ABSTRACT

BERNAL ZUNIGA, OSCAR. Mimicking the Plant Leaf: Cellular Composite Materials for Capturing Solar Energy and Gas-Phase Biocatalysis. (Under the direction of Professor Michael C. Flickinger).

Intact photosynthetic cells have significant stability advantages over isolated photosynthetic pigments and reaction centers and could be used to fabricate highly stable cellular biocomposites that harvest sunlight, absorb CO\textsubscript{2} and produce fuels. What is needed are inexpensive methods to concentrate, stabilize and deposit photoreactive cells into composite sheet-like flexible materials that can be easily illuminated, function for long periods of time in the gas-phase much like natural leaves without dehydration, and can be stored dry without loss of reactivity. This dissertation describes three novel cellular biocomposites for long-term stabilization of concentrated photosynthetic microorganisms. Cells can either be deposited as a thin nanoporous water-borne latex coating on paper, incorporated during the paper making process as an integral highly porous biocomposite (microbial paper) or deposited as a precisely oriented cell monolayer on a surface-modified clear polyester substrate using dielectrophoresis (DEP).

Paper-based biocomposites have a transport network that keeps cells hydrated with nutrients, eliminates waste materials and separates secreted products. CO\textsubscript{2} absorption without cell outgrowth was monitored for >500 hours from cellular coatings of several strains of cyanobacteria coated along with non-toxic latex binder emulsions on chromatography paper at rates that are up to 10 fold superior compared with an equivalent amount of biomass in suspension and are very close to plant leaves under the same experimental conditions. We also observed significant changes of the tolerance to high temperature and light intensity by cyanobacteria in latex coatings, which may pinpoint the need for the development of a new
theoretical framework to describe the behavior of immobilized living cells exposed to high light intensity from a solar simulator, an approach that differs radically from simple comparison with suspension kinetics data.

A logical, yet unexplored extension of the cellular coatings concept on paper was reduced to practice in the form of “microbial paper”, a dry-stabilized cellular biocomposite that exploits all the available surface area within the paper matrix for immobilization. Hydrogen gas production from acetate by the activity highly-concentrated purple non-sulfur photosynthetic bacteria entrapped in microbial paper can be sustained for >1000 hours following drying and rehydration at rates up to 10 fold greater than those of any previous cellular biocomposites developed by our group. By implementing a vacuum-dewatering method during drying and incorporating microfibrillar cellulose (MFC) to a cellulose fiber blend, cell retention was significantly improved which established the conceptual framework for future optimization of mass transfer, cell viability and optical/mechanical properties of this novel biocomposite materials platform for high-intensity cellular biocatalysts immobilization.

A third project involved the fabrication and fundamental characterization of ultra-thin (~1 cell thick) cellular biocomposites of cyanobacteria. We developed a novel cellular coating method based on dielectrophoretic assembly of planktonic cells in suspension followed by controlled deposition on a surface-activated clear and flexible polyester sheet. Cell viability was preserved during deposition and predictions of the most energy favorable cellular arrangements were obtained from numerical simulations of the assembly process. Significant reduction of light scattering and the observed enhanced transmittance of DEP
coatings of cyanobacteria was evidence of reduced self-shading and more efficient cell packaging compared to free settling of suspended cells onto the surface of the substrate.

Understanding the cell-substrate interactions in these biocomposites will enable further intensification, closer cell packing and dry stabilization of virtually any type of cell (prokaryotic and eukaryotic) on any type of support. We envision these highly reactive cellular biocomposites as the cornerstone of the emerging field of continuous bioprocessing and as cost-effective biophotocatalysts that combine intensification, uniform illumination, stabilization of reactivity and separation capabilities for a wide variety of industrial applications.
Mimicking the Plant Leaf: Cellular Composite Materials for Capturing Solar Energy and Gas-Phase Biocatalysis

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

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DEDICATION

This dissertation is dedicated to the loving memory my father,

Luis Eduardo Bernal Alba

Who left this world way too early to see me accomplish this goal,

But gave me absolutely everything I will need to fulfill my dreams

And taught me by example the type of man I want to become

To my mother,

Maria Gladys Zúñiga Murillo

Who gave me the best years of her life to raise me and take care of me,

And also taught me the true nature of unconditional, selfless and infinite love

And to my brother,

Jaime Fernando Cortés Zúñiga

For his genuine admiration, love and support

—

Esta disertación la dedico a la memoria de mi padre,

Luis Eduardo Bernal Alba

Quien partió de este mundo demasiado pronto para verme lograr esta meta,

Pero me dio todo lo necesario para cumplir mis sueños

Y me enseñó con su ejemplo el tipo de persona que quiero ser

A mi madre,

María Gladys Zúñiga Murillo

Quien me regaló los mejores años de su vida para criarme y cuidarme

Y me enseñó la verdadera naturaleza del amor incondicional, desinteresado e infinito

Y a mi hermano,

Jaime Fernando Cortez Zúñiga,

Por su genuina admiración, amor y apoyo

Oscar Iván Bernal Zúñiga, June 2014
BIOGRAPHY

Oscar Iván Bernal Zúñiga was born on June 14th, 1983 in Bogotá, the capital city of Colombia, where he was raised and educated during the first 25 years of his life. Oscar attended the “Colegio Agustiniano de San Nicolás” school for his elementary, middle and high school studies. During these years he showed special interest for math and science, but also pursued music and sports thanks to the continuous influence of his parents Luis and Gladys. In 2001, he decided to pursue a career in Chemical Engineering at the “Universidad Nacional de Colombia”, the largest and most prestigious public university of the country. After three semesters into the program, Oscar decided to transfer to the “Universidad de los Andes”, Colombia’s most recognized and achieved private higher education institution to pursue his true passion: a career combining engineering and basic science. By the end of 2007, Oscar graduated at the top of his class with bachelor’s degrees in Chemical Engineering and Microbiology, and decided to pursue a PhD in Chemical Engineering to complete his preparation for a professional career in the biotechnology field. He joined North Carolina State’s Chemical and Biomolecular Engineering PhD program in 2008 (under the direction of Prof. Michael C. Flickinger), where he obtained a M.Sc. in Chemical Engineering in 2010 and a Masters of Biomanufacturing in 2013 on the way to his doctoral degree. As part of his industry training he interned at Novozymes North America and Novartis Vaccines and Diagnostics, where he joined the downstream technical development team as a Scientist in early 2014. Oscar will graduate from North Carolina State University in the summer of 2014 with a Doctor of Philosophy in Chemical Engineering.
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*Oscar Iván Bernal Zúñiga, June 2014*
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CHAPTER 1

Introduction to Cellular Biocomposites
1.1 Definition: Cellular Biocomposites

This dissertation investigates a new concept that combines elements from biocatalysis, materials science and industrial microbiology: cellular biocomposites. Included is the development of methods for the fabrication and characterization of a new generation of highly-porous, hierarchically structured cellular biocomposites that exploit the advantageous metabolic characteristics of highly concentrated, ordered, photosynthetic non-growing cells, adhesive polymers and fiber-based porous biomaterials.

The term cellular has its root in the Latin word cella, which refers to a small compartment or enclosed space\(^1\). By the end of the XVII century, Robert Hooke, an English natural philosopher used the term for the first time to describe the tiny empty spaces contained by walls he observed while analyzing very thin slices of bottle cork with a rudimentary microscope. What he saw was the highly porous structure of the cork oak vegetal tissue formed by an array of empty non-living cell walls\(^2\). In time, the same term was used to describe the animalcules (“little animals”) observed by Dutch scientist Anton van Leeuwenhoek in a pond water sample just 10 years after Hooke’s discovery. These small organisms were members of the filamentous algae genus Spirogyra, and this observation led later on to the first description of single-celled bacteria, spermatozoa and complex multicellular structures such as muscle fibers and blood vessels\(^3\).

The concept of cellular solid was born as a means to describe materials in which one phase is solid and the other is empty space or a fluid\(^4\). These materials are made up of an “interconnected network of organized struts or plates which form the edges and faces of cells”\(^1\) (in this context, “cells” should be understood as confined empty spaces, not as the
fundamental unit of living beings). Common examples include wood, bone, cork and coral, which are solids that contain structural elements which themselves have a porous structure. It is important to stress the fact that the cellular solids definition does not imply any biological origin of its constituents, and only describes the level of hierarchical organization of the material (Fig. 1.1A). Cellular solids made of ceramics, aluminum, polymers and even carbohydrates have been recently reported. Whenever the phase constituents of a composite cellular solid are either partially or completely derived from a natural source, it can be called a cellular biocomposite. Examples include microfibrillated cellulose reinforced materials, composites of other natural fibers with resins, silica-based hierarchical biocomposites fabricated from mammalian cells scaffolds and diatomaceous earth filtration matrixes.

![Figure 1.1 Examples of cellular solids. A. Polyurethane (scale bar 500 µm). B. Plant leaf vascular system (scale bar 5 µm) – Image by Louisa Howard, Rippel Electron Microscope Facility, Dartmouth College. C. Latex coating of viable E.coli cells (scale bar 4 µm).](image-url)
In this dissertation, the term *cellular* will be used to describe both a high level of organization at the microstructure level and the presence of whole living cells as integral components of the biocomposite. As such, we exploit the natural extension of this concept to the structure of the plant leaf (Fig. 1.1B) and expand it to include those hierarchically structured, multi-phase biomimetic biocomposites featuring stabilized highly reactive cells (Fig. 1.1C). This definition is purposely broad to include any type of cell immobilized in any type of (entirely or partially) synthetic polymer or biologically-derived composite material (matrix + reinforcement phases) and is independent of the fabrication method or final application. Using this definition, some of the examples mentioned previously (wood, coral, cork, bone) are or used to be cellular biocomposites as the empty spaces in their microstructure (“cells”) were at one time occupied by metabolically active and functional living cells. However, these examples are natural materials, not man-made. The methods described in this work involve the precise incorporation of live, non-growing planktonic whole cells that do not exist in a biocomposite in nature into a biomimetic solid material during the fabrication process under controlled conditions to preserve the viability and reactivity of cells as biocatalysts. The next section will develop in detail our case for choosing the plant leaf as our biomimetic inspiration and the advantages of using non-growing cells for biocatalysis.

1.2 Mimicking the structure and functions of a plant leaf

Few (if any) man-made materials or devices have accomplished the level of complexity, functionality, synergy and hierarchical design found in plant leaves. Plant cells have evolved
biopolymers to assemble complex vascular networks, functionalized structures and sensing organs. More importantly, they have developed a highly sophisticated packing strategy that has allowed layered arrangements of photosynthetic cells so that plants can colonize a wide range of habitats, have specialized physiological attributes and photosystem repair pathways to adapt to a highly dynamic environment. In this section, I will discuss the most important functional aspects of biomimetic soft materials design applied to plant leaves structure and organization and how their structure can be used as inspiration to design cellular biocomposites that can exceed the photosynthetic efficiency of natural leaves.

In terms of microstructural architecture, a plant leaf can be defined as a collection of tissues arranged in a hierarchical layered organization. The outermost layer is the epidermis, composed of non-pigmented epidermal cells forming a waxy coating that protects against water loss and regulates gas exchange through small pores called stomata. This external interface can be either hydrophobic or hydrophilic depending upon the relative roughness of the surface and the presence of waxy microbumps. Underneath this protective layer comes the mesophyll, which is considered to be the primary assimilative tissue where photosynthesis takes place. The section closest to the epidermis is called palisade mesophyll, and contains tightly packed array of vertically elongated tubular cells with a high concentration of chloroplasts (the specialized photosynthetic organelles in higher plants). There is enough space between cells to allow for water uptake via capillary action and CO₂ absorption. Full-sun plants feature a multi-layered palisade array (to optimize light harvesting) while shade plants tend to have a single-layered palisade. The spongy mesophyll is beneath the palisade and is composed by round cells in a less tightly packaged
array, which allows for space for the gases captured by the stomata (sub stomatal chambers connect the epidermis with the space between the spongy cells)\textsuperscript{14}. Finally, a complex array of veins runs through the spongy space forming the vascular tissue of the leaf. Each vein is made of a vascular bundle featuring two types of tubes: the xylem that moves water and minerals from the roots into the leaf and the phloem that transports photosynthetic products (sugars) out of the leaf. The tubes are surrounded by a lignin sheath that is responsible for the mechanical strength of the leaf\textsuperscript{17} (Fig. 1.2).

The key feature that makes a plant leaf a cellular biocomposite resides in the plant cell wall. This biocomposite structure is made of 4 basic building blocks: cellulose fibers,
hemicellulose, lignin and pectin\textsuperscript{19}. A matrix of hemicellulose and either lignin or pectin is reinforced by cellulose fibers in one or several layers, while the orientation and volume fraction of these fibers varies in each layer\textsuperscript{1}. The cell wall is secreted by the \textit{living} protoplast (a plant cell with no cell wall), layer by layer, beginning with a primary layer rich in cellulose fibers and hemicelluloses cross-linked by glycoproteins\textsuperscript{20}. Once the cell matures, no further growth occurs and the stiffness and strength of the cell wall increases contributing to the final mechanical stability of the leaf.

Based on the discussion above we can identify several plant leaf structural and functional properties that can be exploited in a biomimetic cellular biocomposite:

- \textit{Cellular organization}: a multi-layered, hierarchical architecture featuring different types of cells and biomaterials for optimal light penetration, gas exchange and mechanical strength.

- \textit{Non-growing cells}: a high concentration of non-growing photosynthetic cells that can devote all their metabolic machinery to the production of desired metabolites in detriment of biomass growth.

- \textit{Vascular system}: a specialized network of channels, running both in open and closed loops across the matrix, in charge of moving water, minerals, photosynthetic products and waste materials in and out of the biocomposite.

- \textit{Highly porous matrix}: the void (superficial pore) space between cells and fibers allows for perfusive flow (acting as a rudimentary vascular system) and allows for transpiration and gas exchange. Superficial pores can act as stomata.
- **Self-tuning capabilities:** the concentration of photosynthetic pigments (as chloroplasts) in leaves is different in full-sun and shade plants. Photosynthetic cells in a cellular biocomposite should be able to self-tune to the available light intensity and photoperiod.

In this dissertation, we studied paper-based model cellular coatings and biocomposites (chapters 2 and 3) as an interim step towards the development of an advanced biomimetic device featuring precisely organized photosynthetic cells (chapter 4) on a microfluidics-based substrate that resembles the functionality and performance of a plant leaf (Fig. 1.3).

![Figure 1.3 Biomimetic cellular biocomposites conceptual framework. A. Model paper-based cellular biocomposite featuring a polymer + cyanobacteria coating and a rudimentary random vascular system. B. An optimized cellular biocomposite coated on a flexible microfluidic channel substrate to supply nutrients to a highly ordered cyanobacteria monolayer.](image)

**1.3 “Non-growing” or “resting” cells as biocatalysts**

Our definition of cellular composite is based on the presence of whole cells as integral part of the material microstructure. These cells are immobilized in a “non-growing” or “resting” physiological state that has several advantages over growing systems and enables unique
biomaterial functionalities. In this section, we will briefly describe the advantages and limitations of this approach, methods for keeping the cells under this arrested growth mode indefinitely and a brief historical background on the use of non growing cells as biocatalysis.

The terms “resting” and “non-growing” cells are used interchangeably in biochemical and bioprocessing literature. They refer to cells that are intact but unable to grow because a particular nutrient essential for growth is either in very low concentration or totally absent from the culture media\textsuperscript{21}. When in this physiological state, cells retain their metabolic machinery for maximum catalytic efficiency and most of the feedstock will be used for synthesis of the desired product or generation of intracellular storage materials instead of metabolized to generate cellular energy to support genome replication, cell division and biomass growth\textsuperscript{22}. Isolation and purification of the desired metabolite is also simplified with resting cultures given that most bioprocesses carried out with growing cells have to deal with other metabolites produced from components present in the media\textsuperscript{21}.

For the specific case of photosynthetic cells, a non-growth state can be achieved by changing the bioavailability of certain elements (N, S, P) or by changes in temperature, light intensity or photoperiod\textsuperscript{23}. Another method involves transferring the cells from growing media to a buffer solution right at the time of the growth phase when the cells are at an optimal composition to perform the desired catalytic function. The pH and salt concentration in this buffer is adjusted in such a way that conditions are optimal for the production of the desired metabolite(s) but not for active genome replication and cell growth\textsuperscript{22}. Resting cells are formed by mitotic division but continued division is not required to sustain biocatalytic activity. This is a critical concept for resting cells should be the most efficient forms of
cellular biocatalysts as they don’t need to germinate (like spores do) or divide to be catalytically activated, which involves additional metabolic energy expenditure. At the morphological level, bacterial non-growing cells can be indistinguishable from vegetative cells but some are altered in size with a darker and denser cytoplasm.

The first reports on the use of non-growing cells for the study of biological processes date back to 1940. By this time, methods for the production of active resting cells of streptococci and other bacteria were being developed for basic research on respiratory activity, antibiotic development, chromosome structure during cell division, ribosome distribution, bacterial toxins and autotrophic metabolism. A classical paper by Bykhovsky and collaborators describe starved resting cells of *P. shermanii* used to elucidate the pathways of vitamin B<sub>12</sub> and penicillin biosynthesis in the early 50’s. Nowadays, the use of non-growing cells is still important as model systems to study the interaction of antibiotics with biofilms along with the production of alcohols, alkanes, hydrogen and monosodium glutamate under growth and non-growth conditions. Resting cells are routinely used by many pharmaceutical, fine chemical and biopharmaceutical companies for chiral biotransformations in order to eliminate enzyme purification costs in combined chemical and enzymatic syntheses of pharmaceutical intermediates. In general, starved resting cells are suitable models for the study of biosynthesis of low-molecular weight compounds without the interference of processes related to culture development, genome replication and cell division.

Recent research has also focused on exploiting the inherent advantages of non-growing cells as immobilized biocatalysts. Given that growing cells may leave the
immobilization matrix and plug the bioreactor, resting cells entrapped in a matrix offer more flexibility and have fewer drawbacks for sustained operation of immobilized systems as well as maintaining a constant concentration of the biocatalyst in the reactor constant. Several examples of industrial processes using immobilized non-growing cells can be found in the literature along with more fundamental studies that address the control, modeling and measuring aspects of growing and non-growing systems for the production of a variety of metabolites.

The Flickinger lab group has developed a comprehensive knowledge on the use of non-growing cells in waterborne latex-derived cellular biocomposites over the last 25 years with the conviction that by using principles of molecular biology, metabolic and protein engineering, materials science, anhydrobiology, soft matter, and process intensification (PI) resting cells can be preserved in advanced composite materials to eventually achieve catalytic half-lives comparable to chemical catalysts. These composites will be used as “off-the-shelf” biocatalysts capable of being stored dry and rehydrated immediately prior to use. What still remains to be understood in detail are the changes in cell physiology that take place at the molecular level when cells are forced into a non-growth or resting state in suspension, on a surface or in a composite microstructure. Only very recently McKinlay and collaborators reported a mechanism that explains the significantly higher H₂ yield of on non-growing N₂-starved cultures of *Rhodopseudomonas palustris*. By using a combination of global transcriptome and biomass composition analysis along with ¹³C labeled acetate tracking they found that non-growing cells shift their metabolism to use the tricarboxylic acid cycle to more efficiently metabolize acetate and generate more reducing power for hydrogen.
production while growing cells use the glyoxylate cycle exclusively\textsuperscript{50}. This recent discovery points towards the future capability to engineer “self-tuning” mechanisms of metabolism in response to nutrient limitation in cells used as nongrowing biocatalysts to dramatically increase the efficiency of the incorporation of a carbon source into the product and minimize other biosynthetic processes related to cell growth. It is also an example of how transcriptome data alone is not sufficient to accurately predict the observed cellular physiology to nutrient starvation which resulted in a 3.5 fold higher hydrogen evolution rate than with growing \textit{Rps. palustris} cells.

\textbf{1.4 Bioprocess intensification}

In section 1.2 we described the desirable biomimetic characteristics found in plant leaves that can be applied on cellular biocomposite design. One of these features is the densely packed array of vegetal cells that make up the leaf structure, which translates into many biocatalytic units per unit area. This allows the plant to \textit{intensify} its solar harvesting capacity, which is an example of process intensification (PI). PI can be defined as any engineering development that leads to a substantially \textit{smaller}, more \textit{productive}, \textit{cleaner}, \textit{safer} and more \textit{energy efficient} technology\textsuperscript{51}. Some of the earliest applications of this concept were made by the Eastman Kodak company in regards to increasing the efficiency of the image development process on plates and using spinning discs to generate thin plastic films\textsuperscript{52}. Most of the early developments in this area were achieved in the field of chemical engineering, where technologies such as rotating devices for enhanced heat transfer (boilers, pipes), separation (centrifuges) and reaction capabilities (fluidized bed and plate reactors) allowed significant
improvements in energy management and production yield. The current emphasis of PI is
directed more towards the optimization of processes in green chemistry, renewable
energy, carbon capture and most recently, bioprocess intensification (BPI).

The Flickinger lab has developed methods for cellular biocomposites fabrication that
exploit the advantages of BPI for engineering the next generation of highly efficient
industrial and environmental biocatalysts. In chapter 2 we describe a latex coating method
that achieves 10 fold photosynthetic intensification factors (defined as the ratio of O₂
production by paper coatings of cyanobacteria and with respect to suspension cultures at the
same cell concentration) by concentrating 100-300 fold a suspension of cells and depositing a
small volume of this highly concentrated slurry (50/50 suspension of cells in latex binder,
75µL) on a very small area (1.5 cm²). In this example all premises of BPI are fulfilled (i.e.
device size reduction, enhanced control over final properties, increased bioreactivity and
process speed) while taking advantage of the non-growth state of the cyanobacteria cells and
the location of the biocatalyst in the gas phase for optimal CO₂ absorption.

1.5 Model photosynthetic systems used in this thesis

Two model photosynthetic organisms were used during this project: strains of cyanobacteria
and a purple non-sulfur bacterium (PNSB). Cyanobacteria are photosynthetic prokaryotes,
formerly known to as blue-green bacteria or blue-green algae and were among the first
organisms capable of harvesting electrons from water and releasing molecular oxygen to the
atmosphere, which make them responsible for most of the current composition of O₂ in air.
They are also the primary nitrogen fixers on earth, putting them on the base of the food chain
as they are responsible for converting atmospheric N\textsubscript{2} to more readily available species like NH\textsubscript{4}\textsuperscript{+} in the soil\textsuperscript{60}. These organisms are almost ubiquitous in every environment on earth: from oceans and freshwater as planktonic cells, forming complex biofilms over rocks or as endosymbionts with higher plants and fungi\textsuperscript{61}.

As the most primitive photosynthetic organisms on earth, cyanobacteria share characteristic traits with both algae and higher plants (Table 1.1). They possess a distinctive system of intracellular membranes known as thylakoids where the photosynthetic and respiration reactions take place. They also have multiple photosynthetic pigments\textsuperscript{62}. Cyanobacteria are also tolerant to desiccation and many strains are halotolerant. Most of the desiccation resistance characteristics of cyanobacteria - critical for long-term stability during dry storage - are related to the thick proteinaceous sheath that surrounds the cells. This protective coating is composed of polysaccharides (glucose, galactose, rhamnose, mannose, arabinose), proteins and negatively charged components (glucoronic acids, phosphate, sulphate)\textsuperscript{63}. Their cell wall is rich in peptidoglycan and non-peptidoglycan carbohydrates (mannosamine, glucose, mannose and glucosamine) and 3-hydroxy fatty acids\textsuperscript{63}.

In contrast, PNSB are a very versatile group of photosynthetic prokaryote microorganisms capable of catalyzing hydrogen production under anaerobic conditions using nitrogenase with no need to protect their photosynthetic apparatus from inactivation from oxygen; in addition, they are capable of fixing atmospheric nitrogen via nitrogenase activity in the process, a surprising characteristic discovered 50 years ago\textsuperscript{65}. 

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Table 1.1 Characteristics of the main groups of photosynthetic organisms

<table>
<thead>
<tr>
<th>Domain</th>
<th>Domain Size (µm)</th>
<th>Genome Size (Mb)</th>
<th>Pigments</th>
<th>Resistance to desiccation</th>
<th>Cellular Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanobacteria</td>
<td>Prokaryote</td>
<td>0.5-4</td>
<td>2</td>
<td>Phycobilisomes, chlorophyll a, carotenoids</td>
<td>High</td>
</tr>
<tr>
<td>Microalgae (unicellular)</td>
<td>Eukaryote</td>
<td>10-12</td>
<td>110</td>
<td>Chlorophyll a, chlorophyll b, carotenoids</td>
<td>Moderate</td>
</tr>
<tr>
<td>Higher Plants</td>
<td>Eukaryote</td>
<td>&gt;1000</td>
<td>160</td>
<td>Chlorophyll a, chlorophyll b, carotenoids</td>
<td>Low</td>
</tr>
</tbody>
</table>

PNSB are easily isolated from soil and water, can grow as aerobes if oxygen is present to generate energy by respiration or as photoautotrophic organisms in the presence of light (they synthesize the light absorbing pigments and acquire a characteristic color depending on the species) with production of H₂ by cyclic photophosphorylation. Over 20 genera of PNSB have been described, but most of the current knowledge about the group comes from studies in four species: *Rhodospirillum rubrum*, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris*, the latter being the most interesting given its extraordinary robustness and the presence of the genes that encode all three nitrogenase isozymes in its genome. Current efforts are focused on photobioreactor design and illumination optimization along with pigment content manipulation at the molecular level to improve H₂ production yields under anoxic conditions.
1.6 Cellular biocomposites fabrication methods

A wealth of methods have been developed for classical cellular solids and cellular biocomposites not containing whole living cells as integral parts of their microstructure based on both biologically and non-biologically-derived polymer materials. Most of these techniques are based on technologies for processing of plastics and general composite materials. Rudimentary, poorly structured biocomposites can be fabricated by compounding\textsuperscript{70}, extrusion\textsuperscript{71}, injection moulding\textsuperscript{72} and co-mingling of thermoplastic polymers with natural fibers\textsuperscript{73}, with a small proportion being assembled by thermosetting biopolymer processing\textsuperscript{74}. Higher levels of structural organization can be achieved by foaming, casting and extrusion\textsuperscript{1}. While these methods can yield beautifully crafted biomimetic materials (i.e. honeycombs and collagen-like fibers), the manufacturing conditions are not, in most cases, compatible with preserving the viability and activity of living cells (high curing temperatures, mechanical shearing, high pressures and toxic resin use). For this reason we custom-tailored three different approaches for fabrication of cellular biocomposites that preserve cellular viability and generate significant levels of hierarchical organization: waterborne cellular coatings on paper, microbial paper and dielectrophoretic deposition of living cells. The following sections describe the historical backgrounds and approaches in the context of living cell biocomposites fabrication. For specific methods the reader should refer to chapters 2, 3 and 4.
Table 1.2 Summary of microbial systems used by the Flickinger lab as latex cellular coatings

<table>
<thead>
<tr>
<th>Organism</th>
<th>Application</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>Biosensor coating</td>
<td>78</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Kinetic modeling</td>
<td>79</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Microstructure assessment</td>
<td>80</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Microstructure evolution</td>
<td>81</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Patch coating</td>
<td>76</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Ink-jet printing</td>
<td>82</td>
</tr>
<tr>
<td><em>Thermotoga maritima</em></td>
<td>High temperature biocatalysis, bimodal blend</td>
<td>83</td>
</tr>
<tr>
<td><em>Gluconobacter oxydans</em></td>
<td>High intensity oxidation, bi-layer coating</td>
<td>84</td>
</tr>
<tr>
<td><em>Gluconobacter oxydans</em></td>
<td>Reactivity modeling</td>
<td>85</td>
</tr>
<tr>
<td><em>Gluconobacter oxydans</em></td>
<td>Ink-jet printing</td>
<td>86</td>
</tr>
<tr>
<td><em>Rhodopseudomonas palustris</em></td>
<td>H₂ production, polyester coating</td>
<td>87, 88</td>
</tr>
<tr>
<td><em>Rhodopseudomonas palustris</em></td>
<td>H₂ production, paper coating</td>
<td>75</td>
</tr>
<tr>
<td><em>Clostridium ljungdahli</em></td>
<td>CO, CO₂, H₂ consumption, paper coating</td>
<td>75</td>
</tr>
<tr>
<td><em>Synechococcus PCC 7002</em></td>
<td>CO₂ capture, paper coating</td>
<td>75, 89</td>
</tr>
<tr>
<td><em>Synechocystis PCC6308</em></td>
<td>CO₂ capture, paper coating</td>
<td>89</td>
</tr>
<tr>
<td><em>Synechocystis PCC6803</em></td>
<td>CO₂ capture, paper coating</td>
<td>89</td>
</tr>
<tr>
<td><em>Anabaena PCC7120</em></td>
<td>CO₂ capture, paper coating</td>
<td>89</td>
</tr>
<tr>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>CO₂ capture, paper coating</td>
<td>75</td>
</tr>
<tr>
<td><em>Kluyveromyces fragilis</em></td>
<td>Ink-jet printing</td>
<td>82</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Convective assembly-deposited coating</td>
<td>90, 91</td>
</tr>
</tbody>
</table>

1.6.1 Waterborne adhesive cellular coatings: microbial paints and inks

Nanoporous, adhesive latex polymer coatings that contain enzymes or viable microorganisms have great potential to be used as biocatalysts. Flickinger and members of his research group pioneered the development of methods for the fabrication of highly reactive acrylate and acrylate co-polymer derived latex waterborne coatings of resting cells of several microorganisms (Table 1.2) on non-porous substrates based on early work by Eastman.
Kodak and their academic consultants. These coatings are stable when rehydrated and catalytically active for 100s to 1000s of hours.

The Flickinger group has recently expanded the nanoporous latex biocoating method to coating paper with other photosynthetic organisms (cyanobacteria and algae)\textsuperscript{75}. Early studies by the Flickinger lab to immobilize \textit{E.coli} cells in small coated patches showed that the pore size of a latex coating is governed by the size and distribution of the latex particles, therefore making it desirable to have bimodal blends of low glass transition temperature ($T_g$) acrylate polymer particles with good wet adhesion and non-film forming polystyrene polymer particles with a $T_g$ high enough ($>100^\circ$C) to avoid particle coalescence into a continuous nonporous film during drying and to block the continuation of polymer particle coalescence following rehydration (wet coalescence)\textsuperscript{76} (\textbf{Figure 1.4A}). Other studies in latex composite membranes for micro and ultrafiltration applications found that in addition to particle size, the porosity of the coating is a function of the structure, size, shape, surface charge and reactivity of the latex particles. In general, latex particles can be modified to have a hydrophilic surface composed mainly of surfactants, surface grafted polymer species (such as hydroxyethyl cellulose) and charged groups derived from the polymerization process\textsuperscript{77}.

The reactivity of nanoporous biocatalytic coatings is a function of coating thickness and microstructure, and the substrate surface energy along with polymer particle chemistry determines the adhesive strength when wet or the capacity of the coating to be delaminated from the substrate as stand-alone biocatalytic films\textsuperscript{13}. On the other hand, the effective coating biocatalytic reaction rate (intensity) is related to the volume fraction of entrapped reactive cells, which needs to be maximized during the process of coating, film formation, drying,
storage and rehydration. However, increasing cell content beyond a maximum determined by cell size decreases overall biocomposite permeability as well as decreasing adhesion to a substrate. Polymer particle coalescence and therefore coating permeability is controlled by polymer-water interfacial tension, capillary forces due to water evaporation, layer compression, interparticle adhesion and the glass transition temperature ($T_g$) of the particles\textsuperscript{92} (Figure 1.4B,C).

Coating functionality and porosity can be further enhanced by different emulsion compositions and drying methods. It is possible to generate multi-layered coatings (top-coated with a nanoporous layer of partially coalesced polymer particles without cells) to protect the microorganisms from harsh environments and avoid cell outgrowth into the media\textsuperscript{84}. Of particular interest for eliminating wet coalescence following rehydration are formulations containing bimodal blends of non-film forming (high $T_g$) hard polymer particles in suspension with smaller, acrylate film-forming (low $T_g$) “soft” latex particles or core shell approaches to avoid permeability loss following rehydration\textsuperscript{83} due to wet coalescence which can cause a significant reduction in diffusivity over months of use as hydrated biocatalytic coatings. The rate of loss of coating porosity by wet coalescence is related to the polymer particle chemistry and incubation temperature\textsuperscript{83}.

Other applications of coatings containing viable microorganisms include biosensors\textsuperscript{78,93}, inkjet printing of living cells\textsuperscript{94} and arrays of metabolically active microorganisms for expression libraries\textsuperscript{95}. Chapter 2 will focus on the microstructure and reactivity characterization of latex coatings of cyanobacteria on chromatography paper\textsuperscript{75}. 
Figure 1.4 A. Schematic of non-porous latex film formation process. B and C: Cryo-SEM micrographs showing different degrees of polymer particle coalescence and particle deformation at the base of a coating (in contact with a non-porous substrate) resulting from arresting polymer particle coalescence during dying.

By using a porous support to keep the cells hydrated from behind (and thus eliminating the need for a bulk liquid phase surrounding cells in suspension and reducing the overall water consumption of the process) and place the photoreactive biomass in the gas phase, illumination is optimized (reduced self-shading) and gas mass transfer resistance is reduced.

1.6.2 Microbial paper: the renewable fiber-based biocatalyst carrier

Our work on paper coatings of cyanobacteria (see chapter 2) along with the recent developments on cellulose nanocomposites led us to the conception of a new type of cellular biocomposite we call microbial paper that addresses the following:
- A cellulose-based matrix as a biomass carrier capable of preserving cell viability when being stored and shipped dry and reactivated upon rehydration.

- Leveraging the expertise of the paper industry to engineer the pore space in the cellulose matrix for immobilization of whole live cells (not just the surface area).

- Exploiting the vast knowledge of paper engineering for fabricating new types of bioactive cellulose-based materials with virtually as many applications as there are industrially-relevant organisms available in nature (as long as the embedded microbes do not degrade the cellulose matrix).

These and many other questions inspired us to develop the concept of microbial paper: a portable, flexible, inexpensive and highly reactive cellular biocomposite that can be stored dry and used for long-term immobilization of virtually any type of living cell.

Much has changed in paper technology since the rudimentary papermaking method developed in ancient China by 200 BCE. Current paper technology is reinventing itself due to the development of micro and nano-scale cellulose fiber materials that offer a wider range of applications given their strength, abundance, low weight, stiffness, optical properties and biodegradability\(^96\). Cellulose micro and nanofibers can be extracted from wood and other agricultural products or can be directly produced as bacterial cellulose (BC) produced by bacteria belonging to the genera *Agrobacterium, Acetobacter, Pseudomonas, Alcaligenes, Sarcina and Rhizobium*\(^97\). Nanocomposites of cellulose with hydrophilic and hydrophobic matrices are currently being investigated to be used as packaging films (due to their exceptional barrier properties), flexible displays (microfibrillated cellulose films are highly transparent) and nanopaper featuring enhanced mechanical properties and low thermal
expansion coefficients. In the field of bioactive materials, nanofibrillated cellulose has found alternative uses as a suitable platform for immobilization of bioactive molecules, drug delivery systems, cell culture, antibacterial agents and wound dressings (Fig. 1.5).

Considering the natural origin of paper pulp, microbes are always present thorough the process and are gradually eliminated by high pressures and high temperatures used to achieve the final mechanical strength of these materials. Our literature survey reveals that under normal manufacturing conditions, most of the contaminating microbial population is eliminated by standard paper manufacturing processing conditions and only spores survive in the final paper product. Aside from one study on the use of Bacillus cereus to produce handsheets of paper under controlled laboratory conditions (drying temperature 80°C) as a model system to obtain risk assessment data in cellulosic fiber-based materials, no reports have been found on cellulose fiber-based materials that include reactive living cells as integral part of their structure.

To our current knowledge, no optimization of the paper pressing and drying conditions has been made specifically to preserve vegetative microbial viability and reactivity during and after the papermaking process. For this reason, the data in this thesis on how to generate reactive microbial paper is already the basis for NCSU invention disclosure 12046 (October 2011) and subsequent IDF's. Our approach incorporates whole living vegetative cells early on during the papermaking process and preserves viability by carefully controlling the pressing and drying conditions during the film formation process.
Figure 1.5 Summary of current and potential applications of bacterial cellulose (BC) and nanofibrillar cellulose (CNF) (adapted from 98). Micro and nanofibers offer great potential for cellular biocomposites given their excellent mechanical and optical properties, biodegradability and biocompatibility.

1.6.3 Dielectrophoretic assembly for directed deposition of living cells

Early on during my graduate research work I realized the unsuitability of the previous “thick”, randomly oriented cellular coating or film fabrication methods to optimize BPI by
relying on drying and self-leveling to determine cell packing, dry thickness (~50µm), adhesion, and latex coatings porosity and microstructure. A more precise method was needed in order to control the final distribution and packing density of the cells in the biocomposite and understand the cell-to-substrate interactions that stabilize the composite material both when dry and following rehydration. Dielectrophoresis (DEP) of charged particles emerged as a tool to address these issues and provided useful additional features and new possibilities to engineer microstructure during biocomposite deposition.

DEP is the “the motion of suspended particles relative to that of the solvent resulting from polarization forces produced by an inhomogeneous electric field”\textsuperscript{114}. The latter characteristic of the electric field is the key element that differentiates DEP from electrophoresis, as particles show electrophoretic mobility under the influence of spatially uniform electric fields when a charged interface between the particle surface and the surrounding fluid is present\textsuperscript{115}. The zeta potential of the particles can be estimated from electrophoretic mobility data and provides with useful data on the surface charge (generally negative for living cells due to the composition of the plasma membrane\textsuperscript{116, 117}) either positive or negative for polymer particles depending upon surface chemistry\textsuperscript{118, 119}.

The use of electric fields for particle manipulation in suspension has several advantages, which explains the current proliferation of publications on the theory and applications of DEP (2000+ papers over the last 10 years)\textsuperscript{120}. The simplicity and inexpensiveness of the equipment and materials needed to build the chips allows for all electrical components to be hard-wired while circuitry can be easily configured for many different applications. Additionally, all the forces exerted on the particles can be fine tuned
and controlled by manipulation of parameters like voltage, frequency, wave shape and electrolyte concentration, which in turn can be adjusted by simple interaction with a single piece of equipment (wave generator) or changes in media composition.

A dielectrophoretic force is generated when a non-uniform AC field is applied to a particle suspension\textsuperscript{120}. The DEP force occurs through the interaction of induced dipoles on the surface of the particles and the gradient of the field, which is different from the classical concept of direct electrostatic interaction between the charges and the sign-changing electrode polarization produced by the alternating field (Figure 1.6A). Given that living cells are generally more polarizable than the media, they tend to be attracted to areas of high electric field intensity (close to the electrodes) where chains and eventually 3D structures are assembled due to the action of the DEP chaining force and gravity\textsuperscript{121} (Figure 1.6B).

The DEP chaining force is proportional to the field strength and depends on the length of the particle chain, and the distance between particles. The magnitude of this force is
maximum when particles align in the direction of the field, which is the case when the system is monodispersed (same particle type) regardless of the magnitude of the particle polarizability compared to the media. For more complex systems (i.e. more than one particle type), particle chains can be formed in other directions, approaching 3D sheet structures as the level of homogeneity among particles decreases. The cellular coating method described in detail in chapter 4 combines this DEP-mediated assembly action with a surface-activated substrate to yield permanently oriented cellular coatings of cyanobacteria. A formal mathematical description of the DEP forces and their interaction with living cells is presented in chapter 4.

Most of the current research efforts involving DEP are based on early attempts to manipulate bacterial cells and genomic material, which eventually gave rise to the modern gel-based separation techniques for nucleic acids. Differences between the membrane polarizability of live and dead cells, or between populations of cells with different membrane receptors allow using DEP to precisely separate cells in continuous systems similar to flow cytometers. DEP has also been used to engineer artificial 3D structures of insulinoma cells that can withstand mechanical shock and fluid flow forces if the DEP force is maintained. More importantly, cells remained viable in all cases and can divide in the presence of the electric field. More recently, DEP has been used to obtain permanent (i.e. structures that do not disassemble after the electric field is turned off) chains and monolayers of yeast cells and functionalized polymer particles that can be manipulated in magnetic fields. These structures may be useful for the fabrication of multitarget pathogen detection...
platforms that can perform filtration, sorting and trapping of cells simultaneously\textsuperscript{130} and for the positioning of target cells prior to electroporation for gene delivery\textsuperscript{131}.

### 1.7 Potential applications of cellular biocomposites

The concept of significantly concentrating and immobilizing whole living cells that do not grow out of an adhesive nanoporous biocomposite which preserves viability, stabilizes and intensifies the reactivity several orders of magnitude (BPI) opens a myriad of possibilities for engineering non-growing industrially relevant microorganisms that could be immobilized on a wide range of suitable supports using colloidal polymer chemistry. An extensive review of all potential applications of this kind of material is beyond the scope of this dissertation, but some feasible examples include (Fig. 1.7):

- **Reactive biofilters:** purification of industrial wastes from a gas (VOCs) or liquid (industrial waste water) could be accomplished by engineered strains of bacteria which can be concentrated and stabilized as components of composite papers or nonwoven textiles. The nonwoven fibers provide both mechanical support for the hydrated cells and serve as a filter to separate the biotransformation products from the waste materials.

- **Intensified surface water bioremediation:** composite landscape fabrics could provide a cost-effective method to clean large volumes of surface water from toxic recalcitrant chemicals (pesticide, fertilizer or herbicide run-off) by incorporating stabilized engineered microbes into the nonwoven fabrics. This engineered cellular composite approach would be more efficient than lagoons and natural biofilters. Biofilm reactors
are low intensity, can be poisoned, starved, cannot be dried and are not able to be engineered to respond quickly to start-up or changing contaminant loads.

Figure 1.7 Potential applications of cellular biocomposites. Engineering of microbial cells and immobilization supports opens a wide range of possibilities for bioprocess intensification (adapted from 91, 132-135)

- Biosensors: Embedded cells can be engineered to sense or report (by color change, fluorescence, luminescence) dangerous levels of contaminants in water or air. This can lead to a new generation of bio-sensing filter materials.

- Specialty papers and smart fiberboard: Papers and fiberboard can be engineered for specific applications as “bio-smart” materials for structural applications to stabilize cellulose from fungal degradation. Smart papers and fiberboard materials could sense
and respond to changes in the environment or protect the contents (of the package or the structure) from decay when wet by engineering cells to secrete environmentally-friendly antimicrobial and antifungal compounds.

- “Biomimetic” leaves: Different photosynthetic microorganisms (and plant cells) with complementary light absorption and biosynthetic capabilities can be combined into one multi-layered composite material to create advanced materials with potentially greater capabilities than natural plant leaves.

- Biophotovoltaics: Multilayered cellular biocomposites of O\textsubscript{2} and H\textsubscript{2} producing bacteria and cyanobacteria can produce electric current from gas flux across a charged membrane. Microfluidics could be incorporated to optimize nutrient delivery and waste removal.

### 1.8 Organization of this dissertation

The original objective of my graduate research work was to develop new methods for the fabrication of monolayer cellular biocomposites containing model live photosynthetic microorganisms. Along the way, I developed novel microstructure characterization and photoreactivity measurement approaches, new types of cellular biocomposites and described in a quantitative basis the reactivity changes that take place after cellular immobilization.

Chapter 2 presents a detailed description and characterization of the wet coalescence method originally developed by the Flickinger lab for deposition of cellular coatings of cyanobacteria on chromatography paper. A detailed analysis of the long term reactivity, specific photosynthetic factors, influence of temperature and light intensity and microstructure is
provided along with intriguing correlations between microstructure and biocomposite performance. Chapter 3 addresses an extension of the cellular coating concept into a novel type of cellular biocomposite that includes whole living cells as an integral part of the microstructure: microbial paper. A discussion on the original proof-of-concept method and data along with an optimized approach for fabricating and characterizing microbial paper films is presented. Chapter 4 focuses on the development of a dielectrophoretic method for the fabrication of highly oriented, 1-cell thick cellular biocomposites of cyanobacteria on a clear substrate. This method provides with ultimate control over the final biocomposite microstructure and constitutes a suitable platform for the basic characterization and simulation of the assembly process. Chapter 5 describes a set of methods developed to support binder screening, microbial culture and reactivity measurements. Finally, chapter 6 provides future directions to expand this work.

References


CHAPTER 2

Specific photosynthetic rate enhancement by cyanobacteria coated onto paper enables engineering of highly reactive cellular biocomposite “leaves”

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Abstract

We describe a latex wet coalescence extrusive coating method that produces up to 10-fold specific photosynthetic rate enhancements by nitrate-limited non-growing cyanobacteria deposited onto paper, hydrated and placed in the gas-phase of small tube photobioreactors. These plant leaf-like biocomposites were used to study the tolerance of cyanobacteria strains to illumination and temperature using a solar simulator. We report sustained CO₂ absorption and O₂ production for 500 hours by hydrated gas-phase paper coatings of non-growing *Synechococcus PCC7002, Synechocystis PCC6803, Synechocystis PCC6308* and *Anabaena PCC7120*. Nitrate-starved cyanobacteria immobilized on the paper surface by the latex binder did not grow out of the coatings into the bulk liquid. The average CO₂ consumption rate in *Synechococcus* coatings is 5.67 mmol m⁻² h⁻¹ which is lower compared to the rate reported in the literature for *Arabidopsis thaliana* leaves under similar experimental conditions (18 mmol m⁻² h⁻¹), but yet surprisingly close considering the simplicity of the coating method. We observed average ratios of oxygen production to carbon dioxide consumption (photosynthetic quotient, PQ) between 1.3-1.4, which may indicate a strong dependence on nitrate assimilation during growth and was used to develop a non-growth media formulation for intrinsic kinetics studies. Photosynthetic Intensification Factors (PIF) (O₂ production by nitrate-limited cyanobacteria in latex coatings/O₂ produced by nitrate-limited cell suspensions) in cyanobacteria biocomposites prepared from wet cell pellets concentrated 100 to 300 fold show 7-10 times higher specific reactivity compared to cells in suspension under identical nitrate-limited non-growth conditions. This is the first report of changes of cyanobacteria tolerance to temperature and light intensities after deposition as a
thin coating on a porous matrix, which has important implications for gas-phase photobioreactor design using porous composite materials. Cryo-fracture SEM and confocal microscopy images of cell coating distribution on the paper biocomposite suggest that the spatial arrangement of the cells in the coating can affect photoreactivity. This technique could be used to fabricate very stable, multi-organism composite coatings on flexible microfluidic devices in the gas-phase capable of harvesting light in a broader range of wavelengths, to optimize thermotolerant, desiccation tolerant or halotolerant cyanobacteria that produce O₂ with secretion of liquid-fuel precursors synthesized from CO₂.

2.1 Introduction

Global energy demand is projected to triple from 13.5 terawatt (TW) in 2001 to 43 TW by 2100¹. Approaches for a more efficient use of energy are needed along with stable, cost-effective technologies with long half-lives to capture solar energy which is the cleanest, most abundant, and most under-utilized energy source². One approach to improve solar energy harvesting capacity is to fabricate inexpensive water-based “cellular biocomposite” materials that mimic or exceed the function and stability of natural plant leaves by ordering one or more layers of closely packed living photosynthetic cells on a surface with a non-toxic adhesive polymer binder. Cellular composite is a term used to describe materials in which one phase is solid and the other is empty space or a fluid ³. Common examples include wood, bone, cork and coral, which are solids that contain structural elements which themselves have a porous structure resembling cells⁴. In the present study, we exploit the natural extension of this concept to the structure of the plant leaf and expand it to include those hierarchically
structured, multi-phase biomimetic biocomposites that include whole living cells as an integral component of their structure. Such biomimetic composites should be fabricated from optimized cells and non-toxic polymer binders deposited onto inexpensive substrate materials, be flexible, contain non-growing cells with complementary functions for light harvesting, CO₂ absorption and net carbon compound biosynthesis, and have stable photoreactivity⁵. The stability must be comparable to natural leaves - from months to many years⁶.

Our focus is to develop inexpensive methods to rapidly assemble composites of adhesive colloidal polymer particles and concentrated, stabilized, ordered unicellular photosynthetic bacteria, cyanobacteria and microalgae as flexible materials that can be stored desiccated (dried) and when rehydrated retain their photosynthetic reactivity for 1,000s of hours and their ability to assimilate carbon dioxide into useful products. We have previously demonstrated this concept with the purple non-sulfur photosynthetic bacteria R. palustris which remained metabolically active without growth in N₂ starved nanoporous coatings for over 4,000 hours producing hydrogen gas from acetate at a constant rate⁷. We have now extended this concept of coating non-growing concentrated photosynthetic cells to microalgae⁸ and cyanobacteria.

The metabolic engineering of microalgae for solar energy trapping and production of fuel precursors is being intensely studied⁹-¹². The next step, the ability to rapidly fabricate composite materials from adhesive layers of spatially-fixed light-absorbing complementary cells will enable engineering of inexpensive, stable biosolar energy trapping materials for a variety of light intensities and wavelengths. These multilayered biocomposites of highly
concentrated cells could significantly exceed the low light harvesting efficiency of cyanobacteria or microalgae in bioreactors. Cyanobacteria have small genomes that can be easily manipulated, can produce H₂ and sequester CO₂ efficiently. Genetically modified strains have been reported that are capable of producing isobutyraldehyde, isoprene, ethanol, 1-butanol, isoprenoid triterpenes, ethylene and organic acids from CO₂, which makes this group of unicellular organisms promising candidates to be incorporated into photoreactive cellular composite materials. Carbon assimilation and hydrogen production by cyanobacteria are also being studied for microbial fuel cells (MFC’s) to produce electric current from H₂ and O₂ flux across an ion exchange membrane.

As a model system to fabricate inexpensive non-growing cyanobacteria composite materials with a micro vascular system to enable photosynthesis in a gas-phase analogous to plant leaves (to provide nutrients, minerals and water to non-growing photosynthetic cells) we report several methods for coating adhesive colloidal polymer particles and photosynthetic cyanobacteria on paper supports that hydrate the cell coating by the fluid in the paper pores below the coating. When placed in a gas-phase these coatings absorb CO₂, and produce O₂ or H₂ for 100s of hours with continuous illumination. These methods are in contrast to attempts by other groups to immobilize viable cyanobacteria for nitrogen fixation using macroporous supports such as polyvinyl and polyurethane foams, hollow fibers, cotton and silk. Cyanobacteria immobilization has also been achieved by adsorption to natural polymers such as chitosan, synthetic polymers and cross-linking or by entrapment in silica sol-gels and alginate. None of these methods can
concentrate or deposit thin, adhesive monolayers of cyanobacteria to fabricate highly structured multi-layered composites that mimic the structure of natural leaves. Also, the microstructure of these materials is irreversibly altered when dried affecting the viability and reactivity of the cells which do not recover when rehydrated.

Previously reported cyanobacteria immobilization methods can be used to screen strains in order to evaluate the cells' photoreactivity after immobilization. However, fabricating many of these materials can be time consuming and there are considerable mass transfer limitations, light scattering and cell shading. In addition, the specific reactivity of cells immobilized in these materials is seldom reported. In order to overcome these limitations, we developed a rapid and inexpensive method for screening of cyanobacteria coatings based on wet coalescence of a suspension of cyanobacteria cell paste mixed with an emulsion of non-toxic adhesive latex polymer particles and protective carbohydrates which can generate small coatings hydrated with non-growth media for rapid gas-phase reactivity measurements.

Determination of changes in specific reactivity between illuminated photoreactive cells suspended in liquid and cells immobilized in a single cell layer (monolayer) on a surface is critical to engineering multilayer and highly structured photobiocomposites. As a first step to predicting how to optimize highly organized multi-layered arrays of non-growing photoreactive cells for efficient illumination and mass transfer and for CO₂ sequestration, we report new methods to characterize the microstructure of latex coatings on paper (an inexpensive nonwoven substrate) and determination of the specific photoreactivity of cyanobacteria after immobilization. One major advantage of this system is that these coatings
can be placed in the gas-phase of small tubes as miniature photobioreactors where the gas phase \( p\text{CO}_2 \) can be controlled and the cells protected from UV irradiation by the glass tube wall. In this system, the illuminated nanoporous cell coatings are continuously hydrated by capillary action from the small volume liquid phase at the bottom of the tubes, and the coating-entrapped cells do not grow out of the coating into the bulk liquid. Products secreted from the cells into the paper pore liquid are accumulated at the bottom of the tube – thus the paper provides both a gas-phase support as well as a separation matrix. The simplicity of this approach allows rapid testing of multiple engineered cyanobacteria or combinations of cyanobacteria with other photosynthetic unicellular organisms (with complementary light harvesting or biosynthetic capabilities), with different chemistries of adhesive colloidal binders to identify the most promising candidates for cellular composite fabrication. What is learned for this system can be applied to coating of microfluidic supports with channels engineered for gas transport and harvesting.

Paper is an inexpensive nonwoven support material to understand how to coat cyanobacteria plus adhesive waterborne polymer binder emulsions on a fiber matrix that can be hydrated and supported in the gas-phase to enhance \( \text{CO}_2 \) mass transfer as well as develop methods to characterize the pore network that remains after latex binder wet coalescence (generating wet adhesion). More importantly, the high density of cyanobacteria coated onto a cellulosic fibre matrix functions as a model vascular system. Understanding the microstructure of model cyanobacteria composites will open new paradigms in the field of water-based, flexible cellular biomimetic composites that are mechanically stable with reactivity engineered to exceed that of natural plant leaves.
Here we report the reactivity of paper coatings of four species of cyanobacteria: the unicellular strains *Synechococcus PCC7002, Synechocystis PCC6308* and *Synechocystis PCC6803* and the filamentous strain *Anabaena PCC7120*. We compare the specific reactivity of these organisms both in coatings on paper in the gas-phase and in suspension under continuous illumination. We have also developed a new method to study the effect of light intensity using a solar simulator as well as the effect of temperature on CO₂ absorption and O₂ production. SEM cryo-fracture and confocal laser scanning microscopy (CLSM) methods are used to visualize coated paper microstructure for elucidation of cell distribution on and within the paper matrix and cellulose fiber pore space.

**2.2 Materials and Methods**

**2.2.1 Cyanobacterial Strains, Media, and Growth Conditions**

Wild type *Synechococcus PCC7002, Synechocystis PCC6308, Synechocystis PCC6803* and *Anabaena PCC7120* were grown aerobically in 250-mL flasks containing 50 mL of BG11 medium at 100 oscillations/min in an orbital shaker with 70 μmol photons m⁻² s⁻¹ cool fluorescent light (light intensity measured using a LI-COR, LI-190SA Quantum Sensor, Lincoln, NE) at 25°C. BG11 was prepared as previously described⁵³. All growth and non-growth coated paper experiments were performed under continuous illumination.

**2.2.2 Adhesive Latex Immobilization**

For all cyanobacteria strains, the latex-cell formulations were prepared from wet cell pellets by centrifuging 45 ml of an OD₅₄₀ ≈ 1, 3-day old culture in 50 ml conical tubes in a bench top
centrifuge for 15 min. at 3,000 x g and 4°C. The wet cell pellets (WCP) were mixed with a commercially available low Tg non-toxic acrylic latex emulsion binder by vortexing (15 s) until homogenous slurries of cells were obtained that concentrated the cells approximately 100 to 300 fold over their concentration in suspension cultures (~1.5 ml WCP from ~150 ml of suspension culture depending on strain). From the slurries 200 µL were transferred to an Eppendorf tube containing 200 µl of SF012 latex (Rhoplex™ SF-012, an organic solvent-free acrylate copolymer latex paint binder, 43.5% solids, maximum viscosity 300cP, minimum film formation temperature 0°C, pH adjusted to pH 7.0, prepared without biocides, Rohm and Haas Co., Philadelphia, PA), and the mixture was mixed by vortexing until homogenous (15 s). Coatings were manually prepared by extruding 75 µl of formulation using a pipet tip (Finntip®250 Universal, Thermo Scientific, San Diego, CA) evenly over a scribed 14 mm circle centered on one end of dry, sterile folded 3MM chromatography paper (3MM Chr, Whatman International Ltd., Maidstone, England) template (2 cm by 14 cm) in triplicate (Fig. 2.1).

2.2.3 Determination of headspace gas absorption or evolution

The coated chromatography paper was immediately placed into vertical Balch tubes containing 10 ml of the BG11 growth medium without waiting for the latex coating to dry; the tubes were sealed and flushed for 5 minutes with a gas mixture of 80% N₂ and 20% CO₂. Abiotic controls were prepared by placing uncoated chromatography paper strips of identical dimensions into Balch tubes containing the same volume of BG11 medium. The paper strip was placed into the tube with the coating 8 cm above the liquid surface (Fig. 2.2A).
Figure 2.1 Extrusive latex coating method. 50 µL of a homogeneous 50/50 (V/V) suspension of cyanobacteria cells and latex binder are aspirated into a 200 µL pipetter tip. A formulation bead is formed by partially expelling part of the formulation from the pipet tip. Cell formulation is applied to demarcated surface of sterile 3MM chromatography paper. The pipet tip does not touch the paper. The latex formulation flows (self-levels) on the paper surface. The process is repeated on a different location while cell formulation is deposited by droplets and allowed to self-level. The final 1.54 cm² coating is ready to be transferred into Balch tube bioreactor.
Figure 2.2 Evaluation of CO₂ absorption by cyanobacteria in response to illumination by a solar simulator. A: Hydrated paper coatings of cyanobacteria in Balch tube bioreactors. B: Solar simulator schematic. The light beam coming from a 300W Xenon lamp is directed to the coating samples using a series of internal and external mirrors. A reflective shield was built around the solar simulator in order to keep UV radiation from escaping to the outside. Temperature inside the shield was controlled by a variable-speed fan.

The entire chromatography paper was completely hydrated by wicking liquid from the bottom of the tube to the top of the paper. The coatings remained adhesive and stable during paper hydration. Visual inspection suggested limited latex coating penetration into the chromatography paper substrate. All tubes were then vented using a water trap back to 1 atm. All coatings were incubated under cool fluorescent light with 100 μmol photons m⁻² s⁻¹ illumination without shaking at 25°C unless they were evaluated using a modified solar simulator (see below). Long-term continuous CO₂ capture and O₂ production was achieved
by flushing the photobioreactors with the gas mixture as soon as CO₂ was depleted from the headspace as indicated by gas chromatography analysis of headspace gas composition. This flushing procedure was periodically repeated for a total incubation time of 500 hours with no additional BG11 medium addition to the tubes.

2.2.4 The effect of temperature and light intensity on coating reactivity

The effect of temperature and light intensity was studied with *Synechococcus PCC7002* coatings prepared using the same procedure described in the previous sections. Tubes containing coatings and BG11 media were incubated in a controlled environment chamber at 5 different temperatures in the range 25-45°C with constant 100 μmol photons m⁻² s⁻¹ illumination for 6 hours. For the effect of light intensity, coatings were incubated in a solar simulator (M-9119, Newport, Irvine, California) equipped with a 300W Xenon lamp (85% emission 400-700nm with discrete peaks in the UV and near-infrared range), a light intensity meter (LI-COR, LI-190SA Quantum Sensor, Lincoln, NE) and modified with a temperature probe (Dickson, TM125 probe, Addison, IL), mirror (Conair, Stamford, CT), and a variable speed fan (Sunbeam, Boca Raton, FL) at light intensities in the range 50-2,000 μmol photons m⁻² s⁻¹ PAR with a constant temperature of 35°C at pH 5.5 for 6 hours (Fig. 2.2B).

2.2.5 Chlorophyll a extraction from coatings

Chlorophyll a was extracted from the latex coating-entrapped cells using 90% acetone. Intact 14 mm diameter coatings were cut from the chromatography paper strip and submersed in 1.5 mL of 90% acetone. Extraction was performed during 30 minutes in the dark at room
temperature and tubes were centrifuged at 15,000 RPM for 10 min. All supernatants were sampled for absorbance at 664, 647, 630 and 750 nm using a Genesys 20 UV-Vis spectrophotometer (Thermo Electron Corporation, Marietta, OH). The process was repeated three times and the supernatants were pooled into a single extract. The amount of chlorophyll a (μg/L) extracted from each coating was calculated as:

\[
Chl_a \left( \frac{\mu g}{L} \right) = \frac{11.85 \cdot (Abs_{664} - Abs_{750}) - 1.54 \cdot (Abs_{647} - Abs_{750}) - 0.08 (Abs_{630} - Abs_{750})}{\rho \cdot V_e} \]

Where \(Abs_{664}\) is the supernatant absorbance at 664 nm, \(Abs_{647}\) is the supernatant absorbance at 647 nm, \(Abs_{630}\) is the supernatant absorbance at 630 nm, \(Abs_{750}\) is the supernatant absorbance at 750 nm (turbidity correction), \(V_e\) is the volume of ethanol extract (mL), \(V_s\) is the sample volume (L), and \(\rho\) is the length of the cuvette parallel to the light path.

2.2.6 Intrinsic kinetics in illuminated suspension cultures with growth limited by nitrate

To compare the reactivity of suspended, illuminated non-growing cells and cells coated on chromatography paper under non-growth conditions, we modified the formulation of the BG11 media by decreasing the concentration of NaNO₃ 10-fold from 17.6 mM to 1.76 mM to arrest cell growth while maintaining metabolic activity. All other media components and concentrations were kept identical.

Cell slurries of Synechococcus PCC7002 were prepared as previously described and washed twice with 45 mL of low-nitrogen BG11. From the same batch of slurry, we extruded
paper coatings and inoculated suspension cultures in triplicate with the same volume of formulation (75 µL). Coatings were hydrated in 10 mL of low-nitrogen BG11, which was the same total volume used for the suspension tubes. Coatings were incubated at 100 μmol photons m⁻² s⁻¹ illumination without shaking at 25°C, while suspension cultures were kept well mixed by mounting the tubes in a shaker (Labquake®, Labindustries Inc., Berkeley, California).

2.2.7 Headspace Gas Analysis

A Hewlett Packard 7890A gas chromatograph containing a Supelco 6’ x 1/8” ID 60/80 mole sieve 5A porous mesh polymer packed stainless steel column and a thermal conductivity detector was used for headspace analysis of H₂, O₂, N₂ and CO₂. Argon was used as the carrier gas at a flow rate of 39 mL/min with injector/oven/detector temperature settings of 160º/160º/250ºC, respectively.

2.2.8 Coating imaging and microstructure

Hydrated chromatography paper coating 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 MellesGriot. Albuquerque, New Mexico, USA). Cyanobacterial natural pigment fluorescence was detected at wavelengths greater than 650 nm (excitation at 488 nm) while paper fiber autofluorescence was visualized at 530 nm (excitation around 400 nm). Samples were imaged using the
green/red channels simultaneously. A stack of 65 CLSM images 2.5 µm apart in the z direction perpendicular to the paper plane were taken to a total depth of ~165 µm into the paper. Image J 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.

Coating microstructure was studied by scanning electron microscopy (SEM) using a Hitachi 3200-N Variable Pressure Scanning Electron Microscope equipped with a 4Pi Isis EDS system for digital image acquisition. 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 surface topography, cell distribution and available pore space. Samples were sputter coated prior to imaging with a thin layer of gold in a mild vacuum (~100 mTorr of Ar gas pressure; 600 V accelerating voltage) for 5 minutes and immediately placed in the SEM vacuum chamber for analysis. Cross-sectional images were obtained by freezing dry coatings in liquid nitrogen and manually cryo-fracturing with a frozen sharp blade while submerged in the liquid nitrogen. These samples were mounted in a cross-sectional sample holder and sputter-coated with gold using the procedure previously described.

2.3 Results and Discussion

This is the first report of latex coatings of *Synechocystis PCC6308*, *Synechocystis PCC6803*, and *Anabaena PCC7120* that retain photosynthetic reactivity for over 500 hours as measured by CO₂ consumption and O₂ evolution when supported by hydrated paper in the gas-phase.
Preliminary reactivity data for *Synechococcus PCC7002* in hydrated gas-phase latex coatings on paper was previously reported by our group\(^3\). All coatings (prepared in triplicate) with the four test strains of cyanobacteria were reactive when incubated in the headspace of the Balch tube miniature bioreactors (Fig. 2.3A,B).

The three unicellular strains were significantly more reactive compared to the filamentous *Anabaena PCC7120*. All organisms showed nearly constant photosynthetic quotient (PQ, molar rates of O\(_2\) production/CO\(_2\) consumption) during the time interval studied. No apparent reactivity decrease (a plateau in a cumulative oxygen production/carbon dioxide consumption plot) was observed during 500+ hours of continuous illumination. *Synechococcus PCC7002* is the most reactive of the cyanobacteria strains tested (Table 2.1), yielding a cumulative CO\(_2\) uptake rate of 5.67 mmol-m\(^{-2}\)-h\(^{-1}\). Under similar experimental conditions, CO\(_2\) uptake rate by plant leaves of wild type *Arabidopsis thaliana* has been reported as 18 mmol-m\(^{-2}\)-h\(^{-1}\). We use this *Arabidopsis* value as a reference because paper coatings are a model of the functionality of natural plant leaves. Further increasing the cyanobacteria biomass concentration in our coatings, multi-layered coatings, and improving the coating method and microstructure will likely lead to coatings of cyanobacteria that surpass the photosynthetic performance of *Arabidopsis* leaves.

In order to further investigate the metabolic characteristics of the strains tested, the photosynthetic quotient (PQ) was calculated as a function of time\(^5\) (Fig. 2.4). The average PQ value for the three freshwater unicellular strains is ~1.3, while the filamentous freshwater strain average PQ is ~1.4 after an initial lag period following immobilization with very low oxygen production.
Figure 2.3 Long-term photoreactivity of hydrated paper coatings. A: Cumulative oxygen production by hydrated paper coatings of cyanobacteria in Balch tube bioreactors. B: Cumulative carbon dioxide consumption by hydrated paper coatings of cyanobacteria in Balch tube bioreactors. Error bars ± 1 Std Dev, n=3
Table 2.1 Average O\textsubscript{2} production and CO\textsubscript{2} consumption rates by 1.54 cm\textsuperscript{2} paper coatings of cyanobacteria. n=3, ± 1 StdDev

<table>
<thead>
<tr>
<th>Organism</th>
<th>O\textsubscript{2} average production (mmol m\textsuperscript{-2} h\textsuperscript{-1})</th>
<th>CO\textsubscript{2} average consumption (mmol m\textsuperscript{-2} h\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synechococcus PCC7002</td>
<td>7.05 ± 0.29</td>
<td>5.67 ± 0.29</td>
</tr>
<tr>
<td>Synechocystis PCC6308</td>
<td>6.58 ± 0.30</td>
<td>5.13 ± 0.23</td>
</tr>
<tr>
<td>Synechocystis PCC6803</td>
<td>4.91 ± 0.10</td>
<td>3.70 ± 0.18</td>
</tr>
<tr>
<td>Anabaena PCC7120</td>
<td>1.66 ± 0.11</td>
<td>1.18 ± 0.09</td>
</tr>
</tbody>
</table>

Figure 2.4 Photosynthetic quotient (PQ) as a function of time by hydrated paper coatings of cyanobacteria in Balch tube bioreactors. Error bars ± 1 Std Dev, n=3

When nitrate is present as the sole nitrogen source, it is reduced to ammonium to become available for assimilation into organic nitrogen compounds. This process produces
two molecules of oxygen per each nitrogen atom assimilated, and the energy required to drive this reaction comes from water photolysis (reducing the amount of energy available for CO$_2$ fixation). No further reduction is necessary if ammonium was the nitrogen source – hence no additional oxygen would be produced - which explains the higher PQ values observed under nitrate-rich conditions$^{57}$. Because nitrate is the nitrogen source in BG11 media, the formulation can be modified for non-growth conditions by reducing the nitrate supply in the media up to levels that maintain cellular metabolic activity but arrest cell growth. Since the unicellular strains can be more conveniently counted, have higher photosynthetic rates and lack a post-immobilization lag period, we investigated *Synechococcus* and *Synechocystis* for intrinsic kinetics studies.

The effect of temperature on photosynthetic rates in microalgae is rarely reported. Furthermore, the effect of temperature on photoreactivity for surface-immobilized photosynthetic biomass has not been characterized. We found that coatings of *Synechococcus PCC7002* had the highest cumulative oxygen evolution with a temperature optimum for CO$_2$ fixation/O$_2$ production of approximately 30°C producing 23.87 ± 3.86 x 10$^{-3}$ pmol O$_2$·cell$^{-1}$·h$^{-1}$ (702.1 ± 24.3 µmol O$_2$·mg Chl$_a$·h$^{-1}$) at a constant light intensity of 100 µmol m$^{-2}$·s$^{-1}$ and a final average pH 5.7 ± 0.2 in the liquid phase for all coatings tested (Fig. 2.5).
Less than $5 \times 10^3$ cells mL$^{-1}$ were present in the liquid phase at the end of the experiment as measured by hemocytometer counting ($>0.03\%$ of the total immobilized biomass). This optimum temperature for coatings (30°C) is significantly lower than the 38°C optimum growth temperature reported for this strain in suspension cultures$^{58}$. It should be noted that these two temperature optima refer to two different physiological processes: cell growth and non-growth gas-phase photoreactivity as coatings. No optimum temperature for photosynthesis has been reported for *Synechococcus PCC7002*, but a reference value at 12°C was reported for *Chlorella*.$^{59}$ Ludwig and Bryant found that genes coding for enzymes involved in CO$_2$ fixation (RuBisCO, carboxysome, carbonic anhydrase, Na$^+$ bicarbonate transporter) are up-regulated at lower temperatures, which might explain the higher reactivity
we observed at 30°C. Even though enzymatic reactions should be slower when the temperature is reduced, CO₂ solubility also increases at low temperatures, which suggests that there is a trade-off in which *Synechococcus* tightly regulates the CO₂ concentration mechanism in response to the concentration of CO₂ in the media in order to avoid over-production of carbon fixation enzymes. At higher temperatures, there are also heat shock responses that have been detected as an increase in the transcripts of genes coding for chaperones that repair thermally damaged proteins. Genes involved in photosynthesis, CO₂ fixation, O₂ evolution, glycolysis and normal metabolic processes are all down-regulated as temperature increases, which in combination with the decreased solubility of CO₂ at high temperatures may explain the observed drop on gas-phase photosynthetic rates.

The specific chlorophyll alpha content of *Synechococcus* cells did not change significantly over the course of this 6-hour experiment (averages of 0.049 ± 0.005 pg Chlₐ·Cell⁻¹ at t = 0h vs. 0.045 ± 0.007 pg Chlₐ·Cell⁻¹ at t = 6h for all tubes tested), but it has been reported that genes that control degradation of accessory pigments such as phycobiliproteins can be up-regulated in response to heat-shock as a means to obtain alternative carbon under unfavorable conditions.

Limited data is available on the effect of light intensity on O₂ production for *Synechococcus* PCC 7002. An optimal light intensity for growth at 250 μmol photons m⁻² s⁻¹ has been reported for suspension cultures, but no saturation value of light intensity for gas-phase surface-immobilized O₂ production was reported. For *Anacystis nidulans*, photosaturation is reached at 250 μmol photons m⁻² s⁻¹, following a trend closely related to nitrogen assimilation from nitrate. In this study, we found that latex-immobilized
**Synechococcus PCC7002** cells exhibit an optimal oxygen production rate of 12.61 ± 1.83 x 10⁻³ pmol O₂·cell⁻¹·h⁻¹ (370.9 ± 10.2 µmol O₂·mg Chl a·h⁻¹) at a constant temperature of 35°C, 100 µmol photons m⁻²·s⁻¹ and final pH 5.6 ± 0.3 for all tubes tested (Fig. 2.6). Less than 5 x 10³ cells mL⁻¹ were present in the liquid phase at the end of the experiment as measured by hemocytometer counting (<0.03% of the total immobilized biomass) and no significant photobleaching was observed after 6 hours of incubation (0.037 ± 0.007 pg Chl a·Cell⁻¹ at t = 0h vs. 0.035 ± 0.009 pg Chl a·Cell⁻¹ at t = 6h, average for all tubes tested). Interestingly, the kinetics of oxygen evolution for coated-cells with continuous illumination does not resemble the characteristic saturation profile commonly reported for growing suspensions of microalgae⁶³. The O₂ evolution rate at 50 µmol photons m⁻²·s⁻¹ is low but responds quickly to a moderate increase in light intensity up to 100 µmol photons m⁻²·s⁻¹ when it reaches its maximum value. If light intensity is further increased, O₂ evolution decreases rapidly and is inhibited at an intensity of 500 µmol photons m⁻²·s⁻¹.

This is an unexpected result given that **Synechococcus PCC7002** has been reported to be extremely tolerant to high light irradiation⁶⁴ due to the protective action of heme-copper oxidases⁶². Saturation of the photosystems in coatings of **Synechococcus** at lower light intensity might be a consequence of the spatial distribution of highly concentrated cells on the surface of the paper. In suspension cultures and photobioreactors, individual cells are constantly being mixed so they are never exposed to uniformly high illumination due to light scattering and considerable self-shading, which may result in an observed “tolerance” of the cell population to higher light intensities given that the cells have a chance to repair their photosystems in the time period they are not exposed to the maximum light intensity.
Figure 2.6 Light intensity dependence of specific oxygen production rate (qO₂) by hydrated paper coatings of *Synechococcus* PCC7002. Error bars ± 1 Std Dev, n=3

When coated onto paper and exposed to constant illumination, cyanobacteria remain in a spatially-fixed orientation, there is no mixing, and therefore each cell is exposed to continuous high light intensity. The only barriers are the thin liquid layer covering the cells and any polymer layers or any cellulose fibres on top of those cells (cells that are immobilized deeper in the paper matrix). It is well known that freshwater cyanobacteria adjust their position in ponds and lakes during the day in order to expose themselves to the optimum light intensity and avoid photosaturation, a process that is not possible in an adhesive coating and might be responsible of the relative intolerance to high light intensities we observed. However, by further development of this method, coating microstructure, light
scattering and shading properties of cells spatially-fixed in multiple layers can be optimized along with photo pigment content to reduce photosaturation\textsuperscript{37}.

It has been reported that microbial nanoporous adhesive latex coatings of microorganisms are several fold more reactive than the same concentration of cells in suspension\textsuperscript{7} when deposited over non-porous polyester substrates. We observed a significant increase of the reactivity of cyanobacteria after immobilization by preparing porous latex paper coatings of three unicellular freshwater cyanobacteria strains and cell suspensions with exactly the same amount of biomass. Both cell coatings and suspensions were incubated under identical conditions of temperature and illumination with shaken suspensions. The cells were growth–limited by nitrate and the absence of growth as confirmed by hemocytometer cell counting (Fig. 2.7). The amount of nitrate in BG11 media was decreased to a tenth of the original concentration. Under these nitrogen-limited conditions cyanobacteria in latex paper coatings are significantly more reactive compared to suspension cultures as measured by oxygen production (Table 2.2 and Fig. 2.8A).

For comparison, we define a Photosynthetic Intensification Factor (PIF) as the ratio of \( \text{O}_2 \) production by nitrate-limited cyanobacteria in latex coatings divided by \( \text{O}_2 \) produced by nitrate-limited cell suspensions (Fig. 2.8B). Due to the very low oxygen evolution reactivity of suspension cultures during the first 120 hours of incubation, the PIF during this interval increases almost exponentially for two of the strains tested. Paper coatings of cyanobacteria are very reactive immediately after rehydration, and do not go through the lag period observed by the suspension cultures. For \( t > 120 \) hours, all three strains reached steady PIF values. The onset of this steady period coincided with the end of the lag period in the
suspension cultures and lasted for 70 hours until pigment bleaching was detected in the suspended cells tubes.

Figure 2.7 Arresting cell growth using nitrate-limited (NL) BG-ll media. Cyanobacteria cell density counted by hemocytometer in BG11 and nitrate limited BG11 media. Cells do not divide but remain metabolically active under non-growth conditions. Error bars ± 1 Std Dev, n=3

Table 2.2 Specific average O₂ production rates (qO₂) by 1.54 cm² paper coatings and suspension cultures of cyanobacteria. n=3, ± 1 StdDev

<table>
<thead>
<tr>
<th>Organism</th>
<th>Immobilized O₂ average production x 10³ (pmol.cell⁻¹.h⁻¹)</th>
<th>Suspension O₂ average production x 10³ (pmol.cell⁻¹.h⁻¹)</th>
<th>PIF O₂ Immobilized/O₂ Suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synechococcus PCC7002</td>
<td>55.4 ± 9.25</td>
<td>5.81 ± 1.09</td>
<td>10.0 ± 2.85</td>
</tr>
<tr>
<td>Synechocystis PCC6308</td>
<td>57.2 ± 10.3</td>
<td>8.22 ± 1.83</td>
<td>8.34 ± 2.32</td>
</tr>
<tr>
<td>Synechocystis PCC6803</td>
<td>40.9 ± 8.36</td>
<td>5.55 ± 3.04</td>
<td>8.05 ± 3.58</td>
</tr>
</tbody>
</table>
Figure 2.8 Photoreactivity Intensification by paper coatings. A: Immobilized biomass oxygen evolution compared to the oxygen evolution of suspended cells by freshwater unicellular cyanobacteria strains. B: Variation of Photosynthetic Intensification Factor (PIF) as a function of time by Synechococcus PCC7002, Synechocystis PCC6308 and Synechocystis PCC6803. Error bars ± 1 Std Dev, n=3
The detailed microstructure of latex paper coatings of several strains of cyanobacteria was determined using cryo-SEM and fluorescent confocal microscopy. We exploited the natural autofluorescence of both the chromatography paper and the cyanobacteria for assessing the distribution of the cell coating on the fiber matrix, while SEM imaging provides higher magnification detail of the alignment of the cells and the distribution of the latex binder. Uncoated chromatography paper (Fig. 2.9A) has an open-pore structure with a broad distribution of flattened cellulose fiber width and thickness that offers high surface area (about 2 m² g⁻¹). SEM images with greater magnification reveal vein-shaped topographic features on the surface of the fibers (Fig. 2.9F). This surface topography combined with the microporous structure of the paper is responsible for the chromatography properties. The pore structure formed by the cellulose fibers on paper coated with latex binder + *Synechococcus PCC7002* (Fig. 2.9B) appears altered by the latex + cyanobacteria coating with open pores still visible between fibres for liquid transport from the bulk liquid phase to the pore-space liquid, and gas diffusion to the cells through the thin liquid layer in contact with the headspace. *Synechococcus* appears to randomly align in the same direction as the paper fibres during the wet coalescence process (Fig. 2.9G) forming sheet-like structures linking adjacent fibres. The cell distribution in the coatings may be related to the directionality of the paper fibre matrix. Latex flow occurs prior to film formation resulting in unequal distribution of polymer on and within the paper matrix. There is evidence of cell movement into deeper layers of the paper during latex film drying, as suggested by the cyanobacteria-shaped void spaces observed on the surface of the coating. These structures may be cells flowing along with the bulk latex formulation into voids in the surface prior to
polymer particle coalescence. *Synechocystis PCC6308*, a strain very similar in shape and size to *Synechococcus PCC7002*, shows a similar behavior when coated. The paper surface (Fig. 2.9C) shows a non-homogeneous distribution of coating formulation, which is a consequence of the manual extrusive coating deposition method which relies on self-leveling of the latex-cell suspension prior to film formation. The self-leveling properties of waterborne latex emulsions during drying can be tailored by emulsion composition and has recently been reported to be altered by bio-generated surfactants in a coating during drying by incorporation of *Pseudomonas aeruginosa* into colloidal polymer systems. Using this simple manual extrusion method for coating latex emulsions + cyanobacteria, some fibers appear to be completely coated with *Synechocystis PCC6308* cells while others show the rough surface observed for SF012-coated paper. More uniform coatings can be achieved by altering the coating method, manipulating the emulsion composition or using ink-jet printing techniques.

A closer examination of the orientation of the latex-entrapped cyanobacteria (Fig. 2.9H) indicates an alignment in the direction of the cellulose fibres and coating of a portion of the paper pore spaces beyond the surface of the paper matrix, very similar to the behavior of *Synechococcus PCC7002* in the same porous substrate. Nanoporous latex coating microstructure, porosity and properties are altered by the size and shape of the embedded cells. For *Synechocystis PCC6803*, which is approximately half the size of the two previous strains, the coating appears to not block a large fraction of the available pore space as revealed by confocal imaging (Fig. 2.9D).
Higher magnification of the grooves of individual fibres (Fig. 2.9I) shows both cells covered by polymer and void spaces (i.e. “tracks”) left by cells flowing along with the latex formulation into the grooves prior to polymer particle coalescence. The size and distribution of the pores in this *Synechocystis PCC6803* coating suggests that some of the latex binder formulation on the paper fiber matrix migrated into deeper layers of the matrix prior to polymer particle coalescence and adhesion. This is also supported by the smaller *Synechocystis PCC6803*. *Anabaena PCC7120* forms long chains, sometimes involving hundreds of individual cells, which should increase the overall cohesiveness of the formulation when mixed with the latex binder. *Anabaena* coatings appear as a continuous sheet covering most of the available pore space on the surface of the paper (Fig. 2.9E), and intact chains follow the topographic profile of the paper. Chains appear to have been dried above and under individual cellulose fibers, but do not appear to follow the directionality of the fibres. Instead, adjacent fibers appear to be pulled together to form small two-dimensional regions where the fibre matrix is homogeneously covered by *Anabaena* (Fig. 2.9J). Little or no evidence of cell sedimentation into the paper was observed in *Anabaena* coatings. All micrographs also feature regions of the chromatographic paper coated only with the latex binder SF012. In this case, much of the porosity of the paper is preserved which suggests that paper fibres are coated individually rather than bulk-coated as a uniformly coalesced layer filling the pore space. A closer look at individual SF012-coated cellulose fibres revealed extensive roughness generated by the ~300nm SF012 partially coalesced latex particles covering the fibre surface.
Cross-section, cryo-fracture SEM images of a *Synechococcus PCC7002* coating revealed penetration of the coating into the paper matrix. At low magnification most of the cell-latex formulation appears to be covering the surface of the paper forming a coating ~20 µm thick. However, what appear to be latex formulation aggregates can be observed in the paper matrix up to 250 µm below the surface, which is a consequence of the random distribution of the coating on the paper fibres (Fig. 2.10A). A layer of cyanobacteria and partially coalesced SF012 particles covers the irregular surface of the chromatographic paper, with some areas being thicker due to irregular leveling (flow) of the polymer prior to film formation and drying. In these areas some of the cyanobacteria move with latex binder prior to film formation and are entrapped underneath the coating surface leaving a void on the surface (Fig. 2.10B). The same phenomenon applies for those aggregates immobilized between fibres in deeper layers of the paper matrix where cells sediment before film formation (Fig. 2.10C). Comparison with the uncoated chromatography paper (Fig. 2.10, Control) reveals only partial closure of the longitudinal pore space in the paper matrix by the cell-latex formulation. However, abundant open pore space is visible underneath the latex coating and between formulation aggregates due to the paper thickness (322 ± 16 µm).²⁷

Both the observed microstructure and the reactivity suggest new hypotheses are needed for explaining the observed reactivity of latex-coated photosynthetic cyanobacteria extruded onto paper. High-resolution images confirmed the non-homogeneous, yet fixed distribution of *Synechococcus PCC7002* on the surface of the chromatography paper, while cross-sectional images show irregular penetration of the coating formulation into the fibre matrix.
Figure 2.10. Cross-section cryo-fracture scanning electron microscopy image of a latex paper coating of *Synechococcus PCC7002*. A. Magnification 250x (scale bar 100μm). B. Detail of the coating surface. Magnification 1000x (scale bar 20μm). C. Detail of the internal paper matrix. Magnification 2500x (scale bar 10μm). Insert: *Synechococcus PCC7002* cell at same magnification for reference.

Analysis of SEM and confocal images suggests several possible cell-latex-fibre motifs on and within the paper matrix, each featuring different implications for the photo and gas adsorbing/evolving reactivity of the biocomposite. In the first case, due to the non-uniform extrusion coating method, there are regions of the paper that are not uniformly
coated resulting in loss of a portion of the available surface area for solar energy trapping (Fig. 2.11A).

**Figure 2.11** Diagram of observed coating microstructure variations from SEM images. A. SF012 coated paper. B. Cell “sheets” between paper fibers. C. “Sinking” cells between paper fibers. D. “Buried” cells on paper fibers. E. “Exposed” cells on paper fibers.
Biocomposite coating uniformity can be improved by using more sophisticated methods for coating nonwovens (i.e. spin coating, roll printing, spraying, convective sedimentation assembly, ink-jet printing) that precisely control the deposited volume, thinness and the formulation distribution on the substrate. Cells can also span a portion of the available micropores on the surface of the paper forming “sheets” in which neighboring cells are linked by latex particles between paper fibres (Fig. 2.11B). In this case, additional photoreactive surface area is created by partially filling some of the paper pores with reactive cells while preserving nonwoven matrix porosity to allow for gas absorption and liquid hydration. These larger pores may function similar to leaf stomata for temperature regulation and gas exchange. Future improvement of the paper coating method should precisely control the latex deposition and adhesive polymer particle coalescence in order to avoid filling or blocking of the available pore space between the cellulose fibers. Interestingly, micropores appear also to be created by cells flowing with the latex binding from the coating surface during the film formation process (Fig. 2.11C), a phenomenon that could be further studied in order to control the final porosity of the biocomposite. Latex formulation additives such as carbohydrates that arrest the polymer particle coalescence process or that modify the viscosity of the latex binder could be used to optimize coating microstructure. With respect to the spatial location of cyanobacteria immobilized on the surface of paper fibres, cells can be either “buried” in polymer (covered by a layer of latex binder) or glued to the cellulose fibres from below without being covered by polymer following the directionality of the paper fibres (Fig. 2.11D & 2.12E). When cyanobacteria are covered by a layer of partially-coalesced latex binder, another barrier for mass transfer and light penetration is created, so it
is desirable to reduce as much as possible any residual polymer on top of the cell layer. Ideally, the microstructure of a highly reactive coating would be where the available paper surface area is entirely covered by a thin coating of photoreactive cells (one to several cells thick) secured to the cellulose fibres by a small amount of adhesive polymer particles surrounding or below the cells.

Our observations suggest that the majority of the latex-immobilized microalgae are exposed to a constant light intensity during continuous illumination, which results in cyanobacteria reaching photo saturation at lower light intensity. Surprisingly, we observe no light saturation profile for paper coatings of cyanobacteria suggesting a decrease in the overall tolerance of the biocomposite to higher light intensities. At low light intensities, latex-entrapped cyanobacteria in deeper layers of the paper (<250 µm in depth) may be light limited, which might explain the low overall paper reactivity observed at 50 µmol photons m⁻² s⁻¹.

Different cyanobacteria strains may be affected by the degree of latex polymer particle wet coalescence during film formation. Diffusivity data for a similar acrylate/vinyl acetate latex binder previously characterized in our group (SF091, Rohm and Haas) revealed a strong dependency of coalescence on the polymer glass transition temperature T_g, the drying and rehydration temperature, drying, relative humidity, and the surface chemistry of the polymer particles. The degree of polymer particle coalescence defines the final nanoporosity, optical properties of the coating, and the permeability to gas transport which may also affect the response of the entrapped cyanobacteria to temperature and light intensity. For this reason further characterization of the nanoporosity generated by wet
coalescence on paper and optimization of both the coating method and latex binder formulation are necessary.

What remains to be understood in detail are the changes in cyanobacteria physiology that take place at the molecular level when cells are starved for nitrogen in a non-growth or resting state in suspension, on a surface or in a biocomposite. Only very recently McKinlay and collaborators reported a mechanism that explains the significantly higher H₂ yield of on non-growing N₂-starved cultures of Rps. palustris. By using a combination of global transcriptome and biomass composition analysis along with ¹³C labeled acetate tracking with simulations of altered pathway flux they found that non-growing N₂ starved Rps. palustris shift their metabolism to use the tricarboxylic acid cycle to more efficiently metabolize acetate and generate more reducing power for hydrogen production while growing cells use the glyoxylate cycle exclusively. This recent discovery points towards future genetic manipulation of “self-tuning” mechanisms of metabolism in response to nutrient starvation to alter the incorporation of a carbon source and metabolite flux in non-growing cells in detriment to biosynthetic processes related to cell growth. We believe this type of adaptation by cyanobacteria at the physiology level combined with the intrinsic reduction of self-shading associated with surface immobilization may result in the significant enhancement of specific photosynthetic rate we observed. More studies at the molecular level on non-growing cyanobacteria physiology will be necessary to confirm this hypothesis.

Paper engineered for minimal acrylate latex binder penetration during film formation without blocking of the paper pores, significant mechanical strength when wet, and optimized optical properties (e.g. brightness, reflectance) may further improve use of paper
as cost-effective nonwoven porous material to support cellular composites for light harvesting and CO$_2$ recycling. Paper-based microfluidic devices have been reported for detection of a wide variety of analytes$^{71,72}$ and for sensing devices when combined with nanostructured materials$^{72,73}$. We foresee coated cellular biocomposites incorporating a microfluidic vascular network to be the next step in engineering biomimetic materials inspired by the plant leaf. These inexpensive flexible composites will have many applications as intensified cellular biosensors, as biomimetic leaves, microbial photo fuel cells, biocatalysts and highly reactive gas or liquid biofilters. The open paper pore structure observed using a low T$_g$ latex binder emulsion and concentrated cyanobacteria cell paste presents a wide range of possibilities for fabricating nonwoven materials that combine engineered photoreactive microorganisms with a porous substrate that provides structural support, a pore network for nutrient and product transport, waste removal, and also functions as a separation device for products secreted by the cells.

### 2.4 Conclusions

Paper is an inexpensive, flexible, porous nonwoven model substrate for supporting latex coatings of photoreactive cyanobacteria in the gas-phase to function as biosolar absorbers or biomimetic leaves, absorbing CO$_2$ and producing O$_2$. We coated four different cyanobacteria strains with diverse morphologies and demonstrated that this simple method of latex binder coating by extrusion is capable of intensifying up to 10 fold the specific photoreactivity of suspended cyanobacteria in non-growth media designed from PQ data. The photosynthetic rates measured from these coatings are very close to those reported in the literature for
Arabidopsis thaliana. Considering that neither the cyanobacteria strains tested or the biocomposite paper microstructure have been optimized, this level of photoreactivity is remarkable and encourages further development of methods to concentrate and coat multiple organisms together to fabricate model highly reactive leaf-like biocomposites with a flexible microfluidic vascular system.

Our results also suggest new correlations between cyanobacteria physiology and biocomposite microstructure. Coating microstructure images provide evidence of partial pore filling and directional immobilization of some strains of cyanobacteria. Intensification of photoreactivity could be further improved by increasing the uniformity of the coating on all cellulose fibers, reducing coating thickness, and controlling latex emulsion flow during drying. By using a solar simulator, we observed significant changes in light intensity and temperature tolerance for nitrate-limited latex-coatings of Synechococcus PCC7002 compared to suspended cells. This is a motivation to further investigate the physiological changes that take place after latex entrapment onto the surface of paper and other nonwoven porous supports. This coating method can be used to fabricate multi-organism coatings that harvest light in a broader region of the light spectrum, or to fabricate inexpensive cellular composite devices that require little more than water and light to sustain long-term photoreactivity.

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CHAPTER 3

Microbial paper: the fiber-based photo-absorber

Oscar I. Bernal, Joel J. Pawlak and Michael C. Flickinger

A version of this chapter will be submitted to

Cellulose
Abstract

Synthetic biology has the potential to engineer photoreactive microbes to have significant stability advantages over isolated enzymes and synthetic photovoltaic materials that harvest sunlight, absorb CO₂, produce O₂, volatile carbon products or H₂. We report the microstructure and reactivity of a novel nonwoven cellulose fiber cellular biocomposite (microbial paper) for long-term stabilization of potentially any type of microorganism. Cells are incorporated during the paper making process as an integral component of a highly porous cellular biocomposite that can be stabilized dry. When rehydrated, the paper pores function as a transport network to keep the entrapped cells hydrated with nutrients, eliminate waste materials and separate secreted products. Hydrogen gas production from acetate by the activity of the nitrogenases in *CGA009 Rps. palustris* entrapped at very high concentration in hand-made microbial paper can be sustained for >1000 hours at a rate of 4.00 ± 0.28 mmol H₂ m⁻² h⁻¹ following rehydration (a rate that is 2x and 10x greater than previously reported H₂ production rates by *Rps. palustris* latex coatings that were dried on polyester and non-dried formulations applied to the surface of paper respectively). By adding vacuum-dewatering and controlled drying steps to the microbial paper making process and incorporating blends of microfibrillar (MFC), soft wood (SW) and hard wood (HW) cellulose fibers we fabricated microbial paper films that produce H₂ gas at 3.94 ± 1.07 mmol H₂ m⁻² h⁻¹ and retain up to 60 mg m⁻² DCW of *Rps. palustris*. MFC content appears to determine the final cell load in the film and may affect gas/moisture mass transfer and optical properties capabilities of the film. SEM images of rehydrated microbial paper film microstructure reveal the distribution of concentrated cells on and between paper fibers that do not clog the pore space, which allows
for perfusive flow through the cellulose fiber matrix. Engineering optimized pulp blends, additives and fiber surface modification methods to generate even more stable cell-cellulose fiber binding will enable intensification and stabilization of other types of cells in cellulosic or cellulose-polymer composite non-woven supports. This cellular biocomposite non-woven paper-based material can be engineered in the future as cost-effective biophotocatalysts for cleaning/absorbing greenhouse gases or efficiently absorbing solar energy that combines intensification, uniform illumination, stabilization of reactivity when dried and separation capabilities for applications in biosolar energy.

3.1 Introduction

Immobilizing a high number of viable and reactive microorganisms on a surface is a simple and inexpensive approach to functionalize catalytically inert materials and intensify bioreactivity several fold. However, if the supporting material is porous, there is an inherent limitation associated with most cellular biocoating methods given that only the most superficial layers of the substrate surface are effectively utilized for immobilization of micron-sized cells, wasting most of the available internal submicron scale pore space and surface area for biocatalyst binding. This intrinsic drawback can be overcome by developing methods that incorporate the biocatalytic biomass during the early stages of substrate manufacture in contrast to as a finishing treatment applied superficially to a pre-made immobilization matrix. This reasoning prompted us to revisit the fabrication method of porous paper as a support for photosynthetic cells to adapt it for the production of cellular
biocomposites. It does, however, introduce potential limitations of diffusion path length, light self-shading and light scattering.

Paper has been present in human history since its recorded existence in China by 200 BCE. The original fiber sources (fishnets, clothing, hemp and bark) have been replaced by wood-derived fibers that can be produced in different sizes and morphologies. The method itself has evolved from using fixed-screen molds, couching (transferring wet paper sheets to felt surfaces) and screw-presses to modern paper machines capable of producing continuous paper strips at very high speeds. The most recent technology development is the use of nanostructured celluloses as building blocks for the development of sustainable functional materials. Cellulose nanofibrils (collectively called microfibrillar cellulose (MFC) or nanofibrillar cellulose (NFC)) have excellent mechanical properties, large surface areas and a variety of surface functional groups suitable to be used as targets for functionalization. The process to obtain cellulose micro and nanofibrils from native cellulose fibers is energy intensive (generally involving an additional refining step followed by several high-pressure homogenizations) but the resulting MFC product (microfibril aggregates, 20-100 nm in diameter and up to several microns long) is capable of imparting novel interesting optical, mechanical, thermal and biological functionalities to composite materials, including as you will see in this chapter the capability to retain high concentrations of viable microorganisms as part of the matrix while preserving viability and reactivity.

Microbial contamination is always present throughout the papermaking process and is gradually eliminated by the high pressures and temperatures used to achieve the final mechanical strength of the material. Contaminating biofilm (slime) samples collected from
the wet end of paper and board machines using recycled fiber or unbleached pulp commonly yield between $2.0 \times 10^{10} - 2.6 \times 10^{12}$ bacterial cells per gram of slime\textsuperscript{7}, and cells are usually embedded in a matrix of polysaccharides. Among these cells, aerobic bacteria are the predominant colonizers, especially members of the genus \textit{Bacillus}, with few or no yeast and molds present\textsuperscript{8,9}. In the final paperboard product, bacteria belonging to the genera \textit{Bacillus}, \textit{Paenibacillus} and \textit{Brevibacillus} are usually found in quantities from $<50$ to $250$ cfu g\textsuperscript{-1} homogenized paperboard\textsuperscript{10-12}, which shows how the paper making process drastically reduces the total viable microbial counts. More interestingly, confocal scanning electron microscopy of coated food-grade papers has showed that most of the surviving microflora accumulates at the interface between the cellulose fibers and the coating (polyethylene, mineral pigments or biodegradable polymer). Soluble nutrients and no antimicrobial compounds were found, which means that the limiting factor for bacterial proliferation in the paper was the access to free water even under conditions of extensive wetting\textsuperscript{13}.

Aside from studies on the characterization of the residual microbial contamination after the papermaking process, there are very limited reports on the deliberate incorporation of live microorganisms into a pulp suspension prior to film formation. In one example, \textit{Bacillus cereus} was used to produce handsheets of paper under controlled laboratory conditions (drying temperature $80^\circ$C) as a model system to obtain risk assessment data in cellulosic fiber-based materials\textsuperscript{14}. Under these conditions, only 5\% of the vegetative cells and spores were retained in the paper. RT-PCR has been used to detect and quantify this same organism in cardboard and paper\textsuperscript{15}. No attempt was made to alter the paper making process or to add protective substances in order to optimize the bioreactivity of the final paper. A low
number of bacilli survive the traditional paper making process when the paper is dried at 80°C. No microbial functionality or reactivity has ever been added to the paper other than to show the presence of bacilli in the final product. In a related, yet significantly different approach, Liu and collaborators were able to encapsulate individual cells of *Pseudomonas*, *Zymomonas* and *Escherichia* in individual triblock polymer fibers that remain viable for months and are able to efficiently exchange nutrients and metabolic products with the environment\textsuperscript{16}. Here, physical entrapment of the cells is achieved by electrospining a suspension of the polymer + cells, yielding low cell retention in the fibers in a lengthy and hard-to-scale process.

The microbial paper manufacture methods described in this chapter are novel, simple and inexpensive, and conceptually compatible with the current papermaking infrastructure in place (suggesting scalability). We report proof of concept reactivity and microstructure data that will open the path for a new generation of sustainable cellular biocomposite materials for diverse applications in biomanufacturing.

### 3.2 Materials and Methods

#### 3.2.1 Bacterial strain, media, and growth conditions

*Rhodopseudomonas palustris* wild type CGA009 was grown anaerobically in 160 mL glass serum bottles (Wheaton, Millville, NJ) containing 100 mL of nitrogen fixing photosynthetic Medium PM(NF)\textsuperscript{17} with an initial headspace pressure of 1 atm N\textsubscript{2}. Cultures were incubated at 30°C under 35 PAR µmol photons m\textsuperscript{-2} s\textsuperscript{-1} (LI-COR, LI-190SA Quantum Sensor, Lincoln, NE) 40 cm below two 60W clear incandescent lights without shaking. PM (NF) is composed
of (per liter): 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 \textit{para}-aminobenzoic acid, 20 mL 1 M sodium acetate, 1 mL concentrated base solution. Concentrated base solution is composed of (per liter): 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$_{24}$•7H$_2$O, 198 mg FeSO$_4$•7H$_2$O, 100 mL metal 44 solution. Metal 44 solution is composed of (per liter): 2.5 g EDTA (free acid), 10.95 g ZnSO$_4$•7H$_2$O, 5.0 g FeSO$_4$•7H$_2$O, 1.54 MnSO$_4$•H$_2$O, 392 mg CuSO$_4$•5H$_2$O, 250 mg Co(NO$_3$)$_2$•6H$_2$O, 177 mg Na$_2$B$_4$O$_7$•10H$_2$O.

\textbf{3.2.2 Hand-made microbial paper: proof of concept method}

Paper pulp was prepared by disintegration and blending of two regular bleached copier paper sheets surface coated with latex (Domtar, New Hill, NC) in 500 mL of diH$_2$O for a final solids concentration of 2% solids using a household blender (Oster, Boca Raton, FL) at maximum speed. This paper pulp was mixed with 100 mL of an OD$_{540}$ ≈ 3, 7-day old \textit{Rps. palustris} culture using three different methods: simultaneous pulping and biomass mixing by maximum-speed blending, mixing at medium speed post-pulping and manual mixing with a glass agitator post-pulping. The resulting bacteria/pulp suspension was left undisturbed for 15 min prior to wet lay formation to allow for incorporation of the biomass into the fiber matrix. \textit{Rps. palustris} microbial paper sheets were manufactured for the first time using a commercial paper making kit (Greg Markim Inc., Milwaukee, WI). A 5.5 x 8.5 in hardwood frame was set on a collection tray along with a mesh screen (Fig. 3.1).
Figure 3.1 Proof of concept method for the fabrication of hand-made sheets of *Rps. palustris* microbial paper. Numbers indicate the stages for small scale microbial paper manufacture and reactivity characterization.
The bacteria/pulp suspension was dispensed on top of the screen and distributed manually to cover the whole mesh area. Another mesh screen was put on top of the suspension along with a hard plastic cover screen sandwiching the suspension in between. The whole set-up was tilted approximately 60 degrees with respect to the tray while gently pushing on the edges and center of the plastic screen to eliminate water from the fiber suspension.

After no bulk water flow was observed coming from the edges of the tray, the wet paper matte (still sandwiched between the two paper making mesh screens) was taken out of the tray and set flat on top of paper towels. Additional paper towels were set on top of the sheet and body-weight pressure was applied using a press bar horizontally, vertically and diagonally. Finally, the two mesh screens were carefully peeled from the wet paper sheet, which was set flat on aluminum foil in an environmentally controlled chamber set at 30°C and 60% RH. Drying was carried out overnight at these conditions. The residual water content of the microbial paper after drying was not determined.

3.2.3 Microbial paper film manufacturing: vacuum dewatering method

The vacuum dewatering method allows for more precise control over the final fiber composition, the thickness and grammage of the microbial paper sheets. Non-latex coated paper pulps were prepared by hand-tearing bleached kraft soft wood (SW) and hard wood (HW) pulp sheets into approximately 2 inch squares and disintegration in a standard 2L disintegrator at 1.2% solids concentration at 3,000 rpm for 5 min\textsuperscript{18}. Final solids concentration was adjusted to 0.1% prior to storage at 4°C. Microfibrillar cellulose was prepared by
disintegration followed by grinding and PFI milling for a final solids concentration of 0.1\%\textsuperscript{19}. Pulp blends of these three fiber types were prepared by manually mixing the 0.1\% pulp slurries in a glass beaker along with 100 mL of the \textit{Rps. palustris} culture. Microbial paper films were manufactured by vacuum dewatering followed by restrained-drying. The pulp blend/bacterial slurries were manually mixed with a glass agitator to ensure proper fibril dispersion and cellular incorporation into the fiber matrix. A Büchner funnel with an 11 cm-diameter Whatman 41 filter and a metallic mesh screen was assembled on a vacuum apparatus. The filter paper was wetted and vacuum-sealed to the mouth of the funnel. The pulp/bacteria slurry was poured into the funnel (making sure it remained leveled at all times) and the vacuum was reapplied until no bulk water flow was observed coming out of the funnel discharge. At this point, vacuum was removed and the film-filter assemblies were removed from the funnel. Films were restrained dried still attached to the filter paper between plastic rings overnight at 30°C, 60\% RH in an incubator. Final films were ~60 g m\textsuperscript{-2} and 50-100\textmu m thick (measured with a rounded-tip micrometer model C112GEB; Mituyo, USA Corporation). Samples of the original \textit{Rps. palustris} cell pool, and filtrates were collected for determination of the dry cell weight to determine the retained dry cell weight in the microbial paper sheets by overnight drying of the cell pool and filtrates in an oven at 70°C.

### 3.2.4 Determination of headspace H\textsubscript{2} production

The microbial paper films were cut into 2 x 5 cm strips and placed into vertical Balch tubes containing 10 ml of the PM(NF) medium; the tubes were sealed and flushed for 5 minutes
with 100% Argon. Microbial paper films were either completely submerged in media for H$_2$ gas production experiments. All tubes were then vented using a water trap back to 1 atm. All films were incubated under cool fluorescent light with 100 μmol photons m$^{-2}$ s$^{-1}$ illumination without shaking at 25°C.

### 3.2.5 Headspace Gas Analysis

A Hewlett Packard 7890A gas chromatograph containing a Supelco 6’ x 1/8” ID 60/80 mole sieve 5A porous mesh polymer packed stainless steel column and a thermal conductivity detector was used for headspace analysis of H$_2$, O$_2$, N$_2$ and CO$_2$. Argon was used as the carrier gas at a flow rate of 39 mL/min with injector/oven/detector temperature settings of 160º/160º/250ºC, respectively.

### 3.2.6 Film imaging and microstructure

Film microstructure was studied by scanning electron microscopy (SEM) using a Hitachi 3200-N Variable Pressure Scanning Electron Microscope equipped with a 4Pi Isis EDS system for digital image acquisition. All films 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 surface topography, cell distribution and available pore space. Samples were sputter coated prior to imaging with a thin layer of gold in a mild vacuum (~100 mTorr of Ar gas pressure; 600 V accelerating voltage) for 5 minutes and immediately placed in the SEM vacuum chamber for analysis. Cross-sectional images were obtained by freezing dry papers in liquid nitrogen and manually
cryo-fracturing with a frozen sharp blade while submerged in the liquid nitrogen. These samples were mounted in a cross-sectional sample holder and sputter-coated with gold using the procedure previously described.

3.3 Results and Discussion

3.3.1 Proof of concept photo reactivity and microstructure data

Preliminary data suggests successful preservation of *Rps. palustris* photoreactivity post-immobilization, drying and rehydration in microbial paper sheets (Fig. 3.2). By controlling the pressing & drying conditions we were able to transfer our expertise on preservation of *Rps. palustris* cellular viability in latex coatings to the fabrication of a paper-based cellular biocomposite. It is also evident from the H₂ production data that the paper manufacturing conditions play a critical role on the final reactivity of the microbial paper sheet. The amount of shear stress the cells are exposed to during the biomass incorporation step as well as the strength and rate of adsorption of the cells to the fibers may affect the final photoreactivity of the paper, which was observed as significant differences between the primary and secondary H₂ production rates by sheets prepared using different blending regimes. The high-shear conditions which occurred when the cells were mixed with the pulp prior to vigorous mixing seem to effect long-term sustained photoreactivity as evidenced by the early plateaus observed in sheets prepared by simultaneous pulping and blending of the biomass. The maximum H₂ production rate recorded from this first set of microbial paper sheets was 4.00 ± 0.28 mmol H₂ m⁻² h⁻¹, which corresponds to post-pulping manual mixing of the biomass with the suspended fibers under low-shear conditions. This rate doubles the H₂ production rate by
latex coatings of *Rps. palustris* on polyester \(^{20}\) (2.08 ± 0.01 mmol H\(_2\) m\(^{-2}\) h\(^{-1}\)) and is almost 10 times greater than the rate from paper coatings\(^{21}\) of the same organism fabricated with the method described in chapter 1 (0.47 ± 0.04 mmol H\(_2\) m\(^{-2}\) h\(^{-1}\)) previously reported by our group. This performance is remarkable considering that this material has not been optimized for photoreactivity, no osmoprotectants were included as part of the pulp formulation and the paper making method is very rudimentary.

**Figure 3.2** Proof of concept microbial paper photoreactivity. Long-term cumulative H\(_2\) production by hand-made *Rps. palustris* microbial paper sheets (Error bars ± 1 Std Dev, n=3).
The microstructure of the initial proof of concept microbial paper sheets provides insight about the reasons for the high photoreactivity of this cellular biocomposite and suggests potential opportunities for improvement. Top-view SEM images of *Rps. palustris* microbial paper (Fig. 3.3A) reveal the distribution of the bacterial cells as clusters between paper fibers (in their characteristic rosette morphology) that do not clog the nonwoven pore space. This allows perfusive flow through the fiber matrix.

Cross sectional images show bacterial cells between fibers acting as a “glue-like” phase connecting adjacent fibers and significant pore space is visible for product and nutrient transport (Fig. 3.3B). This structure provides a nonwoven microenvironment for cells to survive dehydration and remain viable and reactive for long periods of time after rehydration. However the specific cellular mechanisms of dehydration survival and the mechanisms of adhesion between cells and nonwoven fibers are unknown at this point. The micrographs also reveal the presence of a wide distribution of cellulose fiber lengths and thicknesses, which is a consequence of the simple method used for pulping the printer paper used as raw material. As it will be discussed in the next section, a precise distribution of different types of fibers will determine the final mechanical strength, mass-transfer capabilities, optical clarity and bacterial load of the microbial paper sheet, which makes selection of the type of pulp and further improvement of the pulping process critical.
Figure 3.3 Proof of concept microbial paper microstructure. A. Top-view SEM micrograph of a hand-made *Rps. palustris* microbial paper sheet (scale bar 2µm). B. Cross-section (scale bar 10µm)
3.3.2 Vacuum-dewatered microbial paper films

The introduction of MFC in the pulp formulation affects the final cell retention in the microbial paper film. A series of films with MFC content ranging between 0-100% blended with equal parts of SW and HW fibers was prepared in order to characterize the effect of microfibrillar cellulose on reactivity. Filtrate samples from the vacuum dewatering process were obtained to quantify cell retention with respect to the original bacterial pool.

Qualitative inspection of the film-filtrate series (Fig. 3.4A) revealed a positive correlation between the MFC content and the retention of *Rps. palustris* in the film as evidenced by the increased intensity of the pigmentation in the film and the corresponding decreased intensity of the color in the filtrate. The specific H\(_2\) production data revealed some unexpected trends between the MFC content and the photoreactivity of the film (Fig. 3.4B,C). Films in the lower MFC range have the highest specific H\(_2\) production rates but lose specific reactivity as the MFC content is increased. Given that bacterial retention in the film increases with MFC content, this specific reactivity drop might be related to mass transfer limitations, self-shading or enhanced light scattering as the films gain barrier properties in the upper MFC range. It is well known that pure MFC films have poor moisture diffusion coefficients and gas permeabilities\(^{22,23}\), mainly as a consequence of the highly hydrogen-bonded MFC network and strong interactions with the other components of the films.

Further insight into the mass-transfer limitation problem of these blends can be provided by plotting the H\(_2\) productivity data as a function of the film area (Fig. 3.5 A,B). The trend takes a bell shape with an apparent maximum cumulative production rate of 3.94 ± 1.09 mmol H\(_2\) m\(^{-2}\) h\(^{-1}\) around 60% MFC content.
Figure 3.4 Vacuum-dewatered microbial paper films specific photoreactivity. A. Film/filtrate MFC series (numbers indicate MFC content (%). B. Cumulative specific H$_2$ production. C. Specific H$_2$ production rates and bacterial retention. (Error bars ± 1 Std Dev, n=3)
Figure 3.5 Vacuum-dewatered microbial paper films photoreactivity. A. Cumulative H$_2$ production. C. Cumulative H$_2$ production rates. (Error bars ± 1 Std Dev, n=3)
However the standard deviation of all points from 20% MFC to 100% overlap and no statistically significant maximum can be determined from this data. This number is still comparable to the rate observed in films fabricated with the classical hand-made paper making method described in the previous section, but the physical characteristics of the vacuum-dewatered films can be much more easily tuned using this improved method.

The data also shows that the H₂ production rate per m² decreases rapidly as the MFC content is reduced and may be reduced in the high MFC range. At low MFC content, the amount of retained biomass is minimal but mass transfer limitations may be low, which may explain the high specific H₂ productivity rates (cells are not limited by mass transfer) but rather low overall photoreactivity per m². On the other extreme case, as the MFC content approaches 100%, many more cells are retained which may result in reduced mass transfer or optical limitations, which explains the low specific H₂ production rates and relatively high photoreactivity per m². In this case the effect of the lower productivity per cell is counter-balanced by the presence of much higher biomass per unit area.

The previous findings imply that there may be a trade-off between the higher bacterial retention level at high MFC contents and the observable microporosity created by the presence of thicker SW and HW fibers in the biocomposite. Top-view SEM imaging of the *Rps. palustris* films confirm the microstructure changes that take place as the MFC content is varied (Fig. 3.6A, C, E, G, I, K). Micrographs of the films show nearly continuous MFC networks at the higher MFC content with very little (if any) distinguishable microporosity. Individual *Rps. palustris* cells are visible on the surface of the film, but most of the cells appear to be entrapped in the dense MFC matrix or entangled between SW and
HW fibers as the MFC content is decreased. As the MFC content is reduced the structure “opens” and the macropore size increases, as more clearly evidenced in cross sectional micrographs (Figs. 3.6B, D, F, H, L). Additional internal space for perfusive flow is created by the presence of SW and HW fibers, which further explains the higher specific H₂ productivity as the MFC content decreases.

The microbial paper concept is still an early-stage technology with many potential opportunities for improvement. The data presented in this chapter is merely exploratory but should serve as a proof of concept for continued development. For example, our current pulp formulation includes native cellulose fibers that have not been surface-modified to better complement the surface charge of the bacterial cells. The literature reports methods for the chemical generation of positive charges on the cellulose microfibrils surface²⁴ that could create an electrostatic interaction between the bacterial surface and the fibers (a similar approach is described in the next chapter to surface-activate polyester sheets). Surface modification could improve cell retention in microbial papers with a low MFC content, increase cell loading and alter porosity and mass transfer properties. In the search for these optimized cell-fiber interactions, extensive characterization of the microbial papers will be necessary in order to find a suitable tradeoff between optical clarity/reactivity/stability, mechanical strength, porosity and bacterial load. The pulp and paper industry already has methods which should facilitate the adaptation of this technology to the current paper manufacturing infrastructure.
Figure 3.6 Vacuum-dewatered microbial paper films. A, C, E, G, I, K: top views (scale bar 2µm). B, D, F, H, J, L: cross sections (scale bar 10µm).
Microbial papers and cellular biocomposite materials are a new “smart materials” technology platform that offers many potential market applications. No fiber-based material with the capabilities described in this chapter currently exists in the market. The markets for specialty nonwovens and specialty papers continue to expand. This potentially entirely new platform technology will add previously unimaginable functionality to papers, fiberboard and nonwoven materials. There are coatings and inks that contain enzymes, biocides or other photoreactive biomolecules, and microcrystalline cellulose. However, these “smart” biomaterials are not capable of photosynthesis, biosynthesis, absorption of gasses or mineralization of toxic air or water contaminants. Only intact cells have these functions in the context of a biomimetic matrix built around the cells and custom-tailored to intensify their reactivity.

3.4 Conclusions

The microbial paper concept may appear very simplistic – putting a high concentration of engineered microbes into paper. However the combination of custom-tailored pulp blends and careful modification of the paper drying process results in a dry cellular nonwoven cellular biocomposite material that stabilizes and entraps a very high concentration of Rps. palustris cells. Microbial papers are the first biocatalytic nonwovens generated by our group. Our results suggest that these cells regain biological activity following rehydration and produce H₂ gas without outgrowth for 100s to 1,000s of hours at rates that are higher than those of other cellular biocomposites and coatings of this same organism developed in our lab, which shows the great potential of such a promising technology.
Our data also shows the functional and structural complexity of the nonwoven cellular biocomposite we have produced. We explored the effect of some processing conditions and raw material formulations obtaining a wide range of photoreactivities, cell retention and microstructures. Further optimization of this biomaterial will require an interdisciplinary approach by forest biomaterials science, microbiology and chemical engineering. However, the potential market opportunities for using cells as green catalysts could be very large if microbes can be stabilized in a dry form (without needing refrigerated storage or shipping). A new model in which the manufacture site can be separated from the site of use might be feasible with development of highly reactive cellular biocomposites. Nonwoven microbial papers have this dry stabilization functionality. No industry has concentrated and preserved living cells using this dry nonwovens approach. This platform technology will be the prototype of a new generation of inexpensive biocatalyst-on-a-sheet materials in which “libraries” of microbial paper sheets could be stored dry and readily available for being shipped and rehydrated on site and used for a myriad of biomanufacturing, environmental and bioenergy applications.

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References


CHAPTER 4

Dielectrophoretic fabrication of light-harvesting biocomposites of cyanobacteria

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Abstract

We report the investigation of dielectrophoresis (DEP) for the fabrication cellular biocomposites of photoreactive cyanobacteria (*Synechococcus PCC7002*) on flexible polyester sheets (PE). Adhesion is generated by priming a surface-activated polyester substrate with a layer-by-layer (l-b-l) assembled film of alternating charged polyelectrolytes in order to generate a highly positive charged flexible substrate for cell binding. Cell viabilities determined by vital staining up to 95 ± 2% after DEP deposition were observed and the effect of voltage and frequency on chain length was investigated in suspension prior to deposition. COMSOL-based numerical simulations predict the equilibrium structures of the cells in the presence of an external electric field and are in excellent agreement with experimental results. The effect of DEP on the surface coverage efficiency factor (SCEF) was quantified and compared to that of gravity settled cyanobacteria coatings, yielding up to ~60% in the presence of the electric field versus ~40% for free settling, which is remarkably close to the theoretical efficiency of ~70% we estimated from a non-permanent convective assembled monolayer. Coatings remain adhesive after the electric field is turned off due to the electrostatic interaction between the negatively charged cyanobacteria and the positively charged polyelectrolyte layer on the substrate. Photosynthetic pigment integrity in *Synechococcus PCC7002* cells is preserved after DEP immobilization as revealed by confocal laser scanning microscopy and spectrophotometry. Significant reduction of light scattering and enhanced transmittance on DEP coatings of cyanobacteria demonstrate the effect of reduced self-shading compared to suspension cultures. This method was used to assemble a robust, structured cellular biomimetic biocomposite that optimizes cell surface
coverage and preserves cyanobacteria viability after surface attachment while achieving precise control over cyanobacteria orientation and distribution on the PE substrate. This could be a useful technique towards the development of methods to fabricate multilayered biomimetic photo-absorbing materials on flexible media composed of different types of photosynthetic organisms immobilized on microfluidic vascular networks for high-intensity light harvesting “leaves” for biofuel production from CO₂.

4.1 Introduction

Composite materials that mimic or exceed the function of natural plant leaves have been the subject of extensive research efforts over the last several decades. We believe that the next advance in this area will come from the incorporation of live whole photosynthetic cyanobacteria as part of the structure of the material (a cellular biocomposite) given their ease of genetic manipulation and their unique capability to harvest solar light and convert carbon dioxide into useful chemical intermediates and fuels. Our focus is to develop inexpensive methods to rapidly assemble biocomposites of stabilized, ordered, reactive unicellular photosynthetic microorganisms into a structure that has reduced self-shading, optimizes surface coverage, harvests light in a broad portion of the visible spectrum, absorbs carbon dioxide for recycling, and that can potentially surpass the energy harvesting performance of photovoltaics. Current methods for cyanobacteria immobilization include absorption to natural polymers such as chitosan, synthetic polymers and cross-linking or by entrapment in sol-gels such as silica gels and alginate. However, none of these methods is capable of organizing cells into a networked/ordered/layered array.
with adhesive properties for creating a multi-layered structure with optimal light trapping on a flexible substrate, mimicking the distribution of plant cells in a leaf. This task could be accomplished with a method that organizes cells first in suspension and then deposits these assemblies onto an adhesive surface. Several literature reports describe approaches for fabricating freely suspended random cellular aggregates, organized cell assemblies via DEP in AC electrical fields, and methods for promoting cell adhesion to nanopatterned surfaces and polyelectrolyte multilayers separately. There are a few reports on the use of DEP methods for collection and separation of cyanobacteria according to lipid content and viability, but to the best of our knowledge no previous work has been done on DEP-mediated immobilization of viable, reactive closely packed cyanobacteria uniformly on polyelectrolyte coatings.

Dielectrophoresis (DEP) has been extensively investigated for the manipulation and measurement of properties of many types of cells in suspension. It is defined as “the force exerted on the induced dipole moment of an uncharged dielectric and/or conductive particle by a non-uniform electric field.” In the presence of an alternating current (AC) field of frequency $\omega$, a dipole moment is induced in the cells resulting in pearl-necklace type of linear chains. Cells in suspension distort the field in their surroundings inducing a net force on the neighboring cells given by:

$$F_{DEP} = 2\pi \varepsilon_m R^3 Re|K(\omega)|\nabla E_{rms}^2$$

Where $\varepsilon_m$ is the dielectric permittivity of the medium, $R$ is the particle radius and $E$ is the electric field intensity. This force is proportional to the electric field gradient squared and is frequency-dependent through the real part of the Clausius-Mossoti function ($K$):
Here $\varepsilon_p$ corresponds to the dielectric permittivity of the particle, $\sigma_m$ is the conductivity of the media, $\sigma_p$ is the conductivity of the particle and $\tau_{MW} = (\varepsilon_m + \varepsilon_p)/(\sigma_m + 2\sigma_p)$ is the Maxwell-Wagner charge-relaxation time.

Particles align into chains in suspension when exposed to an AC field due to the attractive forces produced by the induced dipoles. This chaining force is proportional to the square of the field intensity but also depends upon the distance between particles and the length of the chain through the $C$ coefficient:

$$F_{\text{chain}} = -C\pi \varepsilon_m R^2 Re|K(\omega)|^2 \sqrt{\varepsilon}, 3 < C < 10^3$$

The chaining force is proportional to the square of the real part of the Clausius-Mossotti function, which makes it very sensitive to the field frequency.

The dielectrophoretic force also has a vertical component $F_{Z,\text{DEP}}$ that acts in the same direction along with the buoyancy force $F_B$ when positive DEP is present and the particle density ($\rho_p$) is greater than the medium density ($\rho_m$):

$$F_B = \frac{4}{3} \pi R^3 (\rho_p - \rho_m) g$$

Where $R$ is the particle radius and $g$ is the gravitational acceleration. The combination of these two forces results in faster settling of the particles onto the substrate. Examples of DEP applications involving live cells include cancer diagnostics,$^{42-44}$ tissue engineering applications$^{45,46}$ and environmental monitoring.$^{32}$

This study describes a new cellular coating deposition method that applies the principles of dielectrophoretic assembly in suspension with adhesion to polyelectrolyte.
multilayers to yield a flexible, light-harvesting biocomposite of viable cyanobacteria. Low cell-density suspensions are used to assess cell viability in suspension after DEP exposure and to evaluate the effect of operating parameters on cell assembly. The numerical simulations of the assembly process provide deeper insight into the cell chaining mechanism and the total electrical energy of the DEP-assembled structures in suspension. These arrays are not permanent in the absence of biospecific linking molecules or functionalized particles and disassemble once the electric field is turned off. Our coating method preserves the organization by creating a permanent electrostatic interaction between the cyanobacteria and the polyelectrolyte-coated substrate. We study the microstructure and in situ viability of these cellular coatings via optical, scanning electron and confocal microscopy after deposition and assess their optical properties side-by-side with suspension cultures.

4.2 Materials and Methods

4.2.1 Materials and substrate activation procedure

Aqueous solutions of poly(sodium 4-styrenesulfonate) (PSS) (Mw=70000 Da) and poly(allylamine hydrochloride) (PAH) (Mw= 15000 Da) (Aldrich Chemical, MO) were prepared with deionized water (Milli-Q system, 18.2 MΩ.cm) at a final concentration of 1mg/mL. A 125 µm thick polyester substrate (DuPont Melinex 454, Tekra Corp, NJ) precut (75 x 25 mm) was wiped extensively with 100% ethanol and partially hydrolyzed with a boiling solution of 15 g/L NaOH for 30 minutes to generate a negative charge on its surface. A polyelectrolyte coating was deposited onto this charged substrate by sequentially exposing the top surface of the polyester to PAH and PSS solutions for 15 min with 2 min rinsing with
deionized water between each deposition. The final activated substrate contains three PAH/PSS bilayers and a final PAH positively charged layer on its surface (Fig. 4.1).

![Figure 4.1 Polyester (PE) substrate activation procedure. A partially hydrolyzed, negatively-charged PE substrate is primed with a layer of positively-charged PAH and subsequently coated with polyelectrolyte layers of PSS and PAH of alternating charge.](image)

### 4.2.2 Cyanobacterium strain, media, and growth conditions

*Synechococcus* PCC7002, was grown aerobically in 250-mL flasks containing 50 mL of BG-11 medium at 100 oscillations/min in an orbital shaker with 70 μmol photons m⁻² s⁻¹ cool fluorescent light (light intensity measured using a LI-COR, LI-190SA Quantum Sensor, NE) at 25°C. BG-11 was prepared as previously described⁴⁷. Suspensions for dielectrophoretic cellular coating deposition were prepared as follows: 1.0 ml of an OD₅₄₀ ≈ 1, 3-day old culture was centrifuged in 1.5 ml Eppendorf tubes in a bench top centrifuge for 15 min. at
3,000 x g and room temperature. The BG11 supernatant was removed by pipetting and the wet cell pellet was resuspended in 1.0 ml of deionized water for a final cell density of ~8.0 x 10^7 cells ml\(^{-1}\) (viability experiments in suspension). For high cell density coating experiments, the wet cell pellet was resuspended in 300 µL of deionized water for a final cell density of ~3-5 x 10^9 cells ml\(^{-1}\). The final pH of all cell suspensions was between 6.8-7.0 and ~1mM final average electrolyte concentration.

4.2.3 Dielectrophoretic coating deposition procedure

Cyanobacteria dielectrophoretic alignment was carried out between two coplanar gold electrodes vapor-deposited on a 25 x 75 mm plain glass slide (Fisher Scientific, NH) with a 3 mm gap in between. The gold electrodes were cleaned with NoChromix (Godax laboratories, MD) overnight, rinsed with water and air-dried prior to DEP deposition. The activated polyester substrate was cut to 10 x 5 mm to fit inside a 20 mm diameter, 1 mm-thick perfusion chamber (Grace Bio-labs, OR) that was used to cover the gold electrodes and house the concentrated cell suspension to be coated. In order to minimize cell attachment to the inner surfaces of the gold electrodes and the perfusion chamber, these elements were treated with 0.5% F-127 Pluronic surfactant (Molecular Probes, OR) for 30 minutes prior to DEP deposition. The gold electrodes were connected to a 33120A 15-MHz square-wave field function generator (Agilent technologies, CO) that provides with a 2-10V peak-to-peak AC signal. This signal was amplified to 25V using a RG-91 voltage amplifier (Burleigh Instruments, NY) connected in series along with a 1 µF capacitor used to filter any DC component of the signal. The circuit was completed with a digital multimeter (Instek, CA)
monitoring the applied electric field which was maintained at 2-10 V mm\(^{-1}\) at 50-250 Hz (Fig. 4.2A). Oriented cellular coating deposition was achieved by turning on the electric field prior to filling the perfusion chamber with the cyanobacteria suspension (Fig. 4.2B). Once the perfusion chamber was loaded with 300 µl of cell suspension, DEP was carried out for 1 hour at constant voltage and frequency. Coatings were rinsed in deionized water prior to imaging in order to remove any unbound cells.

**Figure 4.2** A. Schematics of the experimental apparatus for DEP cyanobacteria coating deposition. B. DEP-mediated cellular coating deposition strategy. Cells are first aligned in suspension and then deposited onto the surface of the activated substrate by a combination of vertical DEP and buoyancy forces

### 4.2.4 Viability assay

To test whether or not the cyanobacteria survived the DEP procedure, a vital staining technique with fluorescein diacetate (FDA) was used\(^{47}\). FDA (Sigma, MO) was dissolved in
100% acetone to a concentration of 0.5% (w/v). This stock solution was diluted with BG-11 media to make a 0.01% FDA solution. Equal volumes of cell suspension and FDA-BG11 solution were mixed in an Eppendorf tube and incubated for 20 minutes prior to imaging. Viable cells appeared bright fluorescent green, while non-viable cells appeared pale red or colorless under fluorescence microscopy (488/530nm excitation/emission respectively). Fluorescence images were captured before turning the field on and during depositions (15, 30 and 60 minutes) with DEP. Image exposure was adjusted manually to achieve optimal contrast and brightness. Cell viability is expressed as the average number of FDA positive (viable) cells divided by the total number of cells in three different fields.

Samples were taken from a culture subjected to DEP at different time intervals and cultured in fresh BG-11 media to determine cell survival after exposure to the electric field. A sterile 24-well plate (Corning, NY) was filled with 1.2 mL, each, of sterile BG-11(N+) media. 20 μL samples were taken every 15 minutes from the DEP chamber for a total time of 1 hour, and were re-suspended in 980 μL of BG-11 media to yield a 1:50 dilution factor. Three wells of the plate were inoculated from each tube on the plate with 300 μL of cell suspension. Non-inoculated wells between inoculated rows served as abiotic controls to check for cross contamination between successive rows. Finally all the wells were mixed with sterile pipette tips and incubated at 25°C, 70 μmol photons m⁻² s⁻¹ and 100 oscillations min⁻¹ for 1 week. Daily 10 μL samples were taken and cell count measured on a hemocytometer (Hausser, PA) after mixing of each well with a sterile pipette prior to sampling.
**4.2.5 Coating imaging and microstructure**

Cyanobacteria alignment in suspension was monitored and characterized using an Olympus BX-61 optical microscope (Olympus America, PA) equipped with transmitted-fluorescence modes and an Olympus DP-70 digital CCD camera. For the experiments in which we evaluated the effect of the DEP operating parameters on chain length we applied the electric field during 2 minutes at the given conditions and took a series of five 40x micrographs covering a line crossing the center microscope field from left to right in the gap between the gold electrodes. The values reported for chain length correspond to an average of the cell counts performed in these five images.

DEP coating microstructure and cell viability were determined from deconvoluted z-plane images obtained by confocal laser scanning microscopy (CLSM) using the Olympus scope and a 515 nm argon ion laser (CVI MellesGriot, NM). Cyanobacterial natural pigment florescence was detected in the red region of the spectra at wavelengths greater than 650 nm (excitation at 488 nm). A stack of 50 CLSM images 0.2 μm apart in the z direction perpendicular to the coating plane were recorded to a total depth of ~10 μm into the coating. Image J software (National Institutes of Health, MD) 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.

Polyelectrolyte coating microstructure was studied by scanning electron microscopy (SEM) using a Hitachi 3200-N Variable Pressure Scanning Electron Microscope equipped with a 4Pi Isis EDS system for digital image acquisition. 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 10000x to characterize surface topography. Samples were sputter coated prior to imaging with a thin layer of gold in a mild vacuum (~100 mTorr of Ar gas pressure; 600 V accelerating voltage) for 5 minutes and immediately placed in the SEM vacuum chamber for analysis.

### 4.2.6 Surface coverage

Image J software was used to estimate coating surface coverage from 40x optical microscopy images at randomized locations in the DEP cellular coatings. These images were converted to 8-bit grayscale, threshold and processed using the “analyze particles” capability of the program. The background subtraction, particle size threshold and circularity were all adjusted manually to detect all cells in the field and estimate the void space between particles. Surface coverage was calculated as the product of the total number of cells detected times the average area per cell divided by the total field area.

### 4.2.7 Optical properties

The optical properties of planktonic *Synechococcus PCC 7002*, the 125 µm thick transparent polyester substrate and DEP generated cellular coatings of *Synechococcus* on the polyester sheet were measured using a 1 ml cuvette filed with BG11 media (for coatings or substrates) or cell suspensions in a Lambda 35 Perkin Elmer UV-VIS spectrophotometer equipped with an integrating sphere (RSA-PE-20, Labsphere, NH) at ambient temperature. A set of larger 4 x 25 mm cyanobacteria DEP coatings were laid flat (vertical) on the back wall of the BG11 filled cuvette. Values for transmittance were obtained between 400-900 nm at 480 nm min⁻¹.
No evidence of cell release or outgrowth to the liquid phase was observed during our experiments as indicated by hemocytometer count.

4.3 Results and Discussion

4.3.1 Substrate activation strategy

Flexible polyester is a suitable substrate for photoreactive cellular coating deposition given its mechanical strength and optical clarity. However, it is also chemically inert, hence the need for an activation step prior to cellular coating deposition. Surface activation was achieved via weak surface hydrolysis of superficial ester bonds in the presence of a concentrated metal alkali hydroxide. This treatment creates negatively charged carboxyl and hydroxyl groups on the surface of the polyester sheet that fix and stabilize the polyelectrolyte coating in place\textsuperscript{48,49}.

The partial alkali hydrolysis pre-treatment was followed by a layer-by-layer deposition of a \~100 nm thick polyelectrolyte coating (thickness measured with a Veeco Dektak D-150 Step profilometer, data not shown) of composition (PAH/PSS)\textsubscript{6}PAH. The first polyelectrolyte layer has a positive charge to bind to the negatively charged partially hydrolyzed polyester substrate and serves as a base layer for the sequential deposition of alternatively charged layers of PSS and PAH. SEM imaging of the raw partially hydrolyzed charged substrate shows a clean polished surface (Fig. 4.3A) primed to be covered by the polyelectrolyte coating. As the coating grows in thickness with subsequent polyelectrolyte layers (Fig. 4.3B), topographic features become more evident and ultimately homogeneously cover the surface of the substrate homogeneously (Fig. 4.3C). The last layer is positively
charged so it can bind to the negative charges on the surface of the cyanobacteria cell wall\textsuperscript{50} and establish a strong electrostatic interaction capable of keeping the cells in place in the absence of an electrical field (Fig. 4.3D). No evidence of cell wall fusion to the polyelectrolyte layer can be observed in these micrographs.

**Figure 4.3** l-b-l polyelectrolyte coating deposition. A. Raw charged polyester substrate. B. Coating at (PAH/PSS)\textsubscript{2}. C. Finalized coating (PAH/PSS)\textsubscript{3}PAH. D. *Synechococcus PCC7002* immobilized on polyelectrolyte coating. Scale bars: 2µm
4.3.2 Effect of voltage and frequency on cell alignment

Our DEP immobilization strategy relies on assembly of cell chains and partial 2-D arrangements in suspension above the polyelectrolyte substrate. We characterized the effect of voltage and frequency on cell chain length in order to choose suitable operating parameters that promote cell assembly in suspension while preserving cell viability. Voltage was varied in a relatively broad range between 10-50V in order to account for the greater influence of the electric field strength on cell surface polarization while frequency was maintained between 50-250Hz to avoid electrohydrodynamic flows at lower frequencies and bulk fluid convective flow at higher frequencies. The frequency used in the experiment was in a narrow range in order to avoid the low-frequency dielectric dispersion (LFDD) limit characteristic of colloidal particles defined by the characteristic relaxation frequency of the cells ($\omega_{\text{char}} = 2DM/R^2$). It has been reported that above this frequency cell chain length decreases due to a dipole magnitude decrease between the ions inside and outside of yeast cells. This parameter depends on the diffusion coefficient ($D$) of the ions in the double layer (mostly Na$^+$ for BG11) and a dimensionless factor ($M$) that represents the effect of the electrosmotic ion flux across the double layer. Our estimate for this frequency is ~680Hz, subject to uncertainty given the lack of specific physical data for this cyanobacteria strain. Because of this, Table 4.1 reports a combination of values either measured or reported for cyanobacteria. The missing cyanobacteria parameters were approximated using those for yeast. All experiments were carried out at 8.0 x $10^7$ cells ml$^{-1}$ cell density, ~1.0 mM electrolyte concentration (final value after cell wet pellet resuspension with deionized water) and pH 7.0.
Table 4.1 Physical parameters for calculation of $\omega_{\text{char}}$ and multi-shell modeling$^{41,51}$

<table>
<thead>
<tr>
<th>Location</th>
<th>Parameter</th>
<th>Symbol</th>
<th>Magnitude</th>
<th>Units</th>
</tr>
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<td><strong>Cytoplasm</strong></td>
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<td>C$^2$ J$^{-1}$ m$^{-1}$</td>
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<td>Transconductance</td>
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<td>S m$^{-1}$</td>
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<td>Rad/s</td>
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<tr>
<td></td>
<td>Voltage</td>
<td>v</td>
<td>25</td>
<td>V</td>
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<tr>
<td></td>
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<td>C$^2$ J$^{-1}$ m$^{-1}$</td>
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<td>K</td>
</tr>
<tr>
<td></td>
<td>Surface charge density</td>
<td>$\sigma_q$</td>
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<td>C m$^{-2}$</td>
</tr>
<tr>
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<td>Effective capacitance of the bound layer</td>
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<td>F m$^{-2}$</td>
</tr>
<tr>
<td></td>
<td>Dimensionless factor</td>
<td>$M = \frac{1 + (q \sigma_q/k_B T C_d')}{C_d'}$</td>
<td>1.02</td>
<td>Unitless</td>
</tr>
</tbody>
</table>
Design Expert Software (Stat-Ease, MN) was used to generate a response surface experimental design that covered the voltage and frequency intervals. The response was the average chain length obtained from five 40x images of the glass electrode gap field after 2 minutes of DEP exposure (Fig. 4.4).

**Figure 4.4** Effect of voltage and frequency on average cell chain length in suspension. Error bars ± 1 Std Dev, n = 5
For all frequencies up to 200 Hz, longer chains are formed as the voltage increases, which correlate well with voltage being the most important parameter for dielectrophoretic assembly. At frequencies above \( \omega_{\text{char}} \), chain assembly is partially disrupted by electrohydrodynamic flow and LFDD, which translates into a lower average chain length. At 50V, cell alignment in the direction of the field is fastest (\(~3\text{-}5\text{s}\)) chains are very close to each other but tend to be shorter due to cell displacement from chain to chain due to the stronger field intensity at the ends the growing chain. Lower frequencies promote longer chain assembly, but the process is significantly slower. The longest chains were observed at 50Hz and 50V (about 13 cells per chain on average), but in this high voltage limit water electrolysis occurs and cell viability is affected. Frequencies above 200 Hz resulted in short chains that tend to collapse with each other forming randomly shaped, short-lived aggregates of cells that quickly dissipate due to significant electro-osmotic currents inside the chamber. From these observations we chose 200Hz and 25V as a suitable set of conditions that allows for rapid chain assembly without significant electrohydrodynamic flow or water electrolysis in the time scale chosen for coating deposition (1 hour).

4.3.3 Cell viability during and after DEP exposure

Despite previous reports on bacteria survival after dielectrophoresis\(^{53}\) it was necessary to directly assess the viability of *Synechococcus PCC 7002* cells after being exposed to 25V at 200 Hz during DEP deposition times up to 1 hour. FDA staining showed that the *Synechococcus* cells remain viable for all DEP exposure times tested (Fig. 4.5A). Image analysis performed comparing the fluorescence pictures with optical microscopy pictures
taken simultaneously yielded viability values greater than 95±5% in all cases, regardless of the alignment degree of the cells or their position relative to the electrodes.

Figure 4.5 *Synechococcus PCC7002* viability assay results. A. Time-course optical micrographs Vs. fluorescence microscopy images of cyanobacteria in suspension exposed to 2-10 V mm⁻¹ field intensity. Scale bar: 50µm. B. Cyanobacteria specific growth rates after exposure to DEP.
To quantify and confirm cyanobacteria viability, cells were also collected from the DEP chamber at each time interval tested with FDA staining and cultured in fresh BG-11 media to assess if the specific growth rate was affected after exposure to the electric field. As can be seen in Fig. 4.5B, all samples were able to resume exponential cell division almost immediately after being transferred to the BG11 filled well, reaching in all cases comparable cell densities (\( \sim 10^8 \) cells ml\(^{-1} \)) and comparable maximum specific growth rates after 1 week of incubation. This correlates well with the cell growth of a non-DEP-exposed control under the same incubation conditions. These results show that the voltage and frequency ranges applied to this cyanobacteria strain are non-lethal and do not affect the growth of the cells or the stability of their photosynthetic pigments. This data correlates well with previous reports in the literature regarding the viability and immune-reactivity of bacterial cells exposed to DEP\(^{53}\).

4.3.4 Numerical simulation of cell assembly in suspension

We performed 2D electrostatics simulations using the COMSOL\textsuperscript{TM} multiphysics modeling package (COMSOL; Burlington, MA) to calculate the total electrical energy of a series of discrete stages simulating an assembly process in suspension. Members of the genus *Synechococcus* are typically described in the literature as spherical cells 1.5-3 \( \mu \)m in diameter\(^{54}\). However, we have chosen to model cyanobacteria as a peanut-shaped particle composed of two-partially fused spherical lobes (\( r = 2.1\mu m \)) aligned in the same plane 2.8 \( \mu \)m apart from each other (measured from the lobe centers) based on confocal imaging measurements of rapidly dividing *Synechococcus PCC7002* cells in culture (Fig. 4.6A). This
geometry better represents the dominant shape of a cellular suspension during exponential growth as the vast majority of cells (>98%) were observed under the microscope as cell pairs connected through a common cell wall portion that elongates as cell division progresses. The simulation box is defined by two conductive boundaries (top and bottom) representing the gold electrodes providing 25V AC (Fig. 4.6B). The box is bound in the left and right sides by insulating non-periodic boundaries with an enclosed area of 9 mm². An aqueous suspension of 1.00 mM electrolyte fills the box \((\varepsilon_m = 80 \varepsilon_0, \sigma_m = 0.015 \text{ S m}^{-1})\).

Living cells have multiple different biomolecular components, each with characteristic electrical properties. For *Synechococcus PCC7002*, we modeled each cell as composed of a 100 nm counter ionic layer, a 0.5 µm-thick cell wall, a thin (0.01 µm) dielectric cell membrane and an aqueous cytoplasm (Table 1, Fig. 4.6C). The complex polarizability of each component was calculated as a function of frequency by using the multi-shell modeling equation \(\varepsilon_{m,c} = \varepsilon_{m,c} - j\sigma_{m,c}/\omega^{41}\) applied to each layer individually. The final result is a simplified model (Fig. 4.6D) treats the *Synechococcus* cell as a single-shelled particle of effective polarizability given by:

\[
\frac{\varepsilon_1''}{\varepsilon_1} = \varepsilon_1 \left[ \frac{R_0^3}{R} + 2 \left( \frac{\varepsilon_2' - \varepsilon_1'}{\varepsilon_2' - 2 \varepsilon_1'} \right) \right] + \varepsilon_{EDL}
\]

Where \(\varepsilon_2' = \frac{c_{m,R}e_2}{c_{m,R} + e_2}\), \(\varepsilon_{EDL} = \frac{2\Delta e_m}{R}\) and \(\sigma_S = 2\sigma_m R_0^{-1}k^{-1} \exp \left( \frac{ze\xi}{2kT} - 1 \right)\). Values for the parameters are provided in table 4.1. Whenever specific values for cyanobacteria were not available, yeast data listed in the literature were used.
The program calculates the electric field distribution in the box by first dividing the space into triangular mesh elements for which the Laplace equation ($\nabla^2 \phi = 0$) is solved independently. The local electric energy density is given by $w_{es} = \frac{1}{2} DE = \frac{1}{2} \varepsilon_0 E^2$ where $D$ is the electric displacement, $E$ is the electric field and $\varepsilon_0$ is the permittivity of vacuum. By
integrating $w_{es}$ over the control space the program calculates the total electrical energy $W_e = \int_V w_{es}dV$ of the system.

A comparison of a dynamic series of micrographs of cyanobacteria assembling into chains in suspension with COMSOL simulations at 200 Hz and 8.3 V mm$^{-1}$ depicting the same cell arrangements reveals mechanistic details of the assembly process (Fig. 4.7A). Given that at this frequency the cells are more polarizable than the media, the electric field intensity is strongest at the poles of the individual cells and increases as the distance between adjacent cells is reduced. The local electric field gradient between cells ultimately pulls them together by positive DEP and promotes chain growth in the direction of the field by addition of neighboring cyanobacteria to the chain tips. These artificial cyanobacteria filaments also grow by addition of shorter chains that are pulled down from the top of the chamber and cause rearrangement of the longer chain at the site of “landing” (neither the vertical component of the DEP force or buoyancy are accounted for in the simulation but are always observed experimentally). Total energy in the simulation box tends to decrease as cells orient in the direction of the field, align in chains and come closer to each other forming 2D arrangements in suspension (Fig. 4.7B). The distribution of the repulsive forces along the equatorial axis of the cells is minimized when aligned into chains, which translates into a lower overall energy level that stabilizes the whole structure. The tendency of chains to aggregate into two-dimensional hexagonal arrays is related to lateral interactions between the dipoles induced in the neighboring particle chains$^{55}$. In the case of microspheres, it has been shown that the electrohydrodynamic interaction of the flows around the particles also plays a
major role on driving the attraction and aggregation of particles into ordered arrays in suspension\textsuperscript{56}.

\textbf{Figure 4.7} A. Snapshots of COMSOL simulation at different stages of cell assembly vs. experimental optical microscopy images of DEP assembly of \textit{Synechococcus PCC7002} taken over a 20 min. time lapse. B. Total electrical energy at discrete assembly stages from random orientation to partial 2D assembly in suspension.
4.3.5 Cellular coating deposition of photosynthetic cells using DEP

Being able to precisely control the physical distribution and orientation of photosynthetic cells in a monolayer on a surface is a key capability for the fabrication of the next generation of flexible biocomposite biophotoreactors. The vast majority of current photobioreactors rely on very large volumes of suspension cultures, where cells self-shade each other limiting the overall light harvesting efficiency\(^5\). By depositing a highly oriented array of cyanobacteria on a flexible flat surface, each cell is illuminated uniformly, light penetration is optimized, illuminated surface-to-volume ratio is significantly increased and the liquid volume is decreased, which translates into cost savings and simplified control schemes. When immobilized in high cell density coatings, nongrowing photosynthetic cells have been shown to be several fold more reactive than suspension cultures and capable of remaining reactive after drying and rehydration for 1000s of hours\(^10\), which makes immobilized biocomposite biophotocatalysts a promising area of study for improving our capacity to harvest sunlight more efficiently and trap carbon dioxide using living cells to recycle gas-phase carbon into valuable products.

Our DEP coating deposition process has two general steps: (1) Cells are aligned in suspension by the action of the positive DEP force that generates movement towards the regions with high electrical field and (2) Cell chains are deposited on top of the activated polyester substrate by the action of a combination of the vertical component of the DEP force (\(F_{Z,DEP}\)) and the buoyancy force (\(F_B\)). These two forces act in the same direction towards the bottom of the chip when positive DEP is present and the particles have higher density than the medium (Fig. 4.2B).
In order to maximize the number of cells that assemble into chains in suspension (optimize chain length), the electric field must be turned on prior to loading the chip with the cyanobacteria suspension. Given that we are not interested in generating very large vertical forces (we just need to guarantee positive DEP to avoid particle levitation under negative DEP conditions), a two coplanar gold electrodes chip was used and the cyanobacteria cells were resuspended in deionized water prior to DEP assembly in order to ensure high cell viability throughout the whole coating deposition process. Accelerated video footage of a low cell density deposition assay shows how chains being pulled down by vertical DEP and buoyancy get attached to the substrate irreversibly as soon as they touch the positively charged polyelectrolyte coating. Additional chains get attracted by the “seed” chains already immobilized and fall in place to increase the length of the growing chain. In the absence of the electrical field, cells settle randomly immediately after the chip is loaded and a significant proportion of the cyanobacteria end up randomly immobilized on the polyester substrate. Once the cell-substrate interaction has been established no further re-orientation of the cells on the substrate is possible with the biocompatible electric field strength used in this study. Further proof of the irreversibility of this procedure was obtained by partially coating only one half of the polyester substrate with polyelectrolyte and leaving the other half untreated. Deposition video shows chains of cyanobacteria pre-assembled at both sides of the polyester substrate, but as the electric field is turned off chains on the right hand side of the interface fall apart while those located on the polyelectrolyte coated side remain aligned. If the field is turned on again chains re-assemble in the uncoated side while those on top of the polyelectrolyte layer are not further affected by the field. Macroscopic inspection of the final
coated polyester yielded a homogeneous coating pattern and with faint green translucent color (Fig. 4.8A) over the entire substrate surface and is stable for manipulation under wet or dry conditions.

4.3.6 Cellular coating microstructure and the effect of DEP on surface coverage

The detailed microstructure of DEP coatings of Synechococcus PCC7002 and gravity settled controls was determined using optical and fluorescent confocal microscopy methods. A reference non-permanent monolayer of Synechococcus PCC7002 cells obtained by convective assembly between to glass slides (Fig. 4.8B) was used as a reference for assessing surface coverage efficiency (capillary forces during liquid phase evaporation pull cells together as close as they can physically be without disrupting the cell walls8, 58). We exploited the natural autofluorescence of cyanobacteria for assessing both the distribution and viability of the cell coating on the polyester substrate. A comparison between a high cell density DEP coating of Synechococcus (Fig. 4.8C) and a randomly settled control deposited from the same source cell suspension (Fig. 4.8D) reveals a higher surface coverage of the available surface area when an electric field is used to assemble the cells together in suspension prior to deposition. Using image analysis and particle detection software we estimated average surface coverage efficiency factors (SCEF, defined as the surface area occupied by cells divided by the total surface area of the field) for the reference non-permanent monolayer, a DEP-deposited coating and a gravity-settled coating on surface-activated polyester (Table 4.2).
Table 4.2. Surface coverage efficiency factors (SCEF) and cell densities after deposition

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<thead>
<tr>
<th>Coating Type</th>
<th>SCEF (± 15%)</th>
<th>Total Cells (cells m$^{-2}$)</th>
<th>Void Space (± 15%)</th>
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<tr>
<td>Convective Assembly (non-permanent)</td>
<td>70%</td>
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<td>Random gravity settled</td>
<td>40%</td>
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<td>Oriented deposition (DEP)</td>
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<td>$3.04 \times 10^{10}$</td>
<td>39%</td>
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</tbody>
</table>

Image analysis of the reference non-permanent monolayer assembled via convective assembly provided with a theoretical limit for SCEF around ~70%. Even though this array might seem to be completely saturated with cells a closer inspection of the micrograph reveals a consistent separation between adjacent cells that creates the free space accounted for in the ~30% void space detected by the program. This separation is likely to be produced by the thick proteinaceous sheath secreted by the *Synechococcus* cells. This protective coating is composed of polysaccharides (glucose, galactose, rhamnose, mannose, arabinose), proteins and negatively charged components (glucoronic acids, phosphate, sulphate). When cells are allowed to freely settle by gravity without the action of any external field, only around 40% of the available space is occupied as there is no driving force to orient the cells and organize them in a more efficient packing array. In the presence of a DEP field, surface coverage improves to ~60% because of two distinct factors: first, the electric field promotes assembly of cell chains and small 2D arrangements that are deposited pre-oriented on the sticky surface of the polyester substrate. As the number of cells in suspension decreases, the vertical component of the dielectrophoretic force along with buoyancy pull down the remaining cyanobacteria and force them to occupy the remaining gaps in the growing
monolayer. Eventually, as the available space becomes scarce, some of the cells end up piling up on top of the first layer, especially at those coating locations where the space between adjacent cells is big enough to allow for partial interaction of the polyelectrolyte coating with a small portion of the cell wall. This phenomenon causes the coating imperfections that can be observed in Fig. 4.8C as isolated coating locations where the array is 2-cells thick. However, the few cells on this second layer are not irreversibly attached to the substrate and eventually fall off, especially in the presence of convective flow across the coating. The second factor has to do with the vertical component of the DEP force which also accelerates the cells in the z direction, which translates into more cells getting deposited on the substrate per unit time compared to gravity sedimentation (at the same cell density in suspension and identical deposition time).

CLSM images of the DEP-deposited coatings of Synechococcus provide further details of the coating microstructure to assess cell viability after deposition. It is well known that live cyanobacteria have bright and distinctive fluorescence in the red region of the spectrum (~680 nm) due to the presence of photosynthetic pigments and while non-viable cells gain fluorescence in the green region (~530) as these pigments break down. As it can be seen in Fig. 4.8E and 4.8F, all the cells in the field have the characteristic red fluorescence which implies that viability is not affected after the deposition process regardless of the presence of an AC electric field as driving force for the deposition process.
Figure 4.8 *Synechococcus* PCC7002 coating microstructure analysis. A. DEP-coated polyester substrate. B. Non-permanent *Synechococcus* PCC7002 monolayer assembled between two glass slides by convective assembly. C. Oriented, tightly packed DEP coating (optical microscopy). D. Gravity-settled random coating (optical microscopy). E. CLSM of deconvoluted images of a DEP coating of *Synechococcus* PCC 7002. F. CLSM of deconvoluted images of a gravity settled coating of *Synechococcus* PCC 7002. Red color corresponds to natural fluorescence of *Synechococcus* PCC 7002 cells. Magnification: 125x. 256² pixels/0.0156 mm² area.
As previously explained, the deposition voltage and frequency did not kill cyanobacteria cells in suspension, and given that PSS and PAH are biocompatible with different types of cells, we were expecting most of the cells to survive the DEP-mediated coating process. The electrostatic nature of the interaction between the cells and the substrate is also not toxic for cells, given that it does not involve chemical cross-linking, membrane fusion or other mechanisms that affect cellular integrity. Cell alignment post-immobilization is more evident using confocal imaging, where it is evident that even those cells immobilized on top of the primary monolayer are deposited as organized chains even if they eventually detach from the coating due to the weaker and interaction of their cell walls to each other compared to cells in direct contact with the polyelectrolyte coating.

These images suggest that the DEP-mediated coating method yields an organized array of viable cells attached to the substrate through a thin polyelectrolyte coating that does not cover the cyanobacteria. This is important because it implies that the gluing phase does not constitute a mass transfer or light transmission barrier, while the cells are fixed in place with their photosynthetic apparatus intact. Furthermore, the fact that there is some residual adhesive interaction for some cells to attach to the primary monolayer in an oriented fashion encourages us to imagine potential applications of this method for assembly of multi-layered, highly structured arrays of different types of photosynthetic cells capable of harvesting light in a broader portion of the spectra. Cells could be “sandwiched” between light-transmitting polyelectrolyte layers and fixed to a porous substrate that allows for nutrient exchange and gas removal. Our group has already proved this concept using a simple extrusion coating method on paper substrates where we were observed up to 10 fold specific photosynthetic
reactivity enhancements due to decreased self-shading. Extension of our DEP approach to other types of substrates seems feasible and potentially highly synergistic.

4.3.7 Optical properties of DEP coatings

A UV-Vis spectrophotometer equipped with an integrating sphere was used to monitor the polyelectrolyte coating deposition process and to characterize suspension cultures along with DEP coatings of *Synechococcus* PCC7002. Photosynthetic organisms absorb light at specific wavelengths depending on the kind of pigment they use for harvesting solar light, and the magnitude of the characteristic peaks can be associated with the viability and photoreactivity of the cells.

As it can be seen in Fig. 4.9, the raw polyester substrate is fundamentally transparent and allows almost 90% of the incident light to be transmitted between 390 and 900 nm (this range includes the visible light spectrum and part of the infrared). The six polyelectrolyte layers roughly account for a 10% further decrease in transmittance, suggesting that there is a limit on the number of layers that can be deposited on a transparent substrate to keep it suitable for use as a support for photosynthetic organisms. The transmittance reduction is symmetrical at all wavelengths and the vertical separation of the lines (related to the thickness of each individual layer) is remarkably similar, which is expected given that each layer was deposited during exactly the same time. Based on these data, it seems up to 6-10 layers of polyelectrolyte is a suitable number to maintain a good trade-off between adhesiveness and light transmission, especially for those bioreactor geometries where the coating(s) is not to be illuminated from the top.
Figure 4.9 Transmission profiles at different coating deposition stages: 1. Multilayered polyelectrolyte coating. 2. A DEP-deposited *Synechococcus* coating on activated polyester. 3. A suspension of *Synechococcus* at the same cell density. Insert: detail of a DEP-deposited *Synechococcus* coating lying flat on the back wall of a BG11-filled cuvette.

The cyanobacteria layer absorbs strongly in the visible range (390-700 nm) and features all the characteristic peaks for chlorophyll a (665 and 430 nm) and phycocyanin (620 nm). Compared to an equivalent number of cells in suspension, the transmission profile is essentially the same, but the magnitude of the peaks is enhanced up to 15% at low wavelengths, which implies enhanced light harvesting activity due to reduced self-shading.
and a more closely packed cell array concentrated in a small area. Hence, by depositing a thin layer of a photosynthetic organism we have functionalized an inert material into a light-harvesting flexible cellular biocomposite.

4.4 Conclusions

We have developed a cellular coating method that combines the best features of both dielectrophoretic assembly in suspension and surface modification via 1-b-1 deposition of polyelectrolyte coatings that allows the attached cells to stay aligned on the surface after the DEP AC field is stopped. This is an inexpensive way to precisely fabricate biocomposites that exploit the light harvesting capabilities of photosynthetic cells and constitutes an attractive functionalization technique to turn inert materials into bio-photoreactive solar energy harvesting devices. We demonstrated that cyanobacteria cells have a very similar behavior compared to other types of cells and particles when exposed to AC fields in suspension. From deposition parameter screening and biocompatibility considerations we identified a set of conditions of voltage, frequency and electrolyte concentration that allowed us to streamline the deposition process while keeping the cells viable. Under these conditions, we were able to exploit the vertical component of the DEP force and buoyancy to accelerate the settling of the DEP-assembled chains without inducing a harmful temperature increase in the sample or water electrolysis.

Our electrostatics simulations indicate that the total electrical energy of the system tends to decrease during suspension assembly under the chosen electric field strength which explains the almost instantaneous response of the cells to DEP and allowed us to deposit pre-
organized structures on the polyelectrolyte coating. The role of DEP during coating deposition is to promote a tighter packing of the cells on the surface as quantified by image analysis of DEP and freely-settled coatings of *Synechococcus*. This translates into more cells per unit area (higher surface coverage efficiency) and an overall higher photoreactivity for the material. Our measurements of diffuse transmittance allowed us to monitor the coating process from surface activation to controlled cell deposition and provided with additional insight regarding the effect of the immobilization process on the relative stability of the photosynthetic pigments compared to cells in suspension. This type of whole photosynthetic cell-based biocomposite material could have exciting applications on biophotovoltaics\(^6\)\(^1\), \(^6\)\(^2\) featuring photosynthetic cells immobilized on a microfluidic vascular network\(^2\) for clean electric energy generation while being biodegradable, self-repairing and sustainable.

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References


CHAPTER 5

Additional methods developed in this dissertation
5.1 Introduction

The fabrication, characterization and optimization of the microstructure and photoreactivity of the biomaterials developed in this dissertation required a combination of methods from different disciplines. The majority of these methods are described in detail in previous chapters. However, much of the preliminary exploratory work carried out at the beginning of this project had to do with screening of polymer lattices contact toxicity, cyanobacteria reactivity in suspension, photobleaching and measurement of fundamental electrical parameters for DEP numerical simulations. These activities helped specify the latex binder for subsequent cellular coating experiments, the model photosynthetic microbial system to be immobilized and the conditions to carry out dielectrophoretic deposition in suspension. This chapter describes those methods not addressed in preceding chapters as a reference for future investigations on different photosynthetic systems or coating methods.

5.2 High throughput latex binder and coating additives toxicity screening using 24-well plates

A preliminary screening of the Flickinger lab latex library and commercial samples of acrylic glues was performed to identify potential binders with suitable film forming and optical properties to be used in coating experiments.
5.2.1 Methods

5.2.1.1 Preliminary latex emulsion polyester binder screening

Samples of neat latex suspensions and dilutions in water (1:10, 1:100) were coated using a
# 0 Mayer rod over a 125 µm thick polyester substrate (DuPont Melinex 454, Tekra Corp, NJ) previously prepared with a holey vinyl mask to generate 2.8 cm² coating patches. These
patches were dried at ambient conditions for 24h, and those that generated an optically clear
coating without cracking or delaminating were rehydrated in water for 48h in a 24-well plate
(COSTAR, New York, USA). Those that retained adhesiveness and did not delaminate or
blistered following rehydration were identified as potential candidates to be used in coating
experiments.

5.2.1.2 Standard latex emulsion contact toxicity assay

250 ml of a culture of Synechococcus PCC7002 incubated under 17.00 PAR µmol photons
m⁻² s⁻¹ at 25°C for 4 days (cell density 1 x 10⁶ cells m⁻¹) were divided into 50 ml aliquots of
the culture into four pre-weighted, sterile 50 mL disposable centrifuge tubes. Cells were
centrifuged 10 minutes at 3000 x g. The cell pellet was washed in 50 mL sterile BG11
medium and centrifuged again at the same conditions described above. The supernatant was
extracted by pipetting and the tube was weighted with the pellet. The weight of the pellet was
determined by subtracting the weight of the pre-weighted tube. The amount of latex, sucrose
and glycerol to be added to the cells was calculated using the formulation ratio 1.2g wet cell
pellet (WCP), 350 µL 0.58 g ml⁻¹ sucrose, 150 µL 100% glycerol and 1mL latex binder¹.
Tubes were numbered 1-4. Table 5.2 describes the distribution of the components in the
tubes. In all tubes, BG11 medium was added in place of any missing formulation components and mixed gently until homogeneous. Tubes were allowed to stand for 30 minutes at room temperature. After this time all tubes were topped off with sterile BG11 to 50 mL and vortexed. 2 mL of each suspension were pipetted in each well of a 24-well plate. Hemocytometer cell counts and chlorophyll extractions were performed at \( t = 0 \) h and subsequently every 24h using the whole content of three wells for a total of 8 data points per plate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latex</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</tbody>
</table>

**Table 5.1. Contact toxicity assay formulation distribution**

### 5.2.1.3 Chlorophyll a extraction from cell suspensions

Cell suspensions were centrifuged at 3000 x g for 10 min. Chlorophyll \( a \) was extracted from the cell pellets by submersion in 1.5 mL of neat EtOH. Pellets were resuspended by vortexing prior to boiling for 1 min at 100°C. Pigment extracts were centrifuged at 15000 RPM for 10 min. All supernatants were sampled for absorbance at 665 and 750 nm using a Genesys 20 UV-Vis spectrophotometer (Thermo Electron Corporation, Marietta, OH). The
process was repeated for a total of three times and the supernatants were pooled in a single extract. The amount of chlorophyll $a$ (μg/L) extracted from each coating was calculated as\textsuperscript{2,3}

$$
\text{Chlorophyll } a \text{ (μg L}^{-1}) = \frac{[11.99 \times (\text{Abs}_{665} - \text{Abs}_{750}) \times V_e]}{\rho \times V_s}
$$

Where $\text{Abs}_{665}$ is the supernatant absorbance at 665 nm, $\text{Abs}_{750}$ is the supernatant absorbance at 750 nm (turbidity correction), $V_e$ is the volume of ethanol extract (mL), $V_s$ is the sample volume (L), and $\rho$ is the length of the cuvette parallel to the light path.

### 5.2.2 Sample results

A total of 21 latex binders were screened for film formation on a polyester substrate after 24h of drying at ambient laboratory conditions (Table 5.2). From these only nine formulations formed a stable, adhesive and optically clear coating over the polyester (neat or diluted with water). The patches belonging to those nine binders were rehydrated in water for 48h. Eight of these formulations remained stable after rehydration with different levels of water incorporation into the coating, and one of them delaminated completely from the substrate. Five of these lattices belonged to the latex library (KAK3945, KAK3947, JP1225, SF091, SF012) while 3 were samples from commercial acrylic glues (Wellbound, T.Medium, Rod. Dodge). Latex binder SF091 obtained biocide-free from Rohm and Haas has been used previously by our laboratory for several types of cells, however it is no longer available. SF012 is the currently available equivalent binder that can be obtained from Dow Chemical. Given that the composition of the commercial products is not accurately provided by the manufacturer and there is no data regarding biocompatibility with cyanobacteria, two binders were chosen from the group of formulations that were successfully screened from the library.
(SF012 and SF091) as they retained some optical clarity after rehydration and did not undergo any undesirable changes (i.e. delamination or blistering) after rehydration. We used these two binders to test the feasibility of using the 24-well plate method to screen lattices and performed a series of experiments in which suspensions of Synchococcus PCC7002 were exposed to the latex formulations according to the standard contact toxicity assay\textsuperscript{4} and were tested for cell proliferation, chlorophyll content and chlorophyll per cell measurements during one week.

\begin{table}[h]
\centering
\caption{Latex screening results}
\begin{tabular}{|l|c|c|c|c|c|c|}
\hline
 & Latex over polyester, 24 h drying & Rehydrated coating in water after 48 h \\
\hline
Latex & Neat & 1:10 water & 1:100 water & Neat & 1:10 water & 1:100 water \\
\hline
HP1055 & White/Crack. & White/Crack. & White/Crack. & White & Transparent & White \\
SF012 & White & Transparent & Transparent & White & Transparent & White \\
KAK3397 & White & White & White & White & White & White \\
KAK3395 & White & White & White & White & White & White \\
KAK3393 & White & White & White & White & White & White \\
JP1225 & Transparent & Transparent & Transparent & Slight White & Slight White & Transparent \\
SF091 & Transparent & Transparent & Transparent & White & White & Transparent \\
KAK3389 & White & White & White & White & White & White \\
KAK3391 & White & White & White & White & White & White \\
KAK3401 & White & White & White & White & White & White \\
KAK3941 & Transparent & Transparent & Transparent & White & Transparent & Transparent \\
KAK3945 & Transparent & Transparent & Transparent & White & Transparent & Transparent \\
KAK3947 & Transparent & Transparent & Transparent & White & White & Slight White \\
Ultra Matte & White & White & White & White & White & White \\
B. Acrylic & White & White & White & White & White & White \\
Slow Dry & Transparent & Transparent & Transparent & Delaminated & Delaminated & Delaminated \\
Liquitex & White & White & White & White & White & White \\
Wellbound & Transparent & Transparent & Opaque & White & Slight White & Slight White \\
T. Medium & Transparent & Transparent & Transparent & White & Slight White & Transparent \\
Bob Ross & White & White & White & White & White & White \\
Rod Dodge & Transparent & Opaque & Opaque & White & Slight White & Slight White \\
\hline
\end{tabular}
\end{table}
Figure 5.1 *Synechococcus* 7002 growth profiles. A. SF012 screening. B. SF091 Screening. L: latex, G: glycerol, S: sucrose. Error bars ± 1 Std Dev, n=3
The growth profiles obtained for SF012 and SF091 are very similar (Fig. 5.1). For SF091, the two treatments that included latex on the formulation yielded lower cell densities compared with the controls, specially the one that included sucrose and glycerol, which might be associated to some degree of sensibility to a component of the formulation or decreased light penetration to the well due to the scattering characteristics of the latex particles. For SF012 the formulation that included latex, sucrose and glycerol reached a higher cell density compared to latex by itself with no cryoprotectants.

Chlorophyll a extraction measurements showed that pigment synthesis begins as soon as the plates are set under the lights, followed by a sharp increase in total chlorophyll alpha concentration as the cells enter their exponential growth phase (Fig. 5.2). Despite the similar behavior of all the treatments up to t = 100h (a lag period with a lower rate of chlorophyll synthesis due to cell acclimation to the new microenvironment in the well), the total final pigment concentration was always greater in the presence of the latex binder and comparable to that of the control. If latex was not present in the formulation, the final Chl\textsubscript{a} concentration tended to be lower as if there was a synergistic effect with respect to the binder but not with the cryoprotectants. This observation led us to modify the coating formulation protocol described in this chapter by excluding both glycerol and sucrose from the formulation so our formulation for making paper coatings of Synechococcus contains only a 50/50 suspension of cells and latex as described in detail in chapter 2.
Figure 5.2 *Synechococcus* 7002 chlorophyll production profiles. A. SF012 screening. B. SF091 Screening. L: latex, G: glycerol, S: sucrose. Error bars ± 1 Std Dev, n=3
The average chlorophyll content in *Synechococcus* cells growing in the 24-well plate system is lower than the 2 pg cell\(^{-1}\) value reported for the shake flask system previously (Fig. 5.3). As can be seen in the plot, the controls came from shake flasks at 2pg chlorophyll cell\(^{-1}\), but after 1 week of incubation in the 24-well plate the average specific pigment content was reduced to 0.5 pg chlorophyll cell\(^{-1}\), which was the same value reached by the cells in the presence of any of the two types of latex (note the sharp bleaching peak in the bottom Fig. 5.3B for the first 24 h). This behavior is likely to be a consequence of the different conditions of aeration and illumination the cells are exposed to in the 24-well plates. While shake flasks have baffles that disrupt the mixing pattern, distribute the liquid in a thin film on the flask wall and create bubbles that enhance gas transfer between the liquid and gas phases, the bottom of the plate wells is flat and the radial displacement of the shaker might not achieve acceptable mixing in the plate. Similarly, the effective light intensity in a shake flask is lower due to the geometry of the incubator (illumination provided from the top) and the greater culture volume in the flasks. The top of the plate is directly exposed to the lights so some cells can reach photosaturation and decrease their specific chlorophyll a content as a self-tuning response to the higher light intensity (Fig. 5.4). Our results on paper coatings of *Synechococcus* also showed that cells tend to modulate their chlorophyll content and saturate at lower light intensities\(^5\) when the geometry of the system is highly restricted (i.e. immobilized in a coating or a small liquid phase at high cell density in the well). These observations served as a motivation to further investigate the effect of intensification (i.e. a high cell density in a small volume or on a small surface area) on tolerance to high light intensity and temperature as reported in chapter 2.
Figure 5.3 *Synechococcus PCC7002* specific chlorophyll production profiles. A. SF012 screening. B. SF091 Screening. L: latex, G: glycerol, S: sucrose. Error bars ± 1 Std Dev, n=3
We performed a series of qualitative patch coating experiments with *Synechococcus* patches to test coating stability upon rehydration in BG11(N+) media. Specifically, we were interested in finding out if any chlorosis could be reproduced in 4.5 cm$^2$ patches incubated in shake flasks and testing suitable patch dimensions to be used on 24-well plates. Pigment bleaching was also observed in *Synechococcus* patches, but the bleaching rate is different depending on the type of latex used. Patches prepared using SF091 degrade faster than SF012 patches, which is clearly visible as a loss of the characteristic green color and the
appearance of blisters on the surface of the coating. Furthermore, by $t = 144$ h, SF091 patches begin to disassemble and all coating integrity is lost (Fig. 5.5).

Figure 5.5 Photobleaching and delamination of $4.5 \text{ cm}^2$ *Synechococcus* sp. patches on polyester sheet incubated in BG11(N+) media under 17 PAR μmol photons m$^{-2}$ s$^{-1}$ at 25°C for 1 week.
Similar photobleaching results were reproduced using smaller 2.8 cm$^2$ patches suitable to be used on 24-well plates (Fig. 5.6). Over the course of 1 week all the patches bleached towards a pale yellow coloration, at different rates depending upon their location on the plate. Visual comparison with suspensions of cells + latex revealed that bleaching also occurs in suspension, but subsequent recovery of the pigment is observed for long periods of time with no fresh media added. Bleaching occurs more quickly in patches and suspensions including SF091 latex which correlates well with the results obtained in shake flask culture with the bigger 4.5 cm$^2$ patches. In general, coatings remain bleached but some cells escape to the media and grow in suspension. Further studies will be necessary to determine if patch bleaching is reversible upon incubation on fresh media as can be achieved using suspension cells. Based on the apparent toxicity of SF091 and the lack of clarity/stability of the remaining binders tested, we chose SF012 as the latex binder to be used for our paper coatings experiments (chapter 2).

5.3 Chlorosis and pigment recovery in suspension cultures

As an integral part of our studies on the metabolic characteristics and behavior of two of the cyanobacteria unicellular strains grown in our lab (Synechococcus PCC7002 and Synechocystis PCC6308), and after analyzing the preliminary bleaching results described in the previous section, we decided to conduct a study with both strains in which we aimed to reproduce at least one complete cycle of chlorosis and pigment recovery under the regular conditions we use for culture maintenance in shake flask culture.
Figure 5.6 Photobleaching screening in 24-well plates. 2.8 cm$^2$ *Synechococcus* patches and suspension + latex binder incubated in BG11(N+) media under 17 PAR μmol photons m$^{-2}$ s$^{-1}$ at 25°C for 1 week.
5.3.1 Methods

5.3.1.1 Chlorosis and recovery protocol

500 mL shake flasks containing 100 mL of BG11 media were inoculated from *Synechococcus PCC7002* and *Synechocystis PCC6308* stock cultures at $10^7$ cells mL$^{-1}$ and incubated in a shaker at 25°C and 17 PAR μmol photons m$^{-2}$ s$^{-1}$ illumination. Chlorophyll a extraction was performed daily according to the protocol described before. Total Chl$_a$ concentration was followed until the yield reached 10-15% of the maximum chlorophyll concentration recorded in the flasks. At this point bleached cultures were transferred to sterile falcon tubes and centrifuged at 3,000 x g for 15 minutes. Cells were resuspended in fresh BG11(N$^+$) media and transferred back to the original culture flasks. Cultures were incubated and tested for total Chl$_a$ concentration under the same conditions until a new pigment peak was observed and subsequent pigment bleaching resumes.

5.3.2 Results

Both strains reached their maximum chlorophyll concentrations in approximately 10 days and started to bleach up to a minimum by day 20 (Fig. 5.7). As shown by the large error bars and the pictures of the flasks during the bleaching phase, cells in different flasks did not not lose their pigment at the same rate, even when incubated under the same light intensity and temperature; this observation differs from the homogeneous rates of chlorophyll synthesis observed during the two growth phases recorded.
Figure 5.7 Chlorosis and recovery profiles in suspension. A. *Synechocystis* PCC6308. B. *Synechococcus* PCC7002.
Once the cells were centrifuged and resuspended in fresh media, a lag phase was observed for both strains, which means that pigment synthesis is not resumed immediately and some time is needed to induce the molecular mechanisms that will make the cells resume synthesizing Chl$_a$. This period of adaptation to the new media took approximately 12 days for both strains after which exponential pigment synthesis resumed. Whether pigment synthesis is induced by replenished nutrients or by light irradiation will need to be further investigated as this experiment was performed under constant illumination.

As it can be seen in the profiles, recovered bleached cultures can reach the same maximum pigment concentration after new nutrients are available in the media. Similarly, once the nutrient concentration in the media decreases, cells begin to lose their pigment in a cyclic pattern. This suggests that the bleaching process does not damage the molecular mechanism of pigment synthesis and that cells are very resilient to changing environmental conditions. The literature reports that some strains of *Synechococcus* can undergo chlorosis due to nitrogen starvation as an adaptation mechanism for long term survival under limited nutrient availability conditions$^6$. Photosynthetic pigments are scavenged to release nitrogen and subsequently pigments are re-synthesized for light harvesting when stronger illumination occurs. It will be really important to further understand the molecular basis of these results as we are interested in ensuring that once the cells are immobilized in a coating they will keep their long term photosynthetic activity. Although some extrapolation should be possible, the bleaching process in coatings has different implications as this is a non-growing system and cells are organized in such a way that their interaction with light is very different compare to suspension cultures.
5.4 Preservation of plant leaves for gas evolution analysis

As previously discussed in chapter 1, the cellular biocomposites concept developed in this dissertation was inspired by the structure and functionality of the plant leaf. In this section we describe a preservation method we developed to obtain O₂ evolution and CO₂ consumption data from live plant leaves to be compared with cyanobacteria suspensions and coatings. In order to account for the different illumination regimes present in nature, we used leaves harvested from trees growing under full sun, partial shade and complete shade light regimes.

5.4.1 Method

~2 g of leaves of water oak (*Quercus nigra* L. – shade tree), flowering dogwood (*Cornus florida* – part-shade tree) and Eastern redbud (*Cercis canadiensis* – full sun tree) were cut at the base of the petiole and immediately placed into horizontal Balch tubes containing 5 ml of sterilized commercial cut flower food (Crystal clear 200, Floralife, SC). The tubes were sealed and flushed for 5 minutes with a gas mixture of 80% N₂ and 20% CO₂. Suspension cultures of *Synechococcus PCC7002* were inoculated from 3-day old shake flask cultures at 1 x 10⁷ cells mL⁻¹ in Balch tubes containing 5 mL of BG11 media and flushed with the gas mixture described above. Preserved plant leaves were incubated at 17 μmol photons m⁻² s⁻¹ illumination without shaking at 25°C, while suspension cultures were kept well mixed by mounting the tubes in a shaker (Labquake®, Labindustries Inc., Berkeley, California). O₂ production and CO₂ production were followed via GC for 170 hours until plant material started to show signs of rotting and/or chlorosis.
5.4.2 Results

The commercial cut flower formulation used to preserve the plant leaves (a combination of citric acid, glucose and antifungal agents) differs significantly from BG11 and any comparisons between the photoreactivity of plant leaves and cyanobacteria cells under these conditions are merely preliminary and qualitative. Preserved plant leaves are more reactive than *Synechococcus* cells in suspension (Fig. 5.8). Photosynthesis resumes as soon as the leaves are placed in the photobioreactor with no lag adaptation period even though they are no longer connected to the plant stem. Since the plant material was cut preserving the petiole, leaves still can absorb nutrients through the vascular system and harvest light through the available foliar surface. No sharp differences were observed between the relative photoreactivity of sun, part-shade and shade leaves in this system up to $t = 100h$. After this point, the sun leaves perform better and reach a better cumulative $O_2$ production by $t = 170$. Given that this experiment was carried out under constant illumination condition, it makes sense to expect a sun tree to tolerate better continuous high light irradiance compared to part-shade and shade trees. Higher plants undergo photoinhibition\(^7\) at high light intensities similar to cyanobacteria as described in chapter 2.
Figure 5.8 O₂ production and CO₂ consumption by preserved plant tissue and *Synechococcus* cells in suspension. Inserts show pictures of the preserved plant material at t = 0 hours and t = 170 hours.
Plotting the photoreactivity of preserved plant cells in a natural leaf structure side by side with suspensions of *Synechococcus* cells is problematic given the different nature of the two systems. Not only the media is different, but a plant leaf is a continuous pectin-sealed multi-layered structure while planktonic cyanobacteria are better modeled as a discrete photosynthetic particle. One way to normalize the units is to express photoreactivity in a specific basis with respect to surface area, gram of biomass or number of cells. Given the differences observed in this experiment and taking into account that cellular coatings are deposited on a fixed surface area, the natural choice of units for the experiments described in chapter 2 was normalized as a function of the coated surface area for long-term photoreactivity assays (for comparison with plant leaves) and as a function of cell number for intrinsic kinetics (for comparison between coating immobilized cells and suspension cultures).

**5.5 Zeta potential measurement**

Given that there are no previous reports on dielectrophoresis of cyanobacteria in the literature, a series of preliminary experiments was carried out to screen the strains previously described for chain formation and zeta potential values. This parameter is related to the surface charge distribution of the cells, and therefore can be used as a first approximation to characterize the particle behavior of these microorganisms when exposed to AC fields.
5.5.1 Method

A Pen Kem System 3000 (Pen Kem, Inc., Bedford Hills, NY, U.S.A.) was used to measure the zeta potential of the cyanobacteria strains previously described. A horizontal, cylindrically focused beam of laser illumination traverses the stationary level of a horizontally mounted parallel plates of an electrophoresis chamber 0.1 by 1.0 cm. Light that is scattered 90° from the beam by the cells undergoing electrophoresis is focused through a microscope objective and serves as a reference to visualize the movement of the cells. Both the laser beam and the microscope are focused on the upper stationary layer, a position at which electro-osmotic effects are zero. A voltage is applied across the electrodes, causing the suspended cells to migrate in the electrical field. If a prism in the optical system is adjusted manually, the electrophoretic movement of the cells can be cancelled on the horizontal direction, and the device automatically computes the average value of the zeta potential for the cells in the chamber. Dilutions (1:100) of BG11 grown cells resuspended in de-ionized water were analyzed this way in triplicate. The same cell suspensions were used in preliminary DEP chaining experiments according to the methods described in chapter 4.

5.5.2 Results

The results show that the polarity of the zeta potential resembles the expected negative charge for biological membranes, and that there are differences on the values among all the tested strains (Fig. 5.9). Such differences can also be seen qualitatively in the pictures where cell chaining was recorded after 30 minutes of DEP for the four unicellular strains tested.
Interestingly, different chain patterns were obtained for the two strains of the genera *Synechocystis* tested; *PCC 6308* shows regular chain structure, with an average chain length of 10±1 (n = 3) cells in close contact with each other and little or no lateral interaction between chains. However, for the strain *PCC 6803*, the chain length is larger (20±1 cells per chain, n = 3) and there was no apparent contact between adjacent cells or chains. This effect could be a consequence of the difference in the zeta potential value compared with the other strains tested (~50% smaller in magnitude), but given the constant distance that is separating the cells and the literature reports regarding the presence of dense exopolysaccharide protective capsules in most species of planktonic cyanobacteria, the most likely reason this
apparent separation is observed is due to a physical, non-conductive barrier between the cells that keeps them from touching each other and cannot be visualized via optical microscopy. The cumulative effect of many of these insulating capsules between cells could translate into the lower zeta potential value recorded, as the difference in potential between the no-slip plane close to the cells and the bulk of the liquid would be smaller assuming there are no conductive components in the capsule.

The marine strain *Cyanothece ATCC 51142* also forms chains in suspension, but the average chain length is lower (5±1 cells per chain, n = 3) and there are significant lateral interactions that cause some clumping between adjacent cells. Previous reports describe a negative correlation between salt concentration and chain length for DEP, which could explain why this marine strain, with a higher salt content in its membranes and cytoplasm, cannot form long chains in the direction of the field lines. However, no osmotic lysis of the cells was observed, even when resuspended in pure water, which proves the robustness of these microbes under harsh environmental conditions.

The longest chains were observed with *Synechococcus PCC 7002*, which is also the strain that aligned in chains most quickly, just seconds after the field is turned on. The chains are homogeneous, and in some cases the chains can cross the whole electrode field with more than 200 cells per chain. As will be discussed in the following section, these chains exhibited strong lateral interactions for longer DEP times, which is a promising characteristic for the fabrication of 2D arrangements in the 4-electrode chamber. For these reasons, *Synechococcus PCC 7002* was chosen as the model organism for the subsequent experiments on DEP assembly of cellular coatings on polyester sheets.
References


CHAPTER 6

Summary and future outlook

Oscar I. Bernal and Michael C. Flickinger
6.1 Summary

The objective of this dissertation was to utilize several model photosynthetic bacteria or cyanobacteria to develop novel methods for the fabrication and characterization of photoreactive cellular biocomposite materials for solar energy harvesting, oxygen production and other applications such as biomimetic leaves. The first part of this thesis is devoted to laying the conceptual framework necessary to define and classify the biomaterials we developed in the context of materials science and biomimetics. By expanding the role of the word “cellular” to biocomposite (i.e. indicating the presence of whole living cells and a hierarchical level of organization of the microstructure) in our definition we created a new concept that describes materials characterized by the presence of live structural elements (cells) that are reactive and can self-tune in response to environmental stimuli. This description is both functionally and conceptually tied to that of the plant leaf, which allowed us to build a case around the advantages of using leaves as a model platform to incorporate a vascular network and a layered distribution of biocatalytic cells into our cellular biocomposites.

Chapter 2 describes a simple extrusion method for the deposition of highly reactive cellular coatings of live cyanobacteria. This approach is an extension of the classic method developed by the Flickinger lab for painting living bacteria onto polyester and other non-porous substrates. By choosing a paper-based nonwoven substrate with high porosity as a model rudimentary vascular system we opened a wide range of unforeseen possibilities and applications that ultimately led to the description of several physiological changes in immobilized cyanobacteria never reported in the literature previously. We observed up to
~10 fold specific photosynthetic rate enhancements in cellular coatings of cyanobacteria compared to the same number of suspended cells along with significant changes in the tolerance to light intensity and temperature that deviates from the characteristic behavior observed in suspension cultures reported in the published literature. This method also proved to be suitable for high-throughput intensification of photosynthetic reactivity, as a single 300x concentration step yielded coatings that are close to matching the specific reactivity (per m²) of plant leaves under the same experimental conditions. These findings suggest that a new paradigm may be available to fabricate and analyze the performance of highly concentrated cellular biocomposites of photosynthetic cells and a significant portion of the current knowledge on the physiology of photosynthetic microorganisms derived from data using suspension cultures may need to be reconsidered in the light of these results.

Chapter 3 describes a novel approach to immobilize live cells as integral components of a fiber-based matrix. We called this new cellular biocomposite *microbial paper (MP)*, a powerful nonwoven wet lay materials concept that was conceived from our work on cellular coatings of cyanobacteria and that could revolutionize the way living organisms are used as “off the shelf” dry stabilized biocatalysts for biomanufacturing. Our first approach to fabricate the MP sheets was based on the traditional method for fabricating hand-made paper sheets and allowed us to reduce to practice this concept by generating preliminary microstructure and reactivity data. These promising results revealed the complex nature of the biocomposite and the effect of manufacturing conditions on the final viability and photoreactivity of the immobilized cells. Further development of the technology was achieved by implementing a vacuum dewatering-based method for MP matte formation and
the incorporation of microfibrillar cellulose (MFC) as part of a more defined paper pulp formulation that also included a blend of hard and soft wood native cellulose fibers. These enhancements allowed us to improve dramatically cell retention and the mechanical strength of the MP sheets when wet. We characterized the effect of the MFC content on the final reactivity of the films, which is an excellent starting point for further improvements to the microbial paper manufacturing method.

Chapter 4 was devoted to the development of a novel cellular coating method that allows the precise manipulation of living cyanobacteria via dielectrophoresis (DEP). This method was conceived to be used as a tool to address fundamental aspects of cellular coating technology, namely surface coverage efficiencies, light scattering, cell-substrate interactions and modeling using monolayer coatings. By using a combination of dielectrophoretic assembly in suspension and controlled deposition on a surface-modified polyester substrate we achieved significant improvements in surface coverage efficiencies compared to free gravity settling in the absence of electric fields. These results provide evidence of high cell viability preservation post dielectrophoretic assembly and deposition using vital staining and *in situ* confocal microscopy, which proves the suitability of the method for manipulation of living organisms. Our numerical simulations extended our understanding of the mechanistic aspects of the assembly process and allowed us to treat and model the cyanobacteria as particles, which opens an interesting range of possibilities for other types of cells with different shapes, sizes and metabolic characteristics. The clear nature of the flexible polyester substrate also allowed us to characterize the optical properties of the coatings and the integrity of the photosynthetic pigments post-immobilization.
6.2 Future outlook

The incorporation of living cells as part of the integral structure of a composite material is a powerful concept that needs to be further developed into a wider range of cell containing biocomposite materials. Considering that some estimates of bacterial species in the world range from $10^7$ to $10^9$ (only bacteria, not even considering yeast, algae, archea and the growing number of industrial strains of engineered insect and mammalian cells) it should be clear that the potential applications of incorporating living cells into composite materials to add complex biocatalytic functionality are limitless. However, in the context of this dissertation there are a couple of extensions of the methods described that will dramatically boost the efforts to take cellular biocomposites to the marketplace.

The first natural extension of the methods described in this dissertation is to use dielectrophoresis to deposit highly oriented monolayer coatings of cyanobacteria on a fiber-based substrate or on filaments that can be made into nonwoven materials. We performed some exploratory experiments to test this possibility by coating a surface-modified polyester monofilament using dielectrophoresis (Fig. 6.1). We surface-activated the polyester monofilament using the method described in chapter 3 and fixed the two ends to the sides of the perfusion chamber. The surface of the monofilament is smooth prior to deposition as shown by optical microscopy (Figs. 6.1A,C). When the cell suspension is added to the chamber and the field is applied, cyanobacteria chains assemble and start to migrate towards the filament, which acts as a physical barrier that divides the chamber in half (Figs. 6.1B,D). When the liquid phase is removed and the polyester is washed with water, a single layer of cells remains attached to the surface and oriented along the axis of the filament (Fig. 6.1E).
**Figure 6.1** DEP-mediated coating of polyester monofilaments with cyanobacteria. A. Raw polyester monofilament (scale bar 200 µm). B. Coating process at $t = 15$ min DEP exposure (scale bar 200 µm). C. Raw polyester monofilament (scale bar 50 µm). D. Coating process at $t = 30$ min DEP exposure (scale bar 50 µm). E. Partially coated washed polyester monofilament (scale bar 50 µm).
It is therefore logical to envision a continuous coating system in which filaments move at a slow speed through the cell suspension and rotate at constant angular speed simultaneously in such a way that the entire surface is exposed to oriented chains and is coated by cyanobacteria bundles by electrostatic attraction of the polyelectrolyte coating with the negatively charged cell surface. The final result would be a photosynthetic filament useful to fabricate a photoreactive fabric (woven or nonwoven) for large-scale light harvesting applications.

Being able to coat a single filament with a photosynthetic organism can be an interim step towards the development of new higher-impact biocatalytic materials technologies in the bioenergy field. The literature reports several examples of cyanobacteria capable of being used as photo-biocatalysts for direct production of electricity in photo-bioelectrochemical cells. These biophotovoltaic devices employ photosynthetic organisms immobilized on the anode to generate electrical power via the water-splitting reaction. The reported immobilization methods rely on drying the cyanobacteria cells on the conductive substrate (carbon cloth, carbon nanotubes, etc.) or in the formation of natural biofilms on the surface of graphite electrodes which translates into short-lived power generation and little or no control of the proliferation, orientation and long term viability of the immobilized biomass. Given that the electric current production magnitude scales with the concentration of immobilized biomass and light intensity, we foresee our cellular biocomposites fabrication methods for cyanobacteria cells to become the workhorse of high-intensity sustained biological power generation. This capability can be further expanded by taking advantage of the atomic layer deposition methods currently available to for making conductive coatings on
nonwoven fiber mats. With this technology, virtually any type of fiber-based material or fabric can be made conductive and suitable for immobilization of current-producing microbes, which may constitute a major breakthrough not only for the characterization of the molecular mechanisms of bioelectricity generation but for large scale biological power generation from solar light.

In yet another potential application in the bioenergy field, CO₂ could be converted into liquid biofuels or fuel precursors using a cellular biocomposite device. When non-growing photosynthetic biofuel-producing microbes are trapped in a cellular biocomposite (superficially or integrally), the product recovery process is simplified, cells are retained for continuous use in the bioreactor and light harvesting is optimized due to reduced self-shading and light scattering. Furthermore, product secreted from the cells can be separated by taking advantage of the porous nature of the immobilization matrix. There are some examples of the use of immobilized whole cell biocatalysts for enzymatic production of biofuels precursors. Wang and collaborators discussed the potential of systems based on solid state fermentation (SSF) and highly heterogeneous biofilms for hydrolysis of lignocellulosic materials. Interestingly, the authors point out the advantages of a layered structure where multiple microbial species are organized to sequentially transform complex substrates into simple sugars susceptible of being cofermented by other members of the population. Furthermore, fungi can also be included in the symbiotic biofilm to allow simultaneous saccharification and delignification. Another system extensively studied is the use of the lipase produced by immobilized cells of Rhizopus orizae for the production of biodiesel via enzymatic methanolysis. Here, most authors focus on trying to make the whole cell biocatalyst
reusable\textsuperscript{10}, expressing more efficient lipases from \textit{Fusarium}\textsuperscript{11} and optimizing the methanolysis operational parameters via design of experiments\textsuperscript{12}.

As pointed out previously, none of the systems described above is photosynthetic, hence no direct CO\textsubscript{2} sequestration takes place. Cyanobacteria and unicellular eukaryotic algae are capable of producing and secreting volatile ethylene or liquid fuel precursors from CO\textsubscript{2} and solar light, as extensively reported in the literature\textsuperscript{13-16}. A cellular biocomposite that secretes a liquid fuel and incorporates several product purification steps in a single device would be the cornerstone to turn a highly energy and water intensive suspension culture photobioreactor or lagoon-based industry into a more energy efficient, sustainable and commercially viable alternative.

Another field that can evolve substantially by incorporating cellular biocomposite technology is environmental remediation. During the course of this dissertation we performed proof of concept experiments in collaboration with Prof. Marc Deshusses’ group at Duke University where we showed successful volatile organic compound (VOC) degradation by cellular coatings of \textit{Pseudomonas putida F1} cells\textsuperscript{17} immobilized on chromatography paper using the methods described in chapter 2. In this short exploratory study we observed up to 10 fold specific toluene biodegradation rates increase compared to agarose-based biofilms, which correlates nicely with our results on CO\textsubscript{2} absorption by cellular coatings of cyanobacteria\textsuperscript{18}. It appears that in non-photosynthetic systems diffusion mass transfer limitations and water activity are the key factors that limit long-term biodegradation activity of biofilters for air-pollution control applications. These results encourage us to envision other potential environmental applications such as high-intensity
gas-phase CO$_x$ absorbers based on coated microfluidic channels or coiled rolls of microbial paper that could simultaneously sequestrate greenhouse effect atmospheric gases and produce second generation biofuels from non-food sources.

All of the previous potential applications could be further improved by the development of a truly continuous bioprocessing platform based on highly concentrated (intensified) immobilized biomass that can be preserved and stabilized when dried. As a result of increased regulatory oversight (requiring safer and more reproducible processes), cost reduction pressures and higher product quality demands, some estimates point out that within 10-15 years this technology will become the standard in the field replacing fed-batch and perfusion bioreactors$^{19}$ (Figure 6.2A). Continuous bioprocessing has some inherent advantages such as streamlined process flow, small equipment size, high volumetric productivity, steady state operation, reduced capital cost and low cycle times$^{20}$. Biopharmaceuticals is among the last industries to incorporate process intensification through conversion from batch to continuous manufacturing if we consider the long tradition of other industries (petrochemical, steel casting, food, paper) on continuous operation$^{20}$. Up to this point most efforts have been focused on the incorporation of perfusion systems for continuous upstream processing$^{21}$ (Figure 6.2B).

Proof of this new philosophy are the efforts carried out by some of the large pharma companies to allocate significant resources for the development of continuous pharmaceutical manufacturing platforms through alliances with academic centers.
**Figure 6.2** From fed-batch to modular continuous biomanufacturing. A. Traditional fed-batch process (upstream + downstream)\textsuperscript{20}. B. Continuous upstream processing (perfusion) coupled with batch capture\textsuperscript{20}. C. High density perfusion coupled with PCC chromatography (complete continuous operation)\textsuperscript{20}. D. Modular continuous manufacturing via cellular biocomposite integrating a whole cell biocatalyst with purification media\textsuperscript{25}.
One good example is the 10-year partnership between Novartis and MIT - the Center for Continuous Manufacturing – that aims to completely redefine the way medicine is made by drastically reducing manufacturing time\textsuperscript{22}. The final product of this collaboration is an all-in-one pilot scale continuous plant that starts from a chemical intermediate for one of Novartis’ best selling drugs and performs “all intermediate reactions, separations, crystallizations, drying and formulation”\textsuperscript{23} resulting in a final tablet ready to be shipped. The whole plant is housed in a 24-foot-long by 8-foot-wide clear plastic case that allows technicians to reach in at certain points and replace parts to adapt the plant to other types of products. This facility is now located in Basel, Switzerland where Novartis is using it as a scale-down model of a commercial size continuous manufacturing site to be opened in 2015\textsuperscript{22}. Other companies like Pfizer, GSK, Amgen and Genzyme are conducting their own continuous manufacturing research programs\textsuperscript{24}.

The pharma experience is slowly permeating into the biopharmaceuticals and biologics industry. A combination of high-density perfusion cell culture and periodic counter current chromatography (PCC) has proved to be useful for continuous capturing of candidate protein therapeutics\textsuperscript{20} (Figure 6.2C). In all cases the final quality of the product was comparable to that of the batch process while several unit operations (clarification and hold steps) were eliminated as a result of the new simplified continuous scheme.

A modular cartridge-type device could be engineered from several layers of functional cellular biocomposite layers to provide true continuous bioprocessing using concentrated non-growing microbes (Figure 6.2D). One layer would contain the stabilized non-growing biomass immobilized in a nanoporous matrix. The next layer could contain
functionalized fiber-based capture media with a custom-tailored surface chemistry to bind cell-derived contaminants and allow the target molecule to flow through. Extra accessory modules for sensing and polishing could be added or removed from the “modular biofactory” as product needs change or individual components age. This conceptual frame could fuse upstream and downstream processing in a single device, eliminate the need for a perfusion cell retention/recycle loop and add further functionality to the manufacturing train.

At the heart of the optimization efforts of the reactivity and half-life of our cellular biocomposites lies the elucidation of the molecular mechanisms that are triggered when non-growing living cells are immobilized at high intensity and are responsible of the specific reactivity enhancements we report in this dissertation. Our preliminary work on the photosynthetic cyanobacteria and the VOC systems suggest some factors (self-tuning, reduced self-shading and mass transfer limitations, water activity) that might also contribute improved performance of biocomposites compared to suspension systems, but only a detailed study at the transcriptome level will reveal which metabolic pathways are up-regulated, bypassed or even shut down under non-growth conditions. This path has very recently been opened by McKinlay and collaborators who investigated the non-growth physiology and hydrogen production rate of *Rps. palustris*\(^26\), but similar studies will be needed for other microbial systems (photosynthetic and non-photosynthetic) with potential to be used as immobilized cellular biocatalysts.
References


