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Prepared by
Sandia National Laboratories
Albuquerque, New Mexico 87185 and Livermore, California 94550

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Helen K. Baca[1], Jeb H. Flemming[2], Carlee Ashley[1], Tamara Hartenberger[3], Monica Manginell[2], Darren R. Dunphy[3], Susan M. Brozik[2], Margaret Werner-Washburne[4], Paul Calvert[5], Michael B. Sinclair[7], Jerilyn A. Timlin[8], Joseph Cesarano III[6], and C. Jeffrey Brinker[1,3] *

[1] Center for Micro-Engineered Materials, University of New Mexico, Albuquerque, NM 87131

[2] Microsensor Science and Technology Department, Sandia National Laboratories

[3] Chemical Synthesis and Nanomaterials Department, Sandia National Laboratories

[4] Department of Biology, University of New Mexico, Albuquerque, NM 87131

[5] Department of Textile Sciences, University of Massachusetts – Dartmouth, Dartmouth, MA 02747

[6] Ceramic Materials Department, Sandia National Laboratories

[7] Microsystems Materials, Tribology and Technology Department, Sandia National Laboratories

[8] Chemical and Biological Sensors, Imaging and Analysis Department, Sandia National Laboratories
P.O. Box 5800
Albuquerque, NM 87185

Abstract follows

* Author to whom correspondence should be addressed: cjbrink@sandia.gov

Abstract

This report summarizes the development of new biocompatible self-assembly procedures enabling the immobilization of genetically engineered cells in a compact, self-sustaining, remotely addressable sensor platform. We used evaporation induced self-assembly (EISA) to immobilize cells within periodic silica nanostructures, characterized by unimodal pore sizes and pore connectivity, that can be patterned using ink-jet printing or photo patterning. We constructed cell lines for the expression of fluorescent proteins and induced reporter protein expression in immobilized cells. We investigated the role of the abiotic/biotic interface during cell-mediated self-assembly of synthetic materials.

Acknowledgements

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1. Introduction.

Emerging world-wide threats of biological and chemical terrorism, including recent experiences with anthrax, have established the need for miniaturized, stand-alone sensor systems for conducting covert reconnaissance missions, for monitoring strategic assets and for battlefield deployment. Toward this end, rapid progress in genomics affords the potential for development of cell-based sensor arrays that effectively utilize a living cell's complex biochemical response pathways to provide unprecedented functional information about chemical and biological warfare agents. Living cells, in a manner analogous to the canaries once used to detect toxic gases in coal mines, respond to unknown toxicants in a physiologically relevant manner[1], incorporating signal recognition, amplification and transduction in a remarkably complex yet compact package. Questions about a chemical's bioavailability, which cellular metabolic pathways an agent will affect, or the functional classification of a novel, engineered pathogen may all be answered directly using model eukaryote cells[2]. Microarray analysis can collect dynamic information on multiple analytes, through both pattern recognition and individual addressability of cells, and will be a fundamental requirement for overcoming current limitations of single-analyte sensors that depend on prior knowledge of a specific threat.

Limitations of cell-based sensors

Currently, non-invasive detection of cellular response to toxic substances relies predominately on measuring metabolic products exported by the cell, changes in cell adhesion, or the response of electrogenic cells such as neural networks. These methods, which are application-driven for specific, identified toxins, lack flexibility and may require complicated microelectronic interfaces[3]. Although cell-based sensors consisting of an array of individual, genetically modified cells from multiple cell lines, could provide unprecedented, rich data about cellular response, serious challenges to the manufacture, maintenance and storage of cell-based arrays exist.

A biosensor capable of a long shelf life must address the need for cell longevity, with preservation of functionality. Cells must be confined to protect them from mechanical, chemical and biological stresses, to sustain them in an environment that promotes cell viability, and to prevent the common problems of cell proliferation and escape. Cells must be accessible to nutrients and analytes, and must be interfaced with abiotic detection systems without loss of functionality. To use the advantages of flexible and complex data from microarray responses for important issues such as validation of pathogen identification and development of real-time appropriate countermeasures, quantification, reliability and predictability of responses must be assured. Sensors should be capable of reutilization and remote or cyclical activation for field deployment. Localization and patterning of cells is necessary for fluid transport requirements and spatial fidelity of cells for individual addressability. Concentration and delivery of pathogens to the sensing elements will also be important to detect low-level chronic or developing acute threats to both civilian and military targets.

Background

Signal Transduction and Detection Through Gene Expression - Single cells can provide efficient signal amplification and transduction through a number of pathways including regulation of metabolic processes, gene expression, and structural changes. Signaling may be initiated either by small molecules that pass through the cell membrane or at protein receptors on the cell surface.

By coupling a sensing element such as a regulatory protein or promoter sequence of plasmid DNA, to a reporter gene through genetic engineering, signal transduction is provided through the production of proteins in the cytosol. Reporter constructs can be designed to respond with high selectivity to a specific analyte, or broader group effects can be targeted, giving important physiological information about the activity of unknown pathogens, chemotoxins or genotoxins. Noninvasive detection of reporter proteins is possible when fluorescence or chemiluminescence allows optical assays of a cell's response.

Challenges to signal transduction using reporter genes and their expressed proteins include identifying constructs that provide a distinct and quantifiable relationship between the analyte and the quantity of protein expressed, and avoiding interference from similar, endogenous proteins. Additionally, systems that rely on optical sensing frequently require the addition of a cofactor to produce a detectable signal, may not be suitable for use in model eukaryote cells, or may express proteins that are not degraded by the cell, thus interfering with sensor reutilization.

Cell Immobilization - Central to the development of functional microarray cytosensors is the localization and confinement of the cell on a sensor platform while maintaining cell viability, sustainability, and functionality. Cell patterning to date relies heavily on immobilizing adherent cells on two-dimensional surfaces, where cellular response may differ considerably from cells in an in-vivo 3-dimensional extracellular matrix[1, 4-6]. Cell washout with continual use and cell proliferation over the surface are frequent problems, and little advantage is offered in cell protection or sustainability[7]. Entrapment of cells in a network, typically a natural or synthetic polymer such as alginate or polyvinyl alcohol, can provide confinement while maintaining cell accessibility but this approach is hampered by loss of cell viability during processing and subsequent polymerization, poor mechanical and chemical strength of the biocomposite, and swelling of the surrounding gel.

Approach

We used *Saccharomyces cerevisiae* as a model cell for genetic engineering of signal transduction pathways. It has many of the same metabolic pathways as humans, carrying out essential cell processes such as replication, protein trafficking, and cell cycle control with proteins that are conserved among the eukaryotes. Yeast cells, with their completely identified genome of approximately 6000 genes, are genetically tractable and easily maintained in a stable G_0, or stationary phase, where a complex process for long term survival without nutrients is activated [8]. In stationary phase, the cell wall thickens, and the cell becomes more stress tolerant, a potential advantage to long shelf life in biosensor applications.

Green fluorescent protein (GFP) is a highly stable, autofluorescent protein that is used as a gene expression reporter, an *in vivo* protein marker for localization, and as a tracer of cell lineages[9]. It can be expressed in yeast cells without the need for additional cofactors, has a high quantum yield, and is detected using fluorescence spectrometry, with its intensity related quantitatively to low levels of specific analytes [10]. GFP is particularly resistant to denaturation by temperature and pH, due to its unique beta-can structure, where strands of beta-sheet surround a central alpha-helix core with the fluorophore protected inside a barrel structure, giving it stability and protection from quenching agents.

Forming a self-assembled inorganic host matrix around cells using soft sol-gel chemistry allowed us to confine cells in a silica network with a bi-modal pore size distribution (**Figure 1**). Micron scale cavities immobilize and isolate the cells, preventing flocculation[11] and uncontrolled growth. A continuous network of smaller, nanometer scale hydrophilic pores in the surrounding matrix allows selective access to small analytes and nutrients, along with removal of waste. Unimodal porosity at the nanometer scale enhances mechanical stability of the biocomposite[12], facilitates flow to the cells, and prevents localized drying of larger pores, maintaining fluidic connectivity. Biocompatible encapsulation and rapid patterning procedures localize the cells in configurations suitable for induction and detection of reporter protein expression.

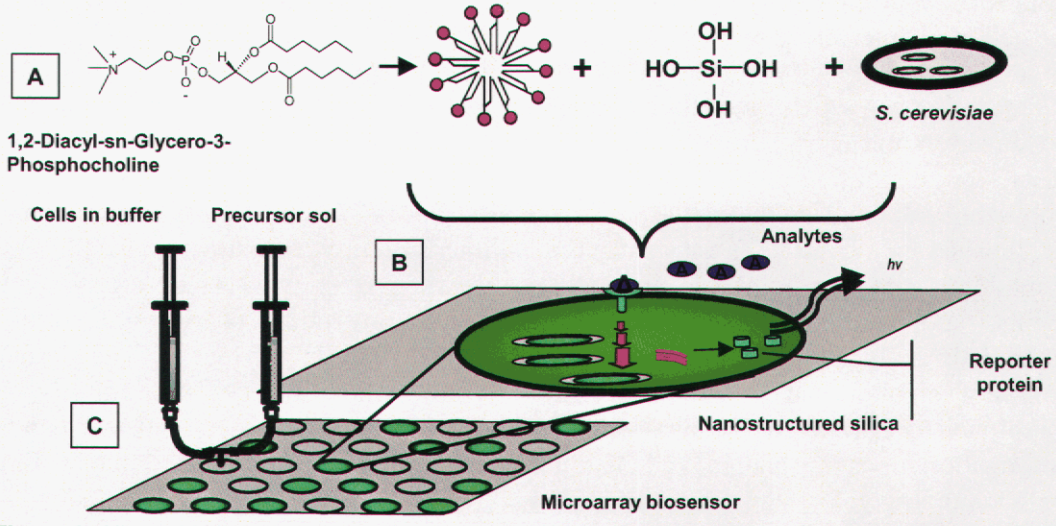


Figure 1. Hierarchical design of responsive, biocomposite materials. **(A)** A porous, nanostructured silica matrix forms around the cell through phospholipid directed self-assembly, providing protection and confinement in a self-sustained, buffered environment and conserving cell functionality. **(B)** At the molecular scale genetically engineered cells provide signal detection, amplification and transduction.**(C)** Biocompatible patterning separates buffered cells from sol components and positions cells for individual addressability

1. Biocompatible cell immobilization.

Attempts to immobilize living cells in sol-gel host matrices began in the early 1990s[13, 14] and met barriers of cytotoxicity during processing. Monolithic biocomposites were limited by low porosity and poor structural integrity of the host matrix, inactivation of guest biological species through pore collapse, dehydration of the cells, low reproducibility, and cell leakage[11, 12, 15-17]. Recent approaches to these problems include aqueous sodium silicate sol-gel chemistry[18, 19], hydrogel encapsulation[5], adaptation of the sol-gel route to yield benign hydrolysis products[20], and addition of protective additives[21]. However, the ability to form robust structures, particularly high porosity thin films, to maintain viability and functionality in ambient conditions, to control the microstructure of the matrix, to encapsulate less robust eukaryotic cells, and to develop cytocompatible patterning methods remain significant challenges.

We have addressed the issues of low porosity, broad pore size distributions, and drying-induced shrinkage of pores by using an amphiphilic template and EISA (evaporation induced self-assembly) to direct the structure of the silica host during encapsulation of cells. Self-assembly typically employs asymmetric molecules that are pre-programmed to organize into well-defined supramolecular assemblies. Most common are *amphiphilic surfactant* molecules or polymers composed of hydrophobic and hydrophilic parts. In aqueous solution above the *critical micelle concentration* (*cmc*), surfactants assemble into *micelles*, spherical or cylindrical structures that maintain the hydrophilic parts of the surfactant in contact with water while shielding the hydrophobic parts within the micellar interior (**Figure 2**). Further increases in surfactant concentration result in the self-organization of micelles into periodic hexagonal cubic, or lamellar mesophases. In addition to surfactant concentration, surfactant shape is also influential in controlling the resultant mesophase due to packing considerations. Surfactant shape may be described by a dimensionless *critical packing parameter* $g = V/a_o l$, where V is the total volume of the surfactant chains plus any co-solvent, a_o is the effective head group area at the micelle surface, and l is the kinetic surfactant tail length[22]. Decreasing values of g favor mesophases with progressively increasing curvature: lamellar→cubic (*Ia3d*)→hexagonal (*p6m*)→cubic (*Pm3n*).

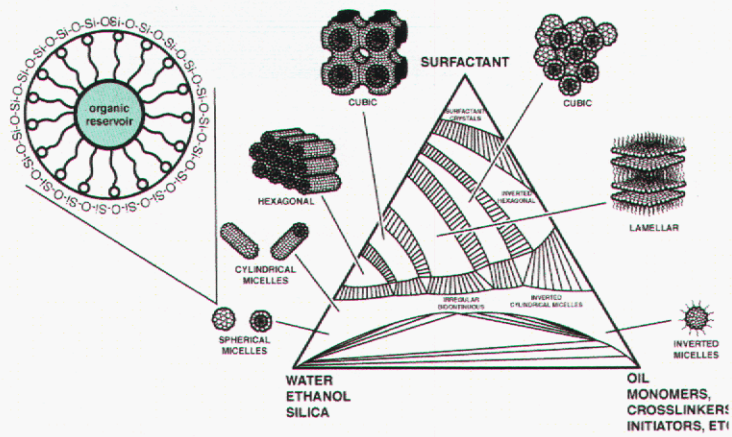


Figure 2. Schematic detergent (surfactant-oil-water) phase diagram. With the introduction of hydrophobic organic (or generally soft) precursors and hydrophilic inorganic (or generally hard) precursors, organic constituents partition within the hydrophobic micellar interiors, while inorganic constituents are organized around the micellar exteriors. Evaporation promotes further self-assembly into lyotropic mesophases, simultaneously organizing organic and inorganic precursors into nanocomposite architectures.

Thus through control of both the shape and concentration of surfactant it is possible to develop predictably a specific mesophase.

Evaporation-induced self-assembly (EISA)[23] provides good control over the formation of silica mesophases through liquid crystalline templating and produces the thin films that are desirable for maximum mass transport to encapsulated biomaterials. Connected unimodal pores, whose size is controlled by the pore template, form a network that spontaneously fills with water, creating and maintaining an aqueous environment for encapsulated species without an auxiliary fluidic system. A surfactant is added, in an initial concentration much below its cmc, to a soluble silica precursor in a solvent/water system. The solvent, frequently ethanol, is chosen for its volatility, and as it evaporates the concentration of the surfactant increases. Micelles begin to form and then self-organize into liquid crystalline domains, nucleating at solid-liquid or liquid-vapor interfaces. Films with long-range order are achieved by adjusting the pH to minimize the rate of siloxane condensation.

Although EISA uses the "soft" room temperature, aqueous conditions of sol-gel synthesis, the biocompatible immobilization and patterning of biological materials presents unique hurdles, with the most stringent requirements governing the immobilization of whole cells. The conditions of processing must allow the cell to be maintained in an appropriate physiological environment, buffered within its pH range[12]. Organic solvents, hydrolysis products, and amphiphilic surfactants may denature integral proteins in the membrane or affect cell membrane permeability and functionality.

Biocompatible EISA - We used a several-pronged approach to provide the biocompatible materials and processing conditions necessary to maintain cell viability while retaining careful control over the microstructure of the inorganic host. Biocompatible templates, high water content, buffered sols, and compartmentalization of sol components within multiple reservoirs prior to coating (**Figure 1**) allowed us to immobilize genetically engineered *S. cerevisiae* cells in a nanostructured silica host and to detect an environmental stimulus through expression of GFP. Sol formulations based on methoxy- or ethoxy- silane precursors were pre-hydrolyzed under acidic conditions, then diluted with water and acidified sufficiently to minimize the silica condensation rate. Since the toxicity of alcohols towards yeast rises with chain length through dodecanol[24], methanol and ethanol were added in minimal amounts to enable consistent nanostructure formation during EISA. Sol formulations had molar ratios that varied from 1 TEOS: 0 to 8.0 C_2H_5OH: 0 to 20.0 H_2O: 0.001 to 0.015 HCl: 0.04 to .015 di_nPC (diacylphosphatidylcholine). The higher concentration ethanol films were spincoated directly on silicon substrates and used for initial phospholipid-templated silica thin film structural analysis, while the films with minimal ethanol were used to immobilize cells. Double reservoirs contained yeast in phosphate buffered saline (PBS) and sol formulations in 1:1 volumetric ratios.

Biocompatible Structure-Directing Agents - Because traditional surfactants are highly toxic to cells in the concentration ranges needed to direct self-assembly, we replaced

them with amphiphilic phospholipids that are integral parts of the cell membrane[25]. Phosphocholines, with zwitterionic headgroups and short, double hydrocarbon tails minimize disruptive electrostatic interactions with cell membranes and can form micelles with significantly high curvature (low critical packing parameter *g*) to give 3-dimensional interconnected phases rather than the usual planar bi-layers found in membrane structures; tail volume was also minimized by using only saturated phosphocholines. We find that diacylphosphatidylcholines, with acyl chains from n=6-10, direct the self-assembly of silica sols, with the morphology of the inorganic phase depending on both

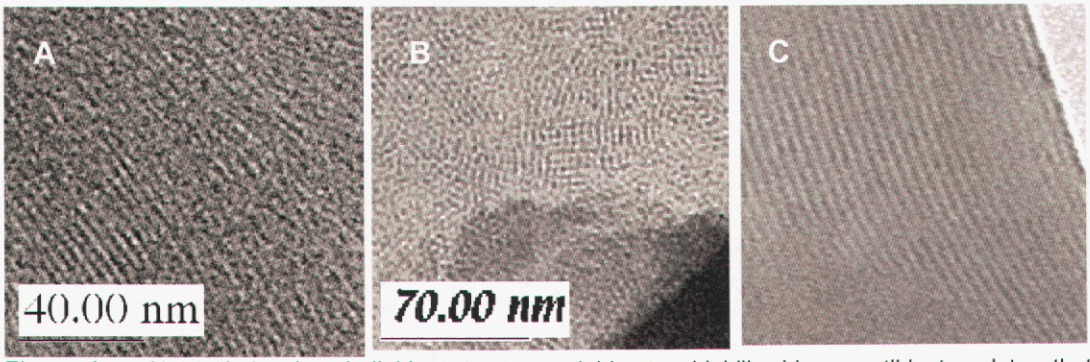


Figure 3. Short chain phospholipids are water soluble, amphiphilic, biocompatible templates that promote self-assembly of silica mesophases. **A.** Transmission Electron Microscopy (TEM) of calcined hexagonal phase, low lipid to silica ratio formulation. **B.** TEM of calcined film, intermediate lipid to silica ratio. **C.** High lipid to silica ratio, uncalcined sample. XRD peak disappears with calcination and TEM appears amorphous, indicating collapse of lamellar structure.

lipid to silica molar ratio and template structure (**Figure 3**). Low lipid to silica ratios, short tail lengths and single tails favor hexagonal and 3-dimensional structures, while longer tails and higher lipid concentrations give lamellar structures. Pore size varies from 2 to 4 nm, increasing with tail length. Additionally, glyceromonoolein (GMO), another biocompatible lipid, will direct the formation of cubic phases at intermediate concentrations (**Figure 4**).

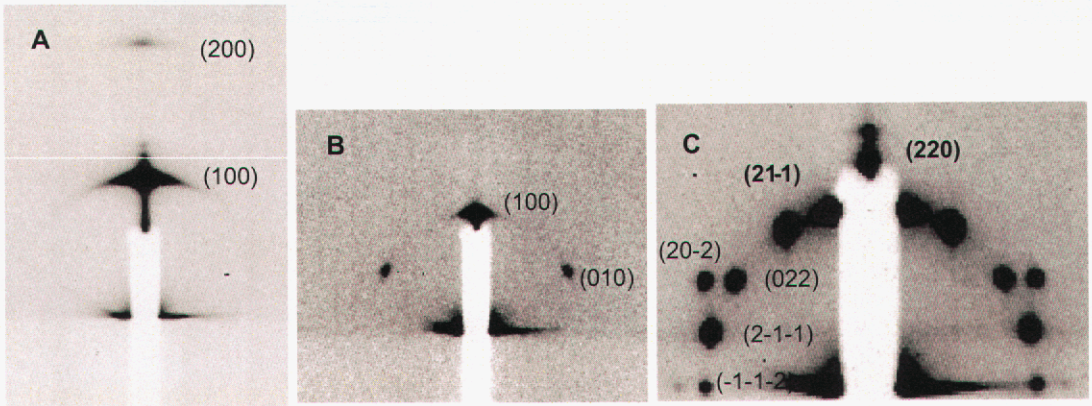
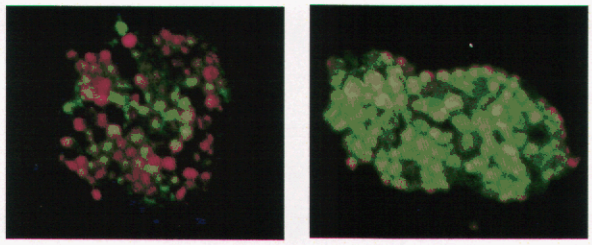


Figure 4. A. Hexagonal phases result from short tail lengths and low lipid concentrations. **B.** Lamellar phases predominate for longer tail lengths and higher lipid to silica ratios. **C.** Cubic phase (Im3m or Pm3n) of GMO.

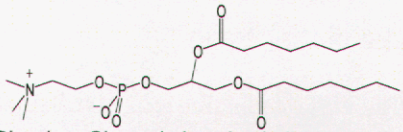
Biocompatibility of these lipids, in both sols and the optically transparent silica host matrices, was confirmed using a two-color fluorescent probe (Fun®1, Molecular Probes) that reports both membrane integrity and cell metabolic activity. [26]. The fluorescent probe, Fun-1, stains the cytoplasm of dead cells a bright green-yellow while cells with an intact membrane will have a diffuse green color. If stained cells are incubated at 30°C, metabolically active cells incorporate the dye into intravacuolar structures that have a red-orange fluorescence when excited at 470-590nm. Cell walls are stained with a fluorescent fungal cell wall marker for visualization. For cells immobilized in silica with lipid, we find markedly increased viability at 4 days compared to cells immobilized in silica alone (**Figure 5**).



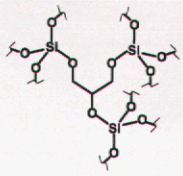
Phospholipid, red cells are viable

No lipid

	%Viability
Immobilized cells w/lipid at 120 min/4 days	75/50
Immobilized cells no lipid at 120 min/4 days	55/(<10)



Diacyl-*sn*-Glycerol phosphocholine



poly(glyceroxysilane)

Figure 5. Cells immobilized in silica with and without phospholipid templates. Viability dyes added 4 days after immobilization show more than 50% of cells are alive in phospholipid directed self-assembled silica while cells immobilized without a template are nonviable.

Cells exposed to phospholipids uptake live/dead assay dyes more quickly than control cells, with no decrease in viability, presumably through membrane permeabilization. Similarly, other researchers[27] have noted that non-ionic surfactants, including some block copolymers, can be used to increase fluorescein uptake through membrane pore formation without further influencing cell structure or function. This observation is consistent with the mild detergent properties of short-chain phospholipids, which will distribute into the lipid bilayer of biological membranes at high concentrations, avoiding interactions with membrane proteins. As expected, cells exposed to sol prepared with the standard surfactant CTAB have zero viability. We find that glycerolysis of TEOS, following partial hydrolysis and condensation, gives a branched poly(glyceroxysilane) network that increases cell viability above that obtained with the control buffer.

As an alternative to liquid-crystalline lipid mesophases, small molecule porogens of biological origin were tested as a pore templating strategy. In addition to their potential biocompatibility, it was believed that these water-soluble low molecular weight compounds would be readily removed from templated materials using a mild aqueous extraction. Two classes of compounds were studied; carbohydrates (glucose, maltose, fructose, di-, tri, and oligosaccharides) and so-called fruit acids (tartaric acid, citric acid).

However, the pore structure formed by these molecules during thin film formation was found to be inappropriate for our purposes. Specifically, pore size was small (for glucose, the mean pore radius after film calcination was 0.8 nm), and pore connectivity was low. Although this last feature prevented the efficient extraction of these porogens from films using solvent extraction, this property may be useful for some applications such as low-k dielectrics where a film with an isolated pore structure is desired. Monosaccharide functionalized surfactants with a glucosamide headgroup for biocompatibility and varying alkyl tail lengths were prepared to examine the micellar properties of these surfactants. The amphiphiles were prepared by either reaction of the acid chloride of the fatty acid with glucosamine or by reaction of the anhydride of the fatty acid with glycosamine. Low solubility of these functionalized surfactants precluded their use in EISA under biocompatible conditions.

3. Cell mediated self-assembly and characterization of the silica/cell interface.

Using confocal fluorescence microscopy of fluorescently labeled lipids (1% of 1-Hexanoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-Glycero-3-Phosphocholine) combined with GISAXS, we find that conducting EISA in the presence of living yeast cells strongly influences the self- assembly pathway, with the cell/silica interface playing a crucial role in both directing the structure of the inorganic phase and maintaining cell viability and functionality. As shown by environmental scanning

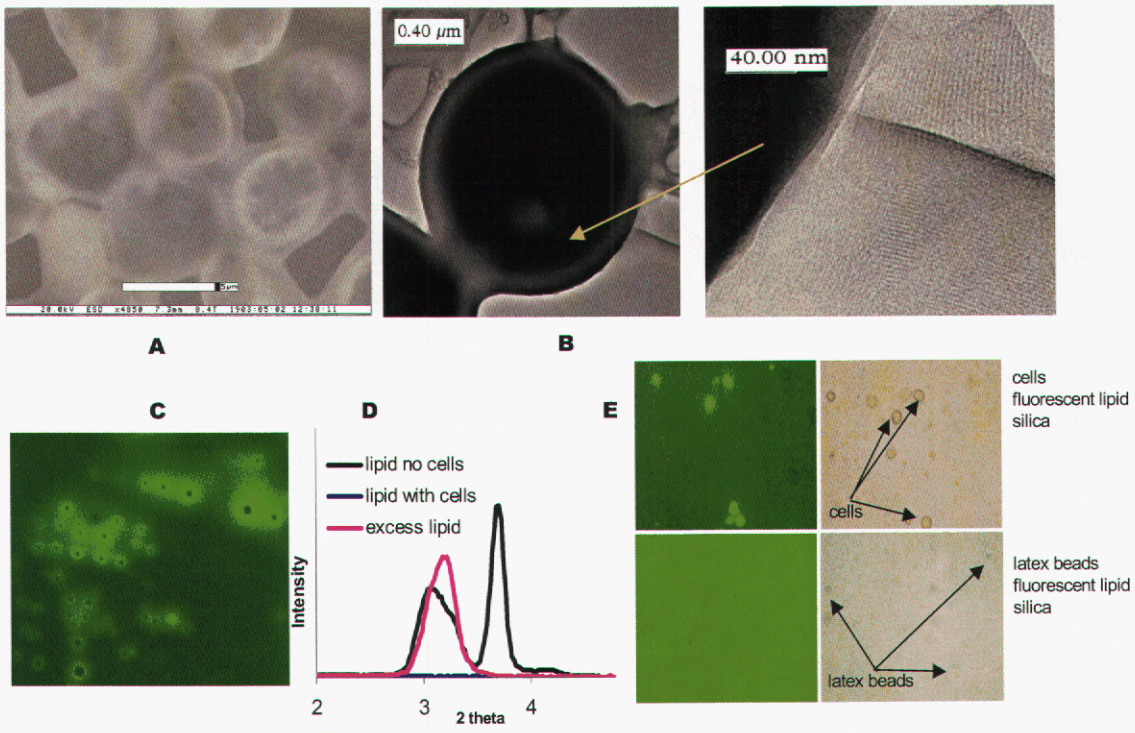


Figure 6. A.ESEM of phospholipid templated silica and cells. **B.**TEM of immobilized cell and surrounding silica nanostructure. **C.** Confocal microscopy of cells immobilized in lipid-templated silica. Using a fluorescent lipid analogue we observe preferential localization of lipids around the cell. The surrounding ordered nanostructured silica matrix contains lipids but at a lower overall concentration. **D.** XRD showing the change in structure of lipid templated films with the presence of cells. Excess lipid must be added during cell-directed self-assembly to compensate for lipid aggregation around the cell. **E.** Phospholipids do not aggregate around hydrophobic and hydrophilic cell models (latex and silica beads).

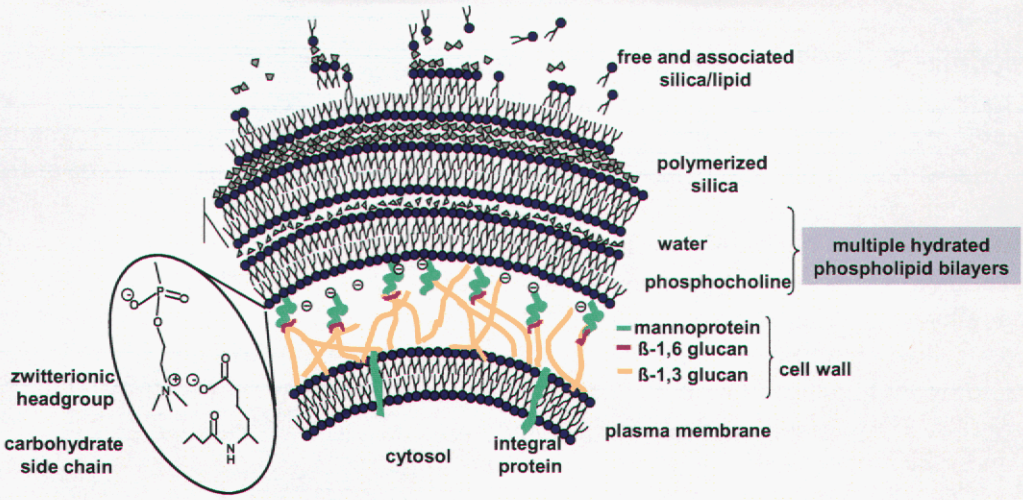


Figure 7. Conceptual model of self-assembly in the presence of living cells

electron microscopy (ESEM), confocal images (**Figure 6**), and GISAXS data, yeast cells are surrounded by a multi-layered organized lipid interface that dictates the structure formed during organization of the silica phase (**Figure 7**). Comparable experiments with surrogate hydrophilic or hydrophobic beads show no such enhanced lipid concentration.

The extended viability of immobilized cells in the presence of phospholipids may result from both a passive, protective effect and a dynamic effect where the cell adapts to its surroundings. In addition to excluding some toxic substances, a layer of phospholipid might be expected to help retain water at the bio-interface. The hydration of lipid bi-layers depends on the nature and fluidity of the hydrocarbon tails and the lipid headgroup[28]. Phosphocholines are superior water absorbers, binding water around the headgroup in a clathrate type structure and forming a fluid layer between adjacent bilayers[29]. Additionally, short chain PCs are in the liquid crystalline phase at room temperature, where the amount of water absorbed per lipid molecule and the width of the interbilayer water layer both increase from the gel phase. Results from FTIR studies of immobilized cells indicate that a cell-based process gradually degrades exogenous lipids in the biocomposite film, with the living cell possibly using surrounding lipids as a nutrient source (**Figure 8**).

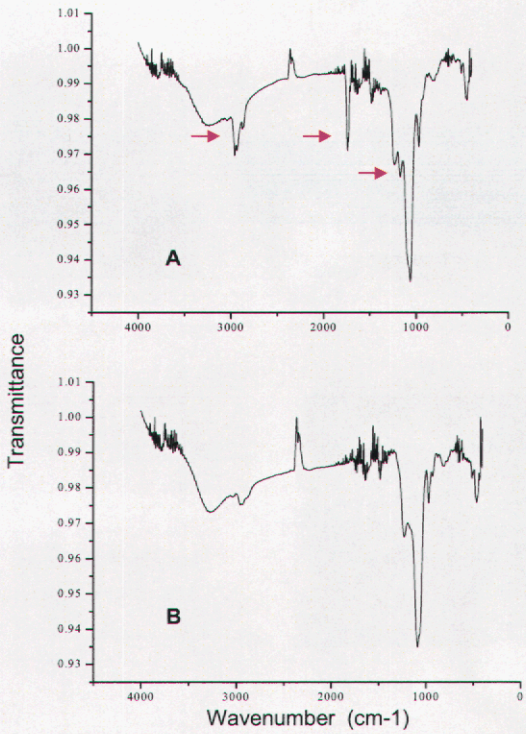


Figure 8. A.FTIR of lipid/ silica/ cell film immediately after deposition. **B**. Spectrum at 4 days. Alkane stretches and ester linkages are markedly reduced. Control film of silica/lipid with out cells shows no change with time.

4. Cell patterning and localization.

Biocompatible EISA, combined with new sequential patterning techniques, has allowed us to address several technical issues associated with patterning of living cells, including precise localization of immobilized cells for array applications, maintenance of accessibility and minimization of both shear stresses and exposure to toxic environmental conditions. As described above, key to preserving cell viability during immobilization is the inclusion of an amphiphilic phospholipid that creates a lipid environment around the cell, providing a continuous fluidic pathway within the silica matrix that insures access to the cells and maintains the cells in a hydrated microenvironment through retention of water. We have demonstrated cell patterning by selective wetting and ink jet printing (IJP). Photo-patterning of photo-sensitive silica mesophases[30] produces optically defined hydrophobic/hydrophilic patterns (**Figure 9A**.). During a second, low-speed, spin coating step, buffered cells preferentially attach to the hydrophilic areas of the pattern. Unexpectedly, cells are centered in the patterned area, with a uniform spacing that depends on the speed of spin coating. Cell attachment seems to proceed through partial hydrolysis and recondensation of the underlying film near the cell, with cells securely attached and viable for more than 72 hours in air.

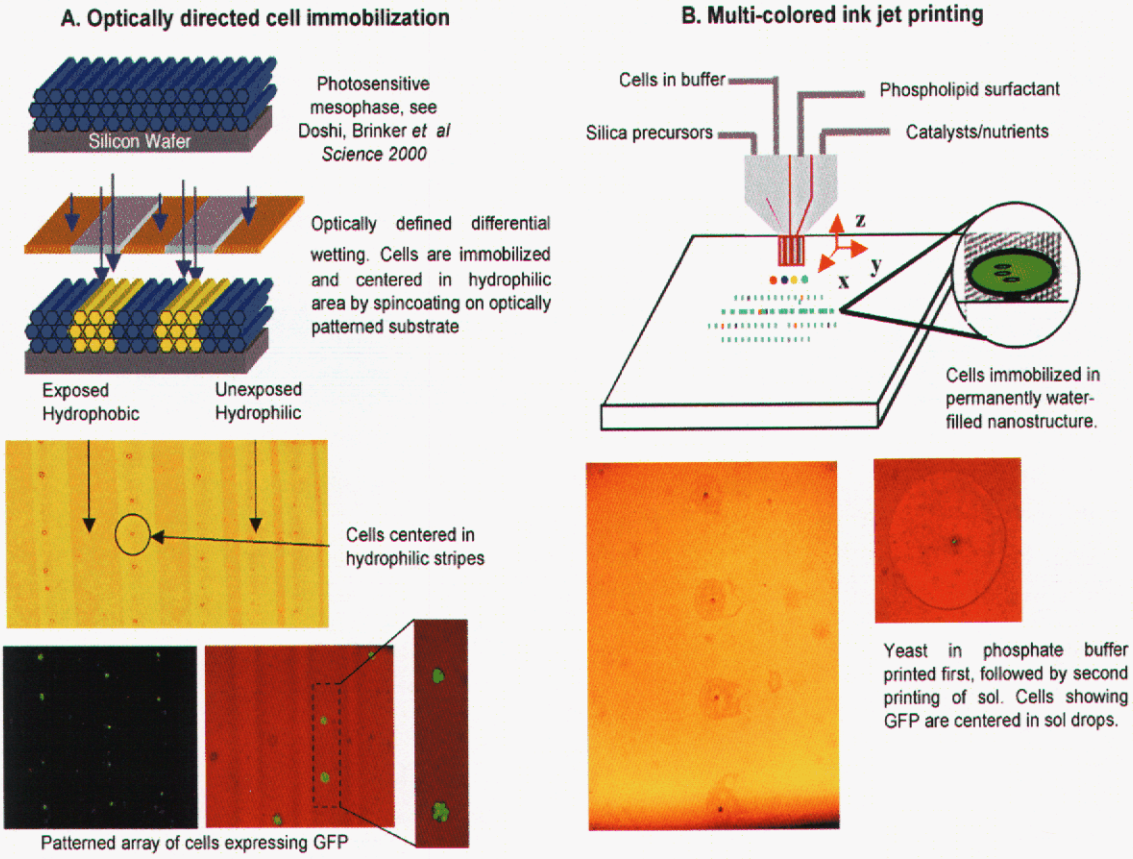


Figure 9. A. Optically directed cell immobilization. Sequential steps of film patterning and cell attachment avoid the exposure of cells to photoinitiators and UV radiation that is required by other photolithographic patterning methods. **B.** Multi-colored ink jet printing. Separating cells and toxic sol components allows retention of functionality.

The development of biocompatible sol-gel systems with appropriate rheology and pH has also allowed us to pattern cells through IJP (ink jet printing). By controlling the extent of condensation through choice of precursors and reaction conditions, the viscosity of the sol can be maintained at a level that allows flow through a printer nozzle, with formation of the polymerized gel stage occurring as the solvent evaporates or is absorbed after printing. Cells and sol are patterned sequentially, with centering of the cell occurring within the sol droplet as the sol coalesces and the silica condenses (**Figure 9B**). The increased spatial resolution provided by centering of yeast cells within the sol droplet extends to other particles and depends on a critical size, surface chemistry and ink composition that allows any particle to become centered.

Microchannels -An alternative approach to cell immobilization that would allow easy optical interrogation and optimal cell viability focused on a mechanical entrapment scheme—forced packing of yeast cells in a microchannel. The channel is a positive poly(dimethylsiloxane) (PDMS) mold poured on a negative master and has an array of posts that acts as a "cell trap"(**Figure 10**) to contain yeast cells for optical interrogation. We have found that induction of reporter protein expression (**Figure 11**) is similar for both packed cells and control cells suspended in buffer. The time to a 100 fold increase in fluorescence (over background) is about 150 minutes, with >75% viability. We found that packed stationary phase cells respond in about 210 minutes, with >75% viability. By using the double reservoir system in series with vacuum assisted micromolding, we have also immobilized cells expressing GFP in an 8μm-deep channel of lipid-templated silica. In this case, cells can be incorporated either by mixing the buffered suspension with the sol or by prior concentration of cells in the channel, followed by infiltration of the sol. Condensation of the silica matrix occurs over 50-60 minutes at 30°C as ethanol preferentially evaporates from the mold.

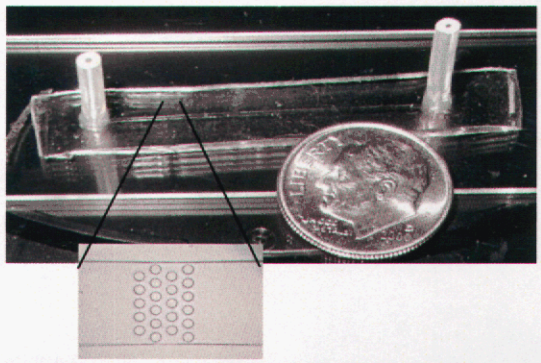


Figure 10. A completed microchannel, 200 μm wide and 35 μm deep, with inlet and outlet posts. The insert shows a top-view of the "cell trap"—35 μm diameter posts.

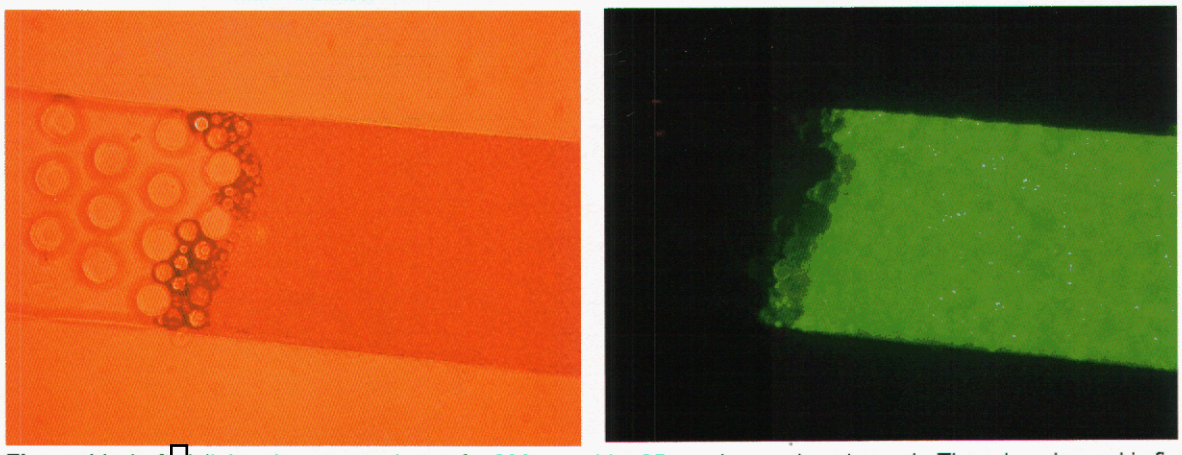


Figure 11. Left: A light microscope photo of a 200 μm wide, 25 μm deep, microchannel. The microchannel is first packed with 20 μm silica beads, followed by 10 μm silica beads, and lastly followed by the 5 μm yeast cells. The stepwise decrease of spheres prevents the yeast cells from flowing downstream. Right: A mercury lamp is passed through a set of filters and excites the green fluorescent proteins in the cells.

Robocasting- We developed processes and automated techniques for precision deposition of materials onto planar and nonplanar substrates. Materials are deposited in three dimensions with resolutions as fine as 100 microns. The delivery can be accomplished by drop-on-demand ink jet printing or with a precisely controlled syringe and orifice. A laser profilometer was attached to the robotic deposition equipment to measure the topology of the substrate. Software was created that can modify a CAD file of the desired pattern to be built in a conformal fashion on the substrate. We demonstrated the precise deposition of inks, ceramics, and polymers.

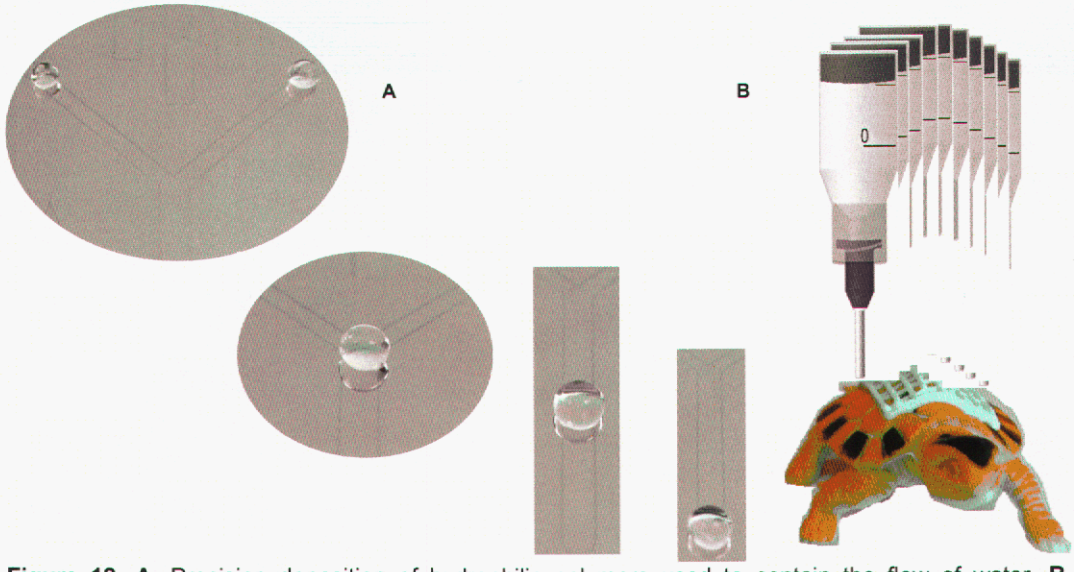


Figure 12. A. Precision deposition of hydrophilic polymers used to contain the flow of water. **B**. Conformal printing on uneven substrate is preceded by *in–situ* measurement of substrate topology and CAD design of the deposition profile.

5. Cell signaling.

Cloning and Gene Expression- Expression of fluorescent proteins in *S. cerevisiae* was achieved using pYES2 (high copy number) expression vector and the CFP and YFP genes present in pDH3 and pDH5, respectively (Yeast Resource Center, Seattle, WA PHS grant # P41 RR11823). The pYES2 expression vector contains the *URA3* selectable marker, a 2μm origin of replication, ampicillin resistance gene, *GAL1* promoter and a *CYC1* transcription terminator separated by a multiple cloning site (MCS). The vector was linearized with *Hind III* and *XbaI*, effectively removing the MCS. The CFP region of pDH3 was PCR amplified using primers designed to add homology to the *GAL1*p and *CYC1* transcription termination regions of pYES2. PCR was accomplished using Expand Long PCR system (Roche, Indianapolis, IN) and fragments were purified using the QIAquick PCR purification kit (Qiagen,Valencia,CA). The purified PCR product (insert) and the linearized vector were co-transformed into *S. cerevisiae* host strain S288C (ura- his-).

Transformation of *S. cerevisiae* was carried out essentially as described by Gietz *et al[31]*. Briefly, cells were grown up in YPD+A liquid medium to exponential phase and collected by centrifugation (2000g, 5 min). They were washed once and resuspended in sterile water. The insert and vector were combined in a 3:1 ratio and added to a solution of 0.1M LiAc, 50% w/v PEG 3500, and denatured salmon sperm carrier DNA. The mixture was then added to the host cells and incubated at 42° for 20 minutes. The cells were collected by centrifugation (2000g, 30seconds) and plated on synthetic medium lacking uracil. A cell line incorporating YFP from pDH5 was constructed in the same way. A yeast strain containing a plasmid with GFP regulated by *GAL1* promoter was obtained from Dr. Sepp Kohlwein (University of Graz, Austria).

Two additional cell lines were constructed in which the fluorescent protein sequence is integrated into the genomic sequence of *S. cerevisiae* S288C and regulated by an *S. cerevisiae* gene. The cell lines were constructed as above using YRC plasmids with the following modifications: The fluorescent protein gene from the plasmids was PCR amplified using primers designed to add 50 nucleotides at the 5' and 3' ends of the target gene open reading frame. Transformants were selected on G418. The new cell lines containing the following regulatory gene/fluorescent protein combinations were constructed: *HST3/CFP* (from pDH3; YRC) and *TIM11*/DsRed (from pTY24; YRC). *NPL3*/GFP plasmid was obtained from the Guthrie lab (CalTech) and transformed into the *S. cerevisiae* S288C strain.

Gene selection for up or down regulation in response to pathogens and BW agents-
S. cerevisiae in stationary phase was exposed to the binding subunit of cholera toxin and a supernatant from media containing anthrax simulants *Bacillus subtilis* and *Bacillus thuringiensis*. After exposure, the cells were lysed and DNA microarray analysis was used to determine changes in gene expression in response to the target agent. The cholera toxin and supernatant were combined as a single cocktail and visualization software was used to identify genes whose induction was specific for one or more agent stimuli versus those whose regulation was more general.

Reporter protein induction-GFP expression in response to a nutrient shift from glucose to galactose was induced in cells immobilized in both templated thin films and μ-molded channels (**Figure 13A**). Films were immersed in a complete synthetic medium lacking uracil, with agitation, to induce GFP expression, while channels were placed in a flow cell. Although previously reported induction of GFP expression in immobilized *E. coli* [32] required short film drying times of 5 minutes to prevent cell inactivation, the presence of a template allowed us to induce expression in films and channels that had condensed and dried in air for over 24 hours. Induction times for immobilized *S. cerevisiae* are similar to times for suspended control cells, confirming a high degree of viability, preserved functionality, and easy accessibility of cells in the biocomposite material. GFP expression in immobilized cells has been mapped successfully using hyperspectral imaging (**Figure 13B 1,2**), confirming the potential for remote sensing and individual addressability of reporter cells.

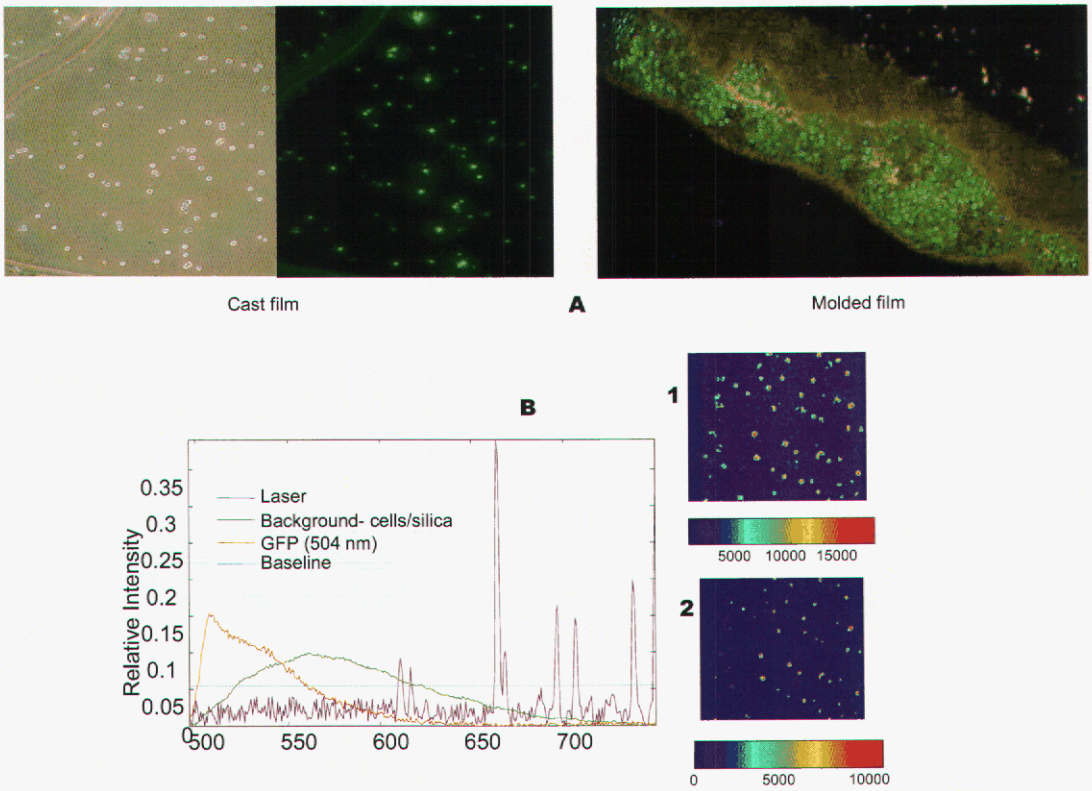


Figure 13 A. GFP expression in cast and molded films, induced more than 24 hours after cell immobilization. **B**.Hyperspectral imaging of cells expressing GFP. The entire emission spectrum is collected as the substrate is scanned, and multivariate data analysis gives information on emission sources at each pixel, allowing a quantitative estimate of fluorophore concentrations. Concentration mapping of the background (includes cells, buffer and silica film) (**1**) and deconvoluted GFP spectrum shows (**2**) GFP localized within cells, appearing as dots in both (**1**) and (**2**).

GFP fluorescence from immobilized cells is still present in biocomposite films that have been exposed to ambient conditions for more than five days, indicating that GFP remains undenatured within its cellular environment. Viability of immobilized cells showing GFP expression has been confirmed for as long as 48 hours and can be extended by applying a second layer of a CTAB templated sol containing a hydrogel monomer, N-Isopropylacrylamide (NIPAAm). The inner phospholipid templated layer provides a

protective, sustaining environment of lipids and buffer while the outer layer contains a swellable polymer, serving as a buffer/nutrient reservoir in fluidic contact with the cells. By adjusting the time allowed for condensation of the inner layer before application of the outer layer, it was possible to retain the individual ordered mesophases of both layers, as evidenced by XRD, and avoid exposure of the cells to the toxic CTAB template. Additionally, the immobilized cells were protected during the 45 minutes of UV exposure necessary to polymerize the NIPAAm.

6. Conclusion.

A single living cell incorporates the abilities to respond to an external stimulus through signal recognition, amplification and transduction. Immobilizing whole cells in a porous, nanostructured silica host matrix through biocompatible evaporation induced self-assembly (EISA) allows the cells to be protected, sustained and confined in a buffered environment while remaining accessible for nutrient, analyte and waste transport requirements. We developed a several-pronged approach to provide the biocompatible materials and processing conditions necessary to maintain cell viability while retaining careful control over the microstructure of the inorganic host. Biocompatible templates, buffered sols and multiple reservoir processing conditions allowed us to immobilize genetically engineered *Saccharomyces cerevisiae* cells in a nanostructured silica host and to detect an environmental stimulus through expression of green fluorescent protein. Using phospholipid templates as amphiphilic structure-directing agents during EISA results in a variety of ordered mesophases, depending on the molar ratio of lipid to silica and the ratio of the lipid's head group surface area to hydrophobic tail volume. Incorporating yeast cells into the inorganic matrix, however, strongly influences the self-assembly pathway. When cells are added to the sol, both the molar ratio of lipid to silica required to form a nanostructured phase, and the morphology of the phase, changes. Adding a fluorescently labeled lipid and using latex and silica beads as hydrophobic and hydrophilic models during self-assembly, allowed us to see the preferential localization of lipid around the cells, consistent with both the cells having a structure-directing role through interaction with lipid templates, and the lipid forming a protective barrier around the cells. Biocompatible EISA, combined with new sequential patterning techniques, has allowed us to address several technical issues associated with patterning of living cells, including precise localization of immobilized cells for array applications, maintenance of accessibility, and minimization of both shear stresses and exposure to toxic environmental conditions. We have demonstrated cell patterning by selective wetting and ink jet printing (IJP).

References

1. Stenger, D.A., et al., *Detection of physiologically active compounds using cell-based biosensors.* Trends in Biotechnology, 2001. **19**(8): p. 304-309.
2. Bousse, L., *Whole cell biosensors.* Sensors and Actuators B-Chemical, 1996. **34**(1-3): p. 270-275.

3. Ziegler, C., *Cell-based biosensors.* Fresenius Journal of Analytical Chemistry, 2000. **366**(6-7): p. 552-559.
4. Curtis, A. and C. Wilkinson, *Topographical control of cells.* Biomaterials, 1997. **18**(24): p. 1573-1583.
5. Koh, W.G., A. Revzin, and M.V. Pishko, *Poly(ethylene glycol) hydrogel microstructures encapsulating living cells.* Langmuir, 2002. **18**(7): p. 2459-2462.
6. Assender, H., V. Bliznyuk, and K. Porfyrakis, *How surface topography relates to materials properties.* Science, 2002. **297**(5583): p. 973-976.
7. Pancrazio, J.J., et al., *Development and application of cell-based biosensors.* Annals of Biomedical Engineering, 1999. **27**(6): p. 697-711.
8. WernerWashburne, M., et al., *Stationary phase in Saccharomyces cerevisiae.* Molecular Microbiology, 1996. **19**(6): p. 1159-1166.
9. Tsien, R.Y., *The green fluorescent protein.* Annual Review of Biochemistry, 1998. **67**: p. 509-544.
10. Shetty, R.S., et al., *Green fluorescent protein in the design of a living biosensing system for L-arabinose.* Analytical Chemistry, 1999. **71**(4): p. 763-768.
11. Bergogne, L., et al., *Bio-encapsulation within sol-gel glasses.* Molecular Crystals And Liquid Crystals, 2000. **354**: p. 667-677.
12. Gill, I., *Bio-doped nanocomposite polymers: Sol-gel bioencapsulates.* Chemistry of Materials, 2001. **13**(10): p. 3404-3421.
13. Uo, M., et al., *Immobilization of yeast-cells in porous silica carrier with sol-gel process.* Nippon Seramikkusu Kyokai Gakujutsu Ronbunshi-Journal Of The Ceramic Society Of Japan, 1992. **100**(4): p. 426-429.
14. Pope, E., *Gel encapsulated microorganisms - saccharomyces-cerevisiae - silica-gel biocomposites.* Journal Of Sol-Gel Science And Technology, 1995. **4**(3): p. 225-229.
15. Conroy, J.F.T., et al., *Cells in sol-gels I: A cytocompatible route for the production of macroporous silica gels.* Journal of Sol-Gel Science and Technology, 2000. **18**(3): p. 269-283.
16. Inama, L., et al., *Entrapment of viable microorganisms by sio2 sol-gel layers on glass surfaces - trapping, catalytic performance and immobilization durability of saccharomyces-cerevisiae.* Journal Of Biotechnology, 1993. **30**(2): p. 197-210.
17. Fennouh, S., et al., *Sol-gel entrapment of Escherichia coli.* Journal Of Sol-Gel Science And Technology, 2000. **19**(1-3): p. 647-649.
18. Bhatia, R.B., et al., *Aqueous sol-gel process for protein encapsulation.* Chemistry of Materials, 2000. **12**(8): p. 2434-2441.
19. Coiffier, A., et al., *Sol-gel encapsulation of bacteria: a comparison between alkoxide and aqueous routes.* Journal of Materials Chemistry, 2001. **11**(8): p. 2039-2044.
20. Gill, I. and A. Ballesteros, *Encapsulation of biologicals within silicate, siloxane, and hybrid sol-gel polymers: An efficient and generic approach.* Journal Of The American Chemical Society, 1998. **120**(34): p. 8587-8598.
21. Nassif, N., et al., *Living bacteria in silica gels.* Nature Materials, 2002. **1**(1): p. 42-4.
22. Israelachvili, J., *Intermolecular & Surface Forces.* 1992, London: Academic Press.

23. Brinker, C., et al., *Evaporation-induced self-assembly: Nanostructures made easy.* Advanced Materials, 1999. **11**(7): p. 579-+.
24. Salter, G. and D. Kell, *Solvent selection for whole-cell biotransformations in organic media.* Critical Reviews In Biotechnology, 1995. **15**(2): p. 139-177.
25. Alberts, B.e.a., *Essential Cell Biology.* 1998, New York: Garland Publishing, Inc.
26. Probes for Yeast Viability, Molecular Probes, 2001. Cells were suspended in HEPES growth media and Calcoflor White M2Tand Fun-1 dyes were added. After a 30-minute incubation period, which removes the cells from stationary phase, the cells were observed with epifluorescence microscopy, using a Xe or Hg lamp. A multi-pass filter set for viewing DAPI, fluorescein and rhodamine allows simultaneous imaging of both dyes. Dead cells appear bright yellow-green, while metabolically active cells form discrete, red Cylindrical Intra Vacuolar Structures CIVS as Fun-1 is processed during an ATP dependent process. Calcoflor is a surface label, staining both dead and live cells fluorescent blue to aid in counting. Cells used for viability studies were *S. cerevisiae*, strain S288C, grown to G_0 or stationary state, where cell walls thicken and stress tolerance increases. They were suspended in phosphate buffer and added to sol formulations in equimolar volumes. Phospholipid templates were added either to the sol or the yeast in buffer as a final step before mixing and immediate spincoating.
27. Laouar, L., K.C. Lowe, and B.J. Mulligan, *Yeast responses to nonionic surfactants.* Enzyme and Microbial Technology, 1996. **18**(6): p. 433-438.
28. Jendrasiak, G. and R. Smith, *What does lipid hydration tell us? A minireview.* Cellular & Molecular Biology Letters, 2000. **5**(1): p. 35-49.
29. McIntosh, T., *Hydration properties of lamellar and non-lamellar phases of phosphatidylcholine and phosphatidylethanolamine.* Chemistry And Physics Of Lipids, 1996. **81**(2): p. 117-131.
30. Doshi, D.A., Huesing, N.K., Lu, M., Fan, H., Lu, Y.F., Simmons-Potter, K., Potter Jr, B.G., Hurd, A.J., Brinker, C.J., *Optically Defined Multifunctional Patterning of Photosensitive Thin-Film Silica Mesophases.* Science, 2000. **290**: p. 107-111.
31. Gietz, R. and R. Woods, *Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method.* Guide To Yeast Genetics And Molecular And Cell Biology, Pt B, 2002. **350**: p. 87-96.
32. Premkumar, J.R., et al., *Fluorescent bacteria encapsulated in sol-gel derived silicate films.* Chemistry of Materials, 2002. **14**(6): p. 2676-2686.

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