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Communication

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Supramolecular Detoxification of Neurotoxic Nanofibrils of Small Molecules via Morphological Switch

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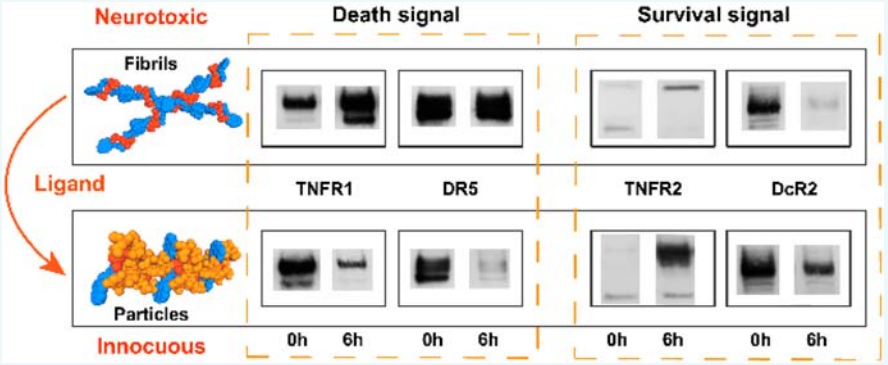

ABSTRACT: Insoluble amyloid plagues are likely cytoprotective, but the cellular mechanism remains less known. To model β-amyloid we use a small peptide derivative to generate cytotoxic nanofibrils that cause the death of model neuron cells (i.e., PC12). The use of supramolecular interaction effectively converts the nanofibrils to nanoparticles that are innocuous to cells. This approach also removes the cytotoxicity of the fibrils to other mammalian cells (e.g., HeLa). Preliminary mechanistic study reveals that, in contrast to the fibrils, the particles promote the expression of TNFR2, a cell survival signal, and decrease the expression of TNFR1 and DR5, two extrinsic cell death receptors. As the first use of ligand−receptor interaction to abrogate the cytotoxicity of nanoscale assemblies of small molecules, this work illustrates an effective way to use supramolecular interaction to control the morphology of supramolecular assemblies for modulating their biological activity.

This communication describes the use of ligand−receptor interaction to convert cytotoxic molecular nanofibrils of small molecules to innocuous particles, illustrating that control over the morphology of aggregates is an effective approach to reduce the toxicity of molecular nanofibrils. Recent advances suggest that soluble β-amyloid (Aβ) oligomers are the most neurotoxic species.[1] Emerging studies also suggest that the early assemblies of misfolded non-disease-associated proteins[2,3] and oligomers of disease-associated proteins (e.g., Aβs)[4] exhibit similar inherent cytotoxicity, suggesting a common mechanism of the cytotoxicity of the molecular aggregates.[5] These new insights imply that the insoluble plaques of Aβ in Alzheimer's disease are neuroprotective[6] and have led to efforts to control the morphology of the molecular aggregates for reducing the cytotoxicity of amyloids.[7−9] For example, Shoichet et al. have demonstrated that colloidal aggregates of small molecules physically sequester proteins or early assemblies of the proteins to nonspecifically inhibit amyloid formation, implying that morphological control of colloids formed by molecular self-assembly may be relevant in more biological milieus.[10,11]

Encouraged by this development, we designed a small molecule **1** containing D-Ala-D-Ala that self-assembles[12,13] in water to result in molecular nanofibrils and to form hydrogels. Below its minimal gelation concentration (mgc), **1** forms nanofibrils that inhibit the proliferation of PC12 cells, a model of neuron cells.[14] Upon the addition of vancomycin (Van)—a well-established ligand of D-Ala-D-Ala,[13,15,16] ligand−receptor interaction effectively converts the fibrils of **1** into particles (Figure 1). The resulting particles are innocuous to PC12 cells and other mammalian cells. Preliminary mechanistic examina-

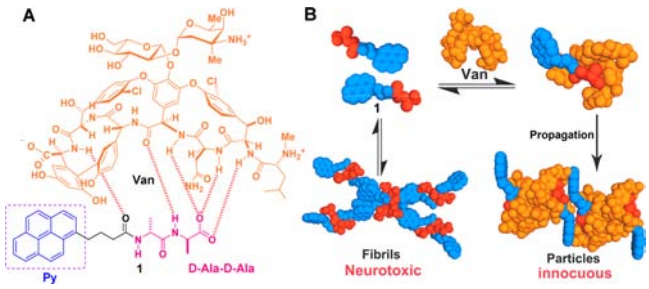

Figure 1. (A) Molecular structures of the ligand (Van), the receptor (a derivative of D-Ala-D-Ala (**1**)). (B) Illustration of the ligand−receptor interaction that transforms the cytotoxic fibrils, formed by self-assembly (SA) of **1**, to innocuous particles made of Van and **1**.

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tion indicates that, while fibrils of **1** suppress the expression of decoy cell death receptors (DcR2[17] and DcR3[18]) and increase the expression of cell death receptors (TNFR1,[19] DR5[20]), particles made of **1** and Van promote the expression of a cell survival signal (TNFR2[21]) and decrease the expression of cell death receptors (TNFR1, DR5). As the first use of the ligand−receptor interaction to abrogate the cytotoxicity of molecular nanofibrils of small molecules via morphological modulation, this work illustrates a new approach to tune the morphology of supramolecular assemblies for modulating their biological activities, and contributes useful insights for the exploration of biofunctional assemblies of small molecules,[10,22−24] an underexplored subject that is increasingly significant in biology and medicine.[25,26]

Our analysis indicates that the volume of aggregates rather than the numbers of individual molecules dictates the cytotoxicity of molecular aggregates, implying that these aggregates cause cell death with rather promiscuous interactions.[5,27] Particularly, our recent work shows that ligand−receptor interaction catalyzes the formation of aggregates of Fmoc-Lys-Gly-Gly-D-Ala-D-Ala (FmocKGGaa) to inhibit the proliferation of cells at 24 h.[28] Considering that **1**, like FmocKGGaa, contains the D-Ala-D-Ala segment, we expect that Van would catalyze the aggregation of **1** to inhibit cell proliferation. According to this rationale, we synthesize **1** and test its cytotoxicity without and with the addition of Van. As shown in Figure 2, **1** exhibits steep cytotoxicity at

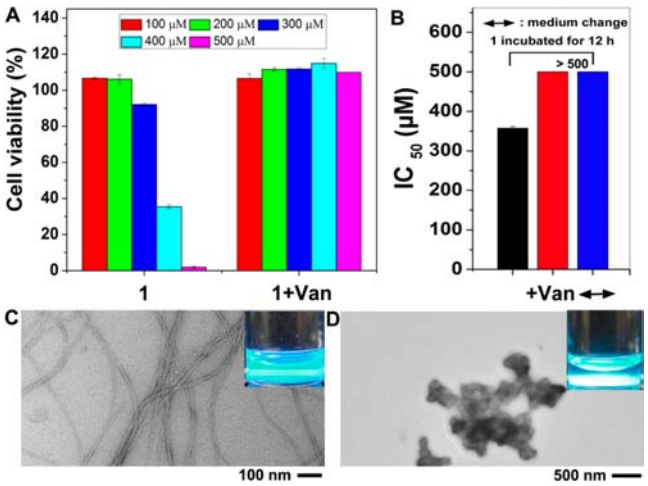

Figure 2. (A) Cell viability of PC12 incubated with **1** and **1**+Van after 5 days, $[\mathbf{1}]_0$: $[\text{Van}]_0$ = 1:1. (B) IC_{50} values of **1**/Van against PC12 cells after 5 days at different conditions: **1** incubated with PC12 cells for 12 h; either adding Van or changing medium (↔) abrogates the cytotoxicity of the nanofibrils. TEM images of (C) solution of **1** and (D) mixture of **1** and Van; insets are corresponding optical images under UV irradiation, $[\mathbf{1}]_0$ = $[\text{Van}]_0$ = 500 μM (enlarged part B in Figure S2).

concentrations above 400 μM (with an IC_{90} between 400 and 500 μM against PC12 cells[29]). This threshold cytotoxicity (Figure 2A) indicates the formation of aggregates of **1** that inhibit cell proliferation, agreeing with reports on the cytotoxicity of nanoscale assemblies.[5,23,24,30−32]

Surprisingly, in contrast to our previous report that Van catalyzes the aggregation of FmocKGGaa to inhibit the cells, the addition of Van completely abrogates the cytotoxicity of the nanofibrils of **1** (Figure 2A). This result, in fact, is similar to the induced formation of plaques to decrease Aβ-associated toxicity[33] and neuronal death occurring at regions (entorhinal cortex and hippocampus) with few Aβ plaques.[34] Optical images under UV irradiation clearly show the transition from a clear solution to a suspension (insets of Figure 2C and D; the bright spots in the bottom of the vial are particles excited by UV). According to visual inspection, the ligand−receptor interaction between Van and **1** results in precipitates, suggesting the formation of particles (Figure S3). Furthermore, transmission electron microscopy (TEM) reveals drastically different morphology of the assemblies of **1** before and after the addition of Van. As shown in Figure 2C, the molecules of **1** self-assemble to form nanofibrils at a concentration of 500 μM (215 μg/mL), which is consistent with light scattering results (Figure S5), implying that the resulting nanofibrils exhibit cytotoxicity. In contrast, the addition of Van completely converts the fibrils to particles (Figure 2D), which are innocuous. This result indicates that ligand−receptor interactions among the small molecules (Van and **1**) convert fibrils of **1** to particles made of **1** and Van, thus abrogating the cytotoxicity caused by assemblies of **1**.

To verify that the elimination of cytotoxicity is due to the ligand−receptor interaction between Van and **1**, we examine the cell viability of PC12 cells incubated with **1**+Van under three control conditions. As shown in Figure 2B, after incubation of PC12 cell with **1** for 12 h, extending the incubation time for another 5 days without any change, the assemblies of **1** are able to inhibit the proliferation of PC12 cells, with IC_{50} value of 357 μM. In contrast, the addition of Van (at the same concentration, i.e., 500 μM, as that of **1**) effectively abrogates the cytotoxicity of assemblies of **1**. Furthermore, after replacing the medium with fresh culture medium, the IC_{50} of **1** against PC12 cells is larger than 500 μM after 5 days, suggesting that it is unlikely for the cytotoxicity to originate from cellular uptake of **1**, but it rather results from assemblies of **1** outside cells. These results further demonstrate that ligand−receptor interactions between Van and **1** are able to abrogate the cytotoxicity of fibrils of **1**.

To further confirm that the morphological modulation by the ligand−receptor interaction is a cell line independent process, we examine the cytotoxicity of **1** with or without the addition of Van against three mammalian cell lines. As shown in Figure 3A, **1** inhibits the proliferation of HeLa, T98G, and HT1080 cells, with IC_{50} values of 293 μM, 321 μM, and 280 μM, respectively. Their corresponding mass concentrations are 126 μg/mL, 138 μg/mL, and 120 μg/mL, respectively, which are comparable to the mass concentration of amyloids (e.g., $A\beta_{42}$: 45 μg/mL)[8,35] in most of the studies. After mixing with Van in equal molar amounts, **1** becomes innocuous to HeLa, T98G, and HT1080 cells even at concentrations much higher than those IC_{50} values.

We synthesize a control molecule (**2**) by replacing D-Ala-D-Ala with L-Ala-L-Ala (Figure S1). As shown in Figure 4, **2** inhibits the proliferation of HeLa cell with or without the addition of Van, with similar IC_{50} values (Figure S7), suggesting that the addition of Van hardly affects the cytotoxicity of **2**. Meanwhile, the IC_{50} of **2** with or without addition of Van against PC12 cells are 303 μM and 285 μM after 5 days; such a little difference further confirms that Van hardly reduces the cytotoxicity of **2**. Moreover, TEM images of solution of **2** and **2**+Van show similar long, flexible nanofibrils (Figure S8). These results agree with negligible interactions between **2** and Van, thus further supporting that the ligand−receptor interaction

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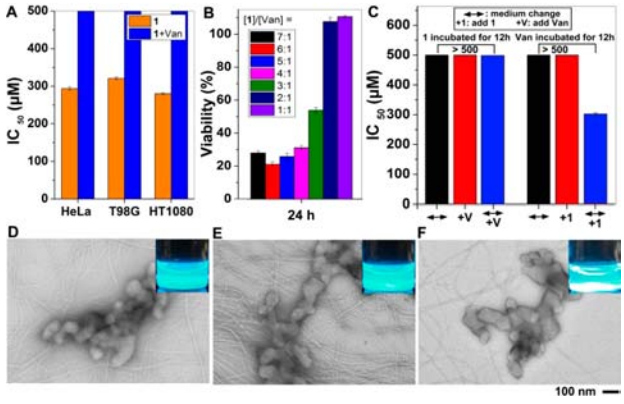

Figure 3. (A) IC_{50} of **1** without and with the addition of Van ($[\mathbf{1}]_0$:$[Van]_0$ = 1:1) against HeLa, T98G, and HT1080 cells for 48 h. (B) At 24 h, the viability of HeLa cells incubated with **1** ($[\mathbf{1}]_0$ = 500 μM) and varying amounts of Van (from 71 μM to 500 μM). (C) IC_{50} of **1**+Van against HeLa cells at different conditions: **1** (or Van), at concentrations of 100 μM to 500 μM, incubated with HeLa cells for 12 h, with (or without) changing medium (↔), then adding Van (or **1**). TEM images of **1** ($[\mathbf{1}]_0$ = 500 μM) and varying amounts of Van (D: 71 μM, E: 100 μM, F: 167 μM), and corresponding optical images under UV irradiation.

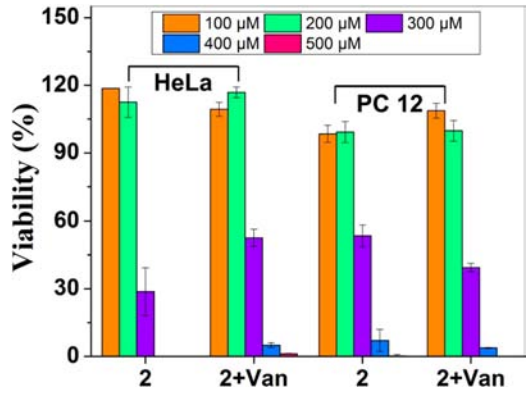

Figure 4. Cell viability of HeLa cells at 48 h, and PC 12 cells at 120 h incubated with **2** and **2**+Van.

modulates the morphology and the cytotoxicity of the fibrils of **1**. To quantify the ligand−receptor interaction, we use isothermal titration calorimetry (ITC) to measure the binding between **1** and Van (Figure S9). Upon titration of Van (8.0 mM) into a solution of **1** (0.7 mM) in PBS buffer, fitting the data using an independent model gives a dissociation constant (K_d) to be 20.7 μM and stoichiometry (n, Van/**1**) to be about 1. The dissociation constant agrees with the binding constants of Van and D-Ala-D-Ala determined by other methods,[36−38] suggesting high affinity between Van and **1** and 1:1 binding of Van and **1**. The control molecule (**2**) barely binds with Van, resulting in an affinity too weak to be measured by ITC (Figure S10). The above results imply the generality that ligand−receptor interaction modulates the morphology of molecular assemblies to abrogate the cytotoxicity of the nanofibrils of small molecules.

To investigate the effectiveness of the ligand−receptor interaction to minimize the cytotoxicity of nanofibrils, we examine the cell viability of HeLa cells incubated with a fixed amount of **1** (500 μM), but with varying amounts of Van (from 71.4 μM to 500 μM). As shown in Figure 3B, when the concentration of Van is above 250 μM ($[\mathbf{1}]_0$:$[Van]_0$ = 2:1), the HeLa cells become almost 100% viable at 24 h. This result confirms that the ligand−receptor interaction between Van and **1** can effectively eliminate the cytotoxicity of the fibrils of **1**. Meanwhile, when the concentration of Van increases (from 71.4 μM to 166.7 μM), the semitransparent solution of **1** gradually becomes a suspension (as shown in the insets of Figure 3D,E,F). Moreover, TEM images of the solutions and suspensions reveal the coexistence of fibrils and particles when the $[Van]_0$ is less than 166.7 μM, implying that the remaining fibrils of **1** are able to inhibit the proliferation of HeLa cells. To further verify that the ligand−receptor interaction abrogates the cytotoxicity of extracellular fibrils of **1**, we measure the cell viability of HeLa cells treated by **1**+Van under three different conditions (Figure 3C). Even after incubation of **1** with the cells for 12 h, equal molar amounts of Van are still able to completely eliminate the cytotoxicity of the fibrils of **1**, similar to the result with PC12 cells. To further establish the generality of ligand and receptor interaction for abrogating the cytotoxicity caused by nanofibrils, we synthesize **3** and **4** by inserting Gly-Gly into **1** and **2**, respectively (Figure S1). Similar to **1**, **3** inhibits HeLa cells, with IC_{50} value of 382 μM. Upon the addition of Van at same molar ratio, the resulting particles are innocuous at concentrations as high as 500 μM (Figure S11). Similar to **2**, molecule **4** inhibits the proliferation of HeLa cells with or without the addition of Van (Figure S12). The nearly identical morphology of assemblies of **4** before and after addition of Van also agrees with negligible interaction between **4** and Van (Figure S13).

To further confirm that the ligand−receptor interaction controls morphologies of supramolecular assemblies that interact with cells, we use a 7-nitrobenzofurazan derivatized vancomycin (NBD-Van) to visualize the aggregates of (**1**+Van) in PBS buffer. After mixing of **1** (500 μM), Van (490 μM), and NBD-Van (10 μM), many yellow spots appear on and away from the cells, indicating the formation of particles (Figure S14) and agreeing with TEM result. When **2** replaces **1**, few yellow spots appear. This result agrees with **2** barely binding with Van. Upon incubation of HeLa cells with **1** and Van, with 1 μM of NBD-Van to visualize the aggregation process, we observe a considerable amount of aggregates formed away from cells, in addition to the yellow spots on the HeLa cell surface (Figure S15; also see the movie of 1+Van_HeLa cell). After we extend the incubation time to 8 h and wash the cells with PBS buffer three times, yellow dots still adhere to cells. There is little cell inhibition, indicating that the aggregates of **1**+Van on the cells are likely innocuous. As expected, no particles appear around the cells in the case of **2** (Figure S15 and S16; also see the movie of 2+Van_HeLa cell), indicating that the fibrils of **2** remain unchanged and still inhibit the cells. Additionally, a similar phenomenon appears in PC12 cells after the same treatment (Figure S17 and Figure S18). Moreover, we use FITC-annexin V and propidium iodide (PI) to stain the PC12 cells. After incubation with the fibrils of **1** for 12 h, most PC12 cells are only stained by FITC-annexin V (Figure S19), which is different from necrotic cells induced by the treatment of 10% DMSO for 8 h (Figure S20). This result indicates that the PC12 cells enter the early stage of apoptosis.[39] However, upon treatment by the same stains, HeLa cells exhibit green and red fluorescence after treatment with **1** (500 μM) for 12 h (Figure S21), suggesting that fibrils of **1** cause the necroptosis of the HeLa cells.[40] Further studies find that pretreatment with necrostatin-1[41] is unable to reduce the cytotoxicity of fibrils of **1** (shown in Figure S22), suggesting that an alternative cell death

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pathway, other than necroptosis, is responsible for the observed cytotoxicity.

In a preliminary mechanistic study, we examine the expression of proteins in the extrinsic pathway of apoptosis of HeLa cells incubated with **1** or **1**+Van (500 μM). As shown in Figure 5, the fibrils of **1** increase the expression of TNFR1

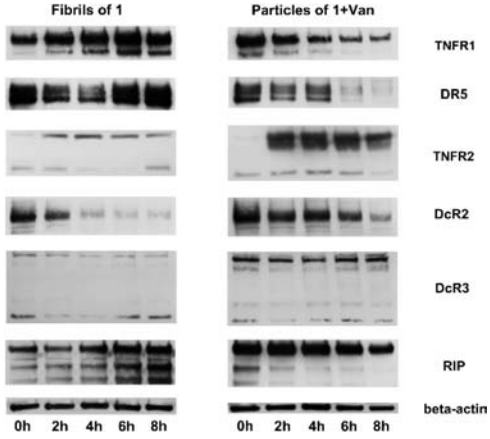

Figure 5. Western blot analysis of the cell lysate of the HeLa cells treated with **1** or **1**+Van at 500 μM for different durations.

and DR5, two extrinsic cell death receptors; decrease the expression of DcR2, a decoy of death receptor; and hardly affect the expression of TNFR2, a cell survival signal, and DcR3, another decoy of death receptor. This observation suggests that the fibrils of **1** likely act as a new type of cell death signal, which is consistent with the downstream fragmentation of RIP and the cytotoxicity of the fibrils of **1**. In contrast, the particles made of **1** and Van decrease the expression of TNFR1 and DR5, increase the expression of TNFR2, and maintain the expression of DcR2 and DcR3. This observation agrees with the decreased fragmentation of RIP and the cytocompatibility of the particles of **1**+Van. These results suggest that morphological change of the assemblies of small molecules is able to modulate cell death signaling, thus controlling cell fate. Since the particles of **1**+Van and aggregates of FmKGGaa and Van show dramatically different cellular responses, we also monitor the changes of apoptotic cell signals (Figure S24) over time. The aggregates of FmKGGaa and Van, also decrease the expression of TNFR1, increase the expression of TNFR2, and maintain the expression of DcR2, DcR3, and DR5. Notably, the aggregates of FmKGGaa and Van significantly increase the expression of Fas and also induce more and more expression of TNFR2 over time. Since Fas is a death receptor and TNFR2 sensitizes cells for cell death, it is reasonable that the aggregates of FmKGGaa and Van lead to cell death. This result indicates that assemblies of FmKGGaa and Van interact with different death receptors from those of **1**+Van, thus resulting in different cell behavior. In addition, we use Z-VAD-FMK, a pan-caspase inhibitor, to treat the HeLa cells in the presence of the fibrils of **1**. The inhibitor only slightly reduces the cytotoxicity of the fibrils of **1** (Figure S25), suggesting that the fibrils of **1** largely induce the caspase-independent cell death (e.g., necroptosis[42]), as recently reported by Wells et al.[23]

In this work, one Van binds with one Py-D-Ala-D-Ala (**1**) to convert the toxic fibrils to innocuous particles; in our previous study, one Van binds with two FmocKGGaa to turn innocuous monomers to toxic fibrils. Such a small difference (between Py and Fmoc) in the receptors results in dramatically different phenotypes of cells. This observation, indeed, provides insights for the paradoxical feature of the molecular aggregates in cellular environment. For example, proteins or oligopeptides bearing Phe and Trp may behave completely differently. The use of enantiomers (e.g., L-Ala-L-Ala versus D-Ala-D-Ala in this work) as the control compounds, in fact, sidesteps the problems of polymorphism associated with the aggregates, thus providing more definitive molecular evidence and cellular mechanism for asserting Van's role in abrogating the cytotoxicity of fibrils of **1**.

Despite the fact that there are many structural characterizations of amyloids (e.g., Aβ_{42}) or aggregates, there are few investigations into the cellular mechanisms. The molecular cell biology characterization of the detoxification of nanofibrils of **1**, in this work, thus provides a much needed understanding of the toxicity of molecular aggregates. Since aggregates of small molecules share many common features with amyloids formed by aberrant proteins or peptides[43] and associated with unsolved and intriguing problems, such as neurodegenerative diseases, the use of ligand−receptor interaction[44,45] effectively converts the neurotoxic nanofibrils to innocuous nanoparticles, which may ultimately lead to approaches that promote the controlled formation of plaques for treating diseases such as Alzheimer's disease.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00356.

Details of the synthesis, cytotoxicity, and confocal images (PDF)

Web-Enhanced Features

Two movies in MPG format for **1**+Van_ cell and **2**+Van_ cell are included in the online version.

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Notes

The authors declare no competing financial interest.

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