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Interactions between cellular proteins and morphologically different nanoscale aggregates of small molecules

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Abstract

Depending on the methods of preparation, amphiphilic small molecules aggregate to form nanostructures with different morphologies that interact with cytosol proteins in a drastically different manner, thus illustrating the first example of morphological dependent protein binding of nanoscale molecular aggregates.

This communication describes the interactions between cytosol proteins of a mammalian cell line (i.e., HeLa cells) and morphologically different nanoscale molecular aggregates formed by small peptidic molecules. In nature, peptides and proteins not only exist as soluble monomers, but also are able to self-assemble to form highly ordered structures. For example, cytoskeletal proteins self-organize to form filaments[1] that act as the cellular scaffolding. Under the proper regulations, certain proteins also give rise to amyloid-like fibrils that are physiologically functional (e.g., melanosomes[2] in mammalian cells). While the controlled and regulated self-assembly of peptides and proteins are essential for biological processes, certain peptides or proteins, if they fail to maintain their native conformation and misfold under pathologically conditions, might aggregate into various intermediate oligomers and further evolve into mature amyloid fibrils under kinetic control.[3] Now, it is increasingly evident that misfolded peptides or proteins in different forms affect cellular process differently and induce even contradictory cellular responses. For example, it is believed that beta-amyloid (Aβ) oligomers are cytotoxic but the plaques of Aβ are neuroprotective.[4] However, little is known about how the different forms of same misfolded peptides or proteins to result in the drastically different cellular outcome. One possible mechanism is that, though composed of the same peptides or proteins, the different aggregates (formed under different conditions or at different stages of aggregation) of the same misfolded peptides or proteins may possess different morphologies or overall conformations, which dictate the interactions of the aggregates with other proteins, thus even resulting in paradoxical phenotypes.

To infer the plausibility of the above hypothesis, we use small peptidic compounds to form the molecular aggregates by changing either the temperature or the pH of the solutions of the molecules that are prone to aggregate, and study the interaction of the aggregates with cytosol proteins. As shown in Scheme 1A, the molecules of **1** or **2** self-assemble to form nanofibers upon the change of temperature. However, upon the change of pH, the molecules of **1** and **2** form precipitates and nanofibers, respectively. The assay[5] of supramolecular hydrogels[6] binding proteins (Scheme 1B) reveals that the nanofibers and precipitates formed by **1** exhibit major differences in both the amounts and profiles of the proteins that they

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bind, and the two types of nanofibers formed by **2** exhibit similar morphologies and profiles of protein binding despite a slight difference in the amounts of the proteins. This work, for the first time, confirms that morphology of kinetically-trapped molecular aggregates directly dictates the proteins bound to the aggregates. Moreover, this work suggests that the emergent properties of the assemblies of simple small molecules (e.g., morphology of the molecular aggregates of small molecules) may play a significant role in cellular environment, an underappreciated inherent, supramolecular process that warrants further investigation for understanding the pharmacological properties of small molecules.

Scheme 1A shows the structures of the two small molecules evaluated in this work. Both molecules contains a di-phenylalanine,[7] the core motif of the most well-known misfolding peptide—beta-amyloid.[8] The incorporation of di-phenylalanine motif in **1** and **2** not only promotes the self-assembly[9] (or aggregation) of the molecules in water, but also allows the aggregates of **1** or **2** to approximate the aggregates of beta-amyloid peptide fragments.**,** a well-established hydrogelator, not only affords a hydrogel by itself, but also serves as self-assembly motif in many other hydrogelators.[10] Being heated to 60 °C then cooled down to room temperature, the solution of **1** (5 mg/mL, pH 7.4, in PBS buffer) turns into a transparent hydrogel (Fig. S1[†]). As shown in Fig. 1A and B, SEM image of the cryo-dried sample of the hydrogel shows fibrillar bundles as the dominated structures, so we denote this sample as **1**-f. The TEM images of **1**-f reveal the diameter of the nanofibers at 24 ± 2 nm, indicating that **1** self-assemble in water to form long, extended nanofibers, which tangle with each other to result in hydrogelation. The change of the pH of the solution of **1** from 8.0 to 7.0 results in precipitates (denoted as **1**-p) that can be separated from the suspension via centrifugation.[†] Fig. 1C shows the SEM image of cryo-dried sample of **1**-p, which mainly consist of microspheres with diameters from 5 μm to around 10 μm. While some of the microspheres have smooth surface, the other microspheres have rough surface (Fig. 1D) in which long nanofibers tangle with each other and resemble a clot of threads.

After the formation of the hydrogel and precipitates of **1**, we examined their interactions with cellular proteins. Recently, we have shown that a hydrogel formed by photo reactive hydrogelator is able to interact selectively with cellular proteins in a pull-down assay.[5] Although it validates that the aggregates of small molecules (in the form of supramolecular nanofibers) selectively bind proteins and supramolecular hydrogels are suitable for protein pull-down, the use of photo reactive residue[5] appears to be unnecessary. Thus, we design an improved molecular hydrogel protein binding assay (Scheme 1B) in which the pull-down of proteins solely relies on non-covalent interactions between the proteins and the nanofibers that act as the matrices of the hydrogel. This kind of protein pulled-down based on the binding affinity between the proteins and the molecular aggregates, which is intrinsically similar as the non-covalent interactions between the proteins and the aggregates of misfolded peptides or proteins in cellular environments. Based on the similar consideration (i.e., to approximate naturally occurring process) and adopting the pull-down assay for protein aggregates, we also establish cytosol protein pull-down assay by the precipitates formed by small molecules (Scheme 1B). Specifically, we incubate **1**-f or **1**-p with cell lysate of HeLa cells, and then use PBS buffer to wash them for three times to remove the proteins that bind non-specifically or weakly to the aggregates. Then, we use Laemmli sample buffer to dissociate the aggregates and release the proteins that specifically bind to the aggregates. Being small and neutral, the hydrogelators hardly interfere with SDS-PAGE. After SDS-PAGE separate the proteins, Coomassie staining of the SDS-PAGE gels indicates **1**-f or **1**-p selectively binds proteins. As shown in Fig. 2, the major bands of protein pulled-

[†]Electronic Supplementary Information (ESI) available: Experimental procedures; images of hydrogels; CD spectra]. See DOI: 10.1039/b000000x/

down by **1**-f are range of 40 to 70 kDa. On the contrary, there are insignificant bands of protein pulled-down by **1**-p at any molecular weight range, indicating that little amount of proteins bind to **1**-p. Fig. 2 shows the comparison of the protein profile of the proteins pulled-down by **1**-f and **1**-p ranging from 40 to 70 kDa. The 16 proteins that have the highest coverage in the sample from **1**-f differ from the 16 proteins from **1**-p. Little overlap of the protein profiles indicates that **1**-f and **1**-p interact with different sets of proteins. Moreover, the sequence coverage of all proteins pulled-down by **1**-p are below the threshold of protein mass spectrometry (15%), which agrees well with the Coomassie staining that shows quite low amount of proteins on **1**-p. The drastic difference in both the amount and profile of the proteins bound to **1**-f and **1**-p is in accord with their distinctly different morphologies. The obviously different surface-to-volume ratio between **1**-f and **1**-p (i.e., the nanofibers in **1**-f have much higher surface-to-volume ratio than the microspheres in the precipitates) likely also contributes to their difference in the amount of protein binding.

Unlike **1**, compound **2**, upon the change of temperature or pH, forms nanofibers, which interact with cellular proteins similarly. Particularly, being heated to 60 °C then cooled down to room temperature, the solution of **2** becomes a transparent hydrogel at 13 mg/mL in PBS buffer. As shown in Fig. 3, TEM image reveal long extended nanofibers (denoted **2**-f-A) as the dominate morphology of the hydrogel. Being slowly changing from pH 8.0 to pH 7.4, the solution of **2** also affords hydrogel (pH 7.4, 13 mg/mL, in PBS buffer). TEM image also displays nanofibers (denoted **2**-f-B) as the dominate morphology of the hydrogel. **2**-fA and **2**-f-B also appears to differ in the range of their diameters (**2**-f-A: 15.6 to 23.2 nm; **2**-f-B: 13.7 to 17.7 nm). Circular dichroism (CD) spectra of **2**-f-A and **2**-f-B (Fig. S2) exhibit peaks with similar shapes but slightly different intensities in both near-UV region (originating from aromatic amino acid side chains)[11] and the far-UV (corresponding to peptide bond)[11], indicating that the molecules of **2** in **2**-f-A and **2**-f-B likely have similar secondary structures. Thus, both TEM and CD suggest **2**-f-A and **2**-f-B exhibit similar morphologies and molecular arrangements.

Following the procedure of the pull-down assay (Scheme 1B), we also use the hydrogels consisting of **2**-f-A and **2**-f-B, to incubate with the cell lysate of HeLa cells. While SDS-PAGE shows the major protein bands at the same range, 40 to 60 kDa, **2**-f-A results in darker bands than **2**-f-B (Fig. 4). The protein profiles reveal that **2**-f-A and **2**-f-B interact with the same sets of proteins, which is in accord with that **2**-f-A and **2**-f-B have the similar morphology. Because protein coverage qualitatively correlates with the amount of proteins, the difference of protein coverage agrees with the result of SDS-PAGE that **2**-f-A binds with more proteins than **2**-f-B does. Although they exhibit similar morphologies, **2**-f-A and **2**-f-B are the consequence of molecular aggregation, which is a kinetic controlled process. Thus, **2**-f-A and **2**-f-B unlikely have identical morphology. Consequently, the amounts of proteins bound to **2**-f-A and **2**-f-B differ.

In conclusion, utilizing supramolecular interactions to form nanoscale aggregates, this study demonstrates that the morphology of kinetically-trapped supramolecular aggregates directly dictate protein binding of the aggregates. Although the definitive mechanism of our observation has yet to be elucidated, the causes of the morphology of nanoscale aggregates to dictate protein binding of the aggregates, we speculate, may arise from one or a combination of the following reasons: i) different morphologies of aggregates results in different surface to volume ratio, as demonstrated by **1**-f and **1**-p. ii) Difference in morphology results in different distribution of the hydrophilic or hydrophobic domains on the surface of the aggregates. For example, **1**-p is more hydrophobic than **1**-f. iii) Difference in morphology might expose different groups on the surface of the aggregates. Since the plaque and the soluble oligomers of amyloids have different morphology, it is not surprising that the protein pull-down by a plague of amyloid correlates poorly with neurodegenerative

diseases, which associate with soluble oligomers of amyloid.[12] Moreover, the results in this work imply that, due to its kinetic nature, the process of the aggregation determines the morphology of the aggregates, which helps explain that different labs, sometimes, give paradoxical results regarding to beta-amyloid oligomers. In addition, the protein profiles demonstrate that protein binding of the supramolecular aggregates is promiscuous. Thus, phenotypic assays (cell or animal based assays),[13] besides target-based assays, are necessary for the verification of protein targets of misfolding protein aggregates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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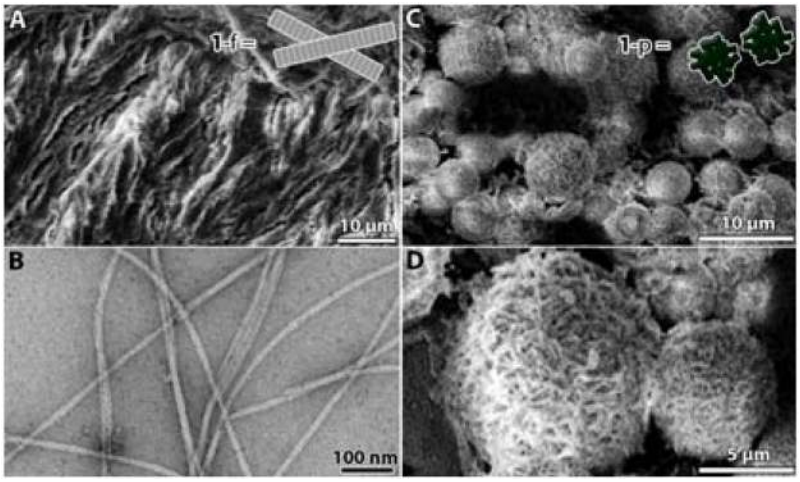


Fig. 1.

Morphologies of **1**-f and **1**-p. (A) SEM image and (B) TEM image of the micro- and nanostructures of **1**-f. (C) SEM image of the microstructure of **1**-p. (D) Enlarged SEM image showing the fine feature of a microsphere with rough surface in **1**-p.

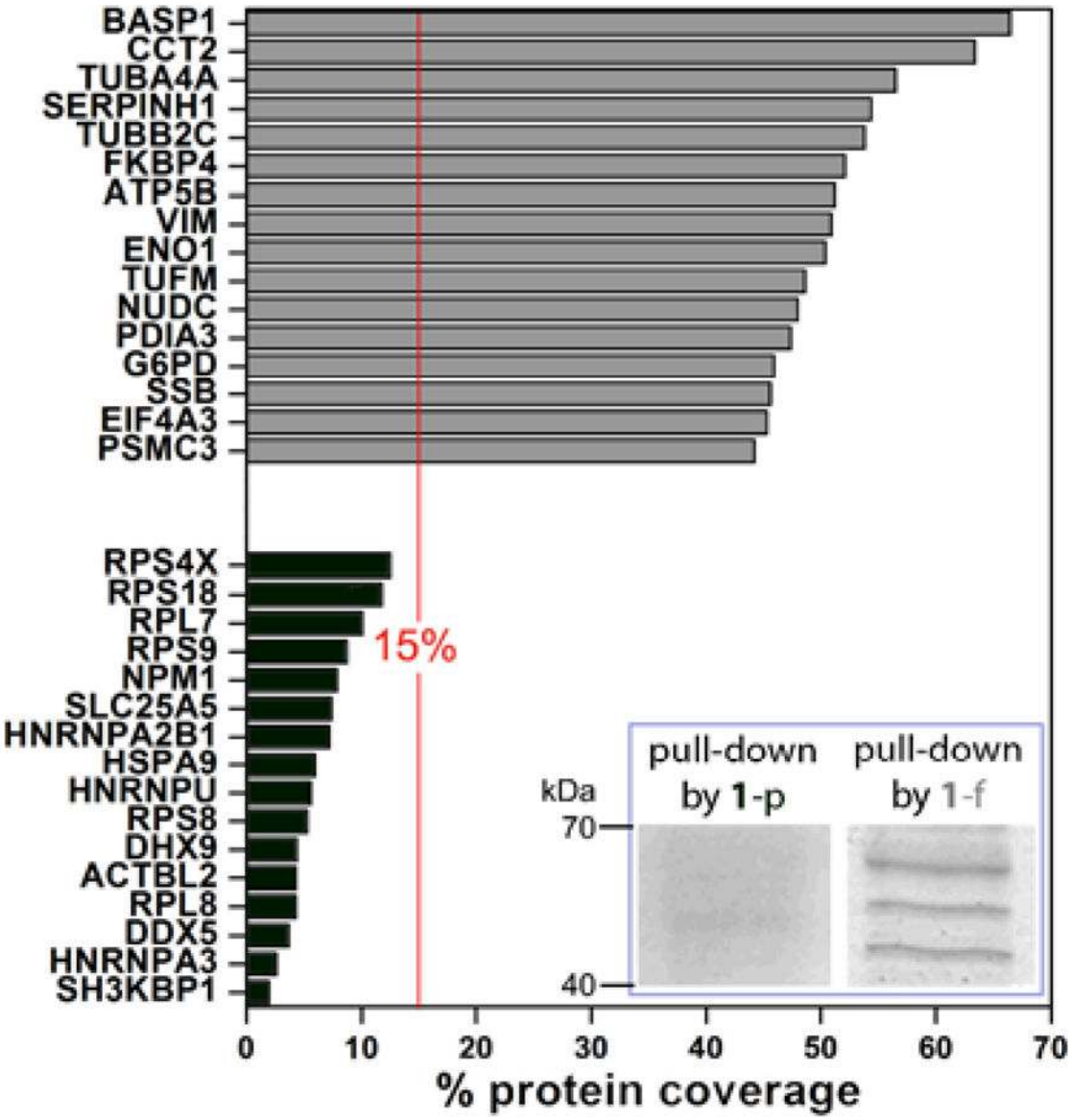


Fig. 2.

The protein sequence coverage analyzed by protein mass spectrometry of the cytosol proteins pulled-down in the range of 40 to 70 kDa by **1**-f (in gray) and **1**-p (in black). Inset is the Commassie blues stain of SDS-PAGE gel of cytosol proteins pulled-down by **1**-f and **1**-p.

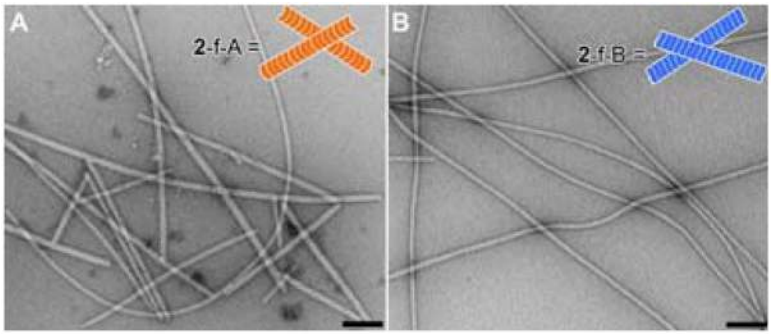


Fig. 3.
Morphologies of **2**-f-A and **2**-f-B. TEM images of the nanostructures of (A) **2**-f-A and (B) **2**-f-B. Scale bar = 100 nm.

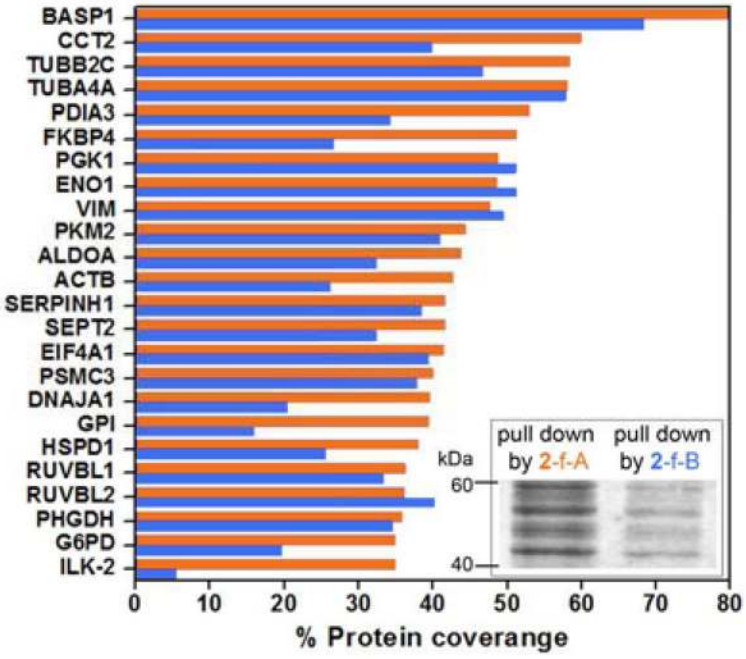
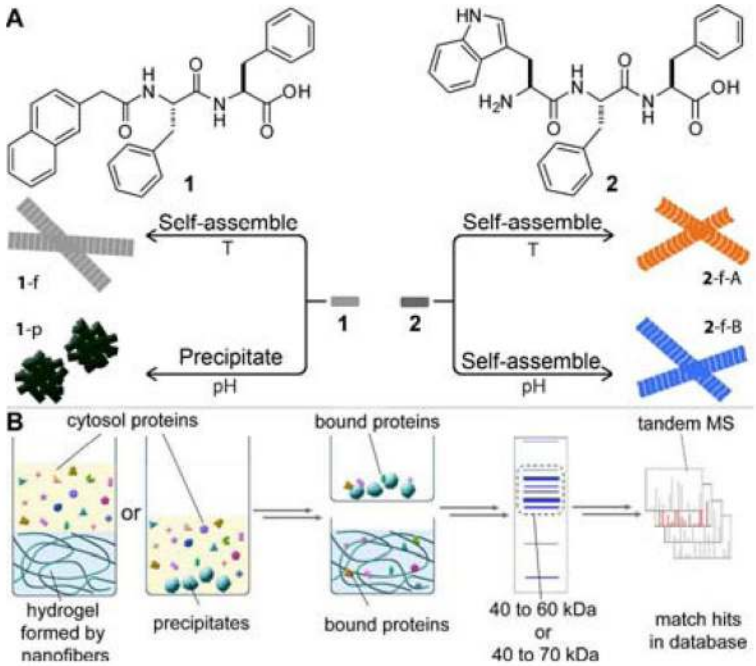


Fig. 4.

Protein sequence coverage of the proteins, pulled-down by **2**-f-A (denoted by orange) and **2**-f-B (denoted by blue), in the range of 40 to 60 kDa. Inset is the Commassie blues stain of SDS-PAGE gel of cytosol proteins pulled-down by **2**-f-A and **2**-f-B at 40 to 60 kDa.



Scheme 1.

(A) Chemical structures of the small peptidic molecules **1** and **2**, and the illustration of nanoscale aggregates formed by self-assembly of **1** and **2**. Upon tuning temperature, **1** forms nanofibers (denoted as **1**-f), which further entangle with each other to result in hydrogel. Changing the pH of the solution of **1** results in precipitates (denoted as **1**-p). Upon changing pH or tuning temperature, **2** affords two types of nanofibers (denoted as **2**-f-A and **2**-f-B), both of which can form hydrogels. (B) Flow chart of cytosol protein pull-down by the hydrogels or precipitates formed by self-assembly of small molecules.