-azo-bis(isobutyronitrile) (AIBN; 98%) was acquired from Merck (São Paulo, SP, Brazil). Acetonitrile, 2-propanol, dichlorometane, HPLC grade, were obtained from J.T. Baker (Phillipsburg, NJ, USA). Elastic nitinol wires (120 μm O.D.) were purchased from Confluent Medical Technologies (Fremont, CA, USA). Water purified in a Milli-Q system (Millipore, São Paulo, Brazil) was used to prepare the aqueous solutions. Artificial CSF and artificial plasma (for protein binding assay) were prepared based on published studies [3,18] (Supplementary Material).

2.2. Preparation of the fiber-in-tube SPME capillary

2.2.1. Synthesis of monomers

To prepare the 1-vinyl-3-(butanesulfonate)imidazolium ($[VIm^+C_4SO_3^-]$) zwitterionic PIL monomer, 20 mmols of 1-vinylimidazole and 20 mmols of 1,4-butane sultone were dissolved in acetone. The mixture was refluxed for 4 days at 50 °C in an inert atmosphere. The monomer was separated by filtration and washed with cold acetone.

The 1,12-di(3-vinylimidazolium)dodecane dibromide ($[(VIm)_2C_{12}]2[Br]$) crosslinker was synthesized by mixing 2:1 (*m/m*) 1-vinylimidazole and 1,12-dibromododecane in 2-propanol. After mixing for 36 h at 55 °C, the reaction medium was cooled to room temperature and the solvent evaporated under vacuum. The dried mixture was then dissolved in water and washed five times with aliquots of dichloromethane.

The solvent was removed under vacuum at 60 °C and the products was dried in a vacuum oven at 45 °C for 2 days prior *in-situ* polymerization.

2.2.2. In-situ polymerization

Nitinol wires (0.120 × 200 mm) were immersed in hydrogen peroxide for 3 h at 72 °C for oxidation of the substrate surface. Then, the wires were dried overnight in a vacuum oven at 80 °C. The oxidized wires were subsequently functionalized with VTMS at 85 °C for 5 h.

For the polymerization process, $[VIm^+C_4SO_3^-]/[(VIm)_2C_{12}]2[Br]$ (2:1, w/w) were dissolved in DMSO/methanol (60:40, v/v) solution. The amount of monomer + crosslinker was 2% (*w/v*) of solvent solution. This mixture was submitted to an ultrasonic bath for 4 min and subsequently degassed with a nitrogen stream for 10 min. AIBN (2.4 mg) was then added to the reaction mixture. The

functionalized wires were packed into fused-silica capillary, which was filled with the reaction mixture. Capillaries were sealed and placed in oven (under an inert atmosphere) at 60 °C for 36 h. The *in-situ* polymerization process was repeated twice to obtain a thicker coating. Finally, the zwitterionic PIL-based fibers were removed from silica capillary and packed into a PEEK tube (0.762 × 200 mm) to obtain the *fiber-in-tube* SPME capillary.

2.3. Characterization of IL monomers and capillaries

The chemical structure of zwitterionic PIL and crosslinker were confirmed by ^{1}H NMR. The fibers were characterized by X-ray dispersive energy (EDX), atomic force microscopy (AFM), and scanning electron microscopy (SEM). Additional details are described in the Supplementary Material.

2.4. Fiber-in-tube SPME extraction optimization

A lab made system was employed for the *offline fiber-in-tube* SPME procedure, Fig. S1. In this system, a pump was used to percolate sample and solution through the capillary. A negative pressure was applied in the system during the draw cycle. The sample percolated through the capillary (upward direction) was collected in a 1000 μL loop (installed at the top of the *fiber-in-tube* SPME capillary). During the eject cycle, a positive pressure was applied to ejected (downward direction). The pump pressure was adjusted in order to obtain adequate sampling rate. The influence of the pH during extraction step was evaluated at pH 2.5, 7.0 and 10.5. Due their compatibility with the mass spectrometer system, 1% formic acid or 1% ammonium hydroxide aqueous solution was used to adjust the pH of the sample. Different cleanup solutions from 0 to 15% (*v/v*) of acetonitrile in water containing 1% of formic acid were evaluated. The elution process was assessed using 1x100 μL and 2x100 μL of different solutions: water/acetonitrile 20:80 (*v/v*) and [water/acetonitrile 20:80 (*v/v*) + 1% of ammonium hydroxide]. The draw/eject cycles from 1 to 10 was evaluated. Finally, the effect of capillary length (10, 15 and 20 cm) and sampling rate (0.5 and 1.0 mL min^{-1}) were investigated. Prior to injection onto the chromatographic system, the extracted analytes were diluted 1:1 (*v/v*) with water to adjust the elution strength of the sample solution. The *fiber-in-tube* SPME capillary was used for multiple extractions. Between each extraction, the capillary was washed with 600 μL of methanol and conditioned with 600 μL of 1% formic acid aqueous solution.

2.5. UHPLC-MS/MS conditions

The analyses were carried out in a Waters ACQUITY UPLC H-Class system coupled to the Xevo® TQ-D tandem quadrupole (Waters Corporation, Milford, MA, USA) mass spectrometer equipped with a Z-spray source operating in the positive electrospray ionization mode (ESI$^+$) with Selected Reaction Monitoring (SRM) mode [19]. The source and operating parameters were optimized as follows: capillary voltage, 3.50 kV; source temperature, 150 °C; desolvation temperature, 500 °C; and desolvation gas flow, 1000 L h^{-1} (N_2, 99.9% purity). Argon (99.9999% purity) was used as the collision gas. The fragments, cone energy, and collision energy were optimized for each analyte, as shown in Table 1. Instrument control, peak detection, and integration were carried out with a MassLynx 4.1 Data System.

Chromatographic separations were carried out on ACQUITY UPLC BEH 300 Å C18 chromatographic column (150 mm × 2.1 mm, 1.7 μm) at 40 °C. Mobile phase A consisted of 0.3% ammonium hydroxide (*v/v*) aqueous solution, and mobile phase B consisted of acetonitrile with 0.3% ammonium hydroxide. At flow rate of 200 μL min^{-1}, the gradient elution started with 20% B (0–1 min), then increasing to 45% B (1–6.5 min) and hold at 45% B for 0.5 min, followed by a final decrease to 20% B for further 5.0 min (re-equilibration time). Fifteen microliters of sample were injected into the chromatographic system.

2.6. Method validation

Analytical curves were generated by linear regression of the ratio between the Aβ peptides and the internal standard (Y) peak areas versus the amyloid beta peptide concentrations (X, ng mL^{-1}). The lower limit of quantitation, LLOQ, corresponded to the lowest concentration on the analytical curve that could be quantitatively measured with acceptable precision and accuracy (within 20%).

Accuracy (relative standard deviation, RSD), precision (coefficient of variation, CV), and relative recovery values were determined by replicate analyses (*fiber-in-tube* SPME and UHPLC-MS/MS assays) of the blank samples spiked with the Aβ peptides from different quality controls (LLOQ, low, medium, and high).

Comparison of the SRM chromatograms of the samples spiked at the LLOQ level with the SRM chromatograms for the non-spiked sample allowed assessment of the selectivity in the developed method. The response of co-eluting interferences should be less than 20% of the response for the analytes and less than 5% of the peak area of the IS.

Carry-over was assessed by injecting aliquots of the same non-spiked sample after analysis of the sample spiked with the analytes at the concentration corresponding to the upper limit of quantification (ULOQ). Carry-over in the non-spiked sample was evaluated immediately after the ULOQ sample should not be greater than 20% of the analyte signals in the LLOQ chromatogram, and not greater than 5% of IS.

Reusability and long-term stability of the capillary were evaluated based on extraction efficiency over multiple extractions (see Supplementary Material).

Matrix effects (ME) were evaluated by the post-column infusion experiments [20]. This experiment uses two pumps: (I) a chromatographic pump to deliver the mobile phase to UHPLC system and (II) an infusion pump to deliver the infusion solution into MS/MS system. An extracted non-spiked sample was injected into UHPLC system (using chromatographic pump) while 500 ng mL^{-1} Aβ peptides solution were delivered at a continuous flow rate (15 μL min^{-1}) into MS/MS system (using infusion pump). The effluent from the chromatographic column combined with the infused analyte and entered into the ESI interface. Presence of interferants from the matrix can be confirmed by considerable suppression or enhancement (>25%) of the infused analyte signal at the retention time of the analyte.

2.7. Protein binding assay

Protein binding assays were carried out based on published studies with some modifications [6]. Three hundred microliters of the biological fluid or aqueous solution (artificial samples without adding HSA) were spiked with Aβ peptides (final concentration of 40 ng mL^{-1}). Samples were kept in incubation process for 1 h at 37 °C with gentle agitation [5]. After incubation, 300 μL of aqueous solution was added into samples and they were submitted to *fiber-in-tube* SPME procedure: 1x600 μL of methanol + 1x600 μL of 1% formic acid aqueous solution (washing and conditioning step), 6 draw/eject cycles of sample (sampling), 1x500 μL of water/acetonitrile 95:5 (v/v) containing 1% of formic acid (cleanup), and 1x100 μL water/acetonitrile 20:80 (v/v) containing 1% of ammonium hydroxide (desorption). Then, water (100 μL) and IS (50 μL, 30 ng mL^{-1}) were added to desorbed solution prior injection into UHPLC-MS/MS system.

Table 1
Ion transitions, instrument settings, and retention times for each studied drug.

Analytes	Precursor ion (*m/z*)[a]	Product ion (*m/z*)	Product ion i.d.	DP (V)	CE (eV)	rt (min)
Aβ38	1033.4	1000.2	b36	45	20	5.7
$^{15}N_{51}$-Aβ38	1046.3	1012.1		45	22	5.7
Aβ40	1082.7	1053.3	b39	55	20	6.1
$^{15}N_{53}$-Aβ40	1096.3	1066.6		45	23	6.1
Aβ42	1128.7	1078.1	b40	55	25	6.5
$^{15}N_{55}$-Aβ42	1142.9	1090.7		50	24	6.5

[a] Precursor $[M+H]^{4+}$.

The sampling rate (1 mL min^{-1}) and draw/eject cycles (8) used in these experiments resulted in adequate sample agitation through the capillary [21], thus the study of protein binding was estimated based on mathematical models developed for SPME [22]. An external calibration curve was applied to quantify the amount of analytes *m* or m_{sample} extracted from aqueous (synthetic matrices without HSA) or sample solution (synthetic matrices with HSA), respectively. The constant *fc* of the extraction capillary was calculated using equation (1).

$$fc = \frac{m}{Cfree} \quad \text{equation 1}$$

Where *Cfree* is the free concentration of the analytes remaining in the solution.

The total final *Ctotal* and free *Cfree sample* concentration in the sample were calculated using equations (2) and (3) respectively.

$$Ctotal = C0sample - \frac{msample}{V} \quad \text{equation 2}$$

$$Cfree\ sample = \frac{msample}{fc} \quad \text{equation 3}$$

Where *C0sample* is initial concentration of the analyte added to the volume *V* of the sample.

Then, the percentage of Aβ peptides binding to HSA was calculated from equation (4).

$$PP\% = \frac{Ctotal - Cfree\ sample}{Ctotal} \times 100 \quad \text{equation 4}$$

3. Results and discussion

3.1. Synthesis of PIL monomers and fabrication of the fiber-in-tube SPME capillary

The zwitterionic and crosslinker monomers were prepared by reacting a nucleophilic reagent (1-vinylimidazole) with an electrophilic precursor (1,4-butane sultone or 1,12-dibromododecane) in an adequate solvent (Fig. S2). Both reactions were carried out in an inert atmosphere and protected from light to avoid premature polymerization of the monomers. The precursors (1-vinylimidazole and 1,4-butanesultone) exhibit higher solubility in acetone. The product, $[VIm^{+}C_4SO_3^{-}]$, is insoluble in acetone and precipitates in the reaction media. On the other hand, the $[(VIm)_2C_{12}]2[Br]$ is very soluble in the reaction solution. Therefore, liquid-liquid extraction was adopted to purify the product. Peaks in the ^1H NMR (Fig. S3) spectra match with the chemical structures of the PIL monomers and demonstrate the high purity of the synthesized products. The monomers were then used for the *in-situ* thermal-initiated polymerization. Impure monomers contain unreacted reagents that could influence negatively in the reproducibility of the *in-situ* polymerization and in the selectivity of the sorbent. Therefore, high purity grade of PIL monomers is required for the synthesis of SPME sorbents.

Before the polymerization step, nitinol wires were treated in two steps: (I) first an oxidation process to create hydroxyl moieties (Ti—OH) and (II) the reaction of Ti—OH groups with VTMS onto nitinol surface. Fig. S4 shows the EDX spectra of the wires before and after step II. The Si peaks (Fig. S4b) belong to VTMS and ascertained the completion of the pretreatment process. VTMS provides vinyl moieties which is important to allow chemical anchoring between the crosslinked zwitterionic PIL sorbent and the surface support.

The amount of $[VIm^{+}C_4SO_3^{-}]/[(VIm)_2C_{12}]2[Br]$ (2:1 or 1:1, *w/w*) was evaluated in the *in-situ* polymerization process. The 1:1 *w/w* ratio produced a rigid zwitterionic PIL coating that was mechanically unstable. On the other hand, the 2:1 *w/w* ratio produced a very stable crosslinked zwitterionic PIL coating featuring no peeling or cracking even after direct immersion in water or organic solvents for several extraction cycles. Similar results with different PILs were observed in our previously study [23]. The proportion (percentage) of monomers/solvent (1, 2, 4 and 10% *m/v*) was also evaluated. The best results were obtained using 2% (*m/v*) of monomers relative to solvent volume. For more concentrated solutions, the synthesis solvent did not completely solubilize the monomers. The fibers obtained using the best synthesis condition were characterized by different spectroscopic techniques. The topography of the pretreated nitinol fibers was evaluated by AFM analysis. The RMS roughness decreased from ~17 nm (Fig. 1a) to 1 nm (Fig. 1b) after the polymerization process. This result is likely due the formation of the coating, which fills the large pores leading to a smoother surface.

The morphology of the pretreated nitinol wires was investigated by SEM using magnifications of 1000 × and 10000 × . Fig. 2a and 2b shows a roughness surface that is related to the oxide film onto pretreated wires. After *in-situ* polymerization, it is possible to see some particles covering the entire surface of the fibers, as shown in Fig. 2c. The micrograph obtained in higher magnification shows a smoother surface, Fig. 2d. The SEM results agree with those AFM analyses and they suggest that the crosslinked zwitterionic PIL films were attached onto the fiber surface.

Next, zwitterionic PIL -fibers were packed into a PEEK tube. Fig. 2e shows a cross-sectional view of the *fiber-in-tube* SPME capillary. These multiple fibers positioned longitudinally inside the capillary ensured high zwitterionic PIL film surface area available for preconcentration.

3.2. Optimization of fiber-in-tube SPME extraction conditions

In order to obtain good extraction efficiency, the following conditions were optimized: sample pH, sample cleanup, desorption solution, draw/eject cycles, capillary length, and sampling rate. The experiments were carried out applying the factor-by-factor approach.

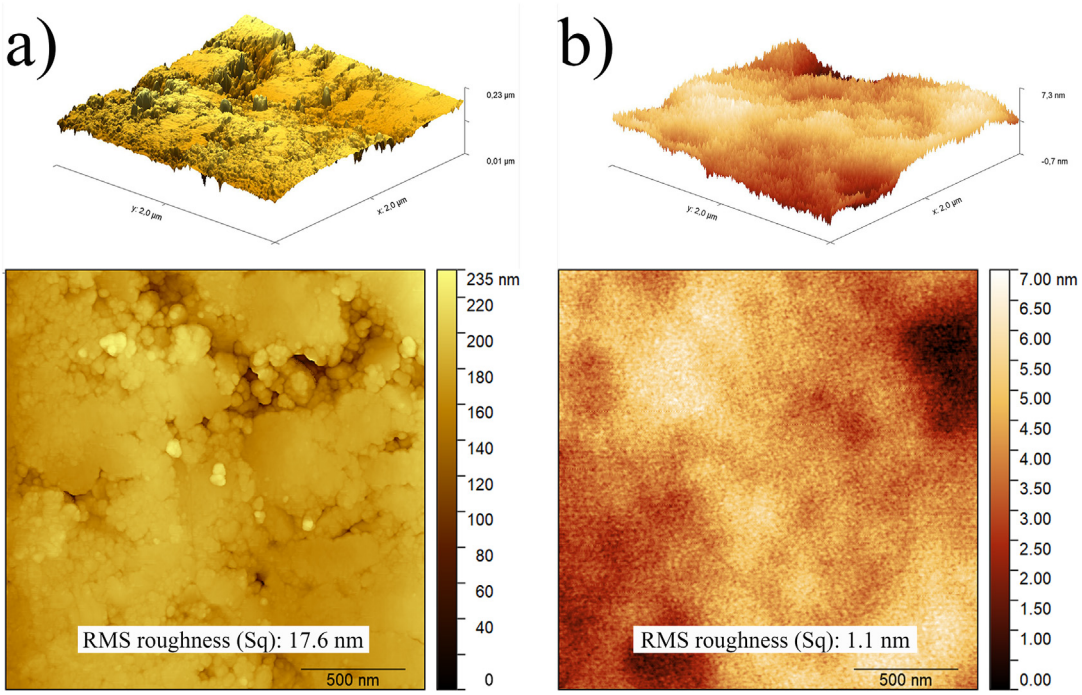

Fig. 1. AFM images of the nitinol wires before (a) and after (b) zwitterionic PIL modification.

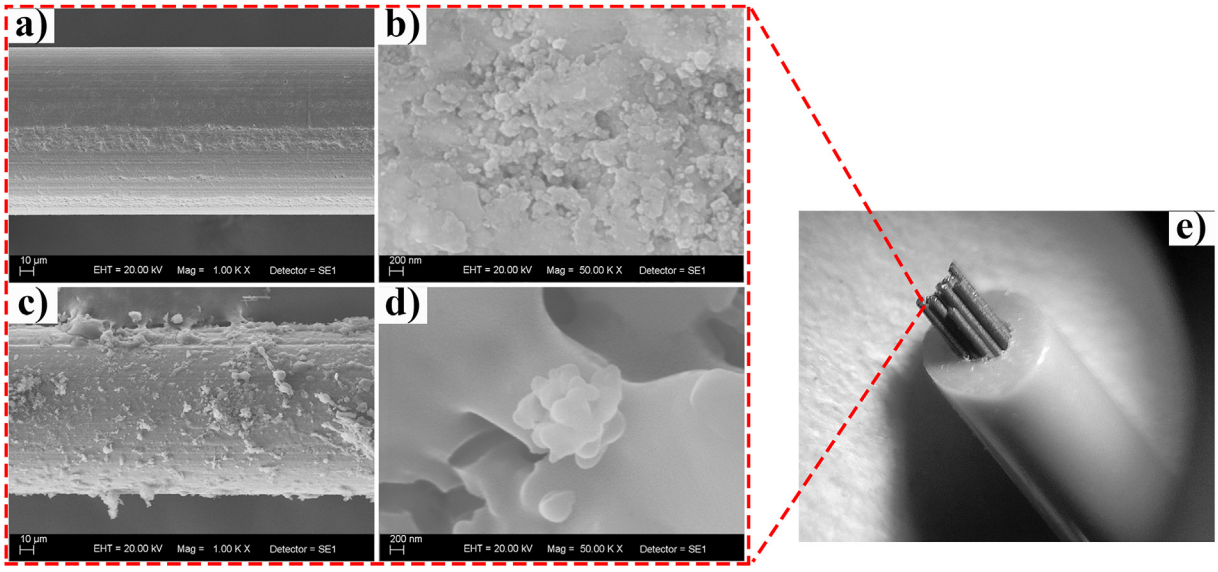

Fig. 2. SEM images obtained with different magnification (1000 and 10000x) of the nitinol wires before (a and b) and after (c and d) the *in-situ* polymerization; and optical image of the fibers packed into PEEK capillary.

Low extraction efficiency was obtained at neutral and alkaline media, Fig. 3a. Chemical structure of Aβ peptides is composed of acidic, neutral, and basic amino acid residues resulting an isoelectric point of approximately 5.2 [19]. Therefore, Aβ peptides can ionize positively in acid media. Considering the chemical structure of the zwitterionic PIL coating, the sulfonic groups exhibit cation-exchange properties. On the other hand, imidazolium cations (paired with halide anion) and alkyl chains can promote hydrogen bonding and dispersive interactions, respectively [24]. Previously, studies have shown that crosslinked PIL-based coatings can extract analytes via a partitioning, which is a non-competitive extraction mechanism [12,25]. Therefore, the better extraction efficiency obtained for amyloid beta peptides in acid media suggests an ion-exchange mechanism through zwitterionic moiety. At the same time, the chemical structure of the crosslinker may contribute with secondary interactions during the extraction process.

Because biological fluids are complex matrices, sometimes endogenous macromolecules can be retained in the sorbent. In this regard, a suitable sample cleanup step is important to ensure effective preconcentration of the analytes in *fiber-in-tube* SPME capillary,

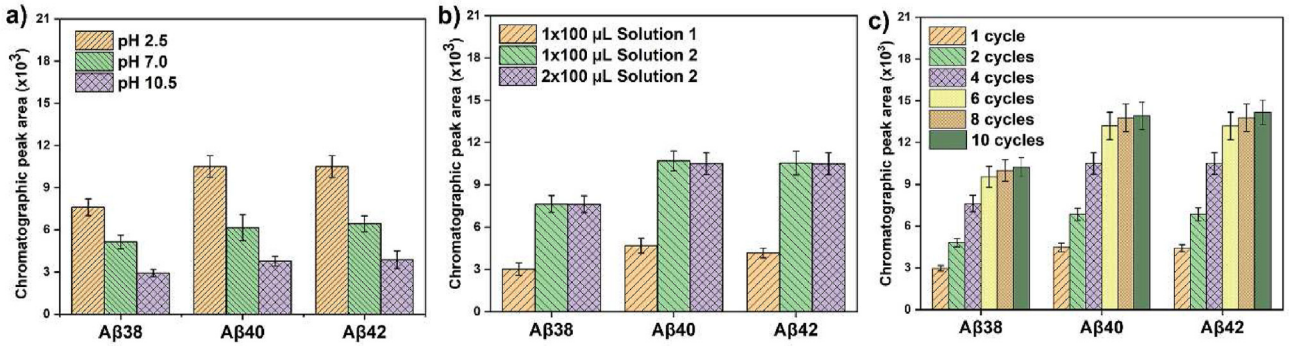

Fig. 3. Optimization of *fiber-in-tube* SPME extraction: (a) sample pH, (b) desorption solution and (c) draw/eject cycle. Solution 1: water/acetonitrile 20:80 (*v/v*), solution 2: water/acetonitrile 20:80 (*v/v*) + 1% of ammonium hydroxide, targets concentration, 15 ng mL^{-1}.

while excluding macromolecules from the biological matrix. Among the different solutions evaluated, water/acetonitrile 95:5 (*v/v*) containing 1% of formic acid was chosen as the optimal condition. As shown in Fig. S5, this cleanup solution was effective to eliminate endogenous compounds that possibly co-elute with the analytes.

Mixtures of water/acetonitrile 20:80 (*v/v*) containing/or not containing 1% of ammonium hydroxide were evaluated as a desorption solution, as shown in Fig. 3b. The solution [water/acetonitrile 20:80 (v/v) + 1% of ammonium hydroxide] was observed to be more efficient. The addition of ammonium hydroxide decreased the affinity of Aβ peptide through the sorbent. On the other hand, water/acetonitrile 20:80 (v/v) was able to elute only a small percentage of analytes. This result is likely explained by dispersive interactions and is corroborated by the mixed-mode interaction mechanism between the zwitterionic PIL sorbent and the Aβ peptides. By increasing the volume of elution solution, the efficacy of the desorption also increased. However, the analytical signal was decreased due to the dilution effects. As shown in Fig. 3b, 100 µL was considered to be a suitable compromise between the amount of analytes eluted and MS/MS signal. To monitor the extraction profiles of Aβ peptides by *fiber-in-tube* SPME, the number of draw/eject cycles was varied from 1 to 10, Fig. 3c. The peak areas of the analytes increased linearly from 1 to 4 draw/eject cycles. The results obtained for 6, 8 and 10 draw/eject cycles were compared using statistical analysis. The *p*-value higher than 0.05 (ANOVA Single Factor) showed that there is no difference among the extraction efficiency of 6, 8 and 10 draw/eject cycles. Therefore, these results suggest that sorption equilibrium was achieved with 6 draw/eject cycles.

Next, the capillary length was also evaluated. With the increase of capillary length, more stationary phase is available in the capillary so the analytes extracted gradually increased as seen in Fig. S6a. A capillary length higher than 20 cm was not possible to evaluate due the high backpressure during SPME procedure. For sampling rate optimization, a slight increase in extraction efficiency was observed using 0.5 mL min^{-1}, Fig. S6b. Considering the possibility of high throughput analysis and extraction time saved, 1.0 mL min^{-1} was selected as a good sample flow rate for next tests. This value is in agreement with reported *fiber-in-tube* SPME methods [26,27].

After each extraction, the *fiber-in-tube* SPME capillary was washed with 600 µL of methanol and conditioned with 600 µL of 1% formic acid aqueous solution. This step was enough to eliminate carryover effects.

3.3. Analytical validation

The main results of the analytical validation of the *fiber-in-tube* SPME and UHPLC-MS/MS are presented in Table 2. The method was linear for all Aβ peptides from the LLOQ to 15 ng mL^{-1} with coefficients of determination that were higher than 0.99, and the calculated *p*-values were higher than 0.05 (Lack-Of-Fit Test). The LLOQs values for Aβ38, Aβ40, and Aβ42 were 0.4 ng mL^{-1}, 0.3 ng mL^{-1}, and 0.3 ng mL^{-1}, respectively. These linear ranges are satisfactory with the expected concentration levels of Aβ peptides in human cerebrospinal fluids [19,28,29].

The method exhibited suitable precision and intra- and inter-assay accuracy with CV and RSD values ranging from 2.1 to 7.3% and −0.3 to 7.4, respectively.

The ME was evaluated by post-column infusion. Fig. S5 revealed no regions of ion suppression/enhancement at the retention times of the analytes. This result confirms that the *fiber-in-tube* SPME and UHPLC-MS/MS method was not affected by ME.

Fig. 4 illustrates chromatograms for sample spiked at the LLOQ concentration and a blank sample. There was no residual carryover in the blank chromatograms.

Capillary-to-capillary reproducibility was evaluated by comparing the extraction efficiency of three new capillaries. The chromatographic peak area obtained using four replicate extractions presented CV values less than 5.1%. The low CV values is an indicative of excellent reproducibility of the synthesis procedure. The capillary was very stable and each capillary was reused for at least 90 extractions and storage at least 10 days (long-term stability) without a significant decrease in extraction efficiency (*p*-value>0,05), Table S1.

3.4. Plasma and CSF protein binding

The *fiber-in-tube* SPME and UHPLC-MS/MS method was successfully applied to estimate the percentage of Aβ peptides bound to proteins from biological fluids. To apply the mathematical equations (1)–(4) it is important to assume that the sampling flow rate into the extraction capillary was enough to guarantee perfect agitation condition [21]. Also, the complex Aβ peptides–HSA is labile [5] and desorption from the protein bound compound occur instantaneous to re-establish the equilibrium between the protein-bound and freely dissolved concentration when the freely dissolved concentration is affected by uptake into the fiber coating. Therefore, measurement of free concentration can be carried out in non-negligible condition [30,31]. To obtain accurate measurements of free analyte concentrations, freshly prepared standard solutions were used to spike the surrogate matrix. Samples were spiked to 40 ng mL^{-1} to guarantee that the amount of extracted analytes were higher than the LLOQ of the method. Protein LoBind® tubes and LoRetention® micropipette tips were used to avoid secondary adsorption of Aβ peptides with vessels surface. The absolute recovery rates of the *fiber-in-tube* SPME process were 26% (Aβ38), 23% (Aβ40) and 24% (Aβ42).

Table 2
Analytical validation parameters.

Analyte	Analytical curve (ng mL^{-1})	LLOQ (ng mL^{-1})	QC[a] (ng mL^{-1})	Precision CV (%)		Accuracy RSD (%)		Relative Recovery (%)
				Intra-assay	Inter-assay	Intra-assay	Inter-assay	
Aβ38	0.4–15	0.4	1.2	6.0	5.0	−3.8	−5.1	95 ± 6
			5	4.5	7.3	0.6	−4.1	101 ± 4
			10	2.5	3.8	−1.4	1.2	98 ± 6
Aβ40	0.3–15	0.3	0.9	6.6	7.1	1.7	−4.8	95 ± 7
			5	3.3	2.3	−0.3	−0.3	99 ± 3
			10	2.4	2.1	1.5	0,9	102 ± 2
Aβ42	0.3–15	0.3	0.9	5.0	2.8	3.9	7.4	104 ± 5
			5	3.1	2.9	−2.9	−5.1	94 ± 3
			10	6.4	4.5	−1.1	2.2	103 ± 4

[a] QC: quality control.

Fig. 5 shows data indicating the percentage of protein binding. Approximately 28% (Aβ38), 33% (Aβ40), 40% (Aβ42) and 83% (Aβ38), 86% (Aβ40), 95% (Aβ42) was bound to HSA in CSF and plasma, respectively. The results showed that Aβ42 exhibited higher affinity

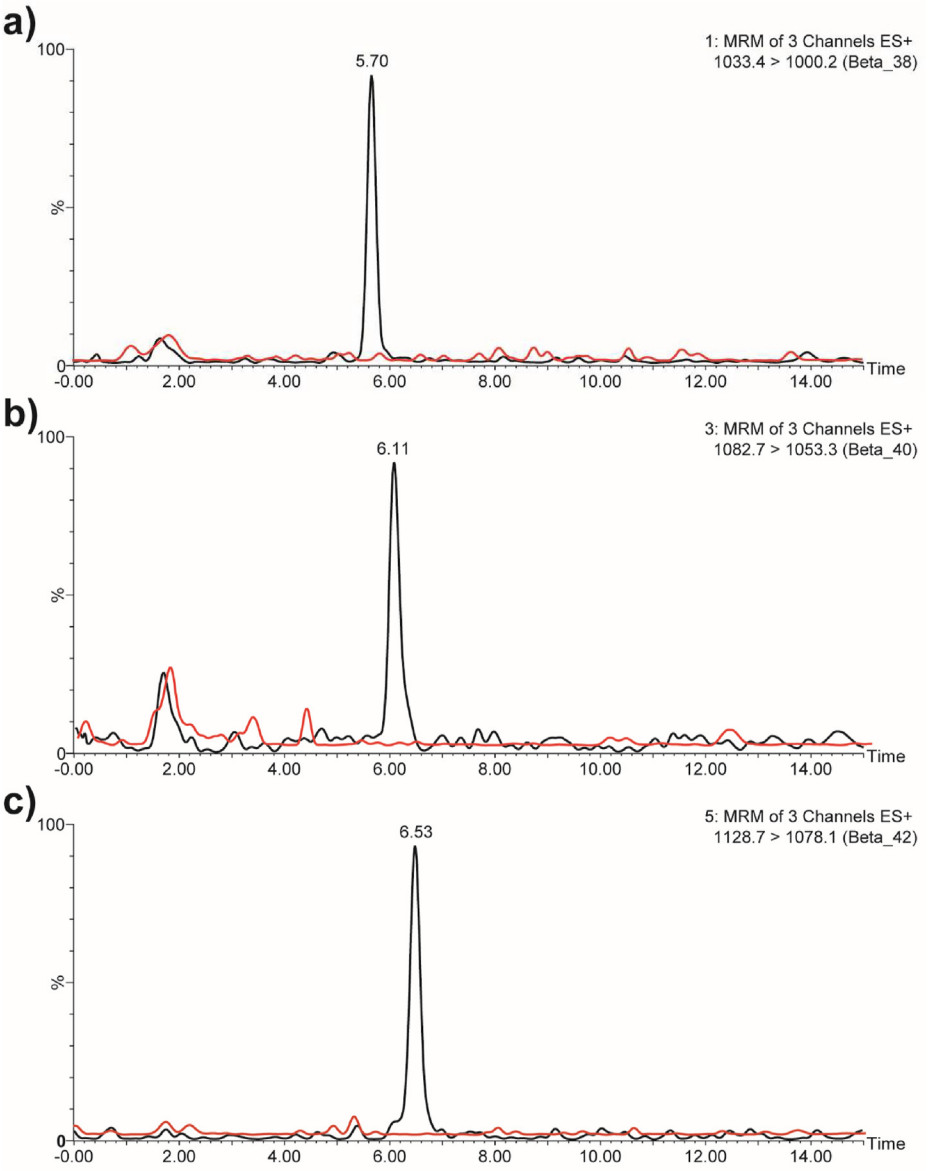

Fig. 4. *Fiber-in-tube* SPME/UHPLC-MS/MS chromatograms of artificial CSF spiked with Aβ at LLOQ concentration (black line) and blank artificial CSF (red line) obtained after analysis of ULOQ. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

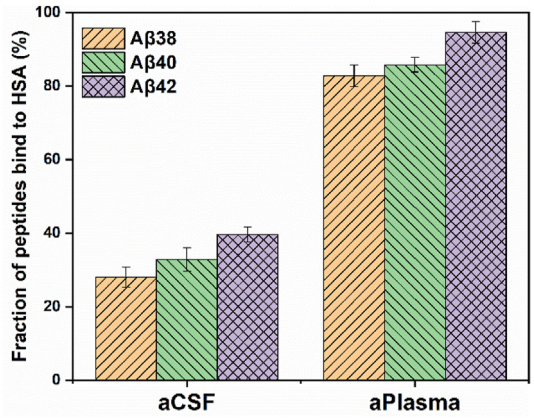

Fig. 5. Percentage of Aβ peptides bound to human serum albumin (HSA) in artificial cerebrospinal fluid and plasma samples. Samples spiked at 40 ng mL^{-1} were incubated for 1 h at 37 °C, and submitted to quantification.

in binding to HSA compared to Aβ40 and Aβ38. It has been suggested that hydrophobic C-terminal residues of Aβ peptide could bind to HSA to form a 1:1 complex with a dissociation constant of approximately 5 μM [3]. Stanyon et al. [3] reported that approximately 40% of Aβ40 and Aβ42 within the CSF were bound to HSA. Rózga et al. [32] applied direct titration of HSA with Aβ40 monitored using dichroism spectroscopy and they found more than 90% of Aβ peptide-bind fraction in plasma samples. Using a Europium immunoassay method, Kuo et al. [5] showed that 95% of Aβ42 present in plasma exists in a bound state at a physiological plasma concentration. In the same study, experiments carried out with real plasma samples from healthy volunteers revealed an average of 64% and 74% of Aβ40 and Aβ42, respectively, binding to HSA. With these in vitro experiments, Kuo et al. [5] also found that the binding equilibrium was sustained for at least 24 h at 37 °C. The interaction in real biological fluids is complex because other molecules compete effectively with the Aβ peptides for the albumin binding sites [5]. However, the essays carried out in the artificial sample is easier and helps to understand this interaction phenomena. The results obtained using the *fiber-in-tube* SPME and UHPLC-MS/MS method are in good agreement with those obtained by applying other described methods [3,5,32]. Therefore, they confirm the applicability of the *fiber-in-tube* SPME and HPLC-MS/MS method to determine Aβ peptides. Compared to immunoassays or spectroscopic techniques applied to determine Aβ peptide-bind fraction, the *fiber-in-tube* SPME and HPLC-MS/MS method is less prone to suffer interference from matrix components and requires less volume of sample.

4. Conclusion

In-situ thermal-initiated polymerization proved to be an efficient route to produce crosslinked zwitterionic polymeric ionic liquid sorbent covalently bonded onto nitinol wires. The *fiber-in-tube* SPME capillary provides dual retention mechanisms based on ion exchange and dispersive interactions. The biocompatible *fiber-in-tube* SPME exhibited high robustness and mechanical strength, which allowed its re-use without significant changes in extraction reproducibility or carryover effects. The protein binding assay carried out using the *fiber-in-tube* SPME and UHPLC-MS/MS method confirms the high ability of HSA to bind of Aβ peptide. The amount of HSA in the biological matrix drastically influences the free concentration of Aβ peptide.

On-going work is devoted to improving the *fiber-in-tube* SPME extraction efficiency to facilitate direct coupling with MS/MS and NanoESI-MS systems. In future experiments, the *fiber-in-tube* SPME-MS/MS method will be applied in the determination of Aβ peptide in real cerebrospinal fluids samples. A bioanalytical study has been designed to study the correlation of Aβ peptide between patients with Alzheimer disease and healthy patients.

CRediT authorship contribution statement

Israel D. Souza: Conceptualization, Methodology, Investigation, Writing – original draft. **Jared L. Anderson:** Conceptualization, Methodology, Writing – review & editing. **Maria Eugênia C. Queiroz:** Resources, Supervision, Conceptualization, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to acknowledge FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, processes 2020/00126-8, 2019/19485-0, and 2017/02147-0) and INCT-TM (465458/2014-9) (Instituto Nacional de Ciência e Tecnologia Translacional em Medicina) for financial support. Also, authors express their gratitude to the Brazilian Nanotechnology National Laboratory (LNNano) at the Brazilian Center of Research in Energy and Materials (CNPEM) for the AFM facilities (AFM-27697). JLA acknowledges funding from Chemical Measurement and Imaging Program at the National Science Foundation (Grant number CHE-1709372).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2021.339394.

References

[1] C. Haass, D.J. Selkoe, Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid β-peptide, Nat. Rev. Mol. Cell Biol. 8 (2007) 101–112, https://doi.org/10.1038/nrm2101.
[2] G. Chen, T. Xu, Y. Yan, Y. Zhou, Y. Jiang, K. Melcher, H.E. Xu, Amyloid beta: structure, biology and structure-based therapeutic development, Acta Pharmacol. Sin. 38 (2017) 1205–1235, https://doi.org/10.1038/aps.2017.28.
[3] H.F. Stanyon, J.H. Viles, Human serum albumin can regulate amyloid-β peptide fiber growth in the brain interstitium, J. Biol. Chem. 287 (2012) 28163–28168, https://doi.org/10.1074/jbc.C112.360800.
[4] E.M. Smoak, M.P. Dabakis, M.M. Henricus, R. Tamayev, I.A. Banerjee, Interactions of amyloid Aβ(1-42) peptide with self-assembled peptide nanospheres, J. Pept. Sci. 17 (2011) 14–23, https://doi.org/10.1002/psc.1284.
[5] Y.-M. Kuo, T.A. Kokjohn, W. Kalback, D. Luehrs, D.R. Galasko, N. Chevallier, E.H. Koo, M.R. Emmerling, A.E. Roher, Amyloid-β peptides interact with plasma proteins and erythrocytes: implications for their quantitation in plasma, Biochem. Biophys. Res. Commun. 268 (2000) 750–756, https://doi.org/10.1006/bbrc.2000.2222.
[6] K.S. Roy, E. Nazdrajić, O.I. Shimelis, M.J. Ross, Y. Chen, H. Cramer, J. Pawliszyn, Optimizing a high-throughput solid-phase microextraction system to determine the plasma protein binding of drugs in human plasma, Anal. Chem. 93 (2021) 11061–11065, https://doi.org/10.1021/acs.analchem.1c01986.
[7] B. Bojko, D. Vuckovic, J. Pawliszyn, Comparison of solid phase microextraction versus spectroscopic techniques for binding studies of carbamazepine, J. Pharm. Biomed. Anal. 66 (2012) 91–99, https://doi.org/10.1016/j.jpba.2012.03.005.
[8] Y. Saito, Y. Nakao, M. Imaizumi, T. Takeichi, Y. Kiso, K. Jinno, Fiber-in-tube solid-phase microextraction: a fibrous rigid-rod heterocyclic polymer as the extraction medium, Fresenius' J. Anal. Chem. 368 (2000) 641–643, https://doi.org/10.1007/s002160000574.
[9] I.D. Souza, I.G.C. Oliveira, M.E.C. Queiroz, Innovative extraction materials for fiber-in-tube solid phase microextraction: a review, Anal. Chim. Acta (2020)

238110, https://doi.org/10.1016/j.aca.2020.11.042.
[10] L. Setkova, S. Risticevic, C.M. Linton, G. Ouyang, L.M. Bragg, J. Pawliszyn, Solid-phase microextraction–gas chromatography–time-of-flight mass spectrometry utilized for the evaluation of the new-generation super elastic fiber assemblies, Anal. Chim. Acta 581 (2007) 221–231, https://doi.org/10.1016/j.aca.2006.08.022.
[11] T.D. Ho, B.R. Toledo, L.W. Hantao, J.L. Anderson, Chemical immobilization of crosslinked polymeric ionic liquids on nitinol wires produces highly robust sorbent coatings for solid-phase microextraction, Anal. Chim. Acta 843 (2014) 18–26, https://doi.org/10.1016/j.aca.2014.07.034.
[12] I. Pacheco-Fernández, M.J. Trujillo-Rodríguez, K. Kuroda, A.L. Holen, M.B. Jensen, J.L. Anderson, Zwitterionic polymeric ionic liquid-based sorbent coatings in solid phase microextraction for the determination of short chain free fatty acids, Talanta 200 (2019) 415–423, https://doi.org/10.1016/j.talanta.2019.03.073.
[13] L. Xu, Z. Chu, H. Wang, L. Cai, Z. Tu, H. Liu, C. Zhu, H. Shi, D. Pan, J. Pan, X. Fei, Electrostatically assembled multilayered films of biopolymer enhanced nanocapsules for on-demand drug release, ACS Appl. Biol. Mater. 2 (2019) 3429–3438, https://doi.org/10.1021/acsabm.9b00381.
[14] L. Xu, H. Wang, Z. Chu, L. Cai, H. Shi, C. Zhu, D. Pan, J. Pan, X. Fei, Y. Lei, Temperature-responsive multilayer films of micelle-based composites for controlled release of a third-generation EGFR inhibitor, ACS Appl. Polym. Mater. 2 (2020) 741–750, https://doi.org/10.1021/acsapm.9b01051.
[15] Y. Biswas, P. Ghosh, T.K. Mandal, Chemical tuning of zwitterionic ionic liquids for variable thermophysical behaviours, nanostructured aggregates and dual-stimuli responsiveness, Chem. - A Eur. J. 24 (2018) 13322–13335, https://doi.org/10.1002/chem.201802367.
[16] K. Kuroda, H. Satria, K. Miyamura, Y. Tsuge, K. Ninomiya, K. Takahashi, Design of wall-destructive but membrane-compatible solvents, J. Am. Chem. Soc. 139 (2017) 16052–16055, https://doi.org/10.1021/jacs.7b08914.
[17] H. Nan, K. Kuroda, K. Takahashi, J.L. Anderson, Examining the unique retention behavior of volatile carboxylic acids in gas chromatography using zwitterionic liquid stationary phases, J. Chromatogr., A 1603 (2019) 288–296, https://doi.org/10.1016/j.chroma.2019.06.021.
[18] S. Hooshfar, B. Basiri, M.G. Bartlett, Development of a surrogate matrix for cerebral spinal fluid for liquid chromatography/mass spectrometry based analytical methods, Rapid Commun. Mass Spectrom. 30 (2016) 854–858, https://doi.org/10.1002/rcm.7509.
[19] M.E. Lame, E.E. Chambers, M. Blatnik, Quantitation of amyloid beta peptides Aβ1–38, Aβ1–40, and Aβ1–42 in human cerebrospinal fluid by ultra-performance liquid chromatography–tandem mass spectrometry, Anal. Biochem. 419 (2011) 133–139, https://doi.org/10.1016/j.ab.2011.08.010.
[20] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds, Rapid Commun. Mass Spectrom. 13 (1999) 1175–1185, https://doi.org/10.1002/(SICI)1097-0231(19990630)13:12<1175::AID-RCM639>3.3.CO;2-S.
[21] J. Pawliszyn, Handbook of Solid Phase Microextraction, Chemical Industry Press of China, Beijing, 2012.
[22] F.M. Musteata, J. Pawliszyn, M.G. Qian, J.-T. Wu, G.T. Miwa, Determination of drug plasma protein binding by solid phase microextraction, J. Pharm. Sci. 95 (2006) 1712–1722, https://doi.org/10.1002/jps.20558.
[23] I.D. Souza, L.W. Hantao, M.E.C. Queiroz, Polymeric ionic liquid open tubular capillary column for on-line in-tube SPME coupled with UHPLC-MS/MS to determine endocannabinoids in plasma samples, Anal. Chim. Acta 1045 (2019) 108–116, https://doi.org/10.1016/j.aca.2018.08.062.
[24] Y. Meng, V. Pino, J.L. Anderson, Role of counteranions in polymeric ionic liquid-based solid-phase microextraction coatings for the selective extraction of polar compounds, Anal. Chim. Acta 687 (2011) 141–149, https://doi.org/10.1016/j.aca.2010.11.046.
[25] T.D. Ho, W.T.S. Cole, F. Augusto, J.L. Anderson, Insight into the extraction mechanism of polymeric ionic liquid sorbent coatings in solid-phase microextraction, J. Chromatogr., A 1298 (2013) 146–151, https://doi.org/10.1016/j.chroma.2013.05.009.
[26] Y. Bu, J. Feng, M. Sun, C. Zhou, C. Luo, Gold-functionalized stainless-steel wire and tube for fiber-in-tube solid-phase microextraction coupled to high-performance liquid chromatography for the determination of polycyclic aromatic hydrocarbons, J. Separ. Sci. 39 (2016) 932–938, https://doi.org/10.1002/jssc.201501103.
[27] J. Feng, X. Wang, Y. Tian, C. Luo, M. Sun, Melamine–formaldehyde aerogel coating for in-tube solid-phase microextraction, J. Chromatogr., A 1577 (2018) 8–14, https://doi.org/10.1016/j.chroma.2018.09.047.
[28] N.S. Schoonenboom, C. Mulder, G.J. Van Kamp, S.P. Mehta, P. Scheltens, M.A. Blankenstein, P.D. Mehta, Amyloid β 38, 40, and 42 species in cerebrospinal fluid: more of the same? Ann. Neurol. 58 (2005) 139–142, https://doi.org/10.1002/ana.20508.
[29] H. Struyfs, B. Van Broeck, M. Timmers, E. Fransen, K. Sleegers, C. Van Broeckhoven, P.P. De Deyn, J.R. Streffer, M. Mercken, S. Engelborghs, Diagnostic accuracy of cerebrospinal fluid amyloid-β isoforms for early and differential dementia diagnosis, J. Alzheim. Dis. 45 (2015) 813–822, https://doi.org/10.3233/JAD-141986.
[30] H. Peltenburg, I.J. Bosman, J.L.M. Hermens, Sensitive determination of plasma protein binding of cationic drugs using mixed-mode solid-phase microextraction, J. Pharm. Biomed. Anal. 115 (2015) 534–542, https://doi.org/10.1016/j.jpba.2015.08.002.
[31] E.H.M. Koster, C. Wemes, J.B. Morsink, G.J. de Jong, Determination of lidocaine in plasma by direct solid-phase microextraction combined with gas chromatography, J. Chromatogr. B Biomed. Sci. Appl. 739 (2000) 175–182, https://doi.org/10.1016/S0378-4347(99)00344-8.
[32] M. Rózga, M. Kłoniecki, A. Jabłonowska, M. Dadlez, W. Bal, The binding constant for amyloid Aβ40 peptide interaction with human serum albumin, Biochem. Biophys. Res. Commun. 364 (2007) 714–718, https://doi.org/10.1016/j.bbrc.2007.10.080.