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Identification and Affinity-Quantification of ß-Amyloid and α-Synuclein Polypeptides Using On-Line SAW-Biosensor-Mass Spectrometry

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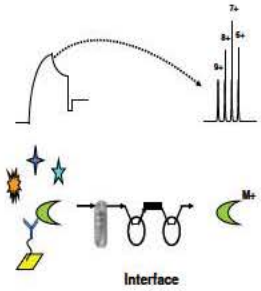

Abstract. Bioaffinity analysis using a variety of biosensors has become an established tool for detection and quantification of biomolecular interactions. Biosensors, however, are generally limited by the lack of chemical structure information of affinity-bound ligands. On-line bioaffinity-mass spectrometry using a surface-acoustic wave biosensor (SAW-MS) is a new combination providing the simultaneous affinity detection, quantification, and mass spectrometric structural characterization of ligands. We describe here an on-line SAW-MS combination for direct identification and affinity determination, using a new interface for MS of the affinity-isolated ligand eluate. Key element of the SAW-MS combination is a microfluidic interface that integrates affinity-isolation on a gold chip, in-situ sample concentration, and desalting with a microcolumn for MS of the ligand eluate from the biosensor. Suitable MS-acquisition software has been developed that provides coupling of the SAW-MS interface to a Bruker Daltonics ion trap-MS, FTICR-MS, and Waters Synapt-QTOF- MS systems. Applications are presented for mass spectrometric identifications and affinity (K_D) determinations of the neurodegenerative polypeptides, ß-amyloid (Aß), and pathophysiological and physiological synucleins (α- and ß-synucleins), two key polypeptide systems for Alzheimer's disease and Parkinson's disease, respectively. Moreover, first in vivo applications of αSyn polypeptides from brain homogenate show the feasibility of on-line affinity-MS to the direct analysis of biological material. These results demonstrate on-line SAW-bioaffinity-MS as a powerful tool for structural and quantitative analysis of biopolymer interactions.

Keywords: On-line bioaffinity-mass spectrometry, Surface acoustic wave (SAW) biosensor, Microfluidic affinity-MS interface, Ion trap-MS, Waters Synapt-QTOF-MS, ß-amyloid, α-Synucleins, ß-Synucleins, Identification, Affinity quantification, Mouse brain homogenate

Introduction

Bioaffinity interactions play a key role in all mechanisms of cellular life. Among a variety of methods, biosensors have recently emerged as powerful tools for the detection and quantification of biomolecular interactions, and have been employed in a number of studies, such as protein-

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peptide, protein-polynucleotide, and antigen-antibody interactions [1–3]. Biosensors have been developed based on different detection principles e.g., surface plasmon resonance (SPR) [4, 5], quartz crystal microbalance (QCM) [6–8], bio-layer interferometry (BLI) [9, 10], and surface acoustic wave (SAW) [11, 12]. Although providing fast and sensitive affinity quantification, a principal limitation of biosensors is the lack of chemical structure information of affinity-bound ligands. The chemical identification of ligands involved in biomolecular interactions, however, is of crucial importance for understanding interaction mechanisms in biochemical and biomedical processes, and in applications such as biomarker development and determination of lead structures for drug development.

We have developed a new interface for on-line bioaffinity-mass spectrometry coupling of a surface acoustic wave (SAW) biosensor to electrospray ionization mass spectrometry (SAW-MS) [13], which provides both affinity detection and quantification and the mass spectrometric chemical structure analysis of ligands. Key element of the on-line biosensor-MS is a microfluidic interface enabling affinity-enrichment, desalting of samples from the biosensor, and transfer of ligands to the ESI-MS source. Here we report a new microfluidic interface that comprises a chip for affinity-enrichment, sample concentration, and a microcolumn for desalting of samples and transfer to MS analysis (Figure 1). Following the initial coupling of the interface to an ion trap- ESI-MS system, the feasibility of the SAW-MS combination to high resolution FTICR-MS [14, 15] and to a Waters Synapt-QTOF-MS is shown in applications to affinity quantifications and mass spectrometric identifications of the neurodegenerative polypeptides, β-amyloid (Aß), and α-Synuclein (αSyn). Moreover, the efficacy of the on-line SAW-MS combination is demonstrated by the direct analysis of αSyn from brain homogenate. These results suggest broad application of on-line SAW-MS as an integrated tool for identification, quantitative affinity determination, and structural characterization of ligands in biopolymer interactions.

Experimental

Antibodies and Proteins

Anti-Aβ-antibodies (mAb, clone 4G8, and 6E10) were obtained from Covance (Princeton, NJ, USA). Anti-αSyn antibody 4B12 was obtained from GeneTex (Irvine, CA, USA), and anti-αSyn antibody pC20 from Santa Cruz (Dallas, TX, USA). Epitopes of the Aβ- and anti-αSyn antibodies were identified by proteolytic excision-mass spectrometry [16, 17]. Lysozyme, calmodulin, and other model proteins were obtained from Calbiochem (Darmstadt, Germany). Recombinant human αSyn, human αSyn (mutant A30P), and human βSyn were expressed in *E. Coli* as previously described [18]. Aβ peptides were synthesized by solid phase peptide synthesis using the Fmoc strategy [19].

SAW Biosensor and Sample Preparation for Affinity Determinations

Bioaffinity and on-line bioaffinity-MS analyses were performed with a K5 S-sens SAW biosensor (SAW-Instruments, Bonn, Germany), consisting of a biosensor unit, an autosampler, and a microchip module with a gold layer sensing surface on a quartz chip. The chip surface was prepared and cleaned before binding and affinity determinations by 45 min washing with a 1:1 mixture (v/v) of concentrated sulphuric acid and hydrogen peroxide (30%). Immobilization of proteins was performed by covalent binding of a monolayer (SAM) of 16-mercaptohexadecanoic acid as previously described [13].

For determining K_D values, polypeptides were immobilized on the SAM by carboxyl-group activation with a 1:1 mixture (v:v) of 200 mM (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and 50 mM *N*-hydroxysuccinimide (NHS) [13]. A 10–30 µM solution of polypeptide in 150 µL PBS was used for coupling, followed by capping of unreacted carboxyl groups with 1 M ethanolamine, pH 8.5. Affinity binding constants (K_D) towards the specific antibodies were determined using ligand concentrations in a wide range (nano- to micromolar), which were selected to comprise the equilibrium concentration. For regeneration of chips, affinity-bound ligands were generally eluted with a 50 mM glycine/0.1 M HCl/acetonitrile solution (pH 2.5) [13]. Binding curves were analyzed using the Origin Pro 7.5 software [11] and pseudo-first order kinetics (k_{obs}) determined using the FitMaster software [11, 13], from which dissociation constants K_D were determined by linear regression. The mathematical model chosen for fitting the binding curves in the Origin software considered an incomplete regeneration, as observed in the experiments.

Interface for On-Line SAW-Affinity-MS

The on-line SAW-MS interface for transfer of the eluted ligand from the biosensor to the ESI ion source consists of a gold chip for affinity-enrichment of ligands, two electric rheodyne 6-port valves, a guard micro-column for desalting (OptiGuard), and a microliter solvent delivery system (Supporting Information, Figure S1b). For transfer of eluted ligands to the MS, a solvent mixture of 5% ACN, 0.3% HCOOH (**A**), and 95% ACN, 0.3% HCOOH (**B**) was generally employed. Connections in the interface were made using 50 µm fused silica capillaries. Each of the two rheodyne valves can be switched independently in two positions, permitting a stable control of solvent flow. The entire interface operation is controlled by the Biosensor2MS software developed in-house [20]. Initially, valve 1 is in position (i) with the flow directed from the SAW biosensor to waste, whereas valve 2 is in position (ii) (Figure 1).

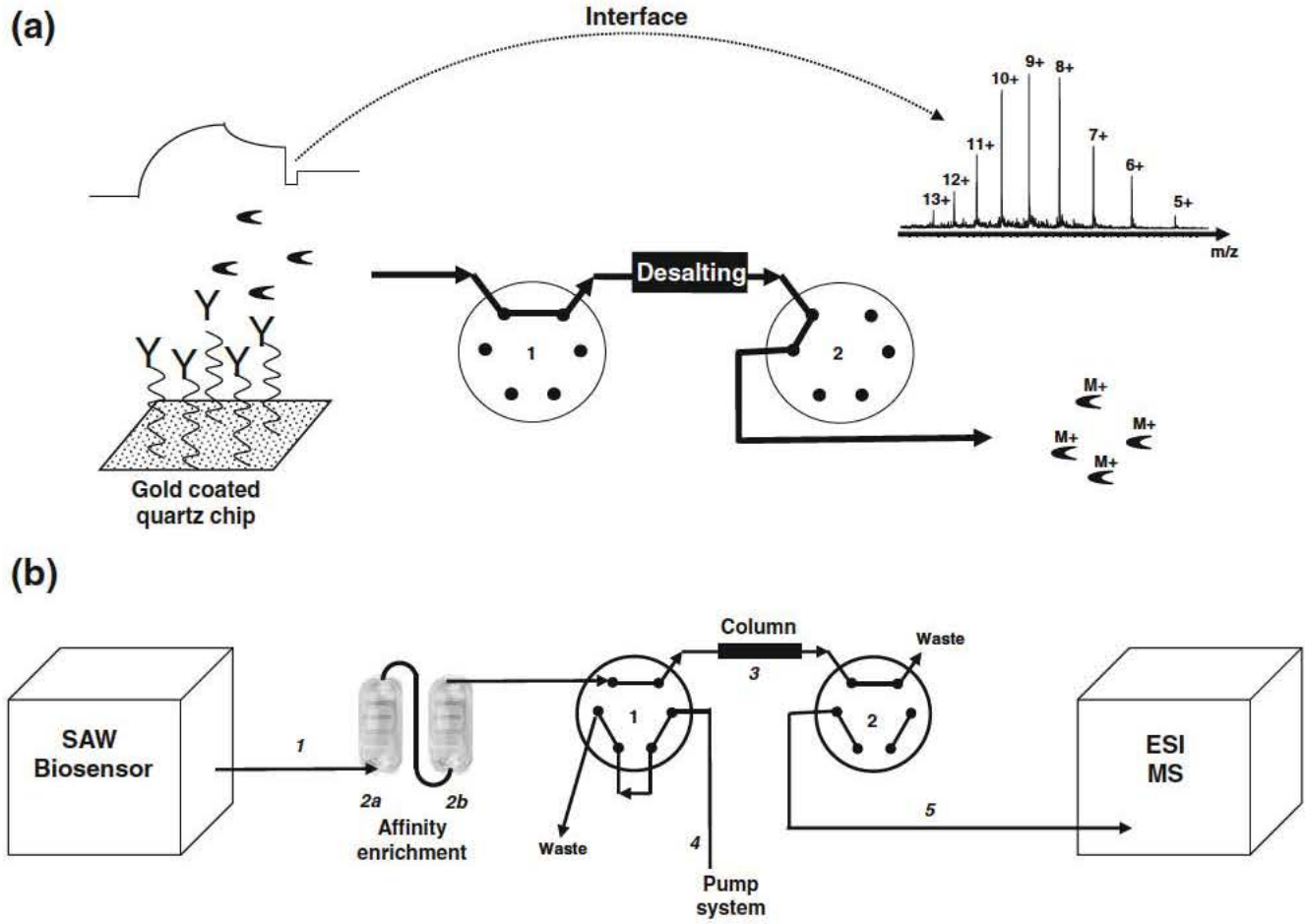

Figure 1. Scheme of the on-line SAW-MS interface. (**a**) Analytical concept showing the interface as an integrating microfluidic component between SAW biosensor and MS. The affinity-isolated, eluted analytes are transferred to the MS via the microfluidic interface. (**b**) Technical scheme showing the interface components. The SAW chip is connected to the interface by a fused silica capillary (*1*). An affinity-enrichment chip is placed into the microfluidic system by a silicone cell (*2*). The analyte is sent via two automatic rheodyne valves to a guard micro-column (*3*) providing buffer desalting, flow rate equilibration, and transfer of eluate to the ESI source (*5*). A precision micropump (e.g., µHPLC pump) (*4*) is used for transfer of solute through the desalting column. Interface, sample transfer, and MS acquisition are operated by the *Biosensor2MS* software

Ligand elution is set by an injection of 150 µL 0.1 M HCl, or 5% ACN in 0.1 M HCl. Upon detecting the elution injection, the Biosensor2MS software switches valve 1 to the microcolumn [position (ii)]; at the end of the elution, valve 1 is switched back to position (i), and a mixture of solvents A and B is applied for desalting the sample. Valve 2 is then switched to position (i) and the flow directed to the ESI source, while solvent B is increased to 70%. The *Biosensor2MS* software then triggers the acquisition of mass spectra; subsequently, the column is washed with solvent B and re-equilibrated.

Determination of affinity constants K_D, including on-line, the affinity-MS experiments were generally performed with immobilized antibodies on the SAM, using the immobilization procedure described above. Generally, a single injection (150 µL) of antibody (200 nM) was found sufficient to saturate the chip surface.

Mass Spectrometry

Several types of mass spectrometers were employed for on-line SAW-MS. ESI- FTICR-MS was performed with a Bruker APEX- II FTICR instrument equipped with an actively shielded 7 T superconducting magnet (Bruker Daltonics, Bremen, Germany) as described [21]. Mono-isotopic masses of singly charged ions generated by XMASS were used for database assignment using http://www.matrixscience.com and http://prowl.rockefeller.edu/.

High resolution oaTOF-MS was performed with a Waters Synapt-HDMS system equipped with an ESI ion source (Waters, Manchester, UK), operated in the positive ion mode. The capillary voltage, cone, and extraction cone voltages were set to 2.8 kV, 30 V, and 5 V, respectively; the source pressure was 2.2 mbar. Source and desolvation temperatures were set to 100°C and 300°C, respectively. The cone and desolvation gas flows were set to 30 l/h and

Table 1. Epitope Analysis and K_D Determination of Neurodegenerative Proteins Using Specific Antibodies

Protein/ polypeptide	MW (Da)	Antibody	Epitope sequence	K_D (10^{-9} M)	Reference
Aβ (1-40)	4326.1	Anti Aβ (17-24) [4G8][a]	(17-24)	20.1±8	[19, 25]
Aβ (1-40)	4326.1	Anti Aβ (1-16) [6E10][a]	(4-10)	16.3±5	[17]
α-Synuclein	14486.2	Anti α-Synuclein [4B12][b]	(81-96)	45.5±3	[16, 27]
α-Synuclein	14486.2	Anti-αSyn pC20	(81-96)	39±11.4	[16, 27]
h Tau (4R/2 N)	45849.9	Anti hTau [TAU5][a]	(210-230)	48.9±12	[28]

[a]Monoclonal Aß-antibody [4G8], Covance Inc., Princeton, USA.
[b]Monoclonal αSyn antibody [4B12], GeneTex, Irvine, USA.

300 l/h, respectively. Trap and transfer collision energies were set to 6 and 4 V; the trap gas flow was to 1 mL/min. On-line SAW-MS experiments were performed with the Synapt used as a standard Q-TOF instrument without using the ion mobility capability. The TriWave cells (comprising the Trap-T Wave, IM-T Wave, and Transfer-T Wave cells) replacing the hexapole collision cell in standard Q-TOF instruments was filled with Argon in the Trap-T and Transfer-T wave cells, and with N_2 in the IM-T wave at $5.04.10^{-3}$ and $2.87.10^{-4}$ mbar, respectively. The bias voltage for entering the T-wave cell was set to 4 V. The instrument was externally calibrated over the *m/z* range 500–5000 Da using a solution of cesium iodide. MassLynx software ver. 4.1 SCN 704 was used for data processing.

Preparation of Mouse Brain Homogenate

The Thy-1-human (A30P)αSyn transgenic mouse model has been previously described [22], by introducing the neuron specific Thy1 promoter for overexpression of human mutant αSyn(A30P). Mice were sacrificed by cervical dislocation, and brains harvested and divided into forebrain left (FB/L)/

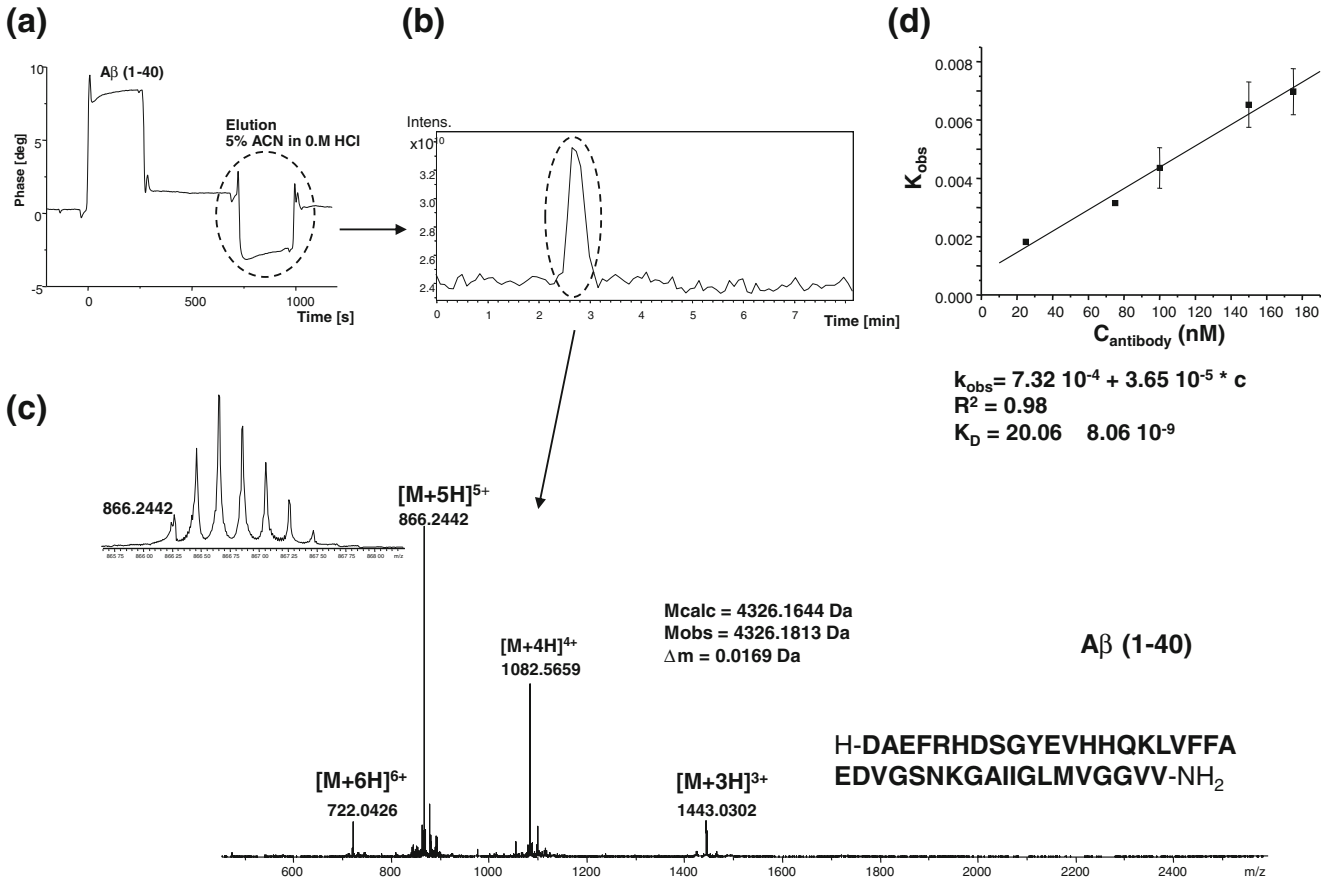

Figure 2. Identification and affinity quantification of an Aß-epitope specific antibody- Aß(1-40) complex by on-line SAW-FTICR- MS. (**a**) SAW binding curve and affinity interaction of the immobilized Aß-antibody (4G8) with pre-aggregated Aß(1-40); (**b**) TIC of the biosensor eluate transferred to the ion source; (**c**) ESI- FTICR mass spectrum of the eluted Aß; (**d**) K_D determination of the Aß- antibody complex. Mass determination accuracy of Aß(1-40) was approximately 6 ppm

right (FB/R), and hindbrain left (HB/L)/right (HB/R). αSyn(A30P) was extracted from brain tissue using multiple steps, by first adding 300 µL buffer 1 (tris-buffered saline plus complete protease inhibitor) to brain pieces, which were then crushed with a tissue- ruptor for 1 min on ice. The brain mixture was rinsed with 200 µL buffer 1, centrifuged at 120,000×*g* for 30 min at 4°C, and the resulting soluble fraction E1 used in this study. Treatment of the pellet with further buffers was used for further protein extraction. Protein quantification was carried out with the BCA assay.

Results and Discussion

Interface for On-Line SAW-MS

Key element for the on-line biosensor-MS combination is an interface that provides sample enrichment and isolation, in-situ desalting, and transfer of the ligand eluate to the ESI- MS. A suitable microfluidic interface incorporating a desalting microcolumn was initially developed for an ESI-ion trap source. The automated coupling interface (Figure 1) used for on-line SAW-FTICR- MS is controlled through a specific software (SAW2MS) for (1), monitoring sample injection in SAW biosensor, (2), detection of elution profile and sample flow, and (3), triggering the MS data acquisition. Epitope- specific anti-Aß-, and αSyn-antibodies [16, 17] were immobilized on the gold chip surface using a 16-mercaptohexadecanoic acid SAM linker, and Aß(1-40) and Aß(12-40) peptides, respectively. Synuclein polypeptides βSyn, αSyn, and overexpressed αSyn(A30P) mouse brain homogenate were eluted at acidic conditions (pH 2.5) for MS analysis.

Since the SAW biosensor employs a microfluidic system with continuous flow, coupling to an ESI ion source is

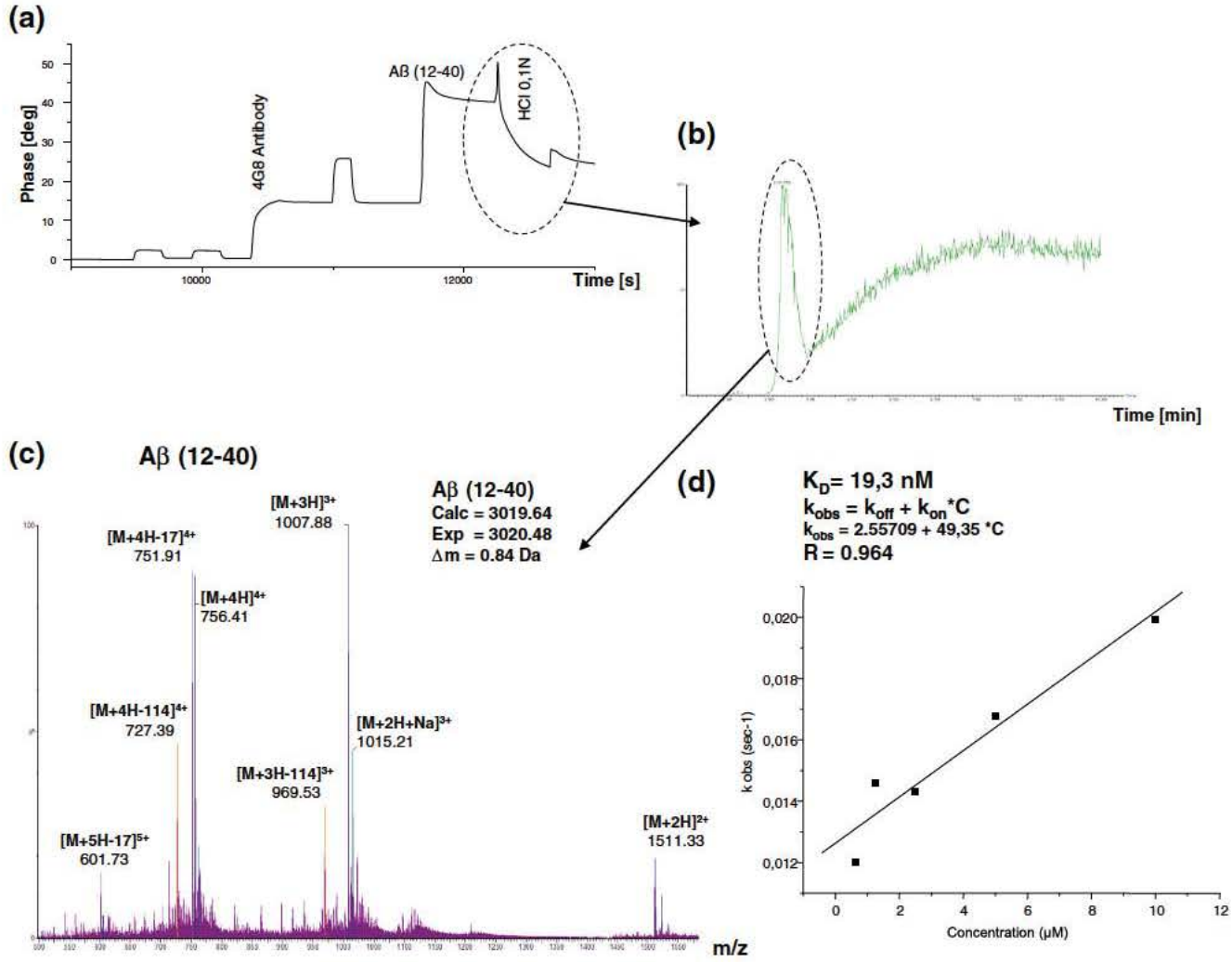

Figure 3. Identification and affinity quantification of an Aß-epitope specific antibody-Aß(12-40) complex by on-line SAW-MS, using the Synapt-HDMS-QTOF instrument. (a) SAW binding curve and affinity interaction of the immobilized Aß-antibody (4G8) with Aß(12-40); (b) TIC of the biosensor eluate transferred to the ion source; (c) ESI mass spectrum of the eluted Aß(12-40); (d) K_D determination of the Aß- antibody complex

straightforward. However, the high-salt PBS buffer concentration used in biosensor binding experiments at physiological solution conditions would present major problems for ESI-MS. Moreover, typical SAW flow rates are not compatible with the standard solvent flow for ESI- MS. These problems were solved by the SAW-MS interface which simultaneously provides (1), desalting by a suitable ESI-compatible solvent mixture; (2), sample concentration by suitable solvent change for release of affinity-ligands; and (3), automated flow rate equilibration between the biosensor (approximately 15 µL/min) and the ESI source (approximately 30 µL/min). Details of the time sequence of the interface operation and operation sequence of the switching valves are described in the Supporting Information, Figures S1 and S2. The BioSensor2MS software was used for monitoring the elution injection, switching the valves for sample transfer, and triggering the MS acquisition once the sample is sent to the ESI source. The software was developed using Visual Basic (Visual Studio 2010) and is compatible with ver. 4.0 of the .NET framework.

The performance of the interface was initially tested by characterization of the interaction of the model system calmodulin–melittin, a calcium receptor involved in a variety of physiological affinity-binding processes [23]. Immobilization of calmodulin and its interaction with melittin were analyzed by K_D determination and by mass spectrometric identification of the eluted peptide ligand.

Characterization of Interactions of Aß-Polypeptides and anti-Aß Antibodies by On-Line SAW-MS

First applications of the on-line SAW-FTICR-MS combination were performed by affinity characterization of antibodies specific for ß-amyloid (Aß), the key polypeptide in Alzheimer's disease (AD), and several other key proteins for neurodegenerative disorders (Table 1). The detailed biochemical pathways and mechanism(s) underlying AD are still unclear; however, extracellular plaques containing aggregated Aβ-peptide have been shown to play a major role, and Aß-oligomers have been identified to exert major neurotoxicity effects [24]. Hence, Aß-specific antibodies capable of disaggregating Aß-plaques or inhibiting Aß-aggregation are gaining increased interest for potential AD immune-therapy [25].

Several epitope-specific Aß-antibodies were studied by on-line SAW-MS for characterization of Aß-peptides and quantification of affinities. The Aß-epitopes recognized by the antibodies were identified in previous studies by proteolytic excision-mass spectrometry [17, 25] (Table 1). The mass spectrometric analysis of the epitope specificity revealed that the aggregation- inhibiting antibody (4G8) binds to a central- to C-terminal epitope, Aß(17-24) [19, 25].

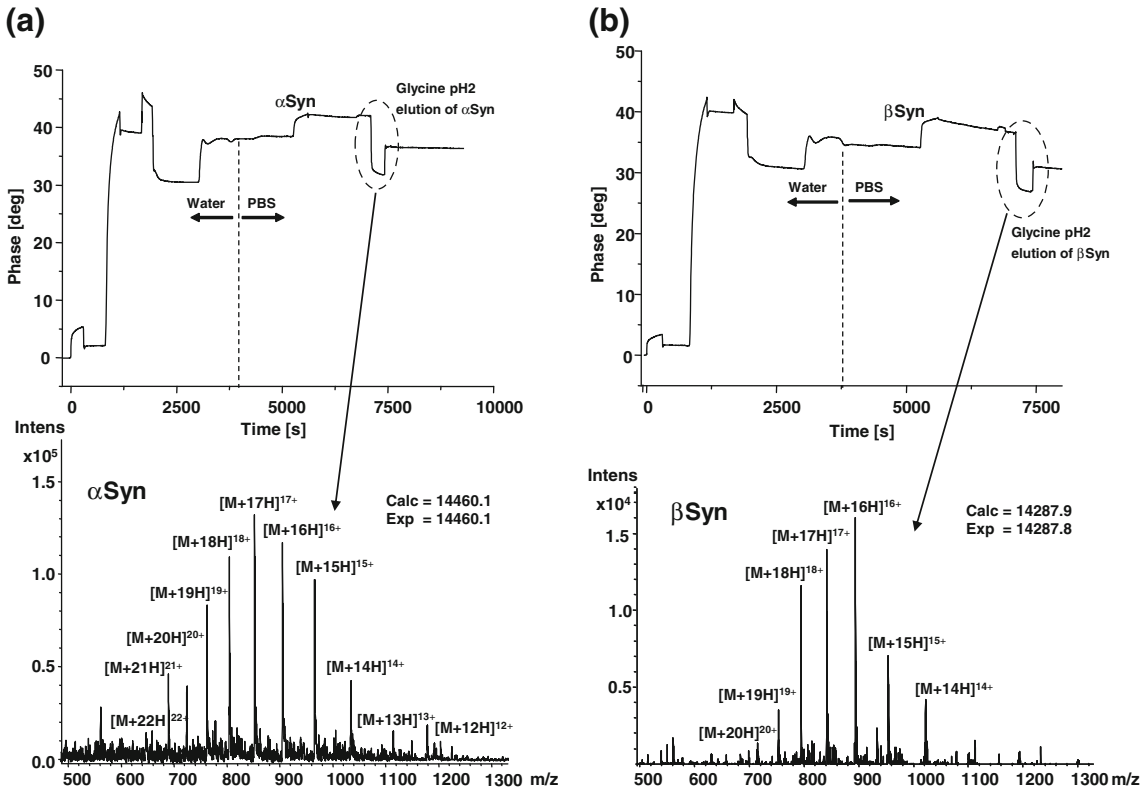

Figure 4. Comparison of affinity binding and mass spectrometric identification of αSyn and βSyn. (**a**) SAW binding curve of αSyn to the immobilized anti-αSyn pC20 antibody and ESI-ion trap mass spectrum of the eluted αSyn fraction; (**b**) SAW binding curve of βSyn to the anti-αSyn pC20 antibody and ESI-ion trap mass spectrum of the eluted βSyn fraction

In contrast, an N-terminal epitope, Aß(4-10) is recognized by a plaque-disaggregating antibody (6E10) [17]. As an example, the on-line SAW-FTICR-MS analysis of a pre-aggregated Aß(1-40) sample from an anti-Aß-antibody (4G8) capable of inhibiting Aß-aggregate formation [19, 25] is shown in Figure 2. Upon interaction with the antibody immobilized on the chip surface and elution from the interface, Aß(1-40) was identified by FTICR-MS (Figure 2a–c). From the binding curve, a dissociation constant K_D of approximately 20 nM was determined (Figure 2d). The on-line SAW-MS analysis of Aß(4-10) (not shown) ascertained that the N-terminal peptide alone had no affinity to the 4G8 antibody. Binding affinities determined by SAW-MS agreed well with previous affinity determinations using conventional methods [17, 19]. No significant change of elution and peak profile was observed in at least five repeated MS analyses and affinity quantifications of Aß(1-40), upon elution from the antibody; no significant increase of background signal was observed upon regeneration of the chip surface (Supporting Information, Figure S3).

The identification and affinity quantification of the Aß-antibody (4G8) complex with Aß(12-40) by on-line SAW-ESI-MS coupling to a Synapt-HDMS instrument is shown in Figure 3. Both the SAW binding curve and affinity elution profile from the immobilized Aß-antibody were very similar to the SAW-FTICR-MS analysis; the ESI spectrum provided unequivocal identification of Aß(12-40) (Figure 3c). The affinity determination provided a K_D of 19.3 nM, which is nearly identical to the K_D of 20.1 nM found for Aß(1-40) (Figure 2), and in agreement with the epitope specificity of the 4G8 antibody in the C-terminal sequence of Aß.

Identification and Affinity Characterization of α-Synuclein from Brain Homogenate

Aggregation of the key protein of Parkinson's disease, αSyn, is thought to proceed via oligomers of high neurotoxicity

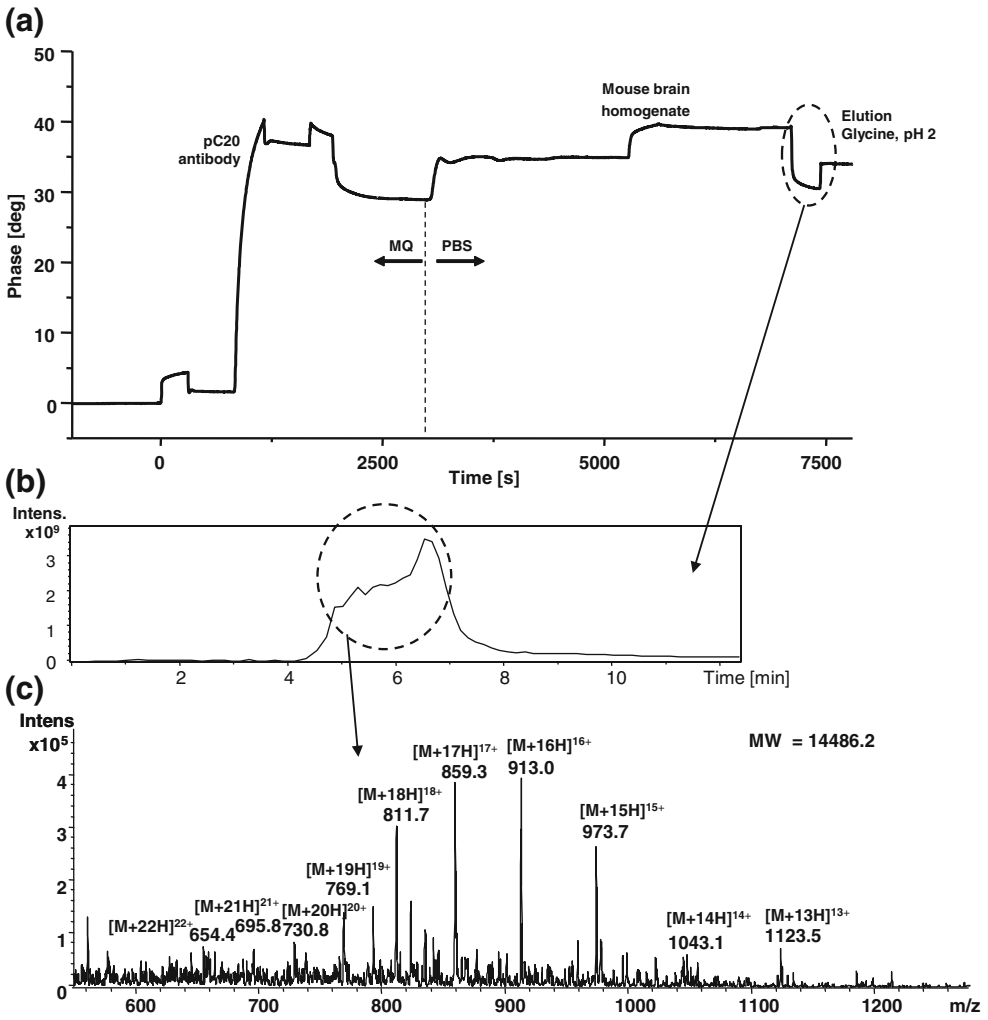

Figure 5. On-line SAW-MS identification of the human αSyn(A30P) mutant from mouse brain homogenate. (**a**) SAW binding curve of αSyn from mouse brain homogenate to immobilized anti-αSyn antibody pC20; (**b**) TIC profile of the eluate from mouse brain homogenate; (**c**) ESI-ion trap mass spectrum of the eluate

[26, 27]. Studies of the in vitro oligomerization- aggregation recently provided the first identification of specific autoproteolytic degradation products; particularly, a highly aggregation-prone C-terminal fragment, (αSyn(72-140), was identified upon incubation of αSyn for several days at physiological pH 7 [16, 18]. In contrast, β-synuclein (βSyn), the non-aggregating, non-toxic synuclein polypeptide in brain with a mutant VFS(70-72) sequence that lacks the cleavage site VVT(70-72) in the central amyloidogenic domain, showed neither oligomerization-aggregation nor any autoproteolytic cleavage. These results afford high interest in the comparative molecular characterization of αSyn and βSyn polypeptides in vivo by affinity-mass spectrometry using synuclein- specific antibodies.

Figure 4 shows the comparison of affinity binding and mass spectrometric identification of αSyn and βSyn by on-line SAW-MS, using an immobilized anti-αSyn antibody (pC20; Table 1). The SAW binding curves were similar for both proteins; the ESI- ion trap mass spectra of the eluted polypeptides provided unequivoval identifications. The affinity determinations yielded similar K_D values of approximately 40 nM for both polypeptides, in agreement with the epitope specificity of the synuclein- antibody in the C-terminal domain (81-96), which is identical for both polypeptides (Table 1).

First applications to synucleins in brain tissue were performed in order to evaluate the feasibility of on-line SAW-MS to the direct analysis of biological material. Using a chip-immobilized antibody recognizing a C-terminal αSyn epitope (4B12; Table 1), direct identifications of α-synuclein and αSyn oligomers were obtained from brain tissue. Figure 5 shows the SAW binding curve and on-line SAW-ESI-MS analysis of the interaction of mouse brain homogenate expressing the human αSyn(A30P) mutant with the anti-αSyn antibody 4B12. A dissociation constant K_D of approximately 45 nM was determined for the αSyn-antibody complex. The mass spectrometric analysis revealed a multiply charged ion series with a molecular mass of 14486 Da, in close agreement with the expected molecular weight of the αSyn mutant (Figure 5a–c).

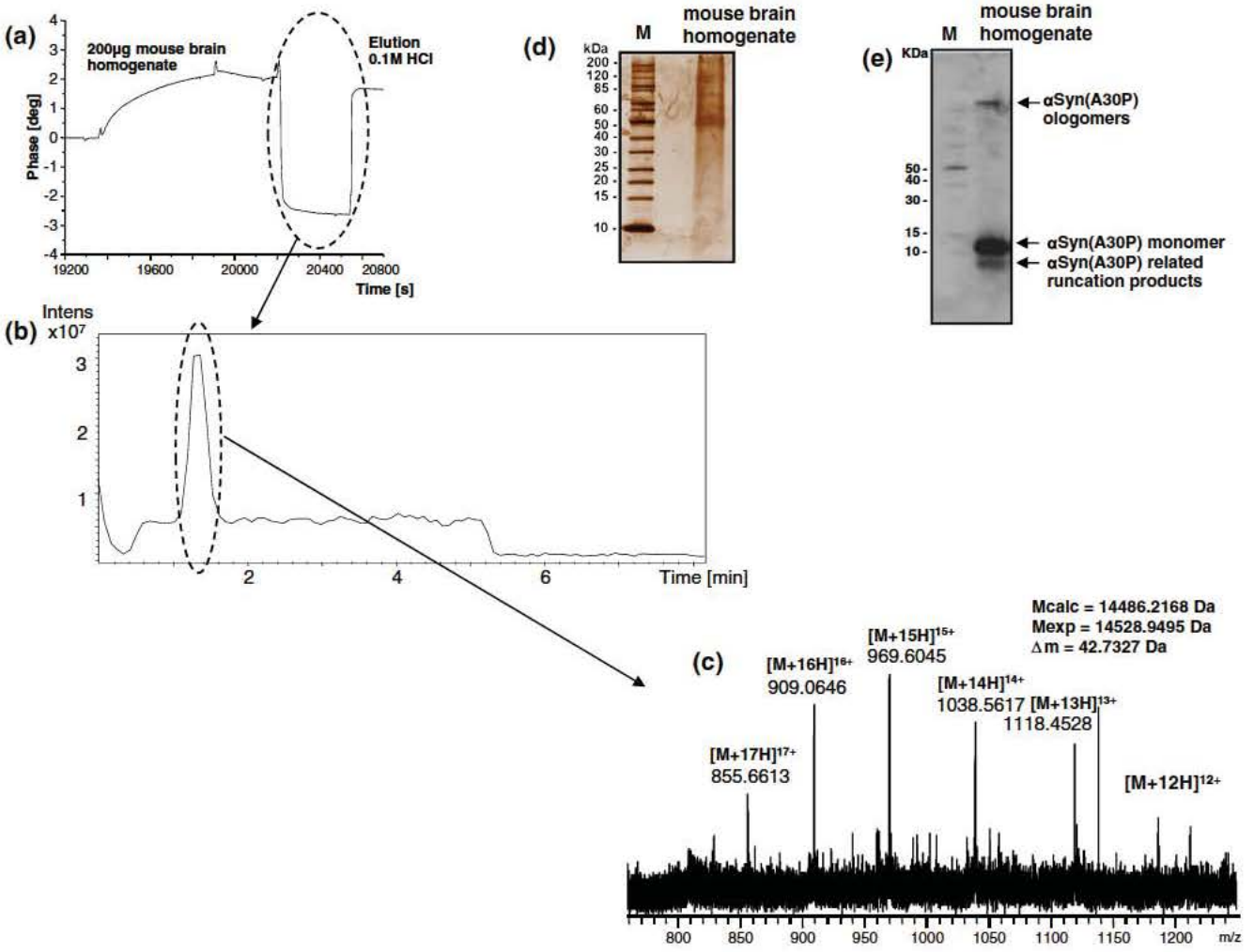

Figure 6. On-line SAW-MS identification of the human αSyn(A30P) mutant from mouse brain homogenate. (a) SAW binding curve of mouse brain homogenate to immobilized anti-αSyn antibody; (b) TIC of the affinity-eluate; (c) FTICR-MS analysis of the eluate from mouse brain homogenate; (d) and (e); gel electrophoresis and Western blot of the mouse brain homogenate sample

A comparison of the analysis of αSyn(A30P) by on-line SAW- FTICR- MS in vitro from mouse brain homogenate is shown in Supporting information, Figure S4; and Figure 6. The SAW binding curve of the immobilized anti-αSyn-antibody, and ESI- FTICR mass spectrum of the affinity-eluted αSyn(A30P) provided a molecular mass of 14486 Da, consistent with the intact protein. In the on-line SAW-MS analysis from mouse brain homogenate, a very similar binding curve and elution profile was obtained. The FTICR-MS analysis of the eluate from brain homogenate yielded a molecular mass of 14528 Da, corresponding to a mass increase by 42 u compared with the mass of 14,486 Da for unmodified αSyn(A30P) determined in vitro. This modification was identified by subsequent sequence determination as posttranslational acetylation [27]. Using the same antibody, a comparative Western blot of the brain sample indicated αSyn(A30P) monomer as a major product together with low levels of possible oligomer and truncation products; however, no possible identification is amenable; for comparison, a standard electrophoresis did not indicate the presence of synuclein or related proteins (Figure 7 d, e).

Conclusions

In the present study, an efficient microfluidic interface has been developed for on-line SAW-bioaffinity-MS, which provides the direct mass spectrometric analysis of biosensor-detected peptides and proteins with high sensitivity, concomitant with the quantitative determination of binding affinities. On-line SAW-MS analyses with high sensitivies (detection limits <1 nmol) were obtained for polypeptides and proteins with a wide range of antibody-affinities (low μM-nM), and suitable MS-acquisition software has been developed for SAW-coupling to several types of mass spectrometers. Moreover, the on-line SAW-MS combination was shown to be directly applicable to the mass spectrometric identification and affinity determination of proteins from biological material. These results suggest on-line SAW-MS as a powerful tool for the molecular characterization and quantification of biomolecular interactions, as diverse as antigen–antibody and protein– carbohydrate interactions.

Acknowledgments

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