



A therapeutic antibody and its antigen form different complexes in serum than in phosphate-buffered saline: A study by analytical ultracentrifugation

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ABSTRACT

During the development of protein therapeutics, characterization of the active pharmaceutical ingredient is performed extensively to ensure the stability, safety, and efficacy of the drug. Little is known, however, about the characteristics of protein drugs circulating in the blood. The recent availability of a fluorescence detection system (FDS) in analytical ultracentrifugation (AUC) instruments enables the characterization of fluorescently labeled proteins in biological fluids. AUC provides information about protein size, shape, self-association, and binding while avoiding many limitations associated with size exclusion chromatography. Furthermore, with the specificity and sensitivity of FDS, measurements can be performed at physiological concentrations directly in serum. In the current study, we used omalizumab, an anti-immunoglobulin E (IgE) monoclonal antibody, to demonstrate the potential of using AUC–FDS for the study of a monoclonal antibody and its complexes directly in human serum. Omalizumab properties were essentially unaltered after labeling with the fluorescent dye Alexa Fluor 488. In addition, omalizumab and IgE formed different complexes in serum than in phosphate-buffered saline in terms of both size and affinity.

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During protein drug development, scientists rely on an array of analytical techniques to assess the stability and efficacy of the protein of interest. In general, stability assessment and characterization are done under storage conditions in a specific container closure system. Although biopharmaceuticals are well characterized *in vitro*, little is known about the physicochemical characteristics of protein drugs once circulating in the blood flow. The detection of potential incompatibilities, degradation, and drug interactions in serum relies solely on expensive, complex, and time-consuming animal studies and clinical trials. The purpose of our work is to extend protein characterization to the events following protein administration and to better understand the characteristics of biopharmaceuticals under physiological conditions.

Analytical ultracentrifugation (AUC),¹ particularly sedimentation velocity, has the ability to provide size, shape, and binding information with a high resolution [1]. AUC is based on first principles

and is a method of choice to study protein association and assembly in solution [2]. Recent advances in AUC hardware brought fluorescence optics to AUC, enabling higher sensitivity but also, and more important, adding selectivity to the detection optics [3]. Fluorescent molecules, whether labeled or intrinsically fluorescent, can be detected in complex matrices, including biological fluids; thus, for the first time, AUC can be used to analyze samples in human serum.

Characterizing therapeutic proteins in human serum should allow the detection of undesirable species formed on injection (e.g., aggregates). In addition, the determination of protein complex size and stoichiometry in serum can provide valuable information about how protein drugs interact with their targets and other proteins. The sensitivity and selectivity of the AUC fluorescence detection system (FDS) allow protein characterization at physiological concentrations in serum.

We used omalizumab (formerly referred to as rhuMABE25 [4]), the active ingredient in Xolair, to examine the feasibility of protein characterization in human serum. Omalizumab is a recombinant humanized monoclonal antibody that selectively binds human immunoglobulin E (IgE) [5] and is currently marketed for the treatment of moderate to severe persistent asthma in adults and adolescents. Omalizumab binds free circulating IgE and inhibits the binding of IgE to the high-affinity IgE receptor (FcεRI) on the sur-

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¹ Abbreviations used: AUC, analytical ultracentrifugation; FDS, fluorescence detection system; IgE, immunoglobulin E; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; UV, ultraviolet; HSA, human serum albumin; SEC, size exclusion chromatography; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; RMSD, root mean square deviation.

face of mast cells and basophils, limiting the release of histamine and other mediators of the allergic response [6]. In vitro studies have shown that omalizumab forms a variety of limited-size complexes with IgE and that the size distribution of omalizumab–IgE complexes is dependent on the molar ratio of the antibody and antigen [4]. In the current study, omalizumab and omalizumab–IgE complexes were characterized in human serum. Because fluorescence labeling of omalizumab was required to perform the measurements, extensive characterization studies were performed on the labeled omalizumab to demonstrate that the labeling procedure did not significantly alter omalizumab's characteristics.

Materials and methods

Materials

Omalizumab (Xolair, rhuMAbE25, molecular mass 150 kDa) was expressed in Chinese hamster ovary (CHO) cells and purified at Genentech (South San Francisco, CA, USA) as described previously [4]. Human IgE (molecular mass 190 kDa) was prepared from a myeloma cell line and purified at Genentech as described previously [4]. Alexa Fluor 488 Protein Labeling Kits were purchased from Molecular Probes (Eugene, OR, USA). Albumin from human serum (fraction V, $\geq 96\%$) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Human serum and plasma were collected from healthy volunteers free of medications. Serum was collected in Vacutainer SST tubes, and plasma was collected in both Vacutainer K₂EDTA and heparin tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Sera and plasma were stored at 2 to 8 °C. AUC experiments were performed in serum, plasma, or phosphate-buffered saline (PBS) pH 7.2: 137 mM NaCl, 27 mM KCl, 8 mM Na₂HPO₄, and 1.5 mM KH₂PO₄. The chemical reagents were analytical grade or better. Water used in the experiments was deionized by a Milli-Q Biocel system (Millipore, Billerica, MA, USA).

Protein concentration determination

Protein concentration was determined by ultraviolet (UV) spectroscopy using a model 14 UV–Vis spectrophotometer (Aviv Bio-medical, Lakewood, NJ, USA). Protein spectra were acquired in 1-mm pathlength quartz cells, the absorbance of the buffer was subtracted, and the concentration was calculated using a molar absorption coefficient at 280 nm of 240,000 M^{−1} cm^{−1} for omalizumab, 304,000 M^{−1} cm^{−1} for IgE, and 34,433 M^{−1} cm^{−1} for human serum albumin (HSA) and a molar absorption coefficient at 494 nm of 71,000 M^{−1} cm^{−1} for the Alexa Fluor 488 dye.

Antibody labeling

Omalizumab was diluted to 2 mg/ml in PBS, and 50 μ l of 1 M sodium bicarbonate was added to 0.5 ml of the 2-mg/ml omalizumab solution. The omalizumab solution was transferred to a vial of reactive dye (Alexa Fluor 488 carboxylic acid, tetrafluorophenyl ester) and incubated for 1 h at room temperature under mild agitation. Labeled omalizumab was purified by size exclusion chromatography (SEC) using BioGel P-30 Fine purification resin (Bio-Rad Laboratories, Hercules, CA, USA) with PBS pH 7.2 (0.1 M potassium phosphate and 1.5 M NaCl with 2 mM sodium azide) as the eluant. The antibody and free dye bands were detected by illumination with a handheld UV lamp. UV spectra (background corrected) of the purified omalizumab were acquired between 225 and 550 nm. The labeled omalizumab concentration was calculated using the following equation:

$$\text{protein concentration (M)} = \frac{[A_{280} - (A_{494} \cdot 0.11)] \cdot \text{dilution factor}}{240000},$$

where A_{280} and A_{494} are the sample absorbance at 280 and 494 nm, respectively, and 0.11 is a correction factor to account for absorption of the dye at 280 nm. The degree of labeling was calculated using the following equation:

$$\text{moles dye per mole protein} = \frac{A_{494} \cdot \text{dilution factor}}{71000 \cdot \text{protein concentration (M)}}.$$

Size exclusion chromatography

SEC was performed on an Agilent 1100 HPLC system (Agilent, Santa Clara, CA, USA). A TSK-Gel Super SW 3000 column (Supelco, Bellefonte, PA, USA) was equilibrated with PBS pH 6.8 (0.1 M potassium phosphate and 0.1 M sodium chloride) at a flow rate of 0.2 ml/min for at least 30 min at room temperature. After at least three omalizumab injections (conditioning runs), unlabeled omalizumab was injected, followed by two injections of labeled omalizumab and one more injection of unlabeled omalizumab. Finally, the elution buffer was injected once. The injection sequence was repeated three times in total. The injection volume was 20 μ l. Antibody samples were diluted to 1 mg/ml in elution buffer before injection. Detection was performed at 280 and 494 nm.

Potency assay

Omalizumab potency was determined using a solid-phase enzyme-linked immunosorbent assay (ELISA) as described previously [7]. Briefly, 96-well high-binding polystyrene plates were coated overnight at 2 to 8 °C with 10 ng of human IgE receptor α -chain immunoglobulin G (IgG) chimera (Fc ϵ RI–IgG) in 100 μ l of carbonate buffer (0.05 M, pH 9.6). The wells were washed, and 200 μ l of assay buffer (PBS, 0.5% BSA, and 0.05% polysorbate 20) was added and incubated for 1 h at 25 °C. In separate polypropylene tubes, the omalizumab samples and standard omalizumab solutions were diluted with assay buffer to reach concentrations ranging from 0 to 6.25 μ g/ml for omalizumab samples and from 0 to 100 μ g/ml for omalizumab standards. Then an equal volume of 10 ng/ml biotinylated IgE was added, and the tubes were vortexed. The well plates were washed, and 100 μ l of the omalizumab–IgE solutions was transferred to the Fc ϵ RI-coated wells; the plates were incubated for 2 h at 25 °C and washed. Captured biotinylated IgE was detected with streptavidin–horseradish peroxidase and tetramethyl benzidine peroxidase substrate. A parallel line bioassay curve-fitting program was used to generate separate log (concentration)–response lines for the omalizumab samples and standards, allowing determination of the potency of the samples relative to the standards. Each sample was tested twice.

Density and viscosity measurements

Buffer and sera densities were measured at 25 °C on a DM5000 densitometer (Anton Paar, Graz, Austria) calibrated each day with dry air and water at 20 °C before use. Buffer and sera viscosities were measured at 25 °C with an AMVn rolling ball viscometer (Anton Paar) at an angle of 30° using a capillary with an internal diameter of 1.6 mm and a ball with a diameter of 1.5 mm and a density of 7.85 g/cm³. The capillary/ball system was calibrated with water at 25 °C.

Analytical ultracentrifugation

Sedimentation velocity experiments were performed in an Optima XL-I analytical ultracentrifuge equipped with absorbance optics, interference optics (Beckman Coulter, Fullerton, CA, USA), and

fluorescence optics (Aviv Biomedical). Experiments were performed in 12-mm graphite-filled Epon centerpieces (Spin Analytical, Durham, NH, USA) at 25 °C using a rotor speed of 40,000 rpm. When using the absorbance optics, scans were acquired at a wavelength of 230 nm and 30- μ m radial increments. When using the fluorescence optics, data were acquired at 20- μ m radial increments and averaging 5 revolutions/scan. The sedimentation boundary was analyzed with SEDFIT (version 11.3 and 11.72c) [8]. The continuous $c(s)$ distribution with 90% confidence level was calculated after optimizing baseline, meniscus, and cell bottom positions by nonlinear regression. All s values obtained with the $c(s)$ distribution in PBS were converted to $s_{20,W}$ with SEDNTERP (version 1.09) using the measured density and viscosity of PBS. The s values obtained with the $c(s)$ distribution in sera were converted to an apparent corrected sedimentation coefficient ($s_{20,W}^+$) with SEDNTERP (version 1.09) using the measured densities and viscosities of the sera.

Statistics

Variances were compared with an F test. If the variances were found to be significantly different ($P < 0.05$), the means were compared with a two-sample unpaired heteroscedastic t test and two-tailed P values were reported. If the variances were not found to be significantly different ($P > 0.05$), the means were compared with a two-sample unpaired homoscedastic t test and two-tailed P values were reported.

Results

Characterization of labeled omalizumab

Characterization of therapeutic proteins in serum by AUC requires labeling of the protein of interest. The conditions used during labeling yielded an average degree of labeling of 4.5 moles dye per 1 mole protein (measured on four separate batches: $SD = 0.3$, $n = 4$). The fluorescent dye reacts efficiently with primary amines, resulting in a labeling of lysine residues and N-terminal amines of omalizumab. The labeling procedure and the dye molecule itself may increase protein aggregation and fragmentation as well as alter the properties of the protein [9,10]. The following sections show that labeled omalizumab had the same potency and physicochemical properties as unlabeled omalizumab.

Size exclusion chromatography

SEC using detection at 280 nm allowed a direct comparison of the aggregate and fragment content of a batch of labeled and unlabeled omalizumab. Unlabeled omalizumab was 98.25% monomeric ($SD = 0.75$, $n = 6$) and labeled omalizumab was 98.77% monomeric ($SD = 0.08$, $n = 6$); the difference between the measurements was not statistically significant ($P = 0.15$). Fig. 1 shows chromatograms of labeled and unlabeled omalizumab. No additional peaks were observed in labeled omalizumab samples. The peaks observed after 20 min retention time are attributed to the buffer. Due to the presence of sodium azide, labeled omalizumab (dotted line) showed a slightly different buffer peak.

The free dye content was determined simultaneously by integrating the area under the peaks at 494 nm; here 4.91% of the dye present in the solution was not associated with omalizumab ($SD = 0.02$, $n = 6$).

Analytical ultracentrifugation

AUC allows assessing the aggregation of protein pharmaceuticals while circumventing the most significant limitations of SEC. AUC is considered as an orthogonal technique to SEC and is exqui-

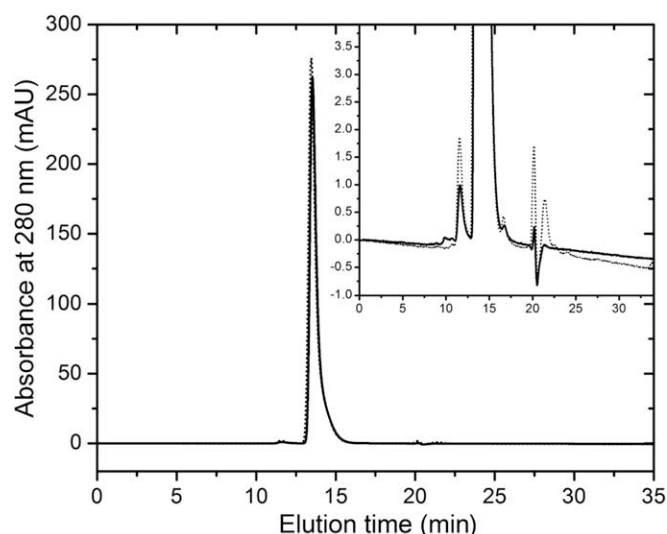


Fig. 1. Characterization of unlabeled (solid line) and labeled (dotted line) omalizumab by SEC. Labeled and unlabeled samples were not found to be significantly different in monomer content (see text). Peaks at more than 20 min retention times are attributed to the buffer.

sitely sensitive to the presence of aggregates that can potentially be adsorbed to the SEC column frits or resin [11,12]. All batches of labeled omalizumab were characterized by AUC at a physiologically relevant concentration of 10.2 nM (Fig. 2). The sedimentation coefficient of omalizumab in PBS, corrected for the standard conditions of water at 20 °C ($s_{20,W}$), was 6.9 S ($SD = 0.1$, $n = 24$). Data obtained by AUC in PBS were in good agreement with those from SEC, showing a monomer content greater than 98% across all batches and confirming that the labeling procedure and fluorescent dye did not cause an increase in aggregation or fragmentation.

Omalizumab-IgE complexes have previously been characterized by AUC, and the size distribution of the complexes formed was shown to be dependent on the omalizumab-IgE molar ratio [4]. In the current work, AUC data were fitted by a direct boundary model, $c(s)$, maximizing resolution and sensitivity [13]. To allow a fair comparison of unlabeled and labeled omalizumab, AUC data were also acquired in PBS with UV optics, with omalizumab con-

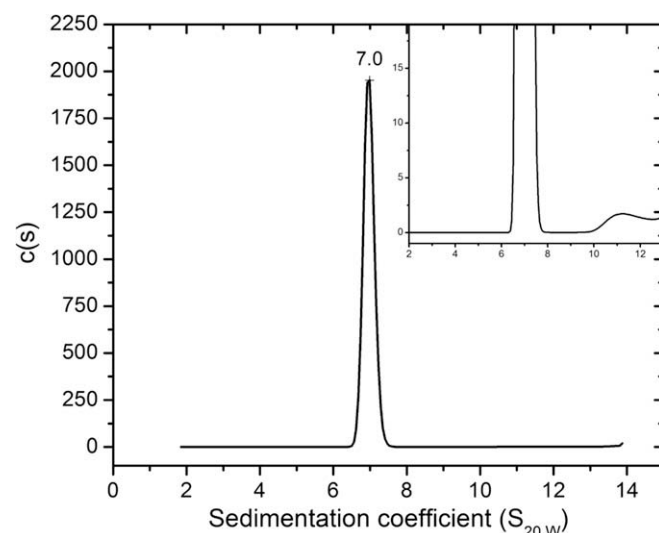


Fig. 2. Continuous sedimentation coefficient distribution of labeled omalizumab in PBS at a concentration of 10.2 nM. Labeled omalizumab was 99% monomeric. AUC did not reveal the presence of aggregates missed by SEC.

centrations (labeled or unlabeled) ranging between 127 and 427 nM. The use of UV optics allowed the detection of omalizumab (labeled or not) and IgE. Fig. 3 shows that labeled omalizumab was able to form the same complexes with IgE as those formed by unlabeled omalizumab, confirming that the labeling process and fluorescent dye did not interfere with the ability of omalizumab to bind IgE.

At a molar ratio of 1:1 (Figs. 3A and B), a significant level of monomeric omalizumab was still observed, as shown by the presence of the peak at approximately 7 S. As expected, the 1:1 molar ratio yielded more of the larger complexes, with a major species at approximately 21 S, corresponding to hexamers [4]. An excess of IgE (Figs. 3C and D) resulted in the formation of smaller complexes and a significant peak at 8.2 S, corresponding to free IgE. Similarly, an excess of omalizumab (Figs. 3E and F) resulted in smaller complexes and a larger peak at approximately 7 S, corresponding to the monomeric omalizumab. At molar ratios of 1:3 and 3:1, IgE and omalizumab did not exhibit the hexamers typical of the equimolar ratio.

To obtain data at low concentrations (10.2 nM), labeled omalizumab was diluted in PBS with IgE present at an equimolar ratio and the sedimentation of the complexes was followed with the AUC fluorescence detection system (AUC-FDS). Fig. 4 shows that even though the sedimentation profile seems to be different at low concentrations (cf. Fig. 3B), the main features of the equimolar mixture are still present: a significant level of monomeric omalizumab (peak at 6.7 S) and the presence of larger complexes (peak at 17.6 S).

Potency

The mechanism of action of omalizumab involves blocking the binding of IgE to its high-affinity receptor (FcεRI) and preventing the subsequent activation of mast cells or basophils [5,6]. The currently used potency assay is designed to mimic the mechanism of action by assessing the inhibition of IgE binding to its high-affinity receptor. Two batches of labeled omalizumab were pooled to obtain enough material to run a potency assay. Unlabeled omalizumab had an average potency of $0.867 \cdot 10^4$ U/mg and labeled omalizumab had an average potency of $0.886 \cdot 10^4$ U/mg, indicating that the labeling process did not significantly compromise the binding activity of omalizumab.

Measurements in human serum and plasma

The sedimentation profile of labeled omalizumab was measured in the sera from three different donors. The initial concentration of labeled omalizumab in human sera was 10.2 nM, a physiologically relevant concentration. The measured densities and viscosities of the sera are presented in Table 1. Two main peaks were identified at approximately 2.6 and 4.4 S (uncorrected sedimentation coefficient values in serum at 25 °C). To correct for the effects and variations in density and viscosity observed between the different sera, as well as to allow the comparison of data obtained in serum with data obtained in PBS, the sera were considered as solvents; the bulk densities and viscosities of sera as reported in Table 1 were used to calculate apparent corrected sedimentation coefficients denoted $s_{20,w}^+$. The two main peaks showed $s_{20,w}^+$ of 4.3 S (SD = 0.1, $n = 18$) and 7.3 S (SD = 0.2, $n = 18$). The first peak at 4.3 S represented 50% of the signal (SD = 5, $n = 28$). Fig. 5 shows a typical sedimentation coefficient distribution of labeled omalizumab in human serum.

The presence of the peak at 4.3 S was unexpected. AUC experiments performed on serum alone, without any added omalizumab or IgE, consistently showed a peak at approximately 4 S (Fig. 6). Control AUC experiments performed on purified bovine and human serum albumin, together with the abundance of albumin in human serum (35–50 mg/ml), indicate that the 4.3-S peak can be

attributed to HSA. Because albumin does not show intrinsic fluorescence when excited at 488 nm, the fluorescence signal must come from an albumin-bound molecule. Binding of natively fluorescent molecules such as flavins and hemoglobin oxidation products to albumin is well documented [14,15]. The fluorescence detection system (FDS) does not subtract the signal of a reference from the sample; therefore, albumin fluorescence cannot be removed by using serum as a reference. Because albumin fluorescence did not interfere with the measurements, we considered that determining which molecule caused the observed albumin fluorescence was beyond the scope of the current study.

To assess the potential influence of clotting factors on our experiments, omalizumab was also analyzed in plasma. Experiments performed in plasma (obtained from K₂EDTA and heparin tubes) gave similar results to experiments performed in serum, but a lower albumin fluorescence was observed; the 4.3-S peak represented 38% of the signal in plasma (SD = 4, $n = 4$).

The 7.3- $s_{20,w}^+$ peak is consistent with the sedimentation of an antibody; however, it is significantly higher ($P < 0.01$) than the 6.9- $s_{20,w}$ sedimentation coefficient reported in PBS (see “Characterization of labeled omalizumab” section above). Interestingly, the sedimentation coefficient of albumin was not higher in serum than in PBS. Overall, data acquired in serum showed higher variability than data acquired in PBS; for example, the standard deviation of the omalizumab sedimentation coefficient was 0.1 S in PBS and 0.2 S in serum. Interpretation of AUC experiments in serum is complicated by the fact that albumin, which sediments during the time course of the experiment, is a contributor to serum’s viscosity and density. Immunoglobulins are also natively present in serum in concentrations greater than 10 mg/ml. We used the bulk densities and viscosities of the sera to calculate apparent corrected sedimentation coefficients ($s_{20,w}^+$). Such an approach can be only an approximation because the equation used for conversion of raw s values to $s_{20,w}$ adjusts sedimentation data for solvent properties and not solution properties. Furthermore, systematic deviations are observed between the fitted lines and the raw data (Fig. 5); the $c(s)$ distribution cannot fully describe the sedimentation of omalizumab in serum. Several fitting parameters can have an impact on the shape of the $c(s)$ distributions and on the calculated s values [16]. Floating the meniscus position and having a fixed f/f_0 of 1.47 during the fitting routine could potentially have introduced an artificial difference between s values obtained in PBS and in serum. We used a subset of our data, as well as data from a fourth serum donor, to assess the influence of meniscus position and f/f_0 on the $c(s)$ distributions. All of the experiments included in this analysis were acquired in the same conditions, at the same concentration (10.2 nM) and in the presence of labeled omalizumab only (no IgE), over a period of 9 months. The meniscus position was manually set at the beginning of the optical artifact for experiments performed both in PBS and in serum. With a fixed meniscus position, s values obtained in serum were still significantly higher than those obtained in PBS, with a more symmetrical distribution of the s values around the mean. Using a fixed meniscus position at the beginning of the optical artifact, we fitted f/f_0 and observed that no significant difference could be observed between omalizumab’s $s_{20,w}$ in PBS and $s_{20,w}^+$ in serum; both converged to 7 S ($P = 0.15$). In serum, the average f/f_0 was 2.1, and minor changes were observed in the shape of the $c(s)$ distributions; the $s_{20,w}^+$ of omalizumab decreased from 7.3 to 7.0, but the shape of the distribution remained similar. The calculated root mean square deviation (RMSD) was marginally lower than with a fixed f/f_0 .

To eliminate the possibility that sedimentation of larger species early in the AUC run could decrease the density and viscosity of serum and cause higher s values to be observed, we measured serum density and viscosity after 5 min centrifugation at 116,480g, corresponding to the mean centrifugal force used during our experiments. The serum density and viscosity were unchanged.

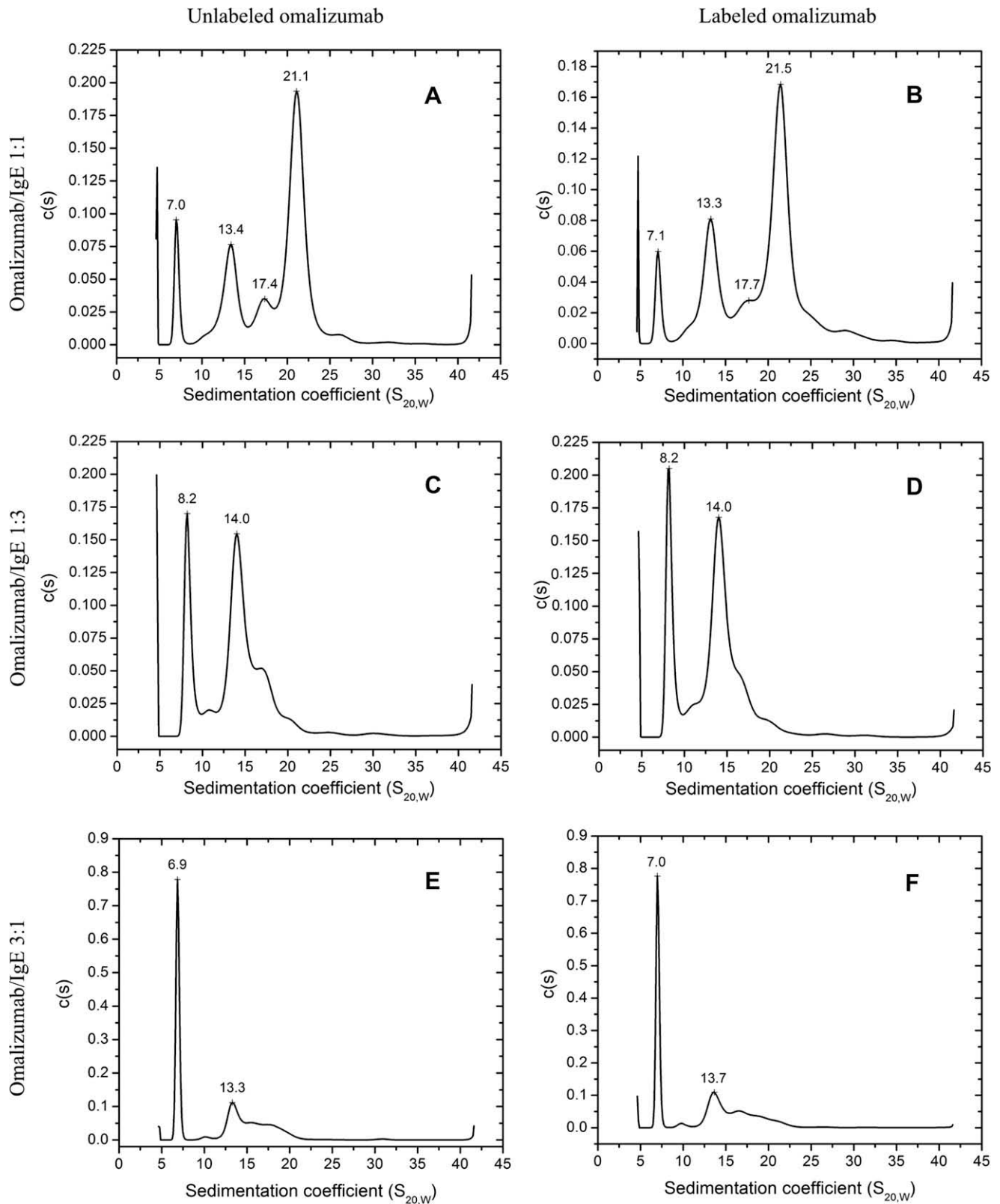


Fig. 3. Omalizumab-IgE complexes formed at various ratios in PBS by unlabeled omalizumab (left panels) and labeled omalizumab (right panels): (A) unlabeled omalizumab-IgE (1:1); (B) labeled omalizumab-IgE (1:1); (C) unlabeled omalizumab-IgE (1:3); (D) labeled omalizumab-IgE (1:3); (E) unlabeled omalizumab-IgE (3:1); (F) labeled omalizumab-IgE (3:1). Total protein concentration was adjusted to obtain an absorbance of approximately 1 at 230 nm: 269 nM omalizumab for panels A and B, 127 nM omalizumab for panels C and D, and 427 nM omalizumab for panels E and F. Unlabeled and labeled omalizumab formed similar complexes with IgE.

Overall, the fitted curves yielded consistent results and represent a reasonable approximation of the signal; the positions of

the two visible boundaries (half-height of the plateaus) were accurately described by the fit.

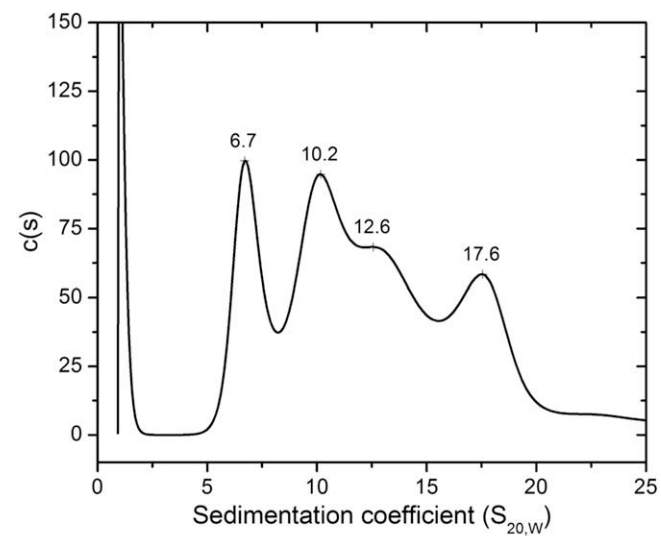


Fig. 4. Omalizumab–IgE complexes formed at an equimolar ratio and low concentration in PBS. The sedimentation profile was recorded using fluorescence optics. The labeled omalizumab concentration was 10.2 nM.

Table 1
Measured densities and viscosities of the sera obtained from three donors.

	Serum density at 25 °C (g/ml)	Serum viscosity at 25 °C (mPa · s)
Donor A	1.02447	1.5500
Donor B	1.02435	1.5533
Donor C	1.02164	1.3967

Omalizumab–IgE complex formation in human serum

The serum concentration of IgE is highly variable among individuals. Asthmatic patients usually have IgE levels between hundreds and thousands of nanograms per milliliter (ng/ml) [17,18]. Healthy volunteers can be expected to have IgE levels between 35 and 170 ng/ml (0.2–0.9 nM), although lower and higher values are not uncommon [19]. To study omalizumab–IgE complexes in human serum, IgE and labeled omalizumab were diluted in human serum, yielding an omalizumab and IgE concentration of 10.2 nM each. The 1:1 molar ratio was expected to yield the largest aggregates according to data obtained in PBS (Figs. 3 and 4), and the IgE concentration (10.2 nM, 1938 ng/ml, 800 UI/ml) was consistent with IgE levels observed in patients [17,18]. A typical sedimentation coefficient distribution of omalizumab and IgE diluted in human serum is presented in Fig. 7. The 4.3-S peak is caused by albumin, as discussed above, and represents approximately 50% of the signal. The two main differences of the *c(s)* distribution between data obtained in serum and data obtained in PBS are the absence of the 21-S peak and the presence of an 8.7-S peak instead of the omalizumab monomer peak observed at 7.3 S in PBS (cf. Figs. 3A, B, and 4). We showed, in the case of omalizumab alone in serum, that the *c(s)* distribution was slightly sensitive to fitting parameters such as meniscus position and *ff*₀. To assess the robustness of the *c(s)* distributions and to ensure that the differences in *c(s)* distributions observed between serum and PBS were not artifactual, several datasets were processed with or without fitting of meniscus and *ff*₀. As in the case of omalizumab alone in serum, fitted *ff*₀ values were large. The meniscus and bottom positions often floated to unreasonable values. Nevertheless, in all cases the main features of the *c(s)* distributions were preserved, both in serum and in PBS. Furthermore, to analyze the sedimentation data with a model-independent method, the same datasets were analyzed with *dc/dt* (data not shown). Despite the lower

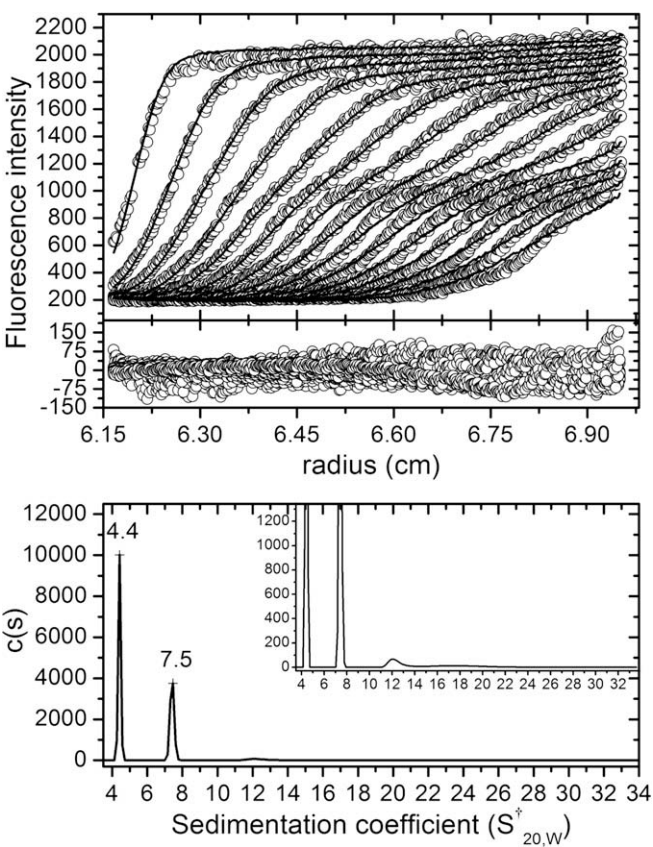


Fig. 5. Sedimentation of labeled omalizumab in human serum at a concentration of 10.2 nM. The upper top panel shows raw data (circles) and best fit (lines). For clarity, only 1 of every 20 scans is plotted. The lower top panel shows fit residuals of the plotted scans. The bottom panel shows the *c(s)* distribution of labeled omalizumab in serum (90% confidence interval). The main peaks are indicated. The first peak was attributed to albumin (see text). The second peak at 7.5 S was attributed to monomeric omalizumab. *s*_{20,W}^{*} denotes an apparent corrected sedimentation coefficient calculated using the serum's density and viscosity.

resolution provided by *dc/dt*, the data showed that the largest 1:1 omalizumab–IgE complexes formed in PBS were not present in serum.

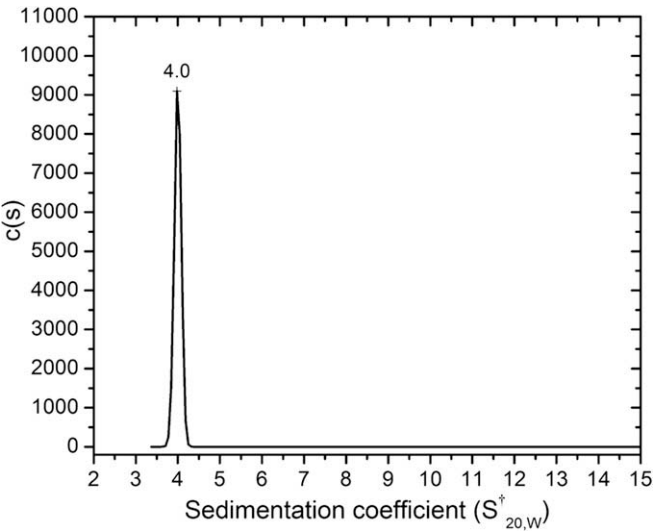


Fig. 6. Continuous sedimentation coefficient distribution of serum alone, determined with the fluorescence detection system. All of the sera that were used in this study showed fluorescence associated with albumin (peak at 4 S). *s*_{20,W}^{*} denotes an apparent corrected sedimentation coefficient calculated using the serum's density and viscosity.

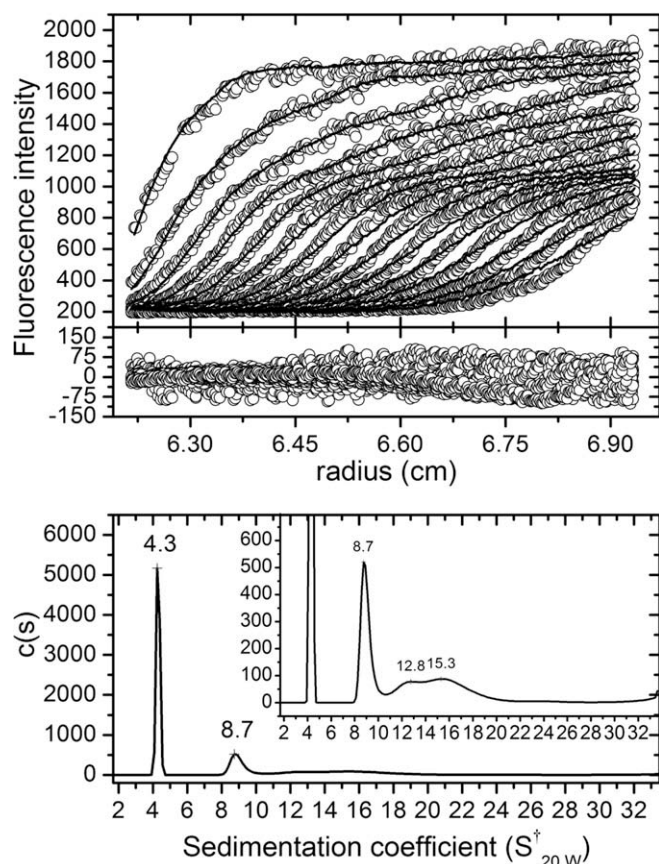


Fig. 7. Sedimentation of omalizumab-IgE complexes in human serum. The omalizumab concentration was 10.2 nM, and the omalizumab-IgE molar ratio was 1:1. The upper top panel shows raw data (circles) and best fit (lines). For clarity, only 1 of every 20 scans is plotted. The lower top panel shows fit residuals of the plotted scans. The bottom panel shows the $c(s)$ distribution of omalizumab-IgE complexes in serum (90% confidence interval). The main peaks are indicated. The first peak at 4.3 S was attributed to albumin. Subsequent peaks represent omalizumab-IgE complexes. Despite the omalizumab-IgE molar ratio of 1:1, no major peak could be observed at 21 S (cf. Figs. 3 and 4). $s_{20,W}^{\dagger}$ denotes an apparent corrected sedimentation coefficient calculated using the serum's density and viscosity.

The absence of the 21-S peak and the different profile observed in serum compared with PBS underline the importance of direct characterization of therapeutic proteins in human serum. Even though endogenous IgE from the blood donor may alter the omalizumab-IgE molar ratio, it is unlikely to shift the ratio enough to cause the disappearance of the 21-S peak given that typical endogenous IgE levels are an order of magnitude lower than the concentrations of added IgE used in our studies (cf. above). Furthermore, data obtained in threefold diluted serum, where endogenous IgE is present at even lower concentrations and so is less likely to alter the omalizumab-IgE molar ratio, also showed the absence of the 21-S peak with omalizumab and IgE at an equimolar ratio (data not shown).

Absence of the 7.3-S peak in serum samples—replaced by an 8.7-S peak—further confirms that the different sedimentation profile reflects distinct behavior in serum compared with PBS. The 8.7-S peak cannot be attributed to monomeric omalizumab (which has a lower sedimentation coefficient), or to IgE (which is not visible in fluorescence AUC experiments because it is not labeled), or to an omalizumab-IgE complex (which would have a higher sedimentation coefficient, as shown by the data obtained in PBS). The 8.7-S peak likely results from a reaction boundary due to rapidly reversible associating systems yielding apparent distributions that have peaks at positions between the s value of the reacting species

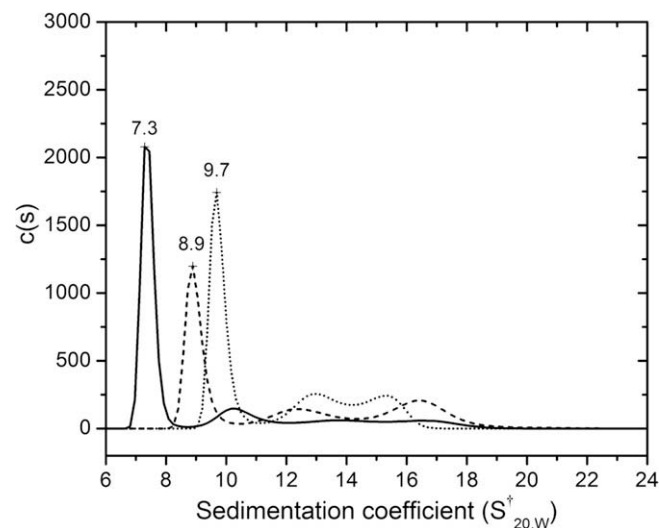


Fig. 8. Continuous sedimentation coefficient distribution of omalizumab-IgE complexes at different molar ratios in human serum. Solid line: omalizumab-IgE (6:1); dashes: omalizumab-IgE (1:1); dotted line: omalizumab-IgE (1:6). The omalizumab concentration was 10.2 nM. The albumin peak at 4.3 S was omitted for clarity. The position of the main peak varied with the molar ratio, indicative of a complex formation taking place on the time scale of sedimentation. The 8.9-S peak corresponds to a reaction boundary, whereas the 7.3- and 9.7-S peaks correspond to monomeric omalizumab and omalizumab-IgE complex, respectively. $s_{20,W}^{\dagger}$ denotes an apparent corrected sedimentation coefficient calculated using the serum's density and viscosity.

and the s value of the complex formed (in our case between the omalizumab s value and the omalizumab-IgE complex s value) [20]. Analysis of different ratios of omalizumab and IgE in human serum confirmed that the 8.7-S peak was attributable to a reaction boundary (Fig. 8). A sixfold molar excess of omalizumab resulted in the expected omalizumab peak at 7.3 S as well as omalizumab-IgE complexes ranging in size from 10 to 18 S. A sixfold molar excess of IgE was able to complex all of the available omalizumab; the main species formed was an omalizumab-IgE complex at 9.7 S, probably a heterodimer [4], as well as several larger complexes between 12 and 18 S. Again, an omalizumab-IgE molar ratio of 1:1 yielded a peak at 8.9 S, an intermediary position between the omalizumab monomer and the omalizumab-IgE heterodimer. The dependency of the peak position on the omalizumab-IgE molar ratio is similar to the concentration dependence observed in protein self-association [20,21] and confirms that the peak can be attributed to a reaction boundary. At this time, the limitations of the $c(s)$ analysis in serum samples probably do not allow a more precise assessment of the interactions taking place between omalizumab and IgE in serum.

The depletion of monomeric omalizumab at a 1:1 omalizumab-IgE molar ratio, even at a concentration of 10.2 nM (Figs. 7 and 8), was observed in serum and contrasted with the data obtained in PBS at higher concentrations (Figs. 3A and B) and at the same concentration (Fig. 4). We hypothesize that the affinity of omalizumab for IgE is higher in serum than in PBS, possibly through the formation of different complexes as shown by the different sedimentation coefficient profiles.

Discussion

Characterization of labeled omalizumab

We showed that labeling omalizumab under the conditions described in this article did not alter its characteristics or its potency. Sedimentation coefficient distributions of unlabeled and labeled

omalizumab did not show significant differences (Fig. 3). Labeling is currently a prerequisite for analysis of proteins by AUC in serum (with the exception of natively fluorescent proteins such as green fluorescent protein), and a detailed characterization of the labeled protein is mandatory to ensure that the data obtained with the labeled protein are relevant. Even though we showed that omalizumab was not altered by the labeling procedure, such results cannot be extrapolated to other proteins. Nevertheless, in our case efficient labeling was obtained without detectable side effects on the protein quality, an encouraging result that paves the way for further studies. Sedimentation coefficient distributions were equivalent in PBS and in serum, and they showed similar fluorescence intensities, indicating that the quantum yield of labeled omalizumab was comparable in PBS and in serum.

Measurements in human serum and plasma

We observed significant albumin-bound fluorescence in human sera. AUC data may have been better fitted without an albumin signal given that the residuals were higher in the boundary region describing albumin (Figs. 5 and 7, lower half of the boundary). The nature of the fluorescent molecule bound to albumin remains unclear but was not further investigated because the albumin peak did not overlap omalizumab or omalizumab–IgE complex peaks on the sedimentation coefficient distributions and did not affect the overall results.

To allow a better comparison of data obtained in PBS and in serum, we corrected the serum s values to an apparent corrected sedimentation coefficient ($s_{20,w}^{\dagger}$) using the sera bulk densities and viscosities. Such an approach is unusual because the equation used in the conversion accounts for solvent properties and not solution properties. Even though our approach might not generate entirely accurate $s_{20,w}^{\dagger}$ values, we found that experimental $s_{20,w}^{\dagger}$ values obtained in serum were in good agreement with $s_{20,w}$ values obtained in PBS. We showed that the fitting parameters could slightly shift the position of the omalizumab peak in serum but that, in general, the corrected s values were comparable between PBS and serum. The potential errors on sedimentation coefficients determined in serum do not affect our conclusions on the different complexes formed in PBS and in serum, which are based on the presence of specific peaks on the $c(s)$ distributions.

In this study, the data were fitted with a $c(s)$ analysis, floating the meniscus and baseline positions while using a fixed f/f_0 of 1.47. The fitting error, which in serum is larger than the random noise, indicates the presence of systematic deviations. We showed that fitting for f/f_0 yielded $c(s)$ distributions of comparable shape but slightly changed the sedimentation coefficients. The large f/f_0 that were obtained in serum caused the meniscus and bottom positions to float to unreasonable values, did not significantly reduce the RMSD, and probably do not have a physical meaning. Despite its shortcomings in analyzing serum data, the $c(s)$ analysis provided reproducible results that were verified by the model-independent dc/dt approach. The $c(s)$ analysis is arguably the most reasonable and practical model available for serum samples; however, the results obtained in serum should not be overinterpreted, especially in terms of s value accuracy and quantitation of minor species. Although more complex models are available (e.g., accounting for nonideality), such models were developed for dilute solutions and probably should not be used until the thermodynamics and hydrodynamics of serum samples are better understood.

We showed that the study of a therapeutic antibody in human serum was feasible; omalizumab was detected in serum having a sedimentation coefficient consistent with its molecular weight and shape, and omalizumab–IgE complexes were successfully characterized. Detailed characterization of biopharmaceuticals in serum may be limited to entities larger than albumin; Kingsbury

and coworkers' work on the transthyretin tetramer, a complex with a molecular weight comparable to albumin, demonstrated the potential of the fluorescence-equipped AUC but the data obtained in serum could not be described by the $c(s)$ model [21]. Sedimentation of the transthyretin tetramer was probably concomitant to albumin sedimentation, rendering the interpretation of the boundary difficult because albumin contributes largely to serum density and viscosity, which are important parameters in analyzing sedimentation velocity experiments. Antibodies and antibody–antigen complexes sediment faster than albumin; therefore, their boundaries are less influenced by albumin sedimentation. Endogenous immunoglobulins may cosediment with omalizumab but are less likely to have a significant impact on the $c(s)$ distribution because of their lower concentration compared with serum albumin. Even in conditions where the species of interest sediments faster than albumin, we observed that the $c(s)$ fits are slightly perturbed by the presence of albumin, as shown by the residuals in Figs. 5 and 7. As discussed above, observation of the raw data and fitted lines seems to indicate that most of the nonrandom residuals can be attributed to the albumin peak (slower sedimenting boundary, lower half of the raw scans) and did not compromise the interpretation of the data even though they may contribute to the higher variability of s values observed in serum.

After spiking sera with omalizumab and IgE, omalizumab–IgE complexes were detected and could be characterized; we were able to confirm that omalizumab and IgE form complexes of limited size [4,6,22]. The low omalizumab concentration (10.2 nM) may have an effect on the size distribution of the complexes (cf. Figs. 3 and 4), but the larger complexes detected in PBS even at low concentration (Fig. 4) were not detected in serum. Omalizumab may adopt a different, more compact conformation in serum; this would explain the absence of hexamers given that the more compact conformation may reduce the flexibility of the molecule and prevent the formation of larger complexes. The serum proteins may act as a crowding agent, and the effects of excluded volume in crowded environments have been shown to favor more compact protein conformations and to enhance protein association [23,24]. The enhanced protein association may also contribute to the higher affinity of omalizumab for IgE observed in serum. An alternate explanation for the differences in $c(s)$ distributions is that in serum Johnston–Ogston effects could distort the areas of the peaks of the $c(s)$ distribution due to the high concentration of background molecules in serum (albumin and immunoglobulins) [25]. In a multicomponent system, the Johnston–Ogston effect results in an increase in the apparent concentration of the slower sedimenting components with a corresponding decrease in the apparent concentration of the faster components. The effect is caused by the sedimentation of the faster components delaying the sedimentation of the slower components [25]. In the case of omalizumab–IgE complexes, Johnston–Ogston effects are unlikely to be of any significance because the omalizumab–IgE complexes sediment much faster than albumin and native serum immunoglobulins. The omalizumab–IgE complexes themselves are present in concentrations too low (nM) to affect the sedimentation of slower species. In a concentration range of 2 to 17 nM, labeled omalizumab did not increase the fluorescence of the albumin boundary (data not shown). Even if Johnston–Ogston effects had an impact on omalizumab–IgE complexes, the probable outcome would be an apparent reduction in the concentration of the larger species (dimers, trimers, and hexamers). On the contrary, we observed an increase in dimers and trimers but a decrease in monomers and hexamers. Johnston–Ogston effects may, however, explain the sharpening of the albumin boundary. Substantial sharpening of the slow boundaries is a typical characteristic of Johnston–Ogston effects [25]. In the case of serum, native immunoglobulins (one of the major faster

sedimenting components) may be slowing down the sedimentation of albumin (the slow component), consistent with a sharpening of the albumin boundary and an apparent increase in albumin fluorescence intensity; however, this should not affect omalizumab–IgE complexes.

In addition to protein sedimentation, lipoprotein flotation is present in serum. The hydrodynamic effect of such a counterflow may perturb the sedimentation of the sample. Our data seem to indicate that the counterflow of lipoproteins did not affect sedimentation of omalizumab in serum; human albumin and omalizumab showed similar corrected sedimentation coefficients in PBS and in serum.

Although we cannot completely exclude the possibility that serum components may weakly bind to omalizumab or omalizumab–IgE complexes, we observed that the corrected sedimentation coefficient of omalizumab was similar in PBS and in serum, indicating that the effect of a hypothetical binding was small enough that omalizumab's sedimentation remained unaffected.

The different omalizumab–IgE complex profiles, as well as the observed higher affinity of omalizumab for IgE in serum, support the idea that the characterization of therapeutic proteins in serum yields valuable biochemical data and is especially relevant for proteins targeted to soluble receptors or antigens. Characterization in serum may allow a more informed selection of protein variants (drug candidates) based on affinity and stoichiometry measurements closely related to the situation encountered in vivo.

This study has demonstrated that characterization of therapeutic proteins in human serum by fluorescence-detected AUC is feasible and yields useful biochemical data not accessible by other methods. The labeling reaction necessary for detection did not alter the characteristics of omalizumab. We showed that the affinity of omalizumab for IgE was higher in serum than in PBS and that the largest omalizumab–IgE complexes observed in serum were smaller than expected. AUC equipped with fluorescence optics has the potential to bring the characterization of biopharmaceuticals to a new level.

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References

- [1] T.M. Laue, W.F. Stafford III, Modern applications of analytical ultracentrifugation, *Annu. Rev. Biophys. Biomol. Struct.* 28 (1999) 75–100.
- [2] G.J. Howlett, A.P. Minton, G. Rivas, Analytical ultracentrifugation for the study of protein association and assembly, *Curr. Opin. Chem. Biol.* 10 (2006) 430–436.
- [3] I.K. MacGregor, A.L. Anderson, T.M. Laue, Fluorescence detection for the XL analytical ultracentrifuge, *Biophys. Chem.* 108 (2004) 165–185.
- [4] J. Liu, P. Lester, S. Builder, S.J. Shire, Characterization of complex formation by humanized anti-IgE monoclonal antibody and monoclonal human IgE, *Biochemistry* 34 (1995) 10474–10482.
- [5] S. Easthope, B. Jarvis, Omalizumab, *Drugs* 61 (2001) 253–260.
- [6] T.W. Chang, The pharmacological basis of anti-IgE therapy, *Nat. Biotechnol.* 18 (2000) 157–162.
- [7] L.G. Presta, S.J. Lahr, R.L. Shields, J.P. Porter, C.M. Gorman, B.M. Fendly, P.M. Jardieu, Humanization of an antibody directed against IgE, *J. Immunol.* 151 (1993) 2623–2632.
- [8] P. Schuck, Size distribution analysis of macromolecules by sedimentation velocity ultracentrifugation and Lamm equation modeling, *Biophys. J.* 78 (2000) 1606–1619.
- [9] G. Hunt, W. Nashabeh, Capillary electrophoresis sodium dodecyl sulfate nongel sieving analysis of a therapeutic recombinant monoclonal antibody: a biotechnology perspective, *Anal. Chem.* 71 (1999) 2390–2397.
- [10] O. Salas-Solano, B. Tomlinson, S. Du, M. Parker, A. Strahan, S. Ma, Optimization and validation of a quantitative capillary electrophoresis sodium dodecyl sulfate method for quality control and stability monitoring of monoclonal antibodies, *Anal. Chem.* 78 (2006) 6583–6594.
- [11] S.A. Berkowitz, Role of analytical ultracentrifugation in assessing the aggregation of protein biopharmaceuticals, *AAPS J.* 8 (2006) E590–E605.
- [12] J.P. Gabrielson, M.L. Brader, A.H. Pekar, K.B. Mathis, G. Winter, J.F. Carpenter, T.W. Randolph, Quantitation of aggregate levels in a recombinant humanized monoclonal antibody formulation by size-exclusion chromatography, asymmetrical flow field flow fractionation, and sedimentation velocity, *J. Pharm. Sci.* 96 (2007) 268–279.
- [13] P. Schuck, M.A. Perugini, N.R. Gonzales, G.J. Howlett, D. Schubert, Size distribution analysis of proteins by analytical ultracentrifugation: strategies and application to model systems, *Biophys. J.* 82 (2002) 1096–1111.
- [14] H.B. White III, A.H. Merrill Jr., Riboflavin-binding proteins, *Annu. Rev. Nutr.* 8 (1988) 279–299.
- [15] G.H. Beaven, A. D'Albis, W.B. Gratzner, The interaction of bilirubin with human serum albumin, *Eur. J. Biochem.* 33 (1973) 500–509.
- [16] J. Liu, J.D. Andya, S.J. Shire, A critical review of analytical ultracentrifugation and field flow fractionation methods for measuring protein aggregation, *AAPS J.* 8 (2006) E580–E589.
- [17] H. Milgrom, R.B. Fick, J.Q. Su, J.D. Reimann, R.K. Bush, M.L. Watrous, W.J. Metzger, Treatment of allergic asthma with monoclonal anti-IgE antibody, *N. Engl. J. Med.* 341 (1999) 1966–1973.
- [18] D.W. MacGlashan Jr., B.S. Bochner, D.C. Adelman, P.M. Jardieu, A. Togias, J. McKenzie-White, S.A. Sterbinsky, R.G. Hamilton, L.M. Lichtenstein, Down-regulation of FcεRI expression on human basophils during in vivo treatment of atopic patients with anti-IgE antibody, *J. Immunol.* 158 (1997) 1438–1445.
- [19] M.J. Baschore, T.D. Howard, L.A. Lange, W.C. Moore, G.A. Hawkins, P.L. Marshik, M.S. Harkins, D.A. Meyers, E.R. Bleecker, A comprehensive evaluation of IL4 variants in ethnically diverse populations: association of total serum IgE levels and asthma in white subjects, *J. Allergy Clin. Immunol.* 114 (2004) 80–87.
- [20] P. Schuck, On the analysis of protein self-association by sedimentation velocity analytical ultracentrifugation, *Anal. Biochem.* 320 (2003) 104–124.
- [21] J.S. Kingsbury, T.M. Laue, E.S. Klimtchuk, R. Theberge, C.E. Costello, L.H. Connors, The modulation of transthyretin tetramer stability by cysteine 10 adducts and the drug diflunisal: direct analysis by fluorescence-detected analytical ultracentrifugation, *J. Biol. Chem.* 283 (2008) 11887–11896.
- [22] J. Corne, R. Djukanovic, L. Thomas, J. Warner, L. Botta, B. Grandordy, D. Gyax, C. Heusser, F. Patalano, W. Richardson, E. Kilchherr, T. Staehelin, F. Davis, W. Gordon, L. Sun, R. Liou, G. Wang, T.W. Chang, S. Holgate, The effect of intravenous administration of a chimeric anti-IgE antibody on serum IgE levels in atopic subjects: efficacy, safety, and pharmacokinetics, *J. Clin. Invest.* 99 (1997) 879–887.
- [23] S.B. Zimmerman, A.P. Minton, Macromolecular crowding: biochemical, biophysical, and physiological consequences, *Annu. Rev. Biophys. Biomol. Struct.* 22 (1993) 27–65.
- [24] A.P. Minton, Influence of macromolecular crowding upon the stability and state of association of proteins: predictions and observations, *J. Pharm. Sci.* 94 (2005) 1668–1675.
- [25] J.J. Correia, M.L. Johnson, G.H. Weiss, D.A. Yphantis, Numerical study of the Johnston-Ogston effect in two-component systems, *Biophys. Chem.* 5 (1976) 255–264.