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Evidence against molecular aggregates in concentrated solutions of X-ray contrast media

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Abstract

In order to evaluate the possible existence of large molecular aggregates in concentrated solutions of X-ray contrast media used for medical imaging, a comprehensive study of the effect of the contrast medium concentration on the osmolarity, light scattering and molecular diffusion has been performed for the two X-ray contrast media, Omnipaque (iohexol) and Visipaque (iodixanol). The measured change in osmolarity and light scattering with increasing concentration of contrast medium was well explained by solvent–solute and solute–solute interactions. Minima were observed for both the concentration-normalized osmolarity and the inverse of the concentration-normalized Rayleigh ratio, observations which are incompatible with the hypothesis of aggregation at higher concentrations. From the observed minima, dynamic clusters containing at maximum two to three molecules were demonstrated. The apparent molecular diameter measured with dynamic light scattering, was found to be well predicted from the thermodynamic expression of the concentration dependency of the mutual diffusion coefficient. The observed scattering intensity was incompatible with an actual increase in the geometric size of the scatterers (i.e. aggregation) with increasing concentration. Contrary to the conclusions made by other investigators, the results presented clearly demonstrate the absence of 'large colloidal-like structures or molecular aggregates' in the X-ray contrast media investigated.

Keywords: X-ray contrast agents; Aggregates; Omnipaque; Visipaque; Iodixanol; Iohexol; Osmolality; Light scattering; Virial coefficients

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1. Introduction

The notion of 'molecular aggregates' in X-ray contrast media presumably stems from their lower than ideal osmolarity at high concentrations (Pietre and Felder, 1980; Skinnemoen, 1987; Eivind-

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vik and Sjøgren, 1995). Recently, results from dynamic light scattering measurements have been taken as further experimental evidence for the existence of 'large colloidal like structures or molecular aggregates' in these concentrated solutions (Schneider, 1996) and it has been speculated that such aggregates could be the cause of the hypersensitivity reactions sometimes observed with these agents (Speck et al., 1997; Lasser and Lamkin, 1997).

The topic of self-association of drug molecules in aqueous solutions is treated extensively by Attwood and Florence (1983), who review results and mechanisms of association for a number of different drug classes. More recently, Zhu and Streng (1996) and Streng et al. (1996) have studied the phenomenon on charged, heterocyclic compounds, mostly by applying calorimetry and conductivity measurements. To the authors knowledge, however, a comprehensive study of the fundamental physicochemical mechanisms behind the observed reduction in osmolarity and increase in apparent molecular size at high concentrations of X-ray contrast media, has not been performed. In the current study we have utilized static and dynamic light scattering techniques for the physicochemical characterization of the X-ray contrast media Omnipaque (iohexol) and Visipaque (iodixanol). We have also investigated the osmolarity of these compounds as a function of concentration by vapor pressure osmometry. The data obtained from these measurements have been evaluated in the context of nonideal thermodynamic expressions which take into consideration the effect of molecular interactions at finite concentrations of solute.

2. Materials and methods

2.1. Materials

The X-ray contrast media used were Omnipaque (300 mg I/ml, equivalent to 0.65 g iohexol/ml) (Haavaldsen, 1980) and Visipaque (320 mg I/ml, equivalent to 0.65 g iodixanol/ml) (Priebe et al., 1995). Samples of lower concentration were made by dilution of these solutions with water. Iodixanol samples of higher concentration were made by evaporation by placing uncapped samples in a heating cabinet at 40°C for up to 48 h.

2.2. Analytical methods

Measurements of osmolality at 37°C were performed using a vapor pressure Osmometer WESCOR 5500 (Wescor Inc., Logan, UT). The instrument was calibrated with 100, 290, and 1000 mmol/kg osmolality standards (Wescor Inc.). Ten μl of tested solution were pipetted onto a paper disc in the instrument sample holder and the sample was conveyed into the instrument sample chamber. For the three highest concentrations of iodixanol solutions investigated the paper disc was fully immersed in sample for 3 min prior to analysis in order to wet the paper completely. The osmolarity (mosm/L) was calculated from the measured osmolality by multiplying with the water content in each sample. Triplicate analyses were performed on each sample preparation.

The content of iodixanol in the high concentration solutions prepared by evaporation was determined from density measurements on an Anton Paar DMA 58 densitometer (Anton Paar, Graz, Austria) and published data on the partial specific volume of iodixanol (Eivindvik and Sjøgren, 1995).

Static and dynamic light scattering experiments were performed on a Malvern 4700 MW/PS (Malvern Instruments Ltd., Malvern, UK) set up with a 7032 Multi 8, 128-channel correlator and equipped with a 70-mW argon ion laser (Cyonics Corp., San Jose, CA, USA) with a wavelength of 488 nm. Samples were filtered through a 0.02-μm Anotop 25 Plus single-use filter (Whatman Lab. Div., Maidstone, UK) immediately before analysis. Experimental conditions were: photomultiplier aperture opening of 200 μm, scattering angle of 90° and temperature of 27°C. Solvent refractive index was set to 1.330, solvent viscosity to 0.85 cP, particle real refractive index to 1.5 and particle imaginary refractive index to 0. From published data on iodixanol (Eivindvik and Sjøgren, 1995), the refractive index increment of iodixanol was calculated to be 0.167 ml/g and the partial specific volume to be 0.55 ml/g. These values were also used for iohexol. Toluene p.a. (E. Merck, Darmstad, Germany) was used as standard for the static light scattering experiments and the Rayleigh ratio of this standard was set to 19×10^{-6} cm^{-1} (Malvern Instruments Ltd., 1993). During the dynamic light scattering experiments, the correlator and experiment duration was set up automatically by the instrument software ('PCS for Windows', version 1.32). As response from the dynamic light scattering analysis, the intensity weighted apparent average molecular diameter calculated with the distribution shape independent algorithm ('multimodal'), was used. One to three replicates of each sample preparation were analyzed.

Light scattering experiments are extremely prone to dust contamination and consequently filtering is the standard procedure during sample preparation. In order to verify that the applied filtering procedure had no effect on the results, an unfiltered sample of 0.65 g/ml iodixanol sample was analyzed as a control. This sample was also used to confirm the absence of scattering angle dependency in both the observed Rayleigh ratio and the apparent molecular diameter, by analysing the sample at eight different angles from 30 to 120°.

2.3. Data analysis

Data on osmolality for iohexol and iodixanol reported by Skinnemoen (1987) and Eivindvik and Sjøgren (1995), respectively, were included in the analysis. Polynomial best fit regressions of results to various theoretical expressions were performed with StatGraphics Plus 3.0 software (Manugistics Inc., Rockville, MD). Intercepts and coefficients from these regressions are stated along with standard error estimates.

3. Theoretical considerations

3.1. Osmotic pressure

At very low solute concentrations, the osmotic pressure (Π) may be expressed by the van't Hoff equation:

$$\Pi = RTc/M \tag{1}$$

where R is the gas constant, T is the temperature, c is the solute concentration and M is the solute molecular weight. Eq. (1) is valid only in the absence of intermolecular interactions, i.e. at infinite dilution of the solute. At finite concentrations in real systems, the osmotic pressure may be expressed as:

$$\Pi = RTc(1/M + A_2c + A_3c^2 + \ldots) \tag{2}$$

where A_2 and A_3 are termed the second and third virial coefficients, respectively (Yamakawa, 1971; Atkins, 1982; Kamide, 1989). The virial coefficients are parameters describing the thermodynamic interactions between the components in the system (i.e. solvent–solute and solute–solute interactions). A_2 describes two-body (pair) interactions, whereas A_3 describes three-body interactions. This form of virial expansion to express concentration dependent deviations from ideal values, is formally analogous to the frequently used expansion of the ideal gas law to describe excluded volume effects and van der Waals interactions in real gases. The concentration normalized (Π/c) version of Eq. (2) has been used extensively for the determination of polymer molecular weight by measuring osmotic pressure at different polymer concentrations and fitting the results to the expression:

$$\Pi/c = RT(1/M + A_2c + A_3c^2 + \ldots) \tag{3}$$

In the special case of a rigid sphere molecule, it has been shown that the third virial coefficient may be expressed in terms of A_2 as (Yamakawa, 1971).

$$A_3 = 5{A_2}^2M/8 \tag{4}$$

3.2. Static light scattering

The time average scattering intensity from a solution containing solute molecules which are much smaller than the wavelength of the incident light, may be expressed through the Rayleigh ratio (ΔR) as:

$$\Delta R = I_sr^2/I_0 = KRTc/(\delta\Pi/\delta c) \tag{5}$$

where I_s is the observed scattering intensity at a distance r from the scattering volume and I_0 is the intensity of the incident light. K is an optical constant dependent on the refractive index of the solvent, the refractive index increment of the solute–solvent system, the wavelength of the incident light and the scattering angle (Katime and Quintana, 1989). Expressing the partial derivate of the osmotic pressure as a power series, by derivation of Eq. (2), and inverting yields

$$Kc/\Delta R = 1/M + 2A_2c + 3A_3c^2 + \ldots \qquad (6)$$

A static light scattering experiment consists of determining the Rayleigh ratio of a solution as a function of solute concentration. Fitting the measured $Kc/\Delta R$ versus c to the power expansion in Eq. (6) yields the weight average solute molecular weight and the virial coefficients for molecular interactions, A_2 and A_3 .

3.3. Dynamic light scattering

Whereas the static light scattering experiment determines the time average scattering from a solution, the dynamic light scattering technique utilizes the time-dependent properties of the scattering signal. The measured quantity is the intensity time auto-correlation function which in turn is used for the calculation of the mutual diffusion coefficient (D_m) of the solute molecules. At infinite dilution the measured diffusion coefficient equals the translation diffusion coefficient (D_t) of the individual molecules and the Stokes–Einstein relation may be used to calculate the spherical equivalent hydrodynamic radius of the solute (R) as

$$R = kT/6\pi\eta D_t \qquad (7)$$

where η is the viscosity of the solution. At finite concentrations the measured diffusion coefficient reflects the mutual or cooperative movement of interacting molecules and the concentration dependency of D_m may be expressed by:

$$D_m = kT/f(1 - vc)(1 + 2MA_2c + 3MA_2c^2 \ldots) \qquad (8)$$

where f is a hydrodynamic friction factor and v is the partial specific volume of the solute (Nystrøm and Roots, 1980; King, 1989). At concentrations where the individual molecules are well separated in the solvent matrix, the friction factor is dominated by solvent–solute friction and for a sphere f is equal to $f_0 = 6\pi\eta R$.

4. Results and discussion

The results from the measurements of osmolarity versus concentration for the two contrast agents iohexol and iodixanol, are shown in Fig. 1. The dotted lines represent ideal values obtained when calculating the osmolarity as a function of concentration, not taking into consideration the effect of molecular interactions at finite concentrations of solute (Eq. (1)). The solid lines are the best fits of the experimental data to the cubic expression in Eq. (2). The results in Fig. 1 confirm the earlier reported deviation from theoretical osmolarities at high concentrations of contrast agent and the relatively larger deviation observed for iodixanol compared with iohexol (Pietre and Felder, 1980; Skinnemoen, 1987; Eivindvik and Sjøgren, 1995). As observed from Fig. 1, the theoretical expression in Eq. (2) which takes into consideration solute–solvent and solute–solute interactions, fits very well with the experimental data.

Fig. 2 shows the concentration normalized osmolarity (Π/c) versus concentration, with the solid lines representing the best fits to the quadratic expression in Eq. (3). According to this equation, the zero intercept of these lines should be equal to $1/M$ and the constants in the calculated best fits should equal the virial coefficients, A_2 and A_3. The estimated molecular weights were 790 ± 30 for iohexol and 1230 ± 80 for iodixanol which are close to actual molecular weights of 821 and 1550, respectively. The estimated virial coefficients (stated in legend to Fig. 2) were significantly different from zero at the 95% confidence level.

The mechanistic significance of the observed negative value for the second virial coefficients (A_2) is simply that two-bodied solute–solute contacts are thermodynamically favored over solvent–solute contacts. At equilibrium conditions, then, there exists a net attractive force between

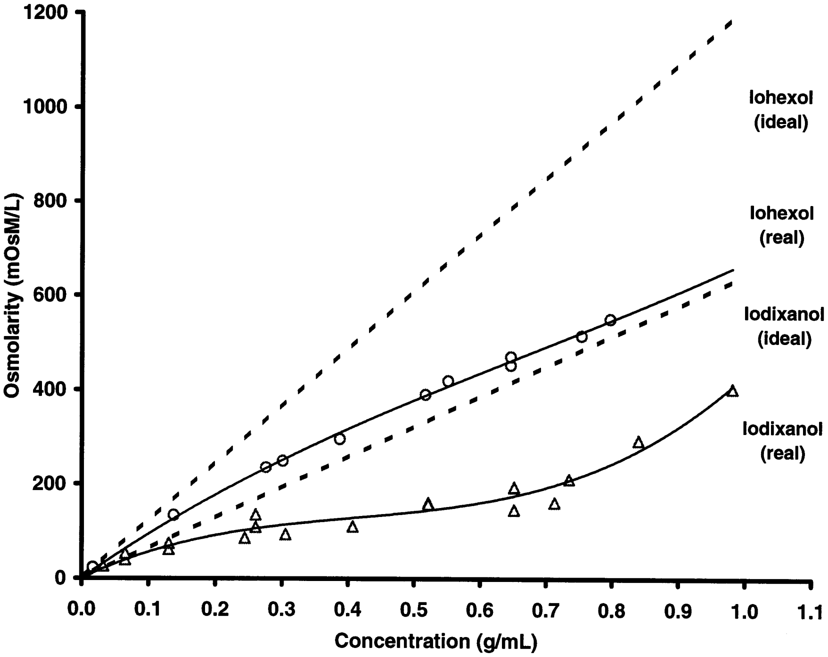

Fig. 1. Osmolarity versus concentration for aqueous solutions of iohexol (○) and iodixanol (△), respectively. Dotted lines are ideal theoretical values calculated from Eq. (1). Solid lines are best fits of the data to the third order polynomial in Eq. (2).

pairs of solute molecules in the system. The mechanistic significance of the third virial coefficient (A_3) is more complex, but formally it describes the thermodynamic interactions in a three-body, solute–solute–solute contact. At low and intermediate concentrations, the net attractive force between pairs of solute molecules is dominant. This force will tend hold the molecules together and causes the osmotic activity (Π/c) to deviate from its ideal value. The attractive intermolecular force will increase the local fluctuations in solute concentration, caused by random Brownian motion and which are present in all solutions, both in magnitude and life time. It is important to notice, however, that this must be a dynamic equilibrium effect, creating short-lived clusters of solute molecules, which are rapidly relaxed by thermal motion and the ensuing differences in chemical potential with neighboring segments. The interaction cannot be strong enough to cause permanent aggregation of molecules, as this would lead to a thermodynamically unstable system and, ultimately, to phase separation. In Fig. 3 is visualized the molecular clustering of closely packed iodixanol molecules at a concentration of 0.65 g/ml. As can be seen from Fig. 2, the osmotic activity falls off from approximately its ideal value at infinite dilution and reaches a broad minimum between 0.4 and 0.8 g/ml for both contrast media. Such behavior can be explained by the increasing importance of a positive third virial coefficient. With increasing concentration the number of three-body contacts become more and more frequent and the third virial coefficient (A_3) becomes a more dominant factor. At a certain point, the two terms balance each other and above this concentration the osmolar activity will increase again with still increasing concentration. As shown in Fig. 2, this effect is indicated for both contrast media. It should be noted that a minimum in osmotic activity is not an uncommon phenomenon and occurs in widely different systems such as aqueous solutions of sodium chloride, lactose and also with the ionic X-ray contrast agent Hypaque (Weast, 1983). From the minimum osmotic activity observed in both systems, the average molecular cluster contains 1.8 and 2.9 molecules for the iohexol and iodixanol system,

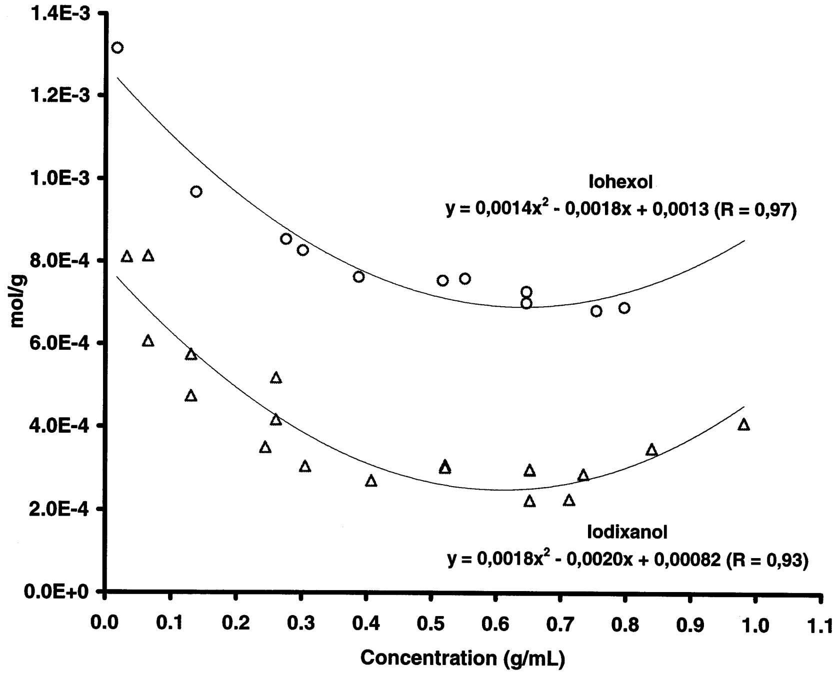

Fig. 2. Concentration-normalized osmolarity versus concentration for aqueous solutions of iohexol (○) and iodixanol (△), respectively. Solid lines and stated equations are best fits of data to Eq. (3). Second virial coefficients (A_2) are estimated to -0.0018 ± 0.0003 and -0.0020 ± 0.0004 mol ml/g^2 and third virial coefficients (A_3) are estimated to 0.0014 ± 0.0003 and 0.0018 ± 0.0005 mol ml^2/g^3, for iohexol and iodixanol, respectively.

respectively. The osmolarity measurements presented clearly demonstrate the absence of increasing molecular aggregation in the contrast medium solutions investigated, as such aggregation would have given a continuous decrease in osmotic activity with increasing concentration, and not a plateau or characteristic minimum as observed.

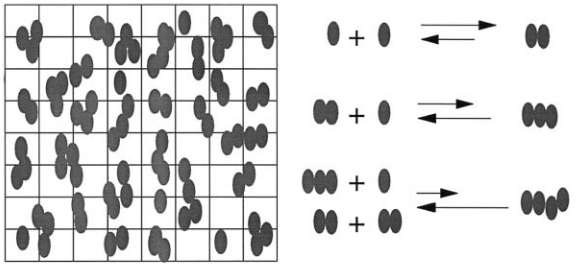

Fig. 3. A visualisation of the molecule density in a 0.65 g/ml solution of iodixanol and of the dynamic equilibrium between individual molecules and molecule clusters. The large square represents a 2-nm deep, 16 × 16-nm section of the solution (the small squares are 2 × 2 nm) and each elliptic body represents a single iodixanol molecule.

The results from the static light scattering experiments showed that the observed Rayleigh ratio was independent of scattering angle and that the plots of $Kc/\Delta R$ versus concentration could be excellently described by the theoretical expression in Eq. (6) (Fig. 4). The static light scattering results are consistent with the results from the osmolarity measurements, although more precise. The estimated molecular weights obtained from the reciprocal of the calculated intercepts were 760 ± 10 and 1480 ± 25 for iohexol and iodixanol, respectively, which are in good agreement with their actual molecular weights. The estimates for the virial coefficients A_2 and A_3 (stated in the legend to Fig. 4) were smaller, but within the 95% confidence intervals of the results from osmometry and all coefficients were significantly different from zero at the 99% confidence level. It should also be noted that the observed values for A_2 and A_3 are in excellent agreement with the relation between the virial coefficients of rigid sphere molecules in solution as stated in Eq. (4). The

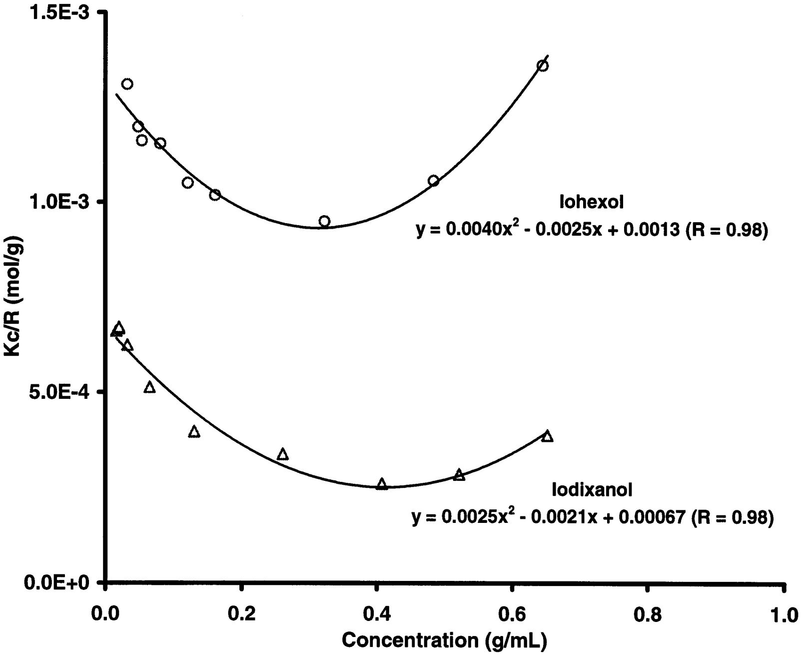

Fig. 4. Inverse of concentration-normalized Rayleigh ratio ($Kc/\Delta R$) versus concentration for aqueous solutions of iohexol (○) and iodixanol (△), respectively. Solid lines and stated equations are best fits of data to Eq. (6). Second virial coefficients (A_2) are estimated to -0.0012 ± 0.0001 and -0.0010 ± 0.0001 mol ml/g^2 and third virial coefficients (A_3) are estimated to 0.0013 ± 0.0001 and 0.0008 ± 0.0001 mol ml^2/g^3, for iohexol and iodixanol, respectively.

clear minima shown in Fig. 4, demonstrate a minimum in osmotic activity (Π/c) (Eq. (5)) at intermediate concentrations, in accordance with the results from the osmolarity measurements. Any aggregation of the contrast medium molecules with increasing concentration would have caused an increasingly negative slope for $Kc/\Delta R$. Again, the minima observed for both iohexol and iodixanol, demonstrate the absence of such aggregation. According to Eq. (6), $Kc/\Delta R$ is proportional to the reciprocal of the molecular weight of the scatterers in the system. From the maximum ΔR values observed, the average molecular cluster contains 1.3 and 2.5 molecules for iohexol and iodixanol, respectively. The consistency between these values and those estimated from osmometry, in fact, demonstrate the absence of a broad distribution of cluster sizes. Whereas the estimate from osmometry is the number weighted average from the cluster size distribution, the estimate from light scattering is volume weighted. The existence of a broad or perhaps bimodal distribution of cluster sizes should lead to higher averages from the light scattering technique than from the osmometry measurements. The fact that the figures from light scattering are slightly smaller, thus demonstrate a narrow cluster size distribution and the absence of higher-order clusters.

The results from the dynamic light scattering experiments are shown in Fig. 5, where the apparent hydrodynamic molecular diameter, calculated from the measured diffusion coefficient using Eq. (7), is plotted against the concentration of iohexol and iodixanol, respectively. The results were independent of scattering angle and sample filtration. The measured correlation function for all samples was well described by a monoexponential decay and no signs of multimodal size distributions were observed. As can be seen from Fig. 5, the hydrodynamic diameter of the individual molecules (extrapolated to zero concentration) was found to approximately 1.1 and 1.4 nm for iohexol and iodixanol, respectively. These values are in good

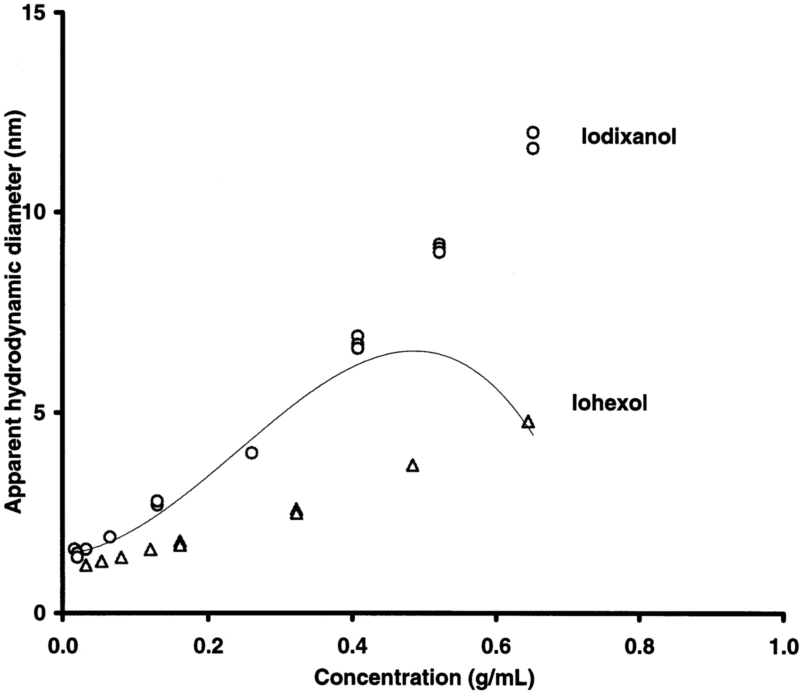

Fig. 5. Apparent hydrodynamic diameter versus concentration for iohexol (○) and iodixanol (△), respectively. The solid line is estimated values for iodixanol based on Eq. (8).

agreement with the dimensions of the molecules obtained from simple molecular modelling. Fully extended, the iohexol molecule fits into a box of approximately 1.0 × 1.2 × 1.2 nm while the iodixanol molecule may be contained in a space of approximately 1.0 × 1.2 × 2.0 nm.

The experimental data shown in Fig. 5 are in good agreement with the observations reported by Schneider (1996). However, the forcing of the diameter versus concentration lines through the origin as performed by this investigator, has no meaning as the results at zero concentration reflect the dimensions of the individual molecules. Also, the evaluation of the observed increase in apparent molecular dimensions with increasing concentration, as evidence for the existence of 'colloidal like structures or molecular aggregates containing several hundred molecules', lacks a theoretical fundamental. The investigator has not taken into consideration the fact that the mutual diffusion coefficient is dependent on concentration as stated in Eq. (8). Unfortunately, the erroneous conclusion from this published light scattering study has resulted in speculations as to a central role of these aggregates in delayed hypersensitivity reactions (Speck et al., 1997; Lasser and Lamkin, 1997).

The solid line in Fig. 5 shows the theoretical change in hydrodynamic diameter for the iodixanol molecule with increasing concentration, using Eq. (8). In the calculation the virial coefficients found from the static light scattering experiment were used and it was assumed that the friction factor f is independent of concentration and equal to f_0. As can be seen from this line, the theoretical expression in Eq. (8) fits well with the experimental results for iodixanol up to a concentration of approximately 0.4 g/ml, with an increase in the apparent molecular dimension of more than four-fold. At 0.4 g/ml, each individual iodixanol molecule is confined to a cube with sides 1.9 nm, if homogeneously dispersed. Clearly, under such conditions solute–solute friction or molecular entanglements will become increasingly important and a constant value (f_0) will no longer apply. Instead solute–solute friction will cause a

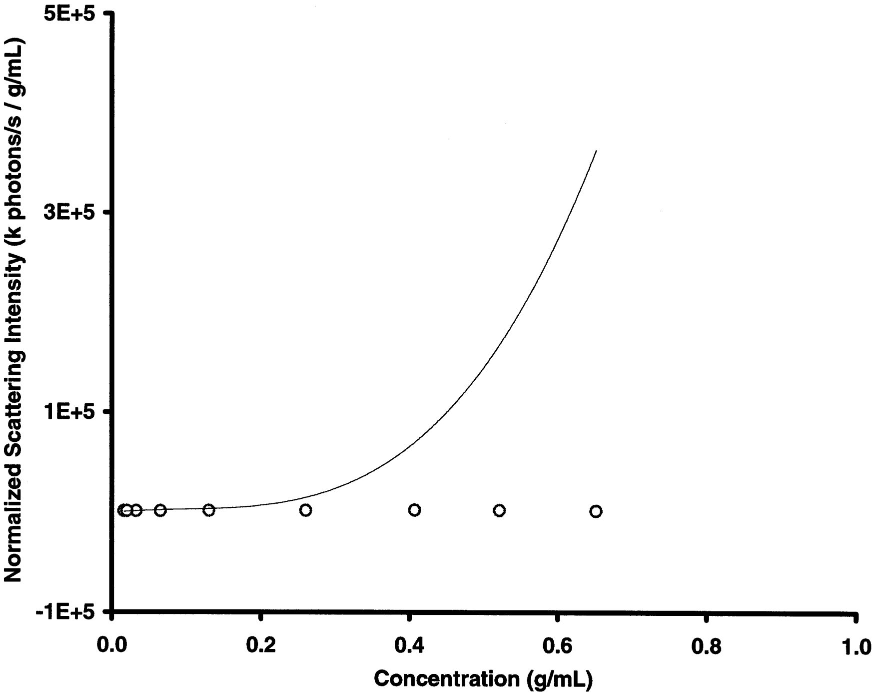

Fig. 6. Concentration-normalized scattering intensity versus concentration for aqueous solutions of iodixanol (○). The solid line represents values calculated from apparent hydrodynamic diameter and assuming that aggregation occurs, according to Eq. (9).

sharp increase in *f* with a resultant decrease in the mutual diffusion coefficient and a continuing increase in apparent molecular diameter with increasing concentration.

The combination of Eq. (5) and Eq. (6) shows that the scattering intensity per unit concentration (I_s/c) is proportional to the molecular weight of the scatterers. As shown earlier (Sontum and Christiansen, 1997), this dependency makes light scattering a very sensitive technique for characterization of aggregation processes. If aggregation occurs, the concentration-normalized scattering intensity should increase strongly with the concentration of contrast medium. Assuming that the molecular weight is proportional to the diameter to the third power, as is the case for a solid sphere, I_s/c should also be proportional to the apparent diameter raised to the third power as according to:

$$(I_s/c)_c = (I_s/c)_{c=0} \times (D_c/D_{c=0})^3 \tag{9}$$

where D_c is the apparent diameter at concentration c. The solid line in Fig. 6 represents the I_s/c values to be expected if the measured increase in the apparent diameter of iodixanol was due to an actual aggregation process. Relative to the expected values, the observed concentration-normalized scattering intensity for iodixanol is practically constant over the whole concentration range studied (Fig. 6). These data clearly demonstrate that the observed increase in the apparent molecular dimension with increasing concentration does not reflect ‘aggregates or colloidal like structures’ present in the solution, but simply the change in the mutual diffusive motion of the iodixanol molecules.

5. Conclusion

We have investigated the effect of the concentration of the two X-ray contrast agents, iohexol and iodixanol, on the osmolarity, light scattering and molecular mutual diffusion coefficient of the solutions. The experimental results were found to be well described by nonideal thermodynamic expressions taking into consideration the effect of molecular interactions at finite concentrations of solute. The concentration dependency of the osmolarity and light scattering was well explained by solvent–solute and solute–solute interaction parameters. Minima for both the concentration-normalized osmolarity (Π/c) and the inverse of the concentration-normalized Rayleigh ratio ($c/\Delta R$) were observed; findings which are incompatible with aggregate formation at higher concentrations. From the observed minima, clusters or associates containing at maximum two to three molecules were demonstrated. The apparent molecular diameter observed with dynamic light scattering was found to be well predicted by the thermodynamic expressions for the expected concentration dependency of the mutual diffusion coefficient. The observed concentration-normalized scattering intensity was incompatible with an actual increase in the geometric size of the scatterers due to aggregation. Contrary to conclusions made by other investigators who have not considered the effect of concentration on the parameters measured, we find that the results presented demonstrate the absence of 'colloidal like structures or molecular aggregates' in the X-ray contrast media investigated,

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