Partial specific volume and solvent interactions of amphipol A8-35

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Abstract

Amphipols are small amphiphilic polymers that can stabilize and keep soluble membrane proteins in aqueous solutions in the absence of detergent. A prerequisite to solution studies of membrane protein/amphipol complexes is the determination of the partial specific volume \( \nu_2 \) and effective charge \( z \) of the polymer. The ratio (\( R \)) of the buoyant molar masses of particles in \( D_2O \) and \( H_2O \) solutions, obtained from sedimentation velocity (\( s_{H}/s_{D} \) method) and sedimentation equilibrium experiments, and their contrast match point (CMP), determined in small-angle neutron scattering experiments, depend on \( \nu_2 \) and \( z \). When \( z \) is known, \( \nu_2 \) can be estimated from \( R \) with a good accuracy as long as \( \nu_2 \) is close to 1. The effects of labile H/D exchange and of polyelectrolyte counter-ion dissociation in general cannot be neglected. The accuracy, advantages, and limits of the \( s_{H}/s_{D} \) method have been studied in detail using model macromolecules (DNA, protein, and polysaccharide). The \( s_{H}/s_{D} \) method appears particularly advantageous for the study of heterogeneous samples. Measurements of density, \( s_{H}/s_{D} \) buoyant molar masses in \( H_2O \), \( D_2O \), and \( D_2^18O \), and CMP of hydrogenated and partially deuterated A8-35, a polyacrylate-based amphipol containing 35 underivatized carboxylates per 100 monomers, led to a consistent description of its buoyancy and charge properties.

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Membrane proteins constitute about one-third of the proteome, but, compared to soluble proteins, few of their structures are known. This is mainly due to their low natural abundance and to the difficulties presented by their extraction from the membrane and their handling in detergent solutions, in which they often have a limited stability [1,2]. This has prompted the search for novel amphiphilic compounds that could be used as alternatives to detergents [3]. Specially designed amphiphatic polymers, called “amphipols,” have been shown to keep in solution and to stabilize a large variety of membrane proteins (for a review, see [4]). Amphipols with different chemical structures have been synthesized and tested (reviewed in [4,5]). Tribet et al. [6] first showed that an anionic derivative of low-molecular-weight polyacrylic acid grafted with octylamine and isopropylamine was able to keep soluble in aqueous solution under their native state four integral membrane proteins: bacteriorhodopsin, a bacterial photosynthetic reaction center, cytochrome b6f, and matrix porin.
Scanning transmission electron microscopy showed that amphipols preserved the original dispersity of the b-f complex in detergent solution [7]. Amphipols protected the sarcoplasmic calcium ATPase from the rapid and irreversible inactivation that follows detergent or calcium removal [8]. The structure, solubility, and functionality of the integral membrane protein diacylglycerol kinase were maintained by amphipols in the complete absence of extraneous lipids or detergent [9]. The comparison of agonist binding kinetics demonstrated that a channel-forming acetylcholine receptor displayed, after trapping with amphipols, unaltered allosteric transitions as compared to the membrane-bound state [10]. The ability of amphipols to keep soluble membrane proteins while maintaining their structure, association state, and functionality motivates structural studies of amphipols and amphipol/protein complexes in solution.

Analytical ultracentrifugation (AUC) and small-angle neutron scattering (SANS) are based on rigorous thermodynamics and can be used to characterize the homogeneity of solutions of macromolecules, their molar masses, and their interactions. The scattering phenomena and the sedimentation of a macromolecule are related to the osmotic work done against the concentration gradient, which can be expressed as a function of the molar mass of the macromolecule, $M_2$. The experimental parameters, buoyant molar mass and forward intensity, are related to $M_2$ through buoyancy or contrast terms, i.e., the increments in mass density or neutron scattering length density. These are functions of the partial specific volume of the macromolecule, $v_2$, and, in the cases of polyelectrolytes (such as charged amphipols) or of multicomponent solvents, of the redistribution of the solvent close to the macromolecule, which determines the value of the preferential solvent binding parameter $\xi_3$ [11–15]. For a polyelectrolyte in dilute salted solvent, $\xi_3$ is directly related to the number of dissociated counter-ions (or effective charge $z$ of the polyelectrolyte). The knowledge of $v_2$ and $\xi_3$ (i.e., $z$) for amphipols is thus a prerequisite for many solution studies of amphipol particles and amphipol/membrane protein complexes. Their determination is the object of the present study.

Amphipol A8-35 has been described previously [6]. Its chemical structure is schematized in Fig. 1. The average molecule has a mass of ~8 kDa and comprises ~70 acrylate monomers. On average, ~35% (mol mol$^{-1}$) of the original carboxylates are underivatized and ~25% carry an octylamine chain. Most of the remaining 40% are derivatized with isopropylamine, but in some samples a small fraction was found to have been blocked by a side reaction (see Materials and methods). For their characterization in solution, amphipols combine the complexities of both polyelectrolytes and polydisperse polymers. However, despite the wide distribution of the masses of individual molecules (typically over a decade around the average mass), amphipols associate in aqueous solutions into relatively well-defined particles (this work and [16,17]). We examine here the contribution of the polyelectrolyte nature of amphipols to their solution properties by confronting AUC, SANS, and density data collected on two isotopically distinct variants of A8-35, an undeuterated form (HAPol), and a deuterated form (DAPol).

Accurate density measurements made at constant solvent composition, i.e., by dissolution of the dry polymer, can be used for the determination of $v_2$, provided that the absolute concentration of amphipol is precisely known. Values of $\xi_3$ can in principle be obtained by complementary density, sedimentation, or scattering experiments made at constant chemical potential of the solvent components [11,18–20]. These methods were not readily practical in the case of amphipols. The samples (for density) have to be prepared using dialysis or gel permeation, with the risk of altering the composition of these heterogeneous mixtures, or the analysis requires prior knowledge of the molar mass of the macromolecule in solution and thus would be difficult to implement in the case of amphipols, which form supramolecular aggregates. As another approach, solvent density variation in sedimentation or SANS experiments with solvents containing H$_2$O, D$_2$O, and eventually D$_2$O$^{18}$O can be used to obtain $v_2$ and $M_2$ in two-component systems [21,22]. (We do not consider the use of cosolvents such as sugars, glycerol, nicodenz, or salts to modulate the density of the solution, because this does not allow a rigorous analysis [23,24]). For a polyelectrolyte, counter-ion dissociation is a special case of solvent interactions, which have to be considered in the analysis of data obtained in H$_2$O/D$_2$O solvents [19,21]. The present paper demonstrates the power of sedimentation analysis in H$_2$O/D$_2$O solvents and applies it to the characterization of amphipols.

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**Fig. 1.** Chemical structure of A8-35. For amphipol A8-35, x, y, and z correspond to 35, 25, and 40%, respectively. HAPol is the hydrogenated form. In DAPol, the alkyl chains are -C$_3$H$_7$ and -C($\text{CD}_3$)$_2$.

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**Abbreviations used:** AUC, analytical ultracentrifugation; SANS, small-angle neutron scattering; CMP, contrast match point; BSA, bovine serum albumin; PAA, polyacrylic acid; NMP, N-methyl-2-pyrrolidone; THF, tetrahydrofuran; DCI, dicyclohexylcarbodiimide; HOBT, 1-hydroxybenzotriazole.
Under Theoretical background, we describe how the ratio \( R \) of the buoyant molar masses obtained in \( D_2O \) and \( H_2O \) solvents from sedimentation velocity or sedimentation equilibrium measurements and the contrast match point (CMP) determined by SANS are related to \( \bar{v}_2 \) and \( \bar{v}_3 \). Under Results and discussion, we first analyze how \( R \) can be used to evaluate \( \bar{v}_2 \) and apparent partial specific volumes and show that the effect of polyelectrolyte counter-ion dissociation in general cannot be neglected. Sedimentation velocity experiments in \( H_2O/D_2O \) solvents using model compounds are used to validate this approach. We then study the partial specific volume and counter-ion dissociation properties of A8-35 in its hydrogenated and deuterated forms, HAPol and DAPol. HAPol was characterized by density measurements at constant composition of solvent components and by equilibrium sedimentation in \( H_2O, D_2O, \) and \( D_2^{18}O \) solvents. Contrast variation experiments in \( H_2O/D_2O \) solvents were performed on HAPol and DAPol using both sedimentation velocity and SANS measurements. By comparing the results of density measurements at constant molality of the solvent components and sedimentation velocity experiments in \( H_2O/D_2O \) solvents, we show that the buoyancy properties of A8-35 are compatible with full dissociation of the counter-ions. Consistent but less accurate estimates were derived from equilibrium sedimentation in \( H_2O/D_2O/D_2^{18}O \) and from SANS measurements. Under Conclusion we discuss the general applicability and limits of the analysis of sedimentation velocity experiments in \( H_2O \) and \( D_2O \) solvents for \( \bar{v}_2 \) and \( \bar{v}_3 \) determination. Finally, we summarize the thermodynamic and operational parameters characterizing A8-35 in solution and discuss how they can be used for the interpretation of AUC and SANS experiments on membrane protein/amphipol complexes.

Theoretical background

Partial specific volume and buoyant molar mass in three-component systems

In the cases of a polyelectrolyte macromolecule or of a multicomponent solvent, solvent redistribution is possible. We therefore consider a three-component system composed of (1) the principal solvent (\( H_2O \) or \( D_2O \)), (2) the macromolecular compound, which consists, in the case of a polyelectrolyte, of the macromolecule itself plus the number of counter-ions needed for electroneutrality (see [11] for a discussion of the components), and (3) the cosolvent (salt, for example) of molar masses \( M_i \) (g mol\(^{-1}\)) and partial specific volumes \( \bar{v}_i \) (ml g\(^{-1}\)), concentration \( c_i \) (g ml\(^{-1}\)) or \( m_i \) (mol kg\(^{-1}\) of \( \bar{V} \)), \( i = 1, 2 \) or 3. The change of the total volume \( \bar{V} \) of the solution is related to \( \bar{v}_i \):

\[
\bar{v}_i = \left( \frac{\bar{V}}{\bar{V}} \right)_{m_i} \frac{m_i}{m_i}.
\]

(1)

The partial specific volume of component \( i \) can be measured by density measurements performed at constant molalities of the components \( j \neq i \). If \( \rho \) and \( \rho^0 \) are the densities of the solution and the solvent,

\[
\left( \frac{\bar{V}}{\bar{V}} \right)_{m_i} = \left( 1 - \frac{\rho^0}{\rho} \right) \bar{v}_i.
\]

(2)

The buoyant molar mass \( M_b \) of the macromolecule in sedimentation experiments is related to the density increment \( (\bar{V}/\bar{V})_{m} \), obtained at constant chemical potential of the diffusive species:

\[
M_b = \frac{M_2(\bar{V}/\bar{V})_{m}}{\rho^0}.
\]

(3)

The index \( \mu \) stands here for \( \mu_1, \mu_3, M_b \) and \( \left( \frac{\bar{V}}{\bar{V}} \right)_{m} \) can be expressed using the preferential binding parameters of the cosolvent with the macromolecule, \( \xi_3 = \left( M_3/M_2 \right) \left( \bar{V}/\bar{V} \right)_{m} \),

\[
(\bar{V}/\bar{V})_m = (1 + \xi_3) - \rho^0 (\bar{v}_2 + \xi_3 \bar{v}_3).
\]

(4)

\( \xi_3 \) (in gram of 3 per gram of 2) or \( (\bar{V}/\bar{V})_m \) (in mole of 3 per mole of 2) represents the hypothetical amount of cosolvent that has to be introduced to or subtracted from the solution when adding component 2, in order to keep the chemical potentials of the solvent components constant. A value of \( \xi_3 \) different from zero corresponds to a redistribution of the solvent related to the presence of the macromolecule.

In a structural analysis, \( (\bar{V}/\bar{V})_m \) and \( \xi_3 \) can be expressed with regard to the water binding (\( B_1 \) gram of water per gram of polymer), the cosolute (here: salt) binding (\( B_3 \) gram of salt per gram of polymer), and the Donnan term (\( E_3 \) gram of salt per gram of polymer), which is related to the counter-ion dissociation of a polyelectrolyte

\[
(\bar{V}/\bar{V})_m = 1 + B_1 + (B_3 - E_3)
\]

\[ - \rho^0 (\bar{v}_2 + B_1 \bar{v}_1 + (B_3 - E_3) \bar{v}_3). \]

(5)

If \( w_3/w_1 \) is the salt to water ratio in the solvent, in gram of salt per gram of water,

\[
\xi_3 = B_3 - E_3 - B_1 w_3/w_1.
\]

(6)

The Donnan contribution is negative. In a dialysis experiment, it corresponds to a flux of salt toward the outside of the bag or a flux of water toward the inside. If \( z \) is the effective number of negative charges on the polyelectrolyte, \( z_+ \) the charge of the solvent salt cation and \( v \) the number of ions composing the solvent salt (\( v = 2 \) for \( NaCl \), \( v = 3 \) for \( MgCl_2 \)), then the Donnan contribution is \( E_3 = (M_3/M_2)z/vz_+ \). For the interested reader, the derivation of this expression was given recently [20]. In the present paper, because the solvent consists of dilute \( NaCl \) solutions (\( w_3/w_1 \approx 0.006 \)), the polyelectrolyte dissociation (Donnan contribution) is the only contribution to \( \xi_3 \) to be considered and Eq. (6) reduces to
\[ \zeta_3 = \frac{(M_3/M_2)}{2}(\tilde{c}m_3/\tilde{c}m_2)_{\mu} = -(M_3/M_2)z/2. \] (7)

Salt binding would entail a smaller value of \( z \). Eq. (7) will be used here for the evaluation of \( \zeta_3 \), \( (\tilde{c}m_3/\tilde{c}m_2)_{\mu} \), and/or \( z \). Its use would be an oversimplification for less diluted solvents (hydration can be detected when the density of the solvent differs from that of water) or for strong cosolute–macromolecule interactions, as observed in the case of halophilic proteins, or in the presence of divalent ions [20], or for membrane proteins in the presence of detergents, for example, where protein–cosolute binding cannot be neglected.

Use of the operational value \( \psi' \)

The operational parameter \( \psi' \) is extensively used to describe the buoyancy properties of macromolecules in multicomponent systems. It is defined as

\[ (\tilde{c}\rho/\tilde{c}c_2)_{\mu} = (1 - \rho_0^b\psi'). \] (8)

It can be used as an apparent partial specific volume to calculate the molar mass of a macromolecule from the buoyant molar mass measured in analytical ultracentrifugation (under the same experimental conditions of solvent and temperature). There is thus a practical interest to determine \( \psi' \). However, \( \psi' \) has no thermodynamic meaning and can be used only in one given solvent condition. It is related in a complex way to \( \rho_0^b, \tilde{c}_2, \zeta_3 \), and \( \tilde{c}_3 \):

\[ \psi' = \tilde{c}_2 + \zeta_3(\tilde{c}_3 - 1/\rho_0^b). \] (9)

Sedimentation of a noninteracting ideal macromolecule

Equilibrium sedimentation measures buoyant molar masses \( M_b \). The Svedberg equation relates the sedimentation coefficient, \( s \), obtained from sedimentation velocity experiments, to \( M_b \), to the hydrodynamic radius, \( R_s \), to the solvent viscosity, \( \eta \), and to the Avogadro number, \( N_A \):

\[ s = M_b/N_A6\pi\eta R_s. \] (10)

In the absence of salt ions in the solvent, the sedimentation velocity of a polyelectrolyte is reduced because the macromolecule and the counter-ions interact and the counter-ions are moving more slowly than the macromolecule. If the solvent comprises 0.1 M NaCl, as was the case in the present experiments, this source of nonideality can be neglected. For dilute samples or for extrapolation to infinite dilution, the Svedberg equation (10) can then be used rigorously.

Obtaining the partial specific volume of a noninteracting ideal macromolecule from measurement of the sedimentation in H2O and D2O buffers

For experiments performed in H2O and D2O solvents, the indices H (sometimes omitted) and D, respectively, are added to the above equations. We define \( R \) as the ratio of the buoyant molar masses in D2O and H2O solvents:

\[ R = M_{bD}/M_{bH}. \] (11)

\( R \) can be obtained from sedimentation velocity experiments if \( R_b \) is assumed to be the same in H2O and D2O solvents:

\[ R = sD\eta_D/sH\eta_H. \] (12)

The mass of the particle increases to \( M_{2D} \) in the D2O solvent, by the substitution of labile H for D. For the solvent as for the particle, we consider that isotopic substitution changes the masses but not the volume properties of the molecules. In consequence, the partial specific volume in D2O can be expressed as a function of \( \tilde{v}_2 (\tilde{v}_2 \) standing for \( \tilde{v}_{2H} \) and \( M_2 \) for \( M_{2H} \)):

\[ \tilde{v}_{2D} = \tilde{v}_{2H}M_{2D}/M_{2H}. \] (13)

We will also restrict the analysis to the case in which the value of \( (\tilde{c}m_3/\tilde{c}m_2)_{\mu} \)—referring the interaction of the macromolecule with the solvent in mole units—is unchanged by the change of the solvent from H2O to D2O. \( M_{bD} \) can then be written using the cosolvent binding parameter \( \zeta_3 \) (\( \zeta_3 \) stands for \( \zeta_{3H} \); it is expressed in gram of component 3 per gram of component 2 as defined for the hydrogenated solvent, i.e., without the consideration of H/D exchange) as

\[ M_{bD} = M_2[(M_{2D}/M_2 + \zeta_3) - \rho_0^b(\tilde{v}_2 + \zeta_3\tilde{v}_3)] \] (13)

and \( R \) can be written as

\[ R = [(M_{2D}/M_2 + \zeta_3) - \rho_0^b(\tilde{v}_2 + \zeta_3\tilde{v}_3)] /[(1 + \zeta_3) - \rho_0^b(\tilde{v}_2 + \zeta_3\tilde{v}_3)]. \] (14)

When \( M_{2D}/M_2 \) and \( \tilde{v}_2 \) are known, \( \zeta_3 \) can be estimated from \( R \):

\[ \zeta_3 = [(M_{2D}/M_2 - \rho_0^b\tilde{v}_2) - R(1 - \rho_0^b\tilde{v}_2)] / [R(1 - \rho_0^b\tilde{v}_2) - (1 - \rho_0^b\tilde{v}_2)]. \] (15)

In a two-component system composed of water and a nonpolyelectrolyte macromolecule, Eq. (14) reduces to \( R = (M_{2D}/M_2 - \rho_0^b\tilde{v}_2)/(1 - \rho_0^b\tilde{v}_2) \) and \( \tilde{v}_2 \) can be extracted from \( R \) using \( \tilde{v}_2 = (M_{2D}/M_2 - R)/(\rho_0^b - R\rho_0^b) \). By analogy, we introduce an apparent partial specific volume \( \psi'_R \):

\[ R = (M_{2D}/M_2 - \rho_0^b\tilde{v}_2)/(1 - \rho_0^b\tilde{v}_2) \] (16)

and

\[ \psi'_R = (M_{2D}/M_2 - R)/(\rho_0^b - R\rho_0^b). \] (17)

Match point in small-angle neutron scattering experiments

The normalized forward scattered intensity \( I(0) \) of an ideal solution of macromolecules can be expressed as a
function of \( M_g, c_2, N_A, \) and the neutron scattering length density increment of the macromolecule \((\partial \rho_N / \partial c_2)_{\mu}\) (in cm g^{-1}) as:

\[
I(0) = \left(1/N_A\right)c_2 M_2(\partial \rho_N / \partial c_2)_{\mu}^2.
\]  

(18)

\((\partial \rho_N / \partial c_2)_{\mu}\) is expressed using the neutron scattering length per gram of solute \( b_2 \) and \( b_3 \) (cm g^{-1} of macromolecule and salt, respectively), the scattering length density of the solvent \( \rho_N^0 \) (cm cm^{-3} of solvent), \( \xi_3, \bar{v}_2, \) and \( \bar{v}_3 \):

\[
(\partial \rho_N / \partial c_2)_{\mu} = (b_2 + \xi_3 b_3) - \rho_N^0 (\bar{v}_2 + \xi_3\bar{v}_3).
\]  

(19)

In a fully or partially deuterated solvent, \( c_2, M_2, \bar{v}_2, \) and \( \xi_3 \) are defined with reference to the fully hydrogenated macromolecule (for example, \( c_2 \) is the weight concentration of the macromolecule prior to H/D eventual exchange). But the percentage of D\(_2\)O, in the solvent \((\%_D)\) determines \( b_2 \) and \( \rho_N^0 \):

\[
b_2 = \left( b_{2D}\%_D + (100 - \%_D)b_{2H} \right)/100
\]  

(20)

and

\[
\rho_N^0 = (\rho_N^0 D\%_D + (100 - \%_D)\rho_N^0 H)/100,
\]  

(21)

where \( b_{2D} \), the neutron scattering length per gram of the macromolecule in the deuterated solvent, is defined in centimeters per gram of the hydrogenated macromolecule and depends on the number of H exchanged for D. If \( EXCH \) is the fraction of exchangeable H exchanged for D and \( b_{2D} \), the neutron scattering length per gram of the macromolecule corresponding to full exchange then \( b_{2D} = b_{2H} + EXCH(b_{2D} - b_{2H}) \). The match point in a H/D neutron titration curve corresponds to the absence of scattering. If CMP is the percentage of D\(_2\)O corresponding to the match point, it corresponds to the condition:

\[
(b_{2D} CMP + (100 - CMP)b_{2H})/100 + \xi_3 b_3 - (\rho_N^0 D\%_D CMP + (100 - CMP)\rho_N^0 H)(\bar{v}_2 + \xi_3\bar{v}_3)/100 = 0.
\]  

(22)

Materials and methods

Samples

Egg white lysozyme and porcine intestinal mucosa heparin (sodium salt) were purchased from Sigma and serum bovine albumin (BSA) was from Bayer. Polyacrylic acid (PAA), MW 5000, was purchased from Aldrich (50 wt% solution in water). This turbid solution was diluted down to 10 wt% in water and warmed up to 90 °C for 10–15 min, prior to freeze-drying. The lyophilized PAA contained about 10 wt% water as determined from the total carbon content of the powder. Gel permeation chromatography analysis of the PAA in its sodium form was performed in 0.5 M LiNO\(_3\) water solution, at 40 °C in Shodex OH pak columns (Waters, MA). A number average molar mass equal to 7500 g mol^{-1} and a polydispersity of 1.6 were obtained (differential viscometry and refractive index double-detection with universal calibration). Octylamine was purchased from Fluka. Deuterated octylamine-D17 (>99% D) was supplied by Isotech (Miami, OH). Isopropanol-D8 (99% D) was purchased from Eurisotop (St. Aubain, France). N-Methyl-2-pyrrolidone (NMP), tetrahydrofuran (THF), and other solvents were from Solvents, Documentations, Synthèses, Peypin, France. Other chemicals including isopropylamine, dicyclohexylcarbodiimide (DCI), and 1-hydroxybenzotriazol (HOBT) were from Aldrich.

Synthesis of isopropylamine-D7

Deuterated isopropylamine was obtained from the corresponding alcohol using the two-stage route reported by Mitsunobu et al. [25], with intermediate formation of N-isopropylphthalimide followed by hydrazinolysis. The alcohol (1.2 g, 20 mmol) was allowed to react at room temperature with one equivalent of triphenylphosphine (5.2 g) and phthalimide (3.0 g) in THF (40 ml). Slow addition of an equimolar amount of diethyl azodicarboxylate (3.4 g, dissolved in 10 ml THF) gave the isopropylphthalimide. THF was evaporated and the reaction mixture redissolved in 98% ethanol (20 ml) with no purification. A 50% solution of hydrazine in water was added to bring the pH above 9.0. The resulting precipitate was washed with ethanol and redissolved in minimum water. Upon addition of concentrated HCl solution to reach pH < 4.0, the isopropy lammonium chloride precipitated and was filtered and washed several times with dry diethyl ether. It was characterized by pH titration, chloride titration (AgNO\(_3\) and silver electrode), and \(^{1}^3\)C NMR.

Polymer modification

Amphipols were obtained by the successive reactions of octylamine and isopropylamine on the PAA precursor dissolved in NMP, in the presence of DCI as activating agent [26]. This procedure affords random grafting of the carboxylic acid groups [27]. A typical synthesis of A8-35 is described, with minor adaptations used for deuterated polymers (see below): the PAA (5 g, 63 mmol acid) was allowed to dissolve in 70 ml NMP for 15 h at 50 °C. Octylamine (2.12 g, 16.4 mmol) solution in 20 ml NMP was added prior to drop-wise supplementation with 3.62 g (17.4 mmol) DCI solution in NMP. The mixture was allowed to react for 2 h at 50 °C and then cooled to 0 °C and filtrated to remove the dicyclohexylurea formed. Successive additions in the reaction bath of NMP solutions containing 1.52 g isopropylamine, 4.0 g
HOBT, and finally 6.0 g DCI yielded, after a 2-h reaction at 50 °C, the amphipol solution under acid form. Two equivalents of sodium methanoate were added to the mixture, which was subsequently diluted in a large excess of water (1.5 L). After filtration of residual dicyclohexylurea, the polymer was precipitated by acidification of the aqueous solution with aliquots of 10% HCl. Purification of the amphipol was achieved by three cycles of redissolution in water–NaOH, filtration, and precipitation by acidification. Finally, the precipitate was redissolved with the minimum amount of NaOH, adjustment of the pH of the solution to pH 8.5–9.0 and freeze-drying, yielding 6.5 g of product.

The synthesis of deuterated compounds differed in the use of isopropylamine under its chlorhydrate form. Isopropylammonium was dissolved in NMP and one equivalent of NaOH (10 M aqueous solution) added. The solution was dried on molecular sieve and NaCl removed by filtration prior to addition in the reaction bath. HOBT and DCI were added and the reaction was allowed to proceed for 30 min at 50 °C. The bath was kept overnight at room temperature before carrying out the purification procedure as described above.

**Chemical analysis of amphipols**

Amphipols were characterized by $^1$H and $^{13}$C NMR, pH titration with HCl, and elemental analysis. Fig. 2 shows $^1$H NMR spectra of HAPol and DAPol, and Fig. 3 shows the $^{13}$C NMR spectrum of DAPol. Table 1 summarizes the results and shows that differences between the chemical composition of HAPol and DAPol are close to the limit of uncertainty on the analyses. The similarity between HAPol and DAPol with regard to octyl, isopropyl, and charge densities appears even more clearly by the comparison of data obtained using the same technique, e.g., $^{13}$C NMR in Table 1. It was therefore considered for the calculation of contrast match points (see Contrast match points of HAPol and DAPol in SANS experiments) that HAPol and DAPol are negligibly different in their composition. Somewhat arbitrarily, though based on the measurements of isopropyl/octyl ratio and charge density having the highest accuracy, a representative composition was fixed at 35% sodium acrylate, 25% octylacrylamide, and 40% isopropylacrylamide units. However, the existence of a small amount of hydrogenated contaminant covalently bound to DAPol was revealed by the presence of a minor peak centered at 3.5 ppm in the $^1$H NMR spectrum (Fig. 2B). Such a peak was essentially absent in the spectrum of the batch of HAPol used for the present study (Fig. 2A). The high degree of modification of PAA required to achieve the A8-35 structure and the corresponding high amount of coupling agents (DCI, HOBT) used during the synthesis increase the risk of side reactions. Several contaminants therefore may become grafted as side groups, including traces of alcohols that may be present in NMP and the side group generated by the translocation of the activated function formed between DCI and carboxylic acid (N-acyl-dicyclohexylurea) [28]. It is worth noting that the spectrum of dicyclohexylurea, a compound comparable to the side-product of reaction with DCI, shows similarly one peak at 3.5 ppm (two protons) and several peaks in the range 1.0–2.0 ppm (methylene of the cyclohexyl rings). In DAPol $^1$H NMR spectra, the integral of the peak at 3.5 ppm yields an estimate of the degree of contamination, namely a molar fraction of 3.3% of the monomer, if ascribed to two protons. The sum of the integrals of the peaks due to the contaminant (labels €

![Fig. 2. $^1$H NMR spectra of HAPol and DAPol. (A) HAPol in D$_2$O/MeOD 20:80 v/v. The spectrum comprises three main regions: that from 0.6 to 1.4 ppm is representative of methyl and methylene protons of alkyl side groups, that from 1.4 to 2.4 ppm is representative of the backbone protons, and that from 3.0 to 4.0 ppm is representative of side group methylene protons in the α position of the amide function. Octyl groups yield three peaks, labeled O1, O2, and O3, accounting, respectively, for 3, 12, and 2 protons per side chain; isopropyl groups yield peaks I1 and I2, accounting, respectively, for 6 and 1 protons per side chain; peaks B1 and B2 account, respectively, for 2 and 1 protons per acrylic unit. The overall degree of derivatization of the polyacrylate can be determined from the integrals of I1, O1, and B2, the relative proportion of octyl and isopropyl groups can be determined from those of O1 and I1 or of I2 and O3. The inset is a zoom of the spectrum obtained in D$_2$O (featuring no methanol signal at 3.3 ppm). It shows the near-absence of α-methylene protons from contaminant side chains (*) in the 3.0–4.0 ppm region (cf. spectrum B). (B) DAPol in D$_2$O. The sharp peaks are due to molecular impurities that are not bound to the polymer. The absence of peaks from the α-methylene of either octyl or isopropyl groups confirms their complete deuteration. The peaks labeled with asterisks correspond to α-methylene (*) and other (**) protons belonging to a small fraction of bound contaminant (see Materials and methods).
** in Fig. 2B) corresponds to a 0.2:1 ratio to the integral of the methine protons along the backbone, yielding an average of 0.067 additional hydrogen atom per monomer unit. While relatively minor compared to the average number of 7.05 deuterium atoms per monomer unit expected from the formula of the nominal per-deuterated form of DAPol, this low level of contamination nevertheless detectably affects (by 6\% D$_2$O) the calculated contrast match point of DAPol in SANS experiments (see below).

**Measurement of the total carbon content of the samples**

The measurements were performed using a Dörrmann DC50 Total Organic Carbon Analyzer (Cincinnati, OH). The samples, at a concentration of ca. 20 g L$^{-1}$ HAPol, were diluted in pure water before injection in the reactor for oxidation in a nitric acid–potassium persulfate aqueous solution in the presence of oxygen and UV light. Oxidation of HAPol yields carbon dioxide which is quantified by infrared absorbance at 1600–1800 cm$^{-1}$ to obtain the total amount of carbon in the sample. Calibration was achieved with potassium phthalate standards.

**Density measurements**

Two sets of density measurements were performed on HAPol. One set used HAPol solubilized at a concentration of 18.8 g L$^{-1}$ (determined by measurement of the total carbon in the sample) and precisely diluted samples in 100 mM NaCl, 20 mM sodium phosphate buffer, pH 6.8. After a short centrifugation, measurements of the density of the solvent and samples (precision: 2 $\times$ 10$^{-5}$ ml g$^{-1}$) were made using a DMA 58 densitometer (Anton Paar, Graz, Austria). A second set of measurements was done in either pure water or 0.1 M NaCl, 20 mM Tris/HCl buffer, pH 8, using polymer powder desiccated on P$_2$O$_5$, the concentrations being determined by weighing. Density measurements were performed at 20.00 °C on a DMA 5000 density meter (Anton Paar, Graz, Austria). The densities of the solvents used in the analytical ultracentrifugation were also measured. The density increment ($\hat{\rho}$/$\hat{c}$)$_{w/p}$, related to $\bar{v}_2$ (Eq. (2)), depends on the difference between the density of the solvent, $\rho^0$, and that of the solution of HAPol at concentration $c_2$ (g ml$^{-1}$), $\rho$:

$$\hat{\rho}/\hat{c}_{w/p} = (\rho - \rho^0)/c_2.$$

**Viscosity measurements**

Viscosity measurements of the solvents were made at 20.0 °C using an AMV 200 rolling ball viscometer with a manually filled capillary of internal diameter 0.9 mm recommended for the range 0.5–5 mPa s (ref. 63872) (Anton Paar, Graz, Austria). The viscosities of 300 l solutions were obtained from the rolling times of the steel ball for the solution measured at three inclination angles (from 40 to 60°) of the capillary. For heparin, BSA and lysozyme, density and viscosity measurements were done on the samples investigated in sedimentation.

**Sedimentation velocity experiments**

Heparin, lysozyme, and BSA samples were prepared by dissolution of the macromolecules at 1–6 g L$^{-1}$ and

![](image.png)

Fig. 3. $^{13}$C NMR spectra of DAPol in D$_2$O. The 170–190 ppm region shows two peaks attributed to the carbon atoms of the carboxylate (1) and amide (2) functions. The ratio of their integrals provides that of the two functions and thus the degree of derivatization of the polycrylate. The ratio of the peaks centered at 18 ppm (3: methyls of the isopropyl groups) and 10 ppm (4: methyls of the octyl groups) yields the isopropyl/octyl ratio.

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**Sedimentation velocity experiments**

Heparin, lysozyme, and BSA samples were prepared by dissolution of the macromolecules at 1–6 g L$^{-1}$ and

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Composition of HAPol and DAPol obtained from NMR, elemental analysis, and titrations</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Free carboxylate (%)</td>
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<td>HAPol</td>
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</tr>
<tr>
<td></td>
<td>28 ± 2\textsuperscript{a}</td>
</tr>
<tr>
<td>DAPol\textsuperscript{e}</td>
<td>29 ± 1\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} $^{13}$C NMR.

\textsuperscript{b} $^1$H NMR.

\textsuperscript{c} Microanalysis.

\textsuperscript{d} pH titration.

\textsuperscript{e} NMR analysis revealed that DAPol additionally contains ~3% of contaminant side chains, which were essentially absent in HAPol (see Materials and methods). Errors indicated for NMR analysis are fitting errors for single spectrum.
subsequent dilution in the appropriate solvent. Heparin was extensively dried on P₂O₅ and dissolved in 0.2 M NaCl, H₂O or D₂O at a concentration of 5.3 g L⁻¹. Subsequent dilutions of heparin were made by weighing. The minimum time between dissolution and sedimentation measurement was 5 h. For HAPol and DAPol, samples were investigated after days of incubation and after SANS measurements. Analytical ultracentrifugation was performed either on a XLA or a XLI centrifuge (Beckman Coulter, Palo Alto, USA) with rotor speeds of 42,000, 57,000, and 60,000 rpm for BSA and lysozyme, heparin, and HAPol and DAPol, respectively, in a double-sector cell of optical path 12 mm, using absorbance or interference optics. For heparin, sedimentation coefficients, s, were evaluated from the displacement of the maxima of the derivatives of the integral distribution and extrapolated to infinite dilution, with linear extrapolation of s or 1/s to zero concentration [29]. The resulting values differed by 0.03 S, and the mean values were used. We used the Sedfit program (available at http://www.analyticalultracentrifugation.com) for two different types of analysis [30,33], which consider globally a set of sedimentation profiles obtained at different times and take advantage of a radial and time-independent noise subtraction procedure [32]. The c(s) analysis determines a continuous distribution of experimental sedimentation coefficients for the proteins and the polymer samples. Finite element solutions of the Lamm equation for a large number of discrete independent species are combined with a maximum entropy regularization to represent a continuous size distribution [30]. Well-resolved distributions are obtained for noninteracting systems [31]. In the c(s) analysis, reasonable hypotheses on the values of the partial specific volume, the frictional ratio f/f₀, the density ρ, and the viscosity η of the solvent allow fixation of a plausible relationship between mass, sedimentation, and diffusion coefficients s and D. The second type of analysis considers the model of one noninteracting species [33]: in that case, s and D are fitted. For the homogeneous lysozyme, it led to sedimentation and diffusion coefficient values that were verified to be independent of the protein concentration (in the investigated range, i.e., up to 0.4 g L⁻¹), yielding a buoyant molar mass that differed by less than 3% from the theoretical mass.

Equilibrium sedimentation of HAPol in solvents containing H₂O, D₂O or D₂¹⁸O

HAPol was dissolved at 20 g L⁻¹ in 0.1 M NaCl, 0.2 M sodium phosphate buffer, pH 7.2. The stock solution was then diluted to 10 and 5 g L⁻¹ in the same buffer, and each of these was diluted 20× in 1 or 0.1 M NaCl in H₂O, D₂O, or D₂¹⁸O. The optical quality of D₂¹⁸O, obtained from Interchim (Montluçon, France) (two samples at 99% D, 95%¹⁸O or at 99% D, 75%¹⁸O), was quite poor (A₃₂₇ = 0.9, decreasing to A₂₈₀ = 0.25, with a maximum of 0.5 at A₁₃₀ = 0.5), which seriously complicated our measurements. The density of the solvents was measured experimentally. 100-μl samples of HAPol at concentrations of 1, 0.5, and 0.25 g L⁻¹ were centrifuged in six-channel centerpieces at 20 °C, at 22,000, 31,000, and 38,000 rpm (in 1 M NaCl) or at 18,000, 22,000, and 42,000 rpm (in 0.1 M NaCl). Sedimentation profiles were obtained using absorbance optics (at 231 nm in H₂O or D₂O and at 315 nm in D₂¹⁸O). The mean buoyant molar masses were estimated in 0.1 M NaCl, from estimates of the absorbance A₀, Aₘ, and Aₙ, i.e., respectively, before sedimentation, and, following equilibration, at the meniscus (rₘ) and at the bottom (rₙ), Mₛ = 2RT(Aₘ − Aₙ)/ω²A₀(ᵣₘ² − rₙ²), (we considered the mean value from the different concentrations and the two lowest rotor speeds), and in 1 M NaCl, from the fit of the equilibrium profiles A(r) obtained at the different concentrations and rotor speeds, r being the radial distance, assuming one sedimenting species, A(r) = A(r₀) exp(ω²Mₛ(r² − r₀²)/2RT) + δ, considering the experimentally obtained baseline δ at 42,000 rpm in H₂O and D₂¹⁸O and δ = 0 in D₂O. In these equations, ω is the angular velocity (s⁻¹) and RT the product of the gas constant by the absolute temperature.

Small-angle neutron scattering

Small-angle neutron scattering experiments were carried out on the D22 beam line at the Institut Laue Langevin (Grenoble, France) at 20 °C. The collimation and sample detector distances were 5 m. The deuterated buffer was made by diluting in D₂O a lyophilized hydrogenated buffer containing 0.1 M NaCl, 20 mM sodium phosphate buffer, pH 7. Lyophilized polymers were resuspended in H₂O or D₂O at a concentration of 100 g L⁻¹ before being lyophilized once again. Powders were then resuspended in either H₂O or D₂O buffer at a final concentration of 10 g L⁻¹. The polymer concentration was checked by measuring the total carbon concentration of the samples. SANS measurements were performed at different percentages of D₂O obtained by mixing the two samples in various proportions. The exact percentage of D₂O in each sample was determined by comparing neutron transmission by the samples and buffers to that of pure H₂O and D₂O. After subtraction of the signal of the solvent and normalization of the data for the geometrical factors of the experiments using the signal of 1.0 mm water [34], the Guinier approximation was used to extrapolate the forward intensity I(0):

\[ \ln I(q)/c_2 = \ln I(0)/c_2 - q^2R_g^2/3. \] (24)

The CMP was derived from the linear regression of \( \sqrt{I(0)/c_2} \) plotted against the percentage of D₂O in the solvent (see Eqs. (18)–(21)). Because of the presence
of a small amount of large aggregates, detected at low angle, two sets of Guinier plots were constructed, one below and one above \( q^2 = 0.001 \) Å \(^{-2} \). A full presentation and analysis of the SANS data will be given elsewhere [17].

**Results and discussion**

In the following, we first analyze how \( R \), the ratio of the buoyant molar masses of a macromolecule in \( D_2O \) and \( H_2O \) solutions, can be used to determine its \( \bar{v}_2 \) and its apparent partial specific volumes, \( \phi'_h \) and \( \phi'_v \), and we evaluate the effects of hydrogen/deuterium exchange and polyelectrolyte dissociation. The use of sedimentation-velocity experiments in \( H_2O \) vs \( D_2O \) solutions is then validated using model compounds. Finally, we determine the partial specific volume and CMP of A8-35 in its hydrogenated and deuterated forms and show that its buoyancy properties are compatible with full dissociation of the counter-ions.

**Factors affecting the value of \( R \)**

\( R \), which can be obtained from sedimentation equilibrium or velocity measurements, is related to three macromolecular characteristics: \( \bar{v}_2 \), \( M_{2D}/M_2 \), and \( \xi_3 \) (Eq. (14)). We evaluate here the effects on \( R \) of each of these three parameters. How is \( R \) related to \( \bar{v}_2 \)? How does \( R \) change with proton exchangeability (via \( M_{2D}/M_2 \)) and with polyelectrolyte counter-ion dissociation (via \( \xi_3 \))? For a polyelectrolyte, how does the apparent partial specific volume deduced from \( H_2O/D_2O \) experiments, \( \phi'_h \), compare with the usual operational one, \( \phi'h \), obtained in \( H_2O \) solvents and related only to \( \bar{v}_2 \) and \( \xi_3 \)?

**General dependency of \( R \) on \( \bar{v}_2 \) in the case of a two-component system.** Fig. 4A illustrates how \( R \) depends on \( \bar{v}_2 \) when the macromolecule does not exchange any of its \( H \) for \( D \) and shows ideal behavior with respect to the solvent. When \( \bar{v}_2 \) increases from 0.4 to 1.25 ml g \(^{-1} \), \( R \) first decreases very slowly (from the limiting value of 1, at \( \bar{v}_2 = 0 \), i.e., an infinitely dense particle) and then more and more abruptly. \( R \) is zero for \( \bar{v}_2 = 1/\rho_D = 0.9 \) ml g \(^{-1} \) (where the sedimentation coefficient \( s_{TD} = 0 \) is zero), and decreases rapidly from this value as \( \bar{v}_2 \) approaches \( 1/\rho_H = 1 \) ml g \(^{-1} \) (where \( s_{TH} \) is zero and \( R \) infinitely negative). For \( 1/\rho_H < \bar{v}_2 < 1/\rho_H \), \( R \) is negative as the macromolecule floats in \( D_2O \) and sediments in \( H_2O \). When \( \bar{v}_2 \) increases above \( 1/\rho_H \), corresponding to flotation in both solvents, \( R \) decreases from infinitely positive to tend again toward its limiting value of 1. Fig. 4A clearly shows that the farther from 1 is the value of \( R \) (particularly for \( \bar{v}_2 \) values, say, between \( \sim0.8 \) and \( \sim1.2 \) ml g \(^{-1} \)), the more accurate is the estimate of \( \bar{v}_2 \). We calculate that a constant experimental uncertainty of \( \pm0.005 \) on \( R \) entails errors on \( \bar{v}_2 \) of \( \pm0.012, \pm0.004 \), and \( \pm0.001 \) ml g \(^{-1} \) for \( R \) values of 0.9, 0.75, and 0.3, respectively. These three \( R \) values are, respectively, representative of those obtained for heparin or nucleic acids, for proteins, and for HAPol (see below, Tables 4 and 5).

**Influence of \( M_{2D}/M_2 \).** Table 2 lists the maximum numbers of exchangeable protons, calculated from the composition of the macromolecules, for BSA, lysozyme,
heparin, and sodium salts of HAPol, DAPol, and DNA and the corresponding maximum values of $M_{2D}/M_2$. Fig. 4B shows the dependency of $R$ on $\bar{v}$ for different values of $M_{2D}/M_2$. When $R$ is close to 1, $\bar{v}$ values derived from it strongly depend on the $M_{2D}/M_2$ ratio. Conversely, uncertainties in $M_{2D}/M_2$ entail smaller errors in $\bar{v}$ when $R$ values move farther away from 1. For example, the values of $\bar{v}$ calculated from $R$ values of 0.9, 0.75, and 0.3 with $M_{2D}/M_2$ values of 1.000 or 1.005 differ by 0.023, 0.013, and 0.007 ml g$^{-1}$, respectively. It is therefore most important to use good estimates of $M_{2D}/M_2$ when measuring low partial specific volumes.

**Influence of polyelectrolyte dissociation on the value of $R$.** The nominal numbers of charges carried by the polyelectrolytes of our study are listed in Table 2. They correspond to $z/M_2$ ratios of 0.0035, 0.0028, and 0.0027 for heparin, HAPol, and DAPol, respectively. The $z/M_2$ value for DNA is 0.0030. The effect of counter-ion dissociation on $R$ was evaluated in the case of $z/M_2 = 0.003$, assuming complete dissociation of Na$^+$-polypelectrolyte in the presence of dilute NaCl (Eqs. (7) and (14)). Fig. 4B illustrates the huge influence of polyelectrolyte dissociation on $R$, particularly for large values of $\bar{v}$ (low $R$ values). It is clear that in the case of a macromolecule interacting with the solvent—such as the polyelectrolyte studied here—this parameter must be taken into account when deriving the value of $\bar{v}$ from that of $R$.

**How are $R$ and $\bar{v}$ related to the apparent partial specific volume $\bar{v}_p$ obtained by AUC?** We have introduced the apparent partial specific volume $\bar{v}_p$ derived from measurements of $R$, to differentiate it from the usual apparent partial specific volume $\bar{v}$ derived from ($\bar{v}_p - \bar{v}_c$)$_m$ measurements. Both are related to $\bar{v}$ and $\bar{v}_c$, while $R$ depends additionally on $M_{2D}/M_2$. The difference between $\bar{v}_p$ and $\bar{v}$ is a complicated function:

$$\bar{v}_p - \bar{v} = \bar{v}_c (\rho_D - \rho_H) ((1 + \bar{v}_c) (\bar{v}_2 - \bar{v}_c))$$

$$\rho_D (1 + \bar{v}_c) - (M_{2D}/M_2 + \bar{v}_c) \rho_H].$$

(25)

It becomes zero for $\bar{v}_c = 0$, which expresses the fact that, in the absence of preferential binding, $\bar{v}_p$ and $\bar{v}$ are equal (in this case, $\bar{v}_p = \bar{v} = \bar{v}_c$). Fig. 4C illustrates the respective evolutions of $\bar{v}_p$, $\bar{v}$, and $\bar{v}_2$ as functions of $\bar{v}$ in the case of a fully dissociated polyelectrolyte with $z/M_2 = 0.003$ in dilute NaCl solution. The difference $\bar{v}_c (\bar{v}_2 - \bar{v}_c)$ between $\bar{v}_p$ and $\bar{v}$ does not depend on $\bar{v}_2$: $\bar{v}_p - \bar{v}_2 = 0.06$ ml g$^{-1}$. The difference $(\bar{v}_p - \bar{v}_2)$, on the other hand, varies continuously. It is zero for $\bar{v}_p = \bar{v}_2 = 1/\rho_H$, less than 0.01 ml g$^{-1}$ for $0.88 < \bar{v}_p < 0.12$ (i.e., $0.83 < \bar{v}_2 < 1.04$ ml g$^{-1}$) and less than 0.02 ml g$^{-1}$ for $\bar{v}_p$ in the range of 0.77–0.88 ($\bar{v}_2$ in the range of 0.73–0.83 ml g$^{-1}$). The difference remains below 0.05 ml g$^{-1}$ for $\bar{v}_p$ and $\bar{v}_2$ values of about 0.45 ml g$^{-1}$. We can calculate that $\bar{v}_p$ varies negligibly with $M_{2D}/M_2$. This is because $\bar{v}_p$ is calculated from $R$ and from the value of $M_{2D}/M_2$. However, the uncertainty in $M_{2D}/M_2$ affects the precision in $\bar{v}_p$ calculated from $R$ and that from $\bar{v}_2$ (see above Influence of $M_{2D}/M_2$).

**Summary.** The experimental determination of the ratio $R$ of its buoyant masses in H$_2$O and D$_2$O buffers can be used to determine the partial specific volume $\bar{v}$ of an uncharged macromolecule in water or, in the cases of a polyelectrolyte or a multicomponent solvent, its operational apparent partial specific volume $\bar{v}_p$. Experimental errors in $R$ and uncertainties in the extent of hydrogen/deuterium exchange do not strongly affect the determination of $\bar{v}$ and $\bar{v}_p$ as long as their values are close to 1 ml g$^{-1}$ (i.e., 1/\rho_H). In the case of a polyelectrolyte, the difference between $\bar{v}_p$ and $\bar{v}$ is roughly proportional to the value of the preferential binding parameter $\bar{v}_c$ and is smaller for $\bar{v}_p$ and $\bar{v}$ close to 1.

**Experimental determination of $R$ from the comparison of sedimentation velocity experiments in H$_2$O and D$_2$O solvents and its interpretation with regard to $\bar{v}$ or $\bar{v}_p$ for heparin and model proteins**

Sedimentation equilibrium in H$_2$O and D$_2$O is often used for the determination of $\bar{v}$ [35–37]. The analysis of the data, however, becomes rapidly ambiguous when the particles under study are not monodisperse. We explore here the use of sedimentation velocity, which constitutes potentially a much more powerful technique for the study of heterogeneous samples. BSA preparations, which are known to contain a small amount of oligomers, were chosen as a model system. Multimers indeed...
were easily upon analysis of the sedimentation profiles, both in H₂O and in D₂O buffers and whether using absorbance or interference optics (Fig. 5). The proportions of the different species found in the two solvents and with the two optical systems are similar, as is the ratio $s_{2}/s_{1}$ for all three peaks, whatever the optical system, as shown in Table 3, which demonstrates the reliability of the analysis. The values of $s$ and $R$ for lysozyme, BSA (monomer peak), and heparin used for the calculation of $\bar{v}_2$ (proteins) or $\phi'_H$ (heparin) are reported in Table 4, along with estimates of the errors and values from the literature. For the three macromolecules, the values determined for $\bar{v}_2$ or $\phi'_H$ are close to those expected, considering the reasonable value of 80–100% H/D exchange (consistent with the typical value of $M_{2D}/M_2 \approx 1.0155$ reported for proteins [38]). Experimental errors are larger when $R$ is close to 1 (low values of $\bar{v}_2$ or $\phi'_H$). The errors for heparin therefore are large, and the results compatible with any extent of counter-ion dissociation (a limited dissociation of the counter-ions was suggested in a previous study [29]). For lysozyme and BSA, the agreement between $\bar{v}_2$ values deduced from $R$ and published data is very good, less than 0.02 ml g⁻¹, i.e., within the experimental uncertainty of 0.04–0.05 ml g⁻¹.

Characterization of amphipol A8-35

The characterization of A8-35 was done by density measurements at constant molality and by sedimentation velocity experiments in H₂O and D₂O solvents. The results were compared to those obtained from sedimentation equilibrium and small-angle neutron scattering.

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**Table 3**

<table>
<thead>
<tr>
<th>Species</th>
<th>$s_1$ (S)</th>
<th>$s_2$ (S)</th>
<th>$s_1/s_0$</th>
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<td>Monomer</td>
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<td>Dimer</td>
<td>Interf.</td>
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<td>6.31</td>
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<tr>
<td>Trimer</td>
<td>Interf.</td>
<td>4.57</td>
<td>3.85</td>
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</table>

*Note. $s_1$s, $s_2$s, sedimentation coefficients in H₂O and D₂O buffers, respectively; Abs., measurements at 278 nm; Interf., measurements by interference. The $c(s)$ profiles are presented in Fig. 5.*

**Table 4**

<table>
<thead>
<tr>
<th>Species</th>
<th>$\rho_H^1$</th>
<th>$\rho_D^1$</th>
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<th>$s_D$</th>
<th>$R$</th>
<th>$H/D$ EXCH</th>
<th>$\bar{v}_2$ or $\phi'_H$</th>
<th>$\phi'$</th>
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<td>1.69</td>
<td>0.924</td>
<td>100</td>
<td>0.45</td>
<td>0.5</td>
<td>0.43</td>
<td>0.01</td>
<td>0.10</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>1.003</td>
<td>1.105</td>
<td>1.207</td>
<td>1.77</td>
<td>1.19</td>
<td>0.811</td>
<td>100</td>
<td>0.71</td>
<td>0.02</td>
<td>0.03</td>
<td>0.05</td>
<td>0.714</td>
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<tr>
<td>BSA</td>
<td>1.013</td>
<td>1.113</td>
<td>1.226</td>
<td>4.17</td>
<td>2.54</td>
<td>0.748</td>
<td>100</td>
<td>0.75</td>
<td>0.01</td>
<td>0.02</td>
<td>0.04</td>
<td>0.733</td>
</tr>
</tbody>
</table>

*Note. Columns (1) to (3); $\rho_H^1$ and $\rho_D^1$, $\eta_H$ and $\eta_D$ are the measured densities and viscosities of the H₂O and D₂O solvents. Columns (4) to (6): the sedimentation coefficients of the macromolecule, $s_H$ and $s_D$, are measured in H₂O and D₂O solvents, respectively, as described in the text. $R$ is $s_H/\eta_H$. H-D EXCH (column (7)) is the percentage of H-D exchange—100% meaning that all the exchangeable hydrogen (see Table 2) is deuterated—\ which was considered for the calculation from $R$ of the partial specific volumes of column (8), $\bar{v}_2$ (proteins) or $\phi'_H$ (heparin), according to Eq. (17), in the case of a polyelectrolyte as heparin. For heparin, $\phi'$ (column (9)) is estimated from $\phi'_H$, assuming full dissociation of the counter-ions, from Fig. 4C (built using Eqs. (7) and (25)), and $\bar{v}_2$ (column (10)) is calculated from $\phi'$ (Eq. (9)). For the errors in $\bar{v}_2$ or $\phi'_H$ from $M_{2D}/M_2$ (column (11)), uncertainties in $M_{2D}/M_2$ of 0.001 and 0.005 for heparin and proteins, respectively, were considered. The uncertainty in $R$, used in the calculation of column (12), was of the order of 0.03 in all conditions, based on uncertainties in $\eta_H/\eta_D$ and $s_H/s_D$ of 0.03 and 0.01, respectively. The total error in $\bar{v}_2$ or $\phi'_H$ in column (13) is the sum of the errors from $M_{2D}/M_2$ and $R$ given in columns (11) and (12) and from $\rho_H^1$ and $\rho_D^1$. The latter two, of 0.002 and less than 0.0005 ml g⁻¹, respectively, considering uncertainties of 0.001 on the densities, are negligible. The last column (14) reports the $\bar{v}_2$ values measured for heparin [29] or calculated from the aminocacid composition.
Determination of the partial specific volume of HAPol from density measurements at constant molality of the solvent components. Fig. 6 shows the density increments, at constant molality of the solvent components, for HAPol in H$_2$O and in 0.1 M NaCl buffered at pH 8 or 6.8 either with 20 mM Tris or with sodium phosphate. Despite the fact that the solvents, the densitometers used for the measurement, the range of HAPol concentrations, and the way that they were evaluated differed from one set of experiments to the other, the results are highly consistent. They yield a partial specific volume $\bar{v}_2 = (\partial V/\partial m)_{w_{\text{sol}}} = 0.809 \pm 0.003$ ml g$^{-1}$. Assuming that HAPol and its deuterated variant DAPol have the same partial molal volume, we expect that for DAPol $\bar{v}_2 = 0.765 \pm 0.003$ ml g$^{-1}$.

Sedimentation velocity analysis of HAPol and DAPol. Sedimentation velocity experiments were performed on HAPol and DAPol at 1 g L$^{-1}$ in H$_2$O and D$_2$O solutions comprising 0.1 M NaCl and 20 mM sodium phosphate, pH 7.1. Initial inspection of the general shape of the boundaries suggests that the particles formed by the two polymers in aqueous solution are rather homogeneous, in keeping with the results of size-exclusion chromatography and SANS experiments [16,17]. Thus, in a first approach, sedimentation profiles were analyzed as reflecting the migration of homogeneous, noninteracting particles (Fig. 7A, B, D, and E). Taking into account the systematic noise in the sedimentation profiles, the fits were rather good, with RMSD values between 0.01 and 0.02. The $s$ values thus obtained yielded a first estimate of the partial specific volumes ($\bar{v}_R$), reported in Table 5. An analysis of the sedimentation profiles in terms of a continuous distribution of particles was then performed, using starting values of the frictional ratio $f/f^0$ ranging from 1 to 1.6 by steps of 0.2. While the details of the final $c(s)$ distribution (the precise position and relative weight of the peaks and thus the apparent homogeneity of the sample with regard to particle size) depended on the choice of $f/f^0$, the mean sedimentation coefficient did not (not shown). The best fits (minimum RMSD) were obtained for $f/f^0$ values of 1.2 and 1.4 for HAPol in H$_2$O and D$_2$O solutions and of 1.0 and 1.2 for DAPol in H$_2$O and D$_2$O solutions, respectively (for example, for HAPol in H$_2$O, RMSD values are 0.0129, 0.0126, 0.0127, and 0.0130 for $f/f^0$ values of 1.2, 1.4, and 1.6). The resulting $c(s)$ distributions are presented in Figs. 7C and F. The analysis suggests the presence of a main population of small particles and of small amounts of larger species. In keeping with SANS observations [16,17], very large aggregates (with $s$ values above 10 and 5 S in H$_2$O and D$_2$O solvents, respectively) may be present but, if so, only in very small amounts. The general features of the distribution are similar for HAPol and DAPol and do not appear to change when moving from light water to heavy water. (We consider that the fact that the main peak appears larger and more asymmetrical for DAPol than for HAPol is unlikely to be significant). No concentration dependency of the size

---

**Table 5**

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Analysis</th>
<th>$s_1$ (S)</th>
<th>$s_0$ (S)</th>
<th>$s_{2D}$ (S)</th>
<th>$R$</th>
<th>$\phi^\prime_R$ (ml g$^{-1}$)</th>
<th>$\bar{v}<em>2$ (from $s</em>{2D}/s_R$) (ml g$^{-1}$)</th>
<th>$\bar{v}_2$ (density) (ml g$^{-1}$)</th>
<th>$z$</th>
<th>$\phi^\prime$ (ml g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAPol</td>
<td>1 species</td>
<td>1.60</td>
<td>0.41</td>
<td>0.26</td>
<td>0.31</td>
<td>0.868 ± 0.008</td>
<td>0.822 ± 0.008</td>
<td>0.809 ± 0.003</td>
<td>−44</td>
<td>± 8 8.666 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>Mean $s$95% of $c(s)$</td>
<td>1.92</td>
<td>0.49</td>
<td>0.26</td>
<td>0.31</td>
<td>0.868</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAPol</td>
<td>1 species</td>
<td>2.27</td>
<td>1.06</td>
<td>0.47</td>
<td>0.57</td>
<td>0.808 ± 0.017</td>
<td>0.769 ± 0.018</td>
<td>0.765 ± 0.003</td>
<td>−38</td>
<td>± 16 0.820 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>Mean $s$95% of $c(s)$</td>
<td>2.45</td>
<td>1.07</td>
<td>0.44</td>
<td>0.53</td>
<td>0.819</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Note: The analysis of the sedimentation profiles was done in two different ways using SediFit: 1 species corresponds to the model of one noninteracting species; mean $s$95% of $c(s)$ corresponds to the analysis of continuous distribution presented in Fig. 7 (only the larger aggregates above 9.3/4.9 and 9.1/2.3 S in H$_2$O/D$_2$O solvents for HAPol in Fig. 7C and DAPol in Fig. 7F were discounted). $s_1$ and $s_0$ represent the sedimentation coefficients obtained in H$_2$O and D$_2$O solvents, respectively. $R = s_{2D}/s_R$ was calculated with $s_R = 1.212$, the measured ratio of the viscosities in D$_2$O and H$_2$O solvents. The apparent partial specific volume $\phi^\prime_R$ was calculated from $R$ (Eq. (17)) using $M_{2D}/M_2 = 1.005$, corresponding to the maximal H/D exchange for the macromolecule, and solvent densities $\rho_R = 1.005$ and $\rho_D = 1.112$ g ml$^{-1}$. The uncertainty was evaluated considering uncertainties of 0.001 on $M_{2D}/M_2$, $\rho_R$, and $\rho_D$ and 0.03 on $s_R/\rho_R$ and $s_{2D}/\rho_R$, the latter uncertainty accounting for about 70% of that on $\phi^\prime_R$. The partial specific volumes $\bar{v}_2$ from $s_{2D}/s_R$ are calculated considering a total dissociation of 35 counter-ions (Eqs. (7 and 14)); that given for DAPol from density measurements is derived from that measured for HAPol, assuming HAPol and DAPol to have the same partial molal volume. The values of $\phi^\prime$ namely the effective charge per 100 monomers, are calculated from $s_{2D}/s_R$ and the $\bar{v}_2$ values obtained from density measurements (Eqs. (7 and 15)). The values of $\phi^\prime$ are from density measurements, assuming full dissociation of 35 carboxylates per 100 monomers (Eq. (7)).
distribution of either HAPol or DAPol particles was evidenced in preliminary sedimentation experiments performed at 5 °C at 0.5 and 1 g L\(^{-1}\) (not shown) nor in SANS experiments performed at 20 °C at DAPol concentrations ranging from 1 to 5 g L\(^{-1}\) [17]. The \(s\) values characterizing the distributions, estimated in various ways, are reported in Table 5. The values of \(R\) obtained either in the one-species analysis or from the integrals of the \(c(s)\) distribution are very close (Table 5). On the other hand, they appear less robust when obtained from the details of the \(c(s)\) distribution: considering only the main species of the \(c(s)\) distributions, for instance, leads to \(R\) values of 0.20 for HAPol and 0.69 for DAPol, compared to 0.31 and 0.53–0.57, respectively, obtained from either the one-species analysis or the integrals of the \(c(s)\) distribution. We have little confidence in the details of the \(c(s)\) distribution, which were found to depend on the choice of the fitting parameters. This is in relation to the limited quality of our experimental data (low signal-to-noise ratio, incomplete sedimentation, incomplete filling of the cells). As a consequence and because of the heterogeneity of the samples and their low \(s\) values, the deconvolution of the systematic time-independent noise and the \(c(s)\) distribution is probably not very robust.

Fig. 7 and Table 5 show that the sedimentation coefficient is more than 4× smaller in D\(^2\)O than in H\(_2\)O for HAPol and 2× smaller for DAPol. The behavior of HAPol is markedly different from that of DAPol. The resulting \(R\) values are therefore very different from 1. As discussed under General dependencies of \(R\) on \(\tilde{v}_2\) in the case of a two-component system, this leads to relatively accurate values of \(\phi_R\), namely 0.868 ± 0.008 ml g\(^{-1}\) for HAPol and 0.808 ± 0.017 ml g\(^{-1}\) for DAPol, despite the rather large uncertainty in \(R\).

**Effects of the charge of A8-35 on its buoyancy properties.** For both HAPol and DAPol, the values of \(\phi_R\) and \(\tilde{v}_2\) differ significantly (Table 5), which is obviously related to the polyelectrolyte nature of the polymer. We combined the value of \(\tilde{v}_2\) from density measurements with that of \(R\) (Table 5), using for the partial specific volume of NaCl \(\tilde{v}_3 = 0.3\) ml g\(^{-1}\) from tabulated density tables, to evaluate \(\xi_3\) values (Eq. (15)), from which were derived values for the effective charges \(z\) (Eq. (7)) of 44 ± 8 for HAPol and 38 ± 16 for DAPol. These values are slightly larger than but quite close to those expected from the nominal chemical composition of the polymer (∼35 carboxylates per 100 monomers). Considering 35 dissociated carboxylates in the sedimentation experiments in H\(_2\)O and D\(^2\)O yields a value of 0.822 ± 0.008 ml g\(^{-1}\) for the \(\tilde{v}_2\) of HAPol (Eqs. (7) and (14)), which is close to that obtained by density measurements (0.809 ± 0.003 ml g\(^{-1}\), Table 5). The corresponding values for DAPol (0.769 ± 0.018 ml g\(^{-1}\) vs
0.765 ± 0.003 ml g⁻¹) are within experimental error of each other. Considering an effective charge of 35, the values for φ² derived from v₂ (Eq. (9)) are very close to those obtained from φ⁺R (Eq. (25)): for HAPol, φ⁺ = 0.866 ml g⁻¹ using v₂ and 0.879 ml g⁻¹ using φ⁺R, and for DAPol, φ⁺ = 0.820 and 0.824 ml g⁻¹, respectively. The values of φ² from density measurements are reported in Table 5. Our results are consistent with the expectation that, in this range of v₂ values, φ² and φ⁺R must be within experimental error of each other (see: How are R and v₂ related to the apparent partial specific volume φ⁺R obtained by AUC?). Thus, the differences between v₂ and φ⁺R or φ² can readily be accounted for by the dissociation of nearly all the counter-ions of the polymer.

Equilibrium sedimentation studies of HAPol using contrast density variation. The partial specific volume of HAPol has been investigated by sedimentation equilibrium experiments performed in 0.1 M NaCl solutions made in either H₂O, D₂O or D₂¹⁸O. Because of the heterogeneity of the samples, which, even though it is limited, prevents the use of global analysis procedures, and the limited quality of some of the data, principally due to the poor optical quality of our samples of D₂¹⁸O, the mean buoyant molar masses are estimated with large and in part systematic errors (up to 50%). Fig. 8 shows the buoyant molar masses as a function of solvent density. A set of experiments was performed in 1 M NaCl to determine more precisely the density match point: the buoyant molar masses are larger than those in 0.1 M NaCl, indicating that the polymer is more aggregated, but the density match points in 0.1 and 1 M NaCl are not significantly different. While the two estimates of the apparent partial specific volume, φ⁺R, obtained by sedimentation equilibrium measurements, 0.859 ± 0.006 ml g⁻¹ in 1 M NaCl and 0.866 ± 0.015 ml g⁻¹ in 0.1 M NaCl, are consistent with those determined by density contrast variation in sedimentation velocity experiments, the accuracy is of the same order of magnitude or even lower, despite the use of the rare and expensive D₂¹⁸O for expanding the range of solvent density.

Contrast match points of HAPol and DAPol in SANS experiments. In SANS experiments, the two polymers appeared as composed of two populations of species, with a major population of small particles and small amounts (a few percent in weight) of very large aggregates (R > 10 nm) [16,17]. We will focus here on the evaluation of contrast match points. Fig. 9 shows the determination of the CMP for the main species of HAPol and DAPol. Table 6 lists their values, error estimates, and interpretation with regard to v₂ and neutron scattering length density. Since the CMP of the large and the main species were identical within experimental uncertainty, a detailed analysis was carried out only on the more precise results for the main (small) species.

The CMP depends on the parameters that define the contrast between the polymer and the solvent, namely the scattering length of the polymer—b₂H in H₂O solutions—and that of the solvent, ρ₀N, the extent of H/D exchange, (ΔnH/ΔnD)₂, and the v₂ of the polymer. Estimates of v₂ therefore can be derived from CMP values, but they are affected by relatively large errors. On the basis of the composition of the polymer given

![Fig. 8. Equilibrium sedimentation of HAPol in aqueous solvents of various densities. Mean buoyant molar mass (M_b) of HAPol in the presence of either 0.1 M NaCl in H₂O or D₂¹⁸O (circles) or 1 M NaCl in H₂O, D₂O, or D₂¹⁸O (squares). Because the samples are not perfectly homogeneous (Fig. 7C) and the quality of the data is limited, due, in particular, to the poor optical quality of the D₂¹⁸O, the mean buoyant molar masses are estimated with large, in part systematic errors (500 g mol⁻¹ in NaCl 0.1 M, 1000 g mol⁻¹ in NaCl 1 M). The estimated density match points are 1.164 ± 0.008 and 1.155 ± 0.020 g ml⁻¹ in 1 and 0.1 M NaCl, respectively, which correspond to apparent partial specific volumes φ⁺R of 0.859 ± 0.006 and 0.866 ± 0.015 ml g⁻¹, respectively. These are close to the more accurate value of 0.868 ± 0.008 ml g⁻¹ obtained by the s₂H/s₁H method (Table 5).](image)

![Fig. 9. Determination of the contrast match points of the hydrogenated and deuterated forms of A8-35 by small-angle neutron scattering. Forward intensities were extrapolated from Guinier plots for the major species (see the text) in a Q² range from to 0.001 to 0.005 Å⁻² and normalized by the total concentration of polymer and geometrical parameters. The value of ±√(N₀(q/₀c₀) = (diH/dc₂D₀) - √M₂ was plotted as a function of the percentage (ν/ν₀) of D₂O in the solvent. Crosses: DAPol (from two different sets of data collected using two different batches); circles: HAPol. The sign of √(N₀(q/₀c₀) was assigned according to that of (diH/dc₂D₀). Solid lines are linear regressions, from which contrast match points were determined.](image)
in Table 1, assuming total dissociation of the carboxylates and complete exchange of the exchangeable protons, $v_2$ values of 0.73 ± 0.10 ml g$^{-1}$ for HAPol and 0.82 ± 0.11 ml g$^{-1}$ for DAPol are yielded. Given the large error margins, whose origins are detailed in Table 6, these values are compatible with those derived from the density and ultracentrifugation measurements.

Theoretical values of the CMP of each polymer can be calculated from their partial specific volume, determined from sedimentation velocity experiments and their chemical composition (Table 6). For HAPol, the agreement with experiment is quite fair (observed, 23.5 ± 1% D$_2$O; expected, 21.9 ± 0.7%). For DAPol, the discrepancy is larger, and it exceeds experimental errors (observed, 85.1 ± 2.5% D$_2$O; expected, 91.4 ± 1.6%, assuming no error on the value of $b_{2H}$ calculated from the composition). This is most likely due to a difference between the expected and the actual chemical composition of the polymer, which affects the value of $b_{2H}$. Such a discrepancy indeed was revealed by NMR spectra (see Materials and methods: Chemical analysis of amphipols), which have shown the presence of about 3% of a hydrogenated impurity. The values of $b_{2H}$ corresponding to the best fit of the CMP for HAPol and DAPol are reported in Table 6. The values of $\left(\overline{\partial \rho / \overline{\partial c_2}}\right)_o$ which define the scattering intensity of the polymer, calculated using $b_{2H}$ determined either from the fit of the experimental SANS data or from the theoretical composition of the polymer are significantly different only for DAPol in D$_2$O solvent: for this particular batch of polymer and in this solvent, the determination of the molar mass of amphipol particles from SANS data therefore would be uncertain.

### Table 6

<table>
<thead>
<tr>
<th>Contrast match point (CMP) of HAPol and DAPol in small angle neutron scattering experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAPol</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td><strong>H/D exchange in D$_2$O, being unchanged from</strong></td>
</tr>
<tr>
<td>Theoretical values of the CMP of each polymer can be calculated from their partial specific volume, determined from sedimentation velocity experiments and their chemical composition (Table 6). For HAPol, the agreement with experiment is quite fair (observed, 23.5 ± 1% D$<em>2$O; expected, 21.9 ± 0.7%). For DAPol, the discrepancy is larger, and it exceeds experimental errors (observed, 85.1 ± 2.5% D$<em>2$O; expected, 91.4 ± 1.6%, assuming no error on the value of $b</em>{2H}$ calculated from the composition). This is most likely due to a difference between the expected and the actual chemical composition of the polymer, which affects the value of $b</em>{2H}$. Such a discrepancy indeed was revealed by NMR spectra (see Materials and methods: Chemical analysis of amphipols), which have shown the presence of about 3% of a hydrogenated impurity. The values of $b_{2H}$ corresponding to the best fit of the CMP for HAPol and DAPol are reported in Table 6. The values of $\left(\overline{\partial \rho / \overline{\partial c_2}}\right)<em>o$ which define the scattering intensity of the polymer, calculated using $b</em>{2H}$ determined either from the fit of the experimental SANS data or from the theoretical composition of the polymer are significantly different only for DAPol in D$_2$O solvent: for this particular batch of polymer and in this solvent, the determination of the molar mass of amphipol particles from SANS data therefore would be uncertain.</td>
</tr>
</tbody>
</table>

### Conclusions

The determination of partial specific volumes by AUC

The theoretical analysis of the sources of uncertainties and errors, along with experimental results obtained with a set of model molecules, was aimed at clarifying the pros and cons of resorting either to sedimentation equilibrium or to sedimentation velocity measurements in H$_2$O vs D$_2$O solutions for the determination of partial specific volumes. While it is more demanding with regard to the number of parameters to be measured, the $s_{D}/s_{H}$ method presents definite advantages compared to equilibrium sedimentation experiments. Accurate knowledge of the density and viscosity is required for the analysis, but their measurement is nondestructive, requires less than 1 ml of solution, and thus can be performed on the same samples as those used for AUC. The great assets of sedimentation velocity measurements include that they reveal much more clearly whether the system under study is homogeneous, how many different species there are, and whether these species interact. Data reduction yields, for each experiment, a distribution of sedimentation coefficients. Comparison of the distribution profiles can be used to check on the condition of unchanged dispersity in H$_2$O and D$_2$O solvents. For heterogeneous samples, determination of the mean $s$ values using sedimentation velocity measurements can be much more accurate than that of the mean buoyant masses in equilibrium sedimentation measurements. Indeed, the latter yields accurate values of buoyant molar masses through a global analysis of a series of experiments and in the framework of a priori models of oligomerization or association with cofactors. Definite models are not always available, e.g., in the present case of a synthetic, heterogeneous polymer. For samples containing a finite number of species, on the other hand, the $s_{D}/s_{H}$ method can be used to separately characterize the buoyancy properties of the different components. From this point of view, the fact that the different oligomers of BSA studied here did feature the same R ratio is quite encouraging. The $s_{D}/s_{H}$ approach ought to be particu-
larly useful for the characterization of complexes comprised of chemically different species, such as protein–amphipol complexes [39].

With both methods, however, the determination of specific volumes by density contrast variation rests on the hypothesis that, in H$_2$O and D$_2$O solutions, the particle has the same composition and, with the sedimentation velocity ($s_{20,w}$) method, the same shape (same hydrodynamic radius). H/D exchange enters into the analysis and uncertainties in its extent increase the margin of error of partial specific volume determinations. Counter-ion dissociation has also to be considered. As a result of the limited range of density achievable by H$_2$O/D$_2$O substitution, the accuracy of the determination of partial specific volumes critically depends on the density of the particle. As summarized in Table 4, for heparin, which is very dense, the method is not accurate (error margin of $\sim 0.11$ ml g$^{-1}$). The accuracy is better for proteins ($\sim 0.05$ ml g$^{-1}$). An excellent accuracy (0.01–0.02 ml g$^{-1}$), on the other hand, can be expected for low-density amphiphilic polyelectrolytes such as amphipols.

Operational parameters for the analysis of the solution behavior of amphipol and membrane protein/amphipol particles

From a practical point of view, the results from this study will be used to derive the molar masses of HAPol and DAPol in solution alone or as a complex with a partner, such as a membrane protein. Using different techniques, we obtain a consistent description of the buoyancy properties of A8-35, in both its hydrogenated (HAPol) and its partially deuterated (DAPol) forms. With the solvent used here (0.1 M NaCl, H$_2$O buffered at pH close to 7), the values of $\bar{v}_2$ (0.809/0.769 ml g$^{-1}$ for HAPol/DAPol) and charge ($z = 35$ per 100 monomers) and the related values of $\phi'$ in sedimentation (Table 5), and of $b_2$ and ($\bar{c}p_{\phi}/\bar{c}c_2$)$_m$ in small-angle neutron scattering (fitted values from Table 6) can be used to derive the molar masses of A8-35 particles [17]. In contrast variation experiments, the match values determined here can be used to mask the contribution of the polymer: in sedimentation experiments, the density $\rho^0 = 1.152$ (for HAPol) and $\rho^0 = 1.238$ g ml$^{-1}$ (for DAPol) derived from $\phi'_m$ (Table 5); in SANS experiments, the CMP values of 23.5 and 85.1% D$_2$O (Table 6). These data have been applied to the study of the composition and organization of bacteriorhodopsin/A8-35 complexes [17,39].

These parameters will depend on experimental conditions (pH, ionic strength, temperature, etc.). The amplitude of change in buoyancy or density contrast can be estimated from the expected variation of parameters $\bar{v}_2$ and $\xi_3$. It has been found that temperature modifies marginally $\bar{v}_2$ (typical shifts for DNA and sodium polyacrylates of $3–6 \times 10^{-4}$ ml g$^{-1}$ K$^{-1}$ have been reported [40]). $\bar{v}_2$ increases when the solvent salt concentration increases, because ion pairs are formed (with a related decrease of $z$) and because water electrostriction due to the charges of the polymer is less pronounced at high salt, as described for DNA (compilations of $\bar{v}_2$ and $\phi'$ are found in [13,40,41]) and for the very acidic halophilic malate dehydrogenase [20]. The maximum effect of electrostriction is 10 and 15 Å$^3$ per carboxylate and per Na$^+$ [20]. For Na$^+$:HAPol, it corresponds to a maximum theoretical increase of $\bar{v}_2$ by 0.06 ml g$^{-1}$. For the hydration, which cannot be neglected when the solvent density is significantly different from that of water, one could expect values of the order of or larger than $B_1 \approx 0.3$ g g$^{-1}$, which are typical of proteins. For salt binding, $B_3 = 0$ g g$^{-1}$ is reasonable. For example, in 1 M NaCl ($\rho^0 = 1.037$ g ml$^{-1}$, $\bar{v}_2 = 0.32$ ml g$^{-1}$, $w_2/w_1 = 0.06$), as compared to 0.1 M NaCl conditions, $\phi'$ of HAPol and DAPol would decrease by 0.043 ml g$^{-1}$ if we consider $B_1 = 1.0$ g g$^{-1}$. The precision with which the molar mass of A8-35 in 1 M NaCl solution can be estimated from $M_1$ would be ca. ±50% in the absence of any experimental information about the hydration of the polymer. Obviously, the experimental $s_0$$w$/H$ method would be recommended to determine molar masses in a rapid and more accurate manner in solvent conditions that differ from that of the present study.

Acknowledgments

Particular thanks are due to F. Giusti (UMR 7099) for his precious help in sorting out the origin of the contaminant bound to DAPol and to D. Charvolin (UMR 7099) and D.M. Engelman (Yale University) for their participation in SANS experiments and in numerous discussions. This work was supported by the CNRS, Université Paris-7, UIF, CEA, CNRS Grant GEOFLEX, EEC Grant Nos. BIO4-CT98-0269, HPRI-CT-2001-50035, and HFSPO Grant No. RG00223/2000-M.

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