ABSTRACT


Advanced composite materials could be revolutionized by the development of methods to incorporate living cells into functional materials and devices. This could be accomplished by continuously and rapidly depositing thin ordered arrays of adhesive colloidal latex particles and live cells that maintain stability and preserve microbial reactivity. Convective assembly is one method of rapidly assembling colloidal particles into thin (<10 µm thick), ordered films with engineered compositions, thicknesses, and particle packing that offer several advantages over thicker randomly ordered composites, including enhanced cell stability and increased reactivity through minimized diffusion resistance to nutrients and reduced light scattering. This method can be used to precisely deposit live bacteria, cyanobacteria, yeast, and algae into biocomposite coatings, forming reactive biosensors, photoabsorbers, or advanced biocatalysts.

This dissertation developed new continuous deposition and coating characterization methods for fabricating and characterizing <10 µm thick colloid coatings – monodispersed latex particle or cell suspensions, bimodal blends of latex particles or live cells and microspheres, and trimodal formulations of biomodal latex and live cells on substrates such as aluminum foil, glass, porous Kraft paper, polyester, and polypropylene. Continuous convective-sedimentation assembly (CSA) is introduced to enable fabrication of larger surface area and long coatings by constantly feeding coating suspension to the meniscus, thus
expanding the utility of convective assembly to deposit monolayer or very thin films or multi-layer coatings composed of thin layers on a large scale.

Results show thin, tunable coatings can be fabricated from diverse coating suspensions and critical coating parameters that control thickness and structure. Particle size ratio and charge influence deposition, convective mixing or demixing and relative particle locations. Substrate wettability and suspension composition influence coating microstructure by controlling suspension delivery and spreading across the substrate. Microbes behave like colloidal particles during CSA, allowing for deposition of very thin stable biocomposite coatings of latex-live cell blends. CSA of particle-cell blends result in open-packed structures (15-45% mean void space), instead of tightly packed coatings attainable with single component systems, confirming the existence of significant polymer particle-cell interactions and formation of particle aggregates that disrupt coating microstructure during deposition.

Tunable process parameters, such as particle concentration, fluid sonication, and fluid density, influence coating homogeneity when the meniscus is continuously supplied. Fluid density modification and fluid sonication affect particle sedimentation and distribution in the coating growth front whereas the suspended particle concentration strongly affects coating thickness, but has almost no effect on void space. Changing the suspension delivery mode (topside versus underside CCSA) yields disparate meniscus volumes and uneven particle delivery to the drying front, which enables control of the coating microstructure by varying the total number of particles available for deposition. The judicious combination of all of
these parameters will enable deposition of uniform, thin, latex-cell monolayers over areas on the order of tens of square centimeters or larger.

To demonstrate the utility of biocomposite coatings, this dissertation investigated photoreactive coatings (artificial leaves) from suspensions of latex particles and nitrogen-limited \textit{Rps. palustris} CGA009 or sulfur-limited \textit{C. reinhardtii} CC-124. These coatings demonstrated stable, sustained (>90 hours) photohydrogen production under anoxic conditions. Nutrient reduction slows cell division, minimizing coating outgrowth, and promotes photohydrogen generation, improving coating reactivity. Scanning electron microscopy of microstructure revealed how coating reactivity can be controlled by the size and distribution of the nanopores in the biocomposite layers. Variations in colloid microsphere size and suspension composition do not affect coating reactivity, but both parameters alter coating microstructure. Porous paper coated with thin coatings of colloidal particles and cells to enable coatings to be used in a gas-phase without dehydration may offer higher volumetric productivity for hydrogen production. Future work should focus on optimization of cell density, light intensity, media cycling, and acetate concentration.
Engineering Multifunctional Living Paints: Thin, Convectively-Assembled Biocomposite Coatings of Live Cells and Colloidal Latex Particles Deposited by Continuous Convective-Sedimentation Assembly

by
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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemical Engineering

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__________________________________________  __________________________________________
Saad Khan                                         Orlando Rojas
DEDICATION

I dedicate this dissertation to my mother and father,

for showing me how to achieve a contented life

without sacrificing my values and humility,

and for wholeheartedly encouraging

every aspiration and endeavor.


Charge the hill!
BIOGRAPHY

Jessica Jenkins was born in Wiesbaden, Germany to parents Anne and Jim, a career Army officer. A typical military child, she was raised in New York and North Carolina with her siblings Jim and Jennifer. After graduating from Terry Sanford High School as one of three valedictorians and a four-season Varsity cross country runner, she attended Lafayette College in Easton, PA as a Marquis Scholar, the college’s most prestigious academic scholarship. While at Lafayette, Jessica participated in a two-year interdisciplinary, bioremediation research project under the guidance of Professors Javad Tavakoli (Department of Chemical and Biomolecular Engineering), Steven Mylon (Department of Chemistry), Arthur Kney (Department of Civil Engineering), and Laurie Caslake (Department of Biology), with her second year of research forming the foundation of her undergraduate thesis *Kinetic Studies of Perchlorate Removal from Saturated DOW3N-Based Ion Exchangers.* She also worked for ARCADIS, an international environmental consulting firm, as an intern in the summer of 2006 and completed a broad range of coursework in environmental chemistry and green engineering. These classes, combined with her internship and research, cemented her decision to pursue a career in the environmental sector of chemical engineering. Jessica graduated from Lafayette College in 2007, having gained a Bachelor of Science in Chemical Engineering, awarded *summa cum laude* and with *thesis* Honors, a minor in Environmental Science, and membership in both Phi Beta Kappa and Tau Beta Pi.
Jessica returned to North Carolina to pursue her graduate studies, joining the Department of Chemical and Biomolecular Engineering at North Carolina State University in 2007 and both the Flickinger and Velev Research Groups in 2008. She completed her first year of graduate studies as an NC State Alumni Association Fellow, an NC State University Provost Fellow, and a College of Engineering Dean’s Fellow and was later awarded a two-year NIH/NCSU Molecular Biotechnology Training Program Traineeship. She interned at the National Renewable Energy Laboratory in Golden, CO under the guidance of Doctors Maria Ghirardi, Michael Seibert, and Sergey Kosourov as part of this Traineeship. Outside of the classroom and laboratory, Jessica served as an officer in the Chemical and Biomolecular Engineering Graduate Student Association and later as a member of the university-wide Graduate Student Association’s Executive Council. She also served as a departmental recruiting captain for the 2009-2010 academic year, participated in the Graduate/Faculty/Staff intramural soccer league, and ran competitively, finishing three half-marathons and several 5K races during her last two years of graduate school. Jessica will graduate from North Carolina State University in the spring of 2013 with a Doctor of Philosophy in Chemical Engineering and a minor in Biotechnology.

*The miracle isn't that I finished. The miracle is that I had the courage to start.*

- John Bingham
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*Keep away from people who belittle your ambitions. Small people always do that, but the really great make you feel that you, too, can become great.*

-Mark Twain

10 October 2012

JSJ
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C) Immobilized Rhodopseudomonas palustris CGA009 produced 5.8 ± 0.5 mmol H₂ m⁻² hr⁻¹ when submerged in the medium compared to when incubated in the headspace of the microbioreactor 0.47 ± 0.04 mmol H₂ m⁻² hr⁻¹ (rates calculated for < 75 hr). Hydrogen production increased to 1.4 ± 0.3 mmol H₂ m⁻² hr⁻¹ when the headspace coatings were submerged in the medium which is comparable to the rate observed over the same period for the coatings which were submerged from the beginning 2.1 ± 0.6 mmol H₂ m⁻² hr⁻¹ (rates calculate for data >75 hr). Arrow indicates when the coating was submerged in medium.

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CHAPTER 1

Introduction and Overview

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1.1 Introduction

Fabricating industrial-scale, ordered arrays of synthetic latex particles and living cells that are highly reactive and mechanically stable when rehydrated requires a deposition strategy that unifies colloid science and biology. Convective assembly meets this need by rapidly depositing suspended particles, including whole cells and latex particles, into thin (typically less than 10 µm thick), organized films with engineered adhesion, compositions, thicknesses, and particle packing. These coatings have a wide variety of applications, including photobiological hydrogen, oxygen or liquid fuel production [1-3], generating clean fuels from sunlight, water, and industrial byproducts [4-6], or non-toxic alternatives to antifouling paints laden with toxic heavy metals, antibiotics, or biocides [7-9], eliminating debris through cell shedding, digestion, or antibiotic secretion [10].

Hyperthermophiles [11], prokaryotes, and yeast [12, 13] are preserved and stabilized when deposited in thin (<50 µm) adhesive films of randomly ordered cells. Cells encapsulated in agar [14], alginate [15], and silica [16] or codeposited with adhesive latex (stable emulsions of polymer microspheres in aqueous medium) have been shown to be more stable and reactive than planktonic cells [17]. However, only latex biocoatings can stabilize desiccated cells, concentrate (500 to 1000-fold) cells to a very high volume fraction, and maintain the integrity of microbial membranes during drying [18]. Once rehydrated, these coatings have been shown to stabilize microbial reactivity and protein synthesis without outgrowth for thousands of hours [2]. Because they significantly improve cell reactivity by
controlling packing and thinness, convectively-assembled coatings are a powerful structure for capturing the diverse catalytic functions of whole cells [14].

Microbial reactivity is also a function of the support matrix’s thinness and nanoporosity which are formed during film formation and dehydration [19]. Latex coatings, unlike gel matrices, maintain adhesiveness and structural integrity when wet or dehydrated and can be readily engineered for improved nanoporosity by altering polymer particle chemistry or co-depositing carbohydrates and glycerol. These porogens protect the entrapped cells from osmotic stress by arresting polymer particle coalescence during coating formation. However, although both the convective assembly and Mayer rod deposition methods preserve microbial viability and reactivity during film formation and dehydration, only the former method can fabricate ordered monolayers (one particle or one cell thick) [20]. These monolayers have improved nutrient diffusion and reduced light scattering, thus overcoming the mass transfer and optical limitations of thicker hydrogels, Mayer rod coatings, and bioceramics (Figure 1.1).

Here we overview the non-biological convective assembly literature, with an emphasis on how the method has evolved from a batch process limited to the creation of monodispersed particle arrays to its current state as a continuous process capable of fabricating biocoatings from a diverse range of particle suspensions. This evolution has helped solve the fundamental challenge of engineering multi-layered coatings with high reactivity, stability, and robustness by clarifying how multiple process, substrate, and particle parameters affect coating structure. We also describe how the method selectively immobilizes live cells to
create biomimetic, artificial leaves and compare these biocoatings to other biomimetic encapsulation systems.

1.2 Background

1.2.1 Coating Fabrication Methods

Methods to generate <75 µm thick adhesive latex plus living microbial biocoatings were pioneered by the Flickinger Group using simple drawdown Mayer rod coating methods, however the limit of coating thinness of this method is ~10 µm thick [21]. This group demonstrated that low T_g acrylate latex binder emulsions can form nanoporous coatings that retain adhesion to coating substrates when rehydrated in media because the latex particles partially coalesce and “glue” the cells to the substrate during film drying while manipulating particle coalescence forming nanopores surrounding the micron-size cells [2, 3, 11]. This method was shown to be capable of fabricating biocomposite coatings containing a very high density of a wide variety of microorganisms without loss of coating mechanical stability (delamination, cracking) or impairment of microbial viability, protein synthesis, and reactivity following rehydration [18, 19]. However, in order to generate highly structured coatings for future applications (nanoporous and perfusive structures), layer-by-layer (LBL) coating methods will be needed to fabricate multi-layered and channeled systems with tailored diffusion, light trapping and reactivity [2]. As such, understanding how to reliably fabricate much thinner monolayer arrays of polymer particles and live cells on rigid and flexible substrates is of significant fundamental interest and practical importance, as
understanding the reactive properties of a layer of single cells ordered into an adhesive biocoating is paramount to the fabrication of the next generation of LBL artificial leaves and other bio-mimetic devices [22].

The Flickinger Group at the University of Minnesota in collaboration with Schriven and Schottel used a hand Meyer rod draw down coating method to deposit composites of microbial cells and nanoscale latex particles in 5 to 100 cm²-scale patches, strips, or sheets on flexible polyester substrates with a range of thicknesses from 10 to 250 µm and a variety of random particle packing configurations [2, 3, 11, 19, 23]. Microbe viability is preserved during film formation and drying by the addition of osmoprotective carbohydrates that also arrest polymer particle coalescence by forming carbohydrate glasses during drying [18]. The method relies on low T_g acrylate co-polymer or non-film forming plus film forming polymer particle blends to alter compaction and arrest coalescence during evaporation to generate nanoporosity [22]. However, this simple coating strategy is limited by wire diameter to coatings of >10 µm thick. Fabrication of reactive multi-layer coatings for prototype devices utilizing adhesive coatings of a variety of prokaryotic and eukaryotic cells [2, 3, 11, 19, 20, 24] will require new methods of polymer particle plus live cell deposition.

In order to extend this early concept to understand the fundamentals of cell-cell and cell-particle interactions in coatings and to engineer coating microstructure, convective assembly deposition was investigated in a collaboration between The Flickinger and The Velev Groups as a method of coating monolayers or very thin multi-layers. Convective assembly combines fluid evaporation, particle transport via fluid flow, and associated meniscus motion
to rapidly and controllably deposit a diverse range of microspheres and cells particles into thinner (typically < 10 µm) highly uniform films [25-31]. The initial collaborative results presented in this thesis suggest that convective assembly could lead to rapid, repeatable fabrication of well-ordered, bio-mimetic arrays of living cells and polymer particles on an industrial scale useful for construction of structured multi-layer systems with enhanced, or even hybrid, functionality (Figure 1.2).

The particle assembly process begins at the periphery of an evaporating fluid film when the film’s height becomes thinner than the diameter of the suspended particles [20]. The menisci formed around these particles induce strong, long-range capillary forces that pull neighboring particles together into two-dimensional nuclei (Figure 1.3). These particle clusters travel with the liquid flux from the bulk suspension to the substrate-air-liquid contact line at the drying front as the fluid evaporates, resulting in the formation of closely packed arrays and subsequent propagation of the coating area (Figure 1.3) [32, 33]. The coating growth rate, $v_c$, is related to the fluid evaporation rate and particle volume fraction by the equation

$$v_c = \frac{\beta j_e l \varphi}{h (1 - \varepsilon)(1 - \varphi)}$$

(1)

where $\beta$ is a particle-particle interaction parameter, $j_e$ is the evaporation rate, $l$ is the drying length, $\varphi$ is the volume fraction of the particles in suspension, $h$ is the height of the deposited coating, and $\varepsilon$ is coating porosity [34, 35]. Values of $\beta$ vary between 0 and 1 and depend on
particle-particle and particle-substrate interactions; for low volume fraction and electrostatically stable particles, $\beta \approx 1$. Once $v_c$ is determined, the length of the thin film where deposition occurs by convection can be calculated using a material flux balance:

$$L_{film} = \frac{v_w d_{cell} (1 - \epsilon)(1 - c_i)}{\beta j c_i}$$

(2)

where $v_w$ is the deposition rate and equal to $v_c$ at steady state, and $c_i$ is the concentration of the bulk suspension at that particular time [34].

The Velev Group adapted convective assembly from a slow, randomized process occurring at the edge of a stationary droplet [32] to a rapid, controllable process occurring at the boundary of a dynamic fluid. Prevo and Velev reported a convective assembly technique that allows for rapid and controllable deposition of coatings from 5-30 µL of suspension (Figure 1.4A) [27]. Up to 30 µL of suspension containing particles at high volume fraction (0.9-35% w/v) is trapped between a horizontal substrate and an inclined top plate or coating knife. Pushing the coating knife top plate at a constant rate along the long axis of the bottom plate by a linear motor spreads the suspension from the meniscus into a thin film across the horizontal substrate, leading to coating fabrication on the substrate by evaporative convective assembly [20]. Both the coating thickness and type of deposited particle layers are readily adjusted by altering the suspension volume fraction, coating knife speed, and knife (or blade) angle and coating knife speed, allowing for precise control in particle packing and coating thickness (Figure 1.4B) [26, 27].
After trying to fabricate close-packed, uniform yeast cell arrays using Prevo’s apparatus, Jerrim and Velev realized that evaporative convective assembly cannot directly generate high-quality cell coatings due to cell sedimentation onto the substrate below the moving meniscus during coating deposition (Figure 1.4C). Yeast cells are at least five times larger than the other types of particles previously deposited using evaporative convective assembly [27, 36-38], leading to inexorable sedimentation that significantly alters coating uniformity [20]. To offset this process, Jerrim and Velev created convective-sedimentation assembly, a modified version of the deposition technique that minimizes sedimentation effects by using variations in the tilt (relative to a horizontal surface) of Prevo’s coating apparatus to direct settling cells toward the drying front during fabrication (Figure 1.4D) [20]. Using this adaptation, Jerrim and Velev obtained thin (<2 cell layers), close-packed yeast coatings in 15-45 min, making the realization of bio-mimetic materials more plausible.

However, although Jerrim and Velev’s modification gave rise to thin, uniform convectively-assembled cell arrays, convective-sedimentation assembly lacks utility on an industrial scale because each array’s total surface area is limited by the finite amount of suspended particles delivered to the drying front by the continuously depleted coating meniscus volume. In this thesis we overcome this shortcoming by reporting continuous convective-sedimentation assembly (CCSA), an adaptation of CSA that uses inline injection to create larger surface area and longer thin films by constantly dispensing suspended, uniformly charged particles (or eventually charged particle and live cell composite suspensions) to the meniscus during coating fabrication (Figure 1.4E) [Chapter 3]. Coating
microstructure is controlled by varying the suspension delivery mode between topside CCSA, in which suspension flows through a capillary from a fluid reservoir to the front of the meniscus along the coating knife’s topside, and underside CCSA, in which suspension flows from a fluid reservoir through tubing fixed to the back of the meniscus along the knife’s underside. Each mode achieves a certain meniscus volume and characteristic particle delivery to the drying front, enabling microstructure control by varying the total number of particles available for deposition (Figure 1.4F) [Chapter 3]. Because CCSA enables continuous coating fabrication without loss of deposition speed, the technique, with further refinement to optimize particle packing, is promising for generating monolayers or very thin live cell + latex polymer coatings for biomaterial applications where a large, highly bioreactive surface is required.

### 1.2.2 Microbial Coating Systems

Microbial latex coatings were introduced in the 1980s by Lawton, Bunning, and Flanagan, who coated solid particles, nylon mesh, membranes, and silica particles with polydispersed acrylate/vinyl acrylate copolymers [39-43]. Cantwell first reported the use of polymer blends of hard and soft polymer particles for microbial entrapment, but did not fabricate thin biocomposite coatings – only flocculates, 1-2 mm aggregates, and 2 mm diameter fibrils [19, 44]. Martens and Hall reported methylmethacrylate and butyl acrylate polymer coatings of photosynthetic *Synechococcus* on a carbon electrode, creating a functional biomimetic device that showed cell viability of “nearly 100%” with photoactivity
after rehydration and exposure to light [19, 45]. These early microbial coating systems suffer from low coating permeability, weak mechanical stability (delamination from the support particles), poor control of coating thickness, uniformity, reactivity and lack of defined coating microstructure (porosity, pore structure) characterization among other limitations [19].

Prevo and Velev first identified the critical parameters responsible for rapid and controlled deposition of crystalline colloidal arrays, thus enabling control over coating thickness and structure and solving the limitations of earlier coating systems (Figure 1.5) [27]. Using operational “phase” diagrams that correlate coating thickness and packing structure against suspension volume fraction and coating deposition speed, Prevo and Velev fabricated high-quality, convectively-assembled coatings from a diverse range of colloids, including monodispersed latex microspheres [27], untreated metallic gold nanoparticles [27, 37], and dielectric nanoparticles like silica [36]. Tessier et al. used convective assembly to deposit gold nanoparticles and latex spheres in a single step [46]. The latex spheres assemble into an ordered crystal array while the nanoparticles simultaneously collect in the void space of the drying array, forming a composite structure that can serve as a template for assembling gold nanoparticles into thin, porous films [46].

Recent collaborations between The Flickinger and Velev Groups have demonstrated that close-packed, convectively-assembled composite coatings can be created from bimodal latex polystyrene suspensions (Figure 1.6A and 1.6B) and blends of Rhoplex™ SFO12 (a commercial film-forming, acrylic copolymer emulsion) and latex polystyrene microspheres
(Figure 1.6C and 1.6D) [22]. Each system’s size ratio controls the convective mixing or demixing of the suspension components during coating fabrication and thus their relative locations in the deposited coating [22].

Current fabrication efforts focus on directed, controlled, and scalable colloidal assembly of live cells and latex microspheres into biocomposite materials with novel structures and functionalities (Figure 1.7) [47, 48]. Jerrim and Velev created thin, mixed layers of Saccharomyces cerevisiae (5.0 µm) and large (10.0 µm) polystyrene microspheres by investigating the fundamental effect of cell sedimentation on the deposition process and developing a means to control the trajectories of the settling cells [20]. By rotating their coating device around a horizontal axis, Jerrim and Velev changed the position of the point at which sedimentation begins to contribute to coating thickness, creating a transition to thicker coatings – inclining the apparatus forward increased the thickness at an earlier point in the coating, improving coating uniformity while inclining the apparatus backward moves the transition farther into the coating, reducing coating uniformity [20].

Using this study as a starting point, we investigated colloidal assembly of polystyrene microspheres (1.0, 5.0, and 8.7 µm) blended with yeast or photosynthetic bacterial or algae cells on nonporous substrates such as glass or polyester sheet. The former investigation demonstrated that simultaneous deposition of cells and latex is possible because the cells behave like simple surface-charged colloidal particles akin to the latex microspheres [22].

Extending the previous work to a porous non-woven substrate coated using continuous convective-sedimentation assembly, the latter study showed how reactive biocomposite
coatings of either *Rhodopseudomonas palustris* (a photosynthetic purple non-sulfur bacteria) or *Chlamydomonas reinhardtii* (a green micro algae) can be fabricated from suspensions containing both non-film forming (photosynthetic cells and 1.0, 5.0, and 8.7 µm latex polymer microspheres) and film-forming solute (Rhoplex™ SFO12 emulsion) of variable sizes and charges [Chapter 4]. Placing these coatings in a gas phase in contact with a low-volume liquid phase which wet the paper pores (by capillary action) and maintained cell hydration by a thin liquid film prolonged its useful life by inhibiting cell outgrowth into the liquid phase and facilitating the escape of gas bubbles (such as H$_2$) from the coating [1]. Variations in microsphere size and suspension composition altered coating microstructure but do not affect coating reactivity. However, despite yielding disparate microstructures, all coatings retained mechanical adhesion and exhibited microbial reactivity after fabrication and rehydration. These coatings also had a higher surface-to-volume ratio than comparable alginate films [49] and suspension cultures, resulting in improved mass transfer and light distribution to all immobilized cells [50]. This partial wetting, combined with the substrate’s low water uptake (2.27x10$^{-4}$ ± 3.72x10$^{-5}$ g/cm$^2$) when completely saturated, suggests that Kraft paper is a robust, suitable substrate for fabricating gas-phase, reactive coatings from water-based live cell + latex polymer microsphere blends. The coating components adhere to the cellulose fibers without plugging the paper pores [1], allowing for nutrient transport from the bulk liquid phase to the immobilized cells on the surface of the paper. The open pore structure may also facilitate escape of gas bubbles (such as H$_2$) from the coating [1].
1.2.3 Microbial Coating Applications: Artificial Leaves and other Biomimetic Devices

Convective assembly allows for the selective immobilization of microbial cells for a diverse range of applications, including foul-release or self-cleaning coatings. Fouling refers to the unwanted accumulation or formation of biomass, sludge, particulates, inhibitors or fats/oils that appreciably degrades a coating’s performance and useful life [18]. Prior studies on fabricating self-cleaning surfaces focused on inorganic antifouling paints containing one or more 1) heavy metals, including cadmium, chromium, silver, and zinc [9], 2) indiscriminate biocides like chlorine [51-53] and mercury [51], or 3) microbial-targeted antibiotics [52, 54]. Because toxic biocides or antimicrobials may endanger human health or cause environmental damage when leached from a coating [8] and pervasive use of antibiotics reduces their efficacy by promoting microbial resistance [54], these paints are increasingly scrutinized and restricted in many countries [9, 55], suggesting the need for nontoxic, “natural” paints that can replace the chemicals commonly used in antifouling coatings [56].

Significant advances in the understanding and fabrication of surfaces with controlled wetting, hierarchical structures, and composition make “natural” foul-release coatings possible [57]. All surfaces can self-clean whenever the adhesion forces between surface debris and the surface are weaker than those between the contaminants and tangential fluid, leading to the debris being carried away in the fluid as it runs off the surface [57, 58]. As such, a diverse range of inorganic and biomimetic antifouling surfaces can be fabricated by tuning the surface properties to weaken this attraction force. Biocomposite coatings may
eliminate surface debris through three mechanisms: 1) *shedding and self-regeneration*, which removes contaminants through cell shedding underneath the debris layer while simultaneously regenerating the depleted top layer by cell proliferation (Figure 1.8A) [20], 2) *digestion*, which uses contaminant-targeting cells to digest contaminating particulates as they diffuse downward through the coating (Figure 1.8B), and 3) *secretion*, which uses bactericidal secretion of antibiotics to kill hostile bacteria on the coating surface (Figure 1.8C) [10]. The literature describing the various inorganic approaches to nonmetallic and nonbiocidal paints is too vast to describe here and thus the reader is directed to other reviews on the topic, especially those by Bhushan and Jung and by Ganesh *et al.*, which thoroughly describe self-cleaning, hydrophilic inorganic surfaces like photocatalytic titanium dioxide and micropatterned silica layers and fabrication of self-cleaning surfaces through engineering of surface hydrophobicity in hierarchical structured films [59, 60].

Previous research suggests both the *secretion* and *shedding and self-regeneration* mechanisms effectively cleanse debris-laden coatings. Jerrim and Velev demonstrated rudimentary self-cleaning by codepositing (using convective-sedimentation assembly) mixed monolayers of 5 µm *Saccharomyces cerevisiae* yeast cells and 10 µm latex particles (Figure 1.9). The larger latex particles settled over the yeast cells, creating a porous topcoat that protects the cells from external perturbations without hindering their access to nutrients and ability to proliferate through the latex topcoat (Figure 1.9B) [20]. When rinsed with a stream of growth media, the outermost cell layer sloughed off the coating surface, taking contaminating debris (simulated by fluorescent microspheres) with it (Figure 1.9C and 1.9D)
One biocomposite coating was stained with FUN1 dye and subsequently analyzed for metabolic activity to assess the viability of the deposited cells; the presence of nonuniform fluorescence within the cells indicated metabolic activity, thus confirming the coating’s ability to self-regenerate. Burgess and coworkers demonstrated the efficacy of the secretion approach by fabricating “natural”, water-based paints that exhibited good activity against marine bacteria, barnacle larvae, and algal spores when coated (without convective assembly) onto Petri dishes [56]. The paint formulations contained extracts from antibiotic-producing marine bacteria (strains giving inhibition zones greater than 11 mm) and a water-based paint resin [56]. Although field trials showed the paints had little effect on the onset and degree of macrofouling in vivo, likely due to rapid leaching from the coating surface, the antifouling efficacy of the laboratory assays suggests that broad spectrum antifouling can be achieved through judicious combinations of metabolites with different leaching rates from painted surfaces and antifouling activities [56].

Another promising use of the convective assembly technique is the fabrication of light-harvesting, hierarchical structures capable of mimicking the photosynthetic reactions of natural leaves – biomimetic leaves. Like plant leaves, these layered devices can capture solar energy, split water into hydrogen and oxygen, and reduce atmospheric carbon dioxide into carbohydrates, thus creating various forms of renewable fuels from solar energy [61]. A diverse range of artificial leaves are reported in the literature, including non-biological, biological (non-cellular), and biological (whole cell) assemblies. Inorganic synthetic leaves are constructed as photoelectrochemical devices that generate hydrogen by combining free
electrons (generated through capture of solar energy) and protons (created through water oxidation at an anode) at a cathode (Figure 1.10) [62]. Some devices achieve light harvesting by mimicking the hierarchical structure of natural leaves while others imitate the natural visible-light response through dye sensitization [61]. Although most artificial leaves possess higher solar energy conversion efficiencies than many crop plants (<1% typically) and bioreactor-grown microalgae (3%) [62, 63], several technical and scientific challenges to developing inorganic, photoreactive leaves remain, including (1) efficient use of the entire solar light spectrum, (2) creation of fabrication techniques that are amenable for mass production, (3) use of inexpensive, abundant materials that are readily scaled-up for bulk production, and (4) development of robust devices with prolonged lifespans [62]. Because inorganic artificial leaves, despite their myriad forms, do not operate on a biological basis, a detailed description of their configurations is beyond the scope of this review, which focuses on convective assembly as a means to fabricate biocomposite structures. Reviews by Bensaid and coworkers and Zhou, Fan, and Zhang offer detailed explanations of the many approaches to constructing inorganic leaves and comprehensive assessments of the many breakthroughs in this research area [61, 62].

The artificial leaf literature also describes attempts to immobilize or entrap photosynthetic components within support matrices, mimicking the light-harvesting pigments embedded within the thylakoid membranes of chloroplasts [64] to create biological (non-cellular) artificial leaves. The support material serves multiple functions, including assisting in the organization and protection of the photoreactive components, allowing for easier
handling of the photoreactive device, and facilitating recycling from the reaction mixture [64-66]. This class of biomimetic leaves includes water-insoluble porphyrins embedded within a lignocellulosic matrix [64], platinized photosystem I protein monolayers immobilized on gold [67], and hybrid complexes of photosystem I and [Ni-Fe]-hydrogenase assembled on gold electrodes [68].

Whole cells are an attractive alternative to immobilization of unstable photoreactive molecules and proteins because whole cell immobilization eliminates the need for expensive reagents and often complex protein extraction and purification, simplifying the fabrication, enhancing the stability of the photosynthetic apparatus and thus reducing the cost of robust artificial leaves. Various types of biological (whole cell) leaves are reported in the coatings literature, including those made from living cells and photoactive materials cross-linked in sol-gel synthetic polymers, sol-gel oxide ceramics, and silica gel glasses [17, 69-76]. For instance, Su et al. created living, biocomposite matrices of silica and photoreactive algae (Chlorella vulgaris or Botryococcus braunii) or Arabidopsis thaliana (plant) cells; the algae and plant systems exhibited photosynthetic reactivity for at least 70 and 30 days, respectively [77, 78]. However, although mechanically robust and sufficiently porous for nutrient diffusion and cell proliferation, most sol-gel ceramics cannot hold more than 5% to 20% (w/w) of cell mass – higher concentrations of living cells reduce the stability of these coatings when in contact with water [19]. Also, many silica networks shrink during lyogel drying, leading to reduced cell viability [19].
Cell-laden alginate gels [49, 79] and hydrogels [80-87] are other possible forms of biological (whole cell) leaves. Researchers at The National Renewable Energy Laboratory have fabricated photoreactive Ca$^{2+}$-alginate films (containing *Chlamydomonas reinhardtii* microalgae) that evolved hydrogen for over 150 hours [49], but these coatings lack the thinness, adhesion and organization of convectively-assembled polymer particle coatings [Chapter 4]. The increased thickness of the latter systems reduces productivity by hindering efficient mass transfer and adequate light distribution to all cells in the hydrogel matrix. Also, alginate films, unlike convectively-assembled coatings, must remain wet during gel cross-linking to prevent the cracking, skinning, shrinkage and other mechanical instabilities that occur when the gels are dehydrated.

Despite myriad publications, hydrogels are not readily useful as coatings because these systems have low mechanical strength and they lack engineered adhesion [19]. Also, hydrogels are hundreds of microns to millimeters thick, resulting in severe mass transfer limitations, and macroporous (pores larger than microorganisms) with thin pore walls, leading to substantial cell release and outgrowth when the gels are exposed to nutrients to regenerate activity and sustain cell viability [19]. These features, combined with an inability to be stored dry or frozen without loss of cell viability [80, 81], suggest hydrogel immobilization lacks sufficient stability, reactivity, adhesion, and preservation of cell viability during gel desiccation [19] to be a suitable method for fabricating biological synthetic leaves on a commercially- or industrially-relevant scale.
The Flickinger Group has pioneered the fabrication of photoreactive, adhesive coatings from bacteria and eukaryotic microbes using nanoporous latex polymer particle coatings [18]. These biocomposite coatings can be fabricated from composite mixtures of latex polymer particles and algae [Chapter 3, [1]], cyanobacteria [1], purple nonsulfur bacteria [Jenkins (unpublished data), [[1-3], or ethanolgens [1]. Of particular note, Gosse and Flickinger fabricated photoreactive biocomposite coatings containing 100- to 1,000-fold concentrated nitrogen-limited purple non-sulfur bacteria that evolved hydrogen gas for over 4,000 hours [2]. We fabricated convectively-assembled biocomposite, algae coatings that demonstrated sustained hydrogen evolution for over 100 hours [Chapter 4]. Optimization of the incident light intensity [88], combined with further development of the production media [49], will likely prolong the useful life of the algal coatings. These coatings prove that intact cells stabilized in thin polymer coatings have the potential to be robust components of advanced light harvesting materials for solar energy generation, thus paving the way for the next generation of biomimetic leaf soft materials with multi-layer architecture containing live cells engineered for optimal light absorption and energy conversion to produce hydrogen gas, alkanes, or liquid fuels (Figure 1.11).

The major challenge facing biological (whole cell) artificial coatings or leaves is the efficient use of solar energy. While photosynthetic cells can be genetically engineered for better conversion efficiency [2, 88], a more direct approach is to fabricate coatings containing multiple cells species, such as co-deposition of algae and photosynthetic bacteria or cyanobacteria. Algae and bacteria capture light in different regions of the electromagnetic
spectrum, suggesting that composite coatings can improve light utilization efficiency through multi-wavelength light harvesting. We demonstrated the validity of this hypothesis by showing that Balch tubes containing a convectively-assembled, multispecies coating (one algae coating and one bacteria coating placed in the same tube) evolved more hydrogen than Balch tubes containing only one algae or bacteria coating, suggesting that multi-species coatings are indeed a promising method for maximizing hydrogen yield.

1.3 Research Goals and Organization of Dissertation

This dissertation explores convective assembly as a means to rapidly deposit large surface area, highly reactive ordered arrays of adhesive colloidal latex particles and living bacteria, yeast, or algae because the fabrication of industrial-scale biocomposite coatings that maintain stability and preserve microbial viability and reactivity remains a challenging problem to both the coatings industry and the biomaterials field [19, 21]. One objective is to understand how the various assembly process parameters and the myriad particle and substrate properties affect coating fabrication and appearance (structural uniformity, thickness, and particle packing). Another objective is to create a coating apparatus that allows for continuous fabrication of large surface area and longer coatings without the loss of structural uniformity and thinness, thereby giving convective assembly industrial relevance. Because biocomposite coatings have the potential to be robust components of biosensors, biocatalysts, and advanced light harvesting materials, the final objective is to identify a coating configuration (cells, latex particles, and substrate) that permanently immobilizes
reactive cells without inhibiting microbial viability and reactivity. To these ends, Chapter 2 demonstrates how the convective assembly technique can be extended to deposit bimodal particle size suspensions of latex microspheres and composite blends of latex microspheres and live cells on glass, plastic, and metal substrates and explains how substrate wettability, suspension composition, particle size ratio and surface charge affect coating assembly and microstructure. Chapter 3 addresses the challenge of fabricating large surface area, longer coatings by introducing continuous convective-sedimentation assembly (CCSA), a deposition method that transforms the technique into a continuous process by constantly supplying coating suspension to the meniscus. To answer the question of how CCSA affects structural uniformity and thinness, the parameter investigations from Chapter 2 are extended to particle concentration, fluid flow-path sonication, suspension density, and meniscus volume. Chapter 4 expands the commercial utility of CCSA by demonstrating that biocomposite coatings deposited on porous Kraft paper are mechanically stable and reactive after rehydration, despite differences in coating suspension, microstructure, and porosity. Finally, Chapter 5 summarizes all original coating fabrication and characterization methods developed in this dissertation.
Figure 1.1: Comparison of (A) a randomly oriented thin biocomposite coating [Image courtesy of H. Ge] and (B) thin ordered biocomposite coatings. Such thin coatings of polymer particles and photoreactive cells (shown in white and green, respectively) enable uniform light distribution (yellow arrows) and nutrient delivery to all surface-immobilized cells.
**Figure 1.2:** Schematic of four coatings deposited using sequential convective assembly to form a multilayer, composite device. Each color represents a different type of particle or cell. Each new layer is deposited after the underlying layer has dried.
Figure 1.3: Schematic of the convective assembly process. Blue and purple arrows denote particle convection and sedimentation in meniscus, respectively. [Adapted from ref. 34]
Figure 1.4: Evolution of convectively-assembled coating fabrication. Mechanistic schematics and summary of major parameter effects for (A, B) evaporative convective assembly [27], (C, D) convective-sedimentation assembly [[20] for D], and (E, F) continuous convective-sedimentation assembly [Chapter 3 for F].
Figure 1.5: Layering transition schematic for monodisperse microspheres deposited using evaporative convective assembly. The different colors of each layer in the experimental micrographs are due to optical interference of the transmitted light; all polygons are drawn to clarify the packing structures. [27], Transition schematic originally reported by [89, 90]
Figure 1.6: Examples of composite coatings assembled from bimodal blends of (A, B) latex polystyrene microspheres [22] and (C, D) Rhoplex™ SFO12 and latex polystyrene microspheres. $R_2/R_1$ is polymer particle diameter ratio. Microsphere sizes are: (A) 5.7 and 8.7 µm, (B) 5.7 and 9.6 µm, (C) 1.0 µm, and (D) 5.0 µm. The SFO12 latex emulsion aggregates on the polystyrene microspheres and forms amorphous bridges between neighboring microspheres. Scales bars are (A, B) 20 µm and (C, D) 2 µm.
Figure 1.7: Examples of advanced biocomposite structures: (A) Bubble-templated “cellosomes” [91, 92]; (B) Composite yeast-polystyrene coating [Jenkins, unpublished data]; (C, D) Composite coatings of photosynthetic cells, Rhoplex™ SFO12, and 1.0 µm polystyrene microspheres for hydrogen evolution [Jenkins, unpublished data]. *Rhodopseudomonas palustris* (purple nonsulfur bacteria) and a collapsed *Chlamydomonas reinhardtii* (algae) are shown in C and D, respectively. Scale bars are (B) 70 µm and (C, D) 2 µm.
Figure 1.8: Self-cleaning strategies for biocomposite coatings containing polymer particles and living cells: (A) Bactericidal secretion, (B) Contaminant digestion, (C) Top layer shedding and self-regeneration.
**Figure 1.9:** Micrographs and schematics demonstrating biocomposite (yeast cell and latex microsphere) coatings with rudimentary self-cleaning properties. (A) Initial coating. (B) Coating after 24-hour incubation in growth medium, allowing for significant cell proliferation. (C) Coating with fluorescent latex artificial debris. (D) Coating following contaminant and cell removal with a stream of growth medium. All scale bars are 50 µm. [adapted from ref. 93]
Figure 1.10: Examples of inorganic artificial leaves: (A) Schematic of a CO-oxygen evolving complex functionalized $npp^+$-silicon single-junction photoelectrochemical cell [94]; (B) Schematic of a TiO$_2$-nanotube array electrode (photocatalyst for water oxidation) and Fe-nanoparticles on N-doped carbon nanotubes (electrocatalyst for CO$_2$-reduction) [62, 95]; (C) Schematic of a membrane that drives positive and negative charges for oxygen and hydrogen evolution when the nano photovoltaic element absorbs sunlight [Credit: Helios SERC (Lawrence Berkley National Laboratory)]; (D) Schematic of nanostructured arrays of membrane-connected anodes and cathodes coated with catalysts that can split water to produce H$_2$ or liquid hydrocarbon fuels [Credit: Lewis Research Group (California Institute of Technology)].
Figure 1.11: Concept of a soft, flexible photobiological fuel cell constructed from biocoatings hydrogen-producing photosynthetic bacteria and oxygen-producing algae (proposed by O. Velev, V. Paunov, and M. Flickinger and modified by J. Jenkins).
References


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CHAPTER 2

Deposition of Composite Coatings from Particle-Particle and Particle-Yeast Blends by Convective-Sedimentation Assembly

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Abstract

The structures resulting from convective-sedimentation assembly (CSA) of bimodal suspensions (4.1 to 10% solids) of strongly charged sulfate latex microspheres (zeta potential -55.9 ± 1.8 mV at pH 8.0) and weakly charged *Saccharomyces cerevisiae* (zeta potential -18.7 ± 0.71 mV at pH 8.0) on glass, polyester, polypropylene, and aluminum foil substrates was evaluated. This study shows how substrate wettability, suspension composition, particle size ratio and surface charge affect the deposition process and resulting coating microstructure (particle ordering and void space). Size ratio and charge influence deposition, convective mixing or demixing and relative particle locations. Substrate wettability and suspension composition influence coating microstructure by controlling suspension delivery and spreading across the substrate. *S. cerevisiae* behave like negatively-charged colloidal particles during CSA. CSA of particle-yeast blends result in open-packed structures (15-45% mean void space), instead of tightly packed coatings attainable with single component systems, confirming the existence of significant polymer particle-yeast interactions and formation of particle aggregates that disrupt coating microstructure during deposition. Further optimization of the process should allow void space reduction and deposition of cells plus adhesive polymer particles into tightly packed adhesive monolayer coatings for biosensors, biophotoabsorbers, energy applications, and highly reactive microbial absorbers.
2.1 Introduction

The development of methods to deposit thin ordered arrays of adhesive polymer particles and live cells, such as bacteria, cyanobacteria, yeast, and algae, will enable the development of a new generation of highly reactive biomaterials that use microbes as biosensors, photoabsorbers, or biocatalysts. Biocatalytic coatings of adhesive latex particles and 50% volume fraction of reactive bacteria have been shown to function as photobiological hydrogen producers [1, 2], generating a clean fuel from light (energy source) and waste organics or water (electron source) [3-5]. Self-cleaning or antifouling cell coatings composed of yeast and polystyrene microspheres are a potential non-toxic alternative to inorganic antifouling paints containing toxic heavy metals, antibiotics, or biocides [6-8]. Currently used toxic biocides or antimicrobials may endanger human health or cause environmental damage when leached from a coating [7], while pervasive use of antibiotics reduces their efficacy by promoting microbial resistance [9]. Biocomposite coatings (made of polymer particles and live cells) can also function as “artificial skin”, where debris and contaminants are either sloughed off the top cell layer by newly dividing cells, absorbed and digested as they diffuse downward through the coating, or antibiotics are secreted from the coating surface killing contaminating bacteria [10]. Other emerging applications include coatings for environmental biosensors, high intensity (greater than 100 g/L coating volume/h) industrial biocatalytic coatings [11, 12] or high reactivity environmental biocatalytic filters. These coating methods can preserve and stabilize hyperthermophiles [13], prokaryotes and yeast
and have been useful for toxicity studies, cell signaling studies, and gradient bioassays [16].

Common immobilization matrices for random entrapment of whole live cells include polymer hydrogels and natural biofilms. However, hydrogels and biofilms lack engineered cell packing and good cohesion; they also disintegrate when the entrapped cell concentration exceeds 30% [17] and are often hundreds of microns to millimeters thick, resulting in severe mass transfer limitations [18]. These matrices cannot be stored dry or frozen for prolonged periods of time without loss of cell viability [17]. Also, when hydrogel- or biofilm-entrapped microorganisms are incubated with nutrients to sustain cell viability and regenerate activity, significant cell release and outgrowth occur because either the gel matrix is macroporous (pores larger than the entrapped microorganisms) and the weak thin gel pore walls can be ruptured by cell growth or the cells die and are sloughed from the surface [18].

The adhesion, viability and reactivity of microorganisms on surfaces are well studied because of the role of bacterial adhesion in pathogenesis, as the initial step in biofilm formation, which can lead to biocorrosion and contamination of water systems. The interaction energy between bacterial cells and surfaces has also been described by Derjaguin-Landau-Verwey-Overbeek (DLVO) and colloid filtration (CFT) theory [19, 20].

Artificial monolayers of randomly oriented bacteria have been fabricated for imaging and measurement of adhesion by atomic force microscopy (AFM) in order to study natural adhesion [21, 22]. Many of these studies utilize non-toxic covalent crosslinking reactions to randomly immobilize the bacteria on glass or mica surfaces via electrostatic forces or by
utilizing the carboxylic acid terminal groups on the cell surface forming semi-stable amine esters [22]. However these approaches are only effective for microbes with sufficient surface carboxylic acid residues to form firm amide bonds to the substrate. Evaporative methods for coating mica surfaces with bacterial films has also been studied to directly measure the adhesive and elastic properties of bacteria to substrates using surface force apparatus (SFA) and AFM methods [23]. One method utilized inclined settling of *Pseudomonas aeruginosa* onto mica followed by drying resulting in microbial deposition at the drying front using paper absorbent to generate capillary action to remove excess bacterial solution. This procedure generates patterned monolayers of bacteria with large void spaces on mica known as honeycomb films [24, 25]. Although these films exhibit some degree of ordering, the observed cellular structure and narrow cell size distribution are the result of random, uncontrolled nucleation and coalescence events [25].

Unfortunately, bacterial settling, coating and bonding methods that do not alter biological activity cannot generate a monolayer coating with engineered adhesion greater than the adhesive force generated by bacterial surface carboxyl groups. These methods also have not been used to generate ordered arrays of cells in monolayers with defined cell packing and interparticle porosity. These methods are not effective for cell adhesion following film rehydration and for coating thin flexible substrates which would be useful for fabricating composite reactive devices.

Biocomposite cell coatings generated from a mixture of reactive cells and non-toxic adhesive (deformable) latex microspheres can have engineered adhesion to a wide variety of
surfaces. Adhesion can be altered by polymer particle glass transition temperature (Tg), latex particle surface chemistry (charge, charge density, surface grafting of crosslinking agents), and the ratio of particle diameter to cell size and polymer particle to cell concentration in the emulsion. Thin (< 20 µm) latex coatings containing carbohydrates stabilize, concentrate (500 to 1000-fold), and preserve the integrity of microbial membranes during drying [18], thereby preserving cell viability resulting in a thin, adhesive coating with minimal diffusion resistance (Figure 2.1). The ability to generate monolayer coatings of photosynthetic microbes or algae as engineered photoabsorbers will allow precise measurements of light absorption by reducing mutual self-shading and protecting the immobilized cells from mechanical degradation and deactivation [1] (Figure 2.1).

Methods to generate monolayers with engineered adhesion are the starting point for the construction of structured multi-layer systems [26]. Following film formation, drying and rehydration, low Tg latex coatings retain adhesion to coating supports when rehydrated in media because the latex particles “glue” the cells to the substrate [1, 13]. Microbial latex coatings were introduced in the 1980s by Lawton, Bunning and Flanagan, who coated solid particles, nylon mesh, membranes, and silica particles with polydispersed acrylate/vinyl acrylate copolymers [27-31]. Cantwell later reported the use of bimodal blends of hard and soft polymer particles to immobilize microbial cells [32]. However, these cells were not deposited in thin coatings, but only in the form of flocculates, 1-2 mm aggregates, and 2 mm diameter fibrils [32]. Martens and Hall immobilized photosynthetic Synechococcus cells on a carbon electrode using a film-forming emulsion of methyldimethacrylate and butyl acrylate
copolymer [33]. More recently, the Mayer rod draw down coating method has been used to deposit various composites of microbial cells and nanoscale latex particles in 5 to 100 cm²-scale patches, strips, or sheets on polyester substrates with a range of thicknesses from 10 to 250 µm [1-2, 13, 34]. Microbe viability is preserved during film formation and drying by the addition of osmoprotective carbohydrates which also serve as porogens arresting polymer particle coalescence by formation of carbohydrate glasses during drying [18]. Simple coating methods such as wire wound rod drawdown are limited by wire diameter to coatings of >10 µm thickness, but have been useful for generating small scale adhesive latex coatings that are biocatalytic using a variety of prokaryotic and eukaryotic cells [1, 2, 12, 13, 16, 34]. This method relies on low T_g acrylate co-polymer or polymer blend particle compaction and arrested coalescence during evaporation to generate nanoporosity resulting in a mixture of particle packing configurations. In contrast, convective assembly utilizes the fluid fluxes generated during evaporation to order particles in wetting films deposited by a coating blade or knife resulting in very thin coatings (< 10 µm) with uniform particle packing. The convective assembly technique has previously been shown to precisely order yeast or polymer particles into closely packed monolayer coatings (one cell thick, < 10µm thickness) with minimal void space (Figure 2.1).

Convective assembly has also been shown to rapidly and controllably deposit a diverse range of particles and cells into multilayer thin films (usually less than 5 layers thick) on solid substrates [35-41], suggesting that development of this method could be used to rapidly fabricate well-ordered composite arrays of living cells and polymer particles on an industrial
scale. The particle assembly process begins when the height of the evaporating fluid film becomes thinner than the diameter of the particles [16]. The menisci formed around these particles give rise to strong and long-range interparticle capillary forces that pull adjacent particles together, forming clusters or two-dimensional nuclei. As the liquid evaporates, liquid flux from the bulk suspension to the substrate-air-liquid contact line at the drying front of the coating replenishes the fluid loss due to evaporation, resulting in particle transport to the drying front, their ordering into closely packed arrays, and subsequent propagation of the coating area [36, 42, 43]. When the meniscus height is larger than the particle diameter, multilayer coatings form [36]. The coating growth rate, $v_c$, is related to the fluid evaporation rate and particle volume fraction by the equation

$$v_c = \frac{\beta j_e l \phi}{h(1 - \epsilon)(1 - \phi)}$$  \hspace{1cm} (1)

where $\beta$ is a particle-particle interaction parameter, $j_e$ is the evaporation rate, $l$ is the drying length, $\phi$ is the volume fraction of the particles in suspension, $h$ is the height of the deposited coating, and $\epsilon$ is coating porosity [44-46]. Values of $\beta$ vary between 0 and 1 and depend on particle-particle and particle-substrate interactions; for low volume fraction and electrostatically stable particles, $\beta \approx 1$ [36]. Once $v_c$ is determined, the length of the thin film in which deposition occurs by convection can be calculated using a material flux balance:

$$L_{film} = \frac{v_w d_{cell} (1 - \epsilon)(1 - c_i)}{\beta j_e c_i}$$  \hspace{1cm} (2)
where \( v_w \) is the deposition rate and equal to \( v_c \) at steady state, and \( c_i \) is the concentration of the bulk suspension at that particular time [45].

Prevo and Velev reported a modified convective assembly technique that allows for rapid and controllable deposition of coatings from 5-30 \( \mu \)L of suspension [37]. Up to 30 \( \mu \)L of suspension containing particles at high volume fraction (0.9-35% w/v) is first trapped between a horizontal substrate plate and an inclined coating knife plate and then the inclined top plate is moved at a constant rate along the long axis of the bottom plate by a linear motor. This delivers and spreads the suspension from the meniscus into a thin film across the horizontal substrate, leading to the formation of a coating on the substrate by evaporative convective assembly [16]. The speed of the coating knife can be adjusted, resulting in precise control of particle packing and coating thickness. The modified technique can also deposit close-packed, thin coatings from pure yeast or pure latex suspensions on glass substrates [16]. However, the principles of fabrication of biocomposite arrays on rigid and flexible substrates by convective assembly have not been examined in depth. This topic is of significant fundamental interest and practical importance, as the reactive properties of thin, ordered cell-latex particle composite coatings are key to the fabrication of the next generation of highly reactive adhesive coatings for biosensors, photoabsorbers, and biocatalysts. Investigation of how biocomposite suspensions convectively assemble into thin, ordered arrays is important for designing bioelectronic devices and biocatalysts and creating prototypes.
We report how convective assembly at high volume fraction can be extended to rapidly deposit bimodal composite blends of either two different-sized latex microspheres (bimodal blend) or latex microspheres and live yeast into ordered arrays on glass, polyester, aluminum foil, and polypropylene substrates. Assembly of yeast cells differs from conventional colloidal assembly because the cells are an order of magnitude larger than the 12 nm to 1 µm diameter polymer particles previously deposited using convective assembly. Thus, sedimentation effects become important during the assembly process [37, 57-59]. Hence, when large (>1.0 µm) particles or cells are deposited by evaporative-convective assembly, the assembly mechanism is denoted as convective-sedimentation assembly (CSA) to account for sedimentation during coating deposition [16]. Other aspects of the assembly process, namely solvent evaporation and particle transport to the drying front, remain identical. As an initial study, high-Tg, non-film forming polystyrene particles (with sulfated surface groups) are used here instead of low-Tg, film-forming particles in order to focus on particle transport, packing and coating void space. Convective assembly has the potential to form thin, ordered biocomposite coatings more rapidly and by a simpler means than any method previously described [46]. However, the effect of the size difference between the components of the suspension (cells or latex microspheres), the interactions between them (cells only or latex-cell blends), and the wettability of the substrate on the convective assembly process must be understood to extend this method to deposit larger surface area uniform monolayer coatings. The effect of bimodal particle size distribution on film formation is well described for hard (high Tg)/soft (low Tg) latex blends [47-52], but not for constant Tg blends nor for latex
particle-live cell mixtures, which is now done here. Subsequent convective assembly studies will utilize low Tg adhesive particles once the fundamentals of generating coatings with minimal void space are known.

2.2 Materials and Methods

2.2.1 Preparation of Coating Suspensions

Single component and composite coating suspensions were prepared from latex microspheres and Saccharomyces cerevisiae. All single particle suspensions were kept at their manufactured percent solids (w/v). All latex-latex and latex-cell suspensions were reformulated as blends at a 1:1 particle (or cell) ratio by combining identical aliquots (same volume and percent solids) of cell or latex suspension.

A 10% (w/w) suspension of 5.0 µm yeast was prepared and adjusted to pH 8.0 (±0.4) as previously described [16] using 0.5 g of Fleischmann’s active dry baker’s yeast (ACH Food Companies, Memphis, TN), 4500 µL of 18.2 mΩ-cm deionized water, and 0.25 g of anhydrous dextrose (Fisher Scientific Chemical Division, Fair Lawn, NJ). The mean cell diameter was estimated by first acquiring a light micrograph of the yeast suspension using an Olympus BX61 microscope equipped with an Olympus DP70 CCD camera and then measuring by averaging the diameters of 75 cells in the micrograph with the NIH ImageJ digital imaging software package. The deionized water was obtained from a RiOs 16 reverse-osmosis water purification system (Millipore Corporation, Bedford, MA). The suspension pH was measured and adjusted every 30-45 min until the percent difference
between subsequent measurements was less than 5%. The cell suspension was sonicated gently in an ultrasonic cleaner (Branson Ultrasonics Corporation, Danbury, CT) for 25-30 seconds to break up any aggregates prior to deposition. Jerrim and Velev reported that the fraction of aggregated yeast in the suspension is decreased both by sonication and by repeatedly adjusting the suspension pH away from the isoelectric point of the yeast [16].

Four sizes (1.0, 5.7, 8.7, and 9.6 µm) of white sulfate latex microspheres (Interfacial Dynamics Corporation, Eugene, OR) were prepared by washing with deionized water to remove residual surfactants and other contaminants produced during particle synthesis. The suspensions were mixed with 18.2 mΩ·cm deionized water and concentrated using a Fisher Marathon Micro A microcentrifuge (3630 RPM for 5 min) or a Fisher accuSpin Micro 17 microcentrifuge (5700 RPM for 10 min) for suspension volumes less than 1000 µL and using a Beckman TJ-6 bench-model centrifuge (3000 RPM for 5 min) for volumes over 1000 µL. All washed suspensions were sonicated in an ultrasonic cleaner to reduce aggregation before deposition.

Cell and particle zeta potentials were measured using a Malvern Zetasizer (Malvern Instruments, Westborough, Massachusetts) equipped with a DTS1060 folded capillary cell and analyzed with the Malvern DTS software package. Zeta potentials were obtained in triplicate using 55 runs per measurement for live *S. cerevisiae* and 1.0 µm latex microsphere suspensions at 0.1% (w/w) and 0.0008% (w/w) percent solids, respectively, using 900-1000 µL of suspension.
2.2.2 Preparation and Characterization of Coating Substrates and Deposition Plates

Fisherbrand 75×25 mm glass microscope slides (Fisher Scientific) were cleaned as previously described [16] first by using NoChromix solution (Godax Laboratories, Cabin John, MD) to remove adsorbed organic molecules and deprotonate surface hydroxyl groups and then washing with 18.2 mΩ·cm deionized water. The method was modified by storing all pretreated slides in separate 100×100 mm Petri dishes (Fisher Scientific) to protect the hydrophilized surface groups from air currents and humidity fluctuations until coating. Separate 75×25 mm strips of 2 mil (0.05 mm) thick polyester (3M, St. Paul, MN), Himont SB 823 polypropylene (Standard Plaque, Inc., Melvindale, MI), and 0.02 mm thick Reynolds Wrap aluminum foil (Reynolds Kitchens, Richmond, VA) were rinsed with 70% (v/v) ethyl alcohol, gently wiped with a KimWipe (Kimberly-Clark, Chantilly, VA) to remove surface contaminants, and dried at the ambient temperature and relative humidity to evaporate any residual alcohol. Aluminum foil strips were flattened using an Orcon smooth action roller (Tools for Floors, Waverly, NY) prior to the alcohol rinse.

Static water contact angles were measured on each substrate surface using a Ramé-Hart 100-00 NRL C.A. goniometer (Mountain Lakes, NJ) and analyzed with the Ramé-Hart DROPimage software package. The advancing contact angles were recorded by injecting 8.0 µL of probing liquid; the receding contact angles were measured by removing 4.0 µL of probing liquid from the dispensed droplet. Advancing and receding contact angles were recorded in duplicate for the left and right side of the droplet and subsequently averaged to obtain advancing and receding contact angles for each substrate.
Surface topography profiles were obtained for each substrate using a surface profilometer (Dektak D150, Veeco, Plainview, NJ) equipped with a 12.5 µm radius and the Dektak v9 software package. Profiles were recorded in triplicate and averaged to obtain a characteristic surface profile for each substrate. The aluminum foil and polyester substrates were affixed to clean glass microscope slides to prevent their surfaces from curling under the stylus force (3 mg). The profile for the polyester and foil is the difference between the measured profiles for the substrate and bare glass.

2.2.3 Deposition of Coatings

All coatings were deposited using the convective assembly method described previously [27]. A clean glass slide and coating substrate (polyester, polypropylene, aluminum foil, or glass slide) were attached to the deposition device (Figure 2.2). The edge of the deposition plate, also referred to as a coating knife, must lie flat against the bottom slide to ensure meniscus formation along the entire length of the knife. 12 µL of coating suspension were injected between the substrate and knife and then spread to form a uniform meniscus. The linear motor pushing the coating knife was operated at 21.1 µm/s, while the suspension was deposited onto the substrate in 15-45 minutes. As previously demonstrated, withdrawal rates approaching 25 µm/s or more yield incomplete or submonolayer coatings for suspension volume fractions of 10% or less deposited using batch convective assembly [37]. All coatings were deposited at an ambient laboratory temperature of 20°C to 30°C and a relative
humidity of 20% to 50%. The temperature and relative humidity above the coating were monitored with a calibrated TM125 Dickson probe (Dickson, Addison, IL).

2.2.4 Dry Coating Characterization

The coatings were imaged by optical microscopy for analysis of the microstructure. Light micrographs were taken with an Olympus BX61 microscope equipped with an Olympus DP70 CCD camera and 5× to 50× objectives. Transparent substrates (glass and polystyrene) were visualized using bright field lighting; all opaque substrates (polypropylene and aluminum foil) were externally illuminated using a Melles Griot IMA101010B0S004 ion laser (CVI Melles Griot Laser Group, Carlsbad, CA) coupled to a FITC filter set (D480/30x, 505, D535/40m). The images for evaluating microstructure homogeneity were collected at four 5 mm intervals across the coating width at a vertical axis located 5 mm from the leading edge of the coating. This protocol allowed for comparison of structure uniformity for multiple coatings. Each micrograph was subsequently analyzed with digital imaging software (Adobe Photoshop 7.0, San Jose, CA) to determine the fraction of the substrate surface coated. Coating length was measured as the longest visibly uniform region of the dried coating.

2.3 Results and Discussion

2.3.1 Coating Structure Dependence on Size Distribution Effects

We first verified that the apparatus can reliably deposit uniform coatings from yeast cells only in the experimental condition studied here (Figure 2.3). In order to determine how to
deposit uniform monolayer coatings from mixtures of large and small particles (or various sizes of microbial cells). 5 suspensions containing 1:1 mixtures of two-sized latex microspheres or mixtures of live cells and polystyrene microspheres were deposited in triplicate onto glass microscope slides using convective assembly. The analysis of the coatings structure using optical microscopy shows that the relative positions of the small and large particles within the monolayers is controlled solely by the size ratio and not blend type (Figure 2.4). This is most apparent in Figures 2.4A and 2.4C. In the former micrograph, the smaller microspheres are collected in a line to the left of the larger particles, an effect phenomenon we have termed as “beaching.” In the latter, the coating has a uniform distribution of particles. The multilayer regions observed in Figure 2.4B and indicated by particles located outside of the focal plane, can form if the meniscus height is greater than the diameter of the larger particle, allowing particles to flow over and settle atop other particles during the assembly process.

Coatings created from the blends of yeast cells and synthetic microspheres (Figures 2.5A and 2.5B) also demonstrate size-selective segregation (Figure 2.5A), resulting in a biphasic appearance – the smaller latex microspheres are clearly separated from the larger yeast clusters. In Figure 2.5B, the smaller yeast is intermixed within the particle clusters. Size-selective segregation is exhibited in both cases, confirming that bimodal suspensions with similar size ratios yield analogous coating structures, regardless of particle component type (yeast or latex particles).
Our hypothesis is that the particle packing distribution on the substrate surface in multimodal coatings is determined by the size-selective segregation that occurs during convective self-assembly. Size-selective segregation is based on the relative size ratio of the particles, and appears to be present in coatings deposited by both conventional convective-evaporative assembly and convective-sedimentation assembly. The micrographs indicate that this segregation depends on the size ratio of the suspended particles. If the relative ratio of the larger and smaller particle diameters is less than 0.25 (determined by the system geometry, see Figure 2.6A), smaller particles pass through the pore spaces between adjacent larger particles and the substrate surfaces and are carried by the evaporating suspension solvent from the bulk particle suspension to the drying region, giving rise to a coating with particle segregation (Figure 2.6B).

Conversely, if the size ratio is greater than 0.25, the smaller particles are too large to pass through the pore spaces to the drying region and remain entrapped and intermixed among the adjacent larger particles, resulting in a coating with uniform particle dispersion (Figure 2.4A). This condition is needed for the deposition of continuous, intermixed single-layer films. For the 0.59 latex blend size ratio, the associated coating appears to exhibit particle segregation rather than particle dispersion. However, close examination of the micrograph (Figure 2.4B) confirms the smaller microspheres are dispersed among the larger particles in the coating interior, in agreement with the predicted coating microstructure for that size ratio.

This particle pseudo-segregation is likely due to stronger viscous drag and surface tension forces on the larger particles that impede their flow to the drying region, allowing the smaller
microspheres to more rapidly flow to and collect along the edges of the coating. When the surface tension force induced by the menisci around the larger particles is large enough, these particles are pressed against the substrate, leading to premature immobilization (due to friction with the substrate) before the particles reach the drying front [42]. The smaller particles, however, remain mobile, leading to convective transport towards the drying region [42].

2.3.2 Coating Structure Dependence on Surface Wettability of Substrate

The effect of substrate wettability on composite coating microstructure is important because the wettability dictates how the liquid film dragged by the coating knife spreads across the substrate surface during convective assembly. When bacteria are present at the surface a minimal moisture layer is required to maintain a smooth surface and measurable contact angle, whose these measurements have been shown to depend on surface moisture content [53-55].

We investigated how to control both coating length and void space by varying surface wettability using a variety of rigid and flexible substrates. Wettability was evaluated by water contact angle. To the best of our knowledge, this is the first time such an investigation has been reported for coatings deposited by convective assembly. A suspension containing *S. cerevisiae* was deposited on glass, aluminum foil, polyester, and polypropylene substrates at constant θ, the angle of the substrate relative to the horizontal, and φ, the angle between the substrate and deposition plate (inclined coating knife), to determine how the surface hydrophobicity influences coating void space and length. The glass substrate was pretreated
with a 70% (v/v) EtOH rinse or NoChromix immersion whereas the aluminum foil, polyester, and polypropylene substrates were all pretreated with 70% (v/v) EtOH.

Because the substrate surfaces are pretreated to homogenize the surface chemistry and decrease roughness and topology effects, the advancing contact angle is considered representative of the equilibrium contact angle in examining the interplay of surface contact angle, coating void space, and coating length. Advancing and receding contact angles were recorded in duplicate for the left and right side of the droplet and subsequently averaged to obtain mean advancing contact angles for each substrate (Table 2.1).

The results indeed indicate that the length and uniformity of a coating deposited by convective-sedimentation assembly can be controlled by varying the wettability of the substrate surface (Figure 2.7). Coating length decreases without a significant change in void space as contact angle varies, suggesting that film thickness controls coating microstructure. For bimodal particle size distributions, higher contact angles promote thicker films, leading to the formation of multiple layers (and increased void space) by allowing particles to stack onto each other. The likely reason is that the hydrophilic surface will have a thinner liquid meniscus, leading to less time for suspended particles to be carried to the drying region by the evaporative and convective fluxes before being trapped between the surfaces of the thinning film [16]. Conversely, because the meniscus is possibly thicker on a more hydrophobic substrate, particles can be transported to the drying region before they sediment onto the substrate surface, leading to a coating with reduced void space and shorter length.
2.3.3 Composite Coating Structure Dependence on Suspension Composition

The structure or void space and length of a composite coating deposited by convective assembly (or convective-sedimentation assembly if large particles or cells are deposited) are not dictated only by evaporation, convection, and surface wettability. Another critical parameter is the effect of the suspension composition (yeast only or latex-yeast blend) on coating void space and length. To determine the effect of the suspension composition on the convective assembly outcome, a 1:1 (by particle number) mixture of yeast and 1.0 µm white sulfate latex microspheres was deposited on the same substrates at the same θ and φ values as the pure yeast suspension, confirming that the convective assembly process can be used to create composite coatings from both pure and composite yeast suspensions on any substrate due to the particle-like behavior of yeast. Three coatings were created and characterized in terms of coating length and void space for each substrate (glass, aluminum foil, polyester, and polypropylene). The measured void spaces and the overall length of the coatings decrease as surface hydrophobicity increases for both the yeast suspension and the latex-yeast blend (Figure 2.7). The cells behave as simple surface-charged colloidal particles akin to latex microspheres – the adhesion of yeast to chemically inert surfaces is unlikely to be controlled by a receptor-adhesion interaction due to the absence of pilus-like projections or flagella on the cell wall [56].

However, the yeast-only and yeast-latex coatings exhibited dissimilar coating lengths and void spaces. Coatings deposited from the yeast suspension were longer whereas coatings deposited from the yeast-latex suspension result in larger void spaces (Figure 2.7) for almost
all substrates, suggesting the coating structure may also depend on the interactions and packing efficiency of the polymer particles and the yeast surface groups and can be controlled by the suspension composition. The relatively large error bars are likely due to the sampling method that accompanies the coating void space analysis – images were collected at four 5 mm intervals across the coating width at a vertical axis located 5 mm from the leading edge of the coating so any local variations in coating uniformity will yield relatively large variations in the reported void space. However, this method allows for quantitative evaluation of coating uniformity for multiple samples.

One cause for the difference in the structures of the pure yeast and yeast-latex coatings is yeast sedimentation during coating deposition, which alters the convective-evaporative assembly mechanism. Jerrim and Velev demonstrated that the assembly of yeast cells by convective evaporation is different in mechanism from conventional colloidal assembly because the cells are at least an order of magnitude larger than the 12 nm to 1 µm diameter polymer particles previously deposited using convective assembly [37, 57-59]. The sedimentation rate of the yeast can be estimated using the Stokes equation [60]:

$$V_s = \frac{2r^2(\rho_{cell} - \rho_{susp})g}{9\mu_{susp}}$$

(3)

where $r_{cell}$ is the radius of the cell or particle, $\rho_{cell}$ is the density of the cell or particle, $\rho_{susp}$ is the density of the suspension media, $g$ is the gravitational acceleration, and $\mu_{susp}$ is the viscosity of the suspension. The average cell density in growing S. cerevisiae cultures is
reported as 1.1 g/mL in the literature [60-64]. Because the cell concentration in the yeast suspension is relatively high at 10 wt% (1.5 billion cells per mL of suspension), its viscosity was adjusted to account for the high particle concentration using data for the relative viscosity versus cell concentration, as previously described [16]. This viscosity adjustment yields a sedimentation rate of 1.16 µm/s for the 5.0 µm diameter yeast (relative viscosity of yeast suspension to water is 1.4) [16]. The sedimentation rate of the cell suspension is almost 10% of the coating knife speed, which was maintained at 21.1 µm·s\(^{-1}\) for all experiments. A significant amount of the suspended yeast in the liquid meniscus will settle out of the meniscus and onto the substrate before being convectively transported to the drying front (the drying region) [16], creating a longer, open-packed coating that is not easily repacked by convective assembly. The biocomposite suspension likely deposits shorter coatings because the repulsive yeast-latex microsphere electrostatic interactions probably stabilize the suspension and allow for more uniform deposition due to the lack of aggregates that may adhere or sediment and disrupt the structure.

Another likely cause of the dissimilar lengths and void spaces of the cell-latex and cell-only coatings is the difference in the electrostatic interactions between yeast cells or a yeast cell and a latex microsphere. At the pH evaluated in this study, both the latex and cell surfaces are negatively charged so electrostatic repulsion predominates [65-68]. The latex particle surface has sulfate functional groups with a net negative charge whereas the yeast surface is has carboxyl (−COOH) groups, proteins, phosphate groups, and other macromolecules [16] whose protonation states vary with solution pH. Each microsphere has
approximately 1.2×10^6 sulfate groups (data by Molecular Probes, Inc.) with an average zeta potential of -55.9 ± 1.8 mV at pH 8.0. The cells have a significantly lower average zeta potential of -18.7 ± 0.71 mV at pH 8.5. Hence, the latex microspheres are more strongly charged than the cells, leading to stronger electrostatic repulsion forces between neighboring yeast and latex microspheres in the suspension (at pH 8.3 typically) than adjacent cells in the yeast suspension. These stronger repulsions yield coatings with higher void spaces for all tested substrates, except aluminum foil, possibly because the net force (the sum of the attractive capillary forces and the negative repulsive forces) between adjacent particles is weaker in the biocomposite suspension. The biocomposite coatings may form a more open-packed structure because the net force is too weak to pull neighboring particles into tight arrays during the assembly process. Also, the 1.0 µm latex microspheres are more buoyant than yeast – the latex is 1/5 the size of the yeast with a density of 1.055 g/mL, making the microspheres less likely to sediment during the assembly process.

However, because electrostatic interactions are only significant at nanoscale distances between adjacent particles, we believe that the disparate sizes of the yeast and latex microspheres also contributes to more open-packed structures in composite coatings by affecting the capillary interactions. It has been demonstrated that the capillary forces between particles of different sizes pull the smaller particles into clusters in the conical space below the air-water interface formed around the larger particles [42]. In the system studied here, this likely disturbs the assembly process by promoting the formation of particle aggregates that disrupt the coating microstructure by preventing uniform spreading.
Because the cell-latex microsphere and cell-only coatings exhibit similar void spaces for all substrates other than aluminum foil, it is not likely that capillary forces and electrostatics alone cause the higher void spaces in the biocomposite coatings. Surface topography profiles were obtained for each uncoated substrate using a Veeco surface profilometer to characterize coating void space in terms of surface roughness to identify any surface variations that may explain why only the aluminum foil yields higher voids for the yeast suspension than for the composite suspension. Because the foil is rolled flat prior to the assembly process, unlike the other substrates, its surface may have depressions and asperities that are not eliminated during the flattening step, providing rough patches on the surface that strengthen particle attachment (data not shown). Profiles were recorded in triplicate and averaged to obtain a characteristic surface profile for each substrate. The absolute heights of all peaks and valleys on the glass and polyester surfaces were less than 0.20 µm whereas the absolute heights of the various peaks and depressions on the aluminum foil surface are as large as 3 µm, providing crevices for cell and latex microsphere attachment either by adhesion to a surface peak or sedimentation into a surface depression (Figure 2.8). No other pretreated substrate has micron-sized surface deformities, preventing particle attachment to the substrate surface other than by evaporative losses during convective assembly. Surface roughness must be quantified to deposit uniform, close-packed coatings because the degree of surface smoothness (where a completely smooth surface is defined as a surface with deformities smaller than one particle diameter) compete with particle ordering.
2.4 Conclusions

The results demonstrate how the convective-evaporative assembly technique for depositing coatings from suspensions containing particles at 4.1 to 10% solids can be extended to deposit bimodal particle size suspensions of sulfated non-film forming latex microspheres and composite blends of latex microspheres and yeast on glass, plastic and metal substrates. Yeast cells are similar to surface-charged colloidal particles like latex microspheres, allowing for deposition of very thin stable biocomposite coatings of bimodal latex-latex and latex-live *S. cerevisiae* blends. Our experiments indicate that the size ratio of the suspension components (cells or particles), the coating substrate wettability, and the net force between particles all influence the assembly process. Probably the most important finding is that the size ratio controls the convective mixing or demixing of the suspension components and thus their relative locations in the deposited coating, suggesting that the components’ relative size ratio can be used to predict coating void space and coating length. In addition, substrate wettability controls coating length and surface coverage by dictating how the coating suspension spreads across the substrate surface during the assembly process and net force may affect void space. The judicious combination of the above parameters allows deposition of latex-cell monolayers over areas on the order of tens of square centimeters or larger.

The moderately open-packed structures (15% to 45% mean void space) of the bimodal coatings observed regardless of component type (cell only or cell plus latex microsphere) and substrate wettability are similar to honeycomb films of bacteria formed by inclined settling
with a three phase contact line without polymer particles [24, 25] where the size of the coating voids is on the order of 50 µm. However, simulations of nanoparticle honeycomb films yield surface coverages of 20% and 35% (or 65% and 80% void space) [25], voids that are approximately 1.5 to 1.7 times greater than the largest mean void space (45%) observed in our bimodal coatings. This disparity suggests coating deposition via CSA is better suited than honeycomb film formation for fabrication of close-packed biocomposite arrays. While cell or particle sedimentation can disrupt the assembly process and increase coating void space, previous work on pure yeast coatings suggests sedimentation effects can be minimized by directing the settling cells toward the drying front of the coating through variations of the coating apparatus configuration [16]. While this strategy was not employed in this study (our focus was the body interactions between the cells, particles, and substrates), Jerrim and Velev obtained thin yeast coatings with low void space, suggesting CSA does not inhibit the creation of uniform monolayers.

Although bimodal coatings deposited using CSA possess fewer voids than honeycomb films, our results show that convective assembly of bimodal systems using strongly charged sulfated latex microspheres does not produce tightly packed highly structured coatings previously observed with single component latex or all yeast suspensions. Thus, a further step forward would be to better identify the means of controlling and mitigating the coating void space by better packing of the yeast and polymer particles during the convective assembly. One way to achieve this could be by manipulating the net force between the particles and yeast and between both particles and the substrate (the sum of the attractive
capillary forces and the negative repulsive forces) for engineering coatings with minimal void space deposited from multi component suspensions. Nevertheless our current results with bimodal suspensions show that convective assembly is a promising method for creating closely packed monolayer bioreactive coatings from non-toxic, non-film forming and adhesive latex particles and live cells such as *S. cerevisiae* [16]. These coatings may serve as functional biomaterials because they can be coated onto rigid and flexible substrates of varied hydrophobicity, transparency, and surface chemistry.
Acknowledgements

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Figure 2.1: Schematic illustrating the structure of polymer particles and reactive photosynthetic cells (shown in white and brown, respectively) in a randomly orientated thick coating produced by Meyer rod deposition (A), or in thin ordered coatings (B and C). Such thin coatings enable uniform light distribution (yellow arrows) and reactivity to all surface-immobilized cells.
**Figure 2.2:** Schematic of the coating apparatus used to deposit coatings from various particle size ratios, various volume fractions, and thicknesses via batch convective assembly. The apparatus is modified after Prevo and Velev, ref. 37.
Figure 2.3: Example of yeast cell coating deposited using batch convective-sedimentation assembly. Coating macrostructure demonstrating uniform particle distribution in the 20×25 mm² thick region (outlined in red dashed line) (A) and microstructure demonstrating close-packed particle layer (B). Scale bar is 50 µm.
**Figure 2.4:** Micrographs illustrating the structures in composite coatings created from polystyrene microsphere blend ratios. Segregation or dispersion is independent of particle type and controlled only by the size ratio of the small ($R_2$) and large particles ($R_1$). All size ratio blends are 1:1 by particle number. Scale bars are 20 µm.
Figure 2.5: Micrographs of particle (and cell) structures in composite coatings assembled from yeast and polystyrene microsphere blend ratios of (A) 1.0 µm latex microspheres and yeast and (B) 8.7 µm latex microspheres and yeast. Segregation or intermixing is independent of particle type and controlled only by the size ratio of the small (R₂) and large particles (R₁). All size ratio blends are 1:1 by particle (or yeast) number. Scale bars are 20 µm.
Figure 2.6: Schematic illustrating the role of particle geometry (A) and interactions in a bimodal suspension during convective assembly. (B) $R_2/R_1 < 0.25$, smaller particles are transported through the spaces between larger particles to the drying region by the convective fluid flow, resulting in segregation of the larger and smaller particles. (C) $R_2/R_1 > 0.25$, the smaller particles are unable to travel to the drying region as they are retained among the adjacent larger particles, resulting in uniform intermixing of the smaller particles and larger particles in the formed coatings.
Figure 2.7: Effect of surface wettability or contact angle on coating length and surface coverage for coatings deposited from a yeast suspension (A and B, respectively) and from a biocomposite mixture of yeast and 1.0 µm sulfate latex microspheres (C and D) on various wettable substrates. Sample size is 3 for all coating lengths, regardless of suspension type (yeast only or yeast-latex microsphere blend), 9 for the yeast-latex microsphere coating void spaces, and 12 for the yeast coating void spaces. Each yeast or yeast-latex coating is analyzed for mean void space and coating length.
Figure 2.8: Surface topography determined by profilometry of the substrates used for the composite coating deposition all pretreated with 70% (v/v) EtOH. Note the significantly larger small-scale and large-scale variations of the aluminum foil substrate.
Table 2.1: Average advancing water contact angles of pretreated substrates

<table>
<thead>
<tr>
<th>Substrate [Pretreatment Method]</th>
<th>Mean Advancing Contact Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass [NoChromix]</td>
<td>10.1 ± 6.5</td>
</tr>
<tr>
<td>Glass [70% (v/v) EtOH]</td>
<td>18.4 ± 1.7</td>
</tr>
<tr>
<td>Aluminum Foil [70% (v/v) EtOH)]</td>
<td>35.0 ± 10.7</td>
</tr>
<tr>
<td>Polyester [70% (v/v) EtOH)]</td>
<td>67.8 ± 13.8</td>
</tr>
<tr>
<td>Polypropylene [70% (v/v) EtOH)]</td>
<td>89.5 ± 11.7</td>
</tr>
</tbody>
</table>
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CHAPTER 3

Continuous Convective-Sedimentation Assembly of Colloidal Microsphere Coatings for Biotechnology Applications

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Abstract
Continuous convective-sedimentation assembly (CCSA) is a deposition method that constantly supplies the coating suspension to the meniscus behind the coating knife by inline injection, allowing for steady-state deposition of ordered colloids (which may include particles or cell-particle blends) by water evaporation. The constant inflow of suspended particles available for transport to the drying front, yields colloidal arrays with significantly larger surface areas than previously described and thus expands the ability of convective assembly to deposit monolayers or very thin films of multiple sizes of particles on large surfaces. Using sulfated polystyrene microspheres as a model system, this study shows how tunable process parameters, namely particle concentration, fluid sonication, and fluid density, influence coating homogeneity when the meniscus is continuously supplied. Fluid density and fluid flow-path sonication affect particle sedimentation and distribution. Coating microstructure, analyzed in terms of void space, does not vary significantly with relative humidity or suspended particle concentration. This study evaluated two configurations of the continuous convective assembly method in terms of ability to control coating microstructure by varying the number of suspended polymer particles available for transport to the coating drying front through variations in the meniscus volume.
3.1 Introduction

Convective assembly is a commonly employed colloidal structuring technique for depositing self-assembled, ordered, thin crystalline coatings (polymer particle arrays) over large areas [1, 2, 3]. Techniques for ordering and assembling colloidal particles into closely packed arrays by solvent evaporation have been thoroughly investigated, including ring formation in drying droplets [4-7] and colloidal coating formation in thin wetting films [8-11] some of which in water borne latex systems contain reactive live cells (bacteria, yeast, cyanobacteria or algae) for future biotechnology applications [9,10]. Reactive microorganisms behave as charged particles in aqueous deposition systems and we have found that net charge leading to repulsion between particles or between particles and cells is an important factor in coating assembly [12]. These findings have resulted in an emerging methods to generate colloidal arrays with varying thicknesses, particle sizes, and types ranging from charged latex particles [8, 11, 13-16] to live cells [9] to composite charged particle plus live cell mixtures [9, 12, 17]. Such convectively assembled arrays are well suited for applications that would benefit from an ordered microstructure, but do not require a completely defect-free, perfect colloidal crystal, such as assembly of microbial photoreactive coatings [10], antireflective coatings [18, 19], electrical circuits [17], chemical sensors [20, 21], and porous membranes [3, 22-24]. Convective assembly could also lead to rapid, repeatable fabrication of well-ordered, industrial-scale arrays that are useful for construction of structured multi-layer systems with enhanced, or even hybrid, functionality (Figure 3.1).
The convective assembly combines fluid evaporation, particle transport via fluid flow, and associated meniscus motion to rapidly and controllably deposit ordered thin films on practically relevant coating surface area scales. The assembly process begins when the thickness of the evaporating fluid film becomes thinner than the diameter of the suspended particles. The menisci formed around these particles give rise to attractive capillary forces that pull adjacent particles together as the liquid evaporates, forming two-dimensional nuclei [25-27]. A liquid flux from the suspension bulk to the substrate-air-liquid contact line at the drying front offsets fluid loss due to evaporation, resulting in particle transport to the drying front as adjacent particles aggregate and subsequent proliferation of the coating [25, 26, 28]. A particle mass balance relates the coating growth rate, \( v_c \), to the fluid evaporation rate and particle volume fraction:

\[
v_c = \frac{\beta j_e l \phi}{h(1-\varepsilon)(1-\phi)},
\]

where \( \beta \) is an interaction parameter, \( j_e \) is the evaporation rate, \( l \) is the drying length, \( \phi \) is the volume fraction of the particles in suspension, \( h \) is the height of the deposited colloidal crystal array, and \( \varepsilon \) is the coating porosity [27, 29]. The values of \( \beta \) vary between \( 0 \leq \beta \leq 1 \) and depend on particle-particle and particle-substrate interactions. For suspensions with low volume fractions and electrostatically stable particles, \( \beta \to 1 \) [28]. Once \( v_c \) is determined, the length of the thin film in which deposition occurs by convection can be calculated using a material flux balance:
where \( v_w \) is the deposition rate and equal to \( v_c \) at steady state, and \( c_i \) is the concentration of the bulk suspension at that particular time [29].

Using an all-colloid system, Prevo and Velev reported a modified convective assembly technique that allows for rapid and controllable deposition of coatings from microliter suspension volumes [8]. 5-30 µL of coating suspension containing particles at high volume fraction (0.9-35% w/v) is trapped between a horizontal substrate plate and an inclined coating knife plate. The inclined top plate is moved at a constant rate along the long axis of the bottom plate by a linear motor. This delivers and spreads the suspension from the meniscus into a thin film across the horizontal substrate, leading to the formation of a coating on the substrate by evaporative convective assembly [9]. The number and type of deposited particle layers are readily adjusted by altering the suspension volume fraction and coating knife speed, allowing for precise control in particle packing and coating thickness [8]. If the meniscus height is less than the particle diameter at the growth front, as in the case of faster knife speeds, the incoming particles form an open-packed structure [28]. Conversely, for slower knife speeds, if the meniscus height is greater than the particle diameter, multilayer deposition occurs [28].

Many methods for depositing colloidal arrays via convective assembly in thin films are variations of the convective assembly apparatus reported by Prevo and Velev that can be optimized by varying the basic process. Kleinert and Velev coupled the convective assembly
mechanism with an electric field to obtain more rapid particle assembly, larger crystal
domains, and reduced structural defects [11]. Park and coworkers proposed a modified
convective assembly technique that uses a dip-coating apparatus to modulate the meniscus
thinning rate, allowing for the formation of well-ordered, multilayered single-component and
binary colloidal crystals [15]. Robinson et al. later presented another variation that more
rapidly deposits crystalline films than conventional convective assembly by altering the fluid
evaporation rate through restricted movement of the meniscus along the top plate [16].
However, while these variations offer greater control over the assembly process, the utility of
convective particle or cell assembly in thin films on an industrial scale is restricted because
the total coating surface area is limited by the amount of suspended particles delivered by the
continuously depleted (batch) coating meniscus volume.

In order to evaluate methods to deposit large surface area composite coatings of polymer
particles and live cell blends in uniform monolayers for future biocoating or biotechnology
applications, we investigated a model colloid system to develop new methods for continuous
convective assembly of thin films on the basis controllable continuous delivery of particle
suspensions to the meniscus. This method allows for steady-state coating deposition by
constantly dispensing suspended uniformly charged particles (or charged particles + live
cells) to the meniscus, resulting in colloidal array coatings with larger surface areas. We call
this method Continuous Convective-Sedimentation Assembly (CCSA) to distinguish it from
conventional batch deposition. Using monodispersed sulfated polystyrene microspheres, we
investigate and report how particle concentration, fluid flow-path sonication, and suspension
density influence the coating microstructure when the meniscus is constantly supplied with particles. We also examine how two configurations of the CCSA apparatus perform in this process: 1) topside CCSA, in which suspension flows through a capillary from a fluid reservoir to the front of the meniscus along the coating knife’s topside, and 2) underside CCSA, in which suspension flows into the meniscus from a fluid reservoir through a capillary fixed to the back of the knife. Finally, we compare batch and continuous convective assembly and discuss how the variation of meniscus volumes affects the coating microstructure by varying the number of particles available for deposition.

3.2 Materials and Methods

3.2.1 Preparation of Coating Substrates and Deposition Plates

Fisherbrand 75×25 and 75×50 mm glass microscope slides (Fisher Scientific) and Dow Corning 75×50 mm glass microscope slides (Fisher Scientific) were pretreated as previously described [9] using NoChromix solution to remove adsorbed organic molecules and deprotonate surface hydroxyl groups (Godax Laboratories, Cabin John, MD). All pretreated slides were stored in separate 100 × 100 mm Fisherbrand Petri dishes (Fisher Scientific) until used in the CCSA deposition device. In order to have a conductive substrate for SEM imaging of coating microstructure, 75×25 mm strips of aluminum foil (Handi-foil of America, Wheeling, IL) were rinsed with 70% (v/v) ethyl alcohol, gently wiped with a KimWipe (Kimberly-Clark, Chantilly, VA) to remove surface contaminants, and dried at the ambient temperature and relative humidity to evaporate any residual alcohol. All foil strips
were flattened using an Orcon smooth action roller (Tools for Floors, Waverly, NY) prior to the alcohol rinse.

3.2.2 Preparation of Coating Suspensions

Sulfate latex microspheres (0.99 ± 0.014 µm) (Interfacial Dynamics Corporation, Eugene, OR) were washed once with deionized water obtained from a RiOs 16 reverse-osmosis water purification system (Millipore Corporation, Bedford, MA) to remove residual surfactants and electrolytes. The washed microsphere suspensions were subsequently concentrated to 8%, 12%, or 16% solids using a Fisher Marathon micro A microcentrifuge (3630 RPM for 5 min) for suspensions less than 1000 µL and using a Beckman TJ-6 bench-model centrifuge (3000 RPM for 5 min) for suspensions over 1000 µL. All suspensions were sonicated gently in an ultrasonic cleaner (Branson Ultrasonics Corporation, Danbury, CT) to reduce particle aggregation before deposition.

Several washed microsphere suspensions were mixed with OptiPrep™ solution (Axis-Shield, Oslo, Norway) to evaluate the role of solution density in convective assembly and coating uniformity. OptiPrep™ is a low viscosity, sterile solution of 60% iodixanol in water with a density of 1.32 g/mL. All hybrid coating suspensions were blended using a Vortex-Genie mixer (Fisher Scientific) to promote solution homogeneity before deposition.

3.2.3 Deposition of Coatings by Continuous Convective-Sedimentation Assembly
All coatings were deposited using the convective assembly method described previously [8] with an added continuous delivery system that feeds the coating suspension to the meniscus. We examine how both topside CCSA or suspension flow to the meniscus front along the knife’s topside and underside CCSA or fluid flow to the meniscus rear along the knife’s underside can deposit larger surface area polymer particle arrays by continuously delivering particles to the coating meniscus. The delivery system was attached to the deposition plate or coating knife and consists of a 1 mL NORM-JECT syringe (4.726 mm ID) (Henke-Sass, Wolf GmbH, Dudley, MA) coupled to 16-18 cm of 0.022 x 0.042 in PTFE microbore tubing (Cole-Parmer, Vernon Hills, IL) through a 3/32 in hose barb to female luer adapter. The tubing was bonded to the adapter’s hose barb with Dow-Corning 732 Multi-Purpose Sealant (Midland, MI) or Dow-Corning Fast Tack 3165 RTV Adhesive Sealant to minimize fluid loss during deposition.

A freshly cleaned coating knife and substrate were attached to the deposition device after the delivery system was connected to the syringe and to the knife (Figure 3.2). The system outlet was placed 1-3.5 mm away from the knife’s edge and 12.5 mm along its length to ensure uniform meniscus formation along the entire length of the knife. Positioning the outlet less than 2 mm from the knife’s edge prevents it from contacting the substrate. Suspension aliquots ranging from 500-1000 µL were pumped from the fluid reservoir to the back of the meniscus along the knife’s underside using a New Era NE-1800 syringe pump (Wantagh, NY). The pump was operated at a priming rate of 100.5 µL/min to rapidly fill the wedge between the substrate and knife and then reduced to a standard rate of 0.5 µL/min.
after the meniscus covered the tubing outlet on the knife’s underside. The meniscus was filled to the tubing outlet to maximize effluent particle delivery and minimize aggregation on the knife’s underside around the tubing outlet. The linear motor pushing the coating knife was operated at a speed between 21-190 µm/s, while the suspension was deposited onto the substrate in 10-60 minutes.

The priming and standard delivery rates were selected by calculating the area of the entrained liquid from a set of equations describing the meniscus shape [9] and calculating the pump flow rate that equilibrates the evaporative flux in the meniscus and the suspension delivery rate. The optical (and reported) monolayer deposition rate was determined by systematically varying the suspension feed rate around the calculated value to identify the rate that deposits the most macroscopically uniform coatings (data not shown).

For topside continuous convective-sedimentation assembly, the delivery system outlet was connected to the knife’s topside, enabling fluid flow from the fluid reservoir to the front of the meniscus along the topside. This configuration hybridizes the inherent advantages of continuous and batch convective assembly – delivering the suspension into the front of the meniscus through the gap between the substrate and knife decreases the required meniscus volume, enabling the use of smaller suspension volumes (a benefit of batch convective assembly) while still constantly renewing the meniscus (a benefit of CCSA). The system outlet was placed no more than 1 mm ahead of the knife’s edge to ensure uniform meniscus formation along the entire length of the knife. Although this results in fluid flow directly onto the substrate surface, the suspension is pulled into the meniscus before the suspended
particles can settle onto the surface, thus eliminating unwanted coating formation in front of the knife. Suspension aliquots ranging from 200-1000 µL were pumped from the fluid reservoir to the gap between the substrate and knife at a constant rate, enabling coating formation in 10-30 minutes. All other experimental aspects were the same as in the underside continuous-sedimentation convective assembly configuration.

All CCSA coatings were deposited inside a Model 510 Benchtop Humidity and Temperature Controlled Environmental Chamber (Electro-Tech Systems, Inc., Glenside, PA) at a chamber temperature of 20-30°C and relative humidity of 40-75%. The relative humidity was maintained with an ultrasonic humidification system (Model 5462 Electro-Tech Systems, Inc.) filled with deionized water. Both the chamber temperature and relative humidity were monitored with a calibrated probe (TM125 Dickson Addison, IL). The syringe pump, tubing, and deposition device were placed inside a topless encasement made from rigid polypropylene sheets (Wilson Jones, Lincolnshire, IL) to prevent the circulating air inside the chamber from disrupting solvent evaporation and associated particle transport during the assembly process.

CCSA uses larger volumes of coating suspension than other convective assembly techniques so Taylor dispersion and particle settling in the suspension delivery system may occur, leading to uneven particle delivery to the meniscus and associated irregularities in coating thickness and particle packing. The apparatus was modified to minimize particle sedimentation by 1) orienting the coating reservoir vertically, ensuring all particle sedimentation collinear with the flow to the outlet and 2) the flow-path tubing was
continuously vibrated at 400 Hz using two 20 mm buzzer piezoelectric elements (Digi-Key Corporation, Thief River Falls, MN) clipped to the delivery system and coupled to a GW Instek GFG-8210 function generator (Good Will Instrument Company, Chino, CA), ensuring that particles were transported to the meniscus rather than settled onto the tubing walls. Many coatings in this study have thick, amorphous particle aggregates along their widths (coating edges perpendicular to the direction of deposition) – these regions are artifacts of the CCSA process that form where the meniscus dries on top of the assembled coating, leading to random, uncontrolled particle coalescence and disruption of the convectively-assembled coating. These edge artifacts can be reduced by additional refinements to the deposition method, such as draining the meniscus at the end of the deposition period.

To reduce variations in coating appearance associated with the knife’s surface roughness or extent of pretreatment, the same knife and delivery system were used to deposit all coatings in each series whenever possible. The knife was dried with a KimWipe and then rinsed with 70% (v/v) EtOH between depositions to remove any residual coating suspension and other debris from the coating edge.

3.2.4 Coating Characterization

All coatings were imaged by digital and optical microscopy for structural analysis. High resolution images were captured with a Canon EOS 5D Mark II SLR digital camera equipped with an EF (Electro-Focusing) 100 mm Macro lens. High magnification micrographs were taken with an Olympus BX61 microscope equipped with an Olympus DP70 CCD camera.
and 5x to 50x objectives. All micrographs were analyzed with Adobe Photoshop software to determine the fraction of the substrate surface that was covered with coating. To analyze the microstructure homogeneity within an individual coating, images were collected at three equally spaced sampling points across the coating width at 15 and 35 mm from the initial contact line. To compare the microstructure homogeneity of multiple coatings, images were collected using a double lattice selection scheme and a 4x4 square frame, as described [30]. All squares were imaged in ten randomly-selected locations with a 20x objective, yielding a sample size for each square that describes almost 25% of that square. The calculation for the overall variation of the double lattice is also described [30], where \( r \) and \( L \) are defined as two and four, respectively.

Random strips of pure suspension (OptiPrep™ or 1.0 µm microspheres at 8% solids) and an 80% (v/v) blend of OptiPrep™-1.0 µm microsphere blend coated onto aluminum foil were selected for structural analysis. Dried coatings were imaged by scanning electron microscopy for structural analysis at the Analytical Imaging Facility at North Carolina State University using a Hitachi 3200-N Variable Pressure SEM equipped with a 4Pi Isis EDS system for digital image acquisition and elemental analysis. All coatings were observed in two or more randomized locations using a 5kV accelerating voltage. Each location was imaged multiple times using sequential magnifications ranging from 30x to 2,000x to characterize surface structure. To increase sample conductivity (and micrograph quality), all samples were sputter coated with a thin layer of gold in a mild vacuum (~100 mTorr of argon gas pressure and 600 V accelerating voltage) prior to imaging.
Surface topography profiles to determine coating thickness were recorded using a Dektak D150 surface profilometer (Dektak D150, Veeco, Plainview, NJ) coupled with the Dektak v9 software package. Standard scans were taken across the full coating width in random locations using a 12.5 μm radius stylus tip with 0.01-0.1 μm resolution and a 1.0 mg stylus force. Profiles were recorded in duplicate for each coating.

3.2.5 Evaluation of Microsphere and Solvent Material Balances

Particle and solvent material balances over the delivery system were used to identify the role of particle and solvent transport during deposition. The microsphere balance was quantified by calculating particle numbers for the meniscus, the coating knife and substrate, and each component of the delivery system after deposition. Fluid fractions from the coating meniscus and from the delivery system’s syringe, adapter, and tubing were characterized using flow cytometry. The particle surface densities for the coated regions of the knife and substrate were estimated by dividing each region by the area of an individual microsphere. All particle counts were performed using a Becton-Dickinson FACSCalibur flow cytometer (Franklin Lakes, NJ) coupled with BD Cell Quest Pro software and calibrated using a mixture of 3.0-3.4 μm Rainbow Calibration Particles (Spherotech, Inc., Lake Forest, IL). All fractions were first diluted to 100 μL with phosphate buffered saline (PBS) solution and then mixed with 100 μL of Rainbow Calibration Particles solution. Fluid fractions for both pure microsphere and microsphere-OptiPrepTM hybrid coating suspensions were collected and counted.
The solvent balance was evaluated by passing 500-1000 μL of deionized water through the delivery system for 16-25 hrs using a syringe pump (New Era NE-1800). The pump was operated at a priming rate of 100.5 μL·min⁻¹ and then reduced to 0.5 μL·min⁻¹ after suspension droplets freely flowed through the delivery system. The water was collected in an aluminum weigh dish (Fisher Scientific) covered with transparent plastic film to minimize water evaporation. The covered dish and a calibrated probe (TM125 Dickson) weighted before and after the pumping period. The delivery system was replaced with a Cadence Science 21G × 6 in standard hub, deflected point needle (Cadence, Inc., Staunton, VA) to minimize fluid loss to the delivery system’s adapter piece [data not shown], improving the accuracy of the water balance. The water was continuously sonicated during the pumping period by vibrating two 20 mm Digi-Key buzzer piezo elements at 400 Hz using a GW Instek GFG-8210 function generator against the needle. Duplicate solvent balances were calculated for two flow conditions – agitated and static fluids – to isolate the effect of continuous sonication on solvent evaporation rate.

3.2.6 Meniscus Volume Characterization

Coating uniformity depends on the meniscus shape at the drying front [31, 32] and on the total number of suspended particles in the meniscus [8]. As we recently demonstrated how both substrate wettability and coating suspension composition control coating length and surface coverage, we did not report here the effect of meniscus volume on coating uniformity [12]. We investigate the effect of meniscus volume on coating structure, analyzed as coating
thickness, macroscopic appearance, and microscopic void space. To estimate the meniscus volume, still images of the meniscus from the side were collected using a Seeker 400 Series Wireless CameraScope Inspection System (Davis Instruments, Vernon Hills, IL) equipped with a removable LCD display and interchangeable 9 and 12 mm diameter camera tipped probes. To quantify the meniscus volume, each image was analyzed with an image processing program (ImageJ, available to the public at http://imagej.nih.gov/ij/) to determine the meniscus height and length in that image. The volume of the entrained liquid was calculated by approximating the meniscus shape as a triangle with a curved edge (Figure 3.3).

The volume is calculated as the product of the net area and the width of the deposition blade:

\[
V_{meniscus} = \left[ \frac{bh}{2} - \frac{r^2}{2} \left( \frac{\theta \pi}{180} - \sin \left( \frac{\theta \pi}{180} \right) \right) \right] x W
\]

(3)

where \( W \) is the knife width, \( b \) and \( h \) are the base and height of the triangle, respectively, and \( \theta \) is the central angle of the circle segment, and \( r \) is the radius of the circle in which the segment is a part.

To account for aberrations in the imaging system and method, menisci with known volumes, namely 10.0, 25.0, 35.0, and 50.0 µL, were deposited via batch convective assembly in triplicate and imaged. All images were analyzed using Eqn (3) and subsequently averaged to obtain a mean estimated volume for each known volume. A calibration curve of
the actual meniscus volumes versus the mean measured ones was constructed (R² value of 0.998).

3.2.7 Evaluation of the Effect of Suspension Deposition Mode and Rate on Meniscus Volume

Washed, 1.0 µm white sulfate-functionalized latex microspheres were first concentrated to 16% solids and then deposited using hybridized forms of the conventional batch and topside CCSA or underside CCSA modes to study the effect of suspension deposition mode and delivery rate on meniscus volume and coating morphology. The deposition mode study used both the topside and underside CCSA configurations whereas the delivery rate study used only the underside CCSA mode. All coatings used the same initial meniscus volume of 24.0 µL and a knife speed of 21.1 µm/s. Any resulting changes in the meniscus volume or coating structure over the coating length can be attributed to the deposition mode or delivery rate. A suspension delivery rate of 1.0 µL/min was used for the deposition mode study whereas the delivery rate was varied between 0 (batch), 1.0, 2.0, 4.0, and 6.0 µL/min for the delivery rate study. For each coating, the meniscus was photographed at 0, 10, and 20 mm from the initial pinning (or contact) line using the side camera, and the initial pinning line was formed ahead of the 0 mm mark to ensure the meniscus was fully developed prior to imaging. To understand the effect of deposition mode on coating structure, coating micrographs were taken at 5, 12.5, and 20 mm from the initial pinning line along two
separate axes, located at 15 and 35 mm along the coating width, using an Olympus BX61 optical microscope coupled with a 50× objective.

3.3 Results and Discussion

3.3.1 Microsphere and Solvent Delivery Material Balances

Material and fluid balances of monodispersed sulfated polystyrene microspheres were calculated to identify particle and solvent losses in the CCSA apparatus that may disrupt the evaporative and convective fluxes that are responsible for coating propagation. The microsphere balance confirms that the delivery system does not inhibit particle delivery to the meniscus, despite losses through particle sedimentation in the delivery system and onto the coating knife of a suspension with 8% solids (Table 3.1). However, the material balance is conservative in that it lacks a factor to account for the multilayered particle aggregation along the coating edges (parallel to the direction of deposition) which is a significant artifact of small surface area coating systems with a large edge/surface area ratio [31]. Several investigators have suggested methods to reduce this artifact as well as meniscus-related edge effects [31]. The particle balance assumes that the number of aggregated particles along the coating edges offsets the number of the particles in the thinner coating interior such that the total number of enumerated particles post-deposition would lead to a complete monolayer under optimal deposition conditions (no edge artifacts). This balance likely undercounts the total amount of particles in the coating, resulting in an estimate of only 2.9% of the particles delivered to the meniscus being deposited in these test coatings.
To evaluate the role of colloid particle sedimentation, the washed microspheres were blended with OptiPrep™ in an 80:20 (v/v) ratio prior to deposition to reduce particle settling in the tubing. OptiPrep™ addition reduced the percent solids in the suspension to ~2%. Because OptiPrep™ has a relatively high density of 1.32 g/mL compared to that of polystyrene latex (1.05 g/mL), the blended suspension has an appreciably higher density than the aqueous latex suspension, making the microspheres more buoyant leading to less particle sedimentation (Table 3.1). This further decreased the percentage of particles delivered to the test coating to ~2.4%.

The results indicate that approximately equal numbers of particles are deposited into the coating for both the pure and OptiPrep™-enriched microsphere solutions (2.6% and 2.4%, respectively). Also, an almost identical number of particles remained in the syringe after deposition (~91.6%) for both suspensions, accounting for the difference between the particle count “pre” and “post-deposition. However, the percentage of microspheres remaining in the fittings, meniscus, and tubing post-deposition, relative to the total particle number in the syringe before deposition, is less for the OptiPrep™ blend compared to the pure microsphere suspension (1% versus 3%, respectively), suggesting that OptiPrep™ inclusion acts favorably to reduce particle sedimentation in the delivery system.

Separate balances were calculated for sonicated and non-sonicated trials to evaluate the effect of continuous piezo sonication of the fluid delivery system on the deposition process. The ambient relative humidity and temperature above the evaporating water were 60-75%
and 22-24°C, respectively. The solvent material balance was corrected for water evaporation during the pumping period (see experimental section and supplemental information).

Fluid material balances generated by pumping fluid through the delivery system into a weighing dish (instead of the coating knife meniscus) confirmed that the modified coating method continuously delivers the coating suspension to the meniscus with minimal solvent loss, regardless of sonication. Sonication appears to decrease the volume of water pumped into the weighting dish – the average volumes in the dish after pumping are 0.52 ± 0.08 and 0.44 ± 0.02 mL when the fluid is stagnant and agitated, respectively. The evaporative loss calculations only account for relative humidity and temperature differences at the air-liquid interface, while they ignore the effects of the sound waves on the evaporation rate of the agitated fluid. However, although sonication affects solvent loss, any evaporative losses from both stagnant and agitated fluids are likely negligible during continuous convective-sedimentation assembly because the suspension is deposited onto the substrate in less than an hour whereas the water was pumped into the weigh dish for 16-25 hours.

3.3.2 Coating Structure Dependence on Suspension Density

Coating structure can also be modified by using a microsphere suspension with a density-modified medium like iodixanol particles (OptiPrep™). Modifying the suspension density allows to increase the buoyancy of the suspended particles and thus minimizes particle sedimentation onto the substrate.
To test this hypothesis, coatings were deposited from either pure microspheres in water \((\rho_{\text{suspension}} = 1.05 \text{ g/mL})\) or an 80:20\% (v/v) composite OptiPrep\textsuperscript{TM}-microsphere solution \((\rho_{\text{suspension}} = 1.27 \text{ g/mL})\) at an ambient chamber temperature of 26.5 ±1.8°C and relative humidity of 49.6 ±1.3\% using underside CCSA and a knife speed of 21.1 µm/s. Visual analysis of both coating macrostructures shows that OptiPrep\textsuperscript{TM} appears to minimize particle clumping across both the coating width and length, improving the overall uniformity (Figure 3.4).

Blending the microsphere suspension with OptiPrep\textsuperscript{TM} changes the dynamics of the assembly process by introducing a transition between long-range and short-range particle ordering. Because the suspended microspheres are more buoyant in an OptiPrep\textsuperscript{TM}-enriched suspension, they are more likely to remain suspended in the meniscus throughout the deposition period (see Figure 3.5). Thus, particles will remain at or near the meniscus surface and will be assimilated into the coating only after the thickness of the evaporating meniscus becomes smaller than the diameter of the suspended microspheres, leading to more uniform particle sedimentation onto the substrate surface.

Overall, the suspension density modification appears to reduce the average coating thickness and produces a more uniform, though less structured, monolayer (Figure 3.6). The observed film is affected by the high concentration (≈80\% (v/v)) of OptiPrep\textsuperscript{TM} necessary to make the solvent denser than the latex microspheres. The iodixanol particles formed during the drying coalesce into a continuous film that fills the void space created by the 1.0 µm microspheres during coating fabrication (Figure 3.6B), leading to reduced striations across
the coating’s surface and improved overall visual appearance. Thus, while OptiPrep does not change microsphere and solvent delivery rate to the meniscus, the iodixanol particles alter the coating’s structure by disrupting the convectively-assembled crystalline array.

We have recently showed that deposition of thin latex polymer particle coatings is a critical precursor to the formation of 1-2 cell layer thick microbial coatings of live cells and latex polymer particles [12]. These biocoatings offer the promise of improved nutrient diffusion to immobilized cells and uniform illumination of the particles or cells, thus overcoming the mass transfer and optical limitations of thicker coating systems.

3.3.3 Coating Structure Dependence on Suspension Sonication during Deposition

Particle sedimentation strongly affects both coating uniformity and the convective assembly process. When sedimentation dominates, most suspended microspheres sediment straight down onto the substrate outside of the drying region [9]. This complicates the deposition of highly uniform coatings by continuously reducing the number of particles transported to the drying front over the deposition period. Sonication was evaluated as means to suppress particle sedimentation during deposition, since sonic standing waves have been used successfully in noncontact manipulation of suspended particles [33-37]. Coatings were deposited using a glass substrate and either a stationary or sonicated continuous delivery system. We observed that changes in vibration frequency up to 1 kHz and piezo positioning underneath the substrate edge had no effect on the coating structure. Sonicating the substrate (rather than the suspension) did not appreciably change the coating structure.
All coatings were deposited using the same knife and delivery system (syringe, fitting, and tubing) to ensure that any deviations in the dried coating appearance are a sonication result. The delivery system was positioned approximately 3 mm from the knife’s leading edge to eliminate the risk of the tubing end affecting the coating uniformity. Coatings were deposited at a chamber temperature of 25.9 ± 2.0°C and relative humidity of 51.6 ± 3.4% using a knife speed of 21.1 µm/s.

The effect of piezoelectric fluid agitation in the delivery system during deposition is shown in Figure 3.7. Although the images and micrographs show regions of dense particle packing in both coatings, the sonicated delivery system has better coating uniformity than the non-sonicated system. Unlike the highly uniform sonicated coating, the coating delivered without piezo vibration of the delivery path ranges from a near monolayer to a submonolayer (high void space) and multilayer particle clusters (see micrographs in Figure 3.7). Also, coatings deposited with the vibrated fluid-flow delivery system show a higher degree of surface coverage (or lower void space) on both the macroscopic and microscopic levels than comparable coatings deposited with the stationary delivery system. In summary, ultrasound sonication of the suspension delivery system improves coating uniformity.

3.3.4 Coating Microstructure Dependence on Suspension Particle Concentration

Coatings from suspensions concentrated to 8% (w/v), 12% or 16% were deposited to determine if the number of particles delivered to the drying front affects the coating void space. The coatings were deposited at 70% relative humidity and a knife speed of 190 µm/s.
as this combination minimizes the particle piling along the substrate edges noticed during the evaluation of the microsphere and solvent delivery material balances. The parameter values were chosen within the relative humidity and deposition speed ranges of 35% to 70% and 21-190 µm/s, respectively. Relative humidity and temperature data varied within 77.3 ± 2.3% and 29.1 ± 1.8°C, respectively. Two surface topography profiles of sections of the coating width were obtained for each coating using a random selection scheme to remove sampling bias. Profiles were grouped by sample number.

The suspended particle concentration strongly affects coating thickness (of 1 to 3 particle layers), but has no appreciable effect on void space (Figure 3.8). The 8%, 12%, and 16% (w/v) suspensions deposit coatings with similar void spaces (Figure 3.8B). However, the 16% suspension yields coatings that are ~1 particle thicker than coatings deposited from the 8% and the 12% suspensions (Figure 3.8A). The large variation in the scans of coating sections, especially for the film deposited from the 12% suspension, shows the coatings are not perfect monolayers and that coating thickness varies by more than one particle diameter with particle concentration. Relatively high fraction of high void spaces (>50% on average) was observed for all coatings. A strong correlation between suspended particle concentration and coating void space cannot be identified due to the overlapping error bars (Figure 3.8B). However, higher suspended particle concentrations will likely result in overall thicker coatings (>3 particles thick) by increasing the height of the liquid film, enabling influent particles or even particle aggregates to flow over and settle atop other deposited particles during the assembly process [12].
3.3.5 Evaluation of Suspension Deposition Mode

To understand how the mode of suspension delivery affects the microstructure of coatings deposited using CCSA, coatings were deposited in triplicate using either the topside or underside CCSA modes at 26.2 ± 2.3°C and 45.9 ± 6.2% relative humidity. All coatings were deposited using a knife speed and suspension flow rate of 21.1 µm/s and 1.0 µL/min, respectively, from an initial meniscus volume of 24.0 µL. Each coating was analyzed for changes in meniscus volume, normalized by the calculated volume at 0 mm, and average void space, as reported in Figure 3.9. Micrographs were collected at three 7.5 mm intervals across the coating length, starting 5 mm from the coating’s leading edge, at vertical axes located 15 and 20 mm from the coating’s top edge. This protocol allowed for comparison of the structural uniformity for multiple coatings.

To estimate meniscus volume, still images of the meniscus profile were collected using a wireless digital camera and analyzed with image processing software to determine the meniscus height and length in each image. The volume of the entrained liquid was calculated by approximating the meniscus shape as a triangle with a curved edge (see Materials and Methods for detailed product information and explanation of calculations). The effect of meniscus volume on coating structure was investigated because this volume dictates both the meniscus shape and total number of suspended particles assembled into the final coating, whether by convective assembly or particle sedimentation.

Analysis of Figure 3.9 shows that the coatings deposited via topside and underside CCSA are dissimilar. The coatings deposited via topside CCSA have a comparatively large
variation in mean void space but a relatively constant meniscus volume whereas underside coatings exhibit a lower variation in mean void space. The continuously decreasing meniscus volume observed by underside delivery indicates that the delivery rate was too slow to maintain a constant volume. The overall lower void space suggests that more particles are transported to the drying region (and incorporated into the propagating coating) in underside CCSA. Conversely, the near constant meniscus volume and high, variable voids in the topside coatings suggest less particles reach the drying region in topside CCSA. This is possible if circulating flow patterns exist in the meniscus [9]. Because the topside CCSA deposition mode and the batch deposition deliver suspension to the front of the meniscus opposite the drying region, it is likely that an eddy also exists in the meniscus during topside CCSA deposition. As such, rather than flowing directly to the drying region at the rear of the interface, particles may become entrapped in the circulating eddy and only move to the drying region when the eddy becomes saturated with particles, a dynamic instability leading to the formation of coatings with variable voids.

3.3.6 Evaluation of the Effect of Suspension Delivery Rate on Meniscus Volume

Although comparison of the topside and underside CCSA deposition confirms the deposition mode affects both meniscus volume and coating microstructure, this analysis failed to explain the effect of the delivery rate itself on the meniscus volume. To understand this relationship, coatings were deposited using a hybridized form of the conventional batch and underside CCSA deposition modes – the meniscus was created by manually injecting an
aliquot of coating suspension between the substrate and coating knife (batch CSA) and continuously replenished with suspension at a constant flow rate (CCSA) – and suspension delivery rates of 0.0 (to simulate batch deposition), 1.0, 2.0, 4.0, and 6.0 µL/min. Elimination of the coating edge effects observed at low knife speeds and relative humidity was not attempted because the study’s purpose was to clarify how suspension flow rate affects meniscus volume and coating microstructure. All coatings were analyzed for variations in coating macrostructure and visual appearance.

The micrographs in Figure 3.10, suggest that, for a given convective assembly deposition mode and initial meniscus volume, coating quality is independent of meniscus volume, at least for the experimental coating apparatus employed in this study. No coating exhibits complete macroscopic uniformity or monolayer thinness – all coatings contain randomly-located thick (intense white) and thin (dull white) regions. For all coatings, the thick zone covers at least 50% of the coated surface area. Also, both the batch mode (0 µL/min) and 1.0 µL/min flow rates exhibit a similar, decreasing meniscus volume across the coating length. The higher flow rates exhibit variable (both increasing and decreasing) meniscus volume across the coating length when utilizing the optical image method for estimating meniscus volume.

3.4 Conclusions
The results demonstrate how continuous convective assembly can be optimized for continuous deposition of polymer particle coatings with larger surface areas than previously described. The modified coating apparatus continuously delivers polymer microsphere suspensions to the meniscus via inline injection. We investigated how two variations of the CCSA apparatus perform in generating larger surface area polymer particle arrays between 1 and 3 particle diameters thick by continuously delivering particles to the drying front for coating assembly. However, changing the suspension delivery mode (topside versus underside CCSA) yields disparate meniscus volumes and uneven particle delivery to the drying front, which alters the coating microstructure by varying the total number of particles available for deposition. For any of these convective assembly deposition modes and the same initial meniscus volume, coating quality was found to be independent of meniscus volume, at least for the coating apparatus employed in this study.

We also investigated the effect of suspended particle concentration, fluid sonication, and density modification on the microstructure of coatings deposited from continuously supplied 1.0 µm microsphere suspensions. Fluid density modification using iodixanol particles and fluid sonication affect particle sedimentation and distribution in the coating growth front whereas the suspended particle concentration affects coating thickness, but has almost no effect on void space. This study did not examine the combined effect(s) of these parameters because our goal was to understand each parameter’s individual role in controlling coating microstructure. The observed trends suggest that the uniformity of any particle, or even live cell, coating can be improved through fluid sonication and suspension fluid density
modifiers. Future practical implementation of the CCSA could examine the combined effect of these parameters on coating microstructure and use such a combination in the deposition of large uniform coatings.

While no CCSA coating mode exhibits macroscopic uniformity or 100% monolayer coating, this study demonstrates that the CCSA technique deposits coatings whose homogeneity and structure are comparable to traditional batch convectively-assembled coatings with decreasing meniscus volume. Noncrystalline or non-uniform CCSA coatings, like batch-assembled arrays, are well suited for numerous applications, including electrical circuits [17], chemical sensors [20, 21], and porous membranes [3, 22-24].

CCSA coatings hold the promise of generating longer, larger surface areas than batch-fed coatings, giving convective industrial relevance. The current study identifies and interpretes the role of the key parameters that control coating appearance (microstructure and thickness) when the meniscus is continuously supplied, laying the groundwork for future studies on coating appearance optimization. Overall, CCSA may be a promising method for generating monolayer or very thin coatings of polymer particles and live cells for numerous biotechnology applications where a highly bioreactive surface is required. It could be extended to engineer multi-layer coatings of particle-bound enzymes or combining layers of different live cells separated by colloid particles to generate coating nanoporosity for composite biocatalytic surfaces, for microbial photo-reactive surfaces or microbial photo absorbers for future solar energy applications [9, 10].
Acknowledgments

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**Figure 3.1:** Schematic of coating microstructures that could be deposited using sequential convective assembly of particle monolayers to form multilayer, composite bioreactive devices. Each color represents a different type of particle or cell. Each new layer is deposited after the underlying layer has dried under controlled temperature and relative humidity conditions.
Figure 3.2: Schematic of the continuous convective assembly coating apparatus. The fluid delivery system pumps the suspension from the fluid reservoir (syringe) to the interface between the deposition plate and substrate. Underside delivery system shown.
Figure 3.3: Schematic of meniscus shape geometry used in the volume evaluations.
Figure 3.4: Coatings deposited from 1.0 µm microspheres (left) and an 80% (v/v) composite blend of OptiPrep™ and 1.0 µm microspheres (right). Scale bar is 5 mm; arrows indicate direction of deposition.
Figure 3.5: Schematic of the proposed variation in coating assembly when suspension fluid density is modified. (A) Aqueous particle suspension process. The particles disperse throughout the meniscus. (B) OptiPrep™-medium with increased fluid density, altering the convective assembly mechanism by delaying particle incorporation into the coating.
**Figure 3.6:** Microstructure of coatings deposited on aluminum foil. (A, B) 80% (v/v) composite OptiPrepTM + 1.0 µm microspheres and (C, D) pure 1.0 µm microspheres. Scale bars are 50 µm in (A, C), and 10 µm in (B, D). The ridges in (C) are an artifact that arises from the limitations of the homemade coating apparatus and the use of glass slides with non-rounded edges.
Figure 3.7: Structure of coatings deposited from a 1.0 µm microsphere suspension when the tubing of the delivery system is vibrated at 400 Hz (left) or without vibration (right). The static coating has uneven particle distribution along its length and width whereas the vibrated coating is highly uniform with no pronounced particle aggregation along the substrate edges. Scale bars for coating images and micrographs are 5 mm and 50 µm, respectively; arrows indicate direction of deposition.
Figure 3.8: Effect of suspended particle concentration on coating (A) thickness and (B) void space for regions of coatings deposited from 8%, 12%, and 16% suspensions of 1.0 µm sulfated polystyrene polymer particles. Thickness profiles determined by profilometry are grouped by sample number to simplify data interpretation. (C) Coating deposited at 16% solids showing minimized particle aggregation along coating edges. Arrow indicates direction of deposition; scale bar is 5.0 µm.
**Figure 3.9:** Variation in (A) meniscus volume and (B) mean void space over coating length during topside and underside CCSA deposition. Insets show sampling scheme for characterizing each parameter; all marked distances are in mm from the beginning of the coating.
Figure 3.10: Structure of coatings deposited from a 1.0 µm microsphere suspension at a volumetric delivery rate of (A) 0.0 (batch), (B) 1.0, (C) 2.0, (D) 4.0, and (E) 6.0 µL/min during underside CCSA deposition. Scale bars for coating images are 5 mm; arrows indicate direction of deposition. Observed ridges are an artifact that arises from the limitations of the homemade coating apparatus and the use of glass slides with non-rounded edges.
Table 3.1: Microsphere counts before and after deposition of a pure microsphere suspension of 8% solids and an OptiPrep™-enriched suspension containing ~2% solids

<table>
<thead>
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<th>Pure Microsphere Suspension</th>
<th>OptiPrep™ + Microsphere Suspension</th>
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<td></td>
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<tr>
<td>Syringe</td>
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<td>6.8x10^{9}</td>
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<td>Coating</td>
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<td>1.1x10^{9}</td>
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<tr>
<td>TOTAL</td>
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<td>1.7x10^{10}</td>
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References


### RAW DATA

**Table R.1:** Microsphere counts before and after deposition

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<td>Meniscus</td>
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<td>5.7x10^9</td>
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<tr>
<td>Tubing</td>
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<td>6.8x10^9</td>
</tr>
<tr>
<td>Coating Knife</td>
<td>0</td>
<td>6.3x10^7</td>
</tr>
<tr>
<td>Syringe</td>
<td>4.2x10^{10}</td>
<td>3.5x10^9</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>4.2x10^{10}</strong></td>
<td><strong>1.7x10^{10}</strong></td>
</tr>
</tbody>
</table>

**Table R.2:** Microsphere counts before and after deposition with OptiPrep™ enrichment

<table>
<thead>
<tr>
<th>Component</th>
<th>Pre-Deposition</th>
<th>Post-Deposition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating</td>
<td>0</td>
<td>1.2x10^9</td>
</tr>
<tr>
<td>Delivery System</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fitting</td>
<td>0</td>
<td>4.1x10^8</td>
</tr>
<tr>
<td>Meniscus</td>
<td>0</td>
<td>1.1x10^8</td>
</tr>
<tr>
<td>Tubing</td>
<td>0</td>
<td>3.1x10^7</td>
</tr>
<tr>
<td>Coating Knife</td>
<td>0</td>
<td>6.4x10^7</td>
</tr>
<tr>
<td>Syringe</td>
<td>5.5x10^{10}</td>
<td>4.6x10^9</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>5.5x10^{10}</strong></td>
<td><strong>6.4x10^9</strong></td>
</tr>
</tbody>
</table>

**Table R.3:** Solvent balance over delivery system

<table>
<thead>
<tr>
<th>Component</th>
<th>Solvent Volume Before Pumping (mL)</th>
<th>Solvent Volume After Pumping (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>Dish</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Evaporation</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Needle</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Syringe</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>1.00</strong></td>
<td><strong>1.00</strong></td>
</tr>
</tbody>
</table>
Table R.4: Solvent balance over delivery system with sonication

<table>
<thead>
<tr>
<th></th>
<th>Solvent Volume Before Pumping (mL)</th>
<th>Solvent Volume After Pumping (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trial 1</td>
<td>Trial 2</td>
</tr>
<tr>
<td>Dish</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Evaporation</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Needle</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Syringe</td>
<td>1.00</td>
<td>0.77</td>
</tr>
<tr>
<td>TOTAL</td>
<td>1.00</td>
<td>0.77</td>
</tr>
</tbody>
</table>

Table R.5: Variation in coating void space during topside and underside CCSA deposition

<table>
<thead>
<tr>
<th>Distance from Initial Contact Line (mm)</th>
<th>Mean Void Space (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Underside CCSA</td>
</tr>
<tr>
<td></td>
<td>15 mm</td>
</tr>
<tr>
<td>5.0</td>
<td>11.0 ± 11.6</td>
</tr>
<tr>
<td>12.5</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>20.0</td>
<td>1.2 ± 1.7</td>
</tr>
</tbody>
</table>
Table R.6: Change in meniscus volume over coating length at variable delivery rate

<table>
<thead>
<tr>
<th>Delivery Rate (µL/min)</th>
<th>Distance from Initial Pinning Line (mm)</th>
<th>Change in Meniscus Volume (x mm/0 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>10</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>1.0</td>
<td>10</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>2.0</td>
<td>10</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>4.0</td>
<td>10</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>6.0</td>
<td>10</td>
<td>2.94</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2.49</td>
</tr>
</tbody>
</table>
SUPPLEMENTAL INFORMATION

S.1 Correction of Solvent Balances for Water Evaporation during Pumping

The solvent material balances were corrected for water evaporation during the pumping period by first calculating the dew point temperature of the air above the solvent pool as a function of relative humidity and dry bulb temperature using the Magnus-Tetens algorithm:

\[
T_d = \frac{b \alpha(T, RH)}{a - \alpha(T, RH)}
\]

where \(a\) and \(b\) are 17.27 and 237.7°C, respectively, \(T\) is the measured dry bulb temperature from 0-60°C, and \(RH\) is the measured relative humidity from 0.01-1.00.

The solvent’s vapor pressures at the dew point and saturation temperatures are calculated using Antoine’s equation:

\[
\ln(P_{sat}) = A - \frac{B}{T + C}
\]

where \(A\), \(B\), and \(C\) are predefined, component-specific parameters. Because the dissolved microsphere concentration is less than 10% solids, the suspension is considered sufficiently dilute to use the predefined parameters for pure water.

The evaporation rate at the pool surface is calculated as a function of vapor pressure, latent heat of vaporization, and local air velocity:
where $P_w$ is the vapor pressure at the saturation temperature, $P_a$ is vapor pressure at the dew point temperature, $V$ is the air velocity at the water surface, and $H_v$ is latent heat of vaporization at the saturation temperature. Air velocity is assumed negligible because 1) the analytical balance’s doors remained closed and 2) the relative difference in the pool and ambient temperatures is too small to generate convective motion.

Once the evaporation rate at the pool’s surface is known, the volume of evaporated solvent is calculated using a macroscopic material balance:

$$W = \frac{3600}{H_v} (P_w - P_a)(0.089 + 0.0782V)$$  \hspace{1cm} (4)

$$N_e = -\frac{p}{S} \left( \frac{dV}{dt} \right)$$  \hspace{1cm} (5)

where $p$ is the density of the coating suspension, $S$ is the available surface area for evaporation, $V$ is the droplet volume, and $t$ is the available time for evaporation. We assume the available surface area for evaporation, $S$, is 5% of the total dish area because the collected pool of water is thin and only covers a small portion of the weigh dish. If $S$ is assumed to be 10% of the total dish area or larger, the volume of evaporated solvent actually exceeds the total volume of water supplied to the delivery system during the pumping period.
S.2 Water Evaporation Rates

Evaporation rates were calculated for five relative humidities between 35% and 70% to determine if variations in relative humidity significantly affect the evaporative flux. The evaporative rate is calculated as a function of droplet volume (and relative humidity) using a macroscopic material balance:

$$ N_e = -\frac{p}{S} \left( \frac{dV}{dt} \right) $$

(1)

where $p$ is the density of the coating suspension, $S$ is the available surface area for evaporation, $V$ is the droplet volume, and $t$ is the available time for evaporation. The average rate for each relative humidity is reported in Table S.1. All rates were analyzed as paired data sets, grouped by relative humidity, at the 95% confidence level using the Independent Samples t-Tests algorithm in the SPSS 17 statistics software package (IBM, Armonk, NY). The results of these t-Tests are reported in Table S.2.
**Table S.1:** Statistical significance of variation in solvent evaporation rate (Nₑ) at 95% confidence level

<table>
<thead>
<tr>
<th>Relative Humidity</th>
<th>Sample Size (n₁, n₂)</th>
<th>Statistical Significance (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35%, 40%</td>
<td>(9, 6)</td>
<td>0.363</td>
</tr>
<tr>
<td>35%, 50%</td>
<td>(9, 6)</td>
<td>0.155</td>
</tr>
<tr>
<td>35%, 60%</td>
<td>(9, 6)</td>
<td>0.041</td>
</tr>
<tr>
<td>35%, 70%</td>
<td>(9, 6)</td>
<td>0.001</td>
</tr>
<tr>
<td>40%, 50%</td>
<td>(6, 6)</td>
<td>0.363</td>
</tr>
<tr>
<td>40%, 60%</td>
<td>(6, 6)</td>
<td>0.363</td>
</tr>
<tr>
<td>40%, 70%</td>
<td>(6, 6)</td>
<td>0.363</td>
</tr>
<tr>
<td>50%, 60%</td>
<td>(6, 6)</td>
<td>0.000</td>
</tr>
<tr>
<td>60%, 70%</td>
<td>(6, 6)</td>
<td>0.193</td>
</tr>
</tbody>
</table>

**Table S.2:** Average evaporation rates

<table>
<thead>
<tr>
<th>Relative Humidity</th>
<th>Evaporation Rate (g/mm²/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35%</td>
<td>1.13x10⁻⁵ ± 2.15x10⁻⁶</td>
</tr>
<tr>
<td>40%</td>
<td>9.03x10⁻⁶ ± 1.49x10⁻⁶</td>
</tr>
<tr>
<td>50%</td>
<td>9.77x10⁻⁶ ± 5.33x10⁻⁷</td>
</tr>
<tr>
<td>60%</td>
<td>9.28x10⁻⁶ ± 4.42x10⁻⁶</td>
</tr>
<tr>
<td>70%</td>
<td>5.41x10⁻⁶ ± 1.13x10⁻⁶</td>
</tr>
</tbody>
</table>
CHAPTER 4

Advanced Photoreactive Gas-Phase Biocatalysts:
Use of Continuous Convective Sedimentation Assembly to
Produce Ultrathin, Highly Ordered Coatings of Photoreactive
Microorganisms and Latex Polymer Microspheres on Paper

Jessica S. Jenkins, Dioncio Rios, Charles B. Mooney,
Orlin D. Velev, and Michael C. Flickinger

A version of this chapter will be submitted to

Green Chemistry (2013).
Abstract

This study is the first report of investigation of the microstructure of convectively-assembled live microbe + latex polymer ultra-thin coatings on a porous nonwoven substrate suitable for use as photoreactive gas-phase biocatalysts or microbial greenhouse gas photoabsorbers for recycling of gas-phase carbon into fuels. These coatings are formed by meniscus waterborne particle delivery to the paper surface followed by rapid particle ordering at the drying front which produces a highly ordered, mechanically stable, adhesive biocoating without loss of microbial reactivity during drying and is photoreactive when rehydrated. Thin biocomposite coatings have a significantly higher surface-to-volume ratio than much thicker alginate hydrogel films, resulting in higher cell density with improved mass transfer and light distribution to the coating-entrapped cells. Model hydrogen gas generating coatings are deposited from a polystyrene and acrylate colloid blend containing: 1) photoreactive *Chlamydomonas reinhardtii* (6.8 ± 1.0 x 7.3 ± 0.9 μm ellipsoid) or *Rhodopseudomonas palustris* (1 x 8 μm rod), 2) low glass transition temperature (Tg) adhesive waterborne latex nanospheres (150 to 300 nm diameter) and 3) 1.0, 5.0, or 8.7 μm latex polystyrene microspheres. The coated cells surrounded by polymer particles are photocatalytically active when rehydrated, but do not grow out of the coating. Since the focus of this work is on the method of coating and coating microstructure of ultra-thin layers, no attempt was made to optimize hydrogen gas evolution in response to illumination. Variations in microsphere size and suspension composition do not affect coating photoreactivity, but do alter coating microstructure. The 1.0 μm microspheres collect around the edges of the cells, while larger
 (>1.0 µm) microspheres form particle clusters over the cells. Highly ordered, thin biocoatings of multiple species of photosynthetic microorganisms engineered to absorb a greater proportion of the near infrared plus visible wavelengths may enable an entirely new method to generate large quantities of hydrogen from light and non-carbohydrate organic carbon.
4.1 Introduction

Living microorganisms can efficiently carry out photosynthesis, detoxify environmental pollutants, transport electrons to electrode surfaces, and produce a diverse range of valuable products, including useful gases like methane, oxygen and hydrogen [1]. Photosynthetic energy generation does not require microbial cell division (growth) and therefore non-growing photosynthetic microbes hold the potential to be the most highly efficient green biocatalysts eliminating the generation of cell biomass as a side product. Engineering microorganisms to capture light and utilize solar energy to produce chemical feedstocks and fuels is a rapidly expanding field of biocatalyst and advanced materials research [2]. The reactivity of these adhesive coatings will be optimized by cellular engineering methods for light absorption, efficiency, stability, and biosynthetic properties. The most important aspect is that engineered coating microstructure preserve cellular biocatalytic activity during drying to enable storage of dry coatings that retain activity when hydrated.

Using coating technology to stabilize and concentrate non-growing light absorbing microbes in thin, nanoporous adhesive latex coatings that remain adhesive when continuously irrigated with a thin water layer can provide an inexpensive method to stabilize cellular photosynthetic reactivity, leading to a new generation of photo biocatalysts with enhanced light absorption and intensity [1]. However, the ability to fabricate biocomposite or live photosynthetic cell + latex polymer coatings without loss of mechanical stability or impairment of microbial viability and reactivity due to the drying process or desiccation by being used in a gas-phase (not submerged in liquid) is a challenging problem to the coating
industry, reactive biofilter [1] and the composite biomaterials field. Solving this problem will significantly expand the use of microbes as photo absorbers for gas-phase recycling of greenhouse gases toxic emission products and allow engineering of photobiological fuel cells for solar energy.

We have recently developed continuous convective-sedimentation assembly methods that use fluid evaporation, particle transport via fluid flow, and associated meniscus motion to rapidly, controllably, and continuously deposit ordered arrays of polymer particles and photosynthetic microorganisms in thin films on practically relevant coating surface area scales [3]. The assembly process begins when the film height of the evaporating fluid becomes thinner than the diameter of the suspended particles (live cells, latex nanospheres, and/or latex polymer microspheres). The menisci formed around these particles give rise to attractive capillary forces that pull adjacent particles together as the liquid evaporates, forming two-dimensional nuclei [4-6]. A liquid flux from the suspension bulk to the substrate-air-liquid contact line at the drying front offsets evaporative losses, resulting in aggregate transport to the drying front and coating proliferation [4, 5, 7].

Techniques for ordering and assembling colloidal particles and reactive microorganisms (which behave as charged particles in aqueous dispersions [8]) into closely packed arrays by solvent evaporation have been thoroughly investigated, including colloidal coating formation in thin wetting films [9-12], and recently in waterborne systems containing reactive bacteria, yeast, cyanobacteria or algae for future environmental or solar energy applications [10, 11, 13]. These methods have resulted in a reproducible technique for generating colloidal arrays
with varying thicknesses, particle size spatial distribution, and coating microstructures from charged latex microspheres [9, 12, 14], anisotropic particles [15-17] or live cells [8, 11] to composite charged microsphere plus cell mixtures [8, 11, 18]. These convectively assembled arrays are well suited for applications that do not require defect-free, perfect microstructures, including a diverse range of environmental and energy applications like microbial photoreactive coatings [10, 13], electrical circuits [18], chemical sensors [19, 20], and porous reactive membranes [21-24].

Here we report the use of a model system of photoreactive purple nonsulfur bacterium *Rhodopseudomonas palustris* (1 x 8 µm rod) and a green microalgae *Chlamydomonas reinhardtii* (6.8 ± 1.0 x 7.3 ± 0.9 µm ellipsoid) as model photosynthetic particles capable of generating hydrogen gas without growth. We demonstrate how continuous convective-sedimentation assembly can be used to fabricate photoreactive live cell + latex polymer coatings that retain mechanical adhesion and microbial reactivity after drying and rehydration when placed into a water-saturated gas-phase. We also show how variations in photosynthetic cell and latex microsphere size affect coating microstructure and porosity. Finally, we show how using this technique we can generate robust coatings containing multiple cell species of different sizes that absorb incident light in complementary wavelengths. This coating technique holds the promise of generating efficient biophotoabsorbers of highly concentrated non-growing microbes capable of producing large quantities of hydrogen gas from light and non-carbohydrate organics or for recycling gaseous organics into fuels.
4.2 Materials and Methods

4.2.1 Preparation of Coating Substrates and Deposition Plates

Dow Corning 75 x 50 mm glass microscope slides (Fisher Scientific) were pretreated as previously described(11) using NoChromix® solution to remove adsorbed organic molecules and deprotonate surface hydroxyl groups (Godax Laboratories, Cabin John, MD). All pretreated slides were stored in separate 100 x 100 mm Fisherbrand Petri dishes (Fisher Scientific) until used in the coating deposition device. In contrast to all previous colloid assembly studies, in this study, all coatings were deposited onto porous Kraft paper (Office Depot, Inc., Boca Raton, Fl) without pretreatment. Porous nonwoven supports enable coatings to be used in the gas-phase without dehydration [13].

4.2.2 Preparation of Coating Suspensions

Microbial cultures were grown in static culture at ambient laboratory temperatures at 20-25°C. Rhodopseudomonas palustris CGA009 (purple nonsulfur bacteria) was obtained from Professor Caroline Harwood (University of Washington, Seattle, WA) and grown anaerobically in 160 mL glass serum bottles (Wheaton, Millville, NJ) containing 100 mL of nitrogen-fixing photosynthetic media [PM(NF)], as described previously [10, 25]. Wild-type Chlamydomonas reinhardtii CC-124 (green algae) was obtained from the Chlamydomonas Resource Center (University of Minnesota, St. Paul, MN) and grown in BD Falcon™ six-well tissue culture plates (Fisher Scientific) agitated at 200 RPM to accelerate the culture’s bulk growth rate. All wells were continuously illuminated using fluorescent daylight bulbs.
with 66 µmol photons/m²/s photosynthetically active radiation (PAR). Incident light intensity was measured as a 15 second average using an LI-250 Light Meter coupled to an LI-190SA Quantum Sensor (LI-COR Environmental Division, Lincoln, NE). Each well contained 5-10 mL of TAP+S media [26, 27].

Three sizes (1.0, 5.0, and 8.7 µm) of monodispersed polystyrene or acrylate polymer microspheres were washed once with deionized water obtained from a RiOs 16 reverse-osmosis water purification system (Millipore Corporation, Bedford, MA) to remove residual surfactants and electrolytes. Both the 1.0 and 8.7 µm microspheres are white sulfate polystyrene particles obtained from Invitrogen (Grand Island, NY); the 5.0 µm microsphere is an acrylic/divinylbenzene copolymer (Rohm and Haas, Philadelphia, PA).

Biocomposite or live cell + latex polymer coating suspensions were prepared from wet cell pastes, multiple latex polymers, and sterile osmoprotectants (sucrose and glycerol). These porogens arrest polymer particle coalescence and improve the cells’ desiccation resistance, creating coating porosity during film formation without killing entrapped microorganisms [1]. All cell pellets were prepared by centrifuging 45-50 mL of cell suspension at high speed for 10-15 min at 4°C and pouring off the supernatant. *Rps. palustris* and *C. reinhardtii* cell pellets were suspended in 1 mL of PM(NF) or TAP-P-S media, respectively, to facilitate transfer by pipette when creating the coating suspensions and mixed by vortexing to create homogeneous cell pastes. All formulations were prepared in 15 mL BD Falcon™ conical centrifuge tubes (Fisher Scientific) to facilitate transfer to the coating deposition device and mixed by vortexing until homogeneous.
All coating formulations for both *Rps. palustris* and *C. reinhardtii* are described in Table 4.1. To simplify the nomenclature, all live cell + latex polymer particle coating suspensions are described only by their cell and microsphere components – for example, 8.7 µm + *C. reinhardtii* defines a biocomposite suspension containing *C. reinhardtii* cells, 8.7 µm latex polymer microspheres, Rhoplex™ SFO12, and sucrose. Cell coatings contain only cell paste and osmoprotectant carbohydrates; cell + Rhoplex™ SFO12 coatings contain cell paste, Rhoplex™ SFO12, and osmoprotectants. All washed microsphere suspensions were concentrated to 8% (w/v) solids prior to blending with the other suspension components. Rhoplex™ SFO12 (Rohm and Haas, Philadelphia, PA) is a film-forming, acrylic copolymer emulsion that improves coating stability by strengthening microsphere and cell adhesion to the substrate.

The zeta potential of the algal cells was quantified using a Malvern Zetasizer (Malvern Instruments, Westborough, MA) equipped with a universal Dip Cell and an analytical software package. Cells were separated from the TAP+S media using centrifugation at maximum speed for 10 minutes and suspended in 1000 µL of TAP-P-S media (TAP+S without sulfur or phosphorous compounds) to create a solution similar to the *C. reinhardtii* cell paste. The suspension was analyzed in triplicate using an effective voltage of 2.28 ± 0.01 V and a suspension conductivity of 2.25 ± 0.01 mS/cm.
4.2.3 Deposition of Biocomposite Coatings by Continuous Convective-Sedimentation Assembly (CCSA)

All coatings were deposited using the underside continuous convective-sedimentation assembly deposition method described previously (3). The syringe pump was operated at a high priming rate (>100.5 µL/min) to rapidly fill the wedge shaped meniscus between the Kraft paper (Vendor, City, State) and coating knife and then the flow rate was reduced to a rate of 0.5 µL/min after the meniscus covered the tubing outlet. All suspensions were deposited onto dry Kraft paper in 25-30 minutes using a knife speed of 21 µm/s. Because the paper is porous, the coating suspension permeated the paper beyond the meniscus’s projected area, leading to depletion of the entrained fluid and a fluctuating volume. To offset this fluid loss, the flow rate was increased (>10.0 µL/min) until the meniscus contacted the tubing outlet and then decreased to 0.5 µL/min. This cycle was repeated, as necessary, until coating fabrication was completed. All coatings were deposited inside a benchtop humidity and temperature controlled environmental chamber (Model 510 Electro-Tech Systems, Inc., Glenside, PA) at a chamber temperature of 20-30°C and relative humidity of approximately 50%. The relative humidity was maintained with an ultrasonic humidification system (Model 5462 Electro-Tech Systems, Inc.) filled with deionized water. Both the chamber temperature and relative humidity were monitored with a calibrated probe (TM125 Dickson Addison, IL).
4.2.4 Evaluation of Coating Reactivity

Coatings were cut into three strips and evaluated for reactivity using hydrogen gas evolution. All strips were transferred to separate sterile, vertical Balch tube containing 10 mL of either TAP-P-S media (TAP+S without phosphorous and sulfur) for *C. reinhardtii* coatings or PM(NF) media for *Rps. palustris* coatings. Each coating was placed in the headspace and hydrated by capillary action from the liquid phase (Figure 4.1). This orientation limits nutrient transport to the immobilized microorganisms, slowing their growth rate and preventing outgrowth [13]. All tubes were sealed with sterile butyl rubber stoppers (BellCo Glass, Inc., Vineland, NJ) coupled with aluminum crimp caps (Fisher Scientific) and subsequently flushed with ultra high purity (UHP) argon gas for 30 min to create an anaerobic headspace (and nongrowth conditions) inside each tube. All coatings remained adhesive during paper hydration and headspace flushing.

Coatings were continuously illuminated at 20-25°C using fluorescent daylight bulbs with 116.7 ± 7.9 µmol photons/m²/s PAR. The light intensity was evaluated by measuring the incident light in three locations over the full length of three, nonadjacent Balch tubes. The headspace of each Balch tube was analyzed for O₂, N₂, and H₂ using 1 mL samples and a Hewlett Packard 7890A gas chromatograph equipped with a Supelco 6’ x 1/8’’ ID 60/80 mol sieve 5A porous mesh polymer-packed stainless steel column and a thermal conductivity detector. Argon was used as the carrier gas at an inlet flow rate of 39 mL/min; the chromatograph’s injector, oven, and detector temperature settings were 160, 160, and 250
°C, respectively. All hydrogen gas production data was normalized by coating area or by chlorophyll a concentration and averaged to obtain the hydrogen output for each coating.

4.2.5 **Coating Structure Characterization**

Although all coating strips were tested for gas evolution, random strips of each live cell + latex polymer microsphere suspension (cells only, cells + SFO12, and three cells + latex polymer microspheres blends) were selected for structural analysis and measured for brightness after confirmation of reactivity. All reactive strips were analyzed for thickness and chlorophyll a concentration (indicative of cell density and light absorption capacity). Dried coatings were imaged by scanning electron microscopy for structural analysis at the Analytical Imaging Facility at North Carolina State University using a Hitachi 3200-N Variable Pressure Scanning Electron Microscope equipped with a 4Pi Isis EDS system for digital image acquisition and elemental analysis. All coatings were observed in two or more randomized locations using a 5kV accelerating voltage. Each location was imaged multiple times using sequential magnifications ranging from 100x to 10,000x to characterize both surface structure and particle-particle interactions between adjacent latex polymer microspheres and cells. To increase sample conductivity (and micrograph quality), all samples were sputter coated with a thin layer of gold in a mild vacuum (~100 mTorr of argon gas pressure; 600 V accelerating voltage) prior to imaging.

Coating brightness was measured at 475 nm using a Brightimeter Micro S-5 TAPPI paper brightness apparatus (Technidyne Corp., New Albany, IN). Dried coatings were analyzed as
single layers. Each coating was sampled in three or more randomized locations. Readings were averaged to obtain a characteristic brightness for each type of live cell + latex polymer microsphere suspension.

Coating thickness was quantified using a digital micrometer (Model ID-C112GEB, Mitutoyo USA Corporation) equipped with a 5-mm diameter convex tip. Each *C. reinhardtii* + latex polymer microsphere coating (cells only, cells + SFO12, and cells + latex polymer microspheres, and three cell + latex polymer microsphere blends) was analyzed. Each coating was sampled in one randomized location. All readings were corrected for the thickness of the uncoated Kraft paper and averaged to obtain a characteristic thickness.

The *Chlamydomonas* density within each coating was approximated by chlorophyll *a* content. Chlorophyll *a* was extracted from the coating-entrapped cells using ethanol extraction. A 6-mm disc was punched from each *C. reinhardtii*, *C. reinhardtii* + SFO12, and *C. reinhardtii* + latex polymer microsphere coating using a 6-mm diameter single-hole paper punch and submersed in 1.5 mL of neat EtOH. All disc-EtOH suspensions were boiled for 1 min at 80-85°C and centrifuged at 15000 RPM for 10 min. All supernatants were sampled for absorbance at 665 nm using a Genesys 20 UV-Vis spectrophotometer (Thermo Electron Corporation, Marietta, OH). The amount of chlorophyll *a* (µg/mL) extracted by each disc was calculated as:

\[
(Chl \ a)_{\text{disc}} = \frac{29.62 (Abs_{665}) (V_{\text{EtOH}})}{(V_{\text{disc}})(L_{\text{cuvette length}})}
\]  

(1)
where $Abs_{665}$ is the supernatant absorbance at 665 nm, $V_{EtOH}$ is the volume of ethanol extract (mL), $V_{disc}$ is the disc volume (L), and $L_{cuvette\ length}$ is the length of the cuvette parallel to the light path. The total amount of chlorophyll a in each coating was calculated as:

$$ (Chl\ a)_{coating} = (Chl\ a)_{disc} \cdot V_{coating} $$

(2)

### 4.2.6 Paper Substrate Structure Characterization

The Kraft paper was characterized for thickness and porosity using digital micrometry and water uptake, respectively. Substrate thickness was quantified using a digital micrometer equipped with a 5mm tip. Three pieces of Kraft paper were sampled in three randomized locations. All readings were averaged together to obtain a characteristic thickness.

The water uptake wet weight was used to characterize paper porosity. Water uptake was quantified by weighing three strips of Kraft paper before and after water saturation. Each strip was soaked in deionized water until a liquid sheen appeared on both sides of the substrate, ensuring complete saturation and an accurate water uptake rate. Each weight was normalized by the area of the corresponding strip to obtain a water uptake rate for that strip. All weights were averaged together to obtain a characteristic water uptake for the Kraft paper.
4.3 Results and Discussion

4.3.1 Role of Coating Substrate Properties and Coating Suspension Formulation in Convective-Sedimentation Assembly of Model Photosynthetic Particles

This study is the first report of convectively-assembled live cell + latex microsphere coatings on a non-woven porous substrate where the cells retain photoreactivity following rapid drying and rehydration. This study is also the first example of convectively-assembled arrays containing non-film forming particles (live cells and latex polymer microspheres) and film-forming particles (a commercially available latex binder emulsion) on paper. The use of a non-woven pervious substrate and multiple waterborne charged particles and sizes may slow coating proliferation compared to our previous studies coating onto non-porous substrates (glass, polyester, metal) by disrupting the assembly process or weakening the mechanical stability of the assembled coating. Porous substrates absorb water from the coating meniscus, likely weakening the liquid flux that carries particles from the suspension bulk to the drying front by reducing evaporative losses [4, 5, 7]. Water uptake may also reduce the mechanical stability of the paper, leading to a coated nonwoven support that deteriorates during coating fabrication. The size ratios and disparate zeta potentials of the suspension particles may affect particle movement and particle-particle interactions during coating fabrication. For example, particle size ratio has been reported to control convective mixing or demixing [8] while zeta potential controls the net force (the sum of the attractive capillary forces and the negative repulsive forces) between neighboring cells and/or latex polymer microspheres.
Kraft paper is not fully saturated with water during coating fabrication because the meniscus volume is kept small by the suspension feed rate and therefore this coating method produces very thin coatings, one particle or one cell thick. Although the meniscus continuously wets the paper outside the projected wetting area underneath the coating knife, the substrate’s underside remains dry (data not shown). This partial wetting, combined with the substrate’s low water uptake (2.27x10^{-4} ± 3.72x10^{-5} g/cm²) when completely saturated, suggests that Kraft paper is a suitable, flexible non-woven substrate for depositing ordered arrays of photosynthetic cells using waterborne latex particle for adhesion only on the surface allowing hydration of the illuminated cells from below the coating by the liquid in the paper pores. This structure allows the cells to remain hydrated by capillary pressure from a small volume liquid phase wicked into the paper pores when the coating is placed in the gas-phase above the liquid phase. The paper support allows reactive coatings that remain hydrated without being submerged below a thick liquid layer for improved gas-liquid-cell mass transfer. SEM images of the rehydrated coated paper indicate that the coating formulation adheres to the cellulose fibers without plugging the paper pores, allowing for water and nutrient transport from the bulk suspension to the immobilized cells through the paper pores. The open pore structure may also facilitate carbon dioxide adsorption and the escape of gas bubbles (such as H₂) from the coating [13].

The bottom of the Kraft paper coated with a C. reinhardtii, 1.0 µm latex polymer microsphere, Rhoplex™ SFO12, and sucrose was imaged using scanning electron microscopy to determine if solute particles or cells are pulled into the substrate pores during
coating fabrication (Figure 4.2). No cells, latex polymer microspheres, or latex emulsion are visible on the bottom paper surface. All visible particles lack the correct size and shape to be *C. reinhardtii* cells), latex microspheres, or Rhoplex™ SFO12 latex (100 to 350 nm). Thus, although Kraft paper is wetted by the suspension, the non-woven substrate traps the cells preferentially on the coated surface. Due to the particle-particle or particle-cellulose fiber interactions, the 1.0 µm microspheres failed to penetrate the much larger paper pores (negating the need to evaluate any coatings containing microspheres larger than 1.0 µm and the 1 x 8 µm *Rhodopseudomonas palustris*).

The zeta potential of the *C. reinhardtii* cells and latex polymer microspheres were determined to determine the net force between the particles in the coating suspension. The zeta potential of the Rhoplex™ SFO12 latex emulsion was not evaluated because the latex is only 5% (v/v) of the coating formulation and binds both to the cells and larger microspheres at ambient temperature, dominating cell- or microsphere-specific electrostatic interactions. The microsphere surface has sulfate groups, yielding a negative zeta potential at neutral pH [8], while the cell surface has hydroxyproline-rich glycoproteins conjugated to arabinose, mannose, galactose, and glucose [28]. These sugars all have pH-dependent protonation states [28, 29], yielding a zeta potential that varies with suspension pH. However, at neutral pH, algae cells have a slightly negative zeta potential of -7.2 ± 0.16 mV. Cellulosic fibers also acquire a negative surface charge when suspended in water [30]. The cell, microsphere, and cellulose fiber surfaces are both negatively charged at neutral pH so electrostatic repulsion (and a negative net force) would be predicted to dominate. However, coating
propagation and solute adhesion to the paper surface appear to be due to convective-sedimentation assembly and not to electrostatic attractive forces between neighboring solute particles and the cellulose fiber surface. The convective-sedimentation coating assembly process remains unchanged, despite the use of multiple non-film forming and film-forming particles in the coating suspension. Also, the negative surface charges of both the latex polymer microspheres and algal cells suggests the cells behave as surface-charged colloidal particles, allowing for deposition of stable live cell + latex polymer microsphere coatings, as we have shown previously for nonporous substrates [8].

4.3.2 Role of Microsphere Particle Size on Coating Structure and Hydrogen Gas Generation

For waterborne latex biocatalytic coatings, generation of stable nanoporosity is essential for preserving microbial reactivity following coating rehydration [1]. Biocomposite coating formulations can be engineered to deposit coatings with stable nanopores by varying the physical properties of the polymer particles (1). Although various polymer particle characteristics can be altered and evaluated for their effects on coating porosity, microsphere diameter is known to have an effect on coating structure. The relative sizes of particles in microsphere + microsphere and cell + microsphere blends affect the deposition pattern of convectively-assembled coatings, leading to convective mixing or demixing of solute during fabrication [8]. Microsphere size can also be varied. Coatings were imaged at the end of hydrogen gas generation studies using scanning electron microscopy to determine microstructure and nanoporosity by varying microsphere diameter (Figure 4.3).
Coating microstructure varies with both polymer particle diameter and coating formulation. The 1.0 µm polystyrene microspheres collect around the edges of the (artificially flattened appearing) ellipse-like *C. reinhardtii* cells (Figure 4.3E) while larger (>1.0 µm) microspheres form particle clusters that sediment onto the cells (Figures 4.3C and 4.3F). Cell morphology and packing does not vary with the addition of Rhoplex™ SFO12 emulsion binder. Cells deposited with and without SFO12 (Figures 4.3B and 4.3D, respectively) appear randomly oriented in the coating layer, difficult to differentiate, and flattened with amorphous edges. The flattened cell images are due to the low pressures used in SEM sample preparation and imaging. The Rhoplex™ SFO12 nanoparticle emulsion binder coats both the cells and the microspheres, acting as an adhesive shell on all particles that creates coating adhesion (and mechanical stability) by binding adjacent particles together during film formation and drying (Figures 4.3B, 4.3C, 4.3E, and 4.3F). Also, all coatings exhibit some nanoporosity though formation of micro cracks (Figures 4.3C and 4.3E) or even macroscale gaps in the coating layer (Figures 4.3B, 4.3D, and 4.3F), despite disparate microstructures.

Unlike coating microstructure, neither non-photosynthetic microparticle size nor suspension composition affects coating reactivity, measured in this model system as hydrogen gas production. As a first approximation to measuring the coated paper optical properties, paper brightness was measured using standard methods employed by the paper coating industry. All coatings are less bright than the bare Kraft paper, confirming enhanced light absorption. The *C. reinhardtii* + Rhoplex™ SFO12 and all *C. reinhardtii* + latex
polymer microsphere coatings exhibit similar reactivity (Figure 4.4) even though they are coated with only a monolayer of photoreactive cells, likely because these coatings are similarly bright when illuminated (Table 4.2). This similarity suggests the coatings absorb equivalent amounts of light when illuminated, despite light scattering by their dissimilar non-absorbing particle sizes and the location of these particles in the coating microstructures. The convectively-assembled microsphere cell coating produces more hydrogen gas than the low Tg latex binder polymer coatings, but less hydrogen than thicker cell-laden alginate films. The enhanced reactivity, relative to the latex binder polymer coatings, results from both a higher cell number (the C. reinhardtii suspension is not diluted with Rhoplex™ SFO12 and latex polymer microspheres, yielding a larger cell fraction than the other formulations) and greater light absorption. Researchers at The National Renewable Energy Laboratory (NREL) have fabricated much thicker (330 µm on average) photoreactive Ca²⁺-alginate films (containing Chlamydomonas reinhardtii microalgae) that generated up to 0.35 mols H₂/m² for over 150 hours [31], but these films lack adhesion and the thinness (to avoid cell-cell shading) and the organization of convectively-assembled biocoatings. As such, alginate films are actually less reactive than biocoatings because the former system’s increased thickness and disorganization inhibits uniform nutrient diffusion and illumination of all the entrapped cells. The disparity in theoretical and experimental results indicates that the effect of coating microstructure on hydrogen gas reactivity of convectively-assembled biocoatings needs further investigation of both their optical and bioreactivity properties.
Algae utilize light energy less efficiency as incident light intensity increases, suggesting the low hydrogen production in the monolayer convectively-assembled cell coatings may result from photoinhibition. Decreased hydrogen-production activity under high light has been reported for both *C. reinhardtii* suspensions [32] and non-adhesive alginate coatings [33]. Laurinavichichene and coworkers reported photoinhibition at 40-60 µmol photons/m²/s PAR for 2 mL *C. reinhardtii* suspension cultures [32]. This finding suggests that, because the live cell and live cell + latex polymer coatings evaluated in this study are no more than 0.011 mL in volume, the immobilized cells are likely photosaturated at the evaluated light intensity, resulting in the observed reduced hydrogen production capability.

The amount of acetate in the media during the gas-evolution phase may also affect hydrogen evolution [31]. Kosourov and Seibert demonstrated that hydrogel strips of algae containing only 400 µg Chl as a measure of cell concentration consumed all of the acetate after two days of nutrient deprivation, preventing maximum hydrogen production [31]. Low acetate levels decrease cellular respiration, leading to intercellular oxygen accumulation and associated deactivation of the hydrogenase enzymes responsible for hydrogen evolution [31]. All *C. reinhardtii* coatings in the current study contain at least 40x more chlorophyll than the hydrogel algal strips evaluated by Kosourov and Seibert (Table 4.3), suggesting the poor reactivity of the former coatings is likely due to rapid acetate depletion at the beginning of the gas-evolution phase. Although optimization of coating reactivity was not attempted in this study, a study of *C. reinhardtii* immobilized in alginate films show that adding acetate to the media after nutrient deprivation improves hydrogen production [31].
4.3.3 Multispecies Coatings with Complementary Light Absorption Produce Hydrogen Gas

Once photosaturated or under high (up to solar) light intensity levels, an algae’s antennae chlorophylls dissipate up to 80% or more of the incident light as fluorescence and heat, leading to low light utilization efficiency [33]. This problem can be addressed by immobilizing algae in thin layers [33], which is the focus of the current study. This low efficiency can also be addressed by co-depositing cells that absorb light in complementary regions of the electromagnetic spectrum, like algae and photosynthetic bacteria. Green algae absorb light at low light intensity [33, 34] in the visible light region (400-700 nm), while photosynthetic purple bacteria absorb light maximally at approximately 875 nm using a light-harvesting antenna system that typically consist of two small polypeptides with a total weight of 10 kDa. [35]. This suggests that hybrid algae + photosynthetic bacterial coatings will absorb a wider fraction of the incident spectrum, leading to enhanced coating photoabsorber reactivity per illuminated surface area. To test this hypothesis, separate C. reinhardtii + 5.0 μm latex polymer microsphere and Rps. palustris + 5.0 μm latex polymer microsphere coating strips were affixed side-by-side in the same tube in the gas-phase and tested for hydrogen evolution using 10 mL of a 50% (v/v) TAP-P-S + PM(NF) media mixture.

The hybrid coating produces more hydrogen than a C. reinhardtii + latex polymer coating with comparable hydrogen gas evolution to a Rps. palustris + latex polymer coatings, despite a longer lag phase than the Rps. palustris coating (Figure 4.5). This suggests that multispecies coatings are a promising method for improving hydrogen evolution and the spectrum of light energy absorbed by coatings of photoreactive cells. Using this method,
reactivity can be further improved by optimizing both media composition, coating microstructure tuned to incident light intensity. Both acetate concentration and light intensity strongly affect photobiological hydrogen production [31, 33]. This is the first known report of a reactive multispecies photobiological cell coating on a porous substrate that enables the coating to remain hydrated when placed in the gas-phase.

4.3.4 Continuous Convective-Sedimentation Assembly as a Photo Absorber Intensifier

Convectively-assembled gas-phase coatings have a higher surface-to-volume ratio (the ratio between the illuminated surface area and coating or suspension volume) than comparable but much thicker alginate films (Figure 4.6), resulting in higher volumetric productivity [36]. The increased thickness of the alginate hydrogel systems theoretically reduces productivity by hindering efficient mass transfer and adequate light distribution to the cells in the lower layers of the support matrix. Although much thicker alginate films are currently more reactive than these prototype cell monolayer paper coatings [33], the gas evolution reactivity of CCSA generated monolayer coatings and multilayer coating generated using this technique can likely be significantly improved through additional optimization of antenna pigment content for light absorption in the cells deposited in each layer, the layer light intensity, light scattering, acetate concentration and use of multiple thin layers of different photosynthetic microorganisms [31, 33]. Also, alginate films, unlike our gas-phase coatings, are not adhesive, they lack structural stability and cannot be placed vertically in a gas phase. They also lose mechanical stability during dehydration, leading to shrinkage, cracking and skinning (see alginate film, Figure 4.6). CCSA deposition with rapid drying of
a very thin meniscus is capable of maximizing volumetric reactivity by concentrating cells on a surface in ordered arrays of multiple cell types and sizes. Surprisingly, this rapid thin liquid film drying also overcomes desiccation resistance which frustrated previous attempts to coating algae and retain activity following drying and rehydration. This method also minimizes the cost of a non-woven porous, flexible support to deliver hydration to the cells by using renewable cellulose fibers which can be engineered or pre-coated to tune surface properties for enhanced wet strength, gas absorption and cell adhesion [37, 38].

4.4 Conclusions

This study is the first report of convectively-assembled monolayers of live photosynthetic cells + latex polymer particles as photoreactive coatings on a porous substrate that retains the hydrogen production reactivity of algae following rapid coat drying and rehydration. These coatings are mechanically stable when rehydrated and have a higher surface-to-volume ratio than comparable alginate films, resulting in improved mass transfer and light distribution to all immobilized cells. Paper coatings will also offer higher volumetric productivity once their non-growth hydrogen production is improved through optimization of cell density, light intensity, light scattering, cell layer thickness and acetate concentration for maximum gas evolution.

This study demonstrates how ultra-thin monolayer coatings can be fabricated using CCSA from suspensions containing both non-film forming (live cells and polystyrene polymer microspheres) and film-forming (low Tg latex emulsions) adhesive binders of
variable sizes and particle charges. Variations in microsphere size and suspension composition do not affect coating reactivity, but both parameters alter coating microstructure. The SEM images reveal that the latex emulsion coats both the cells and the higher Tg microspheres, acting as an adhesive shell on all particles generating coating adhesion (and mechanical stability) during rapid drying, but does not alter cell morphology or packing. The 1.0 µm microspheres collect around the edges of the larger algae cells, while larger (>1.0 µm) microspheres form particle clusters on the top of the cells. However, all live cell and live cell + latex polymer blends adhered to the substrate cellulose fibers without plugging its pores, allowing for continuous nutrient transport from the pore space to the coated cells in the gas-phase for prolonged hydrogen evolution. These findings indicate that continuous convective-sedimentation assembly of live cell + latex polymer blends on non-woven porous substrates is a promising method for generating ultrathin biocomposite solar absorbers and photoreactive biocatalysts for environmental or energy applications where a highly bioreactive and stable photosynthetic surface is required, such as for large surface area greenhouse gas absorption using solar energy, recycling of gas-phase carbon emissions into liquid fuels using microbes or the engineering of efficient photobiological fuel cells.
Acknowledgements

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Figure 4.1: Fabrication of live photosynthetic cell + latex polymer microsphere coatings.  
1) Cells are grown at 20-25°C under ambient temperature with continuous illumination and harvested by centrifugation. (2) Cell pellet is resuspended in media by vortex mixing. (3) Latex microspheres, Rhoplex™ SFO12, and carbohydrate osmoprotectants are gently mixed with cell paste until homogeneous. (4) Coating suspension is deposited onto Kraft paper using continuous convective-sedimentation assembly (CCSA) and dried under controlled relative humidity. (5) Dry coated paper strip inserted into tube headspace and immediately rehydrated by a minimal liquid phase at the bottom of the tube.
Figure 4.2: Underside of Kraft paper coated on the top side with *C. reinhardtii*, 1.0 µm polymer microspheres, Rhoplex™ SFO12, and sucrose. Visible particles lack the size and shape of the polymer particles or algae.
Figure 4.3: Microstructure of (A) uncoated paper; (B) *C. reinhardtii* + Rhoplex™ SFO12 coating; (C) *C. reinhardtii* + 5.0 µm latex polymer microsphere coating; (D) *C. reinhardtii* coating; (E) *C. reinhardtii* + 1.0 µm latex polymer microsphere coating; (F) *C. reinhardtii* + 8.7 µm latex polymer microsphere coating.
Figure 4.4: Hydrogen gas evolution in live cell and live cell + latex polymer paper coatings.

The solid squares are cells coated by continuous convective-sedimentation assembly without polymer additives.
Figure 4.5: Hydrogen gas evolution by individual and multispecies paper coatings. Graphic shows how algae (green) and bacteria (pink) coating strips were assembled adjacent to each other above the liquid phase to form a multicellular coating hydrated by the fluid-filled paper pores.
**Figure 4.6:** Physical comparison of algal paper coatings and alginate films. Dry paper and alginate film coatings are shown as insets. Alginate film is composed of 50% (w/w) cells and 4% (w/w) alginate; film properties from ref. 31. Scale bars are 5 mm.
Paper Coating

\[ V = 23 \, \mu L \text{ per } 6 \, \text{cm}^2 \]

\[ \text{Chl } a = 1.4 \times 10^6 \, \mu g/mL \]

Alginate Film

\[ V = 200 \, \mu L \text{ per } 6 \, \text{cm}^2 \]

\[ \text{Chl } a + b = 2.0 \times 10^3 \, \mu g/mL \]
### Table 4.1: Live cell + latex polymer microsphere coating suspension formulations

<table>
<thead>
<tr>
<th></th>
<th>C. reinhardtii</th>
<th>Rps. palustris</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet cell paste</td>
<td>0.3 g</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Latex polymer microspheres (1.0, 5.0, or 8.7 µm)</td>
<td>200.0 µL</td>
<td>200.0 µL</td>
</tr>
<tr>
<td>100% glycerol (sterile)</td>
<td>0.0 µL</td>
<td>37.5 µL</td>
</tr>
<tr>
<td>0.58 g/mL sucrose (sterile)</td>
<td>87.5 µL</td>
<td>87.5 µL</td>
</tr>
<tr>
<td>Rhoplex™ SFO12</td>
<td>50.0 µL</td>
<td>50.0 µL</td>
</tr>
</tbody>
</table>

### Table 4.2: Characteristics of C. reinhardtii and C. reinhardtii + latex polymer coatings

<table>
<thead>
<tr>
<th>Coating Formulation</th>
<th>Brightness (%)</th>
<th>Thickness (mm)</th>
<th>Volume (cm³)</th>
<th>Chl a (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare paper</td>
<td>19.7 ± 0.4</td>
<td>0.14 ± 0.03</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. reinhardtii</td>
<td>5.5 ± 0.3</td>
<td>0.03 ± 0.04</td>
<td>0.013 ± 0.001</td>
<td>16.0 ± 0.20</td>
</tr>
<tr>
<td>C. reinhardtii + SFO12</td>
<td>9.2 ± 0.7</td>
<td>0.02 ± 0.01</td>
<td>0.013 ± 0.002</td>
<td>21.6 ± 0.25</td>
</tr>
<tr>
<td>C. reinhardtii + 1.0 µm + SFO12</td>
<td>12.2 ± 2.7</td>
<td>0.05 ± 0.02</td>
<td>0.021 ± 0.001</td>
<td>17.8 ± 0.24</td>
</tr>
<tr>
<td>C. reinhardtii + 5.0 µm + SF012</td>
<td>9.8 ± 0.6</td>
<td>0.05 ± 0.04</td>
<td>0.022 ± 0.005</td>
<td>39.5 ± 0.34</td>
</tr>
<tr>
<td>C. reinhardtii + 8.7 µm + SFO12</td>
<td>15.3 ± 1.6</td>
<td>0.03 ± 0.03</td>
<td>0.018 ± 0.003</td>
<td>23.9 ± 0.42</td>
</tr>
</tbody>
</table>
References


[29] L. Bhattacharyya, J.S. Rohrer (Eds.), Applications of Ion Chromatography for


CHAPTER 5

Coating Deposition and Characterization Techniques

This chapter summarizes all original coating deposition and characterization methods developed in this dissertation. The following pages are written as a basic primer for any researchers who continue the projects investigated within this dissertation.
5.1 Introduction

The development of methods to fabricate thin ordered arrays or coatings of adhesive, latex polymer particles and live cells, such as bacteria, cyanobacteria, yeast, and algae, will enable the creation of a new generation of highly reactive biomaterials that use microbes as biosensors, photoabsorbers, or biocatalysts. This dissertation advances the biomaterials field by developing multiple universal protocols for fabricating and characterizing monolayer particle plus microbial coatings from a wide variety of formulations and substrates. These formulations include monodispersed suspensions of latex particles (or microspheres) or cells, bimodal blends of latex particles or live cells and microspheres, and trimodal formulations of latex microspheres, latex emulsion, and live cells. Substrates include nonporous aluminum foil, glass, polyester, polypropylene and porous Kraft paper. This chapter lists all coating deposition and characterization methods developed in this dissertation, gives a brief description of each technique, and highlights the important aspects of each method. Comprehensive protocols for additional techniques are described in other chapters of this dissertation.

This chapter excludes detailed descriptions of common analytical techniques and inherited methods reported by previous group members. However, any modifications to these methods and techniques that are paramount to the efficacy of the methods are described in this chapter. Methods that were not modified and thus omitted from this chapter include:
• Coating fabrication from single component suspensions using simple (non-continuous) convective assembly [1, 2]

• Headspace composition calculations using gas chromatographs [3]


5.2 Coating Deposition Methods

5.2.1 Batch Convective-Sedimentation Assembly of Bimodal Latex Particle Suspensions

Bimodal blends of two different-sized latex polymer microspheres can be rapidly deposited into ordered arrays on glass, polyester, aluminum foil, and polypropylene substrates using convective-evaporative assembly at high volume fraction. Composite blends of live cells and latex polymer particles can also be deposited on a wide variety of substrates, as reported elsewhere [1, 5]. Up to 30 µL of particle suspension containing particle at 0.9-35% w/v is first trapped between a horizontal substrate plate and an inclined coating knife plate and then the inclined top plate is moved at a constant rate along the long axis of the bottom plate by a linear motor. This delivers and spreads the suspension from the meniscus into a thin film across the horizontal substrate, leading to the formation of a coating on the substrate by evaporative-convective assembly [1]. The speed of the coating knife can be adjusted from 0 to 211 µm/s, resulting in precise control of particle packing and coating thickness [1, 2]. Because sedimentation effects become important when large (>1.0 µm) particles are deposited by evaporative-convective assembly [2, 6-8], the mechanism is
denoted as *convective-sedimentation assembly* (CSA) to account for sedimentation during coating deposition [1].

Jenkins and coworkers give a protocol for depositing coatings from bimodal latex particle suspensions and for preparing microsphere suspensions, coating substrates, and deposition plates for coating fabrication [5]. The reader is referred to this protocol for detailed descriptions of the deposition and preparation steps, but the important components of each procedure are discussed here. All coating was carried out in a temperature and humidity controlled chamber. All bimodal latex polymer suspensions are prepared as 1:1 particle blends by combining identical aliquots (same suspension volume and percent solids) of two washed, single component solutions. Also, each bimodal solution is sonicated in an ultrasonic cleaner to reduce particle aggregation before deposition. All glass is cleaned with NoChromix and deionized water as described previously [1]; all other substrates are cleaned with a 70% (v/v) ethyl alcohol rinse. All substrates and deposition plates are stored in separate Petri dishes to protect the hydrophilized surface groups from air currents and humidity fluctuations until coating. Successful coating fabrication requires that the edge of the deposition plate lies flat against the bottom slide to ensure meniscus formation along the entire length of the knife and the use of low withdrawal rates. Rates approaching 25 µm/s or more yield incomplete or submonolayer coatings (coatings with large voids) for suspension volume fractions of 10% or less deposited using batch convective-evaporative assembly [2].
5.2.2 Continuous Convective-Sedimentation Assembly of Single Latex Particle Suspensions on Nonporous Substrates

Continuous convective-sedimentation assembly (CCSA) is a deposition method that constantly supplies the coating suspension to the meniscus by inline injection (Figure 5.1), allowing for steady-state deposition of ordered colloids formed by water evaporation [Chapter 3]. The constant suspension inflow increases the total suspended particles available for transport to the drying front, yielding very thin (1 to several particles thick) coatings with significantly larger surface areas than previously described and thus expanding the utility of convective assembly on a large scale [Chapter 3].

Coatings can be fabricated using three separate deposition strategies, namely topside CCSA, underside CCSA, and “hybrid” CSA-CCSA. In topside CCSA, suspension flows through the delivery system from the syringe to the front of the meniscus along the coating knife’s topside (Figure 5.2A); in underside CCSA, suspension flows from a fluid reservoir through the delivery system to the back of the meniscus along the knife’s underside (Figure 5.2B). In the combined CSA-CCSA method, the meniscus is initially created by manually injecting an aliquot of coating suspension between the substrate and coating knife (conventional CSA) and continuously replenished with suspension at a constant flow rate (CCSA). This hybrid method is used to investigate volume-dependent process parameters like deposition strategy and suspension delivery rate. Because all coatings are fabricated from the same initial meniscus volume, any changes in meniscus volume and coating structure can be attributed to deviations in the process parameter.
Jenkins and coworkers give a protocol for depositing coatings on nonporous substrates from single latex polymer particle suspensions using continuous-convective sedimentation assembly (CCSA) [Chapter 3]. The reader is referred to this protocol for detailed descriptions of the deposition and preparation steps, but the important components of the CCSA process are discussed here and annotated in Figure 5.1. All procedures for preparing microsphere suspensions, coating substrates, and deposition plates for coating fabrication are described by Jenkins and coworkers [5]. All coatings are deposited using the convective assembly method described previously [2] with an added continuous delivery system that feeds the coating suspension to the meniscus.

The position of the tubing outlet varies with the deposition mode. In topside CCSA, the tubing outlet is placed no more than 1 mm ahead of the knife’s edge to ensure uniform meniscus formation along the entire length of the knife. In underside CCSA, the tubing outlet is positioned no more than 2 mm from the edge of the coating knife to ensure the knife contacts the substrate. The meniscus is filled to the tubing outlet to maximize effluent particle delivery and minimize aggregation on the knife’s underside around the tubing outlet.

Because CCSA uses larger volumes of coating suspension than the conventional convective assembly techniques, Taylor dispersion and particle settling may occur in the suspension delivery system, leading to uneven particle delivery to the meniscus and associated irregularities in coating appearance [Chapter 3]. As such, the coating apparatus is modified to minimize particle sedimentation by 1) orienting the NORM-JECT syringe pump vertically inside of the humidity-controlled chamber, ensuring all particle sedimentation is
collinear with the flow to the outlet and 2) continuously vibrating the tubing with piezoelectric elements.

Coating small areas using continuous convective-sedimentation assembly is susceptible to significant edge effects that inhibit coating fabrication, including suspension loss and artifact deposition. To minimize fluid loss (and reduced suspension delivery to the meniscus), the tubing must be bonded to the adapter’s hose barb with adhesive sealant. Coating artifacts – thick, amorphous particle aggregates along the coating edges perpendicular to the direction of deposition – form where the meniscus dries on top of the assembled coating. Coated areas containing artifacts are eliminated from subsequent analysis because the particle aggregates disrupt the underlying convectively-assembled coating. Artifact deposition can be reduced by passing the coating knife or deposition blade over the substrate edge, thereby draining the meniscus, or removing any residual fluid with an absorbent wipe. Another source of coating artifacts is disrupted solvent evaporation and associated particle transport during the assembly process caused by air circulation above the coating apparatus. To prevent this circulation, the syringe pump, tubing, and coating apparatus are placed inside a topless encasement made from rigid polypropylene sheets.

5.2.3 Continuous Convective-Sedimentation Assembly of Live Cell + Latex Polymer Particle Blends on Porous Substrates

Live cell + latex polymer microsphere blends can be readily deposited on porous substrates using continuous convective-sedimentation assembly. Inexpensive flexible
substrates like porous paper offer an approach to decrease a reactive coating’s surface area to volume ratio, reducing the required surface area without loss of productivity [9]. CCSA’s constant suspension inflow increases the total suspended cells delivered to the meniscus, yielding large surface area and very long coatings with high cell densities.

Jenkins and coworkers give a protocol for depositing live cell + latex particle blends on porous Kraft paper using upside CCSA [Chapter 4]. The reader is referred to this protocol for detailed descriptions of the deposition and preparation steps, but the important components of each step are discussed here. All procedures for growing the requisite cell cultures and for preparing all live cell + latex microsphere suspensions, coating substrates, and deposition plates for coating fabrication are described by Jenkins and coworkers [Chapter 4]. To promote coating adhesion and cell survival during coating deposition and drying, all coating formulations must contain Rhoplex™ SFO12 (Rohm & Haas, Philadelphia, PA) and one or more osmoprotectants. Rhoplex™ SFO12 is a film-forming, acrylic copolymer emulsion that improves coating stability by strengthening microsphere and cell adhesion to the substrate. *C. reinhardtii* CC-124 formulations contain only sucrose, but *Rps. palustris* CGA009 suspensions contain sucrose and glycerol. Both compounds are osmoprotectants that concurrently arrest polymer particle coalescence and improve desiccation resistance, creating coating porosity during film formation without killing entrapped microorganisms [10].

The CCSA deposition process must be modified for porous substrates. The coating suspension permeates the substrate beyond the meniscus’s projected area, leading to
depletion of the entrained fluid. To offset this fluid loss (and maintain a meniscus), the flow to the coating knife is increased (>10.0 µL/min) until the meniscus contacts the tubing outlet and then reduced to 0.5 µL/min. This cycle is repeated, as necessary, until coating deposition is completed.

5.3 Coating Characterization Strategies

Reliable microstructure and macrostructure characterization methods are critical for fabricating and engineering thin ordered coatings of adhesive, latex polymer particles and live cells because these techniques clarify how the various process parameters, including the coating substrate, formulation, and assembly process control the microstructure of these coatings. This dissertation developed multiple physical characterization methods for evaluating the effects of process parameters, substrate properties, and formulation characteristics on the coating microstructure (Table 5.1). Comprehensive protocols for each characterization method and detailed product information for all analytical instruments can be found in other chapters of this dissertation and in the literature (Table 5.1).

5.3.1 Chlorophyll a Extraction

Chlorophyll a is a photosynthetic pigment essential for photosynthesis in eukaryotes (algae) and cyanobacteria that is used by cell biologists and microbiologists to approximate the cell density within a cell only or a live cell + latex polymer microsphere coating. Chlorophyll a is extracted from immobilized cells using an ethanol extraction technique. A 6-mm disc is removed from a coating using a single-hole paper punch and submersed in 1.5
mL of neat EtOH. The disc-EtOH suspension is boiled for 1 min at 80-85°C and centrifuged at 15000 RPM for 10 min. The resulting supernatant is green in color and sampled for absorbance at 665 nm using a UV-Vis spectrophotometer. The amount of chlorophyll $a$ (µg/mL) released by the disc is calculated as:

$$\text{(Chl } a\text{)}_{\text{disc}} = \frac{29.62 (Abs_{665}) (V_{\text{EtOH}})}{(V_{\text{disc}})(L_{\text{cuvette length}})}$$

(1)

where $Abs_{665}$ is the supernatant absorbance at 665 nm, $V_{\text{EtOH}}$ is the volume of ethanol extract (mL), $V_{\text{disc}}$ is the disc volume (L), and $L_{\text{cuvette length}}$ is the length of the cuvette parallel to the light path. The total amount of chlorophyll $a$ in a coating is calculated as:

$$\text{(Chl } a\text{)}_{\text{coating}} = \text{(Chl } a\text{)}_{\text{disc}} 	imes (V_{\text{coating}})$$

(2)

where $V_{\text{coating}}$ is the volume of that coating (mL). Multiple strips from the same coating are analyzed for chlorophyll $a$ content and averaged to obtain a characteristic chlorophyll $a$ concentration for each coating (live cells, live cell + Rhoplex™ SFO12, and live cell + latex polymer particle microspheres). Chlorophyll $a$ concentrations are also recorded and averaged for uncoated paper.

5.3.2 Fluid and Solute Balances

Fluid and solute material balances over the delivery system help identify any particle and solvent losses that may disrupt the evaporative and convective fluxes that are responsible for coating propagation. The solute balance involves calculating particle numbers for the
meniscus, the coating knife and substrate, and each component of the continuous convective-sedimentation assembly delivery system before and after coating fabrication. Fluid fractions are collected from the coating meniscus and from the delivery system’s syringe, adapter, and tubing; all fractions are analyzed for solute concentration using a flow cytometer coupled to an analytical software package. Each fraction is blended with a calibration standard containing particles of known size and concentration, enabling calculation of solute concentration (solute conc.):

\[
\text{calibrator conc.} \times \left( \frac{solute}{calibrator} \right)_{\text{event no.}} = \text{solute conc.}
\]

(3)

where solute event no. and calibrator event no. are the number of solute and calibration particles detected by the flow cytometer during analysis and calibrator conc. is the concentration of calibration particles, as specified by the manufacturer. Not all calibration suspensions are compatible with the polystyrene latex polymer particles used for coating fabrication. As such, any potential calibrators should be evaluated for efficacy using a test sample prior to bulk purchase. Because particle enumeration using flow cytometry is a commonly used and widely documented analytical technique, the reader is referred elsewhere, especially to the Flow Cytometry and Cell Sorting Laboratory in the College of Veterinary Medicine at North Carolina State University, for the appropriate protocols. Particle numbers for the knife and substrate are approximated by dividing the coated area of each surface by the area of an individual microsphere. This method is applicable to both
cells and latex polymer particles, but its efficacy for cell counting was not evaluated in this dissertation.

The solvent balance is evaluated by passing a known volume of solvent from a syringe into an aluminum weigh dish through a 21G x 6 in standard hub, deflected point needle. This mimics the CCSA delivery system, but replaces the system’s tubing and leur lock with a needle. This substitution helps close the fluid balance by minimizing observed fluid loss to the delivery system’s adapter piece system during solvent pumping. The pumping volume is selected so that the syringe contains solvent after the pumping period, ensuring the needle is full of water and its fluid volume is analogous to its geometric volume. If CCSA deposition conditions are desired, the solvent is continuously sonicated during the pumping period by vibrating two buzzer piezo elements against the needle. Because the pumping period lasts for multiple hours at moderate relative humidity, the solvent material balance is corrected for fluid evaporation, as outlined in Table 5.2.

The calculations are simplified by several assumptions. First, whenever the dissolved solute concentration is less than 10% solids, the suspension is considered sufficiently dilute to use the predefined parameters for pure water (solvent vapor pressure calculations). Air velocity is assumed negligible in the solvent evaporation rate calculation because 1) the fluid is pumped into a sealed environment 2) the relative difference in the solvent and ambient temperatures is too small to generate convective motion. Finally, the available surface area for evaporation in the final calculation is defined as 5% of the total dish area because the collected solvent only covers a small portion of the weigh dish. If S is assumed to be 10% of
the total dish area or larger, the volume of evaporated solvent actually exceeds the total volume of water supplied to the delivery system during the pumping period.

5.3.3 Hydrogen Evolution

Live cell and live cell + latex polymer microsphere coatings are tested for hydrogen evolution to assess cell survival during convective-sedimentation assembly followed by drying and rehydration and how changes in the coating formulation affect cell reactivity. All coatings are cut into three or more strips to allow for replicate sampling. Each strip is placed in the headspace of a sterile, vertical Balch tube containing 10 mL of either TAP-P-S media (TAP+S without phosphorous and sulfur) for *C. reinhardtii* coatings or PM(NF) media for *Rps. palustris* coatings. A 10 mL, 50% (v/v) mixture of TAP-P-S and PM(NF) media is used for strips containing both *Rps. palustris* and *C. reinhardtii* cells. The vertical coating orientation limits nutrient transport to the immobilized microorganisms, slowing their growth rate and preventing outgrowth [11]. All tubes are sealed with sterile butyl rubber stoppers coupled with aluminum crimp caps (Fisher Scientific) and subsequently flushed with UHP argon gas for 30 min to create an anaerobic headspace (and nongrowth conditions) inside each tube. All coatings are continuously illuminated at 20-25°C under fluorescent daylight bulbs with approximately 110 μmol photons/m²/s PAR.

The headspace of each Balch tube is analyzed for CO₂, H₂, N₂, and O₂ using 1 mL, pressurized samples and an Agilent 7890A gas chromatograph equipped with a Supelco 6’ x 1/8” ID 60/80 molecular sieve 5A porous mesh polymer-packed stainless steel column
and a thermal conductivity detector. Argon is used as the carrier gas at an inlet flow rate of 39 mL/min; the chromatograph’s injector, oven, and detector temperature settings are 160, 160, and 250°C, respectively. Each headspace sample is analyzed for 15 min; H₂, N₂, O₂, and CO₂ peaks appear around 0.41, 0.51, 0.59, and 13.3 min, respectively. However, the CO₂ peak will not appear in any Rps. palustris or C. reinhardtii chromatograph. All hydrogen data is normalized by coating area or by chlorophyll a concentration. Three or more strips of the same coating are evaluated for gas evolution. Hydrogen data is averaged to obtain a characteristic hydrogen output for each suspension.

Hydrogen evolution by C. reinhardtii coatings is susceptible to errors in media formulation and sampling. Only TAP-P-S media is suitable for gas evolution studies! C. reinhardtii cells cannot efficiently resynthesize D1 protein without sulfur, leading to hydrogen production after oxygen consumption (via respiration) surpasses oxygen evolution (via photosynthesis). Although phosphates do not inhibit hydrogen evolution [12], phosphorous compounds are omitted based on data from other investigators. (The hydrogen evolution method was inherited from comparable studies at DOE NREL in alginate films that are mechanically disrupted by phosphate.)

The headspace surrounding the C. reinhardtii coatings need not be sampled within 24 hours after the beginning of sulfur/phosphorous deprivation. Algae cells cannot produce hydrogen until the cellular respiration rate surpasses the photosynthetic rate. This transition occurs approximately 24 hours after the initial sulfur/phosphorous deprivation [13, 14]. As
such, the *C. reinhardtii* chromatographs typically lack quantifiable hydrogen peaks until at least 24 hours after the beginning of nutrient deprivation.

5.3.4 Meniscus Volume and Shape

The use of fiber optic methods with a hand-held wireless device to measure meniscus volume and shape is a novel, inexpensive method for imaging meniscus shape and calculating meniscus volume. Coating uniformity and thickness depend on both the shape and volume of the meniscus at the drying front [15, 16]. The technique uses a wireless digital camera to obtain side views of the meniscus during coating assembly (Figure 5.3). The shape of the meniscus in each image is qualified using visual inspection; the entrained liquid volume in each image is calculated by defining the meniscus shape as a triangle with a curved hypotenuse (Figure 5.4). The volume is calculated as the product of the net area of the entrained liquid and the width of the deposition blade:

\[
V_{\text{meniscus}} = \left( \frac{BH}{2} - \frac{R^2 \left( \frac{\theta \pi}{180} - \sin \left( \frac{\theta \pi}{180} \right) \right) \right) x W
\]

where \( W \) is the knife width, \( B \) and \( H \) are the base and height of the triangle, respectively, \( \theta \) is the central angle of the circle segment, and \( R \) is the radius of the circle in which the segment is a part. Each image is analyzed in ImageJ, an image processing program available for download at http://rsbweb.nih.gov/ij/, to enumerate the values of \( B \) and \( H \) for that image.

Because ImageJ utilizes user-specified measurements to assign values to the meniscus height and base length, this method requires the use of a correction factor to account for
aberrations in the manual analysis. All calculated volumes are corrected using a calibration curve of actual versus calculated meniscus volume. Each meniscus is created by manually injecting an aliquot of coating suspension of known volume between the substrate and coating knife. At least three separate menisci are formed, the images digitalized, and evaluated for each predetermined volume. Calculated volumes are acquired using Equation (5.4) and averaged to obtain a mean calculated volume for each known volume. The calibration curve is constructed from this data and should have a correlation value ($R^2$ value) close to one.

Parameter effects on coating uniformity can be evaluated using the meniscus volume and shape method. The method is particularly useful for determining how variations in the deposition mode and knife withdrawal speed (or deposition rate) affect meniscus volume. The meniscus is always photographed at several distances from the initial pinning (or contact) line using the wireless camera; the initial pinning line is not used as the first imaging location, defined as the 0 mm mark, to ensure the meniscus is fully developed prior to imaging. All meniscus volumes are calculated using Equation (5.4), corrected using the calibration curve, and normalized by the calculated volume at the first imaging location.

5.3.5 Optical Microscopy

Optical microscopy is a facile technique for evaluating how changes in the coating assembly process, formulation, or substrate alter coating voids, microstructure, and surface coverage. Coating uniformity is characterized by quantifying the fraction of bare substrate in multiple micrographs. Light micrographs are obtained from an automated microscope.
equipped with a digital camera and multiple objectives and analyzed using image processing software. The void space in each micrograph is calculated as the percentage of uncoated area (or pixels if Adobe® Photoshop® is used) to the total area of that micrograph. Substrates are illuminated using either bright field or oblique lighting. Opaque substrates can be externally illuminated with a mercury lamp or laser if oblique lighting is insufficient to visualize the coated surface.

Micrographs are collected in accordance with either the grid or double lattice sampling strategy (Figure 5.5). Each method reports an average void space and variance, but the grid sampling strategy is not suitable for comparing multiple coatings. Because the grid is sensitive to local variations in coating uniformity, the method yields a relatively large variance that requires statistical analysis of the data.

As such, the grid method is used to assess the homogeneity and surface coverage within a single coating. Micrographs are acquired at equally spaced sampling points (blue squares in Figure 5.5B) across the coating width and length at user-defined axes. A separate mean void space is reported for each of these axes to evaluate microstructure homogeneity across the entire coating. To simplify data collection, the first column of sampling locations is positioned 5 mm from the coating’s edge and all other columns are located at axes that are multiples of 10 mm from the first column. All rows are spaced 5 mm from each other; the outer rows are positioned 5 mm from the coating’s edges. This scheme not only enables use of the ruler affixed to the microscope stage, but also ensures a large surface area of the coating is sampled.
The double lattice sample strategy facilitates comparison of multiple coatings. The method samples a larger percentage of each coating than the grid method, yielding smaller variances that enable statistical analysis of whatever condition (process parameters, substrate properties, formulation characteristics, etc.) is varied among the analyzed coatings. The sampling scheme is derived from the double lattice selection scheme for a 4x4 square frame originally described by Jessen [17] (Figure 5.5A). All blue and green squares are imaged in ten random locations with a 20x objective, yielding 10 micrographs for each square that describes almost 25% of that square. The overall or reported void space is the mean of all 80 micrographs. The overall variance of this mean is calculated as [17]:

\[
V_{\text{ar}} = \left(1 - \frac{r}{L}\right) \frac{(\bar{y}_A - \bar{y}_B)^2}{2r}
\]  

(5)

where \( \bar{y}_i \) is the average void space of the \( x_i \) micrographs (annotation denoted in Figure 5.5A) and \( r \) and \( L \) are defined as two and four, respectively. To simplify data collection for coatings deposited on transparent substrates, the 4x4 grid can be drawn onto a glass microscope slide with the same dimensions as the coating substrate. The grid’s color scheme is inversed – all colorless squares are blacked out while all blue and green squares remain colorless. The slide is positioned on top of the substrate slide such that the edges of the 4x4 grid are 5 mm from the coating’s edges.
5.3.6 Profilometry

Profilometry is a versatile method for evaluating the topography of bare surfaces and the thickness of coated substrates. Another common method for measuring thickness is digital micrometry, but the instrument lacks the necessary sensitivity to quantify the thickness of ultra-thin convectively-assembled coatings, making profilometry a more suitable method for characterization method. The latter technique characterizes surface roughness and coating thickness by profiling the topography of bare and coated substrate surfaces. In terms of surface roughness, the profile identifies any variations like peaks or depressions that may disrupt coating uniformity by providing localized patches on the surface that strengthen or weaken particle attachment. In terms of coating thickness, the profile both quantifies coating height and characterizes coating surface coverage. Multiple surface topography profiles are obtained for each substrate using a surface profilometer coupled to an analytical software package. Each set of profiles is averaged to obtain a characteristic surface profile for each substrate.

Profilometry scans are susceptible to error from substrate curling, stage tilting, and coating disruption. Because scans longer than 1 mm are prone to stage tilting, long-range (>1000 µm) scans are obtained by compiling shorter scans. To prevent substrate curling, flexible substrates like aluminum foil, polyester, and Kraft paper are affixed with tape to clean glass microscope slides to prevent their surfaces from bending under the stylus force. The profile for these substrates is the difference between the measured profiles for the substrate and bare glass. Coatings deposited from non-film forming polymers like
polystyrene latex particles can be scratched and disrupted by the stylus tip as it profiles the coating surface. Liberated particles often adhere to the stylus tip for the duration of the scan, yielding an inaccurate surface profile. To prevent coating disruption, scan the coating surface with a low stylus force. Alternatively, mix the coating suspension with a film forming emulsion like Rhoplex™ SFO12 prior to coating fabrication to promote particle adhesion to the substrate surface.

5.3.7 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a high-resolution imaging technique that uses emitted electrons to obtain information about surface topography, composition, and other sample properties. SEM can be used to visualize nontransparent samples. As such, the method complements optical microscopy, as SEM micrographs allow for coating visualization whenever optical micrographs cannot be obtained or lack sufficient detail, as demonstrated in Appendix B.

Because sample imaging using SEM is a commonly used and widely documented analytical technique, the reader is referred to the Analytical Imaging Facility at North Carolina State University (www.ncsu.edu/aif/), for a basic protocol. Each user defines the accelerating voltage and sampling scheme. For convectively-assembled coatings, each substrate or coating is observed in two or more randomized locations using a 5kV accelerating voltage. Each location is imaged multiple times using sequential magnifications ranging from 100x to 10000x. This sampling scheme: 1) characterizes both surface structure and particle-particle interactions between adjacent latex polymer microspheres and cells and
2) elucidates how changes in the deposition process, coating formulation, or substrate alter coating microstructure and surface coverage.

Nonconductive substrates and coatings on nonmetallic substrates require pretreatment prior to imaging to ensure high image quality. To increase sample conductivity (and micrograph quality), all samples are sputter coated with a thin layer of gold in a mild vacuum (~100 mTorr of Ar gas pressure; 600 V accelerating voltage) after mounting and prior to imaging. Oblique micrographs – used to analyze internal coating structure – are obtained by examining a sample’s edge at an angle relative to the microscope’s detector. To obtain high quality oblique images, coatings are often frozen in liquid nitrogen and then manually cut with a frozen razor blade to obtain a sample with artifact-free edges.

5.3.8 Water Contact Angle (Goniometry)

Water contact angle quantifies substrate hydrophobicity or wettability by dictating how the meniscus spreads across the substrate surface during convective assembly. Hydrophobicity controls coating length and uniformity, characterized as coating void space or surface coverage. Multiple static water contact angles are measured on each substrate surface using a goniometer coupled to an analytical software package. Advancing contact angles are recorded by depositing the probing liquid (deionized water) on a substrate surface; receding contact angles are measured by removing probing liquid from the dispensed droplet. Separate advancing and receding contact angles are recorded for the droplet’s left and right
sides. Each set of angles is averaged to obtain mean advancing and receding contact angles for each substrate.

Contact angle measurements are susceptible to error from stage tilting. The goniometer uses a user-defined box to locate the left and right sides of a deposited droplet. This box can be adjusted either before or after the droplet is dispensed. The droplet must be positioned inside the rectangular area so that the droplet’s dimensions (and contact angle) are calculated correctly. If the stage is not level, gravitational effects may alter the droplet’s actual shape, yielding inaccurate contact angle values or potentially preventing analysis.

5.3.9 Water Uptake

The water uptake characterizes substrate porosity by measuring how much solvent a substrate can absorb. This value is an important parameter in convective-sedimentation assembly on porous substrates because biocoating suspensions are waterborne and permeate the substrate beyond the meniscus’s projected area, leading to depletion of the entrained fluid and possible termination of coating fabrication unless the meniscus volume is adjusted to offset the fluid loss. A priori knowledge of water uptake thus facilitates coating deposition by enabling proper selection of suitable meniscus volumes (in conventional CSA) or standard rates (in continuous CSA) for each substrate.

Water uptake is quantified by weighing multiple strips of the same substrate before and after water saturation. Each strip must be soaked in solvent until a liquid sheen appears on both sides of the substrate, ensuring complete saturation and an accurate water uptake value.
Each weight is normalized by the area of the corresponding strip to obtain a water uptake value for that strip. All rates are averaged together to obtain a characteristic water uptake value for the substrate.

5.3.10 Zeta Potential

Zeta potential characterizes the charge on particles and is useful to determine the latex particle-latex particle, cell-cell, and latex particle-cell electrostatic interactions in a waterborne coating suspension by quantifying the net surface charges of the suspended cells and polymer particles. These interactions affect coating uniformity by controlling the net force (the sum of the attractive capillary forces and the negative repulsive forces) between neighboring latex particles and cells. Strong repulsions inhibit uniform surface coverage by weakening the net force between adjacent particles and cells, preventing the formation of tightly-packed arrays during coating assembly \(^1\). Conversely, weak repulsions reduce coating void space, creating more uniform coatings, by strengthening the net force between neighboring particles and cells \(^1\). Multiple zeta potentials are measured for each suspension using a Malvern Zetasizer (Westborough, MA) equipped with folded capillary and dip cells and coupled to an analytical software package. Both the capillary and dip cell are compatible with aqueous suspensions, but only the dip cell is suitable for strongly conductive (>5 mS/cm) suspensions. Each set of zeta potentials is averaged to obtain a characteristic zeta potential for each suspension. This method can be used for cell or polymer particle suspensions, but is not suitable for bimodal latex polymer particle solutions and live cell +
latex polymer particle blends, as the surface charge of each particle or cell species cannot be isolated.

Unlike many of the other coating characterization methods, zeta potential measurements are sensitive to other solution parameters. Both suspension pH and the Zetasizer’s effective voltage affect zeta potential. As such, these three values are always recorded and reported. Suspension concentration also affects zeta potential, but this effect is only discernible at relatively high and low solute levels, making zeta potential mostly independent of suspension concentration. Hence, solutions can be concentrated or diluted, as necessary, without loss of accurate zeta potential characterization.
Figure 5.1: Coating apparatus in continuous convective-sedimentation assembly (CCSA) deposition mode. Tubing is positioned underneath knife, forming underside CCSA configuration; coating apparatus is located inside controlled environmental chamber. Function generator and relative humidity display unit are not shown.
Figure 5.2: Continuous convective-sedimentation assembly deposition modes:
(A) topside CCSA and (B) underside CCSA. Red arrows define fluid flow path.
Figure 5.3: Configuration of the Seeker 400 Series Wireless CameraScope Inspection system for capturing image of meniscus volume during coating fabrication. Probe tip is placed against edge of apparatus platform and normal to meniscus. Felt-covered block is also placed normal to meniscus, but on opposite side of probe to enhance image contrast. LCD display unit is not shown.
Figure 5.4: Schematic of meniscus shape and identification of geometrical parameters for calculation of meniscus volume from digital images.
Figure 5.5: Sampling schemes for characterizing coating microstructure:

(A) double lattice sampling scheme and (B) grid sampling scheme.
Table 5.1: Coating characterization methods

<table>
<thead>
<tr>
<th>Characterization Method</th>
<th>Investigated Parameter</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll a extraction</td>
<td>Cell density</td>
<td>Chapter 4</td>
</tr>
<tr>
<td>Fluid and solute balances</td>
<td>Delivery system</td>
<td>Chapter 3</td>
</tr>
<tr>
<td></td>
<td>Fluid sonication</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>Hydrogen evolution</td>
<td>Coating reactivity</td>
<td>Chapter 4</td>
</tr>
<tr>
<td>Meniscus volume and shape</td>
<td>Meniscus volume</td>
<td>Chapter 3</td>
</tr>
<tr>
<td></td>
<td>Deposition mode</td>
<td>Chapter 3</td>
</tr>
<tr>
<td></td>
<td>Deposition rate</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>Optical microscopy</td>
<td>Coating structure</td>
<td>Ref. [5], Chapter 3</td>
</tr>
<tr>
<td></td>
<td>Surface coverage</td>
<td>(both parameters)</td>
</tr>
<tr>
<td>Profilometry</td>
<td>Substrate roughness</td>
<td>Ref. [5]</td>
</tr>
<tr>
<td>Scanning electron microscopy</td>
<td>Coating structure</td>
<td>Chapter 4</td>
</tr>
<tr>
<td></td>
<td>Surface coverage</td>
<td>Chapter 4</td>
</tr>
<tr>
<td>Water contact angle (goniometry)</td>
<td>Substrate wettability</td>
<td>Ref. [5]</td>
</tr>
<tr>
<td>Water uptake rate</td>
<td>Substrate porosity</td>
<td>Chapter 4</td>
</tr>
<tr>
<td>Zeta potential</td>
<td>Solute surface charge</td>
<td>Ref. [5], Chapter 4</td>
</tr>
</tbody>
</table>
Table 5.2: Summary of the calculations for quantifying fluid lost to evaporation

<table>
<thead>
<tr>
<th>Calculation</th>
<th>Equation</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dew point temperature</td>
<td>$\alpha(T, RH) = \frac{aT}{b + T} + \ln(RH)$</td>
<td>$a = 17.7^\circ C; \ b = 237.7^\circ C$;</td>
</tr>
<tr>
<td></td>
<td>$T_d = \frac{b\alpha(T, RH)}{a - \alpha(T, RH)}$</td>
<td>$T = \text{measured dry bulb temperature from 0-60}^\circ C;$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$RH = \text{measured relative humidity from 0.01-1.00}$</td>
</tr>
<tr>
<td>Solvent vapor pressure</td>
<td>$\ln(P_{sat}) = A - \frac{B}{T + C}$</td>
<td>$A, B, \text{and } C = \text{predefined, component-specific parameters}$</td>
</tr>
<tr>
<td>Solvent evaporation rate</td>
<td>$W = \frac{3600}{H_v} (P_w - P_a)(0.089 + 0.0782V)$</td>
<td>$P_w = \text{vapor pressure at saturation temperature};$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$P_a = \text{vapor pressure at dew point temperature};$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V = \text{air velocity at water surface};$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$H_v = \text{latent heat of vaporization at saturation temperature}$</td>
</tr>
<tr>
<td>Volume of evaporated solvent</td>
<td>$N_e = -\frac{P}{S} \left( \frac{dV}{dt} \right)$</td>
<td>$p = \text{density of coating suspension};$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$S = \text{available surface area for evaporation};$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V = \text{droplet volume};$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$t = \text{available time for evaporation}$</td>
</tr>
</tbody>
</table>
References


CHAPTER 6

Conclusions, Future Directions, and Impacts
6.1 Conclusions

This dissertation research examined the deposition of thin, convectively-assembled coatings of particles and live cells in order to determine the fundamental relationships that would allow future engineering of multi-layer biocoatings. These coatings can serve as a platform for developing photobiological fuel cells, self-cleaning surfaces, or chemical sensors, among other diverse biotechnical, environmental, and energy applications where a highly bioreactive surface is required. These coatings were fabricated from diverse suspensions, including single particle suspensions, bimodal latex microsphere blends, live cell + latex polymer blends, using batch and continuous convective-sedimentation assembly on various substrates. This study also identified the critical parameters that control the thickness and structure of each coating class (monodispersed particles, bimodal blends, and live cell + particle blends) for both batch and continuous convective-sedimentation assembly. The conclusions summarize how these process parameters affect coating homogeneity and describe all other major findings of the three experimental thrusts of this dissertation: 1) understanding how surface chemistry, substrate wettability and particle net charge affect batch fabrication of bimodal latex microsphere and live cell + latex microsphere coatings, 2) continuous fabrication of unimodal latex polymer microsphere coatings, and 3) continuous fabrication of photoreactive biocomposite coatings on porous substrates for use in gas-phase biocatalysis.

The originally-planned work to extend the previous self-cleaning studies by the Velev Group could not be completed. Jerrim and Velev recently demonstrated a rudimentary self-
cleaning biocomposite coating made from convective assembly of live yeast cells and sulfate latex microspheres, but the useful life and efficiency of this system have yet to be quantified. We intended to test the useful life of these biocomposite coatings by repeating the self-cleaning process (cell shedding and regeneration) for multiple cycles. Cell attachment number, growth and release, and viability per cycle number would be characterized to assess coating useful life and cleaning efficiency. We also planned to design and implement a novel self-cleaning test system that relies on laminar media flow adjacent to the coating surface to evaluate how fluid shear stress alters cell viability and adhesion for Jerrim and Velev’s self-cleaning coating.

6.1.1 Deposition of Composite Coatings from Particle-Particle and Particle-Yeast Blends by Convective-Sedimentation Assembly

Chapter 2 discussed how substrate wettability, suspension composition, particle size ratio and surface charge control coating microstructure (particle ordering and void space) by changing the deposition process. Both the size ratio and charge of the particles or cells influence deposition, convective mixing or demixing and relative particle locations. Substrate wettability and suspension composition influence coating microstructure by controlling suspension delivery and spreading across the substrate. Probably the most important finding is that the size ratio controls the convective mixing or demixing of the suspension components and thus their relative locations in the coating, suggesting that the components’ relative size ratio can be used to predict coating void space and coating length. The judicious
combination of the above parameters enables fabrication of coatings with areas that are on the order of tens of square centimeters or larger.

Chapter 2 also explained how the convective-evaporative assembly technique for depositing coatings from particle suspensions can be extended to deposit bimodal latex particle suspensions and composite blends of latex microspheres and yeast on glass, plastic and metal substrates. The cells are similar to surface-charged colloidal particles like latex microspheres, allowing for deposition of very thin biocomposite coatings of latex particle + live cell blends. The films can be fabricated on rigid and flexible substrates of varied hydrophobicity, transparency, and surface chemistry. The ability to simultaneously deposit cells and latex particles on various substrates makes convective assembly a promising technique for creating closely packed monolayer latex particle + live cell coatings that may see use as functional biomaterials.

6.1.2 Continuous Convective-Sedimentation Assembly of Colloidal Microsphere Coatings for Biotechnology Applications

Chapter 3 presented a new coating fabrication method, known as continuous convective-sedimentation assembly (CCSA), that constantly supplies formulation to the meniscus behind the coating knife by inline injection, allowing for steady-state deposition of ordered colloids (particles or particle + live cell blends) formed by water evaporation. This method can be optimized using a few key process parameters to continuously fabricate thin coatings
with larger surface areas than previously described, thus expanding the utility of convective assembly.

We show how fluid density, fluid sonication, meniscus volume, and particle concentration influence coating homogeneity when the meniscus is continuously supplied. Fluid density modification and fluid sonication affect particle sedimentation and distribution in the coating growth front whereas the suspended particle concentration strongly affects coating thickness, but has almost no effect on void space. The topside and underside CCSA deposition modes have disparate meniscus volumes, enabling control of the coating microstructure by varying the amount of particles transported to the drying front during coating fabrication. However, for a given convective assembly deposition mode and the same initial meniscus volume, coating quality was found to be independent of meniscus volume (or the rate of fluid delivery to the meniscus). These trends suggest that the uniformity of any particle, or even live cell, coating can be improved through fluid sonication and suspension fluid density modifiers, making CCSA a promising method for depositing monolayer or very thin coatings of polymer particles and live cells for numerous biotechnology applications where a highly bioreactive surface is required.

CCSA also offers promise as a means to fabricate multilayer systems. These composite devices can be constructed by sequentially depositing layers on top of each other, forming a hierarchical structure. Because convective assembly allows for selective immobilization and co-deposition of multiple particles, the device composition, configuration, and function are easily varied. For example, one system can be constructed by stacking biocomposite
coatings, while another device can be made by sequentially stacking cell or latex particle coatings. The biocomposite coatings in the former system can be made from the same cell, giving the composite structure a single function, or from different cells, yielding a structure with multiple functions. Co-depositing cells into the same layer or stacking layers of different cells can also create a synergistic system in which one cell type creates products that are consumed by another cell type as reactants, leading to reduced waste and feedstock needs.

6.1.3 Ultrathin, Dense Biocomposite Coatings of Photoreactive Cells and Latex Polymer Microspheres on Porous Paper

Chapter 4 expanded the field of continuous convective-sedimentation assembly by showing how a model photoreactive biocomposite coating can be continuously fabricated from suspensions containing both non-film forming (live cells and latex polymer microspheres) and film-forming solute (latex emulsion) of variable sizes and charges on a non-woven porous substrate. These coatings are mechanically stable when rehydrated and have a higher surface-to-volume ratio than comparable alginate films and suspension cultures, yielding improvements in nutrient transfer and light access to all immobilized cells.

Microsphere size and suspension composition do not affect coating reactivity, but both parameters alter coating microstructure. However, all live cell and live cell + latex polymer blends adhered to the paper substrate without plugging its pores, allowing for nutrient transport from the bulk suspension to the immobilized cells through the paper pores to
sustain microbial activity for hydrogen gas evolution. Paper coatings will also offer higher productivity, calculated as hydrogen output per coating area, once their reactivity is improved through optimization of light intensity, acetate concentration, and/or sulfur cycling during gas evolution, as discussed in the future directions.

6.2 Future Directions

6.2.1 Convective-Sedimentation Assembly Using Porous Substrates

This dissertation demonstrated the utility of depositing convectively-assembled coatings on porous substrates, but did not optimize the fabrication process for maximal coating uniformity, light absorption, and reactivity. A possible way to achieve these latter goals is to deposit the coatings at higher knife withdrawal speeds and volume fractions. Because Kraft paper and other non-woven porous substrates absorb solvent during coating fabrication, a portion of the liquid film is lost to both evaporation and absorption. These losses disrupt coating uniformity by more rapidly depleting the height of the liquid film on the substrate surface, which likely increases the amount of suspended particles that settle out of the meniscus and onto the substrate outside of the drying region. Increasing the knife withdrawal rate may minimize this disruptive effect on coating uniformity by delivering suspended particles to the substrate-air-liquid contact line at the drying front faster, leading to coating propagation, before the particles settle onto the substrate surface.

Another strategy to offset evaporative and absorptive losses is to increase the concentration of particles suspended in the coating meniscus. A higher volume fraction not
only permits the use of faster knife withdrawal speeds [1], but also augments the number of particles available for transport to the drying front [2]. A coating uniformity versus knife withdrawal speed and volume fraction optimization study would clarify the range of speeds and suspended particle numbers that lead to highly uniform, thin coatings. Film homogeneity can be assessed using scanning electron microscopy and/or profilometry. Unfortunately, the images of cells may be altered by the sample preparation method. This study would also identify any knife speeds that mechanically disrupt the coating through substrate tearing.

Changing solvent density to increase particle buoyancy is another facile way to augment the number of particles delivered to the drying front during coating fabrication. Blending the coating suspension with OptiPrep™, a sterile solution of 60% iodixanol in water with a density of 1.32 g/mL, makes low density particles like latex polymer microspheres (ρ = 1.06 g/mL) and cells (ρ ≈ 1.00 g/mL) more likely to remain suspended in the meniscus during coating fabrication. Thus, the cells and microspheres float at or near the meniscus surface, rather than sediment onto the substrate surface outside of the drying region, and are incorporated into the coating only after the thickness of the evaporating meniscus becomes smaller than the diameter of the floating microspheres. However, preliminary data on live cell + latex polymer coatings shows that OptiPrep™ improves coating uniformity by reducing microsphere aggregation (Figure 6.1) while simultaneously inhibiting coating reactivity (Table 6.1). Coupled coating reactivity and microstructure versus OptiPrep™
concentration optimization studies would identify the range of blend ratios that lead to the deposition of homogeneous, reactive coatings.

6.2.2 *Convective-Sedimentation Assembly Process Modeling*

Convective-sedimentation assembly is controlled by a diverse range of process, solute, and solvent parameters. This dissertation expanded this parameter set by showing how substrate wettability and porosity, particle size ratio and surface charge, and suspension composition and density affect coating fabrication and microstructure at constant temperature and relative humidity. This study also demonstrated how fluid sonication and inline injection can transform conventional convective-sedimentation assembly into a continuous fabrication process. These solute and solvent variables or process modifications have not been considered in any reported convective-sedimentation assembly model. Incorporation of these parameters into previous models – yielding a more universally applicable model that describes continuous convective-sedimentation assembly of bimodal particle and biocomposite blends on porous substrates – would facilitate rapid, accurate prediction of coating properties (thickness, surface coverage, and microstructure). An expanded model would reduce material waste and fabrication time by enabling matching of desired coating characteristics and requisite process conditions prior to deposition.

6.2.3 *Photobiological Hydrogen Production*

This dissertation showed proof-of-concept in that for the first time convectively-assembled live but non-growing *C. reinhardtii* + latex microsphere monolayer coatings retain
microbial reactivity after fabrication, rapid drying under controlled humidity and rehydration, but did not optimize coating reactivity, measured as hydrogen evolution. Cycling reactive coatings between sulfur-deprived and sulfur-rich states to enable protein repair and optimizing the illumination intensity and using light/dark cycles are approaches to reduce photosystem damage are potential ways to improve the coating reactivity. High light intensity, coupled with prolonged sulfur deprivation, damages the photosystem II centers, ultimately curtailing hydrogen evolution [3] in both algal suspensions [4] and coatings [5]. Because the damage results from the absence of the sulfated amino acid residues necessary to replace the damaged D1 proteins [3], the damage can be mitigated by 1) continuously flowing sulfur-limited media through the cells or 2) cycling the cells between sulfur-depleted and sulfur-rich states [6, 7]. Both approaches deliver sulfur to the deprived cells, enabling repair of the photodamaged D1 proteins [7]. Although the former method may not be practical for an anaerobic coating, sulfur cycling is a facile strategy for prolonging the reactivity of oxygen-deprived, immobilized cells – implementation of the cycle only requires that coatings be transferred between Balch tubes containing fresh TAP-P-S or TAP media. Because both sulfur concentration and light intensity strongly affect coating reactivity, coupled gas evolution versus cycle length (retention time in sulfur-rich and sulfur-depleted media) and illumination intensity experiments will identify the requisite cycle length and illumination conditions for maximum hydrogen evolution.

The amount of dissolved acetate during gas evolution also affects hydrogen production [8]. Because low acetate levels decrease cellular respiration, leading to intercellular oxygen
accumulation and associated deactivation of the enzymes responsible for hydrogen evolution [8], poor coating reactivity may result from rapid acetate depletion during the beginning of the gas-evolution phase. However, adding acetate to the media after nutrient deprivation may improve hydrogen production, as demonstrated in a comparable alginate film study [8]. A gas evolution versus acetate concentration and introduction point (after onset of nutrient deprivation) study will identify the optimal additional acetate dosage and introduction time for maximum coating reactivity.

6.3 Impacts

Both the coating industry and biomaterials field face the challenging problem of continuously and rapidly depositing thin ordered arrays of latex particles and live cells that maintain mechanical stability and preserve microbial viability and reactivity [9, 10]. These coatings have the potential to be robust components of advanced light harvesting materials for solar energy generation, thus paving the way for the next generation of biomimetic, artificial leaves capable of evolving hydrogen or oxygen from waste organics and sunlight.

Solving the challenge requires a three-part solution: 1) a detailed understanding of how various process parameters and material properties affect coating thinness, particle-particle packing, fabrication and uniformity, 2) a coating apparatus that allows for continuous fabrication of large surface area coatings without loss of structural uniformity and thinness, and 3) a coating system that permanently immobilizes reactive cells without inhibiting
microbial viability and reactivity. This dissertation has made significant contributions to all three solution components by:

- Demonstrating how substrate wettability, suspension composition, particle size ratio and surface charge affect the deposition process and resulting coating microstructure (particle ordering and void space) of coatings deposited from bimodal latex particle blends and live cell + latex particle composite solutions on glass, polyester, aluminum foil, and polypropylene substrates (Chapter 2);

- Developing and investigating two configurations of a coating apparatus in a humidity and temperature-controlled chamber that evolves convective assembly into a universal, controllable, continuous process (known as continuous convective-sedimentation assembly or CCSA) capable of fabricating coatings from a diverse range of particles, including monodispersed suspensions of microspheres or live cells, bimodal blends of live cells and microspheres, and trimodal formulations of latex microspheres, latex emulsion, and live cells (Chapters 3 and 4);

- Describing how variations in particle concentration, fluid flow-path sonication, suspension density, and meniscus volume influence the microstructure of CCSA coatings (Chapter 3);

- Fabricating photoreactive biocomposite coatings from multiple latex particle and live cell formulations that exhibit mechanical adhesion to a porous substrate and
microbial reactivity (hydrogen production) after rehydration in a gas-phase, despite differences in coating microstructure and porosity (Chapter 4);

- Developing new coating deposition and characterization methods for fabricating and characterizing coatings from a wide variety of formulations – monodispersed suspensions of latex particles (or microspheres) or cells, bimodal blends of latex particles or live cells and microspheres, and trimodal formulations of latex microspheres, latex emulsion, and live cells – and substrates, including aluminum foil, glass, Kraft paper, polyester, and polypropylene (Chapter 5).
Figure 6.1: Microstructures of coatings deposited from (A) C. reinhardtii + 8.7 µm latex polymer microsphere + OptiPrep™ suspension and (B) C. reinhardtii + 8.7 µm latex polymer microspheres. OptiPrep™ appears to reduce particle aggregation, increasing coating porosity. Primary micrographs are 100 µm; inserts are 50 µm.
<table>
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<tr>
<th>Time (hrs)</th>
<th>Hydrogen Production (µmol/g cell paste)</th>
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<tr>
<td></td>
<td>+ OptiPrep&lt;sup&gt;1M&lt;/sup&gt;</td>
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<td>25</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>48.5</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>71</td>
<td>0.96</td>
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<sup>a</sup>Peak was detected, but peak area was too small for quantification
References


APPENDIX A

A Versatile Method for the Preparation of
Hydrated Microbial-Latex Biocatalytic Coatings
for Headspace Gas Absorption and Gas Evolution

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Jessica S. Jenkins, Chris Yeager, Sergey Kosourov,
Michael Seibert and Michael C. Flickinger

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Abstract

We describe a latex wet coalescence method for gas-phase immobilization of microorganisms on paper which does not require drying for adhesion. This method reduces drying stresses to the microbes. It is applicable for microorganisms that do not tolerate desiccation stress during latex drying even in the presence of carbohydrates. Small surface area, 10-65 µm thick coatings were generated on chromatography paper strips and placed in the head-space of vertical sealed tubes containing liquid to hydrate the paper. These gas-phase microbial coatings hydrated by liquid in the paper pore space demonstrated absorption or evolution of H₂, CO, CO₂ or O₂. The microbial products produced, ethanol and acetate, diffuse into the hydrated paper pores and accumulate in the liquid at the bottom of the tube. The paper provides hydration to the back side of the coating and also separates the biocatalyst from the products. Coating reactivity was demonstrated for Chlamydomonas reinhardtii CC124, which consumed CO₂ and produced 10.2 ± 0.2 mmol O₂ m⁻² h⁻¹, Rhodopseudomonas palustris CGA009, which consumed acetate and produced 0.47 ± 0.04 mmol H₂ m⁻² h⁻¹, Clostridium ljungdahlii OTA1, which consumed 6 mmol CO, m⁻² h⁻¹ and Synechococcus sp. PCC7002, which consumed CO₂ and produced 5.00 ± 0.25 mmol O₂ m⁻² h⁻¹. Coating thickness and microstructure were related to microbe size as determined by digital micrometry, profilometry, and confocal microscopy. The immobilization of different microorganisms in thin adhesive films in the gas phase demonstrates the utility of this method for evaluating genetically optimized microorganisms for gas absorption and gas evolution.
A.1 Introduction

Whole-cell biocatalysts immobilized onto insoluble supports are used for biosensors, environmental remediation, biofuel production, and biosynthesis of chiral chemicals [2, 4, 8, 29]. Cell immobilization on membranes or porous supports can concentrate (intensify) biological reactivity and stabilize microbes [10, 13, 14, 17, 33]. Trickle bed and biocatalytic membrane reactors (MBRs) are used in many industries [16]. They are used for generation of H₂, in recycling of gas-phase carbon emissions (COₓ) to fuels, for sulfur (SOₓ) and nitrogen (NOₓ) removal from gases, and in biodesulfurization of petroleum. Interest in microbial bio-catalysts as reactive biofilters that absorb gases (CO, CO₂, CH₄, H₂, NOₓ, SOₓ) or produce useful gases (O₂, H₂, CH₄) will undoubtedly increase with emphasis on low-power input large-scale processes such as conversion of CO-rich synthesis gas (syngas) and capture of waste greenhouse gases to recycle into biofuels [1, 3, 7, 20, 24, 34]. For this reason, there is much interest in the efficiency of gas–liquid–microbe mass transfer in multiphase bioreactors, energy-efficient methods to generate microbubbles, thin-film bioreactors, and optimization of the microstructure of multilayered structured biocoatings of very high density, reactive, but nongrowing microorganisms for large-scale biocatalysis with low power input for mass transfer [12, 17]. All of the above reactors seek to minimize power input for gas-liquid-microbe mass transfer [1, 3, 24, 34]. Entrapping immobilized microorganisms in thin coatings on inexpensive flexible substrates may also be useful as “biomimetic leaves” for uniform illumination of multiple layers of photosynthetic microbes to absorb or produce gases while optimizing light trapping [17].
Microbial latex coatings differ significantly from natural biofilms or cells immobilized in hydrogels. There are fundamental limitations with cell immobilization by growing biofilms to colonize support media in trickle beds, packed beds, biofilters, and membrane bioreactors. Natural biofilms have limited control of cell concentration (intensification) and poor control of film thickness (to reduce the diffusion path), and only support microbes that will adhere to, colonize, and form an extracellular matrix. In addition, natural biofilms release cells, leading to reactor plugging. Techniques which rely on natural or synthetic hydrogel polymers to entrap or immobilize microorganisms suffer from similar problems, and their structure may not remain stable, resulting in release or outgrowth of cells. Hydrogel pores are not stable when dried and therefore cannot pre-serve the reactivity of the entrapped microbes in dry form.

For syngas fermentation, due to the low solubility of H\textsubscript{2} and CO at low pressures, gas–liquid mass transfer rates are very low, even for well-mixed stirred tank bioreactors (STRs) even with microbubble aeration [1, 3, 31]. This process would greatly benefit from a low energy input, high gas volume to liquid volume ratio (>1.0) design to significantly increase gas mass transfer to the microbes; however, valuation of differing reactor designs on laboratory scale is difficult. For example, the lack of a standardized method to screen microbes for conversion of syngas (a mixture of CO, CO\textsubscript{2}, N\textsubscript{2}, and H\textsubscript{2}) to ethanol, acetate, and other fuels often frustrates direct comparison of the biocatalyst reactivity reported by various investigators per unit of gas surface area in contact with the microorganisms [1, 3, 7, 18, 20, 24, 25, 28, 34]. Increasing the agitation power input in STRs to increase syngas
bubbling surface area using high-shear microbubble generators or methods to increase bubble residence time and thus the mass transfer rate may not be economically feasible for very large-scale gas absorbers. As a result, a variety of membrane bioreactors (MBR) with reduced power input for efficient mass transfer have been investigated [1, 7, 20]. In the case of photosynthetic biohydrogen gas production, not only are many candidate microbial systems being investigated (algae, purple nonsulfur bacteria, cyanobacteria), but also a wide variety of bioreactors have been proposed. The most important features are effective contact of the gas phase with the photoreactive microbes and providing sufficient, uniform illumination at high cell density. Examples are tubular or sheet photobioreactors, bubble columns, biofilm coated-membrane bioreactors (sheet or hollow fiber), supported biofilms (rotating biological contactors, stacked array bioreactors, horizontal array bioreactors), trickling packed beds (utilizing a variety of packing materials), and agitated anaerobic bioreactors. These systems all require determination of the effect of microbe concentration, light scattering, and illumination uniformity on productivity. In the case of cell immobilization, key elements such as the pore structure of the immobilization matrix, the thickness of the microbial layer, and the extent to which the cells are retained on the support in the bioreactor are often not accurately measured or reported. Combined, these differences result in difficulty in directly comparing the specific reactivity of many of these experimental systems.

For conversion of sparingly soluble gases, such as H₂ and CO (whose solubility in water is 60 and 4 % that of oxygen, respectively, on a mass basis), the reactivity and
product yield of these systems cannot be optimized by cellular engineering alone. Screening for the rate of gas conversion or production and conversion yield of genetically altered microorganisms must also include a small-scale bioreactor configuration to efficiently contact a high concentration of microbes with gaseous substrates. This is because, at low cell concentrations, the overall reaction rate of these processes is kinetically limited, whereas at high cell concentrations, the kinetics become mass transfer limited. Screening organisms in the laboratory under conditions that have little similarity to an industrial reactor or absorber may not select for optimal biocatalyst traits. It is, however, challenging to scale down reactors and immobilization methods. For this reason, there is much interest in a simple laboratory method for increasing the efficiency of gas-liquid-microbe mass transfer for evaluation of microbes suitable for thin-film bioreactors. This includes optimization of the microstructure of structured (multi-layered) coatings or ordered arrays with a very high density of reactive but nongrowing microorganisms for engineering large-scale bioabsorbers or biophotoabsorbers with low power input for mass transfer and uniform cell illumination [12, 17].

A simple, high cell density, inexpensive, nontoxic, adhesive, nanoporous immobilization method which can be used to evaluate thin films of many different microorganisms in a simple miniature gas-contacting reactor would greatly improve screening of engineered microbes for biofilter, photobioreactor, or energy applications. The method must have controlled microbial deposition and adhesion, and easily measured surface area, and allow accurate determination of the number of microorganisms per unit surface area. Such a
laboratory method could be used to more accurately determine and predict the best combination of microbe and large-scale bioreactor configuration for optimal gas-liquid-microbe contact, illumination, and conversion efficiency. To be most efficient, a microbial gas-phase biocatalyst should be a thin nanoporous insoluble coating that is adhesive to the (porous or nonporous) substrate when hydrated, with nutrient limitation or genetic alteration of the immobilized microorganism to prevent outgrowth (reactor plugging). This coating approach allows uniform distribution of a very high density of microbes in a thin coating that is either in a water- saturated gas phase, is self-hydrating from within, or is hydrated by a porous network behind the coating in order to stabilize microbial reactivity without desiccation. For rapid screening of a variety of microbial systems in the gas phase (for example, the head-space in a tube above the liquid phase), the surface area of the coating should be as large as possible and the gas-phase volume should be small (<25 mL). Similarly, any thin coating immobilization method useful in the gas phase must also be stable when submerged to directly compare conversion of substrates dissolved in the bulk liquid phase to the reactivity of hydrated gas-phase coatings.

Herein we describe a paper coating method using a nontoxic, inexpensive, commercially available latex binder used for paper coating and paints. This emulsion contains latex binder mixed with microorganisms and osmoprotectant carbohydrates. The emulsion is coated onto dry chromatography paper, resulting in adhesion between the cells and the cellulose fibers and thus microbe entrapment in a nanoporous layer by polymer particle wet coalescence without drying. The utility of this approach is demonstrated for immobilization of four
different microbial systems (eukaryotes and prokaryotes) of different size, which either consume or generate gases. This method may be broadly applicable to many different microorganisms to fabricate inexpensive small-scale supported membrane tube bioreactors (SMBs) for screening.

Reactivity data for coatings of *Chlamydomonas reinhardtii* CC124 (eukaryotic microalgal aerobic phototroph), *Rhodopseudomonas palustris* CGA009 (anaerobic bacterial phototroph), *Clostridium ljungdahlii* OTA1 (anaerobic autotrophic acetogenic ethanologen), and *Synechococcus* sp. PCC7002 (cyanobacterial aerobic phototroph) incubated in sealed tubes are reported. The phototrophic microorganisms chosen are representative of the types of organisms being considered for various renewable energy applications, especially green microalgae. *C. ljungdahlii* has received considerable attention as a biocatalyst for conversion of syngas to ethanol. A number of companies are actively working to commercialize syngas fermentation. All of the organisms in this study depend on gas-liquid phase interactions. *R. palustris* requires a soluble organic electron donor, such as acetate, for H2 production; *Synechococcus* requires only micronutrients, CO2, light, and water for O2 evolution, while *C. reinhardtii* requires micronutrients, CO2, light, and water for O2 evolution in addition to possibly requiring acetate. *C. ljungdahlii* uses CO and H2 as substrates for ethanol and acetate production with net CO2 production. Of the model microbial systems evaluated here, the purple nonsulfur bacterium (PNSB) *R. palustris* is the only one that does not consume a gas. Under a N2-free, anaerobic atmosphere such as
argon, *R. palustris* produces hydrogen gas. PNSB are being intensively studied for photoassisted production of biohydrogen from waste organics [17, 30].

**A.2 Materials and Methods**

**A.2.1 Bacterial Strains, Media, and Growth Conditions**

Wild-type *Rhodopseudomonas palustris* CGA009 was grown in 160-ml glass serum bottles (Wheaton, Millville, NJ) containing 100 ml anaerobic nitrogen-fixing photosynthetic medium with 20 mM sodium acetate [PM(NF)20ace] and an initial head-space pressure of 1 atm N$_2$ [17, 18]. Wild-type *Chlamydomonas reinhardtii* CC124 was grown aerobically in a 1-l baffled Erlenmeyer flask containing 250 ml TAP medium with stirring under cool white fluorescent light at 28 °C [19]. Wild-type *Synechococcus PCC7002* was grown aerobically in 250-ml baffled Erlenmeyer flasks containing 50 ml BG-11 medium at 26 °C with agitation (100 rpm) [32]. *Clostridium ljungdahlii* OTA1 was grown with shaking at 100 rpm in 160-ml glass serum bottles containing 50 ml anaerobic 1YCMf medium and initial head-space pressure of 1 atm artificial syngas (10% H$_2$, 20% CO$_2$, 20% CO, 50% N$_2$) at 37°C [35].

PM(NF)20ace, TAP, and BG-11 were prepared as previously described [17-19, 31, 32]. 1YCM contains (in 910 ml) 1 g yeast extract, 50 ml PETC salts (per l, 20 g NH$_4$Cl, 2 g KCl, 4 g MgSO$_4$·H$_2$O, 16 g NaCl, 0.4 g CaCl$_2$·H$_2$O, 2 g KH$_2$PO$_4$), 10 ml PETC modified trace elements (per l, 2 g nitritotriacetic acid, 1.3 g MnCl$_2$·4H$_2$O, 0.4 g FeSO$_4$·7H$_2$O, 0.2 g CoCl$_2$·6H$_2$O, 0.2 g ZnSO$_4$·7H$_2$O, 0.02 g CuCl$_2$·2H$_2$O, 0.02 g NiCl$_2$·6H$_2$O, 0.02 g
Na₂MoO₄·2H₂O, 0.02 g Na₂SeO₃, 0.025 g Na₂WO₄·2H₂O, pH adjusted to 6.0 with 5 M KOH), 10 ml Wolfe’s vitamin solution (per l, 0.002 g biotin, 0.002 g folic acid, 0.01 g pyridoxine, 0.005 g thiamine·HCl, 0.005 g riboflavin, 0.005 g nicotinic acid, 0.005 g pantothenic acid, 0.005 g vitamin B₁₂, 0.005 g p-aminobenzoic acid, 0.005 g lipoic acid), and 0.2 ml resazurin (5 mg/ml), and 5 g of fructose when making 1YCMf. After mixing, the pH was adjusted to 6.8 with 5 M KOH. Serum bottles (160 ml) containing 45.5 ml 1YCM medium were sealed and flushed with syngas (20% CO, 20% CO₂, 10% H₂, 50% N₂), then autoclaved. After the bottles had cooled to room temperature, the medium was reduced with 1 ml each of 2.5% (w/v) cysteine-HCl and 2.5% (w/v) sodium sulfide by anaerobic aseptic addition. Cultures were started with a 5% inoculum of actively growing C. ljungdahlii OTA1 cells [35].

A.2.2 Latex Immobilization

For all cell types, the latex–cell formulations were prepared from wet cell pellets. *R. palustris* CGA009 wet cell pellets were prepared by centrifuging 400 ml culture at 4,424 x g and 4 °C for 15 min, suspending the cells in 40 ml PM(NF) medium without acetate and transferring to sterile 50-ml conical tubes, centrifuging at 5,000 x g at 4°C for 15 min, and then pouring off the supernatant. *C. reinhardtii* CC124 wet cell pellets were prepared by centrifuging 45 ml culture for 3 min at 3,000 x g and 4°C, suspending the cells in 40 ml TAP medium and transferring to sterile 50-ml conical tubes, centrifuging at 3,000 x g at 4°C for 3 min, and then pouring off the supernatant. In some cases,
TAP medium (500 µL) was added to the pellet in order to sufficiently suspend the pellet for transfer by pipette when making the formulation. *C. ljungdahlii* OTA1 wet cell pellets were prepared under anaerobic conditions by centrifuging 200 ml culture at 6,000 x g and 4°C for 15 min, suspending the cells in 40 ml 1YCM medium and transferring to sterile 50-ml conical tubes, centrifuging at 5,000 x g at 4°C for 15 min, and then pouring off the supernatant. *Synechococcus* CC124 wet cell pellets were prepared by centrifuging 45 ml culture for 3 min at 3,000 x g and 4°C, suspending the cells in 40 ml BG11 medium and transferring to sterile 50-ml conical tubes, centrifuging at 3,000 x g at 4°C for 3 min, and then pouring off the supernatant.

The wet cell pellets were mixed by vortexing (15 s) until homogeneous slurries of cells were obtained. From the suspensions for each cell type, 200 µL was transferred to an Eppendorf tube containing 200 µL SF012 latex (Rhoplex™ SF-012, an organic solvent-free acrylate copolymer latex paint binder, 43.5 % solids, maximum viscosity 300 cP, minimum film formation temperature 0°C, pH 7-8, prepared without biocides; Rohm and Haas Co., Philadelphia, PA), and the slurry was mixed by vortexing until homogeneous (15 s). Coatings were subsequently prepared by transferring 50 µL of the cell/latex slurry evenly over a scribed 14-mm circle centered on one end of a dry, sterile, folded 3MM chromatography paper template (2 cm x 14 cm) (Fig. A.1). Before drying, freshly prepared coatings were wetted with the appropriate growth medium by placing the paper strips into vertical Balch tubes containing 10 ml medium and allowing capillary forces to wick the medium from the bottom of the tube to the top of the paper strip.
A.2.3 Imaging and Microstructure Analysis

Freshly prepared strips were frozen at -80°C. The thickness of the chromatography paper, both dry and hydrated, was determined from triplicate frozen coatings using a digital micrometer with rounded tip (model ID-C112GEB; Mitutoyo USA Corporation). Coated paper surface topography and coating thickness were also measured by attaching the 3MM chromatography paper to clean glass slides to prevent the surface from curling under the stylus force (3 mg force) and measuring using a surface profilometer (Dektak D150, Veeco, Plainview, NJ) equipped with a 12.5 µm radius and Dektak v9 software.

Hydrated 3MM chromatography paper microstructure was determined from deconvoluted z-plane images obtained by confocal laser scanning microscopy (CLSM) using an Olympus BX-61 optical microscope (Olympus America, Center Valley, PA) equipped with transmitted-fluorescence modes and a 515-nm argon-ion laser (CVI Melles Griot, Albuquerque, New Mexico, USA). A stack of 50 CLSM images, 4 µm apart in the z direction perpendicular to the paper plane, were taken to a total depth of ~200 µm into the paper. ImageJ software (National Institutes of Health, Bethesda, Maryland) was used to reconstruct the topographic profile of the samples. The Tikhonov-Miller algorithm was used for image deconvolution, noise reduction, and improving image quality.

A.2.4 Headspace Gas Absorption or Evolution

The coated chromatography paper was immediately placed into vertical Balch tubes containing 10 ml of the appropriate medium without waiting for the latex coating to dry; the
tubes were sealed and flushed for 5 min. to achieve the required headspace composition (Table A.1). The paper strip could be placed into the tube either with the coating at the top (above the liquid phase) or with the coating at the bottom, submerged below the liquid surface, depending on the microbial system being evaluated. When the coating was at the top of the tube with the uncoated end of the paper strip submerged in the 10 ml liquid phase, the entire chromatography paper was completely hydrated in < 1 min by wicking liquid from the bottom of the tube to the top of the paper. The coatings remained adhesive and stable during paper hydration. The latex coatings were not observed to penetrate through the 3MM chromatography paper substrate during paper hydration. All tubes were vented using a water trap back to 1 atm. The *R. palustris* coatings were evaluated in paper strip configurations (above liquid phase, completely submerged). In order to duplicate the method as previously reported [18], *R. palustris* coatings were dried at 30°C and 60% relative humidity for 1 h prior to placement into Balch tubes. *Synechococcus* and *Chlamydomonas* coatings were incubated under fluorescent light with 68 µmol photons m⁻² s⁻¹ photosynthetically active radiation (PAR) illumination without shaking at 28°C, and *R. palustris* was incubated under incandescent light with 24 µmol photons m⁻² s⁻¹ PAR without shaking at 30°C. *C. ljunghaldii* was incubated in the dark at 37 °C without shaking.

A.2.5 Head-Space Analysis

A Hewlett Packard 7890A gas chromatograph containing a Supelco 6’ x 1/8” ID 60/80 mol sieve 5A porous mesh polymer-packed stainless steel column and a thermal
conductivity detector was used for head-space analysis of CO, CO$_2$, O$_2$, N$_2$, and H$_2$. Argon was used as the carrier gas at flow rate of 36 cc/min with injector, oven, and detector temperature settings of 160, 160, and 250 °C, respectively.

A.3 Results and Discussion

Reactive latex biocatalytic coatings on hydrated paper substrate were prepared for all four organisms. This is the first report of a latex coating immobilization method for *C. reinhardtii* and *C. ljungdahlii* that retains cell reactivity as measured by oxygen gas evolution or CO consumption, respectively. Previous attempts to immobilize *C. reinhardtii* using latex binders with osmoprotective carbohydrates followed by drying at high relative humidity to promote polymer particle coalescence and adhesion resulted in no observable coating-associated activity, indicating that these microbes may be sensitive to desiccation stress. *Synechococcus, C. reinhardtii, and C. ljungdahlii* immobilized in latex coatings that were not dried prior to rehydration were reactive in the head-space of the Balch tubes. *Synechococcus* coatings and *C. reinhardtii* coatings consumed CO$_2$ (3.60 ± 0.20 and 3.90 ± 0.07 mmol CO$_2$ m$^{-2}$ h$^{-1}$, respectively) and produced O$_2$ (5.00 ± 0.25 and 10.2 ± 0.2 mmol O$_2$ m$^{-2}$ h$^{-1}$, respectively) when incubated in the headspace (Fig. A.2a, A.b). *C. ljungdahlii* coatings, when incubated in the head-space of the microbioreactor, consumed CO and H$_2$ (6 ± 1 mmol CO m$^{-2}$ h$^{-1}$ and 1.84 ± 0.08 mmol H$_2$ m$^{-2}$ h$^{-1}$) while producing CO$_2$ (2.17 ± 0.09 mmol m$^{-2}$ h$^{-1}$); acetate and ethanol accumulated in the liquid phase at the bottom of the tube (Fig. A.2c, A.d). The reactivity of hydrated *R. palustris*
coatings was significantly reduced when incubated in the head-space of the Balch tubes (0.47 ± 0.04 mmol H₂ m⁻² h⁻¹) compared with those submerged immediately after coating in acetate-containing medium (5.8 ± 0.5 mmol H₂ m⁻² h⁻¹). When the headspace-incubated *R. palustris* coatings were submerged in acetate-containing medium, hydrogen production increased (1.4 ± 0.3 mmol H₂ m⁻² h⁻¹), indicating that poor acetate diffusion through either the porous paper or latex nanopores may have limited hydrogen production. This also indicates that oxygen production and carbon dioxide fixation observed for *C. reinhardtii* are independent of acetate present in the TAP medium.

The 3MM chromatography paper was 311 ± 13 µm thick and 322 ± 16 µm when hydrated and measured frozen using the digital micrometer and profilometer methods, respectively (Fig. A.3). Due to the limitations of the manual pipette tip coating method, the porosity and surface roughness of the dry paper substrate, and the small patch surface area, the latex patch coatings were not uniform in thickness across the coated area, resulting in large standard errors in thickness measurements when using a digital micrometer. Frozen *R. palustris* coatings were 18–31 ± 2.0 um thick on dry paper, and frozen *C. ljungdahlii* coatings were 7–15 ± 2.6 um thick on dry paper; both coatings were thinner when the paper was fully hydrated. *Synechococcus* coatings were 35–58 ± 3.6–7.0 um thick on dry paper, and the coating thickness was reduced when rehydrated. Because of the larger size of microalgae, *Chlamydomonas* coatings were 46–68 ± 3.1–5.3 um thick on dry paper and thinner when rehydrated. The surface roughness of the frozen coated paper was determined by profilometry scanning from the coating center outward. The patch thickness
varied by ±25 um, with some outer edges having peaks of >50 um due to the nonuniform manual coating method and the different sizes of the microorganisms. Coatings of C. reinhardtii had the greatest surface variability (data not shown). In spite of the limitations of coating uniformity using this manual deposition method, multiple coatings could be made rapidly, with sufficient cell density to measure gas-phase reactivity. In particular, using this latex coating method, all coatings were <75 um thick, significantly thinner than the 200–500 um thick Calothrix Ca\(^{2+}\)-alginate films recently reported to produce hydrogen gas [23].

Latex binders are commonly used in paper coating, where they partially fill the upper layers of the paper pores to form coatings from 10 to 30 um thick. Visual evidence of 3MM chromatography paper porosity and latex paper coating thickness was obtained from z-plane deconvoluted CLSM images of Synechococcus PCC7002 SF012 latex coatings hydrated in BG11 medium (Fig. A.4). The SF012 latex emulsion does not appear to significantly alter the open paper structure by plugging the pore spaces between the fibers (Fig. A.4b).

The kinetics of wet coalescence of SF012 latex as a function of temperature and time has not been measured. However, the wet coalescence of a similar acrylate/vinyl acetate latex binder (Rovace\(^\text{TM}\) SF-091) was previously measured by our group through the loss of effective diffusivity of delaminated films in a half-cell diffusion apparatus using a nitrate tracer. The kinetics of wet coalescence as a function of temperature from 5 to 30°C was described by an Arrhenius relationship with activation energy of 108 kJ/mol [27]. This
means that wet coalescence can be rapid depending on the polymer particle glass-transition temperature ($T_g$), surface chemistry, and incubation temperature. Determination of the kinetics of latex binder wet coalescence on paper, adhesion to cellulose fibers (or other nonwoven substrates), visualization of the microstructure of hydrated coatings, and accurate determination of the number of reactive microorganisms immobilized on the paper will all be important to further optimize this method. In addition, measurement and control of the hydration film thickness on the surface of the coatings and characterization of the mass transfer of gases through this thin liquid film as resistance to transport into the coating need to be investigated further.

Cellulose fibers (saw dust, shavings, chips, etc.), cellulose-containing plant materials (peat), or compost are common microbe immobilization bioreactor supports for trickling bed bioreactors and biofilters for air pollution control [21]. Paper is used extensively as a substrate for biocomposite devices and for advanced disposable two- and three-dimensional analytical detection devices [9, 11, 15]. Many inexpensive latex polymer particle binders are formulated for good adhesion to cellulose when coalesced and remain adhesive when hydrated. This combination of an inexpensive open nonwoven cellulose fiber paper support and readily available nontoxic inexpensive latex binder currently used for high-speed paper coating may significantly reduce material costs for large surface area, supported membrane absorbers or bioreactors. In addition, the open pore structure observed following latex coating (Fig. A.4b) not only increases gas-liquid-microbe mass transfer but also facilities separation of microbial metabolites (such as acetate and ethanol) secreted into
the liquid-filled pore space which diffuse away from the cells. The open pore microstructure can also facilitate escape of gas bubbles (such as CO$_2$, H$_2$) from the biocatalytic coating.

The pore microstructure and mass transfer resistance of fluid-filled hydrated paper for supporting latex biocatalytic coatings differ from other types of membrane-supported bioreactors. Microporous membranes with liquid-impermeable layers at the gas-membrane interface are used to support natural biofilms on the bulk liquid side of the membrane, but this configuration has the disadvantages of uncontrolled reactivity due to biofilm outgrowth into the liquid phase and pore wetting on the gas-phase side [1]. Stacked or horizontal array membrane bioreactors have a hydrophilic membrane with pore size less than the microorganism size and are used to support growth of a natural biofilm or serve as a support for covalently attached or gel-immobilized biocatalysts covered by a thin liquid film in contact with the gas phase. This leads to extreme pressure drops if the membrane pores are plugged by biomass outgrowth. In contrast, the latex-coated paper method described herein relies on wet coalescence of the latex binder polymer particles to generate adhesion to the cellulose fibers without plugging of the paper pores. The partially coalesced latex particles generate nanoporosity surrounding the microorganisms (pores smaller than the microorganism), and the effectiveness of an additional coating of a thin nanoporous top coat of latex binder to further limit biomass release or nutrient limitation to prevent outgrowth has been demonstrated [12, 14]. In the method described herein, the hydrating liquid film thickness on the paper fiber coating surface in contact with the gas phase is supplied by
the liquid-filled pores beneath the coating in equilibrium with vapor-phase water. The film thickness on the surface of the coating in contact with the gas can thus be controlled by the relative humidity. The mass transfer coefficient of gases in contact with the hydrated paper is directly proportional to the diffusion coefficient divided by the stagnant liquid film thickness. Therefore, generating data with a simple small-scale test system such as described herein where the liquid film thickness and loss of biocatalyst from the coating can both be controlled is a significant advantage for engineering and designing efficient large-scale microbial gas absorbers and biocatalytic membrane bioreactors.

The reactivity of this simple sealed tube system can easily be improved by increasing the coating surface area and cell density in the latex emulsion. When the coating strip is placed in the gas phase, both sides (coated and uncoated) are accessible to gas mass transfer through static liquid in the paper pores. However, due to the thickness of the chromatography paper, the mass transfer rate of gases dissolved in the static liquid in the pores from the uncoated side to the bottom of the latex coating is low, as the uncoated side does not contain cells. Gas consumption significantly increases the transfer rate of CO and CO$_2$ absorption in gas absorbers [5, 6, 26]. Thus, to increase the reactivity, both sides of the paper could be coated. One limitation of stagnant liquid-filled paper pores is that the products of microbial metabolism secreted from the cells accumulate in the fluid-filled pores and must diffuse tangentially down the paper into the bulk liquid at the bottom of the tube. This could be an advantage, as the paper support is acting both as a support and as a separation device. An additional approach to increase the reactivity of these systems is to
promote liquid mass transfer through the paper pores tangential to the coating to minimize inhibition of the microbes in the coating by secreted products. It would be similar in this case to biocatalytic membrane reactors and analogous to falling film or “wetted wall” gas transfer devices described by film theory [6]. Open cross-flow catalyst supports are routinely used for reaction and separation in the chemical industry, and their engineering is well established.

Investigation of the microstructure of inexpensive paper or other nonwoven support materials that exhibit open pore structure following latex coating should lead to development of gas-phase biocatalyst supports capable of simultaneous product separation. These materials may be useful for reducing the accumulation of more toxic microbial products. In particular, we envision that this technology could enhance butanol production from CO, CO$_2$, and H$_2$ by strains of C. ljungdahlii transformed with genes encoding enzymes for butanol biosynthesis [22].

A.4 Conclusions

Although the simple gas-phase latex immobilization method described herein requires little more than cells, a nontoxic latex binder, stabilizing carbohydrates, and chromatography paper, the method has many potential applications. Reactive coatings can now be made using microorganisms that previously did not survive desiccation during latex film formation and drying. It is now possible to determine the molecular mechanisms of preservation of cell viability and reactivity as a function of drying stress, similar to a latex film formation toxicity assay [14], thereby giving a direct means of evaluating how the
coating drying process influences cellular processes that affect the rate of biocatalysis. This method should be useful for screening for viability following coating and the effect of water activity, and in combination with transcriptome or proteome methods may facilitate new cellular engineering approaches to minimize loss of reactivity under the physiological stresses which occur during latex film formation and partial drying. In addition, the reactivity as a function of the mechanism of latex toxicity can be quickly determined for different types of microbes and latex polymer emulsion chemistries.
Acknowledgments

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Figure A.1: Latex immobilization method. All coating steps are carried out according to the requirements of the individual organism being immobilized (for example in an anaerobic chamber for *C. ljungdahlii* coatings) 1. Cells grown and harvested by centrifugation 2. Wet cell pellet resuspended by vortexing 3. Latex mixed with cell suspension until homogeneous 4. Cell formulation applied to demarcated surface of exposed sterile 3MM chromatography paper substrate using a pipette, 3MM filter paper strip wrapped in 2-part foil sleeve for sterilization 5. Substrate folded and removed from remaining sterile foil cover 6. Substrate and coating are inserted into balch tube by either submerging the coating in 10 ml of medium (with coating pointed down) or leaving the coating in the headspace (coating at the top).
**Figure A.2:** Cumulative oxygen or hydrogen evolution and carbon monoxide or carbon dioxide utilization by hydrated paper immobilized organisms in sealed tubes.

A) Immobilized *Synechococcus sp.* PCC7002 utilized 3.6 ± 2.0 mmol CO$_2$ m$^{-2}$ hr$^{-1}$ and produced 5.00 ± 0.25 mmol O$_2$ m$^{-2}$ hr$^{-1}$ when incubated in the headspace.

B) Immobilized *Chlamydomonas reinhardtii* CC124 utilized 3.90 ± 0.07 mmol CO$_2$ m$^{-2}$ hr$^{-1}$ and produced 10.2 ± 0.2 mmol O$_2$ m$^{-2}$ hr$^{-1}$ when incubated in the headspace.

C) Immobilized *Rhodopseudomonas palustris* CGA009 produced 5.8 ± 0.5 mmol H$_2$ m$^{-2}$ hr$^{-1}$ when submerged in the medium compared to when incubated in the headspace of the microbioreactor 0.47 ± 0.04 mmol H$_2$ m$^{-2}$ hr$^{-1}$ (rates calculated for < 75 hr). Hydrogen production increased to 1.4 ± 0.3 mmol H$_2$ m$^{-2}$ hr$^{-1}$ when the headspace coatings were submerged in the medium which is comparable to the rate observed over the same period for the coatings which were submerged from the beginning 2.1 ± 0.6 mmol H$_2$ m$^{-2}$ hr$^{-1}$ (rates calculate for data >75 hr). Arrow indicates when the coating was submerged in medium.

D) Immobilized *Clostridium ljungdahlii* OTA1 consumed 6 ± 1 mmol CO m$^{-2}$ hr$^{-1}$ and 1.84 ± 0.08 mmol H$_2$ m$^{-2}$ hr$^{-1}$ while producing 2.17± 0.09 mmol CO$_2$ m$^{-2}$ hr$^{-1}$. Error bars ± 1 Std Dev, n=3 for all experiments.
Figure A.3: Latex coating thickness determined on frozen coatings for four different microorganisms on 3MM chromatography paper. Dry coatings were not hydrated prior to freezing at -80°C. Hydrated coatings were immediately hydrated after latex deposition (without air drying) by the appropriate medium wicking from the liquid from the bottom of the tube up to the top of the strip (< 1 minute) prior to freezing at -80°C.
Figure A.4: Confocal laser scanning microscopy of deconvoluted z-plane tilted images of 3MM chromatography paper coated with *Synechococcus* PCC 7002 in SF012 latex binder emulsion. Color is natural fluorescence of cellulose fibers. $512^2$ pixels/ 1 mm$^2$ area. A. BG11 medium hydrated chromatography paper. B. *Synechococcus* PCC 7002 latex-coated paper hydrated with BG11 medium.

Table A.1: Model microorganisms used in this study to generate the
biocatalytic coatings and the specific reactor conditions for each

<table>
<thead>
<tr>
<th>Organism</th>
<th>Initial Reactor</th>
<th>Gas</th>
<th>Liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlamydomonas reinhardtii</em> CC124</td>
<td>20% CO₂, 80% N₂</td>
<td>CO₂</td>
<td>Acetate</td>
</tr>
<tr>
<td><em>Rhodopseudomonas palustris</em> CGA009</td>
<td>100% Ar</td>
<td>N/A</td>
<td>Acetate</td>
</tr>
<tr>
<td><em>Clostridium ljungdahlii</em> OTA1</td>
<td>10% H₂, 20% CO, 20% CO₂, 50% N₂</td>
<td>CO, H₂</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Synechococcus sp.</em> PCC7002</td>
<td>20% CO₂, 80% N₂</td>
<td>CO₂</td>
<td>N/A</td>
</tr>
</tbody>
</table>

N/A is not applicable.
References

in: T. Provider, J. Baghdachi J (Eds.), Smart Coatings II (ACS Symposium Series 1002), American Chemical Society, 2009, p. 52.


This appendix contains micrographs of uncoated Kraft paper, uncoated latex polymer microspheres, and various coatings of latex polymer particles or live cells + latex polymer microspheres coated on Kraft paper. These representative images were selected from a larger micrograph set and demonstrate the efficacy of analyzing coating structure and particle (microsphere or cell) interactions using scanning electron microscopy.
Figure B.1: Uncoated Kraft paper. Substrate surface lacks microscale uniformity of other common substrates, likely affecting coating microstructure by interfering with particle movement on surface during convective assembly.
Figure B.2: 8.7 µm polystyrene microspheres affixed to double-sided tape. Microspheres exhibit large size distribution that may affect coating uniformity by interfering with convective assembly.
Figure B.3: 5.0 µm polystyrene microspheres, Rhoplex™ SFO12 latex emulsion, sucrose, and glycerol coated onto Kraft paper. Latex coats *entire* surface of all microspheres, gluing neighboring particles together. Elongated Rhoplex™ SFO12 (latex ropes) between microspheres may result from particle movement after coating rehydration. Coating exhibits nanoporosity, despite dense particle packing.
Figure B.4: 1.0 µm polystyrene microspheres, Rhoplex™ SFO12 latex emulsion, glycerol, and sucrose coated onto Kraft paper.
Figure B.5: 1.0 µm polystyrene microspheres, Rhoplex™ SFO12 latex emulsion, sucrose, and glycerol coated onto Kraft paper. Emulsion only partially coats surface of microspheres. Latex ropes between adjacent microspheres suggest particles move after coating rehydration. Coating exhibits porosity, despite dense particle packing.
Figure B.6: 5.0 µm polystyrene microspheres, Rhoplex™ SFO12 latex emulsion, glycerol, and sucrose coated onto Kraft paper. Microspheres partially cover surface, creating distinct regions of bare and coated substrate.
Figure B.7: 1.0 µm polystyrene microspheres, Rhoplex™ SFO12 latex emulsion, glycerol, and sucrose coated onto Kraft paper. Microspheres are submerged in emulsion. Coating exhibits no porosity, despite lack of particles, suggesting formulation has too much binder emulsion, which rapidly coalesces into a nanoporous coating.
Figure B.8: 5.0 µm polystyrene microspheres, Rhoplex™ SFO12 latex emulsion, glycerol, and sucrose coated onto Kraft paper. Bare or broken portions of microsphere surfaces are locations where another microsphere has detached, likely during coating rehydration.
Figure B.9: Rod-like *Rhodopseudomonas palustris* CGA009, 1.0 μm polystyrene microspheres, and Rhoplex™ SFO12 latex emulsion coated onto Kraft paper. Osmoprotectants are not visible. Cells are dispersed among microspheres. SFO12 binds to microspheres, gluing neighboring particles together.
Figure B.10: Flattened, ellipse-like *Chlamydomonas reinhardtii* CC-124 (cells flattened during sample preparation), 1.0 µm polystyrene microspheres, and Rhoplex™ SFO12 latex emulsion coated onto Kraft paper. Osmoprotectant (sucrose) is not visible. Microspheres collect around cell edges.
Figure B.11: *Chlamydomonas reinhardtii* CC-124, 5.0 µm polystyrene microspheres, and Rhoplex™ SFO12 latex emulsion coated onto Kraft paper. Microspheres form isolated aggregates that sediment onto cells.
This appendix contains detailed recipes for preparing PM(NF) and TAP+S growth media.
C.1 PM(NF) Growth Media for *Rhodopseudomonas palustris*


Prepare the following stock solutions:

I. Concentrated base solution

- 20 g nitrilotriacetic acid
- 28.9 g MgSO$_4$
- 6.67 g CaCl$_2$·H$_2$O
- 18.5 mg (NH$_4$)$_6$Mo$_7$O$_{24}·7$H$_2$O
- 198 mg FeSO$_4·7$H$_2$O
- 100 mL metal 44 solution

Water to 1 L

II. Metal 44 solution

- 2.5 g EDTA (free acid)
- 10.95 g ZnSO$_4·7$H$_2$O
- 1.54 g MnSO$_4·H_2$O
- 392 mg CuSO$_4·5$H$_2$O
- 250 mg Co(NO$_3$)$_2·6$H$_2$O
- 177 mg Na$_2$B$_4$O$_7·10$H$_2$O

Few drops of concentrated H$_2$SO$_4$ to prevent precipitation

Water to 1 L
To make the final media, mix:

25 mL 0.5 M Na$_2$HPO$_4$

25 mL 0.5 M KH$_2$PO$_4$

1 mL 0.1 M sodium thiosulfate

1 mL 2 mg/mL para-aminobenzoic acid

20 mL 1 M sodium acetate

1 mL concentrated base solution

Water to 1 L
C.2 TAP+S Growth Media for *Chlamydomonas reinhardtii*


Prepare the following stock solutions:

I. TAP salts
   - 15.0 g NH₄Cl
   - 1.0 g MgSO₄·7H₂O
   - 2.0 g CaCl₂· 2H₂O
   - Water to 1 L

II. Phosphate solution
   - 28.8 g K₂HPO₄
   - 14.4 g KH₂PO₄
   - Water to 100 mL

III. Hutner’s trace elements (see C.3)

To make the final media, mix the following:

- 2.42 g Tris
- 25 mL TAP salts
- 0.375 mL phosphate solution
- 1.0 mL Hutner’s trace elements
- 1.0 mL glacial acetic acid
- Water to 1 liter
C.3 Hutner’s Trace Elements


Prepare the following solutions:

- 50 g EDTA disodium salt in 250 mL water
- 22 g ZnSO₄·7H₂O in 100 mL water
- 11.4 g H₃BO₃ in 200 mL water
- 5.06 g MnCl₂·4H₂O in 50 mL water
- 1.61 g CoCl₂·6H₂O in 50 mL water
- 1.57 g CuSO₄·5H₂O in 50 mL water
- 1.10 g (NH₄)₆Mo₇O₂₄·4H₂O in 50 mL water
- 4.99 g FeSO₄·7H₂O in 50 mL water

To make the final media:

1. Mix all solutions except the EDTA solution.
2. Bring the mixture to boil.
3. Add the EDTA solution to the suspension (mixture will turn green).
4. Cool the mixture to 70°C after all the compounds dissolve.
5. Keeping temperature at 70°C, add 85 mL of hot 20% KOH solution (20 grams per 100 ml final volume). **Do not use NaOH to adjust the pH.**
6. Bring the final mixture to 1 L total volume (mixture will be clear green).
7. Cover mixture with a cotton plug shake mixture once a day for 1-2 weeks.
Mixture will turn purple and form a rust-brown precipitate. This precipitate is removed by filtration until the mixture is clear.

8. Refrigerate or freeze the mixture.