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RESEARCH ARTICLE

Molecular characterization of the β-amyloid(4-10) epitope of plaque specific Aβ antibodies by affinity-mass spectrometry using alanine site mutation

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Alzheimer disease is a neurodegenerative disease affecting an increasing number of patients worldwide. Current therapeutic strategies are directed to molecules capable to block the aggregation of the β-amyloid(1-42) (Aβ) peptide and its shorter naturally occurring peptide fragments into toxic oligomers and amyloid fibrils. Aβ-specific antibodies have been recently developed as powerful antiaggregation tools. The identification and functional characterization of the epitope structures of Aβ antibodies contributes to the elucidation of their mechanism of action in the human organism. In previous studies, the Aβ(4-10) peptide has been identified as an epitope for the polyclonal anti-Aβ(1-42) antibody that has been shown capable to reduce amyloid deposition in a transgenic Alzheimer disease mouse model. To determine the functional significance of the amino acid residues involved in binding to the antibody, we report here the effects of alanine single-site mutations within the Aβ-epitope sequence on the antigen-antibody interaction. Specific identification of the essential affinity preserving mutant peptides was obtained by exposing a Sepharose-immobilized antibody column to an equimolar mixture of mutant peptides, followed by analysis of bound peptides using high-resolution MALDI-Fourier transform-Ion Cyclotron Resonance mass spectrometry. For the polyclonal antibody, affinity was preserved in the H6A, D7A, S8A, and G9A mutants but was lost in the F4, R5, and Y10 mutants, indicating these residues as essential amino acids for binding. Enzyme-linked immunosorbent assays confirmed the binding differences of the mutant peptides to the polyclonal antibody. In contrast, the mass spectrometric analysis of the mutant Aβ(4-10) peptides upon affinity binding to a monoclonal anti-Aβ(1-17) antibody showed complete loss of binding by Ala-site mutation of any residue of the Aβ(4-10) epitope. Surface plasmon resonance affinity determination of wild-type Aβ(1-17) to the monoclonal Aβ antibody provided a binding constant K_D in the low nanomolar range. These results provide valuable information in the elucidation of the binding mechanism and the development of Aβ-specific antibodies with improved therapeutic efficacy.

KEYWORDS
β-amyloid (4-10) epitope, alanine single-site replacement, Alzheimer disease, functional amino acid residues, high-resolution Fouriertransform-ICR mass spectrometry, immunoassay

1 | INTRODUCTION

The accumulation of senile plaques in cerebral tissue is one of the characteristics associated with Alzheimer disease (AD).[1] The main component of AD plaques is β-amyloid (Aβ), a 39-42 amino acid long peptide derived from the membrane-spanning amyloid precursor protein via proteolytic cleavage by β- and γ-secretases.[2] A soluble form of Aβ is present in the cerebrospinal fluid and plasma of both healthy individuals and AD patients.[3] Under normal circumstances, Aβ generated in the central nervous system is cleared with a half-life of 1 to 2 hours.[4] Although the pathological mechanisms leading to the conversion of Aβ peptides from the soluble to insoluble, aggregated forms are not fully understood, a major therapeutic approach for AD is the development of selective Aβ-aggregation inhibitors.[3,5]

Several therapeutic strategies, including active and passive immunization, have been pursued since the first reports that vaccination of transgenic AD mouse models with fibrillar Aβ reduced the extent and progression of AD pathology.[6] Furthermore, antibodies produced by TgCRND8 mice upon immunization with oligomeric assemblies of Aβ(1-42) have been found to be therapeutically effective, by reducing cerebral Aβ deposits and cognitive impairment.[7] Using proteolytic epitope excision- and epitope extraction-mass spectrometry, we previously identified the epitope recognized by the therapeutically active polyclonal antibodies to be located within the N-terminal domain, amino acid residues (4-10) (^{4}FRHDSGY^{10}). Moreover, mass spectrometric characterization of the epitope recognized by a commercially available monoclonal antibody (anti-Aβ(1-17)) indicated that the identical amino acid residues, Aβ(4-10), are involved in the interaction with this antibody.[8] Our previous work and a study by Bard et al showed that antibodies directed against epitopes within the N-terminal amino acids were capable of recognizing and disaggregating amyloid-related plaques, while antibodies targeting carboxy-terminal sequences of Aβ were unable to bind plaques.[7-9] Bard et al also reported that immunization with a full length Aβ peptide is not required to trigger an efficacious antibody response and that antibodies produced by immunization with small epitope peptides also significantly reduced neuritic pathology. Furthermore, Manea et al showed that bioconjugates generated by attachment of multiple copies of the Aβ(4-10) epitope to carriers consisting of branched chain polypeptides displayed increased antigenicity and thus might be potential immunogens.[10]

These previous findings provided a basis for the present study, which was focused on the identification of the essential amino acids within the Aβ(4-10) epitope sequence, and on the evaluation of their relative contribution to the interaction with Aβ-specific mono- and polyclonal antibodies. In this study, we report affinity-mass spectrometry, enzyme-linked immunosorbent assay (ELISA) using synthetic alanine single site mutated Aβ(4-10) epitope peptides, and SPR affinity determination of the N-terminal Aβ-epitope peptides, to obtain a molecular characterization of the epitope structure.

2 | MATERIALS AND METHODS

2.1.1. | Peptide synthesis

All peptides used in the present study (Table 1) were synthesized by solid phase peptide synthesis on a NovaSyn TGR resin (0.23 mmol/g coupling capacity), using Fmoc/^{t}Bu chemistry with a semiautomated Economy Peptide Synthesizer EPS-221 (Intavis, Köln, Germany). The following side-chain protected amino acid derivatives were used: Fmoc-Tyr(^{t}Bu)-OH, Fmoc-Ser(^{t}Bu)-OH, Fmoc-Asp(O^{t}Bu)-OH, Fmoc-His(Trt)-OH, Fmoc-Arg(Pbf)-OH and Fmoc-Glu(O^{t}Bu)-OH. The protocol of the synthesis was as follows: (1) DMF washing (3 × 1 min), (2) Fmoc deprotection with 20% piperidine in DMF (15 min), (3) DMF washing (6 × 1 min), (4) coupling of 5 equivalents of Fmoc-protected amino acid: PyBOP: NMM in DMF (50 min), (5) DMF washing (3 × 1 min). After completion of the syntheses and removal of the N-terminal Fmoc protecting group, the free amino group was biotinylated using 5 equiv of D-(+)-Biotin, in the presence of PyBOP and N-methylmorpholine. The peptides were then cleaved from the resin using a cleavage solution containing 95% trifluoroacetic acid as cleavage reagent and 2.5% triethylsilane and 2.5% deionized water as scavengers, for 2.5 hours at room temperature. For affinity-mass spectrometry, the peptides were cleaved from the resin before coupling the pentaglycine spacer and biotin.

After cleavage, the peptides were precipitated with cold *tert*-butyl-methyl-ether, the solid material was washed three times with diethylether and dissolved in 5% acetic acid (aqueous solution) prior to freeze-drying. The crude products were purified by reverse phase–high performance liquid chromatography (RP-HPLC) and analyzed by MALDI mass spectrometry.

2.1.2. | Reverse phase–high performance liquid chromatography

Analytical RP-HPLC was performed on a Bio-Rad HPLC (Bio-Rad Laboratories, Richmond, California) using an analytical Nucleosil 300-7 C_{18}

TABLE 1 Amino acid sequences and mass spectrometric characterization of wild type and mutant epitope peptides used in the present study

No.	Peptide acronym	Sequence	[M + H]+ Calculated	Experimental	Δm ppm
1	F4A	DAE**A**RHDSGY	1119.4789	1119.4819	2.6
2	R5A	DAEF**A**HDSGY	1110.4431	1110.4492	5.5
3	H6A	DAEFR**A**DSGY	1129.4875	1129.4914	3.4
4	D7A	DAEFRH**A**SGY	1151.5204	1151.5234	2.6
5	S8A	DAEFRHD**A**GY	1179.4998	1179.5183	15.7
6	G9A	DAEFRHDS**A**Y	1209.5255	1209.5289	2.8
7	Y10A	DAEFRHDSG**A**	1103.4822	1103.4870	4.3
8	BG_5WT	Biotin-GGGGGDAEFRHDSGY	1706.6976	1706.7367	22.9
9	BG_5F4A	Biotin-GGGGGDAE**A**RHDSGY	1630.6663	1630.6948	17.4
10	BG_5R5A	Biotin-GGGGGDAEF**A**HDSGY	1621.6336	1621.6540	12.5
11	BG_5H6A	Biotin-GGGGGDAEFR**A**DSGY	1640.6758	1640.6977	13.3
12	BG_5D7A	Biotin-GGGGGDAEFRH**A**SGY	1662.7078	1662.7124	2.8
13	BG_5S8A	Biotin-GGGGGDAEFRHD**A**GY	1690.7027	1690.7340	18.5
14	BG_5G9A	Biotin-GGGGGDAEFRHDS**A**Y	1720.7132	1720.7395	15.3
15	BG_5Y10A	Biotin-GGGGGDAEFRHDSG**A**	1614.6714	1614.7321	37.6

column (Macherey-Nagel, Düren, Germany) as a stationary phase. Linear gradient elution (0 min 0% B; 5 min 0% B; 50 min 90% B) with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile-water (80:20 *v*/v)) was used at a flow rate of 1 mL/min at room temperature. The samples were dissolved in eluent A and the peaks were detected at λ = 220 nm. The crude products were purified on a preparative C_{18} column (GROM-SIL 120 ODS-4 HE, 10 μm, 250 × 20 mm; Herrenberg-Kayh, Germany) using the same eluents as described above, with a flow rate of 10 mL/min.

2.1.3. | Mass spectrometry

MALDI mass spectrometric analysis was performed with a Bruker APEX II FT-ICR instrument, equipped with an actively shielded 7T superconducting magnet and a cylindrical infinity ICR analyzer cell. The external Scout 100 fully automated X-Y target stage MALDI source was operated with pulsed collision gas (Bruker Daltonik, Bremen, Germany) and a pulsed nitrogen laser (337 nm). A 100-mg/mL solution of 2,5-dihydroxybenzoic acid (Aldrich, Steinheim, Germany) in acetonitrile: 0.1% trifluoroacetic acid in water (2:1 *v*/v) was used as a matrix. A volume of 0.5-μL matrix solution was mixed on the stainless-steel MALDI sample target with 0.5 μL of sample solution and allowed to dry. Calibration was performed with a standard peptide mixture with an m/z range of approximately 5000, using the monoisotopic masses of singly protonated ions of human angiotensin II (1046.542), human bradykinin (1060.569), human angiotensin I (1296.685), human neurotensin (1672.917), bovine insulin β-chain oxidized (3404.651), and bovine insulin (5730.609). Acquisition of spectra was performed with the Bruker Daltonik software XMASS and corresponding programs for mass calculation, data calibration, and processing. For sample preparation, a mixture of 50% methanol and 2% acetic acid in water was used. electrospray ionization mass spectrometry as performed with a Bruker Esquire 3000+ mass spectrometer.

2.1.4. | Preparation of an antibody-immobilized affinity column

Aproximately 100 μg of antibody were dissolved in 0.2 mL of coupling buffer (0.2-mol L^{-1} $NaHCO_3$, 0.5-mol L^{-1} NaCl, pH 8.3). The solution was added to dry N-hydroxysuccinimide-activated 6-aminohexanoic acid-coupled Sepharose 4B (Sigma-Aldrich), and the coupling reaction was performed for 1 hour at 25°C. The Sepharose-coupling product was loaded into a microcolumn (MoBiTec GmbH, Goettingen, Germany) and washed sequentially with blocking solution (0.1-mol L^{-1} aminoethanol, 0.5-mol L^{-1} NaCl, pH 8.3) and washing solution (0.2-mol L^{-1} CH_3COONa, 0.5-mol L^{-1} NaCl, pH 4.0). The blocking solution was used with a total volume of 24 mL (4 times 6 mL) and the washing solution with a total volume of 18 mL (3 times 6 mL). The column was kept for 1 hour at room temperature in blocking solution, followed by a second washing procedure as described above. Thereafter, the column was washed with 10 mL phosphate- buffered saline (5-mmol L^{-1} Na_2HPO_4, 150-mmol L^{-1} NaCl, pH 7.5) and stored at 4°C until use.

2.1.5. | Affinity-mass spectrometry

The peptides were dissolved in PBS at equimolar ratios and allowed to interact with the antibody immobilized affinity matrix for 2 hours at room temperature. The supernatant containing the unbound peptides (washing fraction) was collected in a microreaction cup, and the column was then washed with 10-mL PBS. Affinity bound peptides were eluted with 500 μL 0.1% TFA (aqueous solution). The washing and elution fractions were freeze-dried and stored at 4°C until mass spectrometric analysis. Before MS analysis, the samples were concentrated and desalted using a C18 ZipTip (Millipore).

2.1.6. | Enzyme-linked immunosorbent assay

All assays were performed in 96-well plates coated overnight at 4°C with 150 μL/well of 5-mg/L streptavidin in PBS. Wells were washed once with 0.05% *v*/v Tween 20 in PBS (5mM Na_2HPO_4, 150mM NaCl, pH 7.5). Peptide solutions were prepared and diluted in PBS to a final concentration of 0.5 μM. A volume of 100 μL of each solution was deposited in the wells in triplicate, and the plates were incubated for 2 hours at room temperature. For background subtraction, triplicate wells containing PBS were incubated in a first step for each antibody dilution. The wells were blocked with 5% BSA in PBS (*w*/*v*) for 2 hours and washed with PBS / 0.05% *v*/v Tween 20. The monoclonal antibody (mAb) 6E10 (Chemicon Inc, Temecula, CA; and Sigma, St. Louis, USA; raised against Aβ(1-17) purified IgG) or polyclonal IgG from Aβ(1-42) immunized TgCRND8 mice[7,8] were added in 8 twofold serial dilutions, and the 96-well plates gently shaken for 2 hours to allow complete binding of the antibody. Supernatant unbound antibodies were removed by 4 times washing with PBS/0.05% *v*/v Tween 20. Alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma, Saint Louis, USA; 100 μL at 1 μg/mL in BSA 5% *w*/*v* in PBS) was added and incubated for 2 hours at room temperature. The wells were washed twice with PBS/ 0.05% (*v*/v) Tween 20, and twice with diethanolamine 2.5M, pH 9.5. Wells were finally incubated for 1 to 30 minutes at room temperature with Attophos substrate (Promega, Madison, USA), and the enzymatic reaction monitored as a function of time with a 485/535 (excitation/emission) filter set, using a Victor[2] ELISA plate reader (Perkin Elmer Life/Analytical Sciences, Boston, MA).

SPR affinity determinations were performed with a Reichert-Ametek 2Ch-7500 instrument (Reichert-Ametek, Buffalo, NY, USA). A carboxymethyl dextran hydrogel sensor chip was activated using standard EDC/NHS procedure followed by immobilizing 35 μg of antibody in 10mM sodium acetate buffer pH 5.2; blocking step was performed with 1M ethanolamine pH 8. The calculated K_D was a result of a dilution series of Aβ(1-16) over the chip from 73 nM to 0.5 μM. The TraceDrawer program was used for the fitting of the data. The affinity fit calculates the equilibrium dissociation constant K_D from a signal vs concentration plot using nonlinear regression.

3 | RESULTS

3.1 | Synthesis and structural characterization of N-terminal β-amyloid peptide mutants

Aβ(1-10) decapeptides containing each of the amino acid residues of the Aβ(4-10) epitope, sequentially replaced by alanine, were prepared by solid phase peptide synthesis according to the Fmoc/*t*Bu strategy. For ELISA binding studies, each sequence was prolonged at the

N-terminus with a pentaglycine spacer and biotin, to ensure free accessibility of the epitope to the antibody and efficient immobilization of the peptides to the 96-well plates. The peptides were purified by RP-HPLC and characterized by ESI-MS (Table 1).

3.2 | Affinity-mass spectrometric characterization of alanine mutated Aβ-epitope peptides

The binding of mutant peptides to the anti-Aβ(1-42) polyclonal antibody and the anti-Aβ(1-17) monoclonal antibody was investigated using high-resolution mass spectrometry. Two affinity columns were prepared by immobilizing the antibodies on NHS-activated Sepharose. An equimolar mixture of the peptides 1 to 7 (Table 1) was applied to the antibody columns and allowed to interact with the affinity matrix. The supernatant containing unbound peptides was removed by washing with PBS (Figure 1A), while the peptides bound to the antibody were eluted under acidic conditions. The MALDI-FTICR mass spectrum of the elution fraction (Figure 1B) provided the identification of Aβ(1-10)H6A, Aβ(1-10)D7A, Aβ(1-10)S8A, and Aβ(1-10)G9A. This result indicated that binding to the polyclonal anti-Aβ(1-42) antibody was not abolished by alanine mutations of the H6, D7, S8, and G9 residues. In contrast, mutations causing the loss of peptide binding to the antibody were found to be located at the N- and C-terminal ends of the epitope, amino acids F4, R5, and Y10. In contrast to the polyclonal antibody, the mass spectrum of the elution fraction (Figure 1D) obtained from the monoclonal anti-Aβ(1-17) antibody revealed no signal, indicating that any single-site mutant peptide had lost affinity to the antibody.

3.3 | Enzyme-linked immunosorbent assay

To further characterize the differential effect of the amino acid residues to the binding of the epitope to the monoclonal and polyclonal antibodies, indirect ELISA was performed. To provide accurate background subtraction, triplicate wells of each antibody dilution without antigen were used on each ELISA plate. These results showed that the alanine mutants can be classified into 4 groups. A first group contains the mutants D7A and S8A that display binding properties to the polyclonal anti-Aβ(1-42) antibody, close to that of the wild-type peptide. The Y10A mutant was considered a special case, since it showed a 2.8 times lower binding ability compared to the wild type, without complete loss of binding. In contrast, mutations F4A, R5A, and H6A led to complete loss of antibody binding. For comparison, replacement of glycine with alanine slightly increased the binding ability of the mutant peptide (Figures 2 and 3). The polyclonal anti-Aβ(1-42) antibody presented high affinity to the Aβ(1-16) epitope with a K_D of 22.47 nM. This interaction study provided additional confirmation of the ELISA study.

The binding of the mutant peptides to the monoclonal anti-Aβ(1-17) antibody was analyzed in a similar ELISA experiment. The results shown in Figure 4 clearly indicated that each of the single-site mutations caused complete loss of antibody binding.

4 | DISCUSSION

Previous work in our laboratory on the identification of the epitope recognized by the polyclonal anti-Aβ(1-42) and the monoclonal anti-Aβ(1-17) antibodies using proteolytic epitope excision- and extraction-mass spectrometry showed that both antibodies recognized the identical amino acid sequence located between F4 and Y10. Polyclonal antibodies generally exhibit mixed specificities. Thus, to fully characterize the epitope specificity of the polyclonal anti-Aβ(1-42) antibody, affinity-mass spectrometric and ELISA experiments were performed using mutants that contain single-site mutations of each amino acid within the epitope sequence.

In the polyclonal antibody, mutation of the Aβ(4-10) to alanine of 3 of the 7 amino acids caused loss of antibody binding to the

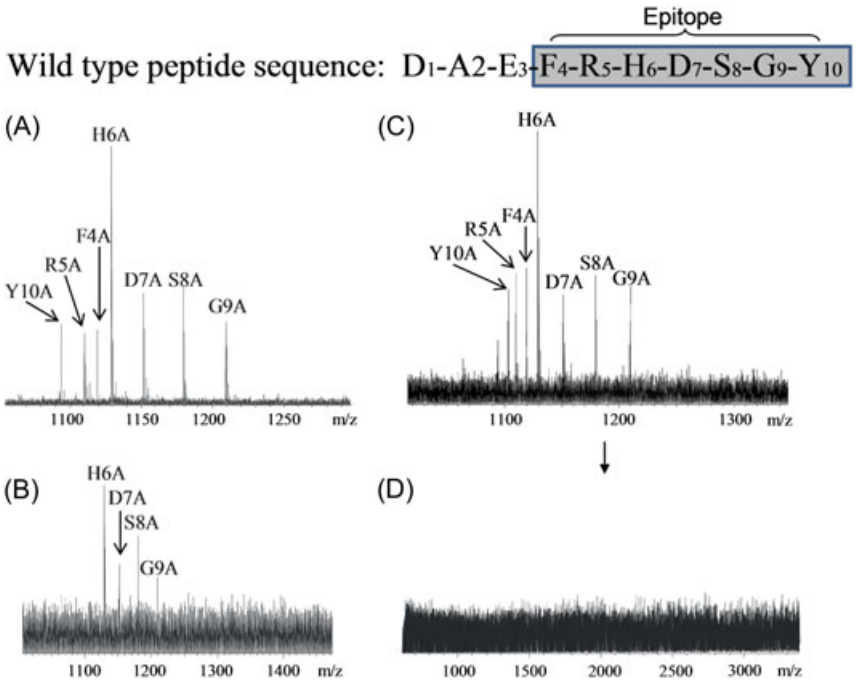

FIGURE 1 Affinity-mass spectrometric identification of the alanine mutants preserving binding to the Aβ antibodies. MALDI-FT-ICR mass spectra of supernatant and elution fractions were collected in the affinity experiment in which an equimolar mixture of mutant peptides was exposed to the polyclonal anti-Aβ(1-42) (A,B) and monoclonal anti-Aβ(1-17) antibody (C,D), respectively

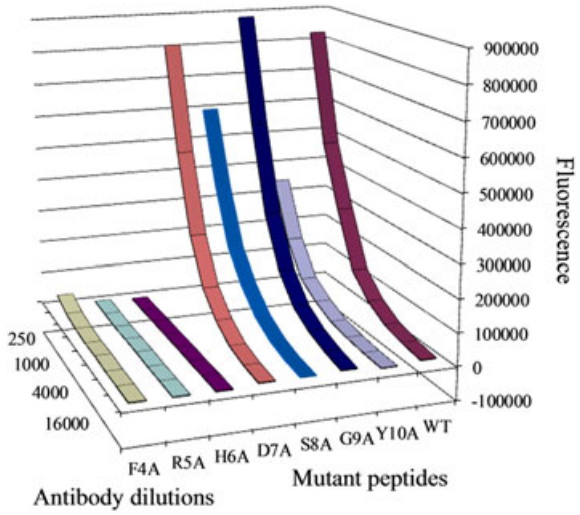

FIGURE 2 Enzyme-linked immunosorbent assay binding study of alanine mutants to the polyclonal anti-Aβ(142) antibody. (s. Figure 1 for assignment of mutants). Experiments were conducted in triplicate; SD was approximately 10%

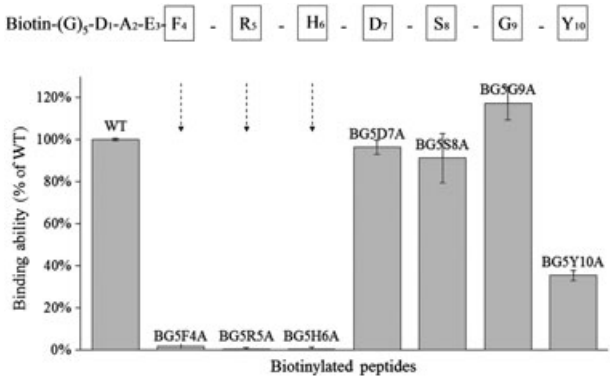

FIGURE 3 Binding ability in percent of the wild-type Aβ peptide for the mutant peptides at an antibody concentration of 1 µg/mL. Experiments conducted in triplicates, with SD of approximately 10%

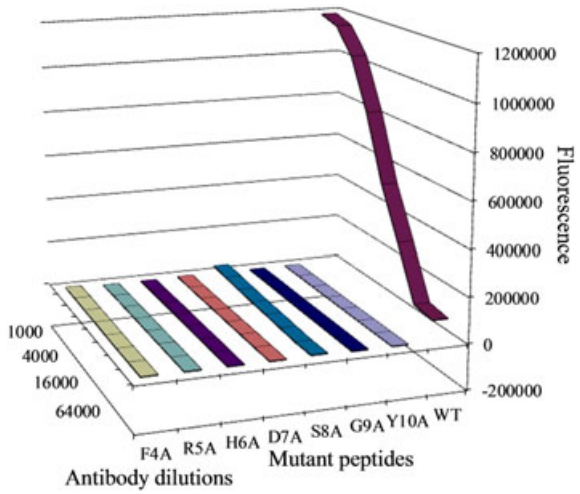

FIGURE 4 Enzyme-linked immunosorbent assay binding study of alanine mutants to the monoclonal anti-Aβ(1-17) antibody. Experiments were conducted in triplicate, with SD of approximately 10%

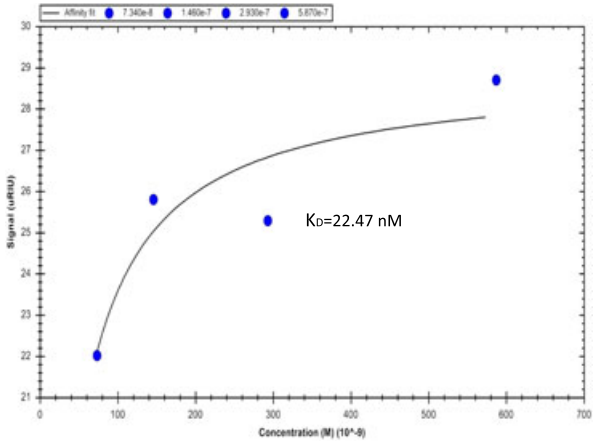

FIGURE 5 Affinity determination of polyclonal anti-Aβ(1-42) antibody interacting with the Aβ(1-16) peptide. From the affinity fitting model, a K_D of 22.5 nM was determined

respective mutant peptide. In contrast, for the monoclonal antibody, all 7 amino acid residues of the epitope were shown to be essential for the binding, consistent with the high selectivity of the antibodies. This is also consistent with SPR biosensor determinations of the binding to the antibodies that showed high affinity with K_D values in the nanomolar range (Figure 5). Three-dimensional structure models of Aβ(1-42) and its interactions have been discussed by Luhrs et al.[11]

In conclusion, in the present study, we identified the amino acid residues that are critical for the recognition of the β-amyloid plaque specific epitope by monoclonal and polyclonal antibodies, suggesting the specific value of the antibodies in the development of therapeutic approaches for AD.

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