Experimental Investigations into Interactions and Collective Behavior in Protein/Polymer Mixtures and Granular Rods

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by

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ABSTRACT

Experimental Investigations into Interactions and Collective Behavior in Protein/Polymer Mixtures and Granular Rods

A dissertation presented to the Faculty of the Graduate School of Arts and Sciences of Brandeis University, Waltham, Massachusetts

by Joshua David Bloustein

Interactions and collective behavior are studied for two examples of protein/polymer mixtures and for dynamic and static ensembles of granular rods. Interactions in protein solutions and protein/polymer mixtures are investigated by measuring virial coefficients. First, a method is presented for measuring virial coefficients using chromatography. After measuring virial coefficients in protein solutions, binary soluble protein/polymer mixtures are investigated. Virial coefficients and effective interactions in these mixtures are determined using light scattering techniques and the results are compared with purely entropic models for binary mixtures. Liquid-liquid phase transition temperatures (cloud points) are measured in these mixtures as a probe of their collective behavior and show that adding large molecular weight polymers raises the cloud point and adding small molecular weight polymers lowers the cloud point. The results from light scattering measurements and liquid-liquid phase transitions temperatures are subsequently compared showing that indeed the interactions determined by light scattering can qualitatively explain the collective behavior seen in the phase transition temperatures. It is also demonstrated that these data are consistent
with attractions between protein and polymer molecules. Interactions in mixtures of a membrane protein associated with detergent moieties and polymer are investigated using the same light scattering techniques as for the soluble protein/polymer mixtures in conditions where these membrane protein/detergent complexes crystallize in order to determine whether or not the crystallizing additive induced attractions between the protein/detergent complexes. It is shown that the addition of polymer to a dispersion of protein/detergent complexes causes these complexes to aggregate and crystallize but does not induce attractions between the individual complexes on the virial coefficient level. It is demonstrated that no phase transition of the detergent moieties occurs in the protein/detergent complex crystallization conditions. The collective dynamic behavior of vortex formation and spontaneous vertical alignment in assemblies of granular rods is investigated by varying the rod aspect ratio and the rods’ material properties. It is shown that vortex formation occurs only for rods with aspect ratio larger than three and that the minimum packing fraction needed for vertical alignment decreases with increasing rod aspect ratio. Vortex formation is observed for rods of different densities and electrostatic charges. Discrete vertical excitations, or taps, cause rods to spontaneously align vertically as does shaking, but tapping does not induce vortex formation. Finally, the co-ordination number of granular rods is measured as a function of aspect ratio in order to probe the structure of static granular rod assemblies and test a statistical model for piles of granular rods. The co-ordination number increases as a function of increasing aspect ratio, but much more slowly than predicted for ellipsoids. In these same piles the collective behavior is quantified by measuring the volume fraction occupied by rods in a pile. The volume fraction and co-ordination number data are combined to show the validity of an excluded volume model for the behavior of piles of granular rods in the long rod limit.
This thesis is partially based on the following publications:


- J. Bloustine, S. Fraden. Is there a crystallization slot for membrane proteins? A CLC-ec1 case study *In preparation*

- J. Bloustine, S. Fraden. On the co-ordination of granular rods. *In preparation*
Contents

1 Introduction ............................................. 1
   1.1 Background ........................................... 1
      1.1.1 Virial Expansion ................................. 1
      1.1.2 Depletion Attraction ............................. 5
      1.1.3 The Random Contact Model ....................... 7
   1.2 Outline ............................................... 9

2 Measuring Protein/Protein Interactions with Chromatography 12
   2.1 Introduction ......................................... 12
   2.2 Theory ............................................... 14
   2.3 Materials & Methods ................................. 19
      2.3.1 Materials ......................................... 19
      2.3.2 Chromatography .................................. 20
      2.3.3 Methods .......................................... 21
   2.4 Results .............................................. 23
   2.5 Conclusion .......................................... 26

3 Light Scattering and Phase Behavior of Lysozyme/PEG Mixtures 28
   3.1 Introduction ......................................... 28
   3.2 Theory ............................................... 29
      3.2.1 Equivalent Hard Sphere Radii .................... 29
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.2</td>
<td>Cloud Point</td>
<td>30</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Static Light Scattering</td>
<td>33</td>
</tr>
<tr>
<td>3.2.4</td>
<td>Dynamic Light Scattering</td>
<td>37</td>
</tr>
<tr>
<td>3.3</td>
<td>Materials &amp; Methods</td>
<td>37</td>
</tr>
<tr>
<td>3.4</td>
<td>Results</td>
<td>40</td>
</tr>
<tr>
<td>3.4.1</td>
<td>Cloud Point Temperature Variation</td>
<td>40</td>
</tr>
<tr>
<td>3.4.2</td>
<td>Light Scattering Data</td>
<td>41</td>
</tr>
<tr>
<td>3.5</td>
<td>Analysis</td>
<td>48</td>
</tr>
<tr>
<td>3.5.1</td>
<td>Need for 3rd Virial</td>
<td>48</td>
</tr>
<tr>
<td>3.5.2</td>
<td>Depletion is Not Sufficient</td>
<td>50</td>
</tr>
<tr>
<td>3.5.3</td>
<td>Light Scattering vs. $T_{\text{cloud}}$</td>
<td>54</td>
</tr>
<tr>
<td>3.6</td>
<td>Conclusion</td>
<td>58</td>
</tr>
<tr>
<td>4</td>
<td>Light Scattering from PDC/PEG Mixtures</td>
<td>59</td>
</tr>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>59</td>
</tr>
<tr>
<td>4.2</td>
<td>Materials &amp; Methods</td>
<td>62</td>
</tr>
<tr>
<td>4.3</td>
<td>Results</td>
<td>64</td>
</tr>
<tr>
<td>4.4</td>
<td>Conclusion</td>
<td>69</td>
</tr>
<tr>
<td>5</td>
<td>On the Mechanisms of Granular Rod Vortex Formation</td>
<td>71</td>
</tr>
<tr>
<td>5.1</td>
<td>Introduction</td>
<td>71</td>
</tr>
<tr>
<td>5.2</td>
<td>Materials &amp; Methods</td>
<td>73</td>
</tr>
<tr>
<td>5.3</td>
<td>Results</td>
<td>73</td>
</tr>
<tr>
<td>5.4</td>
<td>Conclusion</td>
<td>79</td>
</tr>
<tr>
<td>6</td>
<td>On the Co-ordination of Granular Rods</td>
<td>80</td>
</tr>
<tr>
<td>6.1</td>
<td>Introduction</td>
<td>80</td>
</tr>
<tr>
<td>6.2</td>
<td>Materials &amp; Methods</td>
<td>83</td>
</tr>
<tr>
<td>6.3</td>
<td>Results</td>
<td>86</td>
</tr>
</tbody>
</table>
A Expression and Purification of CLC-ec1

A.1 Day1 - Transformation and Plating
  A.1.1 Transformation
  A.1.2 Plating

A.2 Day2 - Cell Growth and Protein Expression
  A.2.1 Cell Growth
  A.2.2 Protein Expression

A.3 Day3 - Protein Extraction and Purification
  A.3.1 Extraction
  A.3.2 Purification I - Cobalt Column
  A.3.3 Purification II - His-tag Cleavage
  A.3.4 Purification III - FPLC
List of Tables

2.1 Comparison of SEC and static light scattering measurements of $B_2$ for lysozyme ................................................................. 25

3.1 Lysozyme and PEG radii determined by dynamic light scattering . 31

3.2 Refractive index increments $dn/dc$ of lysozyme and PEG ........... 39

3.3 Light scattering results for PEG ............................................. 43

3.4 Lysozyme/PEG mixed virial coefficients ............................... 52

3.5 Semidilute cross-over concentration ($c_2^s$) for PEG ............... 52

4.1 Light scattering results for CLC-ec1/OM PDCs ....................... 68

6.1 The co-ordination number ($\langle z \rangle$) as a function of granular rod aspect ratio ................................................................. 88
List of Figures

2.1 Scanning electron micrograph of silica beads .......................... 15
2.2 Calibration curve for PEG in a YMC Diol 200 Column .............. 21
2.3 Lysozyme Chromatograms .............................................. 22
2.4 Lysozyme retention times as functions of injected concentrations ... 23
2.5 \( \ln K_D \) vs. \(< C_I > (1 - K_D)(mg/mL) \) for lysozyme ................. 24
2.6 Comparison of size exclusion chromatography and frontal chromatography measurements for BSA .................................................. 26
2.7 Comparison of size exclusion chromatography and light scattering measurements of \( B_2 \) for lysozyme .................................................. 27
3.1 The refractive index increment of lysozyme ......................... 35
3.2 Cloud point temperatures (\( T_{\text{cloud}} \)) of lysozyme as a function of concentration ................................................................. 41
3.3 Cloud point temperatures (\( T_{\text{cloud}} \)) of lysozyme/PEG mixtures .... 42
3.4 Static light scattering data for PEG1k and 8k solutions ............... 43
3.5 Temperature dependence of second virial coefficients for lysozyme and PEG ................................................................. 44
3.6 Static light scattering data for lysozyme-PEG 8k, PEG 1k and PEG 12k solutions ................................................................. 46
3.7 \( \beta \) for lysozyme/PEG12k mixtures ........................................ 47
3.8 \( \alpha \) as a function of polymer concentration ........................... 49
3.9 $\beta$ as a function of PEG concentration for lysozyme/PEG1k and PEG8k mixtures .......................................................... 51
3.10 Variation of lysozyme’s effective second virial coefficient with polymer concentration .................................................. 54
3.11 Comparison of light scattering and cloud temperature results .... 55
3.12 Variation of $T_{\text{cloud}}$ as a function of lysozyme concentration ... 57
4.1 CMC of OM ................................................................. 65
4.2 Hydrodynamic radius of OM micelles ................................. 66
4.3 $\beta$ for OM micelles as a function of PEG concentration .......... 67
4.4 $\alpha$ of OM micelles as a function of PEG concentration .......... 67
4.5 Pictures of ClC-ec1 crystals ............................................ 69
4.6 Aggregation of ClC-ec1/OM PDCs as a function of time .......... 70
5.1 Shaker schematic .......................................................... 74
5.2 Vortex pictures for different rod aspect ratios ....................... 75
5.3 $\phi_{\text{min}}$ as a function of rod aspect ratio ........................... 76
5.4 Accelerometer traces for shaking and tapping ....................... 78
6.1 Volume fraction of granular rods in a random pile as a function of tap number ........................................................ 84
6.2 Volume fraction of granular rods in random, uncompacted piles as a function of rod aspect ratio ........................................ 84
6.3 Compacted Volume fraction of granular rods in random compacted piles as a function of granular rod aspect ratio ................ 85
6.4 Pictures of a compacted pile of granular rods: boundary and bulk rods 86
6.5 Co-ordination number distribution function of aspect ratio 32 toothpicks 87
6.6 $\langle z \rangle$ as a function of rod aspect ratio. Comparing experiments to simulations ......................................................... 88

xiv
6.7 Plug formation in compacted piles of granular rods ............... 90
6.8 Picture of a plug forming pile of $L/D = 50$ rods ............... 90
6.9 Co-ordination number distribution functions for a plug forming pile at $L/D = 50$ and a non-plug forming pile at $L/D = 32$ ............... 91
6.10 Experimental verification of the random contact model for long, thin granular rods ....................................................... 92
Chapter 1

Introduction

In the Section 1.1, the theoretical background and models motivating and underlying the experiments in this thesis are presented. In Section 1.2 an outline of this thesis is provided.

1.1 Background

1.1.1 Virial Expansion

The equation of state relating pressure and density for any sufficiently dilute gas has a universal form, the Van’t Hoff or ideal gas law: $p/k_B T = \rho$ where $p$ is the pressure, $\rho = N/V$ is the number of molecules per unit volume, $k_B$ is Boltzmann’s constant and $T$ is the absolute temperature. In analogy with dilute gases, the osmotic pressure ($\Pi$) of many sufficiently dilute liquid solutions may be written in the same form $\Pi/k_B T = \rho$ where now $\rho$ is the density of a dissolved solute. At higher solute concentrations the osmotic pressure can be expanded in a power series in the solute density, known as a virial expansion:

$$\frac{\Pi}{k_B T} = \rho + \sum_{n\geq 2} B_n \rho^n$$  \hspace{1cm} (1.1)
The coefficients $B_n$ have units of volume and are known as virial coefficients. The term $B_2$ must be included when interactions between two solute molecules are appreciable. Similarly, the $B_3$ term arises from simultaneous interactions between three solute molecules. Although the term “virial” would appear to have a connection to infectious agents, in this context it does not. It was first used to describe this expansion of the equation of state by H. K. Onnes in 1901 [1, 2] who borrowed the term from the virial theorem in thermodynamics by Clausius.

The collective behavior of an ensemble of molecules is described by a virial expansion as shown by Eq.(1.1), but what is the relationship between the virial coefficients and molecular interactions? This relationship is found by calculating the classical partition function for a dilute solution. In order to calculate the second virial coefficient, a density expansion of the partition function is made. The coefficients in the expansion of the partition function are then related to the virial coefficients. Examples of different techniques for calculating the second virial coefficient are given by Hill [3] and Reif [4]. The resulting relation between intermolecular interactions and the second virial coefficient is:

$$B_2(T) = -\frac{1}{2} \int_V \left[ \exp \left( -\frac{w(\vec{r}, T)}{k_B T} \right) - 1 \right] d\vec{r}$$

(1.2)

where $w(\vec{r}, T)$ is the reversible work needed to bring two solute molecules together from infinitely far apart to the distance $\vec{r}$ in the presence of solute molecules, which is known as the potential of mean force (PMF). The PMF, and therefore $B_2$, depends explicitly on temperature because of the effect of the solvent on solute interactions. In general, the integral in Eq.(1.2) depends on the separation between solute molecules as well as their relative orientations. If the PMF depends only on the separation
between a pair of molecules then:

\[ B_2(T) = -\frac{1}{2} \int_0^\infty [\exp(-w(r, T)/k_B T) - 1] dr \]  

(1.3)

A natural scale for virial coefficients is provided by considering the hypothetical case of hard spheres in solution which cannot overlap but have no other interactions

\[ w(r) = \begin{cases} \infty & r < d \\ 0 & r \geq d \end{cases} \]  

(1.4)

(1.5)

where \( d \) is the diameter of one sphere. The second virial coefficient for hard spheres becomes:

\[ B_2^{\text{HS}} = \frac{16\pi (d/2)^3}{3} = 4\nu_o \]  

(1.6)

where \( \nu_o \) is the volume of one sphere. For realizable experimental situations \( w(\vec{r}) \) takes on positive and negative values. If \( B_2 > B_2^{\text{HS}} \) then the interactions are more repulsive than attractive and the converse is true for \( B_2 < B_2^{\text{HS}} \). The theta temperature \( T_\theta \) is the temperature for which \( B_2(T_\theta) = 0 \).

The second virial coefficients of protein solutions, as probes of protein interactions, have generated a great deal of interest since George & Wilson [5] showed a correlation between protein crystallisability and the second virial coefficient. Their work demonstrated that many proteins crystallize in conditions where the second osmotic virial coefficient becomes slightly negative, indicating net attractive interactions between protein molecules. George & Wilson identified values of \(-8 \leq B_2 \leq -0.8 \times 10^{-4} \) mL mol g\(^{-2}\) as the crystallization slot where soluble proteins crystallize. For less negative or positive \( B_2 \) values in their study, proteins remained as monomers in solution. For more negative \( B_2 \) values, examples of precipitation were found.

A virial expansion may also be made of the excess Gibbs’ free energy \((G)\) of
the solution over that of the pure solvent.

\[ g = \frac{G}{V k_B T} = \rho \ln \rho + B_2 \rho^2 + B_3 \rho^3 + \ldots \]  

This expansion may be extended formally to a system with an arbitrary number of distinct dissolved solutes. The case of a two component solution is relevant for Chapters 3 and 4 of this thesis and is therefore treated explicitly here. The two independent solutes are labelled with subscripts 1 and 2

\[ g = \frac{G}{V k_B T} = \rho_1 \ln \rho_1 + \rho_2 \ln \rho_2 + B_{11} \rho_1^2 + 2B_{12} \rho_1 \rho_2 + B_{22} \rho_2^2 + \]

\[ C_{111} \rho_1^3 + 3C_{112} \rho_1^2 \rho_2 + 3C_{122} \rho_1 \rho_2^2 + C_{222} \rho_2^3 + \ldots \]  

In Eq.(1.8) \( \rho_i = N_i/V \) [Volume\(^{-1}\)] is the number density of component i; \( B_{ij} \) [Volume] are the second virial coefficients and \( C_{ijk} \) [Volume\(^2\)] are the third virial coefficients. The first two terms in Eq.(1.8) represent the ideal entropy of mixing for each component. The terms quadratic in density represent corrections to the mixture’s free energy arising from two particles interacting and the terms proportional to the third power of density describe corrections due to three particles interacting. In multicomponent solutions \( w(r) \) is the potential of mean force between molecules of the same component for \( B_{ii} \) but between different components for \( B_{ij} \). Also \( B_2 \) in Eq.(1.2) has been relabelled as \( B_{ii} \).

The third virial coefficient is given by:

\[ C_{ijk} = -\frac{1}{3V} \int \int \int_V (e^{-u(r_{ij})/k_B T} - 1)(e^{-u(r_{ik})/k_B T} - 1)(e^{-u(r_{jk})/k_B T} - 1) d\vec{r}_i d\vec{r}_j d\vec{r}_k \]  

The osmotic pressure \( (\Pi) \) is found from the free energy by \( \Pi/k_B T = -\partial(G/k_B T)/\partial V \).

Leibowitz [6] solved the Percus-Yevick equation for the equation of state in a binary hard sphere mixture. The virial coefficients for a bidisperse hard sphere
mixture are obtained by expanding the equation of state solved by Lebowitz in a power series of the densities and identifying the expansion which yields:

\[ B^{\text{HS}}_{ij} = \frac{2\pi}{3} (r_i + r_j)^3 \quad ; \quad B^{\text{HS}}_{ii} = (16\pi/3) r_i^3 \] (1.10)

\[ C^{\text{HS}}_{iii} = \frac{5}{16} (B^{\text{HS}}_{ii})^2 \quad ; \quad C^{\text{HS}}_{iij} = \frac{8\pi^2}{27} r_i^2 (r_i^3 + 6r_i^2 r_j + 15r_i r_j^2 + 8r_j^3) \] (1.11)

where \( r_i \) are the hard sphere radii. The conversion of virial coefficients from volume units to experimentally convenient units is given by:

\[ B_{ij}[\text{mL mol/g}^2] = B_{ij}[\text{mL}] \frac{N_A}{M_i M_j} \] (1.12)

\[ C_{ijk}[\text{mL}^2 \text{mol/g}^3] = C_{ijk}[\text{mL}^2] \frac{N_A^2}{M_i M_j M_k} \] (1.13)

where \( M_i[\text{g mol}^{-1}] \) are the solute molecular weights and \( N_A[\# \text{ mol}^{-1}] \) is Avogadro’s number.

### 1.1.2 Depletion Attraction

The addition of small polymers to a dispersion of large colloids can result in precipitation or crystallization of the colloids. Even when the sole colloid/polymer interaction is steric repulsion, an attraction between a pair of colloids is generated by the exclusion of polymer molecules from the region between the colloids. If the polymer concentration is assumed to be larger than the colloid concentration and the polymers are considered to be effectively spherical particles, then the mixture’s entropy is dominated by the polymers’ entropy. When the colloids are far apart the volume accessible to the polymers’ centers of mass is less if the colloids are packed close together. Therefore the entropy increases and the free energy is lowered if the colloids come together. This entropic effect is known as depletion attraction [7, 8]. To model the depletion attraction Asakura & Oosawa [7] considered adding ideal polymers, or
other macromolecules, to a dispersion of spherical colloidal particles. They noted that when two particles approach each other closely, then polymer molecules are excluded from a region near the surface of each particle. This region depleted of polymer has a lower osmotic pressure than the surrounding fluid with polymers. This osmotic pressure difference creates an attractive potential $u(r)$ between the particles, where $r$ is the distance between particles. This attraction has the range $D \leq r \leq D + d$ where $D$ is the colloid diameter and $d$ is the polymer diameter. In the limit where the colloid is much larger than the polymer ($\beta = D/d \gg 1$) the depletion attraction simplifies to $u(r)/k_BT = -3/2\beta \phi x^2$ where $\phi$ is the polymer volume fraction and $0 \leq x \leq 1$ is the reduced inter-colloid distance. The strength of the attraction, as measured by the minimum value of $u(r)$, is linearly proportional to the polymer density. Gast et al. [8] and Hall & Mahadevan [9] employed Asakura & Oosawa’s model to calculate the phase diagrams of colloid/polymer mixtures in the limit where the colloid is much larger than the polymer $r \gg r_g$.

Meijer & Frenkel [10] and Eisenriegler et al. [11] extended this ideal polymer model of the depletion attraction to account for cases when the polymer is larger than the colloid. In that case polymer molecules can wrap partially around colloid particles thereby reducing the polymer/colloid excluded volume. This effect can be accounted for in the framework of an Asakura-Oosawa depletion theory by defining an ideal effective polymer radius ($r_{eff}$) which is smaller than the polymer’s radius of gyration $r_g$ and allowing the effective polymer radius to vary with the ratio of colloid to polymer size. Eisenriegler et al. found a closed formula for relating the effective sphere size ($r_{eff}$) to the protein ($r_{pro}$) and polymer ($r_g$) sizes (see Fig.6 of ref. [11]):

$$y = -x + \frac{x^{1/3}(3\sqrt{\pi} + 6x + x^2 \sqrt{\pi})^{1/3}}{\pi^{1/6}}$$

(1.14)

Here $x = r_{pro}/r_g$ and $y = r_{eff}/r_g$. The case where the polymer molecules are much
larger than the particles \((r \ll r_g)\), has been referred to as the protein limit \([12]\). Tuinier et al. \([12]\) used computer simulations to calculate the polymer concentration profile around colloids as a function of the colloid to polymer size ratio. They calculated effective colloid second virial coefficients and found that adding polymers induces effective attraction only when \(r > r_g\) but that adding polymers never induces repulsion between the colloids. Additionally, they compared their results to the Asakura-Oosawa model and found significant differences, especially when the colloid and polymer sizes are comparable, \(r \sim r_g\). Most notably Tuinier et al. found that the Asakura-Oosawa model severely overestimates the attraction induced between colloids when \(r \sim r_g\). Annunziata et al. \([13]\) used scaled particle theories to calculate the free volume available to idea polymers in protein/polymer mixtures. Their procedure leads to the same results for the variation of the spinodal temperature with polymer concentration as found by using the effective polymer size, Eq.(1.14), as discussed in Section 3.2.2.

All the above mentioned models treat ideal, or non-interacting polymers. These models are expected to be valid for polymers near their theta point but to be invalid for self-avoiding polymers. The protein/polymer mixtures dealt with in Chapters 3 and 4 are composed of self-avoiding polymers. Recently, progress has been made in predicting phase diagrams of colloid/polymer mixtures where the polymers self-avoid. Bolhuis et al. \([14]\) calculated phase diagrams of colloid/polymer mixtures with computer simulations of self-avoiding polymers. Their results show that polymer interactions are more important when the polymer is larger than the colloid.

### 1.1.3 The Random Contact Model

Philipse \([15]\) performed pioneering experiments on piles composed of elongated grains, granular rods. He found that the volume fraction occupied by rods depends inversely
on the rod aspect ratio. In order to model that data, he made a statistical model to
describe piles of granular rods, which he termed the random contact model (RCM).
The RCM considers an ensemble of randomly oriented rod shaped particles. The
orientations available to any given rod are restricted by the presence of that rod’s
neighbors. If we make the assumption that interactions between different pairs are
uncorrelated then the fraction of excluded orientations due to one neighbor is labelled
as $f_{\text{ex}}(\rho, \vec{r})$ and depends on the density of rods $\rho$ and the vector $\vec{r}$ joining the centers of
the two neighboring rods. If we make a mean-field approximation that the density can
be replaced by the average density $\bar{\rho}$ then the average number of contacting neighbors is:

$$\langle z \rangle / 2 = \bar{\rho} \int f_{\text{ex}}(\rho, \vec{r}) d\vec{r}$$

The integral in Eq.(1.15) is the orientationally averaged excluded volume $v_{\text{ex}}$ between
a pair of randomly oriented rods. The volume fraction $\phi$ of rods, with volume per
particle $v_o$, in an assembly is $\phi = \bar{\rho} v_o$. Now Eq.(1.15) can be written as:

$$\langle z \rangle / 2 = \phi \frac{v_{\text{ex}}}{v_o}$$

The volume per particle $v_o$ is taken to be the volume of a right cylinder of length $L$
and diameter $D$ with hemispherical end caps of diameter $D$ so that $v_o = \pi D^3 / 6 +
\pi D^2 L / 4$. The excluded volume between a pair of these spherocylinders was calculated
by Onsager [16, 17]:

$$v_{\text{ex}} = 2L^2 D |\sin \gamma| + 2\pi D^2 L + \frac{4\pi}{3} D^3$$

where $\gamma$ is the included angle between the rods. The last two terms in Eq.(1.17)
are end corrections. Our experiments are carried out on right cylinders without
hemispherical end caps. End effects are not expected to be important in the long rod
limit. For two randomly oriented rods $|\sin \gamma| = \pi/4$ on average. Then the excluded volume becomes $v_{ex} = \frac{\pi}{2} L^2 D + 2 \pi D^2 L + \frac{4\pi}{3} D^3$. Given the volume per particle and the excluded volume we can now simplify the relation between the volume fraction and the co-ordination number.

$$\langle z \rangle / 2 = \phi \frac{L}{D} \left[ 4 \frac{D}{L} + \frac{3(L/D)}{3(L/D) + 2} \right]$$

(1.18)

In the long rod limit ($L/D \gg 1$) this relationship reduces to:

$$\frac{\langle z \rangle}{2\phi(L/D)} = 1$$

(1.19)

In practice, the long rod limit form of Eq.(1.19) is approximately valid for $L/D > 15$. Although Philipse [15] showed that the RCM does capture the scaling of the volume fraction as a function of rod aspect ratio, the important prediction of Eq.(1.19) relating the rod interactions to the collective behavior of rod ensembles remains to be tested.

### 1.2 Outline

The goal of this thesis is to enhance understanding of how interparticle interactions are related to collective behavior in protein/polymer mixtures and in granular rod systems.

In Chapters 2, 3 and 4 protein solutions and protein/polymer mixtures in thermodynamic equilibrium are investigated using chromatography and light scattering to measure virial coefficients and microscopy to look for phase transitions. In Chapters 5 and 6 interactions and collective behavior are investigated for non-equilibrium systems of granular rods.

Chapter 2 describes a novel chromatographic technique for measuring second
virial coefficients using size exclusion chromatography. This technique is implemented using pulse chromatography and commercially available chromatography instrumentation which makes it accessible to any laboratory equipped with chromatography instrumentation. This method also significantly reduces the time and amount of material needed to carry out virial coefficient measurements from the initial implementation. The efficacy of this technique is demonstrated by comparison of measurements of virial coefficients for the proteins lysozyme and bovine serum albumin (BSA) in various solution conditions to previous measurements using established light scattering and chromatography techniques.

In Chapter 3 further investigations into lysozyme interactions are made in lysozyme/polymer mixtures. Chapter 3 evaluates whether or not the water soluble polymer poly(ethylene glycol) (PEG) induces attraction between lysozyme molecules by the depletion mechanism. Light scattering measurements are used to quantify the protein/protein and protein/polymer interactions in lysozyme/PEG mixtures. The collective behavior of these mixtures is investigated by measurements of liquid-liquid phase transition temperatures, cloud points, in these mixtures.

Chapter 4 continues the investigations into the relation between protein/polymer interactions and the collective behavior of protein/polymer mixtures for mixtures of a membrane protein and PEG. A chloride channel integral membrane protein was expressed and purified in order to carry out these studies. Light scattering is used to quantify the interaction between proteins in solution and the effect of adding PEG. Since membrane proteins are soluble only in the presence of detergents, the detergent interactions are investigated.

In the final two chapters of this thesis, driven and static assemblies of granular rods are studied. These granular assemblies are far from thermodynamic equilibrium and so differ markedly from protein and polymer solutions in thermodynamic equilibrium. The same questions asked in Chapters 2-4 about macromolecular systems
are also posed in the context of granular rod assemblies: how do interparticle inter-
actions effect the assembly’s collective behavior and can a statistical description of
the assembly be made which connects the interparticle interactions and the collective
behavior.

In Chapter 5 the effect of varying interactions between granular rods on their
collective dynamic behavior is investigated by examining how dynamic vortex forma-
tion is effected by changing the rod aspect ratio and the material properties of the
rods. It is shown that the formation of dynamic vortices depends significantly on the
rod aspect ratio but not on the specific material properties of the rods indicating that
geometric factors change rod/rod interactions and therefore, their collective behavior.

Chapter 6 studies the interactions between granular rods in static piles by
measuring the co-ordination number of granular rods as a function of the rod aspect
ratio. The collective behavior of rods in these piles is also investigated by measuring
how the density in these piles depends on the rod interactions as determined by the
rod aspect ratio. The validity of the RCM to describe granular rod piles is tested.

An appendix detailing the protocols used to express and purify the membrane
protein CLC-ec1 used in Chapter 4 is included.
Chapter 2

Measuring Protein/Protein Interactions with Chromatography

2.1 Introduction

The most prevalent experimental procedure for measuring $B_2$ is light scattering. Measuring $B_2$ with light scattering is accurate and fairly efficient but requires a specialized instrument not available to many biochemical researchers interested in measuring second virial coefficients of protein solutions. Additionally, sedimentation equilibrium [18], osmometry [19], neutron [20] and x-ray scattering [21], and self-interaction chromatography [22] have been employed to measure $B_2$.

Osmometry requires equipment which is not commercially available and longer than one day to measure $B_2$ [23]. X-ray and neutron scattering both require precious time on specialized beam lines which is not readily available. In separate studies we found that sedimentation equilibrium studies using an analytical centrifuge yield virial coefficient values which depend sensitively on the non-linear fitting parameters used to fit the equilibrium concentration profile. This makes the sedimentation equilibrium mode of analytical ultracentrifugation unreliable for virial coefficient measurements.
Chromatographic separations are used on a daily basis in biochemical separations and purifications. Therefore, a chromatographic method to measure virial coefficients would enable further studies of $B_2$. Tessier et al. [22] developed a method to measure second virial coefficients with self-interaction chromatography (SIC). The SIC technique involves immobilizing the solute molecule of interest on a chromatographic column and then flowing the same solute molecule through the column. Qualitatively, if the solute-solute interactions are more attractive than repulsive, the solutes spend a longer time in the column. We attempted to implement the SIC technique and found that it suffers from a number of technical problems. In order to quantitatively measure virial coefficients, the density of protein bound to the column must be known. In practice, measuring this concentration is difficult and unreliable. We found that protein retention on a column with protein bound depends on many experimental parameters including the injection volume which made quantitating virial coefficients difficult. Since SIC requires protein to be bound on a column it also suffers from the disadvantage that the binding could effect the interaction with free proteins in solution. Due to these technical difficulties with implementing SIC, we set out to look for another efficient chromatographic technique to measure virial coefficients of protein solutions.

In investigating chromatographic techniques we found that Winzor and co-workers [24] developed a method for measuring $B_2$ using size exclusion liquid chromatography (SEC) which is also known as gel filtration chromatography. In SEC the solute retention time on the column depends sensitively on the solute’s size, although no universal calibration for SEC has yet been achieved because solute retention also depends on the nature of the porous beads in the column. In SEC thermodynamic non-ideality leads to concentration dependent retention times, which can be utilized to quantify the second osmotic virial coefficient, $B_2$.

Nichol et al. [24] showed the possibility of measuring $B_2$ with frontal elu-
tion liquid chromatography. Frontal elution chromatography involves saturating a chromatographic column with a given solute while measuring the profile of the concentration when saturating the column and when the saturated region flows out of the column. Although frontal chromatography [25] allows one to fix the solute concentration in the column directly, it requires a large amount of protein (∼0.5 g) and long experiment times (about three hours per column run). In this chapter Nichol et al.’s method is extended to pulse size exclusion high pressure liquid chromatography (HPLC), where a small amount of protein is injected into and subsequently flows down the column. This adaptation drastically reduces the amount of protein (< 25 mg) and time needed (about 15 minutes per column run) to measure $B_2$ by SEC. Our results for $B_2$ obtained with size exclusion chromatography agree well with those from frontal chromatography and from light scattering measurements. It is also demonstrated that SEC can track the evolution of $B_2$ from positive to negative values.

2.2 Theory

SEC is used to separate and identify solutes of different sizes from a mixture. In SEC a solution is flowed through a chromatographic column made up of porous beads [26, 27]. Solute molecules are advected by the flow in the regions between the beads (inter-pore volume or mobile phase volume) and diffuse into the beads. Inside the porous beads (pore volume or stationary phase volume), there is virtually no flow. The beads have a distribution of pores of various sizes. An scanning electron micrograph of beads used for SEC, taken from ref. [28], is shown in Fig. 2.2. Solute molecules larger than the largest pore are unable to penetrate the porous beads and flow through the inter-pore volume. These largest molecules are said to be completely excluded by the column, hence the nomenclature size exclusion chromatography. Solute molecules
smaller than the smallest pore are able to penetrate into every pore and are said to be completely included by the column. Intermediate sized solute molecules penetrate a fraction of the pores depending on their size. Assuming that all solutes have the same energetic interactions with the porous medium, which is not always the case, then the time a solute molecule spends in the pore before diffusing back to the interpore volume depends on the volume accessible to the solute molecule inside the pore. Smaller molecules can access a larger volume and so will stay longer in the pores on average. Therefore, larger molecules will pass through the chromatographic column before smaller molecules.

In chromatography, solute passage through a given column is quantified in volume units. In practice, each given column has a useful range of sizes that can be separated using that column. That range of sizes is given by a calibration curve for a given column which plots the retention volume as a function of solute size, or typically solute molecular weight. A calibration curve for poly(ethylene glycol)
(PEG) dissolved in water eluting through the YMC column described in Section 2.3 is shown in Fig.2.3.2.

Nichol et al.’s [24] model of solute retention in SEC is discussed below which shows how SEC measures $B_2$. We assume a balance of the solute, i.e. protein, chemical potentials ($\mu_p$ and $\mu_i$) between the stationary and mobile phases as the solute is transported through the column. The pore volume (i.e. stationary phase) is labelled with the subscript $p$, and the inter-pore volume (i.e. mobile phase) with the subscript $i$. Equilibrium requires $\mu_p = \mu_i$. We write these chemical potentials by including the standard part $\mu^\circ$, the ideal term, and a term accounting for thermodynamic non-ideality through the activity coefficient $\gamma$:

\[
\begin{align*}
\mu_p &= \mu_p^\circ + RT \ln(C_p \gamma_p(C_p)) \\
\mu_i &= \mu_i^\circ + RT \ln(C_i \gamma_i(C_i))
\end{align*}
\]

where $C_{i,p}$ are the local solute weight concentrations, $R$ is the universal gas constant and $\gamma_p(C_p)$, $\gamma_i(C_i)$ are the thermodynamic activity coefficients of solute molecules in the pore and inter-pore volumes respectively. Rearrangement of these equations yields:

\[
\ln(K_0) = \frac{\mu_i^\circ - \mu_p^\circ}{RT}
\]

\[
\ln \left( \frac{\gamma_i}{\gamma_p} \right) = \ln \left( \frac{C_p}{C_i} \right) - \ln(K_0) \tag{2.1}
\]

where $K_0$ is the partition coefficient of solute molecules between chromatographic phases in the limit of infinite dilution. The relation between weight concentration, $C$, and number density, $\rho$, is $\rho = C \frac{N_A}{M_w}$ where $N_A$ is Avogadro’s number and $M_w$ is the solute molecular mass. Nichol et al. [24] made a virial expansion of the activity
coefficients

\[ \ln \gamma(z) = 2B_2(N_A/M_w)C + \text{higher terms} \quad (2.2) \]

This consideration assumes no difference in the solute-solute interactions in the mobile and stationary phases. The local solute distribution coefficient is \( K_D \equiv \frac{C_p}{C_i} \). If \( K_D \) is independent of concentration, as is the case for pulse chromatography with \( B_2 = 0 \), or if the concentration is constant as in frontal elution chromatography, then [24, 26]

\[
K_D = \frac{C_p}{C_i} = \frac{t_r - t_o}{t_T - t_o} = \frac{V_r - V_o}{V_T - V_o} \quad (2.3)
\]

where \( t_r \) and \( V_r \) are the solute retention time and volume, \( t_o \) and \( V_o \) are the retention time and volume of completely excluded molecules (i.e. the “dead” volume), and \( t_T \) and \( V_T \) the retention time and volume of completely included molecules (i.e. the “total” volume). Inserting the definition of \( K_D \) (Eq. 2.3) and Eq. 2.2 into Eq. 2.1 and keeping only the first order terms in concentration one obtains a relation between \( K_D \), \( B_2 \), and \( C_i \) valid for frontal chromatography where the concentration \( C_i \) is the plateau value of the solute concentration in the mobile phase:

\[
\ln \left( \frac{K_D}{K_0} \right) = 2B_2 \frac{N_A}{M_w} C_i (1 - K_D) \quad (2.4)
\]

To adapt this to pulse chromatography we replace the plateau value with the average concentration \( < C_i > \) of the mobile phase in the pulse:

\[
\ln \left( \frac{K_D}{K_0} \right) = 2B_2 \frac{N_A}{M_w} < C_i > (1 - K_D) \quad (2.5)
\]

Since \( < C_i > \) is not directly accessible in a HPLC experiment, it must be related to measurable parameters. The solute concentration passing through the
outlet of the column is measured as a function of volume. Each solute species passing the column appears in the detector as a peak in the concentration as function of volume curve. The mass of solute molecules in the pulse, or migration zone, \( m_{\text{zone}} \) is determined by integrating the concentration as a function of time over the zone volume, i.e. the peak \( (V_z) \). For columns in which there is no irreversible binding of protein molecules to the column, all the injected molecules are accounted for by integrating the peak. Therefore the total injected mass is the same as the total mass in the zone, \( m_{\text{inj}} = C_{\text{inj}} V_{\text{inj}} = m_{\text{zone}} \), but the concentration of solute in the migration zone is much lower than the injected concentration because the pulse spreads as it is injected into the column. The condition for the conservation of mass of solute molecules in the migration zone (subscript \( z \)) is

\[
m_i + m_p = m_{\text{inj}} \tag{2.6}
\]

\[
<C_i> V_i + <C_p> V_p = C_{\text{inj}} V_{\text{inj}} = m_{\text{inj}}
\]

Here \( V_i \) and \( V_p \) are the mobile (inter-pore) and stationary (pore) portions of the zone volume \( V_z \), with

\[
V_p = (V_T - V_0) \frac{V_z}{V_T}, \quad V_i = (V_0) \frac{V_z}{V_T} \tag{2.7}
\]

We measure the solute zone volume \( V_z \) from the full width \( \Delta t \) at half-maximum of the chromatogram peak using \( V_z = \nu \Delta t \), where \( \nu \) is the flow rate. After substituting the definition of the partition coefficient given in Eq. 2.3 and definitions Eq. 2.7 into Eq. 2.6, one obtains:

\[
<C_i> = \frac{m_{\text{inj}}}{V_z \left( \frac{V_z}{V_T} \right)} \tag{2.8}
\]

In Eq. 2.8, the numerator is the total mass in the zone and the denominator is the volume of the zone accessible to the protein. Thus, the concentration \( <C_i> \) is the
ratio of these terms. In this derivation we have assumed Eq. 2.3 holds, which is no longer the case when both \( B_2 \neq 0 \) and the concentration is changing during transport down the column. However, as we will show below, the changes in \( K_D \) with concentration are small, which may justify our approximation. Eq. (2.8) allows us to extend the method of ref. [24], originally developed using frontal elution chromatography, to pulse HPLC. Alternatively, one could use the maximum concentration \( C_{\text{max}} \) of eluted solute instead of \( < C_i > \) in Eq. 2.5. As shown in Fig. 2.3 \( C_{\text{max}} \) and \( < C_i > \) are approximately equal. Our procedure is then to inject different volumes of samples at various concentrations, measure \( K_D \) from the retention times as given in Eq. 2.3 and then plot \( \ln K_D \) as a function of either \( < C_i > (1 - K_D) \) or \( C_{\text{max}}(1 - K_D) \). The slope of that plot is then \( 2B_2 N_A/M_w \) from which \( B_2 \) is extracted given the solute molecular weight \( M_w \).

2.3 Materials & Methods

2.3.1 Materials

We obtained six times recrystallized, hen egg white lysozyme from Seikagaku America and bovine serum albumin (BSA), from Sigma. Both were used without further purification. All buffer components were obtained from Fisher Scientific. A Millipore Elix system purified water for all the experiments. We prepared potassium phosphate buffers by mixing 50mM solutions of \( K_2HPO_4 \) and \( KH_2PO_4 \), at various NaCl concentrations to adjust the ionic strength, to reach \( \text{pH} = 6.2 \) as measured by an Orion SA520 pH meter. The \( \text{pH} = 4.7 \) of sodium acetate buffers was adjusted by adding concentrated acetic acid to solutions of sodium acetate and NaCl. Additionally all buffers were passed through 0.45 \( \mu \text{m} \) nylon filters, also obtained from Millipore, prior to use. Protein concentrations were measured using a Varian instruments Cary 50Bio spectrophotometer at a wavelength of 278 nm. The extinction coefficient used for
lysozyme was $\epsilon_{278\text{nm}} = 2.64 \text{ mL (mg cm)}^{-1}$, and $\epsilon_{278\text{nm}} = .667 \text{ mL (mg cm)}^{-1}$ for BSA.

### 2.3.2 Chromatography

An 1100 series HPLC system from Agilent Technologies (Wilmington, DE) was used for all chromatographic measurements. Protein retention times were determined using an Agilent differential refractive index detector (RID) and an Agilent diode-array-detector (DAD) by absorbance at 278nm. A TSK-G2000SW (30cm x 0.75cm I.D.) column from TosohBiosep and a YMC-Diol-200AMP (30cm x 0.60cm I.D.) column from YMC were used in the chromatographic measurements. We used a flow rate of 1 mL/min for all measurements. These columns contain a packing of porous silica beads whose surfaces have been made hydrophilic. The manufacturers specified the diameter of a single bead to be $5\mu\text{m}$ for both columns. The average pore diameter is 125 Å for the TSK-G2000SW, and 200 Å for the YMC-Diol-200AMP. We determined the SEC calibration curve for these columns, as shown in Fig. 2.3.2, by using poly-ethylene-glycol (PEG) samples with molecular weights $200 \leq M_w \leq 10^5 \text{ g/mol}$, obtained from Sigma and Fluka. For every run the eluent was the same as the sample buffer. The random run-to-run difference in retention times for our system was less than 0.1%. Any dependence of the dimensionless distribution coefficient $K_D$ for protein molecules between the stationary and mobile phases on the average flow rate $\nu$ would indicate non-equilibrium effects. We found $K_D$ to be independent of flow rate for the experimentally accessible values: $0.1 \text{ mL/min} \leq \nu \leq 1.3 \text{ mL/min}$. Additionally, stop flow experiments have shown that solute retention in SEC is unaffected if the flow is stopped for prolonged periods [27]. We therefore conclude that the solute molecules are in a thermodynamically equilibrated state as they flow through the chromatographic column.
Figure 2.2: The calibration curve for PEG dissolved in water in the YMC Diol 200 column. The retention volume $V_r$ is shown as versus the PEG molecular weight $M_w$. The dashed lines indicate the dead volume and the total volume.

2.3.3 Methods

For each solvent condition we performed a series of HPLC experiments varying solute (protein) injected concentration $C_{inj}$ and using two injection volumes, $V_{inj} = 20 \mu$L and 100 $\mu$L. We identify the protein retention time $t_r$ as the time of the maximum in the RID signal (Fig. 1), where the injection time is $t = 0$. We plot $t_r$ as a function of $C_{inj}$, and find that $t_r$ depends on $V_{inj}$ as shown in Fig. 2.4. In order to apply our modification of Nichol et al.’s method to HPLC, we recalculate the average solute concentration in the pulse, $\langle C_i \rangle$, according to Eq. 2.8, and find that this reassuringly collapses the multiple $t_r$ vs. $C_{inj}$ curves from Fig. 2.4 to a single curve as shown in the insert of Fig. 2.5. The slope of this collapsed curve is proportional to the second virial coefficient according to Eq. 2.4.

In order to calculate $K_D$ according to Eq. 2.3 we must measure the total ($t_T$) and dead ($t_0$) times. We have measured the total time for each run using the solvent peak (these are maximums of the second peaks ($t_T$) in Fig. 2.3). In order to measure
Figure 2.3: Lysozyme chromatograms for $V_{\text{inj}} = 20\mu\text{L}$ and different injected concentrations ($C_{\text{inj}}$) as indicated next to each curve. The average concentrations $< C_i >$ used in the analysis in Fig. 2.5 are dash - 0.74 mg/mL, points - 1.72 mg/mL, dash and points - 2.20 mg/mL, long dash - 2.68 mg/mL, long dash and points - 3.08 mg/mL. The vertical line marks the retention time for the most dilute sample (not shown). The retention time $t_r$ is the time corresponding to the peak of the concentration profile ($C_{\text{max}}$) and increases with increasing concentration. The retention time of completely included molecules (the “total” volume) is marked as $t_T$. The peak at $t_T$ is caused by the very small refractive index difference between the eluent and the solution buffer. The retention time of completely excluded molecules (the “dead” volume) was $t_0 = 6.07$ min (not shown in figure). Note that $C_{\text{max}}$ and $< C_i >$ are similar. The buffer is Sodium Acetate, 50mM, pH 4.7.

The dead time, we used PEG with a molecular weight of $10^5$ g/mol, which is totally excluded from the TSK and the YMC columns. We have measured the dead times for all solvent conditions and injection volumes. It is important to measure $t_T$ and $t_0$ separately for all injection volumes to avoid any instrumental errors associated with precisely identifying the injection time.

Light scattering measurements were performed to determine $B_2$ independently for conditions where results were not found in the literature. Details concerning light scattering measurements are given in Section 3.3.
100 µL
20 µL

Figure 2.4: Lysozyme retention times are plotted versus injected concentrations for two injection volumes. Open circles: $V_{\text{inj}} = 20\mu$L and filled circles: $V_{\text{inj}} = 100\mu$L. Buffer: Sodium Acetate 50 mM pH 4.7.

2.4 Results

We measured the dependance of the retention factor $K_D$ on $C_{\text{inj}}$ and $V_{\text{inj}}$ for lysozyme and BSA in the above mentioned buffers and columns. These buffer conditions were chosen to investigate the cross-over from positive to negative $B_2$ values and to compare with data available in the literature.

Fig. 2.3 shows the RID signal measuring the concentration of the eluted protein versus time for representative lysozyme chromatograms with $V_{\text{inj}} = 20\mu$L. One can see the retention time increase with increasing protein concentration, while $t_T$ remains constant.

In SEC the direction of the shift in the retention time with concentration depends on the sign of $B_2$. For conditions where $B_2 > 0$, $t_r$ increases with increasing protein concentration and where $B_2 < 0$, $t_r$ decreases with increasing concentration. If $B_2 = 0$, $t_r$ is independent of concentration. Previous studies [20, 29, 30, 31] have shown that the sign of $B_2$ for protein solutions depends on the ionic strength of the solution.

Fig. 2.4 shows the dependence of lysozyme retention times on the injected
concentration $C_{\text{inj}}$. The two sets of data correspond to different injection volumes ($V_{\text{inj}}$): 20 $\mu$L and 100 $\mu$L. Following the procedure introduced above for determining the average solute concentration in the mobile phase of the migration zone $< C_i >$, we plot the dimensionless retention parameter, $K_D$, versus $< C_i >$ in the insert of Fig. 2.5. This procedure collapses the data from Fig. 2.4 onto a single curve from which $V_{\text{inj}}$ has been removed as an independent parameter. At the smallest concentrations in the insert of Fig. 2.5, some non-linear dependence of $K_D$ on $< C_i >$ can be observed. We attribute this behavior to errors introduced at the smallest signal to noise ratios. We have not included these points in our fits.

In order to extract $B_2$ from chromatographic data, $< C_i >$ is calculated by Eq. 2.8 and then $\ln K_D$ versus $< C_i > (1 - K_D)$ is plotted. As discussed in Section 2.2 the slope of the linear fit in Fig. 2.5 is proportional to $B_2$. Our results for $B_2$ for lysozyme solutions are listed in Table 2.1.

The protein concentration range typically used to measure $B_2$ by light scattering is approximately $0 < C_i < 30$ mg/mL [20, 29]. In our SEC measurements the protein concentrations $< C_i >$ eluting from the column, correspond to precisely the
Table 2.1: Comparison of size exclusion chromatography (SEC) and static light scattering measurements of the second virial coefficients $B_2 [10^{-4} \text{ mL mol g}^{-2}]$ for lysozyme in Potassium Phosphate Buffer 50 mM, pH=6.2, at various added NaCl concentrations.

<table>
<thead>
<tr>
<th>NaCl (mM)</th>
<th>From SEC</th>
<th>From LS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.4</td>
<td>1.8</td>
</tr>
<tr>
<td>50</td>
<td>1.6</td>
<td>-</td>
</tr>
<tr>
<td>150</td>
<td>-1.0</td>
<td>-1.4</td>
</tr>
</tbody>
</table>

same range, although the injected concentrations are much higher as show in Fig. 2.4. Even with these injected concentrations, the column was never saturated. Such high injected concentrations may not be accessible for other protein systems, and may be avoided by employing larger injection volumes, as shown by the $V_{inj} = 100 \mu$L data in Fig. 2.4.

Fig. 2.6 compares our $B_2$ results for BSA from pulse SEC and those obtained by Nichol et al. using frontal chromatography. Our results show the same slope for $\ln K_D$ as a function of $<C_i>(1 - K_D)$ as those obtained by Shearwin & Winzor [32], which means the $B_2$ values are the same. The solution conditions for the two data sets differ, but other studies [5, 19] have shown that $B_2$ for BSA is insensitive to many changes in solution conditions until crystallizing conditions are approached. Therefore we expect to measure a similar value of $B_2$. We measured different values of $K_D$ than those in ref. [32] because we used a different column.

In order to further validate the extraction of $B_2$ from SEC, our results are compared to those obtained by light scattering in Fig. 2.7, and in Table 2.1. Fig. 2.7 shows the dependence of $B_2$ on solution ionic strength (added NaCl concentration) for lysozyme. Our data agrees quantitatively with those previously obtained over a wide range of ionic strengths. Table 2.1 compares $B_2$ values obtained in a different buffer, Potassium Phosphate 50mM pH 6.2. For this buffer our SEC measurements of $B_2$ also agree with those from light scattering. The differences in magnitude can be attributed to systematic errors associated with light scattering and SEC measurements of $B_2$,
Figure 2.6: A comparison of size exclusion chromatography (SEC) and frontal chromatography measurements for BSA is shown. Open diamonds: BSA in Sodium Acetate 20mM, NaCl 0.18M, pH=4.6, \( B_2 = 1.9 \times [10^{-4} \text{ mL mol g}^{-2}] \) [32]. Black points are for BSA in Potassium Phosphate 50mM, pH=6.2, \( B_2 = 2.0 \times [10^{-4} \text{ mL mol g}^{-2}] \). Injected concentrations are 1.14, 4.85, 10.05, 15.0, 20.7, 25.27, 30.44, 40.72, 50.99 mg/mL. Injection volumes are for squares: 2\( \mu L \); stars: 10\( \mu L \); triangles: 40\( \mu L \); diamonds: 100\( \mu L \).

not statistical variation. Note that previously published results for \( B_2 \) from various groups, as shown in Fig. 2.7, differ by as much or more than the values shown in Table 2.1. These results illustrate the ability of SEC to track the evolution of protein interactions from net repulsive \( B_2 > 0 \) to attractive \( B_2 < 0 \).

2.5 Conclusion

In the experiments described in this chapter we adapted the idea of Nichol et al. and presented measurements of protein second virial coefficients (\( B_2 \)) using size exclusion liquid chromatography implemented with commercially available instrumentation, thereby reducing the cost in time and material of performing \( B_2 \) measurements for protein solutions. After calculating the protein concentration in the solute zone, our results agree with those previously obtained using the independent method of static light scattering.
Figure 2.7: A comparison of size exclusion chromatography (SEC) and light scattering measurements of $B_2$ for lysozyme is shown. $B_2[10^{-4} \text{ mL mol g}^{-2}]$ is plotted as a function of NaCl concentration. Buffer: Sodium Acetate 50 mM pH 4.7. The SEC measurements are denoted by open pentagons. The data for 0, 100, 200, and 300 mM NaCl were taken on a TSK column, and the data for 400 mM NaCl was with a YMC column. The remaining data comes from published light scattering data. Black diamonds: Gripon et al.[30], black triangles: Velev et al. [20], black stars: Kulkarni et al. [31], black rectangles: Muschol et al. [29]
Chapter 3

Light Scattering and Phase Behavior of Lysozyme/PEG Mixtures

3.1 Introduction

The water soluble polymer poly(ethylene glycol)(PEG) has been utilized extensively to induce protein crystallization [33]. PEG is known to be almost completely inert meaning that it does not interact with proteins in solution. Low molecular weight (3350 g mol\(^{-1}\)) PEG is approved by the food and drug administration (FDA) for human consumption in low doses as a laxative. The FDA has approved numerous pharmaceutical products for therapeutic use which contain proteins with attached PEG moieties and PEG is used as an additive to consumer products such as toothpaste.

If PEG is completely inert, then why does adding PEG to a protein solution often induce crystallization? The purely entropic depletion attraction discussed in Section 1.1.2 is one mechanism able to explain how inert PEG molecules could induce attraction between protein molecules subsequently leading to crystallization. Although
the soluble protein lysozyme crystallizes without the addition of PEG, adding PEG does increase the rate of lysozyme crystal nucleation [34]. This chapter addresses the question of whether or not PEG induces attraction between lysozyme molecules.

In addition to a liquid to crystal transition, protein solutions exhibit a metastable liquid-liquid phase transition when cooled as shown by studies on lysozyme [35, 36, 37] and γ crystallins [38, 39]. This is termed the cloud point \(T_{\text{cloud}}\) since at this phase transition a transparent protein solution becomes turbid as liquid droplets of high protein concentration form in a liquid of lower protein concentration. In the protein crystallization literature liquid-liquid phase separation is referred to as “oiling-out”. Numerical simulations by ten Wolde and Frenkel [40] suggested that this metastable liquid-liquid phase transition effects protein crystal nucleation. They predicted that the free energy barrier to crystal nucleation is lowered near a metastable liquid-liquid critical point. Galkin & Vekilov studied lysozyme/PEG mixtures and found that the effect of PEG on \(T_{\text{cloud}}\) depended on the solution’s ionic strength so that no clear evaluation of the depletion effect could be made. The effect of added PEG on the cloud point was studied for two of the γ-crystallin proteins by Benedek and co-workers who found that \(T_{\text{cloud}}\) of γS crystallin increases as PEG is added [39] in agreement with depletion attraction whereas for γD crystallin their results [13] indicate a departure from pure depletion.

In this chapter the free energy of lysozyme/PEG mixtures is modelled by the two component virial expansion given in Section 1.1.1 by Eq.(1.7).

### 3.2 Theory

#### 3.2.1 Equivalent Hard Sphere Radii

The ratio of lysozyme to PEG measured hydrodynamic radii, \(r_H\), in our experiments varied in the range \(3 \geq r_H^{\text{lys}}/r_H^{\text{PEG}} \geq 0.8\). Therefore we need to account for the fact
that PEG molecules, which are nearly as large or larger than lysozyme molecules, can wrap partially around proteins thereby reducing the polymer/protein excluded volume. This is done by employing the effective polymer radius ($r_{\text{eff}}$) defined in Section 1.1.2. In order to use the results outlined in Section 1.1.2, the protein radius and PEG $r_g$ are needed. Our procedure was to equate the protein’s radius with its measured $r_H$. Our procedure to determine the PEG’s $r_g$ is described in the following paragraph.

The PEG radii of gyration for the molecular weights employed here were too small to measure with static light scattering because $r_g$ is much less than the wavelength of light. In order to determine $r_g$ we therefore first measured $r_H$ of the polymers using dynamic light scattering (Section 3.2.4) and then used the relation

$$ r_g/r_H = 1.484M_w^{0.012} $$

(3.1)

which was found from static and dynamic light scattering experiments performed by Devanand & Selser [41] on large molecular weight PEGs $0.8 \times 10^5 \leq M_w \leq 10^6$ g mol$^{-1}$. This should be compared to the theoretical value, $r_g/r_H = 1.56$, for a polymer in a good solvent where the chains cannot interpenetrate [42]. Table 3.1 lists the measured hydrodynamic radii of lysozyme and PEG, the PEG radii of gyration and the PEG effective hard sphere radii. Our measurement of $r_H$ for lysozyme falls within the range of previously reported values by Grigsby et al. [43].

### 3.2.2 Cloud Point

In this section the variation of the spinodal temperature with added polymer concentration is calculated starting from the virial expansion for the free energy of a two component mixture given in Section 1.1.1. In what follows the subscript 1 refers to lysozyme and the subscript 2 refers to PEG. In a single component solution the sta-
Table 3.1: Hydrodynamic radii \( (r_H) \) of Lysozyme and PEG 1k, 8k, 12k and 35k calculated from single particle diffusion constants in the limit of zero concentration by the Stokes-Einstein relation as described in Eq.(3.13), PEG radii of gyration \( (r_g) \) and effective hard sphere radii \( (r_{\text{eff}}) \) of PEG determined as described in Section 3.2, Eqs.(1.14,3.1). \( r_H \) of PEG 1k, 8k and 35k were measured in the phosphate buffer and that of PEG 12k in the acetate buffer. The hydrodynamic radius of lysozyme was the same in both buffers. The radius of PEG needed to reproduce the measured values of \( B_{12} \) shown in Table 3.4 \( (r_{H}^{\text{PEG}}) \) is also shown. \( r_{H}^{\text{PEG}} \) is found by equating the measured \( B_{12} \) with \( B_{12}^{\text{HS}} \) and solving for the polymer radius while holding the protein’s radius fixed.

\[
f(T_{sp}, \rho_1, \rho_2) = \frac{\partial^2 g}{\partial \rho_1^2} \frac{\partial^2 g}{\partial \rho_2^2} - \left( \frac{\partial^2 g}{\partial \rho_1 \partial \rho_2} \right)^2 = 0
\]

Imposing the constraint that the solution remains on the spinodal curve when polymer is added yields:

\[
df = 0 = \frac{\partial f}{\partial T} dT + \frac{\partial f}{\partial \rho_1} d\rho_1 + \frac{\partial f}{\partial \rho_2} d\rho_2
\]

We want to calculate the dependence of spinodal temperature on added polymer concentration at fixed protein concentration. This is done by adding the constraint \( d\rho_1 = 0 \) which yields:

\[
\frac{\partial T_{sp}}{\partial \rho_2} = -\frac{\partial f}{\partial \rho_2} \left( \frac{\partial f}{\partial T} \right)^{-1} \tag{3.2}
\]

Inserting the virial expansion for the free energy density \( g \) ,Eq.(1.8), into Eq.(3.2) one obtains a relation between the virial coefficients and the dependence of the spin-
odal temperature $T_{sp}$ on added polymer concentration in the limit of low polymer concentration:

$$\lim_{\rho_2 \to 0} \frac{\partial T_{sp}}{\partial \rho_2} = -3C_{112} + 2(B_{12} + 3\rho_1 C_{112})^2 \frac{\partial B_{11}/\partial T + 3\rho_1 \frac{\partial C_{111}}{\partial T}}{\partial B_{11}/\partial T}$$

(3.3)

Note that in Eq.(3.3), the mixed virial coefficients have units $B_{ij}$ [Volume] and $C_{112}$ [Volume$^2$]. In all that follows we have set $\partial C_{111}/\partial T = 0$ since experimentally $C_{111} = 0$ at all temperatures (see Fig. 3.6).

We define the numerator of Eq.(3.3) to be

$$\gamma = -3C_{112} + 2(B_{12} + 3\rho_1 C_{112})^2$$

(3.4)

The PEG molecular weight dependence enters Eq.(3.3) only through the virial coefficients. From Eqs.(1.10, 1.11, and 3.3) it can be shown that for a hard sphere mixture $\gamma > 0$ for all sphere sizes $r_1$ and $r_2$ and concentrations $\rho_1$. In the limit of small protein concentration ($\rho_1 \to 0$) the change in the spinodal temperature scales with the ratio of polymer to protein size ($q = r_2/r_1$) as:

$$\lim_{\rho_2 \to 0, \rho_1 \to 0} \frac{\partial T_{\text{cloud}}}{\partial \rho_2} = (B_{11}^{\text{HS}} / 4)^2 \left( \frac{q^3}{2} (12 + 15q + 6q^2 + q^3) \right) / (\frac{\partial B_{11}}{\partial T})$$

(3.5)

Annunziata et al. [13] used scaled particle models to calculate the free volume available to ideal polymer coils in protein/polymer mixtures. From that free volume, the variation of the spinodal temperature was calculated and found to have the same scaling form as in Eq.(3.5).

For a binary mixture the sign of the spinodal temperature variation ($\lim_{\rho_2 \to 0} \frac{\partial T_{sp}}{\partial \rho_2}$) is set by the sign of the temperature variation of the protein second virial coefficient $\partial B_{11}/\partial T$. Thus, if the protein/polymer interaction is due solely to depletion effects then the sign of $\lim_{\rho_2 \to 0} \frac{\partial T_{sp}}{\partial \rho_2}$ cannot change with molecular weight of the added polymer.
3.2.3 Static Light Scattering

In order to explore lysozyme/PEG mixtures further, virial coefficients were determined by light scattering and compared with depletion theory. Light scattering has been used to investigate protein solutions for more than fifty years [45]. Effective virial coefficients in mixtures of proteins and PEG have been measured by light [31, 46] and X-ray [47] scattering along with solubility [48] studies.

We follow the multicomponent fluctuation theory developed by Kirkwood & Goldberg [49] and independently by Vrij [50] to relate light scattering measurements from ternary water, lysozyme and PEG solutions to the virial coefficients of interest. This approach treats the scattering from all solutes on equal footing, thereby eliminating the need to make any assumptions concerning the scattering from a given component. Tong et al. [51] used a similar framework to study the light scattering from mixtures of colloids and polymers. Tong et al. showed that their system behaved qualitatively as a hard sphere mixture and found an expression for the effective second virial coefficient between colloids in the presence of added polymer. Prausnitz and co-workers [46] used light scattering to measure mixed second virial coefficients in lysozyme/PEG and lysozyme/dextran mixtures. Their data show that, depending on the type of salt present in solution, mixed second virial coefficients can increase or decrease with increasing polymer molecular weight. Other analyses of light scattering from protein/polymer mixtures [31] have employed an effective one component analysis, thereby neglecting interference contributions from the polymers’ scattering. A multicomponent approach to X-ray scattering data from protein/polymer mixtures has previously been made by Tardieu and co-workers [52] in order to extract the potential of mean force between the protein urate oxidase and PEG where the protein radius is at least twice as large as the largest PEG employed. Their data are qualitatively consistent with a purely entropic depletion model for the polymer’s effect.
Kirkwood & Goldberg calculated the light scattering from a ternary system such as a mixture with two solute components and a solvent fluid. They showed that the excess light scattering of a two solute system \((R_{1+2})\) over that of a single solute system \((R_2)\) can be written in a simple form:

\[
\frac{Kc_1}{R_{1+2} - R_2} = \alpha + \beta \times c_1
\] (3.6)

Here \(K = 2(\pi n_o n_1)^2/N_A \lambda^4\) where \(n_o\) is the solvent refractive index, \(n_i = \frac{dn}{dc_i}\) is the refractive index increment, \(N_A\) is Avogadro’s number, \(\lambda\) is the wavelength of the incident radiation in vacuum, \(c\) \([\text{mg mL}^{-1}]\) is the weight concentration and \(R\) is the Rayleigh ratio, (Eq.(3.14)).

Experimentally, we find that the solution refractive index depends linearly on the solute concentrations as shown in Fig. 3.1, so that \(n_1\) and \(n_2\) are independent of concentration, although Kirkwood & Goldberg’s treatment can also incorporate concentration dependent \(n_i\). In Section 3.4 and Figures 3.4 & 3.6 we show experimentally the third virial coefficients for the single solute systems, i.e. protein or polymer alone in solution, are zero \((C_{iii} = 0)\) which simplifies the analysis.

Eq.(3.6) has the same form as the equation for the excess scattered intensity of a single component solution over that of the solvent:

\[
\frac{Kc_1}{R} = \frac{1}{M_1} + 2B_{11} \times c_1
\] (3.7)

For a single solute system the intercept of \(Kc/R\) as a function of \(c\) is the inverse of the molecular weight and the slope is twice the solute’s second virial coefficient. For the case of a 2 solute system \(\alpha\) and \(\beta\) depend explicitly on the added polymer properties \((M_2,n_2)\), the polymer concentration \(c_2\), and the thermodynamic interaction between
Figure 3.1: The refractive index increment $dn/dc = 1.85 \times 10^{-4}$ mL/mg for Lysozyme in the phosphate buffer at $T = 30^\circ$C was found from the linear fit to these data points as shown. The refractive index change is linear in the solute concentration. The values of $dn/dc$ listed in Table 3.2 were found from similar graphs for lysozyme in the acetate buffer and all PEGs.

the protein and polymer molecules as represented by the mixed virial coefficients:

$$\alpha = \frac{1}{M_1} + c_2 \frac{M_2 n_2}{M_1 n_1} B_{12}$$ \hspace{1cm} (3.8)

The mixed second virial coefficient $B_{12}$[mL mol g$^{-2}$] is obtained by measurements of the intercept $\alpha$ as a function of polymer concentration $c_2$ shown in Figure 3.8.

In the limit of no added polymer ($c_2 = 0$) Eq.(3.8) simplifies to the familiar one-component solution result, where the Rayleigh ratio is proportional to the solute molecular weight.

The coefficient $\beta$ is given by

$$\beta = 2 \left( B_{11} + c_2 \left[ (3C_{112} - 2B_{12}^2 M_2) + \frac{2(3C_{112} + 2B_{11} B_{12} M_1) M_2 n_2}{M_1 n_1} \right] \right)$$ \hspace{1cm} (3.9)

Performing a linear fit to $\beta$ as a function of $c_2$, as shown in Fig.3.9, we obtain the mixed third virial coefficient $C_{112}$ since all the other quantities in Eq.(3.9) have been
determined: \( B_{11} \) is found from light scattering experiments with component 1 alone; 
\( B_{12} \) is found from Eq.(3.8); the molecular weights \( M_i \) are measured by Eq.(3.7) one component at a time and the refractive index increments \( n_i \) are measured separately.

Adding polymers to a protein solution changes the potential of mean force between protein molecules. Changes in the effective protein interaction can be tracked by considering the following gedanken experiment. Add invisible polymers to the protein solution and ask what is the effect on the second virial coefficient between protein molecules. Experimentally, using an index matching solvent for all polymer concentrations would make the polymer invisible. As discussed by Vrij [53] and Tong et al. [51] in this case the polymer molecules do not scatter light \( (n_2 = 0) \) but still effect the protein interactions. Then

\[
Kc_1/(R_{1+2} - R_2) = 1/M_w + 2B_{\text{eff}} \times c_1 
\tag{3.10}
\]

with the effective second virial coefficient given by:

\[
B_{\text{eff}} = B_{11} + c_2 \left( (3C_{112} - 2B_{12}^2M_2) \right) \tag{3.11}
\]

This effective second virial coefficient between protein molecules in the presence of polymers results from Eq.(1.2) with the potential of mean force \( u(r) \) modified by the presence of polymers. Tong et al. [51] noted that de Hek & Vrij [54] calculated \( B_{\text{eff}} \) using the full Asakura & Oosawa depletion potential, which reduces to \( B_{\text{eff}} \) given in Eq.(3.11) in the limit of low polymer concentration. For a hard sphere mixture Tong et al. [51] found the expression for the effective second virial coefficient in terms of the polymer number density \( \rho_2 \)

\[
B_{\text{eff}} = B_{11}^{\text{HS}} \left[ 1 - \frac{\rho_2 \pi}{6} \frac{(r_1 + r_2)^6}{r_1^3} f(\delta) \right] \quad 0 \leq f(\delta) \leq 1
\tag{3.12}
\]
where $\delta = r_1/r_2$ and $f(\delta) = 1 - (8 + 15\delta + 6\delta^2 + \delta^3)/(1 + \delta)^6$. Since $f(\delta)$ is positive, adding a small amount, $\rho_2$, of hard spheres of radius $r_2$ to a suspension of hard spheres with radius $r_1$ can only decrease the effective second virial coefficient of component 1. In other words, for hard spheres adding a second component always induces attractions. This is consistent with the depletion attraction discussed in Section 1.1.2. Only $3C_{112} > 2B_{12}^2M_2$ will lead to induced repulsion or $\partial B_{\text{eff}}/\partial c_2$.

### 3.2.4 Dynamic Light Scattering

Hydrodynamic radii were measured by dynamic light scattering (DLS). DLS measures the intensity autocorrelation function $g_1(t) = \langle I(t)I(t_0) \rangle$ where $I(t)$ is the scattered intensity as a function of time. For a single solute diffusing the intensity autocorrelation function decays exponentially $g_1(t) = e^{-\Gamma t}$. The diffusion constant ($D$) is calculated from $D = \Gamma/q^2$ where $q = \text{ the amplitude of the scattering vector}$ [55]. $r_H$ is calculated by the Stokes-Einstein relation for spherical particles in the infinitely dilute limit:

$$r_H = \lim_{c \to 0} r_H(c) = \lim_{c \to 0} \frac{k_BT}{6\pi\eta D(c)}$$

(3.13)

where $\eta$ is the solvent viscosity.

### 3.3 Materials & Methods

Six times recrystallized and lyophilized hen egg white lysozyme was purchased from Seikagaku America (Falmouth, MA). The molecular weight of lysozyme based on its amino acid sequence is 14,400 g mol$^{-1}$. We tested the lysozyme purity by size exclusion chromatography and dynamic light scattering and found pure protein existing as a monomer in solution. Lysozyme was used without further purification.

Poly(ethylene glycol) (PEG) was purchased from Sigma (St.Louis, MO) and Fluka (Buchs, Switzerland). We designate the manufacturer’s stated value for the
PEG molecular weight with the nomenclature PEGnk, meaning PEG n×10³ [g mol⁻¹]. The degrees of polymerization (N) for PEG 1k, 8k and 12k are listed in Table 3.4 and were calculated via N = M₂/44 where 44 g/mol is the molecular weight of the PEG monomer.

The protein and PEG were dissolved in two aqueous buffers: a phosphate buffer at pH 6.2 and an acetate buffer at pH 4.6. All buffer components were obtained from Sigma. The phosphate buffer was prepared by mixing solutions of sodium phosphate monobasic (NaH₂PO₄ 0.2M+NaCl 0.5M) and dibasic (Na₂HPO₄ 0.2M+NaCl 0.5M) to reach pH 6.2, where M = [mol L⁻¹]. The acetate buffer was prepared by adding concentrated acetic acid to solutions of Sodium Acetate (NaAc) 50mM + Sodium Chloride (NaCl) 150mM to adjust the pH to 4.6. The acetate buffer was chosen for comparison to previous light scattering studies [31, 29]. The phosphate buffer was chosen because the liquid-liquid phase separation temperatures for lysozyme fall in an experimentally convenient range for this buffer.

A Millipore (Billerica, MA) Elix system produced deionized water for use as the solvent. All solutions were centrifuged at ~12000 × g for 1 hour and then filtered through Millipore Millex 0.22μm syringe filters directly into precleaned, disposable scattering cuvettes obtained from Fisher Scientific (Pittsburgh, PA). The lysozyme concentrations were measured from these centrifuged and filtered solutions by UV absorption measurements on a Varian Instruments (Palo Alto, CA) Cary-50Bio spectrophotometer using an extinction coefficient ε₂₈₀nm = 2.64 mL/mg cm.

Refractive index increments (dn/dc) of lysozyme and PEG in both buffers were measured using a Brookhaven Instruments (Holtsville, NY) differential refractive index detector at λ =620 nm. Although the refractive index of the solution depends sensitively on temperature, dn/dc was found to be independent of temperature for 15 ≤ T ≤ 40°C. Table 3.2 lists the measured values of dn/dc used in all calculations which closely match previously published results.
Table 3.2: Refractive index increments $dn/dc$ of lysozyme and PEG in the phosphate and acetate buffers, measured as described in Section 3.3. $dn/dc$ was found to be independent of the PEG molecular weight and solution temperature for $15^\circ \leq T \leq 40^\circ$. These measurements of $dn/dc$ are commensurate with previously published results [41].

Cloud point temperatures ($T_{\text{cloud}}$) of protein polymer mixtures were determined by optical microscopy as an approximation of the spinodal decomposition temperatures. Rectangular glass capillaries (0.1mm path length) obtained from VitroCom (Mt. Lakes., NJ) were filled with solution, flame sealed and then placed in a custom built temperature controlled microscope stage. The temperature at which homogeneous nucleation of dense droplets occurred was called the cloud point. The temperature of each solution was cycled up and down at approximately $1^\circ C \text{ min}^{-1}$ through the cloud point several times for each measurement. Little or no hysteresis ($\leq 0.5^\circ C$) was observed when comparing cloud points obtained by cooling and heating. This suggests that the cloud point closely approximates the coexistence curve. The cloud point and coexistence curves were shown to agree for $\gamma$-B crystallin [38] protein solutions, when using a similar method.

The static and dynamic light scattering experiments were performed using an ALV (Langen, Germany) goniometer and correlator system with a 22mW HeNe ($\lambda = 633$ nm) laser and an avalanche photodiode detector. The temperature of samples in the goniometer system was controlled to within $\pm 0.1^\circ C$. All of our light scattering experiments were performed using the $vu$ mode in which vertically polarized light is incident upon the sample and all polarizations are detected. Absolute Rayleigh ratios of protein and polymer solutions were determined by using pure toluene as a standard
whose Rayleigh ratio is known to be \( R_{\text{tol}} = 1.35 \times 10^{-5} \text{cm}^{-1} \) at this wavelength and \( T = 20^\circ\text{C} \) [56]. The procedure was to first measure the scattered intensity of pure toluene \( (I_{\text{tol}}) \) filtered through 0.2µm PTFE membranes obtained from Fisher. Then the scattering of the buffer \( (I_{\text{buf}}) \) was measured before measuring the light scattering intensity of a protein solution \( (I_{\text{sol}}) \). The Rayleigh ratio of a protein solution was then determined by:

\[
R_{\text{sol}} = \frac{I_{\text{sol}} - I_{\text{buf}}}{I_{\text{tol}}} R_{\text{tol}} \left( \frac{n_{\text{buf}}}{n_{\text{tol}}} \right)^2
\]

using the refractive index correction factor [45]. The refractive index of toluene was taken to be: \( n_{\text{tol}} = 1.496 \) at \( T = 20^\circ\text{C} \) and a wavelength of \( \lambda = 633 \text{ nm} \). The observed scattering volume varies as \( 1/\sin(\theta) \) where \( \theta \) is the angle between the incident and scattered wave vectors. After correcting for this variation the scattering from toluene was angle independent for \( 30^\circ \leq \theta \leq 150^\circ \) to within \(< 2\%\) which is within the manufacturer’s recommendations for the alignment quality in order to carry out SLS experiments.

### 3.4 Results

#### 3.4.1 Cloud Point Temperature Variation

Figure 3.2 shows the cloud point temperatures \( (T_{\text{cloud}}) \) of lysozyme solutions in the phosphate buffer. The measured dependence of \( T_{\text{cloud}} \) on lysozyme concentration qualitatively parallels that of previous investigations [36, 37]. These studies showed that \( T_{\text{cloud}} \) depends on the solution pH, buffer conditions and added salt concentration. Therefore, the quantitative difference between these previous measurements of \( T_{\text{cloud}} \) and our cloud point temperature measurements is accounted for by the differences in solvent conditions.

The effect of adding PEG on the cloud point temperature is shown in Fig.3.3.
Figure 3.2: Cloud point temperatures ($T_{\text{cloud}}$) as a function of lysozyme concentration are shown for lysozyme in the phosphate buffer. The line is a guide to the eye.

We extracted the dependence of the cloud point on polymer concentration in the dilute polymer limit $\lim_{\rho_2 \rightarrow 0} \frac{\partial T}{\partial \rho_2}$ from these data. For dilute PEG concentrations, low molecular weight PEGs depress the cloud point, i.e. stabilize the solution, and higher molecular weight PEGs raise the cloud point, i.e. destabilize the solution.

### 3.4.2 Light Scattering Data

Static light scattering data for PEG 1k, 8k and 12k solutions at $T=20^\circ\text{C}$ are presented in Fig. 3.4. Table 3.4 shows the PEG molecular weights extracted from these data. These values differ from the manufacturer’s stated molecular weights but are within the manufacturer’s specifications that up to a 20% deviation from the stated mean molecular weight may be present in any given batch. Table 3.4 compares our measured second virial coefficients for PEG to those for hard spheres whose radii are taken to be the PEG radii of gyration ($r_g$) listed in Table 3.1. Here $r_g$ is used as the relevant length scale for PEG/PEG interactions. The measured PEG second virial coefficients are the same order as those for equivalent hard spheres, which shows that the phosphate and acetate buffers are both good solvents for PEG.

Figure 3.6 presents static light scattering data for lysozyme and lysozyme/PEG
Figure 3.3: Cloud point temperatures ($T_{\text{cloud}}$) of lysozyme/PEG mixtures in the phosphate buffer are plotted as a function of PEG concentration for PEG molecular weights ranging from 400 to 35k g mol$^{-1}$ at fixed lysozyme concentrations as labelled. The data in panel c) were measured by T. Virmani. The data in panels a) and b) were repeated by S. Berejnov for verification. The lines are guides to the eye.
Figure 3.4: Static light scattering data for PEG1k and 8k solutions in the phosphate buffer and for PEG12k solutions the acetate buffer at T = 20°C. The filled squares (■) correspond to PEG1k, the open circles (○) to PEG8k and the filled triangles (▲) to PEG12k. The lines are fits to $Kc_2/R_2 = (1/M_2) + 2B_{22} \times c_2$, from which the PEG molecular weights ($M_2$) and second virial coefficients ($B_{22}$) are determined. The results are presented in Table 3.4 and agree well with previous results [41].

<table>
<thead>
<tr>
<th></th>
<th>$M_2$ [10^3 g/mol]</th>
<th>$N$</th>
<th>$B_{22}$ [10^{-3} mL mol/g^2]</th>
<th>$B_{22}/B^{HS}_{22}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG1k</td>
<td>0.97 ± 0.1</td>
<td>22</td>
<td>9.2 ± 1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>PEG8k</td>
<td>10.4±0.5</td>
<td>236</td>
<td>3.35±0.12</td>
<td>0.4</td>
</tr>
<tr>
<td>PEG12k</td>
<td>14.1±2.2</td>
<td>320</td>
<td>3.1±0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Table 3.3: PEG molecular weights ($M_2$) and second virial coefficients ($B_{22}$) taken from the data shown in Fig. 3.4 at T= 20°C are shown in the first and third columns. The equivalent hard sphere values were obtained as described in Section 3.2 using $r_g$ of PEG to determine $B^{HS}_{22}$. The $r_g$ values are listed in Table 3.1. The degree of polymerization $N = M_2/44$ is shown in the second column. The data for the PEG1k and 8k solutions was taken in the phosphate buffer and in the acetate buffer for PEG12k solutions.
Figure 3.5: Temperature dependence of second virial coefficients $B_{ii}$ [mL mol g$^{-2}$] vs $T$ [°C] for lysozyme, PEG 1k and PEG 8k in the phosphate buffer. The variation of $B_{11}(T)$ for lysozyme in the acetate buffer (not shown) is almost identical to that of lysozyme in the phosphate buffer. The lines are linear fits to the temperature variation of $B_{11}(T)$:

- $\partial B_{11}/\partial T = 1.0 \times 10^{-5}$ mL mol g$^{-2}$ °C$^{-1}$; $\partial B_{P1k}^{11}/\partial T = 8.8 \times 10^{-5}$ T mL mol g$^{-2}$ °C$^{-1}$; $\partial B_{22}^{P8k}/\partial T = 9.5 \times 10^{-5}$ T mL mol g$^{-2}$ °C$^{-1}$.

solutions with the scattering ratio $K_{c1}/R_{11}^{c1} - R_{2}$ plotted as a function of $c_1$. As outlined in Eq.(3.6), this ratio should depend linearly on $c_1$. From the fits to the data shown in Fig. 3.6 with no added PEG in the phosphate buffer, panels a) and b), we find $M_1 = 13800 \pm 500$ g mol$^{-1}$ and $B_{11} = -3.95 \pm 0.3 \times 10^{-4}$ mL mol g$^{-1}$ $\approx -0.7B_{11}^{HS}$. For lysozyme in the acetate buffer, panel c), we find that $M_1 = 13700 \pm 140$ g mol$^{-1}$ and $B_{11} = 4.87 \pm 0.2 \times 10^{-5}$ mL mol g$^{-1} \approx 0.1B_{11}^{HS}$. The mixed virial coefficients, $B_{12}$ and $C_{112}$, are obtained from linear fits to $\alpha$ and $\beta$ as functions of PEG concentration as described in Section 3.2 and shown in Figures 3.8 and 3.9. In the limit of no added polymer ($c_2 \rightarrow 0$) $\alpha$ is equal to $1/M_1$. However, Fig. 3.8 shows that we measured an $\sim 10\%$ variation in $\alpha$ for different experiments. We attribute this variation to error in measuring the scattering from the buffer with no solute as a background, $I_{\text{buff}}$ in Eq.(3.14), because the scattering from the buffer is so small. The results of the fits for $B_{12}$ and $C_{112}$ are shown in Table 3.4. We found $B_{12}$ and $C_{112}$ to be temperature
independent within experimental uncertainty. This means that the slopes of the linear fits in Fig. 3.8 are parallel for all temperatures within the large experimental error bars. The slope of the linear fit to the T= 40°C data in Figure 3.8 panel c) is the only line that may indicate some temperature variation of $B_{12}$ in the acetate buffer. The temperature variation of slopes of the linear fits in Fig. 3.9 with polymer concentration are accounted for by the temperature dependence of $B_{11}(T)$, according to Eq. (3.9). Table 3.4 also compares our measured values of the mixed virial coefficients, $B_{12}$ and $C_{112}$, to those of an equivalent hard sphere mixture, $B_{12}^{HS}$ and $C_{112}^{HS}$, calculated from Eqs.(1.10, 1.11). The lysozyme equivalent hard sphere has the radius $r_H$ and the PEG equivalent hard spheres have the radii $r_{eff}$ shown in Table 3.1.

Mixed second virial coefficients have previously been measured for lysozyme/PEG mixtures with light scattering by Prausnitz and co-workers [46] and with solubility experiments by Atha and Ingham [48]. The latter measured virial coefficients of lysozyme-PEG4k mixtures in 50mM Potassium Phosphate and 100mM Potassium Chloride pH=7.0 at T=23°C [48] and found that $B_{12}^{LYs-P4k} = 2 \times 10^{-4}$ mL mol g$^{-2}$. This value is comparable to our result for Lysozyme/PEG8k mixtures in a similar phosphate buffer, as shown in Table 3.4. Light scattering measurements by Prausnitz and co-workers [46] on lysozyme/PEG3.35k and PEG8k mixtures done in 50mM Potassium Phosphate Monobasic (KH$_2$PO$_4$) without fixing the pH found that [46] $B_{12}^{LYs-P3.35k} = 2.87 \times 10^{-4}$ mL mol g$^{-2}$ and $B_{12}^{LYs-P8k} = 3.4 \times 10^{-4}$ mL mol g$^{-2}$. These values are also commensurate with our measurements (Table 3.4). Since virial coefficients are sensitive to salt concentration and salt type the differences in the solvent conditions between our study and those employed by [46] can account for the quantitative difference in $B_{12}$ in these different buffers.

The variation of $\beta$ with PEG12k concentration in the acetate buffer is shown in Fig. 3.4.2 and compared with the data from Kulkarni et al. [31] taken in the same conditions. Our data do not display the non-monotonic variation of $\beta$ with
Figure 3.6: Static light scattering data is shown for lysozyme-PEG 8k and PEG 1k solutions in the phosphate buffer in panels a) and b), and for lysozyme-PEG 12k solutions in the acetate buffer in panel c), at T = 20°C. The lines are fits to Eq. (3.6) from which $\alpha$ and $\beta$ are obtained.
Figure 3.7: The variation of $\beta$ as a function of added PEG12k concentration $c_2$ is shown by the filled symbols for $T = 20, 30^\circ C$ in the acetate buffer. The solid lines represent the linear fits to Eq.(3.9) used to obtain the values of $C_{112}$ listed in Table 3.4. The open symbols were taken from Kulkarni et al. [31].

PEG concentration seen by Kulkarni et al. The multicomponent light scattering analysis given in Section 3.2.3 predicts a linear dependence of $\beta$ on $c_2$ which is seen in our data. It is not known why our data differ so markedly from those of ref. [31]. Kulkarni et al. made two assumptions in their light scattering analysis which may contribute to the discrepancy between our data and theirs. First, they assumed that $\alpha$ was independent of PEG concentration and equal to the inverse of lysozyme’s molecular weight. They used a fixed value of $\alpha$ to fit for the refractive index increment $n_1$ at different PEG concentrations. Secondly, they assumed that lysozyme/PEG mixtures could be treated as an effective one component solution and equated $\beta$ with the effective second virial coefficient whereas the multicomponent analysis of light scattering given in Section 3.2.3 shows that this is not correct.
3.5 Analysis

3.5.1 Need for 3rd Virial

The measured dependence of $\beta$ on PEG concentration shows that a virial expansion truncated at the $B_{ij}$'s is not sufficient. We see this from a contradiction arising by assuming $C_{112} = 0$ and examining the predictions of Eq.(3.9) regarding the light scattering data presented in Figures 3.8 and 3.9 with this assumption. In order to arrive at this contradiction set $C_{112} = 0$ in Eq.(3.9) assuming only pairwise interactions. We label the slope as $\beta'$ to indicate our working assumption that $C_{112} = 0$. Then the expression for polymer dependence of this quantity ($\partial\beta'/\partial c_2$) becomes:

\[
\frac{\partial \beta'}{\partial c_2} = -2B_{12}^2 M_2 + \frac{4B_{11}B_{12}M_1 M_2 n_2}{M_1 n_1} \tag{3.15}
\]

The second virial coefficient for lysozyme alone ($B_{11}$) in the Phosphate buffer is negative, as seen from Fig. 3.6. The mixed second virial coefficients ($B_{12}$) for lysozyme/PEG 1k and 8k mixtures are both positive as shown in Table 3.4. Therefore, the polymer concentration dependence of the slope should be negative for both lysozyme/PEG1k and lysozyme/PEG8k mixtures (i.e. $\partial\beta'/\partial c_2 < 0$). However, Figure 3.9 shows that experimentally $\partial\beta/\partial c_2 > 0$ for Lysozyme/PEG1k mixtures and $\partial\beta/\partial c_2 < 0$ for Lysozyme/PEG8k mixtures. This contradiction is shown graphically in Fig. 3.9. The double dashed line in Fig. 3.9 shows the prediction for $\partial\beta/\partial c_2$ assuming that $C_{112} = 0$ for the $T=40^\circ C$ data. The top panel shows that assuming $C_{112} = 0$ yields qualitatively the wrong slope for lysozyme/PEG 1k mixtures. This contradiction between the results of our assumption and experiments can be resolved by including the mixed third virial coefficient in calculating the scattered light from protein-polymer solutions.

One reaches the same conclusion by considering the polymer dependence of
Figure 3.8: The variation of $\alpha$, Eq.(3.8), as a function of PEG concentration ($c_2$) at different temperatures is shown for lysozyme/PEG1k and PEG8k solutions in the phosphate buffer in panels a) and b), and for lysozyme/PEG12k solutions in the acetate buffer in panel c). The lines indicate the linear fits to Eq.(3.8) used to find the $B_{12}$ values presented in Table 3.4.
the cloud point temperature shown in Fig. 3.3. If we make the same assumption as above ($C_{112} = 0$) then we find for the polymer dependence of the cloud point temperature

$$\lim_{\rho_2 \to 0} \frac{\partial T}{\partial \rho_2} = \frac{2B_{12}^2}{\partial B_{11}/\partial T} > 0$$

Figure 3.5 shows that $\partial B_{11}/\partial T > 0$ for lysozyme in the phosphate buffer. So $\lim_{\rho_2 \to 0} \frac{\partial T}{\partial \rho_2} > 0$ for all PEG molecular weights assuming $C_{112} = 0$. However, Fig. 3.3 shows clearly that this slope is negative for low molecular weight PEG. Therefore we must conclude again that $C_{112} \neq 0$. Including only second virial terms in the free energy of lysozyme/PEG mixtures cannot account for either our light scattering or cloud point temperature measurements.

### 3.5.2 Depletion is Not Sufficient

We now compare our results to the depletion model’s prediction for the effect of adding polymer to a protein solution. The Asakura & Oosawa model of ideal, penetrable polymers is not valid for PEG in our buffers because the PEG/PEG interactions are not negligible as shown by the values of $B_{22}$ in Table 3.4. Therefore, we compare our results for the mixed virial coefficients in lysozyme/PEG mixtures to those of a binary hard sphere mixture [6].

In order to model PEG in our solutions as effective hard spheres the PEG molecules must exist as separated coils in dilute solution, not as overlapping chains in a semidilute solution. The semidilute cross-over concentration for PEG, $c_2^s$, is estimated as the concentration at which there is one polymer molecule per spherical volume defined by the polymer’s radius of gyration [42]:

$$c_2^s = \frac{M_w}{N_A \left(\frac{4\pi}{3}\right)r_g^3}$$

(3.16)

The values of $c_2^s$ are listed in Table 3.5. For the lysozyme/PEG1k mixtures, all the
Figure 3.9: The variation of $\beta$ with polymer concentration ($c_2$) at different temperatures is shown for lysozyme/PEG1k and PEG8k solutions in the phosphate buffer. The lines are linear fits used to obtain the values of $C_{112}$, Eq.(3.9), shown in Table 3.4. The double dashed line shows the dependence of $\beta$ on PEG concentration assuming $C_{112} = 0$ for the T=40°C data as discussed in Section 3.5.1.
Table 3.4: Mixed virial coefficients \(B_{12}, C_{112}\) for lysozyme/PEG solutions obtained from fits to \(\alpha\) and \(\beta\), Eqs.(3.8) and (3.9) are shown. \(B_{12}\) and \(C_{112}\) were found to be independent of temperature. The equivalent hard sphere values were obtained as described in Section 3.2 using \(r_{\text{eff}}\) of PEG along with \(r_H\) of lysozyme to determine \(B_{12}^{\text{HS}}\) and \(C_{112}^{\text{HS}}\). The \(r_{\text{eff}}\) and \(r_H\) values are listed in Table 3.1. The error bars for the measured cross virial coefficients came from the linear least squares fits to the data in Figures 3.8 and 3.9 by Eqs.(3.8) and (3.9). The data for the Lysozyme/PEG1k and 8k mixtures was taken in the phosphate buffer and in the acetate buffer for Lysozyme/PEG12k mixtures.

<table>
<thead>
<tr>
<th></th>
<th>(B_{12}) ([10^{-4}\text{mL mol/g}^2])</th>
<th>(B_{12}/B_{12}^{\text{HS}})</th>
<th>(C_{112}) (10^{-4}\text{mL}^2\text{mol/g}^3)</th>
<th>(C_{112}/C_{112}^{\text{HS}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG1k</td>
<td>12.9±0.2</td>
<td>0.34</td>
<td>16.1±0.3</td>
<td>0.23</td>
</tr>
<tr>
<td>PEG8k</td>
<td>1.7±0.3</td>
<td>0.089</td>
<td>3.13±0.2</td>
<td>0.051</td>
</tr>
<tr>
<td>PEG12k</td>
<td>0.51±0.2</td>
<td>0.023</td>
<td>3.4±0.1</td>
<td>0.044</td>
</tr>
</tbody>
</table>

Table 3.5: Semidilute cross-over concentrations \(c^*_2\) for PEG calculated from \(c^*_2 = M_w/N_A \times 1/(4\pi/3r_g^3)\).

<table>
<thead>
<tr>
<th></th>
<th>(c^*_2) [mg mL(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG1k</td>
<td>223</td>
</tr>
<tr>
<td>PEG8k</td>
<td>45</td>
</tr>
<tr>
<td>PEG12k</td>
<td>28</td>
</tr>
</tbody>
</table>

light scattering data and cloud point measurements were taken well below \(c^*_2\). For the lysozyme/PEG8k and PEG12k mixtures, however, \(c^*_2\) falls in the concentration range used for light scattering measurements, but \(c^*_2\) is still larger than the PEG concentrations used to extract \(\lim_{c_2 \to 0} \partial T_{\text{cloud}}/\partial c_2\). Figure 3.4 shows that the light scattering data for all the PEG molecular weights and concentrations employed here are fit well by the one component light scattering equation, Eq.(3.7), which would not be true for significantly semidilute polymer solutions. These pieces of evidence, and the fact that this is only a crude estimate of \(c^*_2\), indicate that our PEG solutions remain in the dilute concentration range where polymer coils do not overlap.

Table 3.4 list the measured mixed virial coefficients for the lysozyme/PEG mixtures. The lysozyme/PEG mixed virial coefficients \(B_{12}\) for all three PEG molecular weights are less than those of equivalent hard sphere mixtures. Therefore, attractive interactions must exist between the protein and polymer or the repulsion must be
softer than that between hard spheres.

Table 3.4 also lists values for the polymer radius \( (r_2^+) \) obtained by equating \( B_{12}^{HS} \), Eq.(1.10), to the measured value of \( B_{12} \) and solving for the polymer size holding the lysozyme size fixed at \( r_H \). The values of \( r_2^+ \) are smaller than any reasonable measure of the polymer size. In the case of lysozyme/PEG12k mixtures \( r_2^+ < 0 \) demonstrating that purely repulsive interactions cannot account our measured mixed virial coefficients.

This result is consistent with previous work on lysozyme/PEG mixtures by Bhat and Timasheff [57], with Annunziata et al.’s [13] study of cloud point temperatures of bovine \( \gamma \)D crystallin/PEG mixtures and with work by by Abbott et al. [58]. In a series of investigations, Abbott et al. [59, 58] studied the partitioning of various proteins in two-phase PEG/dextran systems and the neutron scattering from BSA/PEG mixtures. Their experimental data are consistent with the presence of weak attractive interactions between BSA and PEG in addition to the depletion effect. Analysis of Abbott et al.’s data leads to the conclusion that a 0.05 \( k_B T \) attractive interaction exists between PEG monomers and BSA. In an earlier investigation Abbott et al. [59] showed that protein partitioning data in two-phase PEG/dextran systems for a number of different proteins also lead to the conclusion that attractions exist between the proteins and PEG. Bhat and Timasheff’s [57] densimetry studies of lysozyme/PEG solutions show that weak attractions compete with the depletion effect to determine the net protein/polymer interaction.

The measured virial coefficients shown in Table 3.4 allow us to calculate the variation of the effective lysozyme second virial coefficient with PEG concentration \( \partial B_{11}^{\text{eff}} / \partial c_2 \). Depletion theory predicts that adding PEG to a lysozyme solution induces attraction between lysozyme molecules leading to \( \partial B_{11}^{\text{eff}} / \partial c_2 < 0 \), as seen from Eq.(3.11). Conversely, if \( \partial B_{11}^{\text{eff}} / \partial c_2 > 0 \) then PEG induces repulsion between lysoyzme molecules. The dependence of lysozyme’s effective second virial coefficient
Figure 3.10: Variation of lysozyme’s effective second virial coefficient, $B_{11}^{\text{eff}}$, with polymer concentration ($c_2$), for lysozyme/PEG1k (●) and PEG8k mixtures (■) in the phosphate buffer. The closed symbols were obtained by using the measured values of the mixed virial coefficients listed in Table 3.4 in Eq.(3.11). The open symbols represent the hard sphere values obtained as described in Section 3.2. The insert shows an expanded view of the lysozyme/PEG1k and PEG8k data. In this view one sees that lysozyme’s effective second virial coefficient increases as a function of added polymer concentration.

$\left(\frac{\partial B_{11}^{\text{eff}}}{\partial c_2}\right)$ on added PEG concentration is shown in Figure 3.10. $\frac{\partial B_{11}^{\text{eff}}}{\partial c_2} > 0$ for both lysozyme/PEG1k and lysozyme/PEG 8k mixtures. We therefore conclude that adding PEG weakens the attraction between lysozyme molecules in contradiction to depletion theory.

### 3.5.3 Light Scattering vs. $T_{\text{cloud}}$

Now that the interactions in lysozyme/PEG mixtures have been determined by light scattering experiments and the collective behavior has been characterized by $T_{\text{cloud}}$, we ask whether or not the interactions combined with thermodynamic theory accurately describe the collective behavior. Figure 3.11 compares the change in the spinodal temperature with added polymer ($\lim_{\rho_2 \to 0} \frac{\partial T_{\text{sp}}}{\partial \rho_2}$) calculated from virial coefficients obtained from light scattering to the measured cloud point temperatures.
Figure 3.11: The symbols depict the mixed virial coefficients obtained by light scattering where the open square (□) corresponds to lysozyme/PEG1k mixtures and the closed square (■) to lysozyme/PEG8k mixtures in the phosphate buffer. The lysozyme concentration was fixed at $c_{\text{lys}} = 48 \text{ mg/mL}$. Cloud point measurements of $\lim_{\rho_2 \to 0} \partial T_{\text{cloud}} / \partial \rho_2$ were determined from the data shown in Fig. 3.3. Sets of $(B_{12}, C_{112})$ were obtained by fitting the measured $\lim_{\rho_2 \to 0} \partial T_{\text{cloud}} / \partial \rho_2$ to Eq.(3.3) using the value of $\partial B_{11}(T) / \partial T$ measured from Fig. 3.5. The solid line shows the contour $\gamma = 0$ and separates the parameter space such that $\lim_{\rho_2 \to 0} \partial T_{\text{cloud}} / \partial \rho_2 > 0$ above the line and $\lim_{\rho_2 \to 0} \partial T_{\text{cloud}} / \partial \rho_2 < 0$ below the line. The experimental $T_{\text{cloud}}$ measurements are shown as the vertically hatched region for PEG8k, where $\gamma > 0$ Eq.(3.4), and the horizontally hatched region for PEG1k, where $\gamma < 0$. The measurements of $\lim_{\rho_2 \to 0} \partial T_{\text{cloud}} / \partial \rho_2$ appear as regions rather than as lines due to experimental error.
at $c_{\text{lys}} = 48 \text{ mg/mL}$. The virial coefficients obtained from light scattering correctly reproduce the sign of $\lim_{\rho_2 \to 0} \frac{\partial T_{\text{cloud}}}{\partial \rho_2}$. However, the virial coefficients do not quantitatively agree with the range of cloud point variation. We attribute this discrepancy partly to the difference between the spinodal temperature ($T_{\text{sp}}$) and the cloud point ($T_{\text{cloud}}$). As discussed previously, $\gamma > 0$ for a hard sphere mixture for all values of $r_1$, $r_2$, and $\rho_1$, whereas Figs. 3.3 & 3.11 show that the sign of $\gamma$ varies with PEG molecular weight. Therefore a hard sphere model cannot account for the experimentally observed variation of the cloud point on polymer concentration, but the virial coefficients measured from light scattering can do so qualitatively.

The combined effect of varying the protein and polymer concentrations on the cloud point temperature is now investigated. The variation of $\lim_{c_2 \to 0} \frac{\partial T_{\text{cloud}}}{\partial c_2}$ with lysozyme concentration measured experimentally and predicted by Eq.(3.3) is shown in Fig. 3.12. Experimentally, $\lim_{c_2 \to 0} \frac{\partial T_{\text{cloud}}}{\partial c_2}$ is nearly independent of lysozyme concentration. This suggests that the physical considerations that determine $\lim_{c_2 \to 0} \frac{\partial T_{\text{cloud}}}{\partial c_2}$ are similar at high and low concentrations. The virial coefficients measured with light scattering predict the much larger variation for the spinodal temperature depicted by the lines in Fig. 3.12, especially for the lysozyme/PEG1k mixtures. The data for the cloud point temperature variation agree with the predicted dependence at the lowest protein concentration, but increasingly diverge as the protein concentration increases. We attribute this to the failure of a virial expansion to describe the free energy of protein/polymer mixtures at high protein concentrations. Therefore Fig. 3.12 demonstrates the need for a more complete thermodynamic model to describe lysozyme/PEG mixtures at all concentrations.
Figure 3.12: The dilute limit of the polymer dependence of the protein spinodal and binodal temperature is plotted vs. protein concentration for lysozyme/PEG mixtures in the phosphate buffer. The symbols depict $\lim_{c_2 \to 0} \partial T_{\text{cloud}} / \partial c_2$ as measured from Fig.3.3, where the filled circles (●) correspond to PEG1k and the filled squares (■) correspond to PEG8k. The lines come from using the cross virial coefficients displayed in Table 3.4 in Eq.(3.2) to calculate $\lim_{c_2 \to 0} \partial T_{\text{spinodal}} / \partial c_2$ as a function of $c_1$, where the dashed line corresponds to PEG1k and the solid line to PEG8k.
3.6 Conclusion

Adding low molecular weight PEG depresses $T_{\text{cloud}}$ whereas high molecular weight PEG raises $T_{\text{cloud}}$. This observation cannot be accounted for solely by the depletion attraction. Lysozyme/PEG interactions were characterized by virial coefficients obtained from light scattering experiments. It is demonstrated that the free energy must include third virial terms to qualitatively explain the data presented in this chapter. Depletion models predict that adding PEG induces an attraction between lysozyme molecules whereas light scattering revealed the opposite: PEG induces repulsion between lysozyme molecules. The measured mixed virial coefficients are smaller than those predicted for an equivalent hard sphere mixture and are consistent with attractions between lysozyme and PEG. Therefore, a complete model of the phase behavior and light scattering of lysozyme/PEG mixtures must account for both the entropic depletion effect and an energetic attraction between protein and polymer. The measured virial coefficients, combined with thermodynamic theory, predict the observed behavior of the cloud point demonstrating the consistency of these two independent sets of experiments and that measured interactions from virial coefficients can be used to predict the collective phase behavior in these protein/polymer mixtures.
Chapter 4

Light Scattering from PDC/PEG Mixtures

4.1 Introduction

As mentioned in Section 2.1, the second virial coefficient of water soluble proteins has shown to be correlated with soluble protein crystallization. Does this correlation also hold for membrane proteins? Hitscherich et al. [60] measured the apparent second virial coefficients of protein/detergent complexes (PDCs) as a function of added PEG concentration. Their results indicate that the PDC second virial coefficient appears to decrease as PEG is added and that crystallization occurs in roughly the same crystallization slot that George & Wilson [5] found for soluble proteins. In Chapter 3 the effect of adding PEG to solutions of a soluble protein, lysozyme, was studied to show that although the addition of PEG increases crystal nucleation rates, it does not induce attractions between lysozyme molecules. In this chapter the effect of adding PEG on PDC second virial coefficients is studied for PDCs composed of a chloride channel protein, ClC-ec1, and a non-ionic detergent, n-octyl-β-maltoside (OM). The interactions between PDCs in solution are studied by light scattering and related to
their collective behavior as represented by the crystallization process.

Detergents are needed to solubilize membrane proteins in aqueous environments due to the hydrophobic nature of membrane proteins’ transmembrane domains. One might then assume that the hydrophobic tails of the detergent molecules associate with the hydrophobic surface residues of the membrane protein. It has been shown that this is not the case for lauryl maltoside associating with bovine heart cytochrome c oxidase (CcO), by NMR studies [61]. These studies demonstrate that the polar heads of the detergent molecules associate with polar CcO residues so that the CcO is dressed with detergent micelles with the same micellar structure as without any protein present. CLC-ec1 was first expressed and purified by Maduke et al. [62] who reconstituted CLC-ec1 into liposomes by adding protein to mixed detergent/lipid micelles and then removing the detergent. This procedure results in CLC-ec1 being embedded in a lipid membrane. Two-dimensional crystals of CLC-ec1 embedded in lipid bilayers were obtained by Midell et al. [63] who used these crystals to perform electron microscopy experiments and obtain the low resolution structure of CLC-ec1. Single channel electrophysiological recordings of CLC-ec1 embedded in lipid bilayers were obtained recently by Accardi et al. [64]. These previous results suggest that that detergent molecules do dress the hydrophobic residues of CLC-ec1.

Rosenbusch [65] has suggested that membrane protein crystallization should be favored in conditions where the detergent moieties in PDCs show a phase transition because in such conditions the detergent micellar interactions are attractive. It has subsequently been shown that in a number of cases PDC crystallization is not associated with any detergent phase transition. Tanaka et al. [66] showed that cytochrome bc1 complex associated with the detergent sucrose monolaurate crystallized although no phase transition of the detergent was observed in the PDC crystallization conditions. Hitscherich et al. [60] note that PDCs composed of OmpF porin and a detergent mixture with n-octyl-β-glucoside and octyl-POE crystallize upon the addi-
tion of PEG but that no phase transition of the mixed detergent micelles is observed in the PDC crystallization conditions.

In addition to static light scattering, dynamic light scattering, NMR and neutron scattering studies have been carried out to investigate the interactions between PDCs in solution. Neutron scattering studies of reaction centers from *Rhodobacter sphaeroides* R-26 associated with the detergents lauryl-dimethylamine-N-oxide (LDAO) or *n*-octyl-β-glucoside (OG) were carried out by Marone et al. [67]. They found that although the addition of PEG to a dispersion of protein/detergent complexes (PDCs) induces crystallization, it does not lead to aggregation prior to crystallization. Tanaka et al. [66] performed dynamic light scattering studies of cytochrome *bc*₁ complex associated with sucrose monolaurate to measure the diffusion constant as a function of PDC concentration for different levels of added PEG. They modelled the PDC concentration variation of the diffusion concentration using the DLVO theory to model interactions and the variation of the diffusion constant. Their experiments find evidence that the addition of PEG induces attraction between *bc*₁ PDCs in crystallization conditions. A static light scattering study of PDCs was performed by Hitscherich et al. [60] on OmpF porin associated with a binary detergent mixture. Their results showed that the apparent second virial coefficient of OmpF porin PDCs decreases as a function of added PEG concentration.

Hitscherich et al. [60] and others [31] have analyzed the light scattering from two solute systems in an effective one solute framework. Such an analysis does not capture the subtlety of a two solute system and can lead to incorrect virial coefficient values as discussed in Chapter 3. In this chapter, the multicomponent analysis of light scattering data presented in Section 3.2.3 is applied to scattering from PDC/PEG mixtures. The non-ionic detergent OM was used to obtain crystals of CLC-ec1 by Dutler et al. [68], and is one of a family of sugar based detergents widely used for crystallization trials of membrane proteins. In all that follows we shall refer to the
PDCs with subscript 1 and PEG with subscript 2.

The two component PDC/PEG solution may also be viewed as an effective one component PDC solution as was done in Chapter 3. Effective interactions between PDCs in solution in the presence of PEG are characterized by the effective second virial coefficient $B_{11}^{\text{eff}}$ from Eq.(3.11).

4.2 Materials & Methods

CLC-ec1 was expressed and purified as described by Dutzler et al. [68] and Accardi et al. [64]. The detailed expression and purification protocols are given in the Appendix. The basics steps are as follows: transform the DNA sequence encoding CLC-ec1 into competent cells. Cell colonies from the transformation plates were then grown to a suitable level for expression. Protein expression was induced and then cells were collected by centrifugation. The cells were disrupted by sonication, and the protein was extracted with detergent. The initial protein purification was performed on a Cobalt column to which the CLC-ec1 C-terminal hexahistidine tag preferentially binds. The protein was then eluted with imidazole which competes for Cobalt binding and reconcentrated. After cleavage of the hexahistidine tag with an endoprotease, the resulting product was loaded on an size exclusion chromatography column where the endoprotease was separated and the detergent was exchanged. The resulting pure CLC-ec1 peak was collected and concentrated for use in light scattering experiments and crystallization trials. This three day expression and purification procedure typically resulted in 5-7 mg of pure protein from 6L of bacteria in growth medium.

In order to verify that our light scattering experiments were performed in CLC-ec1 crystallization conditions, crystals of CLC-ec1 were grown in sitting drops at $T = 20^\circ C$ by equilibrating a 1:1 mixture of protein and reservoir solution against the reservoir. The protein solution consisted of $20 \text{mg mL}^{-1}$ CLC-ec1 in 45mM OM,
75mM NaCl and 10mM Tris-HCl at pH 7.5. The reservoir solution consisted of 320 or 330 mg mL\(^{-1}\) PEG in 50 mM Na\(_2\)SO\(_4\), 50 mM Li\(_2\)SO\(_4\) and Tris 50mM at pH 8.5. As shown in Fig. 4.5 crystals were observed after one week. Crystals were not taken for diffraction studies but were assumed to be protein crystals. Sitting drops without added PEG showed no crystals.

\(n\)-decyI-\(\beta\)-maltoside (DM) (sol-grade) and OM (anagrade) were obtained from Anatrace. Poly(ethylene glycol) (PEG) of nominal molecular weight 400 g mol\(^{-1}\) was obtained from Sigma. The critical micellar concentration of OM in water is 9 mg mL\(^{-1}\) [69].

The protein, OM and PEG were dissolved in the published CLC-ec1 crystallization buffer [68] which is a (1:1) mixture of (75mM NaCl, 10mM Tris-HCl at pH 7.5 : 50mM Na\(_2\)SO\(_4\), 50mM Li\(_2\)SO\(_4\), 50mM Tris at pH 8.5). The CLC-ec1 concentrations were measured by UV absorption using an extinction coefficient \(\varepsilon_{280nm} = 0.85\) mL mg\(^{-1}\) cm\(^{-1}\) calculated from the sequence [62]. It should be noted that the value of the extinction coefficient does not effect the value of \(\beta\) since

\[
\beta = \frac{\partial(Kc/R)}{\partial c} = \frac{\partial(K[a \times c]/R)}{\partial[a \times c]}
\]

Refractive index increments were measured as described in Section 3.3. For PEG400 \(dn/dc = 1.33 \times 10^{-4}\) mL mg\(^{-1}\) independent of \(T\). For OM \(dn/dc = 1.07 \times 10^{-4}\) mL mg\(^{-1}\). For CLC-ec1/OM PDCs \(dn/dc\) was not measured in order not to waste precious PDC sample. Instead, we set \(dn/dc = 2 \times 10^{-4}\) mL mg\(^{-1}\). Using this value of \(dn/dc\) we find that \(M_1 = 1/\alpha = 1.11 \pm 0.04 \times 10^5\) g mol\(^{-1}\). This value for the PDC molecular weight is consistent with the molecular weight of the CLC-ec1 homodimer based on its sequence (1.02\(\times 10^5\) g mol\(^{-1}\)) added to the measured molecular weight of OM micelles to yield 1.16\(\times 10^5\) g mol\(^{-1}\). This procedure does not allow for measurement of PDC molecular weight and can only yield estimates of \(B_{11}\).
However, this procedure does not effect $\partial \alpha / \partial c_2$ or $\partial \beta / \partial c_2$, the necessary quantities for analyzing the effect of PEG on the PDC/PDC interactions.

The static and dynamic light scattering experiments (SLS & DLS) were performed as described in Chapter 3. Hydrodynamic radii $r_H$ were obtained as described in Section 3.2.4. When multiple diffusing species contribute to the time variation of the scattered intensity, the autocorrelation function becomes an integral of multiple exponentials:

$$g_1(t) = \int e^{-\Gamma t} G(\Gamma) d\Gamma$$

where $G(\Gamma)$ is the decay rate distribution function which can also be expressed as a function of decay times as $G'(1/\Gamma)$. The decay time distribution functions shown in Fig. 4.6 were calculated using the ALV-NonLin software which implements the CONTIN algorithm to calculate $G'(1/\Gamma)$ via an inverse Laplace transform of $g_1(t)$.

### 4.3 Results

The detergent used to crystallize CLC-ec1, OM, shows no phase transition as a function of temperature up to concentrations of greater than 500 mg mL$^{-1}$ when dissolved in pure water [69]. All detergent concentrations in this study were less than 50 mg mL$^{-1}$. Samples of OM at concentrations up to 50 mg mL$^{-1}$ in the ClC-ec1 crystallization buffer with added PEG400 165 mg mL$^{-1}$ were observed by bright field microscopy in the temperature range $2 \leq T \leq 95$ °C. No cloud point was observed indicating that no detergent liquid-liquid phase transition is associated with the crystallization conditions of CLC-ec1.

The critical micellar concentration ($CMC$) of OM was determined by SLS using the method described in ref. [60]. The scattered intensity was measured at a scattering angle of $\theta = 90^\circ$ as a function of OM concentration. A linear fit was made to the concentration dependence of the scattering. The $CMC$ was identified as the
Figure 4.1: The scattered intensity $I_s$ in arbitrary units at a scattering angle of ninety degrees from OM dissolved in the CLC-ec1 crystallization buffer with no added PEG at $T = 20^\circ$ C is shown. The $CMC$ was identified from a linear fit to $I_s$ as a function of OM concentration as the extrapolated point where $I_s = 0$. The point shown at an OM concentration of 10 mg mL$^{-1}$ is below the $CMC$ and was not included in the linear fit.

OM concentration where the scattered intensity was extrapolated to zero as shown in Fig. 4.1. The hydrodynamic radius ($r_H$) measured by DLS in the same OM samples was monitored simultaneously. $r_H$ drops sharply to approximately zero at the $CMC$ as shown in Fig. 4.2. For OM in the CLC-ec1 crystallization buffer at $T = 20^\circ$ C the $CMC$ was found to be 10.4 mg mL$^{-1}$ and 9.5 mg mL$^{-1}$ for no added PEG and PEG400 165 mg mL$^{-1}$ respectively. In order to measure the second virial coefficient of OM micelles, the micelle concentration is needed. The micelle concentration was taken to be: $c_{micelles} = c_{OM} - CMC$.

From light scattering experiments on PEG 400 g/mol we measure $M_2 = 400 \pm 100$ g mol$^{-1}$, $B_{22}(T = 20^\circ$ C)$=0.013$ mL mol g$^2$ and $\partial B_{22}/\partial T = -1.6 \times 10^{-4}$ mL mol g$^{-2}$ $^\circ$C$^{-1}$. The PEG 400 g/mol radius of gyration is too small to measure with static light scattering because $r_g$ is much less than the wavelength of light. We therefore used the procedure described in Section 3.2 to determine $r_g$. We measured $r_H = 0.7$
Figure 4.2: The hydrodynamic radius $r_H$ of OM micelles in the crystallization buffer with no added PEG at $T = 20^\circ$ C is shown. The CMC found from Fig. 4.1 and is shown as the dashed line.

nm for PEG 400 g/mol which results in $r_g = 1.1$ nm.

Light scattering experiments on OM at $T = 25^\circ$C without the addition of PEG showed a micellar molecular weight of $14286 \pm 1500$ g mol$^{-1}$ which yields a micellar aggregation number of approximately $N_{OM} = 31$. At this same temperature $\beta = -6 \times 10^{-4}$ mL mol g$^{-2}$ and $\partial \beta / \partial T = 1.7 \times 10^{-5}$ mL mol g$^{-2}$ °C$^{-1}$. The dependence of $\beta$ for OM micelles on PEG concentration and temperature is shown in Figure 4.3. $\beta$ does not vary linearly with PEG concentration indicating that Eq.(3.6) does not accurately capture the light scattering from these mixtures. The OM micelles’ hydrodynamic radius as a function of PEG concentration shows a marked increase at $c_{P400} = 250$ mg/mL in Figure 4.4 panel b) indicating that the OM micelles expand. At the same time, however, $\alpha$ shows a marked increase indicating that the OM micelles’ molecular weight decreases as the micelles become less tightly associated at high added PEG concentration.

The values of $\alpha$ and $\beta$ for CLC-ec1/OM PDCs are given in Table 4.1 without P400 and with the concentration of P400 needed for crystallization. The values of $\beta$
Figure 4.3: $\beta$ for OM micelles is shown as a function of both temperature and PEG 400 g/mol concentration as indicated. $\beta$ displays a non-linear dependence on PEG concentration indicating that Eq.(3.6) does not accurately describe the light scattering from the OM micelles/PEG mixtures.

Figure 4.4: The PEG 400 concentration $c_{P400}$ dependence of $\alpha$ (panel a)) and $r_H$ (panel b)) obtained from light scattering experiments are shown for OM micelles. Both show non-linear dependence on PEG concentration indicating the destabilization of OM micelles at high PEG concentration.
Table 4.1: $\alpha$ and $\beta$ determined by light scattering for CLC-ec1/OM protein/detergent complexes at $T = 25^\circ$C are shown without any added P400 and in the presence of P400 at crystallization conditions. Cross-virial coefficients were determined from these values using Eqs.(3.8,3.9).

<table>
<thead>
<tr>
<th>$c_{P400}$ [mg/mL]</th>
<th>$\alpha$ [$10^{-6}$mol/g]</th>
<th>$\beta$ [$10^{-4}$mL mol/g$^2$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>165</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

both with and without added PEG are positive indicating that $\beta$ for CLC-ec1/OM PDCs does not fall into George & Wilson’s [5] crystallization slot of negative $\beta$ values. Fitting Eqs.(3.8) and (3.9) to the values in Table 4.1 allows calculation of the mixed virial coefficients: $B_{12} = -4.2 \times 10^{-3}$ mL mol$^{-2}$ g and $C_{112} = 4.8 \times 10^{-3}$ mL$^2$ mol$^{-3}$. For any net repulsive interaction $B_{12} > 0$, the measured value of $B_{12} < 0$ indicates that PDCs feel a net attraction to PEG molecules. The measured values of $B_{12}$ and $C_{112}$ allow us to calculate the variation of the effective virial coefficient for CLC-ec1/OM PDCs with added PEG concentration by Eq.(3.12). We find that $\partial B_{11}^{\text{eff}} / \partial c_2 = 2.9 \times 10^{-4}$ mL$^2$ mol$^{-3}$. This positive value for $\partial B_{11}^{\text{eff}} / \partial c_2$ shows that although $\beta$ for PDCs decreases as a function of increasing PEG concentration, this does not necessarily indicate that PDC interactions become more attractive due to the multicomponent nature of the solutions. As noted in Section 3.2.3, since $\partial B_{11}^{\text{eff}} / \partial c_2 > 0$ the PDC interactions become more repulsive as a function of added PEG concentration. Therefore, the depletion attraction discussed in Section 1.1.2 does not apply to these PDC/PEG mixtures. Although the PDC interactions become more repulsive as PEG is added, PEG does induce PDC crystallization as shown in Figure 4.5.

DLS experiments showed that PEG induces formation of aggregates on the time scale of hours whereas macroscopic crystal growth occurs on the time scale of many days. Figure 4.6 displays the decay time distribution function obtained by DLS experiments on CLC-ec1/OM PDCs in the crystallization buffer with a PEG400 concentration of 165 mg mL$^{-1}$. The distribution function approximately 12 hours after the final purification step shows a single peak indicating individual PDCs existing in
solution. As shown in panel b) of Figure 4.6 at later times, a peak at a more than ten times longer decay time appears. This second peak grows with time as the first peak shrinks with time indicating that PDCs aggregate as a function of time with added PEG. With no added PEG the initial decay time peak remains constant and no second peak appears as shown in panel a) of Figure 4.6.

4.4 Conclusion

Protein detergent complexes (PDCs) composed of a chloride channel protein, CLC-ec1, and a non-ionic surfactant, OM, were studied with static and dynamic light scattering experiments in conditions which yielded CLC-ec1 crystals. The apparent second virial coefficients of these PDCs do not fall in the crystallization slot found for soluble proteins. No phase transition of the detergent micelles was associated with PDC crystallization. It was found that PEG400 induces PDC aggregation on
Figure 4.6: The decay time distribution function obtained by dynamic light scattering, Eq.(4.1) for CLC-ec1/OM micelles in the crystallization buffer at $T = 20^\circ C$ is shown in panel a), with out added PEG, and in panel b) with PEG400 165 mg mL$^{-1}$, for different times. Panel a) shows that the distribution function does not change from the initial time (■) to 14 hours after the initial time (open circles). Panel b) shows that adding PEG to a dispersion of CLC-ec1/OM PDCs induced PDC aggregation. In panel b) the decay time distribution is shown two hours after the initial time (open circles) and four hours later (filled triangles). At later times, the peak at the long decay time (3 msec) grows and the peak at the short decay time (0.2 msec) decreases indicating the growth of aggregates possibly associated with crystallization. The initial time in both panels (■) corresponds to approximately 12 hours after the final purification step and to the start of the crystallization trials.

short times scales compared to the crystal growth time scales. PEG400 was found to induce repulsion rather than attraction between non-aggregated PDCs indicating that the depletion attraction does not describe the effect of adding PEG to these PDC solutions.
Chapter 5

On the Mechanisms of Granular Rod Vortex Formation

5.1 Introduction

Driven granular materials form a variety of patterns and display a host of dynamic instabilities [70, 71, 72]. Many experimental studies of granular materials have focused on spherical or grain-like particles such as glass beads, sand and metal powders. Recently, a number of studies of anisometric granular materials have expanded the gamut of observed behaviors and generated interest in understanding the differences between the behavior of spherical and anisometric particles. As discussed in Section 1.1.3 and Chapter 6 Philipse [15] studied random granular rod packings and found that the aspect ratio controls the packing fraction. Villaruel et al. [73] investigated the packing of short rods in a narrow container and found that the walls induce vertical alignment and subsequently a layered packing. Stokely et al. [74] studied piles granular rods confined to two dimensions showing that jamming leads to voids on many length scales and that the number of voids decreases with increasing void size. As discussed in Chapter 6, random packings of granular ellipsoids were investigated
by Donev et al. [75] who showed that ellipsoids can pack to higher filling fractions than spheres and that such packings of ellipsoids have higher average co-ordination than sphere packings. Compaction of granular rods in very narrow containers was studied by Lumay & Vandewalle [76] who showed that the packing fraction of rods has a minimum for containers whose size is equal to the rod length and that long rods align along container walls much like spaghetti in a pasta box. The dynamic patterns formed by vertically vibrated single layers of rods in wide containers were studied by Blair et al. [77] who observed that rods spontaneously align vertically and undergo vortex motion. Blair et al. mapped the phase diagram of vertical alignment and vortex formation for aspect ratio twelve rods as a function of excitation strength and rod filling fraction. They measured the fraction of vertically oriented rods as a function of time, mapped out the velocity field of rods in a vortex and measured the phase diagram of rods for one aspect ratio as a function of excitation strength and rod filling fraction. Blair et al. proposed a void filling mechanism for the spontaneous vertical alignment of rods. Small voids can be filled by vertically aligned rods whereas large voids can be filled only by horizontally aligned rods. Blair et al. noted that if the initial number of voids decreases with void size, as it does in two-dimensions [74], then rods will spontaneously align vertically as a function of time filling the small voids. Following Blair et al.’s study of vortices Volfson et al. [78] performed detailed investigations of the motion of tilted rods in an annulus and found that the friction between a tilted rod and the driving plate leads to the vortex motion. In this chapter experimental investigations of the crucial role of rod geometry and material properties of the rods on vertical alignment and vortex formation are made.
5.2 Materials & Methods

Experiments were performed in a square container with 9.1 cm side lengths and a circular container with a 6 cm diameter. Therefore the container size ($l_{\text{cont}}$) to rod length ($L$) ratio varied from $l_{\text{cont}}/L=30$ for $L/D = 4$ rods to $l_{\text{cont}}/L=4$ for $L/D = 48$ rods where $D$ is the rod diameter. Granular rods were cut from round aluminum wire whose density is $\rho_{\text{Al}} = 2.6$ g mL$^{-1}$ and nylon fishing line (Berkeley) whose density is $\rho_{\text{nylon}} = 1.25$ g mL$^{-1}$. A Schleuniger (RC 3250) cable cutter cut the wire into pieces of well defined length $L$. The measured polydispersity in length was $\Delta L/L \leq 1\%$. The rods were vertically excited by an electromagnetic shaker. A schematic of the experimental apparatus is depicted in Fig. 5.1. A key control parameter is the aspect ratio of the rods $L/D$. Another control parameter is the dimensionless filling fraction of rods $\phi = N/N_{\text{max}}$ where $N$ is the number of rods in the container and $N_{\text{max}}$ is the number of rods corresponding to one layer of triangularly packed, vertically aligned rods. For a circular container $N_{\text{max}} = (\pi/\sqrt{12})(l_{\text{cont}}/D)^2$. An accelerometer (Analog Devices) measured the vertical acceleration of the container as a function of time. We denote the dimensionless acceleration as $\Gamma(t) = a(t)/g$ where $g$ is the acceleration of gravity. The maximum downward acceleration is labelled as $\Gamma_{\text{max}}$.

5.3 Results

We find that for $L/D < 4$ vertical alignment does not occur at any $\phi$. For $L/D \geq 4$ we find that rods spontaneously align vertically. We tested alignment up to $L/D = 48$. Vortex formation occurs for all $L/D \geq 5$. No long lived vortices were observed for $L/D = 4$ and we were unable to determine whether or not transient vortices form at the highest densities for $L/D = 4$. Figure 5.3 shows that $\phi_{\text{min}}$ decreases as a power law with increasing $L/D$. This dependence was best fit by: $\phi_{\text{min}}(L/D) = (L/D - 3)^{-1/3}$. We find no discernable dependence of $\phi_{\text{min}}$ on container size. The data points shown
Figure 5.1: A schematic of the electromagnetic shaker used to vertically vibrate granular rods is shown. $a(t)$ represents the accelerometer which measures the vertical acceleration as a function of time. L.B. represents a rigid linear bearing (Nippon Bearing) used to eliminate horizontal motion of the shaft connecting the shaker to the sample holder.
Figure 5.2: Pictures of vortices and vertically aligned rods for $L/D = 7$ at $\phi = 0.63$ in panel a) and for $L/D = 36$ at $\phi = 0.32$ in panel b).

in Fig. 5.3 at $L/D = 6$ and 12 for two container sizes $l_{\text{cont}}/L=10$ and 15 showed the same $\phi_{\text{min}}$ within experimental error demonstrating that $\phi_{\text{min}}$ does not depend on $l_{\text{cont}}/L$ when the container size is large enough. We expect $\phi_{\text{min}}$ to depend on container size for small containers. Note that we find $\phi_{\text{min}} = 0.48$ for $L/D = 12$ at $\Gamma_{\text{max}} = 4$ whereas Blair et al. [77] found $\phi_{\text{min}} = 0.35$ for the same $L/D$ and $\Gamma$ with oxidized copper rods. We attribute this to the difference between the surface properties of the aluminum rods in our experiments and the copper rods used by Blair et al.

All the rods we tested and those used by Blair et al. (copper, aluminum and nylon) vertically align and form vortices but the $\phi$ at which vertical alignment and
vortex formation occur depend on the specific rod material. We performed experiments on \( L/D = 12 \) rods cut from nylon fishing line. We purposefully did not control for static charge and found that highly charged nylon \( L/D = 12 \) rods vertically aligned and formed vortices only at \( \phi_{min} = 0.8 \), a higher value than for rods cut from aluminum or copper rods. However, the addition of bronze powder (Acupowder grade 61A) reduced the minimum packing fraction for vertical alignment for nylon rods to \( \phi_{min} = 0.6 \). Therefore, friction between rods affects \( \phi_{min} \). Additionally, we investigated vertical alignment in two binary rod mixtures of \( L/D = 8 \) and 16 and \( L/D = 8 \) and 24 rods. In these mixtures rods vertically align and form vortices and stay mixed. Blair et al. [77] showed that vertical alignment and vortex formation for \( L/D = 12 \) rods occurs in a narrow region of the \( \Gamma \) vs. \( \phi \) phase space. The results presented in this chapter show that vertical alignment and vortex formation occur over a wide range of \( L/D \) values and rod properties.
Vertical vibration experiments were repeated using discrete vertical excitations, taps, in place of continuous vibration, shaking, to test whether or not continuous rod motion is necessary for vertical alignment and vortex formation. Accelerometer traces are shown in Fig. 5.4 for shaking in panel a) and tapping in panel b). Tapping was implemented using a single period of a sine wave followed by approximately thirty periods of no excitation. During the period of no excitation, all granular rods come to complete rest. After coming to rest, the rods are accelerated by the subsequent tap. We found that tapping induces vertical alignment of granular rods for the same values of $L/D$ as for shaking. However, no vortex formation was observed when tapping. Tapping led to significantly more heaping and convection of the rods than shaking did although it is unclear why this is so. Heaping is when the rods preferentially pile up on one side of the container. It was observed that heaping occurs even when the container is unlevelled so that movement to the heaped side is uphill. The term convection refers to situations where convection rolls form in the rod samples. In convection rods move primarily along these convection rolls. Small (10 rods) and transient regions appear which qualitatively resemble vortices when tapping but heaping and convection destroy these regions before they form fully developed vortices. We therefore conclude that the specific dynamics of vertical excitation do not affect the spontaneous vertical alignment of granular rods, but that competing affects do not allow vortex formation.

It was observed that tapping prepares samples of rods for vortex formation. Tapping aligns the rods. If the form of excitation is subsequently changed from tapping to shaking we observe that the time between the initiation of shaking and vortex formation decreases significantly compared to when shaking is used with an initially unaligned rod sample. We therefore conclude that the specific dynamics of vertical excitation do not affect the spontaneous vertical alignment of granular rods.

Air pressure can have a significant effect on driven granular systems [72, 79]
Figure 5.4: Accelerometer traces of the normalized acceleration as a function of time ($\Gamma(t)$) are shown for continuous shaking in panel a) and tapping in panel b).
which are many grain sizes deep. Experiments on size segregation in driven systems of spherical grains by Möbius et al. [72] show that the interstitial air between the grains can play a large role due to the high pressures which are developed when the grains impact the container and compact. In order to investigate whether or not air pressure has an effect on vortex formation, we repeated the experiments for $L/D = 12$ rods in the circular container after evacuating the container to a final air pressure of approximately 6 torr. Since all these experiments were performed on single layers of rods, we expect the interstitial air to play a much less significant role than for dense samples which are many layers thick. The lowered air pressure did not effect the vertical alignment of rods or the formation of vortices as expected.

5.4 Conclusion

Dynamic vortex formation and vertical alignment in single layers of granular rods were studied as a functions of rod aspect ratio, rod packing fraction and rod material. A minimum rod aspect ratio of $L/D = 4$ was found to be necessary for vortex formation and the minimum rod packing fraction needed for vertical alignment was found to decrease as a function of increasing rod aspect ratio. Vertical alignment and vortex formation were found to occur with rods of different densities, friction coefficients and charges. Air pressure was found not to effect vortex formation or vertical alignment. Additionally, it was found that continuous vibration was needed for vortex formation, but rods vertically align when all rods are allowed to come to rest between vertical excitations. Therefore, rods’ geometry is shown to strongly influence the collective dynamic behaviors of spontaneous alignment and vortex formation whereas the specific nature of the rods’ surfaces plays a secondary role.
Chapter 6

On the Co-ordination of Granular Rods

6.1 Introduction

In any random packing, the number of touching neighbors per particle, or the co-ordination number is an important quantity because contacts between particles provide the necessary mechanical constraints to ensure a stable pile. Recently it has been shown that random packings of nearly spherical ellipsoids show a significantly different co-ordination number than that of spheres [75]. In this chapter the average co-ordination number for random packings of rods is measured as a function of the rod aspect ratio. The average co-ordination number measures the number of touching neighbors rods have in a packing. The only interactions in a static granular packing occur through contacts, therefore the co-ordination number captures information about the interactions in a given packing. A statistical theory of granular rods is needed in order to relate the co-ordination number to the collective behavior of the packing. The random contact model discussed in Section 1.1.3 provides this theoretical framework. The co-ordination number measurements will be coupled with
measurements of the collective behavior of granular rod packings and subsequently compared with the predictions of the random contact model.

The average co-ordination number for a given pile is:

$$\langle z \rangle = \frac{\sum_z n_z z}{\sum_z n_z}$$

(6.1)

where $n_z$ is the number of rods with $z$ neighbors. The fraction of rods which have a given co-ordination number is $\gamma_z = n_z / \sum_z n_z$ so that the normalization condition $\sum_z \gamma_z = 1$ is met. $\gamma_z$ as a function of $z$ is the co-ordination number distribution function.

In an isostatic, or minimally rigid, pile the number of constraints, or contacts per particle, equals the number of force and torque balance equations [80]. For an isostatic packing of frictionless spheres in $d$ dimensions the number of contacts equals the number of force balance equations $\langle z \rangle = 2d$. For frictional spheres each contact determines two equations so that $\langle z \rangle = d + 1$ [81]. This result holds for frictional particles of any shape so that in 3 dimensions, the isostatic frictional limit yields $\langle z_{\text{iso}}^{\text{fric}} \rangle = 4$ [82]. For frictionless rods in 3 dimensions: $\langle z_{n}^{\text{rod-iso}} \rangle / 2 = 3 + 2$ so that $\langle z_{n}^{\text{rod-iso}} \rangle = 10$.

Bernal & Mason [83] measured $\langle z_{\text{sph}}^{\text{sph}} \rangle = 6.4$ for a random packing of spheres. Their result for $\langle z_{\text{sph}}^{\text{sph}} \rangle$ is close to the isostatic limit for frictionless spheres. Their method was to pour spheres into a container, pour paint into the container, drain the paint, allow the pile to dry and then count the number of spots on each sphere without any paint. Such spots represent touching neighbors. This is a tedious process. More than forty years later Donev et al. [75] made the subsequent experimental measurement of $\langle z_{\text{M&M’s}}^{\text{M&M’s}} \rangle = 9.8$ using Bernal & Mason’s method on oblate ellipsoids with an aspect ratio of 2 (M&M’s Candies [84]), which is near the isostatic limit for frictionless spheroids. These two results on random packings of spheres and ellipsoids...
indicate that either excluded volume rather than friction provides the stability of these packings or that these are very overdetermined frictional packings. Donev et al. [75] also performed frictionless simulations of random packings of ellipsoids and showed that $\langle z \rangle$ has a minimum for spheres and increases sharply as spheres are deformed into ellipsoids. Their simulations demonstrate that $\langle z \rangle \sim 10$ for maximally jammed random [85] packings of frictionless prolate and oblate ellipsoids at an aspect ratio of approximately two where the aspect ratio of an ellipsoid is defined as the ratio of its major to minor axes. Their simulation results lend further evidence to the possibility that excluded volume rather than friction provides stability in random packings.

Simulations of granular rods in two dimensions [74] show that $\langle z \rangle$ decreases as the rod aspect ratio increases towards an asymptotic value of $\langle z^{2d} \rangle = 3.2$. Philipse & Verberkmoes presented a frictionless and purely geometric argument for random packings of rods in three dimensions and calculated a lower bound of $\langle z \rangle = 5$ [86]. Williams & Philipse [87] performed frictionless monte carlo simulations of random rod packings in three dimensions and found that $\langle z \rangle$ decreased towards the frictional isostatic limit with increasing rod aspect ratio.

As mentioned in Section 1.1.3, Philipse [15] presented experimental data on compacted piles of granular rods showing that the product of the pile’s volume fraction ($\phi$) and the ratio of the rod’s length (L) to the rod’s diameter (D) is a constant $\phi \times (L/D) = c$, for long, thin rods ($L/D > 20$) meaning that low volume fractions can be achieved experimentally ($\phi \approx 0.06$ for $L/D = 96$). As discussed in Section 1.1.3 Philipse [15] showed that the random contact model, described in Section 1.1.3, predicts this scaling. In this chapter the RCM is experimentally tested by measuring all the quantities in Eq.(1.19).
6.2 Materials & Methods

Compaction experiments were performed to measure \( \phi \) as a function of \( L/D \) by pouring rods into a container and then compacting the pile by tapping, as described in Chapter 5 at a maximum acceleration of five times gravity until the height of the pile reached a steady state value. Cut pieces of tinned bus wire were used for compaction experiments and for the co-ordination number experiment with \( L/D = 3 \). A Schleuniger RC 3250 cable cutter cut spools of round wire to make the rods. These wire pieces have \( \Delta L/L = 1\% \) polydispersity in length. Each experiment was repeated three times and the volume fraction was taken to be the average of the three trials. A steady state was reached after typically 500-1000 excitations. The evolution of the piles’ volume fraction as a function of number of excitations is shown in Fig. 6.1. Note that the rods in these piles never aligned vertically in a layered packing as did the \( L/D = 3 \) rods in the experiments of Villaruel et al [73]. Long rods form highly jammed piles in which local particle rearrangements are constrained. Rods in the bulk of such piles do not rearrange when the pile is vertically vibrated. Of course, even large aspect ratio rods will flow align when poured into very small containers as do pieces of spaghetti in a thin box [76]. The volume fraction of the compacted piles scales as \( \phi_{\text{comp}} \times (L/D) = 5.4 \pm 0.3 \) as shown in Fig. 6.3, which is consistent with previous results [15], and for uncompacted piles as \( \phi_{\text{uncomp}} \times (L/D) = 4.2 \pm 0.2 \) as shown in Fig. 6.2.

The co-ordination number \( \langle z \rangle \) for random rod packings was measured using Bernal & Mason’s method. Wooden toothpicks and bamboo skewers with circular cross-sections were used as the granular rods in piles for which \( \langle z \rangle \) was measured. The toothpicks measure 0.2 cm in diameter and 6.5 cm in length with pointed ends. The bamboo skewers measured 0.26 cm in diameter and were cut to 4 and 13 cm lengths to provide \( L/D=15 \) and 50 rods. The measured polydispersity in the toothpicks’ length was approximately 2\% and 3\% for the bamboo skewers. We used Jujube
Figure 6.1: The volume fraction $\phi$ of piles of granular rods is shown as a function of the number of vertical excitations for $L/D = 12, 32, 64$ and 96.

Figure 6.2: The dependence of the initially poured, uncompacted volume fraction ($\phi_{\text{Uncomp}}$) as a function of $L/D$ is shown. The data from bulk compaction experiments are shown as open circles and the data from co-ordination number experiments are shown as filled squares. The solid line is $\phi \times (L/D) = 4.2$. 
Figure 6.3: The dependence of the final, compacted volume fraction ($\phi_{\text{Comp}}$) as a function of $L/D$ is shown. The data from bulk compaction experiments are shown as open circles and the data from co-ordination number experiments are shown as filled squares. The solid line is $\phi \times (L/D) = 5.4$.

candies [88] as $L/D=1$ rods. Jujubes approximate the shape of right cylinders of $L/D=1$. The polydispersity of Jujubes is $\Delta L/L = 20\%$ and $\Delta D/D = 10\%$. Those rods which contacted the container walls or exposed upper surface of the pile were categorized as boundary particles and those which did not were categorized as bulk particles. The compacted pile of toothpicks used to measure $\langle z \rangle$ after removal from the container is shown in Fig. 6.4. The boundary particles are clearly aligned whereas the bulk particles appear to be randomly oriented. The measured co-ordination number distribution for this pile of toothpicks is shown in Fig. 6.5. The co-ordination number distributions are qualitatively similar for all rod aspect ratios and pile volume fractions. The center of the distribution shifts as a function of aspect ratio but the width of the distribution remains constant within experimental error. As shown in Fig. 6.5, the co-ordination number distribution for boundary particles is shifted to a lower value of $\langle z \rangle$ relative to that for bulk particles and is slightly asymmetric due to the lower limit on $z$. Rods which contact the boundary must have fewer contacts.
Figure 6.4: The compacted pile of $L/D = 32$ rods is shown after painting, drying and removal from the container. Panel a) shows the entire pile and panel b) shows the pile after the boundary rods have been removed. The scale bars indicate 1 cm.

with other rods than rods which do not contact the boundary which explains the shift to a lower $\langle z \rangle$ value. The asymmetry in the co-ordination number distribution for boundary rods is due to the presence of a lower cutoff: no rods were observed to have $z < 2$, whereas no upper cutoff independent of $L/D$ was observed. Table 6.1 lists the measured values of $\langle z \rangle$ for compacted and uncompacted piles of different aspect ratio rods as indicated including only the bulk rods.

### 6.3 Results

Fig. 6.6 shows the dependence of $\langle z \rangle$ on aspect ratio for our experimental data as well as for the data on spheres from Bernal & Mason [83], on ellipsoids from Donev et al. [75] and Williams & Philipse’s simulation results [87]. The average co-ordination number for random rod packings increases as a function of aspect ratio from $\langle z \rangle \sim 6$ for small aspect ratio rods and asymptotes at $\langle z \rangle \sim 10$ for $L/D > 30$. As shown in Table 6.1 we also find that $\langle z \rangle$ increases when a random packing of rods is compacted. Although $\langle z \rangle \sim \langle z^\text{rod-iso} \rangle$ for compacted piles of long aspect ratio rods, these piles are certainly not isostatic because the rods are frictional and the piles were compacted. Amazingly, the pile of $L/D = 50$ rods at $\phi = 0.105$ has the same value of $\langle z \rangle$ as the pile of aspect ratio two oblate M&M’s at $\phi = 0.685$ which is more than six
times more dense. Fig. 6.6 shows that $\langle z \rangle$ for rods increases gradually as a function of aspect ratio whereas Donev et al. show that $\langle z \rangle$ increases approximately ten times more steeply for ellipsoids. Why do packings of rods and ellipsoids behave so differently? One hypothesis is that the geometrical difference between a cylindrical and an ellipsoidal particle leads to the observed differences in $\langle z \rangle$ for random packings. Another possibility is that friction between rods leads to this striking difference in the dependence of $\langle z \rangle$ on aspect ratio for rods and ellipsoids. Simulations which could systematically vary the friction would be able to discriminate between these two hypotheses.

Philipse [15] noted that random packings of sufficiently high aspect ratio rods form solid-like plugs. One of the original motivations for performing the experiments described in this chapter was to test whether or not the value of the co-ordination number is related to plug formation. We observed plug formation only for compacted piles composed of rods with $L/D > 44$ as shown in Fig. 6.7. The plugs become more
Table 6.1: The co-ordination number $\langle z \rangle$ is listed as a function of rod aspect ratio $L/D$. Only the bulk rods are included. The column labelled as “Uncomp” corresponds to the uncompacted random rod packings whereas the column labelled as “Comp” corresponds to the compacted packings.

<table>
<thead>
<tr>
<th>$L/D$</th>
<th>Uncomp</th>
<th>Comp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>6.2</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>5.7</td>
</tr>
<tr>
<td>15</td>
<td>6.5</td>
<td>7.0</td>
</tr>
<tr>
<td>32</td>
<td>7.2</td>
<td>9.8</td>
</tr>
<tr>
<td>50</td>
<td>8.3</td>
<td>9.8</td>
</tr>
</tbody>
</table>

Figure 6.6: The co-ordination number $\langle z \rangle$ is shown as a function of the aspect ratio on a logarithmic scale. The closed symbols represent experimental data from random packings and the open symbols represent simulation data. The squares are from our compacted piles and the diamonds represent our experimental data for uncompacted piles. The bowtie is taken from Bernal & Mason’s experiment on spheres [83]; the inverted triangles are from Williams & Philipse’s simulation of rods [87] and the circles are data from Donev et al.’s experiments and simulations on ellipsoids [75].
rigid as the aspect ratio of the rods increases. For all the plugs it was observed that rods are free to translate along their long axis. Rotation of a plug results in many rods falling out along the direction of their long axis. We measured \( \langle z \rangle \) for the plug forming pile of bamboo skewers at \( L/D = 50 \) shown in Fig. 6.8. The data in Table 6.1 show that \( \langle z \rangle \) is larger for the plug forming pile at \( L/D = 50 \) than for the uncompacted pile which did not form a solid-like plug. However, this value of \( \langle z \rangle = 9.8 \) was observed for the compacted pile at \( L/D = 32 \) which did not form a plug and for M & M’s with aspect ratio of two [75] which shows that a certain value of \( \langle z \rangle \) is necessary but not sufficient for plug formation. The co-ordination number distribution functions for the compacted piles at \( L/D = 32 \) and 50 are compared in Fig. 6.9. These distribution functions are almost identical with the only difference between the plug forming pile at \( L/D = 50 \) and the non-plug forming pile at \( L/D = 32 \) being the appearance of a second peak in the distribution function for \( L/D = 50 \). The distribution functions shown in Fig. 6.9 are similar to the distribution function measured for M&M’s [75] so the appearance of this second peak in \( \gamma(z) \) for \( L/D = 50 \) is likely not significant.

We conclude that the mechanism of plug formation in piles of granular rods remains unilluminated by these \( \langle z \rangle \) measurements.

The data from Figs. 6.2, 6.3 and 6.6 allow us to answer whether or not the RCM’s scaling prediction, Eq.(1.19), is borne out by experiments. The left-hand side of Eq.(1.19) is plotted in Fig.6.10 for bulk particles in compacted piles and demonstrates that experimental piles of granular rods follow the RCM scaling in the long rod limit. The RCM assumes that contacts between rods are uncorrelated and that rods are randomly oriented. Fig.6.10 shows strong deviation from the RCM scaling for short rods meaning that either the rods in these piles are not oriented randomly or that contacts between the rods are correlated. We attribute the deviation from RCM scaling for short rods to correlations of contacts between rods since we did not observe any qualitative ordering of the piles. However, the orientational
Figure 6.7: Piles of granular rods of sufficiently large aspect ratio ($L/D > 44$) form solid-like plugs as mentioned by Philipse [15]. Panel a) shows a compacted pile of $L/D = 40$ rods which does not form a plug. Panel b) shows a compacted pile of $L/D = 48$ rods which does form a plug which displays extensional rigidity. Note that in panel b) the surface normal of the plug deforms with mild applied stress as indicated by the lines. Panel c) shows a compacted pile of $L/D = 64$ rods which forms a more rigid plug than the pile of $L/D = 48$ rods. The pile of $L/D = 64$ rods can be rotated as a solid body as shown and the surface normal does not deform under mild applied stress.

Figure 6.8: The compacted pile of $L/D = 50$ rods forms a solid plug. The pile is shown before $(z)$ was measured. The white scale bar indicates 5cm.
Figure 6.9: The co-ordination number distribution function $\gamma(z)$ is shown for the compacted pile of $L/D = 32$ rods (filled squares) and for the compacted, plug forming pile of $L/D = 50$ rods (open circles). As shown in Table 6.1, the measured value of $\langle z \rangle$ is the same for these two piles. Comparing their co-ordination number distribution function shows only one statistically significant difference: the appearance of a second peak in $\gamma(z)$ for $L/D = 50$. The small increase at $\gamma(z = 15)$ for $L/D = 50$ is not statistically significant.
Figure 6.10: The ratio \( \frac{\langle z \rangle}{2\phi(L/D)} \) from co-ordination number experiments is shown as a function of \( L/D \) for compacted piles. The RCM predicts that this ratio equals unity for long rods which is validated by the experimental data.

distribution function of rods was not measured.

### 6.4 Conclusion

The average co-ordination number \( \langle z \rangle \) for piles of granular rods was measured as a function of rod aspect ratio as an indicator of rod interactions. \( \langle z \rangle \) increases with increasing aspect ratio. This increase of \( \langle z \rangle \) with rod aspect ratio was markedly slower than that observed in simulations of ellipsoidal particles. The co-ordination number reaches \( \langle z \rangle \sim 10 \) in the large aspect ratio limit, \( L/D \geq 30 \). Independent measurements were made of the piles’ bulk volume fraction \( \langle \phi \rangle \) in these random rod packings as a probe of the collective behavior of these piles. Coupling these two sets of data together shows that the random contact model accurately describes these piles in the long rod limit.
Appendix A

Expression and Purification of CLC-ec1

In this appendix the procedure used to express and purify the membrane protein CLC-ec1 is stated. These protocols were developed in Professor C. Miller’s laboratory at Brandeis University following the protocols developed by R. Dutzler in Professor R. MacKinnon’s laboratory at Rockefeller University. These protocols require three days to complete. The protocol for the first day should be commenced in the afternoon and requires approximately 1.5 hours. The second day’s protocol begins in the morning and requires most of the day but not constant attention. The third day should be started early in the morning and requires the entire day and constant work. The third day is busy. Make sure to read through the entire protocol carefully before beginning as many of the steps are time sensitive.
A.1 Day1 - Transformation and Plating

A.1.1 Transformation

1. Transform competent D3 cells according to protocol given by the cells’ provider using approximately 10ng of DNA (C terminal His tag eriC Wild Type) for 50μL of cells. Cells stored in $T = -80^\circ$C freezer.

1b. Alternatively, use pretransformed cells stored in glycerol stocks. These cells may be obtained from members of C. Miller’s laboratory.

A.1.2 Plating

1. Plate approximately 200 μL of suspended cells in LB or TB on each LB-Ampicillin agar plate. Use one plate per liter of cell growth media.

2. Incubate plates at $T = 37^\circ$C about 12 hours, but not more than 16 hours.

A.2 Day2 - Cell Growth and Protein Expression

A.2.1 Cell Growth

1. Check the plates from Day1 to make sure that the cell colonies have grown on the agar plates. Use 1L of TB in 2L flasks for cell growth.

2. Inoculate TB by adding 2mL of Ampicillin (50 mg/mL) solution to each 1L of TB. Preheat TB flasks in $T = 37^\circ$C incubator.

3. Scrape cell colonies from plates using TB or LB making sure to suspend cell colonies homogenously in media. Place equal amount of suspended cells into each TB flask.

4. Measure absorbance in each flask. Grow cells until the absorbance is $1.6 \leq abs \leq 1.9$. Cell growth usually takes approximately 3 hours. Note that cell growth is exponential so monitor absorbance closely near the end of the growth period.
A.2.2 Protein Expression

1. When cells have grown to the proper absorbance, induce with 1mL of αTC (0.2mg/L) and again add Ampicillin as before.
2. Induce for 3 hours. Measure absorbance again after induction. Absorbance should stay at the same level as after growth or decrease slightly indicating that cells have not grown during induction. If absorbance increases during induction it means that protein was not expressed properly because cells continued to grow instead of expressing protein.
3. After expression, spin cells down at 5000 r.p.m. for 20 minutes.
4. Discard supernatant and remove pellet from centrifugation bottles with a metal spatula into 100 mL steel beaker or just leave in centrifugation bottles. Place pellet in T = 4°C environment overnight.

A.3 Day3 - Protein Extraction and Purification

**Stocks:** 200 mM Tris-HCl pH 7.5 5M NaCl 4M Imidazole-HCl pH 7.5 pepstatin and leupeptin (0.1 mg/mL each in 95% Ethanol

**Buffers:** Breaking Buffer (BB) 50mL 50mM Tris-HCl / 100mM NaCl (spatula each of lysozyme and DNase I

2X Cobalt Wash Buffer (2XWB) 50mL 40mM Tris-HCl / 200mM NaCl / 20mM sol-grade decyl-maltoside (DM) pH 7.5

Cobalt Wash Buffer (WB) 50mL Dilute 2XWB with equal volume of water

Cobalt Imidazole 30mM Buffer (Im30) 40mL Make this from 4M Im stock ,2XWB and water

Cobalt Imidazole 400mM Buffer (Im400) 15mL Make this from 4M Im stock, 2XWB and water

FPLC Buffer (FB) 150 mL 10mM Tris-HCl / 150mM NaCl / 45mM octyl-maltoside
A.3.1 Extraction

1. Lift the soft pellet from the centrifuge tubes with metal spatula and place into 100 mL steel beaker which is resting on ice. Scrape and wash the remainder of the pellet off the bottle with 20mL BB.

2. Make smooth suspension of pellet with a 25mL stereological pipette and electric pipetter. Then add the remainder of the BB and the lysozyme and DNase. Record the total volume which should be about 60-70mL.

3. Sonication - high-intensity ultrasound is used to break the cell membranes. Check that the sonicator is tuned properly. Before sonication make add 4mg of PMSF protease inhibitor to 0.5mL of the pepstatin-leupeptin stock and ready it to add into the beaker during sonication. Place sonicator tip so that it stays near the bottom of the beaker without contacting the beaker. Make sure beaker is well surrounded by ice to avoid heating. Sonicate for 1 minute intervals, 5 times stopping between each interval to allow the suspension to cool completely. During the first interval add the 0.5mL of pepstatin-leupeptin-PMSF. Sonication should not cause much foam to appear but should visibly disturb the suspension’s surface. If foam appears, readjust sonicator tip.

4. Weigh out sol-grade DM for 50mM final concentration in suspension. Place sonication suspension into rhomboid tubes and add the weighed out DM into these tubes.

5. Extract by gentle shaking for 2 hours. It will take a few minutes for the dry DM to fully dissolve.

A.3.2 Purification I - Cobalt Column

1. After extraction, pour the mixture into JA-20 rotor tubes (or others) and centrifuge at 16,000 r.p.m. for 45 minutes.
2. While the extraction mixture is spinning, prepare the Cobalt column: Use approximately 1mL of Cobalt chromatography slurry per liter of bacteria growth media so 6mL for a 6L preparation. Pour column by hand and flush with water. Also flush absorbance detector with water.
3. Equilibrate Cobalt column with 5-10 column volumes of WB with DM.
4. When the extraction mixture has finished the centrifugation step, pour the supernatant off which contains the protein. Discard the pellet.
5. Load the supernatant from extraction onto the Cobalt column at \( \frac{2}{7} \text{mL/minute} \) flow rate. When the entire supernatant volume is on the column, switch back to flowing WB.
6. When the brown color clears from the eluted flow, start flowing into the absorbance detector and start the chart recorder to measure the absorbance at 280nm wavelength.
7. When the absorbance settles down to a baseline value switch to Im30. Run 30-40 mL of Im30 through at a flow rate of 2mL/min. You should see a peak of nonspecific protein eluting. ClC-ec1 will remain bound to the column.
8. Now elute the CLC-ec1 by switching to Im400 and collect the peak of CLC-ec1 into a prewashed (with WB) 15mL centricon 50K cutoff centrifugal concentrator tube.
9. Centrifuge the centricon tube at 3200 rpm for 20-60min until the protein volume is reduced to \( \frac{0.4}{10} \text{mL} \).
10. While the protein solution is concentrating flush the column and absorbance detector with water, then a 20% ethanol mixture. Leave the column apparatus in air and close off the absorbance detector with the 20% ethanol solution inside.

A.3.3 Purification II - His-tag Cleavage

Add LysC protease, 0.5U total (100\( \mu \text{L} \)) from aliquot in freezer. Let the protease cut for 1 hour at room temperature. Sample is now ready to load on the FPLC Superdex 200 column to separate CLC-ec1 from any remaining contaminants including the C
terminal histadine tag cleaved by the LysC protease.

A.3.4 Purification III - FPLC

1. Make sure to follow all the Miller lab protocols associated with the FPLC. Someone from the Miller lab should guide you through the FPLC usage in order to ensure proper procedures are followed.
2. Before Cobalt column step start flushing Superdex 200 column with water and then equilibrate the column with filtered and degassed FB.
3. Inject no more than 0.25mL of sample for each run. Run sample through column at a flow rate of 1mL/min
4. Main CLC-ec1 peak elutes at 11-13 mL. Collect the rising phase of the peak and the falling phase of the peak in different tubes. Use only the rising phase to ensure maximum purity.
5. Flush column according to prescribed FPLC running procedures.
6. Concentrate final, purified CLC-ec1 sample using a 50K cutoff centrifugal filter to reduce the volume to less than 0.5mL. Sample is now ready for crystallization or light scattering studies.
7. If PEG is to be added, this may be done by dialyzing overnight against a buffer containing PEG with an appropriate dialysis membrane. If PEG is added light scattering studies must be carried out as soon as possible as PEG causes irreversible aggregation of the CLC-ec1/OM complexes.
Bibliography


[84] M &M’s Candies are a registered trademark of Mars Inc.


[88] Jujubes are a registered trademark of The Hershey Company.