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Neutral polymers as coatings for the high resolution electrophoretic separation of Aβ peptides on glass microchip

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This study reports a comparison of the performances of two neutral polymers, poly ethylene-oxide (PEO) and poly (dimethylacrylamide-*co*-allyl glycidyl ether) (EpDMA), in glass microchips to achieve zone electrophoresis separation of several truncated forms of Aβ peptides, sharing very similar structures. The peptides were derivatized by the fluoprobes 488 NHS to allow their fluorescence detection. Two protocols based either on PEO or EpDMA led to good pH stabilities in addition to a significant reduction of the electroosmotic flow. These two polymer coatings allowed repeatable analyses and high resolution for the simultaneous analysis of three beta-amyloid (Aβ) peptides, Aβ 1-38, Aβ 1-40 and Aβ 1-42, considered as potential biomarkers of Alzheimer's disease. A recovery study showed that EpDMA was superior in reducing the adsorption of the Aβ peptides on the coated inner wall. Finally, the separation method relying on the EpDMA coated microchips was validated as linear using a calibration curve and the LOD was estimated to be close to 200 nM. Despite very short migration distances, different N-term or C-term truncated Aβ peptides, corresponding to promising biomarker combinations for future diagnostic, were fully resolved. The method was successfully applied to detect these peptides in a spiked cerebrospinal fluid and provides a first achievement toward the development of a microsystem that would integrate preconcentration and separation steps.

1 Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly [1] and it is becoming a paramount issue throughout the world. Today, AD affects about 35.6 million people worldwide, and recent projections estimate this number to increase to 115.4 million by 2050 [2]. The neuropathological hallmarks of AD are amyloid plaques, mainly composed of beta-amyloid 1-42 (Aβ1-42) [3] and neurofibrillary tangles (NFTs) containing hyper phosphorylated Tau [4]. To date, cognitive tests such as Mini Mental State Evaluation and brain imaging are mainly used for diagnosis while determination of Aβ1-42, total tau and phospho-tau-181 levels in cerebrospinal fluid (CSF) are often used to help diagnosis [5] [6]. However, these assays alone are not sufficient to diagnose AD with certainty [7] [8]. Furthermore, the major challenge is to diagnose the disease at its earliest stages, before irreversible brain damages or mental decline have occurred [9]. Consequently, research on new strategies to allow either more efficient or earlier biochemical diagnosis is among the most active areas in Alzheimer's science [10].

Among these strategies, proteomic [11], genomic [12] and metabolomic [13] have permitted biomarkers to be identified for early stages of AD. Very recently, the potential of metabolomic [14] and in particular lipidomic [15] to predict the progression of AD was reviewed. Small molecules such as amino acids or lipids have been also proposed as potential CSF biomarkers for this disease as some differences between AD and non-AD samples were described in CSF. Several metabolites, among which amino acids, amino acid derivatives and di- or tri-peptides were, identified as possible disease progression biomarkers [16]. A set of ten lipids from peripheral blood has been discovered and validated, predicting phenoconversion to either amnestic mild cognitive impairment or Alzheimer's disease with over 90% accuracy [17]. In a very recent study, a panel of 10 plasma proteins predicting progression to AD was validated with an accuracy of 87% [18].

Amyloid beta-peptides represent a family of peptides produced by the enzymatic cleavage of amyloid precursor protein, which are found in both plasma and CSF [19] [20]. They consist of 43 amino acids (untruncated form) or less (truncated at the N or C-terminal amino acid sequence). CSF Aβ 1-40, Aβ 1-38 and Aβ 1-42 are the most abundant forms [21]. According to Bibl et al. [22], the average total abundance of Aβ peptides in CSF is around 2.5 nM in AD

patients. The main change observed in CSF of AD patients compared to controls is the decrease of Aβ1-42 levels [5]. Indeed, the Aβ 1-42 which has a high tendency to polymerize and form insoluble fibrils in the brain plays a pivotal role in the pathogenesis of AD. That is why it is considered as a relevant biomarker of this disease. However, its measurements in CSF lead to limited specificity and poor discrimination of AD toward normal aging and other neurologic disorders [23]. Only its combination to other biomarkers, such as Tau and p-Tau, produces a more specific diagnosis [24]. Several groups have investigated the diagnostic power of the simultaneous quantification of Aβ 1-38, Aβ 1-40, and Aβ 1-42. Some authors demonstrated that the combination of Aβ 1-42 with Aβ 1-38 and Aβ 1-40 could enhance the accuracy of Aβ 1-42 determination in CSF alone [25]. In particular, the Aβ1-42/Aβ1-38 ratio was found to allow for a slightly better discrimination between AD patients and non-demented (nD) controls than Aβ1-42/Aβ1-40 ratio [25]. A promising ratio combination, Aβ 1-42/(Aβ1-42 + Aβ 1-40 + Aβ1-38), producing a high discrimination between AD and nD patients has been also reported [26]. Besides these conventional Aβ peptides, N-truncated ones have recently attracted the attention of several research groups (see ref. [27] for a review). In 2012, Wilftang's group reported an AD-specific decrease of Aβ 2-42 in comparison to frontotemporal dementia, and proposed Aβ 2-42 as a candidate biomarker. Indeed, CSF levels of Aβ 2-42 and 1-42 have been shown to be similarly decreased in AD [28]. In addition, Esselmann *et al.* have filed a patent on the use of quantitative ratio of Aβ peptide (1-42, 2-40 and 2-42) to diagnose patients suffering from an early stage of Alzheimer's disease [29].

From the analytical point of view, different methods have been developed to analyze simultaneously several Aβ peptides from CSF such as capillary electrophoresis-laser induced fluorescence [26], solid phase extraction coupled to ultra performance liquid chromatography-tandem mass spectrometry [30] or ultrafiltration/liquid chromatography-matrix-assisted laser desorption mass spectrometry [31]. ELISA assays, based on specific antibodies commercially available are frequently used but cannot determine individual level of each of the amyloid peptides. Very recently, Haussmann *et al.* [32] developed a novel capillary isoelectric focusing (CIEF) immunoassay for the detection and discrimination of amino-terminal Aβ variants. The sensitivity of the method was sufficient to detect total Aβ peptides starting with Asp(1) in human CSF after desalting/buffer exchange and without prior enrichment. Nevertheless, CIEF based assays do not allow to separate C truncated Aβ peptides such as Aβ 1-38, Aβ 1-40, and Aβ 1-42, which exhibit the same isoelectric point. That is why alternative separation techniques are still required to evaluate the CSF level of each form composing the main promising biomarker combination. Microchip capillary electrophoresis (MCE) is emerging as a reliable analytical technique for proteins and peptides analysis [33, 34, 35, 36] showing significant advantages compared to conventional capillary electrophoresis (CE) such as speed and low sample consumption, providing also a strong potential for automation and integration [37]. To date, the only reported application of MCE for the analysis of Aβ peptides relies on a gel-based MCE method to separate Fluoprobes 488 (FP488) labeled Aβ peptides in a PDMS microchip. In this previous work, we reported for the first time a reproducible analysis in MCE of C-truncated Aβ peptides [35]. However, only three of the C-truncated variants were separated. In addition, due to the poor resolution obtained, quantification was not possible at this stage. In parallel, miniaturized immunocapture modules for Aβ peptide preconcentration have been also proposed [38], [39].

Surface treatment of microchannels is often essential to achieve high resolution and reproducible peptide analyses in MCE, not only to limit the adsorption of peptides to the glass wall of the microchip, but also to tune the EOF in order to obtain the highest resolution. In the present work, we focused on two polymers as coatings for glass microchannels: poly ethylene-oxide (PEO) and poly (dimethylacrylamide-*co*-allyl glycidyl ether) (EpDMA). A non-permanent coating based on PEO can be easily obtained but needs to be regenerated between runs [39]. PEO has been widely used in glass microchip, but mostly as a sieving matrix for the separation of proteins [41] and *DNA* fragments [42]. More recently it was added to a triblock copolymer, Pluronic F127 (PEO-PPO-PEO), to coat a highly hydrophobic PDMS microchip channel, and showed to highly reduce the non-specific adsorption of proteins [43]. Despite its potential to reduce adsorption, no work focusing on capillary zone electrophoresis (CZE) separation of protein or peptide on a glass microchip coated with physically adsorbed PEO has been reported yet. Besides this polymer, Chiari's group reported EpDMA as an efficient co-polymeric coating showing high ability to reduce non-specific adsorption of both acidic and alkaline proteins, while reducing the EOF on a wide pH range. It has been first reported in conventional capillary electrophoresis demonstrating a high chemical stability [44]. Later, EpDMA has been used as coating for the separation of DNA fragments [45] and more recently for the analysis of milk protein by CIEF on glass and PDMS microchips [46]. EpDMA has also been used as part of a two layer coating for PDMS microchannel for the analysis of Aβ peptides heretofore mentioned [38].

The final objective of the present study was to develop a method to achieve the simultaneous separation in microchip of several relevant Aβ peptides for the AD diagnosis. The MCE method had to be compatible with a coupling to an on line preconcentration module. The aim of the present work was essentially to compare the performances of the two polymers PEO and EpDMA as glass microchip coatings.

2. Materials and methods

2.1. Chemicals and Reagents

Amyloid peptides (Aβ 1-38, Aβ 1-40, Aβ 2-40, Aβ 2-42) were purchased from Anaspec (Fremont, CA, USA) with the exception of Aβ 1-42 which was from American peptide (Sunnyvale, CA, USA). PEO (MW 200 000), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) sodium salt, Boric Acid, Sodium hydroxide, hydrochloric acid (HCl), and DMSO were obtained from Sigma (St. Louis, MO, United States). The Fluoprobes 488 NHS was obtained from Interchim (Montlucon, France). EpDMA was synthesized in ICRM, Milano, Italy, according to the procedure previously described [47].

2.2. Material and Apparatus

Simple cross glass microchips (PS-SC) were obtained from Micralyne (Edmonton, Canada). Reservoirs, adhesive rings, and gaskets were purchased from Upchurch (Oak Harbor, WA, USA).
The microchip design used in these experiments consisted of a simple cross channel with a separation channel (50 µm wide and 20 µm deep) of 8.1 cm from the injection cross to buffer waste (BW). The distance from sample (S) and sample waste (SW) reservoir to the injection cross was 0.48 cm. The length from buffer reservoir (B) to the injection cross was 0.94 cm. A schematic diagram of the microchip is presented in Figure 1. Platinum electrodes were

inserted into the reservoirs, providing electrical contact from the power supply to the electrolyte solutions.

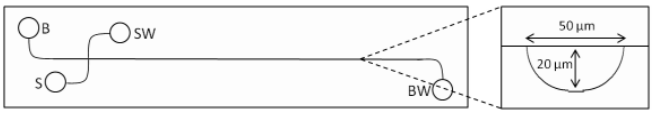

Fig. 1 Schematic diagram of the single cross glass microchip and of its cross section (right). B represents the buffer reservoir, BW the buffer waste reservoir, S the sample reservoir and SW the sample waste reservoir.

A HVS448 6000 D power supply from Labsmith (Livermore, CA, USA) controlled by a workstation was used for the microchip capillary electrophoresis. An inverted fluorescence microscope Axio Observer A1 from Carl Zeiss (Marly le Roy, France), equipped with a 20X objective lens (Carl Zeiss) was used to detect in real time fluorescently labeled peptides. Illumination was done by a HXP 120 C mercury arc lamp from Carl Zeiss. A CCD camera Orca-03G02 from Hamamatsu (Hamamatsu, Japan) was used to capture images of the detection zone located at 70 mm from the injection cross. HCImage software (Hamamatsu, Japan) was used to optimize the framerate, light sensitivity, and to define the region of interest. Azur software (DATALYS, France) was used to convert the mean grey scale from CCD as a function of time into electropherograms.

2.3. Buffers and solutions

The 100 mM (concentration) HEPES buffer was prepared by dissolving appropriate amounts of HEPES sodium salt in deionized water. A solution of HCl (1M) was then added to reach pH 8.0. The Borate buffer (pH 10.5, 40 mM ionic strength (IS)) was prepared by mixing appropriate volumes of sodium hydroxide (1 M) and boric acid (0.5 M). The 40% saturated ammonium sulfate solution was prepared by adding 0.242 g of ammonium sulfate to 1mL of water. The labeling stock solution (10 mg/mL) was prepared by dissolving 1 mg of Fluoprobes 488 NHS ester in 100 µL of DMSO. This solution was stored at -20 °C and in the dark until used. Two different EpDMA polymer formulations were used for the experiments on glass chip coating. EpDMA was first dissolved in deionized water at 1.2% (m/v). Once completely dissolved, the solution was diluted twofold by adding either deionized water or a solution of ammonium sulfate (40% sat.). The PEO polymer solution was prepared by first dissolving the PEO powder in deionized water at 0.22% (m/v) and by vigorously vortexing the solution at ambient temperature. Before the coating step; HCl was added to the solution in order to obtain a PEO solution at 0.20% (m/v) in 0.1 M HCl. PEO and EpDMA polymer solutions were prepared freshly before each coating process. All buffers and solutions were stored at 4 °C. HEPES and labeled peptides samples were protected from light.

2.4. Peptide dissolution and storage, CSF samples

Upon reception, standard Aβ 1-42 was dissolved in 0.16% (m/v) ammonium hydroxide aqueous solution at a concentration of 2 mg/mL and then divided into several aliquots which were individually stored at −20 °C. All other amyloid peptides were dissolved in 0.10% (m/v) ammonium hydroxide and at the same concentration.

The CSF aliquots analyzed in this work were taken from one patient attending the department of Neurology in Ulm (Department of Neurology, University of Ulm, Pr Markus Otto), who obtained a lumbar puncture to exclude meningitis or bleeding. Basic CSF parameters (albumin ratio, cell count, oligoclonal band) were normal. Collection and analysis of the CSF sample was approved by the Ethics Committee in Ulm. A signed written informed consent form was obtained in each and every case from the patient himself or his relatives. CSF was aliquoted within 2 h after collection, and stored at −80 °C.

2.5. Methods

2.5.1. Fluorescence derivatization of standard Peptides and spiked CSF samples

Aliquots of peptide solution were defrosted, eventually mixed with others Aβ peptides and then diluted 2-fold in borate buffer (pH 10.5, 40 mM IS). Then, the mixtures were lyophilized to remove the ammonium hydroxide. The resulting powder was dissolved in borate buffer (pH 10.5, IS 40 mM) to reach the desired concentration of the peptide. 2 µL of Fluoprobes 488 NHS stock solution were added to 98 µL of the peptide solution. The sample was then gently mixed with a pipette and incubated at room temperature for 5 minutes.

Three different CSF samples (48 µL) were prepared using the same original CSF aliquot. One sample was filtered on a 3 kDa centrifugal unit. This filtrated sample and another non-filtrated one were diluted 2-fold in a borate buffer (pH 10.5, 40 mM IS) containing a mixture of the three Aβ peptides (1-38, 1-40 and 1-42) at 1µM concentration while the third one was just diluted 2-fold in a borate buffer. Then, we added 2 µL of sodium hydroxide 0.1 M to each 96 µL sample to reach pH 10.5. Finally, 2 µL of Fluoprobes 488 NHS stock solution were added to each CSF sample. The samples were then gently mixed with a pipette and incubated at room temperature for 5 minutes before analysis.

2.5.2. Coating of the microchip channels

Before the first use, new microchips were systematically flushed with 1 M NaOH for 10 min, 0.1 M NaOH for 10 min and deionized water for 10 min by applying vacuum.

To perform the PEO coating (PEO 1), the microchip was first rinsed with 0.1 M NaOH for 3 min, deionized water for 3 min, and HCl 1 M for 3 min. Then, three reservoirs (B, SW and BW) were filled with PEO solution (0.2% (m/v) in HCl 0.1M) and vacuum (30 mbar) was applied for 5 min to the S reservoir in order to fill all the microchannels. The microchip was then rinsed with the BGE. Another PEO coating protocol (PEO 2) was tested using the same protocol except that the rinsing with HCl 1M was omitted.

For EpDMA coating, three protocols were tested. The first protocol (EpDMA 1) was performed by rinsing the microchannels with NaOH 1 M for 5 min, then with deionized water for 5 min, followed by a 10 min rinsing with HCl 0.1 M, and finally 5 min of deionized water. The polymer solution in ammonium sulfate was then hydrodynamically flushed (2 mL/hr) in the channel for 30 min using a syringe pump fixed at the BW reservoir and filled by the polymer solution. The microchip was then rinsed extensively with water and dried at room temperature for a few hours. Two alternative protocols adapted from Rech *et al*. [45] were also tested. For EpDMA-2 protocol, the microchip was rinsed for 10 min with NaOH 0.1 M before the introduction of the polymer solution as in protocol EpDMA 1. The chip was then rinsed extensively with water and finally the microchannels were emptied by applying vacuum at BW reservoir. The coated chip was then directly used after filling the channels with the BGE. The EpDMA 3 protocol was the same than EpDMA 2 except that the polymer was dissolved in plain water.

2.5.3. Microchip capillary electrophoresis

Before each analysis, the coated microchip was rinsed for 5 min with the BGE. To load the peptide samples and to separate the analytes, a set of electrical voltages was applied to the four reservoirs.

The set of voltage depended on the kind of polymer coating employed to modify the glass channel surface (PEO or EpDMA). In all cases, the pinched mode was performed for the injection. For PEO coated microchips, the sample was loaded by applying -200 V to S, -100 V to B, +300 V to SW and -700 V to BW. After 2 minutes, the set was switched to separation step by applying +600 V to S, 0 V to B, +650 V to SW and 2000 V to BW. PEO coating protocol was repeated after three successive Aβ peptides analyses performed in the same microchip. For EpDMA coated microchips, the sample was loaded by applying 0 V to S, -100 V to B, +350 V to SW and -1200 V to BW. After 90 seconds, the set was switched to separation step by applying +350 V to S, -500 V to B, +400 V to SW and 2000 V to BW. For EpDMA, no coating regeneration was needed. After four successive Aβ peptides analyses, the microchannels were just rinsed with water (5min) and then with BGE (5 min) before a new analysis series. For uncoated microchips, the sample was loaded by applying 200 V to S, 100 V to B, -300 V to SW and 700 V to BW. After 90 seconds, the set was switched to separation step by applying -600 V to S, 0 V to B, -650 V to SW and -2000 V to BW. The microchannels were rinsed with NaOH 0.1 M (5 min), water (5min) and then with BGE (5 min) before each new analysis.

2.5.4. Measurement of the electroosmotic mobility:
As very low electroosmotic flow was generated in the coated microchips, we applied the constant effective mobility method as described by Wang *et al.* [48] to estimate the residual EOF mobility (μ_{EOF}) inside the channels. The apparent mobility (μ_{app}) of the fast migrating and negatively charged Fluoprobes 488 NHS, was measured in the coated glass microchip. In a separate experiment, the effective mobility of this compound (μ_{ep}) was determined by CE using a capillary (thermally controlled at 25°C) in which the EOF was already known, thanks to the analysis of a neutral marker (thiourea). The μ_{EOF} in the coated microchip was then calculated by subtracting the μ_{ep}, obtained from the CE experiments from the μ_{app} of the Fluoroprobes experimentally measured in microchip. The microchip experiments were performed in a separate room where the temperature was set at 21°C.

2.6. Validation of the methods

The repeatability of the methods was estimated on both PEO 1 and EpDMA 2 coated glass microchip by measuring RSD of migration times and peak areas obtained for Aβ 1-38 at a concentration of 10µM. Four successive analyses of the peptide were performed without any rinsing step between runs.
The separation performance has been evaluated on both PEO 1 and EpDMA 2 coated glass microchip. A mixture of Aβ1-38, 1-40 and 1-42 amyloid peptides (20µM each) was analyzed using the two methods and the Rs between Aβ 1-38 and Aβ 1-40 and between Aβ 1-40 and Aβ 1-42 were calculated.

2.7. Recovery, calibration curve and LOD:

Standard solutions containing the Aβ peptide 1-38 at concentrations ranging from 0.48 µM to 96.8 µM were employed to evaluate the linearity of the response. The samples were analyzed in triplicate for each concentration level, 96.8 µM, 48.4 µM, 12.1 µM, 2.42 µM, 1.21, 0.61 µM and 0.48 µM. T-test and Anova were performed to confirm the linear regression.
Possible adsorption of the peptides to the channel wall was estimated by recovery studies according to a method adapted from Preisler *et al.* [49] and described in detail by Tran *et al.* [50]. This equation is in accordance with the more general one proposed by Espinal *et al.* for any kind of distances employed [51]. The recovery of Aβ1-38, 1-40 and 1-42 amyloid peptides was determined by analyzing them individually at a concentration of 20 µM and by comparing the peak areas obtained during the same analysis at two detection points (35 mm and 70mm).

The recovery percentage (X) was then deduced from the equation (1):

$$X\ \% = 100 \times (A_L/A_S)^{L/(L-S)} \qquad (1)$$

where L is the longest effective length (70mm), S is the shortest one (35mm), and A_L and A_S are the peak areas of peptides detected at 70 mm and 35 mm, respectively.

3. Results and discussion

3.1. Optimization of the coating protocol

In order to select the most efficient polymer coating and protocol to achieve a reproducible and high resolution separation of the amyloid peptides in glass microchip, we have compared several procedures relying on two neutral polymers: PEO and EpDMA. These polymers have already been described for reducing EOF or protein adsorption during electrokinetic separations by CE [52] [53]. In particular, EpDMA has been reported one time for MCE of proteins, but using the IEF separation mode [46] while PEO has been employed only in conventional CE [54]. Critical parameters such as microchip pretreatment, polymer solution composition and nature, and coating rinsing steps were investigated. Based on previous works performed by CE [49] [50] [55], we selected first 0.2% (m/v) of PEO to perform the coating. Other concentrations from 0.1% to 0.4% did not improve the coating performances. Concerning the EpDMA solution, a concentration of 0.6 % (m/v) was chosen, as described in a previous work [47].

Coating protocols were evaluated by measuring the electroosmotic mobility (μ_{EOF}) in the resulting coated microchannels. RSDs of the μ_{EOF} were also compared to estimate the stability of the coatings after 5 and 10 successive analyses. Using EpDMA, three protocols (EpDMA 1, EpDMA 2 and EpDMA 3) were evaluated. The EpDMA 1 procedure integrates a protonation step, by rinsing the microchip channels with HCl 0.1 M before flushing it with the polymer solution, while EpDMA 2 and 3 protocols entail a deprotonation step, by rinsing the glass surface with NaOH 0.1 M EpDMA 2 and EpDMA 3 are equivalent except that the medium of the polymer solution is ammonium sulfate in EpDMA 2 and water in EpDMA 3.
With PEO, it is possible to coat silica capillaries with or without acidic pretreatment for high performance as shown by Iki *et al*. in 1996 [40]. So far, only the coating protocol with acidic pretreatment has been adapted to glass microchip, but only for μ_{EOF} reduction purposes [56]. The two procedures have been therefore tested. Table 1 summarizes the μ_{EOF} obtained with different protocols and the resulting RSD for μ_{EOF} measured at pH 8.0 in HEPES buffer.

Table 1 Electroosmotic mobilities and their corresponding RSD after 5 (n=5) and 10 (n=10) consecutive analyses for uncoated or coated glass microchip using EpDMA or PEO and different coating protocols (see materials and methods section for more details). The measurements were performed in 50 mM HEPES at pH 8.0

	µEOF		
Protocols	**intensity** ($m^2\ V^{-1}\ s^{-1}$) 10^{-08}	**%RSD** (n=5)	**%RSD** (n=10)
Uncoated	3.84	2.7	
EpDMA 1	1.26	8.7	15.1
EpDMA 2	1.06	1.3	3.5
EpDMA 3	1.03	0.9	18.4
PEO 1	1.33	1.8	-
PEO 2	1.30	5.2	-

- Not determined since coating regeneration was required every 5 runs for EOF marker analysis

We first noticed that all the protocols led to a 3 or 4 fold decrease of μ_{EOF} compared to uncoated glass microchip at pH 8.0. By comparing the μ_{EOF}, we deduced that the best protocols for efficient shielding of surface charges were EpDMA2 and 3 ones. EpDMA 1 coating protocol produced high RSDs for μ_{EOF} which were even amplified after 10 analyses. The EpDMA 1 coating is less stable. Probably the protonation of the silanol groups before the polymer adsorption hinders the formation of covalent bonds between the epoxy groups of the polymer and the surface silanols by nucleophilic addition. In the absence or with a reduced number of covalent bonds, the stability of the coating could be seriously compromised at extreme pH values. On the contrary, EpDMA 2 and 3 protocols, performed with a polymer solution dissolved in ammonium sulfate or water, but after a deprotonating rinsing step, gave very good RSDs. However, after 10 successive analyses, the RSD reached 18% with EpDMA 3 protocol (table 1). These results are in accordance with those of Chiari's group which demonstrated that ten runs were sufficient to deteriorate an EpDMA coating when polymer is dissolved in water and that the use of anti-chaotropic salts in the coating solution generated a more stable coating overtime [45].

For PEO, as the coating was not stable, a regeneration of the coating was required after 4 successive analyses. In comparison to EpDMA 2 and 3, the μ_{EOF} were less decreased. The successive measurements led to much lower RSD using PEO 1 protocol than PEO 2 one. We concluded that the protonation of the silanols before the adsorption of the polymer ensures a higher number of hydrogen bounds with PEO and then a better stability of the coating. The overall results indicated that EpDMA 2 and PEO 1 procedures provided the best coating stability and efficiency, and further studies were carried out using these two surface treatment protocols.

3.2. μ_{EOF} as a function of buffer pH:

During electrokinetic separations, μ_{EOF} is greatly dependent on the efficiency and stability of the coating. Extreme pHs may remove partially the coating. This may result in peptide adsorption and lack of reproducibility. The μ_{EOF} values in EpDMA 2 and PEO 1 coated and uncoated channels were evaluated at different pHs ranging from pH 3 to 9. RSDs for μ_{EOF} were also estimated at each pH. Figure 2 illustrates the μ_{EOF} variation in the three conditions. As expected, the μ_{EOF} in native glass chip increased from pH 3 to pH 9 due to ionization of silanols [57]. Using both polymer coatings, the μ_{EOF} was markedly decreased over the entire range of investigated pHs.

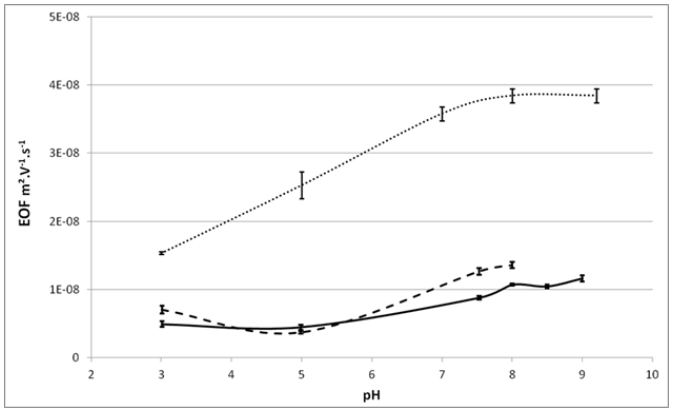

Fig. 2 Evolution of EOF mobilities in PEO (dash line), EpDMA (solid line) coated and uncoated glass microchip (dotted line) as a function of pH. Bars indicate RSD of the μ_{EOF} calculated from 5 successive measurements.

PEO 1 and EpDMA demonstrated a good stability until pH 8.0 and 9.0, respectively. Indeed, both coating protocols showed very low RSD for μ_{EOF} (less than 2.5 %) for most pHs, excepted at the most acidic and alkaline ones (with RSD reaching 5.0% in that cases).

For PEO, the polymer molecules are held to the microchannel walls only by weak interactions, such as Van der Waals forces and hydrogen bonds. EpDMA has a strong adhesive character with formation of hydrogen bonds between silanol groups of PDMA backbone. Even though oxiranes stabilize the coating by covalently reacting with silanols, still the pH of the BGE may be a critical parameter to ensure polymer adhesion through hydrogen bounds. Although not completely suppressed, the EOF was significantly lower than that of the uncoated capillary in both cases. This is an interesting feature, as residual EOF can, in principle, help in reaching high resolution. From this preliminary study, we concluded that the two coating procedures give stable and efficient coatings and could be used with high repeatability using BGE from pH 3 to 8 for PEO 1 and from pH 3 to 8.5 for EpDMA 2.

3.3 Analysis Repeatability and Resolution performance

In order to further compare the two methods, we have performed a repeatability study for the analysis of the FP488 labeled Aβ 1-38 peptide. The separation was performed in reverse polarity, as the µep of the peptides are higher than the µEOF at pH 8.0. Figure 3 shows the corresponding electropherograms obtained from successive analyses of Aβ 1-38. Repeatability data obtained for PEO 1 (n=3) and EpDMA 2 (n=4) coated glass microchips under the same separation conditions revealed good repeatability of migration times and peak areas with both polymers. Slightly better results were obtained using EpDMA 2 with RSD of 0.5 % and 2.1 % for migration times and peak areas, respectively. In addition, EpDMA 2 allowed at least four successive analyses without any rinsing or coating regeneration while PEO 1 was not stable over 3 analyses of Aβ peptide samples.

Three Aβ peptides, 1-38, 1-40 and 1-42, have been selected to evaluate the resolving capacity of the microchip coated by the two methods in comparison to an uncoated glass microchip. These peptides exhibit the same charge at pH 8 (theoretical pI of 5.41 for the three peptides) and differ only by their length (from one or two

amino acids). Their separation was already achieved by CZE with a separation distance of 50 cm [26]. However, obtaining a good resolution was quite challenging using separation microchannels of only a few centimeters. Figure 4 presents the electropherograms obtained from the analysis of the peptide mixture with PEO-1 and EpDMA-2 coated glass microchips or uncoated glass microchip, using the same optimized separation buffer. The two methods, PEO-1 and EpDMA-2, allowed a good resolution of the three peptides, while with the uncoated chip, unresolved peaks and a lack of repeatability were observed. This confirmed the massive adsorption of analytes in the uncoated microchip. The resolution was slightly better with EpDMA 2 based method with Rs of 1.7 and 1.1 between 1-38 and 1-40 and 1-40 and 1-42, respectively, while PEO 1 led to a resolution of 1.5 and 1.0 respectively. The Rs obtained between these close migrating species, using EpDMA 2 method, are slightly lower than the ones obtained previously by CE [26], but they remain sufficient for a simultaneous quantification of the three peptides, which is mandatory for a high performance diagnosis tool based on Aβ biomarkers.

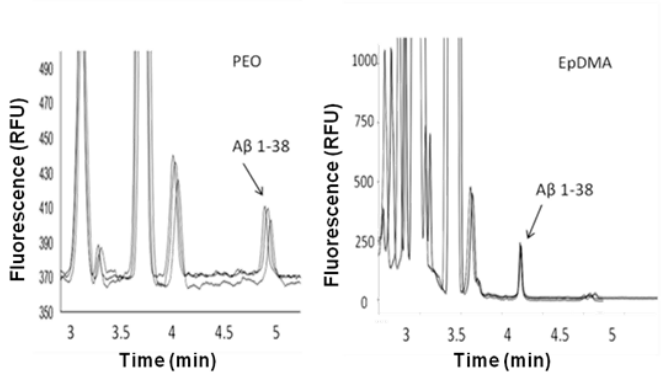

Fig. 3 Microchip electropherograms obtained from successive analyses of labeled Aβ 1-38 using PEO 1 (n=3) or EpDMA 2 (n=4) procedures to coat the glass microchip. BGE: HEPES/HCl Buffer, pH 8.0, 100 mM. Sample: Aβ 1-38 (10 µM) diluted in Borate buffer pH 10.5, 40 mM. Fluorescence Detection: 488 nm at 70 mm from the injection cross.

Using PEO-1, we also noticed a change in the migration time of the peptide Aβ 1-38, by 7.35%, between two sets of experiment performed on two different coated microchips. This indicates a poor inter-microchip repeatability of the PEO-1 coating procedure. In contrast, the inter-chip repeatability coating protocol of EpDMA-2 seemed to be much better, as the migration times of the peptide did not vary more than 0.5%. This observation confirms the higher performance of EpDMA-2 as coating for the separation of Aβ peptides in glass microchip.

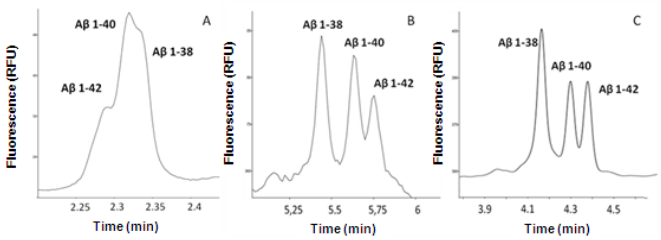

Fig 4 Separation of three Aβ peptides (Aβ 1-38, Aβ 1-40 and Aβ 1-42) at 20 µM using uncoated glass microchip (A), PEO coated (B) or EpDMA coated (C) glass microchip. Conditions are same as in Figure 3.A.

3.4 Recovery, calibration curve and limit of detection:

To ensure precision and accuracy for the quantification of biomarkers from biological fluids, it was mandatory to check that negligible adsorption of the peptides occurred on the coated channels walls. A recovery study has been performed in order to estimate the possible loss of analyte after adsorption to the inner wall during the separation step. The recovery percentages of Aβ 1-38, Aβ 1-40 and Aβ 1-42 and in the two coated microchips were determined by comparing the peak areas obtained for each peptide at two distances from their injection point and using the equation described in experimental section.

Very good recoveries for Aβ 1-38 and Aβ 1-40 with values close to 100% were observed with both methods (Table 2). The recovery for Aβ 1-42 was lower, especially when the PEO coating was employed. This can be explained by the higher hydrophobicity of Aβ 1-42 and its ability to self-aggregate at micromolar concentrations as recently described [32]. The recovery value for this peptide increased to an acceptable value of 93% with the EpDMA 2 coating. EpDMA has indeed a polydimethyl acrylamide backbone with oxirane groups attached to the skeleton. The oxirane groups enhance the hydrophilicity of the copolymer while at the same time maintaining the excellent adsorptive properties of the EpDMA on glass even at pH 8.0. This specific polymeric structure allows an excellent limitation of proteins or peptides adsorption on the coated microchannel wall.

Table 2 Recovery % of Aβ1-38, Aβ1-40 and Aβ1-42 on EpDMA 2- and PEO 1 -coated glass microchips. Detection performed at 35 mm and 70 mm from the injection cross. BGE: HEPES/HCl, 100 mM, pH8.0.

Peptides	PEO	EpDMA
Aβ 1-42	84	93
Aβ 1-40	101	98
Aβ 1-38	97	98

Finally, considering the excellent performance of the method based on EpDMA coating in terms of reproducibility, recovery, and resolution, the EpDMA coated glass microchip method was validated in terms of linearity and limit of detection for the Aβ 1-38. The linearity of the detection was assessed by performing triplicate analyses of Aβ 1-38 solutions at concentrations ranging from 0.48 to 100 µM. The determination coefficient R^2 was equal to 0.990 (see ESI). On a significance level of 0.05, the t- test demonstrated the variance homogeneity at each concentration. Then, ANOVA test demonstrated that the linear regression fitted the experimental data for concentrations ranging from 0.48 to 10 µM. The detection limit, considered as the minimum analyte concentration yielding a signal-to-noise equal to three, was 240 nM, while the value obtained statistically through the regression data was 185nM.

3.5 Application to amyloid peptides mixture and to spiked CSF

Finally, we applied the validated EpDMA coated MCE method to the separation of different mixtures containing truncated amyloid peptides of relevance for the diagnosis of AD [12, 13]. As shown in Figure 5.A, full separation of Aβ 1-38, Aβ 1-40, Aβ 1-42 is achieved for the first time in microchip capillary zone electrophoresis. In addition, a successful separation of three different N and C truncated variants of Aβ1-42 (Aβ 1-42, Aβ 2-40 and Aβ 2-42), recently patented for their diagnosis value in AD [29], is also presented in Figure 5.B. Finally, the method was applied to a real sample of CSF to evaluate the compatibility of the method with this biological medium. Indeed, the complexity of the CSF matrix was expected to deteriorate the resolution. In order to investigate the impact of the high salt concentration of CSF on the method performance, the CSF was analyzed before and after a desalting step

performed by a filtration on a 3 kDa centrifugal unit and results were compared in term of resolution between the three Aβ peptides. As our high resolution separation MCE method is not sensitive enough to detect Aβ peptides directly from CSF, both CSF samples were, therefore, spiked with the same concentration (1 µM) of a mixture of three Aβ peptides (Aβ 1-38, Aβ 1-40, Aβ 1-42). In addition, a non-spiked labeled CSF sample was also analyzed to check for potential interferences in the migration region of interest. The resulting profiles are compared in Figure 6. It clearly indicates that the presence of salts in the sample impacts on the amount of peptides injected in the separation channel as the peak areas were lower for the non-filtered CSF. The most important result is that the presence of salts does not seem to affect the resolution. The non-spiked CSF results pointed out that no interfering species were migrating in the relevant region.

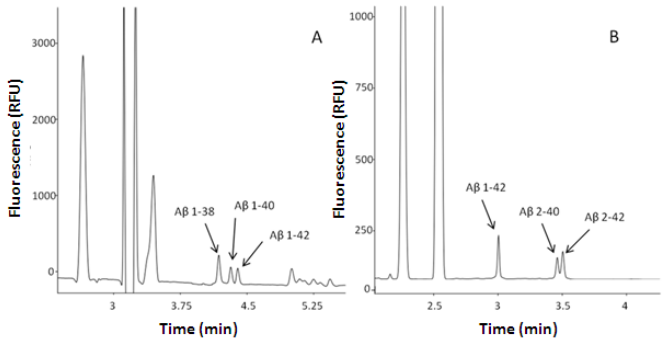

Fig. 5 Electrokinetic separation of two mixtures of Aβ amyloid peptides performed on a EpDMA coated glass microchip. MCE separation conditions: as in figure 3 for (A) and (B) with the exception in (B) of the BGE pH which was changed to pH 7.5 and voltages applied for the separation step: B (-1000V) and BW (3000V). Detection at 75 mm from injection cross.

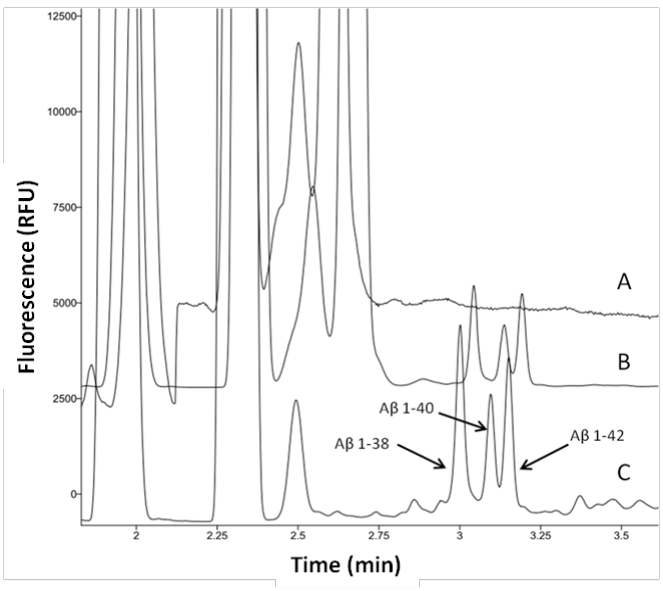

Fig. 6 MCE analysis of human CSF sample, derivatized with Fluoprobes 488 NHS (A), the same human CSF spiked with 1 µM of Aβ 1-38, Aβ 1-40 and Aβ 1-42 (B), CSF filtered on a 3 kDa membrane and then spiked with 1 µM of Aβ 1-38, Aβ 1-40 and Aβ 1-42 (C). MCE conditions are the same as Figure 3.A. except voltages applied for the separation step: B (-1000V) and BW (3000V). Detection at 75 mm from the injection cross.

Conclusions

We have developed for the first time a microchip capillary zone electrophoresis method able to achieve high resolution separation of closed migrating Aβ peptides in a coated glass microchip. This simple method has been validated in terms of linearity and repeatability and was applied successfully to two mixtures of Aβ peptides described as relevant combinations for discriminating AD from nD patients. The fully validated method was applied then to spiked CSF samples with no loss of resolution between the three Aβ peptides (Aβ 1-38, Aβ 1-40, Aβ 1-42). Compared to the conventional CE method previously published [26], this MCE strategy provides ease and fast surface modification allowing six times faster separations of Aβ peptides while keeping excellent repeatability, recovery and resolution for simultaneous quantification. The miniaturization solves the first bottleneck of the development of a diagnostic tool, by demonstrating the possibility to separate with still high resolution these peptides in microchip. The sensitivity of detection in this method is not yet sufficient for the direct detection of Aβ peptides in CSF, as a preconcentration factor of at least 200 is needed. Efforts are currently directed toward integrating a preconcentration step before this separation.

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Notes and references

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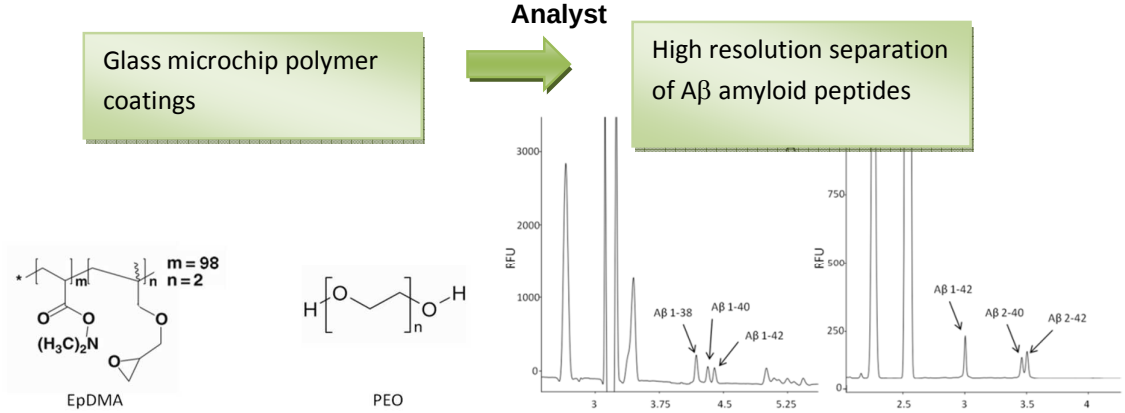

EpDMA , an efficient coating for glass microchip to achieve high resolution separation of relevant Aβ peptides for Alzheimer disease diagnosis.