Development of a magnetic immunosorbent for on-chip preconcentration of amyloid β isoforms: Representatives of Alzheimer's disease biomarkers

Zuzana Svobodova,¹ Mohamad Reza Mohamadi,² Barbora Jankovicova,¹ Hermann Esselmann,³ Romain Verpillot,⁴ Markus Otto,⁵ Myriam Taverna,⁴ Jens Wiltfang,³ Jean-Louis Viovy,² and Zuzana Bilkova^{1,a)}

¹*Department of Biological and Biochemical Sciences, University of Pardubice, Pardubice, Czech Republic*

²*UMR 168, Curie Institut/CNRS/Universite Pierre et Marie Curie, Paris, France*

³*Department of Psychiatry and Psychotherapy, LVR-Hospital, University of Duisburg-Essen, Essen, Germany*

⁴*Faculte de Pharmacie, UMR 8612-LPNSS, University of Paris Sud 11, Chatenay Malabry, France*

⁵*Department of Neurology, University of Ulm, Ulm, Germany*

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Determination of amyloid β ($A\beta$) isoforms and in particular the proportion of the $A\beta$ 1-42 isoform in cerebrospinal fluid (CSF) of patients suspected of Alzheimer's disease might help in early diagnosis and treatment of that illness. Due to the low concentration of $A\beta$ peptides in biological fluids, a preconcentration step prior to the detection step is often necessary. This study utilized on-chip immunoprecipitation, known as micro-immunoprecipitation (μ IP). The technique uses an immunosorbent (IS) consisting of magnetic beads coated with specific anti- $A\beta$ antibodies organized into an affinity microcolumn by a magnetic field. Our goal was to thoroughly describe the critical steps in developing the IS, such as selecting the proper beads and anti- $A\beta$ antibodies, as well as optimizing the immobilization technique and μ IP protocol. The latter includes selecting optimal elution conditions. Furthermore, we demonstrate the efficiency of anti- $A\beta$ IS for μ IP and specific capture of 5 $A\beta$ peptides under optimized conditions using various subsequent analytical methods, including matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), capillary electrophoresis, microchip electrophoresis, and immunoblotting. Synthetic $A\beta$ peptides samples prepared in buffer and spiked in human CSF were analyzed. Finally, on-chip immunoprecipitation of $A\beta$ peptides in human CSF sample was performed. © 2012 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4722588>]

I. INTRODUCTION

Alzheimer's disease (AD) is a progressive, fatal neurodegenerative disorder characterized by deterioration in cognition and memory, progressive impairment in the ability to carry out activities of daily living, and a number of neuropsychiatric and behavioral symptoms.¹ AD is giving rise to serious socioeconomic problems, as the number of AD patients requiring long-term care increases each year.^{2,3} An important step toward addressing these issues in relation to AD would be to develop diagnostic tools for early detection of AD biomarkers in preclinical phase.⁴

At present, clinically relevant biomarkers used for AD diagnosis in cerebrospinal fluid (CSF) are amyloid β ($A\beta$) peptides 1-42,^{5,6} total tau, and hyperphosphorylated tau protein.^{2,7-9} Our work focused on $A\beta$ peptide biomarkers. Various isoforms of the $A\beta$ peptide, usually between 37 and 43 amino acids (AA) in length, occur naturally in our body liquids and are derived by proteolysis

^{a)}Electronic mail: zuzana.bilkova@upce.cz.

of a larger protein known as the amyloid precursor protein (APP).¹⁰ The A β 1-42 isoform (4 kDa) is the major species found in the senile plaques. It is regarded as a key molecule in AD pathology and more prone to aggregation than are the shorter A β isoforms.¹¹

The concentration of A β 1-42 analyzed by A β -SDS-PAGE/immunoblot was reported by Wiltfang *et al.* to be in the range of 0.91–1.57 ng/ml in the CSF of AD patients but 1.56–2.88 ng/ml in the CSF of controls without dementia disease.¹² The decrease of A β 1-42 level compared to other isoforms in the CSF of AD patients is presumably due to its lower clearance from the brain into the CSF. This might be explained by the fact that A β 1-42 creates senile plaques in brain tissue, and it could also be considered as a first sign of conversion from mild cognitive impairment to AD.¹³ Therefore, it is important not only to determine total concentration of A β peptide in CSF of dementia-affected patients but also the proportions for each of the A β isoforms.¹²

Differential quantification of the various A β peptides at such low concentration in a complex biological matrix is challenging. Various methods for preconcentration of target analyte can be applied. Impressive results were reported in Refs. 14–16 using isotachopheresis (ITP) where fluorescently labeled or unlabeled samples were enriched up to 10 000 fold from whole volume of simple mixture of standard proteins or amino acids. It is very efficient technique applicable mainly for pharmaceutical, food, and/or lipoprotein analysis.¹⁷ However, according to Lion *et al.*¹⁸ ITP suffers from one drawback: the sample conductivity must be carefully controlled, which is not always that easy when dealing with “real” biological samples. Therefore, we have decided in this work to use immunoprecipitation (IP) since as reported by numerous other authors,^{12,19–21} IP using specific antibodies is one of the most efficient methods for isolation and preconcentration of target biomarkers from complex biological samples enabling its subsequent determination.^{22,23} Although this method accomplishes in our hands lower preconcentration level compare to ITP, we can gain especially high selectivity which is substantially desired in such cases and, moreover, the analytes are after IP presented directly in reagent compatible with subsequent detection method. IP is very powerful tool especially when antibody providing appropriate affinity and specificity to target antigen is at hand. Recent advances in microfluidics have enabled development of on-chip immunoprecipitation (μ IP), which offers such advantages as lower sample and reagent consumption, easier sample handling, and reduced protein loss due to surface adsorption.²⁴

Magnetic or nonmagnetic beads can be used for creating an IP microcolumn inside a microfluidic device. Nonmagnetic beads can be fixed mechanically by a frit or weir in the microchannel, but this generally requires high pressures and furthermore reproducible packing is difficult to achieve. Thus, capturing magnetic beads in a microchannel by the action of two strong magnets has proven an efficient and convenient alternative.²⁵ The principle of experiment setup based on specific capture of target peptides on microaffinity self-assembled column formed by magnetic anti-A β immunosorbent (IS) which is followed by release of captured peptides from the column is described here (see Fig. 1). By this method, we are able to enrich from CSF sample the 5 A β peptides using N-terminated monoclonal antibody immobilized on magnetic particles.

FIG. 1. Scheme of experiment setup in macro- and micro-scales. (a) Prepared magnetic anti-A β immunosorbent is inserted in PDMS microchip using syringe pump where they are self-assembled into affinity microcolumn. (b) Sample containing A β peptides is applied into the chip and (c) the specific immunocapturing is performed. After proper washing (d) the specifically captured peptides are eluted using compatible eluting reagent according to subsequent method (e).

In general, different immobilization techniques, either non-covalent or covalent, may be applied for attaching antibodies onto the magnetic beads and thus for magnetic immunosorbent preparation. Nevertheless, covalent binding provides the strongest linkages compared with such other non-covalent immobilization methods as biospecific, ionic and/or affinity adsorption, and leakage of ligand from the beads is thus often minimized.²⁶ In our work, various site-groups on magnetic beads enabling random or site-specific covalent binding of Ab were compared for their immobilization efficiency.

Two types of specific anti-A β antibodies were attached to beads with highest immobilization efficiency: (i) rabbit polyclonal IgG to amyloid β (1-14 AA), and/or (ii) mouse monoclonal IgG1 to amyloid β (1-16 AA). Both antibodies were directed against an N-terminus of A β peptide which is common for all 5 isoforms (1-37, 1-38, 1-39, 1-40, and 1-42), and hence all tested isoforms are presumed to be immunocaptured. Proportional immunocapture of both ISs, anti-A β IS (pAb) or anti-A β IS (mAb) for all 5 isoforms was investigated. When successful preconcentration of synthetic A β peptide mixtures on prepared ISs was confirmed, IP with spiked or native human CSF samples of non-AD patients was performed.

Preliminary testing of the prepared magnetic IS and its use under micro-immunoprecipitation (μ IP) conditions for 3 synthetic A β peptides with urea/tricine/tris SDS-PAGE detection had been described in our previous work.²⁷ Mohamadi *et al.* also had introduced application of such immunocapture in combination with microchip electrophoresis in detecting 5 A β isoforms.²⁸ It was concluded that a preconcentration step was essential for sensitive detection of such low-abundance analytes. In the present paper, therefore, results from an extensive study on the development of a magnetic anti-A β IS and its characterization and validation for A β peptides using various analytical tools ranging from conventional western blotting to newly developed microchip electrophoresis are presented.

II. EXPERIMENTAL SECTION

A. Materials and reagents

SiMAG-active, maghemite-core, superparamagnetic, nonporous silica beads, 1 μ m in diameter and with various active groups (SiMAG-PGL, SiMAG-Amino, SiMAG-Carboxyl, SiMAG-Hydrazide, SiMAG-Thiol, SiMAG-Cyanuric, SiMAG-Oxiran and SiMAG-Hydroxyl), were acquired from Chemicell (Berlin, Germany). Nonspecific human IgG isolated from serum and goat anti-mouse antibody marked with horseradish peroxidase were from Sigma-Aldrich (St. Louis, MO). Two anti-A β antibodies were used: rabbit polyclonal IgG to amyloid β (1-14 AA) from Abcam (Cambridge, UK) and mouse monoclonal IgG1 to amyloid β (clone 6E10, 1-16 AA) from Covance (Princeton, NJ). Synthetic A β peptides 1-37, 1-38, 1-39, 1-40, and 1-42 were purchased from Sigma-Aldrich (St. Louis, MO), Anaspec (Fremont, CA), and/or Apronex (Vestec, Czech Republic). Other chemicals, such as D-glyceraldehyde, sodium periodate, acrylamide, bis-acrylamide, EDAC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride), 2-(N-morpholino)ethanesulfonic acid sodium (MES), Tween 20, sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (SMCC) and acetonitrile, were from Sigma-Aldrich (St. Louis, MO). α -cyano-4-hydroxycinnamic acid (CHCA) was from LaserBio Labs (Sophia-Antipolis, France). MicrospinTM G-25 columns were from GE Healthcare (Buckinghamshire, UK). BCA Protein Assay Kit was from Pierce (Rockford, IL). ECL Plus western blot detection reagents were from Amersham Biosciences (now GE Healthcare, Buckinghamshire, UK). Trifluoroacetic acid (TFA) was from Fluka (St. Louis, MO), NH₄HCO₃ and urea from Lachema (Brno, Czech Republic), and formic acid and NH₄OH from Merck & Co (Whitehouse Station, NJ). Laemmli and tricine sample buffer were from Bio-Rad (Hercules, CA). Sodium dodecyl sulfate (SDS) was purchased from Penta (Prague, Czech Republic) and FluoProbe 488 NHS ester from Interchim (Montluçon, France). CSF samples of non-AD patients were provided by Professor Marcus Otto from the Department of Neurology at the University of Ulm (Ulm, Germany). Collection and analysis of the CSF samples were approved by the Ethics Committee at the University of Ulm.

B. Immobilization of antibodies onto SiMAG-active beads with various reactive groups

Selection of a suitable reactive group for antibody immobilization on the beads' surface was performed first on a model ligand using human nonspecific serum IgG (hu IgG). We used 1 mg of beads and 100 μ g of hu IgG in a 1 ml reaction volume. The immobilization was carried out according to the manufacturer's instructions. SiMAG-PGL and/or SiMAG-Cyanuric form a covalent bond through their terminal autoreactive polyglutaraldehyde/cyanuric groups. For immobilization to SiMAG-Amino and SiMAG-Carboxyl, a two-step carbodiimide method was used. SiMAG-Hydroxyl beads were activated by CNBr, and the ligand was bound through its amino group. SiMAG-Thiol beads form a covalent bond with IgG through a sulfhydryl reactive group using a maleimide method, and SiMAG-Oxiran beads use the epoxide groups for IgG immobilization. SiMAG-Hydrazide beads use site-directed covalent coupling of IgG through their carbohydrates after oxidation by sodium periodate. In this case, the supplier's recommended protocol was slightly modified. Antibodies were oxidized in 0.02 M sodium periodate, and the reaction was stopped by addition of 0.4 μ l of ethylene glycol. The unreacted reagent was removed using G-25 micro columns. The coupling process ran overnight at RT under stirring, then the IS was repeatedly washed and stored in phosphate buffered saline (PBS; pH 7.4) with 0.05% sodium azide at 4–8 °C. Different covalent immobilization techniques were compared according to the amount of immobilized IgG molecules on the beads' surface using SDS-PAGE and BCA assay. The difference between IgG concentration in the samples before and after immobilization was measured. Binding capacity was expressed as the percentage of hu IgG inputted to the reaction. When the most suitable beads were selected, the specific anti-A β antibodies were immobilized.

C. Batchwise immunoprecipitation

An A β sample (1 ml) was added to the specific anti-A β IS (1 mg) previously equilibrated with PBS (pH 7.4). After 1.5 h incubation at RT under stirring, the unbound proteins/peptides were removed by three washing steps. Each step consisted of 5 $\times$ 1.5 ml changing 0.1 M phosphate buffer (pH 7.0) with three different NaCl molarities (0.2 M—1 M—0 M). Subsequently, immunospecifically captured A β peptides were released by adding 3 $\times$ 200 μ l of elution reagent followed by 15 min incubation at RT under stirring. The elution fractions were concentrated in a speed vac and analyzed off-line.

D. On-chip immunoprecipitation (μ IP)

The single channel microchip device with one inlet and one outlet was fabricated by rapid prototyping and PDMS (polydimethylsiloxane) technology at Institut Curie (Paris), as previously described by Slovakova *et al.*²⁹ The microchannel dimensions were 1 mm $\times$ 0.250 mm $\times$ 20 mm. The anti-A β immunosorbent suspension (1 mg) was injected into the channel using a syringe pump from KD Scientific (Holliston, MA) under flow rate 2 ml/h. The suspension was passed by two strong magnets placed along the channel in 20° angle position where the magnetic beads were reversibly trapped in the magnetic field and formed a compact dense plug. Once a microcolumn was created, equilibration with PBS at pH 7.4 (100 μ l, 500 μ l/h) followed as described in our previous work.²⁷ The flow rate conditions were slightly modified. A sample containing A β (100 μ l) was pumped into the chip under flow rate 50 μ l/h at RT. The unbound peptides were removed by washing with 50 μ l of 0.1 M phosphate buffer (pH 7.0) with three different NaCl molarities (0.2 M – 1 M – 0 M) at flow rate 200 μ l/h at RT. Immunospecifically captured A β peptides were eluted by 100 μ l of elution reagent under flow rate 200 μ l/h. The collected elution fractions were concentrated in a speed vac and analyzed off-line.

E. Dot blot monitoring of μ IP

The A β concentration level during each particular step of the μ IP was studied by dot blot. A β 1-42 was selected to simplify the experiment's interpretation. Each 10 μ l of liquid which

passed through the chip during the binding, washing, and elution phases was collected and applied onto nitrocellulose membrane to determine the A β concentration in the dots. Then, the membrane was blocked by 1% bovine serum albumin in PBS-T (PBS, pH 7.4, with 0.05% Tween 20) for 1 h and after a quick wash in PBS-T it was incubated overnight in a solution containing specific monoclonal mouse anti-A β primary Ab (1:1000). After washing in PBS-T, it was incubated with the secondary antibody goat anti-mouse marked with horseradish peroxidase (1:5000). The signal was developed using an enhanced chemiluminescence detection kit (ECL, Roche, France) and the image was captured using an LAS-3000 mini Fuji imager (Fuji-film Life Science, Dusseldorf, Germany). The image was processed using ImageJ and the concentration of A β 1-42 in each dot of the samples and the calibration curve in concentration range 1–50 μ g/ml were determined.

F. Urea/tricine/tris SDS-PAGE of immunocaptured A β peptides

We used a 16.5%T/3%C tricine/tris SDS-PAGE gel modified with 8 M urea for batchwise analysis of IP A β peptides according to Klafki *et al.*³⁰ The gels of 0.75 mm thickness were run in a Mini-PROTEAN Tetra electrophoresis cell from Bio-Rad (Hercules, CA) in tricine/tris/SDS (0.1 M tris, 0.1 M tricine, 0.1% [w/v] SDS) buffer. Samples were mixed with tricine sample buffer (1:1) and boiled for 2 min at 100 °C.

G. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of immunocaptured A β peptides

The preconcentrated A β samples were detected using a 4800 MALDI TOF/TOFTM Analyzer mass spectrometer from Applied Biosystems (Foster City, CA) in reflectron positive mode with laser intensity 3000 and range m/z 800–5000. A 1 μ l volume of each sample was spotted onto the target MALDI-plate, air-dried, then covered with 1 μ l of matrix solution (α -cyano-4-hydroxycinnamic acid, 50 mg/ml in 33% acetonitrile, 0.3% TFA). The mass spectra were evaluated using the program DATA EXPLORER (Applied Biosystems, Carlsbad, CA).

H. Capillary electrophoresis (CE)

The conditions for analyzing preconcentrated A β samples were described in detail by Verpillot *et al.*³¹ The Beckman Coulter PA 800 ProteomLab employed for off-line analysis of A β samples was equipped with UV and photodiode array detection (DAD). The uncoated silica capillary had 50 μ m inside diameter and 57 cm total length and was thermostated at 25 °C. The samples were introduced into the capillary by hydrodynamic injection under 3.4 kPa. The peptides were detected by UV detection (214 nm) or DAD (190 nm). The separations were carried out at 30 kV with positive polarity at the inlet and using 40 mM borate buffer (pH 9) containing 3 mM of diaminobutane as the electrolyte. The data acquisition and instrument control were directed using KARAT 7.0 software. Detection limits ranged from 300 to 500 nM depending on the A β peptide isoform.

I. Microchip electrophoresis (MCE)

A chip-based method for A β profiling recently described by Mohamadi *et al.* was performed.²⁸ The electrophoresis chip has channel cross section dimensions of 100 μ m $\times$ 50 μ m $\times$ 35 mm. The channel surface was coated by introducing a 0.2% solution of a home-made copolymer PDMA-AGE in ddH₂O. For separation, methylcellulose at various concentrations (at least 0.2%) with 0.01% Tween 20 was added to the electrophoresis buffers. The fluorescently labeled samples containing synthetic A β 1-37 and A β 1-42 peptides (1:1) preconcentrated on anti-A β IS were injected into the electrophoresis channel by pinched injection method. An Olympus IX71 inverted fluorescence microscope equipped with a 10 $\times$ 0.3 NA objective lens (Olympus, Tokyo, Japan) was used to detect fluorescent intensity of labeled A β peptides in real time within the detection zone using a Nikon digital DS-Qi1 camera. Illumination was by 100 W mercury arc lamp.

J. Immunoblotting analysis

CSF aliquots of 0.5 ml from non-AD patients were received from the University of Ulm on dry ice and then stored at -20°C until analysis. Before analysis, the CSF sample was thawed and diluted 1:1 (v/v) with working buffer and the batchwise IP or μIP was immediately performed on anti- $\text{A}\beta$ ISs. The $\text{A}\beta$ samples for analysis were dried on a speed vac and sent at 4°C to the University of Duisburg-Essen for analysis. The $\text{A}\beta$ -SDS-PAGE/immunoblot according to Wiltfang *et al.* was used for $\text{A}\beta$ isoforms quantification.¹²

III. RESULTS AND DISCUSSION

A. Magnetic microbeads for μIP

Nonporous silica superparamagnetic microbeads $1\ \mu\text{m}$ in diameter were selected for IS preparation, as they were supplied with a wide range of terminal reactive groups on their surfaces (hydrazide, polyglutaraldehyde, cyanuric, hydroxyl, amino, carboxyl, oxiran, and thiol). In order to obtain high density of Ab on the bead surface, the amount of IgG immobilized on each type of beads was determined by SDS-PAGE or BCA test. The protocols for ligand immobilization differed in their process requirements, the reagents used, and the orientation of immobilized ligand (Fig. 2). Therefore, the final decision as to which site-group would be used for μIP was based upon how simple was the protocol providing random or site-specific binding of IgG, whether the reagents were or were not harmful, and what was the IgG capacity on 1 mg of the beads. Our results from SDS-PAGE (Fig. 3) and the bicine-choninic acid (BCA) test (Fig. S-1)³² show high binding capacity of immobilized IgG was achieved using hydrazide, cyanuric, or thiol beads. In the end, microbeads with the hydrazide site-group were determined to be most suitable for further experiments with specific anti- $\text{A}\beta$ IgG because they afforded a site-specific, covalent type of IgG binding in combination with high density and reasonable binding protocol.

B. Optimization of elution step in IP of $\text{A}\beta$ peptides

Immunocaptured analytes are supposed to be released from the anti- $\text{A}\beta$ IS by using an elution reagent which ideally is compatible with the subsequent detection method. Toward this end, various elution conditions using 0.05% TFA (pH 2.3), 0.25% formic acid (pH 2.35), 0.16% ammonium hydroxide (pH 11.0), 100 mM ammonium bicarbonate (pH 8.0), 6 M urea (pH 8.2) and 2% SDS (pH 8.2) were tested. The determining criteria for evaluating elution efficiency were the concentration of batchwise immunoprecipitated $\text{A}\beta$ peptides (1-38, 1-40, 1-42) in elution fractions as analyzed by urea/tricine/tris SDS-PAGE (Fig. 4). Chaotropic reagents such as urea and SDS in such concentrations were not compatible with subsequent analytical methods, but efficient elution of all tested $\text{A}\beta$ isoforms was achieved using the other tested reagents. Therefore, elution reagents employed for releasing of captured peptides were adapted according to the analytical method that followed: For MALDI-TOF-MS analysis, 0.05% TFA

FIG. 2. Scheme of immobilization techniques employed for IgG on magnetic beads with various site-groups

FIG. 3. Binding efficiency (%) of SiMAG-active Chemicell microbeads (1 mg) with different site-groups for human serum IgG (100 μ g) immobilization as determined by SDS-PAGE (3 replicates)

was used, while for CE, chip electrophoresis and/or immunoblotting 0.16% NH_4OH was preferred. For CE, moreover, 100 mM NH_4HCO_3 or 0.25% formic acid also was suitable.

C. On-chip $\text{A}\beta$ preconcentration

On-chip IP (μ IP) runs on anti- $\text{A}\beta$ IS self-organized in the channel when using two permanent magnets to generate an affinity microcolumn (Fig. 5). The volume of the plug formed in the channel was calculated from its height (0.25 mm) and width (1 mm) and length (2.33 mm). The length may vary, thence 3 different images underwent the image analysis using software NIS Elements AR (Nikon, Tokyo, J), where the length was measured in 5 different places of plug and the average of the 15 measurements were calculated (2.33 ± 0.44 mm). Final volume of plug was determined as 0.58 mm^3 .

Although batchwise IP and μ IP include a number of the same steps, such as binding, washing, and elution, there also are some basic differences. What is considered as reaction or incubation time in batchwise IP experiments can be transposed into flow rate in on-chip immunocapture (μ IP). Hence, the μ IP process was monitored by dot blot to confirm that the μ IP produced the same results as did conventional batchwise IP. This technique was selected because it is a sensitive and quantitative method enabling easy monitoring of $\text{A}\beta$ 1-42 concentration at each particular step of μ IP. The record of $\text{A}\beta$ 1-42 concentrations in collected fractions (10 μ l) from the chip device under optimized flow rates is presented in Fig. 6. This result clearly shows that mostly all of the $\text{A}\beta$ 1-42 applied into the chip was biospecifically captured during the binding step and efficiently released during the elution step.

Concentration factor of μ IP setup may vary and depends on different parameters, such as primer concentration of sample, sample volume, amount of the beads, flow rate, and volume of

FIG. 4. 16.5% urea/tricine/tris SDS-PAGE of $\text{A}\beta$ peptides (1-38, 1-40, 1-42) eluted by the various elution reagents: 0.05% trifluoroacetic acid (TFA; pH 2.3), 0.25% formic acid (FA; pH 2.35), 0.16% ammonium hydroxide (OH; pH 11.0), and 100 mM ammonium bicarbonate (NH_4HCO_3 ; pH 8.0); molecular weight standard 1.4–26.6 kDa (MW, BioRad).

FIG. 5. (a) Master for PDMS molding, (b) scheme of PDMS microfluidic device, and (c),(d) detail of anti-A β plug/affinity microcolumn in microfluidic channel (1 mm $\times$ 0.25 mm $\times$ 20 mm) formed by magnetic microbeads self-organized using 2 permanent magnets.

the elution reagent. In this case, we were able to preconcentrate the A β peptide on the IS surface from the volume 100 μ l to volume 0.58 μ l which means at least 170 fold and obtain peptide in high purity presented in reagent suitable for subsequent separation and/or detection method.

D. Detection of A β peptides preconcentrated by IP or μ IP

Several methods were utilized for comparing and confirming immunocapture efficiency of the two prepared ISs (mAb and pAb) for the 5 A β peptide isoforms: First MALDI-TOF-MS for qualitative analysis, next CE or MCE for A β profiling, and then western blotting for the final quantitative and sensitive analysis of A β peptides preconcentrated from human CSF.

1. MALDI-TOF-MS

MALDI-TOF-MS is a qualitative method suitable for preliminary IS evaluation and allowed us to check whether the desired peptides were present in samples preconcentrated by IP. Its results did not give us information, however, about the proportions of the different A β peptides in samples, and, at least in our hands, the method was not sufficiently sensitive to detect A β peptides when their concentrations were close to the common levels in CSF samples. We therefore tried CSF samples spiked with A β synthetic peptides (6 μ g/ml of 1-37, 1-38, 1-39, 1-40, and 1-42 A β isoforms). The A β isoforms 1-37 and 1-39 were not detectable in the samples preconcentrated by IP (Fig. S-2).³² Thus, to evaluate A β immunocapture in biologic samples, other methods were utilized.

2. CE detection

In their work, Verpillot *et al.* had presented A β peptide separation and detection by ultra-violet visible CE (CE-UV). Compatibility with 0.16% NH₄OH and 100 mM NH₄HCO₃ elution reagents allowed analysis of the preconcentrated A β peptides. Original samples (OS), binding

FIG. 6. Monitoring of on-chip A β 1-42 immunocapture on anti-A β IS (pAb) by dot blot on nitrocellulose membrane. The process consists of A β 1-42 capture (0–100 μ l; flow rate 50 μ l/h), washing (110–260 μ l; flow rate 200 μ l/h), and elution of captured A β 1-42 (270–370 μ l; flow rate 200 μ l/h) by 0.05% TFA.

FIG. 7. CE-UV of A β mixture of three synthetic peptides (1-38, 1-40, 1-42) from IP performed on anti-A β IS (pAb): original sample (OS; sample before IP), binding fraction (BF; sample with non-IP A β peptides), and three successive elution fractions (E1–3; fractions with IP A β peptides eluted by 0.16% NH₄OH). E1–3 show preferential capture of A β 1-42. Analytical conditions were according to Verpillot *et al.* (2008).

fraction (BF; supernatant with non-IP A β peptides), and three successive elution fractions (E1–3) from batch IP on anti-A β IS (pAb) were analyzed by CE-UV (Fig. 7). Despite there being the same initial concentration of each A β isoform, we observed higher peak intensity for eluted A β 1-42 (66%) than for the shorter A β isoforms 1-40 (23%) and 1-38 (9%). These results were confirmed also by urea/tricine/tris SDS-PAGE, where the proportions of eluted A β peptides were 74% for A β 1-42, 21% for A β 1-40, and 5% for A β 1-38 (Table S-I).³² We thus concluded that the anti-A β IS (pAb) showed preferential capture of A β 1-42. To confirm this tendency, other methods for A β detection were also employed.

3. MCE for comparing magnetic anti-A β IS (mAb and pAb)

In our previous work, we presented an MCE method with fluorescent detection for profiling of 5 A β isoforms.²⁸ Here, the MCE method was used for monitoring the μ IP process by comparing the ratio of A β isoforms in the sample before the μ IP and in the elution fraction using two types of immunosorbents: one coated with mAb and one with pAb anti-A β IgG (Fig. 8(a)). The results presented in Figure 8(b) (average of 3 repetitions) show that when sample consisting of A β 1-37 and A β 1-42 (A β 1-37/A β 1-42 = 1.6) was immunoprecipitated, the A β peptides were eluted with different ratios according to the Ab-coated immunosorbents: pAb (0.6) and mAb (0.8). In other words, the results show that both anti-A β ISs (pAb and mAb) demonstrated preferential capture of A β 1-42 over A β 1-37. Such tendency might be caused by their structural difference and higher hydrophobicity of A β 1-42 in comparison to the other 4 isoforms.^{33,34} This feature might possibly change accessibility of the epitope for Ab, which is known as epitope

FIG. 8. (a) Electropherogram from MCE of fluorescently labeled A β 1-37 and 1-42 from μ IP on anti-A β IS (mAb); FP = FluoProbe. (b) Comparison of fluorescence intensity (FI) ratio of A β 1-37/1-42 peptides in samples obtained from μ IPs on anti-A β IS (pAb) and anti-A β IS (mAb) (averages of 3 repetitions). OS = original sample (before IP).

FIG. 9. A β peptides separated by A β -SDS-PAGE/immunoblot on polyvinylidene fluoride membrane (according to Wiltfang *et al.* (2002)). (a) Batchwise IP of CSF sample on anti-A β IS (pAb) and anti-A β IS (mAb). CSF = CSF sample before IP; IP (pAb) = batchwise IP on anti-A β IS (pAb); IP (mAb) = batchwise IP on anti-A β IS (mAb). (b) μ IP of CSF sample on anti-A β IS (mAb). A β ST = A β standard peptides mixture corresponding to levels in CSF; μ IP = μ IP on anti-A β IS (mAb).

masking. These observations were discussed by Wiltfang *et al.* in their study that compared their results from A β -SDS-PAGE/immunoblot with those of other authors determining A β concentrations in CSF.¹² They reported finding that differences in A β isoforms concentration were due to the different types of antibodies that had been used. Thus, the origins of different antibodies, and despite their having the same specificity, could create different reactivity with immunoprecipitated peptides.

4. Immunoblotting of A β captured by μ IP from human CSF

Previous testing of both anti-A β ISs was done with samples containing synthetic A β peptides or spiked CSF samples in concentration range higher than is usually found in CSF samples. The ability of both anti-A β ISs (pAb and mAb) to capture A β peptides from real human CSF samples of non-AD patients was thus tested by immunoblotting since this method affords sensitivity compatible with the concentration level of these peptides in biological samples. The two ISs were compared to show proportional immunocapture of A β isoforms in human CSF samples (Fig. 9(a)). Both antibodies used were presumed to immunocapture all 5 A β isoforms. Significant differences between the two ISs were observed, however, as shown in the previous results, with anti-A β IS (pAb) having higher affinity for the A β 1-42 isoform than for the other A β isoforms. The anti-A β IS (mAb) provided a more uniform and efficient capture of all 5 A β isoforms from CSF.

The ability of anti-A β IS (mAb) to preconcentrate the A β isoforms from CSF on-chip was also confirmed. The μ IP of CSF sample was performed and the preconcentrated A β peptides were analyzed by immunoblotting (Fig. 9(b)). An IS with monoclonal antibodies showed a representation of all A β isoforms in proportions nearly similar to those present in the initial CSF sample, albeit still with a slight preference for the A β 1-42 isoform (see Table I).

TABLE I. Quantification of amyloid β (A β) isoforms in human CSF before and after μ IP.

A β isoforms	CSF before μ IP (ng/ml)	CSF after μ IP (ng/ml)	CSF before μ IP (%)	CSF after μ IP (%)
1-37	2.32	0.15	6.0	6.1
1-38	4.81	0.34	12.4	13.7
1-39	2.60	0.14	6.7	5.7
1-40	24.25	0.88	62.7	35.6
1-42	4.69	0.96	12.1	38.8

IV. CONCLUSION

Micro-immunoprecipitation is a suitable method for isolation and preconcentration of A β peptide biomarkers occurring in low concentrations from CSF. Variability in elution reagents allows releasing the target molecules in a medium compatible with the subsequent detection method, be that performed by MALDI-TOF-MS, CE, MCE and/or immunoblotting. CE provided the best combination of sensitivity, convenience, and resolution, with a baseline resolution of the three isoforms investigated. Microchip electrophoresis, however, revealed promising potential for on-line connection and/or integration of immunocapture and detection into a single microfluidic device. A certain rate of A β 1-42 preferential capture was observed for both investigated antibodies (mAb or pAb), although with anti-A β IS (mAb) the representation of A β peptides was much closer to the composition of the initial human CSF sample. The proportional capture of A β isoforms has great significance for the clinical diagnosis of AD and in research as to the disease's pathogenesis. Hence, this effect will be considered in our next investigation. Our future work will also focus on developing an integrated lab-on-chip device for preconcentration, separation, and detection of 5 A β isoforms. In the aim to enhance the sensitivity of the analysis, an additional preconcentration component is now under testing to be integrated into the microfluidic device. Such system will be evaluated on samples from patients affected by AD or other dementias and which might contribute in the future to rapid A β profiling of patients suspected of having AD and to their diagnosis.

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