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Peptide Oligomerization Memory Effects and Their Impact on the Physical Stability of the GLP-1 Agonist Liraglutide

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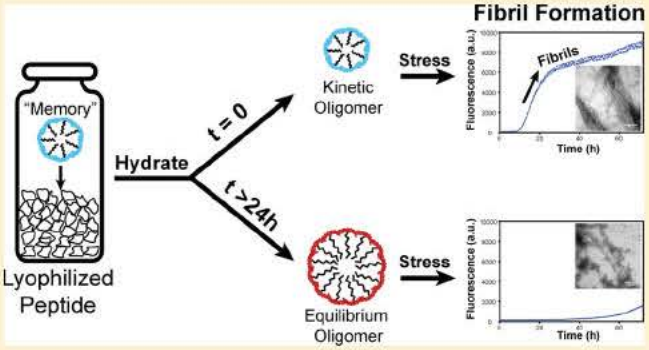
Supporting Information

ABSTRACT: Peptides and proteins commonly have complex structural landscapes allowing for transformation into a wide array of species including oligomers, aggregates, and fibrils. The formation of undesirable forms including aggregates and fibrils poses serious risks from the perspective of drug development and disease. Liraglutide, a GLP 1 agonist for the treatment of diabetes, is a conjugated peptide that forms oligomers that can be stabilized by pH and organic solvents. We have developed an analytical toolkit to overcome challenges inherent to Liraglutide's conjugated acyl chain and probed the impact its oligomers have on its physical stability. Our studies show that Liraglutide's oligomer states have significant and potentially detrimental impacts on its propensity to aggregate and form fibrils as well as its potency. Liraglutide delivered as a synthetic peptide is able to maintain its oligomerization state in dried lyophilized powders, acting as a memory effect from its synthetic process and purification. Through Liraglutide's oligomer memory effect, we demonstrate the importance and impact the process for synthetic peptides can have on drug development spanning from discovery to formulation development.



KEYWORDS: *fibrillation, aggregation, secondary structure, size exclusion chromatography, CD spectroscopy, bioassay*

Peptides are important and complex modalities for pharmaceutical development, and approvals of new molecular entities for therapeutic treatments have rapidly risen over the past decade.[1−3] Continued breakthroughs in chemistry for peptides and proteins will enable greater exploration of an expanded chemical space for developing new therapeutics.[4] A key structural element common to peptide based therapeutics is the addition of a conjugate to the peptide core to enhance its pharmaceutical properties. Some benefits of conjugation can include tuning the peptide's in vivo half life or improving solubility.[5] In addition to enhancing pharmaceutical properties, incorporation of conjugates can significantly impact the physical state and stability of the peptide in the final formulated drug product ultimately dosed in patients. Over the course of a formulated peptide's shelf life, there is potential risk that the peptide may have a propensity to aggregate or form fibrils.[6] These pathways of physical instability are undesirable due to their potential risk of immunogenic responses and impacting bioavailability and efficacy.[6,7] The complex structural landscapes available to peptides and their associated risks necessitate that robust analytical methods are developed to characterize and under stand their stability to ensure stabile formulated drug products are delivered to patients.

A major and currently expanding class of therapeutic peptides are those that target the GLP 1 receptor for the treatment of diabetes.[8−11] Several peptides that are marketed or in development are conjugated to allow dosing for different durations ranging from once daily to once weekly.[11] For example, the GLP 1 agonist Liraglutide's primary structure mimics the natural GLP 1 hormone with the exception of a single amino acid mutation at its N terminus and the inclusion of a C16 acyl conjugation (Figure S1). The natural GLP 1 peptide hormone, that which several therapeutic peptides resemble, is known to have a complex structural landscape including a high propensity to aggregate and form fibrils.[12−14] Recently, it was shown that the GLP 1 peptide is one of a few known peptides reported in the literature to have unique pH dependent fibrillation properties.[14] Generally, peptides that undergo fibrillation do so in a concentration dependent manner where increasing peptide concentration results in enhanced fibrillation kinetics.[15,16] In stark contrast, the GLP 1 peptide shows an inverse concentration dependence at pH < 7, where it has a tendency to form fibrils more rapidly at low concentrations. Beyond the unique aspects of the GLP 1 peptide's pH dependent fibrillation properties, Liraglutide has been shown to form soluble oligomers that vary in size as a

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function of pH.[17] Similar to the GLP 1 fibrillation concentration dependence, the oligomerization state of Liraglutide differs depending on if the solution pH is acidic or basic.

Here, we sought to probe whether Liraglutide, with the addition of its conjugation and pH dependent oligomerization, maintains the unique fibrillation properties that have recently been identified for GLP 1. To characterize Liraglutide, we have deployed an analytical toolkit including size exclusion chromatography (SEC) coupled with light scattering detection, kinetic fibrillation/aggregation experiments, transmission electron microscopy, circular dichroism spectroscopy, and bioassay. Through our characterization studies, we show that similar to GLP 1, Liraglutide exhibits unique concentration dependent fibrillation and aggregation kinetics as a function of pH.[14] During development of our characterization techniques, it became apparent that control of Liraglutide's pH dependent oligomerization state was crucial for its physical stability. Our studies show that Liraglutide is able to maintain its oligomer state in lyophilized powders, the form in which most synthetic peptides are delivered. Liraglutide's oligomer states can significantly impact its propensity to form fibrils, aggregate, and its biological potency. Previous work on recombinant GLP 1 peptides has shown the synthetic process can have impacts on solubility and generation of undesirable aggregates.[18,19] Given Liraglutide's oligomer memory, our studies highlight the importance of controlling and understanding the process used in synthetic peptide synthesis and purification and its potential downstream impacts that span from discovery to preclinical development and formulation activities.

EXPERIMENTAL SECTION

Materials. Liraglutide was purchased from Bachem (Torrance, CA) with a potency of 97.5% and used without further purification. The Bachem sourced Liraglutide was used for all studies with the exception of those specifically noted. Liraglutide was purchased from Achemblock (Burlingame, CA) with a purity of 99.4% and used without further purification. Liraglutide peptide powder was reconstituted with 50 mM sodium phosphate adjusted to the desired pH with sodium hydroxide. All other chemicals used in this study were reagent grade or better.

Size-Exclusion Chromatography. Experiments were carried out on a variety of HPLC/UHPLC systems including Waters Acquity UPLCs, Aglient 1290s, and Agilent 1200s controlled using Empower 3. The columns used for SEC experiments included the Sepax Unix C 300 Å pore size 4.6 × 150 mm (UHPLC) and Sepax SRT C 300 Å pore size 7.8 × 300 mm (HPLC). The mobile phase was 10 mM sodium phosphate at a pH of 6.4 or 8.1. Note, because of the slow kinetics of the oligomer transition, the populations of States A and B were not significantly impacted by the mobile phase pH. Flow rates were 0.3 and 1.0 mL/min for UHPLC and HPLC, respectively. The LC sample tray was set to 5 °C for all sample analysis with the exception of 25 °C for incubation studies. UV detection for UHPLC/HPLC was carried out at 280 nm, and corresponding peak areas were used for oligomer population determination. HPLC SEC experiments included online detection of multiangle light scattering (MALS), quasielastic light scattering (QELS/DLS), and refractive index using Wyatt Dawn Heleos II, Wyatt Nanostar, and Wyatt Optilab Trex detectors. We measured and used the refractive index increment of dn/dc = 0.186 mL/g for Liragluitde (matches within 2% of a recent study) with a Wyatt Optilab Trex.[17] Analysis of light scattering data was carried out using Wyatt ASTRA 6.1.7.15.

Lyophilization. Liraglutide samples were prepared by reconstituting the Liraglutide lyophilized powder with 50 mM sodium phosphate at the desired pH (6.4, 6.7, 8.1). Samples were allowed to incubate at 25 °C for 43 h to achieve their equilibrium oligomer distribution. The equilibrated samples were then lyophilized using an SP Scientific VP 60X lyophilizer. The lyophilized samples were reconstituted with water and immediately assayed by SEC.

Fluorescence and Kinetic ThioT Experiments. Liraglutide samples were prepared by reconstituting the lyophilized peptide with 50 mM sodium phosphate at the desired pH (6.4−8.1) at a concentration of 8 mg/mL with gentle stirring until the peptide went into solution. The additional concentrations of 1−4 mg/mL were prepared by diluting the 8 mg/mL Liraglutide stock with 50 mM sodium phosphate at the desired pH. Samples were then transferred to a 96 well plate with a total sample volume of 200 μL with 5 μM thioflavin T (ThioT). Physical stress and fluorescence detection (excitation = 440 nm and detection = 480 nm) were carried out using a Spectramax M2. Samples were constantly stressed by shaking at 25 °C, and ThioT fluorescence was recorded every 5 min.

Transmission Electron Microscopy. Images of peptide fibrils were obtained on an FEI Tecnai Spirit Biotwin transmission electron microscope at a voltage of 120 kV. All peptide solutions were diluted to 1 mg/mL as necessary with water. A 5 μL aliquot of the 1 mg/mL peptide solution was deposited on the surface of a 200 mesh carbon coated copper grid. After 1 min, excess liquid was blotted away with filter paper, and the grid was rinsed briefly with 5 μL of water. The rinsewater was wicked away with filter paper, and 5 μL of 1% uranyl acetate was then added to the grid as a negative stain to enhance contrast. After 1 min, excess stain was blotted away, and the grids were imaged immediately.

Circular Dichroism Spectroscopy. Circular dichroism spectra were acquired on a Chirascan qCD Spectrophotometer (Applied Photophysics, Surrey, UK). The temperature was maintained at 25 °C during the course of the measurement, consisting of two repetitions scanning from 200 to 280 at 1 nm bandwidth. Solid Liraglutide was dissolved to a concentration of 1 mg/mL in 50 mM sodium phosphate at the desired pH value (6.4, 6.7, and 7.2). These solutions were allowed to stand at room temperature over the course of the experiments. Aliquots (75 μL) were removed from the solution and further diluted 4× with buffer to a final concentration of 0.25 mg/mL before acquisition of CD spectra. This dilution was necessary to bring the total absorbance below 1 AU for the entire spectral range. The final time course spectra were normalized to the initial time point before comparison. For the experiments pertaining to addition of organic solvent, the same procedure was followed with the exception of the initial Liraglutide solutions, which were made with 10% of either EtOH or TFE. For analysis of kinetic data, we assumed Liraglutide CD spectra to be a linear combination of States A and B

$$\theta_{exp} = \alpha \cdot \theta_A + \beta \cdot \theta_B \quad (1)$$

where θ_{exp} is the experimental CD spectrum, α is the percentage of State A, θ_A is the State A reference CD spectrum, β is the percentage of State B, and θ_B is the State B

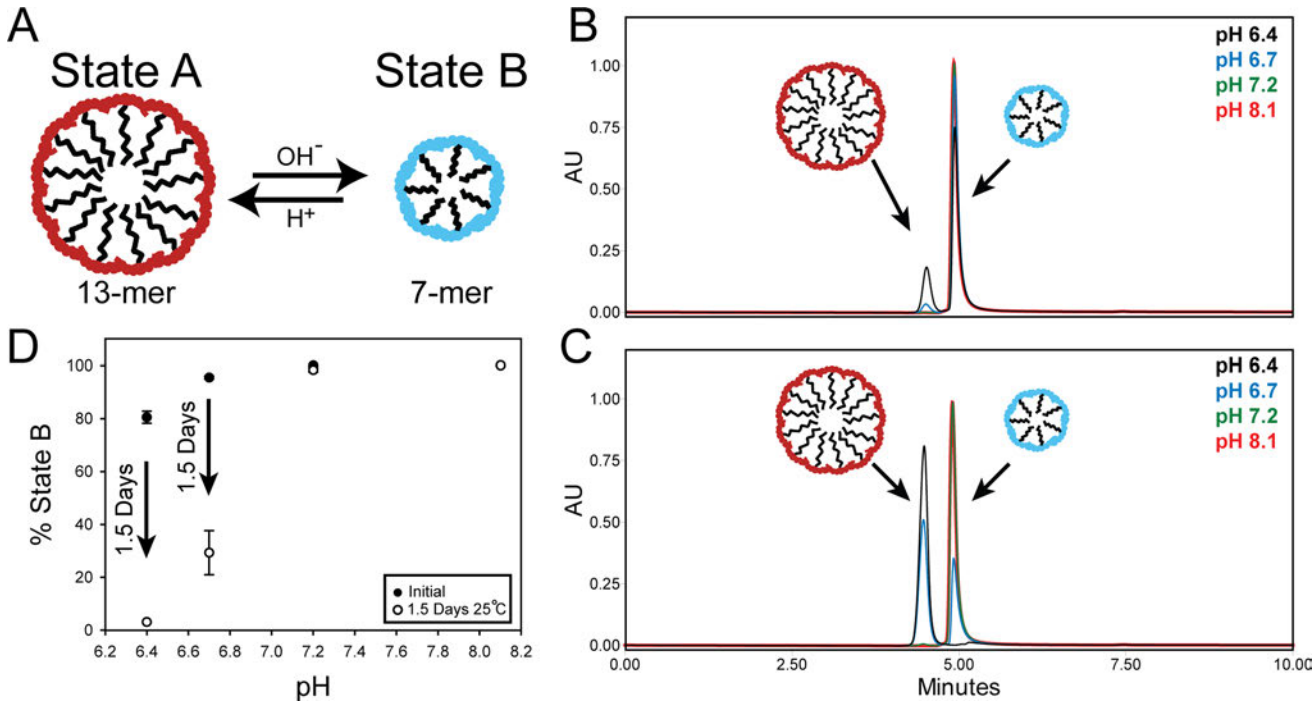

Figure 1. Characterization of Liraglutide oligomerization by SEC. (A) Liraglutide exists as two different oligomerization states that are stabilized by acidic or basic conditions. (B) SEC chromatograms of 4 mg/mL Liraglutide solutions at pH 6.4−8.1 recorded immediately after preparation. (C) SEC chromatograms of 4 mg/mL Liraglutide solutions at pH 6.4−8.1 recorded after incubation at 25 °C for 1.5 days. (D) Population of State B determined by SEC peak areas immediately after preparation (filled circles) and after incubation at 25 °C for 1.5 days (open circles).

reference CD spectrum. Eq 1 was fit using the "Non LinearModelFit" function built into Wolfram Mathematica 8.

Cell Based Potency Assay. The bioassay method used was based on Cisbio's HTRF technology (Homogeneous Time Resolved Fluorescence) for quantitative measurement of cyclic AMP using Cisbio's cAMP Dynamic 2 reagents with Chinese hamster ovary (CHO) cells stably expressing the glucagon like peptide 1 receptor (CHO GLP1R) cells. As a G protein coupled receptor, GLP 1 receptor mediated signaling involves activation of the adenylate cyclase component of GLP 1 receptor, which increases the level of cAMP (adenosine 3′,5′ cyclic mono phosphate).[20]

The bioactivity of samples was tested in a bioassay immediately after preparation of Liraglutide samples at pH 6.4 and 8.1 that primarily populate State B. The pH 6.4 sample was in a kinetic oligomer distribution at pH 6.4, primarily populating State B (Figure 1D). Bioactivity was also tested for samples that had been allowed to incubate at 25 °C for 3 days. Here, the pH 6.4 sample had achieved equilibrium primarily populating State A. A freshly prepared sample at pH 8.1 was used as a reference to compare the potency values of the samples at pH 6.4 (immediate and 3 day, 25 °C) and 8.1 (3 days, 25 °C). Percent relative potency values (reference IC_{50}/sample IC_{50} × 100) were determined from the comparison of the reference and test sample using a 4 PL curve fit. The IC_{50} value obtained from the curve represents the concentration of Liraglutide that inhibits 50% of the maximum response.

RESULTS AND DISCUSSION

Characterization of Liraglutide Oligomerization. It was recently reported that Liraglutide can populate two oligomerization states that are stabilized by varying pH (Figure 1A).[17] At pH < 7, Liraglutide was shown to exist as a 12 mer by dynamic light scattering (DLS) and static light scattering (SLS). While under basic conditions, Liraglutide has been shown to exist as either an 8 mer[17] or 7 mer[21] depending on the technique used (light or small angle X ray scattering (SAXS)). While DLS, SLS, and SAXS were able to provide deep insights into the oligomerization of Liraglutide, the interpretation and deconvolution of light/X ray scattering data has significant limitations for cases such as Liraglutide, where it is possible that multiple states with differing size can simultaneously exist in solution at near neutral pH.[22−25]

To overcome challenges inherent to light scattering measurements of bulk solutions, we sought to develop a size exclusion chromatography method able to separate the oligomerization states of Liraglutide. Achieving separation of the oligomerization states would allow for accurate determination of their populations and enable additional on or offline analysis by techniques such as light scattering or mass spectrometry. Separation of conjugated peptides is notoriously challenging due to their conjugation chemistries having a tendency to cause undesirable secondary interactions with column stationary phases. This is particularly the case for acylated peptides where a greasy carbon chain (C16 for Liraglutide) is covalently attached. Addition of organic solvents in some cases can help to minimize secondary interactions but are not ideal, because they could disrupt or generate aggregates/oligomers on column. Initial SEC method screening with a pure aqueous mobile phase showed a challenge in achieving conditions that minimized secondary interactions between Liraglutide and the stationary phase (Figure S2). Through our screen, we identified that by utilizing Sepax "C series" columns (UHPLC/HPLC) we could achieve conditions with minimal secondary interactions, good peak shape, and most importantly separation of the oligomerization states of Liraglutide. The stationary phase of these columns has a

proprietary lay down nanometer film chemically bonded to the silica particles likely minimizing Liraglutide's undesirable secondary interactions.

With the Sepax SEC method in hand, we were able to demonstrate the separation of two distinct oligomerization states (Figure 1B) that are stabilized as a function of pH consistent with a recent report by Wang et al.[17] State A is stable at pH < 7, and State B is stable at pH > 7 (Figure 1A). Separation of the individual states allowed us to probe their structure with online DLS and MALS detection (Figure 1B). The hydrodynamic radius for each state ($R_{h,A}$ = 3.4 ± 0.3 nm and $R_{h,B}$ = 2.6 ± 0.2 nm) was in agreement with the radii recently reported for each species by bulk DLS,[17] confirming that the oligomerization states of States A and B observed on column are the same as in bulk solution. Molecular weight analysis of the individual species using MALS showed that State A exists as a 13 mer (13.0 ± 0.5), and State B exists as a 7 mer (6.7 ± 0.6), consistent with the sizes recently reported as 12 mer (State A) and 8 mer or 7 mer (State B).[17,21] The differences observed in reported molecular weights are likely attributable to error and analysis differences between SEC MALS, bulk SLS, and SAXS. The combined light scattering data supports that the species observed by SEC are representative of the oligomers in bulk solution. Further, it was previously reported that the kinetics of the transformation between States A and B is slow, occurring on the time scale of days.[17] Our SEC results were consistent with pH driven transitions being slow on the SEC time scale, where the mobile phase pH did not significantly impact the populations of States A and B calculated from the oligomer peak areas.

Surprisingly, upon preparation of Liraglutide samples expected to favor State A at pH 6.4 by reconstitution with sodium phosphate buffer, we observed State B to be the initial primarily populated state (Figure 1B). After the samples were allowed to equilibrate at room temperature for several days, the system achieved equilibrium with Liraglutide transitioning to fully populate the thermodynamically favored State A (Figure 1C). We tested synthetic Liraglutide sourced from another vendor and saw a similar effect (Figure S3). The observation of an initial kinetic distribution of Liraglutide upon reconstitution of the lyophilized peptide powder led to the hypothesis that either the thermodynamically unfavored State B could be kinetically favored upon hydration of the dry powder or that the Liraglutide oligomer immediately populated in solution is a memory effect from its prelyophilized state. In order to distinguish between the two scenarios, we prepared Liraglutide solutions that populated different distributions of States A and B (pH 6.4, 6.7, 8.1) and lyophilized the solutions after they had achieved their oligomer thermodynamic equilibrium. Similar to our initial experiment, we assayed the oligomer distribution immediately after reconstitution of the lyophilized samples with water. Interestingly, samples that had favored State B upon reconstitution of the "as is" purchased lyophilized powder now populated their thermodynamically favored oligomer distributions observed prior to our lyophilization (Figure 2). Through the lyophilization process, the oligomer distribution was not impacted by any sodium phosphate acidic pH swing that occurred during freezing[26] because of the slow oligomer transformation kinetics that are likely even slower at subzero temperatures. This demonstrates that Liraglutide has an oligomer "memory effect", where the oligomerization states in solution prior to lyophilization are maintained in the lyophilized powder and populated upon hydration into solution.

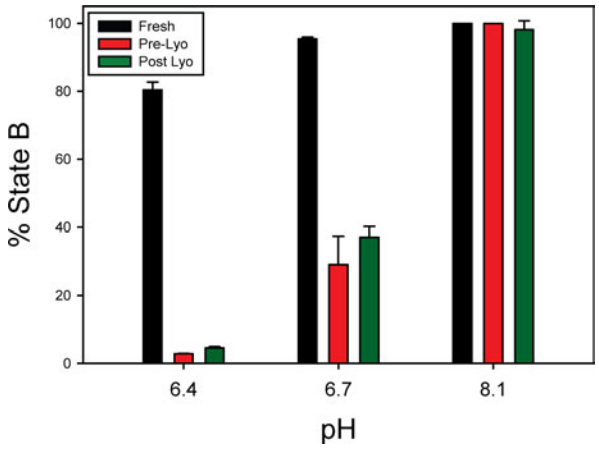

Figure 2. Populations of Liraglutide State B before and after lyophilization at varying pH. Fresh (black): Liraglutide population after reconstitution of the peptide powder with 50 mM sodium phosphate; Pre Lyo (red): Liraglutide State B population after incubation at 25 °C for 43 h prior to lyophilization; Post Lyo (green): Liraglutide State B population immediately after reconstitution of the Lyo cake with water.

Oligomerization Impact on Physical Stability. Given our observation of the oligomer "memory effect", we sought to probe the impact oligomer states could have on the physical stability of Liraglutide. To examine the physical stability and tendency to form fibrils and aggregate, we carried out studies catalyzing fibrillation and aggregation with physical stress using ThioT fluorescence as a reporter of fibril formation. Liraglutide stress studies were done using concentrations of 1−8 mg/mL at pH 6.4−8.1 employing physical stress by shaking at 25 °C. Shown in Figure 3 are the resulting fluorescence profiles for stressed Liraglutide at 25 °C for pH 6.4 and 8.1 where fluorescence increases upon ThioT binding fibrils or aggregates. Liraglutide exhibits an interesting concentration dependence at pH 6.4 (Figure 3A) where fibrillation occurs more rapidly as concentration decreases. This is similar to a recent report on the natural GLP 1 hormone, for which a similar concentration dependence is observed at pH < 7.[14] It is interesting that Liraglutide is able to maintain this unique property despite the addition of a bulky conjugate, mutation at the N terminus, and formation of oligomers in solution. This rare inverse concentration dependence has only been reported for a few peptides.[14,27,28] At a basic pH of 8.1 (Figure 3B), Liraglutide exhibits more rapid fibrillation with increasing concentration, which is the classical concentration dependence typically observed for fibrillation.[15,16,29]

In addition to the unique concentration dependence observed at pH 6.4, the oligomerization state of Liraglutide plays a significant role in its tendency to form fibrils and aggregate. Utilizing our knowledge of Liraglutide oligomerization from SEC and our ability to control the populations of each oligomer, we carried out studies to probe the impact of the initial oligomer state of the system (kinetic or equilibrium) on aggregation and fibrillation. First, we compared the ThioT profile for Liraglutide samples immediately prepared at pH 6.4 that exist in a kinetic oligomer distribution where State B is primarily populated (populated at ~80%) and samples that had been allowed to achieve equilibrium fully populating State A prior to stress. As shown in Figure 3A, the ThioT profiles are

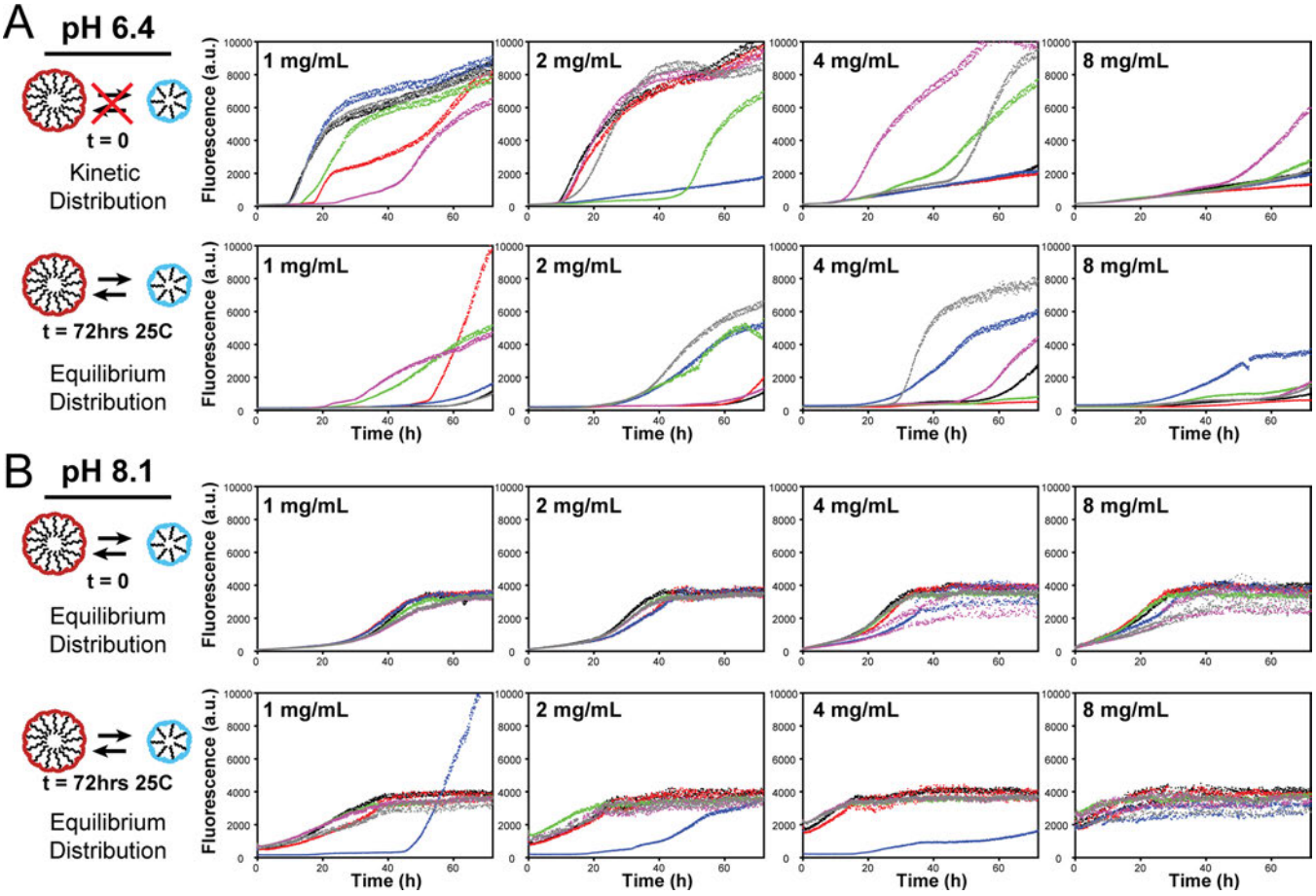

Figure 3. Fibrillation/aggregation of 1−8 mg/mL Liraglutide solutions upon immediate sample preparation and after incubation at 25 °C for 3 days. Six replicates were recorded, and each replicate is a different color. (A) ThioT fluorescence profile recorded during physical stress of Liraglutide solutions at pH 6.4 immediately after preparation (top) and after incubation at 25 °C for 3 days (bottom). (B) ThioT fluorescence profile recorded during physical stress of Liraglutide solutions at pH 8.1 immediately after preparation (top) and after incubation at 25 °C for 3 days (bottom).

drastically different between the samples in kinetic or equilibrium distributions particularly at low concentrations. Samples that were stressed while in a kinetic oligomer distribution at 1 mg/mL underwent more rapid changes in fluorescence than those that were allowed to achieve their equilibrium state prior to stress (Figure 1). We also observed a similar effect at pH 6.7 where samples stressed beginning with a kinetic distribution had much more rapid increases in fluorescence compared to those with an equilibrium oligomer distribution (Figure S4). In contrast to the samples that contain State A (pH 6.4 and 6.7), samples at a pH > 7 that exclusively populate State B upon reconstitution and at equilibrium became less stable as the samples aged. At pH 8.1, samples stressed immediately after preparation underwent ThioT fluorescence changes slower than samples allowed to incubate at 25 °C for 3 days (Figure 3B). SEC MALS analysis of pH 8.1 samples aged at 25 °C for 3 days showed that high molecular weight aggregates were present at the initiation of the fibrillation experiments, having formed during the incubation period. It is possible that these aggregates could give rise to the higher initial fluorescence and more rapid fibrillation. Liraglutide samples at pH 7.2, only populating State B, also showed decreasing stability after the samples had aged for 3 days (Figure S4).

These combined data from pH 6.4−8.1 show that Liraglutide's fibrillation and aggregation characteristics are modulated both by the populations of each oligomer state and whether the system is in thermodynamic equilibrium prior to initiating physical stress. Systems (pH 6.4 and 6.7) with a kinetic oligomer distribution were significantly less stable where they were undergoing oligomer conformational trans formations during physical stress. The ThioT profiles are complex particularly at low pH having variable lag times, multiple phases in fluorescence increase, and a lack of signal plateauing. These complexities preclude global fitting of the data to a specific fibrillation or aggregation mechanism.[30] However, it is clear at low pH that when the oligomer distribution is not at equilibrium, Liraglutide has enhanced physical instability with an inverse concentration dependence. When under kinetic oligomer conditions, transformation from State B to State A may enable faster fibrillation and aggregation because of the presence of a catalyzing intermediate state. The inverse concentration presence for fibril formation could be from high concentrations of State B that play an inhibitory role in the fibrillation/aggregation process favored at acidic pH's.

The unique ThioT profiles observed for Liraglutide necessitated a deeper look with orthogonal methods to better understand the species that had formed upon stress. Increases in ThioT fluorescence are indicative of conformational

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