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Semi-automated microplate monitoring of protein polymerization and aggregation

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Abstract

Static light scattering (SLS) is a commonly used technique for monitoring dynamics of high molecular weight protein complexes such as protein oligomers or aggregates. However, traditional methods are limited to testing a single condition and typically require large amounts of protein and specialized equipment. We show that a standard microplate reader can be used to characterize the molecular dynamics of different types of protein complexes, with the multiple advantages of microscale experimental volumes, semi-automated protocols and highly parallel processing.

Keywords

protein dynamics; protein aggregation; protein assembly; chaperone; cytoskeleton

Static light scattering (SLS) techniques such as multi-angle (MALS) and right-angle (RALS) measure the light deflected from particles in solution that are larger than the wavelength of the light emitted. Such methods typically require expensive fluorimetry equipment, consume large amounts of purified protein, and may be unsuitable for high-throughput assays [1, 2]. For example, a fluorimeter equipped with a stirrable, temperature-controlled cell holder is required for RALS analysis, and the cost of such equipment may be prohibitive for many laboratories. Furthermore, such devices are limited to single cell measurements, and the need to maintain solution homogeneity through mechanical stirring dictates mL-scale volumes per experiment. If proteins are tested at nano- or micromolar concentrations, significant amounts of purified or purchased proteins are required.

We describe here an alternative method for tracking the formation of higher molecular weight complexes to study polymerization and aggregation of proteins that yields results comparable to SLS. This approach utilizes a microplate reader with temperature control, along with 96-well half area microplates that allow for multiple reactions to be analyzed

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simultaneously in a low reaction volume. While previous applications of microplate technology for following protein dynamics in high-throughput molecular screens have been described [3-5], we demonstrate here the sensitivity and range of this technique by measuring protein polymerization, protein aggregation and aggregate prevention by a molecular chaperone. The method described here measures, in real-time, the increase in turbidity that occurs as high molecular weight protein complexes form in an aqueous buffer. Our overall goal was to show that a method for sensitive, rapid and reproducible comparative monitoring of protein assembly dynamics in small volumes can be accessible to molecular biologists.

The molecular dynamics and biochemical requirements of cytoskeletal proteins such as eukaryotic actin and tubulin and their bacterial homologs can be characterized by measuring their assembly into large ordered complexes to understand how these filaments contribute to cell shape and cell division [6, 7]. FtsZ, a prokaryotic homolog of eukaryotic tubulin, serves as a scaffold and potential force generator for bacterial cell division. FtsZ polymerizes into a ring-like structure at mid-cell beginning the assembly of the cell division machine, a multi-protein complex that divides daughter cells [8]. The GTP dependent polymerization of FtsZ has been well studied in vitro and is influenced by many factors including pH, cations such as K^+ and Ca^{2+} and temperature [9, 10]. Upon FtsZ polymerization, GTP is hydrolyzed, followed by disassembly of the protofilaments. We measured the polymerization of purified FtsZ in the presence of different concentrations of $CaCl_2$ and GTP. FtsZ was purified as described [11], and resuspended in FtsZ polymerization buffer (50 mM morpholine ethanesulfonic acid ([4] pH 6.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 10% sucrose) for storage and polymerization assays. Polymerization assays were conducted in a Synergy MX Microplate Reader (BioTek). FtsZ (15 μM) was equilibrated to room temperature in FtsZ polymerization buffer with 0, 2.5, and 5 mM $CaCl_2$ in a total volume of 180 μl in wells of a 96 well, half area, UV-transmissible plate (675801, Greiner Bio-One). Absorbance (decreased transmittance of light due to increased turbidity in the sample) was measured at 320 nm at 30 sec intervals for 3 min. The protein solutions must be equilibrated to the temperature and buffer conditions of the assay, as an abrupt shift in buffers or temperature can result in an artificial increase in absorbance. GTP was added at a concentration of 1 mM to each well and mixed thoroughly for 5 sec using a multichannel pipettor, taking care not to introduce bubbles, and measurements were collected for 120 min. The average of the first 3 min of reads was subtracted from all reads for each condition to normalize the data. All assays were performed concurrently on a single microplate.

Our results were comparable to data published using RALS, although we tested a slightly higher protein concentration (15 μM vs 12.5 μM) at lower temperature (25°C vs 30°C) [9, 10]. Polymerization followed by depolymerization was observed for all conditions, although as expected, depolymerization was incomplete in 5 mM $CaCl_2$ after 120 min (Fig. 1A). To verify that the microplate reader was detecting typical FtsZ polymers, we examined FtsZ protein incubated with 5 mM $CaCl_2$ and 1 mM GTP at the time of maximal polymerization by transmission electron microscopy. FtsZ (15 μM) was incubated in FtsZ polymerization buffer with 5 mM $CaCl_2$ for 10 min at room temperature prior to adding 1 mM GTP and incubating at 25°C for 35 min. Grids were prepared, negatively stained and imaged as

described [12]. As expected for these buffer conditions, single ~5 nm thick protofilaments of FtsZ were visible together with protofilament bundles (Fig. 1B).

In addition to verifying the usefulness of the microplate technique in reproducibly monitoring FtsZ polymerization, we also assessed whether this microplate reader assay would be adaptable to study the ability of molecular chaperones to modulate protein aggregation. Molecular chaperones help to maintain cellular proteomes by interacting with unfolded or partially folded proteins, preventing their aggregation and stabilizing polypeptides until native conformations are achieved [13]. Various aggregation-prone model proteins including rhodanese and citrate synthase have been used to elucidate biochemical features of chaperone function and specificity [14-17]. These and other commonly employed substrates rapidly aggregate when diluted from a denaturing solution into an aqueous buffer unless accompanied by molecular chaperones [18, 19]. Rhodanese and citrate synthase were incubated in denaturing buffer (6 M guanidinium chloride, 5 mM dithiothreitol) at concentrations of 13.3 and 11.6 μ M, respectively for 1 hr at room temperature [2, 17, 19]. Refolding buffer (25 mM Tris pH 7.5, 100 mM NaCl) or denaturing buffer were pre-equilibrated at 25°C in a 96-well, half area plate for 5 min and baseline absorbance was determined. After equilibration, chemically denatured substrate was added to a final concentration of 150, 300, 600, or 900 nM into the refolding buffer or 900 nM into the denaturing buffer to a final volume of 180 μ L. The samples were mixed thoroughly and absorbance was measured at 320 nm at 30 sec intervals for 30 min. Changes in absorbance were calculated after subtracting baseline absorbance at time zero, and all experiments with a given substrate were performed concurrently on a single microplate.

The aggregation of chemically denatured rhodanese (Sigma) was tracked by measuring absorbance at 320 nm (Fig. 2A). Our results indicate that this method successfully detects increasing aggregation over time, using concentrations of denatured rhodanese in line with those previously published [2, 16]. Similar results were obtained using chemically denatured citrate synthase (Sigma) [15, 20]. Aggregates formed by either substrate were detected at concentrations as low as 150 nM and the change in absorbance increased with greater chaperone concentrations, consistent with increased light scattering due to the formation of aggregates (Fig. 2A).

Citrate synthase is recognized as a substrate by the Hsp110 class of chaperones that stabilize the unfolded protein to prevent aggregation [14, 21]. We analyzed the aggregation dynamics of citrate synthase in the presence of the yeast Hsp110 chaperone, Sse1. Hexahistidine tagged Sse1 was purified from *Escherichia coli* by chemical lysis in buffer B (50 mM Tris pH 7.5, 200 mM NaCl, 2 mM $MgCl_2$, 5 mM imidazole). The cell lysate was incubated with His-Pur Cobalt Resin (Thermo Scientific), washed with buffer B and C (50 mM Tris pH 7.5, 600 mM NaCl, 2 mM $MgCl_2$, 10 mM imidazole), and eluted with Buffer E (50mM Tris pH 7.5, 700 mM NaCl, 2 mM $MgCl_2$, 200 mM imidazole). Sse1-containing elution fractions were combined, buffer exchanged (25 mM Tris pH 7.5, 100 mM NaCl), and further purified by size exclusion chromatography using Sephacryl S-100 (GE Healthcare). For analysis of protein aggregates in the presence of a chaperone, refolding buffer was equilibrated with 0, 100, 200, 400, or 800 nM Sse1 or denaturing buffer in the absence of Sse1 at 25° C in a 96-well, half area plate for 5 min while absorbance was measured. After equilibration,

chemically denatured citrate synthase was added to 200 nM, chosen as a minimal aggregating substrate concentration, into the refolding buffer or into the denaturing buffer, with and without Sse1, to a final volume of 180 μ L. The samples were mixed thoroughly for 5 sec and absorbance was measured at 320 nm every 30 sec for 30 min at 25°C. Consistent with reported results, increasing Sse1 concentrations promote solubility of the aggregate-prone substrate (Fig.2B, C). Notably, absorbance changes due to the aggregation of citrate synthase correlated with transfer of the substrate from a high-speed sedimentable fraction to a soluble state as assessed by SDS-PAGE of endpoint samples followed by Coomassie Blue staining (Fig. 2C).

We have described an accessible, low- to high-throughput alternative to SLS that provides similar results at levels of detection comparable to single-cell analyses. It should be noted that a few seconds of data are lost during insertion and calibration of the plate when using a plate reader, making this technique unsuitable for analysis of initial burst dynamics. However, kinetic analyses to determine aggregation parameters may be undertaken using standard calculations [22]. Nonetheless, the technique presented here is a simpler, quicker, and more efficient way to simultaneously compare the dynamics of high molecular weight protein assemblies across multiple samples and conditions. As microplate readers can be outfitted with both injection systems and ambient temperature controls, this approach can allow for concurrent analysis of multiple experimental variables.

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Figure 1.

FtsZ protein polymerization and calcium induced bundling. (a) Turbidity of FtsZ (15 μ M) in FtsZ polymerization buffer with the indicated CaCl_2 concentrations after the addition of 1 mM GTP at 25°C. Average data points from independent experiments were plotted ($n=3$) with the following standard deviations: 0.003 (no CaCl_2), 0.002 (2.5 mM CaCl_2), and 0.008 (5 mM CaCl_2). (b) A representative electron micrograph of negatively stained FtsZ (15 μ M) in the presence of 1 mM GTP and 5 mM CaCl_2 . Scale bar, 100 nm.

Figure 2.

Protein aggregation and protection by molecular chaperones. (a) Aggregation of denatured substrate (rhodanese $n=4$, or citrate synthase $n=2$) at the indicated concentrations was measured at 25 °C by turbidity. (b) Denatured citrate synthase was diluted into assay buffer with increasing concentrations of Sse1 chaperone ($n=3$). For all light scattering analyses, data shown are the average of independent experiments (standard deviation = 0.003). (c) Samples were collected from the end points of experiment (b) and the soluble and aggregated fractions of CS and Sse1 were separated into supernatant and pellet through differential centrifugation followed by SDS-PAGE and staining with Coomassie blue.