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Engineered non-fluorescent Affibody molecules facilitate studies of the amyloid-beta (Aβ) peptide in monomeric form: Low pH was found to reduce Aβ/Cu(II) binding affinity

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ABSTRACT

Aggregation of amyloid-beta (Aβ) peptides into oligomers and amyloid plaques in the human brain is considered a causative factor in Alzheimer's disease (AD). As metal ions are over-represented in AD patient brains, and as distinct Aβ aggregation pathways in presence of Cu(II) have been demonstrated, metal binding to Aβ likely affects AD progression. Aβ aggregation is moreover pH-dependent, and AD appears to involve inflammatory conditions leading to physiological acidosis. Although metal binding specificity to Aβ varies at different pH's, metal binding affinity to Aβ has so far not been quantitatively investigated at sub-neutral pH levels. This may be explained by the difficulties involved in studying monomeric peptide properties under aggregation-promoting conditions. We have recently devised a modified Affibody molecule, $Z_{Aβ3}$(12–58), that binds Aβ with sub-nanomolar affinity, thereby locking the peptide in monomeric form without affecting the N-terminal region where metal ions bind. Here, we introduce non-fluorescent Aβ-binding Affibody variants that keep Aβ monomeric while only slightly affecting the Aβ peptide's metal binding properties. Using fluorescence spectroscopy, we demonstrate that Cu(II)/Aβ(1–40) binding is almost two orders of magnitude weaker at pH 5.0 (apparent $K_D = 51$ μM) than at pH 7.3 (apparent $K_D = 0.86$ μM). This effect is arguably caused by protonation of the histidines involved in the metal ligandation. Our results indicate that engineered variants of Affibody molecules are useful for studying metal-binding and other properties of monomeric Aβ under various physiological conditions, which will improve our understanding of the molecular mechanisms involved in AD.

1. Introduction

The current dominant hypothesis for Alzheimer's disease (AD)-related cognitive dysfunction and neuropathogenesis is the "Amyloid Cascade Hypothesis," according to which the brain pathology is caused by aggregation of 39 to 43 amino acids long amyloid-β (Aβ) peptides, first into possibly toxic oligomers and then into amyloid plaques [1–3]. The aggregation of these peptides is strongly pH-dependent, and as inflammatory conditions leading to physiological acidosis appear to be involved in AD progression, it becomes important to study how the properties of the Aβ peptides vary with the pH.

Aβ peptide aggregation and amyloid formation are modulated by copper, zinc, and iron ions that are over-represented in brains of AD patients [4,5]. It is well established that Cu(II) ions and low pH in combination strongly accelerate the fibrillization kinetics of Aβ peptides [6,7], perhaps due to metal ion coordination by two or more Aβ peptides acting in concert [8,9]. Distinct pathways for Aβ peptide aggregation in the presence of different Cu(II)-to-peptide ratios have recently been demonstrated [10]. Binding of redox-active Cu(II) ions to the Aβ peptide furthermore appears to increase the toxicity of the peptides, possibly through formation of toxic reactive oxygen species (ROS) [7,8,11,12]. Taken together, this suggests that metal ions may play a role in the progression of AD [13,14]. However, our understanding of the role of metal ions in AD remains limited [8,15] and measurements of metal concentrations in brain tissue are often compromised by the sample preparation steps [16]. Metal chelation therapy has been tried and evaluated as a potential AD treatment, but the results are not conclusive [17,18], and the debate continues about whether the therapeutic goal should be removal of excess Cu(II) or re-distribution of Cu(II) between extra- and intracellular environments [19].

Various specific ligands to the metal ions have been proposed [20], and our previous NMR studies of Aβ(1–40) at pH 7.4 have shown a major binding site for Cu(II) located to H6, H13, H14 and the N-

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terminus [21]. This histidine ligandation model is supported by theoretical calculations [22], although the ligandation appears to vary with the pH and between different metal ions [23–26]. A wide range of micromolar to attomolar binding affinities between Cu(II) and the monomeric Aβ peptide have previously been reported [21,27–29], which partially can be explained by the measurements having been carried out with different techniques. Another plausible explanation for the large variation in observed binding affinity is the difficulties involved in keeping the aggregation-prone Aβ peptide in monomeric form during measurements and titrations (metal ions seem to display stronger binding to aggregated Aβ peptides than to the Aβ monomer). The difficulties involved in carrying out studies of the monomeric Aβ peptide under conditions that facilitate aggregation may also explain why binding affinities of metal ions to the Aβ peptide so far have not been determined at sub-neutral pH's [25].

However, previous research by us and others has shown that an engineered Affibody molecule, $Z_{Aβ3}$, binds the Aβ peptide with nanomolar affinity and keeps it in monomeric form, thereby preventing aggregation [30–33]. This Affibody molecule binds to the central and C-terminal parts of the Aβ peptide, and does not seem to interact with the N-terminal part where the primary binding site for metal ions is located.

In this work, we created and evaluated modified variants of a shortened $Z_{Aβ3}(12–58)$ Affibody molecule. By substituting the fluorescent-active tyrosine 18 residue with various non-fluorescent amino acids, Affibody variants could be created that do not interfere with fluorescence spectroscopy measurements but still keep the Aβ peptide in monomeric form. We then used fluorescence spectroscopy to measure the fluorescence from Tyr-10 in the Aβ peptide upon titration of Cu(II). The fluorescence quenching induced by the Cu(II) ions allowed us to determine the binding affinity between Cu(II) and the Aβ(1–40) peptide at pH 7.3 and at pH 5.0, both with and without the Aβ peptide bound to an Affibody molecule. This approach made it possible to measure the pH-dependency of copper binding to the Aβ peptide in monomeric form, and to evaluate the usefulness of non-fluorescent $Z_{Aβ3}(12–58)$ variants as tools for keeping the Aβ peptide in monomeric form during spectroscopic measurements.

2. Materials and methods

2.1. Amyloid-beta sample preparation

Aβ(1–40) peptides were bought lyophilized from AlexoTech AB (Umeå, Sweden), and fresh samples were prepared as previously described [34,35] before each fluorescence or NMR spectroscopy measurement. In short, the peptide was dissolved in 10 mM cold NaOH to a concentration of 1.0 mg/ml, sonicated in an ice bath, diluted to half the final sample volume with cold distilled water, sonicated again, and diluted to the final concentration with either cold citrate (pH 5.0) or cold sodium phosphate (pH 7.3) buffer.

2.2. Synthesis, purification and dimerization of Affibody molecules

Based on the $Z_{Aβ3}(12–58)$ Affibody molecule, with the sequence AGGEIVYLPNLNPDQLCAFIHSLHDDPSQSANLLAEAKKLNDAQAPK, two non-fluorescent variants were created by substituting the fluorescent-active tyrosine at position 18 (based on the numbering of the original $Z_{Aβ3}(1–58)$ Affibody molecule [31,33]) into either leucine (Y18L) or arginine (Y18R) (Table 1). These two Affibody variants, $Z_{Aβ3}(12–58)$Y18L and $Z_{Aβ3}(12–58)$Y18R, together with the unmodified $Z_{Aβ3}(12–58)$ molecule, were synthesized using standard solid phase peptide synthesis (SPPS) according to procedures described in the supplementary material.

The crude products were then purified by reversed phase (RP)-HPLC using a semipreparative column (Reprosil GOLD C18 300, 250 mm × 10 mm, 5 μM particle size). The correct molecular weights were verified by liquid chromatography electrospray ionization mass spectrometry (LC–ESI–MS).

The three purified and lyophilized Affibody variants were dissolved in 100 mM $(NH_4)_2CO_3$ buffer, pH 9.2, and oxidized at RT overnight to allow formation of homodimers via Cys-28–Cys-28 disulfide linking. In addition, the heterodimer of $Z_{Aβ3}(12–58)$Y18L and $Z_{Aβ3}(12–58)$Y18R was easily prepared by first protecting/activating the thiol of $Z_{Aβ3}(12–58)$Y18L, using 2,2′-dipyridyl disulfide to form a pyridyl disulfide [36]. This protected/activated monomer, denoted $Z_{Aβ3}(12–58)$Y18L-Pys, readily reacted with the free thiol of the other monomer to form the heterodimer $Z_{Aβ3}(12–58)$Y18L/R. All dimers were then separated from traces of monomers using semi-preparative RP-HPLC, followed by verification of the molecular weights using LC–ESI–MS.

Because the engineered Affibody molecules are devoid of both tyrosine and tryptophan residues, the absorbance at 280 nm could not be used for concentration determination. Instead, Affibody dimer concentrations were determined via a colorimetric assay using bicinchoninic acid (BCA protein assay, Thermo Fisher Scientific Inc., carried out according to the manufacturer's instructions).

2.3. Binding evaluation of Affibody molecules

A Biacore 2000 (GE Healthcare) surface plasmon resonance (SPR) instrument was used to study the real-time binding interaction between the Affibody dimers and the Aβ peptide. A six carbon long chain (LC) linker was used to immobilize an Aβ(1–40)-LC-biotin construct (AnaSpec, CA) on a streptavidin sensor chip to approximately 175 response units (RU). The four Affibody dimer variants were dissolved in PBS (phosphate buffered saline) and then diluted to 100 nM concentration in either HBS-EP (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.05% surfactant P20, pH 7.4) for studies at pH 7.4 or in citrate buffer (20 mM citrate supplemented with 0.05% surfactant P20, pH 5.0) for studies at pH 5.0, and injected over the chip surface at a flow rate of 30 μl min^{-1}. The binding affinities of the different Affibody dimers were ranked based on their dissociation curves, which were normalized to the initial response of the dissociation phase (i.e. at $t = 650$ s in the sensorgrams).

For full analysis of the binding kinetics of dimeric $Z_{Aβ3}(12–58)$Y18L, the Affibody molecule concentration was determined by amino acid analysis (Aminosyraanalyscentralen, Uppsala, Sweden). Five concentrations ranging from 1.6 to 25.5 nM diluted in either HBS-EP or citrate buffer were injected in duplicates. The dissociation constants were determined using BIAevaluation 3.2 software (GE Healthcare) and a 1:1 Langmuir model of binding interaction.

Table 1
Sequences for the different $Z_{Aβ3}(12–58)$ Affibody variants. The underlined residues were double-coupled during the synthesis. Since the Affibody molecules are derived from the original $Z_{Aβ3}(1–58)$ molecule [31], the numbering begins at position 12.

Affibody molecule	Sequence
	12 20 30 40 50 58
$Z_{Aβ3}(12–58)$	AGGEIVYLPNLNPDQLCAFIHSLHDDPSQSANLLAEAKKLNDAQAPK
$Z_{Aβ3}(12–58)$Y18L	------L----------
$Z_{Aβ3}(12–58)$Y18R	------R----------

All Biacore experiments were performed at 25 °C with association and dissociation times of respectively 500 and 1300 s, using HBS-EP as running buffer at pH 7.4 and citrate buffer at pH 5.0. After each injection the chip surface was regenerated using 0.05% SDS.

2.4. Fluorescence spectroscopy

A Jobin Yvon Horiba Fluorolog 3 (Longjumeau, France) unit was used to record fluorescence emission spectra of 10 μM Aβ(1–40) peptide at +5 °C between 290 and 350 nm (excitation wavelength 276 nm). The excitation and emission slits were set at 4 nm. A 4 mm pathlength quartz cuvette was used to hold 1 ml of Aβ(1–40) sample, either in 20 mM sodium phosphate buffer at pH 7.3, or in 20 mM citrate buffer at pH 5.0. Cu(II) ions (copper acetate) were added stepwise to final concentrations of 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390, 420, 450, 480, 510, 540, 570, 600, 630, and 660 μM. The buffers were chosen to facilitate comparison with previous studies on Aβ/Cu(II) binding [21,25].

The fluorescence data were fitted to a binding model (Eq. (1)) that assumes a single binding site, and which contains a parameter (k) accounting for quenching by non-bound copper (the details of this equation are further explained in the supplementary material):

$$\frac{I}{I_0} = 1 - k\cdot[\mathrm{Cu}] - q\cdot\frac{1}{2[\mathrm{A}\beta]}\cdot\sqrt{(K_\mathrm{D} + [\mathrm{A}\beta] + [\mathrm{Cu}])^2 - 4\cdot[\mathrm{A}\beta]\cdot[\mathrm{Cu}]} \qquad (\mathrm{Eq.1})$$

Here, I is the measured fluorescence intensity at each titration step, I_0 is the starting intensity before addition of copper, k is a linear constant accounting for fluorescence quenching caused by non-bound copper, [Cu] is the total copper concentration in the solution, [Aβ] is the concentration of Aβ peptide (which remains constant during the titration), and K_D is the dissociation constant of the copper ions. Since they depend on the buffer conditions, the K_D's derived from the experiments should be considered apparent dissociation constants, i.e. $K_\mathrm{D}^{\mathrm{App}}$.

2.5. NMR spectroscopy

A Bruker Avance 500 MHz spectrometer equipped with a triple-resonance cryogenically cooled probehead was used to record two-dimensional $^1\mathrm{H}^{15}\mathrm{N}$-HSQC (heteronuclear single quantum coherence) spectra at 5 °C of 100 μM ^{15}N-labeled Aβ(1–40) peptide in 1:1 complex with the unlabeled $Z_{A\beta3}$(12–58)Y18L Affibody dimer in 20 mM sodium phosphate buffer at pH 7.3 (90/10 H_2O/D_2O), both in absence and presence of 250 μM copper acetate.

3. Results and discussion

The three Affibody variants $Z_{A\beta3}$(12–58), $Z_{A\beta3}$(12–58)Y18L, and $Z_{A\beta3}$(12–58)Y18R were successfully synthesized and homodimerized. The heterodimer $Z_{A\beta3}$(12–58)Y18L/R was also created. LC–ESI–MS analysis showed that all four different dimeric proteins were obtained in high purity and with the correct molecular weights.

The Y18L and Y18R substitutions were chosen based on the original phage display selection of Affibody variants [37], where variants with other residues than tyrosine in position 18, including leucine and arginine, also were found to bind the amyloid-β peptide. Consequently, it was not surprising to find that the three modified Affibody dimers bind the Aβ(1–40) peptide almost as efficiently as the non-modified $Z_{A\beta3}$(12–58) (Supplementary Fig. S1). SPR-based biosensor (Biacore) measurements showed that for the three modified molecules, $Z_{A\beta3}$(12–58)Y18R had the fastest off-rate, both at pH 7.4 and pH 5.0. The heterodimer $Z_{A\beta3}$(12–58)Y18R/L had the slowest off-rate at pH 7.4, which may be explained by differential interactions of the two sub-units: the solution structure of the original $Z_{A\beta3}$(1–58)/Aβ(1–40) peptide complex indicates that glutamic acid 22 of the Aβ-peptide forms a hydrogen bond with tyrosine 18 of one of the sub-units, while the tyrosine of the other sub-unit appears to contribute generally to the hydrophobicity [31]. At pH 5.0, the homodimer $Z_{A\beta3}$(12–58)Y18L had the slowest off-rate, which together with its simpler production route prompted us to select this variant for further use.

SPR-based binding kinetics analysis yielded dissociation constants (K_D) of 5.5 nM for $Z_{A\beta3}$(12–58)Y18L binding to the Aβ(1–40) peptide at pH 7.4, and 0.55 nM at pH 5.0 (Fig. 1; Table 2). The value at pH 7.4 is a magnitude higher than our previously reported K_D value of 0.7 nM for $Z_{A\beta3}$(12–58) [33], and thus more similar to the 9.5 nM K_D reported for the originally selected and longer $Z_{A\beta3}$(1–58) Affibody molecule [31,33]. We conclude that the Y18L substitution slightly decreases the binding affinity to the Aβ peptide. The one magnitude lower K_D for $Z_{A\beta3}$(12–58)Y18L at pH 5.0 compared to pH 7.4 (Table 2) is readily explained by the pI values of 5.3 for the Aβ(1–40) peptide and 4.9 for $Z_{A\beta3}$(12–58). At neutral pH both molecules are negatively charged, leading to electrostatic repulsion, while at pH 5.0 Aβ(1–40) is positive and $Z_{A\beta3}$(12–58)Y18L slightly negative, leading to electrostatic attraction. Preliminary results suggest that this pH-effect is less pronounced for the unmodified $Z_{A\beta3}$(12–58) molecule (Supplementary material Figs. S3 and Table S1), which is something we plan to further investigate in future studies.

The amide region of the $^1\mathrm{H}^{15}\mathrm{N}$-HSQC NMR spectrum of ^{15}N-labeled Aβ(1–40) peptide in one-to-one complex with the unlabeled $Z_{A\beta3}$(12–58)Y18L Affibody dimer is shown in Supplementary Fig. S2, both in absence and presence of copper ions. The HSQC spectrum differs slightly from the corresponding spectrum for Aβ(1–40) in complex with the non-mutated $Z_{A\beta3}$(12–58) molecule [33], indicating that the Y to L substitution induces local chemical and structural changes. Hence, transfer of the peak assignment for the Aβ(1–40)/$Z_{A\beta3}$(12–58) complex was not successful for every cross-peak in the spectrum. Nevertheless, the NMR data clearly show that all assigned N-terminal cross-peaks (i.e. E3, F4, R5, G9, Y10, and V12) have disappeared after the addition of copper (Supplementary Fig. S2). On the other hand, a number of Aβ residues in the 17–35 region (e.g. G25, K28, G29, A30, and G33), which are bound to the Affibody molecule [31], are unaffected by the copper ions. The assigned C-terminal cross-peaks, i.e. G37, G38, and V39, display reduced intensity but are still visible. This strongly indicates that the copper ions specifically bind the N-terminus of the Aβ(1–40) peptide, just like they do for free Aβ peptide [21]. This is not surprising, as the Affibody complex does not seem to involve or interfere with the Aβ N-terminus where the histidine copper binding ligands are located.

The results of the fluorescence spectroscopy measurements are shown in Fig. 2. Copper was titrated to Aβ(1–40) alone or to Aβ(1–40) in complex with the $Z_{A\beta3}$(12–58)Y18L Affibody dimer, both at pH 7.3 and at pH 5.0. Thus, four titration curves were obtained. It is immediately obvious that i) the quenching effect of copper is more pronounced at pH 7.3 than at pH 5.0, suggesting a marked pH-dependence of the copper binding to the Aβ(1–40) peptide, and ii) the presence of the

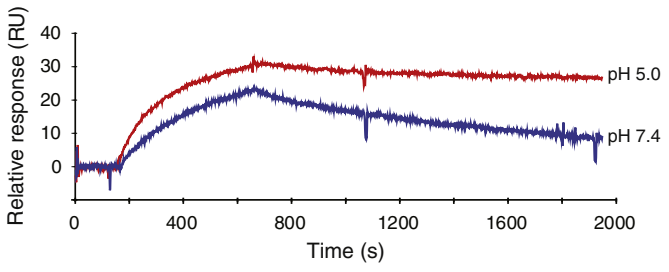

Fig. 1. SPR sensorgram showing the responses for the Affibody molecule $Z_{A\beta3}$(12–58)Y18L homodimer injected at pH 5.0 (red line) or pH 7.4 (blue line) over a chip surface with Aβ(1–40) peptides linked via a streptavidin/biotin system. The concentration of the $Z_{A\beta3}$(12–58)Y18L homodimer was 25 nM in both injections.

Table 2
Kinetic data for $Z_{A\beta3}$(12–58)Y18L binding to the Aβ(1–40)-peptide, obtained from SPR measurements performed at pH 5.0 and pH 7.4.

pH	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (M)	χ^2
5.0	3.1×10^{5}	1.7×10^{-4}	5.5×10^{-10}	0.7
7.4	1.3×10^{5}	7.2×10^{-4}	5.5×10^{-9}	0.2

Affibody dimer slightly reduces the copper quenching effect, both at pH 7.3 and 5.0.

All titration curves nicely fit our theoretical model, indicating that little aggregation occurs during the titration. This is particularly important given a recent statement that metal binding to Aβ cannot be analyzed without accounting for the aggregation of the peptide [8 p. 255]. In fact, the large variation in previously measured K_D values (ranging from 10^{-6} to 10^{-18}) for Cu(II)/Aβ binding [21,27–29] can probably be at least partially attributed to different degrees of Aβ aggregation before and during measurements. To the best of our knowledge no systematic studies of Cu(II) binding to aggregated Aβ have been conducted, but the high amounts of copper (and zinc) deposited in amyloid plaques of aggregated Aβ peptide in the brains of AD patients [5,38] indicate that aggregated Aβ can successfully compete for Cu(II) binding with the natural copper chaperones [39], which display K_D's in the pico- to attomolar range [40,41]. Furthermore, the lowest K_D values for metal binding to Aβ [27] were reported before the aggregation properties of the Aβ peptide were fully understood. Today we know that Aβ aggregation is promoted by numerous factors, such as high peptide concentration, high temperature, low pH, agitation/stirring, and presence of metals [42]. Yet, even though monomeric Aβ peptide samples can be prepared with these considerations in mind, it is perhaps no surprise that ITC measurements, which involve continuous stirring for an extended period of time, typically yield lower K_D values than the quicker fluorescence measurements [28,29,43 and references therein]. The lack of noticeable aggregation effects in our fluorescence measurements, which were carried out at low temperature and without stirring during the course of circa 15 minutes each, suggests that the aggregation-inhibiting effects conferred by the Affibody molecule might be most useful for longer fluorescence spectroscopy experiments and for studies carried out with e.g. NMR and ITC techniques.

Fitting the fluorescence data to Eq. (1) yields the apparent binding constants presented in Table 3. At pH 7.3 the K_D^{App} is 0.86 μM, which is in general agreement with our previously reported K_D^{App} of 0.4 μM for Cu(II) binding to Aβ(1–28) at pH 7.4 [21]. At pH 5.0 the K_D^{App} is 51 μM, suggesting an increase in K_D by approximately a factor of 50 when the pH is decreased from 7.3 to 5.0 (Table 3). As both pH variation and the presence of various metal ions are known to affect the aggregation pathways of Aβ peptides, this decreased binding affinity of Cu(II) to the Aβ peptide at lower pH might play a role in AD pathology. The reduced binding affinity can probably be attributed to protonation of the histidines that coordinate the copper ions, i.e. H6, H13, and H14. However, a complicating factor with the measurements is that the observed (apparent) K_D values are likely affected by buffer interactions, since Cu(II) can form various chelates with citrate buffer and insoluble Cu(II)-hydroxido-phosphato complexes with phosphate buffer [20,44,45]. Unfortunately it seems virtually impossible to avoid buffer effects of that kind, as even the more inert zwitterionic buffers such as HEPES are known to form complexes with Cu(II) [8,20]. When these types of fluorescence measurement are corrected for buffer composition, absolute binding constants in the range 30–60 nM are typically obtained at neutral pH [8,28,46]. Nevertheless, the observed K_D difference by a factor of 50 between pH 7.3 and 5.0 is likely to be relatively correct, as the buffer effects to some extent cancel out when two measurements in buffers of similar strengths are compared. Furthermore, although determination of buffer-corrected absolute K_D values is of scientific importance, elucidating the AD mechanisms requires comparisons of K_D values obtained under physiological conditions. Hence, future studies may benefit from measuring apparent K_D values at even higher salt concentrations—perhaps as an effort to study Aβ/copper ion interactions in systems that mimic extra- and intracellular environments [19]. Because different metal ions (copper, zinc, iron) appear to compete for the same Aβ peptide binding site [8,21,25], the important issue might ultimately not be how a shift in pH or ionic strength affects the K_D values in absolute terms, but rather how the binding affinities of different metal ions change relative to each other. Thus, it is unclear whether the observed pH-dependent decrease in Aβ peptide/Cu(II) binding would be expected to promote or hamper AD progression—more research on the subject is clearly needed.

When the $Z_{A\beta3}$(12–58)Y18L Affibody molecule is present, Cu(II) binds the Aβ peptide/Affibody complex with a K_D^{App} of 5.4 μM at pH 7.3, and 198 μM at pH 5.0 (Table 3). Thus, the apparent K_D values are about 5 times higher when the Affibody molecule is present, for both pH values, and the K_D difference between the two pH values consequently remains roughly a factor of 50. There are various possible explanations for this increase in K_D. First, a secondary weaker metal binding site has been suggested between Asp^{23} and Lys^{28} of the Aβ peptide [21,47]. This secondary binding site is obviously made inaccessible by the Affibody molecule. Second, the Affibody molecule might slightly interfere with the binding to the primary metal site—either sterically or by altering the local charge distribution. However, the truncated $Z_{A\beta3}$(12–58) Affibody was created by removing the 11 first aa of the original $Z_{A\beta3}$(1–58) molecule, as these were not involved in binding the Aβ peptide. Thus, removing these 11 dangling residues created a tighter Affibody/Aβ complex where the Affibody molecule has a lower opportunity for interaction with the free N-terminal of the Aβ peptide. Third, the Affibody molecule locks the Aβ peptide in a β-strand hairpin, i.e. the conformation in which the Aβ peptide aggregates into fibrils [48]. Without the Affibody molecule present, monomeric Aβ peptides preferably adopt a random coil structure

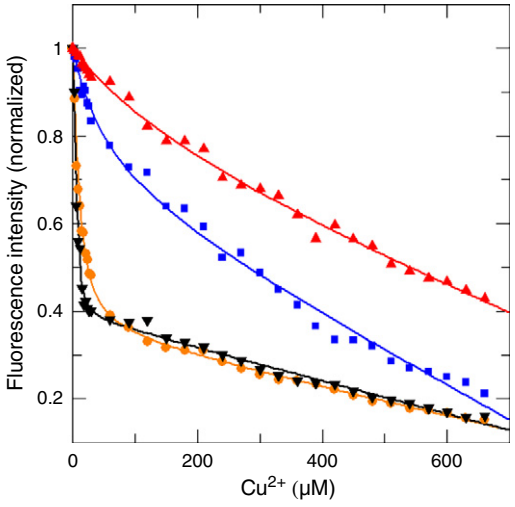

Fig. 2. Fluorescence spectroscopy data showing the fluorescence quenching when Cu(II) ions were titrated onto 10 μM free Aβ(1–40) at pH 7.3 (black triangles) and at pH 5.0 (blue squares), and onto 10 μM Aβ(1–40) in 1:1 complex with the $Z_{A\beta3}$(12–58)Y18L Affibody homodimer at pH 7.3 (orange circles) and at pH 5.0 (red triangles).

Table 3
Binding constants between Cu (II) ions and Aβ(1–40) peptide alone and in 1:1 complex with the $Z_{A\beta3}$(12–58)Y18L Affibody molecule, at pH 5.0 and 7.3, obtained by fluorescence spectroscopy measurements.

	pH 5.0	pH 5.0 with Affibody	pH 7.3	pH 7.3 with Affibody
K_D	51 μM	198 μM	0.86 μM	5.4 μM

in solution. This conformational change might affect also the metal binding properties. Although we presently cannot discriminate between these possible explanations, the overall effect of the Affibody molecule on the copper binding is rather small, and furthermore consistent. It therefore appears possible to control for the effect of the Affibody molecule. This suggests that the $Z_{A\beta3}(12–58)$ molecule and variants thereof have huge potential as tools for studying the metal-binding and other properties of the N-terminal part of the Aβ monomer without concern for aggregation.

Such studies are particularly interesting as the hairpin structure of the Aβ peptide in the Affibody/Aβ complex may be regarded as a soluble and stable variant of the Aβ building blocks in fibrillar and possibly also oligomeric Aβ aggregates [see review 49]. The overall nature of these building blocks is a β-strand hairpin formed by Aβ residues 15–37 with an unstructured N-terminal segment 1–14 [50,51]. But even though residues 1–14 are generally unstructured, at least in the absence of e.g. metal ions, the importance of this segment for the biological impact of Aβ has been firmly established. As the Aβ residues 1–14 are equally free and unstructured outside the Affibody/Aβ complex, this system may allow for detailed studies of the Aβ N-terminal in a biologically relevant Aβ conformation.

4. Summary and conclusions

In this study we show that the binding of Cu(II) ions to the Aβ(1–40) peptide involved in Alzheimer's disease is almost two orders of magnitude weaker at pH 5.0 ($K_D^{App} = 51$ μM) than at pH 7.3 ($K_D^{App} = 0.86$ μM). A straightforward explanation for this effect is protonation at low pH of the Aβ histidines H6, H13, and H14, which coordinate the copper ions. Given that i) metal ions influence the Aβ peptide aggregation pathways, ii) acidotic conditions appear to be involved in the progression of AD, and iii) cell toxic effects increase when redox-active Cu(II) ions bind the Aβ peptide, our findings may be important for understanding the molecular mechanisms of AD progression. Further, we created modified non-fluorescent versions of the $Z_{A\beta3}(12–58)$ Affibody molecule that bind the Aβ peptide and prevent its aggregation. These Affibody dimers lock the Aβ peptide in a hairpin structure without affecting the N-terminal segment where the metal ions bind. The hairpin conformation with an unstructured N-terminal mimics the structure adopted by Aβ peptides in the amyloid plaques found in brains of Alzheimer's disease patients. We therefore believe that this Affibody molecule, and engineered versions of it, will prove very useful for elucidating the properties of the monomeric Aβ peptide—in particular the metal-binding and other properties relating to the Aβ N-terminal.

Abbreviations

Aβ	amyloid beta
AD	Alzheimer's disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSQC	heteronuclear single quantum coherence
K_D^{App}	apparent dissociation constant
LC	long chain
LC–ESI–MS	liquid chromatography electrospray ionization mass spectrometry
RP-HPLC	reversed phase high performance liquid chromatography
SPPS	solid phase peptide synthesis
SPR	surface plasmon resonance

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jinorgbio.2012.11.005.

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