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# Pro-apoptotic Peptide Amphiphile Self-assembled with the Assistance of Polycations

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Herein, we describe how a peptide amphiphile, consisting of a pro-apoptotic peptide and a hydrophobic tail connected by a reduction-responsive cleavable spacer, forms self-assembled nanostructures. In the presence of polycations, this amphiphile exhibits loosely entangled nanofiber morphology with a length of several hundred nanometers.

Supramolecular nanostructures constructed through the self-assembly of peptide amphiphiles have been investigated for various applications in biomedicine, including drug release, tissue engineering, and regenerative medicine.[1] Nevertheless, the morphologies of these nanostructures were still limited to simple spherical, fibrous, or tubular structures. Thus, there is urgent demand for developing a control strategy to construct nanostructures with preferred morphologies.[2]

Recently, the Smac (second mitochondria-derived activator of caspase) protein[3] and an AVPI tetrapeptide,[4] located at the N-terminal of the Smac protein, have been shown to enhance the intended activity of anticancer drugs through the inhibition of IAPs (inhibitor of apoptosis proteins).[5] The IAPs, which are over-expressed in several cancer cells, suppress apoptosis at least in part by inhibition of caspases that are key players in apoptosis.[6] To address the limitation that the pro-apoptotic AVPI tetrapeptide is not cell permeable, the AVPI peptide was conjugated to a carrier peptide[4] or, alternatively, AVPI mimetic molecules[7] were developed.

With these in mind, we set out to develop a peptide amphiphile termed **AVPI-NP-C12**, which would consist of the AVPI tetrapeptide linked with a hydrophobic dodecyl (C12) chain through a nitrophenyl (NP)-containing spacer moiety, as shown Figure 1A. Herein, we describe how, in the presence of polycations, the peptide amphiphile forms self-assembled nanostructures with loosely entangled nanofiber morphology with a length of several hundred nanometers. Moreover, we expected that the AVPI tetrapeptide could be released from a self-assembled nanostructure consisting of **AVPI-NP-C12** upon reduction of the nitro group of the spacer based on the mechanism shown in Figure 1B. Recently, it has been revealed that the microenvironments around cancers, i.e. the so-called hypoxic environments, are reductive because of low oxygen supply and trigger reduction of nitro compounds.[8]

The synthesis of **AVPI-NP-C12** was carried out based upon a standard solid-phase peptide synthesis of the AVPI tetrapeptide, and subsequent coupling with NHS-ester of a NP-C12 derivative (diastereomeric mixture) at the N-terminal amino group of the peptide (see Supporting Information for details). The ability of **AVPI-NP-C12** to form nanostructures in aqueous media (50 mM HEPES-KOH at pH 7.6) was evaluated by measurement of optical transmittance. As shown in Figures 2A and S6, the optical transmittance (%T) decreased with an increase in **AVPI-NP-C12** concentration in the presence of cations such as $Ca^{2+}$ (1 mM) and poly-L-lysine (PLL) (5 mM monomer unit, $PLL_{4000}$: $M_w = 4000–15000$, $PLL_{70000}$: $M_w =$

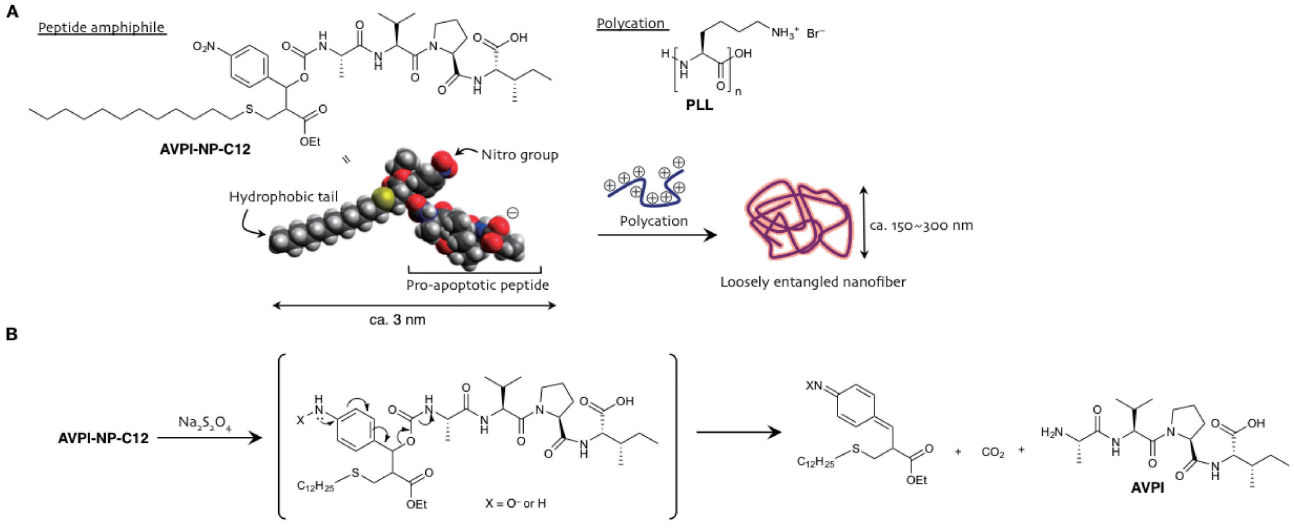


**Figure 1.** (A) Schematic representation showing self-assembly of a pro-apoptotic peptide amphiphile (**AVPI-NP-C12**) assisted by a polycation (not to scale) and their chemical structures and (B) plausible mechanism showing the reduction-responsive degradation of **AVPI-NP-C12** to release a pro-apoptotic AVPI peptide.

  

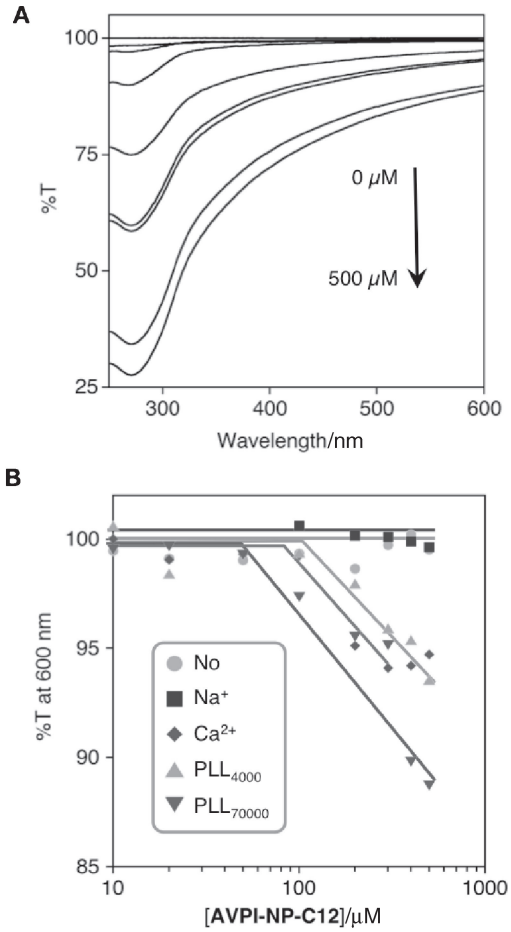


**Figure 2.** (A) Change in optical transmittance (%T) by increasing the concentration of **AVPI-NP-C12** in the presence of $PLL_{70000}$ (5 mM monomer unit) and (B) Plots of %T (600 nm) against concentration of **AVPI-NP-C12** to evaluate CAC. *Conditions*: [**AVPI-NP-C12**] = 0, 10, 20, 50, 100, 200, 300, 400, and 500 μM, $[Ca^{2+}] = 1$ mM or [PLL (monomer unit)] = 5 mM or $[Na^{+}] =$ 100 mM, 50 mM HEPES-KOH (pH 7.6), 25 °C.

70000–150000). To prevent overgrowth of the assembled structures to micrometer scale, the concentration of cationic species was set to be higher than that of **AVPI-NP-C12**. In contrast, in the absence of polycations or the presence of the monovalent cation $Na^{+}$ (100 mM), almost no decrease in %T except for that around 270 nm (assignable to an absorption band of NP group) was observed up to 500 μM of **AVPI-NP-C12** (Figure S6). From the plot of %T at 600 nm against the concentration (Figure 2B), apparent critical association concentrations (CACs) of **AVPI-NP-C12** in the presence of $Ca^{2+}$, $PLL_{4000}$, and $PLL_{70000}$ were evaluated to be ca. 100, 100, and 50 μM, respectively. We presume that one proline moiety in the middle of the peptide sequence, which could destabilize intermolecular hydrogen-bonding interactions to induce self-assembly, could prevent **AVPI-NP-C12** from forming assembled nanostructures without the polycations, at least below the concentration of 500 μM. We also observed no decrease in %T for the AVPI tetrapeptide without the hydrophobic C12 chain upon the addition of cations. To assess the size of the nanostructures above the CACs, dynamic light scattering

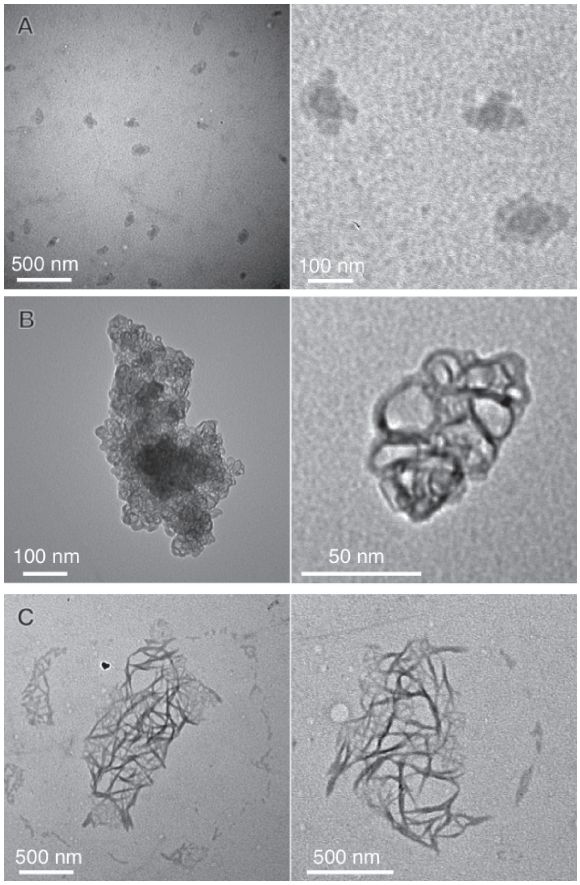


**Figure 3.** Unstained TEM images of **AVPI-NP-C12**·cation nanostructures (cation for (A) $Ca^{2+}$, (B) $PLL_{4000}$, and (C) $PLL_{70000}$).

measurement was carried out. The size of the nanostructures of **AVPI-NP-C12** complexed with $Ca^{2+}$, $PLL_{4000}$, and $PLL_{70000}$ were evaluated to be $149 \pm 93$, $159 \pm 58$, and $282 \pm 144$ nm, respectively (Figure S7). These results indicate that the polycations and divalent cation such as $Ca^{2+}$ can induce the assembly of **AVPI-NP-C12** to form nanostructures, most probably owing to electrostatic interactions between a cation and C-terminal carboxylate anion of **AVPI-NP-C12**.

To obtain insight into the morphology of the nanostructures, transmission electron microscopy (TEM) experiments were carried out. As shown in Figure 3A, ill-defined nanostructures were observed in the presence of $Ca^{2+}$. In sharp contrast, interestingly, dramatically different morphologies were observed in the presence of PLL. Nanostructures consisting of loosely entangled nanofibers with a fiber diameter of several tens of nanometers were observed in the presence of both $PLL_{4000}$ (Figure 3B) and $PLL_{70000}$ (Figure 3C). In the case of shorter $PLL_{4000}$, fibrous structures wound and packed more closely compared with $PLL_{70000}$. Although additional, more detailed studies are required to elucidate the self-assembled mode of **AVPI-NP-C12** with PLL, we speculate that PLL would define the growth of the self-assembled structures of **AVPI-NP-C12** through electrostatic interactions along the main chain of PLL and hydrophobic interactions, mainly among dodecyl chains of **AVPI-NP-C12**.[9]

To investigate their reduction-responsive property, **AVPI-NP-C12**·$Ca^{2+}$ nanostructures were incubated with sodium dithionite (100 μM) in HEPES buffer (pH 7.6) at 37 °C. Following the process with reverse-phase HPLC (RP-HPLC), it was revealed that **AVPI-NP-C12** decomposed almost completely within 2 h. Then, to detect the peptide product (AVPI-tetrapeptide) released from the nanostructures by RP-HPLC, the reaction mixture was centrifuged and the resultant supernatant was treated with 2,4,6-trinitrobenzenesulfonic acid[10] (TNBS), which labeled the N-terminal amino group of the peptide to give TNB-peptide (Figure S8). The presence of released AVPI-tetrapeptide was confirmed by comparing a HPLC chart of a control sample prepared from AVPI-tetrapeptide and TNBS as well as MS spectrum.[11] These results support our view that the reduction of the nitro group of **AVPI-NP-C12** can trigger the cleavage of the linkage between the AVPI-tetrapeptide and the hydrophobic tail, and eventually release the peptide from the nanostructures (Figure 1B).

In summary, we demonstrated that a pro-apoptotic peptide amphiphile bearing a reduction-responsive cleavage site was capable of forming self-assembled nanostructures, with the assistance of polycations. Interestingly, nanostructures comprising entanglements of nanofibers were obtained in the presence of poly-L-lysine. Preliminary experiments also revealed the reduction stimulus-responsive degradation of the peptide amphiphile to release a pro-apoptotic peptide. We believe that the nanostructures created in the present study could find future potential applications as nanomedicines for encapsulating and delivering drugs, as well as for sensing reductive environments such as caused by hypoxia. Exploration of such functions and modifications of component molecules to improve or modulate stimuli response is currently underway in our laboratory.

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Supporting Information is available electronically on J-STAGE.

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10 R. B. Freedman, G. K. Radda, *Biochem. J.* **1968**, *108*, 383. TNBS can label amino groups (side chain and N-terminal) of PLL in addition to AVPI-tetrapeptide, which would hamper the HPLC analysis of AVPI release. Reduction responsive property was thus investigated for **AVPI-NP-C12**·$Ca^{2+}$ nanostructures in the present study.

11 MALDI-TOF MS spectrum (negative); Calcd. for [TNB-AVPI ($C_{25}H_{35}N_7O_{11}$) – H]$^-$: $m/z = 608.2$; Found 608.7 (Figure S8). The overall yield was evaluated to be ca. 5% from the peak area of HPLC.


 