Formation of Functionalized Nanowires by Control of Self-Assembly Using Multiple Modified Amyloid Peptides

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Amyloid peptides have great potential as building blocks in the creation of functional nanowires due to their natural ability to self-assemble into nanofibrillar structures and because they can be easily modified with various functional groups. However, significant modifications of an amyloid peptide generally alter its self-assembly property, making it difficult to construct functionalized fibrils with a desired structure and function. In this study, a very effective method to overcome this problem is demonstrated by using our structure-controllable amyloid peptides (SCAPs) terminated with a three-amino-acid-residue cap. The method consists on mixing two or more structurally related amyloid peptides with a fraction of modified SCAPs which co-assemble into a fibril. This SCAP-mixing method provides remarkable control over the self-assembly process both on the small oligomers level and the macroscopic fibrils level. Furthermore, it is shown that the modified peptides imbedded in the resulting fibril can subsequently be functionalized to generate nanowires with the desired properties, highlighting the importance of this SCAP method for nanotechnology applications.

1. Introduction

Functionalized nanowires possess unique properties that make them valuable materials for the design of next generation devices but also systems with never before seen functions. Adding functional groups to nanowires allows to mix both the structure and electrical properties of nanowires with the recognition of catalytic properties of biomolecules. These added properties give them great potential in nanoelectronic applications to work as novel sensors, field-effect transistors, photodiodes and LEDs,[1–7] creating a high demand for this nanoscale material.

One practical approach to create these functionalized nanowires is to use the bottom-up strategy which consists on assembling molecular building blocks into a variety of functionalized nanostructures. Several materials like peptide amphiphiles, carbon, and DNAs have been identified as good building block candidates to make nanowires.[5–9] However, despite their ability to successfully form fibrils, these materials are very limited in the type of functional nanowires they can form, or require complicated synthetic procedures. Therefore, attention has been focused on the use of amyloid peptides as building blocks since, not only they can naturally self-assemble into uni-dimensional fibrils of exceptional stability and mechanical strength,[10–20] but given their peptidic nature, it is easy to assign them various functionalities with rather simple engineering techniques.

The most common problem when making fibrils out of amyloid peptide building blocks is the difficulty in controlling the structure of the resulting fibrils (for example length, height, and homogeneity of the ensemble). This feature is especially present when functionalized amyloid building blocks are employed, because the modification of amyloid peptides seriously alters their self-assembling property.[21] Therefore, this fact highlights the importance of developing effective methodologies to control the self-assembly of modified amyloid building blocks.

Recently, we have demonstrated that the attachment of a three-amino-acid-residue unit to the N-termini of amyloid peptides drastically affects their self-assembly formation.[22] These peptides can accelerate fibril formation but also can yield fibrils with quite different architectures, depending on the properties of attached amino acids. Therefore, the added N-termini of these structure-controllable amyloid peptides (SCAPs) offer an additional binding interface that helps stabilize the

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DOI: 10.1002/adfm.201300577

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self-assembly formation. Furthermore, the fact that in natural systems interactions among multiple amino acid residues support folding or stabilization of protein structures suggests that employing two or more SCAPs as a mixture would allow effective control of fibril formation and structure. This would also make it possible to introduce functional molecules to the building blocks without any disruption of the fibril formation.

In this study, we describe an efficient and straightforward way to control the self-assembly of peptides by mixing multiple SCAPs and also present a method to make functionalized nanowires (**Figure 1**). Mixing multiple SCAPs allowed control of the fibril formation. Detailed analysis of ion mobility spectrometry-mass spectrometry (IMS-MS) studies[23–25] demonstrated a unique structural evolution of peptides at a small oligomer level in the mixed SCAP system. We also show that probing one of the SCAPs in the mixture does not disrupt the structure or the self-assembly of the fibrils and is successful in yielding probed embedded nanowires. Using these probed nanowires, we were able to functionalize them with biomineralization peptides, which were then transformed into titania nanowires.

2. Results

2.1. Drastic Enhancement of Fibrillation by Mixing Multiple SCAPs with Different Amino Acid Units

Two types of SCAPs (Supporting Information, Table S1) were synthesized by adding three amino acids to the N-terminus of the TTR(105–115) amyloidogenic fragment of transthyretin:[26,27] one with a triple lysine (K_3-TTR) and the other with a triple glutamic acid (E_3-TTR). When these two SCAPs and unmodified TTR(105–115) peptide were incubated by themselves in acidic water at pH 2.0 as described elsewhere,[27–29] only E_3-TTR was able to form several-micrometers-long fibrils with a height of 4.56 ± 0.45 nm (**Figure 2**a–c; Supporting Information, Figure S1). However, mixing K_3-TTR and E_3-TTR at a 1:1 molar ratio was able to form extremely long fibrils of well over 40 μm long and 5.11 ± 0.69 nm high (Figure 2d). These very long fibrils, to our knowledge, have the highest aspect ratio (length/thickness >8000) to date, and therefore can be useful as nanowire templates. This dramatic difference in fibrillation suggests that mixing SCAPs can provide particular self-assembling property to peptides.

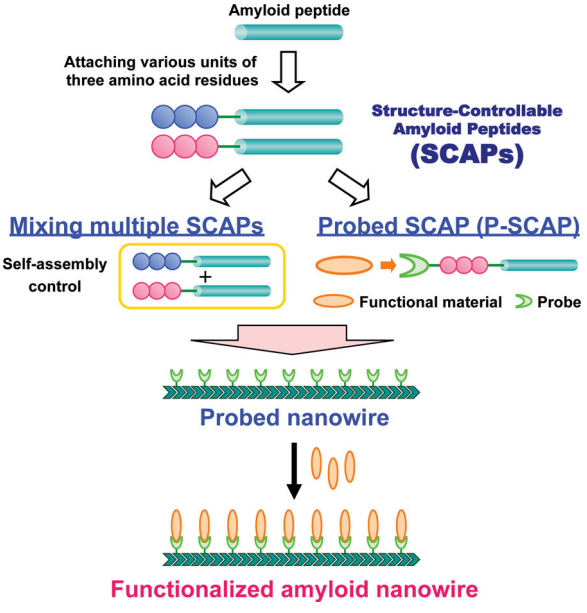

Figure 1. Schematic picture of our strategy for forming a wide variety of functionalized amyloid nanowires. Mixing multiple SCAPs containing two different units controls the self-assembling property of amyloid peptides. When probed SCAP (P-SCAP), which has a specific probe moiety at the N-terminus for subsequent functionalization, is added to the SCAPs mixture, nanowires form with probe molecules embedded. Many kinds of probes can be incorporated into fibrils and the probed nanowires can be easily functionalized by associating the corresponding functional materials with the nanowires via a specific interaction with the probe.

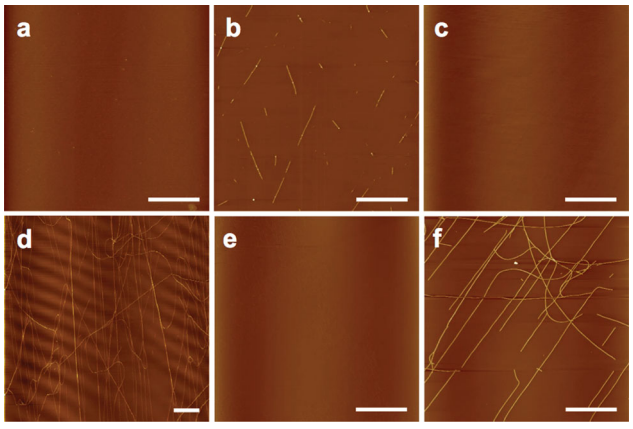

Figure 2. Control of fibril and probed nanowire formation by mixing multiple SCAPs. AFM images of a) unmodified TTR(105–115), b) E_3-TTR, c) K_3-TTR, d) K_3-TTR:E_3-TTR = 1:1, e) Mal-E_3-TTR and (f) K_3-TTR:Mal-E_3-TTR:E_3-TTR = 5:1:4. Each image represents a sample incubated for 4 days. Scale bars are 5 μm.

2.2. Characteristic Self-Assembling Property of Oligomers Demonstrated by Ion Mobility Spectrometry-Mass Spectrometry

To characterize the effect of mixing SCAPs on the enhanced fibrillation, we analyzed oligomer self-assembly stage, which is the first step of self-assembly, by employing IMS-MS. **Figure 3** represents the electrospray ionization (ESI) mass spectra of K_3-TTR, E_3-TTR and their 1:1 mixture solutions. The most abundant peak for each sample has an $n/z = 1/2$ (where n = oligomer number and z = charge of the ion). The ^{13}C isotope separation of $\Delta(m/z) = 0.5$ for the double-charged $n/z = 1/2$ peak (Figure 3a–c) reveals the dominant species in each sample corresponds to the monomeric form ($n = 1$). However, it should be noted that there is a big difference between the oligomer distribution in the solution of the single peptides and that of the peptide mixture. K_3-TTR and E_3-TTR data show oligomer peaks, $n/z = 2/3$, 3/5 and/or 3/4, while the 1:1 mixture data shows multiple oligomer peaks that represent additional higher order oligomers ($n/z = 4/5$ and 5/6), as well as a much more intense $n/z = 2/3$ peak. In the high-resolution mass spectra, the $n/z = 2/3$ peak of the mixture is apparently in the middle of

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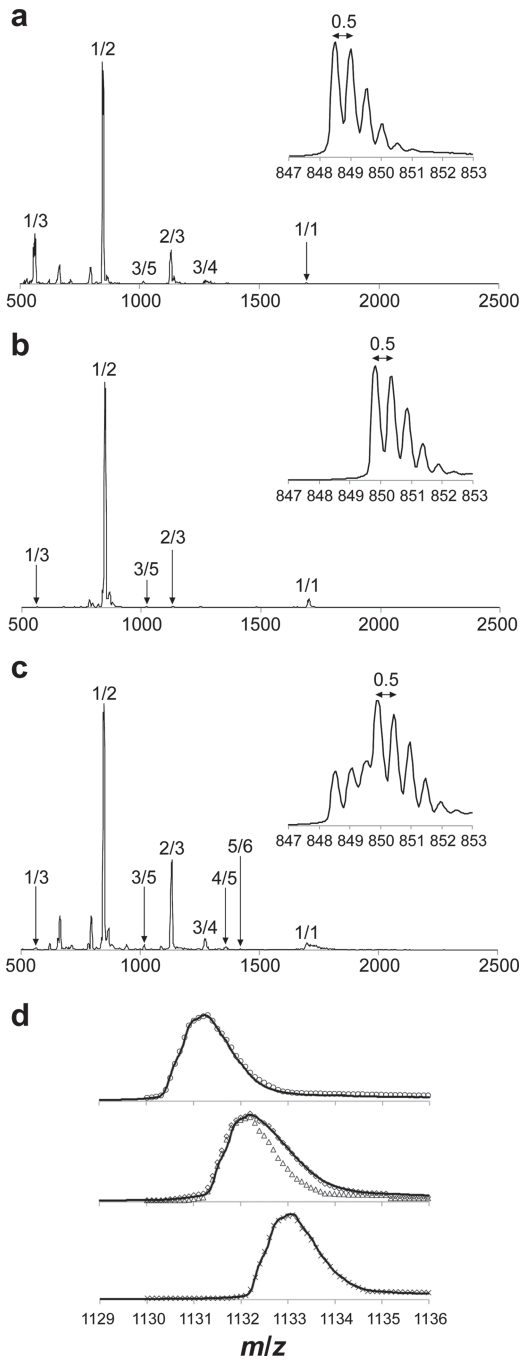

Figure 3. a–c), ESI-Q mass spectra (positive ion mode) of a) K_3-TTR, b) E_3-TTR and c) a 1:1 mixture of K_3-TTR and E_3-TTR. The peaks are labeled with the ratio of oligomer number *n* to charge *z*. The insets show high-resolution ^{13}C isotope patterns for the $n/z = 1/2$ peaks. The $n/z = 1/2$ peak shape of the 1:1 mixture shows an overlapped pattern of the K_3-TTR and E_3-TTR spectra. d) High-resolution mass spectra of the $n/z = 2/3$ charge states for K_3-TTR (top), K_3-TTR:E_3-TTR = 1:1 (middle) and E_3-TTR (bottom). The symbols represent a fitting analysis for the abundance of each dimer species. Fits to K_3-TTR (○) and E_3-TTR (x) spectra describe the spectrum patterns of K_3 and E_3 homodimer, respectively. The triangle (△) is an average of both homodimers representing pure heterodimer. A 1/20/5 ratio of the K_3 homodimer/heterodimer/E_3 homodimer (◇) gives the best fit to the experimental spectrum of the 1:1 mixture.

both homodimers (Figure 3d). A fit to the experimental spectrum of the mixture reveals the 1:20:5 abundance ratio of the K_3 homodimer to heterodimer to E_3 homodimer, whereas a 1:2:1 ratio is expected based on statistics. Therefore, the SCAP mixture obviously prefers heterodimer formation. These results suggest that mixing SCAPs also enhances self-assembly of oligomers through the formation of heterooligomers.

Figure 4 represents the arrival time distributions (ATDs) of the mass-selected ions corresponding to $n/z = 2/3$ at different peptide concentrations. The collision cross sections of the major peaks for K_3-TTR and E_3-TTR (544 and 510 Å^2, respectively) are about $2^{2/3}$-fold larger (yielding values of 538 and 519 Å^2, respectively) than the corresponding monomer cross sections. This indicates isotropic growth from the monomers to the dimers with globular structures in both cases. Interestingly, however, the $n/z = 2/3$ dimer of the 1:1 mixture has a cross section of 558 Å^2, which is ≈6% larger than the expected isotropic dimer (525 Å^2), suggesting the formation of an extended fibrillar structure (Figure 4c). Moreover, the dimer cross section of the mixture increases to 574 Å^2 (≈10% larger than the isotropic cross section) at higher concentrations, which was not the case for K_3-TTR or E_3-TTR. Similarly, this increase was also observed in the ATDs of the $n/z = 3/4$ and 3/5 peaks (Supporting Information, Figures S2,S3). Further, the ATDs of the $n/z = 3/5$ peaks show that the 1:1 mixture is more likely than either K_3-TTR or E_3-TTR alone to form a parallel fibrillar structure (Supplementary Information, Figure S3). Thus, it is suggested that an extended parallel fibrillar structure is strongly stabilized upon mixed-SCAP system. This is further supported by the presence of $n/z = 4/5$ and 5/6 peaks in the mixture only. The cross sections of the 4/5 and 5/6 ions follow the same linear correlation with oligomer number, *n*, as the dimer and trimer cross sections of the mixture (Supporting Information, Figure S4), demonstrating that the size of these oligomers grows linearly and not isotropically. Linear growth with *n* is expected for one-dimensional growth towards fibrillar structures.[23] These results clearly show that mixing SCAPs significantly influences the self-assembling property of very small oligomers to form the parallel fibrillar structure, which helps form higher number of oligomers, and thus should be related to the long fibril formation. Hence, mixing SCAPs can provide remarkable control over the self-assembly process.

2.3. Control of the Formation of Probed Fibrils by Mixing SCAPs

Of great interest is to control the self-assembly of functionalized amyloid building blocks. The significant impact of mixing SCAPs on self-assembly would allow controlling fibril formation in the presence of functionalized peptides. A versatile way to yield various functional nanowires is employing probed SCAP (P-SCAP), where a certain compound is covalently coupled to the SCAP to recruit functional nanomaterials via specific interaction after fibrillation. We synthesized a P-SCAP with a maleimide group, which is readily able to recruit any thiol compounds, at the N-terminus of E_3-TTR (Mal-E_3-TTR). As shown in Figure 2e, this peptide formed no fibrils by itself probably because of the effect of the attached maleimide group on self-assembly. As expected, however, very long nanowires with a

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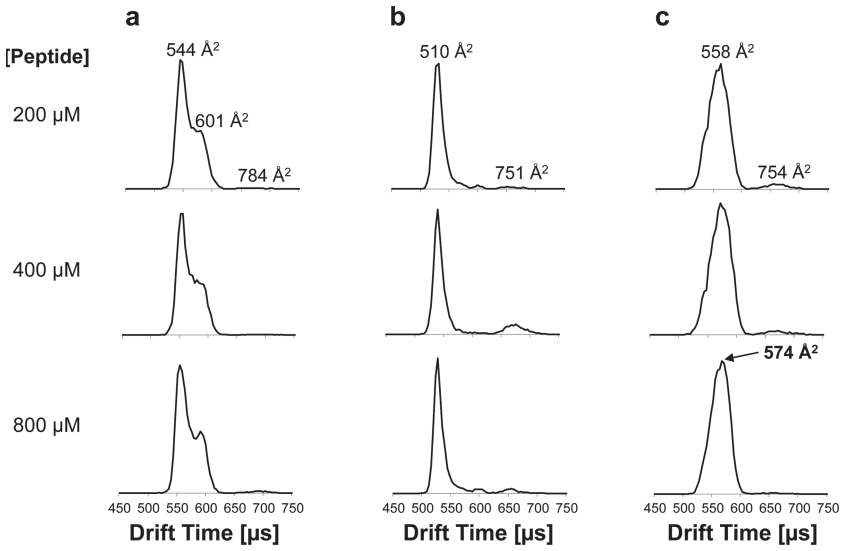

Figure 4. Arrival time distributions (ATDs) of a) K_3-TTR, b) E_3-TTR and c) K_3-TTR:E_3-TTR = 1:1 for n/z = 2/3 charge states at three different peptide concentrations. The labels are the measured collision cross sections. The weak peak with a cross section of 750–780 Å^2 could be an antiparallel β structure, in which the peptides are associating through the native TTR(105–115) region (Supporting Information).

height of 5.09 ± 0.60 nm were formed from a mixture of Mal-E_3-TTR with the two SCAPs at a molar ratio of K_3-TTR:Mal-E_3-TTR:E_3-TTR = 5:1:4 (to give a 1:1 ratio of K_3 to E_3) (Figure 2f). These nanowires have a quite similar morphology to that of the 1:1 K_3-TTR:E_3-TTR system described above but should have probes on their surfaces. Thus, mixing SCAPs is clearly effective for controlling self-assembly of modified building blocks.

2.4. Functionalization of Probed Nanowires and Transformation of the Fibrils to Inorganic Nanowires via Biomineralization

To functionalize the maleimide-probed nanowires, we synthesized a multi-functional peptide (Fluorescein-G-RKLPDA-GG-C-amide, or F-TBP-Cys) containing a cysteine residue, a fluorescent label, and a sequence derived from minTBP-1 (RKLPDA) which shows biomineralization activity.[30] An aqueous solution of this peptide was incubated with the nanowires formed from K_3-TTR:Mal-E_3-TTR:E_3-TTR = 5:1:4 on a glass surface. These probed nanowires were readily functionalized with the specific F-TBP-Cys, which was demonstrated by the observation of unambiguous fluorescent fibrils under fluorescence microscopy (**Figure 5**a). On the other hand, no fluorescence was observed after the incubation of F-TBP-Cys with maleimide-free fibrils of 1:1 K_3-TTR and E_3-TTR (Supporting Information, Figure S5). This confirms that the maleimide probes were definitely incorporated into nanowires, and that F-TBP-Cys was successfully arranged via maleimide probes imbedded.

The biomineralization activity of minTBP-1, which specifically promotes nanoparticle formation of titania, silver and silica,[31] enables us to yield such inorganic nanowires with our functionalized fibrils via biomineralization of F-TBP-Cys. We let titania mineralize onto the nanowires by using a K_2TiF_6 solution at neutral pH for two hours. After incubation, our fibrils were successfully transformed to titania nanowires observed by transmission electron microscopy and identified by energy dispersive X-ray spectroscopy (Figure 5b,c). It is therefore demonstrated that the F-TBP-Cys conjugated onto the fibrils were actually able to carry out their function, and that our SCAP method has usefulness for creating inorganic nanowires as well.

3. Discussion

In this report we have developed an effective method for creating functionalized amyloid nanowires by control of self-assembly using our SCAPs. The unique self-assembling features of SCAP mixture observed both at a small oligomer and macroscopic fibril levels clearly show that mixing SCAPs can control whole self-assembly processes. The fact that mixing SCAPs also enhanced fibril formation in the presence of P-SCAP demonstrates the great advantage of our method for controlling self-assembly of probed nanowires. Our method to fabricate functional nanowires was successful in the modification of the probed nanowires with biomineralization peptide, followed by transformation into titania nanowires.

A very interesting finding in AFM and IMS-MS study is that those self-assembly conditions which lead reproducibly to the extremely long fibrils (1:1 mixtures of E_3 and K_3-based TTR peptides) also lead to the formation of a higher number of oligomers with remarkably extended peptide conformation. Hence, the enhanced fibril length would substantially result from self-assembling property of oligomers altered by mixing SCAPs.

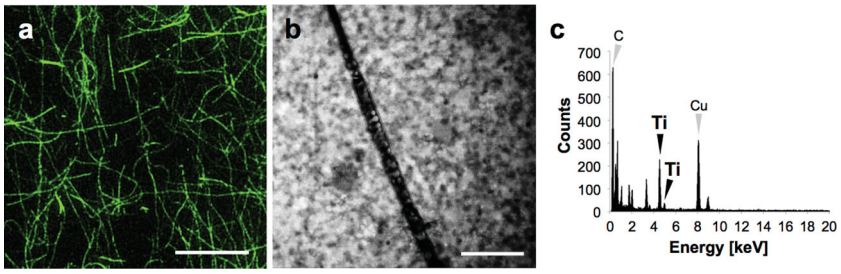

Figure 5. Functionalization of maleimide-containing nanowires. a) A fluorescence microscopy image of F-TBP-Cys peptides aligned onto K_3-TTR:Mal-E_3-TTR:E_3-TTR = 5:1:4 fibrils. b) A transmission electron micrograph of a titania nanowire formed after biomineralization with K_2TiF_6 and F-TBP-Cys-conjugated nanowires. c) Energy dispersive X-ray spectrum obtained from the titania nanowire shown in (b). Two peaks attributed to titanium were observed. The carbon and copper peaks are derived from the grid. The scale bars represent 5 μm in (a) and 200 nm in (b).

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Oligomers observed by IMS-MS under these conditions are dimers through pentamers and the data indicate a non-isotropic one-dimensional growth from monomer to dimer up to the pentamer. This suggests that a fibril-like arrangement of the peptide units is already present at the oligomer stage. This insight may find use in tailoring fibrillar assemblies. However, the relationship between the oligomeric and resulting fibrillar structures still needs to be further explored. Understanding the mechanism of self-assembly in these systems will allow to control and fine-tune the assembly process. The parallel fibrillar arrangement known to exist in the fibrils of TTR(105–115)[28] may be the structural element responsible for the extreme length these fibrils can assume. We believe that this structural element may also be present in the fibrils formed under our mixed-SCAP conditions including conditions involving P-SCAPs. Perhaps the interaction of the E_3 and K_3 units with each other provides an appropriate binding mode, which mediates formation of a crucial structural element that promotes fibril growth to great lengths. This same interaction is possibly responsible for the extended shape of small oligomers observed by IMS-MS. The significant findings in IMS-MS study also indicate the potential importance of introducing other SCAPs with different N-terminal units in this system, to provide novel binding modes. This could allow further control of fibril architecture, which is beneficial for developing functional nanomaterials.

The density of modification on fibril surface is of importance for the property of functional nanowires. Three groups have reported a similar approach to ours, where a fraction of modified peptides were co-assembled with unmodified peptide.[32–34] However, only a maximum of about 1% of modified peptides could be co-assembled with the amyloid-forming peptide. In contrast, our method allowed assembling probed nanowires with 10% P-SCAP, which should provide 1–2 nm distribution of probes on the surface based on the calculation according to the reported TTR(105–115) fibril structure in which four peptides exist in each 4.7 Å unit.[28,29] The contents of embedded probes can be further tuned, if required, just by adjusting the mixing ratios.

Limited types of functionalization to amyloid fibrils have been reported to date because most of those studies focused on the arrangements of metal nanoparticles bound to cysteine residues,[35–37] or of fluorophore-containing amyloid peptide.[38–41] Our method of incorporating a maleimide group attached to P-SCAP embedded in the fibril allows functionalization by any material containing thiol groups. Furthermore, our method is not limited to just the maleimide group as a probe. Various types of probed nanowires with lipoyl, biotin and azide groups were successfully formed (Supporting Information, Figure S6). These nanowires can be used to yield fibrils functionalized with gold-, avidin protein- and alkyne-containing materials, respectively. Therefore, our method has great versatility allowing to create various functionalized nanowires such as light-harvesting nanowires, conductive nanowires, and nanowires modified with proteins, catalytic compounds, or biomineralization peptides.

Many biomineralization sequences with specificities to various inorganic elements have recently been identified.[42–44] This fact makes our method more important because the use of such specific biomineralization peptides could enable fabrication of a variety of inorganic nanowires suitable for nano-electro devices. Previous studies have shown that it is feasible to grow fibrils composed of a certain amyloid peptide from a seed fibril prepared with a different kind of peptide.[45,46] Combining that technique with ours may potentially allow fabrication of patterned functional nanowires, which would have many applications in nanotechnology.

4. Conclusions

In conclusion, we report a novel straightforward method for the preparation of functionalized amyloid nanowires. We demonstrate that mixing multiple SCAPs strongly enhances fibrillation, resulting in very long fibrils. It appears β-sheet formation may occur early, at the dimer, suggesting that the mixed SCAPs controls the self-assembling property of the peptides over fibrillation processes. Furthermore, we successfully controlled the formation of probed nanowires by mixing P-SCAP with multiple SCAPs at an overall ratio of K_3:E_3 = 1:1. Finally, these probed nanowires can easily be functionalized with the attachment of probe-specific molecules, and subsequently transformed into inorganic nanowires through a biomineralization technique.

5. Experimental Section

Peptide Synthesis: All peptides (Supporting Information,Table S1) were synthesized by a standard Fmoc strategy as described previously.[22] Modifications with maleimide and fluorescein to the N-termini of peptides were carried out on the resins prior to deprotection. The peptides were obtained in ≥95% purity determined by reverse-phase HPLC.

Fibril Formation: All peptides were initially dissolved at a concentration of 8 mM into 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to make stock solutions. The stock solution of each peptide mixture was prepared by mixing the stock solutions of respective single peptides. To initiate fibril formation, each stock solution was diluted by a factor of 40 in aqueous solution at pH 2.0 (adjusted with a small amount of hydrochloric acid), and incubated in plastic tubes at 37 °C. To assess fibril formation, samples incubated for 1, 2, or 4 days were observed on a mica surface using AFM in air. Samples that showed no fibril formation after 4 days incubation were incubated further (up to 16 days).

Ion Mobility Spectrometry-Mass Spectrometry (IMS-MS): All IMS-MS experiments were carried out on a home-built instrument as described elsewhere.[23,47] Briefly, the samples were prepared by diluting the HFIP stock solution with acidic water (pH 2.0) to the appropriate concentration. The solution was then loaded into a gold-coated nano-ESI capillary and subsequently sprayed in the electrospray source of the IMS-MS instrument. Ions generated in the spray were focused and stored in an ion funnel and then pulsed into a drift tube filled with ≈3 Torr of helium gas for the IMS measurement. The ions passed through the drift tube with constant velocity under the influence of a weak electric field. The velocity (v_D) is determined by the balance between the force of the electric field (E) and the retarding force of friction, thus v_D is proportional to E with the proportionality constant (K), termed the ion mobility:

$$v_D = KE \quad (1)$$

After exiting the drift cell, ions of a particular oligomeric state are selected by mass in a quadrupole mass filer and detected as a function of their arrival time. From the position t_A of a peak in these arrival time

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distributions (ATDs) the respective ion mobility or ion-He collision cross section (σ) can be evaluated[48,49] using the relationship

$$\sigma = 1.3 \left(\frac{q^2 E^2 T}{\mu k_B p^2 N^2 l^2} \right)^{1/2} (t_A - t_o) \quad (2)$$

where q is the charge of the ion, T the temperature, μ the reduced mass of the ion-He collision, k_B the Boltzmann constant, p the He gas pressure, N the He number density at STP, l the drift cell length, and t_0 the time the ion spends outside of the drift cell. All of the quantities in Equation 2 are either known or measured for each experiment; therefore σ can be accurately determined for each species. An additional mass spectrometer, a Q-TOF instrument with higher mass resolution (Waters, UK) equipped with a nano-ESI source, was used to measure the ^{13}C isotope separation.[50] However, no IMS data were obtained on this instrument.

Arrangement of Functional Peptide onto the Maleimide-Containing Nanowires: 10 µL of diluted (1/20) suspension of K_3-TTR:Mal-E_3-TTR:E_3-TTR = 5:1:4 fibrils deposited onto a cover glass (Micro Cover Glass 22 mm × 32 mm, Matsunami Glass, Osaka, Japan), followed by washed, to adsorb the fibrils. 10 µL of peptide solution (10 µM F-TBP-Cys, 10 mM phosphate, pH 7.4) was subsequently incubated on the surface for 10 min. After incubation, the surface was washed three times with 1 mL of ultra-pure water and dried in air.

Biomineralization on Fibrils: For titania biomineralization, a polymer-coated Cu grid (high resolution carbon substrate, carbon thickness <15 nm, STEM 100-Cu grids, Okenshoji, Tokyo, Japan), on which F-TBP-Cys-conjugated fibrils had been deposited, was immersed in a solution of 20 mM K_2TiF_6 containing 10 mM sodium phosphate (pH 7.4) for 2 h. After mineralization, the grid was washed by immersing in ultra-pure water for 30 s.

Microscopy: AFM measurements were performed as described previously.[22] Fluorescence microscopy was performed using a BIOREVO BZ-9000 confocal microscope (Keyence, Osaka, Japan) with a GFP-BP filter. Shutter speed was set at 3.5 s. Transmission electron microscopy and energy dispersive X-ray spectroscopy were conducted using an HD-2000 ultra-thin film evaluation system (Hitachi, Tokyo, Japan).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was supported in part by "Molecular and System Life Science" Promotion of Novel Interdisciplinary Fields Based on Nanotechnology and Materials from MEXT (to K. U. and K. S.), Grant-in-Aid for Scientific Research (B) (No. 24310152) from JSPS (to K.S.), Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists from JSPS (No. 23–7100) and the Global COE Program (No. B01) from MEXT, Japan (to H. S.). The electron microscopy and energy dispersive X-ray spectroscopy were carried out at the OPEN FACILITY, Hokkaido University Sousei Hall. We thank Mathieu Lussier-Price for assistance with preparing the manuscript. The work at UCSB was done with the support of US National Science Foundation Grant CHE-0909743 (MTB). MTB also acknowledges the Waters Corporation for the generous donation of the Synapt prototype.

Received: February 14, 2013
Revised: March 14, 2013
Published online: April 23, 2013

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