Direct Attachment of Microbial Organisms to Material Surfaces Through Sequence-Specific DNA Hybridization

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Due to their potential for the production of fuels and energy-intensive products, the metabolic pathways of many microbial organisms have been the subject of extensive research. Typically, these studies are carried out using bulk culture experiments, in which large numbers of cells are dispersed throughout the culture medium. These experiments have provided important information regarding the overall metabolism of the organisms when grown under various conditions and in the presence of different carbon sources. However, there are limits to which one can specify the environment that is presented to each individual cell, making it highly challenging to obtain quantitative data under fully controlled conditions. These experiments also provide data at single or a limited number of time points, and are difficult to carry out using multiple organisms at once.

In order to gain a more precise understanding of metabolic pathways and the often complex factors affecting them, it would be highly advantageous to combine strictly controlled growth conditions with real-time monitoring capabilities in a microfabricated device setting. Additionally, enhanced metabolite production has been observed for cells (such as biofuel or polymer production by bacteria, algae, and fungi) that have been immobilized using agarose, alginate, and other polymers. In previous studies, mammalian cells have been captured in small-volume channels that allow the nutrient flux, temperature, and other conditions to be systematically controlled. Microelectrodes have also been incorporated into these platforms for real-time product monitoring. Unfortunately, these studies have been difficult to extend to the study of microbial organisms due to their smaller cell sizes and lack of readily available controlled surface adhesion strategies. Some organisms can be cultured as biofilms on the electrodes of microbial fuel cells, but this process typically takes several days and can lead to heterogeneous and poorly characterized interfaces. As an alternative strategy, some examples have appeared in which non-mammalian cells have been covalently attached to functional groups extending from material surfaces. However, the success of these chemical approaches varies extensively from one organism to another, creating a significant need for a universal and specific adhesion strategy that can be used to capture virtually any microbial cell type of interest into a device format.

Single-stranded DNA (ssDNA) has been used as an attachment strategy for many biomolecules, such as proteins and lipid vesicles, for the development of sensitive detection assays, biosensors, nanostructured supramolecular devices, and microarrays based on DNA complementarity. DNA and biotin mediated immobilization has also been used to anchor and/or micropattern vesicles in 2 and 3-dimensions as model systems for cell membranes and cells. In 2009, a new method was reported for the adhesion of mammalian cells to surfaces through the use of synthetic single-stranded DNA molecules that were attached to the amine groups of proteins extending from the cytoplasmic membranes. This endowed them with the ability to bind to complementary sequences extending from the surface of glass slides, metal oxide electrodes, AFM cantilevers, and capture surfaces within complex microfluidic devices. However, the chemical strategies for the introduction of the DNA strands on mammalian cell surfaces have proven largely unsuccessful when applied to most microbial organisms, likely because there are too few solution exposed amines extending from their outer membranes or cell walls. To address these cell types, we report herein a new oxidative strategy for the introduction of nucleic acid strands on microbial cell surfaces. This new approach results in their strong, efficient, and specific binding to surfaces displaying the sequence complement without apparent losses in cell viability. The resulting ability to incorporate a wide range of new organisms into device platforms will give access to more quantitative information regarding the influence of environmental factors on production levels of molecules and products by a variety of cells of interest, such as those listed in Figure 1b. An important additional benefit of this technique is its ability to combine multiple organism types into complex 2- and 3-dimensional patterns through the use of multiple binding sequences. This provides a valuable new tool for the exploration and evaluation of symbiotic relationships, in which metabolites are transferred among different organisms with predefined spatial relationships.

Since a variety of microbes with metabolic functions of interest (such as fungi, algae, and bacteria) display carbohydrates on their surfaces, we envisioned a two-step method to attach ssDNA to these cell types based on the initial oxidation of the 1,2-diols in the carbohydrates with sodium periodate. This step would then be followed by condensation of the resulting aldehyde groups with hydrazides to form hydrazones. Figure 1a. For the first step in this protocol, several organisms were exposed to sodium periodate at concentrations of 0.5 to 5 mM for 45 min or less. Figure 1b lists the specific

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DOI: 10.1002/adma.201104336
conditions used for each organism, and full experimental protocols are provided as Supporting Information. In each case, the presence of new aldehyde groups on the cell surfaces was confirmed qualitatively through exposure to AlexaFluor 488 alkoxyamine. Cell viability was determined using a 5-cyano-2,3-di-4-tolyl-tetrazolium chloride (CTC) stain or by returning the cells to culturing conditions. Though previous studies involving the oxidation of mammalian cell glycans have not observed appreciable declines in cell viability, periodate concentrations in excess of 5 mM greatly lowered cell viability for many of the microbial organisms in this study, even after only 5 min of exposure. Viability seemed to be further reduced for some cell types when high oxidant concentration was combined with increased reaction temperatures of 37 °C. By using lower concentrations and times, we were able to develop more optimal oxidation conditions for all of the cell lines listed in Figure 1b, allowing the introduction of large numbers of aldehyde groups without causing any observable detrimental effects on cell viability or the ability to reproduce.

After rinsing excess periodate away from the cells, the resulting aldehyde functional groups then served as attachment points for commercially available 5′-hydrazide-terminated ssDNA (I-Linker DNA, IDT) bearing 3′-fluorescein dyes. Hydrazone formation was facilitated by the addition of aniline (10–25 mM) to the reaction mixture as a catalyst. Hydrazone formation reaction times were determined by first allowing the cell modification reactions to proceed overnight (12–16 h). If this reaction time was sufficient to result in...
dense cell patterning on glass slides displaying complementary ssDNA (Figure 1d–l, cell densities appear in Supporting Information (SI), Figure S1), shorter times were screened. This was the case for A. vinelandii and S. cerevisiae. If 12 h was not sufficient to create cells that patterned with centrifugation, longer times were screened until the modified cells created dense patterns visible by the naked eye on ~5 mm complementary DNA spots. The resulting cells exhibited strong fluorescence as a result of DNA-FITC attachment, as shown for S. cerevisiae in Figure 1c,d. When the oxidized microbe surfaces were exposed to hydrazide-ssDNA with no aniline catalyst, little or no hydrazide formation (SI), Figure S1), shorter times were screened. This was the case for A. vinelandii, the number of DNA strands that were attached to each cell was determined using flow cytometry. A fluorescence calibration curve was first generated using standardized polymer spheres displaying a known number of fluorophores. These standards take into account any potential self-quenching due to a high fluorophore density. Comparison of a bacterial sample labeled with fluorescent ssDNA as described above indicated that about 26,000 molecules had been attached to each cell (mean fluorescence intensity (MFI) = 8.08), meaning there are approximately 1000 molecules of ssDNA per μm². 

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After optimizing the cell modification scheme for ssDNA, we next examined the ability of the strands to mediate surface adhesion. Glass slides were prepared with circular patterns of DNA-modified yeast continued to divide during this time, with daughter cells exhibiting replication with little loss of surface coverage. The cell denaturation step (Sorvall Legend Mach 1.6R, 5 min at 3000 rpm) was also counterstained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) for whole cell visualization. Both stains were qualitatively assessed using fluorescence microscopy. This assay was also used to arrive at the optimal oxidation conditions listed in Figure 1b, with viability considered to be unaffected when the red fluorescence intensity per cell of the modified group was approximately equal to that of unmodified cells.

For S. cerevisiae, S. oneidensis, and C. reinhardtii, viability was instead assessed by returning the modified cells to culturing conditions. In all cases, use of the optimized modification conditions led to no appreciable loss in viability either before or after glass surface attachment. In the case of yeast, dense patterns of cells have been maintained in culture for 9 days, exhibiting replication with little loss of surface coverage. The yeast continued to divide during this time, with daughter cells (which possess a de novo synthesized cell wall) being released into solution.

Normal cell morphology and phenotype were also maintained after ssDNA modification and culturing (SI, Figure S2a–d). A. vinelandii and S. cerevisiae were modified with hydrazide ssDNA, after which no changes in cell morphology were observed. The cells were then returned to the culture medium for two days. After this time the ssDNA modified cells and a sample of unmodified (control) cells were again imaged at 100X using DIC microscopy. Consistent with the initial observations, there were no apparent morphological differences between the control and ssDNA modified cells at this time point.

After establishing that the cell modification chemistry and immobilization had negligible effects on viability, the effects of DNA modification on ammonia production were investigated using the diazotrophic bacteria A. vinelandii, in order to verify that the cell modification chemistry does not interfere with normal cell processes. Cultures of oxidized (control) and ssDNA modified A. vinelandii were cultured in a medium lacking a nitrogen source. Samples were collected after 5 min and 8 days of growth. Ammonia production was then analyzed using an NH₃ ion selective electrode (ISE). For the purpose of
small bacterial cells using a microscope.\textsuperscript{[19]} PCV measurements also account for biomass accumulated by the cells.

ISEs are reported to be insensitive to nitrogen in biopolymers, such as DNA or proteins. This fact was confirmed experimentally in our lab, but some response to the aniline used to catalyze hydrazone formation was observed. The ammonia quantified in the 5 min samples indicated that little if any aniline was carried over from the surfaces of the microbes after modification and rinsing (Figure 2e). After 8 days, the control and ssDNA modified cells produced approximately the same amount of NH$_3$, demonstrating that nitrogen fixation, an important and industrially useful biological function, was not affected negatively by the modification procedure. 

\textit{A. vinelandii} is a commercially interesting source for fixed N$_2$ due to its remarkably fast metabolism and ability to fix N$_2$ in the presence of atmospheric concentrations of O$_2$, potentially providing a much less energy intensive method for NH$_3$ production\textsuperscript{[5a, 20]} than the Haber–Bosch process\textsuperscript{[21]}. This prospect could be further explored by using this ssDNA modification strategy to immobilize \textit{A. vinelandii} in small scale devices, providing a useful platform to study real-time ammonia production values as well as a means to optimize ammonia production output through alteration of the environmental and nutrient conditions\textsuperscript{[22, 23]}.

One unique strength of the DNA-based adhesion method is its ability to immobilize multiple cell types through the use of different DNA capture sequences\textsuperscript{[6b, 11, 13, 24]}. When combined with lithographic patterning techniques, this can lead to the formation of complex 2- and 3-dimensional patterns comprising multiple types of organisms. As one example, we have used this technique to generate the 3-dimensional layered cell microculture shown in Figure 3a–d. To generate this assembly, DNA-labeled \textit{Synechocystis} (red autofluorescent, strand A\textsuperscript{′}) was bound to a glass slide bearing 60 μm spots of the sequence complement (strand A) using the centrifugation protocol described above. A sample of \textit{A. vinelandii} (DAPI stained) was labeled with two different DNA sequences (A and B\textsuperscript{′}) in a single step, providing a set of strands that could bind to the remaining A\textsuperscript{′} strands on the immobilized \textit{Synechocystis} cells, Figure 3b. In a final step, the B\textsuperscript{′} set of \textit{A. vinelandii} strands was hybridized to sample of \textit{R. rubrum} (BacLight Green labeled) cells bearing strands with sequence B. Multicolor fluorescence imaging (Figure 3a–d) clearly showed colocalization of the three cell types.
types, with little-to-no background binding in the non-DNA printed regions. It should be noted that dense patterning was required in this experiment to form vertical layers, rather than arranging the cells side-by-side.

The application of this technique to the formation of 2-dimensional patterns with arbitrary complexity is shown in Figure 3e. In this case, an aluminum “lift off” technique was used to pattern three DNA capture strands on the surface of a glass slide. This pattern was exposed sequentially to *Synechocystis* (red, autofluorescent) labeled with the first sequence, *A. vinelandii* (blue, DAPI labeled) modified with the second DNA strand, and *S. cerevisiae* (blue-green, double labeled with BacLight Green and DAPI stain). Double labeling
test was necessary to achieve a strong fluorescent signal. After each exposure, the sample was centrifuged to facilitate cell binding, and then the non-DNA captured cells were rinsed away with gentle shaking in PBS solution. All three organisms exhibited efficient binding to the appropriately patterned location and showed little non-specific adhesion in other areas. The ability of ssDNA modified cells to pattern only on glass modified with the complementary strand was further verified by attempting to immobilize *A. vinelandii* modified with sequence C on patterns of mismatched strands A and B (See SI for specific sequences).

Acknowledgements

This work was supported by the Director, Office of Science, Office of Basic Energy Sciences, of the US Department of Energy under Contract No. DE-AC03-76SF00098. The authors would like to thank Prof. Anastasios Melis and his lab (UC Berkeley Plant and Microbial Biology), Caroline Ajo-Franklin (Lawrence Berkeley National Lab), Prof. Douglas Clark (UC Berkeley Chemical Engineering), Prof. Krishna Niyogi (UC Berkeley Plant and Microbial Biology), Prof. Carolyn Bertozzi (UC Berkeley Chemistry) for cell lines and many helpful discussions. Prof. Daniel Arp (Oregon State Botany and Plant Pathology) is gratefully acknowledged for the *A. vinelandii* hup- and wild-type strains, as well as for helpful discussions. Photolithography was done in the Marvell Nanofabrication Laboratory at UC Berkeley.

Received: November 11, 2011
Revised: December 12, 2011
Published online:


