The Pennsylvania State University

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Department of Chemical Engineering

EXAMINING INITIAL BACTERIAL ADHESION:
ORIENTED ADHESION AND SURFACE NANODOMAINS

A Thesis in

Chemical Engineering

by

Joseph F. Jones

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The thesis of Joseph F. Jones was reviewed and approved* by the following:

Darrell Velegol  
Associate Professor of Chemical Engineering  
Thesis Advisor  
Chair of Committee

Seong H. Kim  
Assistant Professor of Chemical Engineering

Costas Maranas  
Donald B. Broughton Professor of Chemical Engineering

John M. Regan  
Assistant Professor of Environmental Engineering

Andrew L. Zydney  
Walter L. Robb Family Chair and Professor of Chemical Engineering  
Head of the Department of Chemical Engineering

*Signatures are on file in the Graduate School
ABSTRACT

Initial bacteria adhesion is the first step in the process of biofilm formation, affecting areas as diverse as infection on biomedical implants to the efficiency of bioremediation strategies to industrial biofouling. *Escherichia coli* were studied to better understand the orientations, time scales, and length scales of initial adhesion. The *E. coli* K-12 D21 strain was found to initially adhere via a single end of the rod-shaped cells. This adhesion was found to be instantaneous (< 1 second). By comparison with strains of varying lipopolysaccharide (LPS) layer, the LPS molecules were determined not to be the adhesive molecules. As well, with Brownian dynamics simulations, the end-on adhesion found for D21 could not be explained simply by geometric effects. The existence of bacterial polarity, a localization of molecules at the pole of the cell, was supported with surface charge nonuniformity measurements. The extent of adhesion within a population of D21 cells was found to be $15.9 \pm 3.4\%$ via the end, and the development of a single adhesive end was connected to the cell division process. The surface of D21 was hypothesized to be more complex than a localization of molecules at a single end with smaller nanodomains distributed across the cell body. The threshold area of the adhesive end and the smaller nanodomains was estimated as $0.016 \, \mu m^2$. Though *E. coli* D21 predominantly adhere end-on via a single end, the surface characteristics of D21 were shown to change significantly with differing growth conditions. The end-on adhesion effect did not readily extend to two *Burkholderia cepacia* strains, but the paradigm of examining the bacterial surface as discrete nanodomains when discussing adhesion remains supported.
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Though the action is by no means a worthy enough one, I dedicate this work to Francis M. Jones.


Chapter 1

Motivation and Research Goals

1.1 Motivation

Bacteria are ubiquitous organisms with estimates of $4 - 6 \times 10^{30}$ cells globally (15), existing in practically every known location and climate. Though the majority of prokaryotes are found free floating in the ocean, a significant amount of bacteria are adhered to solid surfaces (15). From implanted biomaterials and infections (3) to industrial equipment fouling (1) and ship hull colonization (7), adhered bacteria, particularly in the form of biofilms, have a significant environmental, medical, and economic impact.

The advancement of biomedical technologies has led to the nearly commonplace surgical implantation of artificial devices in humans. These implants are often plagued with the adhesion of cells, causing blood clots, rejection of implants, and most often, infection (8). Surfaces must be developed that passively enter the body and nondestructively remain operational. As well, the healing process and the means of infection must be understood to provide an effective implementation of biomedical devices without harm to the recipient. A clear understanding of how bacteria adhere to surfaces is necessary for such developments.

Demand for potable water has become a global issue on a scale exceeding the natural availability of fresh water supplies (14). Traditional wastewater treatment
methods are no longer able to meet the demands in some areas, leading to the exploration
and implementation of indirect wastewater reuse projects (2, 13). The impact of bacteria
in aquifers used for water reuse as both natural treatment agents and as pathogenic agents
needing removal is significant (13). Bacterial adhesion is important for both and
underlies the effective production of safe potable water via traditional means or via
augmentation of existing water supplies with reused water.

Bacterial adhesion is pivotal in the development of effective strategies for the
bioremediation of aquifers. Many bacteria are capable of degrading pollutants. However, limitations in bacterial transport through soils, i.e. bacteria do not travel far
from the point of injection into the soil (4, 5, 9), prevent the economical and efficient
application of this method of pollution cleanup. The strong adhesion of bacteria to soil
particles is often thought to be the cause of this limitation. The ability to probe and
understand the molecular level of environmental systems is essential for solving the
complex environmental problems of this type (11).

Additionally, bacterial adhesion to industrially relevant surfaces causes significant
problems in efficient operation of many systems. In marine environments, bacteria
adhere to the hulls of ships causing increased drag and decreased fuel efficiency (7). As
well, bacteria aid in the degradation of surfaces subjected to marine exposure. A surface
layer of bacteria is the precursor to the attachment of larger organisms, which also cause
increased drag and surface degradation. Bacterial adhesion extends to industrial
manufacturing surfaces from the food industry to chemical heat exchangers (1), again
causing significant logistical and economical concerns.
Bacterial adhesion research is fueled by the simple fact that bacteria appear capable of adhering and surviving at almost any solid surface. Though the majority of bacterial adhesion issues are associated with biofilm formation, biofilms tend to be robust structures not responsive to antibiotics (3) and other removal methods (12). The prevention and control of bacterial adhesion potentially exists with the initial stages of bacterial adhesion prior to biofilm formation. Understanding the mechanisms and time and length scales of bacterial adhesion might allow for the control of bacterial adhesion. This control might occur either by the engineering of surfaces for adhesion or the bacterial surface.

1.2 Research Goals

This research aimed to add to the understanding of initial bacterial adhesion. The overall question posed for this research is how does initial adhesion occur for the model bacterium *Escherichia coli* to nonbiological surfaces not treated for specific interactions? The specific goals of this research were to determine:

1) **The orientation of initial adhesion and the likely molecules involved in this adhesion for *E. coli* bacteria.**

2) **The geometric contributions to initial adhesion orientation by developing and applying a Brownian dynamics simulation (BDS) of the interaction of rod-shaped and spherical colloids.**

3) **The extent of end-on adhesion within a population of cells.**

4) **The time scales and length scales involved with end-on adhesion.**
5)  The variability of end-on adhesion under differing growth conditions and among other species.

A multitude of research tools, both experimental and computational, were used in this research, and the research draws from many bodies of knowledge. The diversity in approach to accomplish the proposed research goals is reflective of the complexity involved in bacterial adhesion.

1.3 Overview of Thesis

This thesis follows a somewhat chronological development of the research involved in understanding the initial adhesion of *E. coli* bacteria. Chapters 4, 5, 6, and 7 are derived directly from published, submitted, or soon to be submitted technical manuscripts. The structure of the thesis is such to accommodate the nature of these publications. A brief literature review is provided in Chapter 2, adding to the literature reviews present in each major chapter. As well, a material and methods section in Chapter 3 expands upon and adds clarity to the material and methods explained in each major chapter. All relevant copyright information is contained in each chapter. Where needed, a “Preface” section has been added to each chapter to add fluidity and clarity to the overall document. For the summaries of each chapter to follow, the abstract from the technical publication is used.
1.3.1 Chapter 4: Oriented Adhesion of *E. coli* to Polystyrene Particles

The adhesion of non-flagellated *Escherichia coli* K12 strain to polystyrene (PS) latex spheres or glass capillaries has been observed using several techniques. Attention was focused on the orientation of the rod-shaped bacteria as they adhered to the surfaces in 100 mM PBS. Data show that PS particles adhered to the ends of the bacteria more than 90% of the time. Moreover, the PS particles adhered to one end only, never both. Similarly, for experiments with bacteria adhering to glass, the bacteria adhered end-on. In order to determine whether the end of a bacterium had a different charge density from the middle, rotational electrophoresis experiments were used. These experiments indicated no measurable charge nonuniformity. In order to examine how strongly adhered the bacteria were to the surfaces, differential electrophoresis was used. Almost always, bacteria were found to be irreversibly adhered to the PS spheres. The cause of the oriented adhesion is not likely due to surface lipopolysaccharides (LPS), since the three strains of K12 that were used – each having a different length of LPS – showed similar behavior. The results are discussed in terms of bacterial cell polarity. The data indicate that nanodomains on the bacterial ends are important, and that the time scale for irreversible adhesion is short.

1.3.2 Chapter 5: Orientation Effects for the Irreversible Adhesion of Spherical Particles to Spheroidal Collectors

When a sphere adheres to a spheroid irreversibly, it is often of interest to know the orientation on the spheroid where the sphere adhered. We have performed Brownian
dynamics simulations to predict the orientation of adhesion, with variables being the dimensions of the sphere and spheroid. As expected, when the spheroid has a high rotational diffusion coefficient, the spherical particle adheres to the end of the spheroid. We tested our model for two experimental systems: 1) oppositely-charged colloidal systems (spheres and spheroids) and 2) *E. coli* K-12 D21 adhesion to spheres, for which the spheres have previously been reported to adhere only to one end of the bacterium. Experiments agree with predictions for case (1), supporting the simulations, while case (2) is shown not to agree with predictions. The case (2) result shows that the end-on adhesion of the bacteria is not purely a physical phenomenon.

### 1.3.3 Chapter 6: Laser Trap Studies of End-on *E. coli* Adhesion to Glass

Rod-shaped *Escherichia coli* K-12 D21 bacteria were previously found to adhere by their ends (poles) (10). In the current study we used a Nd:YAG 1064 nm laser trap to quantify the fraction of adherent bacteria and the time scale for the adhesion to occur. For the *E. coli* studied, 15.9 ± 3.4% of the bacteria adhered when presented end-on for 15 seconds to a cleaned glass surface that was not treated for specific interactions. These bacteria were found to adhere either instantaneously (approximately < 1 second) or not at all, and the adhesion was shown to be independent of power (force) of the laser trap. Additionally, for a given bacterium, either 0 or 1 ends were adhesive, never both ends. It is hypothesized that the end-on adhesion of D21 is related to bacterial polarity that dynamically results from the division process. We studied the reattachment of cells after adhesion and subsequent removal, finding that most bacteria reattach, some at least 5
times. However, a small fraction of D21 did not reattach after the first removal. Bacterial cells with observable division planes were tested for end-on adhesion; none of the 18 cells studied adhered by either end. On the other hand, we examined 50 daughter cells immediately after division, and 4 of the cells were adhesive. End-on adhesion is shown to be an important initial adhesion strategy for the *E. coli* strain via a single end with adhesion occurring instantaneously. Knowledge about adherent nanodomains (here, on one end) on bacteria will lead to better predictions of sticking coefficients and bacteria transport through porous media.

1.3.4 Chapter 7: End-on Adhesion Effect in Initial Bacterial Adhesion: Bacteria Odds and Ends

The initial adhesion of *E. coli* bacteria has been shown to occur predominantly via a single adhesive end of the rod-shaped cell with bacterial polarity hypothesized as a probable cause(10). Charge nonuniformity light scattering (CNLS) is used to measure charge nonuniformity on the surface of *E. coli* D21 cells. These bacteria are shown to exhibit significant charge nonuniformity at mid-exponential growth with the symmetric zeta potential difference ($\zeta_2 - \zeta_1$) equal to $7.9 \pm 0.40$ mV. The D21 bacteria also exhibit charge nonuniformity in stationary growth, but to a lesser extent with a $\zeta_2 - \zeta_1$ of $3.8 \pm 0.43$ mV. In addition to the bacterial polarity of D21, supported by CNLS studies, the existence of molecular localizations (of the same molecules) of smaller area are hypothesized to exist on the body of the cell. Particle adhesion orientation studies show an abrupt shift in adhesion with decreasing particle radius, i.e., a significant reduction in
end-on adhesion, around a particle radius of 0.5 microns consistent with this hypothesis. 

The threshold area of these smaller localizations is estimated as roughly 0.016 \( \mu m^2 \).

Examining end-on adhesion for other species, *Burkholderia cepacia* strains G4 and ENV435 were tested for adhesion orientation to sulfated polystyrene (PS) latex particles, exhibiting end-on adhesion of 75.5% and 79.8%, respectively, for the adhesive cells. However, *B. cepacia* exhibited end-on adhesion to a lesser extent than *E. coli*, and particles adhered to both ends in many cases. Though bacterial polarity consistent with a single adhesive end for D21 is supported, this phenomenon is dependent on growth conditions and does not readily extend to other species.

### 1.3.5 Summary of Appendices

The appendices add more detail to the content of the main thesis. Detailed information on bacteria growth and safety is contained in Appendix A. The algorithms and analyses used in this work are contained in Appendix B. Finally, the development and validation of the charge nonuniformity light scattering (CNLS) technique is detailed in Appendix C. Appendix C is derived from a published manuscript, and the abstract from that manuscript follows in Section 1.3.6.

### 1.3.6 Appendix C: Charge Nonuniformity Light Scattering

Classical models for colloidal forces assume that the particles are uniformly-charged. However, charge nonuniformity can decrease suspension stability and reduce
the accuracy of colloidal assemblies. Our lab group has previously seen that polystyrene latex particles are often nonuniformly-charged, through the experimental technique of “rotational electrophoresis” (6). Even though rotational electrophoresis is unique in finding zeta potential nonuniformity on individual particles, the technique is currently time-consuming and for practical reasons is limited to particles larger than 1 micron in size. We now introduce a modification of the rotational electrophoresis technique: “charge nonuniformity light scattering” (CNLS). CNLS takes advantage of two effects: 1) nonuniformly-charged particles will rotate in an applied electric field, and 2) light scattering from anisotropic particles changes when the particles align, an effect already used in the electric light scattering technique. By “visualizing” rotational electrophoresis with light scattering and interpreting the results with electrokinetic modeling, CNLS enables a measurement of zeta potential distribution for a colloidal suspension of particles with anisotropic geometry. In order to test the method, we synthesized model colloidal doublets composed of spheres with zeta potentials $\zeta_1$ and $\zeta_2$. Using translational electrophoretic mobility experiments, we found the difference $\zeta_2 - \zeta_1$ for our doublets. We then conducted experiments with CNLS, which gave the same value of $\zeta_2 - \zeta_1$, verifying the accuracy of the technique. Thus, CNLS allows for much more rapid measurements of charge nonuniformity and will soon be applied to more challenging systems.
1.4 References


Chapter 2

Literature Review

2.1 Introduction

Chapters 4 – 7 contain individual sections accounting for a “Literature Review” section typically in the form of an “Introduction.” The content of each “Introduction” addresses the literature appropriate to the individual chapters. This general “Literature Review” section expands upon and adds clarification to the content of the sections to follow. In particular, the concept of initial adhesion, the details of the cell surface membrane, and the relevant techniques used to study bacterial adhesion are addressed in this section. Table 2-1 details the various topics contained in the individual chapter literature reviews, and these sections should be referenced for the various topics listed.

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Table 2-1: Table of literature review information contained in each chapter. Chapters 4-7 are derived directly from published, submitted, or to be submitted manuscripts, and therefore contain literature reviews relevant to each chapter.
2.2 Initial Bacterial Adhesion

Bacterial adhesion has been studied scientifically for over 70 years. Mudd and Mudd first recognized the differences in adhesive characteristics associated with bacterial species by studying phase partitioning of cells (40). Claude Zobell pioneered research in marine microbiology and biofilm formation during the 1930’s and 40’s (76, 77), truly beginning the study of bacterial adhesion. The research of bacterial adhesion spans many disciplines as well, from colloid science to dental research to wastewater treatment.

The process of bacterial adhesion has been often described to have an initial, reversible phase followed by a long-term, irreversible phase (36, 75, 76). The approach of a bacterium to a surface followed by adhesion to the surface by physiochemical forces details the events of initial adhesion. Long-term adhesion is affected by such processes as the manufacture of extracellular polymeric substances (EPS) (66, 72), changes in surface organization (12), production of DNA (71), cell-signaling (17), and/or bond aging (61), which aid in permanently adhering a bacterium to a surface and begin the process of biofilm formation. Interactions between bacterial surface appendages (pili, flagellae) (20, 59) or bacterial surface polymers (LPS) (7, 27) and the surface may aid in this transition.

Focusing on initial adhesion, the DLVO theory, borrowed from colloidal science, has often been used to describe bacterial adhesion (46). This theory couples electrostatic (52) and van der Waals forces (34, 44), providing repulsion and attraction respectively, in a distance dependent force model. Additionally, the DLVO theory has been extended in
its application to bacterial adhesion to include acid base interactions (42), and this theory has been applied to bacterial systems (41, 70). Since most bacteria and the natural and manmade surfaces to which bacteria adhere have negatively charged surfaces, the electrostatic forces are repulsive (46). Additionally, hydrophobic forces often provide attractive forces between bacteria and surfaces (43, 54), though these forces are less well understood. All of the above forces constitute non-specific forces. Specific forces are receptor ligand interactions (8). However, for systems involving bacteria and inanimate surfaces, interaction forces are generally assumed to be entirely non-specific (8).

The initial adhesion of bacteria is the first step in the formation of biofilms. A biofilm is a colony of bacteria embedded in a polymer matrix, consisting of extracellular polymeric substances (EPS) exuded by the cells, which are adhered to a surface (3, 35). The biofilm community provides structure, nutrient accessibility, and some protection from detrimental environmental elements to the individual bacteria (35). It is the robustness of biofilms that causes the significant concern over bacterial adhesion.

The ultimate prevention and control of biofilm formation and bacterial adhesion resides in the understanding of initial bacterial adhesion. This research worked with model systems in controlled environments in order to begin to discern the process of initial adhesion. The adhesion of rod-shaped bacteria to nonbiological surfaces (polystyrene particles and glass), not treated for specific interactions, was studied. The mechanisms of adhesion as well as the time and length scales of initial adhesion are the focus of this work.
2.3 Bacterial Outer Membrane

This research involves the study of adhesion, and therefore an important consideration is the surfaces involved. The surfaces to which the bacteria adhere are polystyrene colloidal particles and glass surfaces, both of which are not treated for specific, i.e., stereochemically specific (8), interactions and are used as model surfaces. The other major surface involved is the bacterial cell surface.

2.3.1 Gram Negative Cell Envelope

When discussing the surfaces of bacteria, the Gram positive and Gram negative cell membranes must be distinguished. Exploiting differences in size of the peptidoglycan (a molecule giving rigidity to the cell) layer allows differentiated visualization of the two types of cells using the well-known Gram staining procedure (33). All bacteria used for experimentation in this research are Gram negative, and thus, the Gram negative cell membrane will be discussed exclusively. Gram negative bacteria (*E. coli, B. cepacia*) have an internal lipid bilayer surrounding the cytoplasm (cell interior), a thin (several nanometers) peptidoglycan layer, and then an outermost lipopolysaccharide (LPS) layer. A schematic of the Gram negative cell membrane is shown in Figure 2-1. The LPS layer is roughly a phospholipid bilayer with phospholipid molecules towards the cell’s interior and LPS molecules extending into the bulk. Lipopolysaccharides consist of lipid molecules (forming the bilayer) bonded to a polysaccharide chain extending into the bulk. These molecules comprise approximately
75% of the Gram negative outer membrane (48) with the rest proteins. Thus, the Gram negative cell surface is charged, hydrophobic, and heterogeneous in composition.

Figure 2-1: Schematic of Gram negative bacterial cell wall (33). The interior of the cell would be below the diagram with a lipid bilayer, peptidoglycan layer, and lipopolysaccharide layer surrounding the interior.

### 2.3.2 Bacterial Polarity and the Cell Surface of *Escherichia coli*

Bacterial polarity is the localization of surface structures or particular molecules at the ends (poles) of rod-shaped bacteria (55). This type of localization is readily apparent for cells with larger surface structures like polar flagella, but more difficult to determine for cells with localized molecules at the ends. Bacterial polarity on a molecular scale has been shown to be important in cell division (26) and cell life cycle
17

(2). The differences in molecular composition presented by the bacterial polarity provide a heterogeneous surface composition which may facilitate adhesion. Adhesion caused by bacterial polarity has been shown for specific adhesion events (12, 13, 32, 78), but has not been shown for nonspecific adhesion (the topic of this thesis).

Bacterial polarity has been found for *E. coli*, where several proteins in the outer membrane of the cell were shown to be retained at the poles with fluorescent labeling (14). This localization was at least partially connected to the metabolism of murein and inert regions of murein at the poles (16). Additionally, outer membrane proteins and murein have been shown to localize at the poles of *E. coli* and to follow spiral patterns across the length of the cell (22, 74). *E. coli* also contains localizations of peptidoglycan (15, 16), proteins (14, 39), and phospholipids (38) in the cell wall of the bacterium.

### 2.3.3 Bacteria Cell Division: Binary Fission

Bacteria divide by the process of binary fission, where one cell elongates, forms a septum in the cell center, and divides into two cells. The process involves a complex interplay of molecules and organization to provide both daughter cells with the necessary DNA and molecules to survive, as well as to divide symmetrically. Bacterial polarity has been shown as a result of the cell division process (26, 51, 57). This polarity results on the internal structure of the cell membrane. Inert regions of peptidoglycan result at the poles of *E. coli* as a result of division, and these patches are very stable (74). The peptidoglycan layer is closely associated with the outer-most LPS layer, specifically
through proteins. Thus, it is likely that polarity in the peptidoglycan layer may very well be reflected in polarity of the outermost membrane.

2.4 Techniques to Study Bacterial Adhesion

Techniques to study bacterial adhesion typically fall into two main categories: techniques involving the use of shear forces to determine the amount of adhesion or techniques studying individual bacteria from a population of cells. Though significant research has been conducted in the area of bacterial adhesion, in general, little agreement has been found for trends with adhesion and macroscopic properties or mechanisms of adhesion (6, 46, 50). Bacterial adhesion studies began with the early experiments of Zobell examining the adhesion of marine organisms to glass slides (76, 77).

However, incubation and rinse experiments of this type have continued (21, 50, 65), examining surface free energy and hydrophobicity effects on adhesion. More direct use of shear to study bacterial adhesion is utilized in column flow experiments and various flow chamber experiments. Column experiments generally examine the flow of bacteria through porous media and the retention of bacteria within the column (7, 49, 53, 60). Flow chamber experiments take two basic forms: parallel flow chamber experiments (6, 24, 45, 64) and radial stagnation point flow (23, 69, 70). These experiments measure the adhesion of bacteria as a function of shear forces and correlate these results with such macroscopic properties as hydrophobicity (6), surface charge (69), and predicted DLVO forces (70). All of the experiments involving shear typically
examine populations of bacteria and draw generalized conclusions about particular species under controlled conditions.

Three main techniques have been employed to study the adhesion of individual bacteria from a population: electron microscopy, atomic force microscopy (AFM), and laser (optical) traps. Electron microscopy has been used in examining the surface structure of bacteria (5, 37) and specific adhesion mechanisms to nanocolloid probes (12, 13). However, electron microscopy requires extreme conditions (dry sample and high vacuum), which can cause significant damage to the bacterial surface. Atomic force microscopy overcomes the limitations of electron microscopy, allowing for bacteria to be studied in aqueous suspension. This instrument has been applied to study the effects of specific surface molecules on adhesion (1, 9, 56). As well, AFM has been used to compare microscopic force data with macroscopic surface measurements (62, 63). However, atomic force microscopy has not been used to characterize the ends of rod-shaped bacteria (10, 47). Additionally, the AFM has significant limitations in studying bacterial adhesion. The question arises about whether adhesion is accurately studied in AFM experiments, since the bacterium must first be adhered to a support surface. The non-adhered portions of the surface, which the AFM tip contacts, may undergo conformational and compositional changes resulting from adhesion with the substrate. As well, the surface adhered to the substrate is inaccessible to the AFM tip. The AFM tip is only able to probe the middle of a bacterium, since bacteria are rounded at the edges (10). Finally, results from the application of AFM to bacterial membranes are difficult to conduct and interpret (67, 68). However, some success has been made in understanding
AFM data from bacterial surfaces (29, 30), as well as measuring the flexibility of such surfaces (73).

The laser trap is another technique capable of studying bacterial adhesion on an individual bacterium basis. This technique overcomes most of the difficulties of AFM, since laser traps are able to both manipulate bacteria (4) and quantify attractive forces of bacteria to surfaces (11, 19, 28, 31, 58). This technique has been applied to spherical bacteria (11, 28, 58). Work with rod-shaped bacteria has entailed measurement of specific interactions (19, 31), viability studies (18), and movement (4) or placement (25) of bacteria. A detailed discussion of the application of laser trapping to bacterial adhesion and the limitations of this application are contained in Sections 6.1 and 6.2.2, respectively.

### 2.5 References


Chapter 3

Materials and Methods

Chapters 4 – 7 contain individual “Materials and Methods” appropriate to the content of the individual chapters. This general “Materials and Methods” section is not intended to be redundant to these other sections, but expands upon and adds clarification to the content of the sections to follow. In particular, the bacteria and growth conditions used for all experiments, the details and setup of the laser trap apparatus, and the details and setup of the CNLS apparatus are addressed in this section.

3.1 Bacteria and Growth

Two species of bacteria are used for experimentation: *Escherichia coli* and *Burkholderia cepacia*. Both species are Gram negative and rod-shaped. The *E. coli* bacteria are roughly 1 micron by 3 microns in size, and the *B. cepacia* are of equivalent size. Both species were chosen as model organisms, *E. coli* K-12 being perhaps the most scientifically studied bacteria and *B. cepacia* being a well-studied organism for bioremediation. *E. coli* K-12 D21 and D21f2 bacteria were obtained from the *E. coli* Genetic Stock Center (Department of Biology, Yale University, New Haven, CT), and *E. coli* K-12 JM109 was donated by Shahriar Mobashery (Wayne State University, Detroit, MI). *B. cepacia* G4 and Env435 were obtained from Mary DeFlaun at Envirogen Corp.
### 3.1.1 Strains

Three strains of *E. coli* K-12 were studied: D21f2, D21, and JM109. These strains differ in the length of the outer LPS layer. A schematic of the composition and length of the various LPS is shown in Figure 3-1. The interior of the cell is toward the right in the diagram with the LPS molecules extending towards the left into solution. The JM109 strain contains the longest LPS molecules with the full o-antigen (an additional repeating unit sugar chain) at the end of the molecules. The D21 strain does contain the o-antigen portion of the LPS, and the D21f2 strain only contains the core lipid A portion of the LPS.

![Figure 3-1: Schematic of the structure of the lipopolysaccharide (LPS) layer of three *E. coli* K-12 bacteria: D21f2, D21, and JM109. The interior of the cell would be towards the right with the LPS molecules extend left away from the cell. D21f2 contains the shortest LPS molecules only comprising the inner lipid A structures. D21 has more typical LPS molecules. JM109 has the longest LPS molecules, containing an additional o-antigen chain of sugar residues.](image)

The two *B. cepacia* strains G4 and ENV435 also differ in the lengths of LPS. By the use of gel-electrophoresis, the ENV435 has been shown to lack the o-antigen layer. The G4 strain contains the full o-antigen and is more adhesive than the ENV435 strain.
The EVN435 is a genetically modified strain specifically designed to be less adhesive. These bacteria are important for the degradation of such pollutants as tri- and tetrachloroethylene and were chosen as model organisms, since this research was partially motivated by improving bioremediation strategies using such organisms.

### 3.1.2 Growth Conditions

All bacteria were grown under aerobic batch conditions in a temperature controlled incubator and were constantly rotated on a shaker table at 150 RPM. The temperatures chosen were the temperatures for optimum growth, which were 37°C for *E. coli* and 30°C for *B. cepacia*. The liquid growth medium used for all bacteria was Luria Broth, prepared from powder at 50 g of powder per liter of MilliQ water. The growth medium was autoclaved prior to use and stored in a refrigerator. The detailed growth procedure for all bacteria as well as the necessary sterilization and safety precautions are contained in Appendix A.

In general, bacteria were grown to the mid-exponential phase of growth for experimentation. Under batch condition conditions, bacteria follow four phases of growth. Figure 3-2 shows the batch-wise growth of a bacteria culture versus time. Bacteria initially enter a lag phase of growth where the bacteria are adjusting to the growth conditions. Then, the bacteria enter an exponential phase of growth where the growth rate of new bacteria exceeds the death rate; this phase is the healthiest phase of growth for bacteria. Once waste products begin to build up and the molecules necessary for growth begin to become scarce, the bacteria enter the stationary phase of growth
where the growth rate of new cells equals the death rate. The stationary phase is a more stressed growth phase characterized by genetic and compositional changes in bacteria. Finally, the bacteria enter a death phase where growth molecules are completely used, and the death rate of cells is greater than the growth of new cells.

Figure 3-2: Graph of a batch growth of bacteria with number of bacteria versus time.

Growth curves were measured for all bacteria used experimentally. The absorbance of light at a wavelength of 600 nm was measured for bacteria samples with a UV-vis spectrophotometer. The absorbance is directly proportional to the number of bacteria in solution. Growth curves were conducted until evidence of the stationary phase was found by a leveling off of the absorbance with time. These curves were then used to calculate the mid-exponential growth time for each bacteria strain. The mid-
exponential growth time is simply the time for a batch growth of bacteria to grow half the
time of the full exponential phase.

Growth curves for *E. coli* D21f2, D21, and JM109 are shown in Figure 3-3 a), b),
and c), respectively. The mid-exponential growth times for the three *E. coli* strains are all
2.5 hours. Growth curves for *B. cepacia* G4 and ENV435 are shown in Figure 3-4 a) and
b), respectively. The mid-exponential growth times for the two *B. cepacia* strains are 5.0
and 3.5 hours, respectively. For stationary growth of *E. coli* D21, used in later
experiments, a growth time of 12 hours was used.
Figure 3-3: Growth curves for *E. coli*. Panes a), b), and c) show the growth curves for D21f2, D21, and JM109, respectively.
Figure 3-4: Growth curves for *Burkholderia cepacia*. Panes a) and b) show the growth curves for ENV435 and G4, respectively.
3.2 Laser Trap

Originally developed by Arthur Ashkin, the single beam, optical gradient laser trap is a device capable of precise manipulation of colloidal species in three dimensions with the capacity to measure forces of interaction at piconewton resolution \((1, 2)\). A laser trap of this type is effectively a single collimated laser beam shined through a high numerical aperture (NA) microscope objective in reverse. This setup results in the laser beam focused to a diffraction limited spot. The high focal angles of the laser light concentrated at a small area create an optical trap in three dimensions, allowing a colloidal species of about 10 microns or smaller with a higher index of refraction than the surrounding medium to be held at the focal point. A laser beam with a Gaussian intensity profile is used to create a gradient of light intensities across the area of the focal spot. This gradient in turn provides stability to the optical trap by creating a net force towards the center of the focal spot for any small movements (less than about a micron) away from the trap center. By combining the laser trap with a motorized microscope stage, a colloidal species may be manipulated in three dimensions.

Figure 3-5 schematically shows examples (panes A), B), and C)) of a colloidal sphere moving a small distance away from the optical center of the trap. Given that light has momentum, as light diffracts through the colloidal sample a change in momentum is observed. At the center of the optical trap, the forces created due to all the changes in momentum are balanced. However as a spherical particle moves in any direction away from the center of the trap (Figure 3-5), a correcting (balancing) force exists, which acts to move the particle back to the center of the trap. Thus, a colloidal species is held at the
center of the trap. For rod-shaped species, such as the bacteria used in this research, the
rod-shaped object always aligns with the direction of the laser light as shown in Figure 3-
5 D). This orientation results as the most stable state to balance the relevant optical
forces.
Figure 3-5: Schematic of a colloidal particle moving away from the laser trap center (A), (B), and (C)) (1) and schematic of a rod-shaped colloid aligned in a laser trap. The diagram of the particles moving away from center is reproduced from ref (1). The correcting forces, created from the changes in momentum, are shown with arrows in Figure 3-5 A), B), and C).
The laser trap setup involves a laser beam directed into the rear optical port of a Nikon TE 300 Eclipse inverted optical microscope (objective is below the sample) connected to video equipment (Cohu 4010 CCC camera, TV monitor, and DVD recorder). The laser light from a 2.5 W 1064 nm Nd:YAG laser (Coherent) is shined through a 20X beam expander and collimator (Newport). This produces a beam cross sectional area of roughly 2.5 cm. This area is such that it completely covers the area of the epi (rear) port entering the back of the microscope. The beam is then reflected off of a dichroic mirror through the 100X microscope objective (NA = 1.3) and is focused into the sample cell. The dichroic mirror (ideal for a wavelength of 933 nm) is mismatched with the laser wavelength, allowing for enough reflected laser light to create an effective trap as well as transmitting enough light for easy alignment of the laser beam.

The laser trap system is aligned by visual observation. First, the laser beam is shined directly onto the dichroic mirror. The laser spot is visible via a CCD camera and monitor from the laser light transmitted through the dichroic. This spot is aligned at the center of the viewing area (center of the objective) and adjusted for maximum intensity. The beam expander is then placed in the light path and adjusted for central alignment and maximum intensity. Finally, small adjustments may be made once the sample is in place, by positioning the trap center over the sample chamber surface, where interference rings are observed. The optics may again be adjusted for central positioning of the focal point and maximum intensity. A detailed explanation of the intensity of the laser light reaching the sample plane is contained in Section 6.2.2. This laser trap setup is slightly modified from a setup described in detail by Aron Parekh, please refer to his thesis entitled “The Anisotropy of Collagen Gels Remodeled by Fibroblast Traction Forces (4)”.

3.3 Charge Nonuniformity Light Scattering

Charge nonuniformity light scattering (CNLS), explained in detail in Appendix C, utilizes a small angle light scattering apparatus for data collection. An image (with labels) of the light scattering setup is shown in Figure 3-6. A 17 mW HeNe laser (Coherent Incorporated) at a wavelength of 623.8 nm is expanded 10 times with a beam expander (Coherent Incorporated) and directed through an iris (Newport), adjustable to the appropriate sample chamber size. For all experiments, the beam size is reduced to 3 mm before the sample cell.

![Figure 3-6: Image of small angle light scattering apparatus used for CNLS.](image)

The laser then passes through the sample cell. A picture and diagram of the sample cell are shown in Figure 3-7. The cell is U-shaped and allows for a platinized electrode to be inserted on either side of the cell, shown in Figure 3-7 a. The electrodes allow for the application of a (dc) linear electric field via a Keithley source meter. The sample cell is glass, and the shape is designed to prevent any bubbles from passing through the horizontal portion through which the laser passes.
Scattered light from the sample is collected for a single scattering angle of 2.9°. The scattering angle is determined by two circular anodized slit plates (cut to 270°) with diameters of 2.20 and 4.40 cm (Chemical Engineering Machine Shop, Don Lucas, Penn State). The slit plates are placed at the appropriate distances apart to obtain the desired scattering angle. A piece of black electrical tape, placed on the first slit plate in the light path, is used to block the incident beam for better signal resolution. The scattered light is then passed through a double plano-convex lens (Newport). This lens focuses the scattered light onto the 1 cm² sensing region of a head-on photo multiplier tube (PMT) (Hamamatsu). Prior to reaching the PMT, the scattered light is directed through a 632.8 ± 2.4 nm band pass filter (Edmund Optics) to filter out any ambient light. As well, the

Figure 3-7: Image of the CNLS sample (a) with accompanying schematic (b). The image is reproduced from Gretchen Holtzer’s thesis (3).
entire light scattering device is cloaked in black cloth to prevent ambient light and dust from entering the system.

The signal gain for the PMT is set manually via the PMT power supply (Hamamatsu) through an input voltage set at 0.9 V (the maximum allowable voltage for consistent signal acquisition and safe operation of the PMT). The current from the PMT is collected with a Keithley source meter and acquire with a Dell computer using LabVIEW 7.1 software. All optics for the scattering device, including the laser and PMT, are mounted and aligned on a 2 m optical rail (Newport). For a detailed parts and price list for the small angle light scattering apparatus used in this research please refer to Gretchen Holtzer’s thesis entitled “Particle Force Light Scattering – A Technique for Measuring Interparticle Forces(3)”.

3.4 References


Chapter 4
Oriented adhesion of E. coli to polystyrene particles

4.1 Introduction

Bacterial adhesion and biofilms are a critical problem for in situ bioremediation (26,12), heat exchanger fouling (18), biomaterial infections (9), and ship hull drag increase (18,8). Prior to the “anchoring” of a bacterium on a surface (29), a number of initial adhesion events must occur as a bacterium approaches a surface. First the bacterium must overcome the repulsive electrostatic forces (40,36). This is at least partly done by the attractive van der Waals forces (35,28), although the hydrophobic forces – less well understood – are often important in causing attractive forces between bacteria and surfaces (47). These initial adhesion events occur over length scales of O(100 nm) [i.e., “over length scales of the order of 100 nm”] and time scales of O(seconds), rather than over a few nanometers and hours, like long term adhesion processes (39). Not until these initial events have occurred can long-term events such as surface conformational changes (11), extracellular polymeric substance (EPS) production (44,53), DNA production (52), and cell signaling (14) dominate the adhesion process.

In this work we show that bacterial surface nonuniformities play an important role in adhesion. Such nonuniformities could result from bacterial polarity, which means that specialized structures or dynamic molecular localization occurs at or near the ends of the cell (42). While bacterial polarity has been shown to be important in cell division (25)
and cell life cycle (2), bacterial polarity has been studied with regard to adhesion primarily for specific adhesion events (20), not for physical forces only. The mechanism of how bacterial polarity affects adhesion is not known. One possibility is surface charge nonuniformity, which is the localization of charged regions on a length scale of O(10 nm) or O(100 nm). Charge nonuniformity has been shown to affect adhesion in colloidal systems (17), and it could arise on bacterial surfaces if molecules at the bacterial surface localized on a larger than molecular scale.

Techniques to measure and observe initial bacterial adhesion events are usually macroscopic in nature, better suited to examining populations than probing effects of bacterial cell surface polarity. Incubation and rinse (19,46), column flow (38,4), and flow chamber experiments (22,45) cannot study bacteria on a submicron level. The technique of atomic force microscopy (AFM) is a powerful tool that has been used to study individual bacteria and bacterial adhesion, and recently researchers have examined polymer and EPS attachment strength related to bacterial adhesion (7,41). However, the method has not been used to characterize the adhesion of the ends of rod-shaped bacteria (37,6). There are several challenges to using AFM to study bacterial adhesion in more depth: a) The adhered portion of the bacterium faces away from the AFM tip and therefore is very difficult to probe. b) Only the central region of the bacterium can be studied, since the tip slides off the edges. c) Bacterial measurements on soft surfaces are difficult to conduct and to interpret (51,50).

The literature contains several references concerning oriented bacterial adhesion to particles or surfaces. Several researchers have examined the role of flagella, which can cause oriented bacterial adhesion. Marshall et al. observed the preferential orientation,
dependent upon motility, of a flagellated *Pseudomonas* strain attaching to a surface (29). A *Pseudomonas* strain was observed by Fletcher to attach to hydrophilic surfaces via flagella, while assuming random orientations on hydrophobic surfaces (21). McClaine and Ford have found that flagella rotation increases attachment rates of *E. coli* bacteria to glass (30,31), although it could not be ascertained whether the bacteria were adhering by their cell body or by their flagella. Other researchers have observed polar (oriented) adhesion due to specific mechanisms (5), such as adhesin-mediated adherence to fibronectin (10), lectin-mediated adherence to Sepharose beads covalently derivatized with lactose (27), or pili adherence to tracheal cells (54). Using a synthesized wettability-gradient surface for non-flagellated bacteria, Ellen et al. have shown that *Treponema denticola* adhere flat onto hydrophobic surfaces, but adhere end-on to hydrophilic surfaces (15). More recently, Haruff et al. caused rod-shaped *Klebsiella pneumoniae* to adhere in specific orientations by positioning them with a laser trap (23). In the present manuscript we examine the oriented adhesion of non-flagellated *E. coli* to polystyrene latex spheres having only sulfate charge groups and not treated to promote adhesion.

We combine four methods for examining bacterial adhesion. 1) Video microscopy orientation experiments reveal not only whether a bacterium is adhered to a particle or a surface, but they also reveal the precise location on a bacterium where the adherence occurs. 2) Differential electrophoresis allows us to measure bacterium-particle attractive forces with sub-piconewton resolution, up to a force of about 50 pN. This technique can thus discern “irreversible” adhesion. 3) Rotational electrophoresis enables us to determine whether the bacteria are uniformly-charged, an important point since we
are considering bacterial polarity. 4) Shear swaying experiments enable us to visualize whether bacteria adhere to a flat surface by their ends, or by their entire surface.

4.2 Materials/Methods

4.2.1 Bacteria

Three strains of *Escherichia coli* K12 – D21f2 and D21 obtained from the *E. coli* Genetic Stock Center (Department of Biology, Yale University, New Haven, CT), and JM109, donated by Shahriar Mobashery (Wayne State University, Detroit, MI) – were studied. The wild type is D21. The D21f2 strain has a shorter LPS chain on the surface, while the JM109 strain has a longer LPS chain. All bacteria were grown in a shaker incubator at 150 RPM and 37 °C in Luria broth (Miller’s LB broth), and they were harvested in mid-exponential growth. All three strains were non-motile and non-flagellated. While *E. coli* K-12 have peritrichous flagella under many conditions (1), in our experiments we saw no motility other than Brownian motion, and neither transmission electron micrographs nor scanning electron micrographs revealed flagella on the bacteria under our growth conditions (M. Elimelech, personal communication).

Doubling times were determined from growth curves conducted in our laboratory. These were 39 min for JM109, 35 min for D21, and 28 min for D21f2. Cells were prepared for experimentation by washing in the suspending media three times with centrifugation (Sorvall Biofuge Primo) at 5000 RPM (3466 g) for 10 minutes. The bacterial suspension was then prepared for experimentation. All three strains were found
to be >90% viable after 30 min (the approximate time required for our experiments) in solution using Live/Dead BacLight (Molecular Probes; Eugene, Oregon; kit L-7007; lot 02A1-3). The temperature during the experiments was typically 20 to 25 °C, with the temperature holding constant to within 0.5 °C during each experiment.

4.2.2 Experimental techniques

A Nikon TE 300 Eclipse inverted optical microscope coupled with video equipment (e.g., Cohu 4010 CCD camera, VHS) was used for all experiments with a 100× magnification oil objective and differential interference contrast (DIC) for visualization of the bacteria. Experiments were conducted in 100 mM phosphate buffered saline (PBS) solutions consisting of potassium hydrogen-phosphate and potassium dihydrogen-phosphate dissolved in Milli-Q water. Glassware for the experiments was cleaned using sonication, soaked in 16 N nitric acid for 24 hours, and rinsed with Milli-Q water.

Bacteria were mixed by hand-shaking with 1.5 µm sulfated polystyrene (PS) particles (Interfacial Dynamics Corporation; Portland, Oregon; batch # 695,1). The bacteria and PS particles were then able to undergo Brownian aggregation in a 0.2 by 2.0 mm (inside dimensions) capillary tube (Vitrocom, Mountain Lakes, New Jersey). The capillary was mounted on a standard 25×75 mm microscope slide, and we observed the resulting couplets using video microscopy, recording whether the PS particles adhered to the middle or ends of the bacteria. A motorized stage (Prior) allowed a linear progression across the length of the capillary tube so that couplets were not recorded twice.
For observations of bacteria adhering to glass surfaces, solutions containing only bacteria in PBS were placed in the glass microelectrophoresis cell (48) and allowed to contact the glass surface. A low electric field (~1 V/cm) was applied and then reversed; the electroosmotic flow within the electrophoresis cell produced a gentle shear field that caused many bacteria to sway back and forth. Thus we could determine whether the bacteria were adhered, and moreover whether they were adhered on one end.

For the D21 strain, adhesion forces were measured between the bacterial cells and the PS sphere in many couplets using “differential electrophoresis”. Differential electrophoresis is a technique that exploits differences in zeta (\(\zeta\)) potential of colloidal species to measure the attractive forces between the two moieties (48,3,43). Since the bacterium and the PS sphere composing the couplet have different \(\zeta\) potentials, they want to move at different velocities in an applied electric field (\(E_0\)). As the magnitude of \(E_0\) increases, a stronger force pulls the couplet apart. In our experiments we located a particle-bacterium couplet, applied an electric field (\(E_0\)), and incrementally increased its strength. The couplet was then followed visually. This process was continued until either the couplet broke or the couplet escaped the viewing plane. Figure 4-1 shows the only particle-bacterium couplet that broke in all our experiments with PS particles. From the value of \(E_0\) and the \(\zeta\) potentials of the two parts of the couplet, a value for the attractive force (\(F\)) holding together the bacterium and particle can be estimated using Eq. 4.1 (48)

\[
F = 8.76\pi\varepsilon_0|\zeta_2 - \zeta_1|E_0
\]  

4.1
where \( a \) is an “average” particle radius; \( \varepsilon \) is the fluid permittivity for water, and 1 and 2 represent the bacterium and the particle. This equation was derived for two spherical particles, and therefore can give only estimates for the bacterium-PS sphere couplets. Importantly, this relation does not depend upon any particular model for interparticle forces. In order to interpret our experiments, we chose \( a = 0.75 \, \mu m \). \( \zeta \) potentials on the bacteria and the PS particles were measured using a Brookhaven Zeta PALS analyzer.

The fact that bacteria adhere to one end indicated that perhaps the bacteria were nonuniformly-charged, and so we used the technique of rotational electrophoresis to measure the charge nonuniformity on the bacterial surfaces (49,16,17). The essence of this technique is that particles that are uniformly-charged do not rotate by electrophoresis, regardless of shape (34). However, nonuniformly-charged particles will rotate by electrophoresis, and we have recently developed the experimental and theoretical tools to measure and interpret charge nonuniformity (49,16,17). Thus, if we see bacteria rotating in an applied electric field, we know that this bacterium is nonuniformly-charged, and we can interpret the angular velocity in terms of a standard deviation of \( \zeta \) potential over the bacterial surface.

Figure 4-1: Differential electrophoresis image of PS particle/bacterium couplet breaking. The images are taken approximately 0.5 seconds apart. Of the 25 couplets that we have observed, this is the only couplet that broke.
4.3 Results

4.3.1 Orientation Observations

Initially from observations with differential electrophoresis, the bacteria (*E. coli* D21) were found to adhere to the colloidal polystyrene particles most often in an end-on fashion (Figure 4-2 b-d). Only rarely did the PS particles adhere to the central section of a bacterium (Figure 4-2 a). Based on the size comparison of the particles to the bacteria, adhesion to the end of the bacteria versus the middle was visually unambiguous. In the systematic studies, we found that an overwhelming majority (>90%) of all three strains of bacteria adhered end-on to the PS particles (Table 4-1). Furthermore, only one end of the bacteria adhered to the PS particles; out of 246 couplets observed, no bacterium was found with particles adhered at both ends. In fact, for many couplets, several particles were adhered to one end of a bacterium (Figure 4-2 c-d), but never both.
Attachment of the bacteria to the glass capillary was also observed to occur in an end-on geometry. This geometry was observed by applying a small electric field and observing the bacteria swaying. Figure 4-3 a shows two bacteria attached to a glass surface in the absence of flow, while Figure 4-3 b-c show the same bacteria in the presence of a flow field. The bacteria are anchored by one end, but otherwise sway in the flow.
Table 4-1: Adhesion statistics for *E. coli* K12 to polystyrene latex spheres. The columns indicate whether the PS latex sphere adhered to the middle of the bacterium, or on the ends. Furthermore, the Table distinguishes between having one sphere adhered on the end, versus multiple spheres. Note that in no case did PS spheres ever adhere on both ends.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Middle</th>
<th>Single PS on end</th>
<th>Multiple PS on end</th>
<th>PS on both ends</th>
<th>Total on end</th>
<th>Total couplets</th>
<th>% on end</th>
</tr>
</thead>
<tbody>
<tr>
<td>D21</td>
<td>9</td>
<td>122</td>
<td>18</td>
<td>0</td>
<td>140</td>
<td>149</td>
<td>93.3</td>
</tr>
<tr>
<td>D21f2</td>
<td>4</td>
<td>47</td>
<td>3</td>
<td>0</td>
<td>50</td>
<td>54</td>
<td>92.6</td>
</tr>
<tr>
<td>JM109</td>
<td>3</td>
<td>39</td>
<td>1</td>
<td>0</td>
<td>40</td>
<td>43</td>
<td>93.0</td>
</tr>
</tbody>
</table>

**4.3.2 Force measurements**

Differential electrophoresis was done on 25 of the bacterium-PS couplets in order to quantify adhesion forces. The technique can apply roughly 50 pN or less, which is more than sufficient for measuring the weak, reversible (often sub-pN) forces associated
with adhesion in a DLVO secondary energy minimum (24). Although the literature does not define “irreversible bacterial adhesion” in quantitative terms, we here define “irreversible” as an adhesion force greater than 10 pN, based on calculations from classical colloid theory. However, from experiments on 25 couplets, only one couplet broke. This strong adhesion is not typical of DLVO forces, but rather much stronger forces (e.g., hydrophobic forces, specific interactions). Zeta potential values in 100 mM PBS, used in calculating the applied force of differential electrophoresis, were $-26 \pm 1$ mV for D21 and $-73 \pm 2$ mV for PS.

4.3.3 Charge nonuniformity

All three E. coli strains were tested using rotational electrophoresis for surface charge nonuniformity. No rotation was found for any strain, indicating that charge nonuniformity was too small to be measurable with this technique. That is, the angular velocities could not be distinguished from the Brownian motion of the bacteria. Figure 4-4 shows the rotation angles of a D21f2 bacterium in an electric field as compared to a B. subtilis bacterium with polar flagella. While the B. subtilis rotated with the electric field to a steady angle and then rotated back when the field was reversed at 20 seconds, the E. coli exhibited only small, random changes in angle associated with Brownian motion. The B. subtilis had a quantifiable surface charge nonuniformity based on its angular velocity, while the E. coli did not. The experiments represented in Figure 4-4 were conducted in 1 mM Tris buffer. However, for all three E. coli strains, these experiments
were repeated in 100 mM PBS, and again no measurable charge nonuniformity was observed.

Table 4-1 clearly shows that *E. coli* adhere end-on to PS latex spheres. This result is complemented by the observations on bulk glass of bacteria adhered on end. One end of the bacterium clearly showed different adhesion properties than the middle of the bacterium. One explanation of the data is bacterial cell polarity (42). In *E. coli* this bacterial polarity is caused by the localization of peptidoglycan (13), proteins (33), and phospholipids (32) on the surface of the bacterium. Differences in the surface

---

**Figure 4-4**: Measuring charge nonuniformity on bacteria using rotational electrophoresis. The open circles represent an *E. coli* bacterium. $\theta$ is the angle in degrees between the applied electric field and the long axis of the bacterium. Since this bacterium is undergoing only random Brownian rotation, its charge nonuniformity is too small to be measured. On the other hand, the filled circles represented a *B. subtilis* bacterium, which rotated in the electric field. When the field direction was switched at about 20 seconds, the rotation reversed since this bacterium was nonuniformly-charged.

**4.4 Discussion**

Table 4-1 clearly shows that *E. coli* adhere end-on to PS latex spheres. This result is complemented by the observations on bulk glass of bacteria adhered on end. One end of the bacterium clearly showed different adhesion properties than the middle of the bacterium. One explanation of the data is bacterial cell polarity (42). In *E. coli* this bacterial polarity is caused by the localization of peptidoglycan (13), proteins (33), and phospholipids (32) on the surface of the bacterium. Differences in the surface
composition and chemistry at the bacterium end could result in preferential adhesion at the end.

Nevertheless, the rotational electrophoresis experiments revealed no charge nonuniformity on the bacteria, and this remains puzzling. Although no charge nonuniformity could be discerned, localization of particular surface molecules must still be occurring. The small nanodomain where the PS latex particles adhere is a small fraction of the total bacterial surface area, and so based on geometry, it is highly improbable that 90% of the PS particles would adhere to the end. More importantly, the PS particles adhere to one end, never both. This bacterial polarity apparently is not exhibited through charged functionalities, or at least the differences in charge across the surface are not detectable by rotational electrophoresis. To maintain the integrity of the membrane structure, it is unlikely that a large localization of molecules other than LPS occurs. However, the combination of several patches of molecules at a polar end could result in significant differences in adhesion properties.

Although the three strains of \textit{E. coli} had differing lengths of LPS surface molecules, the adhesion orientation of these bacteria did not change, remaining at 90% end-on adhesion. This result indicates that LPS does not seem to be the important molecule involved in adhesion. Our differential electrophoresis experiments showed that the initial adhesion process was most often irreversible, consistent with the findings of Rijnaarts et al. \cite{39}. The one couplet (out of 25) that did break in our experiments had an attractive force that was roughly <10 pN.
4.5 Conclusions

Polar adhesion of *E. coli* bacteria was shown to occur to both polystyrene latex particles and to standard glass microscope slides. Greater than 90% of the adhering bacteria adhered on end, which cannot be accounted for by geometric considerations. The adhesion of bacteria was found to occur at only a single end, indicating different functionalities in those regions. And yet, rotational electrophoresis measurements revealed no charge nonuniformity on the *E. coli*. Since the three strains of K12 – each with a different length of LPS – behaved similarly in the experiments, it is possible that polarity of protein molecules are causing the polar adhesion. This adhesion is essentially irreversible, since the classical colloidal force models cannot explain the large attractive forces measured using differential electrophoresis.

We are currently working to apply the techniques we used in this research, in particular the electrophoresis and video microscopy techniques, to other bacteria. Bacteria adhere to surfaces by a variety of mechanisms, and by using these techniques, we will examine the generality or specificity of end-on adhesion for various strains of bacteria. As of yet we do not know, for instance, whether the polar adhesion results from the division process. But our aim is to isolate and identify the molecular composition that causes the adhesion in the “sticky” nanodomains, with the long term goal of controlling the expression of these molecules.
4.6 Acknowledgments and Copyright Information

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4.7 References


Chapter 5

Orientation Effects for the Irreversible Adhesion of Spherical Particles to Spheroidal Collectors

5.1 Introduction

Classical Smoluchowski flocculation theory describes the time required for spherical particles to aggregate (4, 8). The model is based on the diffusion of particles in either homogeneous or heterogeneous flocculation. The commonly-used versions of the theory often contain corrections for interparticle potentials and changes in particle mobility from the lubrication hydrodynamics for nearly-touching particles (4).

The role of particle orientation is not commonly considered in these calculations. Particle orientation might be important for a sphere aggregating with a spheroid, or even for two spheres aggregating if one region of the sphere has different surface properties from another part. For example, our lab group showed that rod-shaped *E. coli* bacteria often adhere by the hemispherical ends to spherical colloids (6). This preferential adhesion is thought to result from bacterial polarity (6), the localization of molecules or surface structures at the end of the bacterium (9). However, we sought evidence that the end-on adhesion observed with these bacteria did not result from simple statistical effects. That is, we sought to verify that the bacterial ends truly had adhesive properties that were causing end-on sticking.

Our investigation uses a Brownian dynamics simulation (BDS) for the adhesion of spheroidal colloids to spherical colloids. The simulation is supported with
experimental data using a model colloidal system. The results of the BDS are compared to rod-shaped *E. coli* bacteria adhesion results to determine whether the observed end-on adhesion can be contributed to physical phenomenon or arises from more complex interactions.

### 5.2 Experimental Materials and Methods

#### 5.2.1 Orientation Experiments

Orientation experiments involving the adhesion of *Escherichia coli* K-12 D21 bacteria (D21) to 1.5 μm diameter sulfated polystyrene (PS) particles have been described and published previously (6). Briefly, bacteria are mixed in suspension with PS particles in 100 mM phosphate buffered solutions (PBS). The suspension is observed with video microscopy, and the orientation of spherical particles adhering to bacteria is recorded, i.e. adhering to the hemispherical end or cylindrical middle. Experiments with D21 and 0.81, 0.99, or 4.9 μm diameter sulfated PS spheres were conducted in the same manner in 100 mM PBS solution.

We have also done experiments with well-characterized colloidal particles. Similar experiments were carried out with stretched sulfated PS particles and spherical amidine PS particles. Visualization was achieved with a Nikon TE 300 Eclipse inverted optical microscope coupled with video equipment (Cohu 4010 CCD camera, DVD recorder, etc.). A 100X oil objective (NA = 1.3) was used for all experiments.
Orientation experiments with stretched particles (see Section 5.2.2 on synthesis of spheroidal particles) were conducted in 1 mM PBS. Spherical particles were mixed with stretched particles in suspension. A sample of this mixture was then transferred to a rectangular capillary tube (0.2 by 2.0 mm ID, Vitrocom, Mountain Lakes, New Jersey), and the tube was mounted on a standard 25 by 75 mm microscope slide. The solution was observed using video microscopy, and movement through the capillary was achieved with a motorized xyz stage (Prior). The adhesion of spherical particles to the stretched particles was noted as well as the orientation of this adhesion.

Figure 5-1 shows a diagram of an ellipsoidal (stretched) particle, a rod-shaped bacterium, and a spherical particle. The stretched particle is divided into three regions similar to the rod-shaped bacterium: end 1, middle, and end 2. These end regions are of size r in axial dimension matching the radius r of the hemispherical ends of the rod-shaped species. The middle region of the ellipsoid is of size 2L in the long axial dimension, where L is half the length of the cylindrical portion of the rod-shaped species, and 2r in the small axial dimensions. These dimensions mimic the dimensions of the cylindrical portion of the rod-shaped species. For further discussion of size dimensions, r and L will be used exclusively. Orientation of adhesion refers to which of these regions a spherical particle adheres. This determination was made visually and was conducted by the same researcher for all similar experiments, i.e. the observer was the same for all stretched particle experiments.
5.2.2 Production of Spheroidal Particles

Spheroidal colloids were produced via an indirect synthesis method, i.e. mono-disperse spherical colloids were physically deformed. The method of this work follows the techniques of Nagy and Keller (7) and Ho et al. (5). Polyvinyl alcohol was dissolved in a suspension of MilliQ water and 1.6 μm sulfated PS particles in a 5% w/w concentration at a temperature of 80° C. The suspension was then poured in a Petri dish to be dehydrated. The result is a uniformly thin polymer film dispersed with particles. A rectangular 2 by 5 cm section of the film is cut for stretching. The film must be thick
enough so as to not degrade during stretching; films on the order of 1 mm thick were found to be suitable.

The apparatus for stretching allowed for the film to be stretched linearly in one dimension. The polymer film section was clamped at both ends and stops were placed to achieve the desired stretch ratio. A stretch ratio of 3:1 corresponds to stretching the film to 3 times its length and correspondingly stretching the PS spheres to a length 3 times the original diameter of the particles. The apparatus was designed for simplicity and to ensure safety when immersing the film into a heated oil bath for stretching. An oil bath (paraffin oil) heated to 200º C was used to raise the PS particles above the glass transition temperature (Tg ~ 105º C) and to make the polymer film pliable enough for stretching. The film was inserted into the heated oil bath for 3-4 seconds to equilibrate and then was immediately stretched and removed in 1-2 seconds. After allowing the film to cool at room temperature for several minutes, the film was washed with acetone to remove any excess oil. The center portion of the film was harvested for experimental use to ensure monodispersity of the stretched particles. This piece of film was placed in water at 80º C to dissolve the PVA polymer matrix. The solution was then centrifuged and washed in MilliQ water multiple times to remove any excess polymer. The stretched particles were observed with optical microscopy to verify stretching and size, and then were used for experimentation.

Spheroidal particles were produced from sulfated PS particles of radii 0.8 µm with a stretch ratio of 3:1. Assuming no volume change for the particles and a full stretch (the long axis is 3 times the original radius), the resulting prolate ellipsoids had a long axis radius of 2.4 µm and small axes radii of 0.46 µm, resulting in an r of 0.46 µm and an
L of 1.94 μm. The size of the stretched particles was verified by comparison to spheres of known size. Optical microscopy images of the stretched particles are shown in Figure 5-2. With optical microscopy, a largely uniform distribution of stretched particles was observed. Particles which were not stretched to full length were disregarded during orientation experiments.

5.3 Brownian Dynamics Simulations of Particle Adhesion

The Brownian motion of a sphere and a spheroid were simulated in a confined space. A single sphere and a single ellipsoid were allowed to diffuse within a spherical volume. The spherical volume was defined with radius (R), which estimates the concentration of particles used during experimentation.
The translational diffusion coefficient of a Brownian sphere is given as (Eq. 5.1)
\[ D_T = \frac{kT}{6\pi \eta a} \]  
where \( k \) is the Boltzmann constant, \( T \) is temperature, \( \eta \) is the viscosity of water, and \( a \) is the radius of the sphere. The diffusion was simulated in time steps \( \Delta t \) with random movements in the \( x \), \( y \), and \( z \) directions from a normal distribution of standard deviation (Eq. 5.2)
\[ \sigma_x = (2D_T \Delta t)^{1/2} \]  
with an average of 0. The rotational diffusion for the sphere was irrelevant for this simulation since the sphere is uniform in both geometry and for the purposes of adhesion. However, its inclusion would be straightforward.

The translational diffusion of an ellipsoid was simulated over the same \( \Delta t \) with a translational diffusion coefficient (10) (Eq. 5.3)
\[ D_{T,i} = \frac{kT}{16\pi \eta} \left( \chi + \alpha_i a_i^2 \right) \]  
for each axis of radius \( a_i \), where \( \chi \) is defined as Eq. 5.4
\[ \chi = \int_0^\infty \frac{d\lambda}{\Delta(\lambda)} \]  
\[ \Delta(\lambda) = \left[ (a_1^2 + \lambda)(a_2^2 + \lambda)(a_3^2 + \lambda) \right]^{1/2} \]  
and \( \alpha_i \) is defined as Eq. 5.5
\[ \alpha_i = \int_0^\infty \frac{d\lambda}{(a_i^2 + \lambda)\Delta(\lambda)} \]
for each axis. Similarly, the movement is simulated in three dimensions as a random movement at each time step from a normal distribution with the above standard deviation and an average of 0.

In addition, the rotational diffusion of the spheroid was simulated using a rotational diffusion coefficient (11) (Eq. 5.6)

$$D_{\theta} = \frac{3kT}{16\pi\eta}\left[\frac{a_1a_1^2 + a_2a_2^2}{a_1^2 + a_2^2}\right]$$ for \(a_1\neq a_2\neq a_3\)  

5.6

The rotational diffusion is simulated using the time step \(\Delta t\) and random movements from a normal distribution with the average of 0. The standard deviation for the distribution of \(\theta\) (\(\theta\) is here defined as the angle with the z-axis using spherical coordinates) is the same as for the translation diffusion. However, the standard deviation for the distribution of \(\phi\) is defined (Eq. 5.7)

$$\sigma_{\phi} = \left(\frac{2D_{\theta}\Delta t}{\sin^2\theta}\right)^{1/2}$$  

5.7

in terms of \(\theta\).

Thus, the spherical particle and the spheroid are allowed to undergo Brownian motion within a spherical volume of radius \(R\) until the two come into contact. In order to follow the positions of each species, a vector is defined from the origin (the origin is defined as the center of the sphere constraining the movement of the sphere and spheroid with radius \(R\)) to the center of each species. The distance between the sphere and the ellipsoid is determined by the magnitude of subtracting these vectors. A local orientation vector for the ellipsoid is also established by \(\theta\) and \(\phi\) to determine the appropriate
translational movement along each ellipsoidal axis and to define the volume of the ellipsoid.

When determining the contact of the sphere and the ellipsoid, the ellipsoid is defined as a cylindrical species with two hemispherical ends which will be referred to as “rod-shaped” as shown in Figure 5-1. This definition is for simplification and for direct comparison to rod-shaped bacteria of the same geometry. The ellipsoid has the same axial dimensions as the rod-shaped species defined by the values \( r \) and \( L \). Contact occurs between the sphere and the ellipsoid when their positions are such that the surface of the sphere is in contact with the surface of the rod-shaped species. The rod-shaped species is divided into the three regions as shown in Figure 5-1, such that the region of contact is recorded when adhesion occurs. The simulation was conducted for multiple spherical particle sizes for a given ellipsoid size. Again, it is important to note that this simulation models the Brownian motion of the anisotropic species as an ellipsoid, but models the adhesion as a rod-shaped species of the same axial dimensions. These simulation conditions were used for simplicity and ease of calculation, since the Brownian motion of a rod-shape and the adhesion to a spheroid are nontrivial, and were supported experimentally.

In addition to experimental support, the simulation was compared with known flocculation theory. The rapid flocculation time \( t \) for Brownian doublet formation of spheres can be calculated (8) (Eq. 5.8)

\[
t = \frac{\pi \eta a^3}{\phi kT} \tag{5.8}
\]
where $\eta$ is the fluid viscosity, $a$ is the radius of the spheres, $\phi$ is the volume fraction of the spheres, $k$ is the Boltzmann constant, and $T$ is temperature. Simulations of sphere-sphere adhesion provided consistent values for rapid flocculation times with theory. The simulated rapid flocculation time was calculated as the average number of time steps for sphere-sphere adhesion multiplied by the time step $\Delta t$. Additionally, simulated rapid flocculation times for sphere-spheroid adhesion were the same order of magnitude as values for sphere-sphere adhesion. As a final test of the simulation, the percents adhesion for each end of the spheroid for a given size sphere were compared, resulting in statistically identically values for all cases. These results would be expected since the adhesion is treated as irreversible, and the ends are geometrically the same.

5.4 Results

Orientation experiments were conducted with the stretched ellipsoidal particles and varying sized amidine PS spheres suspended in 1 mM PBS. The results from these experiments are shown in Table 5-1. Due to the positive surface charge of the amidine particles and the negative surface charge of the sulfated stretched particles, the adhesion of these particles was favorable and irreversible. However, this condition resulted in the adhesion of multiple amidine particles to a single sulfated stretched particle in some instances. A low salt suspending medium was used in order to minimize these multiple adhesions via electrostatics. The multiple adhesion result was noted in the observations as seen in Table 5-1. For the calculation of the percentage of particles that adhered to the middle of the ellipsoidal particles, all instances where a spherical particle adhered to an
ellipsoidal particle were included, e.g., an ellipsoidal particle having a spherical particle adhered to the middle portion and an end is counted as having two adhesions: one middle adhesion and one end adhesion. This condition was used since simulations only included cases of a single sphere adhering to an ellipsoid, and the experimental conditions allow for multiple spheres to adhere to a single ellipsoid, though these occurrences are much less than single spheres adhering to single ellipsoids. For the majority of spherical particle sizes, the percentage of middle adhesion declines with increasing particle size.

<table>
<thead>
<tr>
<th>Spherical Particle Radius (micron)</th>
<th>Adhesion of Spheres to Ellipsoid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single End</td>
</tr>
<tr>
<td>0.26</td>
<td>19</td>
</tr>
<tr>
<td>0.385</td>
<td>30</td>
</tr>
<tr>
<td>0.5</td>
<td>49</td>
</tr>
<tr>
<td>0.75</td>
<td>103</td>
</tr>
<tr>
<td>1.25</td>
<td>51</td>
</tr>
<tr>
<td>1.65</td>
<td>87</td>
</tr>
</tbody>
</table>

The experimental results match well with simulations, as shown in Figure 5-3. For these simulations, the dimensions $r$ and $L$ were chosen as 0.46 and 1.94 µm, respectively, to match the size of the stretched particles. The time step chosen ranged from 0.01 to 0.005 seconds. The time step for each size was chosen as the largest time step which showed no significant change in results from smaller time steps. The simulations show a decrease in the percentage of middle adhesion with increasing particle size as well.
After finding that simulations match well with experimental results, the experimental results of rod-shaped bacteria adhering to spherical particles were compared to simulations. These results are shown in Figure 5-4, comparing the adhesion of D21 to varying sized sulfated PS particles with simulations. For the bacteria simulations, r and L were chosen as 0.5 and 1 µm to resemble the average size of D21 bacteria, and the Δt chosen ranged from 0.01 to 0.001. Similar to Figure 5-3, the simulations show a decrease in the percentage of middle adhesion with increasing particle size. However, not all experimental results with bacteria match well with simulations. Specifically, the 0.75 micron radius sphere results differ significantly from the simulated results.

Figure 5-3: Comparison of simulations to experimental results for the adhesion of spherical amidine PS particles to ellipsoidal sulfated PS particles. The experimental data is depicted as grey triangles, while the simulation results are shown as black circles. Error bars indicate 95% confidence intervals.
5.5 Discussion

By observing Figure 5-3 and Figure 5-4, it is apparent that the size of spherical particles has a significant effect on adhesion orientation to ellipsoidal colloids or rod-shaped bacteria with the percentage of spherical particles adhering to the middle of these species decreasing with increasing size of the spheres. As expected, the simulations begin to approach percentages of middle adhesion that are comparable to the ratio of middle surface area to total surface area as the size of the spherical particles decreases. The middle surface area ratio is 81% for the ellipsoid simulations and 67% for the

Figure 5-4: Comparison of simulations to experimental results for the adhesion of spherical sulfated PS particles to rod-shaped D21 bacteria. The experimental data is depicted as grey triangles, while the simulation results are shown as black circles. Error bars indicate 95% confidence intervals.
bacteria simulations (note that both cases are treated as rod-shaped for the purposes of the simulation). This limit is expected since the Brownian motion of the small particles far exceeds the Brownian motion of the ellipsoid or bacterium, allowing effectively uniform adhesion. For the larger sized spherical particles, the middle adhesion percentages begin to level off due to the Brownian motion of the sphere becoming very small and the size of the sphere approaching a planar surface.

For the case of stretched ellipsoidal particles, the experimental results match well with simulations. However for the smallest and largest experimental cases, the data appear to deviate from simulation results. In order to test the statistical similarity of these data, simulations were conducted for 4 sets of 50 ellipsoidal particles each to mimic experimental data and to obtain standard deviations. These simulated data were then compared to experimental results, as shown in Table 5-2, using the unpaired Student’s t-test. In all cases, the experimental data are statistically the same with 95% confidence as simulations, except for the smallest case of 0.26 μm spheres.

The discrepancies for this size may result from observational difficulties, i.e., the size of the particle is approaching the resolution limit of the optics, making it difficult to distinguish orientation of adhesion. As well, the assumption of the ellipsoid as a rod-shaped species in the simulation may underestimate the ratio of middle adherence because the size of the “ends” is overestimated. For an ellipsoid and a rod-shaped species both with dimensions r of 0.46 μm and L of 1.94 μm, the ends surface area and the total surface area are greater for the rod-shaped species. The surface area (S) of an ellipsoid is described as (12) (Eq. 5.9)
where $a$ is the short axis radius and $c$ is the long axis radius such that $c > a$. Also, the ratio of the total ends surface area to the total surface area is 44% greater for the rod-shaped species (0.192 versus 0.108). Thus, the possible adhesion area at the ends is significantly overestimated by the ellipsoid simulation as compared to the experimental conditions. Finally, the simulation does not account for surface interactions or interparticle forces (see Section 5.7). The motion is entirely Brownian diffusion and adhesion occurs when the species come into physical contact.

\[ S = 2\pi a \int_{-1}^{1} \sqrt{1 + \frac{(a-c)(a+c)z^2}{c^4}} \, dz \]

Table 5-2: Statistical comparison of simulated experiments to actual experimental data for adhesion of spherical amidine PS particles to ellipsoidal sulfated PS particles. The percents of spherical particles which adhered to the middle of spheroids are shown with standard deviations. The number of trials (experimental or simulated) for each percentage value is shown in brackets. The unpaired Student’s t-test used in this analysis compares a calculated t-value to an expected theoretical t-value. If the calculated t-value is less than the expected, the two means of the results (middle adhesion percentage) are said to be statistically the same. If the calculated t-value is greater than the expected, the two means are statistically different. The listed t-values correspond to a 95% confidence level for comparison.

<table>
<thead>
<tr>
<th>Spherical Particle Radius (micron)</th>
<th>Experimental Middle Adhesion ± SD (%)</th>
<th>Simulated Middle Adhesion ± SD (%)</th>
<th>t-value</th>
<th>Expected t-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.385</td>
<td>69.3 ± 15.23 [2]</td>
<td>57 ± 7.39 [4]</td>
<td>1.08</td>
<td>2.78</td>
</tr>
<tr>
<td>0.5</td>
<td>54.3 ± 18.82 [3]</td>
<td>53.5 ± 2.52 [4]</td>
<td>0.07</td>
<td>2.57</td>
</tr>
<tr>
<td>0.75</td>
<td>37.6 ± 18.72 [3]</td>
<td>43.5 ± 7.55 [4]</td>
<td>0.51</td>
<td>2.57</td>
</tr>
</tbody>
</table>
The simulation procedure, supported by the ellipsoidal particle experiments, was then used for comparison with rod-shaped bacteria adhering to spherical colloids. As seen in Figure 5-4, the experimental data for D21 bacteria appear to deviate from simulations as the spherical particle size is decreased. A similar comparison as with the ellipsoidal particles (Table 5-2) was conducted with the bacteria to find statistical similarities to simulations. Table 5-3 shows the comparison of D21 experimental results to simulated results of 4 cases of 50 bacteria for each size of spherical particle. Again, an unpaired Student’s t-test was performed showing that the results for the 0.75 radius PS spheres with D21 differ significantly from the simulated results with 95% accuracy. The 2.45 μm radius particles do not differ from simulations, and the smaller sizes of 0.495 and 0.405 μm radius particles also do not differ from simulations.

<table>
<thead>
<tr>
<th>Spherical Particle Radius (micron)</th>
<th>Experimental Middle Adhesion ± SD (%)</th>
<th>Simulated Middle Adhesion ± SD (%)</th>
<th>t-value</th>
<th>Expected t-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.405</td>
<td>54.3 ± 11.20 [2]</td>
<td>44.0 ± 2.83 [4]</td>
<td>1.28</td>
<td>2.78</td>
</tr>
<tr>
<td>0.495</td>
<td>32.0 ± 6.12 [2]</td>
<td>36.5 ± 4.73 [4]</td>
<td>0.91</td>
<td>2.78</td>
</tr>
<tr>
<td>0.75</td>
<td>7.1 ± 5.94 [4]</td>
<td>30.5 ± 4.43 [4]</td>
<td>6.30</td>
<td>2.45</td>
</tr>
<tr>
<td>2.45</td>
<td>11.1 ± 0.00 [2]</td>
<td>12.5 ± 5.00 [4]</td>
<td>0.56</td>
<td>2.78</td>
</tr>
</tbody>
</table>

These results agree with the conclusion that the end-on adhesion effect results from surface characteristics and not purely physical phenomena (6). For the cases of 0.75 and 2.45 micron radius particles, the extent of middle adhesion, and accordingly end-on
adhesion, for the D21 bacteria remains constant. These results deviate significantly from simulations as the spherical particle size decreases as evidenced by the 0.75 micron spherical particles results. If a single adhesive end were the only available area for adhesion, the constant value for end-on adhesion would remain as spherical particle size was decreased further. However, the smaller sized particles show a sharp increase in middle adhesion that then matches simulated results. This agreement with simulations (for the smaller sized spherical particles) is hypothesized to result from patches of localized molecules distributed across the bacterial cell of the same composition as the hypothesized patches at the ends of the D21 bacteria thought to cause end-on adhesion, but of a much smaller size. Thus as shown in Figure 5-5 a), larger particles are able to adhere only to the adhesive regions at the end of the bacterial cell. However, smaller particles are able to adhere to both the large regions at the end of the cells and smaller regions along the rest of the cell (Figure 5-5 b)), creating a more uniform surface for adhesion and matching simulated BDS results. This type of nonuniform distribution of surface molecules has been observed for E. coli with larger localizations of proteins at the cell ends and smaller spiral patterns on the cell body (1).
5.6 Conclusions

Geometric considerations are not solely responsible for end-on adhesion of *E. coli* to spherical particles. Rather, bacterial polarity appears to be playing a significant role in the adhesion of the cells to the spherical particles. Our BDS model has been tested against model sphere-spheroid particles of non-biological origin, and there is significant agreement between experiment and theory.

We are now exploring the possibility that we can control end-on adhesion of particles without the use of specific adhesion. That is, if we have particles with a rapid rotational diffusion coefficient, it might be possible to create end-on adhesion without doing anything specific to the ends of the particles. This would provide a type of...
colloidal molecule in which the nanoscale features of the end-groups are produced not chemically, but physically.

### 5.7 Notes on Brownian Dynamics Simulation

For the BDS presented previously, it should be noted that no interparticle forces or close particle interactions were included in the simulation. Only Brownian motion was used to determine adhesion orientation, where adhesion was treated as irreversible and occurred at first physical contact of the two surfaces (sphere and ellipsoid). Interparticle forces were not included because a DLVO calculation (including electrostatics and van der Waals forces) showed that they were irreversibly attractive (< -100 kT) for the case when the sphere and the spheroid were oppositely-charged, and the potentials were prohibitively repulsive for the case when the sphere and the spheroid were negatively charged (> +100 kT). The experimental zeta potentials on our spheres were -73 mV, while the zeta potentials on the bacteria were -26 mV. The experiments were done in 100 mM PBS. If the zeta potentials were of smaller magnitude and of the same sign, such that the interparticle barrier were perhaps +5 kT, then the interparticle forces could have been included through use of known equations describing the interactions of spheres and cylinders (2, 3). The hydrodynamics could have been accounted for using lubrication hydrodynamics, but since the hydrodynamic effect seldom changes the adhesion time by more than a factor of two, we did not include it for simplicity reasons. However, it is noted that for the same-sign (zeta potential) sphere-spheroid interactions, both effects
(i.e., interparticle forces and lubrication hydrodynamics) will influence particles to appear on the end more than in the middle, especially for larger spheres.

5.8 Acknowledgements and Copyright Information

This chapter is a modified version of a publication to be submitted:


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5.9 References


6.1 Introduction

Understanding initial bacterial adhesion would allow the development of strategies to prevent biofilm formation, which would be important in reducing the failure of implanted biomaterials (4), improving bioremediation and water treatment processes (7, 17), and preventing industrial biofouling (11). Given the heterogeneity and dynamics of the bacterial cell surface, specific nanodomains on the surface are quite possibly important for initial adhesion. Based on previous work from our lab (15), bacterial polarity of \textit{Escherichia coli} was hypothesized to cause preferential polar adhesion to polystyrene and glass surfaces. The presence of a nanodomain, or molecular localization, at the pole(s) of rod-shaped bacteria is referred to as bacterial polarity (23). End-on (polar) adhesion is the preferential adhesion of a rod-shaped bacterium to a surface by the hemispherical pole of the bacterium.

For specific adhesion events, bacterial polarity has been connected to the oriented adhesion of \textit{Bradyrhizobium japonicum} to lactose (20), \textit{Pseudomonas aeruginosa} to canine tracheal cells via pili (27), and \textit{Treponema denticola} to fibronectin (5). Furthermore, Dawson and Ellen have shown an increase in the number of fibronectin adhesins located at the poles of \textit{Treponema denticola} over time in the presence of fibronectin (6). However, bacterial polarity has not been connected to adhesion for
nonspecific adhesion. Morisaki qualitatively observed the end-on adhesion of a *Bacillus* species to a quartz surface by noting the rotation of adhered cells about a single end during the reversal of an applied flow (21). Bacterial polarity resulting from cell division has been implicated for the anchoring of chromosomes to the poles of *Bacillus subtilis* (3) and shown for certain cell life cycles, with specialized structures forming at specific poles (1). Furthermore, several proteins in the outer membrane of *E. coli* were shown to be retained at the poles with fluorescent labeling (8). This localization was at least partially connected to the metabolism of murein and inert regions of murein at the poles (9). Additionally, outer membrane proteins and murein have been shown to localize at the poles of *E. coli* and to follow spiral patterns across the length of the cell (12, 13). The *E. coli* presented in this manuscript are hypothesized to have adhesion related to bacterial polarity at only one pole. We also hypothesize that the polarity is linked to the division process (15).

In this study we used a laser trap, which allowed both the manipulation of bacteria and the measurement of adhesion force values, to study the initial end-on adhesion of rod-shaped *E. coli* bacteria. Rod-shaped species align with the direction of the laser such that a bacterium points end-on toward the glass surface. Thus, this instrument is well-suited for the study of end-on adhesion of rod-shaped bacteria, since the bacteria in this research have been shown to adhere predominantly by a single end (15). Several studies have shown the ability of a laser trap to manipulate bacteria, to sort bacteria (2), and to position bacteria at surfaces in defined orientations (14). Researchers have used a laser trap to measure the adhesion forces of *E. coli* adhering to mannose self-assembled monolayers on gold films (18), *E. coli* adhering to the receptor sugar galabiose via pap
pili (10), and *Staphylococcus aureus* adhering to fibronectin coated microspheres (24). However, for these systems a specific interaction mediated the adhesion. Repulsive forces for nonspecific adhesion have also been measured with a laser trap for *S. aureus* at glass surfaces in low salt concentration (1 mM) (16). But the adhesion and adhesion forces of bacteria adhering nonspecifically to surfaces have not been characterized using a laser trap.

Laser trapping is capable of studying end-on initial adhesion of bacteria and adhesion forces with a force resolution less than 1 piconewton. For this work the end-on adhesion of *E. coli* to glass capillary surfaces not treated for specific adhesion was studied and quantified with a laser trap. In particular, the frequency of adhesion and the time scales of the adhesion were measured. The possibility of end-on adhesion being caused by bacterial polarity was investigated, as well as the hypothesis that division brings about end-on adhesion.

### 6.2 Materials and Methods

A nonmotile strain of *Escherichia coli* K12: D21, obtained from the *E. coli* Genetic Stock Center (Department of Biology, Yale University, New Haven, CT) was studied. The D21 is the wild type strain containing the lipid A and core region of the lipopolysaccharide (LPS) layer, but not the α-antigen region. Growth was at 37°C and 150 RPM in Luria broth, and bacteria were harvested in mid-exponential growth. Bacteria were washed in 100 mM phosphate buffered saline (PBS) solutions (consisting of potassium hydrogen-phosphate and potassium dihydrogen-phosphate dissolved in
Milli-Q water) three times with centrifugation (Sorvall Biofuge Primo) at 5000 RPM (3466 g) for 10 minutes. Solutions of PBS were tempered to 37°C prior to addition of the bacteria. After washing, the suspension of bacteria was diluted further with 100 mM PBS, maintaining ionic strength, but reducing the bacterial concentration from roughly \(10^8\) cells/mL to \(10^7\) cells/mL.

### 6.2.1 Experimental Techniques

For all laser trapping experiments, a Nikon TE 300 Eclipse inverted optical microscope was used along with a 100× magnification Nikon Plan Fluor CFI oil objective (NA = 1.3). This microscope was operated in bright field mode with video equipment (e.g., Cohu 4010 CCD camera, VHS) for recording experiments and a motorized stage (Prior) to control xyz movements. A 2500 mW (maximum power), 1064 nm Nd:YAG laser was expanded 20 times before being sent through the epi port of the microscope to form a laser trap. The microscope was Koehler-illuminated, and the laser trap was aligned to the center of the objective prior to each experiment. This preparation insured the most efficient laser trap as well as the alignment of the optical viewing plane and the plane of the center of the laser trap (i.e., a trapped bacterium was visually in focus).

Rectangular capillaries (Vitrocom) with 0.2 by 2.0 mm inside dimensions and 0.2 mm wall thickness were mounted on glass microscope slides with paraffin wax for experimentation. Glassware was cleaned by sonication in an Alconox solution for 20 minutes, and then the glassware was washed with 70% ethanol. Finally, the glassware
was soaked in 16 N nitric acid or 6 N hydrochloric acid for at least 1 hour, after which it was rinsed with Milli-Q water.

### 6.2.2 Laser Trapping of Bacteria

Bacteria were trapped and studied one at a time using the laser trap. A bacterium always aligns with the direction of the laser light, perpendicular to the glass surface, such that the end of a bacterium is always presented to the surface. Laser powers of 100 mW were used for all bacterial manipulations, unless otherwise noted. The actual power of the laser trap was reduced significantly by the light path to the specimen plane. A transmittance of 30–40% was measured (Coherent Lasermate-Q) for all experiments (at 100 mW) for the path up to the objective, including beam expander and dichroic. The Nikon Plan Fluor CFI objective (NA = 1.3) used for all experiments was measured to have a transmittance of 61% by Neuman et al. for a 1064 nm laser wavelength (22). Thus, the total transmittance through the optical path to the specimen plane is estimated to be 20% or 20 mW for a measured laser power of 100 mW. Larger laser powers were only used temporarily to remove adhered bacteria from the glass surface when appropriate, and the laser power was immediately reduced to 100 mW upon removal. Bacteria were held in the laser trap only while being tested for adhesion or being moved. The time of exposure to laser light was minimized as much as possible.

An important consideration when using a laser trap with biological samples is the effect of the focused laser light upon the physiology of the sample. Every effort was taken to minimize damage to the bacterial cells by the laser light, such that ideally the
laser trap acted as an “invisible hand” manipulating the cells. For *E. coli* cells, exposure to a 1064 nm laser at 50 mW in the specimen plane showed a three fold increase in the time to cell death compared to cells not exposed to the laser for aerobic conditions (22). However, the trapping time was roughly 1000 seconds, and the cloning efficiency of cells was reduced to 45% after 5 minutes of trapping at 88 mW in the specimen plane (22). The laser powers used for the current study are roughly 20 mW in magnitude at the specimen plane with exposure times of order 100 seconds or less. Both the power and time of exposure are several times less than the exposure mentioned previously; thus, it is expected that minimal damage was incurred by the bacteria due to the laser light. The damage from a laser trap likely results from oxidation it induces, and it occurs in a linear fashion (22). Thus, some damage to the cell is inevitable. However, reattachment studies presented in this paper show that bacteria could be attached multiple times to a surface after removal with a laser trap, lending support to limited effect of the laser on the adhesion of the bacteria.

### 6.3 Brownian Randomization of Orientation

The time of bacteria to rotate from 0 to 90° was estimated by the inverse of the rotational diffusion coefficient ($D_r \sim 0.2 \text{ sec}^{-1}$) for a rod-shaped bacterium 1 by 3 microns (26), giving a time of rotation on the order of 5 seconds. Detailed Brownian dynamics simulations were performed to determine the average time necessary for bacteria to rotate 90° from vertical. This time was found to be roughly 7.5 seconds. Allowing 15 seconds (to account for variations in size) for a bacterium to randomize its orientation allowed us
to use the laser trap to test both ends in a statistical manner. Thus, if the probability is about 0.50 that a given end will point downward, the probability that it has not been tested after 5 randomizations is \((0.5)^5 = 0.031\), and after 10 randomizations it is 0.00098. Hence for all experiments involving the release and recapture of bacteria in the laser trap, the bacteria were released for 15 seconds before being recaptured.

6.4 Results

Planktonic bacteria were manipulated and brought to the surface of the capillary with the laser trap (100 mW). No differentiation was made about the physiology of each bacterium, i.e., all cell types (dividing, newly divided, cells in between) were tested at random. Each bacterium was held near to the surface, such that the bacterium could contact the surface, for 15 seconds. The distance from the surface was determined using Newtonian interference rings resulting from the laser interacting with the sample and the surface. The size of these rings was evaluated against the focus of objects obviously adhered to the surface, such that bacteria in the laser trap could be consistently and verifiably placed at the surface to test adhesion by maintaining a constant size of the interference rings for each bacterium. After the bacterium was held at the surface, the laser trap was then moved out of the capillary (i.e., through the glass) in the \(z\)-direction (perpendicular to the plane of the glass), and the focus was repositioned to observe the bacterium without trapping the bacterium. Of the 233 bacteria examined over 8 experimental samples (4 separate growth flasks on different days and 2 samples from each flask), 15.9 ± 3.4% (standard error) were found to adhere. Adhesion was observed
by the significant reduction in Brownian motion when a bacterium adheres at the glass,
by the bacterium not diffusing away from the glass surface, and by verifying both these
conditions for 15 seconds. For this particular experiment, the bacteria were observed
qualitatively to either adhere quickly, within the first second at the surface, or not at all.

To further examine the effect of time on adhesion, bacteria were tested for
adhesion after 1, 15, and 100 seconds at the surface. This experiment was conducted
similarly to the previous experiments; however, after a bacterium adhered, the testing of
adhesion was stopped (e.g., if a bacterium adhered at 1 second, it was not tested at 15 or
100 seconds). Of the 95 bacteria examined over 7 experimental samples (7 separate
growth flasks, each on different days), 20% ± 4.6 (standard error) adhered after 1 second
(consistent with the previous data), 6.3% ± 2.2 (of the original 95) after 15 seconds, and
8.4% ± 2.7 after 100 seconds. This result is shown in Figure 6-1 compared with the
expected results extrapolated from the previous experiment, assuming adhesion is
instantaneous (< 1 second) and a bacterium has only a single adhesive end. In other
words, we expected that 15.9 ± 3.4% would adhere as before after 1 second. Of those
that did not adhere after one second, half should be caught in the opposite orientation by
the laser trap, since the bacteria are released from the trap and allowed to undergo
Brownian motion between each run, and the resulting adhesion percentage should be
roughly 8% (of the original number) at 15 seconds. Similarly for 100 seconds, 4%
should adhere.
The effect of laser power (force) on the adhesion of the bacteria was also determined. Bacteria were held at the glass surface for 15 seconds and then released. A single bacterium was released for 15 seconds and then recaptured and retested five times.

Figure 6-1: Fraction of bacteria adhering to the glass surface versus time held at the surface by the laser trap. Each bacterium was tested for 1, 15, and 100 seconds in that order or until adhesion occurred. The grey shows the experimental results, while the hashed shows the extrapolated predicted results from previous experiments. The predicted results assume a single adhesive end and equal probability of vertical orientations in the laser trap at each time.
This process was conducted for laser powers of 100 and 500 mW, in order. Only bacteria that did not adhere any of the 5 times tested at 100 mW were tested at 500 mW. This restriction was to insure that both ends of the bacterium were tested for adhesion and that neither end was adhesive at 100 mW. For 10 bacteria tested, all bacteria that were not adhesive under 100 mW of laser power were not adhesive at 500 mW. Thus, the force of the laser used to present the bacteria to the surface did not play a role in the adhesion. Conversely, bacteria not under the influence of an applied laser force (0 mW) were often observed to adhere end-on to the glass surface. This result was observed in previous research with applied fluid flows (15).

The end-on adhesion of cells with visually-apparent division planes was also tested. In order to test both ends of these cells, the cells were brought to the surface and held for several seconds, then released as above. Non-adherant cells were allowed to undergo Brownian motion for 15 seconds, then retrapped and retested for adhesion, and the process was repeated 10 times. Of the 18 cells with obvious division planes studied, none adhered, i.e., neither end of each of the 18 dividing cells was adhesive.

Bacteria were then tested for the ability to re-adhere by an already adhered end. A bacterium was presented to a surface, allowed to adhere, released from the trap, and then grabbed by the trap at higher power and removed. Removal was accomplished by placing the laser trap center slightly above the center of the bacterium and increasing the laser power until the bacterium came off the surface. The process was then repeated in different locations on the glass surface until the bacterium no longer adhered, up to a maximum of 5 times. The majority of the bacteria studied were able to adhere multiple times. Some bacteria adhered only once, some at least 5 times. The laser power used to
remove the bacteria showed no discernable trend with number of times adhered. The results are summarized in Table 6-1. Based on these results, it was not conclusive for any individual bacteria whether an adhesive end would readhere after being removed from the surface, although most did readhere.

Table 6-1: Results of reattachment study of D21 bacteria with laser trap. Each bacterium was allowed to adhere to the glass surface, removed with the laser trap, and attempted to reattach. The number of times adhered by the process and the laser power range necessary to remove the bacterium are shown. Laser power roughly corresponds to force as 2 pN per 50 mW, obtained from a calibration in our lab.

<table>
<thead>
<tr>
<th>Bacterium Number</th>
<th>Times Adhered</th>
<th>Laser Power to Remove (mW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>&gt;250</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>100-250</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>&gt;500</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>150</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>150</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>770</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>350</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>100-330</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>790-860</td>
</tr>
</tbody>
</table>

Finally, the adhesion was tested for newly-divided daughter cells. Cells with visually-apparent division planes were observed while free floating in suspension until the cells divided into daughters cells. The two daughter cells for each divided cell were tested for adhesion as with previous experiments. Adhering cells were left at the surface, while nonadhering cells were tested for adhesion by holding the bacterium at the glass surface for a few seconds, then releasing for 15 seconds, and re-testing for up to a total of
5 times. Of the 50 daughter cells tested (25 dividing cells over 13 experimental trials), 4 of the cells adhered. We note that only a few experimental cases could be studied during each experimental trial, which required two days of preparation. Hence, these experiments are tedious and demand considerable time to acquire significant data.

### 6.5 Discussion

Previous research with D21 concluded greater than 90% of the cells that adhered to sulfated PS spheres adhered end-on (15). However, no quantification of end-on adhesion within a total population of cells was made. The laser trap is well-suited to study end-on adhesion, since rod-shaped bacteria will always align vertically in the trap and bacteria can be manipulated individually from a population. By using a laser trap to position individual bacteria at a glass surface, we found that 15.9 ± 3.4% of our D21 were adhesive at one end.

From our particle adhesion studies, we have hypothesized that a single end being important for adhesion (15). Since roughly 1/6 of the bacterial ends are adhesive, it is likely that roughly 1/3 of the bacteria would adhere via a single end to the glass surface, since the laser trap is only able to sample the adhesion of single end at a time. The experimental results from the multiple time experiments agree well with predicted values for end-on adhesion from the 15 second experiments (which found 15.9 ± 3.4% of D21 cells to be adhesive by the ends) as expected for 1 and 15 second times, shown in Figure 6-1. This agreement supports the presence of a single end important for adhesion on the D21 bacteria. However for 100 seconds held at the surface, the percentage of bacteria
adhering is slightly higher than expected (Figure 6-1). This result may be influenced by a life cycle time-dependency of the adhesive end; the time experiment takes several minutes to complete for the 100 second time trial. This increased adhesion may also result from the influence of the bacterium being at the surface, e.g. bond aging (25).

To test the time dependency and possible division effects on adhesion, bacteria with visually observable division planes were isolated from a population and tested for end-on adhesion. Of 18 such dividing bacteria studied, none adhered to the glass surface via the ends. With dividing cells not adhering by either end, we hypothesized that the adhesive end originates in the division plane. A possible schematic for the production of a propagating single adhesive end is shown in Figure 6-2 a). The adhesive nature of the single end of a cell diminishes as the cell begins to divide (again, cells with obvious division planes were not found to adhere by either end), resulting ultimately in two daughter cells with single adhesive ends.
Qualitative observations during the course of experimentation found many cells with observable division planes adhering by the middle of the cell, i.e., by the division plane region. Figure 6-3 shows images of this phenomenon. From panes a) to c), the dividing bacterium is rotated about its center using the laser trap (unbounded bright spot at upper end of cell). Based on this observation, daughter cells from observed dividing cells were tested for adhesion. Of the 50 daughter cells tested only 4 were adhesive. However, of the 4 adhesive daughter cells, only 2 were from the same dividing cell (as in Figure 6-2 a). The other 2 adhesive daughter cells were cases where only one of the daughter cells from the original dividing cell was adhesive (as in Figure 6-2 b)). Thus, the hypothesis in Figure 6-2 a) has experimental support but for only one dividing cell out of 25. These results show single adhesive ends resulting immediately after division,
though they do not fully explain the resulting extent of adhesion (15.9 ± 3.4%) seen in a population of cells with previous experiments. An additional hypothesis for the development of single adhesive ends through division is that the adhesive end may result some time after division. However, no experimental evidence of this hypothesis was obtained and experiments to test this hypothesis are very challenging.

6.6 Conclusions

Using a laser trap to position bacteria at a glass surface, 15.9 ± 3.4% of the *E. coli* bacteria studied were shown to adhere. This adhesion occurred instantaneously (< 1 second) via a single adhesive end, i.e., at most only one end of the rod-shaped bacterium is adhesive. The adhesion was shown to be independent of applied force (laser power).
Cells were found to re-attach multiple times after removal from the surface, although this result was not universal and showed no noticeable trend with force necessary to remove an adhered bacterium. Bacteria with observable division planes were not adhesive by either end, but 4 of 50 daughter cells adhered by a single end immediately after division. This result supports the hypothesized development of daughter cells with a single adhesive end occurring right after division in the division plane (Figure 6-2). However, it does not account fully for the extent of end-on adhesion seen in the population (15.9±3.4%).

An important ramification of this work concerns the prediction of bacterial sticking coefficients ($\alpha$) (19). Currently these coefficients are measured for various bacteria in particular porous media. The bacterial surface is normally considered to be chemically uniform and geometrically smooth, and adhesion is treated as empirical and by chance. However, with a knowledge that a particular region on the bacterial surface is “sticky” – perhaps by independent, microscopic techniques – we would be able to use geometric and hydrodynamic considerations to estimate accurate values of $\alpha$. Thus, knowing the nanoscale bacterial chemical properties would enable one to predict $a\ priori$ the adhesion properties of the bacteria for a variety of porous media and solution conditions, and potentially enable researchers to design improved schemes for reducing or enhancing bacterial adhesion.

6.7 Acknowledgments and Copyright Information

Chapter 4 is a modified version of the author’s submitted manuscript:

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6.8 References


Chapter 7

End-on Adhesion Effect in Initial Bacterial Adhesion: Bacteria Odds and Ends

7.1 Introduction

The initial adhesion of bacteria is the first step in the process of biofilm development, affecting such diverse areas as infection on biomedical implants (9) to efficiency of bioremediation strategies (6, 13). For E. coli K-12 D21 bacteria, the initial adhesion to colloidal polystyrene particles and glass surfaces has been shown to occur predominantly via a single adhesive end (10). This single end has been hypothesized to occur due to bacterial polarity (10), or the localization of molecules at the end or pole of the rod-shaped bacterium (16). As well, smaller molecular localizations distributed not just at the end of the cell but across the entire cell have also been hypothesized for E. coli (Chapter 5).

Many instances for bacterial polarity of E. coli and other bacteria exist (1, 2, 4, 5, 8, 15, 16, 19). Furthermore, the heterogeneity of the surface due to bacterial polarity would likely result in a heterogeneous charge distribution on the surface of the bacteria. Measurements of charge nonuniformity on the surfaces of colloidal suspensions have been supported for the technique of charge nonuniformity light scattering (CNLS) (11). Similar techniques using the electrooptic effect have been applied to measure dipoles of bacteria (17). Thus, CNLS is well-suited to indirectly validate the existence of bacterial
polarity manifested in a single adhesive end by measuring charge nonuniformity of *E. coli* bacteria.

End-on adhesion has been shown for many bacteria involving specific (i.e., stereochemically specific) molecular interactions (2, 3, 14, 15, 19), but beyond *E. coli* bacteria (10) no quantification of end-on adhesion has been made for other bacteria via nonspecific adhesion. In this study, the end-on adhesion of *Burkholderia cepacia* bacteria, useful for the degradation of tri- and tetrachloroethylene pollutants (7), is studied. These bacteria are Gram-negative, rod-shaped bacteria similar in size to *E. coli*.

### 7.2 Materials and Methods

#### 7.2.1 Bacteria

Three strains of bacteria were used for experimentation: *Escherichia coli* K12 D21, *Burkholderia cepacia* G4, and *Burkholderia cepacia* ENV435. The D21 strain was obtained from the *E. coli* Genetic Stock Center (Department of Biology, Yale University, New Haven, CT), while the *B. cepacia* strains were obtained from Mary DeFlaun at Envirogen Corp. The D21 is a nonmotile strain containing the lipid A and core region of the lipopolysaccharide (LPS) layer, but not the o-antigen region. The G4 contains the full LPS layer, and the ENV435, a genetic mutant of G4, also lacks the o-antigen region. All bacteria are rod-shaped, similar in size, and non-motile.
Growth for D21 was at 37°C and 150 RPM in Luria broth for 2.5 hours for bacteria harvested in mid-exponential growth and 12 hours for bacteria harvested in stationary phase growth. Growth of G4 and ENV435 was in Luria broth at 30°C and 150 RPM for 5 and 3.5 hours, respectively for mid-exponential growth. Bacteria were washed in suspending medium for experimentation three times at 5000 RPM (3466 g) for 10 minutes. The suspending medium was tempered to the growth temperature prior to addition of the bacteria. After washing, the suspension of bacteria was either used directly or diluted further with the suspending medium. For all orientation adhesion experiments, the suspending medium was 100 mM phosphate buffered solution (PBS) (consisting of potassium dihydrogen phosphate and potassium hydrogen phosphate), and the suspending medium was 10 mM PBS for all CNLS experiments.

7.2.2 Orientation Adhesion Experiments

Orientation experiments for *B. cepacia* were conducted similarly to previous research (Chapter 4) (10). Sulfated polystyrene (PS) particles (1.6 micron diameter) and bacteria were mixed in suspension. Video microscopy was used to observe the orientation in which the particles adhered to the bacteria (10), i.e. to the hemispherical end or the cylindrical middle. Instances where multiple particles adhered to a single bacterium were observed and recorded. Each instance of a particle adhering to a bacterium was counted as adhesion, i.e., if two particles adhere to a bacterium, one at the end and one in the middle, both the end-on adhesion and middle adhesion are included separately in the calculation of percent adhesion.
7.2.3 Charge Nonuniformity Light Scattering (CNLS)

The technique of charge nonuniformity light scattering (CNLS) is described extensively elsewhere (Appendix C) (11). However, a brief summary of the technique and relevant details to this research are included in this chapter. The CNLS technique combines the effects of scattering intensity varying with the orientation of an anisotropic species and nonuniformly charged species rotating in an applied electric field. By observing changes in intensity with changes in an applied electric field, the charge nonuniformity of a population of homogenous anisotropic colloidal species can be measured in terms of $\zeta_2 - \zeta_1$, a measure of charge nonuniformity as a symmetric zeta potential difference.

For the CNLS experiments, the electric fields used were $\pm 2, \pm 4, \pm 6, \pm 8, \pm 10, \pm 12, \pm 14, \pm 16, \pm 18$, and $\pm 20$ V/cm. Data points were acquired at a rate of 8 per second. In each case, the electric field was applied for 15 seconds. An electric field of 0 V/cm was applied at the beginning and end of each experiment for 15 seconds as well as between each nonzero value of the electric field. Every experimental sample was tested three times for each of three repetitions of the same bacteria and conditions. Intensity ratios were averaged for each system over the 9 experimental runs. All other necessary calculations to acquire intensity values are the same as previously described.

The goal of our experiments was to measure charge nonuniformity on the surface of D21 bacteria as a validation of the existence of bacterial polarity on the surface of
these cells. These bacteria are rod-shaped, and the intensity of scattered light will be approximated by the scattering of an ellipsoid of equal dimensions. In order to relate the measured intensity of scattering to \( \zeta_2 - \zeta_1 \), ratios of intensity differences are related to simulated intensity difference ratios. The experimental ratios are fit to the simulated ratios for the best \( \zeta_2 - \zeta_1 \) via a Monte Carlo statistical method.

### 7.3 Experimental Results and Discussion

Significant charge nonuniformity was measured for D21 bacteria in the mid-exponential and stationary growth phases, suspended in 10 mM PBS. Although for comparison the CNLS experiments would ideally be conducted using 100 mM PBS, electrolysis and inconsistent scattering results were obtained for suspensions in 100 mM PBS with the electric fields applied. Therefore, 10 mM PBS was used for all CNLS experiments. The \( \zeta_2 - \zeta_1 \) for each phase were measured as 7.9±0.40 mV and 3.8±0.34 mV, respectively. The experimental CNLS results for D21 in the mid-exponential phase compared with simulated results are shown in Figure 7-1. The experimental data match very well with simulations. The experimental CNLS results for D21 in the stationary phase compared with simulated results are shown in Figure 7-2. Again, the experimental data match very well with simulations. Previous research with video microscopy rotatational electrophoresis (VMRE) found no charge nonuniformity on the surface of D21 (10). However, the measurement of charge nonuniformity for D21 with VMRE likely falls within the limitations of the technique, which are overcome by CNLS. Specifically, the rotational Brownian motion of D21 bacteria is of similar magnitude as
the electrophoretic rotation for the electric fields used for VMRE. Therefore, it is very difficult to visually discern rotation due to charge nonuniformity for these bacteria, rendering the VMRE observations moot.

The charge nonuniformity found for the surfaces of D21 bacteria is consistent with bacterial polarity hypothesized for these bacteria. A localization of molecules, differing from the molecules covering the rest of the cell surface, at a single end of a bacterium would result in a nonuniform charge distribution. Though significant charge nonuniformity was seen for cells grown to the mid-exponential phase as well as the stationary phase, the $\zeta_2 - \zeta_1$ for the mid-exponential phase was double that found for the stationary phase.

Radial stagnation point flow experiments with a fluorescent modified D21 show greater adhesion for cells in the stationary phase than in the mid-exponential phase(18). The differences in surface charge nonuniformity found for D21 in differing phases of growth likely correlate with changes in the amount and distribution of surface molecules. This surface modification affects the extent of adhesion of *E. coli*, and it is likely that these changes in adhesion would extend to differences in end-on adhesion. Thus, the adhesion of bacteria, specifically *E. coli*, is dependent on growth phase and correlates with changes in the surface characteristics of the bacterial cell.
Figure 7-1: Graph of experimental CNLS measurements of D21 bacteria in the mid-exponential phase of growth suspended in 10 mM PBS compared with simulated results.

Figure 7-2: Graph of experimental CNLS measurements of D21 bacteria in the stationary phase of growth suspended in 10 mM PBS compared with simulated results.
In addition to characterizing differences in bacterial surfaces with changes in growth phases, *B. cepacia* bacteria, rod-shaped Gram-negative bacteria similar in size to *E. coli* (1 by 3 microns), were tested for end-on adhesion. Particle adhesion orientation study results for ENV435 and G4 are presented in Table 7-1. The percentages of 1.6 micron diameter sulfated PS spheres adhering end-on to the two *B. cepacia* strains are 79.8% and 75.5%, respectively. Additionally, for both bacteria particles were found to adhere to both ends of the cells in several cases. This result does not support a single adhesive end for these bacteria.

Though the adhesion of *B. cepacia* was found to be largely end-on, the end-on adhesion of G4 and ENV435 was not as predominant as D21. Furthermore, both *B. cepacia* strains were found to have particles adhering to both ends of a single bacterium in several instances. Thus, a Gram negative bacterial strain of the same shape and of similar size showed significantly different results for end-on adhesion. Even though end-on adhesion is found for other species of bacteria, it is not likely that the molecular causes are the same or that this effect is a predominant adhesion strategy for many bacteria. However, the practicality of studying the adhesion of bacteria in terms of specific areas or nanodomains on the bacterial surface seems warranted by the end-on adhesion effect seen for *E. coli*, given that this adhesion is probably resulting from bacterial polarity.
Using previous particle adhesion results for D21 (Figure 5-4), some estimation of the area of the hypothesized nanodomains on the cell surface of D21 can be made. The adhesion of varying sized sulfated PS particles to D21 bacteria is shown in Figure 7-3. The middle adhesion remains constant at roughly 10% for larger sized particles, but then sharply rises around a particle radius of roughly 0.5 microns. The constant adhesion for larger sized particles is consistent with bacterial polarity of a single end causing adhesion, as shown in Figure 7-3. The sharp rise in middle adhesion is predicted to occur from the existence of smaller area localizations all over the bacterial cell of the same molecules that cause end-on adhesion (Chapter 5).

<table>
<thead>
<tr>
<th>Particle Type (diameter)/Bacteria</th>
<th>Number of Bacteria Adhering to PS Particles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>end</td>
</tr>
<tr>
<td>1.6 µm sulfated PS/ENV 435</td>
<td>76</td>
</tr>
<tr>
<td>1.6 µm sulfated PS/G4</td>
<td>102</td>
</tr>
</tbody>
</table>

Table 7-1: Results for adhesion of 1.6 micron diameter sulfated PS particles to B. cepacia ENV435 and G4 bacteria.
Given the dramatic rise in middle adhesion at roughly a radius of 0.5 microns, this particle size may be used to estimate a threshold area of the smaller patches likely to exist on the middle cell surface. Coincidentally, the radius of the hemispherical end is also roughly 0.5 microns. Thus, an estimated threshold area size indicates the largest area the smaller patches can comprise as well as the smallest area the end localization can comprise. To estimate this area, the adhesion of a sphere to a planar surface will be used. This estimation is valid for a bacterium adhering end-on to a planar surface, as seen with laser trap experiments (Chapter 6), since the end of the cell is hemispherical and the glass is flat. However, for the adhesion of particles to the middle of the bacterial cell, this estimation may slightly overestimate the area, since the cell middle is cylindrical in

---

**Figure 7-3:** Graph of particle adhesion studies involving the adhesion of spherical sulfated PS particles to D21 bacteria. This graph is reproduced from data shown in Figure 5-4.
geometry, not planar. For the adhesion of a sphere to a planar surface, the diameter \((L)\) of the circular area of interaction is given by Eq. 7.1

\[
L = (8ah)^{\frac{1}{2}} \tag{7.1}
\]

where \(a\) is the sphere radius and \(h\) is the perpendicular distance from the planar surface to the surface of the sphere (when the sphere and planar surface are in contact) where the area of interaction begins. In the case of a particle adhering to a bacterium or a bacterium adhering to a flat surface, \(h\) is defined by electrostatics (the screening length of electrostatic interactions) and physical constraints to adhesion such as the length of lipopolysaccharide (LPS) molecules on the bacterial surface preventing access to the adhesive area, shown in Figure 7-4. Again, based on previous research (10), LPS molecules are not the adhesive molecules; proteins are likely the adhesion molecules. Using a particle radius of 0.5 microns and setting \(h\) as 5 nm (an estimation of the length that the LPS molecules extend into solution (12)), the estimated threshold area size for adhesion is 0.016 microns squared \((L = 140\) nm).
7.4 Conclusions

The existence of bacterial polarity of a single end of E. coli D21 is supported with charge nonuniformity results. Cells grown to the stationary phase exhibited less charge nonuniformity than was found for cells grown to the mid-exponential phase of growth. The change in surface charge nonuniformity likely manifests as changes of the localization of molecules, resulting in changes in adhesion, specifically end-on adhesion. Finally, the threshold patch sizes of adhesive molecular localizations on the surface of E. coli D21 were found to be 0.016 $\mu m^2$. 

Figure 7-4: Schematic of a spherical particle adhering to a bacterial surface. Lipopolysaccharide molecules are shown extend from the bacterial surface. The grey region the surface denotes the adhesive region. The height $h$ indicates roughly the height of the LPS molecules or the distance relevant to the accessibility of the particle to the adhesive region. $L$ is the diameter of the area projected onto the surface by the particle at height $h$. 

LPS molecules
The existence of nanodomains on the surface of bacteria, which are important for adhesion, seems an important consideration when examining adhesion. Studying the bacterial surface as map of discrete areas may provide a useful strategy for solving bacterial adhesion problems. However, effects such as the end-on adhesion observed for D21 may not readily extend to other bacterial species. The mechanisms of adhesion for a bacterial species or group of bacteria may not be broadly applicable.

### 7.5 Acknowledgments and Copyright Information

Chapter 7 is a modified version of the author’s soon to be submitted manuscript:


Pending publication, the content of this chapter is protected under the copyrights of AEM and should not be reproduced or used in any way without proper citation or express permission of the publisher when necessary.

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7.6 References


8.1 Contributions of this Research to Bacterial Adhesion

The main goal of this research was to study the initial adhesion of a model bacterial species *Escherichia coli* in order to understand the mechanisms and time and length scales involved in the initial adhesion of this organism to nonbiological surfaces not treated for specific interactions. The specific contributions of this research are addressed according to the research goals described in Section 1.2.

1) *Determine the orientation of initial adhesion and the likely mechanisms involved in this adhesion for E. coli bacteria.* The initial adhesion of *E. coli* K-12 D21 was found to occur predominantly via a single end of the rod-shaped cells with particle orientation studies. This effect was observed qualitatively on glass. By using various strains with differing LPS lengths on the surface, no difference in adhesion was found indicating that LPS molecules are not the important molecules for adhesion. Bacterial polarity was hypothesized as the reason for adhesion via a single end. Charge nonuniformity results supported the existence of single end bacterial polarity for D21. A single adhesive end was again supported by laser trap experimental results. These results also indicated the development of a single adhesive end as a result of the division process.
2) *Determine the geometric contributions to initial adhesion orientation by developing and applying a Brownian dynamics simulation (BDS) of the interaction of rod-shaped and spherical colloids.* A BDS was developed for sphere to spheroid adhesion. This simulation was supported by experimental results with ellipsoidal and spherical polystyrene particles. Results for D21 adhesion to spherical particles were found to deviate significantly from BDS results. This deviation indicated that geometric effects could not fully explain the single end adhesion effect. Furthermore by varying the size of the spherical colloids, experiments supported the existence of not only a single adhesive end on the surface of the D21 bacteria, but also smaller adhesive patches distributed across the entire bacterial surface.

3) *Determine the extent of end-on adhesion within a population of cells.* Laser trap experiments found 15.9 ± 3.4% of D21 bacteria within a population in the mid-exponential phase adhere by a single end.

4) *Determine the time scales and length scales involved with end-on adhesion.* The D21 bacteria were found to adhere instantaneously (< 1 second) via the end to glass surfaces. The adhesion increased slightly from expected values when held at the surface for 100 seconds than for shorter times. The threshold size for the adhesive area on the end of the cells as well as for the smaller localizations on the body of the cell was estimated as 0.016 µm².

5) *Determine the variability of end-on adhesion under differing growth conditions and among other species.* The first part of this goal is accomplished indirectly. The charge nonuniformities of D21 cells in the mid-
exponential and stationary phases of growth were found to be significantly different, indicating differences in the surface heterogeneity of these cells. These differences would likely result in differences in the end-on adhesion effect as with differences in overall adhesion of these cells in differing growth phases seen by other researchers (2). The adhesion results of *Burkholderia cepacia* G4 and ENV435 were found to largely occur via the end for particle adhesion studies. However, the extent of this adhesion was not as significant as *E. coli* nor was it found to occur via a single end.

The broad reaching contribution of this research was to examine bacterial adhesion in terms of nanodomains or discrete regions on the surface. Typically bacterial adhesion is thought of in terms of a population of cells, the cell surface as a whole, or individual molecules on the surface. However, studying individual bacteria cells within a population, and specifically looking at regions on the surface important for adhesion, seems to provide a good framework for examining the initial adhesion of bacteria.

### 8.2 Final Thoughts Concerning Bacterial Adhesion

The original motivation for the research in this thesis was to add to the development of a theory for bacterial adhesion. Much like colloidal theories, the idea was to develop some framework generally describing the phenomena of bacterial adhesion so as to broadly apply such a theory to describe most cases of bacteria adhering to surfaces. However, bacteria are biological organisms capable of responding to changes in the environment, evolving over time to different conditions, and varying
significantly from cell to cell within a given population, much less from species to species. The surfaces of bacteria are heterogeneous and dynamic in nature, making simplifying assumptions such as surface uniformity, thermodynamic equilibrium, and smooth surfaces difficult if not fully incorrect. Even the definition of the “surface” of a bacterium with its polymers reaching into solution, membrane flexibility, and conformational diversity requires careful consideration and is ambiguous.

Work by Bakker et al. highlighted the diversity of adhesion mechanisms from bacteria isolated from different niches or environments (1). Their work also pointed out that the literature attempting to develop broad generalizations about bacterial adhesion tends to be limited in scope, focusing on a few organisms (1). This thesis supports differences in adhesion strategies for only two species with similar characteristics. Thus, it seems very unlikely that an overall theory of bacterial adhesion is possible or even worth pursuing.

Based on the results of this thesis which indicate that nanodomains on the surface of bacteria are important for adhesion, it seems possible to practically apply the lessons and experience from research with one species to problems involving other bacteria. The key fundamental difference is that the research should be focused from an engineering perspective and less a scientific one, i.e., to examine problems related to bacterial adhesion on a case by case basis. In problems where bacterial adhesion is an important consideration, research should be conducted to develop a solution for particular bacteria for particular adhesion conditions. Bacterial adhesion seems too complex a problem to be resolved in a sound and broad reaching theory.
8.3 Future Work

The work of this research should continue in two stages. First, the specific molecules involved in the adhesion of *E. coli* to nonbiological surfaces should be resolved. Techniques such as fluorescence resonance energy transfer (FRET), fluorescence recovery after photobleaching (FRAP), colloid probe analysis with electron microscopy, total internal reflection aqueous fluorescence (TIRAF), and flow cytometry may be useful in determining these molecules. An important consideration of such research is to directly show that the molecule in question causes the adhesion. Second, the adhesive molecules should be manipulated in some way to change the adhesion (eventually to control the adhesion). Genetic modification of the bacterial surface, indirect modification of either the bacterial surface or the substrate (e.g., changing ionic strength or pH, adding surfactants or polymers), and/or modification of the substrate should result in the development of control of the adhesion. However, it is still the opinion of the author that a more practical approach to the problems involving bacterial adhesion (perhaps even using the methods just outlined) may be ideal.

8.4 References

Appendix A

Bacteria Growth and Safety Procedures

A.1 General Safety for Biosafety Level (BSL) 1 and 2 Bacteria

1. Appropriate personal protection equipment must be worn at all times when working with bacteria.
   a. Wash hands with antimicrobial soap prior and post to working with any bacteria.
   b. Safety glasses, latex gloves, and closed toe shoes must be worn.
   c. Clean latex gloves at regular intervals (especially any time bacteria solution may have spilt on gloves) with 70% ethanol solution.

2. Conduct general operations with bacteria solutions under a laminar flow hood.
   a. All transfer of bacteria solution or work with bacteria in open containers for BSL 2 bacteria (*B. cepacia, P. aeruginosa*) must be conducted under an operating laminar flow hood.
   b. Work with BSL 1 bacteria (*E. coli, B. subtilis*) can be conducted on a bench top once the bacteria have been harvested. All work with these bacteria prior to the wash procedure must also be conducted under a laminar flow hood.
   c. Place bacteria solutions and glassware in bins if transporting between 178 Fenske and 57A Fenske. Hold the bin with a gloved hand and open doors with a clean bare hand.
d. Thoroughly clean all workspaces with 70% ethanol solution prior and post to working with bacteria.

e. UV-sterilize the laminar flow hood on a regular basis. Leave the UV lamp on for approximately 20 minutes with the sash fully closed. NOTE: UV sterilization only works at the surface level. Containers will not be sterilized.

3. Dispose of all bacteria waste in marked biohazard waste containers.
   
a. All solutions containing bacteria must be disposed of in an appropriate waste container. Rinse all vessels containing bacteria with 70% ethanol (also dispose of in biohazard waste container) and autoclave (see autoclaving section, #5).
   
b. Dispose of all solid waste contacting bacteria solutions in the appropriate solid waste containers.
   
c. Store all waste containers for BSL 2 bacteria under the laminar flow hood and autoclave and dispose of on a regular basis. Waste containers for BSL 1 bacteria can be stored outside of the hood, but must also be autoclaved and disposed of on a regular basis.
   
d. Cover any spills of bacteria solution with paper towels doused in 10% bleach solution. Then clean with paper towels, while wearing the appropriate PPE. Dispose of all solid waste in the appropriate biohazard waste container.

4. Grow all bacteria in the appropriate incubator.
   
a. Grow both BSL 1 and BSL 2 bacteria in the incubator in 178 Fenske.

5. Autoclave glassware containing bacteria solutions. NOTE: All caps must be left on loosely while autoclaving.
a. Fill all culture flasks with deionized water after rinsing with 70% ethanol solution.

b. Then autoclave this glassware on the liquid cycle for 20 minutes.

c. Wash this autoclaved glassware with water and Alconox, rinse with deionized water, and dry.

d. Autoclave this glassware once more on the dry cycle for 20 minutes.

e. Autoclave all liquid waste containers on the liquid cycle for 20 minutes. Cover loosely with tin foil cap. After autoclaving, pour the sterilized liquid waste down the sink.

f. Autoclave all solid waste in its biohazard waste bag for 20 minutes. Put autoclave tape on the containers prior to autoclaving. EHS picks up sterilized solid waste weekly.

### A.2 Growing Bacteria

1. Revive cells

   a. Clean laminar flow hood surface with 70% ethanol solution. NOTE: Ethanol solution is extremely flammable. Do not store ethanol bottle near flame. Make sure all surfaces in the vicinity of the flame are dry (free of ethanol), including hands and gloves.

   b. Transfer 30 mL Luria broth (LB) into 50-mL autoclaved flask with sterilized pipet.

   c. Sterilize tweezers over gas flame.
d. Remove cane of culture beads from cryogenic storage vessel. Wear cryogenic safety gloves when working with the cryogenic vessel. Vials of culture beads are labeled.

e. Take a culture bead out of its vial and drop it into the flask of LB with the sterilized tweezers.

f. Sterilize tweezers again.

g. Place flask on the proper shaker table for 18 hours at:

\[37^\circ C \text{ for } E. \text{ coli and } P. \text{ aeruginosa,}\]

\[30^\circ C \text{ for } B. \text{ cepacia and } Bacillus \text{ subtilis.}\]

Use the incubator in room 178 for both BSL 1 and BSL 2 bacteria.

h. Make sure the gas is off and clean the surface with 70% ethanol solution.

If using frozen liquid stock instead of culture beads (B. cepacia G4), replace steps c – f with:

c. Set vial from cryogenic storage vessel on hood surface and let the frozen stock melt. Wear cryogenic safety gloves when working with the cryogenic vessel.

d. Transfer 600 µL of the liquid stock into the 30 mL of LB using an autoclaved pipet tip.

e. Dispose of the vial in a biohazard container.

2. Grow cells

a. Clean laminar flow hood surface with 70% ethanol solution.

b. Transfer 100 mL LB into 250-mL autoclaved flask with sterilized pipet.
• For *B. cepacia* and *Bacillus subtilis*, temper this broth for 15 minutes by placing this flask in the incubator.

c. Add 1 mL of revived cells using autoclaved pipet tip or 1 mL sterilized pipet.

d. Place flask on appropriate shaker table and harvest at mid-exponential time shown in Table A-1 (concentration $\approx 10^8$ cells/mL).

e. Clean surface with 70% ethanol solution.

3. Safety and procedural notes

a. Wear safety goggles, gloves, and closed-toe shoes. Wear cryogenic safety gloves when working with the cryogenic storage vessel.

b. Conduct all work under the laminar flow hood.

c. Dispose of trash (gloves, pipets, pipet tips, paper towels) into marked biohazard containers.

d. Clean surfaces with 70% ethanol solution. Clean major spills with 10% bleach solution.

e. LB broth:

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Optimal Growth Temperature (°C)</th>
<th>Growth Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. Coli</em> (D21, D21f2, JM109)</td>
<td>37</td>
<td>2.5</td>
</tr>
<tr>
<td><em>B. cepacia</em> G4</td>
<td>30</td>
<td>5.0</td>
</tr>
<tr>
<td><em>B. cepacia</em> ENV435</td>
<td>30</td>
<td>3.5</td>
</tr>
</tbody>
</table>
• 25 grams powder LB broth + 1 L DI water (fill to the fill-line on the orange-capped containers), mark with autoclave tape, autoclave for 20 minutes on the liquid cycle with cap loosened, mark with dated and initialed tape, store in refrigerator capped tightly.

**A.3 Wash and Loading Procedure for Bacteria**

1. Once bacteria have been grown for the appropriate time (mid-exponential point) bacteria are washed in the appropriate suspending medium (e.g., PBS) for experimentation as follows:
   
a. Place a given volume (usually 25 mL) of bacteria broth solution in centrifuge tube by pouring from culture flask. NOTE: For bacteria of BSL 2 (e.g., *B. cepacia* and *P. aeruginosa*), all transfer of liquid or opening of containers containing bacteria must be done under laminar flow hood using appropriate safety precautions. Bacteria of BSL 1 can be worked with on a bench top, minding the appropriate safety precautions.
   
b. Seal centrifuge tube by hand tightening screw cap.
   
c. Turn on centrifuge.
   
d. Open centrifuge and remove metal cover. Place tube in centrifuge, being sure to balance the centrifuge.
   
e. Replace centrifuge cover, close centrifuge, and begin centrifuging (5000 rpm for 10 minutes).
f. At end of cycle, remove centrifuge tube from centrifuge. Remove screw cap and pour liquid contents of tube off into biohazard liquid waste container. A bacteria “pellet” should remain at the bottom of the tube. NOTE: Again, all transfer of liquids containing bacteria of BSL 2 must be done under a laminar flow hood.

g. Fill tube with appropriate suspending medium to the same level as previously in tube.

h. Seal centrifuge tube by hand tightening screw cap.

i. Vortex tube until bacteria pellet at bottom of tube is resuspended.

j. Repeat steps 1d-i twice more.

k. Resuspended bacteria solution is now ready for experimental use.

l. Dispose of centrifuge tube in biohazard waste container.

2. Once the bacteria solution is ready for experimentation, the appropriate experimental cell (electrophoresis cell or capillary cell) can be loaded with bacteria.

a. To load the electrophoresis cell, first make sure the cell has been appropriately cleaned (sonicated, 24-hour nitric acid soak, and rinsed with deionized water) and contains no leaks.

   i. After checking that valves on cell are closed, fill both sides of electrophoresis cell with bacteria solution to roughly the middle of the ground glass fittings.

   ii. Carefully place appropriate electrodes (one by one) into ground glass fittings creating an airtight fit. NOTE: Some bacteria solution may escape from cell as the electrodes are inserted. Be sure to catch this
excess with a paper towel and clean any surfaces or gloves with ethanol.

iii. Now that the electrophoresis cell is sealed, the cell may be used for experimentation. However, until the cell is sealed all work with BSL 2 bacteria must be conducted under a laminar flow hood.

b. To load capillary cell, first make sure capillary is properly cleaned (sonicated in beaker with Alconox and deionized water solution, 24-hour nitric acid soak, and rinsed with deionized water) and secured to microscope slide with silicone sealant or paraffin wax.

i. Pipet roughly 20 μL of bacteria solution at the edge of one capillary opening. Allow capillary action to draw the fluid into the capillary.

Add more solution, if necessary, to fill capillary completely.

ii. Wipe any excess solution from slide.

iii. Capillary cell is now ready for experimentation.

iv. Dispose of slides in biohazard sharps container.

A.4 Preparation of Phosphate Buffered Solution (PBS)

1. To make a 100 mM ionic strength phosphate buffered saline (PBS) solution (pH ~ 7.3), use the following procedure:

a. Measure out 1.4088 g of potassium dihydrogen phosphate (KH2PO4) and 5.2049 g of potassium hydrogen phosphate (K2HPO4).
b. Carefully pour both salts into 1 liter volumetric flask. Be sure to wash all salt into the flask, since small masses are being used.

c. Fill to fill mark with deionized water.

d. Cap with ground glass fitting and shake until all salt is dissolved.

2. To make a 1 mM ionic strength PBS stock solution (pH ~ 7.3), use the following procedure:

a. Measure out 2.6178 g of potassium dihydrogen phosphate (KH$_2$PO$_4$) and 4.6892 g of potassium hydrogen phosphate (K$_2$HPO$_4$).

b. Carefully pour both salts into 1 liter volumetric flask. Be sure to wash all salt into the flask, since small masses are being used.

c. Fill to fill mark with deionized water.

d. Cap with ground glass fitting and shake until all salt is dissolved.

e. This solution must be diluted 10 times (99 parts deionized water to 1 part solution) to produce the final 1 mM ionic strength PBS solution.

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**A.5 Center for Disease Control (CDC) Description of Biosafety Level 1 and 2 and Necessary Precautions**

**Biosafety Level 2** is similar to Biosafety Level 1 and is suitable for work involving agents of moderate potential hazard to personnel and the environment. It differs from BSL-1 in that (1) laboratory personnel have specific training in handling pathogenic agents and are directed by competent scientists; (2) access to the laboratory is limited

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when work is being conducted; (3) extreme precautions are taken with contaminated
sharp items; and (4) certain procedures in which infectious aerosols or splashes may be
created are conducted in biological safety cabinets or other physical containment
equipment. The following standard and special practices, safety equipment, and facilities
apply to agents assigned to Biosafety Level 2:

1. Standard Microbiological Practices

   a. Access to the laboratory is limited or restricted at the discretion of the laboratory
director when experiments are in progress.

   b. Persons wash their hands after they handle viable materials, after removing
gloves, and before leaving the laboratory.

   c. Eating, drinking, smoking, handling contact lenses, and applying cosmetics are
not permitted in the work areas. Food is stored outside the work area in cabinets
or refrigerators designated for this purpose only.

   d. Mouth pipetting is prohibited; mechanical pipetting devices are used.

   e. Policies for the safe handling of sharps are instituted.

   f. All procedures are performed carefully to minimize the creation of splashes or
aerosols.

   g. Work surfaces are decontaminated on completion of work or at the end of the day
and after any spill or splash of viable material with disinfectants that are effective
against the agents of concern.
h. All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method such as autoclaving. Materials to be decontaminated outside of the immediate laboratory are placed in a durable, leak-proof container and closed for transport from the laboratory. Materials to be decontaminated off-site from the facility are packaged in accordance with applicable local, state, and federal regulations, before removal from the facility.

i. An insect and rodent control program is in effect.

2. Special Practices

a. Access to the laboratory is limited or restricted by the laboratory director when work with infectious agents is in progress. In general, persons who are at increased risk of acquiring infection, or for whom infection may have serious consequences, are not allowed in the laboratory or animal rooms. For example, persons who are immunocompromised or immunosuppressed may be at increased risk of acquiring infections. The laboratory director has the final responsibility for assessing each circumstance and determining who may enter or work in the laboratory or animal room.

b. The laboratory director establishes policies and procedures whereby only persons who have been advised of the potential hazards and meet specific entry requirements (e.g., immunization) may enter the laboratory.

c. A biohazard sign must be posted on the entrance to the laboratory when etiologic agents are in use. Appropriate information to be posted includes the agent(s) in use, the biosafety level, the required immunizations, the investigator's name and
telephone number, any personal protective equipment that must be worn in the laboratory, and any procedures required for exiting the laboratory.

d. Laboratory personnel receive appropriate immunizations or tests for the agents handled or potentially present in the laboratory (e.g., hepatitis B vaccine or TB skin testing).

e. When appropriate, considering the agent(s) handled, baseline serum samples for laboratory and other at-risk personnel are collected and stored. Additional serum specimens may be collected periodically, depending on the agents handled or the function of the facility.

f. Biosafety procedures are incorporated into standard operating procedures or in a biosafety manual adopted or prepared specifically for the laboratory by the laboratory director. Personnel are advised of special hazards and are required to read and follow instructions on practices and procedures.

g. The laboratory director ensures that laboratory and support personnel receive appropriate training on the potential hazards associated with the work involved, the necessary precautions to prevent exposures, and the exposure evaluation procedures. Personnel receive annual updates or additional training as necessary for procedural or policy changes.

h. A high degree of precaution must always be taken with any contaminated sharp items, including needles and syringes, slides, pipettes, capillary tubes, and scalpels.
i. Needles and syringes or other sharp instruments should be restricted in the laboratory for use only when there is no alternative, such as parenteral injection, phlebotomy, or aspiration of fluids from laboratory animals and diaphragm bottles. Plasticware should be substituted for glassware whenever possible.

ii. Only needle-locking syringes or disposable syringe-needle units (i.e., needle is integral to the syringe) are used for injection or aspiration of infectious materials. Used disposable needles must not be bent, sheared, broken, recapped, removed from disposable syringes, or otherwise manipulated by hand before disposal; rather, they must be carefully placed in conveniently located puncture-resistant containers used for sharps disposal. Non-disposable sharps must be placed in a hard-walled container for transport to a processing area for decontamination, preferably by autoclaving.

iii. Syringes which re-sheathe the needle, needleless systems, and other safety devices are used when appropriate.

iv. Broken glassware must not be handled directly by hand, but must be removed by mechanical means such as a brush and dustpan, tongs, or forceps. Containers of contaminated needles, sharp equipment, and broken glass are decontaminated before disposal, according to any local, state, or federal regulations.
i. Cultures, tissues, specimens of body fluids, or potentially infectious wastes are placed in a container with a cover that prevents leakage during collection, handling, processing, storage, transport, or shipping.

j. Laboratory equipment and work surfaces should be decontaminated with an effective disinfectant on a routine basis, after work with infectious materials is finished, and especially after overt spills, splashes, or other contamination by infectious materials. Contaminated equipment must be decontaminated according to any local, state, or federal regulations before it is sent for repair or maintenance or packaged for transport in accordance with applicable local, state, or federal regulations, before removal from the facility.

k. Spills and accidents that result in overt exposures to infectious materials are immediately reported to the laboratory director. Medical evaluation, surveillance, and treatment are provided as appropriate and written records are maintained.

l. Animals not involved in the work being performed are not permitted in the lab.

3. Safety Equipment (Primary Barriers)

a. Properly maintained biological safety cabinets, preferably Class II, or other appropriate personal protective equipment or physical containment devices are used whenever:

i. Procedures with a potential for creating infectious aerosols or splashes are conducted. These may include centrifuging, grinding, blending, vigorous shaking or mixing, sonic disruption, opening containers of infectious materials whose internal pressures may be different from ambient
pressures, inoculating animals intranasally, and harvesting infected tissues from animals or embryonate eggs.

ii. High concentrations or large volumes of infectious agents are used. Such materials may be centrifuged in the open laboratory if sealed rotor heads or centrifuge safety cups are used, and if these rotors or safety cups are opened only in a biological safety cabinet.

b. Face protection (goggles, mask, face shield or other splatter guard) is used for anticipated splashes or sprays of infectious or other hazardous materials to the face when the microorganisms must be manipulated outside the BSC.

c. Protective laboratory coats, gowns, smocks, or uniforms designated for lab use are worn while in the laboratory. This protective clothing is removed and left in the laboratory before leaving for non-laboratory areas (e.g., cafeteria, library, administrative offices). All protective clothing is either disposed of in the laboratory or laundered by the institution; it should never be taken home by personnel.

d. Gloves are worn when hands may contact potentially infectious materials, contaminated surfaces or equipment. Wearing two pairs of gloves may be appropriate. Gloves are disposed of when overtly contaminated, and removed when work with infectious materials is completed or when the integrity of the glove is compromised. Disposable gloves are not washed, reused, or used for touching "clean" surfaces (keyboards, telephones, etc.), and they should not be
worn outside the lab. Alternatives to powdered latex gloves should be available.

Hands are washed following removal of gloves.

4. Laboratory Facilities (Secondary Barriers)

a. Provide lockable doors for facilities that house restricted agents (as defined in 42 CFR 72.6).

b. Consider locating new laboratories away from public areas.

c. Each laboratory contains a sink for hand washing.

d. The laboratory is designed so that it can be easily cleaned. Carpets and rugs in laboratories are inappropriate.

e. Bench tops are impervious to water and are resistant to moderate heat and the organic solvents, acids, alkalis, and chemicals used to decontaminate the work surfaces and equipment.

f. Laboratory furniture is capable of supporting anticipated loading and uses. Spaces between benches, cabinets, and equipment are accessible for cleaning. Chairs and other furniture used in laboratory work should be covered with a non-fabric material that can be easily decontaminated.

g. Install biological safety cabinets in such a manner that fluctuations of the room supply and exhaust air do not cause the biological safety cabinets to operate outside their parameters for containment. Locate biological safety cabinets away from doors, from windows that can be opened, from heavily traveled laboratory
areas, and from other potentially disruptive equipment so as to maintain the biological safety cabinets' air flow parameters for containment.

h. An eyewash station is readily available.

i. Illumination is adequate for all activities, avoiding reflections and glare that could impede vision.

j. There are no specific ventilation requirements. However, planning of new facilities should consider mechanical ventilation systems that provide an inward flow of air without recirculation to spaces outside of the laboratory. If the laboratory has windows that open to the exterior, they are fitted with fly screens.
Appendix B

Algorithms and Analyses

B.1 Algorithm for Brownian dynamics simulations

The MathCad code containing the Brownian dynamics simulation used in Chapter 5 is shown below. Explanation of symbols and the algorithm is contained in the text of the code. For additional explanation, see Section 5.3.
\[ T := 273 \quad k := 1.38 \cdot 10^{-23} \quad \eta := .001 \quad \Delta t := 0.1 \]

\[ L := 1 \quad r := 0.5 \quad a := 1.65 \quad R := 20 \]

\[ a1 := L + r \quad b := r \]

\[ \Delta(\lambda) := \left[ \left( a1^2 + \lambda \right) \left( b^2 + \lambda \right) \left( b^2 + \lambda \right) \right]^2 \]

\[ \chi := \left( \int_0^\infty \frac{1}{\Delta(\lambda)} \, d\lambda \right) \quad \alpha a1 := \left( \int_0^\infty \frac{1}{\left( a1^2 + \lambda \right) \cdot \Delta(\lambda)} \, d\lambda \right) \]

\[ \alpha b := \left( \int_0^\infty \frac{1}{\left( b^2 + \lambda \right) \cdot \Delta(\lambda)} \, d\lambda \right) \]

\[ \chi = 2.493 \]

\[ D_e := \frac{k \cdot T}{16 \cdot \pi \cdot \eta} \left( \chi + \alpha a1 \cdot a1^2 \right) \cdot 10^6 \]

\[ \Delta t_e := D_e \cdot \Delta t \quad \text{Translational motion of ellipsoid} \]

\[ DT1 := \frac{k \cdot T}{16 \cdot \pi \cdot \eta} \left( \chi + \alpha b \cdot b^2 \right) \cdot 10^6 \]

\[ \Delta t_{T1} := DT1 \cdot \Delta t \]

\[ DT2 := \frac{k \cdot T}{16 \cdot \pi \cdot \eta} \left( \chi + \alpha b \cdot b^2 \right) \cdot 10^6 \]

\[ \Delta t_{T2} := DT2 \cdot \Delta t \]

\[ DR := \frac{3 \cdot k \cdot T}{16 \cdot \pi \cdot \eta} \left[ \frac{\left( \alpha a1 \cdot a1^2 + \alpha b \cdot b^2 \right)}{a1^2 + b^2} \right] \cdot 10^{18} \]

\[ \Delta t_\vartheta := DR \cdot \Delta t \]

\[ \Delta t_\phi := DR \cdot \Delta t \]
joe(N) :=

for i ∈ {1..N}

\[
\begin{align*}
\theta & \gets \pi \\
\phi & \gets 0 \\
V_s & \gets \begin{pmatrix} 0 \\ 0 \\ 5 \end{pmatrix} \quad \text{Starting position of sphere} \\
V_c & \gets \begin{pmatrix} 0 \\ 0 \\ -5 \end{pmatrix} \quad \text{Starting position of ellipsoid} \\
\text{flag}_{i,0} & \gets 0 \quad \text{Flag to track adhesion} \\
\text{flag}_{i,1} & \gets 0 \quad \text{Flag to track adhesion time steps} \\
\end{align*}
\]

while \( \text{flag}_{i,0} = 0 \)

\[
\begin{align*}
\sigma_\theta & \leftarrow \left(2 \cdot \Delta_\tau_\theta\right)^2 \\
\Delta_\theta & \leftarrow \text{morm}\left(1,0,\sigma_\theta\right)_0 \\
\eta & \leftarrow \text{round}\left(\theta / \pi, 0\right) \\
\theta & \leftarrow \theta + \Delta_\theta \\
\sigma_\phi & \left\{ \text{if } (\theta = n \cdot \pi), 10^{-6}, \left[2 \cdot \left(\Delta_\tau_\phi / (\sin(\theta))^2\right)^2\right] \right\} \\
\Delta_\phi & \leftarrow \text{morm}\left(1,0,\sigma_\phi\right)_0 \\
\sigma_s & \leftarrow \left(2 \cdot \Delta_\tau_s\right)^2 \\
\Delta_\Sigma & \leftarrow \text{morm}\left(1,0,\sigma_s\right)_0 \\
\sigma_{T1} & \leftarrow \left(2 \cdot \Delta_\tau_{T1}\right)^2 \\
\Delta_\Sigma_{T1} & \leftarrow \text{morm}\left(1,0,\sigma_{T1}\right)_0 \\
\sigma_{T2} & \leftarrow \left(2 \cdot \Delta_\tau_{T2}\right)^2 \\
\Delta_\Sigma_{T2} & \leftarrow \text{morm}\left(1,0,\sigma_{T2}\right)_0 \\
\end{align*}
\]

Random jumps for rotational and translational motion
\[ \begin{align*}
\sigma_s & \leftarrow \left(2 \Delta \tau_s\right)^2 \\
\Delta x & \leftarrow \text{rnorm}(3, 0, \sigma_s) \\
Vs & \leftarrow Vs + \Delta x & \text{Position vector of sphere} \\
\phi & \leftarrow \phi + \Delta \phi \\
e & \leftarrow \begin{pmatrix}
\cos(\phi) \sin(\theta) \\
\sin(\phi) \sin(\theta) \\
\cos(\theta)
\end{pmatrix} & \text{Orientation vector of ellipsoid} \\
\text{ee} & \leftarrow \begin{bmatrix}
\left(\cos(\phi) \sin(\theta)\right)^2 & \left(\sin(\theta)\right)^2 \cos(\phi) \sin(\theta) \cos(\theta) \\
\left(\sin(\theta)\right)^2 \cos(\phi) & \left(\sin(\phi) \sin(\theta)\right)^2 \\
\sin(\phi) \sin(\theta) \cos(\theta) & \left(\cos(\phi)\right)^2
\end{bmatrix} \\
A & \leftarrow \text{runif}(3, -1, 1) \\
I & \leftarrow \begin{pmatrix} 1 & 0 & 0 \end{pmatrix} \\
B & \leftarrow (I - \text{ee}) \cdot A & \text{Perpendicular vector to e} \\
C & \leftarrow e \times B \\
Vc & \leftarrow Vs + \Delta S^e + \Delta ST1 \left[ \frac{B}{(|B|)} \right] + \Delta ST2 \left[ \frac{C}{(|C|)} \right] \\
Vc1 & \leftarrow Vc + L \cdot e & \text{Vectors defining center point of hemispherical ends of rod shape} \\
Vc2 & \leftarrow Vc - L \cdot e \\
dc & \leftarrow |Vc| \\
ds & \leftarrow |Vs| \\
dc1s & \leftarrow |Vc1 - Vs| \\
dc2s & \leftarrow |Vc2 - Vs| \\
\text{diag} & \leftarrow \left(2L^2 + (r + a)^2\right)^{\frac{1}{2}} \\
\text{adjc} & \leftarrow 2 \cdot |Vc - R \cdot \frac{-Vc}{|Vc|} \\
Vc & \leftarrow \text{if}(dc > R, Vc + \text{adjc}, Vc) \\
\text{adjh} & \leftarrow 2 \cdot |Vs - R \cdot \frac{-Vs}{|Vs|} \\
Vs & \leftarrow \text{if}(ds > R, Vs + \text{adjh}, Vs) \\
\text{h} & \leftarrow dc1s \cdot \text{sin} \left( \arccos \left( \frac{\text{dc2s}^2 + (2L)^2 - \text{dc1s}^2}{2(2L) \cdot \text{dc2s}} \right) \right)
\end{align*} \]
<table>
<thead>
<tr>
<th>$\text{flag}_{i,0}$</th>
<th>$\leftarrow 1$ if $[(dc1s \leq r + a) \cdot (dc2s \geq \text{diag})]$</th>
<th>Stuck on end 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{flag}_{i,0}$</td>
<td>$\leftarrow 2$ if $[(dc2s \leq r + a) \cdot (dc1s \geq \text{diag})]$</td>
<td>Stuck on end 2</td>
</tr>
<tr>
<td>$\text{flag}_{i,0}$</td>
<td>$\leftarrow 3$ if $[(h \leq r + a) \cdot (dc1s \leq \text{diag}) \cdot (dc2s \leq \text{diag})]$</td>
<td>Stuck in middle</td>
</tr>
<tr>
<td>$\text{flag}_{i,1}$</td>
<td>$\leftarrow \text{flag}_{i,1} + 1$</td>
<td></td>
</tr>
</tbody>
</table>

$t := \frac{\pi \eta R^3}{2 k \cdot T} \cdot 10^{-18}$ \hspace{1cm} \text{Rapid flocculation time for two spheres}
B.2 Randomization of bacterial orientation

The MathCad code used for the randomization of bacterial orientation used in Chapter 6 is shown below. Explanation of symbols and the algorithm is contained in the text of the code. For additional explanation, see Section 6.2.3.
\[ T := 273 \quad k := 1.38 \cdot 10^{-23} \quad \eta := .001 \quad \Delta t := .001 \]

\[ a_1 := 2.5 \quad b := 0.5 \]

Definitions and explanations same as previous

\[ \Delta (\lambda) := \left[ (a_1^2 + \lambda)(b^2 + \lambda)(b^2 + \lambda) \right]^{\frac{1}{2}} \]

\[ \chi := \left( \int_0^{\infty} \frac{1}{\Delta (\lambda)} d\lambda \right) \]

\[ \alpha a_1 := \left[ \int_0^{\infty} \frac{1}{(a_1^2 + \lambda) \cdot \Delta (\lambda)} d\lambda \right] \]

\[ \alpha b := \left[ \int_0^{\infty} \frac{1}{(b^2 + \lambda) \cdot \Delta (\lambda)} d\lambda \right] \]

\[ DR := \frac{3 \cdot k \cdot T}{16 \pi \eta} \left[ \frac{(\alpha a_1 \cdot a_1^2 + \alpha b \cdot b^2)}{a_1^2 + b^2} \right] \cdot 10^{18} \]

\[ \Delta \tau_0 := DR \cdot \Delta t \quad \Delta \tau_0 := DR \cdot \Delta t \]

\[ j(n) := \begin{array}{l}
\text{for } i \in 1..n \\
\theta \leftarrow 0 \\
\text{flag}_{i,0} \leftarrow 0 \\
\text{flag}_{i,1} \leftarrow 0 \\
\text{while } \text{flag}_{i,0} = 0 \\
\sigma_0 \leftarrow \left( 2 \cdot \Delta \tau_0 \right)^2 \\
\Delta \theta \leftarrow \text{morn}(1,0,\sigma_0)_0 \\
\theta \leftarrow \theta + \Delta \theta \\
\text{flag}_{i,0} \leftarrow 1 \text{ if } \theta > \frac{\pi}{2} \\
\text{flag}_{i,0} \leftarrow 1 \text{ if } \theta < \frac{-\pi}{2} \\
\text{flag}_{i,1} \leftarrow \text{flag}_{i,1} + 1
\end{array} \]
**B.3 Simulation of scattering of population of doublets or ellipsoids**

The MathCad file used for the simulation of the scattering intensity of a population of doublets or ellipsoids is shown on the following pages. Explanation of symbols and the algorithm is contained in the text of the code. For additional explanation, see Section C.2.5.
\[ a_s := 5 \cdot 10^{-7} \]
\[ n := 5000 \]
\[ j := 500 \]
\[ \lambda := 632.8 \cdot 10^{-9} \]
\[ \theta := \frac{2.9}{180} \pi \]
\[ i := 1..n \]
\[ T := 298 \]
\[ N := 0.64 \]
\[ \Delta \zeta := .001 \]
\[ b := 5 \cdot 10^{-7} \]
\[ k := 1.38 \cdot 10^{-23} \]
\[ \eta := 0.001 \]
\[ a := 1 \cdot 10^{-6} \]
\[ \varepsilon := 7.1 \cdot 10^{-10} \]
\[ Kr := 8 - 1.72 \cdot 1 + 23.64 \cdot 1^2 \]
\[ \eta := 0.001 \]
\[ Dr := \frac{k \cdot T}{Kr \cdot \pi \cdot \eta \cdot (a_s)^3} \]
\[ \Delta t := 0.005 \]
\[ \Delta \tau_\theta := Dr \cdot \Delta t \]
\[ \Delta \tau_\phi := Dr \cdot \Delta t \]

\[ eE_i := \begin{bmatrix} 1 \\ 0 \\ 0 \end{bmatrix} \]

\[ \text{for } h \in 0..25 \]
\[ \phi \leftarrow \text{runif}(1,0,2\pi) \]
\[ \theta \leftarrow \text{runif}(1,0,\pi) \]
\[ \text{flag} \leftarrow 0 \]
\[ E \leftarrow h \cdot 100 \]
\[ \text{Electric field} \]
\[ \frac{(e \cdot \Delta \zeta \cdot \varepsilon) \cdot N}{\eta \cdot a_s} \]

\[ \text{Peclet number} \]
\[ \text{Pe} \leftarrow \frac{Dr}{Dr} \]

\[ \text{while } \text{flag} < j \]
\[ \sigma_\theta \leftarrow \left(2 \cdot \Delta \tau_\theta \right)^2 \]
\[ \Delta \theta \leftarrow \text{rnorm}(1,0,\sigma_\theta) \]
\[ \text{Brownian rotation} \]
\[ \Delta \theta_{ep} \leftarrow -\text{Pe} \cdot \text{Dr} \cdot \sin(\theta) \cdot \Delta \tau_\theta \]
\[ \text{Electrophoretic rotation} \]
\[ \theta \leftarrow \theta + \Delta \theta + \Delta \theta_{ep} \]
\[ n \leftarrow \text{round} \left( \frac{\theta}{\pi}, 0 \right) \]
\[ \sum_{i=1}^{n} eE_i \]

\[ \text{Pavg} := \frac{1}{n} \]

\[ h := 0..25 \]

\[ \text{Pavg}_0 = 1 \]

\[ \Delta \text{P}_h := \text{Pavg}_{h,0} - \text{Pavg}_{0,0} \]
Appendix C

Charge Nonuniformity Light Scattering

C.1 Introduction

Colloids and nanocolloids commonly serve as building blocks for more complex metamaterials and devices. These materials range from traditional materials like coatings and ceramics (31, 33), to modern devices like circuits (9, 26), sensors (6), barcodes (37, 39), photonic (8, 40, 56), and colloidal crystals (19, 25). In processing particles, two critical goals are 1) to control particle aggregation and 2) to promote accurate particle self-assembly. Traditional mechanisms for stabilizing particles include electrostatic (45) and electrosteric forces. Electrostatic forces can arise from dissociated surface groups (e.g., sulfates on polymer latices, silanol groups on silica). The charge groups often give zeta potentials ($\zeta$) of magnitude greater than 50 mV, and classical DLVO calculations predict electrostatic repulsive barriers of order 100 to 1000 kT between micron-size particles. Thus, particle stability to aggregation is *predicted* to be essentially permanent (2, 3, 52).

Industry, however, continues to stabilize particles using electrosteric dispersants (38) [2] (e.g., Daxad [3], Tamol [4]) or other passivation techniques (e.g., pluronics [5],

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[3] Daxad consists of napthalene sulfonic acids, which adsorb by hydrophobic part (napth). It is produced by Dow.
PVA \[^5\], even soya lecithin). Dispersants are used because despite prevailing theory, electrostatic forces alone do not impart sufficient dispersion \[^7\] for most applications. In the past few years, our group has examined one hypothesis for why electrostatics alone do not keep particles dispersed: charge nonuniformity. The classical picture of a colloidal particle is of a uniformly-charged surface. This assumption has led to the prevailing models for colloidal forces and ordering into crystals. In the past 15 years, researchers have hypothesized that surfaces can be nonuniformly-charged \(20, 22, 23, 29, 30\), and in the past three years, our lab group has measured that in fact particles are often quite nonuniformly-charged \(14-16\) – usually in an undesirable random distribution that sacrifices colloidal stability \(10, 21, 24, 35, 47, 50, 54\). This nonuniform charge contributes to the need for a dispersant to maintain stability. As our data have shown, a uniformly-charged particle has much greater stability than a nonuniformly-charged particle \(50, 54\).

Measurements of charge nonuniformity are scarce in the literature. A few measurements of charge nonuniformity exist for planar surfaces \(5, 17\). Most of the measurements are done using atomic force microscopy (AFM), which is very challenging to use on spherical surfaces. One existing measurement of charge nonuniformity on spheres used 50 \(\mu\)m glass beads \(46\), a radius that is more than an order of magnitude

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\[^4\] Tamol 731A has polycarboxylic acid, and it acts as an anionic dispersing agent. It is produced by Rohm & Haas.

\[^5\] Pluronics are a PEO-PPO-PEO diblock copolymer made by BASF. There are various versions, some better for wetting, some better for dispersing particles.

\[^6\] Dispersant-20 consists of PVA polymeric stabilizer, made by Shenzhen Huasu.

\[^7\] Lecture by Steven Reznek (Cabot Corporation) at the American Chemical Society Colloid & Surface Science Symposium (Ann Arbor, Michigan, 2002). He stressed the critical importance of having reliable stability for particles.
larger than the spheres examined in this paper. More recently, investigators used AFM to find 50-100 nm domains on micron-size particles, although the particles were dried (49). Several challenges exist with using AFM to measure charge nonuniformity on colloidal particles: 1) the particle must be fixed to a surface, meaning that much of the particle is inaccessible to the AFM tip, 2) the AFM tip tends to “slide off” the sides of the particle, again limiting the accessible area, and 3) the effective size of the AFM tip is usually large (e.g., >50 nm), when including the radius of curvature of the tip and the electrostatic screening length in the solution. The various spectroscopic and electron microscopy techniques also have difficulty in measuring nanoscale charge nonuniformity. Two common challenges of these techniques are spatial resolution (often micron-scale resolution) and the need for high vacuum (i.e., aqueous samples cannot be used).

One technique that has measured charge dipoles on particles is the electric light scattering technique (48). The charge dipole on disk-like clays, bacteria, and viruses have been measured using this powerful electro-optic technique, although data from the technique have not been related to measurable surface parameters like zeta potential. Our technique does not use the high electric fields and high frequencies common in this technique, but we do use light scattering to assess particle orientation.

In the past few years, our lab group has developed “video microscopy rotational electrophoresis” (VMRE) as a technique to measure charge nonuniformity. VMRE is the first technique able to assess zeta potential nonuniformity on the surface of individual colloidal particles. By using this technique, we have measured significant charge nonuniformity on the surfaces of polystyrene latex doublets consisting of two “identical” 4.3 µm particles suspended in KCl solutions. While VMRE has been used to measure
charge nonuniformity, the technique has three significant limitations. 1) The measurements are currently time-consuming, often taking more than half a day to get proper statistics for a single sample. 2) The visualization of small (<500 nm) particles is not yet possible, due to the resolution limits of optical microscopy (about 200 nm). 3) For small particles (e.g., less than a micron), Brownian motion makes it challenging to separate rotation due to electrophoresis from rotation due to Brownian motion, since rotational Brownian motion scales as the inverse cube of particle size.

The purpose of this paper is to introduce the technique of charge nonuniformity light scattering (CNLS). CNLS combines light scattering “visualization” of colloidal doublet rotation with interpretation using precise electrokinetic modeling, in order to obtain charge nonuniformity measurements. CNLS overcomes the three limitations mentioned in the previous paragraph, since CNLS can sample a population of particles (100’s of nanometers to over a micron) in a time on the order of minutes using light scattering. The primary data in this paper show that for model colloidal heterodoublets, we obtain the same measurement of charge nonuniformity using classical translational electrophoresis as we do using CNLS. Thus, not only does CNLS rely on sound theory, but the method has been verified against traditional electrophoresis techniques. As a result we will soon be using CNLS to examine bacteria and sub-micron particles of various materials.
C.2 Materials and Methods

C.2.1 Formation of model heterodoublets

An important step in testing the validity of CNLS is obtaining model colloidal doublets, in which one sphere of the doublet has zeta potential $\zeta_1$ and the other has $\zeta_2$. Surfactant-free polystyrene latex spheres were obtained from Interfacial Dynamics Corporation (Portland, OR). Particles with diameters of 1.00 µm were purchased already functionalized with amidine or sulfate groups (Batch Numbers 701,2 and 3990,1, respectively). A suspension of model doublets was prepared through a controlled synthesis process that will be described elsewhere. Briefly, amidine functionalized latex particles were coated with PSS (polystyrene sulfonate) in such a way that one defined amidine functionalized region per particle remained free from PSS. Thus, the entire particle was negative, except for the amidine region which was positively-charged. The single amidine patch on the particle was then free to bind to an oppositely-charged 1.00 µm sulfate functionalized latex particle. The resulting heterodoublets consisted of one PSS coated particle (with a negative zeta potential) and one sulfate functionalized particle (with a different negative zeta potential). During the synthesis, pH $\approx 5.5$ and ionic strength $\approx 0$ to 0.06 M PBS.

C.2.2 Sample preparation and characterization

Suspensions of doublets were diluted in 10 mM KCl for experimentation. This solution was examined under 100× video microscopy to insure the presence of only
singlets or doublets, with a lack of higher order aggregates in any significant amount. Higher order aggregates make the interpretation of CNLS data difficult. For all experiments involving video microscopy, a Nikon TE 300 Eclipse inverted optical microscope coupled with video equipment (e.g., Cohu 4010 CCD camera, DVD) was used with a 100× oil immersion objective (NA = 1.3). Capillary tubes with inside dimensions 0.2 × 2.0 × 50 mm (Vitrocom) were used for observation as a component of a home-built microelectrophoresis cell. Electric fields were applied using a Keithley source meter with platinum black electrodes.

For the two types of spheres comprising the doublets, the zeta potential difference was measured by two methods. First, the difference in translational velocities of singlets in the experimental solution under an applied electric field was measured using video microscopy. Using the Smoluchowski equation, a measured difference in electrophoretic velocity \(U_1 - U_2\) between two independently translating particles can be used to calculate a difference in zeta potential \((\zeta_1 - \zeta_2)\) between the particles (Eq. C.1),

\[
U_1 - U_2 = -\varepsilon \frac{(\zeta_1 - \zeta_2)E_0}{\eta}
\]

where \(\varepsilon\) is the fluid permittivity and \(\eta\) is the fluid viscosity. The observations are for two particles in the same plane of view, and by subtracting their speeds we also cancel contributions to velocity from electroosmotic flow in the electrophoresis cell, or from buoyancy-driven flows that sometimes result. Figure C-1 shows two singlets translating at different velocities in an applied electric field. Further experiments were done to obtain \(\zeta_2 - \zeta_1\); a Brookhaven Zeta PALS analyzer was used to measure the zeta potentials of the free PSS coated particles and sulfate functionalized particles.
C.2.3 Video Microscopy Rotational Electrophoresis (VMRE)

The essential physics of VMRE is that while uniformly charged particles with thin electrical double layers do not rotate by electrophoresis, regardless of their shape (36), nonuniformly-charged particles do rotate (Figure C-2) in an applied electric field ($E_0$) (13). Thus, rotational electrophoresis uses microscopy observations of the rotation to quantify charge nonuniformity of colloidal particles.

The technique of VMRE is described extensively elsewhere (13-16). However, some key features of the technique will be discussed relevant to this research. Previous research has focused on the rotation of doublet particles (15), due to the ease of observing and measuring rotation. Doublets will be observed in this research for similar reasons.
Orientation changes of spheres have been experimentally visualized by others (e.g., tagging with fluorophore (32) or Perrin’s classic experiments (43)). However, the use of doublets requires no visualization marker that could affect the charge nonuniformity we seek to measure. We emphasize that translational electrophoresis cannot detect charge nonuniformity since by far the dominant contribution to translational motion is the average zeta potential (\(\zeta_0\)) (1). For rotational electrophoresis the dominant term is due to charge nonuniformity.

\[
\Omega = -\frac{d\theta}{dt} = \frac{\varepsilon (\zeta_2 - \zeta_1) E_0}{\eta L} N \sin \theta
\]  \hspace{1cm} \text{C.2}

Figure C-2: Rotation of the nonuniformly-charged model doublets used in this paper. The times are \(t_a = 0\) sec, \(t_b = 0.33\) sec, \(t_c = 0.83\) sec, and \(t_d = 0.97\) sec. The heterodoublets consist of 1.00 micron diameter spheres, and the applied electric field was 4 V/cm for the doublets in this figure. Many such doublets appear in the microscope, and the rotation shown is easily and commonly seen.

The angular velocity (\(\Omega\)) of colloidal heterodoublets can be related to the difference in zeta potential of the two particles (\(\zeta_2 - \zeta_1\)) (12, 53) (Eq. C.2)

where \(\varepsilon\) is fluid permittivity, \(E_0\) is applied electric field, \(\eta\) is the fluid viscosity, \(L\) is the center-to-center distance for the doublet (\(L = 2a\) for touching spheres), \(N\) is 0.64 for rigid doublets, and \(\theta\) is angle formed by the electric field vector and the doublet center-to-
center vector. This paper focuses on heterodoublets, which are doublets formed from two
different spheres. We have previously examined the rotational electrophoresis of
*randomly*-charged doublets (14, 15). These are formed from homogenous suspensions,
although the individual spheres will in general have some level of charge nonuniformity.

The rotation due to Brownian motion is characterized by a rotational diffusion
coefficient (51) (Eq. C.3):

\[ D_r = \frac{kT}{K'\pi \eta a^2} \]  

C.3

where \( K' = 29.92 \) for rigid colloidal doublets. The electrophoretic rotation and Brownian
rotation of a doublet can be compared through a Peclet number (Pe) for the maximum
electrophoretic rotation rate (which occurs at \( \theta = \pi / 2 \)) (Eq. C.4):

\[ Pe = \frac{\epsilon (\zeta_2 - \zeta_1)E_0 N}{\eta LD_r} = \frac{30.08\epsilon (\zeta_2 - \zeta_1)E_0 a^2}{kT} \]  

C.4

where the last part of the equation is written for rigid doublets. The Pe can be used to
predict the applicability of VMRE in measuring angular velocity. For our previous
VMRE experiments, Pe was greater than 100, such that Brownian rotation was negligible
(15, 16). However, Pe varies as \( a^2 \), making the observation of rotation due to
electrophoresis difficult to see compared with rotation due to Brownian rotation for small
particles.
**C.2.4 Charge Nonuniformity Light Scattering**

Light scattering measurements were taken with a small angle light scattering device based on designs from the literature (34, 41) [Ref (41) shows a nice schematic of the device]. The incident beam is a 17 mW vertically polarized HeNe Laser ($\lambda = 632.8$ nm) which impinges on a cylindrical glass sample chamber (microelectrophoresis cell) with an inside diameter of 0.5 cm. The scattering is measured at an angle of 2.9° and is collected by a photomultiplier tube (Hamamatsu) that is interfaced with a computer via LabView software for data acquisition. The sample cell remains stationary during all experimental runs for a given sample. The entire apparatus is shrouded in black cloth so as to prevent collection of ambient light. Transmitted incident light from the sample is blocked for better resolution of the scattering signal. The electric field is applied perpendicular to the incident light.

For the CNLS experiments, various $E_0$ were used: ±1, ±2, ±4, ±10, ±20, and ±25 V/cm. These electric fields were applied for 15 seconds with an $E_0 = 0$ V/cm for 15 seconds in between each value. This process was repeated for three trials for each sample. Intensity values for a given electric field were averaged over the range of data points for about 10 seconds in the middle of each 15 second application. The intensity value for $E_0 = 0$ was averaged over all cases where intensity was measured with no applied electric field. Finally, intensity differences were averaged for both positive and negative applications of a given electric field.

The goal of our experiments is to measure with light scattering the changes in orientation of a population of colloidal doublet particles in the presence of an applied
electric field ($E_0$). The resulting changes in intensity will be used to quantify $\zeta_2 - \zeta_1$, which for our model system represents the charge nonuniformity on the doublets.

The intensity of scattered light for an anisotropic particle is not only dependent on size and composition (as is the case for spheres), but also on orientation. This effect is described by the form factor, $P(\phi)$. We use $\phi$ as the scattering angle since we are already using $\theta$ as the angle from $E_0$ to the doublet center-to-center vector ($e$). We approximated the angular dependence of light scattering due to a doublet by the angular dependence on a spheroid. The equations for light scattering of a spheroid are (28, 42) (Eq. C.5 and Eq. C.6)

\[
P(\phi) = \frac{9\pi}{2} \frac{J_{3/2}^2(u)}{u^3}
\]

\[
u = \frac{4\pi a}{\lambda} \left( \sin \frac{\phi}{2} \right) \left( \cos^2 \beta + \frac{b^2}{a^2} \sin^2 \beta \right)^{1/2}
\]

The minor axis of the spheroid has length (a), while the major axis has length (b). The angle between the incident light and ellipsoid direction vectors is $\beta$; the scattering angle is $\phi$; and the wavelength of light is $\lambda$. $J$ represents a Bessel function of the first kind. The intensity $I(\phi)$ is proportional to $P(\phi)$.

We approximate the doublet (consisting of two touching particles with radius a) with a spheroid having a minor axis (a) and a major axis (b = 2a). Since the average scattering from a doublet is known (28, 42), we did compare the average scattering from a doublet with an equivalent spheroid as we defined it. The results matched to better than 1%.
C.2.5 Modeling of rotational electrophoresis

For a given scattering angle, the intensity of scattered light is a sum of the various contributions to scattering (Eq. C.7):

\[ I = I_{\text{glass}} + I_{\text{solution}} + I_{\text{spheres}} + \sum_{j=1}^{n_d} I_d \beta_j + I_{\text{higher}} \]  

C.7

where \( I_d^{0} \) is the scattering constant for doublets and \( n_d \) is the number of doublets. The intensity due to the glass (\( I_{\text{glass}} \)), the solution (\( I_{\text{solution}} \)), and all the singlet spheres in solution (\( I_{\text{spheres}} \)) is assumed constant for all values of an applied electric field. Additionally, the number of larger scatterers (e.g., triplets, 4-lets, etc.) is assumed to be much less than \( n_d \), making higher order contributions to scattering \( I_{\text{higher}} \) negligible. The electric fields that we apply are not likely to break up doublets or cause the formation of aggregates, so \( n_d \) and \( I_{\text{spheres}} \) remain constant, as we see in our experiments for \( E_0 = 0 \) at various times.

When the intensity of scattering is measured with and without an applied electric field, the difference in scattering becomes (Eq. C.8):

\[ \Delta I(\beta_j) = I_d \beta_j \left[ \sum_{j=1}^{n_d} P(\beta_j^E) - \sum_{j=1}^{n_d} P(\beta_j^\alpha) \right] \]  

C.8

An average of the various form factors can be taken by dividing by \( n_k \), and the ratio of intensities for the intensity difference at two different non-zero electric fields (\( Pe > 0 \)) becomes (Eq. C.9):

\[ \frac{\Delta I(Pe_1)}{\Delta I(Pe_2)} = \frac{\langle P(\text{Pe}_1) \rangle - \langle P(\text{Pe} = 0) \rangle}{\langle P(\text{Pe}_2) \rangle - \langle P(\text{Pe} = 0) \rangle} \]  

C.9
In this relation, neither $n_d$ nor $I_d^0$ needs to be known. Furthermore, the experimental data and simulated data are related through a single Peclet number (Pe).

In order to interpret the raw data obtained from the CNLS, a simulation for the measured system was created. The numerical simulation accounts for rotation due to both electrophoresis and Brownian motion. For a given time step ($\Delta \tau = tD_\tau$), the Brownian rotation is modeled as that of a spheroid with semi-axes $a$ and $2a$, with $(a)$ taken as the radius of one sphere in a doublet. The electrophoretic part of the rotation is given by Eq. C.2. The Brownian rotation is taken from the probability distribution for Brownian motion (55), which gives

$$f(\Delta \theta) = \frac{1}{\sqrt{4\pi \Delta \tau}} \exp\left(-\frac{\Delta \theta^2}{4\Delta \tau}\right)$$  \hspace{1cm} \text{C.10}$$

Typically, 2000 doublets were used for the simulations. The doublets started with random orientations, and then the doublets were stepped with $\Delta \tau = 0.005$. At each time step, the value of rotation due to Brownian motion was taken from the Gaussian distribution given by Eq. C.10. The amount of rotation due to electrophoresis is given by (Eq. C.11)

$$\Delta \theta = -\Omega \cdot \Delta \tau$$  \hspace{1cm} \text{C.11}$$

with $\Omega$ given by Eq. C.2. After a long time $\tau = 2.5$, we had 2000 doublets with many angles. The light scattering due to each doublet was calculated by Eq. C.5 and Eq. C.6. Thus, the total scattering from 2000 doublets being oriented with a Peclet number (Pe) was found, for Pe from 0 to 45.
From the experiments we obtain the scattering (I) as a function of the applied electric field (E₀). At this point we do not know the experimental Peclet number, because we do not know the experimental ζ₂ - ζ₁. However, ζ₂ - ζ₁ is the parameter that we seek to find from the experiments, and therefore by comparing the experimental ratios of \( \Delta I(Pe)/\Delta I(Pe_{\text{max}}) \) (as given by Eq. C.9) to theoretical values for this ratio (which depend only on Pe), we obtain the experimental Pe and therefore the experimental ζ₂ - ζ₁. All intensity differences (ΔI) in this research are in reference to an averaged measured intensity for E₀ = 0, and Pe_{max} is taken as E₀ = 25 V/cm, above which changes in intensity leveled out.

The uncertainty of the best fits for ζ₂ - ζ₁ were analyzed using a well-known Monte Carlo statistical method (4). In this method we measure each I(E₀) a number of times and find the experimental uncertainty. We obtain one value for the best fit of ζ₂ - ζ₁ by fitting the average intensities using Eq. C.9. Then we obtain a “new set” of intensities by adding a Gaussian random number, with uncertainties taken from the standard deviations for the I(E₀), to the average I(E₀). This “new set” of I(E₀) is fit for the best ζ₂ - ζ₁. We make a number of these “new sets” of I(E₀), therefore obtaining a number of best fits for ζ₂ - ζ₁, and then we take the average and standard of the best fit ζ₂ - ζ₁ as the actual values for the charge nonuniformity.
C.3 Experimental Results

Intensity measurements from CNLS for the 1:1 micron doublets are shown for electric fields of ±4 V/cm and ±10 V/cm in Figure C-3. As expected, obvious changes in intensity occurred with the application of an electric field as the zeta potential dipoles of the doublets aligned with the electric field, biasing the orientation distribution and increasing the scattering intensity. When the electric field was turned off, the scattering intensity returned to a baseline value for the intensity of a random distribution of orientations.

Figure C-3: Raw data for changing intensities of light scattering for various electric fields. The intensity for \(E_0 = 0\) is given at approximately 5.02 arbitrary units. At ±4 V/cm (listed at the top of the figure), a rise in the intensity is seen. An even larger rise is seen at ±10 V/cm, although at this electric field the signal is starting to reach a maximum value.
As a check to assure that the observed differences are due to orientation effects, the light scattering was measured for a suspension of only 1.0 micron singlet (spherical) particles with a similar volume fraction of particles. Figure C-4 shows that the application of an electric field has a statistically insignificant effect on the scattering intensity of spheres. The differences shown can be attributed to instrument noise.

Figure C-4: Intensity variations for colloidal spheres in arbitrary units. As expected, spherical particles do not change their light scattering in an electric field like nonuniformly-charged doublets do. As the error bars show, differences are very close to zero, and there is no systematic variation with increasing $E_0$.

Figure C-5 shows the experimental intensity difference ratio data for the 1:1 micron doublets. The Pe number was varied until the simulated result for intensity ratios matched the data. The value of $\zeta_2 - \zeta_1$ that gave the best fit is $13.8 \pm 0.67$ mV. This number can be compared to two other values, both from translational electrophoresis. The first is using a video microscope to obtain difference in mobilities at the same plane
in an electrophoresis cell. This method gives accurate values for differences using Eq. C.1. The difference for 5 pairs of spheres was found to be $\zeta_2 - \zeta_1$ to be $14.7 \pm 1.1$ mV, fairly close to the result from CNLS. We did not obtain absolute values for the zeta potentials from the video microscopy experiments, but measurements from a ZetaPALS instrument gave values of $-82 \pm 2.6$ mV for the sulfated particles and $-66 \pm 4.0$ mV for the coated amidine particles, resulting in a $\zeta_2 - \zeta_1$ of $16 \pm 4.8$ mV.

Figure C-5: CNLS data used to find the charge nonuniformity on the model heterodoublets. The dots indicate the data, and the line gives the best-fit result from simulation. For our doublets the best fit occurred at $13.8 \pm 0.67$ mV, which matches well with translational electrophoresis experiments.
C.4 Discussion

The technique of CNLS is shown to accurately predict the $\zeta_2 - \zeta_1$ of model heterodoublets. The predicted $\zeta_2 - \zeta_1$ value agrees well with values from translational velocity measurements and Zeta PALS measurements. Thus, CNLS enables us to overcome two key limitations of previous rotational electrophoresis experiments, since measurements can be done quickly and can be done on sub-micron (even down to 100 nm) particles.

Figure C-5 shows that $I(E_0)$ levels off at high values of the electric field (or equivalently, high values of Pe). Using the values from Figure C-5 along with Eq. C.9 enables us to use difference ratios to find $\zeta_2 - \zeta_1$ without knowing the number of doublets in solution, as long as the doublets are of the same size.

Although, CNLS has been shown to be accurate in measuring charge nonuniformity in the form of $\zeta_2 - \zeta_1$ for the given system, several limitations currently exist for the technique. In order for the scattering equations to be applicable, the requirement that $\frac{4\pi a}{\lambda} \sin \frac{\phi}{2} << 1$ must hold (i.e., we must have small particle sizes or small scattering angles). Though this technique is limited to particles smaller than about several microns, the upper limit of this assumption was approached with good results, and the technique will be extended to sub-micron sizes. Another potential limitation is the sensitivity of the technique. We will soon try CNLS for random distributions of zeta potential nonuniformity (14-16). Since particles with random charge nonuniformity will in general rotate much more slowly than the heterodoublets used in this study, the
scattering signal differences from these systems are expected to be low and more difficult to measure. A time averaged approach for multiple applications of an electric field value will be taken in order to combat this limitation.

C.5 Conclusions

CNLS has been shown to be a technique capable of measuring charge nonuniformity of colloidal heterodoublets with a finite $\zeta_2 - \zeta_1$. The size regime studied was about 1 micron; however, it is predicted that the size regime of this technique may extend as low as roughly 100 nm. This technique is much faster and efficient than VMRE, and measurements and data interpretation can be done in a few minutes instead of a day. Forthcoming work will focus on measuring charge nonuniformity on particles with random distributions of charge nonuniformity, with smaller diameters, and with different chemical compositions (e.g., silica, PMMA, bacteria).

Charge nonuniformity measurements can impact a number of scientific disciplines, a point appreciated by Stoylov (48) and others in the electro-optics community. In colloid science interparticle forces have just begun to be understood in terms of charge nonuniformity (35, 50). Charge nonuniformity is especially important for nanoparticles, since they have so few total surface charges. A key advance in our research is that since the charge nonuniformity is related to surface potential nonuniformity, models for interparticle forces can be quickly applied (54). In microbiology the impact of nonuniformities in the lipopolysaccharide layer or surface proteins of bacteria will enable the assessment of adhesion with different tools (18, 27).
In polymer science knowing the origins of charge nonuniformity (e.g., pearl necklace effect) (7, 11, 44) will enable control of the pattern of charging in polyelectrolytes, and therefore also on latex particles.

C.6 Acknowledgments and Copyright Information

Appendix C is a modified version of the author’s published manuscript:


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C.7 References


VITA

JOSEPH F. JONES

EDUCATION
Doctor of Philosophy in Chemical Engineering (December 2005)
The Pennsylvania State University, University Park, PA

Bachelors of Science in Chemical Engineering (May 2001)
Clemson University, Clemson, SC

PUBLICATIONS
Please see the front of this thesis for a complete list of publications.

ADDITIONAL TECHNICAL EXPERIENCE
Undergraduate Researcher (January 2000 – May 2001)
Clemson University, Department of Chemical Engineering, Clemson, SC
Water Uptake Effects on L-Phenylalanine Solubility in Single Solvents.

NSF-REU Program (May 2000 – August 2000)
University of South Carolina, Department of Chemical Engineering, Columbia, SC
Characterization of Dendrimer Encapsulated Metallic Nanoparticles

SELECTED PRESENTATIONS

LEADERSHIP EXPERIENCE
Graduate Recruiting Coordinator (Fall 2003 – Spring 2004)
Penn State Chemical Engineering

President, Graduate Student Council (Fall 2002 – Fall 2003)
Penn State Chemical Engineering

Project Leader (January 2001 – July 2001)
Clemson University in South Africa Project