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### Liquid-Liquid Phase Separations in Aqueous Solutions of Globular Proteins

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**Liquid-Liquid Phase Separations in Aqueous  
Solutions of Globular Proteins**

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LIQUID-LIQUID PHASE SEPARATIONS IN AQUEOUS  
SOLUTIONS OF GLOBULAR PROTEINS

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A simple statistical-mechanical theory, known as the random-phase approximation, is applied to study liquid-liquid phase separations in solutions of globular proteins. Phase-separation may be induced by addition of non-ionic polymer or/and ordinary electrolytes. In this analysis, the osmotic-attraction mechanism whereby the depletion of "solvent" particles between two proteins causes an attractive force, is primarily responsible for phase-separation. For one-component models of protein solutions, the theory yields simple algebraic expressions for the equation of state and for the chemical potential of the protein. This analytical theory describes the observed solubility behavior of proteins, including the effect of protein and polymer size, protein charge and concentration, and concentration of simple electrolytes.

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## 1. Introduction

Several experimental studies (Vrij, 1976; de Hek and Vrij, 1981; Vincent et al., 1980; Sperry et al., 1981; Atha and Ingham, 1981; Haire et al., 1984; Forciniti et al., 1991) and several theoretical studies (Asakura and Oosawa, 1954 and 1958; Vrij, 1976; Joanny et al. 1979; Middaugh et al., 1979; de Hek and Vrij, 1981; Gast et al., 1983; Mahadevan and Hall, 1990 and 1992; Lekkerkerker, 1990) have described phase separation of colloids and globular proteins by addition of a non-adsorbing polymer (e.g. polyethylene glycol) to the aqueous solution; here non-adsorbing means that there are no attractive forces between polymer and protein. These studies are motivated by an increasing demand for pure proteins in pharmaceutical and related industries (Bjurstrom, 1985). In this context, charge-stabilized colloids, having electrochemical properties similar to those of aqueous globular proteins, play an important role. Interactions in colloidal dispersion are less complex than those in protein solutions; therefore, colloids may serve as model substances to test theoretical predictions for aqueous solutions of globular proteins. The one-component model applied in this work, frequently used to describe interactions in colloidal dispersions (Verwey and Overbeek, 1948; Asakura and Oosawa, 1958; Ottewill and Richardson, 1982; Gast et al. 1983; Grimson, 1983; Vincent and Hansen, 1984), may be useful for modeling aqueous solutions

of globular proteins (Mahadevan and Hall, 1990 and 1992; Vlachy and Prausnitz, 1992).

In an aqueous polymer solution, experimental observations indicate that the solubilities of proteins depend on their size: larger proteins precipitate at lower concentration of polyethylene glycol (PEG). Further, protein solubilities depend on the size of the polymer; the solubility of proteins decreases with rising molecular weight of PEG. The electrochemical properties of the solution are also important: protein solubility falls as solution pH approaches the protein's isoelectric point. Protein solubility also falls with the solution's rising ionic strength.

In their seminal work, Asakura and Oosawa (1954,1958) have suggested that phase separation is caused by the depletion of polymer particles in the region between two colloids. The osmotic pressure exerted by the polymer molecules in the space between two colloidal particles is smaller than its bulk value; the result is a net osmotic attraction between the colloidal particles. This idea has been explored in several theoretical papers (Vrij, 1976; de Hek and Vrij, 1981; Gast et al., 1983; Mahadevan and Hall, 1990 and 1992; Lekkerkerker, 1990); comparison with experimental data suggests that "osmotic" attraction is indeed the primary mechanism for phase separation in these systems.

Recent theoretical studies of polymer-induced phase separations in aqueous and nonaqueous colloidal suspensions (Gast et al., 1983), combine the osmotic attraction model and the perturbation theory of Barker and Henderson (1967). The theory predicts existence of a very dense (solid-like) flocculated phase. However, some experimental data (de Hek and Vrij, 1981) suggest liquid-liquid phase separation rather than flocculation. Nevertheless, the perturbation theory correctly predicts general trends observed in experimental studies.

Important theoretical and experimental studies of protein precipitation by nonionic polymer have been presented by Hall and coworkers (Mahadevan and Hall, 1990 and 1992; Forciniti et al., 1991). The effective protein-protein interaction due to the presence of polymer is related to the osmotic-attraction model of Asakura and Oosawa (1954,1958). Again, the perturbation theory of Barker and Henderson (1967) is used to calculate the solubility curves for varying protein-polymer diameter ratio. Mahadevan and Hall (1990) have also been able to incorporate the effect of parameters such as pH and ionic strength in the theory. Recently the theory is extended to a mixture of two proteins in aqueous solution (Mahadevan and Hall, 1992). The theoretical predictions are in accord with experimental observations. The only disadvantage of the perturbation theory (Gast et al., 1983; Mahadevan and Hall, 1990 and 1992) is that it requires

substantial numerical work, which makes an extension to the multicomponent cases, e.g. study of protein fractionation, very difficult. Equally (numerically) demanding seems to be an extension of the model to regard the polymer molecules as separate species in the protein-polymer mixture.

In this paper we apply another statistical-mechanical theory, the random-phase approximation (RPA) (Evans and Sluckin, 1981; Grimson, 1983; Victor and Hansen, 1984), to study protein (or colloid) phase separation induced by addition of non-adsorbing polymer or simple electrolyte. An important attractive feature of RPA is its simplicity; the theory yields algebraic expressions for the osmotic pressure and chemical potential of the protein in the mixture. The study presented here is based on a one-component model, where the major contributions to the effective pair potential between two protein molecules are osmotic attraction (Asakura and Oosawa, 1954), electrostatic repulsion and dispersion interactions (Verwey and Overbeek, 1948). The theory predicts phase separation similar to vapor-liquid transition in simple liquids. The concentrations of protein in the coexistent dilute and dense phases have been calculated to obtain the protein partition coefficient as a function of protein size and concentration, pH of the solution (net charge on the protein) and electrolyte concentration.

The paper is organized as follows. After this introduction (Section 1), we discuss effective interactions in the one-component model of the protein-polymer-electrolyte mixture (Section 2). Sections 3 and 4 introduce the necessary statistical-mechanical framework and the random-phase approximation; the equations for the osmotic pressure and chemical potential are also derived. Section 5 describes phase separations in protein-polymer and protein-polymer-electrolyte mixtures. Section 6 discusses the influence of the osmotic attraction force on the phase separation in protein solutions induced by addition of a simple electrolyte. Conclusions are summarized in the final section.

## 2. Model interactions in protein solutions

Aqueous solutions of colloids or globular proteins are multicomponent systems which are too complicated for a complete description on the molecular level. However, many experimental properties of colloidal dispersions and aqueous solutions of globular proteins can be explained using a simple one-component model wherein a pseudo-solvent (electrolyte, water and polymer) modifies interactions between the protein molecules (Verwey and Overbeek, 1948, Asakura and Oosawa, 1954; de Hek and Vrij, 1981;

Gast et al. 1983; Mahadevan and Hall, 1990 and 1992; Vlachy and Prausnitz, 1992).

According to this model, the mixture is described as a fluid of single-component macroparticles with diameter  $d_2$  interacting via the potential  $u(r)$  (Gast et al. 1983; Mahadevan and Hall, 1990 and 1992):

$$u(r) = u_R(r) + u_A(r) + u_{OA}(r) , \quad (1)$$

where  $u_R(r)$  is the repulsive interaction,  $u_A(r)$  is the attractive (dispersion) potential and  $u_{OA}(r)$  is the potential due to osmotic attraction derived by Asakura and Oosawa (1954,1958). The screened Coulomb repulsion is given by

$$\begin{aligned} u_R(r) &= \beta^{-1} A / r \exp(-\kappa r), & r > d_2, \\ u_R(r) &= \infty, & r < d_2, \end{aligned} \quad (2)$$

$$A = z_2^2 L_B \exp(\kappa d_2) / (1 + \kappa d_2 / 2)^2$$

$$L_B = \beta e^2 / (4\pi \epsilon_0 \epsilon_r)$$

In Equation (2)  $r$  is the distance between macroion centers,  $z_2 e$  is the charge on a polyion and  $\beta = (k_B T)^{-1}$ ,  $T$  is the absolute temperature and  $k_B$  is Boltzmann's constant;  $\kappa^{-1}$  is the Debye screening length ( $\kappa^2 = 8\pi L_B N_A I$ ;  $I = 0.5(z_+^2 n_+ + z_-^2 n_-)$   $I$  is ionic strength and  $N_A$  is Avogadro's number) and  $\epsilon_r$  is the relative permittivity

of the solution. All properties of water and electrolyte are subsumed in  $\kappa$ . An attractive van der Waals term,  $u_A(r)$ , is added to account for short-range attraction.

$$u_A(r) = H/36(d_2/r)^6 \quad (3)$$

Eq. 3, where  $H$  is the Hamaker constant, is the simplified form of the dispersion interaction potential (Grimson, 1983), which is only correct at large reduced distances  $r/d_2$ . Because dispersion interactions play a minor role in these phase separations, a limiting form of  $u_A(r)$  is chosen to keep the theory analytical. The third term in Eq. 1,  $u_{OA}(r)$ , represents the osmotic attraction contribution to the total potential (Asakura and Oosawa, 1954, Asakura and Oosawa, 1958). This term is essential to describe phase transitions induced by a non-adsorbing polymer (Vrij, 1976; de Hek and Vrij, 1981; Gast et al., 1983; Mahadevan and Hall, 1990 and 1992), but it may also be important at higher concentrations of simple electrolytes (Vlachy and Prausnitz, 1992). For our approximate purposes (a more realistic polymer model is used by Joanny et al., 1979), we use the potential proposed by Asakura and Oosawa (1954, 1958).

$$\begin{aligned} u_{OA}(r) &= \infty, & \text{for } r < d_2, \\ u_{OA}(r) &= -4\pi\beta^{-1}d_{23}^3\rho_3/3[1-3r/4d_{23}+r^3/16d_{23}^3], & \text{for } d_2 < r < 2d_{23}, \\ u_{OA}(r) &= 0, & \text{for } r > 2d_{23}. \end{aligned} \quad (4)$$

In Eq. 4,  $d_3$  and  $\rho_3$  are the diameter and the number concentration of the particles (polymer or electrolyte) exerting the osmotic force and  $d_{23}=(d_2+d_3)/2$ . For separations  $r < d_2+d_3$ , polymer molecules cannot penetrate into the region between two macroions and the depletion of polymers in this region causes a net attraction between the two macroions. The attraction potential vanishes for  $r > d_2+d_3$ , when the concentration of polymer molecules in the space between two proteins becomes equal to that in the bulk. Eq. 4 is an approximation; more accurate calculations of Henderson (1988) show that the osmotic interaction potential is an oscillatory function of  $r$ , if the volume fraction of small spheres (solvent species) is high. The recent article of Heno and Regnault (1991) discusses this topic using integral-equation theories.

### 3. Structure and stability of simple liquids

An important class of statistical-mechanical theories uses the Ornstein-Zernike equation

$$h(r) = c(r) + \rho \int c(r')h(|r-r'|)dr' \quad (5)$$

as a starting point to calculate thermodynamic properties for the system of interest (Hansen and McDonald, 1986). Eq. 5 relates the total correlation function  $h(r)$  to the direct correlation function  $c(r)$ . The correlation function  $h(r)$  is related to the radial distribution function  $g(r)=h(r)+1$ . The Fourier transform of  $h(r)$  is given by

$$h(k) = \int dr c(r) \exp[ik \cdot r], \quad (6)$$

where  $k$  is the wave vector;  $h(k)$  can be determined by light-scattering or neutron-scattering experiments. These scattering data yield important information about the "structure", i.e. correlations between the particles in charged colloidal dispersions, micellar solutions and globular proteins (Ottewill and Richardson, 1982). An important quantity is the polyion-polyion structure factor  $S(k)$

$$S(k)^{-1} = [ 1 - \rho c(k) ] \quad (7)$$

Upon applying the Ornstein-Zernike equation,  $S(k)$  can also be expressed in terms of  $h(k)$ . The connection between the structural properties (i.e. correlation functions) and thermodynamics comes in the limit as  $k \rightarrow 0$  (Hansen and McDonald, 1986). The  $k=0$  limit is equivalent to integration over the volume:  $C \equiv \lim_{k \rightarrow 0} c(k) = 4\pi \int c(r) r^2 dr$ . For the one-component

system (and only in this case)  $C$  [and  $S(0)$ ] are related to the isothermal compressibility,  $\chi_T$  (Hansen and McDonald, 1986).

$$(\rho\chi_T)^{-1} = (\partial P/\partial \rho)_T = \beta^{-1} [ 1 - \rho C ] \quad (8)$$

For colloidal solutions, described by a one-component model [McMillan-Mayer approximation (Hansen and McDonald, 1986)],  $\chi_T$  is an "osmotic compressibility" which can be determined experimentally (Ottewill and Richardson, 1982). Utilizing angular light scattering (scattering angle  $\theta$  is proportional to the wave vector  $k$ ), it is possible to obtain the structure factor  $S(k)$  (Ottewill and Richardson, 1982). By extrapolation of  $S(k)$  to  $k \rightarrow 0$  ( $\theta \rightarrow 0$ ), it is then possible to determine  $\chi_T$  and, if this quantity is measured as a function of the colloid concentration, also the osmotic pressure.

Eq. 8 is important for assessing the stability of a one-component system. The isothermal compressibility  $\chi_T$ , reflecting concentration fluctuations in a liquid, becomes infinite when the system is approaching phase separation (Hansen and McDonald, 1986).

#### 4. The random-phase approximation

We apply the random-phase approximation (Evans and Sluckin, 1981; Grimson, 1983; Victor and Hansen, 1984) to study phase separation in a protein solution induced by addition of neutral polymer and/or simple electrolyte. This approximation has been used previously by Evans and Sluckin (1981) to study the liquid-vapor transition in the Lennard-Jones fluid and by Grimson (1983) for a system similar to ours.

RPA is a perturbation theory which approximates the direct correlation function  $c(r)$  by

$$c(r) = c_0(r) - \beta u_1(r), \quad (9)$$

where  $c_0(r)$  is the direct correlation function for the reference system and  $u_1(r)$  is the perturbation part of the total potential  $u(r)$ . By taking the Fourier transforms of both sides of Eq. 9 in the  $k=0$  limit, we obtain

$$C = C_0 - \beta U_1 \quad (10)$$

where  $C_0 = 4\pi \int c_0(r) r^2 dr$  and  $U_1 = 4\pi \int u_1(r) r^2 dr$ . From Eqs. 8 and 10 we obtain the equation of state in the form

$$\beta P/\rho = \beta P_0/\rho + \rho\beta U_1/2, \quad (11)$$

where  $P_0$  is the contribution of the reference system to the total pressure  $P$  and  $\rho$  is the number concentration of protein particles in a one-component system. Eq. 11 is a version of the Van der Waals equation (Hansen and McDonald, 1986).

The Gibbs-Duhem equation relates the chemical potential of the protein  $\mu$  to  $C$  through

$$\rho(\partial\mu/\partial\rho)_T = \beta^{-1} [1 - \rho C]. \quad (12)$$

Integration of Eq. 12 yields a simple expression for the chemical potential of the protein in the random-phase approximation (Grimson, 1983):

$$\beta(\mu - \mu') = \ln \rho + \beta\mu_0 + \rho\beta U_1 \quad (13)$$

In Eq. 13  $\beta\mu' = \ln(\Lambda^3)$  and  $\Lambda^2 = \beta h_p^2/2\pi m$ , where  $h_p$  is Planck's constant and  $m$  the mass of the molecule. Further,  $\beta\mu_0$  is the contribution of the reference system to the chemical potential of the protein. The reference system is specified below.

Eqs. 11 and 13 give the pressure and the chemical potential of a one-component fluid. To implement these equations, it remains to split the pair potential  $u(r)$  into the reference part

$u_o(r)$  and perturbation part  $u_1(r)$ . Following Grimson (1983), we choose a hard-sphere reference system:

$$\begin{aligned} u_o(r) &= \infty, & u_1(r) &= u(r_{\min}) & \text{if } r < r_{\min}, & \text{ and} \\ u_o(r) &= 0, & u_1(r) &= u(r) & \text{if } r > r_{\min}, & \end{aligned} \quad (14)$$

where  $r_{\min}$  is the position of the first minimum in the potential. To avoid complications in the perturbation theory due to the "softening" of the hard-core when the screened-Coulomb potential (Eq. 2) is included in calculation, we use the additional approximation,  $r_{\min} = d_2$  where subscript 2 refer to the protein (Grimson, 1983). As discussed by Victor and Hansen (1984), this is a valid approximation when the concentration of simple electrolyte is high (large  $\kappa$ ).

Eq. 14 indicates that the reference system is a hard-sphere fluid, where  $c_o(r)$  in Eq. 8 is the direct correlation function and  $P_o$  and  $\mu_o$  the pressure and chemical potential of the hard-sphere fluid.  $P_o$  is given by the Carnahan-Starling equation (Carnahan and Starling, 1970)

$$\beta P_o / \rho = (1 + \eta + \eta^2 - \eta^3) / (1 - \eta)^3, \quad (15)$$

where volume fraction of protein is  $\eta \equiv \eta_2 = \pi \rho d_2^3 / 6$ , and the hard-sphere contribution to the chemical potential of protein  $\mu_o$  is given by

$$\beta \mu_o = \eta (8 - 9\eta + 3\eta^2) / (1-\eta)^3 . \quad (16)$$

Finally, to obtain the total pressure and chemical potential for the system of interest here, Eqs. 11 and 13, we need to evaluate integral  $U_1 = 4\pi \int u_1(r) r^2 dr$ . From Equations (1-4) we obtain

$$\begin{aligned} \rho_2 \beta U_1 = & 8\eta_2 z_2^2 L_B / d_2 [1 + 3/(\kappa d_2) + 3/(\kappa d_2)^2] / (1 + \kappa d_2 / 2)^2 \\ & - 4/9 \eta_2 \beta H - \eta_{23} \eta_2 [(d_{23}/d_2)^3 - 3(d_2/d_{23}) + 2(d_2/d_{23})^3], \end{aligned} \quad (17)$$

where  $\eta_{23} = \pi d_{23}^3 \rho_3 / 6$ .

The approximations of the RPA and its relation to other perturbation theories have been analyzed by Victor and Hansen (1984). They have shown that RPA, given by Eq. 9, can be obtained from a more exact theory for  $c(r)$  (Eq. 18),

$$c(r) = c_o(r) - \frac{1}{2} \beta u_1(r) \partial^2 [\eta^2 g_o(r; \eta_o)] / \partial \eta^2, \quad (18)$$

by making a mean-field approximation for the contribution of the perturbation to the thermodynamic properties; i.e. by replacing  $g_o(r; \eta_o)$  by 1 in Eq. 18. This approximation can easily be

relaxed, giving a more accurate, but unfortunately, nonanalytical theory (Victor and Hansen, 1984). Eq. 18 is first-order thermodynamic perturbation theory which, in combination with Eq. 14, yields accurate results for thermodynamic properties of simple liquids (Weeks et al., 1971; Hansen and McDonald, 1986).

## 5. Phase separations by non-adsorbent polymer

We first consider phase separation of colloidal particles induced by polymer in a nonpolar solvent where electrostatic effects are negligible. Phase separations in dispersions of spherical, lyophilic, monodisperse silica particles and polystyrene molecules in cyclohexane at theta temperatures were studied experimentally and theoretically by de Hek and Vrij (1981). They found that the concentration of polymer needed to induce liquid-liquid separation decreases, i) with rising molecular weight of polymer, ii) with rising diameter of colloidal particles, and iii) with increasing concentration of particles. We apply the random-phase approximation to the system studied by de Hek and Vrij (1981).

Only osmotic attraction and hard-sphere interactions (Eq. 4) are important in the non-aqueous systems studied by de Hek and

Vrij (1981); accordingly,  $z_2$  and  $H$  in Eq. 17 can be set to zero. Figure 1 presents the equation of state, i.e.  $P$  as a function of the colloid volume fraction  $\eta_2$ , at several volume fractions of polymer  $\eta_3$ . At low values of  $\eta_3$ , the osmotic pressure is a single-valued increasing function of colloid  $\eta_2$ . Above a "critical" value of  $\eta_3$ , the curves exhibit familiar van der Waals loops; in a well-defined region, which corresponds to unstable states,  $P$  is a decreasing function of  $\eta_2$  and the isothermal compressibility of fluid is negative. The locus of points connecting the maxima and minima is the spinodal curve which is sometimes used as a criterion for the onset of phase separation (de Hek and Vrij, 1981).

A one-component system is stable against spinodal decomposition if the isothermal compressibility is a positive quantity, i.e. if  $1 - \rho_2 C > 0$ . The molar concentration of added polymer  $n_3^*$ , needed to destabilize the system (limiting concentration), may be calculated from the condition  $\chi_T^{-1} = 1 - \rho_2 C = 0$ . This spinodal criterion has been used by de Hek and Vrij (1981) to predict trends in phase separation in mixtures of colloidal silica spheres and polystyrene molecules in cyclohexane. For a polymer-induced phase separation in a colloid-electrolyte-polymer mixture, we obtain the analytical expression:

$$\begin{aligned}
n_3^* = & (6/\pi d_{23}^3 N_A) \{ \beta (\partial P_o / \partial \rho_2)_T - 4/9 \eta_2 \beta H + 8 \eta_2 z_2^2 I_B / d_2 \\
& [1 + 3/(\kappa d_2) + 3/(\kappa d_2)^2] / (1 + \kappa d_2 / 2)^2 \} / \\
& \{ \eta_2 [(d_{23}/d_2)^3 - 3(d_2/d_{23}) + 2(d_2/d_{23})^3] \}, \quad (19)
\end{aligned}$$

where  $\beta (\partial P_o / \partial \rho_2)_T = (1 + 4\eta_2 + 4\eta_2^2 - 4\eta_2^3) / (1 - \eta_2)^4$ . Figures 2 and 3 show results for the limiting concentration of polymer needed for onset of phase-separation  $n_3^*$  (H and  $z_2$  are zero) as a function of polymer size  $d_3$  and as a function of  $\eta_2$ , respectively.  $n_3^*$  decreases upon raising the polymer diameter (molecular weight)  $d_3$ , or by increasing the colloid concentration  $\eta_2$ . Eq. 19 also predicts the decrease of the limiting polymer concentration upon raising the size of the colloid. All these trends have been observed in the measurements of de Hek and Vrij (1981).

We now turn to phase separation in water where electrostatic effects and dispersion forces cannot be neglected. Protein phase separations may be induced by addition of a non-adsorbent polymer to aqueous protein solutions containing ordinary electrolyte. If the molar concentration ( $n$ ) of low-molecular electrolyte is not high ( $n < 0.8$  M), we can use Eq. 19 to predict the limiting polymer concentration as a function of electrolyte concentration (or ionic strength  $I$ ) and pH ( $z_2$ ). For high electrolyte concentrations, Eq. 19 in its present form is not applicable, because the osmotic attractive force due to the presence of electrolyte may become important (Vlachy and Prausnitz, 1992), in

addition to the polymer contribution. The case of high salt concentration (no polymer present) is discussed in Section 6.

Figure 4 presents  $n_3^*$  as a function of the electrolyte concentration in the range  $0.05M \leq n \leq 0.6M$ , at two values of  $d_3$ . The limiting polymer concentration is a decreasing function of  $n$ . Eq. 19 predicts an increase of  $n_3^*$  with increasing charge on the protein  $z_2$ . These results reflect the functional form of the potential, Eqs. 1-4; by decreasing the electrostatic repulsion between the charged particles, osmotic attraction increases producing a decrease of solubility. In agreement with experimental data, proteins exhibit minimum solubility at the isoelectric point. The influence of the dispersion interactions can also be found from Eq. 19; as expected, a higher value of the Hamaker constant yields a lower value of the limiting polymer concentration.

Eq. 19, based on the condition  $1 - \rho_2 C = 0$ , can only be used to predict general trends with respect to the parameters of the model. We expect phase separation to occur for the values of  $\eta_2$  between the spinodal and binodal (coexistent) curve; the binodal curve connects the points in both phases having equal  $P, T$  and  $\mu_2$ . To locate the coexistence points, we need to calculate the chemical potential of protein  $\mu_2$  given by Eq. 13.

Using Eqs. 11, 13 and 15-17, we can calculate the volume fractions of the protein in the dilute  $\eta_2(v)$  and in the dense phase  $\eta_2(l)$ ; the two phases have equal chemical potential  $\mu_2$ , pressure and temperature. We are particularly interested in the partition coefficient  $K$ , here defined as  $K=\eta_2(l)/\eta_2(v)$ . We want to determine how  $K$  varies as a function of protein and polymer size ( $d_2$  and  $d_3$ ), electrolyte concentration  $n$  and other parameters of the model.

Figure 5 shows results for the partition coefficient  $K$  as a function of the size of the protein. Other parameters are:  $z_2=5$ , electrolyte concentration  $n=0.6M$ ,  $d_3=1.5$  nm and  $\beta H=5$ . In accord with Eq. 19,  $K$  increases upon raising  $d_2$  while other parameters are kept constant. Although not shown here (cf. Figure 2), partition coefficient  $K$  decreases upon raising  $d_3$ , the size of polymer. The results obtained by the random-phase approximation are consistent with those from other theoretical (Mahadevan and Hall, 1990) and experimental studies (Atha and Ingham, 1981).

## 6. Phase separation induced by electrolyte: Influence of the osmotic attraction

We now consider phase separation caused by addition of electrolyte (no polymer present). Liquid-liquid transitions in

colloidal systems have been observed experimentally by Cowel and Vincent (1982). The experimental work has been followed by theoretical studies of Grimson (1983), Victor and Hansen (1984), and Kovačič and Vlachy (1991). The observed "liquid-vapor" type of phase separation is believed to be a result of the sensitive balance between attractive van der Waals forces (Eq. 3) and repulsive electrostatic interactions (Eqs. 2) (Grimson, 1983; Victor and Hansen, 1984). The concentration of simple electrolyte plays here a role similar to that of temperature in the liquid-vapor transition of simple liquids.

Previous studies (Grimson, 1983; Victor and Hansen, 1984; Kovačič and Vlachy, 1991) are based on the one-component model which does not take into account the effect of the finite size of ions. In our recent study (Vlachy and Prausnitz, 1992) we have demonstrated that, for high electrolyte concentrations ( $n > 1$  M), the osmotic attraction term given by Eq. 4 must be included in Eq. 1 to reproduce correctly the results of the more realistic multicomponent model. Our present study indicates that the osmotic-attraction term may provide an important driving force for the phase separation induced by the addition of low-molecular electrolyte.

Since the mechanism of phase separation induced by addition of electrolyte is not known, we compare the results for two suggested models. The first one is that of Grimson (1983); we

call it the "dispersion-interaction" model, which models the interactions in the colloidal dispersions as a sum of the screened Coulomb potential (Eq. 2) and the attractive van der Waals (Eq. 3) potential. When this model is used with the random-phase approximation, it yields the following results (Grimson, 1983): i) the magnitude of  $z_2$  has little effect on the shape of the coexistence curves, ii) the value of the Hamaker constant is crucial in determining the phase diagram, iii) the size of the interacting species has marginal (colloid,  $d_2$ ) or no (ion,  $d_3$ ) effect on the shape of the coexistence curve and therefore on the partition coefficient  $K$ .

The second model is essentially the same as that described in Section 5. The interaction potential between the two colloid particles in the colloid-electrolyte mixture is given by Eq. 1. The contribution of dispersion interactions (Eq. 3) is assumed to be less important here; the primary driving force for phase transition is osmotic attraction (Asakura and Oosawa, 1954). Concentration  $\rho_3$  in Eq. 4 is now the number concentration of simple ions in the solution. Calculated partition coefficients  $K$  are therefore similar to those obtained in Section 5. In contrast to the first, "dispersion interaction" model, we found that the shape of the coexistence curves (and partition coefficient  $K$ ) strongly depends on the sizes of interacting species. To illustrate, Figure 6 shows  $K$  plotted as a function of  $d_2$ . Figure 6 shows that the partition coefficient  $K$  increases

strongly with rising size of the protein; larger particles separate more efficiently. Figure 7 presents the partition coefficient  $K$  as a function of electrolyte concentration  $n$  (ionic diameter is  $d_3=0.4$  nm), showing the strong effect of electrolyte concentration on phase separation. Also, in accord with the results of the previous section, larger ions yield better separation. These calculations indicate that the two suggested models for phase separation yield qualitatively different behavior with respect to some of the pertinent parameters.

## 7. Conclusions

The statistical-mechanical random-phase approximation has been applied to simple models for phase separation caused by addition of non-adsorbing polymer to solutions of globular proteins. The effective protein-protein interaction due to the presence of the polymer is related to the osmotic-attraction potential of Asakura and Oosawa (1954, 1958). The virtue of the random-phase approximation is its simplicity. The theory yields analytical equations for the pressure and chemical potential; therefore, the partitioning of the colloid (protein) between the two coexisting phases can be easily studied for a variety of experimental conditions. Although the theory is simple, it

reproduces all the experimentally observed dependencies with respect to protein size, polymer size, protein concentration, polymer concentration, electrolyte concentration and charge on the precipitating particles.

We show that, under certain conditions, the osmotic-attraction mechanism plays a significant role in electrolyte-induced separation. The two different mechanisms, one based on dispersion interactions (Grimson, 1983) and the other on osmotic attraction (Asakura and Oosawa, 1958), yield qualitatively different results with respect to the parameters of the model including protein and electrolyte size. The relevance of this or other models (Taratutta et al, 1990) of phase separation for protein solutions can be assessed by comparison with experiment. Comparison with some experimental data taken in this laboratory (Shih et al., 1992), indicates that the simple theory suggested here is in essential agreement with protein-precipitation experimental results. However, other experimental results (Arakawa et al., 1990) indicate that the solubility of protein in aqueous electrolyte solution, governed by the interactions between solvent components and proteins, may not obey simple electrostatic theory. The effects of electrolyte on the stability of protein are not always entirely electrostatic in nature; little dependence on protein charge has been found in some cases (Arakawa et al., 1990). The model, given by Eqs. 2

and 3, is not able to describe effects due to restructuring of solvent around charged groups on the protein.

The random-phase approximation used in this paper may not be as accurate as the perturbation theory of Barker and Henderson (1967) used by Gast et al. (1983) and Mahadevan and Hall (1990,1992). However, the random-phase approximation also has some advantages versus numerically much more demanding perturbation theory: i) it can be easily generalized to a multicomponent case to treat a mixture of proteins, ii) the polymer can actually be included as a separate species into the model, and iii) the protein-association, known to be an important feature of many proteins, can be incorporated into the RPA theory. All these refinements require little additional numerical work. However, an obvious first step in improving the theory would be to replace the mean-field approximation in Eq. 18 [ $g_o(r;\eta_o)=1$ ] with a more realistic approximation. Victor and Hansen (1984) have discussed the approximations in the random-phase theory and suggested a more accurate approach which, unfortunately, is not analytical. Evans and Sluckin (1981) found that RPA gives a reasonable estimate of the critical point for Lennard-Jones fluid; it is somewhat less accurate than the Percus-Yevick approximation. The accuracies of different statistical-mechanical approximations can be evaluated by comparison with computer simulation data but these are not now available.

On the other hand, it may not be worthwhile now to use a more demanding statistical-mechanical theory until the pertinent forces responsible for protein-protein and protein-ion interactions are better identified. In their present form even the more advanced perturbation theories neglect specific salt effects and protein-protein association leading to semi-stable dimers and higher aggregates; these forces are known to be important for at least some protein solutions. An increase in mathematical sophistication is not helpful unless that increase corresponds to a comparable improvement in our quantitative understanding of intermolecular forces.

#### Acknowledgment

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## Notation

$c(r)$  = direct correlation function

$d$  = diameter of a molecule, m

$e$  = elementary charge, C

$g(r)$  = radial distribution function

$H$  = Hamaker's constant, J

$h(r)$  = total correlation function

$h_p$  = Planck's constant

$I$  = ionic strength of solution, mol/dm<sup>3</sup>

$K$  = partition coefficient

$k$  = wave vector, m<sup>-1</sup>

$k_B$  = Boltzmann's constant, J/K

$L_B$  = Bjerrum's length, m

$N_A$  = Avogadro's number of molecules, mol<sup>-1</sup>

$m$  = mass of the molecule

$n$  = molar concentration, mol/dm<sup>3</sup>

$P$  = osmotic pressure, Pa

$r$  = intermolecular distance, m

$S(k)$  = structure factor

$T$  = absolute temperature, K

$u(r)$  = intermolecular pair potential, J

$z$  = valence of ion

## Greek Letters

$$\beta = (k_B T)^{-1}$$

$\epsilon_r$  = relative permittivity

$\epsilon_0$  = permittivity in vacuum, C/Vm

$\eta$  = volume fraction

$\theta$  = scattering angle

$\kappa$  = inverse Debye length,  $m^{-1}$

$\mu$  = chemical potential, J/mol

$\rho$  = number concentration,  $m^{-3}$

$\chi_T$  = isothermal compressibility,  $Pa^{-1}$

## Subscripts

o = reference system

1 = perturbation

2 = protein

3 = polymer

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## Figure captions

Figure 1. Osmotic pressure in colloid-polymer mixtures (Pascal units) as a function of the volume fraction of colloid  $\eta_2$ ;  $z_2=0$ ,  $H=0$ ,  $d_2=9.0$  nm and  $d_3=1.5$  nm. The volume fractions of polymer  $\eta_3$  are 0.20 (o), 0.225 ( $\blacktriangle$ ) and 0.25 ( $\square$ ).

Figure 2. Molar concentration of polymer  $n_3^*$  needed for onset of phase-separation, calculated using Eq. 19, as a function of the polymer diameter  $d_3$  at  $\eta_2=0.1$ . In this calculation  $z_2=0$ ,  $H=0$  and  $d_2=9.0$  nm.

Figure 3. Molar concentration of polymer  $n_3^*$  needed for onset of phase-separation, calculated using Eq. 19, as a function of the volume fraction of colloid  $\eta_2$ . Here  $z_2=0$  and  $H=0$ ;  $d_2=8.0$  nm and  $d_3=1.2$  nm.

Figure 4. Molar concentration of polymer  $n_3^*$  needed for onset of phase-separation, calculated using Eq. 19, as a function of electrolyte concentration  $n/M$  ( $M$  in mole/dm<sup>3</sup>) for  $d_3=1.2$  nm (open squares) and  $d_3=1.5$  nm (filled squares). In this calculation  $z_2=5$ ,  $\beta H=5$ ,  $d_2=8.0$  nm and  $\eta_2=0.1$ .

Figure 5. Phase separation induced by addition of neutral polymer: partition coefficient  $K$  as a function of  $d_2$ . Other parameters are:  $z_2=5$ ,  $\beta H=5$ ,  $d_3=1.5$  nm and  $n=0.6$  M.

Figure 6. Phase separation induced by addition of electrolyte: partition coefficient  $K$  as a function of  $d_2$ . Other parameters are:  $z_2=5$ ,  $\beta H=5$ ,  $n=3.0$  M and ionic size  $d_3=0.4$  nm.

Figure 7. Phase separation induced by addition of electrolyte: partition coefficient  $K$  as a function of electrolyte molar concentration  $n/M$ . Other parameters are:  $z_2=5$ ,  $\beta H=5$ ,  $d_2=5.0$  nm and ionic size  $d_3=0.4$  nm.

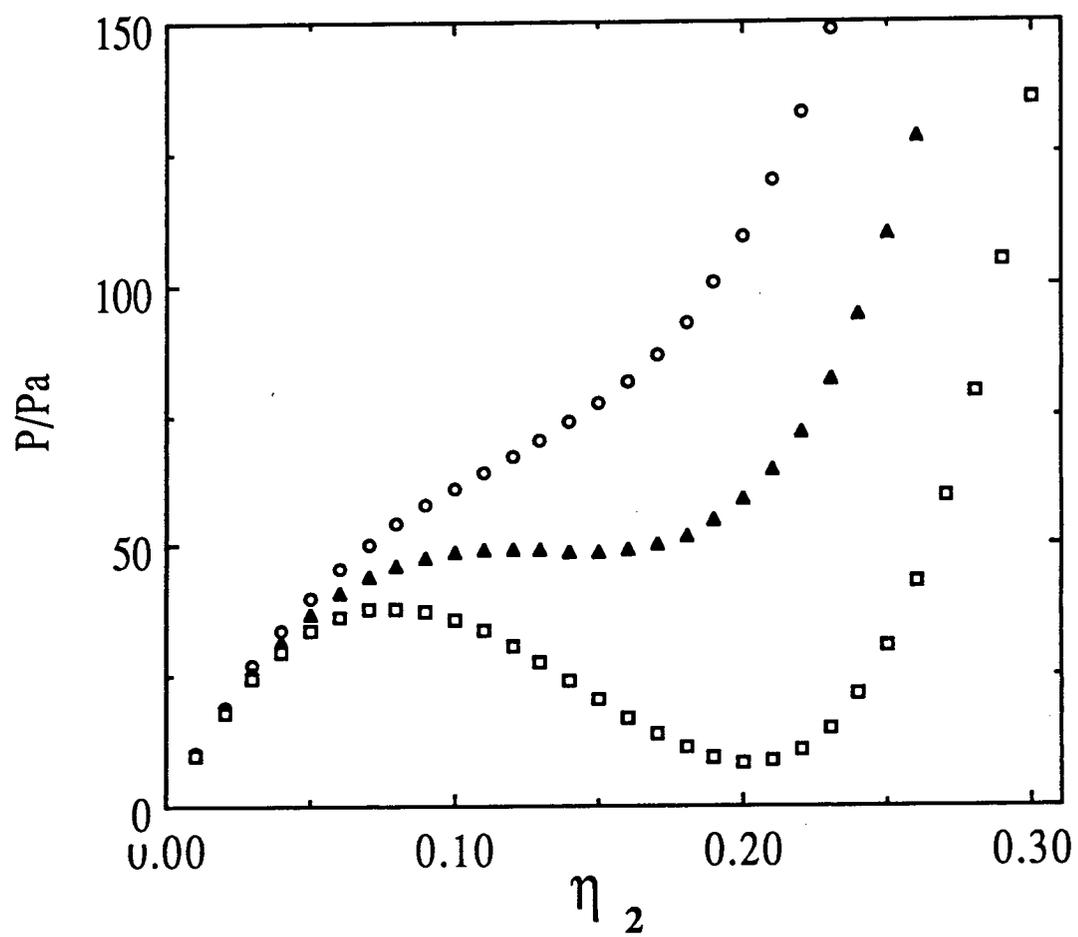


FIGURE 1

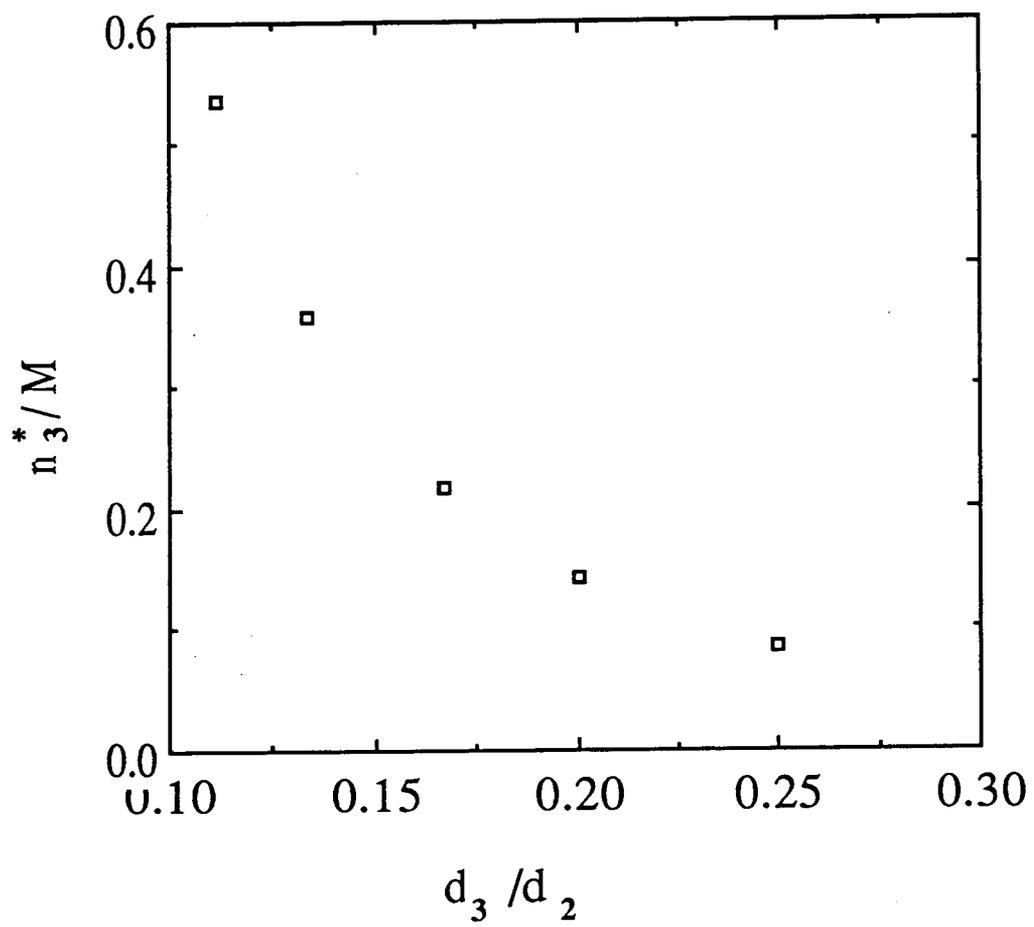


FIGURE 2

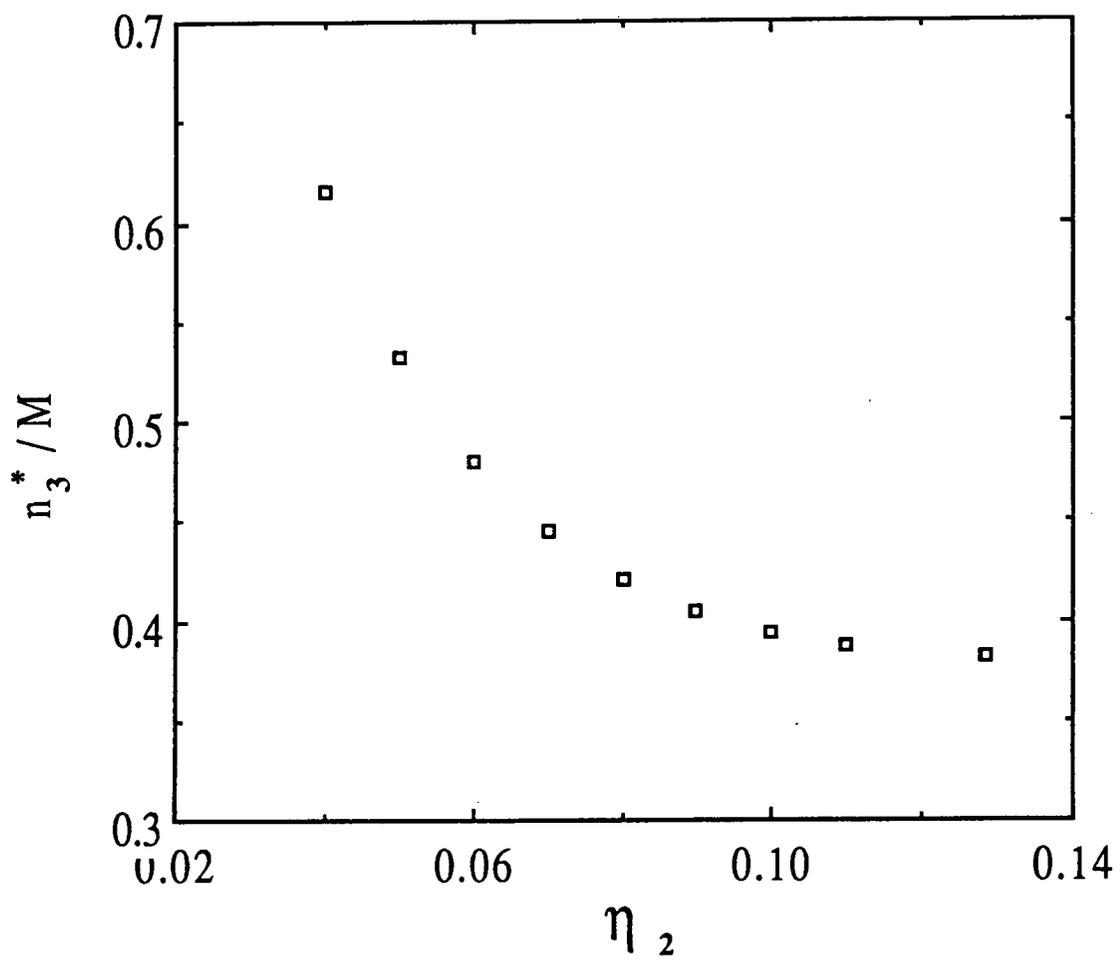


FIGURE 3

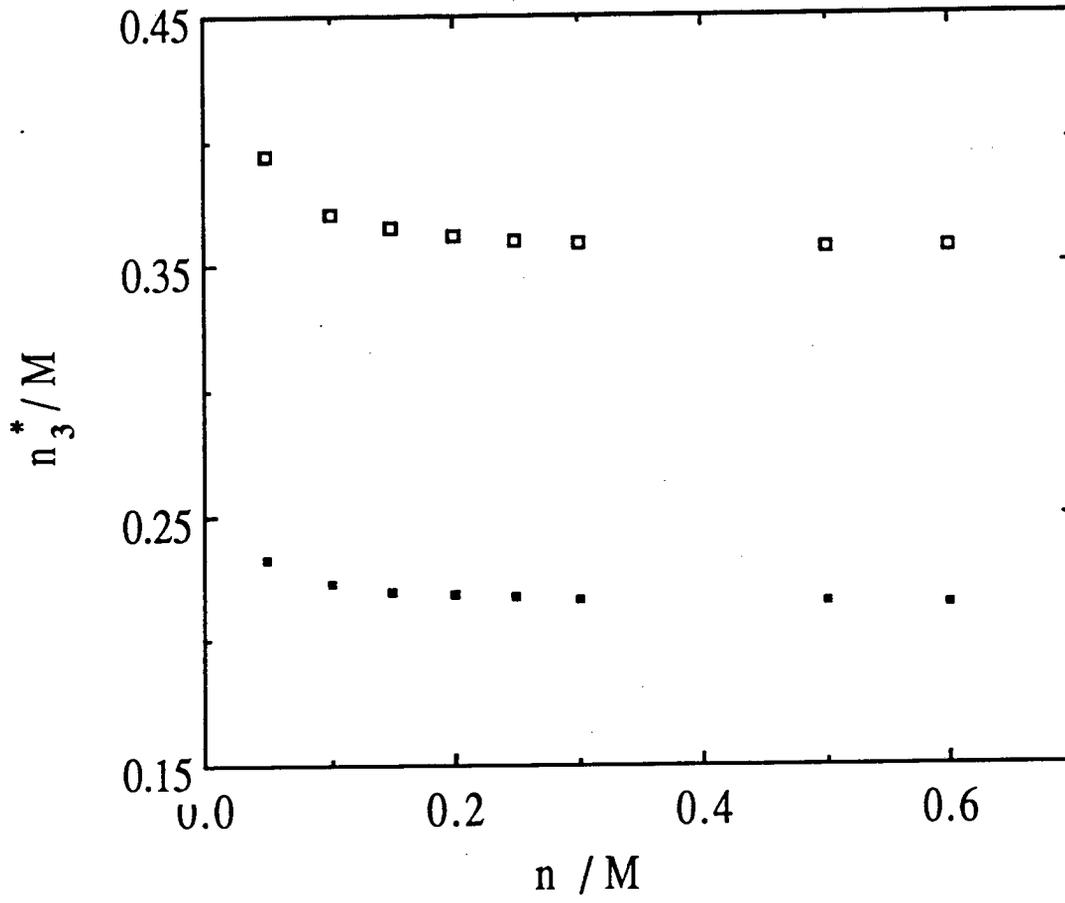


FIGURE 4

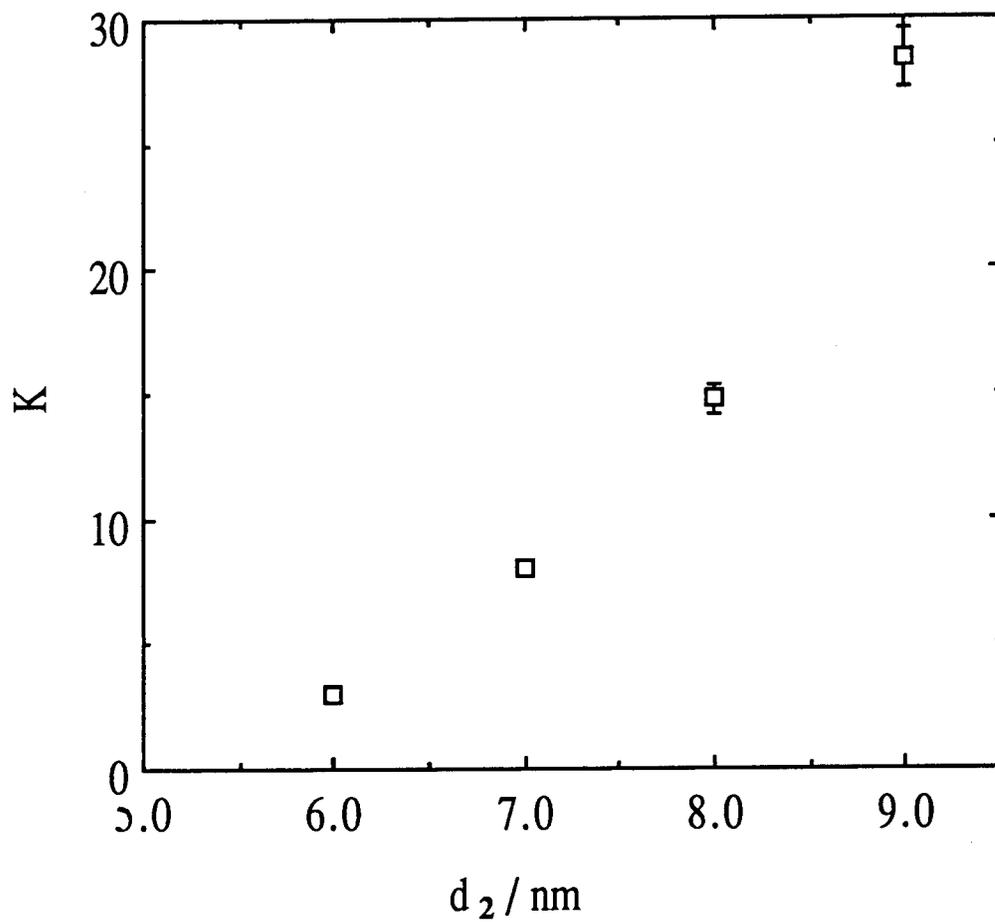


FIGURE 5

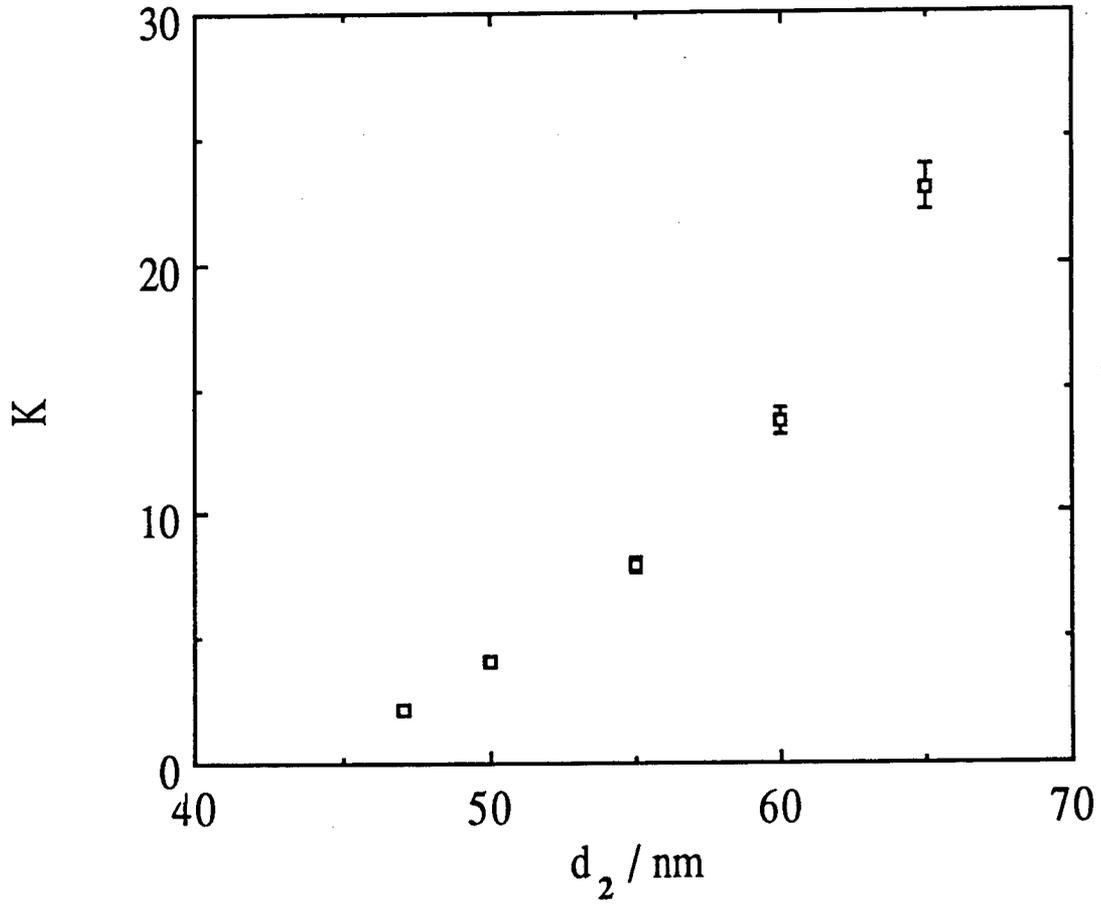


FIGURE 6

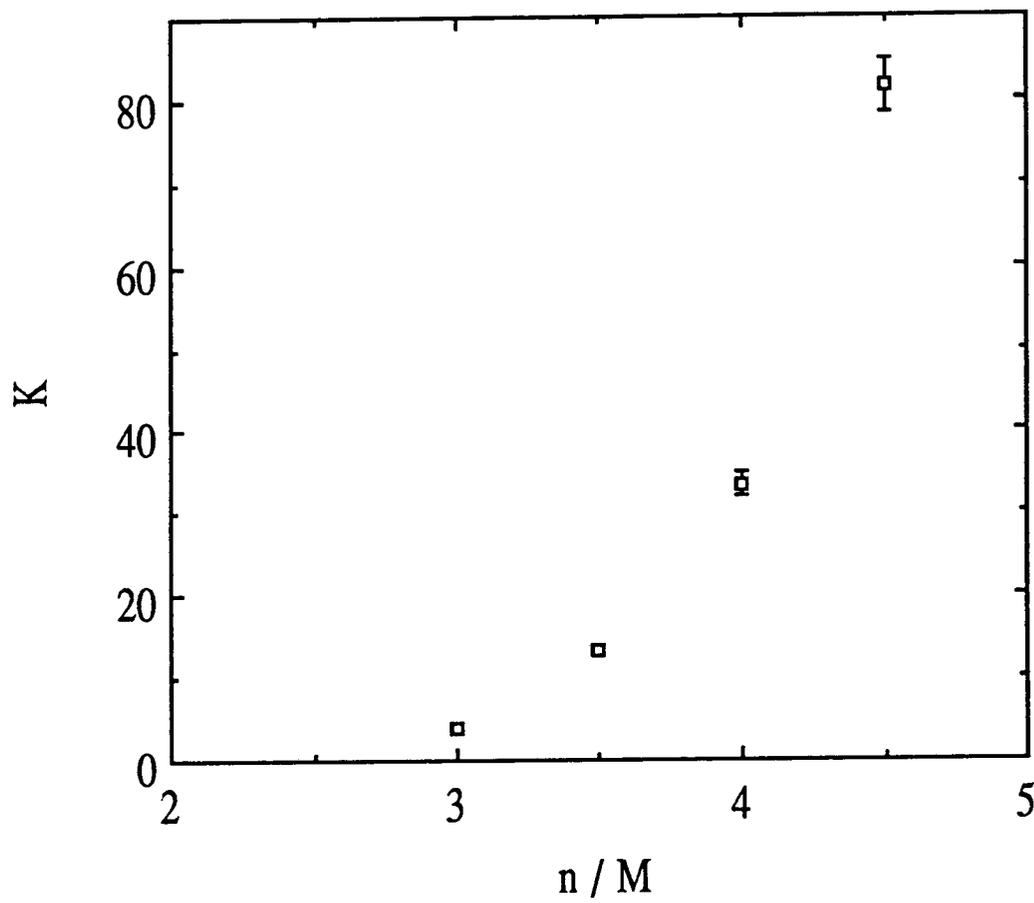


FIGURE 7

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