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Comparison of free solution capillary electrophoresis and size exclusion chromatography for quantitating non-covalent aggregation of an acylated peptide

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Abstract

There are few methods available for the rapid and precise quantitation of non-covalent aggregation. The very methods used to measure the aggregation can easily disrupt the weak forces holding an aggregate together. This paper describes the novel application of free solution capillary electrophoresis (CE) for the quantitation of a biologically inactive non-covalent aggregate of C8GLIP (Des-amino-histidine-7-arginine-26 N^{ε}-octanoyl-lysine-34-human glucagon-like insulinotropic peptide), an acylated peptide. The CE results are compared to a more traditional approach using size exclusion chromatography (SEC). Under the conditions explored in this paper, SEC showed a significantly slower apparent rate of aggregation than CE. This is due to the disruption of the aggregate during the SEC process. The cause of the disruption is complex and is potentially related to the separation process itself, on-column dilution effects, and/or interactions of the aggregate with the column packing or SEC components. Analysis times and dilution are greatly reduced by CE, and, because there is no potentially interactive stationary phase and because both the protein and the walls of the capillary are negatively charged, potential disaggregation due to surface interactions is reduced. Thus, CE is shown to be superior to SEC for this peptide in that disruption of the aggregate is minimized.

Keywords: Aggregation; Capillary electrophoresis; Size exclusion chromatography; GLP-1; Protein; Peptide

1. Introduction

Aggregation is one of the major degradation pathways that has been widely reported for a large number of proteins [1–4]. Aggregation, as defined in these reports, describes a number of physical and chemical states of association including covalent and non-covalent interactions. Because aggregation potentially can affect therapeutic activity and/or cause immunological reactions in patients using protein products, methods to assess the degree of aggregation are required to assure the safety and potency of these protein drug products. A number of methods have been successfully used to monitor and/or

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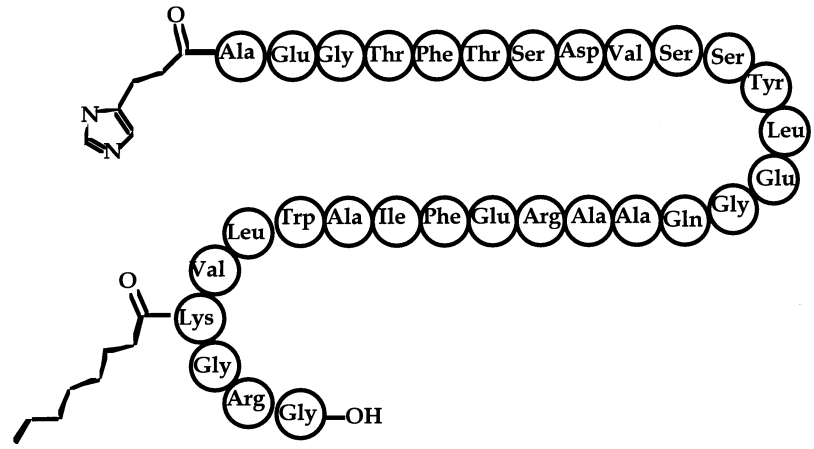

Fig. 1. The structure of des-amino-histidine-7, arginine-26 N^{ε}-octanoyl-lysine-34-human glucagon-like insulinotropic peptide(7-37)-OH (C8GLIP).

quantitate covalent aggregation. These include dynamic light scattering, low angle and multiangle laser light scattering, ultracentrifugation, viscometry, reversed phase chromatography, gel electrophoresis, and size exclusion chromatography (SEC). Light scattering and ultracentrifugation can provide excellent information on the aggregate size, but offer only a limited amount of quantitative information concerning the molecular weight distribution in a sample [1,2]. Because of their ease of use and ability to separate and quantitate aggregates of various sizes, reversed phase chromatography [3] and, to a much greater extent, size exclusion chromatography have been widely been used for the analyses of covalent aggregation states [4].

However, non-covalent associations formed as a consequence of much weaker intermolecular interactions (e.g. electrostatic, hydrophobic, and hydrogen bonding), are much more difficult to quantitate. Several authors have reported the difficulties of using chromatography for such aggregate and/or micelle quantitation [3–9]. Disaggregation has been reported due to on-column dilution in the chromatographic technique, disturbing the monomer:aggregate ratio. The on-column dilution depends on the size and type of the column used and the time on-column [10]. If the rate of disaggregation is fast when compared to the time scale of the separation, the aggregation levels may be seriously altered [10,11]. Interactions with the SEC column itself may disrupt the aggregation state, [10,12] and are difficult to eliminate [13,14]. Various methods have been used to alleviate the deficiencies described above, including reducing on-column time and dilution effects by increased flow rates and/or decreased column lengths [10–12].

CE has recently been used for studying non-covalent association. It has been applied to the determination of the critical micelle concentration for sodium dodecyl sulphate [15], to the characterization of liposomes [16], to the determination of the dimerization constants for glycopeptide antibiotics [17], to the folding pathway for trimeric P22 tailspike [18], and to the evaluation of drug-protein binding constants[19,20]. To our knowledge, free solution CE has not previously been applied to the quantitation of non-covalent protein aggregates.

C8GLIP (Des-amino-histidine-7-arginine-26 N^{ε}-octanoyl-lysine-34-human glucagon-like insulinotropic peptide) (Fig. 1), is a potential therapeutic agent for the treatment of type II diabetes. Evidence suggests that it can exist in various association states that are strongly dependent on the concentration, ionic strength, and temperature in the solution state. In 5 mM phosphate buffer, pH 7.5, C8GLIP is an α-helical form with a tetrameric association state. In phosphate-

buffered saline (PBS) the secondary structure is mainly β-sheet with an association state that is highly temperature dependent. The α-helical form is bioactive. The β-sheet form is initially bioactive with a slightly longer biological time of action than the α-helical state. Finally, the β-sheet form can form a highly aggregated biologically inactive form [21].

In this paper, we describe the separation and quantitation of the biologically inactive non-covalent aggregate of C8GLIP from the non-aggregated form, using SEC and CE, and compare the results and suitability of the two techniques.

2. Experimental

Size exclusion chromatography was performed using Shimadzu HPLC gradient equipment including a Sil 10 autosampler, two 10AT gradient pumps, and a SPD-10A UV detector. Five different SEC columns were investigated including a Toso Haas TSKG4000 SWXL 300×7.8 mm (MW range 20–7000 kDa), a Toso Haas TSK G6000 PWXL 300×7.8 mm (MW range $<$ 200 000 kDa), a Pharmacia Superose 6 10/30 (MW range 5–5000 kDa), a Pharmacia Superose 12 10/30 (MW range 1–300 kDa), and a Zorbax GF250 9.4×250 mm (MW range 4–400 kDa). The detector was set at 214 nm (except as noted) and the flow rate was typically 1 ml min^{-1} with a mobile phase 14 mM sodium phosphate dibasic adjusted to pH 7.4 with phosphoric acid (this is equivalent to 16 mM phosphate).

Capillary electrophoresis was performed on a Beckman P/ACE 5000 using a 25 μm $\times$ 47 cm silica capillary (except as noted) at a temperature of 23°C. The capillary was rinsed 5 min with 0.1 N NaOH daily and 1 min with run buffer between injections. The run buffer was 16 mM phosphate pH 7.4 (identical to the SEC mobile phase). The running voltages were 25–30 kV (except as noted). The detector was set at 214 nm and the sample injections were 20–30 s using the pressure (0.5 psi) mode (except as noted). The CE peak areas were normalized by dividing by the migration times.

Dynamic light scattering studies were performed using a Protein Solutions DynaPro-801 instrument. All samples were filtered with 0.2 μm Whatman, Anotop 10 inorganic membrane filters prior to analyses. The light scattering intensity was normalized versus the light scattering intensity at 24 h. This is the light scattering intensity at 100% aggregation.

The C8GLIP was typically prepared at a concentration of 2 mg ml^{-1} in the mobile phase/running buffer. Samples were incubated for various times using the SIL10 autosampler set at the appropriate temperature.

3. Results and discussion

3.1. Comparison of initial SEC results and CE results

A typical SEC chromatogram showing the change in aggregation with time for a 2 mg ml^{-1} sample at 23°C is shown in Fig. 2. Note that the aggregate peak is totally excluded from the pores of the packing and elutes at the exclusion limit for this column, equivalent to a molecular weight of $\geq$ 400 000. A typical electropherogram of a similarly prepared sample of C8GLIP is shown in Fig. 3. In CE, the separation is based on the differences in mobility. The large size of the aggregate is evidently more than compensated by its greater negative charge, resulting in greater mobility in opposition to the electroosmotic flow; thus, the aggregate elutes after the monomer.

As seen in Fig. 3, the peak shape by CE (as with SEC), particularly of the 'monomer', is poor. It is, however, consistent and readily integrated. No modifications were made to the run buffer or sample solvent to improve peak shape (e.g. via stacking) since our intent was to have no changes in run buffer which could impact the rate of aggregation, and to keep the SEC mobile phase and CE run buffer identical to each other. There is a shoulder on the later migrating peak in CE (e.g. 2.2 and 3.0 h timepoints in Fig. 3), which may indicate that the 'aggregate' represents more than one species. However, our focus in this paper was comparison of the rate of aggregation as

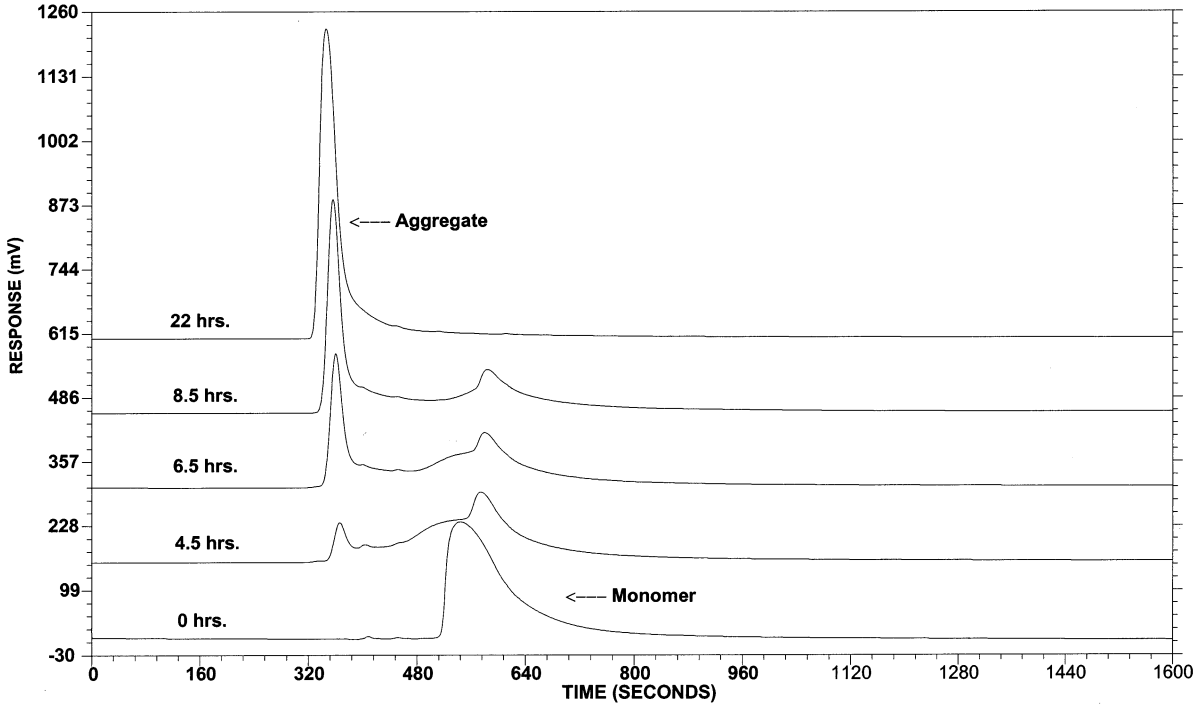

Fig. 2. Typical chromatograms from a time-course study showing the formation of aggregate for a 2 mg ml^{-1} sample in 16 mM phosphate pH 7.4 at 23°C on a Zorbax GF250 column.

ascertained by CE versus SEC, and we therefore integrated the 'monomer' and 'aggregate' peaks as complete entities for ease of comparison between the two techniques. It may be that CE is capable of separating intermediate species which are not distinguished by SEC; however, that potential is not investigated further here. Finally, there are some very small unidentified peaks between the two major peaks by CE (Fig. 3). At no point were these peaks significant contributors to the total area and so they were not integrated.

A time-course study at room temperature (23°C) for a 2 mg ml^{-1} sample was run using SEC and CE. As can be seen in Fig. 4, the SEC curve for aggregation lags significantly behind the curve obtained using CE. The CE shows a much faster rate of aggregation and a slightly higher aggregation end-point. Various experimental parameters were therefore investigated to evaluate and better understand the differences obtained by SEC and CE, and to determine which was giving more accurate results.

3.2. Choice of sample solvent and running buffers

16 mM phosphate pH 7.4 was initially chosen as a potential formulation diluent. Since we were interested in the aggregation state under the potential formulation conditions, the phosphate buffer was used as the sample diluent for all of our studies. In addition, to minimize the effects on the monomer:aggregate equilibrium, the phosphate buffer was used as the SEC mobile phase and the CE running buffer.

Organic modifiers (such as acetonitrile, isopropanol, or methanol) have typically been used to minimize hydrophobic interactions with the SEC packing [13]. However, these modifiers will also rapidly disrupt the C8GLIP hydrophobic aggregates. For example, adding 30% acetonitrile to the SEC mobile phase causes initially aggregated and non-aggregated samples to co-elute. Since it was known that the non-covalent forces holding the aggregate together are easily disrupted, it was decided to match the eluting condi-

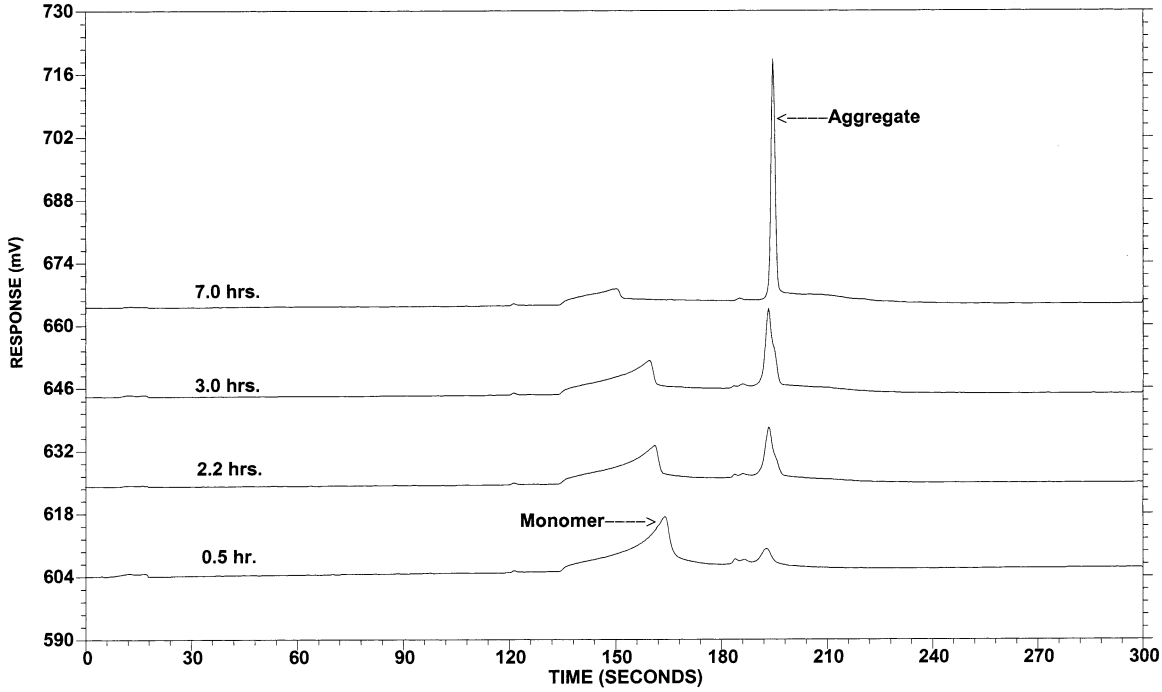

Fig. 3. Typical electropherograms from a time-course study on a 25 μm × 47 cm silica capillary at 30 kV showing the formation of aggregate for a 2 mg ml^{-1} sample in 16 mM phosphate pH 7.4 at 23°C.

tions as closely as possible to the sample solvent to minimize potential solvent effects. These facts led to the choice of the phosphate buffer without organic modifier.

3.3. Confirmation that SEC is separating aggregates

Fractions were collected of the monomer and aggregate peaks from SEC (Fig. 2) and assayed by dynamic light scattering in order to confirm that the peaks corresponded to C8GLIP aggregate and monomer respectively. The early eluting peak showed a mean aggregated radius of 22 nm corresponding to an estimated molecular weight of approximately 5 000 000 Da. The later eluting peak showed a hydrodynamic radius of less than 1 nm (limit of determination) corresponding to a molecular weight of less than 8000 Da. When 30% ACN was added to the aggregated fraction, DLS showed a resulting hydrodynamic radius of less than 1 nm.

3.4. CE analyses of SEC fractions

To confirm that the aggregate peak as determined by SEC was equivalent to the aggregate peak as determined by CE, a partially aggregated sample was fractionated on the SEC system. The fractions were re-injected on the CE system and confirmed the correlation of aggregate and monomer peaks on the two systems.

In an effort to further explore the column effects upon aggregation, samples at various levels of aggregation were examined by CE before and after passing through the SEC column. A sample of C8GLIP was prepared at 2 mg ml^{-1} in 16 mM phosphate and incubated at 23°C. This sample was analyzed by CE and SEC after 0, 2, 3, 4, 5 and 22 h. (Typical SEC runs were done using 20 μl injections. However, to enhance sensitivity for post-column CE analyses of the samples, 100 μl injections were used for this experiment). Samples at each time point were injected onto the SEC system and fractionated at 1 min intervals over a

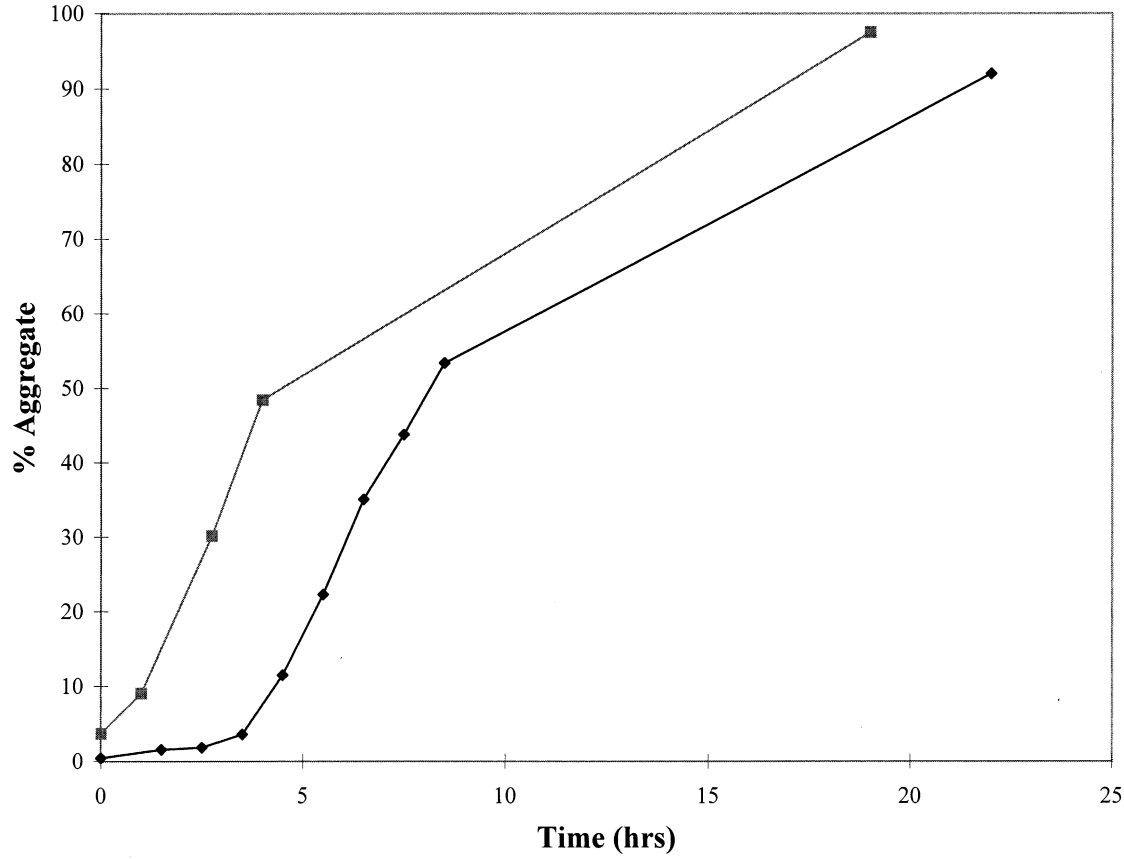

Fig. 4. Comparison of the rates of aggregation by capillary electrophoresis (■) and size exclusion chromatography (◆) for a 2 mg ml^{-1} sample in 16 mM phosphate pH 7.4 at 23°C. SEC and CE conditions were the same as those use in Figs. 2 and 3.

10 min period until all of the peaks had completely eluted. Thus, 10 fractions were collected at each time point. These fractions were analyzed by CE. An example of the resulting electropherograms for the 5-h time point is shown in Fig. 5. From the integrated CE peaks for all fractions, we could determine the apparent extent of aggregation of the samples before and after SEC (Table 1). The CE data shows that the extent of aggregation after passing through the SEC system was significantly less than was observed prior to SEC. Also, even the earliest-eluting SEC fraction consistently showed some levels of monomer. These data suggest that the SEC system itself is disrupting the aggregate.

A time-course study at 23°C was performed by CE on the first fraction (5-h timepoint) which had been collected from the SEC column. Within the first 30 min after collection from the SEC, the amount of aggregate in the fraction ranged from 39 to 31%; however, after 16 h, the fraction contained 54% aggregate and after 40 h it contained 77% aggregate. The results showed that the sample re-aggregated after removal from the SEC system despite the on-column sample dilution. Thus, dilution alone does not explain the disaggregation seen by SEC.

3.5. Effects of pressure and tubing

The effects of pressure have been reported to alter the aggregation state of some proteins [15]. To explore the possibility that the pressure encountered in SEC was promoting C8GLIP disag-

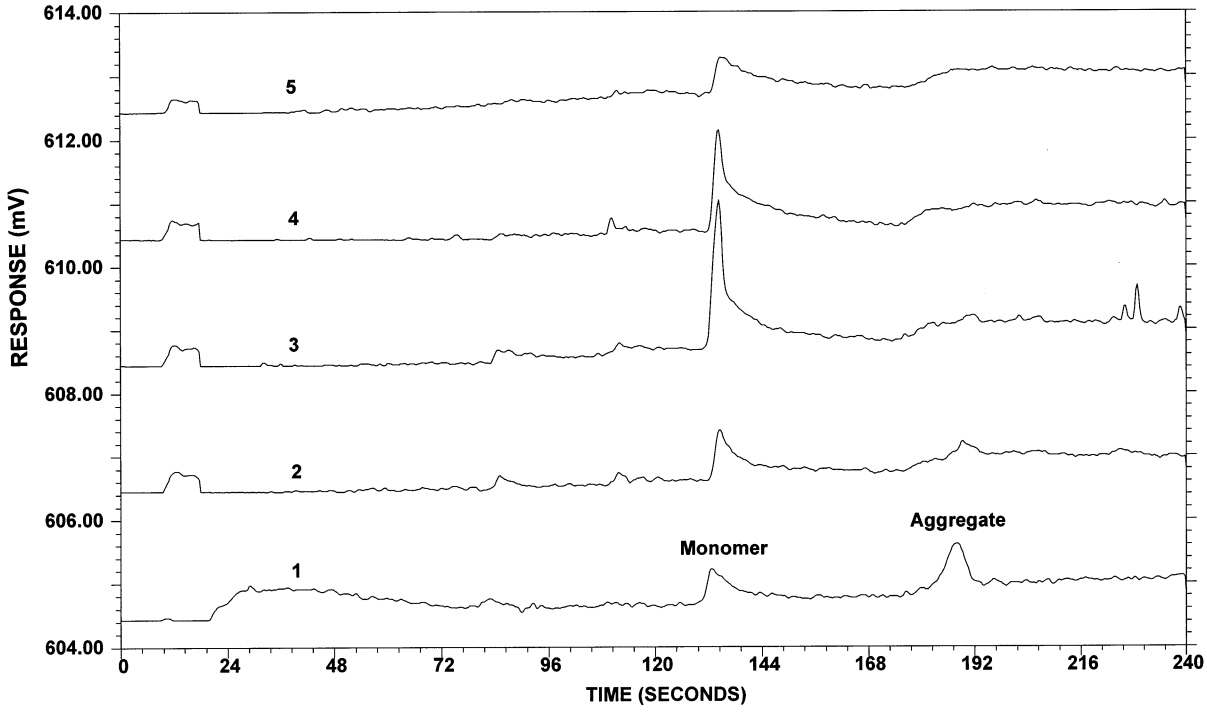

Fig. 5. Electropherograms of fractions 1–5 collected from the SEC system for the 5-h sample. Fraction numbers (collected at 1 minute intervals) are indicated. CE conditions were the same as those in Fig. 3, except $\lambda = 200$ nm, injection = 30 s (to increase sensitivity).

gregation, the SEC column was removed and a back-pressure restrictor was placed in line with the chromatographic pump and injector. The flow rate was adjusted to vary the pressure experienced by the sample. Two aggregated samples were prepared by placing a 2 mg ml^{-1} solution of C8GLIP at 23°C for 2 h (partially aggregated) and at 35°C for 20 h (fully aggregated). 100 μl of each sample was injected at three flow rates 0.4, 0.8 and 1.2 ml min^{-1}. This generated respective back-pressures of 70, 135 and 190 bar. (The typical back-pressure seen with SEC was about 70 bar.) The samples were collected as they eluted from the back-pressure restrictor (each within 1 min) and were examined by CE. In no instance was there a significant increase in the level of monomer detected. This suggests that pressure is not a significant cause of disaggregation for this sample.

Table 1
Extent of aggregation as found by CE

Time (h)	% Aggregate before SEC	% Aggregate after SEC (Total from all fractions)
3	35	~0 (n.d.)
4	45	3.7
5	57	9.5
21	99	82

The tubing or other SEC components apparently promote disaggregation of C8GLIP. A fully aggregated sample was injected onto the SEC system with the column replaced with a 4 ml stainless steel loop (no packing). The eluting peak profile was broad with a significant degree of tailing, indicating interaction of C8GLIP with the tubing or other components. Fractions were collected as they eluted from the system and monitored for aggregate formation by injection onto the CE system. The fractions as they eluted from the column showed steadily increasing percentage of monomer (from 3.1 to 28.9% for fractions 1

and 10, respectively. These data demonstrate that disaggregation occurs even in the absence of the column, showing that the tubing or SEC components may be significant contributors to disruption of the aggregate and retention of the monomer.

3.6. On-column load and dilution

As was previously noted, various authors have reported that non-covalent aggregates dissociate due to on-column dilution. Typically, the sample is diluted 20 to 40-fold on the SEC column. In CE, the aggregate is typically diluted 3 to 10-fold. Several experiments were performed to investigate the effects of dilution and on-column loads on the extent of aggregation as determined by SEC and CE.

To investigate the SEC results with respect to injection volume, on-column load, and sample dilution prior to injection, the following experiment was performed. An aggregated sample was prepared by storing a 2 mg ml^{-1} solution of C8GLIP in phosphate buffer at 35°C for 2 h. The SEC on-column load was varied by using injection volumes of from 1 to 400 μl, or by diluting the sample by a factor of from 2 to 400 and then injecting a constant 400 μl portion. The apparent % aggregate was determined from the SEC peak areas for each column load. The less sample injected on-column (lower on-column load), the lower the % aggregation: ranging from 77% to as low as 9% aggregate (Fig. 6). Variations in sample dilution or injection volume had much less effect if the on-column load was kept constant. The aggregated C8GLIP sample was diluted by factors of from 10 to 400. The on-column load was held constant at 2 μg per injection by using the appropriate injection volumes. The extent of aggregation ranged only from 9 to 18%. (Some of this difference can be accounted for by the lack of precision obtained at this low sample load). These results suggest that the on-column load is more critical to the apparent % aggregation than is sample dilution prior to SEC.

To further investigate the effects of sample dilution upon the aggregation state, a fully aggregated sample (22 h at 23°C) was diluted 50- and

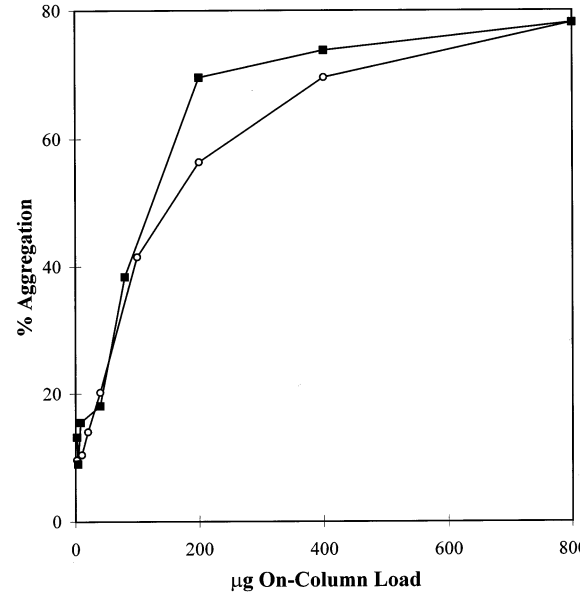

Fig. 6. Levels of aggregation as a function of SEC column load. The column load was varied by (a) injecting increasing volumes of sample at constant concentration (o), or (b) by injecting a constant volume (400 μl) of increasing concentrations (■). The sample was prepared at 2 mg ml^{-1} in 16 mM phosphate pH 7.4 and stored for 2 h at 35°C, then diluted as necessary for case (b).

200-fold. Conversion of the aggregate to monomer was monitored by CE. A gradual shift was observed for the 50-fold dilution in which the aggregate peak area diminished to about 80% of its initial value after 2 h while the monomer peak showed a corresponding increase. These levels did not change further over the next 2 h. The 200-fold dilution also showed a similar decrease in aggregate, although any monomer was below the detection limit. Thus, while some disaggregation occurred due to dilution, the rate was slow on the timescale of CE and SEC, and much remained aggregated.

As indicated above, sample load plays a major role in the % aggregation measured by SEC. In order to investigate the role of sample load in apparent aggregation by CE, the following experiments were performed. An aggregated sample was prepared at a concentration of 2 mg ml^{-1} in 16 mM phosphate by storing it at 35°C for 2 h. The CE on-capillary load was varied using injection

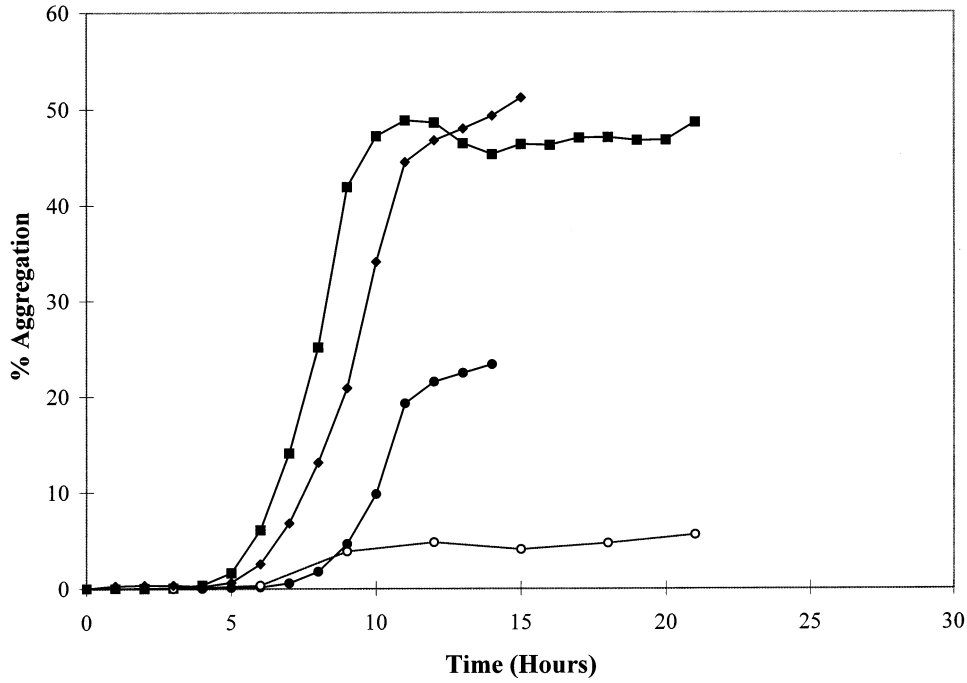

Fig. 7. Time course study showing the differences in the rates of aggregation at 23°C as determined by SEC on a Superose 12 column at different flow rates: 1.0 ml min^{-1} (■), 0.5 ml min^{-1} (●), 0.2 ml min^{-1} (○), and 1.0 ml min^{-1} with PEEK tubing (◆). The sample was prepared at 2 mg ml^{-1} sample in 16 mM phosphate.

times from 1 to 50 s. The % aggregate observed for all of these capillary loads ranged from 88.0 to 96.6%. To extend the lower end of the range loaded on the CE, the 2 mg ml^{-1} fully aggregated sample was diluted 50-fold. Again, the on-capillary load was varied using the following injection times: 2, 5 and 10 s. The data follows the trend that a smaller load yields slightly lower apparent % aggregate, but was still 84% or greater. This data is consistent with the data discussed above showing a slight increase in monomer levels upon dilution. These results showed that the variability due to sample loading is less significant for the CE method than for the SEC method.

3.7. Effects of time on-column

The dependence of the extent of aggregation on SEC flow rate was investigated. The % aggregation was monitored at three flow rates: 1 ml min^{-1}, 0.5 ml min^{-1}, and 0.2 ml min^{-1} on a Superose 12 column. Fig. 7 shows the time response curve for aggregation at the three flow rates. At reduced flow rates, both the apparent extent and rate of aggregation were greatly reduced, indicating significant disaggregation on-column.

For CE, the amount of time on-capillary was varied by adjusting the voltage at which the separation was run. A single sample (2 mg ml^{-1}) was monitored (% aggregation vs. time) at both 30 and 10 kV. The migration time of the aggregate was approximately three times longer at 10 kV (9.7 min vs. 3.2 min), but the % aggregation results were virtually identical.

Next, effects of the capillary length on the apparent rate of aggregation were explored. An aggregated sample was monitored using capillary lengths of 27, 37 and 57 cm (Fig. 8). Such increases in capillary length help to asses the impact of any interaction with the silica, and the impact of greater time of separation on the equilibrium. The results showed very little reduction in the apparent rate of aggregation in increasing the

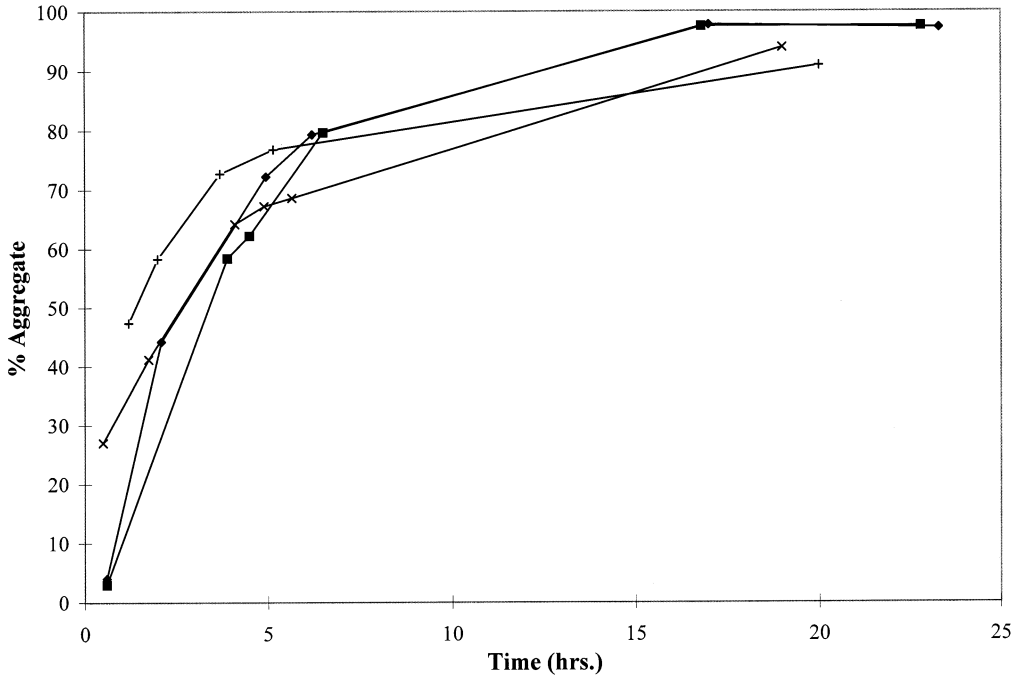

Fig. 8. Impact of CE capillary length on apparent rate of aggregation: 27 cm (◆ and +), 37 cm (■), and 57 cm (x). Evaluations were conducted on two days: day 1 (27 vs. 37 cm) and day 2 (27 vs. 57 cm). CE conditions as in the Experimental section (injection times ranged from 15 to 27 s, depending on day and capillary length). Samples (2 mg ml^{-1}) were stored at ambient conditions during the studies.

capillary length from 27 to 37 cm with a slight reduction observed for the 57 cm capillary. Thus, CE results do not appear to be distorted unless unnecessarily long capillaries are used, and are much less sensitive to time on-column than is the case for SEC.

3.8. Other factors affecting SEC performance

To further explore the potential relationship between the column packing material and disaggregation, various SEC columns were investigated. Table 2 lists the columns investigated and the levels of aggregation and recovery determined by SEC using 16 mM phosphate pH 7.4. Recovery was determined by measuring the total peak area for each of three samples (unaggregated, partially aggregated, and fully aggregated) with and without the column. Recovery from these columns (with the exception of Zorbax GF250 special) for all samples was greater than 88%. Aggregation levels in these samples were also determined by CE. In each case, the apparent % aggregation from SEC was significantly less than that determined by CE, indicating that all SEC columns used resulted in disaggregation. In addition, there was a large discrepancy among the different SEC columns in the % aggregation measured for the partially and fully aggregated samples. These results indicate that the column type can play a major role in the extent of aggregate disruption.

3.9. Other factors affecting CE performance

Although CE is much less prone than SEC to cause disaggregation of C8GLIP, one significant means of disrupting the aggregate in CE is by excessive current. In order to increase sensitivity, a 75 μm × 27 cm capillary was investigated. However, the increase in capillary i.d. greatly increased the current and Joule heating at high voltages.

Table 2
% Aggregation as determined by different SEC columns and compared to CE[a]

Sample[b]	Aggregate (CE)	Aggregate superose 12	Aggregate superose 6
Initial	0	0	0
Partially aggregated	77	28	40
Fully aggregated	97	78	77

Sample[b]	% Aggregate (CE)	% Aggregate G6000 PWX	% Aggregate G4000SWXL
Initial	16	0	3
Partially aggregated	70	0	4
Fully aggregated	98	51	10

[a] The mobile and running buffer used in all experiments was 16 mM phosphate, pH 7.4.
[b] Samples were prepared at 2 mg ml^{-1} in 16 mM phosphate, pH 7.4. The initial was stored at 5°C until analysis. The partially aggregated sample was stored for 2 h at 23°C and then stored at 5°C until analysis. The fully aggregated sample was stored for 2 h at 35°C and then stored at 5°C until analysis.

Under such conditions, dramatic and rapid disaggregation occurred. Fig. 9 shows the increase in % monomer for a given sample as the current was increased. A plot of current versus voltage (Ohm's Law plot) for the capillary indicated non-linearity (Joule heating) above 30 μA. At currents below this value no disaggregation was observed. Off-line experiments confirmed that heating (75–80°C) indeed promoted disaggregation. By contrast, a 25μm × 27cm capillary yields a linear

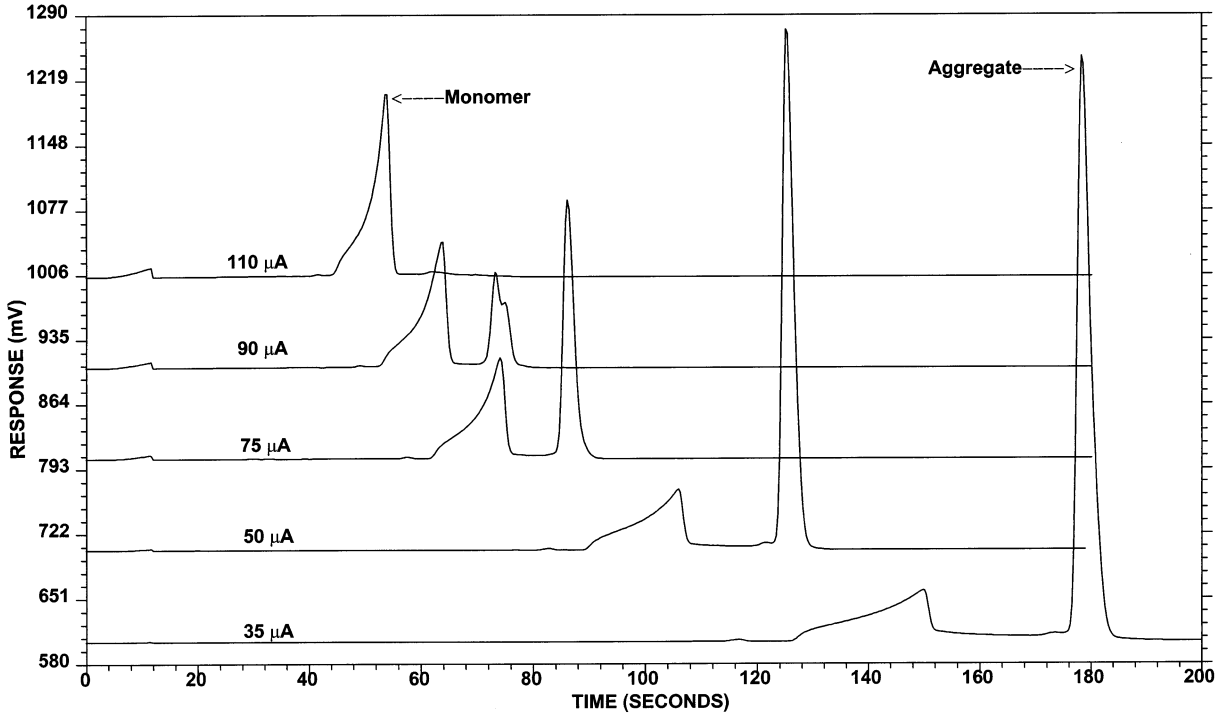

Fig. 9. Effect of CE current on the % aggregation. CE conditions: 75 μm × 27 cm silica capillary, 23°C, 1 s injection, sample (2 mg ml^{-1} ~7 h ambient), $\lambda = 214$ nm, with the current controlled at 35, 50, 75, 90 and 110 μA.

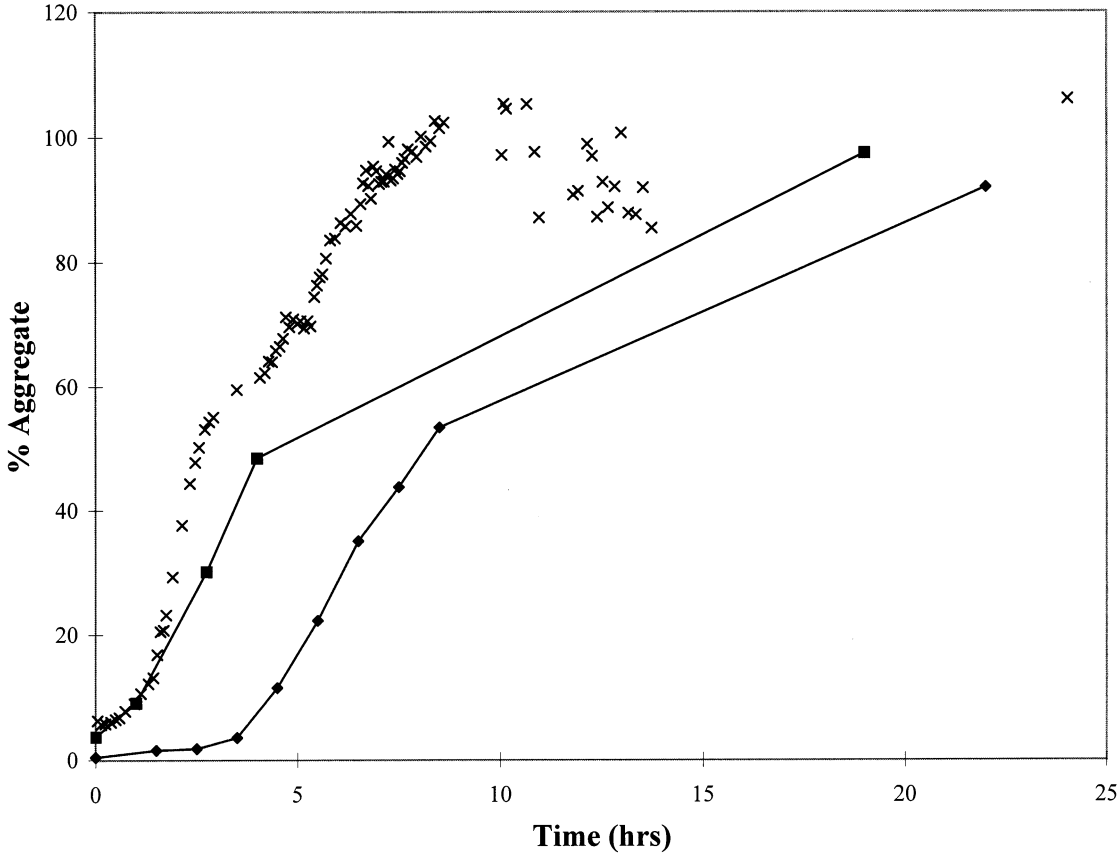

Fig. 10. Comparison of the rates of aggregation by capillary electrophoresis (■), dynamic light scattering (x), and size exclusion chromatography (◆) for a 2 mg ml^{-1} sample in 16 mM phosphate pH 7.4 at 23°C. SEC and CE conditions were the same as those use in Figs. 2 and 3.

Ohm's Law plot and has a current of only 18 μA at 30 kV. Therefore, disaggregation due to Joule heating is not an issue for the CE methodology used throughout this work. However, it does restrict the range of capillary diameters and buffer concentrations which may be used.

3.10. Dynamic light scattering

A solution of C8GLIP was prepared at 2 mg ml^{-1} in 16 mM phosphate pH 7.4 and assayed for aggregation rates using dynamic light scattering. Assuming a bimodal distribution as was evident from the SEC and CE data, the light scattering intensity is proportional to the concentration of the aggregate. As can be seen in Fig. 10, the light scattering data (consistent with the CE data) shows a rapid onset of aggregation, which increases rapidly until it reaches a plateau at approximately 100% aggregation (12–24 h). The aggregate continues to slowly increase in size, doubling its radius after an additional 48 h (72 h from the initial). After 4 days the solution contains visible precipitate. The DLS data during the initial 24 h time period is more consistent with the data obtained by CE than the data obtained by SEC (Fig. 10). The SEC data shows a significant delay in the rate of aggregation. The comparability of CE and DLS versus SEC is particularly evident for the early timepoints of the aggregation curves (e.g. the 3 h time point that shows approximately 35% aggregate by DLS, 30% by CE, and

less than 5% by SEC). Thus, DLS is supportive of the conclusion suggested above that CE is less disruptive of aggregate formation than is SEC.

4. Conclusions

The exact cause of the disaggregation of the C8GLIP during analyses has not been fully determined. However, it is clear that the results obtained by SEC are significantly affected by the separation process itself. This may be due to a variety of factors including effects of dilution and the separation mechanism on the monomer:aggregate equilibrium and the possible disaggregation due to interactions of the aggregate with SEC contact surfaces. For C8GLIP, free solution CE shows significantly less variability from operational parameters and is more consistent with dynamic light scattering data, showing rapid aggregation.

Free solution CE provides an alternative technique to SEC which can be less disruptive of non-covalent aggregates, with significantly reduced analyses times. Like SEC, it can provide quantitative information about aggregation not provided by other techniques (e.g. light scattering and ultracentrifugation) by a simpler, faster, and less expensive means.

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