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Painting and Printing Living Bacteria: Engineering Nanoporous Biocatalytic Coatings to Preserve Microbial Viability and Intensify Reactivity

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Latex biocatalytic coatings containing ~50% by volume of microorganisms stabilize, concentrate and preserve cell viability on surfaces at ambient temperature. Coatings can be formed on a variety of surfaces, delaminated to generate stand-alone membranes or formulated as reactive inks for piezoelectric deposition of viable microbes. As the latex emulsion dries, cell preservation by partial desiccation occurs simultaneously with the formation of pores and adhesion to the substrate. The result is living cells permanently entrapped, surrounded by nanopores generated by partially coalesced polymer particles. Nanoporosity is essential for preserving microbial viability and coating reactivity. Cryo-SEM methods have been developed to visualize hydrated coating microstructure, confocal microscopy and dispersible coating methods have been developed to quantify the activity of the entrapped cells, and FTIR methods are being developed to determine the structure of vitrified biomolecules within and surrounding the cells in dry coatings. Coating microstructure, stability and reactivity are investigated using small patch or strip coatings where bacteria are concentrated 10^2- to 10^3-fold in 5−75 μm thick layers with pores formed by carbohydrate porogens. The carbohydrate porogens also function as osmoprotectants and are postulated to preserve microbial viability by formation of glasses inside the microbes during coat drying; however, the molecular mechanism of cell preservation by latex coatings is not known. Emerging applications include coatings for multistep oxidations, photoreactive coatings, stabilization of hyperthermophiles, environmental biosensors, microbial fuel cells, as reaction zones in microfluidic devices, or as very high intensity (>100 g·L^{-1} coating volume·h^{-1}) industrial or environmental biocatalysts. We anticipate expanded use of nanoporous adhesive coatings for prokaryotic and eukaryotic cell preservation at ambient temperature and the design of highly reactive "living" paints and inks.

1.0. Introduction

Many microorganisms in nature are associated with surfaces in biofilms. Biofilms are hundreds of microns to several millimeters thick and have complex structure formed by secreted extracellular polymeric materials, and the embedded microbes are biologically active. The biological activity of biofilms is time-dependent because microbes colonize, grow, die, and are eventually sloughed from the surface. However, not all microbes are capable of forming biofilms. It would be useful to mimic natural biofilms in order to engineer efficient whole-cell biocatalysts on surfaces. This requires engineering nontoxic adhesive polymers capable of permanently entrapping and stabilizing a high density of *any* viable cell to *any* surface in a thin (<100 μm thick) nanoporous coating with minimal diffusion resistance. These thin composite polymer coatings, known as biocatalytic coatings and microbial inks, could revolutionize the use of microbes as biocatalysts (*1*, *2*).

Polymer coatings are used in many commercial products for direct energy savings and resource conservation (*3*); however, they can also be used as nanoporous supports to mimic the design of heterogeneous chemical catalysts (*4*). The development of waterborne latex coating technologies over the past 2 decades with lower volatile organic content (VOC) in order to comply with environmental regulations has produced emulsions with low solvent toxicity (*3*). The polymer chemistry and film-forming properties of these waterborne emulsions can be

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modified to generate adhesive nanoporous latex coatings for permanent entrapment of living cells (*1*).

Reactive latex polymer coatings containing biological molecules such as enzymes or antibodies were first developed more than 25 years ago by Eastman Kodak for use in layered dry reagent clinical chemistry (*5*, *6*). Latex biocatalytic coatings containing enzymes include hygienic, antifouling, biodecontamination, biosensing or biodetection coatings, and coatings used to stabilize, concentrate, intensify, and miniaturize enzymes for biocatalysts. However, nanostructured coatings containing enzymes in most cases are capable of only single-step hydrolytic reactions (*7*−*13*). Many isolated enzymes cannot carryout oxidizations or reductions because they require expensive cofactors (such as ATP for phosphorylation or release of energy by hydrolysis) and electron donors (reduced co-enzymes NADH, FADH, or reduced ferredoxin). Although numerous in vitro cofactor regeneration systems including cofactor "tethering" have been reported for immobilized enzyme systems (*14*), it remains more cost-effective to use robust living cells to regenerate cofactors, and industry still uses slowly growing, immobilized, or non-growing "resting" microbes for chiral oxidations and reductions to produce chemical intermediates and for waste treatment.

1.1. Why Develop Coatings of Non-Growing Microorganisms Preserved at Ambient Temperature? Microbes can be genetically manipulated to dramatically alter their capabilities as biocatalysts (*15*, *16*). However, living cells as biocatalysts are seldom available as "off-the-shelf" (stabilized) highly reactive (concentrated) reagents, cannot be stored without loss of activity unless refrigerated or frozen, are used in dilute suspension in the same reactor as they were propagated, and have insufficient stability (active half-life) and specific reactivity (intensity) to replace most chemical catalysts for industrial processes.

Preservation of living cells at ambient temperature in adhesive polymer coatings provides an inexpensive method to stabilize their viability and activity so that highly concentrated microbes can be used with minimal mass transfer limitations on surfaces. Engineering nanoporous coatings for preservation of the reactivity of living partially desiccated cells without refrigeration is a challenging composite materials problem. Solving this problem will not only expand the use of whole microbial cells as biocatalysts but may also be useful for transporting and preservation of eukaryotic cells without refrigeration. Understanding how nanoporous coatings stabilize cell viability during film formation (drying) as well as during storage will allow the design of biocatalysts with dramatically increased volumetric reactivity (process intensity). In addition, by using systems biology, metabolic and protein engineering methods to understand nutrient-limited gene expression and optimize in vivo enzyme stability, in the future non-growing microbes could be genetically optimized for use as "off-the-shelf" reagents with reactive half-lives comparable to those of chemical catalysts (thousands of hours) (*4*).

1.2. Development of Robust Porous Materials Containing Living Microorganisms. ***1.2.1. Biocers.*** Living cells have been entrapped in many types of modified cross-linked sol−gel synthetic polymers, in sol−gel oxide ceramics (biocers) and silica gel glasses (*17*−*24*). Biocers (biological ceramics) are formed by freeze-casting methods and are mechanically robust ceramic coatings (hard, transparent, often reinforced using fibers) made from silica or inorganic nanosols. However, these sol−gels often kill the majority of the embedded microbes as a result of alcohol toxicity during cross-linking to form lyogels. Cell viability is further reduced during lyogel drying or freeze drying to form stronger xerogel films as a result of shrinkage of the silica network. Alcohol toxicity can be minimized by using aqueous nanosols produced by metal-catalyzed silicon or metal alkoxide hydrolysis in water. The addition of glycerol (*24*) as well as the addition of pore-forming monosaccharides such as sorbitol is important to retain high cell viability (∼70% viable cells) and high coating porosity (50−60% measured by mercury intrusion). Average pore diameters measured by BET are 7−10 nm (*23*). However, the major disadvantage of sol−gel ceramics for cell entrapment is that only 5−20% by weight of cell mass can be incorporated. Higher concentrations of cells reduce coating stability when hydrated. Therefore their reactivity per square meter is very low and their ability to retain entrapped cell viability after storage at 4 °C is poor, <5% cell viability after 9 days (*23*). Recently Desmone (*25*) reported a hydrophilic silicon oxide matrix for preservation of *E. coli* for recombinant protein production, but only as a starter culture.

1.2.2. Hydrogels. There are many publications describing hydrogel-based microbial entrapment systems (gel beads, slabs or gel-coated beads) because they are easily made in the laboratory, have high porosity and entrap cells with minimal loss of viability. Unfortunately hydrogels are not useful as coatings because they are not adhesive, have poor mechanical strength, and are hundreds of microns to millimeters thick, resulting in severe mass transfer limitations. Hydrogels are macroporous (pores larger than the microorganisms), have thin pore walls, and most hydrogels cannot be stored dry or frozen for long periods of time without loss of cell viability (*26*). When hydrogel-entrapped microorganisms are incubated in the presence of nutrients to sustain cell viability and regenerate activity, significant cell release and outgrowth occurs from microcolonies. Hydrogels require covalent cross-linking to enhance their mechanical stability and disintegrate when the cell concentration exceeds 30% (*26*). For this reason, despite the numerous publications and monographs (*26*−*32*), most hydrogel immobilization systems are not sufficiently stable, reactive, or robust, nor do they retain a sufficiently high concentration of cells for the process intensity and active half-life needed as industrial biocatalysts.

1.2.3. Early Studies of Latex Microbial Coatings. Microbial latex biocatalytic coatings were originated in the 1980s by Lawton, Bunning, and Flanagan, who were the first to report the use of polymer latexes to coat solid particles, nylon mesh, membranes, and silica (*33*−*37*). These studies used polydispersed acrylate/vinyl acetate copolymers (particle size ∼260 nm, T_g = 13 °C). Coating porosity was generated by integrating calcium carbonate that was later leached from the coatings with acid to form pores for colonization by microbial growth. Cantwell (*38*) first reported the use of polymer blends of hard and soft polymer particles having a T_g range of −60 to 60 °C for microbial entrapment to stabilize enzyme activity. However, this study did not coat these blends in thin coatings, only flocculates, 1−2 mm diameter aggregates and 2 mm diameter fibrils. No data was presented on cell viability following entrapment or aggregate permeability. Martens and Hall (*39*) reported the use of methylmethacrylate and butyl acrylate copolymer coatings of *Synechococcus* to generate photoreactive coatings on a carbon electrode. Upon rehydration and exposure to light, photosynthetic activity was measured in an electrochemical cell. Coating diffusion properties were measured using a rotating disc electrode. Cell viability was reported to be "nearly 100%". The major difficulties of these early investigations, however, were low coating porosity, coating instability (delami-

nating from the support particles, creep), poor control of coating thickness, and lack of methods to accurately measure the number of viable cells that survived the latex coating/drying/rehydration process. When these studies were performed there was little knowledge of the mechanism of waterborne film formation and how these polymer coatings might preserved microbial viability during coat drying at ambient temperature. In none of these early investigations was the nanoporosity, pore structure, uniformity, or the cell viability measured, nor was the physiological response of the latex-embedded microorganisms to film formation investigated.

Swope and Flickinger were the first investigators to demonstrate gene expression and β-galactosidase enzyme synthesis in 80 μm thick bilayer acrylate/vinyl acetate latex *E. coli* coatings using emulsions containing glycerol to generate porosity and preserve viability at ambient temperature in dry coatings and when rehydrated in nitrogen-limited media (*40*). *E. coli* retained viability and the ability to synthesize β-galactosidase for several weeks following coating on a polyester sheet, drying, and storage, with retention of the cells in the coating by a nanoporous sealant latex top coat (*41*). Differential viability staining and laser scanning confocal microscopy (LSCM) were used to determine that the viability of the entrapped *E. coli* was as high as 95% for several weeks; however, this method had limited penetration into the coatings due to photobleaching (*41*−*43*). Subsequent work by Lyngberg using *E. coli* coatings with a nanoporous top coating showed that the addition of sucrose or trehalose with glycerol could be used to increase coating porosity (*44*, *45*). A simple patch coating method was developed (*46*, *47*) using a perforated vinyl mask on a polyester substrate, and formulations were developed to make ink-jet inks and multilayer coatings from 5 to 65 μm thick. These early studies established laboratory methods to generate thin adhesive latex patch coatings ($\sim$2 to $\sim$75 μm thick, 2 orders of magnitude thinner than cross-linked hydrogels), overcoming the severe mass transfer limitations of hydrogel entrapment of living cells, and to increase latex coating porosity by incorporation of carbohydrate porogens. These studies also established that latex coatings maintained their adhesive properties and integrity when rehydrated at cell concentrations as high as 50% by volume.

1.3. Engineering Microbial Biocatalytic Coating Reactivity. ***1.3.1. Generating Nanoporosity in Latex Coatings.*** The reactivity of microbial latex coatings is a function of the entrapped viable cell density following coat drying and rehydration and nanoporosity. Latex paint emulsions are formulated for rapid polymer particle coalescence followed by polymer diffusion to generate a *nonporous* high molecular weight coating to protect the surface (substrate) from water. The rate and degree of polymer particle coalescence must be quenched or arrested at the point of film formation (particle ordering and partial polymer particle welding) in order to generate a porous coating that can be completely hydrated. The initial focus of investigations by our group was on generating and controlling the degree of coating nanoporosity (pores $<$50 nm) by quenching coalescence using temperature, the addition of porogens, bimodal blends, or core−shell approaches (*45*, *48*−*51*). Nanoporosity is generated in low glass transition temperature (T_g) polymer coatings by the addition of glycerol and carbohydrates to arrest polymer particle coalescence during film formation; however, when these low T_g coatings are rehydrated, porosity is slowly lost as a function of time and temperature as a result of wet coalescence. Bimodal polymer blend (*51*) and core−shell approaches can minimize wet coalescence following coating rehydration; permanently porous latex coatings useful for arresting wet coalescence have recently been described (*52*). In addition to nanoporosity, coatings must be adhesive (to substrate, layer to layer) and contain a very high volume fraction of microorganisms below the critical concentration that would disrupt polymer particle coalescence. However, the presence of cells can decrease porosity and blocks polymer particle coalescence depending on cell size and the interaction between the cell surface and the surface of the polymer particles (*44*, *45*).

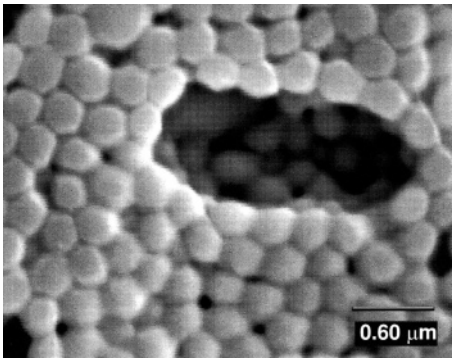

Figure 1. High magnification cryo-SEM of nanopores surrounding a void resulting from entrapment of *E. coli* ZK211 in a partially coalesced Rovace SF091 latex coating. (Reproduced with permission from ref 46. Copyright 1999 John Wiley & Sons).

1.3.2. Perfusive Latex Biocatalytic Membranes. An alternative form of diffusive biocatalytic coatings is a perfusive standalone trilayer membrane with microorganisms permanently entrapped between two thin nanoporous polymer layers or coated onto a microporous membrane substrate (*44*, *45*). Trilayer all-latex structures can be generated by multilayer coating onto a non-adhesive substrate and delamination with rehydration. With efficient mass transfer, the reactivity of perfusive biocatalytic membrane structures is limited only by the permeability of the entrapped microorganisms. Jons (*53*) reported fabricating porous latex membranes; however, the methods used organic solvents that are not compatible with microorganisms. Development of perfusive biocatalytic membranes containing viable microorganisms or latex coatings for membranes would enable the use of highly concentrated non-growing microbes as biocatalysts at phase boundaries (gas/liquid, aqueous/nonaqueous) in multiphase membrane bioreactors because the two surfaces of the trilayer membrane could have different polymer chemistry.

1.3.3. Activating Microbial Coatings Without Out-Growth. Latex-entrapped microorganisms are physically constrained because they are surrounded by nanopores with thick pore walls (the diameter of the partially coalesced polymer particles) (Figure 1). Most biocatalytic coating studies use nitrogen-limited conditions to inhibit microbial out-growth from single or bilayer patches or strip coatings (described below). A thin nanoporous sealant polymer-only topcoat is used in some systems to retain the cells embedded in the layers below. Phosphate and an energy source such as pyruvate, organic acids, or light sustain cell viability without significant growth. Although the physiology of latex-entrapped microbes is still unknown, up-regulation of gene expression and protein synthesis by the embedded microbes has been used to activate the coatings or to generate biosensing coatings (*47*). Gene induction can increase coating specific reactivity, increase sensitivity, and alter response kinetics. The demonstration of β-galactosidase expression over a period of 17 days induced by lactose or IPTG in non-growing latex-entrapped *E. coli* without out-growth was the first report of in situ activation of a microbial latex coating (*40*).

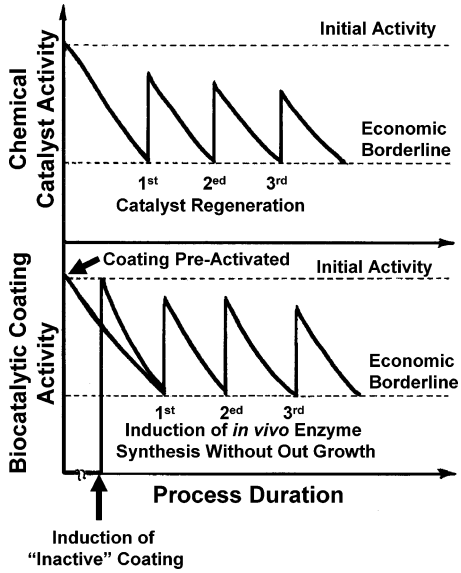

Figure 2. Comparison of chemical catalyst regeneration to biocatalytic coating activation and reactivation by induction of in vivo enzyme synthesis without outgrowth. (Reproduced with permission, adapted from ref 4. Copyright 1999 Wiley-VCH).

The ability to activate and reactivate biocatalytic coatings allows several approaches to significantly extend coating reactive half-life beyond that of suspended non-growing "resting cells" in an way identical to chemical catalyst regeneration (Figure 2) (*4*). In the first approach, biocatalytic coatings are cast pre-induced or "pre-activated". The microbes are induced to accumulate high levels of intracellular enzymes so that they contain the desired reactivity prior to coating. This approach has been used when the enzymes for the desired reactivity are membrane-associated (*51, 54*−*57*). Membrane-associated enzyme activity has recently been shown to be significantly stabilized in latex coatings (*54, 56*). However, as a result of in vivo protein turnover, intracellular enzyme activity will slowly decay. The potential exists to periodically induce new enzyme synthesis in coating-entrapped cells accompanied by limiting additions of a source of nitrogen thus reactivating or regenerating the coatings to maintain their activity above an economic level (Figure 2).

In the second approach, microbial biocatalytic coatings can be made, stored, and shipped without the cells having the desired enzymes for a particular application, i.e., an "inactive" coating. Following rehydration at the site of use, expression of the genes encoding the enzymes for that particular application can be induced by a suitable inducer along with the addition of a nitrogen source and the coating "activated" without growth. This approach is favored for cytoplasmic or periplasmic enzymes. In contrast to the first approach, microbial biocatalytic coatings will not lose in vivo enzyme activity during storage and shipping prior to use because they are manufactured in an inactive (uninduced) state. In use, these coatings can also be periodically induced and reactivated to maintain an economic level of activity (Figure 2).

2.0. Methods for Investigating Microbial Biocatalytic Coatings

2.1. Polymer Contact and Film Formation Toxicity. ***2.1.1. Toxic Polymer Synthesis Residues.*** A variety of acrylate, methacrylate, vinyl acetate, butyl acetate, butadiene and polystyrene latex polymers, copolymers and polymer blend emulsions with good coating and adhesion properties have been evaluated for toxicity to bacteria, yeast and fungi (*34*, *35*, *44*, *54*). In our work, both 30 min contact toxicity assays (CTA) followed by viable plate counting and a film formation toxicity assay (FTA, see below) are used to measure toxicity (*55*). The toxicity of latex emulsion components (surfactants, dyes, viscosity modifiers, residues from polymer synthesis, salts), many of which are concentrated in the coating pores surrounding the microorganisms during film formation, may also be evaluated individually. Characteristic polymer synthesis residues present in a monodispersed acrylic/vinyl acetate copolymer latex (e.g., Rovace SF091 ∼300 nm average particle diameter, T_g ∼10 °C, Rohm & Haas) have been evaluated to determine their toxicity to bacteria, spores and yeast. Characteristic residues for this type of latex emulsion include vinyl acetate (1400 ppm), acetaldehyde (150 ppm), methyl propionate (130 ppm), butyl ether (160 ppm) and butyl propionate (65 ppm). In general, many strains of Gram-negative bacteria are not killed by these residues. Some polymer synthesis residues, however, are toxic to Gram-positive bacteria and yeast. Where cytotoxicity to a specific microorganism is observed, the conditions of emulsion polymerization can be altered to eliminate the residue or the latex emulsion dialyzed or diluted to reduce toxicity. Subsequent reformulation of the dialyzed emulsions may be necessary to maintain coating properties.

2.1.2. Biocides. Commercial latex emulsions contain antimicrobial and antifungal biocides such as, for example, mixtures of isothiazolones (ACS Registery nos. 26172-55-4, 2682-20-4) that can be chemically inactivated prior to use with microorganisms. Latex preparations are also available without biocides, but this reduces their shelf life. Most Gram-negative bacteria added at the high concentrations used in biocatalytic coating formulations (∼10^{11} colony forming units/mL emulsion) are not very sensitive to the presence of biocides added at a level of 15 or 30 ppm. Gram-positive bacteria and fungi may be more sensitive. Dialysis may not remove all biocides because they may be weakly bound by polymer particle surface groups. In general, the density of microorganisms used in biocatalytic coating preparations is very high (50% v/v) and the number of cells killed by biocides is low.

2.1.3. Measuring Film Formation Toxicity. In order to evaluate the effect of concentration of nonvolatile polymer synthesis residues and biocides on the entrapped microorganisms, a film formation toxicity assay (FTA) was developed (Figure 3) (*55*). This assay measures cell viability during film (coating) formation as a function of emulsion formulation, temperature, relative humidity, and time. It can detect toxicity due to substances concentrated in the coating pores surrounding the microbes. In this method, a mixture of latex emulsion plus wet cell paste is rapidly coated into a thin channel formed by a mask on a perforated polyester substrate. Coated squares of polyester are periodically removed, and the cells and noncoalesced latex are immediately redispersed in buffer so that the viability of the redispersed microorganisms can be determined by viable plate counting (Figure 3). At the point of film formation, the coating cannot be redispersed and the cells are entrapped (the few microorganisms detected in the buffer after film formation are released from the edges and top of the newly formed coating). For some microorganisms, significant viability is lost as a result of film formation. This may occur if the coating has low porosity or when the microbes are osmotically stressed or starved for oxygen during film formation. Monitoring the change in cell viability as a coating is formed using the FTA assay can be combined with other measurements of microbial activity such as enzyme activity or the ability to carry out gene

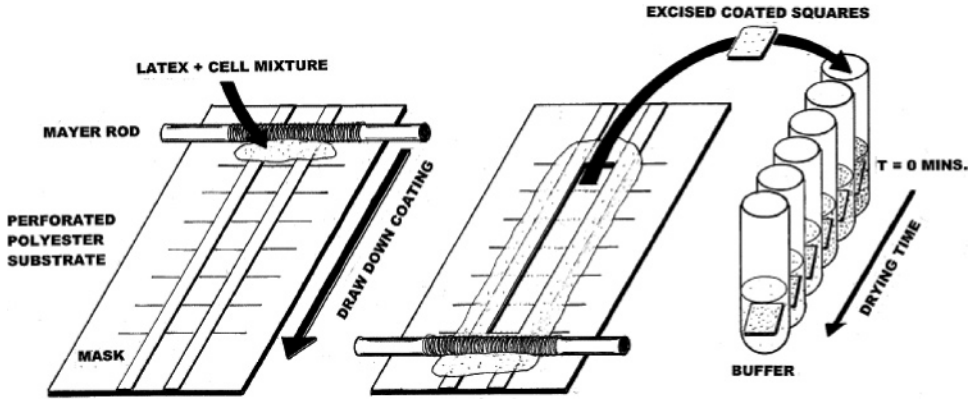

Figure 3. Latex film formation toxicity assay (FTA).

expression and protein synthesis. A particularly useful approach is evaluating whether stress-induced promoters are activated during film formation (see 3.2.2. below).

2.2. Laboratory Coating, Cell Viability, and Microbioreactor Techniques. ***2.2.1. Mayer Rod Draw Down Coating.*** Wire-wound or Mayer rod draw down coating methods (*58*) are used with masks to make small biocatalytic coatings on polyester, aluminum or stainless steel as sheets, strips, or patches with edges sealed by a sealant nanoporous topcoat (*45*, *46*, *56*, *57*) (Figure 4). However, because of the small size of theses coatings, edge effects and non-uniform coating thickness can be significant and need to be monitored (*44*, *56*). Coatings can be made in filtered air (such as within a laminar flow hood), in an anaerobic hood, or in a contained coating laboratory with both humidity and air-borne particulate control. Coating thickness can be altered by Mayer rod wire diameter (*58*), emulsion formulation (percent solids), rod pressure and mask thickness. Filaments also can be coated for use in non-woven filament biofilters (*59*, *60*). Rehydrated patches of 12−12.7 mm or 35−50 mm in diameter have patch thickness ranging from <10 to ~75 μm thick resulting in coating volumes of <3 to 125 μL. Strips sized to fit into tubes can vary from 1 to 12.5 cm^2 (*57*). Essentially any thickness and size patch or strip can be made to entrap viable microorganisms as long as the drying temperature and relative humidity are controlled. Relative humidity during coat drying should be >50% for optimal retention of viability. At 50% volume fraction of wet cell paste, 12.7 and 35 mm diameter patches contain $>1 \times 10^8$ and 1×10^{10} viable bacteria, respectively, but this varies with cell size and shape. Because of the very high microbial density, the sealant topcoat, and use in nutrient-limited non-growth media, coatings can be cast on non-sterile substrates (cleaned with 70% ethanol) using non-sterile latex emulsions (adjusted to neutral pH) under non-aseptic conditions.

2.2.2. Determination of Microbial Viability Following Coating Rehydration. Without a sealant topcoating of porous polymer, microbes can be released from the patches by shaking in buffer and mild sonication, depending upon the interaction between the cell and polymer particle surface. This "shake out" method was used by Lyngberg et al. (*46*) to estimate the number of cells that survived the coating, drying and rehydration process by viable plate counting (CFU). However, the interaction between the surface of the cells and the surface groups on the polymer particles that affect polymer particle coalescence and thus coating porosity is unknown and is different for different microorganisms. For example, hydroxyethyl cellulose (HEC), a nonionic polymer derivative of cellulose that is used as a thickener and stabilizer in paint to alter latex emulsion rheology (*61*), can affect coating porosity (*45*, *54*) and the interaction between the cell surface and the polymer particle surface that can be used to formulate redispersible coatings (*57*). Some latex coatings containing embedded bacteria can be disrupted with mild sonication, releasing large coating fragments, clumps of cells embedded in many latex particles that settle (*55*, *56*). Measuring cell viability by sonication of latex coatings followed by viable plate counting (CFU) usually results in an artificially low estimate of cell viability because not all the cells are released as single cells. For example, the attachment of two cells to a single latex particle would result in an observed loss of viability by the CFU method of up to 50%. Estimation of cell viability by ATP methods and vital staining in sonically disrupted latex coatings has been shown to be not as accurate as CFU (*54*, *56*). Recently, latex blend coatings of *Rhodopseudomonas palustris* easily redispersible without clumps with mild sonication have been developed (*57*) in order to more accurately determine the total number of viable cells that survive film formation and use embedded in latex strips.

2.2.3. Coating Microbioreactors. Because latex biocatalytic coatings are adhesive and contain a very high number of bacteria, many types of small volume bioreactors containing single patches or strip coatings have been devised for reactivity and kinetic studies. Initial *E. coli* studies of bilayer coatings used 1.3 cm squares of coated polyester with sealed edges (*40*−*43*) or 45 cm^2 circular coatings divided into sectors on the surface of 72 mm diameter aluminum plugs in a stirred, jacketed cell−thin film plug reactor (TFPR) (*62*). Oxygen uptake was used as a measure of *E. coli* viability following coat drying under different conditions and rehydration (*62*, *63*). Subsequently, 12.7 and 47 mm diameter *E. coli* patches were made using pressure sensitive vinyl holey masks formed by circular punches (*46*, *54*), which simplified sealing of the edges (Figure 4). The reactivity of 12.7 mm diameter bilayer patches can be investigated in shaken scintillation vials (*41*, *46*, *47*), Petri dish bioreactors (*54*−*56*), 24-well plates (Figure 4G), static anaerobic bottles (*51*), or in 10 mL highly aerated microbioreactors (*56*, *64*). Punches to create masks to coat patches of any diameter on polyester or other substrates are available, and a slightly larger diameter punch can be used to excise the substrate with the patch edges sealed with a topcoat (Figure 4**G**). Large 47 mm diameter patches can fit into polycarbonate membrane filter holders with flow distributors to generate small, inexpensive membrane bioreactors. These same filter housings can be used to make nonwoven latex-coated filament biocatalytic filter (FBF) bioreactors (*59*, *60*).

Strip coatings each 1 cm wide by 12.5 cm long have been made by Mayer rod coating in an anaerobic hood using a large rectangular mask mounted on precut polyester substrate to generate 13 identical coatings for Balch tube microphotobiore-

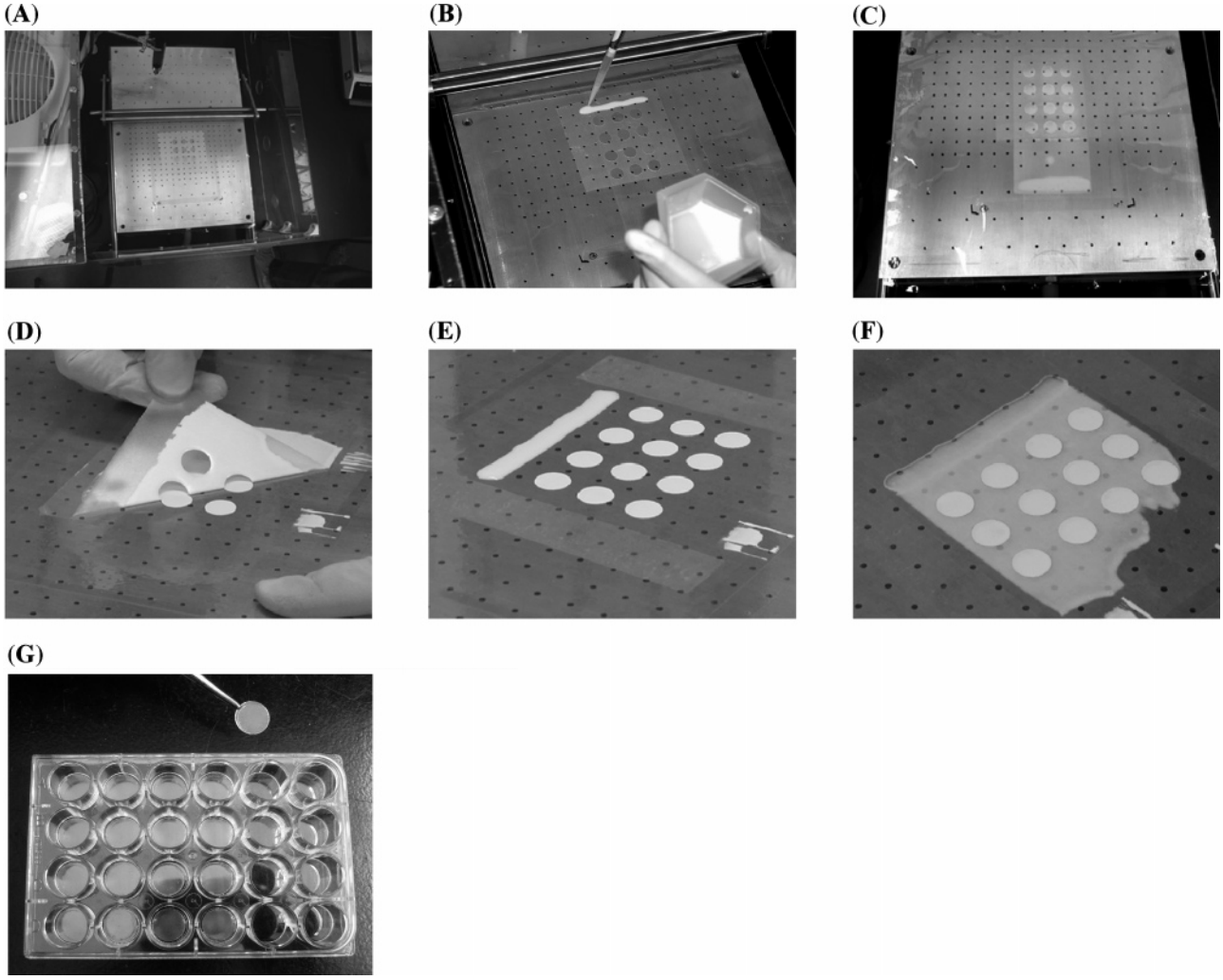

Figure 4. Bilayer latex patch coating method on polyester substrate (*44*). (A) Perforated pressure-sensitive vinyl mask on top of a polyester substrate on a vacuum table for draw down coating in humidity controlled chamber. B) Delivery of latex formulation (wet cell paste, glycerol and carbohydrate porogens). (C) Cellcoat material coated over mask by constant pressure Mayer rod draw down. (D) Removing the mask (opaque polymer used to show patches). (E) Application of side spacer strips and pool of top coat formulation. (F) Topcoat material after draw down coating. (G) Bilayer patch (12 mm diameter) punched and placed in a 24-well plate for measurement of reactivity.

actors (MPBs) for screening photosynthetic anaerobes (*57*). Both single layer and bilayer (top coated, sealed edge) strips of any size can be made by cutting appropriate pressure sensitive masks.

2.3. Measurement of Coating Porosity. ***2.3.1. Estimation of Porosity by Tracer Diffusivity.*** Biocatalytic coating reactivity is directly related to coating porosity, which has been estimated as tracer diffusivity in delaminated coatings mounted as films in a diffusion cell (*45*, *48*, *49*, *51*, *54*−*56*). In biocatalytic coatings, the microbial cells can also significantly decrease porosity depending on their size, shape and volume fraction. *E. coli* mass fractions as high as 150 g cell dry weight/L of coating volume have been reported (*45*).

Coatings are formulated and dried under controlled humidity and temperature to generate maximum diffusivity without compromising their mechanical and adhesive properties and without compromising their stability, i.e., their substrate adhesion and ability to retain a high density of entrapped microorganisms. The top sealant coat is a nanoporous sterile barrier that must be as thin as possible to reduce diffusion resistance and without coating flaws to retain the cells in the coating. For estimation of coating porosity, latex coatings are cast on stainless steel and dried for 1 h at 22−26 °C, 50% relative humidity and delaminated in 5 °C distilled water for 30 min. Stand-alone films 35 mm in diameter are punched from the delaminated coatings, and the effective diffusivity of a tracer molecule with strong UV absorption (such as nitrate or riboflavin) is measured at 30 °C by mounting the film between two well-stirred 14.5 mL chambers. The tracer is injected into the donor cell, and the UV absorbance of the receiver cell is monitored with a flow-through detector. The effective diffusion coefficient (D_{eff}) is calculated by Nightingale's equation based on a pseudo-steady state approximation (*65*). The diffusivity of latex films is expressed as the ratio of D_{eff} and the diffusivity of the tracer in water (D).

2.3.2. Measuring Changes in Coating Nanoporosity. Several methods can be used to quench coalescence and generate coating porosity during film formation using low T_g latexes without killing the entrapped microorganisms. These include decreasing capillary pressure by adding glycerol, coating at low temperature followed by a short high-temperature "welding" of the polymer particles, or arresting polymer particle coalescence by the addition of porogen carbohydrates such as sucrose, sorbitol or trehalose (*45*). Incorporation of sucrose into the coating increases the nitrate diffusivity from $D_{eff}/D = 0.002$ to $D_{eff}/D = 0.072$. At sucrose levels of 0.7 g/g latex, film formation is interrupted and the polymer particles redisperse (*45*, *54*, *56*).

Rehydration of low T_g latex coatings, which incorporate glycerol or carbohydrate porogens to arrest polymer particle coalescence, results in dissolution of the entrapped vitrified carbohydrate in the pore space. Unfortunately this can result in loss of porosity due to wet coalescence. The temperature dependence of wet coalescence of SF091 coatings ($T_g \sim 10$ °C) has been determined by Solheid (*54*) and described by an Arrhenius relationship with an activation energy of 108 kJ/mol (*51*). Arresting wet coalescence of rehydrated latex coatings is important for engineering a stable microstructure that maintains high porosity over time. This can be accomplished by using bimodal polymer blends, core shell or non-film-forming high aspect ratio polymer (HARPS) in blends. The porosity of polymer blends and core shell coatings is a function of blend ratio and shell thickness, respectively. The non-film-forming cores will not change diameter when the coating is rehydrated. The addition of sucrose to a bimodal blend with non-film-forming 800 nm polystyrene particles combined with a low T_g latex used to coat *Thermotoga maritima* (*51*) did not change rehydrated coating permeability, indicating that formation of carbohydrate glasses in the pore space does not contribute to porosity in bimodal blends of large non-film-forming and smaller film-forming particles (*50*, *51*, *54*). Bimodal blend coatings showed stable porosity over a period of 5 days in seawater at 80 °C (*51*).

2.3.3. Imaging Coating Microstructure and Nanoporosity. Cryogenic scanning and field emission scanning electron microscopy (cryo-SEM, cryo-FESEM) methods pioneered by Davis, Scriven, and coworkers for monitoring film formation in low-VOC waterborne latex coatings and particle shape following coating rehydration have been used to confirm arrested polymer particle coalescence and reveal extensive nanoporosity surrounding the embedded microorganisms (*46*, *49*, *56*). The large dark pores in Figures 1, 5, and 6 are spaces occupied by bacteria before they were released from the coating, and in general the size of these spaces does not differ significantly from the size of the microorganisms measured in suspension (*45*). The nanopores formed by arresting coalescence of these polymer particles are <20 nm in diameter. Fissures of <50 nm in width are also observed in some coatings (Figure 7A). Lyngberg (*45*) correlated the observed pore structure of the top of a coating using image analysis with diffusivity measurements. Coatings cast on silicone wafers and plunged into liquid nitrogen can be broken on a cryo-stage to produce an image of the cross section of the entire coating (Figures 6 and 7). Field emission cryo-SEM images of the fracture surfaces of latex coatings show characteristic star-shaped polymer particle ductile cryo-fracture pull-outs observed at high magnification by Ma (*50*) and Ge (*67*) as well as the structure of the nanopore space surrounding the entrapped microorganisms in the fracture plane (Figure 7B).

3.0. Model Systems for Engineering Coating Reactivity

3.1. *Gluconobacter oxydans* Coatings for Oxidation of D-Sorbitol to L-Sorbose. A model system for casting coatings of "pre-activated" microbial cells to engineer a bilayer latex coating to maintain the viability and enzyme activity of the strictly aerobic acetic acid bacterium *Gluconobacter oxydans* during film formation, coat drying and storage has recently been reported (*56*). Acetic acid bacteria such as *Gluconobacter* are used for quantitative oxidation of polyols to ketones, ethanol to acetic acid, and sugars to acids (*68*) but are very sensitive to oxygen starvation (*69*). This organism is used for the industrial oxidation of D-sorbitol to L-sorbose, a biological step in L-ascorbic acid (vitamin C) production. The reactivity, kinetics and effectiveness factor (η) of the quantitative oxidation of D-sorbitol to L-sorbose by *G. oxydans* ATCC621 was determined in a non-growth nitrogen-limited medium composed of sorbitol, pyruvate and phosphate buffer to eliminate growth of cells released from latex coatings (*56*). The kinetics were determined using 6 μL volume 12.7 mm diameter acrylate/vinyl acetate bilayer latex patches as a function of cell coat and topcoat thickness. The activity of individual patch coatings can easily be studied in Petri dish bioreactors or oxygenated micro bioreactors (Figure 8). With topcoatings of greater than 12 μm thicknesses, <0.05% of the cells were released during the course of the experiments, allowing for accurate determination of the effectiveness factor without a significant contribution to the observed reaction rate from released cells. Even though the coatings were optimized for porosity, the viability of this strict aerobe in latex patches was reduced to 51% for cellcoat thicknesses of >10 μm, indicating the sensitivity of this microbe to oxygen starvation during film formation and coat drying. The addition of the sealant polymer topcoat layer further reduced viability. For bilayer coatings, the cell viability was reduced from 51% down to 30% and 11% as the top sealant coat thickness was increased from 9 to 20 μm, respectively (*55*, *56*). The active half-life for the oxidation of D-sorbitol to L-sorbose of bilayer 12.7 mm diameter *G. oxidans* latex patches made with glycerol and 0.4 g sucrose/g polymer was determined to be ~430 h compared to only ~28 h for suspended cells.

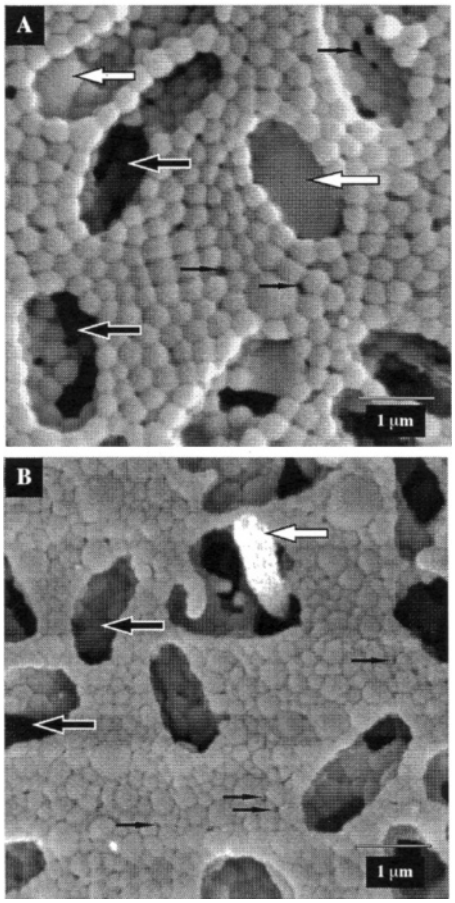

Figure 5. Cryo-SEM images of coating voids (large black arrows) created by entrapment of *E. coli* and pore spaces between polymer particles (small arrows). White arrows: *E. coli*. (A) Coating generated with monodispersed acrylate/vinyl acetate copolymer with sucrose (SF091) and glycerol. (B) Coating generated with polydispersed acrylate/vinyl acetate copolymer (RES661) plus glycerol (Reproduced with permission from ref 49. Copyright 1999 Elsevier).

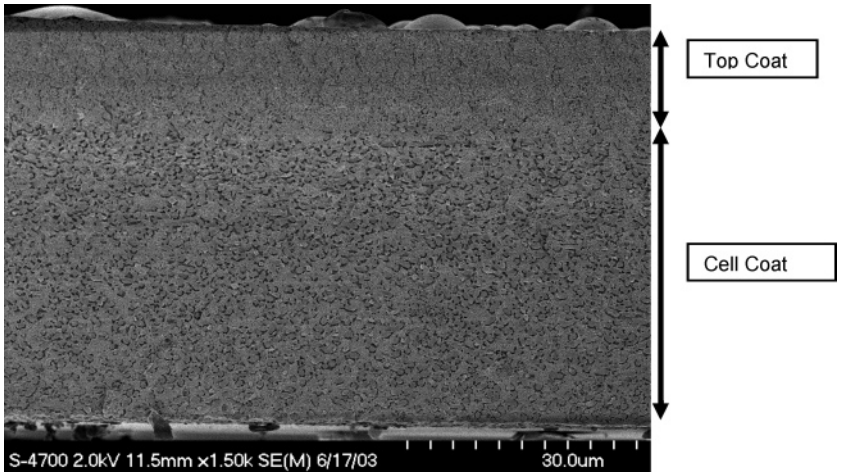

Figure 6. Freeze-fracture cryo-SEM image of a *G. oxydans* bilayer coating. Cell coat thickness 35 μm; topcoat thickness 15 μm. (Reproduced with permission from ref 56. Copyright 2006 John Wiley & Sons).

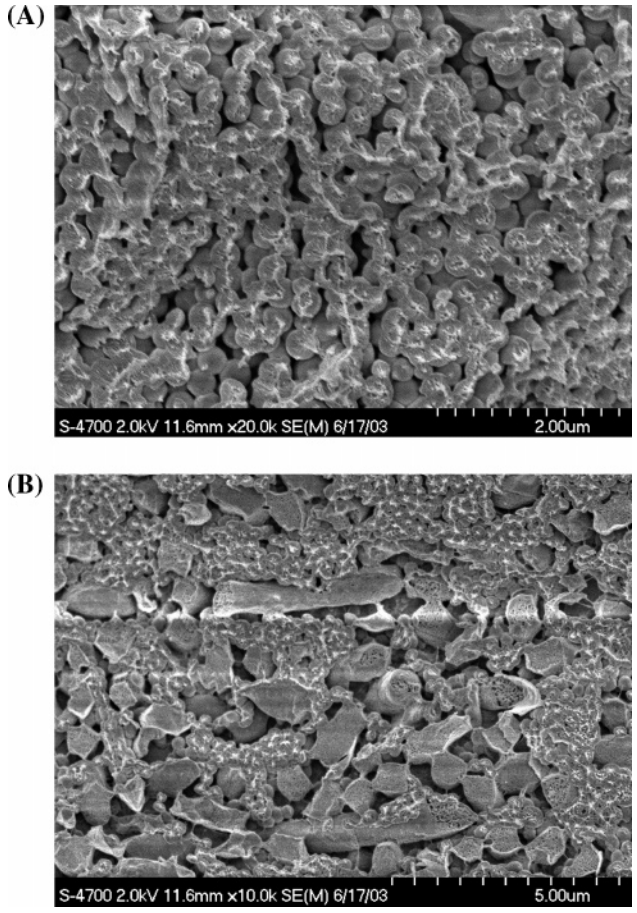

Figure 7. Cryo-fracture FESEM image of a *G. oxydans* coating. (A) Top coat showing fissures and polymer particle pull-outs. (B) Cell coat showing pore space surrounding the latex-embedded *G. oxidans.* (Panel B reproduced with permission from ref 56. Copyright 2006 John Wiley & Sons).

Hydrated *G. oxydans* patches could be stored for at least 7 weeks at 4 °C without loss of reactivity (*54*).

A bilayer diffusion reaction model was developed to predict the effectiveness factor (η) as a function of cellcoat thickness and topcoat thickness (*55*, *56*) in order to optimize the cell concentration and to predict the oxygen concentration profile and oxygen consumption rate in the cellcoat layer as a function of bulk liquid oxygen concentration. The experimental data and simulations using the diffusion reaction model were in good agreement, indicating that extremely high coating reactivity can be achieved, especially using thin topcoats of <10 μm thickness. L-Sorbose volumetric production rates of >100 g/L of coating volume·h can be achieved (Figure 9) if both D-sorbitol and oxygen can be continuously supplied at a sufficient rate to not limit the reaction velocity. These rates are significantly higher than any previously reported suspended or immobilized *Gluconobacter* oxidation rates for this same oxidation (*70*). Using single 6 μL volume patches (Figure 8), effectiveness factors of 0.22 to 0.24 were measured, which are 20-fold higher than previous values reported by Müh (*70*). The observed effectiveness factors were higher than the model-predicted values of η because of the nonlinear dependence of viable *G. oxydans* density in the cellcoat as a function of oxygen availability as a result of topcoat thickness. This study confirms that very thin microbial biocatalytic coatings can be engineered for very high volumetric reactivity (intensity).

In order to exceed L-sorbose volumetric production rates of 100 g/L of coating volume·h, higher oxygen transfer rates would be needed than are achievable in aerated suspension bioreactors. Monolithic microchannel reactors with high surface to volume ratio are used in the chemical industry for very high gas−liquid−solid mass transfer such as for hydrogenations (*71*−*74*). The biological equivalent of these systems is a gas−liquid reaction catalyzed by a biocatalyst immobilized on the wall of a microchannel. The above diffusion reaction model has been recently combined with mass transfer models of multiphase (gas−liquid−solid) microchannel reactors. Simulations of the oxidation of D-sorbitol to L-sorbose in a model 4 meter 1,000 μm diameter microchannel coated with a 21 μm latex coating of *G. oxydans* operated in the kinetically controlled regime with segmented Taylor flow (intermittent gas bubbles and liquid slugs) indicates that with polymer topcoats of less than 5 μm, reaction velocities of >200 g/L·h could be easily obtained (*75*).

3.2. Reactive Coatings as Biosensors or To Detect Microbial Stress During Film Formation. ***3.2.1. Regulating Gene Expression in Non-Growing Latex-Entrapped E. coli.*** Investigation of gene expression, protein synthesis and in vivo protein activity have been carried out in latex-entrapped *E. coli* because of the ease of constructing plasmids encoding promoters fused to *lacZ*, *lux* or *gfp*. Using gene fusions, the kinetics of non-growth-associated inducible gene expression can be monitored nondestructively in latex patches using color changes (β-galactosidase cleavage of a non-colored substrate to produce a colored compound), luminescence or fluorescence. The *plac*

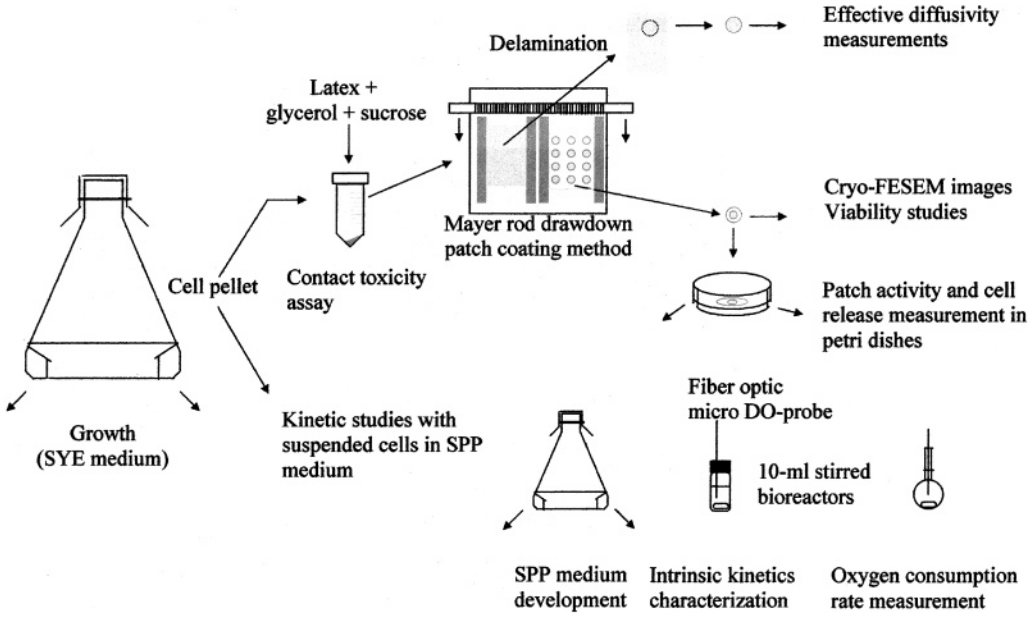

Figure 8. Steps in preparing and investigating the diffusivity and reactivity of *G. oxydans* latex patch coatings. (Reprinted with permission from ref 56. Copyright 2006 John Wiley & Sons).

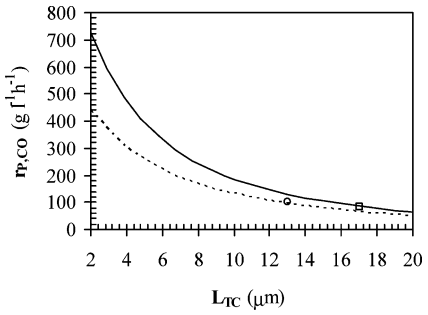

Figure 9. Simulated and experimental L-sorbose volumetric production rate ($r_{P,CO}$) per liter of coating volume in bilayer latex coatings as a function of topcoat thickness (L_{TC}) at different cellcoat thicknesses (L_{CC} = (—) 12 or (- - -) 21 μm) calculated by a diffusion reaction model. Symbols: ○, □, experimental data. (Reproduced with permission from ref 56. Copyright 2006 John Wiley & Sons).

promoter was initially found to be capable of regulating β-galactosidase gene expression in *E. coli* coatings incubated in a nitrogen-starvation buffer (*40*). Subsequently it was found that latex-entrapped *E. coli* contains both σ^{70} and σ^{S} RNA polymerase forms (*76*), suggesting that promoters capable of regulating gene expression either during vegetative growth or during stationary phase might be useful in entrapped non-growing cells.

3.2.2. Promoter-Fusions for Monitoring Gene Expression in Latex-Entrapped E. coli. Of the promoters investigated for non-growth-associated gene expression in latex coatings, the mercury(II)-inducible *pmerR* mer operon promoter is especially active in latex patches (100,000-fold upregulation) (*47*). This lead to subsequent development of a single-use latex patch biosensor for bioavailable mercury using *E. coli* HB101 pRB28 encoding a mercury-inducible *pmerR-lux* fusion developed by Selifonova (*77*). Recently, a *pmerR-gfp* green fluorescent protein fusion protein has been constructed (*78*) to study the kinetics of spatial gene expression in a 35 μm thick nanoporous bilayer coating using laser scanning confocal microscopy (LSCM). To orient the location of the GFP-expressing *E. coli* cells within the coating, fluorescent Estapor Y microspheres (555/570 nm excitation/emission) were added to the topcoat, and Flash Red fluorescent microspheres (660/690 nm excitation/emission) were added to the cell coat. Because of the different excitation and emission properties of these microspheres relative to GFP (488/509 nm excitation/emission), all three fluorescent molecules could be detected and quantified simultaneously. The analysis indicated that initially the *E. coli* closest to the topcoat−cellcoat interface showed a higher level of GFP induction relative to cells at the bottom of the coating (Figure 10). Higher concentrations of mercury used as inducer resulted in increased GFP fluorescence in the bottom layers of the coating (Figure 10), and therefore the observed gradient of mercury-induced GFP fluorescence is likely due to the properties of Hg^{+2} diffusion through the coating. This system can be used to optimize latex coating reactivity as biosensors and activation of biocatalysts with regard to cellcoat and topcoat thickness and the diffusion of oxygen, nutrients and inducers.

3.2.3. Stress-Inducible Promoters for Monitoring the Microbial Response to Film Formation. Altered gene regulation can occur during film formation as a result of the physical forces of polymer particle coalescence, partial desiccation, changing pH, ionic strength, osmolarity, and the effect of concentration of toxic polymer synthesis residues or biocides. In order to begin to measure the cellular response to film formation, a library of *E. coli* stress-inducible promoters fused to *lux*CDABE was evaluated along with the *pmerR* promoter under non-growth nitrogen-limited conditions in latex coatings with different chemical "stresses" at 25 and 37 °C. Some of these promoters are recognized by RNAPσ^{70} and some by RNAPσ^{s} (*79*). The different kinetics of luminescence induction, luminescence intensity and duration, as well as response to temperature indicates the potentially broad range of selective reactivity of microbial latex coatings (*79, 80*) (Figure 11). For example, the *pmerR* promoter responds slowly in latex patches to the presence of Hg^{+2} (maximal response in 500 min), whereas the *pkatG* promoter responds to the presence of H_2O_2 in ~50 min (Figure 11A). These differential responses indicate that coatings can be formulated containing either a single microbe or a mixture of microbes engineered for differential sensitivity to a wide variety of conditions in their environment (temperature, salt, toxic metals, oxidizers such as H_2O_2, ethanol, DNA-damaging agents, oxidative conditions, osmotic shock, desiccation, the presence of toxins, etc.) or for drug screening. The only

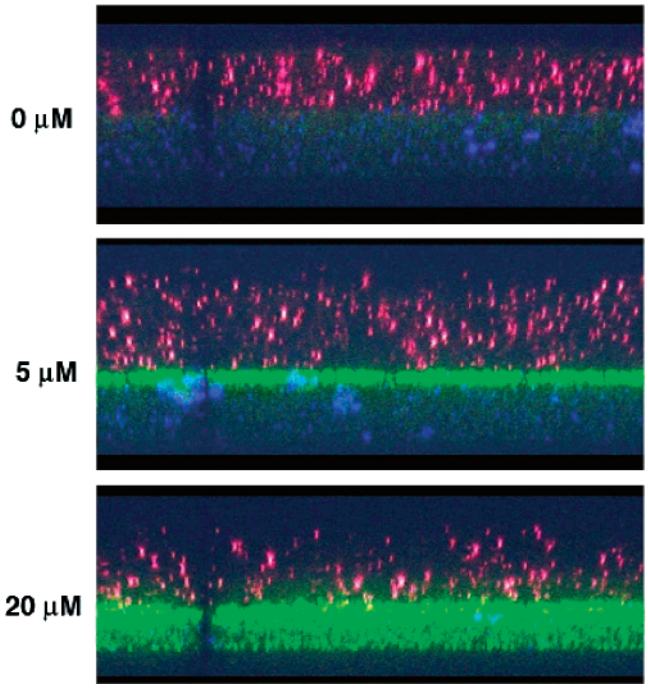

Figure 10. Z-plane confocal microscopy images of mercury-induced expression of GFP in 35 μm thick bilayer latex coatings of *E. coli* containing *pmerR-gfp* as a function of Hg^{+2} concentration, incubated at 30 °C for 42 h. Diffusion of Hg^{+2} from the top (*78*).

difference would be the mechanism of regulation of gene transcription of the reporter constructs of the entrapped strains.

In addition, microbial stress-inducible promoters can be used to detect intracellular responses to latex film formation. A sodium chloride responsive promoter, *pwza*, appears to be partially induced in a latex patch when rehydrated, indicating that *E. coli* "sensed" increased salinity during film formation (Figure 11B). These results indicate that the embedded microbes themselves could be the most sensitive indicators for detecting diffusion barriers and optimizing coating microstructure and drying conditions for cell viability.

3.3. Formulation of Reactive Microbial Inks. Latex coating formulations containing glycerol and sucrose have been modified to reduce viscosity below ~4 cp for piezoelectric droplet-on-demand layer-by-layer delivery of a high density of viable microbes in <5 μm thick coatings onto bioelectronic or microfluidic devices or for miniature high throughput microbial drug/toxicity screening. Ink formulations were evaluated in different ink-jet printing devices with 25, 50, or 64 μm nozzle apertures (*2*, *44*). Droplet sizes ranging from 0.1 to 0.5 nL were used to generate miniature square latex wells filled with latex + *pmerR-lux E. coli* or 10 by 10 arrays of latex dot microstructures (1−5 droplets per dot, 100 or 250 nL total volume printed) containing *pmerR-lux E. coli* (Figure 12A). Recovery of viable *E. coli* (CFU) by sonication indicated ~87% of the original cell viability even though ink drying was very rapid (<0.5 s at >50% relative humidity). LUX luminescence was induced with 100 nM Hg^{+2} and arrays responded fully within 400 min (Figure 12B). Printed *E. coli* density was 3.8−9.4 × 10^4 CFU per dot (25 or 50 μm tip, respectively) resulting in a printing density of ~1.5−2 *E. coli* μm^{-2} (*2*). Optical microscopy and profilometery were used to analyze dry dot thickness and shape (Figure 12, A and C). The structure of the dry dots indicates depressions (craters) caused by surface tension gradients during drying and substantial fluid flow prior to ink drying. Further development of latex ink formulations is needed

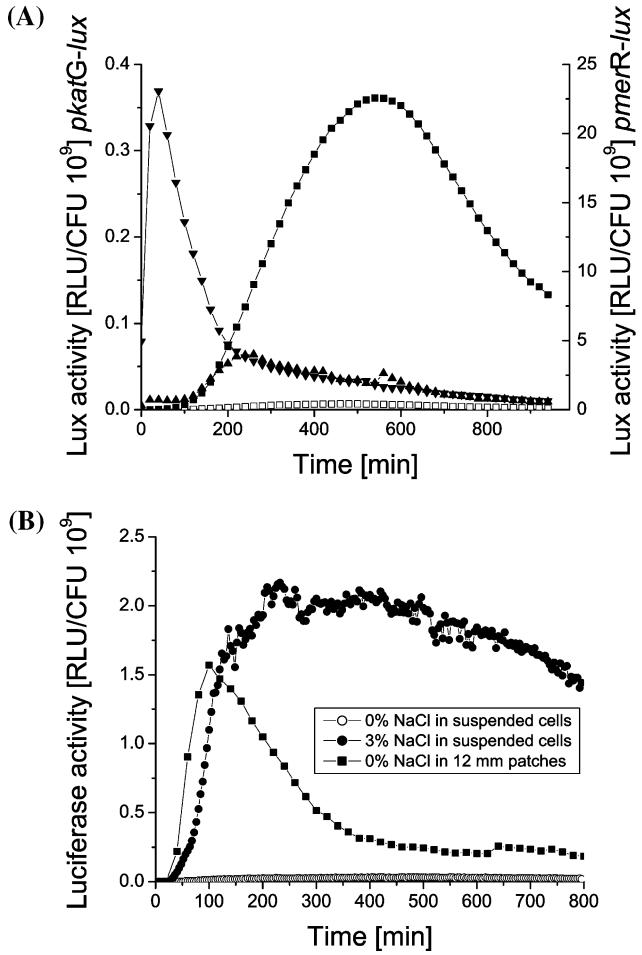

Figure 11. Relative luminescence response of *E. coli* stress-inducible promoters fused to *lux* in 12 mm diameter latex patches under non-growth conditions (nitrogen-limited, $n = 3$). (A) *pkat*G-*lux* at 37 °C: (▲) 0% H_2O_2, (▼) 0.006% H_2O_2. *pmerR-lux* at 25 °C: (□) no Hg^{+2}, (■) 5 μm Hg^{+2}. (B) *pwza-lux* inducible with NaCl at 37 °C (*80*).

to minimize surface tension-driven fluid flow producing gradients and stress on the entrapped microbes prior to film formation. Ink jet printing of microbial inks directly onto sensor arrays could be used to rapidly detect the composition of contaminants in complex fluids (liquids or gas) or to rapidly detect differential gene expression.

3.4. Engineering Photoreactive Coatings for Light Adsorption and Hydrogen Production. A potentially valuable application of latex coatings is to preserve and distribute photosynthetic microbial, cyanobacteria or algal cells for uniform illumination. This was first reported for *Synechococcus* by Martens and Hall (*39*).

Nitrogenase activity under non-growth conditions of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris* CGA009 is being evaluated in acrylate/vinyl acetate 1 cm × 6.25 cm latex strip coating micro photobioreactors (MPBs) with an argon atmosphere (*57*). Under these conditions *Rps. palustris* catalyzes the photodecomposition of acetate (and other organic acids as well as phenolic compounds) with generation of hydrogen gas. Single layer ~60 μm thick 6.25 cm^2 coatings of *Rps. palustris* containing ~10^{10} cells produce more hydrogen per square meter than the same quantity of settled cells. Hydrogen evolution is light-dependent (Figure 13A) and stable at 30 °C. Latex-entrapped *Rps. palustris* strips can be stored frozen at −80 °C for 1 year without loss of photoreactivity, and coating microstructure (porosity, the presence of osmoprotectants, glycerol, sucrose) affects hydrogen

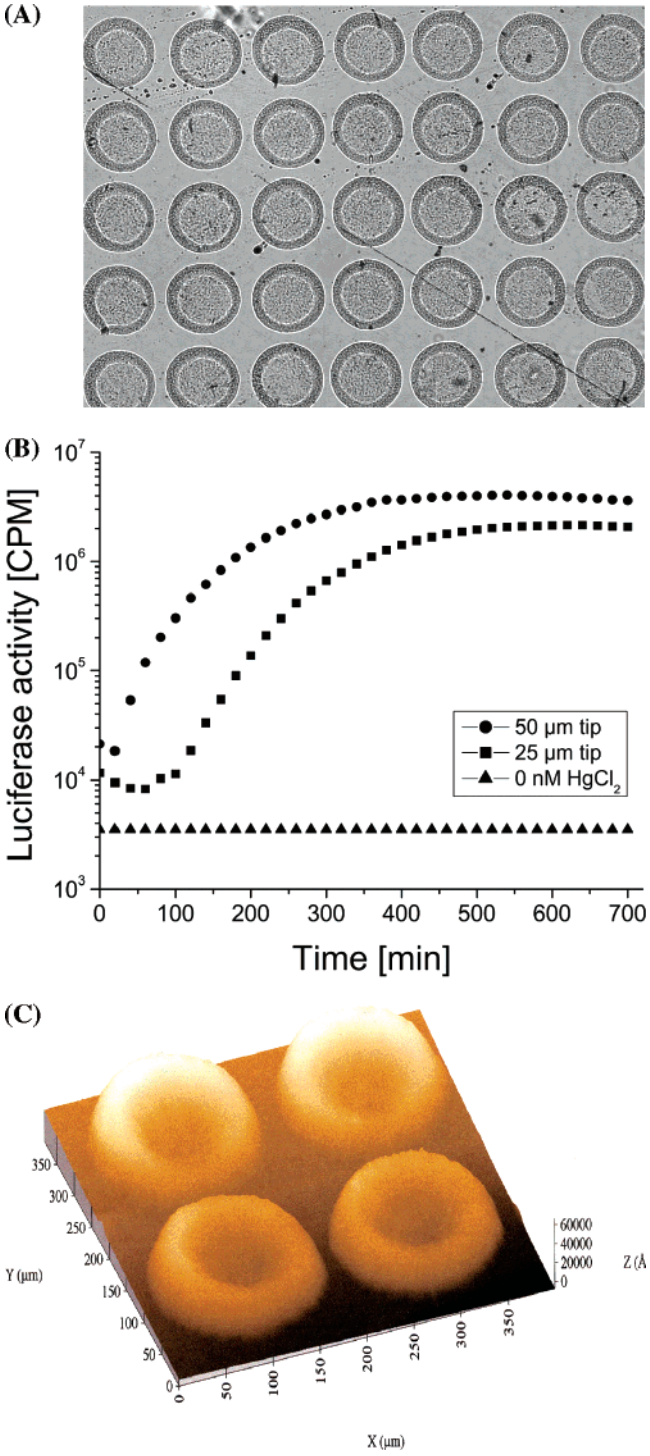

Figure 12. Ink-jet deposited *E. coli pmerR-lux* dot arrays using an acrylate/vinyl acetate latex ink formulation. (A) Dots printed on polyester sheet using 50 μm diameter piezo tip, 5 droplets per dot, ~9 × 10^4 *E. coli* per dot, ~170 μm dot diameter, ~2 cells/μm^2. (B) Luminescence response of 10 × 10 dot arrays of printed *E. coli* induced with 100 nM Hg^{+2}: (●) 250 nL total volume (2.5 nL per dot) printed with 50 μm tip, ■, 100 nL total volume (1 nL per dot) printed with 25 μm tip (*2*). (C) Profilometer image of *E. coli* latex dots showing craters. Dot thickness: center ~2 μm, rim ~6 μm.

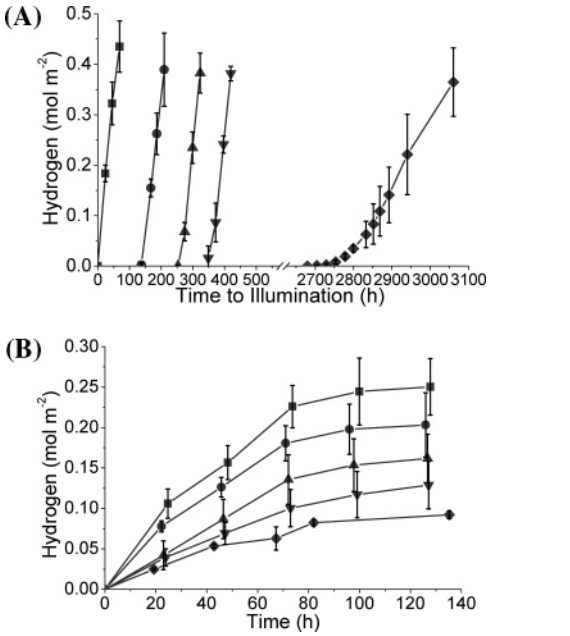

Figure 13. Hydrogen production by 54 μm thick latex coatings of *Rps. palustris* CGA009 in an argon atmosphere, 20 mM acetate. (A) As a function of storage time without light (foil-covered tubes) at 30 °C. Hydrogen production begins immediately upon illumination (removing of the foil covering) at 34 μmol photons/m^2•s. Initial rate following illumination (mmol H_2 m^{-2} h^{-1}): (■) 6.3; (●) 5.3; (▲) 5.6; (▼) 5.3; (◆) 1.6. Error bars ± 1 SD, n = 3. (B) Hydrogen production from *Rps. palustris* latex strips coated with emulsions containing different porogens. Coating formulations contain: (■) glycerol + sucrose; (●) sucrose only; (▲) glycerol only; (▼) no porogens; (◆) settled cells. Error bars ± 1 SD, n = 3. (Reproduced with permission from ref 57. Copyright 2007 American Chemical Society and American Institute of Chemical Engineers).

production rate (Figure 13B). The genome sequence of *Rps. palustris* CGA009 encodes three functional nitrogenase with different reactivity (*81*) and a mutated uptake hydrogenase (*82*). Thus this coating system potentially can be engineered to optimize photon adsorption and hydrogen evolution per surface area using multiple layers of coatings containing different nitrogenase and photopigment mutant strains optimized spatially for light adsorption by wavelength and intensity (Flickinger et al., PCT WO 2005/014805 A1, US 2005/0176131 A1). These photoreactive coatings may be useful for generation of hydrogen for fuel cells or as inexpensive biological photoadsorbers for distributed solar generation of hydrogen from organic wastes.

3.5. Bimodal Blend Coatings of the Hyperthermophile *Thermotoga maritima*: A Latex Coating That Retains Porosity at 80 °C. Hydrogel matrices can be unstable above 65 °C, and therefore high T_g polystyrene-based latex blends may be useful to concentrate and stabilize hyperthermophile microorganisms on surfaces for use a biocatalysts in extreme chemical and thermal environments. Using an 800 nm diameter polystyrene (T_g = 94 °C) mixed with 20% (v/v) of 158 nm diameter acrylate/styrene particles (T_g = −5 °C), a bimodal blend coating was developed, which when coated at 30 °C, had stable porosity (D_{eff}/D > 0.04) at 80 °C in seawater for 5 days for entrapment of the marine hyperthermophile *Thermotoga maritima* (Figure 14) (*44*, *51*). Starch hydrolysis by the toga-sheath-associated amylase of *T. maritima* for over 200 h was used as an indication of the biocatalytic potential of this high-temperature latex coating, which concentrated this microbe on polyester to 49 g wet cell weight/L of coating, 25-fold greater than the density achieved in anaerobic liquid culture (*51*).

3.6. Coating *Geobacter sulfurreducens* on Electrodes for Electrochemistry and Microbial Fuel Cell Studies. Microbial coating technology may also be a useful tool to study microbe-electrode interactions and to harness bacteria as electricity-generating or electricity-consuming biocatalysts. Anaerobic bacteria capable of utilizing metals as electron acceptors can also utilize electrodes as electron donors or acceptors (*83*−*85*).

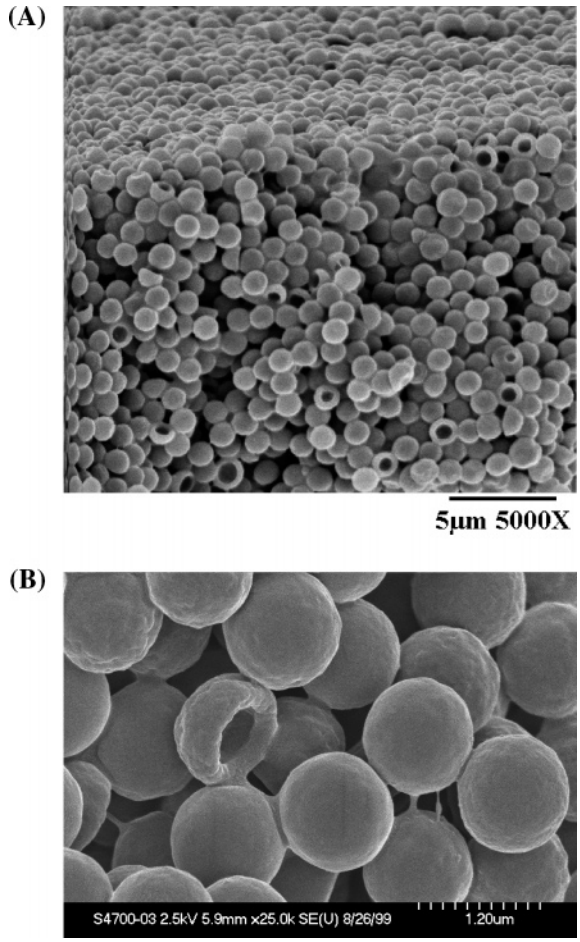

Figure 14. Cryo-SEM images of a polymer blend coating of 0.8 (v/v) 800 nm polystyrene particles (Ropaque 1055) and 0.2 (v/v) 158 nm acrylate/styrene particles (JP1232) dried at 30 °C and stable at 80 °C in seawater for entrapment of *T. maritima*. (A) Top view and fracture surface. (B) High-resolution view of fracture edge showing low T_g latex coating the polystyrene particles and at the particle contact surfaces. (Reproduced with permission from ref 51. Copyright 2005 Springer-Verlag).

The most common approach to study this interaction is a carbon electrode suspended in a bioreactor inoculated with bacteria. Characteristics of this "microbial fuel cell" are monitored over days to weeks, following attachment and growth of a biofilm on the electrode surface. Deposition of adhesive nanoporous thin microbial coatings could avoid the need to precolonize electrodes, create uniform electrodes for controlled studies, and minimize release of bacteria. *Geobacter sulfurreducens* produces fragile extracellular pili structures, thought to be required for attachment to substrates and electron transfer to electrodes (*86*). Washed cultures of *G. sulfurreducens* immobilized on carbon paper electrodes using 4% pectin (cross linked with $BaCl_2$) immediately demonstrated electron transfer to electrodes, as monitored via cyclic voltametry and electrochemical impedance spectroscopy (*87*). Subsequent incubation of *Geobacter*-coated electrodes demonstrated sustained electrical output, rising to 1.75 A/m^2 within 24 h. Electrochemical features of these coatings were identical to electrodes colonized by a biofilm in a microbial fuel cell for weeks (*87*). Further investigation of electron-transfer phenomena and development of latex-based polymer coatings that immobilize bacteria while allowing them access to insoluble minerals or conductive substrates could lead to development of current-generating microbial coatings for conversion of waste organic materials into electricity.

4.0. Microbial Latex Coatings and Inks: Challenges and Future Applications

A variety of latex microbial coatings are being investigated as model systems in order to determine the fundamental properties for engineering high reactivity coatings. Many embodiments of this method may be possible (Figure 15). Still to be exploited are adhesive microbial coatings with biosynthetic capabilities that continuously secrete for example enzymes, peptides, antibiotics or protective metabolites from surfaces. Multilayer and pattern coatings of microorganisms that combine layers or reaction zones containing different microbes, biological reactivity and physical properties are also feasible.

4.1. What Is the Chemical Environment, Distribution and Structure of Water in the Dried Coating Pore Space? Critical to future applications of this technology is the molecular understanding of preservation of microbial viability at ambient temperature when cells are entrapped in nanoporous coatings. Biopreservation research started with the discovery of higher than normal levels of certain molecules in the cytoplasm of desiccation and freeze-tolerant species. When these organisms were exposed to gradual changes in temperature, salinity or humidity, osmolytes were accumulated (ions, proteins, carbohydrates, amino acids or urea) that helped them survive the harsh conditions (*88*). However, the molecular role of intracellular osmolytes in desiccation tolerance is not known, although they likely modify the activity and state of intra/extra cellular water. It is hypothesized that they either replace structural water in the immediate vicinity of proteins and membranes, enabling these structures to maintain their native configuration (*89*), or are preferentially excluded from the surface to help preserve structural water (*90*). Much literature exists on preservation of viable cells by glycerol, by vitrification of carbohydrates to produce extracellular glasses, the effect of salts concentrated during drying, and processing parameters (drying temperature, relative humidity, drying rate, drying time) (*89*), but these effects have not previously been studied in coatings where the effect of the pore surface will be significant. It is believed that this same biomolecular structural transition from a low viscosity liquid to a glassy state occurs within cells when dried, freeze-dried or frozen (*89*). In addition, microbes such as *E. coli* respond to desiccation and osmotic stress by synthesis of cytoplasmic osmolytes such as glycine betaine (*91*−*93*) and yeasts produce trehalose (*94*), so the physiological response of the latex-entrapped microbes may be the most sensitive measure of how to optimize coating microstructure for retention of viability as a result of changing water activity.

For the particular case of confinement of biomolecules and water in coating nanopores where surface effects dominate, it is likely that water activity is altered. This is due to the dipolar nature of water, which causes it to change its residence time as a function of its immediate environment (*95*). For example, the freezing temperature of water when confined in nanopores decreases with pore size (Figure 16A) (*96, 97*). Therefore confinement in coating nanopores has the same effect as exposure to osmolytes with respect to water activity and reduction of biochemical reaction rates. The phase change behavior (*95*), T_g and crystallization rates of solutions are also affected by confinement (*98, 99*). Changes in the mobility of water and solvents may be the mechanism of stabilization of living cells in nanoporous coatings resulting from increasing fluid viscosity and entropic confinement during film formation and simultaneous pore formation by arrested polymer particle coalescence. A microfluidic device on the stage of an FTIR (Figure 16B) has recently been reported as a tool for directly

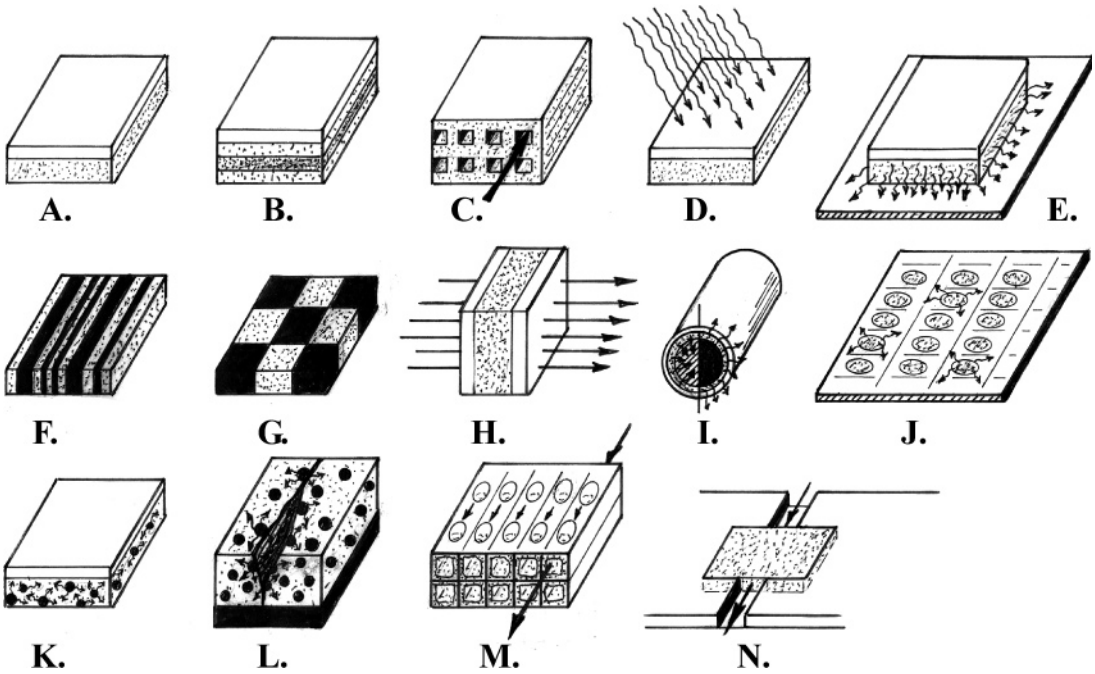

Figure 15. Embodiments of latex biocatalytic coatings. A. Bilayer. B. Multilayer containing different microorganisms. C. Structured perfusive coating. D. Photoreactive. E. Current generating. F. Pattern coated microbial "bar code". G. Pattern coated with different microorganisms. H. Perfusive trilayer membrane. I. Coated filaments: cladding which can be sensing (generating fiber optic signals) or reactive filaments. J. Ink-jet printed latex microstructure arrays printed on a detector surface. K. Nanocomposite. L. Self-healing (protective). M. Wash coatings on monolithic microchannels, segmented Taylor multiphase flow. N. Microfluidic reaction zone.

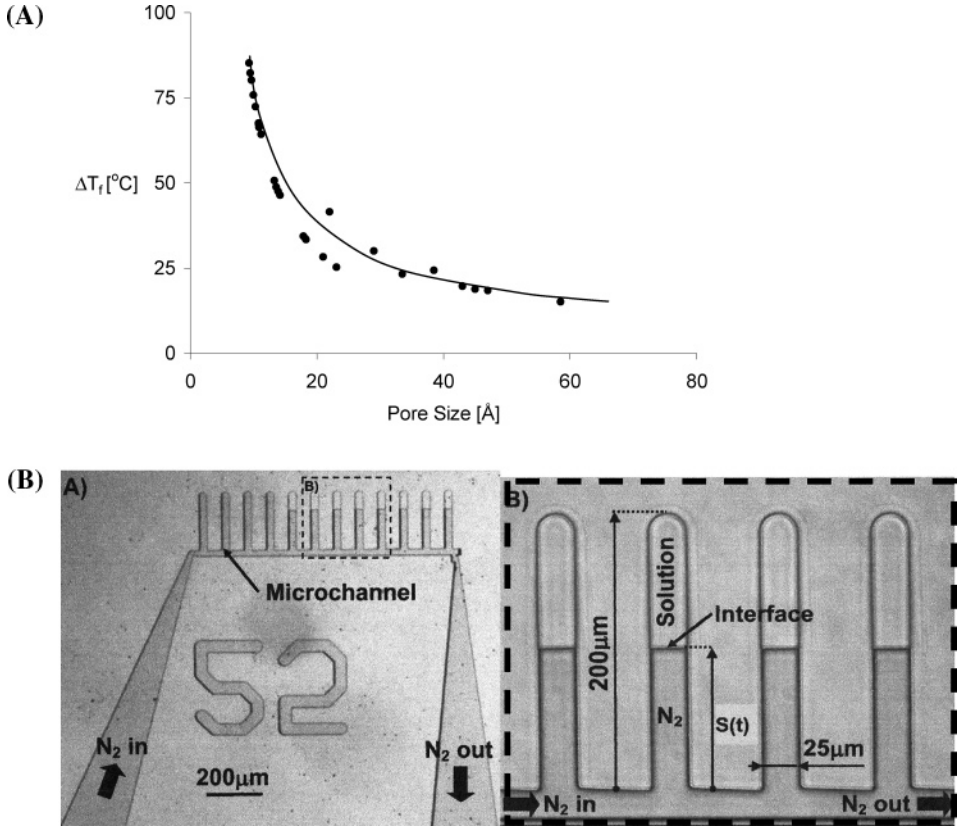

Figure 16. Investigation of the structure of water during vitrification in nanopores. (A) Change in the freezing temperature of water as a result of confinement in nanopores. Compiled from data from refs 96 and 97. (B) Model microfabricated pore mounted on the stage of an FTIR for direct monitoring of vitrification. (Reproduced with permission from ref 100. Copyright 2006 American Institute of Physics).

measuring the change in extracellular viscosity and microbial membrane thermodynamic behavior (membrane phase transition) as a function of water content during vitrification (*100*), which may be useful to confirm the mechanism of viable cell stabilization during latex film formation.

4.2. Physiology of Non-Growing Microorganisms. The biology of latex-embedded microorganisms that are not growing (nutrient-limited) or growing very slowly is poorly understood. This includes the redox and energy balance in non-growing cells, DNA repair mechanisms active in the absence of DNA

synthesis, ribosome stability, RNA polymerase and mRNA stability, regulation of protein turnover and new protein synthesis, all of which affect in vivo protein stability and therefore coating reactivity. By understanding how to manipulate the regulation of gene expression without growth, the specific reactivity of biocatalytic coatings could be increased by several orders of magnitude. In addition, understanding the regulation of in vivo proteolysis in nitrogen-limited non-growing microorganisms could lead to strategies to alter protease activity in latex biocatalytic coatings and the design of proteins with long in vivo functional half-lives.

4.3. Intensification, Miniaturization and Composite Coating Materials for Cell Preservation. Of parallel importance with increasing the specific reactivity (process intensity) and active half-life of biocatalytic coatings is the development of combinations of both nanoporous and nonporous latex with nanostructured organic or inorganic materials to produce mechanically stable highly structured coatings with increased stability, abrasion resistance, or electrical properties and for photoreactive coatings, with optimal light adsorption (*101*). Development of mechanically strong highly structured coatings with microchannels, stand-alone composite latex biocatalytic membranes and non-woven filters that retain porosity for very long periods of time with low-pressure drop, even when exposed to extreme temperature and reaction conditions will facilitate the industrial use of extremophile microbes that thrive in harsh environments as industrial biocatalysts.

An extension of this coating technology is miniaturization by ink-jet fabrication of very small coatings and latex microstructures containing living microorganisms by improving formulations for piezoelectric droplet deposition. Using printing technology, precise deposition of a high density of living microorganisms onto the sensing elements of integrated circuits is possible so that the metabolism of microorganisms can be incorporated into the design of bioelectronic devices.

What we learn from investigation of the molecular mechanisms of preservation of viable microorganisms in dry latex coatings at ambient temperature can likely be translated to higher eukaryotic cells to improve the preservation of more fragile mammalian cells in partially desiccated adhesive coatings. Many types of mammalian cells and cell lines can be frozen and thawed with cryoprotectants without loss of viability. It is likely that some of the same mechanisms of cryoprotectants that preserve the viability of mammalian cells during freezing can be incorporated into the design of cell-preserving nanoporous coatings in the future for medical applications such as cell transplants, transport of living cells without refrigeration, and cell repositories.

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