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Detection of aggregate formation during production of human immunoglobulin G by means of light scattering

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Abstract

In human immunoglobulin preparations with a concentration of 50 mg/ml aggregate formation below 0.3% is difficult to quantify. Such small traces may later be responsible for reduced stability and therefore this generation during the process must be prevented. The influence of process conditions on the conformational changes and subsequent aggregation of immunoglobulins were assessed by size-exclusion chromatography (SEC), UV and static light scattering (LS) detection. This work focused on pH-adjustment experiments since several pH adjustments are required during the production of intravenous immunoglobulin G. Experiments in a labscale were made varying process conditions in a narrow range. It was possible to detect differences concerning the formation of aggregates dependent on these small variations of process conditions.

Keywords: pH effects; Light scattering detection; Detection, LC; Aggregate formation; Immunoglobulins; Proteins

1. Introduction

Intravenous immunoglobulin G (IVIG) has been used to treat a variety of disorders such as primary and secondary immunodeficiencies, autoimmune and inflammatory diseases and infectious diseases [1]. High requirements on safety, purity and on a minimal aggregate content are requested from health authorities. In particular, aggregates can be responsible for undesirable side effects in patients such as the unspecific complement activation which can lead to tachycardia, dyspnea and exanthema [2]. The standard separation method analyzing the aggregate content is high-performance size-exclusion chromatography (SEC) combined with UV detection. This method meets the detection limits of aggregates present in IVIG preparations which are relevant for the release of IVIG preparations. Therefore, more sensitive methods concerning aggregate detection are required to optimize the process steps leading to improved protein stability of the final product.

IVIG preparations are primarily produced by cold ethanol precipitation and the yielded paste is further processed by clear filtration, ultra-/diafiltration, several chromatographic steps and virus inactivation/removal steps [3]. Each preparation step can influence the chemical and physical properties of the IgG molecules. The slightly denatured molecules may expose more hydrophobic amino acids at the surface and are therefore susceptible to partial aggregation. These so-called precursor aggregates can be responsible for posterior aggregation. Minimizing the aggregate formation during production can be a key achieving a better long-term stability of the final product. It is clear that the final IgG solution will always undergo aggregation with time since the folded state of any protein is not infinitely stable in solution [4]. Hence a careful examination of stability influencing factors may help to reduce protein instability, one of the major problems in pharmaceutical industry [5].

The purpose of this work was to characterize process-induced changes concerning the aggregate formation with another detection method in addition to conventional methods like SEC or sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Describing differences in the aggregate composition of IVIG preparations is extremely difficult since the aggregate content is often lower than 0.3% of the total IgG content. Light scattering (LS) is a very sensitive detection method for large molecules [6], enabling the comparison of different IVIG preparations concerning the aggregate pattern [7].

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In this work the influence of the pH itself and different process conditions during pH adjustments were analyzed concerning aggregate composition. There are several studies describing pH effects on IVIG preparations [8–10]. One of the major effect of increasing the pH is the spontaneous formation of dimer complexes in human IVIG preparations. The majority of these dimers are thought to be idiotype (Id)/anti-Id complexes that associate noncovalently via the Fab fragments [11,12]. It has also been reported that the aggregate content is reduced by lowering the pH. A turbidity study showed that the highest turbidity was achieved at pH 8 for an IVIG preparation [10]. At pH 7 the turbidity was already ten times higher than at pH 4.25. These findings are in agreement with studies showing the isoelectric point (p*I*) range of human IgG [13]. It has been reported that IgG molecules have an isoelectric point varying from 4.35 to 9.95, where the majority has a p*I* between pH 7 and 9. Since aggregation is increased near the isoelectric point because of reduced electrostatic repulsion it is not surprising that human IgG tends to aggregate at physiological pH. In the present study SEC combined with online SLS detection should enable a more detailed description of aggregation during pH adjustments of human IgG solutions. The case of non-optimal process conditions during pH adjustment can increase the tendency of aggregate formation. In the present study it was possible to identify differences of low aggregated IgG preparations from pH 4.0 to 6.8 concerning aggregate formation.

2. Theory

The basic light scattering equation [6] is:

$$\frac{Kc}{R_{(\theta)}} = \frac{1}{M_w P_{(\theta)}} + 2A_2 c + \cdots, \quad (1)$$

where K is an optical constant equal to $[4\pi^2 n^2 (\mathrm{d}n/\mathrm{d}c)^2]/(\lambda^4 N_\mathrm{A})$, c the solute concentration in mg/ml, $R_{(\theta)}$ the excess intensity of scattered light at the angle θ, λ is the wavelength, n the refractive index of the solvent, ($\mathrm{d}n/\mathrm{d}c$) the refractive index increment, N_A the Avogadro's number, M_w the weight-average molecular mass, A_2 the second virial coefficient and $P_{(\theta)}$ is the shape factor which is calculated as follows:

$$\frac{1}{P_{(\theta)}} = 1 + \frac{16\pi^2 {R_\mathrm{G}}^2}{3\lambda^2} \sin^2\theta, \quad (2)$$

where R_G is the radius of gyration.

3. Experimental

3.1. Materials

Human IVIG preparations with a protein concentration of 30–60 mg/ml were obtained from Octapharma Pharmazeutika (Vienna, Austria). For HPLC analysis samples were diluted to 5 mg/ml with water and filtered through a 0.20 μm minisart RC 15 filter (Sartorius, Göttingen, Germany).

3.2. Determination of protein concentration

The protein concentration was determined by UV-absorption at 280 nm using an extinction coefficient of 1.4 cm^2/g.

3.3. Size-exclusion chromatography and laser light scattering analysis

High-performance size-exclusion chromatography analysis was performed using a TSK G3000SW column (60 cm × 7.5 mm i.d., TosoH Biosep, Montgomeryville, USA) connected to an HPLC workstation (Agilent HP 1100 system). The separation was performed at a flow rate of 0.6 ml/min at room temperature. An aqueous buffer consisting of 20 mM sodium phosphate, 0.1 M sodium chloride at pH 6.8 was used as eluent. Elution of protein was monitored by a UV detector, a laser light scattering detector (PN3020, Postnova Analytics, Landsberg, Germany) and a differential refractive index detector (PN 3120, Postnova Analytics). The protein concentration of the injected samples was 5 mg/ml and the sample volume was 50 μl. All reagents used were of analytical grade from Merck (Darmstadt, Germany).

3.4. pH adjustments of an IVIG preparation

The pH of 200 g of an IVIG preparation was adjusted from pH 4.0 to 6.8 with a sodium hydroxide (Merck) solution in a stainless steel container with an internal diameter of 80 mm at 7 °C. Afterwards the pH of the IgG solution was readjusted to pH 4.0 with a hydrochloride solution (Merck, Darmstadt, Germany). Process conditions were varied concerning the strength of added base or acid, flow rate of added base and acid and mixing speed. Base and acid were added with a P1 pump from Pharmacia (Uppsala, Sweden) and for mixing a propeller mixer with a diameter of 45 mm was used. During experiments samples were taken and analyzed by SEC–UV–SLS.

3.5. Short storage experiments of IVIG preparations

Three hundred milliliters of suspended IgG paste (from ethanol precipitation) at pH 4.6 were tempered at 2 °C and at 10 °C for 10 h. During storage samples were taken and analyzed by SEC–UV–SLS.

4. Results and discussion

During manufacturing of IVIG preparations several pH adjustments between pH 4.0 and 6.8 are required. In this work the influence of the pH itself and of the used process

Table 1
Process conditions during pH-adjustment experiments

Experiment[a]	NaOH (mol/l)	Flow rate (ml/min)	Mixing speed (U/min)	Experiment[b]	HCl (mol/l)	Flow rate (ml/min)	Mixing speed (U/min)
1	0.1	0.09	200	5	0.3	0.068	200
2	0.1	0.3	75	6	0.3	0.3	75
3	0.5	0.09	200	7	0.5	0.068	200
4	0.5	0.3	75	8	0.5	0.3	75

[a] Experiments 1–4: after reaching pH 6.8 the pH was readjusted to pH 4.0 with a 0.3 M HCl solution, a flow rate of 0.068 ml/min and a mixing speed of 200 U/min.
[b] Experiments 5–8: the pH was adjusted from pH 4.0 to 6.8 with a 0.1 M NaOH solution, a flow rate of 0.09 ml/min and a mixing speed of 200 U/min and afterwards process conditions were varied.

conditions on the aggregate composition should be characterized by light scattering detection. It has already been reported that at higher pH the aggregate content of human IgG solutions is higher compared to pH of 4.0 [8].

The pH-adjustment experiments were performed by varying the molarity and the flow rate of the added base or acid and the mixing speed in a narrow range (Table 1) to observe changes in aggregate composition. The IVIG preparation used was obtained from the flow through of an ion-exchange chromatographic step, which had a very low aggregate content [7] at the starting pH 4.0. The material was obtained from a pilot scale batch, which was produced according to a protocol previously described by Buchacher et al. [3]. The use of this highly purified IVIG preparation should guarantee that influences concerning the aggregate pattern result exclusively from the pH itself and the applied process conditions (Table 1).

A typical SEC chromatogram derived from these experiments is shown in Fig. 1. The UV signal shows monomers and dimers clearly, and a very small peak representing aggregate 2 is detectable. In this example the sample consisted of 96.066% monomers, 3.886% dimers and 0.048% aggregate 2. In addition, two small aggregate peaks are visible in the SLS signal, which were attributed to be aggregates 1 and 2. The aggregates eluting within the aggregate 1 peak are very high-order aggregates which are present in traces and can only be detected with the SLS signal. Aggregate 2 represents the aggregate content with a lower molecular mass compared to aggregate 1 but it is present in higher concentrations in the IVIG preparations. The detection limit for aggregate 2 was 0.02% of the total IgG content with the UV signal. Unfortunately, the concentration is too low to calculate the accurate molecular mass of these aggregates.

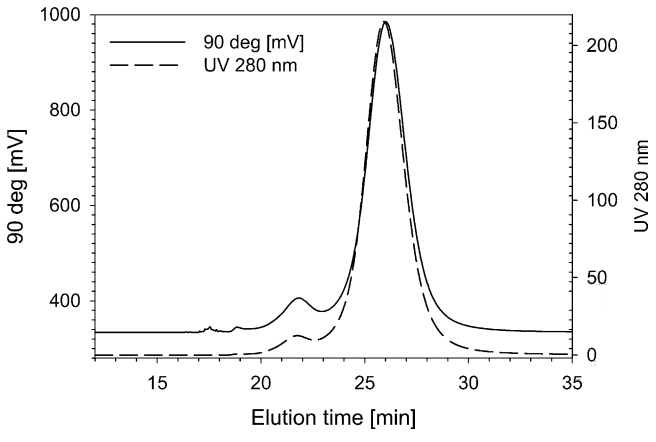

Fig. 1. SEC chromatograms of human IgG from experiment 8 at pH 5.0. The concentration of the sample was 5 mg/ml, 50 μl of the sample were injected onto TSK G3000 SW column (TosoH Biosep) at pH 6.8, 0.1 M salt at a flow rate of 0.6 ml/min. The solid line is the SLS signal, the dashed-dotted line is the UV signal.

In Fig. 2 an example is illustrated how aggregates and dimers behave over a pH range from 4.0 to 6.8. It can be seen clearly that the amount of dimers increases with a higher pH, which has already been reported for human IgG dimer complexes [14] due to the formation of Id/anti-Id complexes [12]. The formation of these dimers follows a linear correlation with increasing pH and is completely reversible by lowering the pH to 4.0 (Fig. 3). As mentioned above, our major interest was directed towards the formation of aggregates. Interestingly, the two aggregate peaks are behaving in an opposite manner (Fig. 2): on the one hand the aggregate 1 peak decreases with a higher pH and on the other hand the aggregate 2 peak increases with a higher pH. The increase of the peak area of aggregate peaks can be due to two reasons or a combination of both (see Eqs. (1) and (2)): (1) the concentration of the aggregates increases and/or (2) the molecular mass of the aggregates increases. The reason

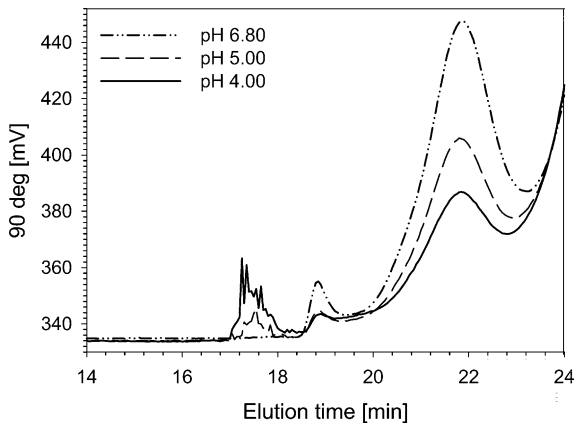

Fig. 2. SLS signal of SEC chromatograms drawn to a larger scale showing aggregates and dimers of an IVIG preparation at different pH from experiment 8.

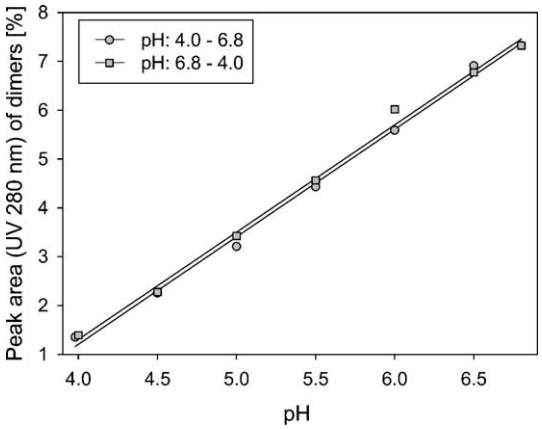

Fig. 3. Formation of dimers with increasing pH and resolution of dimers with decreasing pH.

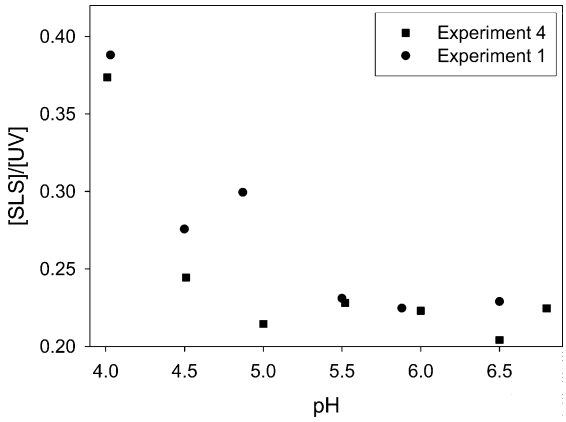

Fig. 5. Trends of the ratio [SLS]/[UV] with the pH.

for the increase of aggregates can only be explained for aggregate 2 of which the concentration can be determined. If the molecular mass does not change significantly, the ratio of the peak areas of the SLS and the UV signals will stay in the same range. The reason why the peak area of aggregate 1 is reduced by increasing the pH cannot be determined due to the missing concentration signal. This phenomenon could be explained by the acid sensitive Fc fragment of the IgG molecule. It has been reported that at a treatment at pH 3.5 the Fc fragment undergoes denaturation [15]. It is possible that arising precursor aggregates due to lowering the pH to 4.0 can already be detected with SLS.

Fig. 4 summarizes the pH-adjustment experiments where process conditions were varied concerning the pH increment. For illustrating data concerning aggregate 2 the SLS signal was evaluated. The percentage of the peak area of aggregate 2 (total IgG = 100%) is illustrated against the pH. It can be seen clearly that the use of a stronger base (Fig. 4C and D) led to an increased formation of aggregate 2 compared to the experiments with a weaker base (Fig. 4A and B). The process conditions of experiments 1 and 2 did not show a significant difference concerning the formation of aggregates although different flow rates and mixing speeds were used. The maximum values for the area percentage of aggregate 2 at pH 6.8 were 0.81% for experiment 1 and 0.89% for experiment 2.

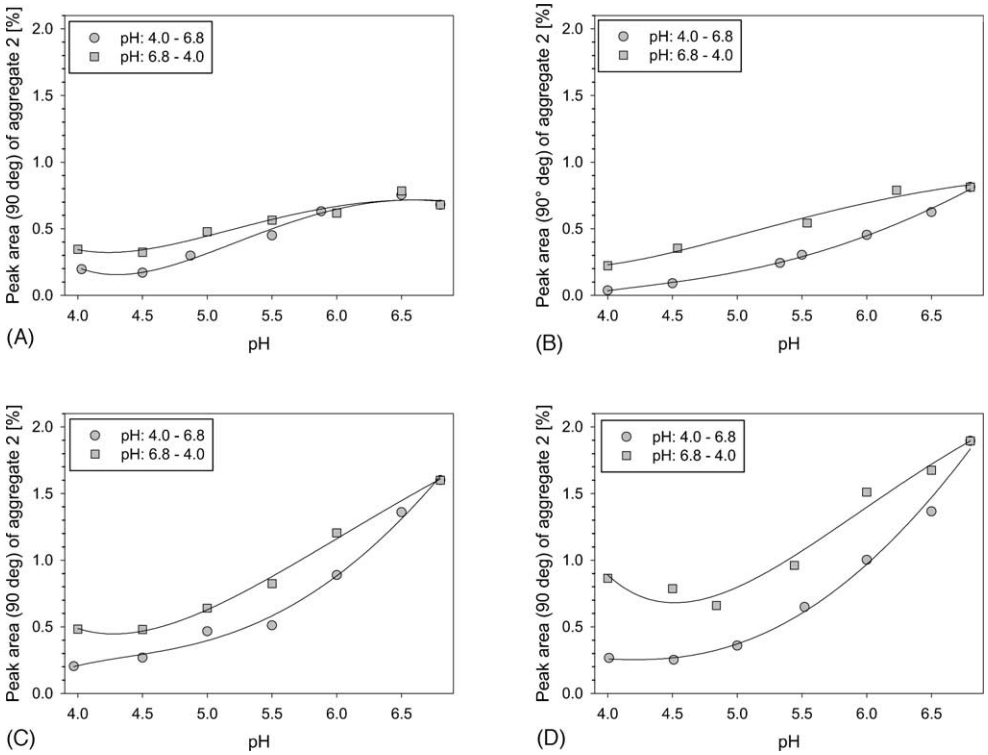

Fig. 4. Formation of aggregate 2 with increasing pH at different process conditions (percent aggregate 2 of total IgG). (A) Experiment 1, (B) experiment 2, (C) experiment 3, (D) experiment 4.

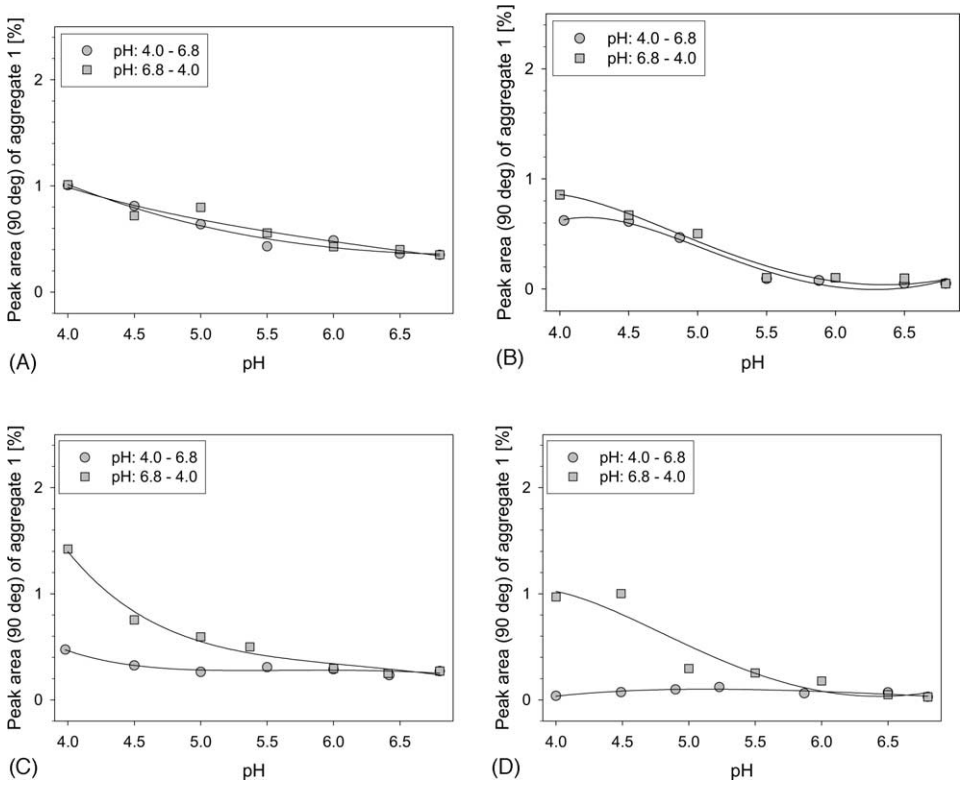

Fig. 6. Formation of aggregate 1 with decreasing pH at different process conditions. (A) Experiment 5, (B) experiment 6, (C) experiment 7, (D) experiment 8.

For experiment 3 the maximum value of the area percentage of aggregate 2 at pH 6.8 was 1.6 and 1.9% for experiment 4. These values are approximate two times higher than the values from experiments 1 and 2. Comparing experiments 3 and 4 the use of a different flow rate and mixing speed had an influence on the formation of aggregate 2. The combination of higher flow rate and reduced mixing speed in experiment 4 led to a higher increase of aggregate 2 compared to experiment 3 due to higher local alkaline conditions.

The same evaluation was done with the UV signal where similar curves were obtained (data not shown). The great difference between the SLS and the UV signals is the response on aggregate 2. In the SLS signal the area percentage for aggregate 2 is in a range between 0.04 and 1.9% whereas in the UV signal it is about 0.02–0.3% aggregate 2 of the total IgG content. During these experiments the ratio of the peak areas of the SLS and UV signals was changing indicating variances of the molecular mass of these aggregates. The trends are shown in Fig. 5 for experiments 1 and 4. The addition of base led to a decreased molecular mass of aggregate 2 and an increased concentration of aggregate 2. The respective aggregates eluted closely to void volume, thus a resolution of the various molecular sizes is not possible with SEC. Therefore, the ratio of the peak areas of the SLS and UV signals only provides information about the change of the molecular mass of the aggregates.

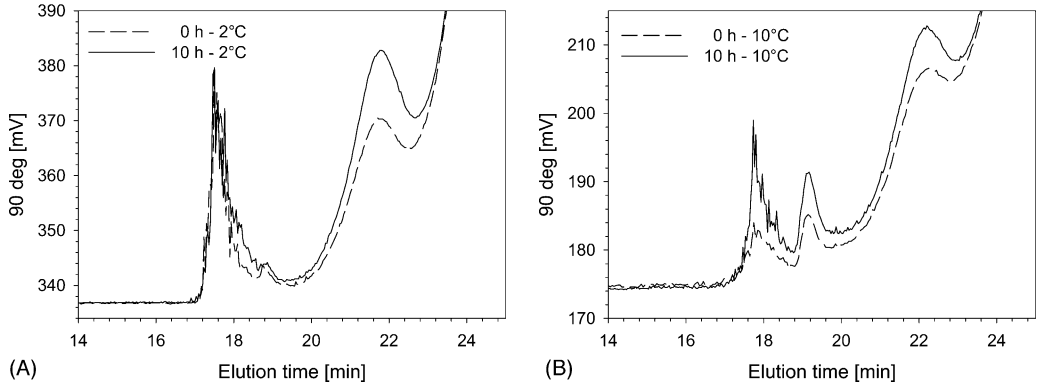

Fig. 7. SLS signal of SEC chromatograms drawn to a larger scale showing aggregates and dimers of short storage experiments for 10 h at 2 and 10 °C.

In Fig. 6 the results for experiments concerning the influence of acidic conditions on aggregate 1 are summarized. Comparing the four graphs in Fig. 6, it is obvious that an enhanced growth of aggregate 1 was caused by the use of a stronger acid (Fig. 6C and D). Although process conditions were chosen in a very narrow range (Table 1) a significant difference in aggregate 1 formation could be shown.

One important factor influencing protein aggregation is the temperature. Two suspensions of the yielded IgG paste from the cold ethanol precipitation were stored at 2 and 10 °C for 10 h (Fig. 7). The aim was to demonstrate that in a relatively short time a difference in aggregate content can be shown. At 2 °C the dimerization was increased significantly whereas the aggregate compositions showed no distinct difference in the SEC chromatogram. In contrast at a storage temperature of 10 °C the growth of both aggregate peaks was observed after 10 h.

5. Conclusions

Light scattering detection allowed significant distinction between samples derived from different process steps concerning their aggregate composition. pH-adjustment experiments showed that a 0.5 M NaOH or HCl solution already led to an increased aggregate formation which can cause posterior aggregation at some later time.

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