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A Luminescence-Based System for Identification of Genetically Encodable Inhibitors of Protein Aggregation

Travis J. Nelson, Shuo Liang, and Cliff I. Stains*

ABSTRACT: Molecules that disrupt protein aggregation represent potential tool compounds for the investigation of numerous human disease states. However, the identification of small molecules capable of disrupting protein aggregation has proven challenging. Larger biomolecules such as antibodies and proteins are promising alternatives due to their increased size. Despite the promise of protein-based inhibitors, generalizable assays are needed to more readily identify proteins capable of inhibiting aggregation. Herein, we utilize our previously reported self-assembling NanoLuc luciferase fragments to engineer a platform in which both detection reagents are expressed from the same plasmid, enabling facile co-transformation with a genetically encodable inhibitor. This streamlined system is capable of detecting changes in the solubility of amylin, huntingtin, and amyloid-β (Aβ) proteins in response to mutations, small-molecule inhibitors, and expression of genetically encodable inhibitors. This improved platform provides a means to begin to identify protein-based inhibitors with improved efficacy.

INTRODUCTION

Protein aggregation and the formation of insoluble protein fibrils are associated with numerous human diseases. This has motivated several efforts to identify small-molecule inhibitors of protein aggregation. Although powerful tools, small-molecule inhibitors suffer from relatively limited surface areas, hindering their ability to disrupt protein–protein interactions. Alternatively, protein-based inhibitors provide the potential to disrupt interactions involving large surface areas. However, a lack of assays capable of identifying protein-based inhibitors of aggregation that function in cellular environments has limited progress in this area.

Early strategies for the detection of protein aggregates relied on staining with small molecules, such as thioflavin T and congo red, capable of producing a change in optical signal in the presence of aggregates. These small-molecule probes remain powerful tools to analyze protein aggregation in vitro but have limited utility in cellular applications and can produce false positives when screening for inhibitors of fibrillization. To address this issue, genetically encodeable reporters of protein aggregation have been developed. These reporters generally rely on using the aggregation of an appended protein-of-interest to modulate the function of a reporter (Figure 1a). In an elegant example, a GFP-based folding reporter has been used to identify small-molecule inhibitors of Aβ aggregation. As a complementary approach to monitor protein aggregation, we have utilized self-assembling fragments of NanoLuc luciferase (Nluc). Nluc is a small (19 kDa), engineered luciferase and provides a robust platform for engineering luminescence reporter assays. We have previously identified Nluc fragments termed N65 (residues 1–65) and 66C (residues 66–171) that are capable of spontaneous reassembly to afford functional enzyme. Fusion of a protein-of-interest (POI) to the N-terminus of N65 results in a change in the amount of N65 available for reassembly that is proportional to the solubility of the POI. Using this approach, relative changes in the solubility of the POI as a result of point mutants or treatment with small-molecule inhibitors can be assessed (Figure 1b). Our previous platform relied on the coexpression of POI-N65 and 66C reporter constructs from different plasmids, complicating the identification of genetically encodable inhibitors. Herein, we reengineer this system using a single plasmid to drive expression of both reporter components (Figure 1c). This re-engineered system is used to monitor the solubility of amylin, huntingtin, and Aβ proteins and is capable of reporting on the relative influence of mutations, small-molecule inhibitors, and protein-based inhibitors on aggregation.
**RESULTS AND DISCUSSION**

In order to investigate the ability to identify protein-based inhibitors of aggregation, we first examined whether coexpression of our reporter system from the same plasmid was feasible. For this purpose, we chose the commercially available pETDuet-1 vector, which is compatible with P15A, Mini-F/RK2, CloDF13, RSF1030, or ColA replicons. We examined whether mutations known to increase the solubility of amylin could be detected in this new system. Importantly, we have previously shown that our split-Nluc fragments are capable of reporting on the relative increase in the solubility of the I26P mutant of amylin when expressed from separate plasmids. Accordingly, wild-type (wt) amylin or the I26P mutant were fused to the N-terminus of N65 in the S′ multiple cloning site of pETDuet-1 (Table S1). The 66C Nluc fragment was cloned into the 3’ multiple cloning site of pETDuet-1 (Table S1). These coexpression constructs were transformed into bacteria, expression was induced by addition of IPTG, and samples were normalized to cell density prior to luminescence analysis in intact cells. The I26P mutant showed an increase of 2.3-fold in normalized luminescence in cells incubated with their respective inhibitors that was consistent with our previously reported data using two reporter plasmids (Figure 3). These experiments indicate that our re-engineered coexpression platform can be utilized to identify small-molecule inhibitors of protein aggregation.

Encouraged by these results, we sought to determine whether the re-engineered platform was also capable of assessing the influence of genetically encodable inhibitors on protein aggregation. As a proof-of-concept system, we chose the evolved miniprotein known as TJ10 (Table S1). TJ10 is a variant of the hyperthermophilic mutant of the IgG-binding protein (HTB1) that was evolved via phage display to bind to Ab oligomers and disrupt aggregation in vitro. We have

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**Figure 1.** Cell-based assay systems for detecting protein solubility. (a) A reporter capable of producing an observable signal is fused to the C-terminus of a protein-of-interest (POI). The activity of the reporter protein is modulated by the equilibrium between the folded and unfolded states. (b) A POI is fused to the N-terminus of N65 (blue). The equilibrium between folded and unfolded protein dictates the amount of N65 available for reassembly with 66C (red). Reassembled N65/66C produces a luminescent signal that is proportional to the amount of soluble POI. (c) The previously described split-Nluc assay system was based on two expression plasmids for POI-N65 and 66C. The re-engineered split-Nluc assay system utilizes a single plasmid to drive the expression of both POI-N65 and 66C proteins, allowing for the interrogation of genetically encodable inhibitors.

**Figure 2.** Monitoring the influence of mutations on amylin solubility using the single-plasmid split-Nluc assay. (a) The amino acid sequence of amylin is shown with the mutation site indicated in red. (b) A dramatic increase in the luminescence of bacterial cells expressing the I26P mutant is observed relative to cells expressing wild-type (wt) protein using the Nano-Glo Live Cell Assay reagent. Error bars represent the standard deviation of three biological replicates assayed in triplicate. *** indicates a p-value of <0.001.

**Figure 3.** Monitoring the influence of small molecules on protein solubility using the single-plasmid split-Nluc assay. (a) The structure of silibinin, a known inhibitor of amylin aggregation, is shown. (b) Luminescence from bacterial cells expressing amylin-N65/66C in the presence or absence of silibinin using coelenterazine as the substrate. (c) The structure of cystamine, a known inhibitor of Htt aggregation, is shown. (d) Luminescence from bacterial cells expressing Htt97Q/N65/66C in the presence or absence of cystamine using coelenterazine as the substrate. Error bars represent the standard deviation of three biological replicates assayed in triplicate. *** indicates a p-value of <0.01 and ** indicates a p-value of <0.001.
previously reported the ability of the split-Nluc assay system to detect changes in \( \alpha \beta_{1-42} \) aggregation upon treatment of lysates with o-vanillin. However, this small-molecule inhibitor was found to be toxic to living cells at the concentrations required for inhibition of \( \alpha \beta_{1-42} \) aggregation. In the long term, we hypothesize that our re-engineered system may allow for the identification of protein-based inhibitors of \( \alpha \beta_{1-42} \) aggregation with reduced toxicity. Toward this goal, \( \alpha \beta_{1-42} \) was fused to the N-terminus of N65 in the dual-expression vector and TJ10 was subsequently cloned into the 5′ cloning site of the pRSFDuet-1 vector (Table S1). We hypothesized that the use of a higher-copy plasmid as well as a T7 promoter for the complementary expression vector, pRSFDuet-1, containing a plasmid (Figure S1). Interestingly, expression of TJ10 in the N-terminal expression vector yielded a similar luminescence increase to the single-copy site of pRSFDuet-1, the resulting dual-TJ10 expression vector capable of producing a >10-fold change in luminescent signal depending on the solubility of the protein fused to N65, thus we expect that the systems described herein can be used directly for identification of inhibitors. Efforts to further improve the dynamic range of the assay are focused on increasing the reassembly efficiency of N65 and 66C, which is currently ~0.1%. Given the ability to generate DNA libraries of mutant proteins and peptides, we anticipate that these new assay systems could potentially be used in their current form to identify protein-based inhibitors of protein aggregation.

**CONCLUSIONS**

We have re-engineered a split-Nluc platform to utilize a single coexpression plasmid to drive production of the detection constructs. This platform is capable of detecting changes in protein solubility resulting from mutations as well as addition of small molecules. The assay retained its functionality for detecting amylin, huntingtin, and \( \alpha \beta \) protein aggregation. Building upon the ease of co-transformation in this system, we demonstrated the ability to interrogate the influence of genetically encoded inhibitors on protein aggregation. Previous work has demonstrated that the split-Nluc system is capable of producing a >10-fold change in luminescent signal depending on the solubility of the protein fused to N65, thus we expect that the systems described herein can be used directly for identification of inhibitors. Efforts to further improve the dynamic range of the assay are focused on increasing the reassembly efficiency of N65 and 66C, which is currently ~0.1%. Given the ability to generate DNA libraries of mutant proteins and peptides, we anticipate that these new assay systems could potentially be used in their current form to identify protein-based inhibitors of protein aggregation.

**METHODS**

**Small-Molecule Inhibitor Stocks.** Silibinin (amylin aggregation inhibitor) was prepared as a 200× stock solution at a concentration of 1 mM in dimethyl sulfoxide (DMSO). Cystamine (Htt aggregation inhibitor) was prepared as a 1000× stock solution at 50 mM in water.

**Cloning of N65 Fusions and 66C into pETDuet-1.** Sequences for \( \alpha \beta_{1-42} \)-N65, amylin-N65, Htt97Q-N65, and 66C were amplified from previously described vectors. DNA sequences encoding for N-terminal fusions to N65 were cloned into the first multiple cloning site of the pETDuet-1 vector (Novagen, TB337) using primers containing 5′ NcoI and 3′ NotI restriction sites. The DNA sequence corresponding to the C-terminal Nluc fragment (residues 66−171) was then incorporated into the second multiple cloning site using primers containing 5′ Ndel and 3′ XhoI restriction sites. Ligated DNA was transformed into XL1-Blue competent cells (Agilent, #200249) and amplified on LB plates containing ampicillin (100 μg/mL). The amylin I26P mutant was generated using site-directed mutagenesis according to the manufacturer’s protocol (QuickChange XL Site-Directed Mutagenesis Kit, Agilent, #200517). Plasmids were verified by DNA sequencing.

**Cloning of TJ10 into pRSFDuet-1.** A DNA sequence encoding for TJ10 was cloned into the first multiple cloning site of the pRSFDuet-1 vector (Novagen, TB391) using primers containing 5′ BamHI and 3′ NotI restriction sites. Where indicated, a second copy of TJ10 was cloned into the second multiple cloning site of the vector using primers containing 5′ Ndel and 3′ KpnI restriction sites. Ligated DNA was transformed into XL1-Blue competent cells and amplified on LB plates containing kanamycin (50 μg/mL). Plasmids were verified by DNA sequencing.

**Solubility Assays.** Samples were prepared according to previous procedures. Briefly, BL21-Gold(DE3) cells (Agilent, #230132) were transformed with the indicated constructs and grown overnight in terrific broth (TB, 5 mL) with shaking at 37°C in the presence of the appropriate antibiotics (ampicillin at 100 μg/mL and kanamycin at 50 μg/mL). The number of the pRSFDuet-1 plasmid, we investigated whether TJ10 was present at saturating concentrations within cells. This was confirmed by cloning a second copy of TJ10 into the 3′ site of pRSFDuet-1, the resulting dual-TJ10 expression vector yielded a similar luminescence increase to the single-copy plasmid (Figure S1). Interestingly, expression of TJ10 in the amylin and Htt97Q reporter systems resulted in 20 and 46% increases in luminescence, respectively (Figure S2). This may indicate that TJ10 recognizes common oligomeric structures shared by these proteins and \( \alpha \beta_{1-42} \) similar to previously described oligomer-specific antibodies. Taken together, these experiments demonstrate that our re-engineered assay platform can be utilized for the straightforward interrogation of genetically encoded inhibitors of protein aggregation.

![Figure 4](https://dx.doi.org/10.1021/acsomega.0c00779)
optical density of each sample was measured at 600 nm, and samples were diluted to an OD_{600} = 0.1 in 5 mL. The cells were grown in a shaker at 37 °C until they reached an OD_{600} = 0.6–0.8 and were then induced with IPTG (0.2 mM final concentration). Small-molecule inhibitors were then added as indicated. The cells were subsequently incubated overnight, with shaking at 16 °C. The next morning, the OD_{600} of the sample was measured and each culture was normalized to an OD_{600} = 3.0 in 1 mL of TB, except in Figure 2b where each culture was normalized to an OD_{600} = 0.003 in 1 mL of TB due to the use of the more-sensitive Nano-Glo Live Cell Assay reagent (see below). Cells were then harvested by centrifugation at 700g for 10 min at 4 °C and resuspended in 200 μL of 1X Nluc assay buffer (50 mM 2-ethanesulfonic acid (MES) pH = 6.0, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 75 mM KCl, 1 mM 2-mercaptoethanol, and 17.5 mM thiourea) or 200 μL of nanopure water when using the Nano-Glo Live Cell Assay reagent. The cells were then mixed with either 200 μL of Nano-Glo Live Cell Assay reagent (Promega, N2011) prepared according to the manufacturer’s protocol or 200 μL of 1X Nluc assay buffer containing either furimazine (Promega, N1110, 2% v/v) or coelenterazine (GoldBio, C2Z2.5, 25 μM final concentration) as indicated. Relative changes in luminescence obtained in assays using either furimazine or coelenterazine are comparable.\(^\text{20}\) The resulting mixtures were then loaded into a 384-well assay plate (Corning, 3824, white, low volume, 10 μL sample per well). Luminescence intensities were measured 30 min after addition of the substrate using a SynergyH1 hybrid reader (BioTek). Relative luminescence data are shown and represent the average of triplicate experiments conducted on three biological replicates. Error is reported as the standard deviation. Reported p-values were calculated using a two-tailed, unpaired t-test.

### ASSOCIATED CONTENT

1. **Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c00779.

One copy of TJ10 saturates the Δβ_{2−42} system; TJ10 may increase the solubility of amylin and Htt97Q; amino acid sequences for constructs (PDF)

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### Notes

The authors declare no competing financial interest.

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