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Magneto-immunocapture with on-bead fluorescent labeling of amyloid-β peptides: Towards a microfluidized-bed-based operation

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Abstract

A new sample treatment approach for sensitive determination of three amyloid-β peptides (Aβ 1-42, Aβ 1-40 and Aβ 1-38) with capillary electrophoresis coupled with laser induced fluorescent detection is reported herein. These Aβ peptides are considered an important family of biomarkers in the cerebrospinal fluid (CSF) for early diagnosis of Alzheimer's disease (AD). Due to their extremely low abundance in CSF (down to sub nM ranges), batch-wise preconcentration via magneto- immunocapture with enrichment factors up to 100 was implemented. The Aβ peptides were first captured onto magnetic micro-beads. Then, on-beads fluorescent labeling of the captured Aβ peptides were carried out to avoid the unwanted presence of extra fluorescent dye in the eluent as in the case of in-solution labeling. Finally thermal elution was performed and eluted labeled peptides were analyzed off line with CE-LIF. The Aβ-capturing efficiencies of different commercially available antibodies grafted onto magnetic beads were tested. Aβ peptides in CSF samples collected from AD's patients and healthy persons (used as controls) were measured and evaluated. As a proof of concept, the developed strategy was adapted into a miniaturized fluidized bed configuration that has the potential for coupling with a microchip separation system.

1. Introduction

Amyloid β (Aβ) peptides having from 37 to 43 amino acids (AA) are naturally produced in our body fluids, mainly cerebrospinal fluid (CSF), via proteolysis of a larger protein known as the amyloid precursor protein (APP) [1]. Among these Aβ isoforms, Aβ 1-42 and Aβ 1-40 are considered well-established and internationally validated biomarkers for early diagnosis of Alzheimer's disease (AD) [2]. CSF is a relevant biological fluid for the monitoring of AD evolution as it reflects molecular events occurring in the brain thanks to its direct contact with the extracellular brain space [3]. In AD patients, the CSF levels of Aβ 1-42 (< 500 pg/mL) are lower than those in normal people (about 800 pg /mL) due to its aggregation in the brain's senile plaques [2]. On the other hand, low levels of Aβ 1-42 in CSF may be also associated with other neurodegenerative diseases (NDs), notably dementia with Lewy body (DLB), Parkinson's disease (PDD) or Creutzfeld-Jacob disease (CJD) [4]. The determination of Aβ 1-42 together with other biomarkers has therefore been implemented for more precise discrimination of AD from other NDs. Different proposed biomarker combinations for AD include Aβ 1-42 and Tau / p-Tau protein [5, 6], Aβ 1-42 / Aβ 1-40 ratio [6, 7], Aβ 1-42, Aβ 1-40 and Aβ 1-38 [8, 9], the ratio of Aβ 1-42 / (Aβ 1-42 + Aβ 1-40 + Aβ 1-38) [10], the ratio Aβ 1-40/42 and those between Aβ 1-37, Aβ 1-38 and Aβ 1-39[11,12] and Aβ 1-42 together with Aβ 2-42 [13]. Esselmann *et al.* described in their patent the use of quantitative ratio of Aβ 1-42 , Aβ 2-40 and Aβ 2-42 for early diagnosis of AD [14].

In the context of determination/quantification of different AD's biomarkers in general and Aβ peptides in particular in CSF, the most frequently employed analytical approaches are immunoassays [15-19], mass spectrometry [16, 20-23] and Western blot [11, 24-26]. Like the two other techniques, the immunoassay based ones (MSD, xMAP, ELISA) can now detect different AD's biomarkers at the same time as they can employ simultaneously different antibodies

specific for each biomarker [15, 27]. However, all these methods, albeit being well established and having been used as reference techniques, are hardly convertible into miniaturized formats and therefore have little or no potential for production of point-of-care devices for early AD diagnosis purpose.

One alternative to the aforementioned techniques which has a high potential for miniaturization, automation and integration while offering also peptide separation capabilities is electrokinetic separation. Capillary zone electrophoresis (CZE), being the simplest mode of this technique, was applied successfully for separation and quantification of a mixture of different Aβ peptides down to 300 – 500 nM with UV detection [28] and 35 nM with laser-induced fluorescent detection (LIF) [10], respectively. Downscaling of this approach to microchip platforms has also been reported by our group with detectable levels of Aβ peptides close to 200 nM [9, 29]. These detection limits however were not sufficient for analyses of CSF samples where concentrations of Aβ peptides are only at sub nM ranges. Recently Wiltfang 's group presented a novel method based on capillary isoelectric focusing (CIEF) immunoassay which was capable of detecting total Aβ in CSF after desalting / buffer exchange [26]. This method nevertheless is not applicable to C-truncated peptides possessing the same isoelectric point such as Aβ 1-38, Aβ 1-40 and Aβ 1-42. To compensate for the insufficient sensitivity of Aβ peptides determination via electrokinetic techniques, inclusion of a forefront sample treatment module based on magnetic immuno-capture, so-called immuno-precipitation (IP) has been proposed [10, 26, 29, 30]. This technique belongs to the sample treatment strategies that are based on bio-functionalized magnetic nanoparticles. They are particularly suitable for peptides pre-concentration due to their large surface area, biocompatibility and ease in manipulation [31]. To further enhance the detection sensitivity, one option is to combine the magnetic pre-concentration step with fluorescent chemical labeling to allow then LIF detection. Fluorescent derivatization of Aβ peptides was already carried out off-line prior to

the magnetic pre-treatment step [29, 30]. However this labeling - capture - elution sequence was hardly adaptable to the microchip platform due to at least two following limitations: 1) the requirement of one additional microchamber containing no microbeads for fluorescent labeling of Aβ peptides prior to their magneto-immunocapture and 2) the difficulty in well mixing the laminar flows of sample and fluorophore solutions in microchannels. In addition, the previous studies employed magnetic micro-beads that were kept stationary in the form of tiny columns inside the micro-chip. This may not offer optimal contact between Aβ peptides and the beads' surface which is needed for higher capture efficiency.

Herein we reported for the first time an integrated capture - label - elution strategy that allows Aβ peptides enrichment via magnetic-bead-based immunocapture followed by their in-situ fluorescent-labeling directly on beads prior to CE-LIF operation. One of the advantages of this novel sample treatment strategy is that the unwanted presence of extra fluorescent dye and fluorescent side products [9, 10] are avoided in the electrophoresis profile. In an effort to enhance IP efficiency, different antibodies with varying specificities toward several Aβ peptides were investigated. The optimized whole sample treatment process developed in batchwise mode, including magnetic-bead-based capturing, on-bead fluorescent labeling and thermal elution offered enrichment factors up to 100. The batch-wise mode was then downscaled into a micro-fluidized bed for perspective coupling with microchip electrophoresis operation. The magnetic beads in this case were made circulated inside the microchannel rather than kept stationary during the passage of the sample flow for improvement of contact between Aβ peptides and magnetic beads and to increase the analysis throughput.

2. Experimental

2.1. Chemicals, reagents and samples

Amyloid peptides Aβ 1-38 and Aβ 1-40 were purchased from Anaspec (Fremont, CA, USA) whereas Aβ 1-42 was obtained from American peptide (Sunnyvale, CA, USA). Boric acid, formic acid, phosphate buffered saline (PBS 10X), bovine serum albumin (BSA), ammonium hydroxide 28.1 % (m/V), sodium hydroxide, dimethyl sulfoxide (DMSO, 99.9% purity), triethanolamine (TEA), IgG from murine serum (reagent grade, ≥ 95%) and diaminobutane chloride (DAB) were provided by Sigma (St. Louis, MO, United States). The Fluoprobe 488 NHS (FP-488) was purchased from Interchim (Montluçon, France) and was dissolved in DMSO to obtain aliquots of 10 mg / mL which were then stored at -20°C in the darkness. Dimethyl pimelimidate dihydrochloride (DMP) was purchased from Thermo Scientific (Rockford, USA). All buffers were prepared with deionized water purified with a Direct- Q3 UV purification system (Millipore, Milford, MA, USA).

Magnetic micro-particles (diameter of 2.8 μm) surface-bound with sheep anti-mouse IgG (Dynabeads M-280, 10 mg/mL) and different monoclonal anti-Aβ antibodies (6E10, 12F4 and 4G8) were purchased from Covance, Emeryville, CA. The magnetic microbeads employed in our work were the same as those reported in ref [10]. All CSF samples were taken by the department of Neurology, university of Ulm (Ulm, Germany), aliquoted and stored at – 20°C until use. The sampling procedure was detailed elsewhere [9, 10]. Our CSF samples were provided by the University of Ulm. Their collection and analysis were approved by the Ethics Committee at the University of Ulm.

2.2. Materials and Apparatus

All CE-LIF analyses were implemented with a Beckman Coulter PA 800 ProteomLab coupled with a LIF detection system (a 3.5 mW argon-ion laser having an excitation wavelength of 488 nm and a 520 nm band-pass filter for collection of the emitting beam light). Data acquisition and instrument control were realized with the Karat 7.0 software. Bare fused silica capillaries of 375 µm o.d. and 50 µm i.d. were purchased from Phymep (Paris, France). pH values of solutions were controlled with an inoLab WTW series pH 730 meter. Amicon ultra 0.5 centrifugal filters (3 kDa) were purchased from Millipore Ireland (Cork, Ireland). Thermal operation was carried out with a GC oven (GC 5890, series II from Hewlett Packard, USA).

The micro-fluidized bed employed in this work was adapted from the design reported recently [32, 33]. The chips were fabricated from cyclic olefin copolymer (COC) material to withstand temperature increase during the elution step [34]. Before the loading of magnetic beads, the COC channel was coated with BSA 1% to prevent / minimize any possible accumulation of the beads onto the channel wall. More details on the setup and operation of this micro-fluidized bed can be referred to [32, 33].

2.3. Methods

Peptides preparation and storage

Stock Aβ 1-42 was prepared in ammonium hydroxide 0.16 % (m/V) whereas other amyloid peptides were dissolved in ammonium hydroxide 0.10% (m/V). Aliquot solutions (10 µL) of individual peptides were prepared at a concentration of 2 mg / mL and subsequently lyophilized to remove all traces of ammonia. These lyophilized aliquots were then stored at -20 °C until use. For preparation of standard solutions (STDs), the lyophilized Aβ peptides were diluted with borate buffer (pH 10.5, ionic strength IS 40 mM) to obtain desired

concentrations. These solutions were freshly prepared and stored at 4°C for use within one day.

CE-LIF conditions

The procedure for in-solution labeling of Aβ peptides with FP-488, as well as details on CE-LIF conditions for analyses of the fluorescence-labeled Aβ peptides can be found in our previous publication [10]. To minimize the adverse effect of CE-inherent migration time fluctuation on peak identification and quantification, the migration-time-based electropherograms of Aβ peptides after the capture-label-elution process were converted into electrophoretic-mobility-based ones. The peak of FP-488 nonspecifically adsorbed on the beads surface during the labeling process, which always appeared before those of Aβ peptides in the electropherograms, was employed as a reference for this profile correlation.

Magneto-immunoprecipitation with fluorescent labeling

Magnetic micro-particles (Dynabeads M-280, 10 mg in 1 mL) were separately coated with 40 µg of different monoclonal anti-Aβ antibodies (*i.e*. 6E10, 12F4, 4G8 and IgG) according to the manufacturer protocol. Briefly, the Dynabeads M-280 (10 mg) and the antibodies (40µg) of selected type were suspended in ~~a~~ 1 mL of PBS 1X solution containing 0.1 % (m/V) BSA, and were incubated over night on an orbital shaker (VXR basic Vibrax, Ika, Staufen, Germany) at 4 °C. The mixture was then allowed to react with DMP for 1h at room temperature under a basic condition (0.2 M TEA, pH 10.8) to crosslink the bound antibodies to the surface of magnetic microbeads via the covalent binding between this noncleavable imidoester and primary amines in the antibodies. The antibodies-bound magnetic beads were subsequently washed with PBS 1X containing BSA 0.1% and then re-suspended in PBS 1X containing BSA 0.1% and NaN_3 0.02% for storage at 4°C. These beads (10 mg / mL) did not

aggregate and performed well even after several months of stokage. Addition of any unnecessary compound other than the reaction medium, including the manufacturer-recommended washing buffers citrate and EDTA, in any step of the capture-label-elution procedure (even for the beads - washing purpose) was avoided to prevent ~~the potential~~ any possible triggering of aggregation of the easily-aggregable amyloid peptides, especially Aβ 1-42 and also to keep the same buffer composition for labeling, elution and separation. A neodymium magnet (Adem-Mag MSV from Ademtech, Pessac, France) was employed to retain the magnetic beads during removal or addition of a suspension solution.

For magnetic immunocapture in batch, the suspension of antibodies-coated magnetic beads (10 mg / mL) was vortexed for 3 min for homogenization before withdrawal of 50 µL aliquots. The solution was removed from the aliquot and a volume of 800 µL of either an STD solution (prepared in PBS 1X and BSA 0.1%) or CSF sample was incubated with this 500 µg of magnetic beads coated with the desired antibodies on a shaker at 4°C for 15 hours. The beads were then washed once with PBS 1X / BSA 0.1% and twice with borate buffer (pH 10.5, IS 40 mM). Subsequently, 50 µL of borate buffer containing FP-488 (0.2 – 1mg / mL) was poured into the washed beads and the suspension was vigorously orbitally shaken for 5 - 60 min at room temperature. This optimization was implemented based on our previous work on in-solution fluorescent labeling of Aβ peptides[10]. Several parameters were changed upon optimization for on-beads fluorescent labeling, including the shaking time (from 5 to 60 min) and the concentration of FP-488 (from 0.2 to 1 mg /mL). The optimized on-beads labeling conditions (FP-488 at 0.6 mg / mL, shaking time of 30 min, see section 3.1.2) were employed throughout all capture and elution experiments. The beads were then washed twice with borate buffer to remove all un-reacted FP-488, followed by elution of the labeled Aβ peptides bound on beads. Different elution approaches were tested, including chemical elution with

borate buffer (pH 10.5) or 0.1M formic acid (pH 2.2) at ambient temperature and thermal elution at 95°C. The eluents were subsequently subjected to CE-LIF analyses without further dilution. Note that the eluent volumes were dependent on the desired enrichment factors. For the tested enrichment factors of 40 and 100, the used eluent volumes were 20 µL and 8 µL respectively.

For immunocapture with the fluidized bed platform, 50 – 100 µg of magnetic beads were injected in the microchip. The cross diameters of its input and output channels were 110 µm and 100 µm, respectively. The microbeads - containing chamber had a conic form that reached a diameter of 1800 µm at its widest width. The total volume of the chamber was 0.46 µL whereas that occupied by the microbeads was 0.085 µL. 100 µL of an STD solution of Aβ peptides (60 nM) was injected in the chamber with a flow-rate of 1 µL / min. The beads were then washed with 20 µL of borate buffer at a flow-rate of 1.5 µL / min, followed by passage of 15 µL of borate buffer containing FP-488 at 0.5 µL / min. After this labeling step, the extra and unreacted fluorescent dye was washed out of the chamber with a flow of borate buffer (2 µL / min for 20 min). For thermal elution, the chip was heated to 70 °C once the flow inside the chamber was completely stopped. The neodymium magnet was always positioned at the bottom of the fluidized bed to prevent the magnetic beads from escaping out of the system. Upon conclusion of the elution step, the solution inside the fluidic chamber was pushed into a PEEK tubing (length of 30 cm, internal diameter of 250 µm) connected at the output of the micro-fluidic chip. The solution inside this reservoir tubing was then collected for subsequent CE-LIF analyses.

3. Results and Discussion

3.1. Magnetic-beads-based sample treatment optimization

The sample treatment operation can be divided into three steps: 1) capture of the Aβ peptides onto the antibody-grafted magnetic beads, 2) chemical fluorescent labeling of the Aβ peptides by the fluoprobe FP488 and 3) elution of the peptides from the immunosupport into a small and defined eluent volume for analyte preconcentration and further electrokinetic separation. These steps can in principle be carried out with different orders. We selected the capture - labeling - elution sequence as it was expected to simultaneously satisfy efficient Aβ peptides enrichment, removal of extra fluorophore and possible translation from batchwise into microfluidic fluidized bed format. Compared to the method of static interaction where the antibodies are immobilized onto a planar surface, the circulation of the magnetic beads in our approach is expected to facilitate the dynamic interaction between the antibodies and the Aβ peptides, which in turn should improve the immune-capture efficiency. This also is the case for subsequent on-beads fluorescent labeling. In addition, the surface area provided by a microbead of diameter D (Area = $\pi \cdot D^2$) is much larger than that of a square planar surface having the same length D (Area = D^2). As a result, for the same antibody immobilization efficiency, a higher density of antibodies is expected for micro-beads, allowing better immuno-capture and higher enrichment gains.

3.1.1. Capture of Aβ peptides onto magnetic beads

The capture of Aβ peptides onto the magnetic beads is based on specific interaction between Aβ peptides with the antibodies grafted onto the surface of the beads [29, 30, 35, 36]. The binding yields of the antibodies to the magnetic microbeads and their specificity towards the targeted peptide epitope play a crucial role in determining efficiency of the immune-capture process. Accordingly, three commercial monoclonal mouse antibodies from Covance, namely 6E10, 4G8 and 12F4, which are reactive to amino acid residues 1-16 (N-terminus), amino acid residues 17-24 and the C-terminus of beta amyloid, respectively were tested. 12F4 is specific

for the peptide isoform's ending at the 42th amino acid and was already used in a silicon-platform-based high-sensitivity immunoassay for Aβ 1-42 [17]. Non specific IgG from murine serum was employed to check for non specific capture. Magnetic beads grafted with the different antibodies were subjected to the same sample treatment process, *i.e.* capture – labeling – elution. Eluted fractions with the enrichment factor of 40 were directly analyzed with CE-LIF (Fig. 1). With the antibodies 6E10 and 4G8, Aβ 1-42 and Aβ 1-40 peaks could be detected whereas only that of Aβ 1-42 appeared in the case of 12F4. In the case of 6E10, the area ratio of Aβ 1-40/Aβ 1-42 was 0.44, which means that the original concentration ratio of Aβ 1-42 (8 nM) / Aβ 1-40 (15 nM) was preserved after the immunocapture process. The peaks obtained with 6E10 and 12F4 are much higher (approximately 4 times) than those with 4G8. Clearly, 6E10 and 12F4 exhibit the best performance in terms of capture of Aβ 1-40 and Aβ 1-42 for the former and Aβ 1-42 for the latter respectively under the tested conditions. To our opinion, the poorer result with 4G8 is possibly associated with: 1) low binding yields of antibodies to the beads surface, leading to an unsatisfactory on-bead antibody density and 2) less or no accessibility of primary and / or Lysine's amino groups which are needed for subsequent fluorescent labeling once the Aβ peptides are on-bead-immobilized. The efficient binding of 4G8 immobilized onto a silicon microarray platform to underivatized Aβ 1-42 and Aβ 1-39, as reported by Gagni *et al.* (see ref [17]), excludes the possibility that the immobilized 4G8 antibody shows low affinity for the concerned peptides.

3.1.2. On-bead fluorescent labeling of Aβ peptides

Considering the aforementioned preliminary results, the optimization of on-bead fluorescent labeling with FP488 was then performed with 6E10 as the capture antibody. Borate buffer (pH 10.5) was employed to provide a basic medium needed to produce mainly ditagged species formation. Dittaged species display indeed higher fluorescent intensities and are better

resolved by CE-LIF than monotagged species [10]. The alkaline pH required for achievement of desirable di-tagged peptides still belongs to the pH range for pH-independent fluorescence emission of FP-488 which is 4 - 10 according to the manufacturer. In our previous work reporting in-solution fluorescent labeling, it was found that a 5-min static incubation of Aβ peptides with 0.2 mg / mL FP-488 (molar ratio of FP-488 to Aβ peptides more than 4) was sufficient for complete in-solution peptide labeling with Aβ concentrations ranging from some dozen up to at least a thousand nM [9, 10]. The direct application of this in-solution labeling conditions however led to unsatisfactory fluorescent tagging of Aβ peptides when they were bound on biofunctionalized beads. FP-488 is a fluorescent dye that binds covalently to amino groups of the peptides. Each intact beta amyloid possesses three conjugable amino groups, *i.e.* one primary and two on Lysine's residues. Nevertheless, they can become less accessible after the binding of Aβ peptides on beads. In the case of 6E10, the primary amine of Aβ peptides might partially lose its availability once 6E10 binds to their 1-16 amino acid residues. Another obstacle to efficient on-bead Aβ labeling came from the employment of a basic medium (pH 10.5). While this medium is expected to facilitate ditagged labeling [10], it can also accelerate hydrolysis of the FP-488, which in turn could degrade the labeling efficiency over time. To overcome these problems, two optimizations were performed. Firstly, a prolongation of incubation time to 30 min in combination with vigorous agitation was carried out to favor the contact between FP-488 and on-beads bound Aβ peptides. Secondly, an increase in FP-488 concentration was implemented to compensate for the amount of FP-488 lost due to hydrolysis in the basic medium and to maintain the reaction towards stable conjugate formation over the prolonged period. Accordingly, it was found that a shaking incubation with an elevated FP-488 concentration of 0.6 mg / mL in borate buffer at room temperature for 30 min offered the best on-bead fluorescent labeling of Aβ peptides. The labeling reaction occurred under the presence of DMSO at small amounts (2 - 6 % v/v) when FP-488 prepared

in DMSO was added into the mixture. Nevertheless, the effect of DMSO on the established binding equilibrium between Aβ peptides and on-beads-immobilized antibodies (if any) was considered insignificant due to its small volumetric percentage. An efficient on-beads capture of Aβ peptides under such presence of DMSO was already reported in the label-capture-elution approach developed by Svobodoba *et al.* [30].

3.1.3. Elution of the bound and labeled Aβ peptides

Different approaches for elution of the captured Aβ right after magnetic-immunoprecipitations were already reported, including chemical elution with a large volume of ammonium hydroxide [10, 30], formic acid [35, 36]or thermal elution in the presence of a neutral buffer [26]. While these methods exhibited satisfactory performance in a two-step capture - elution procedure, the inclusion of an on-bead fluorescent labeling process in between may render the Aβ peptides elution more difficult due to the hindrance of FP-488 dyes non-specifically adsorbed onto the beads during this labeling step. Furthermore, the elution medium had to be optimized taking into consideration the subsequent CE-LIF analysis as it would significantly influence the analytical performance in terms of Aβ peak shape and sensitivity. This situation is reflected in Fig. 2 with electropherograms of fluorescently labeled Aβ 1-40 prepared in different media. The Aβ 1-40 peptide was labeled by a 5-min static incubation of Aβ 1-40 with 0.2 mg / mL FP-488 prepared in DMSO according to the procedure reported in our previous publication[10]. High and sharp peaks were obtained with alkaline media while an evident decrease in peak height was observed with a shift of pH towards acidic conditions. Based on these considerations, different elution options were then investigated and their capacities of desorbing labeled Aβ out of the magnetic beads were compared (Fig. 3). Regardless of the medium used and its pH, the chemical elution at room temperature did not lead to appearance of Aβ peaks in the electropherograms. Elution with ammonium hydroxide

0.16 - 1 % or citric acid 100 mM was tested as well, but led to no detectable peaks with CE-LIF. This confirms that the on-beads bound Aβ were not eluted by the basic medium (pH 10.5) employed during the fluorescent labeling process. Only thermal elution at 95°C for 5 min allowed detecting Aβ peptides with CE-LIF. The heating is therefore critical in breaking the interaction between Aβ peptides and the antibodies. No observable thermally induced modification of labeled Aβ peptides conformation was found, as no electrophoretic mobilities shift was observed for the thermally eluted peptides compared to the in-solution labeled ones. With this thermal elution approach, the antibodies-coated magnetic beads could be used only once because the antibodies could be denatured by such high temperature. Due to its favorable properties in terms of sharp peak shapes (Fig. 2) and high compatibility with the subsequent CE analysis, borate buffer (pH 10, IS 40 mM) was selected as the elution medium in this thermal approach. In addition the use of the same buffer compositions for thermal elution and for the fluorescent labeling is expected to facilitate the subsequent translation of this batchwise operation into micro-fluidic fluidized bed format. By employing the borate buffer, three consecutive steps, including fluorescent labeling, beads washing and elution can share the same medium.

3.2. Separation and sensitive detection of Aβ peptides in CSF samples

Excluding all washing steps, the optimized batchwise procedure of magnetic-bead-based sample treatment prior to CE-LIF comprised 15 hours of magneto-immunocapture at 4°C, 30 min of on-bead fluorescent labeling at room temperature and 5 min of thermal elution at 95 °C. With this protocol using 6E10 antibody, the salient performance data were presented in table 1. Calibration curves were acquired with satisfactory linearity (correlation coefficients more than 0.96) for the concentration ranges of 40 - 400 nM for Aβ 1-42 and 40- 800 nM for Aβ 1-38 and Aβ 1-40 respectively. Over these ranges no further increase in peak heights was

observed. The reproducibility of the inter-batch measurements of corrected peak areas and migration times was found to be about 12% and 1%, respectively. They deemed acceptable considering that these RSD values are due to the accumulation of errors of all operations*, i.e.* sample preconcentration, labeling, elution, injection and separation. In the case of repeated CE-LIF measurements of the same batch, the intra-batch RSD values for peak areas were improved to around 6 %. An enrichment factor of 100 (calculated from the sample to eluent volumes ratio) for the Aβ 1-38, Aβ 1-40 and Aβ 1-42 peptides could be obtained (table 1). The calculation of preconcentration gains based on a referenced peak area was not possible due to the unavailability of the (commercial) standard fluorescently ditagged Aβ peptides containing no extra FP-488. Deduction of the concentrations of fluorescently labeled peptides from those of the standard peptides was not done neither because the precise yield of Aβ peptides fluorescent di-tagging could not be determined. For this reason, while the relative ratios of different peptides captured on micro-beads could be provided (see section 3.1.1), information on the absolute recovery was not obtainable. The smallest Aβ concentrations that were detectable by LIF detection, in other words the detection limits without preconcentration, where 8 - 10 nM, which are almost 4 times lower than that reported in the precedent work on CE-LIF [10]. This is mainly due to the absence of exceeding fluorescent dye in the final sample solution, which is only made possible with on-bead labeling approach. Further improvement in LODs could be achievable just by increasing the amount of magnetic beads in the batchwise capture process. This however was not envisaged as diminution of beads amount would be needed for a prospective adaptation towards a microfluidic fluidized bed platform. The detectable levels of Aβ peptides by LIF after our developed immune-capture - on-beads labeling - thermal elution procedure with an enrichment factor of 100 were 0.08 - 0.1 nM which are compatible with the Aβ peptides' levels in the CSF. In terms of sensitivity, our method has not reached yet the ELISA performance whose quantifiable level

of Aβ 1-42 is 0.03 nM [15]. On the other hand, our method offers some advantages over ELISA, including 1) concurrent separation and determination of different structurally close Aβ peptides without recourse to simultaneous employment of different antibodies of high specificity towards each Aβ peptide of interest [9, 10] and 2) distinguishing of monomeric and oligomeric forms of Aβ 1-42 [37].

The concentrations of Aβ 1-38, Aβ 1-40 and Aβ 1-42 in CSF samples were determined using the aforementioned sample treatment method followed by CE-LIF analyses. Electropherograms of CSF samples obtained from cognitive normal (controls) or AD patients are shown in Fig. 4. Both the standards and the CSF samples were subjected to the same enrichment - fluorescent derivatization for appropriate comparison. The filtration through 3kDa filters was employed to remove all small unwanted species from the sample matrix while retaining the concerned Aβ. Much better baseline with no signal drifting was achieved with sample pre-filtering. Among the three Aβ peptides, Aβ 1-40 in CSF samples was present at the highest concentrations of 6 - 8 nM. Aβ 1-38 in these CSF samples was found to be at the order of 1 - 2 nM. The most challenging was the determination of Aβ 1-42 due to its extremely low abundance in CSF samples, as well as the presence of some unknown peaks adjacent to that of Aβ 1-42 (see Fig. 4). However identification of these other compounds was not envisaged in the scope of this work. Recourse to immunocapture with 12F4 was then realized for selective determination of Aβ 1-42, as demonstrated in Fig. 5. The employment of this antibody specific for the C-terminus of Aβ 1-42 led to a clear visualization of Aβ 1-42 peak only. A decrease in Aβ 1-42 concentration could be observed in the CSF sample from an AD patient compared to that of a control one. The relative concentrations ratios of Aβ peptides in CSF samples obtained with our approach were in agreement with those reported by Svobodova *et al.* [30] who used the same antibodies and CSF samples provided from the

same source (Ulm hospital) but not from the same patient. Pre-filtering of CSF samples would probably increase the performance of our system. Nevertheless, our main goal was to conceive a microsystem that integrates all pre- and analytical steps. As filtering in microsystems is not trivial, this additional sample treatment step was not further considered in this work.

3.3. Operation in a microfluidic fluidized bed: A proof of concept

While the batchwise mode of the developed magnetic immunocapture exhibited satisfactory performance for the determination of Aβ peptide mixture in CSF samples, its long operation time with manual solution switching may pose some inconvenience to the operator. Our prospective objective is therefore to integrate all capture, label, elution and MCE-LIF separation steps into an automated procedure in a microfluidic platform. The preliminary stage towards this objective relies on the implementation of the capture-label-elution protocol inside a microfluidic fluidized bed whose configuration was reported recently [32, 33]. Inside its chamber, magnetic microparticles continuously re-circulate-thanks to two counter driving forces, *i.e.* pressure-driven hydrodynamic and magnetic attraction forces. The contact of magnetic beads with Aβ peptides (during the capturing step) and with FP-488 (during the on-bead labeling process) is therefore expected to be much improved in this case compared to the previous microchip design where magnetic microbeads were kept stationary inside the microchannel [29, 30]. Indeed, with the previous microchip design, fracture in the magnetic plug could occur over a certain flowrate. In case of fracture the liquid could preferentially flow through this fracture of lower hydrodynamic resistance and thus reduces drastically the bed surface in contact with the sample. With our device we obtained a homogeneous contact of the liquid with all the beads and we could thus expect a better efficiency. Compared to batch experiment, this micro-fluidized bed configuration allows an efficient stirring and should

enhance the mass transfer between the solid and the liquid phase. Accordingly, better capture efficiency and labeling interaction are expected to be achievable within shorter operation duration. The capture - label - thermal elution procedure was then carried out in the microfluidic fluidized bed and the fluorescent signal obtained at its output microchannel after thermal elution is shown in Fig. 6A. This fluorescent fraction was subsequently collected and subjected to CE-LIF separation for visualization of Aβ 1-42, Aβ 1-40 and Aβ 1-38 as shown in Fig. 6B. The fluorescent signal shown in Fig. 6A was indeed the total signals of the released FP-488 and labeled Aβ peptides that were well separated with CE-LIF as displayed in Fig. 6B. The operation time was shortened from more than 15 hours in the batch-based mode into only around 3 hours with the fluidized bed configuration. To avoid saturation of the microchannel, the amount of magnetic beads was tenfold reduced. Even under this condition, successful capture-label-elution operation in this microfluidic fluidized bed was still achieved with an enrichment factor of 20. Higher preconcentration gains are expected if dilution of the eluent at the output of the microfluidized bed (at the nL range) into a collectable volume (some μL) for subsequent CE-LIF operation could be avoided. This would be the case when integrating the preconcentration step to the microchip electrophoresis operation will be realized. We demonstrated therefore that this novel approach which integrated for the first time Aβ capturing and fluorescent labeling on the same immunosupport could be downscaled. In the case of offline labeling followed by immunocapture [29, 30], manual batchwise in-solution labeling in the absence of magnetic beads can hardly be downscaled into the microfluidic fluidized bed platform. Operation integration and automation in the magnetic-beads based microfluidic system for perspective point-of-care device production are therefore more achievable with the developed capture-label-elution strategy where the sample capture and labeling can be both implemented on-chip in the same microchamber. Furthermore, the undesirable preferential capture of fluorescently labeled Aβ 1-42 over the other Aβ peptides

encountered in the offline labeling - immunocapture technique reported by Svobodova *et al*. [30] was not observed with our capture-label-elution approach. For successful perspective development of a point-of-care device that can quantitatively reach the extremely low level of Aβ 1-42 in CSF samples, three processes have been envisaged, including: 1) improvement of LIF detection limit from 35 nM to 8 nM without the preconcentration step by the removal of the exceeding fluorophores in the sample matrix after the labeling, by using microbeads 2) pre-concentration of Aβ peptides with microbeads-based immunocapture and 3) integration of all steps, *i.e.* preconcentration - labeling - elution - separation on a microfluidized bed coupled with a separation microchip to eliminate the volume loss and variation at each step and to significantly reduce the elution volume for better enrichment gain. At the present stage, the presence of Aβ 1-42 in CSF samples was detected with the first two aforementioned approaches.

4. Conclusions

This work contributed significantly to three achievements. Firstly, an MCE / CE-LIF compatible technique of Aβ peptides enrichment was successfully developed based on magneto-immunocapture operation. Secondly, the successful fluorescent labeling of Aβ peptides was for the first time done on-beads, leading to the removal of all unwanted extra fluorescent dyes. These pre-concentration and fluorescent labeling steps which act as a forefront sample treatment process prior to CE-LIF render the separation and sensitive detection of Aβ 1-42, Aβ 1-40 and Aβ 1-38 in CSF samples possible. The FP-488 that was non-specifically adsorbed onto microbeads during the labeling process and then released into the eluent during the elution step was profited as the internal standard for calculation of relative migration times of Aβ peptides in CE-LIF profiles. Finally, a proof of concept of micro-fluidized bed based operation for enrichment and fluorescent-labeling of Aβ was

successfully demonstrated. This opens the door for production of hand-held devices for facile and high-throughput probing of Aβ peptides in CSF samples based on lab-on-chip electrokinetic separation. Perspective work on coupling the fluidized bed with microchip electrophoresis will be soon envisaged.

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Table 1. Linearity of the response, detection limits (LODs) and reproducibility for the determination of preconcentrated Aβ peptides by CE-LIF. CE-LIF conditions as in Fig. 1.

Analyte	Range (nM)	Correlation coefficient	LOD[a] (nM)	LOD-P[b] (nM)	RSD % T_m[c] (n=4)	RSD % A_{inter}[d] (n=4)	RSD % A_{intra}[e] (n=3)
β 1-42	40-400	0.9675	10	0.1	1.31	14.03	7.39
Aβ 1-40	40-800	0.9712	8	0.08	1.63	11.79	5.29
Aβ 1-38	40-800	0.9882	10	0.1	1.03	11.95	6.34

[a] Based on peak heights corresponding to 3 times the baseline noise.

[b] LOD - P: detectable concentrations with the calculated enrichment factor F = 100.

[c] Migration time, corrected to the reference peak of FP-488. RSD was measured with standard concentration of 15 nM.

[d] RSD % A_{inter}: Inter-batch RSD values for peak areas (comparison of 4 different batches). The peak areas were calculated from the mobility-based electropherograms that were converted based on the migration times of FP-488 that was used as the referenced peak.

[e] RSD % A_{intra}: RSD values for peak areas obtained with the same batch. The batch was measured three times with CE-LIF. The peak areas were calculated from the mobility-based electropherograms that were converted based on the migration times of FP-488 that was used as the referenced peak.

Figure captions:

Fig. 1. CE-LIF electropherograms of a mixture of Aβ 1-42 (8 nM) and Aβ 1-40 (15 nM) after magnetic-beads-based sample treatment (capture – label – elution) obtained with different anti-Aβ antibodies. The batch-based capture and label conditions were detailed in the 'Experimental' section. For elution, the magnetic beads were heated up to 95°C for 5 min in the presence of borate buffer (pH 10.5, IS 40 mM). Enrichment factor of 40. CE-LIF conditions: fused silica capillary with effective length (l_{eff}) of 38.4 cm and total length (L) of 48.2 cm; BGE: borate buffer (pH 9.25, IS 40 mM) added with DAB 3.25 mM; LIF detection with excitation wavelength (λ) of 488 nm.

Fig. 2. CE-LIF electropherograms of fluorescently labeled Aβ 1-40 (1000 nM) prepared in different media. The Aβ 1-40 peptide was labeled by 5-min static incubation of Aβ 1-40 with 0.2 mg / mL FP-488 prepared in DMSO according to the procedure reported in our previous publication[10]. SM: sample medium. CE conditions as in Fig. 1.

Fig. 3. CE-LIF electropherograms of the captured and labeled Aβ 1-42 (8 nM), Aβ 1- 40 (15 nM) and Aβ 1- 38 (15 nM) after chemical and thermal elutions. Capture was done with magnetic micro-beads coated with either 6E10 or IgG (for blank control). CE conditions as in Fig. 1. A) Capture with 6E10 and elution with formic acid 0.5% at room temperature for 15 min; B) Capture with 6E10 and elution with borate buffer (pH 10.5, IS 40 mM) at room temperature for 15 min; C) Capture with 6E10 and elution with Borate buffer (pH 10.5, IS 40 mM) at 95°C for 5 min and D)

Capture with IgG and elution with borate buffer (pH 10.5, IS 40 mM) at 95°C for 5 min (Blank).

Fig. 4. CE-LIF electropherograms of CSF samples after magnetic immune-precipitation using the antibody 6E10 (enrichment factor of 100) and on-beads fluorescent labeling. Thermal elution conditions: 95 °C for 5 min in the presence of borate buffer (pH 10.5, IS 40 mM). CE conditions as in Fig. 1. A) Sample treatment without pre-filtering of CSF samples; B) sample treatment with pre-filtering of CSF samples using 3K Dalton filters. Peak identification: (1) Aβ 1-42, (2) Aβ 1-40, (3) Aβ 1-38. CSF – AD stands for CSF samples from AD patients; CSF – C stands for CSF samples from cognitive normal people used as controls; STD 1: standard solution of Aβ 1-42 (0.2 nM), Aβ 1-40 (0.2 nM) and Aβ 1-38 (0.2 nM); STD 2: standard solution of Aβ 1-42 (2 nM), Aβ 1-40 (8 nM) and Aβ 1-38 (8 nM). The indicated concentrations in the brackets were those of the standards before immune-enrichment. Both standards and CSF samples were subjected to the same immunocapture - label - elution procedure.

Fig. 5. CE-LIF electropherograms of CSF samples after magnetic immune-precipitation using the antibody 12F4 (enrichment factor of 100) and on-beads fluorescent labeling. Thermal elution conditions: 95 °C for 5 min in the presence of borate buffer (pH 10.5, IS 40 mM). CE conditions as in Fig. 1. Sample treatment was carried out without pre-filtering of CSF samples. CSF-AD: CSF sample collected from an AD patient; CSF-C: CSF sample from a cognitive normal person (used as control); STD: standard Aβ solution (8 nM). The indicated concentration in the

brackets was that of the standard before immune-enrichment. Both the standard and CSF samples were subjected to the same immunocapture - label - elution procedure.

Fig. 6. A) Fluorescent observation at the output channel of the micro-fluidized bed during the passage of the eluent after thermal elution. The sample is 100 μL of 60 nM standard peptides. The capture - label - elution procedure can be found in section 2.3.

B) CE-LIF electropherogram obtained for the eluent collected at the output channel of the micro-fluidized bed after thermal elution. CE conditions as in Fig. 1.

Figure 1

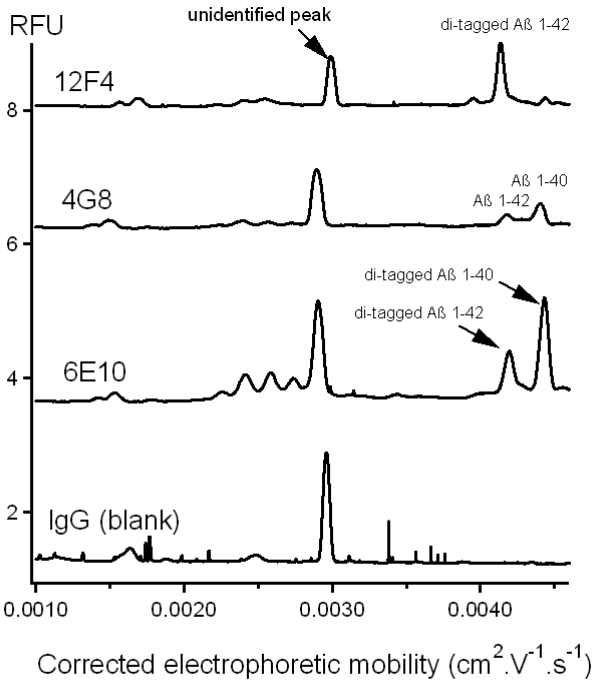


Figure 2

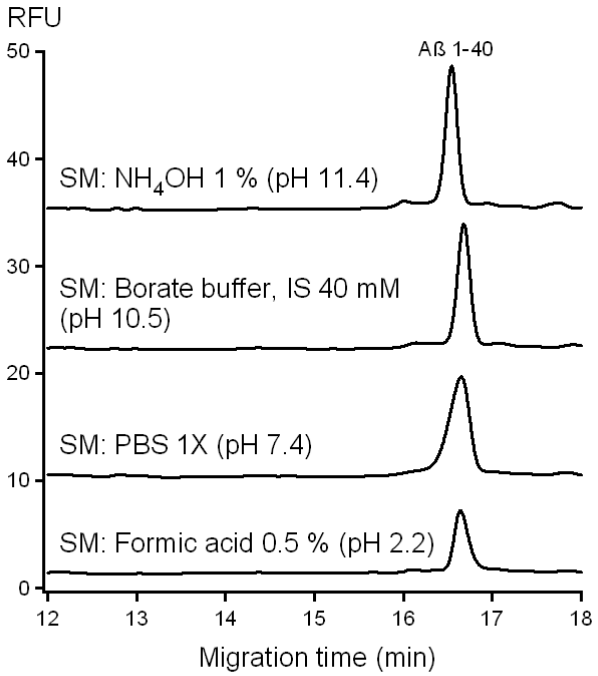


Figure 3

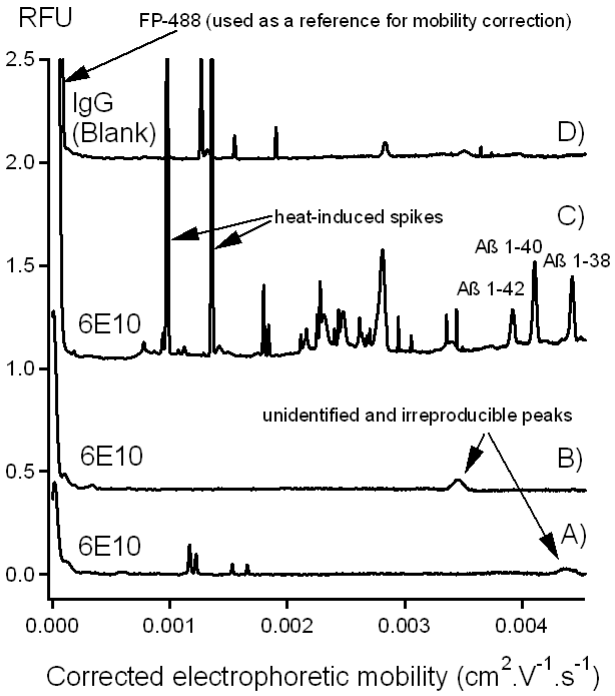


Figure 4

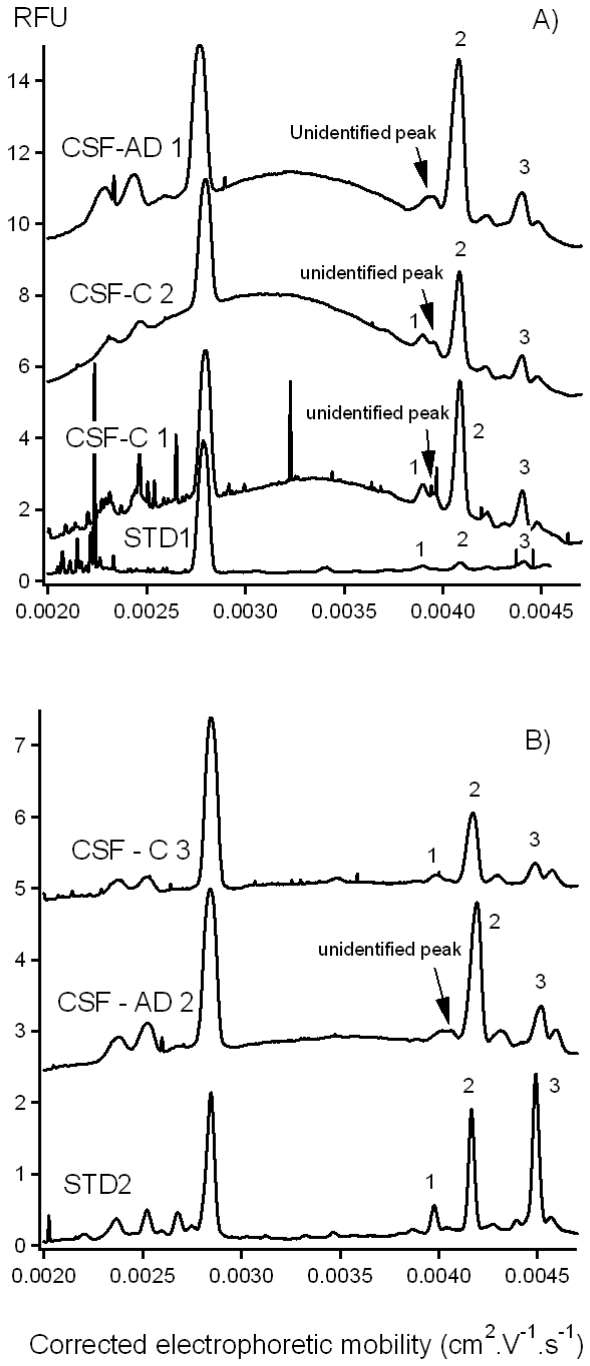


Figure 5

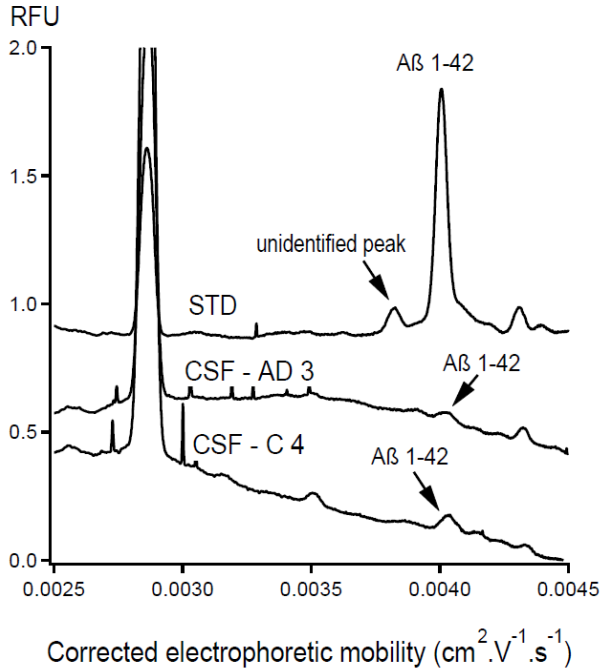
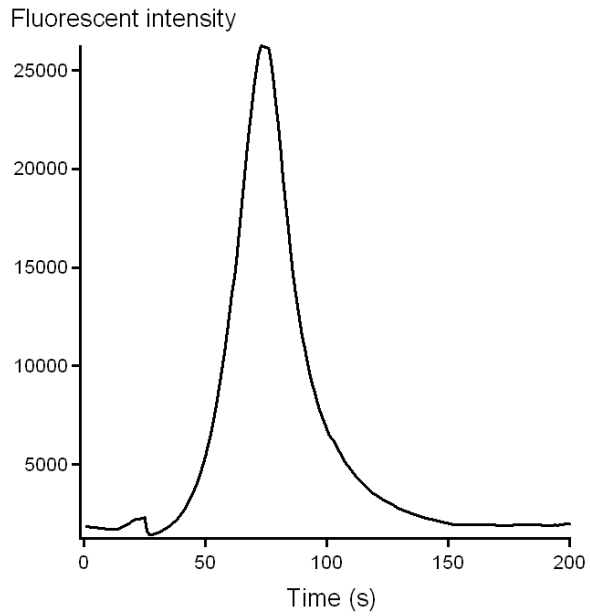


Figure 6

6A



6B