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High-Temperature Self-Assembly of Peptides into Vertically Well-Aligned Nanowires by Aniline Vapor**

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The self-assembly of peptide-based building blocks into nanostructures is an attractive route for fabricating novel bio-inspired materials because of their capacity for molecular recognition and functional flexibility as well as the mild conditions required in the fabrication process.[1–4] Among various peptide-based building blocks forming nanostructures, the simplest building blocks are aromatic dipeptides like diphenylalanine, which can readily self-assemble into nanotubes in aqueous solutions at ambient conditions.[2,5–7] According to literature, the peptide nanotubes could be used in versatile applications for casting conducting metal nanowires,[8] enhancing the sensitivity of electrochemical detection of biomolecules,[9] and fabricating nano-fluidic channels[10] or peptide liquid crystals.[11]

Although the self-assembly of peptides into nanostructured materials had been extensively studied, little progress had been made in the alignment and positioning of peptide nanostructures on a solid surface. Major obstacles include the complexity of current 'solution-based' approaches to peptide nanofabrication, causing dispersion and agglomeration problems,[6] which also require the chemical modification of surface and peptide nanostructures.[12] In the present study, we report a novel solid-phase growth of crystalline peptide nanowires at high temperatures driven by aniline vapor under anhydrous conditions. The formation of vertically well-aligned peptide nanowires on a solid surface were investigated through multiples tools, such as X-ray diffraction (XRD), scanning electron microscopy (SEM), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, and thermal analytical tools like the differential scanning calorimeter (DSC) and thermogravimetric analysis (TGA).

We prepared an amorphous peptide thin film by drying a drop of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) solution containing diphenylalanine on a Si substrate. We conducted the experiment under strictly anhydrous conditions in a vacuum desiccator because water vapor could affect the final surface structure of the peptide thin film.[13] We could control the thickness of the film from a few μm down to $\sim$50 nm precisely by simply changing the diphenylalanine concentration in HFIP solution. According to our SEM and XRD analysis, the thin peptide film exhibited no surface features (Fig. 1) and no characteristic diffraction peaks (Fig. 2), which indicate the 'amorphous' nature of the film. From the amorphous peptide film as a starting point, we were able to successfully grow vertically well-aligned peptide nanowires by aging the film at temperatures above 100 °C with aniline vapor. Figure 1 shows the electron micrographs of vertically well-aligned, rigid peptide nanowires. Peptide nanowires were uniformly formed over the entire region of the whole surface (10 mm × 10 mm). The average diameter of the nanowires measured by SEM was found to be about 150 nm, but if we consider the effect of conductive coating for SEM, the actual diameter of these nanowires should be less than 150 nm. Note that the peptide nanowires have a very high aspect ratio of at least 100 and a long persistence length over 10 μm. The rigid and long shape suggests that our peptide nanowires may have high mechanical strength, but further experimentation is needed to verify this. The formation of peptide nanowires occurred in a significantly different way from the report by Reches and Gazit[13a] who formed peptide nano-forests by simply drying a diphenylalanine solution in the air.

According to the XRD analysis of the film (Fig. 2), the intensities of diffraction peaks increased with aging time. The intensity changes were most prominent at 2θ of 6.8° and 20.5°. This result demonstrates that the crystallinity of the peptide film increased with time through the aniline vapor aging. The time evolution of SEM images (Fig. 2) also shows the gradual growth of peptide nanowires from the film with time. In order to compare the XRD pattern of our peptide nanowires with that of peptide nanotubes formed in aqueous solution,[2] we collected a XRD-pattern of the peptide nanowires after removing the substrate. The diffraction pattern was significantly different from that of peptide nanotubes (see Fig. S1 in the Supporting Information). Based on the results from the XRD and SEM analysis, we propose a surface-initiated nucleation in the initial stage of high-temperature aniline vapor aging, which should guide the growth of vertically well-aligned peptide nanowires. Our hypothesis is further supported by a recent paper[14] that reported a two-step process for the fabrication of polysiloxane nanofibers through surface initiated, vapor-phase polymerization of organotrichlorosilanes.

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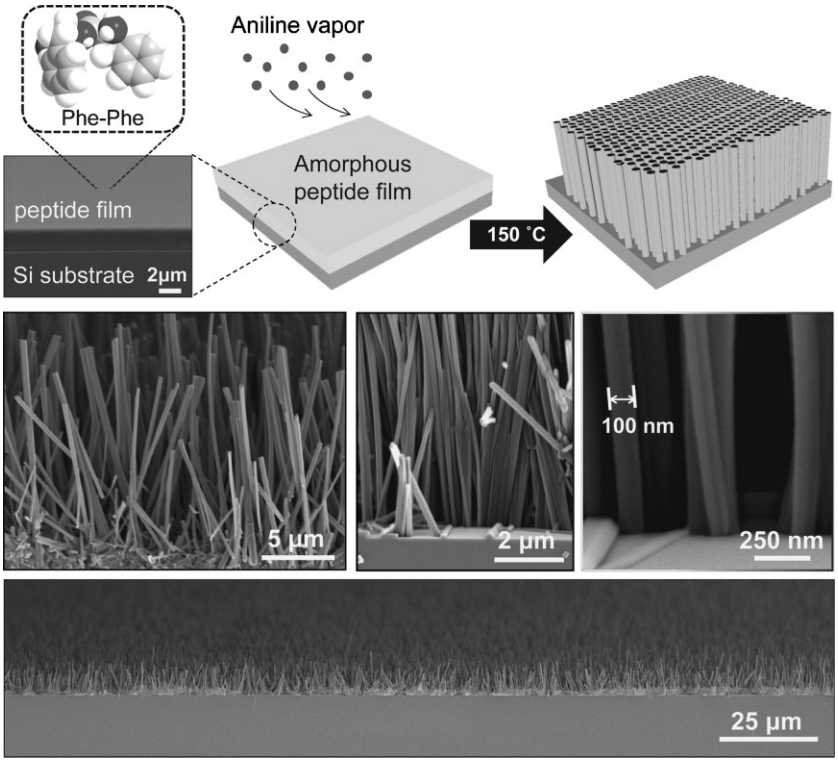

Figure 1. Experimental scheme and cross-sectional electron micrographs showing the growth of vertically well-aligned nanowires from amorphous peptide thin film by high-temperature aniline vapor aging.

Further, we investigated whether aniline was contained in the resultant nanowires by measuring the UV/visible absorbance and the MALDI-TOF mass spectrum of the peptide film before and after the high-temperature aging with or without aniline vapor. To avoid any saturation of absorbance in the UV region by aromatic rings, we made the peptide thin film as thin as possible (~50 nm). The UV/visible absorbance spectrum of the film without high-temperature aniline vapor treatment was significantly different from that with the treatment (Fig. S2). The film treated with aniline vapor at 150 °C presented a new absorption peak at around 280 nm, which was likely caused by the presence of aniline, as indicated by Pavia et al., 2001.[15]

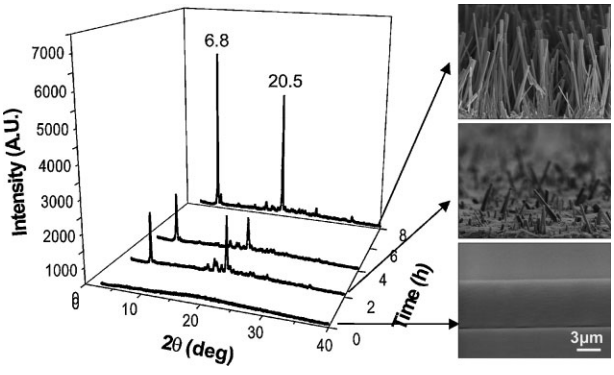

Figure 2. The evolution of powder XRD patterns and cross-sectional electron micrographs with aging time in the presence of aniline vapor at 150 °C.

Further analysis of the peptide nanowires with MALDI- TOF mass spectrometry also confirms the presence of aniline in the peptide nanowires grown under high-temperature aniline-vapor conditions (Fig. 3). We were able to obtain a simple mass spectrum from diphenylalanine not treated with aniline vapor, which had a strong, protonated molecular ion peak at m/z of 313.27 (Fig. 3B). On the contrary, diphenylalanine exposed to aniline vapor at 150 °C for 12 h exhibited a complex mass spectrum (Fig. 3C). We observed peaks corresponding to not only fragmented diphenylalanine ($[M–OH]^+$, 295.20) but also protonated aniline ($[ANI + H]^+$, 94.12) and matrix molecules ($[DHB–OH]^+$, 137.08; $[DHB + H]^+$, 155.09). However, we were not able to identify any peak for higher molecular weight species that could be formed through the chemical reaction of diphenylalanine and aniline vapor. We speculate that the fragmentation of diphenylalanine and the appearance of matrix molecule in the mass spectrum originate from residual aniline contained in the nanowires.

In order to observe the effect of aging temperature on peptide nanowire growth, we incubated the amorphous diphenylalanine film with or without aniline vapor at different temperature (50, 100, and 150 °C), respectively. As shown in Figure S3 (Supporting Information), the presence of aniline vapor had a significant effect on the shape of resultant nanostructures for the temperatures we tested. At 50 °C, no structural change was observed in the absence of aniline vapor (i.e., only dry air), whereas vertically-aligned, thick 'nanorods' rather than 'nanowires' were formed under aniline vapor at that temperature. The average diameter of nanorods measured by SEM was found to be about ~200 nm and their length reached over ~3 μm. At elevated temperatures of 100 and 150 °C, one-dimensional nanostructures were formed regardless of the presence of aniline vapor, but with different shapes. While the high-temperature aniline vapor aging resulted in the formation of uniform and well-aligned peptide 'nanowires,' dry air aging without aniline at the high temperatures induced the growth of highly flexible 'nanofibrils' with an irregular shape.

In the following experiments, we investigated whether structural analogs of aniline such as benzene and toluene could also induce the self-assembly of dipeptide into nanowires as aniline had done. These solvents were chosen based on their vapor pressure and structural similarity to aniline. According to our results, the amorphous diphenylalanine films aged under benzene or toluene vapor remained unchanged even after incubation for 20 h (Fig. S4A). XRD analysis of the resultant film also supports the SEM observation (Fig. S4B). While the amorphous film aged under either benzene or toluene vapor presented no diffraction peaks, aniline vapor-treated film

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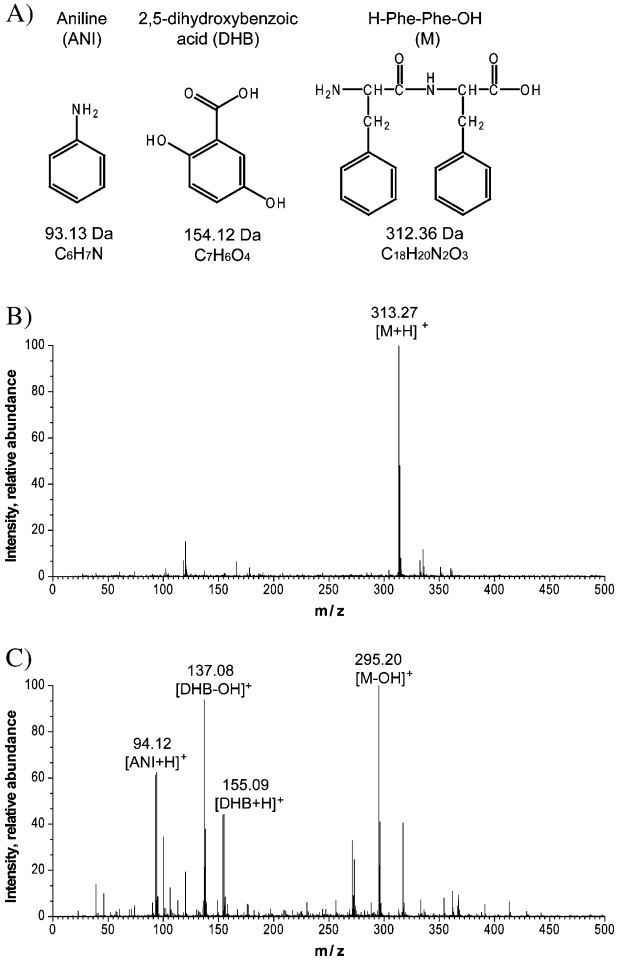

Figure 3. Chemical structures (A) of aniline (ANI), 2,5-dihydroxybenzoic acid (DHB), and diphenylalanine (M) and their molecular weight. Mass spectra of diphenylalanine before (B) and after (C) high-temperature aging with aniline vapor at 150 °C for 12 h were obtained by a MALDI-TOF mass spectrometer.

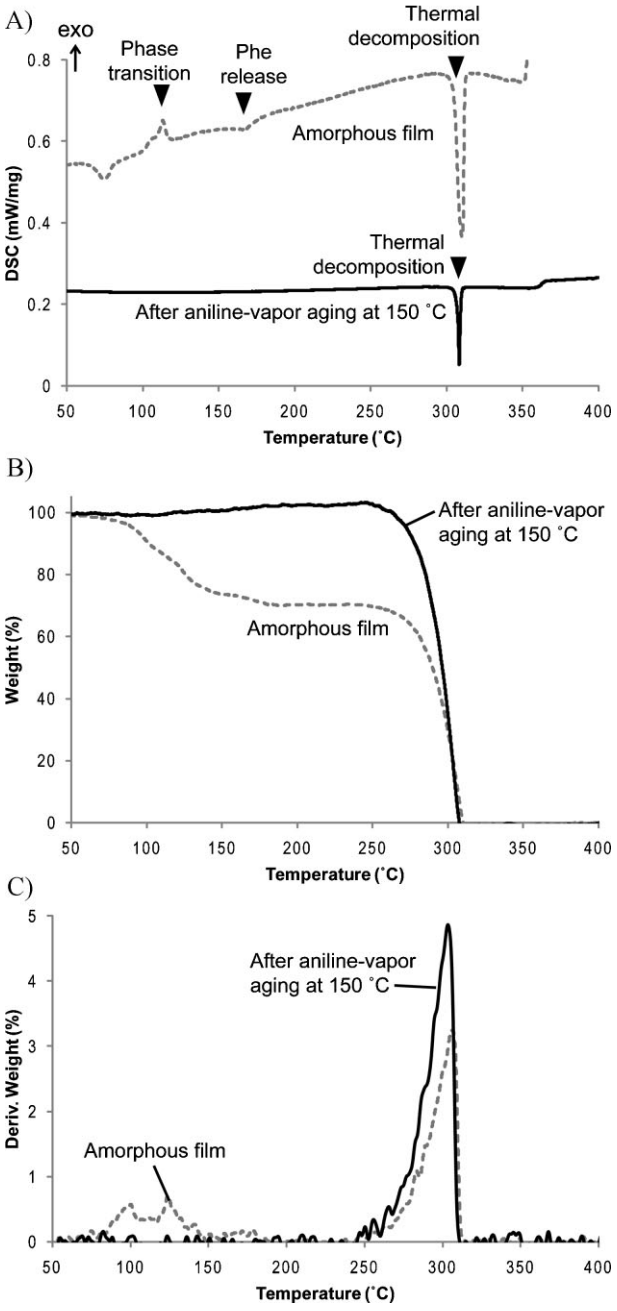

Figure 4. Thermal analysis of amorphous diphenylalanine films before and after high-temperature aniline vapor-aging at 150 °C. Amorphous and nanowire films were characterized by DSC (A) and TGA (B). The derivative TGA curves (C) for both films are plotted against temperature.

exhibited several characteristic diffraction peaks, most prominently at 2θ of 6.7 ° and 20.4 °. We speculate that the differential effect of aromatic solvents on the self-assembly of diphenylalanines results from the presence of the amine group in aniline, which can act as a hydrogen-bond donor. According to recent papers,[13b,16,17] the formation of hydrogen bonds between solvent (e.g., water) and peptide molecules is the driving force for the self-assembly of diphenylalanines. Nevertheless, the underlying mechanism for the exact role of aniline molecules in the growth of vertically well-aligned peptide nanowires needs to be verified further.

In the following experiments, we investigated the thermal properties of the amorphous peptide film and the peptide nanowires grown through high-temperature aniline vapor aging by using DSC and TGA. Both the DSC (Fig. 4A) and TGA (Fig. 4B) thermograms show that a thermal decomposition of diphenylalanine completed at 308 °C. Amorphous diphenylalanine film presented an endothermic DSC peak at 175 °C and an exothermic DSC peak at 115 °C, which should correspond to the release of phenylalanine from diphenylalanine[18] and phase transition, respectively. Note that an endothermic peak centered at 73.5 °C can be attributed to the evaporation of residual HFIP from the amorphous

peptide film. On the contrary, the peptide nanowires formed through high-temperature aniline aging presented no phase-transitional behavior, demonstrating only a negligible weight change upon heating to 200 °C (Fig. 4C). In our SEM analysis (data not shown), we did not observe any structural change in the nanowires upon heating to 200 °C. Our peptide nanowires self-assembled under aniline vapor had much higher thermal stability than peptide nanotubes formed in aqueous solutions, which start to lose their structural integrity at 100 °C.[18]

Placing nanostructured materials onto a pre-determined surface with the alignment in the desired orientation for their applications is crucial.[6,19–21] In the case of nanostructures formed from peptides, however, little research has been conducted because of the complexity of the current solution-based method, which causes many problems for fabrication.[6,12] In this work, we combined the high-temperature aniline vapor aging method with a simple soft-lithographic technique[22] to fabricate a micro-pattern of the vertically grown peptide nanowires (Fig. 5A). We used a microchanneled polydimethylsiloxane (PDMS) mold (channel spacing: 50 μm) to make a micropattern of amorphous peptide film on a substrate. Briefly, after the spontaneous formation of conformal contact between the PDMS mold and the substrate, a drop of diphenylalanine solution in HFIP was placed at the entrance of the microchannels, which were readily filled with the diphenylalanine solution in a few second by capillary force. Then, the microchannels were allowed dry in a vacuum desiccator for 30 min. After the complete evaporation of volatile HFIP, the PDMS mold was removed from the substrate, and we obtained a micro-patterned amorphous peptide film. By incubating the substrate further under aniline vapor for 12 h at 150 °C, we were able to grow vertically-aligned peptide nanowires only in the areas defined by the PDMS microchannels (Fig. 5B). Unlike peptide nanowires grown on the bulk substrate, those from the patterned substrate showed a position-dependent ordering or alignment behavior. While the nanowires located in the middle of microchannels were vertically well aligned, the nanowires on the edge were less ordered. The fabrication of the micro-pattern of peptide nanowires demonstrates that our high-temperature aniline vapor aging method can simplify the peptide-based nanofabrication process.

We report herein a solid-phase growth of vertically well-aligned peptide nanowires having a high aspect ratio (at least 100) and a long persistence length (more than 10 μm) through the high temperature aniline vapor aging process, starting from an amorphous peptide thin film. The self-assembly of peptide nanowires from the film depended on both the aging temperature and the nature of the solvent vapor used. The peptide nanowires were thermally stable up to 200 °C without any loss in weight or structural integrity. Furthermore, we were able to simultaneously fabricate a micro-pattern of peptide nanowires by combining a simple soft-lithographic technique and the high-temperature aniline vapor-aging process. We believe that the dry process will provide a new horizon for peptide-based nanofabrication because it can minimize problems occurring in conventional solution-phase methods.

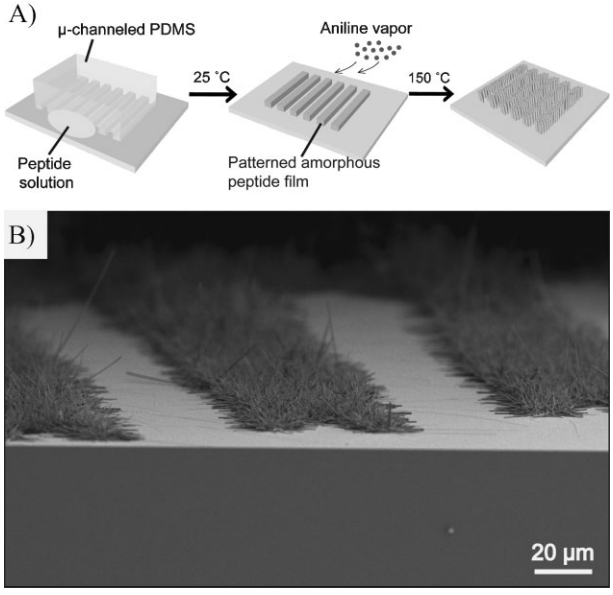

Figure 5. A) A schematic illustration for the micro-patterned growth of vertically well-aligned peptide nanowires. Microchanneled PDMS mold was used to prepare a micro-pattern of amorphous peptide thin film. The film was then aged at 150 °C under aniline vapor. B) Electron micrograph of the resultant micro-pattern of peptide nanowires on a silicon substrate.

Experimental

Materials: Diphenylalanine (FF) peptide in a lyophilized form was obtained from Bachem AG (Bubendorf, Switzerland). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), aniline, benzene, toluene, 2,5-dihydroxybenzoic acid (DHB), and acetonitrile were obtained from Sigma–Aldrich (St. Louis, MO). Polydimethylsiloxane (PDMS) (Sylgard 184 silicone elastomer kit) was purchased from Dow Cowning Korea (Seoul, Korea).

Solvent Vapor-Aging of Amorphous Film of Diphenylalanine: Fresh diphenylalanine solution was prepared by dissolving as-received diphenylalanine in HFIP at a concentration ranging from 10 to 100 mg ml^{-1}. To avoid the formation of any aggregates during storage, diphenylalanine solution was always freshly prepared just before use. A transparent and amorphous diphenylalanine film was obtained by placing a drop of diphenylalanine solution at a desired concentration onto a substrate such as silicon wafer, slide glass, or quartz plate, and drying the film in a vacuum desiccator in the absence of water vapor at room temperature. The amorphous diphenylalanine film was then aged in a glass Petri-dish having two separated compartments. The amorphous diphenylalanine film and a fixed volume of solvent were loaded into each compartment, respectively, allowing only solvent vapor, not liquid, to reach the film through the headspace in the petri-dish. Then, the Petri-dish was sealed with Teflon tape and aluminum sealing film. Samples were then aged at a constant temperature of 25, 50, 100, or 150 °C.

Patterned Vertical Growth of Peptide Nanowires: Microchanneled PDMS mold (channel width, 50 μm; channel height, 10 μm) was

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prepared by mixing the PDMS precursor and the curing agent with a ratio of 10:1 on a Si master. The silicon master was kindly provided by Dr. Min-Gon Kim at Korea Research Institute of Bioscience and Biotechnology (KRIBB). A detailed description about the casting of PDMS mold is available elsewhere [22]. After filling the microchannels enclosed by the PDMS mold and a substrate with the diphenylalanine solution by capillary force, the peptide solution was allowed to dry in a vacuum dessicator to form a micro-pattern of amorphous diphenylalanine film. The patterned diphenylalanine film was then aged under aniline vapor condition at 150 °C for 12 h.

Electron Microscopy: For electron microscopy, samples were prepared on Si wafer (10 mm × 10 mm). After coating the samples with platinum thin film using SCD005 Pt-coater (Bal-Tec AG, Liechtenstein), they were imaged by an S-4800 field emission scanning electron microscope (Hitachi High-technologies CO., Japan) at an acceleration voltage ranging from 1 to 3 kV.

X-ray Diffraction (XRD): For powder diffraction analysis, amorphous diphenylalanine film was prepared with a 50 μl drop of diphenylalanine solution (50 mg ml^{-1}) on a slide glass (15 mm × 18 mm). Structures of amorphous diphenylalanine thin film before and after solvent vapor-aging were analyzed with a Rigaku D/MAX-IIIC powder X-ray diffractometer (Rigaku, Co, Japan) equipped with a Ni filter under the following conditions: scan speed, 3 ° min^{-1}; Cu Kα radiation, $\lambda = 1.5418$ Å; scan range, 3–50 °.

UV/Visible Absorption Spectroscopy: Amorphous diphenylalanine film was prepared by drying a 10 μl drop of diphenylalanine solution (2 mg ml^{-1}) in HFIP on quartz plate (20 mm × 20 mm). Then, the high-temperature aging of the diphenylalanine film was carried out at 150 °C for 12 h with or without aniline vapor. UV/Visible absorption spectra of the film before and after the aging were obtained using a Biospec Mini spectrophotometer (Shimadzu Co., Japan).

Mass Spectrometry: The diphenylalanine peptides treated with or without high-temperature aniline vapor were dissolved (10 mg ml^{-1}) in pure HFIP, respectively. DHB matrix solution was prepared by dissolving DHB (10 mg ml^{-1}) in a 50% aqueous solution of acetonitrile. After mixing the DHB matrix solution and the diphenylalanine solution in a 1:1 ratio, a drop of the resultant solution was allowed to dry onto a steel target to obtain a thin film mixture of the peptides and the matrix by evaporation. Then, their mass spectra were measured by a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA). MALDI-TOF mass spectrometer was operated under the following conditions: a pulsed nitrogen laser with a wavelength of 337 nm; pulse length, 3 ns; accelerating voltage, 20 kV; reflection-mode, positive-ion reflection.

Thermal Analysis: Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) of amorphous diphenylalanine film before and after aniline-vapor aging were carried out using DSC 204 F1 (NETZSCH) and thermogravimetric analyzer Q50 (TA Instrument, DE). After equilibration at 40 °C for 30 min, samples were heated up to 400 °C at a constant rate of 10 K min^{-1} under nitrogen environment.

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