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# Characterization of antigen–antibody complexes by size-exclusion chromatography coupled with low-angle light-scattering photometry and viscometry

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**Abstract**

In this paper, the molecular masses ($M_r$s) of the complexes of monoclonal anti-BSA (antibody to bovine serum albumin) (clone: 33) and monomer BSA were determined on-line by using size-exclusion chromatography (SEC) coupled with a low-angle laser light-scattering (LALLS) detector and two concentration detectors, ultraviolet (UV) and refractive index (RI) (SEC–LALLS/UV/RI system). Also, the size and $M_r$s of the complexes were evaluated by the SEC–LALLS/UV/viscometer (VISC) system. This study demonstrated that, for small size macromolecules, the combination of light scattering and viscosity detection was a suitable choice for determining their $M_r$s and sizes. © 1997 Elsevier Science B.V.

*Keywords:* Detection, LC; Molecular mass determination; Antigens; Antibodies; Proteins; Albumin

## 1. Introduction

Many physiological properties of soluble antigen–antibody (Ag–Ab) complexes, including clearance from the circulation, complement fixation, adherence to phagocytes, and differential tissue deposition, depend on the size and composition of these complexes [1–4]. Therefore, information about the $M_r$ and size of Ag–Ab complexes is desirable. One way to estimate the composition of an Ag–Ab complex is to determine the $M_r$ of the complex, assuming that the $M_r$ of individual components are known.

Static light scattering for the mixture of Ag and Ab can provide information that can be used to deduce the size and $M_r$ of complexes by complex mathematical calculations [5]. For a slow dissociation Ab–Ag complex (usually high affinity of Ab to Ag), the (1:2 and 1:1) complexes can be separated and isolated. If the complexes are separated by a chromatography system coupled with multi-angle light-scattering (MALS) detection, the size and $M_r$ of the complexes can be determined and directly interpreted.

However, for compact biopolymers of $M_r$ up to 1 000 000, there is no applicable size dependence of the scattering function [6]. Many studies have found [5,7,8] that light-scattering measurements alone *cannot* determine the radius of gyration of small molecules. Theoretical calculations show that the size of a globular molecule with a $M_r$ of 10 600 000 is about

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11.3 nm [9], which is much smaller than the typical wavelength, 632.8 nm, used by many commercially available light-scattering detectors. Therefore, MALS may not *always* be a suitable method for determining the size of soluble complexes of anti-bovine serum albumin (anti-BSA) and BSA, for example.

Any isocratic high-performance liquid chromatography (HPLC) method, most often size-exclusion chromatography (SEC), combined with on-line light-scattering (any mode of light-scattering, such as right-angle, low-angle or multi-angle) and viscosity detection, can determine the size and $M_r$ of polymers, simultaneously. This method is valid for the polymer size range that is below the limit for which MALS works [10]. Therefore, for size determinations of biopolymers, a combination of two molecular mass-sensitive detectors, light-scattering and viscometry, is a preferable method to that using only a multi-angle light-scattering detector.

In this study, a low-angle laser light-scattering (LALLS) detector was used. Compared with other modes of light-scattering, LALLS serves equivalent functions for small size macromolecules, even though it does not have any advantages. The $M_r$ of the BSA–anti-BSA complexes was determined by SEC–LALLS/UV/RI. Concentrations of injected complexes for the $M_r$ calculation were estimated by using the refractive index (RI) signal and a calibration constant for this detector. To determine the size of complexes, a SEC–LALLS/UV/VISC system was used, and the concentration of a complex was estimated by using its UV signal and a calibration constant for this detector. Also, a conventional calibration curve for proteins was used to estimate the $M_r$s of complexes.

## 2. Experimental

### 2.1. Apparatus

Two systems were used for this study. The first, for determining molecular masses, consisted of a Model CM 4000 multiple solvent delivery system (Thermo Separation Products (TSP), Riviera Beach, FL, USA), a Rheodyne Model 7125 injector with a 100-μl injection loop, a TSK gel G3000 $SW_{XL}$ column (5 μm, 30 cm×7.8 mm I.D.) (The Nest Group, Southboro, MA, USA), a Model KMX-6 LALLS detector (632.8 nm), a variable-wavelength UV–Vis detector (all from TSP) and a *modified* Model refractoMonitor IV, RI detector (TSP). The second system for determining $M_r$ and size was the same as the first, except that a Model H-50 viscometer (Viscotek, Houston, TX, USA) replaced the RI detector.

Off-line differential RI ($dn/dc$) determinations were performed with a Model KMX-16 laser (632.8 nm) differential refractometer (TSP). A Model Spectronic 1201 UV/VIS spectrophotometer (TSP) was used to measure the absorbance of BSA or anti-BSA solutions.

A Model 59A Micro-Centrifuge (Fisher Scientific, Fair Lawn, NJ, USA) was used to concentrate sample solutions with a Microcon-30 microconcentrator (Amicon, Beverly, MA, USA).

A TSK gel G 2000 $SW_{XL}$ column (5 μm, 30 cm×7.8 mm I.D., TosoHaas, Tokyo, Japan) was used to isolate BSA monomer from its aggregates, and a Protein G cartridge (PerSeptive Biosystems, Framingham, MA, USA) was used to isolate the Ab from anti-BSA mouse ascites fluid.

### 2.2. Chemicals

HPLC-grade water was purchased from Fisher Scientific. ACS-grade sodium chloride and monobasic sodium phosphate were also from Fisher Scientific. Dibasic sodium phosphate was from Aldrich (Milwaukee, WI, USA). BSA, monoclonal anti-BSA mouse ascites fluid (Product No. B-2901) and pig IgG were all from Sigma (St. Louis, MO, USA).

### 2.3. Mobile phase

Phosphate-buffered saline (PBS) was prepared from HPLC-grade water, and consisted of 50 m*M* phosphate and 150 m*M* sodium chloride, pH 7.1–7.2. The mobile phase was filtered through a 0.2-μm Nylon 66 filter from Supelco (Bellefonte, PA, USA) and was degassed before use.

### 2.4. Sample preparation

#### 2.4.1. Preparation of anti-BSA solution

The Ab to BSA (anti-BSA) was purified using a

Protein G cartridge. The concentration of Ab was estimated from its extinction coefficient, at 280 nm, of 1.4 ml $mg^{-1}$ $cm^{-1}$. Prior to mixing with the Ag, the purified Ab was concentrated using a microconcentrator unit and centrifugation at 600 *g*.

*2.4.2. Isolation of monomer BSA*

The BSA monomer was isolated by SEC using a TSK G2000 $SW_{XL}$ column. Approximately 1–2 mg of BSA were injected onto the column and the BSA monomer fraction was collected manually. The SEC mobile phase was PBS, set at a flow-rate of 0.5 ml/min. The concentration of the BSA monomer fraction was determined by measuring its absorbance at 280 nm and using an extinction coefficient of 0.62 ml $mg^{-1}$ $cm^{-1}$. Prior to mixing with the anti-BSA Ab, the BSA monomer was reinjected onto the SEC column to check for possible formation of BSA aggregates.

*2.4.3. Preparation of anti-BSA and BSA complexes*

Anti-BSA and BSA complexes were prepared by mixing the purified and concentrated anti-BSA Ab solution and the monomer BSA solution in various proportions. These mixtures were incubated for at least 30 min at room temperature before injection.

*2.5. Procedure*

*2.5.1. dn/dc determinations*

The d*n*/d*c* of BSA and Pig IgG were determined off-line with the KMX-16 differential refractometer. The average d*n*/d*c* of five different concentrations for each of the proteins, ranging from 1–4 mg/ml, was determined and plotted vs. their respective concentrations. The SEC–UV/RI system described previously was used for on-line d*n*/d*c* determination [11].

*2.5.2. SEC–LALLS/UV/RI*

A solution of the Ab–Ag complex was injected into the SEC–LALLS/UV/RI system. The concentration of this complex was determined on-line by RI detection using the detector calibration constant, $K_{RI}$ ($K_{RI}$ was determined in a separate experiment, as outlined in the operational manual). The two complexes (1:2 and 1:1 Ab–Ag) were collected and concentrated using a microconcentrator unit. The LALLS, UV and RI signals were collected simultaneously and later processed to determine the $M_r$s of the complexes. In a separate experiment, the RI detector was replaced with the VISC and the signals from the VISC, LALLS and UV were used to determine the radius of gyration ($R_g$) of the complexes.

*2.5.3. SEC calibration curve of proteins*

A SEC calibration curve was generated using standard proteins. A calibration plot was generated of log $M_r$ vs. the retention volume of the standards. The data was fit using regression analysis.

## 3. Theory

*3.1. Determination of the concentration of the Ag–Ab complex*

The procedure used to check the concentration of a sample is usually to create a concentration (or more accurately a mass) calibration plot. The area under the concentration trace is directly related to the mass [conc. (mg/ml)×injection volume (ml)] of the sample injected. This relationship between the concentration, the RI signal and the specific experimental parameter can be easily deduced from the following equation (Eq. (1)):

$$c_o = (k_2 f/k_1) \times \left( \int du\, dt \right) / (dn/dc) V_{inj} \quad (1)$$

where $c_o$ is the concentration of the injected sample solution, $k_1$ is a constant and a function of the RI detector cell design, $k_2$ is a constant that is dependent on the detection range setting and the full scale output of a RI detector, $f$ is the flow-rate (ml/min), $(k_2 f/k_1)$ is the so-called calibration constant for RI detection, $K_{RI}$, d*n*/d*c* is the specific refractive index increment of the sample, $V_{inj}$ is the injection volume and $du \cdot dt$ is the area of the concentration signal. Eq. (1) is valid only when the concentration is low, as is generally the case in SEC.

A similar equation can be derived for the UV detector,

$$c_o = K_{UV} \times \left( \int du\, dt \right) / E\, V_{inj}. \quad (2)$$

where $K_{UV}$ is the calibration constant for a UV detector, which is a function of detection cell geometry, the detector output scale, the detector setting range and the flow-rate (ml/min), and $E$ is the absorption coefficient of the sample at the detection wavelength.

### 3.2. Molecular mass determination by LALLS

For Rayleigh scattering at low angles, the following equation is valid.

$$(K^*c)/R_\varphi = 1/M_r + (2A_2c) \quad (3)$$

where

$$K^* = (2\pi n^2/\lambda^4 N)(dn/dc)^2 \quad (4)$$

and, where $c$ is the concentration of scattering species, in g/ml, $M_r$ is the weight-average molecular mass of scattering species, $n$ is the refractive index of the solution, $\lambda$ is the wavelength, $N$ is Avogadro's number, $A_2$ is the second virial coefficient and $R_\varphi$ is the excess Rayleigh factor.

Typical $A_2$ values for proteins are in the range of $10^{-3}$ to $10^{-5}$ ml mol/g$^2$. Since the concentration of proteins eluting off a SEC column is generally low, the second term on the right-hand side of Eq. (3) has little effect on the $M_r$ determination and can be ignored. However, in order to determine the $M_r$ of the complexes, the $dn/dc$ and concentration of the complexes must first be determined.

According to the additivity rule, $dn/dc$ of a copolymer is a sum of the products of $dn/dc$ and mass fraction for the particular monomer or homopolymer [12–14]:

$$(dn/dc)_{copolymer} = \sum W_i(dn/dc)_I \quad (5)$$

where $\Sigma W_i = 1$. This equation is particularly true in the case of block copolymers [15]. This relation was used to determine the $M_r$ of bioconjugates and biocopolymers using LALLS by Watanabe et al. [16] and Kato et al. [17].

For the complexes of BSA and anti-BSA, Eq. (5) may be written as:

$$(dn/dc)_{complex} = (1 - W_{BSA}))(dn/dc)_{anti-BSA} + W_{BSA}(dn/dc)_{BSA} \quad (6)$$

where $W_{BSA}$ is the mass percentage of BSA in the complexes.

Using Eq. (6) for determining the $dn/dc$ of the complexes, one needs to know not only the $dn/dc$ of BSA and anti-BSA, but also the mass percentage of the components in the complex. Typically, $dn/dc$ values of proteins increase with increasing $M_r$ and reach an asymptotic limit for $M_r$ values greater than about 20 000 [8]. The $dn/dc$ of proteins of $M_r$ >20 000 may be expected to be almost the same. This assumption is consistent with earlier results on the specific $dn/dc$ of biopolymers [18,19] and is evident from Table 1. Using similar reasoning, the $dn/dc$ of protein–antibody complexes could be considered to be equal to that of proteins. This assumption was used to determine the $M_r$s of the BSA–anti-BSA complexes.

### 3.3. Size determination

In the SEC–VISC–LS experiments, the molecular size at every elution volume can be calculated in addition to the $M_r$. The hydrodynamic radius of the molecule ($R_h$) can be expressed as [20]:

$$R_h = \{(3/4\pi)([n]M/0.025)\}^{1/3} \quad (7)$$

where $[n]$ is the intrinsic viscosity of a sample, and $M$ is the $M_r$ of the sample.

Table 1
*dn/dc* determination of proteins

| Sample | $dn/dc$ (ml/g) (On-line) | R.S.D. (%) ($n$) | $dn/dc$ (ml/g) (Off-line) | R.S.D. (%) ($n$) |
|---|---|---|---|---|
| BSA | 0.169 | 3 (6) | 0.166 | 0.6 (6) |
| Pig IgG | 0.170 | 3 (6) | 0.168 | 2 (5) |
| Anti-BSA | 0.175 | 6 (3) | | |

R.S.D. (%): Percentage relative standard deviation.
$n$: Number of $dn/dc$ determinations.

For linear flexible chain polymers, this can be related to the radius of gyration, using the Flory-Fox and Ptitsyn-Eisner equation [21,22]:

$$R_g = (1/6)^{0.5} \times ([n] M/F)^{1/3} \quad (8)$$

where, $R_g$ is the radius of gyration, $F$ is the Ptitsyn-Eisner function and is expressed as:

$$F = 2.86 \cdot 10^{21}(1 - 2.63e + 2.86e^2) \quad (9)$$

where $e$ is the expansion coefficient and is expressed as:

$$e = (2a + 1)^{1/3} \quad (10)$$

and $a$ is the exponent of the Mark-Houwink equation:

$$[n] = KM^a \quad (11)$$

In the TriSEC software, calculations of $R_g$ for the different models (random coil, rod and sphere) all use Eq. (8). However, for a different protein model, a different default value of $a$ in Eq. (11) is used to calculate $R_g$. For a sphere model, a default value of 0.0 is assumed [23].

## 4. Results and discussion

Table 1 shows the results of the $dn/dc$ determinations. The $dn/dc$ values of BSA and pig IgG from the off- and on-line determinations were very close [±R.S.D. (%)]. Although the $dn/dc$ of anti-BSA from the on-line determination was slightly larger than others, it was still in agreement considering the different experimental procedures. The average value of the measured $dn/dc$ of BSA and pig IgG, 0.167, was used as the $dn/dc$ of the complexes.

The SEC–LALLS/UV/RI system was used for the $M_r$ determinations of the complexes. Figs. 1 and 2 show the chromatograms of BSA monomer and anti-BSA, respectively. Fig. 3 shows the chromatogram of the mixture of anti-BSA and BSA monomer, when BSA was in excess. There was a new peak that appeared in this SEC chromatogram, and it was assigned as complex 1. The measured $M_r$ (Table 2) was close to that of a 2:1 (BSA–anti-BSA) complex (297 000 vs. 282 000) [24–26]. Considering the experimental error, it seemed that complex 1 was a

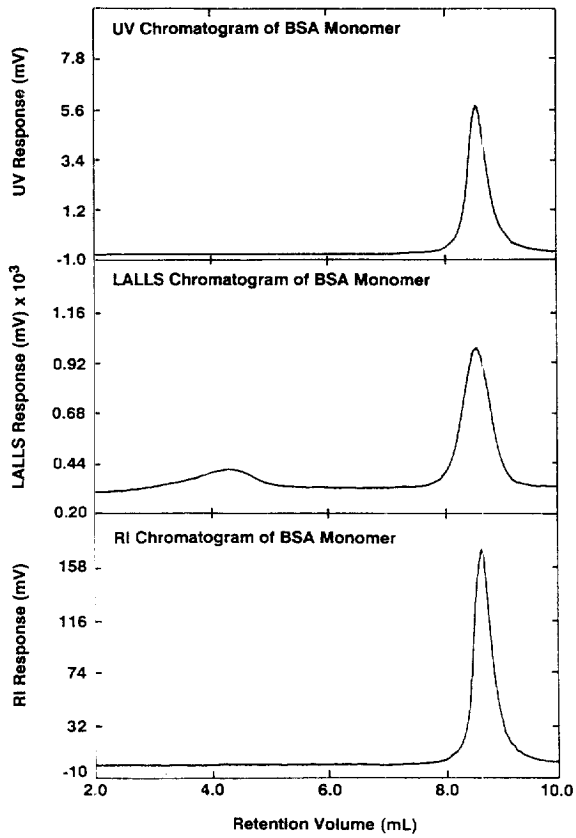


Fig. 1. SEC chromatogram of BSA monomer. Column, G 3000 SWXL; mobile phase, PBS, pH 7.1–7.2; flow-rate, 0.4 ml/min; loop, 100 μl; UV, 280 nm, 0.2 aufs; RI range, 0.1 ($\times 10^{-3}$ $d$RI); LALLS, 6–7° annulus, 0.2 nm field stop, and $G_0 = 200$ mV with two, three, four attenuation.

BSA to anti-BSA 2:1 complex. Upon re-injection of complex 1, a single peak was observed with a retention volume and $M_r$ equal to those of complex 1.

When anti-BSA was in excess, there were two new peaks that appeared in the chromatogram (Fig. 4). These were not well resolved. The elution volume of the earlier-eluting peak corresponded to complex 1. The $M_r$ of the second peak, assigned as complex 2, was in between that expected for the 2:1 and 1:1 (Ag–Ab) complexes. Due to the poor resolution of these two peaks, worsened by the dead volume of an in-line filter and LALLS cell volume, the complex 2 peak included a percentage of complex 1. The overlap of these peaks led to the

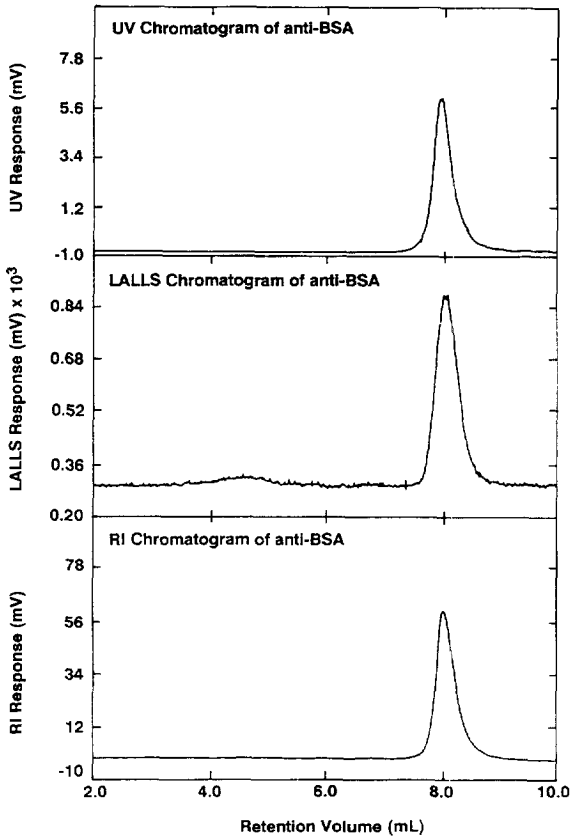


Fig. 2. SEC chromatogram of anti-BSA. All conditions were the same as those in Fig. 1.

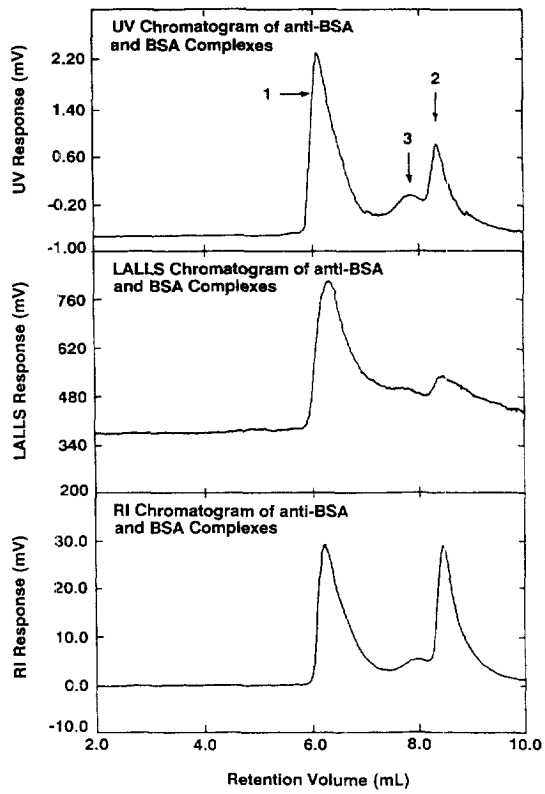


Fig. 3. SEC chromatogram of the mixture of BSA and anti-BSA. All conditions were the same as in Fig. 1. BSA was in excess.

determined $M_r$ of complex 1 being lower (255 000) than that previously determined (297 000, Table 2). Based on the $M_r$ and the elution volume, complex 2 was most likely a 1:1 species. It was impossible to obtain a fully resolved 1:1 complex, since the equilibrium between the two species favored the 2:1 ratio.

The SEC–LALLS/UV/VISC system also determined the size ($R_g$) of the BSA, pig IgG and the Ag:Ab complexes. The calculated values of the $R_g$ of BSA and IgG were consistent with their literature values (Table 3) [24–26]. Due to the poor resolution of the two complexes, the $R_g$ of the 1:1 complex could not be determined. To simplify the size determination of the 2:1 complex, excess BSA was used to form the 2:1 complex exclusively (Fig. 5).

Fig. 6 shows a calibration curve for a set of standard proteins. The $M_r$ values of anti-BSA and its complexes were: 101 900, 191 900 (1:1 complex) and 260 000 (2:1 complex). The expected values were 150 000, 216 000 and 282 000, respectively. The lower than expected $M_r$ for anti-BSA may have been due to a difference in the shape of anti-BSA from that of globular proteins or from non-specific SEC interactions.

Table 2
$M_r$ determination using the SEC–LALLS/UV/RI system

| Sample | $M_r$ | R.S.D. (%) ($n$) | Literature value |
|---|---|---|---|
| Anti-BSA | 150 800 | 5 (3) | 150 000 |
| BSA | 66 700 | 4 (6) | 66 000 |
| Complex 1 | 297 300 | 3 (3) | – |
| Complex 2 | 242 000 | NA (2) | – |

NA = not applicable, too few data points to determine R.S.D. (%).

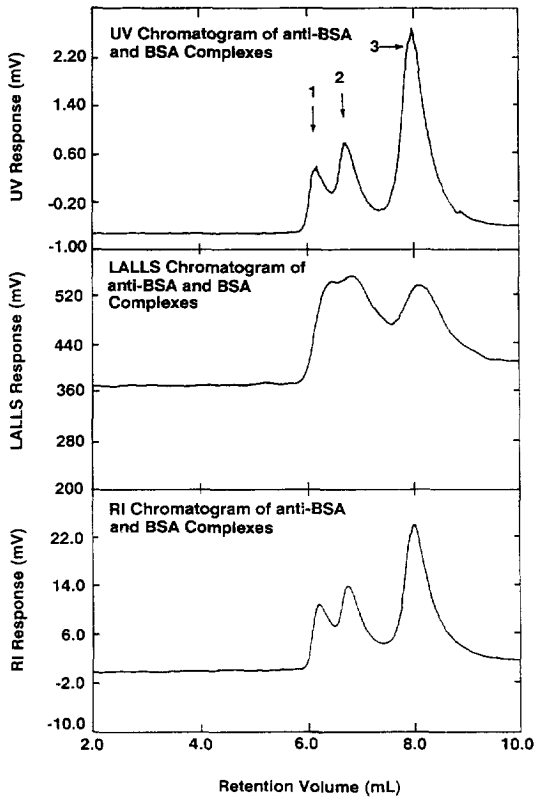


Fig. 4. SEC chromatogram of the mixture of BSA and anti-BSA. All conditions were the same as in Fig. 1. Anti-BSA was in excess.

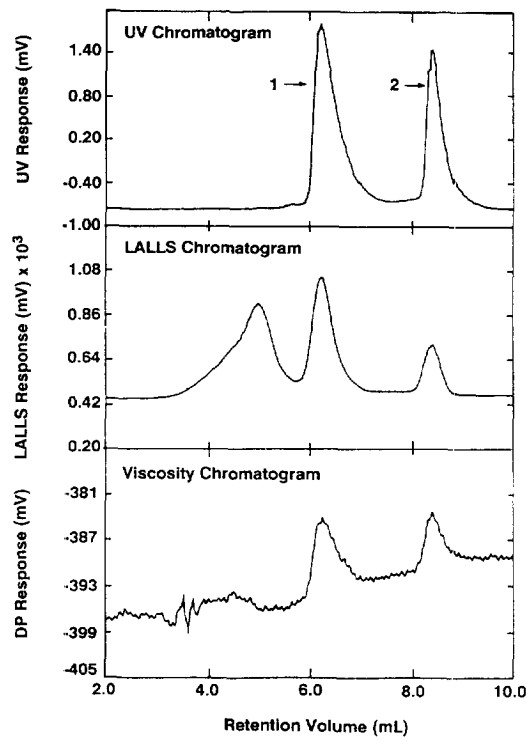


Fig. 5. SEC chromatogram of the mixture of BSA and anti-BSA. All conditions were the same as in Fig. 1, except that the RI detector was replaced by a viscometer. BSA was in excess.

## 5. Conclusions

These results confirmed that the separated complexes were Ag (BSA) to Ab (anti-BSA), 1:1 and 2:1 complexes, respectively. For protein–protein complexes, the $dn/dc$ of proteins with $M_r$s above 20 000 is approximately constant, independent of species. This can be used to deduce the $dn/dc$ of complexes.

Table 3
Size determination

| Sample | $R_g$ [$n$, R.S.D. (%)] | $R_g$ (literature) |
|---|---|---|
| BSA monomer | 3.5 nm (3, 6%) | 3.6 nm |
| pig IgG | 5.8 nm[a] (4, 1%) | 5.6 nm |
| 2:1 complex | 6.5 nm (3, 6%) | – |

[a] Calculated according to the rod model in TriSEC software.

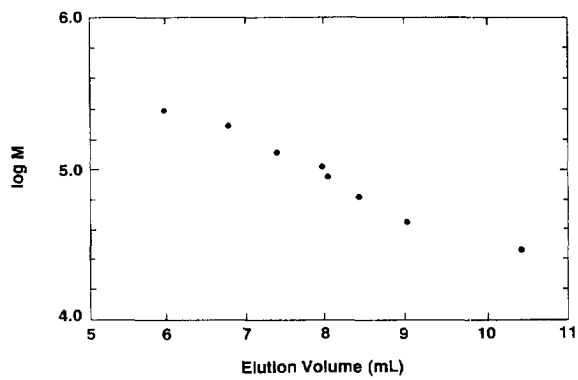


Fig. 6. Calibration curve for proteins. Column, G 3000 $SW_{XL}$; mobile phase, PBS, pH 7.1–7.2; flow-rate, 0.4 ml/min; loop, 40 μl; UV, 280 nm, 0.1 aufs.

We have developed a procedure to use the change of the RI value and $dn/dc$ to calculate the concentration of the complex. This was then combined with LALLS signals for the $M_r$ determinations. This is a new and convenient way to characterize these types of complexes. There are several practical advantages to using this procedure, such as, it avoids problems with the peak mass not corresponding to injected mass, due to $<100\%$ recovery.

VISC detection combined with LALLS can be used to determine the $M_r$ and size of complexes, simultaneously. This combination is especially useful for a protein or biopolymer whose size is smaller than 15 nm, where multiple-angle light-scattering cannot always succeed.

## 6. Abbreviations

| | | |
|---|---|---|
| Ab | = | antibody |
| Ag | = | antigen |
| aufs | = | absorbance units full scale |
| Ag–Ab | = | antigen–antibody complex |
| anti-BSA | = | antibody to BSA |
| BSA | = | bovine serum albumin |
| dRI | = | (differential) refractive index |
| $dn/dc$ | = | differential refractive index |
| $E$ | = | Epsilon (extinction coefficient) |
| HPLC | = | high-performance liquid chromatography |
| LALLS | = | low-angle laser light-scattering |
| LS | = | light scattering |
| MALS | = | multi-angle light-scattering |
| $M_r$(s) | = | molecular mass(es) |
| nm | = | nanometer |
| PBS | = | phosphate-buffered saline |
| R.S.D. (%) | = | percentage relative standard deviation |
| $R_g$ | = | radius of gyration |
| SEC | = | size-exclusion chromatography |
| TSP | = | Thermo Separation Products |
| UV | = | ultraviolet |
| Vis | = | visible |
| VISC | = | viscometer or viscometry |

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