Ligand−Receptor Interaction Catalyzes the Aggregation of Small Molecules To Induce Cell Necroptosis

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S *Supporting Information*

ABSTRACT: Because they exhibit important biological functions, from unfolding proteins to activating enzymes to controlling cell fates, aggregates of small molecules are able to serve as functional molecular entities in cellular environments. However, the inability to precisely control their production has hampered the understanding and exploration of their biological functions. Here we show that the well-established ligand−receptor interaction between vancomycin and D-Ala-D-Ala catalyzes the aggregation of a D-Ala-D-Ala-containing small peptide derivative in water. The resulting aggregates largely adhere to the cell surface to induce cell necroptosis. Mutation of D-Ala-D-Ala to L-Ala-L-Ala or removal of the aromatic group in the derivative results in innocuous compounds, confirming that the aromatic−aromatic and ligand−receptor interactions are responsible for the formation and corresponding cytotoxicity of the aggregates. In addition to being the first example of ligand−receptor interaction-catalyzed aggregation of small molecules on the surface of mammalian cells, this work provides useful insights for understanding the cytotoxicity of molecular aggregates of small molecules.

Although intensive research efforts have been focused on aggregates of aberrant proteins or peptides because of their association with neurodegenerative diseases,[1] recent studies have also identified aggregates of proteins (e.g., nonpathogenic prions of cytoplasmic polyadenylation element binding protein, mitochondrial antiviral-signaling protein, or T-cell-restricted intracellular antigen 1) that have beneficial or even essential functions in cells.[2] In addition, aggregates of partially unfolding α-lactalbumin and oleic acid have found application in cancer therapy.[3] Similar to discoveries in research on protein aggregates, emerging evidence over the past decade from several unrelated fields (e.g., biomaterials,[4] high-throughput drug screening,[5] and neurodegenerative diseases[6]) has highlighted the significance of aggregates of small molecules in biology and medicine. With their ability to sequester enzymes or unfold proteins,[7] block β-amyloid formation,[6] activate enzymes,[8] inhibit cancer cell growth,[9] or recruit mRNAs to form cell-free RNA granules,[10] aggregates of small molecules constitute a new class of functional molecular entities in cellular environments.[11]

Studies of such aggregates, however, have frequently suffered from inconsistency and irreproducibility.[12] One major reason is the lack of precise control over the production of the aggregates.
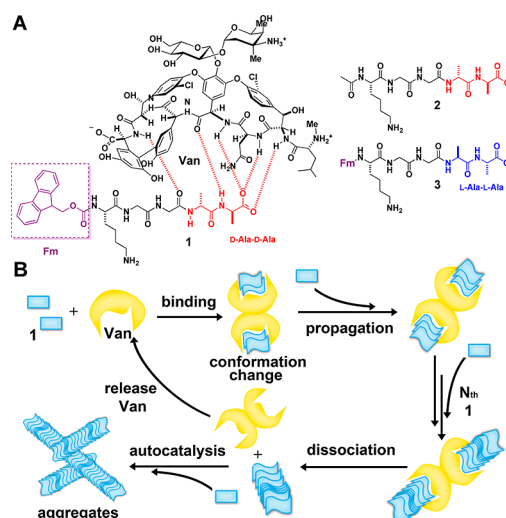
Figure 1. (A) Structures of the ligand (Van), the receptor (a D-Ala-D-Ala derivative), and the relevant controls. (B) The ligand−receptor interaction-catalyzed molecular aggregation.

Because they are hydrophobic, molecules that form aggregates in water usually require dissolution in an organic solvent (e.g., hexafluoroisopropanol[13] or dimethyl sulfoxide (DMSO)[12a]) before being dispersed in water. As a poorly controlled kinetic process, this type of dispersion usually results in different aggregates (i.e., polymorphism), even for the same molecules under the same conditions.[12a] For example, the polymorphism exhibited by Aβ amyloids likely contributes to conflicting reports on their neurotoxic[14] and neuroprotective[15] properties. Despite this problem, there are few studies that aim to generate these aggregates in a consistent and reproducible manner. One approach is to use an enzymatic reaction to catalyze the self-assembly of small molecules for generating the aggregates.[16] Although this approach is relatively successful and effective, the requirement of an enzyme still limits its application. Thus, we choose to explore the use of ligand−receptor interactions to promote the formation of aggregates of small molecules, because the formation of prion aggregates,[17] in essence, can be viewed as a result of ligand−receptor interaction (here the ligand and receptor are the same protein with different conformations[18]).

Specifically, we choose vancomycin (Van) as the ligand to promote the aggregation of D-Ala-D-Ala derivatives (as the

receptors) for three reasons: (i) The binding of Van and D-Ala-D-Ala is not only a well-established ligand–receptor interaction,[19] but also able to promote molecular self-assembly to form nanoscale aggregates (e.g., nanofibers),[20] as demonstrated by Walker et al.[20b] (ii) A mechanistic study by Williams et al. suggests that the binding of Van with D-Ala-D-Ala not only promotes the dimerization of Van and D-Ala-D-Ala,[21] but also generates a conformation change upon ligand–receptor interaction.[22] (iii) Unlike the cases of other receptors (antibodies, glutathione S-transferase), it is relatively easy to modify D-Ala-D-Ala to generate appropriate derivatives and control compounds. Thus, we designed and synthesized a D-Ala-D-Ala derivative (**1**) to interact with Van (Figure 1A).

Our results show that Van catalyzes the aggregation of D-Ala-D-Ala derivatives via ligand–receptor interactions, likely via two Van binding with four molecules of **1** to catalyze the aggregation of **1** (Figure 1B). The aggregation process is autocatalytic. Furthermore, cell viability tests indicate that the resulting aggregates inhibit cell growth, probably by necroptosis[23] of the cells. Fluorescence microscopy suggests that most of the aggregates adhere to the cell surface. The result of cell viability tests under various incubation conditions confirms that aggregates catalyzed by ligand–receptor interactions result in cell death. Mutation of D-Ala-D-Ala to L-Ala-L-Ala or removal of the aromatic group in the derivative results in innocuous compounds, confirming that the aromatic–aromatic and ligand–receptor interactions are indispensable for the formation and cytotoxicity of the aggregates of the D-Ala-D-Ala derivatives. As the first example of the use of ligand–receptor interaction to catalyze the aggregation of small molecules to inhibit cell growth, this work illustrates a fundamentally new approach to generating molecular aggregates in a consistent manner for controlling the fate of cells.

Based on the remarkable capability of fluorenyl-9-methoxycarbonyl group (Fm) to enhance the self-assembly of small molecules[24] via aromatic–aromatic interactions,[25] we designed molecule **1** to study the aggregation triggered by ligand–receptor interactions in phosphate-buffered saline (PBS). With D-Ala-D-Ala at its C-terminal, **1** is able to bind with Van via five hydrogen bonds (Figure 1A).[21,26] To evaluate the roles of stereochemistry and of the Fm group, we also designed two control molecules: **2** (without Fm) and **3** (L-Ala-L-Ala replacing D-Ala-D-Ala). After the synthesis of **1** via a solid phase peptide synthesis,[27] we examined the interaction of **1** with Van and found the formation of large aggregates (>5 μm, as shown in Figure S1) after mixing solutions of **1** ([**1**]$_0$ = 300 μM) and Van ([Van]$_0$ = 300 μM) at pH 7.4. To understand how ligand–receptor interaction catalyzes the formation of aggregates, we monitored this process by measuring the amount of aggregate **1** via the UV absorption change of the supernatant. As shown in Figure 2A, without Van a negligible amount of **1** forms aggregates after 12 h. After 84 h, <5.0 wt% of **1** forms aggregates, even with an initial concentration of **1** as high as 3 mM (Figure S2). Upon increasing [Van]$_0$ from 60 to 150 μM, the aggregation of **1** increases from 19.7% to 54.7% (relative to the total amount of **1**) after 14 h. This result indicates that Van promotes the formation of aggregates of **1**. Strikingly, when [Van]$_0$ is increased to 200 μM (2:3), 48% of **1** forms aggregates after 0.5 h, implying that the ligand–receptor interaction is an efficient approach to catalyzing the formation of the aggregates of **1**. Furthermore, the amount of Van in the aggregates decreases with time (Figure S3), indicating the release of Van from the aggregates during this process. This result confirms that Van acts as a catalyst for the aggregation process. In
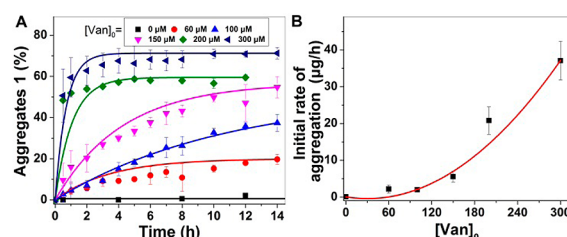
Figure 2. (A) Aggregate production as a function of time with varying initial concentration of Van ([Van]$_0$) over 14 h ([**1**]$_0$ = 300 μM). (B) Initial rate of aggregation versus [Van]$_0$ at [**1**]$_0$ = 300 μM. Data are fitted with a quadratic model.

Figure 2B, we show the initial rate of aggregation as a function of the initial concentration of Van ([Van]$_0$) when the initial concentration of **1** is 300 μM. This rate increases from 2.2 to 5.6 μg/h when [Van]$_0$ is raised from 60 to 150 μM and reaches 37.1 μg/h at [Van]$_0$ = 300 μM. The rate of formation of aggregates of **1** thus increases quadratically (Figure 2B) with the amount of Van, which agrees well with the model proposed in Figure 1 that two molecules of Van bind with four molecules of **1**. This result further supports the catalytic role of Van in the aggregation of **1**, and suggests that formation of the aggregates is autocatalytic, similar to the formation of prions.[28] We also simulated the formation of aggregates of **1** and obtained the best fit (Figure S4) when the initial binding step involves two molecules of Van and four molecules of **1** and the final step is autocatalytic. This kinetic analysis supports the scheme proposed in Figure 1B. The model used in the simulations is described in the Supporting Information.

To explore the molecular detail of how D-Ala-D-Ala derivatives bind with Van, we used isothermal titration calorimetry (ITC) to examine the interactions between **1** and Van. Figure 3 shows the
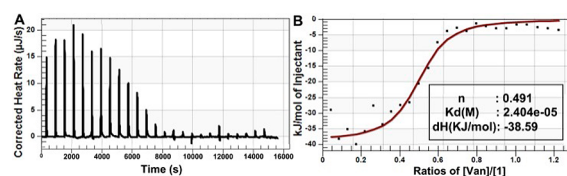
Figure 3. Isothermal titration of **1** with Van at 25 °C for the determination of dissociation constant (K_d) and stoichiometry (n).

heat flow of each injection during the titration of Van (8.0 mM) into a solution of **1** (2.0 mM) in PBS (pH 7.4). After correction of the raw data, data fitting using an independent binding model gives the dissociation constant (K_d) to be 24.0 μM and the ratio of binding (n) of Van to **1** as 0.5. This result is consistent with the high affinity between D-Ala-D-Ala and Van,[22] and also indicates that one molecule of Van binds with two molecules of **1** (a plausible mode of interaction is shown in Figure S5); such binding likely stems from hydrogen bonding and the intermolecular aromatic–aromatic interactions between the Fm groups in **1**. After removal of the Fm group, the control molecule, **2**, binds with Van in a 1:1 ratio (n = 1, Figure S6), and K_d = 134.6 μM. This result confirms that the aromatic group (Fm) promotes the dimerization of **1**, thus allowing two molecules of **1** to bind with one Van, which constitutes the molecular basis for Van catalyzing the aggregation of **1**. As expected, after mutation of D-Ala-D-Ala in **1** to L-Ala-L-Ala, the resulting molecule (**3**) barely binds with Van, thus resulting in a binding too weak to be determined by ITC (Figure S6).

To investigate the cellular response to the aggregates catalyzed by Van, we used an MTT assay to examine the viability of HeLa cells incubated with **1**, Van, or **1** plus Van. As shown in Figure 4A,
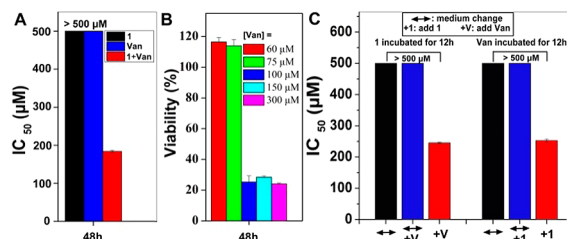
Figure 4. (A) IC$_{50}$ of **1** without and with Van ($[1]_0/[\text{Van}]_0 = 1:1$) against HeLa cells for 48 h. (B) At 48 h, the viability of HeLa cells incubated with **1** ($[1]_0 = 300 \, \mu M$) and varying amounts of Van ($60-300 \, \mu M$). (C) IC$_{50}$ values of (**1**+Van) against HeLa cells at different conditions: **1** (or Van) incubated with HeLa cells for 12 h, with (or without) changing the medium ($\leftrightarrow$), then adding Van (or **1**).

1 or Van alone is innocuous to HeLa cells even when the concentration of **1** or Van is as high as 500 μM. After mixing with Van in equimolar amounts, **1** is able to inhibit HeLa cell growth (IC$_{50}$ = 184 μM). We examined the cell viability of HeLa cells incubated with **1** (300 μM) and varying amounts of Van ($60-300 \, \mu M$). As shown in Figure 4B, when the ratio of the initial concentrations of Van and **1** is equal to or greater than 0.33, the aggregates catalyzed by Van inhibit the proliferation of HeLa cells, suggesting that Van serves as a catalyst for aggregation. It is necessary that Van catalyzes the formation of the aggregates of **1** *in vivo*, because the addition of preformed aggregates of **1** to cell culture is unable to inhibit cells (Figure S7). Incubation of Van and **1** with HT1080 cells gives ~90% cell death at 48 h ($[1]_0 = 300 \, \mu M$, $[\text{Van}]_0/[1]_0 = 1/5$, Figure S8), further supporting the claim that ligand—receptor interaction is an efficient approach to catalyzing the formation of aggregates that inhibit proliferation of cells.

To confirm that the cytotoxicity of (**1**+Van) originates from the aggregates catalyzed by Van, we examined the cell viability of HeLa cells incubated with (**1**+Van) under different incubation conditions. As shown in Figure 4C, after incubation of HeLa cells with **1** (or Van) for 12 h, addition of Van (or **1**) at the same concentration as **1** (or Van) results in inhibition of the HeLa cells, with IC$_{50}$ values of 245 μM (or 252 μM) at 48 h. In contrast, after incubation of HeLa cells with just **1** (or Van) for 12h, changing the culture medium to a fresh medium containing Van (or **1**) at the same concentration barely affects the viability of the HeLa cells. This result not only confirms that ligand—receptor interaction catalyzes the formation of the aggregates and induces cell death, but also suggests that cell death is caused by extracellular aggregates. Moreover, **2** scarcely inhibits the proliferation of HeLa cells with or without addition of Van at 500 μM, further confirming that the aromatic group is essential for the formation and cytotoxicity of the aggregates. We also added **2** (300 μM, 900 μM, 1.5 mM, and 2.7 mM), as a competitor of **1** to bind with Van, to the mixture (**1**+Van) at 300 μM just before treating the HeLa cells. We found that the addition of **2** (~2.7 mM) abrogates the cytotoxicity caused by (**1**+Van) (Figure S10), suggesting that **2** can occupy the binding site on Van, so that Van is unable to catalyze the aggregation of **1** to form enough aggregates to induce cell death. When we add Van to a solution of the control molecule **3**, the mixture (**3**+Van) is also innocuous to the cells (IC$_{50}$ > 500 μM, Figure S11). These

results, collectively, verify that aromatic—aromatic and ligand—receptor interactions are essential for the formation of aggregates of **1** catalyzed by Van and the corresponding cytotoxicity of the aggregates.

To verify that aggregates in the extracellular environment inhibit cell growth, we used a 7-nitrobenzofurazan-derivatized vancomycin (NBD-Van) to stain the aggregates of **1**, because small amounts of Van are able to bind with the aggregates (Figure S2). As shown in Figure S12, when **1** (300 μM), Van (294 μM), and NBD-Van (6 μM) are mixed together, we observe the appearance of yellow dots (1 min) and the formation of large yellow spots (20 min), indicating the formation of aggregates. In contrast, there are few yellow dots under the same conditions when **3** replaces **1**. This result further confirms that ligand—receptor interaction catalyzes the aggregation of **1** and indicates that NBD-Van can help visualize the aggregates of **1** in a cellular environment. As shown in Figure S13A, after incubation of HeLa cells with **1**, Van, and NBD-Van for 1 min, a few yellow dots appear. After 10 min, the number of yellow spots around the cells increases significantly. With extended incubation (30 min), many yellow spots form and accumulate on the cell surface. This result unambiguously confirms that aggregates of **1**, formed via catalysis by Van, adhere to the cell surface, which is also consistent with the result in Figure 2A that 51% of **1** forms aggregates after catalysis with Van for 30 min. Consistent with the results of the cell-free experiments, there are few fluorescent aggregates in the cell culture when **3** replaces **1**. Incubating HeLa cells with NBD-Van (6 μM) shows few aggregates on the cells (Figure S14). Together with the cytotoxicity data, these results demonstrate that ligand—receptor interaction catalyzes the formation of aggregates, which adhere to the cell surface and cause cell death.

To obtain a preliminary insight into the process of cell death, we used FITC-conjugated annexin V and propidium iodide (PI) to stain the HeLa cells. FITC-conjugated annexin V has a high affinity for phosphatidylserine, which is exposed during apoptosis or necrosis. The membrane-impermeable PI, as a nucleic acid dye, is able to discriminate live or early apoptotic cells from late apoptotic or necrotic cells that lose membrane integrity. Figure S13B shows that most of the HeLa cells are stained by both dyes after incubation with **1** and Van for 24 h, which is similar to necrotic cells that are induced by incubation with DMSO for 8 h (shown in Figure S15). However, some of the HeLa cells exhibit only green fluorescence (Figure S13B). These results indicate that the process of cell death caused by aggregates of **1** is rather heterogeneous (as evidenced by HeLa cells entering the late apoptosis or secondary necrosis stage). Thus, the aggregates of **1** likely cause necroptosis of cells.[23] Furthermore, the lack of cell specificity (Figure S16) also indicates necroptosis. To demonstrate the generality of the idea that ligand—receptor interaction catalyzes aggregation and leads to cell death, we designed molecules **4** and **5** by removing the lysine from **1** and **2**, respectively (Figure S17). As shown in Figure S18, **4**, like **1** also forms aggregates upon treatment with Van and inhibits cell growth (IC$_{50}$ = 157 μM), while **5** is innocuous to cells with or without Van.

In conclusion, we report the first use of ligand—receptor interaction to catalyze the aggregation of small molecules. The resulting aggregates not only act as autocatalysts, but also inhibit cell growth. Amphiphilic peptides,[29] as broadly targeting, also serve as auto-catalysts for accelerating their own formation and creating supramolecular structures.[30] Moreover, recent meta-analysis reveals that protein aggregates and small-molecule aggregates exhibit similar cytotoxicity (i.e., most IC$_{50} \approx 0.2$ mg/

mL) to cells.[31] Thus, the insights revealed in this work may provide useful hints for understanding and controlling cytotoxic protein aggregates, which are plausible causal agents of many neurodegenerative diseases. This result also provides supporting evidence for cytotoxicity caused by disruption of membranes.[32]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details of the synthesis, simulations, TEM images, ITC data, NMR and LC-MS data, cytotoxicity, and confocal images. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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