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Analysis of aggregates of human immunoglobulin G using size-exclusion chromatography, static and dynamic light scattering

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Abstract

Large aggregates (M_r: 10^6–10^7 g/mol) of human immunoglobulins are present in extremely small concentrations in IgG preparations (<0.1%). Traces of large protein aggregates cannot be determined by conventional size-exclusion chromatography (SEC) using UV detection due to limitations in sensitivity. The conventional analysis of IgG by SEC is limited to dimers and oligomers. Using light scattering it is possible to determine significant differences concerning the aggregate composition and the extent of protein aggregation in samples of different process steps. Two different pilot preparations were analyzed by SEC with UV and static light scattering detection and compared to dynamic light scattering in the batch mode. The change of large aggregates could be monitored and data were corroborated by dynamic light scattering.

Keywords: Hydrodynamic radius; Light-scattering detection; Detection, LC; Immunoglobulins; Proteins

1. Introduction

When particles with a diameter longer than 1 nm are present in a solution the incident light is scattered. The extent of scattering is dependent on concentration, size and shape of the molecule and the design of the experimental set up. During static light scattering (SLS) the incident light beam is scattered and the intensity of the scattered light is measured at different angles. SLS is used for determination of the absolute molecular mass. The molecules also undergo Brownian motion that is related to the hydrodynamic radius, which can be described with the Stokes–Einstein equation [1]. Fluctuations in scattered light intensities caused by the Brownian motion result in a time-varying signal, which can be monitored by a high sensitivity detector. This process is called dynamic light scattering (DLS). The larger particles diffuse slower than the smaller ones. Intensity signals are recorded by a photon correlator. Since the size of molecules can be assessed SLS and DLS are useful tools in a wide field of applications in protein sciences. Depending on the mathematical model the molecular mass or radius of gyration and/or geometrical shape can be determined. The method can also be applied for optimization of process chromatography and provides further insight how the eluates are composed.

There are numerous studies dealing with aggregation and dissociation processes [2], unfolding and refolding experiments [3,4], molecular mass estimations in combination with size exclusions [5], crystallization studies [6] and the biophysical characterization of the influence of salt concentration [7,8], pH

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[9] and concentration [10] on protein behavior in solutions. A prime application of DLS is studying protein aggregation. Thermal denaturation and aggregation of β-lactoglobulin [11,12] and human immunoglobulin G (IgG) [13] were studied. Association and dissociation of monomeric and dimeric or even higher multimeric forms are often of interest because of biological activity and can be studied with light scattering (LS). For example, the aggregation of recombinant human Factor VIII was studied with DLS to investigate aggregate formation [14].

The major applications of LS is the molecular mass estimation, because SLS provides a direct measure of molecular mass. High-performance size-exclusion chromatography (SEC) is a simple method for molecular mass estimation and therefore widely used. This method suffers from a few restrictions that prevent correct molecular mass determination. Retention difference in SEC is caused by different diffusivities of the proteins into pores. In addition electrostatic interaction and hydrophobic interaction with the matrix may give rise to aberrant retention. Obviously, all these factors could affect the accuracy of the calculated molecular mass. With the combination SEC–LS these restrictions can be circumvented and the method enables a direct determination of the molecular mass [5,15–17].

The purpose of this work was to find a sensitive detection method for process-induced changes of aggregate composition during IgG processing. Aggregation of proteins is a major problem in pharmaceutical industry because of a possible loss of potency and visual appearance of the product. Although large aggregates are present in extremely low concentration, they may have a big impact on the quality of the product. SEC has been widely used to characterize protein aggregates present in human IgG preparations [18]. The analysis of large IgG-aggregates in highly concentrated protein solution is extremely difficult since these large aggregates are present in low abundance; often smaller than 0.3% of the total IgG content. Therefore a sensitive method to determine large aggregates in a reproducible manner is required. Light scattering is the method of choice. Since differences in the aggregate composition can be hardly assessed with conventional SEC using UV monitors. Currently SLS and DLS are not frequently used in pharmaceutical industry for monitoring process-induced changes on protein solutions. One application of SEC–LS concerning the extent of protein aggregation during manufacture was shown in food industry with whey protein concentrates [19]. Attention has to be paid on the fact that the separation of aggregates by SEC may influence aggregate composition, since aggregation is highly concentration dependent. The equilibrium may shift towards less aggregated forms upon dilution. In addition to SEC–LS measurements a method was required which allows analyzing of high concentrated IgG solutions without any sample preparations like dilution. DLS measurements are extremely sensitive to changes in the aggregate composition. Aggregation can be directly measured without dilution by DLS. In the present paper SEC–SLS and DLS were used characterizing in process samples during manufacturing of human IgG.

2. Theory

In 1871 Rayleigh developed the theory of light scattering. When polarized monochromatic light passes through a solvent containing macromolecules, the excess light scattered by the the molecule of an angle to the incident beam over the scattered light by the solvent alone is directly proportional to the molecular mass.

The basic light scattering equation [15] is:

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

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

$$\frac{1}{P_{(\theta)}} = 1 + \frac{16\pi^2 R_G^2}{3\lambda^2} \cdot \sin^2 \theta \quad (2)$$

where R_G is the radius of gyration. For the evaluation of the molecular mass for proteins, containing

no carbohydrates and an $M_w < 5 \times 10^7$ g/mol, the ''two-detector method'' [5] can be applied for estimating the molecular mass. This simplified method is usually provided by the software of the instrument. The molecular mass can be determined from the ratio of the area from the light scattering detector and the refractive index detector:

$$M = K' \cdot \frac{(\mathrm{LS})}{(\mathrm{RI})} \tag{3}$$

where K' is the instrument calibration constant:

$$K' = \frac{K_{\mathrm{RI}}}{K_{\mathrm{LS}}\left(\frac{\mathrm{d}n}{\mathrm{d}c}\right)} \tag{4}$$

with the refractive increment ($\mathrm{d}n/\mathrm{d}c$).

The constant K' was determined by using following proteins of known molar mass: bovine serum albumin ($M_r = 66\,500$ g/mol; Sigma, St. Louis, MO, USA) and α-lactalbumin ($M_r = 14\,500$ g/mol; Sigma,). The proteins thyroglobulin (Sigma) and β-amylase (Sigma) with a higher molecular mass were used to check the instrument constant.

DLS is concerned with the investigation of correlation of photons. The objective of DLS is to find any peculiar properties of the scattered signal which can be used to characterize the random ''noise'' of the signal, and the correlation curve is used to achieve this objective. The autocorrelation function is given by the following equation:

$$G(\tau) = \int_0^\infty I(t)^* I(t+\tau)\mathrm{d}t \tag{5}$$

where *I(t)* is the intensity measured at $t = 0$ and $I(t+\tau)$ is the measured intensity at some later time. The scattering intensity at the detector is dependent upon the position of the macromolecule relative to the detector. The correlation is still present as long as the diffusional volume is finite. The particle position is defined by the degree of Brownian motion. Therefore the measured intensity correlation curve is an indirect measure of the diffusion coefficient of the particle. For a typical diffusion processes the correlation function has the form 1 plus an exponential decay function:

$$G(\tau) = 1 + \exp(-\gamma t) \tag{6}$$

where γ is the decay constant and is representative of the diffusional properties of the macromolecule. The diffusion coefficient is used to calculate the hydrodynamic radius via the Stokes–Einstein equation:

$$D = \frac{kT}{6\pi\eta R} \tag{7}$$

where k is the Boltzman constant, T is the absolute temperature, η is the viscosity of the solvent and R is the hydrodynamic radius.

For evaluating DLS data the softwares PrecisionDeconvolve and PrecisionDeconview were used. The algorithm used is a proprietary algorithm from Precision Detectors (Bellingham, USA).

3. Experimental

3.1. Materials

Human IgG 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 running buffer and filtered through a 0.20 μm minisart RC 15 filter (Sartorius, Göttingen, Germany).

3.2. Concentration determination

The protein concentrations were 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 SEC analysis was performed using a TSK G3000SW column (60 cm × 7.5 mm I.D., TosoH Biosep, Montgomeryville, PA, USA) connected to a high-performance liquid chromatography (HPLC) workstation (Agilent HP 1100 system). The separation was carried out at a velocity of 0.6 ml/min at room temperature. An aqueous buffer consisting of 20 m*M* sodium phosphate, 0.1 *M* sodium chloride at pH 6.8 was used as eluent. Elution of protein was monitored by UV detection,

laser light scattering detection (PN3020, Postnova Analytics, Eresing, Germany) and differential refractive index detection (PN 3120, Postnova Analytics). The protein concentration of injected samples was 5 mg/ml and the sample volume was 20 μl. All reagents used were of analytical grade from Merck (Darmstadt, Germany).

3.4. Dynamic light scattering

DLS measurements were performed using a PD2000DLS dynamic light scattering detector combined with a PDDLLS/Batch platform from Precision Detectors. The system consisted of an optical unit, equipped with a laser (100 mW output at 800 nm) plus a cuvette unit, and digital correlator with a maximum of 256 channels configurable within 1024 positions. All measurements with the PDDLS/Batch system were done at a fixed angle of 90°. The time correlation function was computed by the digital correlator and these data were analyzed using commercial data inversion with the software PrecisionDeconvolve or PrecisionDeconview. The DLS data were illustrated in the form of the intensity dis-

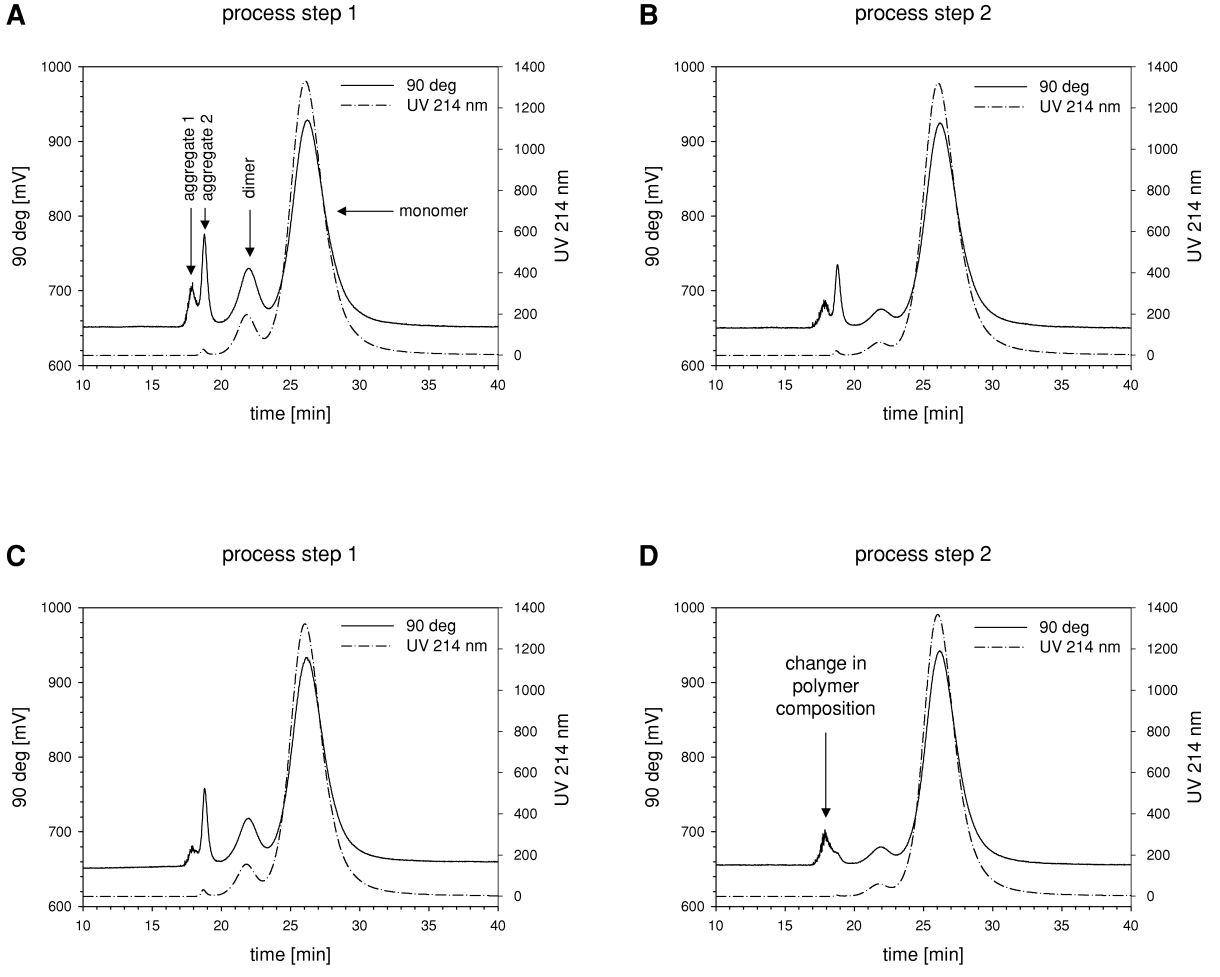

Fig. 1. SEC chromatograms of human IgG. The concentrations of samples were 5 mg/ml, 20 μl of each sample was injected onto the TSK G3000 SW column (TosoH Biosep) at pH 6.8, 0.1 *M* salt at a flow-rate of 0.6 ml/min. The solid line is the SLS signal, the dashed–dotted line is the UV signal. (A) Process step 1 of preparation A, (B) process step 2 of preparation A, (C) process step 1 of preparation B, (D) process step 2 of preparation B.

tribution function $D_i(Rh)$. The IgG samples were directly filtered in the cuvette through a 0.20 μm minisart RC 15 filter (Sartorius).

4. Results and discussion

Immunoglobulin G was initially produced by the ethanol precipitation according to Cohn (see Ref. [20]) or Kistler-Nitschmann (see Ref. [20]). Nowadays the ethanol precipitation methods have been refined and virus inactivation methods such as heat treatment, treatment with a combination of tri-*n*-butyl phosphate and detergents, and/or nanofiltration are used [21]. Chromatographic steps have also been implemented into IgG production processes. Regulations from health authorities request a minimal content of polymers, such as dimers, trimers and higher aggregates. Current production methods meet these quality requests, but the knowledge higher aggregates may be an indicator on the history of the whole production procedure.

Two different preparations of human IgG were analyzed concerning their aggregate composition showing the influence of different process steps on aggregate formation in IgG solutions. Pilot scale preparation A is a conventional procedure consisting from ethanol fractionation, ultrafiltration and virus inactivation using solvent detergent method. Preparation B is identical to A with the exception that an additional ion-exchange chromatographic step was used.The ion-exchange step is performed in a negative mode. The flow through contains IgG and is further processed.

Fig. 1 shows the chromatograms of two sequential process steps of the two different preparations denoted as A and B. The chromatograms obtained from the 90° light scattering signal and the UV at 214 nm are superimposed. The pilot scale preparation would meet the level of maximal polymer content. In addition in preparation A (Fig. 1A and B) two large aggregate peaks could be observed with the 90° LS signal, whereas the UV signal could only detected one minute peak eluting between 15 and 20 min. Although the concentration of aggregate 1 was too low for detection in the UV signal, a clear peak in the SLS signal was detected. Because the scattering intensity is highly dependent on the radius of the particle, the light scattering detector is very sensitive for higher-order multimers (see Eq. (2)). The ratio of the peak area from aggregate 1 to aggregate 2 (Table 1), calculated from the SLS signal with the software Discovery32, was determined to compare the samples from different process steps. In the second process step of preparation A change in aggregate

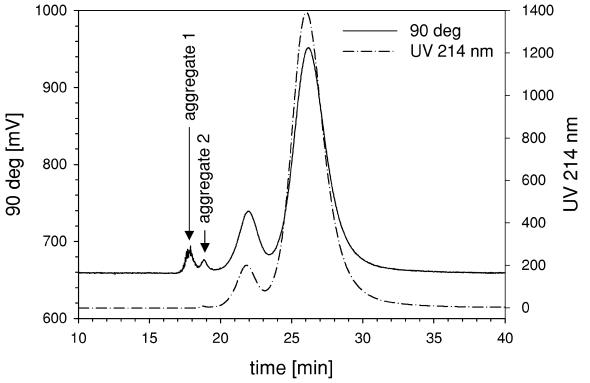

Fig. 2. SEC elution pattern of the flow through from IEX from preparation B. The solid line is the LS signal, the dash–dotted line is the UV signal.

Table 1
Overview of the SEC–LS–UV results of preparations A and B

	Ratio of the large aggregates detected by 90° LS signal		Area percentage calculated from UV signal at 214 nm		
	Process step	Peak area aggregate 1/ peak area aggregate 2	% Aggregates	% Dimers	% Monomers
Preparation A	1	0.515	0.32	7.58	92.10
	2	0.554	0.26	2.50	97.25
Preparation B	1	0.282	0.35	6.29	93.36
	Flow through IEX	2.607	0.09	7.81	92.10
	3	3.531	0.06	2.41	97.53

composition could not be observed. The difference of the ratio is within the error of measurement. Process step 2 reduced dimer content and increased monomer content, while the large aggregates remained almost constant (Table 1). In preparation B a significant difference concerning the aggregate composition was observed (Table 1). The second process step reduced the large aggregate denoted as aggregate 2, while the largest aggregates increased. This reduction of aggregates was achieved by the introduction of an ion-exchange chromatography (IEX) between process step 1 and 2 (Fig. 2). IEX is performed in the negative mode. The flow through was then further processed in step 2. With UV adsorption these large aggregates (1) cannot be detected.

In addition to the SEC experiments, DLS was performed. The samples were measured un-diluted to ensure that the sample composition is maintained in its original state. Process step 1 from preparation A showed a very wide intensity distribution (Fig. 3A), which suggested the presence of aggregates with

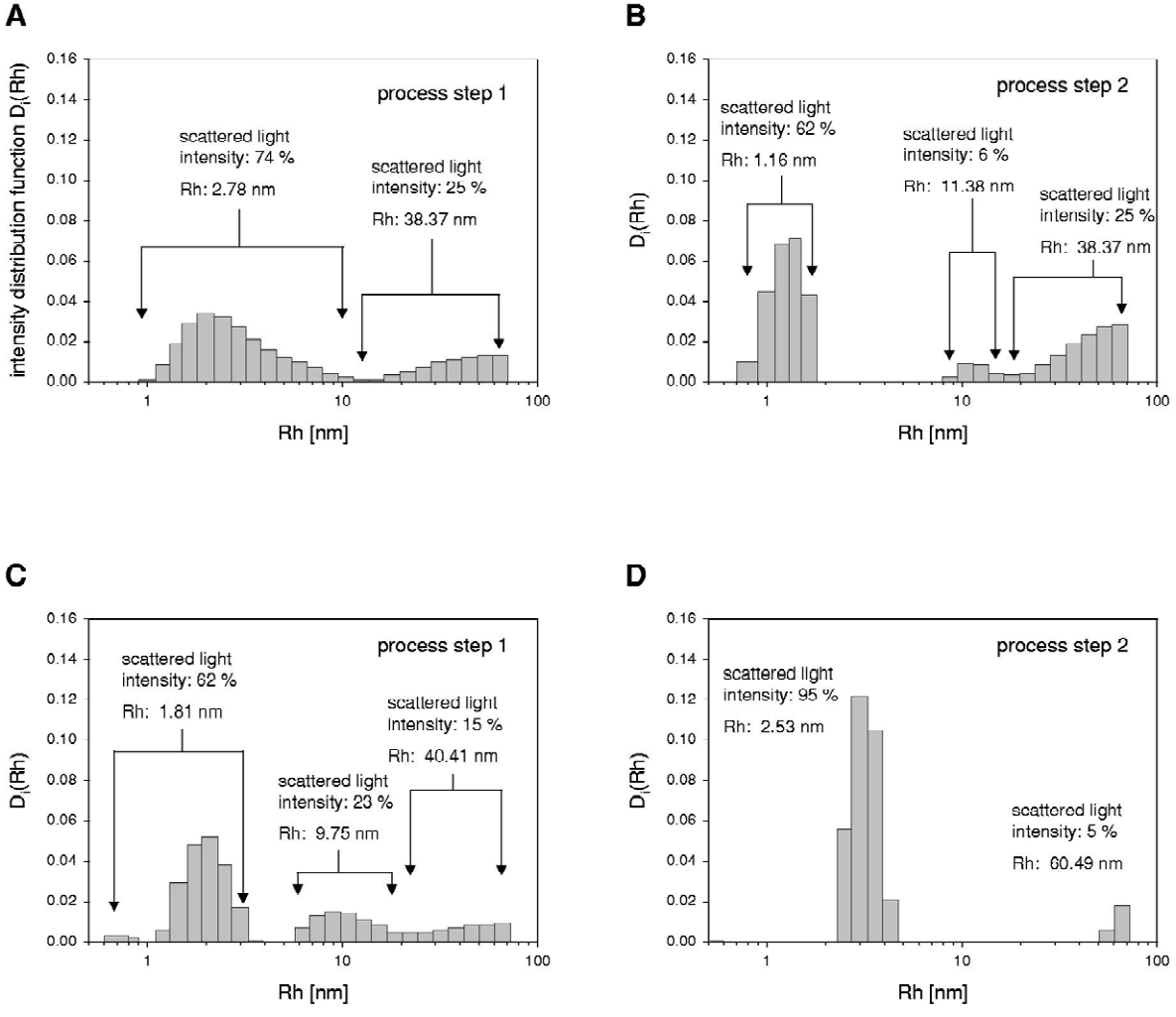

Fig. 3. Distributions of different particle sizes of human IgG. DLS measurements were made on PDDLS instrument (Precision Detectors) at room temperature. (A) Process step 1 of preparation A, (B) process step 2 of preparation A, (C) process step 1 of preparation B, (D) process step 2 of preparation B.

many different molecular masses. By the following process step (Fig. 3B) only a slight improvement of the sample composition concerning the aggregate pattern could be achieved. For interpreting DLS data it is always necessary considering that the larger molecules produce a stronger signal than smaller ones [22]. The intensity distribution function must be further transformed in order to get the distribution based on mass. The transformation was performed with following equation:

$$M_w = k\mathrm{Rh}^{\alpha} \quad (8)$$

where M_w is the molecular mass, k is an empirical factor, Rh is the hydrodynamic radius and α is an exponent dependent on the molecular type: 1 is used for rigid (rod) molecules like polymers, 2 is used for random coiled or disc shaped proteins and 3 is for globular proteins. UV and SLS monitoring indicated that more than 99.9% monomeric and dimeric immunoglobulins were present in the samples. Thus it is obvious to select a value of 3 for the exponent α. The transformation in this particular case resulted that 62% of scattered light intensity from the first size distribution represent more than 99.9% of the sample composition. Due to the regularization algorithm the monomer and dimer cannot be resolved. Therefore the first distribution represented the scattered light intensity from both monomer and dimer. In comparison to preparation A, process step 2 of preparation B (Fig. 3D) contained only small traces of aggregates, because the scattered light intensity of the aggregates contributed just 5% of the total scattered light intensity. This improvement is due to the implemented IEX step, which removed most of the aggregates. These results are in good correspondence with the results obtained from SEC–UV–SLS analysis, where a reduction of the lower molecular mass aggregates was clearly visible.

The drawback of the DLS measurements is that highly concentrated protein solutions lead to a loss of light scattering intensity because of multiple scattering effects and therefore the interparticle interactions are no longer negligible. Predominating repulsive interaction can cause an increase of the diffusion coefficient and hence a decrease of the hydrodynamic radius can occur [10]. Fig. 4 shows that the diluted sample had a higher scattering intensity and a larger hydrodynamic radius than the concentrated

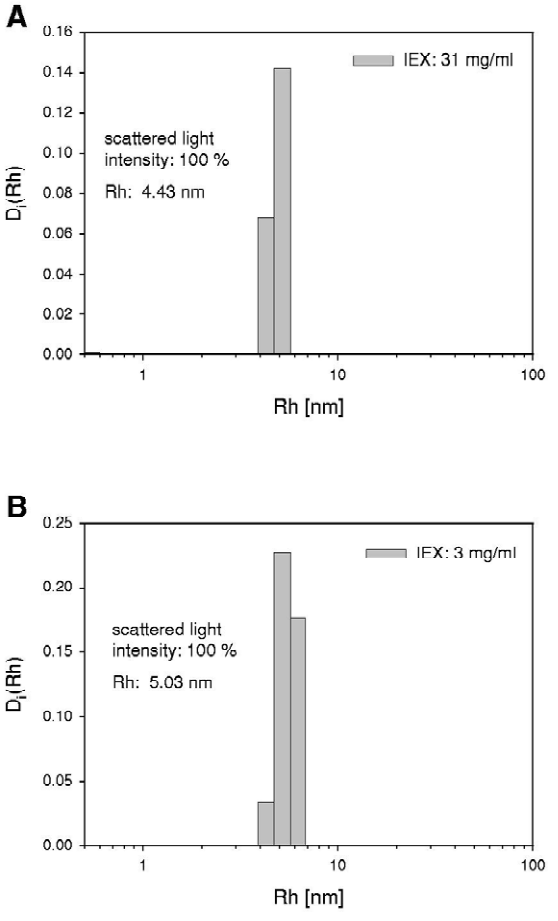

Fig. 4. Intensity distribution of the flow through from the IEX preparation step. (A) Protein concentration 31 mg/ml, (B) protein concentration 3 mg/ml.

sample and is in good correspondence for the IgG molecule [13,23]. Another drawback of DLS is the interference of scattering by large molecules [10]. Fig. 5A shows the correlation function from a sample containing a population of molecules with a broad molecular mass distribution. Whereas Fig. 5B illustrates a perfect correlation function from which the hydrodynamic radius can be calculated correctly. Due to the heterogeneity of the analyzed samples one could hardly determine a perfect correlation function for estimating absolute Rh values. For this reason the analyzed samples were only qualitatively compared concerning the aggregate pattern and the contributed light scattering intensity from the several size distributions.

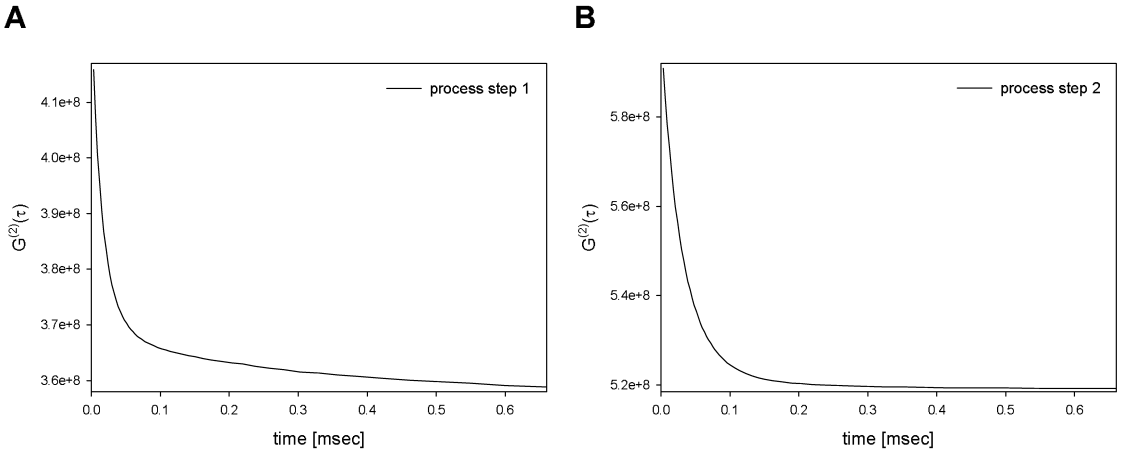

Fig. 5. Autocorrelation function from IgG samples of preparation B. (A) Process step 1, (B) process step 2.

5. Conclusions

SEC with on-line SLS detection allowed a significant distinction between samples of several process steps concerning their aggregate composition. Traces of large aggregates in human immunoglobulin preparations can be detected with dynamic and static light scattering. SEC with on-line LS detection allows a separation of aggregates, whereas the UV signal can only monitor one aggregate peak. DLS in the batch mode is a useful tool to detect small traces of aggregates in IgG samples from different process steps without previous sample preparation, e.g., diluting. A drawback of DLS is that a homogenous distribution is required to calculate the correct Rh.

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