N-terminal engineering of amyloid-β-binding Affibody molecules yields improved chemical synthesis and higher binding affinity

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Abstract: The aggregation of amyloid-β (Aβ) peptides is believed to be a major factor in the onset and progression of Alzheimer's disease. Molecules binding with high affinity and selectivity to Aβ-peptides are important tools for investigating the aggregation process. An Aβ-binding Affibody molecule, $Z_{Aβ3}$, has earlier been selected by phage display and shown to bind Aβ(1–40) with nanomolar affinity and to inhibit Aβ-peptide aggregation. In this study, we create truncated functional versions of the $Z_{Aβ3}$ Affibody molecule better suited for chemical synthesis production. Engineered Affibody molecules of different length were produced by solid phase peptide synthesis and allowed to form covalently linked homodimers by S-S-bridges. The N-terminally truncated Affibody molecules $Z_{Aβ3}$(12–58), $Z_{Aβ3}$(15–58), and $Z_{Aβ3}$(18–58) were produced in considerably higher synthetic yield than the corresponding full-length molecule $Z_{Aβ3}$(1–58). Circular dichroism spectroscopy and surface plasmon resonance-based biosensor analysis showed that the shortest Affibody molecule, $Z_{Aβ3}$(18–58), exhibited complete loss of binding to the Aβ(1–40)-peptide, while the $Z_{Aβ3}$(12–58) and $Z_{Aβ3}$(15–58) Affibody molecules both displayed approximately one order of magnitude higher binding affinity to the Aβ(1–40)-peptide compared to the full-length Affibody molecule. Nuclear magnetic resonance spectroscopy showed that the structure of Aβ(1–40) in complex with the truncated Affibody dimers is very similar to the previously published solution structure of the Aβ(1–40)-peptide in complex with the full-length $Z_{Aβ3}$ Affibody molecule. This indicates that the N-terminally truncated Affibody molecules $Z_{Aβ3}$(12–58) and $Z_{Aβ3}$(15–58) are highly promising for further engineering and future use as binding agents to monomeric Aβ(1–40).

Keywords: amyloid; protein engineering; Alzheimer's disease; solid phase peptide synthesis; NMR spectroscopy

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Introduction

The amyloid-β (Aβ) peptides are small aggregation-prone cleavage products from the amyloid precursor protein (APP). They vary in length between 39 and 43 amino acid residues, where Aβ(1–40) is the most abundant peptide and Aβ(1–42) is generally considered to be the most toxic species.[1,2] The peptides were first identified in 1984 in the meningeal blood vessels of patients suffering from Alzheimer's disease (AD) and in individuals with Down's syndrome.[3,4] It was later shown that the thread-like structures found in the postmortem brains of patients with AD predominantly were built up by Aβ peptides.[5] Although the underlying mechanism of AD progression still remains unknown, massive research indicates that the Aβ peptides play an important role in the development and progression of the disease.[6,7] Elevated levels of high-molecular-weight β-amyloid oligomers have been reported in the cerebrospinal fluid of AD patients,[8] and numerous studies have shown that Aβ peptides are toxic to cells *in vitro*[9–11] and that animals exposed to soluble oligomers of Aβ peptides display reduced cognitive function.[12]

Still today, more than one hundred years after AD was first discovered, there is no effective treatment for the disease. Since the Aβ peptides are strongly associated with AD, different antibody-based strategies directed toward reducing the amount of Aβ peptides in the brain have been investigated. Aβ-specific antibodies have also found important applications in the diagnosis of AD, and have been suggested as diagnostic probes for molecular imaging of AD patients.[13,14] A vaccine against AD, AN-1792, has shown promising results in various studies, but severe side effects have also been reported.[15–17] Thus, an alternative and perhaps safer approach might be to treat patients with Aβ-binding antibodies by so called passive immunization.[18,19]

Another class of affinity proteins with potential advantages in terms of size, cost, and stability is Affibody molecules, which are based on the engineered domain Z derived from the B domain of staphylococcal protein A.[20,21] An Affibody molecule, $Z_{Aβ3}$, that selectively binds soluble, nonaggregated Aβ peptides with high affinity has previously been selected using phage display.[22] $Z_{Aβ3}$ has shown potential to efficiently inhibit Aβ aggregation, and recent studies have shown that $Z_{Aβ3}$ is also capable of dissolving preformed Aβ oligomers.[2,23] When coexpressed in *Drosophila melanogaster*, the molecule attenuated the neurotoxic effect of Aβ(1–42).[2] Structure analysis showed that $Z_{Aβ3}$ binds as a disulfide-linked homodimer to the Aβ peptide, and that the original three-helix bundle structure of the Z domain is not retained in the $Z_{Aβ3}$ Affibody molecule. Instead, the first α-helix of the parental protein becomes partially unstructured in the free form of $Z_{Aβ3}$,[24] and upon binding to the Aβ peptide residues 15–18 adopt a β-strand conformation. Residues 1–13 of $Z_{Aβ3}$ are unstructured in the complex and do not participate in binding the Aβ peptide.[23,24] When bound to $Z_{Aβ3}$ the Aβ(1–40) peptide adopts an antiparallel β-hairpin structure held together through intramolecular hydrogen bonds, allowing the two subunits of the $Z_{Aβ3}$ dimer to form a four-stranded antiparallel β-sheet together with the Aβ peptide. This β-sheet is anchored against Affibody helix 3 through nonpolar interactions involving both Val-17 and a salt bridge between Glu-15 and Lys-49 in the Affibody molecule. Since amyloid fibers appear to be formed of parallel β-sheets consisting of Aβ peptides in loose β-hairpin conformation held together by intermolecular hydrogen bonds, Aβ peptides in β-hairpin conformation may be the primary unit involved in Aβ aggregation.[25,26] To the best of our knowledge, $Z_{Aβ3}$ is the only molecule known to lock Aβ peptides in this interesting conformation.

The Aβ-binding Affibody molecule could be used *in vitro* for AD diagnosis, as a research tool for studies of the dynamics and function of the Aβ peptide in the potentially toxic β-hairpin conformation, and possibly also for *in vivo* therapy. For many of these applications it would be advantageous if the Affibody molecule could be produced chemically, since this would facilitate chemical modifications such as the introduction of unnatural amino acids or site-specific labeling with reporter groups. It has previously been shown that the 58 aa Affibody molecule can be prepared using solid phase peptide synthesis (SPPS), albeit with a low yield.[27–30] Even though the outcome of a synthesis is sequence-dependent and can be difficult to predict, longer peptides typically display lower yields due to incomplete coupling and deprotection reactions and the accumulation of side products.

In this study, we aim to create shorter but still fully functional versions of the $Z_{Aβ3}$ Affibody molecule better suited for production by chemical synthesis. Since the NMR structure indicates that the N-terminus of $Z_{Aβ3}$ is unstructured and does not participate in binding to the Aβ peptide, it was hypothesized that N-terminal truncation would yield functional Affibody molecules that can be synthesized with higher efficiency. Six different N-terminally truncated variants of the $Z_{Aβ3}$ Affibody molecule and the corresponding full-length molecule were synthesized by SPPS and evaluated in terms of synthetic yield using reversed-phase ultra high performance liquid chromatography (RP-UHPLC) and mass spectrometry (MS). The biophysical properties of the truncated Affibody variants and their interaction with the Aβ(1–40) peptide were characterized by circular dichroism (CD), surface plasmon resonance (SPR), and nuclear magnetic resonance (NMR) spectroscopy.

Results

Solid phase peptide synthesis of the truncated Aβ–binding $Z_{Aβ3}$ Affibody molecules

Six N-terminally truncated variants of the $Z_{Aβ3}$ Affibody molecule (Fig. 1 and Table I) were successfully

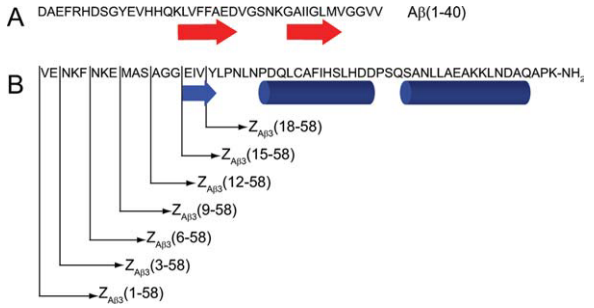

Figure 1. The amino acid sequences of the Aβ(1–40) peptide (A) and the $Z_{Aβ3}$(1–58) Affibody molecule (B). The arrows indicate the sites of truncation giving rise to the six different variants produced by solid phase peptide synthesis (SPPS). Note that the $Z_{Aβ3}$(1–58) sequence has an Asp^2Glu substitution compared to the previously published $Z_{Aβ3}$ Affibody molecule.[22] Block arrows indicate β-strands, while the cylinders indicate α-helical structure, as determined by the structure analysis of the complex done by Hoyer *et al*.[23] [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

synthesized using standard Fmoc SPPS. All truncated variants were found to be soluble, and their synthetic yields were determined by analytical RP-UHPLC (Table II). The shorter variants $Z_{Aβ3}$(18–58), $Z_{Aβ3}$(15–58), $Z_{Aβ3}$(12–58), and $Z_{Aβ3}$(9–58) were obtained in high yields, whereas the longer variants $Z_{Aβ3}$(6–58), $Z_{Aβ3}$(3–58) and the full-length Affibody molecule $Z_{Aβ3}$(1–58) were obtained in much lower yields (Table II). The bulky, trityl-protected asparagine residues in positions 3 and 6 in the N-terminal region of the Affibody molecules have earlier been found difficult to couple[27] and this likely contributes to the observed sudden drop in synthesis yield in the longer $Z_{Aβ3}$ variants. All Affibody variants were dimerized through Cys-28—Cys-28 disulfide linking and purified by removing traces of monomeric forms. The end products were therefore highly pure dimers, and in the following all $Z_{Aβ3}$ Affibody molecules referred to in the text are understood to be disulfide-linked dimers.

Circular dichroism spectroscopy

The CD spectra of the four different Affibody molecules have local minima at 208 and 222 nm, consistent with a largely alpha-helical secondary structure (Fig. 2). Upon titration with Aβ(1–40) the resulting Affibody/Aβ(1–40) systems displayed different secondary structure transformations. The CD spectrum of the $Z_{Aβ3}$(18–58)/Aβ(1–40) system (Fig. 2(D)) was virtually identical to the CD spectrum of the $Z_{Aβ3}$(18–58) Affibody molecule alone, indicating little difference in secondary structure. The CD signal of free Aβ(1–40) peptide was found to be small compared to the signal of the Affibody molecules, both at low and high temperatures, reflecting the unstructured nature—close to random coil—of free Aβ(1–40), which is characterized by a weak CD signal. For $Z_{Aβ3}$(1–58), the CD signal decreases rather uniformly upon addition of Aβ(1–40), indicating a lower helical content in the $Z_{Aβ3}$(1–58)/Aβ(1–40) system compared to the free Affibody molecule (Fig. 2(A)). For $Z_{Aβ3}$(12–58), the CD signal around 208 nm decreases significantly while the signal around 222 nm decreases somewhat less, suggesting both lower helical content and increased β-strand structure in the Affibody/Aβ system compared to the free $Z_{Aβ3}$(12–58) molecule (Fig. 2(B)). For $Z_{Aβ3}$(15–58), the CD signal around 208 nm decreases while the signal around 222 nm increases, indicating increased β-strand content in the system when Aβ(1–40) is added, possibly together with reduced helicity (Fig. 2(C)). Since the titrations were carried out using identical protocols and titration steps, it is clear that the different responses to added Aβ peptide reflect differences in interaction between the Aβ(1–40) peptide and the four Affibody variants. Although free Aβ(1–40) has a weak CD signal, the peptide may display a more defined secondary structure and a stronger CD signal when bound to an Affibody molecule, and the increased β-strand content observed in some Affibody/Aβ systems may originate from structural transitions either in the Affibody molecule or in the Aβ peptide. The observed reductions in helical content, on the other hand, must originate from a loss of helical structure in the Affibody molecules.

Melting profiles of the four Affibody variants were recorded with CD spectroscopy at 220 nm both in presence and absence of Aβ(1–40) peptide. The melting temperatures (T_m) of free $Z_{Aβ3}$(18–58) and the $Z_{Aβ3}$(18–58)/Aβ(1–40) system were rather

Table I. *Sequences of the Full-Length and Truncated $Z_{Aβ3}$ Affibody Variants*

Protein	Sequence
$Z_{Aβ3}$(1–58)[a]	VE NKF NKE MAS AGG EIV YLPNLNPDQLCAFIHSLHDDPSQSANLLAEAKKLNDAQAPK
$Z_{Aβ3}$(3–58)	NKF NKE MAS AGG EIV YLPNLNPDQLCAFIHSLHDDPSQSANLLAEAKKLNDAQAPK
$Z_{Aβ3}$(6–58)	NKE MAS AGG EIV YLPNLNPDQLCAFIHSLHDDPSQSANLLAEAKKLNDAQAPK
$Z_{Aβ3}$(9–58)	MAS AGG EIV YLPNLNPDQLCAFIHSLHDDPSQSANLLAEAKKLNDAQAPK
$Z_{Aβ3}$(12–58)	AGG EIV YLPNLNPDQLCAFIHSLHDDPSQSANLLAEAKKLNDAQAPK
$Z_{Aβ3}$(15–58)	EIV YLPNLNPDQLCAFIHSLHDDPSQSANLLAEAKKLNDAQAPK
$Z_{Aβ3}$(18–58)	YLPNLNPDQLCAFIHSLHDDPSQSANLLAEAKKLNDAQAPK

The underlined residues were double-coupled during the SPPS synthesis.
[a] $Z_{Aβ3}$(1-58) has an Asp^2Glu substitution compared to the original $Z_{Aβ3}$ sequence.

Table II. *Synthesis Results for the Different $Z_{A\beta3}$ Affibody Variants*

Protein	Length (aa)	Theoretical MW[a] (Da)	Experimental MW[b] (Da)	Synthetic yield[c] (%)
$Z_{A\beta3}$(1–58)	58	12610.0	12610.8	8[d]
$Z_{A\beta3}$(3–58)	56	12153.6	12154.0	7
$Z_{A\beta3}$(6–58)	53	11374.6	11375.0	9
$Z_{A\beta3}$(9–58)	50	10631.8	10632.7	20
$Z_{A\beta3}$(12–58)	47	10053.2	10053.7	29
$Z_{A\beta3}$(15–58)	44	9682.8	9683.0	30
$Z_{A\beta3}$(18–58)	41	9000.0	9000.0	35

[a] Molecular weight of the disulfide-linked dimer.
[b] Obtained from LC-ESI MS measurements.
[c] Obtained from RP-UHPLC elution profiles at 220 nm.
[d] From a separate synthesis.

similar, suggesting a lack of specific interaction between the two molecules (Fig. 3, Table III). The melting temperatures of the other three Affibody/Aβ(1–40) systems were markedly higher than the T_m's of the respective free Affibody molecules, indicating formation of complexes that are more stable than the Affibody molecules themselves. In their free form the truncated Affibody versions $Z_{A\beta3}$(12–58) and $Z_{A\beta3}$(15–58) display slightly lower thermal stability than the full-length version $Z_{A\beta3}$(1–58), but the higher ΔT_m of $Z_{A\beta3}$(12–58) and $Z_{A\beta3}$(15–58), that is, +16.4 and +14.7°C, respectively, compared to +7.6°C for $Z_{A\beta3}$(1–58), suggests a stronger binding of the two truncated Affibody versions to the Aβ(1–40) peptide (Fig. 3, Table III).

Surface plasmon resonance-based biosensor analysis

SPR-based biosensor (Biacore) analysis was carried out at 25°C with different Affibody molecules being injected over a chip surface harboring an immobilized biotin-LC-Aβ(1–40) peptide. The kinetics of the interactions was monitored in real time and dissociation constants could be determined from the measured rate constants k_a and k_d (Fig. 4, Table III). The dissociation constant (K_D) of the full-length control Affibody dimer $Z_{A\beta3}$(1–58) was found to be 9.5 nM, which is close to the published value for $Z_{A\beta3}$ (K_D = 17 nM) determined by isothermal titration calorimetry (ITC).[23] A difference between the two studies is that the previously published work was performed

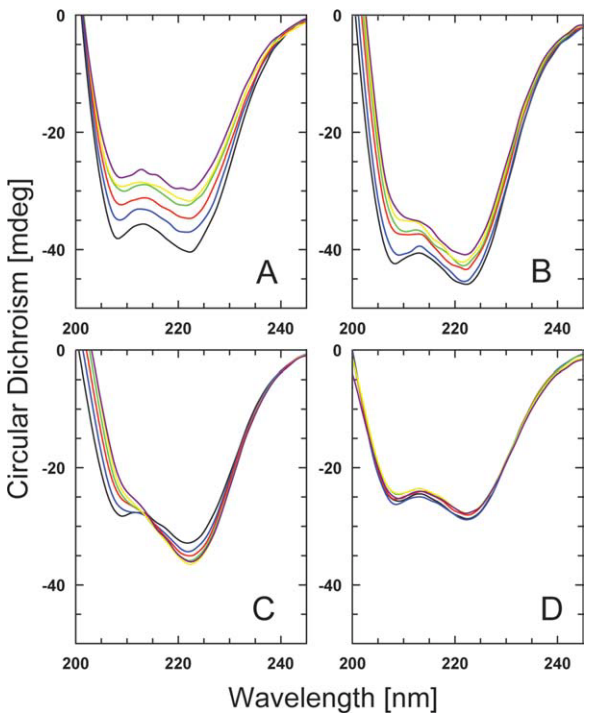

Figure 2. CD spectra of titrations of Aβ(1–40) onto the four Affibody homodimers $Z_{A\beta3}$(1–58) (A), $Z_{A\beta3}$(12–58) (B), $Z_{A\beta3}$(15–58) (C), and $Z_{A\beta3}$(18–58) (D). Measurements were conducted at 25°C in 10 mM Na-phosphate buffer, pH 7.4, with an Affibody dimer concentration of 10 μM. Black line–1:0 Affibody dimer/Aβ(1–40) ratio; Blue line–1:0.2 ratio; Red line–1:0.4 ratio; Green line–1:0.6 ratio; Yellow line–1:0.8 ratio; Purple line–1:1 ratio.

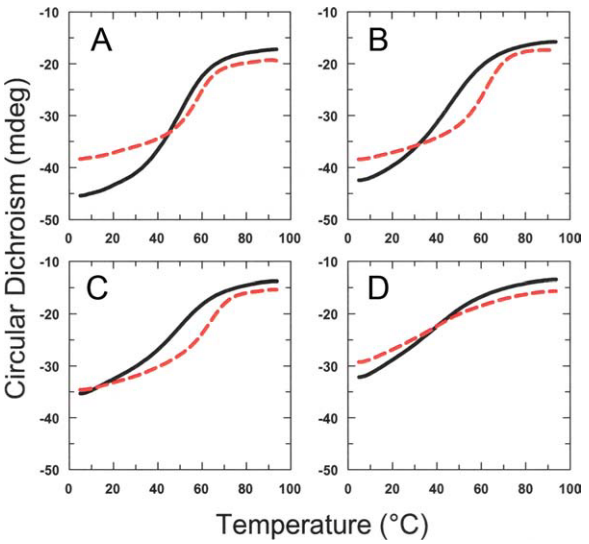

Figure 3. Thermal melting profiles from 5 to 94 °C for different Affibody homodimers recorded with CD spectroscopy at 220 nm. Affibody dimer concentrations were 12 μM in 10 mM Na-phosphate buffer at pH 7.4. (A) $Z_{A\beta3}$(1–58); (B) $Z_{A\beta3}$(12–58); (C) $Z_{A\beta3}$(15–58); (D) $Z_{A\beta3}$(18–58). Solid profiles are for Affibody dimers alone; dashed profiles are for Affibody dimers in 1:1 complex with the Aβ(1–40) peptide.

Table III. *Kinetic and Thermodynamic Data for Binding of the Aβ(1–40) Peptide to the Affibody Homodimers $Z_{Aβ3}$(1–58), $Z_{Aβ3}$(12–58), $Z_{Aβ3}$(15–58), and $Z_{Aβ3}$(18–58), Obtained from CD and SPR Measurements*

Affibody	T_m free Affibody (°C)[a]	T_m Affibody in complex with Aβ-peptide (°C)[a]	ΔT_m (°C)	k_a ($M^{-1}s^{-1}$)[b]	k_d (s^{-1})[b]	K_D (M)[b]	χ^2
$Z_{Aβ3}$(1–58)	50.5	58.1	7.6	4.1×10^4	3.9×10^{-4}	9.5×10^{-9}	0.8
$Z_{Aβ3}$(12–58)	46.2	62.6	16.4	4.5×10^5	3.1×10^{-4}	6.9×10^{-10}	0.5
$Z_{Aβ3}$(15–58)	49.1	63.8	14.7	6.3×10^5	3.0×10^{-4}	4.8×10^{-10}	1.0
$Z_{Aβ3}$(18–58)	37.9	40.7	2.8	n/a	n/a	n/a	n/a

[a] Determined from CD spectroscopy melting profiles at 220 nm.
[b] Determined by SPR measurements.

using a recombinant Affibody molecule with an N-terminal hexahistidine affinity tag, that is, starting MGSSHHHHHHLQVDN, whereas in this study the full-length Affibody molecule is composed of a 58 aa sequence starting VEN, where N is amino acid number 3 in the original Affibody sequence (Table I). No binding to the Aβ(1–40) peptide could be detected for the $Z_{Aβ3}$(18–58) variant, but $Z_{Aβ3}$(12–58) and $Z_{Aβ3}$(15–58) showed high binding affinities with K_D values of respectively 0.69 and 0.48 nM. These higher binding affinities seem to be related to faster on-rates for $Z_{Aβ3}$(12–58) and $Z_{Aβ3}$(15–58) compared to the full-length dimer $Z_{Aβ3}$(1–58). Similar results were obtained with an Aβ(1–40)-LC-biotin peptide, indicating that the interaction between Aβ(1–40) and the Affibody molecules is the same regardless of whether the biotin is attached to the N- or the C-terminus of the Aβ peptide (data not shown).

Nuclear magnetic resonance spectroscopy

The $^1H^{15}N$-HSQC spectrum of ^{15}N–labeled Aβ(1–40) together with unlabeled $Z_{Aβ3}$(18–58) is virtually identical to the HSQC spectrum of Aβ(1–40) alone, indicating that no interaction takes place between Aβ(1–40) and $Z_{Aβ3}$(18–58) (Fig. 5). On the other hand, the HSQC spectra of Aβ(1–40) mixed with a slight molar excess of either $Z_{Aβ3}$(12–58) or $Z_{Aβ3}$(15–58) are quite different from the HSQC spectrum of Aβ(1–40) alone, showing that both truncated Affibody dimers bind to and induce significant structural changes–and corresponding chemical shifts–in the Aβ(1–40) peptide (Fig. 5).

The HSQC spectra for Aβ(1–40) in complex with dimers of respectively $Z_{Aβ3}$(12–58) and $Z_{Aβ3}$(15–58) are very similar, but not identical. The assigned HSQC peaks for Aβ(1–40) in complex with $Z_{Aβ3}$(12–58) (Fig. 6) were consistent with the previously published assignment of Aβ(1–40) in complex with the full-length Affibody molecule $Z_{Aβ3}$.[23] The chemical shift differences between Aβ(1–40) alone and Aβ(1–40) in complex with respectively $Z_{Aβ3}$(12–58) and $Z_{Aβ3}$(15–58) are shown in Figure 7. For both truncated Affibody variants, the chemical shifts of Aβ(1–40) residues 18–24 and 30–36 are in good agreement with β-strand formation in the Aβ(1–40) peptide upon complex formation, as revealed by previous structural studies.[23]

The average chemical shift difference between Aβ(1–40) in complex with $Z_{Aβ3}$(12–58) and Aβ(1–40) in complex with full-length $Z_{Aβ3}$[23] was found to be 0.048 ppm, while the average chemical shift difference between Aβ(1–40) in complex with $Z_{Aβ3}$(15–58) and Aβ(1–40) in complex with full-length $Z_{Aβ3}$[23] was found to be 0.061 ppm. Even though the chemical shift difference is slightly higher for the Aβ(1–40)/$Z_{Aβ3}$(15–58) complex, the chemical shifts induced when Aβ(1–40) binds to the truncated versions $Z_{Aβ3}$(12–58) or $Z_{Aβ3}$(15–58) are generally similar to those induced when Aβ(1–40) binds to $Z_{Aβ3}$. This indicates that the first 11 or first 14 amino acids of $Z_{Aβ3}$ can be removed without significantly altering the structure[23] of the Affibody molecule/Aβ(1–40) complex (Fig. 5).

To further characterize the Aβ(1–40)/Affibody interaction, the $Z_{Aβ3}$(12–58) dimer was carefully titrated onto Aβ(1–40). At substoichiometric concentrations of $Z_{Aβ3}$(12–58) two sets of peaks are visible in the $^1H^{15}N$-HSQC spectrum of Aβ(1–40), corresponding to free and bound forms of Aβ. This shows that the binding is relatively strong and that exchange takes place on a slow time-scale (Fig. Supporting information-1).

Discussion

The previously published NMR structure of the disulfide-linked homodimerized $Z_{Aβ3}$ Affibody molecule in complex with the Aβ(1–40) peptide indicates that

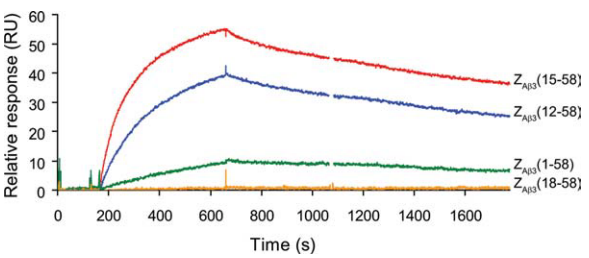

Figure 4. SPR sensorgram showing the responses when different Affibody homodimers were injected over a chip surface to which Aβ(1–40) peptides were linked via a streptavidin/biotin system. Concentrations: $Z_{Aβ3}$(18–58) 200 nM; $Z_{Aβ3}$(15–58) 20 nM; $Z_{Aβ3}$(12–58) 20 nM; $Z_{Aβ3}$(1–58) 20 nM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

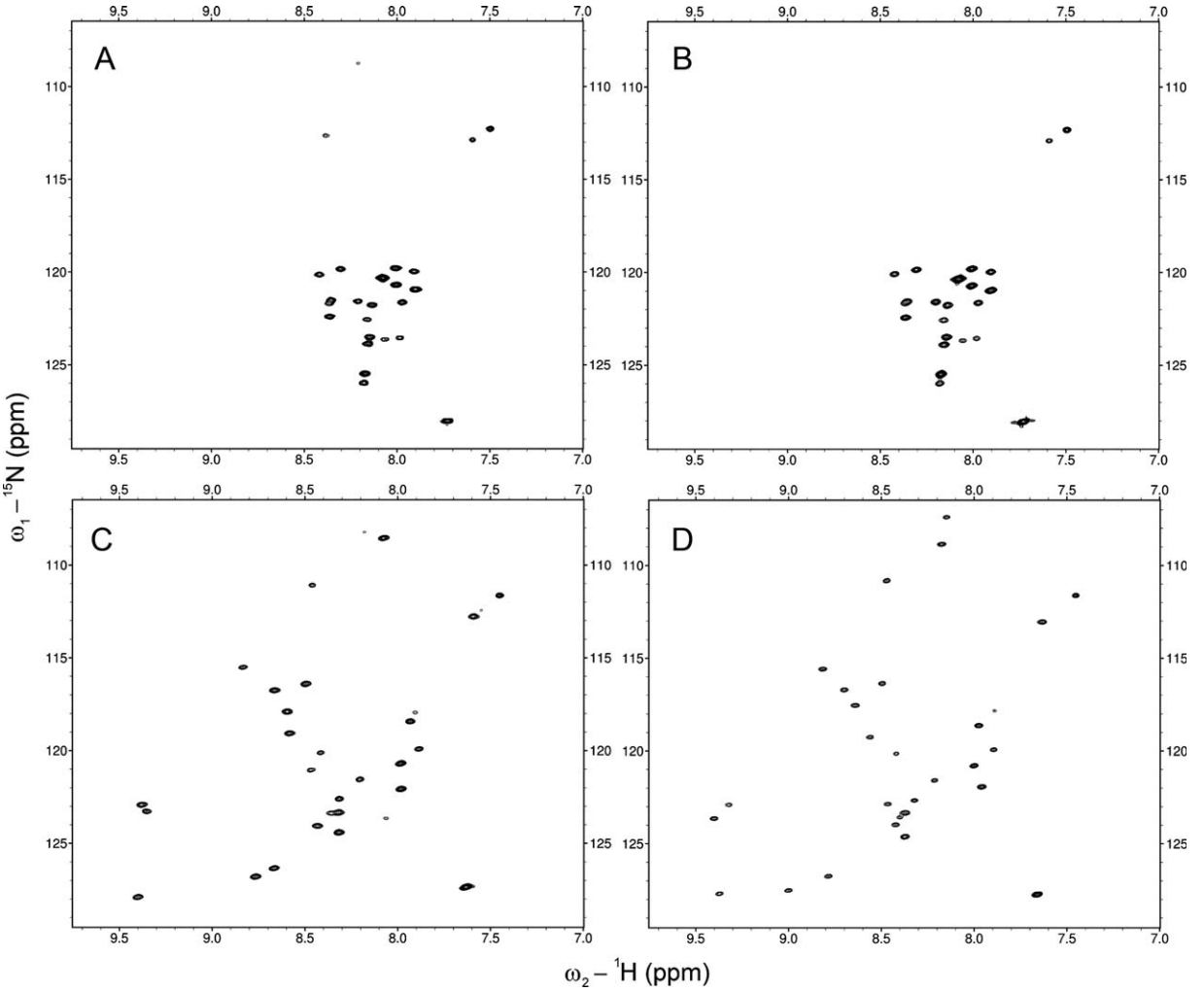

Figure 5. $^{1}H^{15}N$-HSQC spectra of 100 μM ^{15}N-labeled Aβ(1–40) at 25°C in 10 mM Na-phosphate buffer, pH 7.4, either alone (A) or in 1:1 complex with the unlabeled Affibody homodimers $Z_{Aβ3}$(18–58) (B), $Z_{Aβ3}$(15–58) (C), and $Z_{Aβ3}$(12–58) (D).

residues 1–13 of $Z_{Aβ3}$ are disordered and not involved in the binding interaction.[23] Based on this three-dimensional structure, six different N-terminally truncated $Z_{Aβ3}$ Affibody molecules were designed (Fig. 1) to study the effect of N-terminal truncation on solubility, stability, and interaction with the Aβ peptide. The six truncated variants and the full-length $Z_{Aβ3}$ Affibody molecule were produced by chemical synthesis using standard Fmoc chemistry. All truncated variants were found to be soluble under the work-up and assay conditions, and the synthetic yields varied from 35% for the shortest variant to 7–8% for the full-length $Z_{Aβ3}$, clearly demonstrating the impact of sequence length on the performance of solid phase peptide synthesis. CD spectroscopy showed no specific interaction between the most truncated derivative $Z_{Aβ3}$(18–58) and the Aβ(1–40) peptide. This was expected, since three amino acids (Glu^{15}-Ile^{16}-Val^{17}) previously shown by the NMR structure to form a β-strand that interacts with the Aβ peptide through hydrogen bonds are missing. The absence of interaction was confirmed by SPR analysis and NMR spectroscopy. In contrast, the same techniques showed that both $Z_{Aβ3}$(12–58) and $Z_{Aβ3}$(15–58) dimers bind efficiently to Aβ(1–40) peptides. NMR results demonstrated that the complexes formed between Aβ(1–40) and either of $Z_{Aβ3}$(12–58) and $Z_{Aβ3}$(15–58) have the same overall chemical shift pattern, and therefore the same overall structure, as the complex between Aβ(1–40) and the full-length Affibody molecule $Z_{Aβ3}$.[23] In particular, the Aβ(1–40) peptide appears to form a β-hairpin also when bound to $Z_{Aβ3}$(12–58) or $Z_{Aβ3}$(15–58). Interestingly, the Aβ residues that form β-strands in the presence of Affibody molecules, that is, aa 18–24 and 30–36, are approximately the same that form alpha-helices in SDS micelles,[31] showing a general tendency of these residues to participate in intramolecular hydrogen bonding. Given the above results, detailed spectroscopic analysis of the longer variants $Z_{Aβ3}$(3–58), $Z_{Aβ3}$(6–58), and $Z_{Aβ3}$(9–58) was not performed.

SPR analysis of the binding between the truncated Affibody variants and the Aβ peptide showed

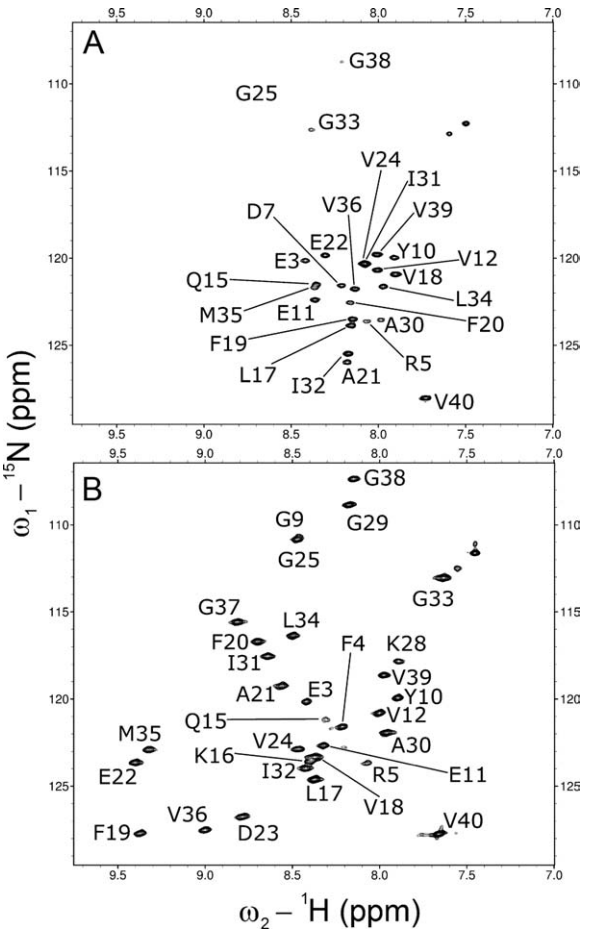

Figure 6. Assignments of $^{1}H^{15}N$-HSQC spectra of 250 μM ^{15}N-labeled Aβ(1–40) at 25°C in 10 mM Na-phosphate buffer, pH 7.4. (A) Aβ(1–40) alone; (B) Aβ(1–40) in 1:1 complex with the unlabeled Affibody homodimer $Z_{Aβ3}$(12–58).

that the affinities of $Z_{Aβ3}$(15–58) and $Z_{Aβ3}$(12–58) dimers to Aβ(1–40) were stronger and improved by one order of magnitude compared to the affinity of the full-length $Z_{Aβ3}$(1–58) Affibody dimer (Table III). We conclude that removing N-terminal amino acids previously shown not to participate in the binding resulted in two truncated Affibody molecules with improved binding affinities to the Aβ(1–40) peptide. This affinity difference can mainly be attributed to the truncated versions having faster on-rates than the full-length $Z_{Aβ3}$(1–58) molecule (Table III). CD analysis showed that $Z_{Aβ3}$(1–58), and to a smaller extent also $Z_{Aβ3}$(12–58), loses helical structure upon binding to Aβ(1–40) (Fig. 2). This is in agreement with previous results showing that the N-termini of free $Z_{Aβ3}$ dimer display a partially helical structure,[24] whereas the N-termini of $Z_{Aβ3}$ dimer in complex with Aβ(1–40) appear unstructured or heterogeneous on the NMR timescale.[23] The results are further supported by the Agadir algorithm,[32] which predicts a 52% helix content in the 14 first amino acids of the full-length Affibody sequence. Hence, to bind the Aβ peptide the partially helical N-termini of the Affibody dimer must first unfold, and the energy barrier of the unfolding step may explain the slower on-rate for the full-length $Z_{Aβ3}$(1–58) molecule. This could explain why $Z_{Aβ3}$(15–58) displays the fastest on-rate. However, the slower on-rates of the longer Affibody variants might also be related to the longer N-termini sterically interfering with the binding to the Aβ peptide.

The $Z_{Aβ3}$ Affibody molecule may be used for *in vivo* and *in vitro* applications. A purely synthetic production expands the range of chemistry that may be applied for conjugation of different probes, and facilitates exploratory clinical studies by avoiding fermentation development and host derived contaminants of the product. The synthetic yield for $Z_{Aβ3}$(12–58) and $Z_{Aβ3}$(15–58) is very similar, 29% and 30%, respectively. Although both $Z_{Aβ3}$(12–58) and $Z_{Aβ3}$(15–58) appear to bind Aβ(1–40) in approximately the same way as the full-length dimer $Z_{Aβ3}$ does, the NMR data indicates that the $Z_{Aβ3}$(12–58)/Aβ(1–40) complex best resembles the previously determined structure of the $Z_{Aβ3}$/Aβ(1–40) complex. This suggests that $Z_{Aβ3}$(12–58) may be the preferred molecule to use in further studies despite a somewhat lower affinity, at least if for example chemical modifications to the Affibody molecule are to be devised based on the $Z_{Aβ3}$/Aβ(1–40) solution structure.

Materials and Methods

Solid phase peptide synthesis of truncated Affibody molecules

Based on the sequence and structure of the previously selected Affibody molecule $Z_{Aβ3}$, six N-

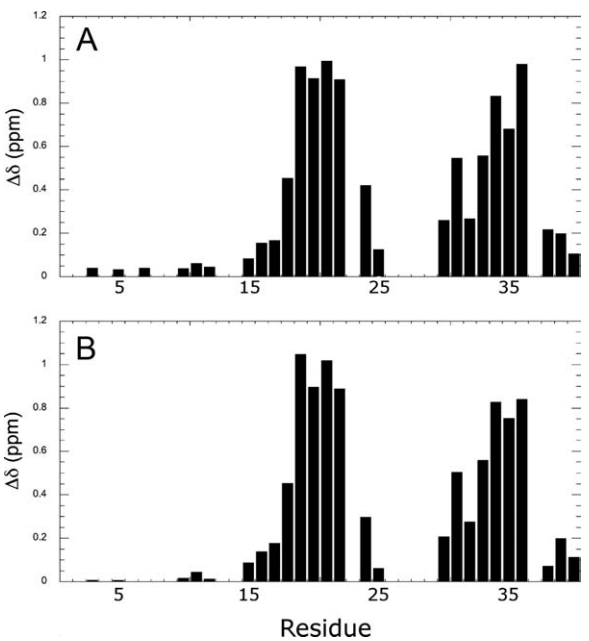

Figure 7. Chemical shift differences ($\Delta\delta = (((\Delta\delta_N/5)^2 + (\Delta\delta_H)^2)/2)^{1/2}$) between HSQC spectra of ^{15}N-labeled Aβ(1–40) in its free form and bound to the Affibody homodimers $Z_{Aβ3}$(12–58) (A) and $Z_{Aβ3}$(15–58) (B).

terminally truncated variants were designed (Fig. 1). The full-length and the truncated peptides (Table I) were synthesized by solid phase peptide synthesis (SPPS) on a 433 A Peptide Synthesizer (Applied Biosystems, Foster City, CA) on a 0.1 mmol scale using standard Fmoc chemistry. An acid-labile Fmoc amide resin was used as solid support throughout the synthesis (Rink Amide MBHA Resin (100–200 mesh), loading 0.7 mmol g^{-1} (Novabiochem)). Acylation reactions were performed with a 10-fold molar excess of amino acid in NMP (*N*-methylpyrrolidone, Merck), activated with 1 equiv of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronoium hexafluorophosphate (HBTU) (IRIS Biotech), 1 equiv of 1-hydroxybenzotriazole (HOBt) (IRIS Biotech) and 2 equiv of diisopropylethylamine (DIEA) (Applied Biosystems). Single couplings for 10 min were used for all amino acids, except for some residues that generally are difficult to couple and therefore were double-coupled, as shown in Table I. Any remaining unreacted amino groups were capped with acetic anhydride (0.5*M* acetic anhydride (AlfaAesar), 0.125*M* DIEA, 0.015*M* HOBt in NMP) for 5 min. Following every coupling, deprotection of the Fmoc group was performed by treatment with 20% piperidine (Sigma-Aldrich) in NMP for 10 min. All amino acids were introduced with standard side chain protection groups (*tert*-butyl (*t*Bu) for Asp, Glu, Ser, and Tyr, *tert*-butyloxycarbonyl (Boc) for Lys, and trityl (Trt) for Asn, Gln, His and Cys).

The different peptide lengths were obtained by removal of a fraction of the peptide-resin before the synthesis was continued using the rest of the material. The full-length control peptide was synthesized using the same protocol, but in a separate synthesis from the six other variants. The $Z_{A\beta3}$ Affibody molecule originally selected by phage display contains aspartic acid in position 2, but this residue was here substituted with glutamic acid to prevent the side reaction of aspartimide formation in the synthesis.[27] After completed synthesis, the peptides were cleaved from the solid support and simultaneously deprotected by treatment with TFA/EDT/H_2O/TIS (94:2.5:2.5:1) (TFA: trifluoroacetic acid (Apollo), EDT: 1,2-ethanedithiol (Aldrich), TIS: triisopropylsilane (Aldrich)) at RT for 2 h with occasional mixing. After TFA treatment, the peptides were extracted three times using 20% acetonitrile (Merck) in water and *tert*-butyl methyl ether (Merck). The aqueous phases were then combined, filtered, and lyophilized.

Analysis, purification, and dimerization

The crude products were analyzed on reversed phase ultrahigh performance liquid chromatography (RP-UHPLC) using an analytical column (Zorbax Eclipse XDB-C18 Rapid resolution HT, 4.6 × 50 mm column, particle size 1.8 μm) and a gradient of 6–36% B (A: 0.1% TFA-H_2O; B: 0.1% TFA-CH_3CN) over 25 min at a flow rate of 2.2 mL min^{-1}. The correct molecular weights of the peptides were verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) on a MALDI-MS Biflex IV (BRUKER Daltonics, Leipzig, Germany) and liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) on a 6520 Accurate Mass Q-TOF LC/MS (Agilent Technologies). The crude peptides were then purified by semipreparative RP-HPLC (Reprosil GOLD C18 300, 250 × 10 mm, 5 μm particle size) and a gradient of 32–35% B over 30 min at a flow rate of 2.3 mL min^{-1}, followed by lyophilization.

The purified and lyophilized products were dissolved in 0.1*M* $(NH_4)_2CO_3$ buffer, pH 9.2, and oxidized at RT with air continuously being introduced into the solution to facilitate dimerization through Cys-28—Cys-28 disulfide linking. The dimer formation was followed by LC-ESI-MS or RP-HPLC and found to be complete after 5–16 h for the different variants. Although the dimerization frequently was close to quantitative, after oxidation the dimers were separated from traces of monomeric peptides by RP-HPLC (Reprosil GOLD C18 300, 250 × 4.6 mm, 3 μm particle size) using a gradient of 34–40% B over 25 min at a flow rate of 0.8 mL min^{-1}. The end products were therefore dimers of very high purity. The correct molecular weights of the final products were verified by LC-ESI-MS (Table II) and the concentrations were determined by amino acid analysis (Aminosyraanalyscentralen, Uppsala, Sweden).

Aβ(1–40) peptide preparation

Aβ(1–40) peptide was bought either unlabeled, ^{15}N-labeled, or $^{13}C^{15}N$-labeled from AlexoTech AB (Umeå, Sweden) and prepared according to previously described protocols.[31,33]

Surface plasmon resonance biosensor studies

The binding kinetics of the full-length $Z_{A\beta3}$(1–58) Affibody dimer and the truncated variants $Z_{A\beta3}$(12–58) and $Z_{A\beta3}$(15–58) were analyzed by real-time biospecific interaction analysis (BIA) on a Biacore 3000 instrument (GE Healthcare). A streptavidin sensor chip (GE Healthcare) was used for directed immobilization of either biotin-LC-Aβ(1–40) peptide or Aβ(1–40)-LC-biotin peptide (AnaSpec, CA), where LC is a 6-carbon long chain (LC) linker. Approximately 200 response units (RU) were obtained on both surfaces. The different Affibody variants were dissolved in HBS-EP (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.05% Surfactants P20, pH 7.4) and injected over the sensor surface at a flow rate of 30 μL min^{-1}. The association time was 500 sec and the dissociation time was 1100 sec. All experiments were run in duplicates with HBS-EP as running buffer at 25°C and the surfaces were regenerated with 0.05% SDS after each injection. A

surface without immobilized Aβ peptide was used as a reference, and the response from this reference surface was subtracted from all curves before evaluating the results. Five concentrations ranging from 0.625 to 10 nM were used for $Z_{A\beta3}$(12–58) and $Z_{A\beta3}$(15–58), while five concentrations ranging from 6.25 to 100 nM were used for $Z_{A\beta3}$(1–58). The dissociation constants were determined using the BIAevaluation 3.2 software (GE Healthcare) and a 1:1 Langmuir model of binding interaction.

Circular dichroism spectroscopy

A Chirascan CD unit from Applied Photophysics was used to record CD spectra and thermal melting profiles of the $Z_{A\beta3}$(1–58), $Z_{A\beta3}$(12–58), $Z_{A\beta3}$(15–58), and $Z_{A\beta3}$(18–58) dimers. The Affibody molecules were dissolved in 10 mM Na-phosphate buffer at pH 7.4 to final dimer concentrations of 10 μM. CD spectra were recorded using a 2 mm quartz cuvette holding 400 μL of sample. Aβ(1–40) peptide was titrated to each of the four dimeric Affibody variants until a 1:1 ratio was reached, and CD spectra between 190 and 270 nm were recorded for each titration step. For the thermal melting profiles, a CD signal was recorded at 220 nm while the temperature was increased from +5 to +94°C at a rate of 0.5 centigrades per second. Melting profiles were recorded for 12 μM samples of Affibody dimers alone and in presence of surplus amounts (1:1.2 ratio) of Aβ(1–40) peptide. Melting temperatures (T_m) were calculated from the second derivatives of the melting profiles.

Nuclear magnetic resonance spectroscopy

Bruker Avance 500 and 700 MHz spectrometers and a Varian Inova 600 MHz spectrometer were used to record NMR spectra at 25°C of isotope-labeled Aβ(1–40) peptide in 90/10 H_2O/D_2O and 10 mM sodium phosphate buffer at pH 7.4, both in the absence and presence of unlabeled truncated Affibody dimers of different lengths. All three machines were equipped with triple-resonance cryogenically cooled probeheads. On the Varian 600 MHz unit, $^1H^{15}N$-HSQC spectra of 100 μM ^{15}N-labeled Aβ(1–40) peptide were recorded in absence and in presence of the unlabeled Affibody dimers $Z_{A\beta3}$(12–58), $Z_{A\beta3}$(15–58), and $Z_{A\beta3}$(18–58), which were added in slight molar excess (1:1.2 molar ratio) to the Aβ(1–40) peptide samples. Chemical shift differences between the spectra of monomeric Aβ(1–40) and Aβ(1–40) in complex with the different Affibody constructs were calculated as the standard weighted average, i.e. $\Delta\delta = (((\Delta\delta_N/5)^2 + (\Delta\delta_H)^2)/2)^{1/2}$.[34] On the Bruker 500 MHz unit, HSQC spectra of 75 μM ^{15}N-labeled Aβ(1–40) peptide were recorded in presence of increasing amounts of unlabeled $Z_{A\beta3}$(12–58), that is, concentrations corresponding to 0, 15, 65, and 120% of the Aβ(1–40) peptide concentration, to further characterize the interaction between these two molecules. In addition, a series of HSQC spectra of monomeric 75 μM ^{15}N-labeled Aβ(1–40) peptide were recorded at temperatures between 5 and 25°C, in steps of 3°C, allowing the previously published assignment[35] of this spectrum at low temperature to be transferred to the 25°C spectrum. On the Bruker 700 MHz unit, HNCO, HN(CA)CO, HNCA, and HN(CO)CA spectra[36] of 250 μM ^{13}C ^{15}N-labeled Aβ(1–40) peptide were recorded in presence of 290 μM unlabeled $Z_{A\beta3}$(12–58) to assign the Aβ(1–40) peptide resonances in complex with $Z_{A\beta3}$(12–58). The completed assignment was deposited with entry number 17159 in the BioMagResBank (BMRB) database. The spectrum of $Z_{A\beta3}$(15–58)-bound Aβ(1–40) was then assigned by direct transfer of the $Z_{A\beta3}$(12–58)-bound Aβ(1–40) assignment (Fig. 6). All spectra were referenced to the water signal, processed with nmrpipe, and analyzed using Sparky software.[37,38]

Conclusions

Removal of respectively the 11 or the 14 first amino acids of $Z_{A\beta3}$ yields truncated Affibody molecules $Z_{A\beta3}$(12–58) and $Z_{A\beta3}$(15–58) with higher binding affinity to the Aβ(1–40) peptide than the full-length $Z_{A\beta3}$(1–58). The higher affinity is mainly due to a higher on-rate for the N-terminally truncated Affibody variants. This may be related to the full-length Affibody dimer having partially helical N-termini, which must undergo an unfolding step prior to binding the Aβ peptide. In analogy with full-length $Z_{A\beta3}$, the truncated versions lock the Aβ(1–40) peptide in a biologically relevant β-hairpin conformation, and prevent the peptide from aggregating into fibrils. The shorter Affibody molecules can be produced by standard SPPS in significantly higher synthetic yields than the full-length molecule. This is an important advantage, since chemical production expands the number of ways of making conjugates and heterodimeric molecules for further studies of the Affibody molecule/Aβ peptide complex.

Acknowledgments

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