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"Click Peptide": pH-Triggered in Situ Production and Aggregation of Monomer Aβ1–42

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The intense and uncontrollable self-assembling nature of amyloid β peptide (Aβ) 1–42 is known to cause difficulties in preparing monomeric Aβ1–42; this results in irreproducible or discrepant study outcomes. Herein, we report novel features of a pH click peptide of Aβ1–42 that was designed to overcome these problems. The click peptide is a water-soluble precursor peptide of Aβ1–42 with an O-acyl isopeptide structure between the Gly25–Ser26 sequence. The click peptide adopts and retains a monomeric, random coil state under acidic conditions. Upon change to neutral pH (pH click), the click peptide converts to Aβ1–42 promptly ($t_{1/2} \approx 10$ s) and quantitatively through an O-to-N intramolecular acyl migration. As a result of this quick and irreversible conversion, monomer Aβ1–42 with a random coil structure is produced in situ. Moreover, the oligomerization, amyloid fibril formation and conformational changes of the produced Aβ1–42 can be observed over time. This click peptide strategy should provide a reliable experimental system to investigate the pathological role of Aβ1–42 in Alzheimer's disease.

Introduction

Amyloid β peptides (Aβs) are the main proteinaceous components of amyloid plaques found in the brains of Alzheimer's disease (AD) patients.[1] Amyloid plaques contain abundant fibrils formed from Aβs that have been found to be neurotoxic in vitro and in vivo.[2] Although many studies support the notion that the self-assembly of Aβs is crucial in the pathogenesis of AD, the toxicological mechanisms of Aβs in neuronal cells are unclear and remain controversial. One major obstacle in elucidating these mechanisms is that differences in the manner of Aβ monomer preparation apparently lead to irreproducible or discrepant experimental results in studies of the pathological significance of Aβs.[3–8]

To investigate the toxicology of Aβ, samples should be used in their monomeric random coil states, because the neurotoxicity and kinetics of Aβ aggregation are directly linked with the assembly states (monomer, oligomer and aggregate). However, chemically synthesized Aβs often contain various oligomeric forms,[6,9] because Aβs undergo self-aggregation in aqueous trifluoroacetic acid (TFA)/acetonitrile during HPLC purification.[10] In addition, the quantitative ratio of each oligomeric state is different among the sources (lots) of synthesized Aβs. Various pre-treatments have been adopted to minimize problems with Aβs. For example, dimethylsulfoxide (DMSO),[11–13] hexafluoroisopropanol (HFIP),[14] TFA,[15] NaOH[16] and NH_4OH[17] have been used to solubilize and/or disaggregate Aβs. Moreover, ultrafiltration,[16] ultracentrifugation[17] and size-exclusion chromatography (SEC)[18] have been performed to obtain low molecular weight Aβs. These various pretreatment methods lead to different results in physicochemical or biological studies of Aβs. Moreover, the obtained monomer Aβs sometimes assemble during storage prior to their use. Thus, the problem of irreproducible or discrepant study outcomes due to different sources (lots), pre-treatments and storage conditions is predominantly associated with the intense and uncontrollable self-assembling nature of Aβs.

Hence, we conceived the idea that an in situ production system that affords only monomer Aβs with a random coil structure would be a superb tool in understanding the pathological role of Aβs. We envisioned that such a system would require 1) a water-soluble precursor peptide possessing no self-assembling nature and 2) an ability to produce monomer Aβs under physiological conditions.

We previously developed an "*O*-acyl isopeptide method"[19,20] for the synthesis of peptides containing difficult sequences. We disclosed that the presence of an *O*-acyl instead of the native *N*-acyl residue at a hydroxyamino acid residue (for ex-

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ample, Ser and Thr) in the peptide backbone drastically changed a physicochemical property of the native peptide. Moreover, the target peptide was generated through an O-to-N intramolecular acyl migration. This method was applied to the synthesis of Alzheimer's Aβ1–42, the low yield and purity of which were caused by self-aggregation during solid-phase peptide synthesis and HPLC purification. Namely, modification of the *O*-acyl-isomerized structure between Gly25–Ser26 of Aβ1–42 resulted in an efficient synthesis of 26-*O*-acyl isoAβ1–42 (**1**; Scheme 1).[21] Due to the ionized N^{α}-amino group of Ser26, **1** was 100-fold more water-soluble than Aβ1–42.[21,22] Consequently, **1** was easily purified by HPLC. Moreover, Aβ1–42 could then be obtained from **1** through a pH-dependent, O-to-N intramolecular acyl migration under neutral conditions (Scheme 1). These features are not only beneficial for efficient synthesis but also for physicochemical/biological studies used to examine the role of Aβ1–42 in AD. In this context, we developed analogues of **1** that converted into Aβ1–42 by photoirradiation under physiological conditions.[23] These isopeptides were expected to give intact Aβ1–42 with a monomeric random coil structure. Recently, we[19–28] and other groups[29–34] have applied the method to various fields of research.

In this article, we describe novel features of isopeptide **1** (designated "pH click peptide") that are useful in studying AD. These features were verified by various physicochemical experiments. Click peptide **1** clearly adopted and retained a monomeric state with a random coil structure under acidic conditions. Upon a change to a neutral pH (pH click), **1** promptly and quantitatively converted into Aβ1–42 in situ ($t_{1/2}$ ~10 s). Thus, we succeeded in establishing an in situ system predominantly comprised of monomer Aβ1–42 as a result of a quick and irreversible conversion of monomer **1** upon a pH change (pH click). Moreover, oligomerization, amyloid fibril formation and conformational changes of the in situ-produced monomer Aβ1–42 were observed over time.

Results

Stability of pH click peptide 1 and its conversion to Aβ1–42

The stability of pH click peptide **1** under acidic or neutral conditions was examined by HPLC. Compound **1** was stable (>95%) in 0.1% aqueous TFA at 37 °C for 24 h, at room temperature for 6 days and at −80 °C for 1 month. However, upon addition of phosphate buffer (pH 7.4) to **1** in 0.1% aqueous TFA (final concentration of **1** was 40 μM, See the Experimental Section) and incubation at 37 °C, **1** was converted to Aβ1–42 through the O-to-N intramolecular acyl migration with a $t_{1/2}$ of ~10 s (Figure 1). The conversion was mostly complete after 2 min and no precipitates from the peptide were observed. A similar conversion profile was observed at room temperature (15 s: 54%, 30 s: 72%, 1 min: 85%, 2 min: 91% and 5 min: 94%). In our previous study, the conversion of **1** to Aβ1–42 took place in 1% DMSO containing phosphate buffered saline (pH 7.4) with a $t_{1/2}$ of 1 min;[21b] this suggests that 1% DMSO slowed the O-to-N migration. Consequently, **1** was stable in 0.1% aqueous TFA and enabled a quick and quantitative in situ production of Aβ1–42 upon a pH change (pH click) to pH 7.4.

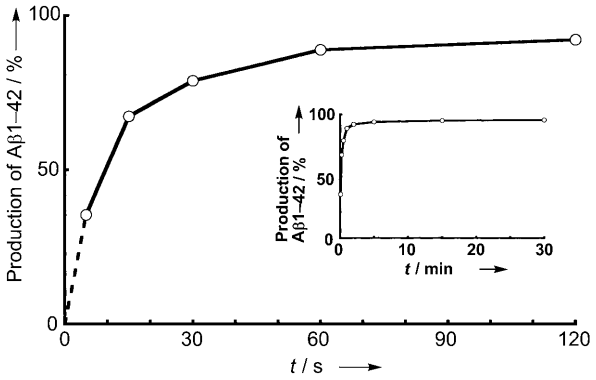
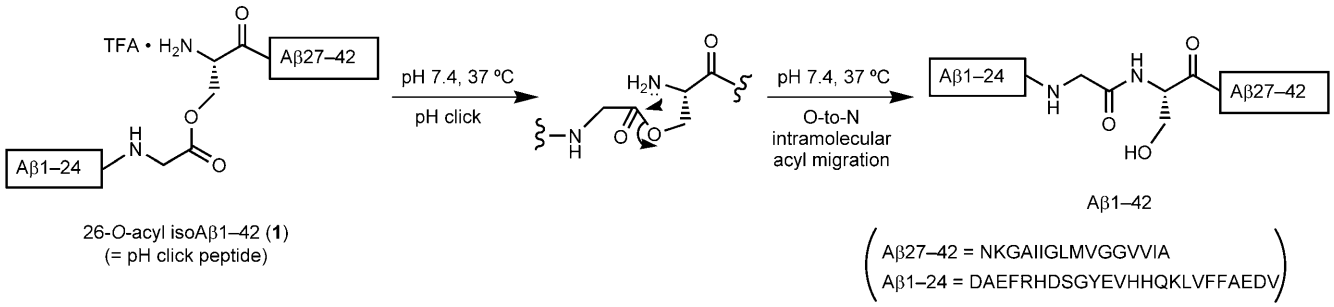


Figure 1. Time course of Aβ1–42 production from pH click peptide **1** (40 μM) through an O-to-N intramolecular acyl migration in pH 7.4 buffer. The inset shows a longer time scale.

Aggregation assay of pH click peptide 1 under acidic conditions

pH click peptide **1** (30 μM) in 0.1% aqueous TFA , which was prepared by ultra-centrifugation (see the Experimental Section), was incubated at 37 °C. The oligomerization, amyloid fibril formation and secondary structure of **1** were then periodically investigated by SEC, a thioflavin-T (ThT) assay and circular



Scheme 1. In situ production of Aβ1–42 from 26-*O*-acyl isoAβ1–42 (designated pH click peptide **1**) through an O-to-N intramolecular acyl migration triggered by pH change (pH click).

dichroism (CD) spectroscopy (Figure 2).[35] In the SEC analysis, the peak area at elution time 28 min, which corresponds to a monomeric molecular mass, was not significantly affected

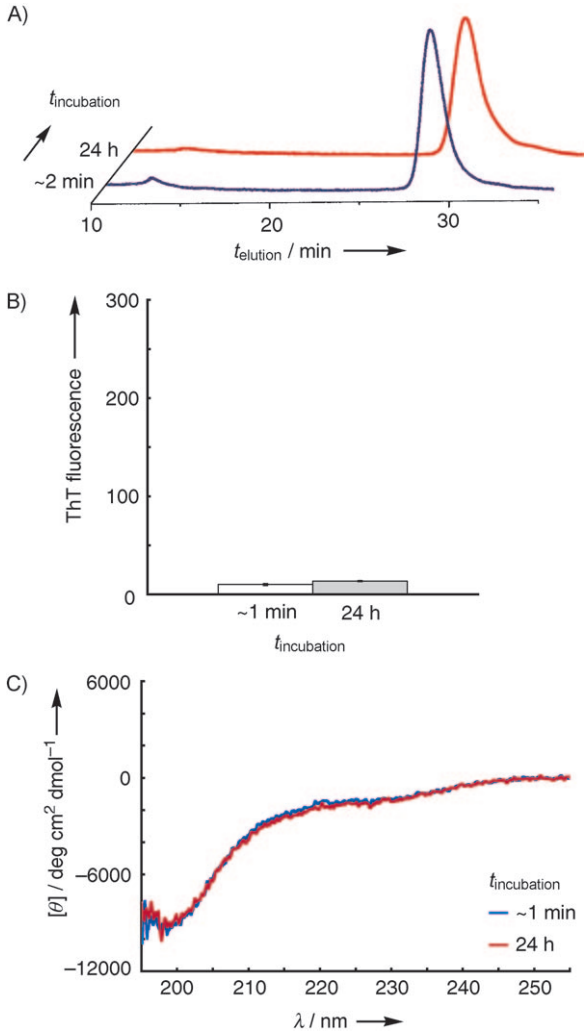


Figure 2. A) Size-exclusion chromatography (SEC), B) thioflavin-T (ThT) assay and C) circular dichroism (CD) spectroscopy of pH click peptide **1** (30 μM) in 0.1% aqueous TFA (before pH click), incubated at 37 °C.

during a 24 h incubation. A peak corresponding to the oligomer was not detected (Figure 2A). In addition, no significant change was observed after the incubation of **1** in the ThT fluorescence intensity corresponding to the amount of oligomer and/or amyloid fibril (Figure 2B). Moreover, the CD spectrum of **1** in 0.1% aqueous TFA showed a negative maximum at approximately 200 nm, which is characteristic of a random coil structure, in both the ~1 min and 24 h incubations (Figure 2C). These results clearly demonstrate that **1** adopts a monomeric, random coil structure without aggregation in an aqueous solution with 0.1% TFA, and the state of **1** was clearly maintained over a reasonably time period to allow for its storage for various experiments.

In the case of Aβ1–42 under the same conditions, a peak area corresponding to the monomer decreased after 24 h by SEC analysis (Figure S1A). In the ThT assay, a drastic increase in ThT fluorescence intensity indicated the oligomerization of and/or amyloid fibril formation by Aβ1–42 (Figure S1B). In CD spectroscopy, a secondary structure transition (random coil to β-sheet) was observed (Figure S1C). These results agree with the previous observation that the *O*-acyl isopeptide structure in **1** suppresses the self-assembling nature of Aβ1–42 by modifying hydrogen-bond interactions.[21,23]

Aggregation assay of Aβ1–42 from pH click peptide 1 under neutral conditions

A solution of pH click peptide **1** (140 μM) in 0.1% aqueous TFA was neutralized by dilution with phosphate buffer (pH 7.4) to 30 μM of **1** (See the Experimental Section) and incubated at 37 °C. The self-assembling nature of the converted Aβ1–42 was periodically investigated by SEC, the ThT assay and CD spectroscopy.[35] At an incubation time of ~2 min, in which the conversion was mostly complete as described above, only a peak at elution time 28 min (corresponding to the monomer) was observed without any oligomer peak in the SEC profile (Figure 3A). This profile showed that monomer Aβ1–42 dominated the system. A ThT assay and CD experiment of the sample with a similar incubation time (~1 min) indicated that the produced Aβ1–42 did not form any amyloid fibrils and adopted the random coil structure immediately after neutralization to pH 7.4 (pH click), as shown in Figure 3B and C.

In the SEC analysis, a newly generated peak (elution time 17 min) corresponding to an oligomer (>octamer) of Aβ1–42 appeared after 1 h of incubation and increased with incubation time at the expense of the monomer peak (elution time 28 min) (Figure 3A). The fluorescence intensity in the ThT assay also increased with time (Figure 3B). These results indicated that oligomers and/or amyloid fibrils of Aβ1–42 were periodically formed. Moreover, in the CD experiments, the [θ] values in the range of 195 to 200 nm increased, and the values at approximately 217 nm decreased with incubation time (Figure 3C). These changes suggested that the conformation of the in situ-produced Aβ1–42 shifted from a random coil to a β-sheet structure in pH 7.4 buffer. As for the case of an authentic Aβ1–42, oligomerization, amyloid fibril formation and conformational change were also observed under similar conditions (Figure S2). These results indicate that monomer Aβ1–42 can be prepared from **1** through a quick and irreversible transformation, and both aggregation and conformational changes of the monomer Aβ1–42 proceed over time in situ.

Discussion

pH click peptide **1**, an *O*-acyl isopeptide of Aβ1–42 with a β-ester bond between the native Gly25–Ser26 sequence, is a water-soluble precursor peptide of Aβ1–42. Compound **1** can produce an intact Aβ1–42 by a pH trigger. In 0.1% aqueous TFA, **1** was chemically stable without any hydrolysis or chemical rearrangement. In addition, **1** in 0.1% TFA clearly adopted a monomeric state with a random coil structure and did not exhibit self-assembly, while Aβ1–42 itself was highly aggregative

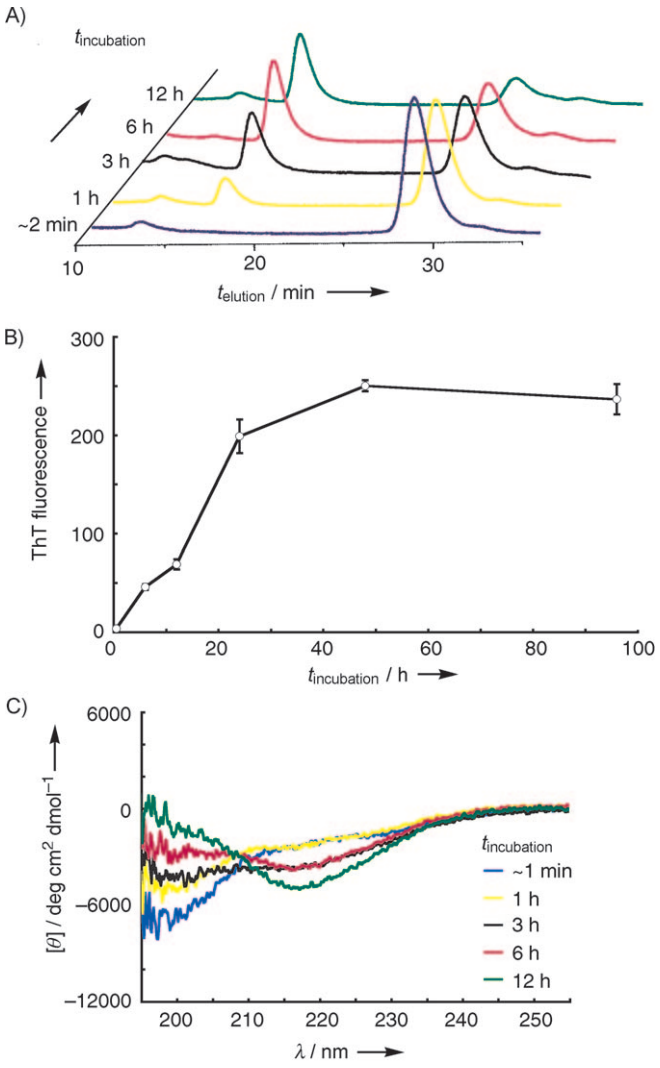


Figure 3. A) Size-exclusion chromatography (SEC), B) thioflavin-T (ThT) assay and C) circular dichroism (CD) spectroscopy of Aβ1–42 from pH click peptide **1** (30 μM) in pH 7.4 buffer (after pH click), incubated at 37 °C.

under similar conditions. These results demonstrated that the *O*-acyl isopeptide structure in **1** plays a key role in the suppression of the self-assembling nature present in Aβ1–42. Upon a pH change to neutrality (pH click), a very quick O-to-N, intramolecular acyl migration in **1** ($t_{1/2}$ ~ 10 s) occurred to give intact monomer Aβ1–42 with a random coil structure. The in situ-produced Aβ1–42 exhibited time-dependent oligomerization, amyloid fibril formation and a conformational change from a random coil to a β-sheet structures (Figure 4).

The intense and uncontrollable self-assembling nature of Aβ1–42 during synthesis and storage has led to difficulties in preparing monomeric Aβ1–42. The difficulties have brought irreproducible or discrepant experimental results. To overcome these problems, **1** was designed and displayed the following advantages: 1) the isopeptide was efficiently obtained due to the suppression of aggregation during peptide synthesis and HPLC purification, 2) the 100-fold higher water solubility of **1** over that of Aβ1–42 enabled the preparation of solutions with higher concentrations, which would allow for the diversification of experiment types, 3) the monomeric, random coil structure of **1** was stable under acidic conditions, and **1** could be stored as a stock solution and 4) monomer Aβ1–42 could be quickly produced in situ upon being triggered by a pH click of **1**. Thus, the use of **1** should overcome the handling problems of Aβ1–42 in investigating its pathological role in AD.

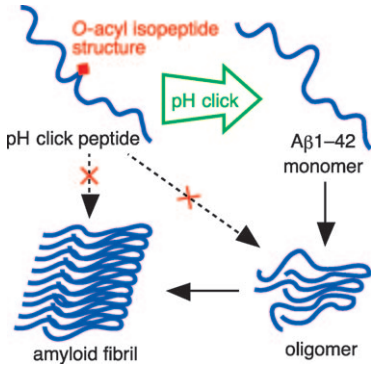


Figure 4. In situ production of monomer Aβ1–42 from pH click peptide **1** and the following aggregation of the produced Aβ1–42.

Conclusions

In the present study, novel features of pH click peptide **1** were shown by various physicochemical experiments. From these features, we established an in situ system in which monomeric **1** quickly converted into monomer Aβ1–42 with a random coil structure in neutral pH buffer. Moreover, the produced Aβ1–42 monomer underwent self-assembly and conformational changes under physiological conditions. The click peptide could reproducibly provide a means to recreate the assembly events that originate from monomer Aβ1–42. The study of these events is important in the elucidation of the pathological role of Aβ1–42 in AD. Thus, this click peptide strategy could provide a reliable experiment system in Aβ-related AD research and help to more clearly explain the functions of Aβ1–42. We are currently pursuing studies on the pathophysiological significance of Aβ1–42 using this click peptide.

Experimental Section

Peptide synthesis: pH click peptide **1** and Aβ1–42 were synthesized according to our previous report.[21b] Homogeneity and identity of synthesized peptides were confirmed by analytical HPLC, MALDI-TOF MS and amino acid analysis. Analytical HPLC was performed on L-7000 series units (Hitachi High-Technologies Corporation, Tokyo, Japan) using a C18 reversed-phase column (4.6× 150 mm, YMC Pack ODS AM302; YMC Co., Ltd., Kyoto, Japan) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (40 °C), detected at 230 nm. MALDI-TOF MS spectra were recorded on a Voyager DE-RP (Applied Biosystems, Foster City, CA, USA) using α-cyano-4-hydroxy cinnamic acid as a matrix. Amino acid analyses were performed at Peptide Institute, Inc. (Osaka, Japan). Standard chemicals were purchased from commercial suppliers such as Wako Pure Chemical

Ind., Ltd. (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), Watanabe Chemical Ind., Ltd. (Hiroshima, Japan) and Sigma–Aldrich.

Stability of 1 in 0.1% aqueous TFA: pH click peptide **1** (140 μM) in aqueous TFA (0.1%) was stored at −80 °C, or the solution (30 μM **1**) was incubated at rt or 37 °C. Each solution was analyzed by analytical HPLC as described above.

Conversion of 1 to Aβ1–42 in phosphate buffer: pH click peptide **1** (140 μM) in aqueous TFA (0.1%) was diluted with an equal volume of phosphate buffer (0.2 M, pH 7.4). Additional phosphate buffer (0.1 M, pH 7.4) was immediately added to obtain 40 μM of the peptide solution (pH 7.4). The solution (100 μL) was incubated at rt or 37 °C, mixed with HFIP (100 μL) at the desired time points (5, 15 and 30 s and 1, 2, 5, 15 and 30 min) to quench the migration reaction, and analyzed by analytical HPLC.

Preparation of peptide solution:[17,35] pH click peptide **1** (280 μM) was dissolved in aqueous TFA (0.1%), while Aβ1–42 (180 μM) was dissolved in aqueous NH_4OH (0.02%) due to the low water-solubility of Aβ1–42. Each solution was ultra-centrifuged (435 000 or 541 000 *g*) at 4 °C for 3 h on a TL-100 (Beckman Instruments, Inc., Palo Alto, CA, USA) with a TLA-100.1 or TLA-100.3 rotor (Beckman Instruments, Inc., respectively). The upper three-quarters fraction was collected. The peptide concentration of each solution was estimated from a UV absorption at 280 nm on a BioSpec-1600 (Shimadzu Co., Kyoto, Japan), using a Tyr extinction coefficient of 1490 $M^{-1}cm^{-1}$.[36] The solution of **1** was further diluted with aqueous TFA (0.1%, 140 μM **1**), while the solution of Aβ1–42 was diluted with aqueous NH_4OH (0.02%, 80 μM Aβ1–42). Each solution was stored at −80 °C until use. Just before the experiments, the stock solution of **1** or Aβ1–42 was thawed and diluted with an equal volume of phosphate buffer (0.2 M, pH 7.4). Then additional phosphate buffer (0.1 M, pH 7.4) was immediately added to obtain the peptide solution (30 μM, pH 7.4).

In the preparation of each peptide solution under acidic conditions, **1** (280 μM) was dissolved in 0.1% aqueous TFA, while Aβ1–42 was saturated in 0.1% aqueous TFA. The solution of **1** or Aβ1–42 was applied to ultra-centrifugation in a similar manner as described above. Each obtained solution was further diluted with 0.1% aqueous TFA to obtain 30 μM of **1** or Aβ1–42.

Size-exclusion chromatography (SEC):[35] pH click peptide **1** or Aβ1–42 (30 μM each peptide) in aqueous TFA (0.1%) was incubated at 37 °C. The aliquot (35 μL, 1.1 nmol) was directly applied to SEC at the desired time points (~2 min and 24 h). Compound **1** or Aβ1–42 in a pH 7.4 buffer was also incubated and applied to SEC at the desired time points (~2 min and 1, 3, 6, 12 and 48 h). SEC was performed on an ÄKTA explorer 10S (GE Healthcare UK Ltd., Buckinghamshire, England) instrument using Superdex 75 HR 10/30 (GE Healthcare UK Ltd.) with an isocratic solvent system: phosphate buffered saline (pH 7.4) at a flow rate of 0.5 mL min^{-1}, detected at 220 nm. The column was pre-treated with excess bovine serum albumin (BSA; Sigma–Aldrich) to block non-specific binding of peptides. Molecular mass was estimated with FITC-dextrans (M_W 4000, 20 000 and 40 000; Sigma–Aldrich) as standards. A voiding time of 16 min was estimated by an elution time of blue dextran (av. M_W 2 000 000; Sigma–Aldrich).

Thioflavin-T (ThT) assay:[35] pH click peptide **1** or Aβ1–42 (30 μM each peptide) in aqueous TFA (0.1%) was incubated at 37 °C. The solution was applied to ThT assay at the desired time points (~1 min and 24 h). **1** or Aβ1–42 in a pH 7.4 buffer was also incubated and applied to ThT assay at the desired time points (~1 min and 6, 12, 24, 48 and 96 h). The aliquot sample (69 μL, 2.1 nmol) was added to ThT (5 μM, Sigma–Aldrich Inc.) containing glycine buffer (2 mL, 50 mM Gly, pH 8.5). ThT fluorescence at 480 nm was measured at an excitation wavelength of 440 nm at RT on a VersaFluor (Bio-Rad Laboratories Inc., Hercules, CA, USA) instrument. ThT fluorescence intensity was calculated using a standard fluorescence intensity (1000) of a 20 μM calcein solution (Dojindo Laboratories, Kumamoto, Japan) in phosphate buffered saline (pH 7.4). Each assay was done in triplicate.

Circular dichroism (CD) spectroscopy:[35] pH click peptide **1** or Aβ1–42 (30 μM each peptide) in aqueous TFA (0.1%) was incubated at 37 °C. The solution (210 μL, 6.3 nmol) was applied to CD spectroscopy at the desired time points (~1 min and 24 h). Compound **1** or Aβ1–42 in a pH 7.4 buffer was also incubated and applied to CD spectroscopy at the desired time points (~1 min and 1, 3, 6, 12 and 48 h). CD spectra were measured at 37 °C on a J-720WI (JASCO Corporation, Tokyo, Japan) instrument using a 1.0 mm path length quartz cell. Eight scans were averaged for each sample. Averaged blank spectra were subtracted, respectively.

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Keywords: acyl migration · aggregation · amyloid beta peptides · click peptides · isopeptide · rearrangement

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