The Dynamics of Surface Detachment and Quorum Sensing in Spatially Controlled Biofilm Colonies of *Pseudomonas aeruginosa*

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**ABSTRACT**

Biofilms represent a highly successful life strategy of bacteria in a very broad range of environments and often have negative implications for industrial and clinical applications, as their removal from surfaces and the prevention of biofouling in the first place represent formidable and to date unmet challenges. At the same time, biofilms modulate important natural processes, including nutrient cycling in rivers and streams and the clogging of porous materials. Biofilm development is a dynamic process, dependent on a host of cellular and environmental parameters that include, among others, hydrodynamic environment and the communication among cells (QS).

Here we used microfluidics and micro-contact printing, paired with video-microscopy and image analysis, to study several aspects relating to the temporal dynamics of bacterial biofilms. Beyond reporting on specific findings from these experiments, we demonstrate how these innovative technologies can aid in obtaining a new layer understanding on biofilm processes and biofilm removal, thanks to unprecedented control over the biofilm’s microenvironment.

In a first set of experiments (chapter 2), we cultured *Pseudomonas aeruginosa* biofilms in a microfluidic channel for different times, after which we used the passage of an air plug as a mechanical insult to drive detachment. We found that the adhesion properties of an early-stage biofilm have a strong correlation with the time of growth and that biofilm detachment can occur in a spatially heterogeneous manner characterized by a regular pattern of ‘holes’ in the original biofilm. The resulting spatial distribution of bacteria correlates with the distribution of the extra polymeric substance (EPS) matrix before the insult, indicating that the locations of the holes correspond to where there was the least EPS. These results demonstrate that the detachment mechanism is a competition between the shear force exerted by the external flow and the local adhesion force of a given patch of biofilm, in turn governed by the local amount of EPS. This mechanism, and the observed heterogeneity in the detachment, imply that even at rather high
shear rates, where the bulk of the biofilm is removed, local strongholds survive detachment, and may represent seeds for the colony to reform.

In a second set of experiments (chapter 3), we examined the effects of patch size and hydrodynamic environment on QS induction on spatially defined patches of Pseudomonas aeruginosa biofilm. We found that the smaller biofilm patches start QS earlier than those in the larger patches. However at later times the proportion of auto-induced bacteria (normalized by the surface area covered by the cells) is higher in the larger patches. We expanded on these findings by investigating the contribution of flow to QS induction on the patches. The effect of ambient fluid flow was to accelerate the induction of quorum sensing compared to static conditions at moderate flow rates, due to the increase in the convective supply of nutrients and to quench quorum sensing at high flow rates, due to the autoinducer signal being washed out by flow. These findings establish microfluidics as a new tool in the study of biofilms, which enables both accurate control over microenvironmental conditions and direct observation of the dynamics of biofilm formation and disruption.

Chapter 4 deepens the exploration of the role of micro-spatial heterogeneity on microbial processes by presenting a numerical model of how heterogeneity and microbial behavior (chemotaxis) affect trophic in a microbial food web. Results show that the intensity of the trophic transfer strong depends on the motile behavior of the different trophic levels: trophic transfer is enhanced when directional motility towards resource patches outweighs the random component of motility inherent in all microbial locomotion.

Finally, in the Appendices, we demonstrate how the methods developed in this thesis can help in the assessment of the antifouling capabilities of new-generation surfaces, designed to prevent fouling and in the assessment of the cell adhesion on surfaces, fabricated with an arrangement of spatially localized hydrophobic patterns.

In summary, this thesis demonstrates that use of new micro-technology and associated mathematical modeling enables new insights into the colonial life form of microorganisms and may provide impetus for new approaches to prevent biofouling on surfaces or remove biofilms from surfaces.

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rhIR, which means the las QS system controls RhlR at the transcriptional level. AII also controls the activity of RhlR at the post-translational level by binding to RhlR, which decreases AII2 binding to RhlR, therefore inhibiting expression of rhlA and other genes. Presumably, this action ensures that the LasI/LasR circuit is established prior to the establishment of the RhlI/RhlR circuit. The Pseudomonas quinolone signal (PQS) is an additional regulatory link between the Las and Rhl QS circuits.

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Figure 4-9 Increase in consumer patchiness due to chemotactic motility. Shown is the patchiness index, \( P_{BB} \), at time \( t = 0.02 \) for a two-level food chain (A, B), two values of the biomass transfer rate, (a) \( \eta_B = 1 \) and (b) \( \eta_B = 10 \), and a matrix of motility values, \( \kappa_B \) and \( \chi_B \). The case \( \chi_B = \kappa_B = 0 \) corresponds to a passive food chain, while cases with \( \chi_B = 0 \) correspond to ‘diffusive’ consumers (motile but non-chemotactic).

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Chapter 1  Introduction and Background

1.1 Microbial Biofilms

Microorganisms are ubiquitous in the environment. Despite their tiny size, their combined biomass is enormous. For example, a milliliter of seawater contains a million bacteria, making for a total of $10^{29}$ bacteria in the world’s oceans $^1$, alone. Concentrations of bacteria are even higher in other environments, including the subsurface and the human body, and it is now well accepted that this huge bacterial biomass performs a plethora of crucial functions in nearly every environment.

Many microbes are associated with solid surfaces. These interfacial microbial communities are termed ‘biofilms’ and are structurally and dynamically complex biological systems (Fig. 1.1). The structures consist primarily of a matrix of extracellular polysaccharide substance (EPS) that the bacterial cells produce and within which they are embedded $^2$. The EPS matrix provides several functional purposes for the biofilm, including protecting bacteria from environmental threats, providing mechanical stability, and degrading macromolecules to be used by the cells $^3$. 

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Biofilm formation is a sequential process, consisting of a series of steps that occur when cells encounter favorable nutrient and surface conditions. The steps are:

(i) transport of microbes to a surface by swimming, sinking or flow

(ii) initial reversible attachment, affected by hydrodynamic conditions, where bacteria move on the surface by means of flagellar swimming or pili-mediated twitching motility \(^4\), seeking a niche to settle \(^6,7\)

(iii) irreversible attachment, accompanied by profound physiological changes, including
rapid down-regulation of flagellar synthesis \(^{3,8-10}\), reduced growth rates and up-regulation of alginate synthesis to produce EPS, which promotes cell adhesion and formation of 3D cell clusters, protecting cells from desiccation, antibiotics, bacteriophages and predators \(^{7,11}\).

(iv) biofilm maturation, when cell clusters grow to thicknesses of order 100 \(\mu\)m and complex architectures emerge, with water channels transporting nutrients across the biofilm.

(v) motility-mediated dispersion \(^{2,5,12}\) or shear induced erosion \(^{13}\) of cells from the biofilm, possibly to colonize new habitats and initiate secondary infections \(^{14}\).

Fully developed biofilms have complex architectures and have been compared to ‘cities of microbes’ \(^{15}\). Mushroom-shaped microcolonies, tens of microns thick \(^{16,17}\), are encased in an EPS matrix and criss-crossed by interstitial water channels \(^{2,9}\). Their spatial heterogeneity harbors a range of microenvironments, providing niches for multiple phenotypes \(^{18}\), and affects community structure and productivity \(^{19}\). This environment is dynamic: structures appear and disappear as new cells attach, others migrate among colonies \(^{20}\), cooperate for biofilm stability or compete for nutrients \(^{21}\), and ultimately disperse \(^{9}\).

Biofilms are a major component of world-wide bacterial biomass \(^{22,23}\) and they often have
deleterious effects in natural, industrial and medical settings. In natural settings, they can impact environments on a global level, including producing and consuming atmospheric gases; mobilizing toxic elements such as mercury, arsenic and selenium causing stream or soil contamination\textsuperscript{24}; and producing toxic algal blooms and creating oxygen depletion zones in lakes, rivers and coastal environments\textsuperscript{25}. In many industrial processes, the formation of biofilms on nearly every surface is responsible for huge economics losses (in the billions of dollars yearly in the US alone\textsuperscript{26}) resulting from biofouling and biocorrosion\textsuperscript{27}, which leads to equipment clogging and damage, product contamination and energy losses\textsuperscript{28}. In medicine, biofilms cause deleterious effects in many medical devices including catheters and prosthetic heart valves\textsuperscript{23}.

There are, however, applications that take advantage of biofilm growth. The ability of the biofilm polymer matrix to entrap minerals and nutrients is exploited in purification of drinking water\textsuperscript{29}, detoxification of oil spills, removal of heavy metals and biodegradation of hazardous xenobiotics in contaminated waters and soils\textsuperscript{30}, as well as environmental monitoring using biosensors\textsuperscript{31}. Many biofilms play an important role in the ecology of the earth and the sustainability of life. In soil, for instance, almost every chemical transformation involves active contributions from microorganisms, many of which are surface-attached. In particular, microbes play an active role in soil fertility by mediating the cycle of nutrients, like carbon and nitrogen,
which are required for plant growth. An understanding of the mechanisms underlying biofilm formation, development and detachment is a crucial step towards elucidating the role of biofilms in ecosystems and developing new strategies for biofilm control.

1.2 Biofilm Detachment

Of these phenomena, one of the least understood is detachment from a surface, even though understanding detachment mechanisms has great significance for controlling biofilms. Detachment refers to the release of microbial cells, and their associated matrix polymer (EPS), from a solid surface into the bulk fluid. The detachment process is an important component of the biofilm life cycle and plays a fundamental role in dissemination and contamination and ultimately the long-term survival in either natural or man-made settings of industrial and medical importance. Some of the factors that have been suggested to be important in biofilm detachment include matrix-degrading enzymes, microbially generated gas bubbles, nutrient levels and microbial growth status, fluid shear stress and quorum-sensing signals. Passing air-liquid interfaces (air bubbles or air plugs) have been demonstrated to yield extremely efficient detachment forces, as in the oral cavity during eating, speaking, drinking and swallowing, on the eye and on contact lenses during blinking, and on rocks and ship hulls in
marine environments. Since biofilm age increases biofilm adhesive strength due to EPS production, which in turn increases the biofilm’s resistance to cleaning treatments and protects biofilm cells against antimicrobial agents and desiccation, biofilms become gradually more difficult to remove over time. This is a huge problem also because, even if a small part of the biofilm survives the cleaning treatment, it can represent a seed from which the biofilm rapidly grows back. Thus, treatment should ideally be applied during the early stage of biofilm development before the biofilm is strongly sheltered by its EPS.

In Chapter 2 of this thesis, I present experiments to understand how the age of the biofilm affects removal by shear, applied through passage of an air plug. These experiments led to the discovery of a new ‘detachment mode’, in which biofilm removal is highly heterogeneous and characterized by a semi-regular pattern of holes, from which cells are removed, separated by regions in which the biofilm remains unscathed. High-resolution observations in space and time, enabled by the microfluidic model system, are used to rationalize the detachment dynamics, leading to the conclusion that the local amount of EPS sets the ability of any given area of the biofilm to withstand removal by mechanical forces.
1.3 The Role of Cell Signaling in Biofilm Dynamics

Molecular ‘quorum sensing’ (QS) systems are an important mechanism by which bacteria form and maintain complex biofilms through the ability to communicate and to alter behavior in response to the presence of other bacteria. Bacterial cells produce signaling molecules that are secreted into the environment, from which they can disperse by diffusion or advection. The producer cells respond to their own signals, which are therefore called autoinducers. Although the signaling mechanisms differ, QS systems are utilized by both gram positive and gram negative bacterial species. In general to sense their population density gram negative bacteria use small chemical molecules called acylated homoserine lactones (AHLs) and gram positive bacteria use oligo-peptides, both collectively referred to as autoinducers (AIs). Typically, an autoinducer molecule induces the transcription of a set of genes that includes the gene encoding the autoinducer-producing enzyme, which results in a positive feedback loop. The autoinducer can trigger a response by the cell if the autoinducer concentration—regulated by the rates of production, decay and mass transfer integrated over time—reaches a threshold at the cell's location (Fig. 1-2).
Figure 1-2 LasI/LasR-RhlI/RhlR quorum sensing system in *Pseudomonas aeruginosa*. The LasI protein produces the homoserine lactone signaling molecule N-(3-oxododecanoyl)-homoserine lactone, autoinducer 1 (AI1, blue circles), and the RhlI protein synthesizes N-(butryl)-homoserine lactone, autoinducer 2 (AI2, red triangles). Activated LasR (with AI1) activates transcription of rhlR, which means the las quorum sensing system controls RhlR at the transcriptional level. AI1 also controls the activity of RhlR at the post-translational level by binding to RhlR, which decreases AI2 binding to RhlR, therefore inhibiting expression of rhlA and other genes. The *Pseudomonas* quinolone signal (PQS) is an additional regulatory link between the Las and Rhl quorum sensing circuit.

The existence of a threshold cell concentration for the production of bioluminescence in the marine symbiotic bacteria *Vibrio fischeri* \(^5^5\), the formation of fruiting bodies in myxobacteria \(^5^9\) and the development of competence for genetic transformation in *Streptococci* \(^6^0\), are among the lines of evidence for cell-to-cell signaling systems in bacterial communities. At present, QS is defined as a cell-density-dependent bacterial intercellular signaling mechanism that enables
bacteria to coordinate the expression of certain genes to coordinate group behaviors.

Several physical, biological, and chemical factors have the potential to influence QS in biofilm systems \(^6^1\). For example, genetically modified mutants of *Pseudomonas aeruginosa* unable to perform quorum sensing do not form the complex mushroom-shaped structures typical of wild-type biofilms \(^5^0\), which suggests that the production of EPS in *Pseudomonas aeruginosa* is influenced by QS. Other studies have shown that QS regulates components of EPS which contribute to the sticky level, thus impacting the structural components of biofilm matrix. \(^6^2,^6^3\).

The hydrodynamic environment can also influence QS. Janakiraman et al. \(^6^4\) studied biofilm growth and QS in uniform microfluidic chambers and developed a one-dimensional mathematical model. They found that the flow rate greatly affects QS: at high flow rates, the biofilm thickness is smaller due to detachment, and the transport rate of AHL out of the biofilm by fluid flow can be so high that the AHL concentration does not reach the threshold required for induction. Purevdorj and Costerton \(^6^5\) emphasize the importance of transport processes, in particular highlighting that the washout of signal molecules from the bulk liquid surrounding bacterial microcolonies is expected to increase the concentration gradient between the biofilm and the bulk liquid, in turn driving a larger diffusive flux of signal molecules out of the biofilm. Their work indicates that diffusion through the biofilm under different hydrodynamic conditions
is strongly correlated with the AHL signal concentration in microcolonies. Muller et al. \(^6\) mathematically studied QS induction on biofilm patches. They found that induction can occur for a lower number of cells in a patchy biofilm than in a homogeneous biofilm, because the cell density (and consequently, AHL concentration) when cells are compacted in small clusters.

Building on this evidence of the key roles of the hydrodynamic environment and patchiness for QS in biofilms, I expand on these findings by investigating the contribution of flow to QS induction on spatially defined patches of biofilm. These experiments, presented in chapter 3, take advantage of the technique developed as part of this thesis for controlling and monitoring biofilms in microfluidic devices, including their spatial arrangement on a solid surface. Although QS plays an important role in biofilm formation and maturation \(^6\), the methods currently available for creating and studying biofilms do not provide reproducible, spatially controllable biofilms for comparative analysis to study the chemical, physical, and environmental factors that influence biofilm development in a statistically relevant experimental format. The major advantage of the technique that I present and apply in this thesis is that it allows placement hydrophobic chemical groups with a well-defined structure on a solid surface, enabling control over the amount of adsorbed cells via the density of these hydrophobic groups.
1.4 Microfabrication

Methods used to study biofilms should ideally enable tight control of physical conditions. Traditional approaches based on 96- or 384-well plates are only suitable for studying biofilm development in static conditions. However, the environment that microbes inhabit is anything but static. Microorganisms are exposed to a range of flow environments, from creeping flow in soil, to variable flow conditions in rivers and streams, to the strong shear experienced in stirred bioreactors and industrial pipelines. The presence of flow not only affects how microorganisms are transported and dispersed, but also their ability to interact with their local habitat, including attaching and detaching from surfaces, casting chemical signals for chemical communication, and resisting penetration by antibiotics.

Microfabrication is a flexible and powerful approach to create and control microenvironments in which cell behavior can be studied. The ability to culture cells in microfluidic systems offers numerous advantages over traditional benchtop-scale technologies. Bioreactors used for conventional cell culture typically operate in a batch or semi-batch mode that requires frequent media changes and the use of mechanical stirring to enhance mass transfer. These processes introduce mechanical stresses and steep concentration gradients that deviate significantly from those encountered in natural microbial habitats. Consequently, the cellular microenvironment is
either severely disrupted or completely unmaintainable, altering the observed growth and behavior in culture.

Since flow and nutrients transport is precisely controllable in a microfluidic system, cells are exposed to an environment that, for many aspects, more closely mimics their natural one. Furthermore, the optical access enabled by the transparency of microfluidic devices means that single cells or collections of cells can be directly observed microscopically as they respond to stimuli under conditions that are more consistent with actual physiological settings. Microsystems also offer the capability of performing studies in a highly parallel manner by incorporating a few to several dozens of cell culture chambers within a single chip.

In this work, I used microfabrication to produce experimental systems that allowed me (i) spatial control over biofilm formation; (ii) accurate control over fluid flow rates and nutrient delivery rates; (iii) control over environmental insults, such as air plugs; (iv) the direct monitoring of biofilm dynamics through automated imaging and image analysis, producing unprecedented amounts of data on the spatial and temporal dynamics of biofilms; and (v) simultaneous, parallel experiments to include on-chip controls as well as on-chip variation of relevant parameters. This work, this, contributes to establish microfabrication and microfluidics as an ideal tool for biofilm research.
1.5 μContact Printing for Biofilm Research

Most bacteria attach to surfaces in spatially heterogeneous or ‘patchy’ patterns, because distributions of hydrodynamic shear on surfaces and important chemical constituents are heterogeneous in natural systems, and because of natural variability in the time of colonization of different areas on a surface. Therefore, it would be desirable to study properties (e.g., quorum sensing, detachment) of biofilms as a function of their spatial structure, and thus to be able to control and manipulate this spatial structure.

Many approaches to bacterial patterning have been reported, including dip-pen lithography, inkjet printing, photolithography, and spotting. Although spotting, photolithography, and inkjet printing can create micropatterns of cells and dip-pen lithography is suitable for fabricating nanopatterns, these methods depend heavily on specialized microfabrication facilities and, as a result, have been used very little to address fundamental questions about biofilm dynamics. Also, these robot-controlled printing methods are serial and require long processing that is undesirable to prepare single-use, disposable devices for flexible experimentation. Microcontact printing (μCP) provides an alternative method, which affords great flexibility to readily alter the size and shape of features that control cell adhesion, density and geometry.

Pioneering work in μCP was performed by Whitesides’ group (Harvard University) for the
patterned transfer of thiols onto gold surfaces by means of a microstructured poly(dimethylsiloxane) (PDMS) stamp. The process of μCP is schematically depicted in Fig. 1-3 (left-hand side). PDMS is the material most frequently used to make stamps, as a slab of polymer that bears a microscale relief pattern on one side, since it can be molded using a master. PDMS is flexible enough to make conformal contact for μCP even with rough surfaces but still has enough mechanical stiffness to reproduce patterns in the micrometer range. The PDMS stamp is “inked” and put in contact with the substrate surface. Ideally, the ink is transferred from stamp to substrate only in the area of contact. The successful application of μCP of chemicals from a microstamp onto a surface is dependent upon the time required to progress from drying the stamp to printing onto substrates, and upon the property of the immobilizing surfaces. Insufficient pressure might result in poor printing and excessive pressure will induce sagging of the stamp, causing chemicals to print outside of the features.

Microcontact printing to produce spatially controlled biofilm patches

As part of the work for this thesis, I created bacterial biofilm patches within microfluidic channels by patterning hydrophobic substrate patches within the channels onto which biofilm-forming bacteria preferentially attached. I successfully created arbitrarily-shaped hydrophobic patches on a glass surface encased within a PDMS microfluidic chamber. Fig. 1-3 (left-hand side)
shows the schematic of the process. This surface treatment has been obtained through micro-contact printing (μCP) of organosiloxane (R-Si-Xₙ, where X is a hydrolysable group such as halogen, alkoxy, acyloxy, or amine) self-assembled monolayers (SAMs) onto glass substrates, by using a PDMS stamp. Specifically, the organosiloxane I found to be optimal for this procedure is octadecyltrichlorosilane (OTS), which is an amphiphilic molecule consisting of a long-chain alky group (C₁₈H₃₇⁻) and a polar head group (SiCl₃⁻), because it can be easily covalently attached to the microscopic glass slide and it has excellent stability and resistance to high shear flow and ambient light. The OTS-modified surfaces were found to be appropriate for the task since they have a –CH₃ group that makes them hydrophobic and hydrophobic surfaces typically favor bacterial attachment.⁷⁸,⁷⁹

Two layers of testing have demonstrated the viability of this method to produce biofilm patches. First, I have tested the performance of the microstamping process by assessing the presence and integrity of OTS patches using bovin serum albumin (BSA) labeled with fluorescein isothiocyanate (FITC-BSA), as shown in Fig.1-3 (right-hand side). The epifluorescence images of FITC-BSA adsorption indicate that the non-specific protein adsorption can be attributed to the hydrophobic interactions between the OTS-modified regions and the hydrophobic portions of BSA molecules, which are commonly accepted as the dominant driving
force for adsorption between the substrate and proteins.

Figure 1-3 (Left) Schematic of the microcontact printing (µCP) technique used for depositing hydrophobic patches onto glass substrates. (a) Wet inking is achieved by placing a patterned PDMS stamp onto the OTS ink pad. (b) After inking, the stamp is left in a chemical hood for 2 min to let the solvent (hexane) evaporate. (c) By bringing the inked stamp in conformal contact with the glass surface, the hydrophobic long-chain (C-18) silane is transferred to the glass surface. Due to the patterned structure of the stamp, only the areas with protrusions contact the glass surface, and the silane is area-selectively transferred according to the pattern of the stamp. (d) The microfluidic channel is positioned on top of the patterned substrate. (Right) Fluorescent images of FITC-labeled bovin serum albumin (BSA) absorbed onto the hydrophobic patches, demonstrating the specificity of the µCP technique. The top row shows squares of 20 µm and 50 µm size. The bottom row shows lines of 100 µm thickness and the MIT logo.

Second, I have tested the ability of this method to create well-defined biofilm patches by flowing a suspension of Pseudomonas aeruginosa through the microchannel and determining the spatial distribution of adherent cells. Results show that this technique allows the creation of
patches of biofilm of different shape and size, with high specificity (Fig. 1-4), in that there is strong preference for cells to attach within, but not outside, the patches. Furthermore, the degree of cell adhesion can be controlled by varying the surface wettability, through control over contact printing time of OTS.

Cells preferentially adhere to the hydrophobic patches, via hydrophobic interactions between the bacterial cell wall and the SAM, and the degree of cell adhesion can be controlled by varying the surface wettability. Since wettability and surface chemistry are supposed to be the key parameters that control the adhesion of bacteria, I investigated various alkylsilane self-assembled monolayer inks to find the best candidate for stamping hydrophobic patterns onto glass substrates. I have achieved the best selectivity in the adhesion of bacteria on patches by using octadecyltrichlorosilane (OTS), and found that thin layers of this compound possess high chemical and mechanical stability under shear stress, even when the latter is strong. Thus, using the technique described here, it is possible to create biofilm patches with defined size and shape, without the chemical substrate being washed away by flow. Furthermore, this approach seamlessly allows replication of patterns in arbitrary arrangements and in large numbers over a surface (Fig. 1-4), without any additional effort beyond the initial computer-based design of the stamp.
Figure 1-4 Phase-contrast images of *P. aeruginosa* PA01 selectively attached on hydrophobic patches of different shapes and sizes. The top row shows squares and the bottom row shows vertical lanes of biofilm. Note the strong difference in the density of attached cells between the patches and the regions of the surface outside the patches, demonstrating the specificity achieved by this patterning process.

Since this approach creates reproducible biofilm patches, it overcomes a limitation of traditional methods in which biofilms are typically grown from cells that have adsorbed to surfaces through a process that is largely stochastic and nearly impossible to control or reproduce. Thus, the patterning of bacteria on surfaces described here provides a unique opportunity to study biofilm formation in controlled, spatially heterogeneous environments. This technique has allowed me to create spatially controlled, functional surfaces that capture and localize cells.
within selected regions, while limiting nonspecific adsorption of cells to adjacent, ‘background’ regions.

1.6 Motivation and Thesis Scope

The motivation of this research is to contribute to a better understanding of bacteria attached to surfaces, in view of their importance in a broad range of settings and in particular of their heightened antibiotic resistance compared to planktonic cells. In nature, microbial biofilms are characterized by strong heterogeneity and are continuously subject to fluid flow and the resulting environmental insults (e.g., detaching shear forces). This heterogeneity ranges from the physiological differences between each of the myriad of cells in the biofilm, to the individual colonies of the same species or of different species that make up the biofilm, to the variability imposed by the topography of the supporting surface. Nevertheless, biofilm studies often assume that environmental conditions are homogeneous, as this small-scale spatial variability is difficult to model and control in a laboratory setting. Thus, the effect of microscale heterogeneity on the development and stability of biofilms is largely unknown. Fluid flow is similarly ubiquitous in biofilm environments, because completely quiescent environments are exceedingly rare, and even relatively slow fluid motion at the macroscale can generate strong velocity gradients (shear)
at the microscale. The resulting hydrodynamic shear, coupled with the inherent variability within the biofilm (e.g., as will be seen, the local strength of adhesion), can determine the detachment dynamics of the biofilm from the surface.

In this thesis, I investigate the role of heterogeneity and of fluid flow on bacterial biofilms. To address these questions, I devised a new microfluidic technique (sections 1.5 and 2.4) to precisely control spatial biofilm structure and hydrodynamic conditions. With this system in hand, I have investigated the effect of patch size and fluid flow on biofilm dynamics in *Pseudomonas aeruginosa* PA01 – an opportunistic pathogen in humans and a model organism for biofilm studies – including the susceptibility to disruption by shear (Chapter 2), and the ability to perform quorum sensing (Chapter 3).

In Chapter 2, I demonstrate a novel mode of detachment of a biofilm from a surface. After the passage of an air plug, the break-up of the residual thin liquid film scrapes and rearranges bacteria on the surface, such that a ‘Swiss cheese’ pattern of holes is left in the residual biofilm. I demonstrate that this pattern results from the competition between hydrodynamic forces from shear, tending to dislodge cells, and adhesion forces originations from the local amount of EPS produced by the cells. This new detachment mode needs to be considered when shear-based removal strategies are designed, as biofilm removal might be confused with biofilm
rearrangement, and persisting biofilm strongholds could rapidly seed biofilm regrowth, rendering treatment vain.

In Chapter 3, I examine the effects of biofilm colony size and ambient fluid flow on the QS induction in a developing *Pseudomonas aeruginosa* biofilm. I hypothesize that as the fluid flow rate over a biofilm increases, the amount of biofilm biomass required for the induction of QS within the population also increases. I found that quorum sensing sets in earlier in smaller biofilm patches, yet its intensity at long times is greater in larger patches. The effect of ambient fluid flow was to accelerate the induction of quorum sensing compared to static conditions at moderate flow rates, due to the increase in the convective supply of nutrients and to quench quorum sensing at high flow rates, due to the autoinducer signal being washed out by flow. These findings establish microfluidics as a new tool in the study of biofilms, which enables both accurate control over microenvironmental conditions and direct observation of the dynamics of biofilm.

Heterogeneity is a broad feature of microbial populations, and can have multiple consequences on microbial processes beyond those specific to biofilms. As a first attempt to characterize the trophic consequences of heterogeneity, in Chapter 4 I present a spatially explicit mathematical model to better understand how heterogeneity affects nutrient transfer across different trophic
levels. I find that chemotaxis accelerates nutrient transfer when chemotactic sensitivity exceeds a threshold, as otherwise random motility acts to slow nutrient transfer. I find that nutrient transfer is enhanced by chemotaxis by up to 10-fold, and that chemotaxis can increase patchiness within the microbial community. While in its current implementation the model is only conceptually linked to heterogeneity in biofilms, as the model focuses specifically on planktonic communities, this chapter broadly addresses the role of heterogeneity on the interactions between different populations of microbes, focusing on predator-prey interactions. It is hoped that, in the future, models of this kind could provide guidance also for the analysis of heterogeneity on biofilm dynamics.
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Chapter 2  Disruption of biofilm patches by an air plug reveals microscale heterogeneity

Bacteria often adhere to surfaces, where they form communities known as biofilms that display high resistance to both chemical and mechanical insults. Despite the importance of biofouling in health and ecology, the mechanisms governing biofilm development remain poorly understood, particularly for what concerns biofilm disruption by mechanical forces. Here we present experiments with surface-treated microfluidic devices that reveal a new mode of biofilm disruption by fluid flow. We show that, after the passage of an air plug, the break-up of the residual thin liquid layer scrapes and rearranges bacteria on the surface, resulting in a characteristic, semi-regular pattern of holes in the biofilm. We demonstrate that this pattern depends on the local biofilm age and correlates with the spatial distribution of the extracellular polymeric matrix, revealing that biofilm detachment can be highly heterogeneous and is governed by the microscale variability in adhesion strength. Because few survivors suffice to regrow a biofilm, these results point at the importance of considering microscale heterogeneity when designing and assessing the effectiveness of biofilm removal strategies by mechanical forces.
2.1 Introduction

Biofilms are surface-associated microbial communities encased in a self-secreted matrix of extracellular polymeric substances (EPS). Biofilms account for the largest fraction of bacterial biomass on the planet, and often have deleterious effects in natural, industrial and medical settings. In the environment, biofilms can mobilize heavy metals such as mercury and arsenic causing stream or soil contamination, and create oxygen depletion zones in lakes, rivers and coastal habitats. In industrial processes, the formation of biofilms is responsible for huge economic losses (in the billions of dollars yearly in the US alone) resulting from biofouling and biocorrosion, which leads to equipment clogging and damage, and product contamination. In medicine, biofilms represent the major source of infections associated with catheters and implanted devices. Despite the importance of finding effective methods for biofilm removal in these and other applications, our understanding of biofilm development and in particular of the mechanisms responsible for biofilm detachment remains far from complete.

Detachment refers to the release of bacterial cells from the surface-associated biofilm into the bulk fluid. Several factors can contribute to detachment, including matrix-degrading enzymes, nutrient levels and microbial growth status, fluid shear stress and quorum-sensing signals. Mechanical forces associated with fluid flow have long been used to detach biofilms from surfaces, with a prominent example being potential, effective approach to remove biofilms.
from surfaces involves the passage of bubbles or air plugs over the surface \(^1\). Bubbles pluck bacteria off a surface when the three-phase line (separating the liquid, the air and the solid surface) contacts the bacteria \(^2\). By producing high shear stresses, traveling air-liquid interfaces generate large detachment forces in a broad range of environments, including in the oral cavity during eating, speaking, drinking and swallowing \(^3\), on the eye and on contact lenses during blinking \(^4\), and on rocks and ship hulls in aquatic systems \(^5\). However, previous research on biofilm detachment by air plugs has focused on endpoint measurements to quantify the net amount of biofilm removed, whereas the mode of biofilm disruption has remained unexplored.

Here we show that, for early-stage biofilms (i.e., when bacterial colonies are essentially organized as monolayers), insult by mechanical forces results in a new phenomenon, whereby the passage of an air plug opens regular holes in the biofilm but fails to completely remove it. We rationalize this finding in terms of the competition between dislodging shear forces and the spatially varying adhesion strength resulting from intrinsic heterogeneity in the age and matrix production of different areas within a biofilm.
2.2 Results and Discussion

We studied the formation and disruption of controlled Pseudomonas aeruginosa PA01 biofilm patches on the glass bottom of a microfluidic channel (Fig. 2-1 a).

(a)

![Diagram](image)

(b)

![Images](image)

Figure 2-1 An air plug creates a characteristic pattern of holes in a biofilm. (a) Schematic of the microfluidic setup, showing the geometry of the microchannel and the experimental method. Pseudomonas aeruginosa bacteria preferentially attached to the hydrophobic patches. After that a certain growth time (4 h, 8 h, or 12 h), a controlled air plug was injected in the channel at the mean flow speed of 250 μm/s. (b) Residual biofilm after the passage of the air plug, showing the semi-regular hole pattern for different patch sizes (from left to right: 400, 300, 200 and 100 μm). The biofilm growth time was 8 h. Scale bar = 100 μm.
Biofilm patches formed by the preferential adhesion of *P. aeruginosa* cells to hydrophobic square patches, previously created on the glass by microcontact printing (μCP; 18-20; Fig. 2-2; Methods).

Figure 2-2 Schematic of the microcontact printing (μCP) technique used for depositing hydrophobic patches onto glass substrates.

A dilute bacterial suspension (optical density OD$_{600}$ ~ 0.2) was injected in the channel and incubated under quiescent conditions for 1 h, allowing cells to attach to the channel’s surfaces.
Then, the bacterial suspension was replaced by a minimal culture medium (M63), which was flown continuously at 3 μl/min (average flow velocity = 250 μm/s) to supply adhering cells with nutrients. Over the course of a few hours, *P. aeruginosa* cells progressively covered the surface of the hydrophobic patches. While some bacteria attached to the surface outside of the patches, most bacteria adhered onto the patches (Fig. 2-3 a,b,c, ), where cell adhesion is greatly favored by the substrate's strong hydrophobicity 21,22. The concentration of adhering cells could be controlled by varying the concentration of the chemical (OTS; see Methods) used in printing the patches (Fig. 2-4).

To determine the effect of a mechanical insult on an early-stage biofilm, biofilm patches were exposed to the controlled passage of an air plug after 8 h of growth. The air plug was created by rapidly switching injection from media to air, creating a 2.5 mm long air plug traveling at 250 μm/s (Methods). The air plug traveled over each patch in less than 10 s, causing a dramatic and highly characteristic disruption of the original biofilm patch: the resulting biofilms were in the shape of a semi-regular pattern of holes, from which bacteria had been entirely removed, separated by 'bacterial levees', consisting of a concentrated monolayer of cells (Fig. 2-1 b).
Figure 2-3 The effect of the air plug strongly depends on biofilm age. (a-f) Biofilms grown for different times (4 h, 8 h, 12 h), shown before (a-c) and after (d-f) the passage of an air plug. The air plug traveled from left to right. (g) Surface coverage (fraction of the surface of a patch covered by bacteria) before and after the passage of an air plug, for biofilms of different age (grayscale bars). The red curve shows the normalized change in surface coverage. (h) Free surface distance between cells (grayscale bars) and fractal dimension of the cell distribution (red symbols) measured before and after the passage of an air plug, for biofilms of different age.
Figure 2-4 *P. aeruginosa* adsorption on the OTS step-wise gradient. The contact-printing time of OTS is gradually increased from inner out. The color plot (top figure) was generated using the value of fluorescence intensity over the distance (bottom figure). The higher intensity indicates the higher adsorption density of *P. aeruginosa*.

Image analysis showed that holes, which varied in shape but displayed no obvious asymmetry associated with the flow direction, had an equivalent radius of 6.5 ~ 8 μm and the porosity of the end-state biofilm was ~68%. This pattern was highly consistent among different patches within the same microchannel and among replicate experiments (Fig. 2-5). Experiments with patches of different size (100, 200, 300 and 400 μm squares) showed that the pattern and its porosity were independent of patch size within the range tested (Fig. 2-5).
Figure 2-5 Porosity and equivalent hole radius on the size of patches. The top four panels correspond to the size of patches.

To confirm that the holes in the biofilm resulted from the detachment of bacteria, rather than the detachment of the underlying OTS layer, we scanned the ruptured biofilms using atomic force microscopy (AFM). Results showed that the height of the surface inside the holes was greater than the uncoated glass surface outside the patches by an amount corresponding to the original thickness of the OTS layer (Fig. 2-6), indicating that cell detachment – not OTS detachment – was responsible for the hole patterns. In addition, chemical analysis of the surface using scanning electron microscopy coupled with energy dispersive X-ray spectroscopy (SEM/EDX) showed that the OTS coating is stable even under high shear flows (Fig. 2-7).
Figure 2-6 AFM characterization of a ruptured *P. aeruginosa* biofilm surface. (a) The residual biofilm is shown in yellow, the region from which bacteria have detached is in brown. (b) The height profile of the surfaces along the white line in panel (a). The red and green arrowheads correspond to the same symbols in panel (a). Note the different height of the surface in the hole (left green arrow) and on the glass surface (right green arrow), showing that OTS coating in the hole has not detached.
Figure 2-7 (a) Secondary electron micrographs with spot EDX analysis of *P. aeruginosa* biofilm patches. EDX is an analytical technique used for the chemical characterization of a sample. Briefly, the interaction between X-ray excitation and the sample surface is used to determine the surface composition. Carbon-containing substances are dominant in the detached regions of cell patches (a and b, top plot), whereas silicon-containing substances are dominant in the uncoated glass regions (a and b, bottom plot). Since the biofilm holes do not show abundant Si, it infers that the regions are still covered by OTS or by cells.

The adhesion properties of an early-stage biofilm are strongly dependent on the biofilm growth time. This is clearly revealed by comparing experiments in which the air plug was injected after 4, 8 and 12 h of biofilm growth, respectively (Fig. 2-3). For 4 h old biofilms, the air plug reduced the surface coverage within patches by nearly two thirds, from 32% to 13%. For 8 h old biofilms, the reduction in surface coverage caused by the air plug was considerably smaller.
(from 45% to 39%). For 12 h old biofilms, the reduction was essentially negligible (from 49% to 47%) and the air plug produced no visible change of the biofilm structure.

This trend is supported by an analysis of the mean distance between individual cells in the biofilm before and after the air plug passed. Imaging at 40X allowed us to resolve individual cells in the biofilm monolayer. The mean distance between cells was computed as

$$L = \frac{\sum_{i=1}^{N} \sqrt{\frac{4A_i}{\pi}}}{N}$$

after having measured the area $A_i$ of each hole, for all $N$ holes in a patch, via image analysis (Methods). This analysis revealed that the mean distance between cells changed considerably upon passage of the air plug for the younger biofilms: for 4 h and 8 h old biofilms, $L$ increased from 4.1 μm to 7.9 μm and from 3.1 μm to 5.8 μm, respectively. In contrast, $L$ remained nearly unchanged for the 12 h biofilm (from 2.1 μm to 2.3 μm). Taken together, these measurements indicate that an air plug largely removes 4 h old biofilms, primarily rearranges cells in 8 h old biofilms without detaching them, and has little effect on 12 h old biofilms. The rearrangement of the 8 h old biofilm is evident from the imaging (Fig. 2-3e) and is further supported by a considerable change in the fractal dimension of the cell distribution, from 1.92 to 1.68.
Figure 2-8 Biofilm holes and hole formation dynamics. (a) Close-up view (60X objective) of the bacterial distribution at the edge of a 400 x 400 μm² biofilm patch after the passage of an air plug. The dotted line denotes the edge of patch. (b) Zoomed-in view of the dynamics of hole formation at 1 s intervals. The boundaries of three holes are identified with red, blue and yellow at each of the four time points. (c) Time course of the hole area, for the three holes shown in (b). Black symbols denote the average of the three measurements.
Imaging of the hole formation dynamics at high temporal resolution (50 frames/s) revealed that the hole pattern resulted from the rupturing of the residual thin liquid film between the channel surface and the air plug at discrete locations. The ensuing movement of the contact line scraped bacteria outwards from the holes, to form ‘bacterial levees’ between adjacent holes (Fig. 2-8).

An air plug traveling over a solid surface remains separated from it by a thin layer of liquid, whose thickness depends on the capillary number, \( Ca = \mu U/\sigma \), which measures the relative importance of viscous forces and capillary forces. Here, \( \mu \) is the dynamic viscosity of the liquid, \( U \) is the air plug velocity, and \( \sigma \) is the interfacial tension between liquid and air. In our experiments, the air plug traveled at \( U = 250 \, \mu \text{m/s} \) in water \((\mu = 10^{-3} \, \text{Pa s}; \sigma = 72 \times 10^{-3} \, \text{N/m})\), resulting in \( Ca = 3.5 \times 10^{-6} \). For \( Ca \ll 1 \), the liquid film thickness, \( h \), follows Bretherton’s law\(^{22,24} \), \( h/H = Ca^{2/3} \), where \( H = 50 \, \mu \text{m} \) is the microchannel’s height\(^{24} \). This results in an estimated liquid film thickness of \(< 0.1 \, \mu \text{m} \). Thus, the air plug would create, on a flat surface, a liquid film that is thinner than the thickness of the bacteria \((\sim 1 \, \mu \text{m})\). This suggests that the uneven carpet of surface-adhering bacteria, over which the liquid film must travel, represents a collection of comparatively tall obstacles for the advancing liquid film, providing a mechanism for its rupture.
Calculations show that the rupture of the thin film is consistent with an evaporation-driven instability. An evaporation rate of $5 \times 10^{-5}$ Kg/m$^2$ s$^{-1}$, determined assuming room temperature and 50% relative humidity (RH) $^{25}$, indicates that the 0.1 μm thick water film evaporates in ~2 s. A sensitivity analysis on the relative humidity, RH, which is unfortunately unknown in our experiments, reveals that our conclusions are robust for even large variations in RH. For RH values ranging from 10% to 90%, the evaporation rate ranges from $10^{-5}$ to $10^{-4}$ Kg/m$^2$ s$^{-1}$, which gives an evaporation time of 1s to 4s for a 0.1 μm thick water film $^{26}$. These times are in all cases smaller than the ~10 s passage time of the air bubble, indicating that, irrespective of the precise value of the RH in the air bubble, the proposed mechanism allows the evaporation of the entire film over the time of the bubble's passage. Thus, over the 10 s that the air plug resides over a given point on the surface (the plug’s length divided by its speed), the thin film can completely evaporate. Its evaporation timescale is consistent with the dynamics of hole opening (Fig. 2-8b) and indicates that evaporation can rapidly thin the liquid layer, leading to the deformation of its free surface in the voids between bacteria and to its ultimate rupture. The resulting three-phase contact line moves radially outward form the point of rupture to minimize surface energy. This process is akin to “confined dewetting lithography”, where the thinning and rupture of thin liquid film is used to arrange surface-residing colloidal particles into defined patterns $^{26}$. Because
bacteria are thicker than the liquid film, they protrude above the air-liquid interface of the
dewetting hole’s receding contact line: the resulting capillary force can dislodge individual cells,
which are transported outwards by the receding contact line until the latter becomes pinned when
the accumulated bacteria form a levee between the opening hole and an adjacent hole (Fig. 2-8b).
The key role of evaporation in this process is supported by the observation that the hole’s area
grows linearly with time (Fig 2-8c). This quasi-linear dependence of the hole area on time is
broadly consistent with a role of evaporation in driving the rupture. This mechanism works at
intermediate adhesion strengths (8 h old biofilm), whereas for younger biofilms cells are swept
away and for older biofilms the capillary force is insufficient to scrape cells across the surface.

While microorganisms maintain their individual functioning during biofilm development, EPS
formation initiates irreversible attachment. The EPS matrix provides several functions for
the biofilm, such as protecting bacteria from environmental threats, providing mechanical
stability, and degrading macromolecules to be used by the cells. Some reports indicate that
EPS content is not associated with biofilm adhesion strength. This apparent discrepancy is
likely due to the complex dependency of detachment on temperature, nutrient availability,
hydrodynamics, and presence of chemical toxins. Alternation in extracellular components
during biofilm development can have a potential effect on biofilm detachment.
A correlation analysis shows that the spatio-temporal distribution of EPS is the primary determinant of the local strength of adhesion, strongly suggesting that it is therefore responsible for the observed heterogeneous patterns. To quantify the distribution of EPS, we injected fluorescently labeled lectins (wheat germ agglutinin (WGA) conjugated with tetramethyl rhodamine isothiocyanate (TRITC); \(^{32}\)) in the microchannel at different times of the biofilm's growth (2 h, 4 h, 6 h and 8 h). Interestingly, although the distribution of cells in each patch is rather uniform (Fig. 2-9a), the distribution of EPS before passage of the air plug is heterogeneous (Fig. 2-9c), likely reflecting intra-population variability in EPS production and different surface colonization times, in line with recent discoveries about the role of EPS in guiding the formation of micro-colonies \(^{33}\). Furthermore, there is a significant correlation between the pre-air-plug EPS distribution and the post-air-plug cell distribution, and this correlation is 5-fold larger than the correlation between the pre-air-plug EPS distribution and the pre-air-plug cell distribution (Fig. 2-9e-f). Furthermore, we found that the passage of the air plug had a negligible effect on the distribution of EPS on the surface (Fig. 2-9d), which further confirms the role of the EPS in protecting biofilm-forming bacteria from mechanical forces.
Figure 2-9 The hole pattern correlates with the EPS distribution. (a-b) Distribution of fluorescently-labelled *P. aeruginosa* after 8 h of biofilm growth, immediately before (a) and immediately after (b) the passage of an air plug. (c-d) Distribution of EPS, fluorescently labeled using lectins (Methods), at the same times and position as (a-b). (e) Overlay of the EPS distribution before the air plug (red) and the bacteria distribution after the air plug (green). Data are from a different experiment than (a-d). (f) Normalized cross-correlation between the EPS distribution before the air plug and the cell distribution before (second bar from left) and after (fourth bar from left) the air plug. Error bars correspond to the standard error over three replicate experiments. The first and third bars represent results from a bootstrapping analysis (BS), where the EPS distribution before the air plug is correlated with random permutations of the cell distribution before (first bar) and after (third bar) the air plug. Note the lack of correlation in these controls.
These results indicate that holes in the biofilm structure formed where there was the least amount of EPS before the air plug arrived, and that cells from regions of low EPS concentration were scraped into the regions of highest EPS concentration, where they – together with the EPS – formed levees that prevented further hole expansion.

2.3 Conclusion

These findings may have significant impact and potential applications when shear-based removal strategies are designed, as biofilm removal might be confused with biofilm rearrangement. In the past, biofilm has been assumed to be homogeneous and biofilm models have been developed based on that assumption. However, the results presented in this paper show that the heterogeneous distribution of extracellular matrix play a crucial role in the detachment mode of biofilm under a mechanical insult or hydrodynamic shear. A recent study showed that *P. aeruginosa* secretes Psl during migration. The resulting Psl trails influence the motions of succeeding *P. aeruginosa* cells on the surface. Characteristic length in separated holes in structures showed ~10 μm at 5h, which is similar to our finding, 6.5–8μm hole radius after the bubble passage on 8h biofilm growth. Thus, the report supports that where more matrix is initially deposited, that is where the biofilm is strongest at the microscale, and those will be the strongholds that will resist mechanical insult, even acting as collection points for other bacteria.
This study demonstrates that high fluid shear can be effective in stimulating bacterial detachment. The addition of air bubble to the flow allows the detachment of tenaciously adhering bacteria not detached by flow alone. The principles outlined are expected to have general validity for bacterial detachment from surfaces by fluid flow and passing air bubble.

2.4 Materials and Methods

**Materials.** Octadecyltrichlorosilane (OTS) \([\text{CH}_3(\text{CH}_2)_{17}\text{SiCl}_3]\) (Aldrich, 97%), hexane (Aldrich, anhydrous, 99%), and fluorescein isothiocyanate-bovine serum albumin (FITC-BSA) and tetramethyl rhodamine isothiocyanate-wheat germ agglutinin (TRITC-WGA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polydimethylsiloxane (PDMS, Sylgard 184) was purchased from Dow Corning.

**Bacterial strain and growth condition.** The wild-type strain of *Pseudomonas aeruginosa* PA01 was used for this study (courtesy of George O'Toole, Dartmouth University). For the preparation of the cell culture, cells from freezer stocks were inoculated in LB medium (10 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone) at 30°C under shaking (150 rpm). Cells were resuspended in fresh LB medium and incubated at 37°C under shaking (180 rpm) up to \(\text{OD}_{600} = 0.2\), corresponding to early exponential phase. An aliquot of this cell suspension was injected for 1 h in the microfluidic channel. Thereafter, continuous injection of M63 minimal medium (100
mM of KH$_2$PO$_4$; 15 mM of (NH$_4$)$_2$SO$_4$; 1.8 uM of FeSO$_4$; 1.0 mM of MgSO$_4$) and 0.5% glucose at a constant volumetric flow rate of 3 µl/min began, and was maintained for 4, 8 or 12 hours, to produce biofilms of different stage of maturation. This flow rate corresponds to an average flow velocity of 250 µm/s and a shear rate at the bottom glass wall of ~30 s$^{-1}$.

**Air plug generation.** To introduce controlled air plugs in the microchannel, we used a three-way valve: one inlet for the bacteria solution (used for initial injection), one inlet for the bacteria-free M63 media (used for 4 h, 8 h or 12 h), and one inlet for atmospheric air. To control the velocity of the air plug, the valve and a syringe pump were connected to a microchannel inlet and outlet. By opening and closing the valve port, air plugs were introduced into the microchannel.

**PDMS stamp fabrication for surface patterning.** PDMS stamps were fabricated by curing the prepolymer on silicon masters patterned with SU-8 photoresist (SU-8 2050, MicroChem, MA, USA) using conventional photolithography. The masters used for patterning had recessing features, which resulted in PDMS replicas with protruding features. To assist in removal of cured PDMS from the masters, the SU-8 masters were silanized overnight by exposure to the vapor of 1,1,2,2-tetrahydrooctyl-1-trichlorosilane, CF$_3$(CF$_2$)$_6$(CH$_2$)$_2$SiCl$_3$. To cure the PDMS prepolymer, a mixture of 10:1 silicon elastomer and the curing agent was poured on the master and held at 65°C for 2 h. The PDMS replica was then peeled from the silicon master.
Generation of patterned hydrophobic coatings. Hydrophobic patterns of OTS on the glass substrate were made by using microcontact printing (μCP), as shown in Fig. 2-2. The PDMS stamp was inked with a 2 mM hexane solution of OTS and dried in air for 5 min, then placed in contact with the glass substrate at room temperature for 30 s. The stamp was carefully peeled off and the substrate was rinsed with 2-propanol (IPA) and DI, then dried. Because the trichlorosilane reagents are sensitive to the water content and temperature of the printing ambient, the relative humidity and the temperature of the room were kept constant at ~50-55% and ~22-24°C, respectively. Additional experiments showed that the concentration of OTS alters the adsorption density of *P. aeruginosa* (Fig. 2-3). The contact-printing time of OTS is gradually increased from inner out to generate the OTS step-wise gradient.

Microscopy. Conventional epifluorescence microscopy imaging was performed using an inverted microscope (Nikon TE-2000E) fitted with GFP and RFP filter sets. Images were acquired with 40x and 60x objectives and an Andor iXon CCD camera (50 frame / s) cooled to -65°C. Image analysis was performed using built-in plugins of the ImageJ software (http://rsbweb.nih.gov/ij/).
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Chapter 3  The Effect of Patch Size and Fluid Flow on Quorum Sensing in Biofilms

Biofilm forming bacteria are industrially and medically relevant organisms that are exceptionally resistant to a wide variety antimicrobial treatments. This resistance is due in part to a biofilm forming bacteria’s ability to sense and communicate with neighboring bacterial. As a result of this intercellular communication, i.e. Quorum Sensing (QS), bacteria are able to cooperate as a complex community. This communication system is used to modulate important facets of biofilm behavior and thus is an attractive target for biofilm control and potential antimicrobial agents. Understanding the significance of hydrodynamics in QS is crucial if we consider the habitats in which biofilms tend to form, where fluid flow is often prevalent.

3.1 Introduction

Quorum sensing (QS) is an important mechanism by which bacteria form and maintain complex biofilms through the ability to communicate and to alter behavior in response to the presence of other bacteria \(^{1-3}\). Bacterial cells produce signaling molecules that are secreted into the environment, where they disperse by diffusion or advection. In general, to sense their population density, gram negative bacteria use small chemical molecules called acylated homoserine
lactones (AHLs) and gram positive bacteria use oligo-peptides, both collectively referred to as autoinducers (AIs). Typically, an autoinducer molecule induces the transcription of a set of genes that includes the gene encoding the autoinducer-producing enzyme, which results in a positive feedback loop. The autoinducer can trigger a response by the cell if its concentration - regulated by the rates of production, decay and mass transfer integrated over time - reaches a threshold at the cell's location (Fig. 3-1).

**Figure 3-1** LasI/LasR-RhlI/RhlR quorum sensing system in *Pseudomonas aeruginosa*. The LasI protein produces the homoserine lactone signaling molecule N-(3-oxododecanoyl)-homoserine lactone, autoinducer1 (AI1, blue circles), and the RhlI protein synthesizes N-(butryl)-homoserine lactone, autoinducer2 (AI2, red triangles). Activated LasR (with AI1) activates transcription of rhlR, which means the las QS system controls RhlR at the transcriptional level. AI1 also controls the activity of RhlR at the post-translational level by binding to RhlR, which decreases AI2 binding to RhlR, therefore inhibiting expression of rhlA and other genes. Presumably, this action ensures that the LasI/LasR circuit is established prior to the establishment of the RhlI/RhlR circuit. The *Pseudomonas* quinolone signal (PQS) is an additional regulatory link between the Las and Rhl QS circuits.
The existence of a threshold cell concentration for the production of bioluminescence in the marine symbiotic bacteria *Vibrio fischeri*\(^7\), the formation of fruiting bodies in myxobacteria\(^8\) and the development of competence for genetic transformation in Streptococci\(^9\), are among the lines of evidence for cell-to-cell signaling systems in bacterial communities. At present, QS is defined as a cell-density-dependent bacterial intercellular signaling mechanism that enables bacteria to coordinate the expression of certain genes to coordinate group behaviors.

Several physical, biological, and chemical factors have the potential to influence QS in biofilm systems\(^10\). Among these, the hydrodynamic environment can have a significant influence on QS. Janakiraman et al.\(^11\) studied biofilm growth and QS in microfluidic chambers and with a one-dimensional mathematical model, at low Reynolds numbers (Re<<1). They found that the flow rate greatly affects QS: at high flow rates, the transport rate of AHL out of the biofilm can be so high that the AHL concentration does not reach the threshold required for induction. Purevdorj and Costerton\(^12\) emphasize that the washout of signal molecules from the bulk fluid surrounding bacterial microcolonies is expected to increase the concentration gradient between the biofilm and the bulk liquid, in turn driving a larger diffusive flux of signal molecules out of the biofilm. Muller et al.\(^13\) mathematically studied QS induction in biofilm patches. They found that induction can occur for a lower number of cells in a patchy biofilm than in a homogeneous
biofilm, because the cell density (and consequently, the AHL concentration) is higher in small cell clusters.

QS is gaining increasing interest as a promising treatment target, e.g., for pathogenic bacteria\(^{14}\), which raises the interest in understanding environmental controls on QS. QS may depend on the spatial properties of the environment. In particular, it has been recognized that not only a high population density but also a confined space favor QS\(^{15,16}\). This feature cannot be captured by most studies of QS, which focus on batch liquid cultures. Here we explore the effect of the size of the biofilm on QS.

Although QS plays an important role in biofilm formation and maturation\(^{17}\), the combined effect of hydrodynamics and spatial heterogeneity are still poorly understood, mostly due to experimental difficulties. We present here microfluidic experiments to investigate the effects of biofilm size and fluid flow on QS induction in *Pseudomonas aeruginosa* biofilms.

### 3.2 Results and Discussion

**Comparison of GFP intensity between static and flowing conditions**

We used three *P. aeruginosa* PA01 strains, which are wild-type (WT), PA01 carrying pSMC21 (constitutively expressing GFP), PA01 carrying pMH520 (only expressing GFP when QS system is activated. The model organism *P. aeruginosa* PA01 mini Tn5-rhlA::GFP (carrying pMH520)
excretes a variety of fluorescent compounds, including pyocyanin, pyoverdine, and pyorubin. Since the experiments presented here rely on the detection of GFP expression as a measure of QS, we needed to differentiate green fluorescence signals from the different sources in order to reliably use GFP expression as a measure of QS. Under non-flowing culture conditions, the wild-type PA01 strain (green triangles in Fig. 3-2a) shows considerable green fluorescence, with an intensity that is similar to that of a QS marker construct (orange circles), but weaker than the fluorescence displayed by PA01 pSMC21 (blue squares), which constitutively expresses GFP.

Conversely, when these strains are grown in a microchannel under flow conditions (mean speed = 250 μm/s), fluorescence intensities for both the wild-type PA01 strain and the QS marker construct are considerably weaker than the PA01 pSMC21 strain (Fig. 3-2c). These observations demonstrate that under the flow conditions used in the patch-biofilm experiments, pyocyanin and other green fluorescent compounds are washed away by flow and contribute negligibly to overall fluorescence intensity. Comparison of the static and flowing conditions allows us to conclude that the fluorescence measured under fluid flow in the microchannels can be attributed to and used as a metric of QS.
Figure 3-2 Comparison of fluorescent intensity under static (a, b) and flowing (c, d) conditions for three *P. aeruginosa* strains: PA01 mini Tn5-rhlA::GFP (QS marker; yellow symbols), PA01 wild-type (WT; green symbols), and PA01 pSMC21 (constitutively expressing GFP; blue symbols). Under static conditions, fluorescent intensities increase over time for all three strains, with the greatest increase expressed by the PA01 pSMC21 strain. This is shown both using quantitative fluorescence data (a) and visually in an assay plate (b). In contrast, under flowing condition, the fluorescence of PA01 pSMC21 was much higher and was expressed more rapidly than the other two strains, and PA01 WT displayed negligible fluorescence. This is shown via quantitative imaging (c) and visually in microscopic snapshots taken after 18, 42, and 45 h of biofilm growth in the microchannel.
The effect of patch size on QS

Fluorescence measurements were used as a metric for QS in biofilm patches of different size, monitored in microfluidic channels over time. Results are compared for 3 patch sizes in Figure 3-3, including square patches of 50, 150 and 400 μm side length. We found that bacteria in the smaller biofilm patches start QS earlier than those in the larger patches. However, at later times the QS signal (normalized by patch size) is higher in the larger patches.

Specifically, we observed that the smaller patches (50 and 150 μm) are the first to show the induction of QS, which first occurs at $t = 500$ min for the 50 μm patches and at $t = 600$ min for the 150 μm patches. At $t = 700$ min, the QS signal from the 150 μm patches overtakes the QS signal from the 50 μm patches, and remains larger until the end of the experiment at $t = 2000$ min. Meanwhile, the QS signal from the 400 μm patches has a considerably slower start, beginning only at $t = 1000$ min. Thereafter, however, the 400 μm patches exhibit the fastest growth in the QS signal. At $t = 1400$ min, the QS signal from the 400 patches overtakes the QS signal from the 50 μm patches. At $t = 1600$ min, the QS signal from the 400 patches overtakes also the QS signal from the 150 μm patches. Thereafter, the QS from the 400 μm patches remains the strongest among all three patch sizes, until the end of the experiment at $t = 2000$ min.
Figure 3-3 Quorum sensing signal (GFP fluorescent intensity) for square biofilm patches of different sizes, for a flow rate of 1 µl/min corresponding to a Peclet number of 8.4. The top panel shows the time series of the fluorescent intensity for each patch size, as the mean and standard deviation over 6 patches. The lower set of four panels shows the fluorescent intensity at four time points. In each of the lower panels, the upper row corresponds to the raw fluorescent data, and the lower row presents the same data in pseudo-coloring, to clarify differences. In the pseudo-coloring, purple to purple-blue tones correspond to weaker QS, whereas yellow to red tones correspond to stronger QS. The left column in each of the lower set of four panels corresponds to a patch size of 50 µm, the middle one to a patch size of 150 µm, and the right one to a patch size of 400 µm.
These observations reveal a clear and consistent pattern, never reported before, which applies to the comparison among any two of the three patch sizes tested. The smaller patch always begin to exhibit QS earlier, but is then overtaken in terms of the strength of the QS signal by the larger patch, and after this cross-over it is the larger patch that continues to exhibit the strongest QS signal for the entire duration of the observations.

That larger patches exhibit stronger QS at long times corresponds to one’s intuitive understanding of QS, which is favored by higher cell numbers in the same region. In virtue of their larger surface area, the larger patches provide greater real estate for cell growth, hence for a greater concentration of QS molecules. What is not intuitive and, to the best of our knowledge has not been reported previously, is the dominance of smaller patches in terms of QS at earlier times. This transient dominance of smaller patches represents a new feature of QS and highlights the importance of taking explicitly into account the size of a biofilm when investigating QS. In the paragraphs that follow, we present a conceptual model that, while not entirely verifiable with the data at hand, provides a reasonable framework with predictions that are in line with the unique cross-over we have observed.

The initial cell densities are highly comparable among all patch sizes. The vertical confinement, represented by the depth of the microchannel (50 μm) is also the same for all patch
sizes. The main difference between the different patch sizes, then, is the higher ratio between perimeter and surface area of the smaller patch sizes. This varies from $200 \, \mu m / 50^2 \, \mu m^2 = 0.08 \, \mu m^{-1}$ for the $50 \, \mu m$ patches, to $0.027 \, \mu m^{-1}$ for the $150 \, \mu m$ patches, to $0.01 \, \mu m^{-1}$ for the $400 \, \mu m$ patches, an 8-fold variation overall. Because the vertical dimension is equal or smaller than the horizontal extent of the patches, this suggests that cells in the smaller patches are on average more readily supplied by nutrients in the surface-parallel (x,y) direction, and that cells in the largest patches are on average (and particularly at the center of the patch) limited by nutrient supply. This argument suggests that biofilms in the smaller patches should grow faster than biofilms in the larger patches. While this conflicts with early-time measurements of biofilm surface coverage, these early times are more likely to be dominated by vertical nutrient supply, thus not showing the predicted nutrient limitation. Unfortunately, we lack data on biofilm biomass at later times to corroborate this assumption.

A closely related same argument allows us to speculate on the reason for the stronger QS signal in the larger patches at later times. The argument is again based on the differential magnitude of lateral (i.e., surface-parallel) transport, only in this case the transport of the autoinducer molecules. Because of the higher perimeter to surface area ratio, we speculate that the autoinducer molecules produced by the small patches more readily escape from the region.
directly above the cells, whereas they remain more ‘trapped’ in the case of the larger patches. Together, these two arguments – based on a single mechanism applied to the two solutes that affect QS, i.e., nutrients and autoinducer molecules – indicate that smaller patches are less limited by nutrient supply at early times, thus growing faster and expressing QS earlier, whereas larger patches grow more slowly, but the autoinducers that they express remain more strongly localized, thus leading to stronger QS at later times. The differential perimeter to surface area ratio, then, can explain the crossover between the QS signals for different patch sizes observed in the experiments. We envisage that a mathematical model of QS and mass transport would confirm this prediction, but this model is beyond the scope of this thesis.

The effect of fluid flow on QS

Here we investigate the effect of fluid flow on QS. Relative to static conditions, flow has two primary roles: (1) it contributes to biomass growth by increasing the convective supply of nutrients, which enables increased cell growth and thus accelerates the induction of QS; and (2) it contributes to AI mass transfer. Convective transport of AI molecules can affect the onset of QS through the following: (1) AI molecules that are produced in a biofilm colony and diffuse from the colony into the aqueous phase are transported downstream (i.e., locally removed),
which delays local up-regulation of QS; (2) downstream colonies receive Al molecules from those upstream, which enhances up-regulation of QS in downstream colonies.

The effect of fluid flow on the transport of Al is best quantified in comparison to the transport of Al by diffusion alone, i.e., the no-flow case. This comparison is captured by a dimensionless parameter called the Peclet number, defined as $Pe = \frac{UL}{D}$, where $U$ is a characteristic fluid velocity (here taken to be the mean speed of the flow), $L$ is a characteristic length scale (here taken to be the depth of the microchannel), and $D$ is the molecular diffusivity of the Al. For Al in water, $D_{Al} \approx 4.9 \times 10^{-10} \, \text{m}^2/\text{s}$ $^{10}$. Here we performed experiments over a range of values of the Peclet number, $Pe = 8.4, 25.5, 102.0$, capturing a broad range of scenarios, from one in which transport by fluid flow is predicted to be of comparable importance to transport by diffusion, to two cases ($Pe = 25.5, 102.0$) in which flow is predicted to trump diffusion.

To study the effects of flow on QS, time series of fluorescent intensity were recorded within biofilm patches for different flow velocities, corresponding to different Peclet numbers (Fig. 3-4).

This was done for two different patch sizes. Panel a in Figure 3-4 corresponds to the case analyzed above (Fig. 3-3, top panel), and the discussion here centers around differences compared to the previous case.
Figure 3-4 Time series of QS intensity for different flow rates. The intensity of QS was measured as the GFP fluorescence intensity, reported here as a percentage of QS induction. Flow rates corresponded to average flow velocities of (a) 83 μm/s, (b) 250 μm/s, and (c) 1,000 μm/s. In each graph, the Peclet number is given, which expresses the relative importance of transport by advection and by diffusion (see text). For each flow rate, two different patch sizes were investigated (150 μm, 400 μm). Data correspond to means and standard deviations over six biofilm patches, for each case. Panel a corresponds to the top panel in Figure 3-3. Panel d corresponds to the situation in which flow was stopped after the data in panel c (Pe = 102.0) were collected, and shows that QS resumed upon stopping the flow.

First, for a decrease in flow rate (Pe=8.4, Fig. 4a), the 150 μm patches exhibit a later onset of QS and the 400 μm patches also exhibit a later onset of QS compared to the case of Pe=25.5. The
two patch sizes, in this case, display onset of QS at around $t = 600$ min, $t = 1000$ min. Still, as before, the bigger patches ‘catch up’, and the QS signal from the 400 µm patches overtake that from the 150 µm patches at $t = 1600$ min, a cross-over time that is not substantially different from the Pe=25.5 case (Fig. 3-4b).

Second, for an increase in flow rate (Pe=102.0, Fig. 3-4c), none of the patches begins QS, and the fluorescent signal decays over time (no increase in fluorescent intensity). This likely indicates that the advective transport by fluid flow washed out the AI molecules, preventing them from accumulating to a sufficient degree to initiate QS. Stopping the flow, in this latter experiment, led to the induction of QS, confirming that a reversible process, such as the washout of the AI molecules, was responsible for the lack of QS at $Pe = 102.0$.

**Measurement of the QS intensity per unit biomass by confocal reflection microscopy**

The results presented here demonstrate the importance of hydrodynamics in modulating biofilm structure and QS expression. An important aspect that warrants further attention in this respect is the quantification of biomass in the biofilms, in particular to determine whether the observed QS results from increased biomass (e.g., due to higher nutrient supply) or from increased QS expression per unit biomass (e.g., due to higher accumulation of autoinducer molecules).
The biofilms' biomass can be estimated at early times from the area coverage using phase or bright-field microscopy. However, these methods are not suitable past the time at which the biofilm grows beyond a monolayer. For this reason, confocal microscopy techniques were used to determine the structure and biomass of the biofilms in three dimensions over time.

The technique presented here combines confocal reflection microscopy (CRM) and fluorescent confocal laser scanning microscopy (FCLSM). CRM is a variation of reflection microscopy that employs a confocal laser scanning microscope, permitting the visualization of three-dimensional samples without fixation and fluorescent labeling. This is a technique that has not been widely employed in biofilm research in recent times, but is very powerful for obtaining a quantitative measure of biomass in three dimensions, via a non-intrusive approach (no fixation, no labeling). For CRM, biofilms were illuminated with a 488 or 514 nm argon laser and the reflected light was collected through a 470-500 or 505-530 nm band pass filter, respectively, to avoid the influence of autoflourescence. Time-lapse observations of reflectance (CRM) and fluorescence (FCLSM) were performed simultaneously to examine the relation between biofilm biomass (CRM) and QS expression (FCLSM). Conveniently, illumination with a single laser generates both reflectance and fluorescence, which are quantified separately using band-pass filters. Measurements were acquired at 3 h intervals.
The biomass was quantified in terms of the biofilm's bio-volume. For this purpose, CRM images were analyzed using the COMSTAT software, which runs in MATLAB. Thresholding an image stack (which makes up the three-dimension CRM measurement of the biofilm at a given point in time) results in a three-dimensional matrix with a value of one in positions where the pixel value in the original image exceeds a threshold (presence of biofilm), and zero otherwise (absence of biofilm). The cumulative number of biomass pixels ('ones') in all images of a stack is multiplied by the voxel size (pixel size times vertical distance between images) and divided by the cross-sectional area of the image stack. The resulting value is the biofilm's volume divided by the surface area (μm^3/μm^2), which can be interpreted as an equivalent average biofilm thickness. Quorum sensing intensity was measured in terms of the intensity of GFP expression, using FCLSM as customary in biofilm research.

Figure 3-5a shows how the temporal dynamics of the biofilm biomass, measured in 3D using CRM, is correlated with the temporal dynamics of QS, measured in 3D using FCLSM. The QS intensity is negligible for the first 18 h, even though the biomass more than doubles during that time, going from an equivalent biofilm thickness of 1.0 to over 2.0 μm (Fig. 3-5b, top panel).
Figure 3-5 Simultaneous confocal measurements of biofilm biomass and QS intensity. (a) Eight time-lapse images showing both biomass (left, shown in grayscale) and the corresponding fluorescent intensity (right, shown in green) captured by CRM and FCLSM, respectively. The patch size is 150 μm × 150 μm and the flow rate was 1μl/min, corresponding to a Peclet number of 8.4. The voxel size is (0.29 μm)x × (0.29 μm)y × (1.31 μm)z = 0.11 μm3. (b) The temporal evolution of the biofilm’s biomass (red) and fluorescent intensity (green) recorded at 3 h intervals (top), and the fluorescent intensity normalized by the biomass (bottom).

The QS intensity per unit biomass (Fig. 3-5b, bottom panel), therefore, is very low for the first 18 h of biofilm growth. The picture changes dramatically thereafter. While the biomass still increases, it does so at a markedly slower rate, as the biofilm increases in mean equivalent thickness by another micrometer over 27 h, reaching a mean equivalent thickness of ~1 μm at the end of the observations (t = 45 h). Meanwhile, the fluorescent intensity that measures QS expression takes off at t ~ 18 h, and thereafter increases at a rapid, constant rate until the end of
the observations. From 18 to 45 h, the fluorescent intensity goes from ~5% to ~120%, a greater than 20-fold increase. Together, these observations result in a strong increase in QS expression per unit biomass (Fig. 3-5b, lower panel). This indicates that (i) the increase in fluorescent intensity is not due simply to an increase in the biofilm’s biomass, but to an actual increase in the per-unit-biomass (i.e., per cell) QS signal; and (ii) there is a rather clear point in time (18 h) at which this increase in QS activity sets in. In summary, these results confirm that the fluorescent signal used as a metric for QS in the first sections of this chapter is indeed to be attributed to QS induction (presumably, after AI concentration reach the threshold to activate the QS reporter gene), and not to a simple increase of population density. Thus, fluorescence intensity increases in microchannel experiments are very likely due to upregulation of QS among *P. aeruginosa* and not simply to an accumulation of biomass.

### 3.3 Conclusion

These findings establish microfluidics as a new tool for the study of biofilms. This new tool enables both accurate control over microenvironmental conditions and direct observation of the dynamics of biofilm formation. Specifically, we have here reported observations that shed new light on the role of physical parameters of biofilms on the biological process of quorum sensing.
First, we have reported the counterintuitive discovery that QS in smaller biofilms can be triggered earlier than in larger biofilms, and have proposed a rationalization of this discovery based on the limitations in the transport of both nutrient molecules and autoinducer molecules. Second, we have demonstrated that intermediate flow rates are optimal for the onset of QS, and have proposed a rationalization of this finding that is again based on mass transport: intermediate flow rates alleviate nutrient transport limitations associated with molecular diffusion alone, but without strongly washing out the autoinducer molecules as higher flow rates do. These two set of experiments, then, rest on a common theme: that the interplay of nutrient transport and autoinducer transport – both controlled by the same physical parameters (biofilm size, fluid flow, etc) – is a crucial determinant of fundamental biofilm processes such as quorum sensing. We speculate that a broad range of other phenomena within biofilms will be affected by the processes elucidated here, and that microfluidics and direct, real-time imaging will provide a highly valuable approach for the investigation of these dependencies.

3.4 Materials and Methods

**Materials.** Octadecyltrichlorosilane (OTS) \([\text{CH}_3(\text{CH}_2)_{17}\text{SiCl}_3]\) (Aldrich, 97%), hexane (Aldrich, anhydrous, 99%), fluorescein isothiocyanate-bovine serum albumin (FITC-BSA) and tetramethyl rhodamine isothiocyanate-wheat germ agglutinin (TRITC-WGA) were purchased.
Poly(dimethylsiloxane) (PDMS, Sylgard 184) was ordered from Dow Corning.

**Strains, Growth Conditions, and Microfluidic Experiments.** For the QS experiments, we used *P. aeruginosa* PA01 mini Tn5- rhlA::GFP strain (courtesy of George O'Toole, Dartmouth University) harboring the QS reporter, which expresses GFP when it is actively undergoing QS. Using epifluorescent microscopy, we monitored QS by measuring the fluorescent intensity over time in biofilm patches of different size, fabricated in microfluidic channels with 50 μm height and 4 mm width. In a typical experiment, we first inject for 1 h in the microfluidic channel a low-density bacterial solution (OD₆₀₀ = 0.2-0.3), which is grown in 5 ml tryptone broth (TB) in a rotary shaker incubator at 180 rpm at 37 °C for 4 h, after being inoculated with 50 μl of an overnight culture (OD₆₀₀ ~ 1.0) in TB broth in a rotary shaker incubator at 150 rpm at 30 °C. After 1 h, the injection is switched from the bacterial solution (in Trypton broth) to pure culture medium (minimal M63 medium) at a constant flow rate of 3 μl/min, which corresponds to a mean flow velocity of 250 μm/s and wall shear rate of ~30 s⁻¹. This flow condition is maintained for up to 48 h, during which time the biofilm grows without the further external addition of cells. The same protocol and media are simultaneously applied to separate (up to 5) microchannels, on the same chip (i.e., glass slide), each having square hydrophobic patches of differing size (50,
150 and 400 μm, respectively). This parallel approach permits a direct comparison among patches of different size, since the bacterial suspension, its age, and the environmental conditions (e.g., temperature) are identical among all channels. Initial cell attachment and surface coverage are monitored using phase-contrast microscopy, using computer-controlled routines to move the microscope stage and automatically monitor all microchannels over time. The QS signal is monitored using epifluorescent illumination and a GFP filter, with a 20 ms exposure. The fluorescence intensity is quantified in terms of GFP signal strength.

**PDMS stamp fabrication for surface patterning.** PDMS stamps were fabricated by curing the prepolymer on silicon masters patterned with SU-8 photoresist (SU-8 2050, MicroChem, MA, USA) using conventional photolithography. The masters used for patterning had recessing features, which resulted in PDMS replicas with the opposite sense. To assist in removal of cured PDMS from the masters, the SU-8 masters are silanized by exposure to the vapor of 1,1,2,2-tetrahydrooctyl-1-trichlorosilane, CF₃(CF₂)₆(CH₂)₂SiCl₃ overnight. To cure the PDMS prepolymer, a mixture of 10:1 silicon elastomer and the curing agent was poured on the master and placed at 65°C for 2 h. The PDMS replica was then peeled from the silicon master.
Bibliography

biological, and physical environment on quorum sensing in structured microbial communities.


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Chapter 4  Enhanced Trophic Transfer Rates in a Chemotactic Food Chain

4.1  Introduction

In most natural habitats, predator-prey interactions occur in a spatially heterogeneous environment. One of the major consequences of this heterogeneity is patch formation of resources and organisms, which results in increased persistence of the prey population, compared to homogeneous conditions, and increased consumption by higher-level organisms \(^1,2\). Benefits conferred by patchiness include a higher probability of mating, but patchiness can be harmful by favoring predator foraging success. In the marine zooplankton *Nyctiphanes australis*, swarming is seasonal and linked to breeding \(^3,4\), suggesting that organisms aggregate in order to find mates. Ritz has speculated that the hydrodynamics in aquatic crustacean schools might optimize the capture rate of suspended prey \(^5\). Modelling studies also suggest that collective motion can improve ability to forage in a noisy resource field \(^6,7\).

There are several negative effects associated with patch formation. An obvious cost is that available resources are shared with other group members. When in aggregation, krill are more vulnerable to large predators that have evolved efficient strategies to detect and exploit patches \(^8,9\). In the St-Laurent estuary, for example, mammals including rorqual whales are attracted by aggregations of krill \(^10-12\). Whales are observed in regions where krill densities are generally high and patchy \(^10,13\). Dense aggregations also favor the spread of infectious diseases, which can lead to mass mortality \(^14\). On the other hand, organisms able to sense the approach of predators can avoid patches to decrease predation risk. In the Southern Ocean, schools of Antarctic krill are
observed to disperse rapidly when approached by underwater vehicles\textsuperscript{15,16}, which is thought to be a strategy for escaping predator attacks by confusing the predator.

Grünbaum proposed a non-dimensional indicator, the Frost number $Fr$ as a predictor of the performance of an organism in exploiting patches in its resources\textsuperscript{17}. The Frost number expresses the ratio between the timescale of patch dissipation and the timescale characterizing the predator’s quest for and exploitation of the patch. When $Fr \geq 1$, prey patches are highly available to foragers. Conversely, $Fr \leq 1$ indicates that prey patches dissipate too rapidly for foragers to exploit them\textsuperscript{18}.

Although spatial heterogeneity has significant implications for ecological processes, population models in the ocean are often spatially homogeneous\textsuperscript{19}. Marine ecologists, however, increasingly recognize that a consideration of heterogeneous spatiotemporal dynamics is indispensible because there is growing evidence that nutrient patchiness is pervasive at all scales in the ocean\textsuperscript{20}. Recent work has shown that patchiness extends down to the microscale ($\mu$m-$mm$), where motile marine microbes are believed to forage preferentially on point nutrient sources\textsuperscript{21-24}.

Motility in the microbial world is characterized by a random, diffusive component, used to explore the environment, e.g., for nutrients, and a directional, chemotactic component used to exploit nutrient patches upon detection. Chemotaxis is the ability of consumers to direct their movement toward regions of high resource concentration. The performance of a chemotactic organism depends on the timescale of chemotaxis relative to dispersion of the resource. This concept is akin to the Frost number introduced by Grünbaum. Since planktonic microbes in the microbial loop play an important role in energy flow and biogeochemical cycles\textsuperscript{21,25-28}, investigating the foraging abilities of the microbes is a key issue to quantify their effects on trophic transfer and biogeochemical rates in the environment. Here we present a modeling study
that suggests that patchy interactions at the microscale can have major effects on macroscopic processes.

Blackburn & Fenchel (1999) showed that planktonic microbes can find nutrient patches by chemical cues over distances of a few millimeters to a centimeter \(^{29}\). The nutrient patches are often ephemeral, limited by the erosive effects of diffusion. The capacity of planktonic microbes to actively locate and exploit the nutrient patches will be important in environment within \(Fr \geq 1\). Seymour \textit{et al.} (2008) observed strong responses to microbial patches by three sequential levels of marine microorganisms in the microbial food web: a phytoplankton (\textit{Dunaliella tertiolecta}), a heterotrophic bacterium (\textit{Pseudoalteromonas haloplanktis}), and a phagotrophic protist (\textit{Neobodo designis})\(^{30}\). Due to technical complexities associated with measuring the foraging behaviour of multiple microbes simultaneously, they quantified the chemotactic response of each microorganism separately, resulting in a clear and intense patch of each microorganism, corresponding to the initial position of the nutrient patch. Such patches lasted in the order of 20 minutes. These scales will guide our choices in the simulations, where we will explore times from \(10\) to \(10^3\) seconds, domains from \(L = 1\) to \(10\) mm, and diffusion coefficients appropriate for low-molecular-weight organic substrates, \(D_A = 10^{-9}\text{ m}^2\text{ s}^{-1}\). The experiments by Seymour \textit{et al.} (2008) directly inspired the simulations presented here, which provide a model for a food web where each trophic level responds by chemotaxis to the population of its prey. Contrary to Seymour \textit{et al.} (2008), the modeling framework allows us to directly explore the simultaneous presence of all three trophic levels, and – by comparison with a non-chemotactic food web – the consequences on resource transfer through the food web.
4.2 Methods

Mathematical Model

We modelled a chemotactic food chain as a set of coupled partial differential equations, one for each trophic level. The lowest level represents dissolved nutrients, of concentration $A$ (but could also represent a concentration of non-motile prey organisms). The subsequent levels have concentrations of organisms denoted by $B$, $C$, $D$. We are assuming that all concentrations have been normalized to the same units, e.g., expressed in terms of carbon concentration (mol$_{C}$/m$^{3}$). The length of the food chain is varied in the simulations, from 2 (A,B) to 4 (A,B,C,D) trophic levels. Equations are given in vector form, for application to 1D, 2D and 3D domains. This work focuses on 3D domains, except where indicated, to simulate aquatic environments. Because we are primarily interested in the comparison among different scenarios (active, passive, uniform), rather than the prediction of absolute quantities, we adopt a number of simplifications intended to make the model simpler and reduce the number of parameters.

To illustrate the model we focus on the 2-level food chain, for which the equations governing diffusion, chemotactic motility, and predation read:

$$\frac{\partial A}{\partial t} = \mu_A \nabla^2 A - \beta_A A B$$  
(1a)

$$\frac{\partial B}{\partial t} = \mu_B \nabla^2 B - \nabla \cdot (\mathbf{V}_B B) + \gamma_B \beta_A A B$$  
(1b)

Bold symbols denote vector quantities. The first term on the right hand side models diffusion. For the dissolved nutrient (A), this is molecular diffusion and diffusivity is $\mu_A$. For the organisms (B), diffusion arises from the ‘random motility’ component of swimming and is characterized by a diffusivity $\mu_B$. The latter can be estimated by modelling the movement pattern as a random walk with swimming speed $U_B$ and characteristic reorientation time $\tau$, yielding $\mu_B = \tau U_B^2/3$. The
second term on the right hand side of equation (1b) represents chemotaxis. $V_B$ is the chemotactic velocity of the organisms towards the resource (A) and is here modeled as 

$$V_B = U_B \tanh \left( \frac{|\nabla A|}{g_A} \right) \frac{\nabla A}{|\nabla A|},$$

where $U_B$ is again the organisms’ swimming speed. Strictly speaking, $U_B$ should be the maximum chemotactic speed, which is a fraction of the swimming speed. Their theoretical ratio is of order 1 (e.g., 2/3 for bacteria, see Ahmed & Stocker, 2008), hence we will here take $U_B$ to be equal to the swimming speed for simplicity. The hyperbolic tangent accounts for an increasing response with increasing magnitude of the resource gradient and saturation beyond a threshold gradient $g$ (Rivero et al., 1989). The magnitude of $V_B$ is 76% of $U_B$ when $|\nabla A| = g$. This chemotaxis model is an extension of the classic one by Keller and Segel, with provision for the saturation of the chemotactic velocity. The term $\nabla A / |\nabla A|$ sets the direction of the chemotactic velocity to be along the resource gradient.

The last term in equations (1a,b) models resource transfer between trophic levels. The shape of this term depends on the transfer mode, for example osmotrophic uptake versus predation. For simplicity, we adopt a transfer function that is linearly proportional to encounter rate between organisms and their resources (here, $AB$) and describe the transfer process generally as ‘predation’. The magnitude of predation is measured by $\beta$ and a fraction $\gamma_B$ (assimilation efficiency) of the prey biomass is turned into predator biomass. Because $\gamma_B$ is typically of order 1 (0.1-0.6; e.g., Fenchel 1982, Kirchman et al. 1992, Conan et al. 1999, Cole & del Giorgio 2000), we use $\gamma_B = 1$ for simplicity.
Higher trophic levels (C and D) were modeled in the same manner as B, including random motility, chemotaxis and predation. Equations were then made dimensionless by rescaling concentrations A, B, C and D with their initial values $A_0$, $B_0$, $C_0$ and $D_0$; lengths with the domain length scale $L$; time with the nutrient diffusion time scale $L^2/\mu_\delta$; and thus velocities by $\mu_\delta/L$. The full equations for a four-level food chain, in dimensionless form, are then:

\[
\frac{\partial A}{\partial t} = \nabla^2 A - \eta_A AB 
\]  
\[
\frac{\partial B}{\partial t} = \kappa_B \nabla^2 B - \chi_B \nabla \cdot \left( \tanh \left( \frac{\nabla A}{\delta_A} \right) \frac{\nabla A}{\nabla A} \right) + \omega_B \eta_A AB - \eta_B BC 
\]  
\[
\frac{\partial C}{\partial t} = \kappa_C \nabla^2 C - \chi_C \nabla \cdot \left( \tanh \left( \frac{\nabla B}{\delta_B} \right) \frac{\nabla B}{\nabla B} \right) + \omega_C \eta_B BC - \eta_C CD 
\]  
\[
\frac{\partial D}{\partial t} = \kappa_D \nabla^2 D - \chi_D \nabla \cdot \left( \tanh \left( \frac{\nabla C}{\delta_C} \right) \frac{\nabla C}{\nabla C} \right) + \omega_D \eta_C CD 
\]

Where the parameters are

\[
\kappa_B = \frac{\mu_B}{\mu_A}, \quad \kappa_C = \frac{\mu_C}{\mu_A}, \quad \kappa_D = \frac{\mu_D}{\mu_A} 
\]  
\[
\chi_B = \frac{U_B L}{\mu_A}, \quad \chi_C = \frac{U_C L}{\mu_A}, \quad \chi_D = \frac{U_D L}{\mu_A} 
\]  
\[
\eta_A = \frac{B_A \beta_A L^2}{\mu_A}, \quad \eta_B = \frac{C_0 \beta_B L^2}{\mu_A}, \quad \eta_C = \frac{D_0 \beta_C L^2}{\mu_A} 
\]  
\[
\delta_A = \frac{g_A L}{B_0}, \quad \delta_B = \frac{g_C L}{C_0}, \quad \delta_C = \frac{g_D L}{D_0} 
\]  
\[
\omega_B = \frac{A_0}{B_0}, \quad \omega_C = \frac{B_0}{C_0}, \quad \omega_D = \frac{C_0}{D_0} 
\]
These dimensionless parameters govern the population dynamics and trophic transfer. $\kappa$ is the ratio of the organisms' diffusivity by random motility relative to the nutrient diffusivity; $\chi$ is a chemotactic Peclet number, describing the ratio of time scales for organism chemotaxis relative to nutrient diffusion (akin to the Frost number $^{23}$), $\eta$ is a dimensionless biomass transfer coefficient, $\delta$ is a dimensionless saturation gradient and $\omega$ measures the ratio of initial organism concentrations.

**Numerical solution, initial conditions and parameter values**

Equations (2a-d) were solved using a finite element methods software (Comsol Multiphysics, Natick, MA). The spatiotemporal evolution of A, B, C and D was computed subject to Neumann boundary conditions (no-flux) at the edge of the domain. We computed solutions for different domain dimensionalities (1D, 2D and 3D), but focus primarily on the 3D case here. Polar and spherical symmetry were assumed in the 2D and 3D domains, respectively, such that the solution only depends on the distance $r$ from the origin. The computational domain is therefore the radial axis. The volume of the domain is $2, \pi r^2$ and $4\pi r^3/3$ in 1D, 2D and 3D, respectively.

The radial axis was discretized into 9676 elements, with higher density of elements at smaller values of $r$, where sharper changes were expected. Convergence was tested by ensuring that differences in the results were negligible upon refining the mesh by a factor of four. The simulations time step was automatically set by the numerical solver, ensuring accuracy and stability. Data were stored at time intervals $dt = 10^{-4}$ (from $t = 0$ to 0.005) and $dt = 10^{-3}$ (from $t = 0.005$ to 0.02).
The initial condition for the nutrients is a Gaussian patch of half-width $\sigma$ and total initial mass of 1:

$$A(r, t = 0) = \frac{1}{(\sqrt{2\pi} \sigma)^n} e^{-\frac{r^2}{2\sigma^2}}$$

(7)

where $n$ is the dimensionality of the system (1, 2 or 3). We chose $\sigma = 0.02$ for all simulations. This corresponds for example to a 200 $\mu$m nutrient patch in an $L = 10$ mm domain. For comparison, we also run cases with a uniform distribution of $A$, again with total initial mass of 1.

The initial distribution of B, C and D was uniform with total biomass of 1, i.e., $B_0 = C_0 = D_0 = 1/2, 1/\pi$ and $3/(4\pi)$ in 1D, 2D and 3D, respectively.

We explored a wide range of values for $\kappa$, $\chi$, and $\eta$, estimated from typical values of biophysical parameters and movement characteristics of marine microorganisms and their environment. Scales of interest ($L$) range from 1 to 10 mm. Nutrient diffusivity $\mu_A$ ranges from $10^{-9}$ m$^2$s$^{-1}$ for low molecular weight substrates (e.g., sugars and amino acids) to $10^{-11}$ m$^2$s$^{-1}$ for high molecular weight ones (e.g., polysaccharides and carbohydrates) (Benner et al, 1997, 2003). Swimming speeds range from 10 to 1000 $\mu$m s$^{-1}$, and random motility coefficients from $10^{-10}$ to $10^{-8}$ m$^2$s$^{-1}$ (Visser & Kiorboe, 2006). The strength of predation, $\beta$, approximately ranges from $10^{-4}$ to $10^{-3}$ s$^{-1}$. The threshold gradient, $g$, ranges from $10^{-2}$ to $10^2$ mm$^{-1}$ (Rivero et al, 1989). This resulted in estimates for the range of $\kappa_1 (10^{-1}$ to $10^3)$, $\chi_1 (10^1$ to $10^6)$, $\eta_1 (10^{-4}$ to $10^3$), and $\delta_1 (10^{-1}$ to $10^4$). We further assumed that each trophic level initially had the same biomass ($\omega_i = 1$).

To quantify the degree of patchiness of each trophic level, $i$, and the correlation between two connected trophic levels (which determines uptake), we computed the zero-lag correlation and the cross-correlation metrics at any given time, $t$. 

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\[ P_i(t) = \sqrt{E\left[ \frac{\Delta i^2}{\bar{i}} \right]} \quad P_j(t) = \sqrt{E\left[ \frac{\Delta i \Delta j}{\bar{i} \bar{j}} \right]} \]

\( \bar{i} = E[i], \quad \Delta i = i - \bar{i}, \quad \bar{j} = E[j], \quad \Delta j = j - \bar{j}, \)

where \( E[\cdot] \) is a spatial average over the domain, \( \bar{i} \) and \( \bar{j} \) are the mean values of biomass in trophic levels \( i \) and \( j \), and \( \Delta i \) and \( \Delta j \) are the spatial deviations from those averages. We call \( P_i \) the patchiness index and \( P_j \) the resource-consumer correlation index.

4.3 Results

We investigated chemotaxis-mediated trophic dynamics in three-dimensional space. This represents an appropriate model, for example, for aquatic ecosystems away from surfaces, where diffusion is typically not constrained by boundaries. For other situations, including spreading of microorganisms on surfaces or gamete encounters at air-water interfaces, a two-dimensional approach would be more appropriate. The concentration profiles generated by diffusion depend on the dimensionality of the problem. This is shown in Fig. 4-1, where the radial concentration profiles of the dissolved nutrient A are plotted at time \( t = 0.005 \) in 1D, 2D and 3D scenarios. Higher dimensionality results in stronger gradients. On the other hand, gradients in higher dimensions fill a smaller volume (the fraction of the domain volume within distance \( r \) from the origin is \( r^3 \), \( r^2 \) and \( r^3 \) in 1D, 2D and 3D, respectively) and thus are available to a smaller fraction of a (uniformly distributed) population of organisms. As chemotaxis hinges on the response to gradients, a quantification of the effect of chemotaxis on trophic transfer requires first a choice of system dimensionality. While the essential process described here – the enhancement of trophic transfer rates by chemotaxis – occurs independently of the dimensionality of the system, an
estimate of its quantitative impact is dimension-dependent. Therefore, with aquatic ecosystems in mind, where microbial interactions occurs mostly in the unbounded water column, we focus here on three-dimensional systems, and use the appropriate mathematical expressions for the vector operators in Eq. (1).

Figure 4-1 Effects of dimensionality and domain size. The diffusion of a resource patch generates different profiles of concentration (\(A\)) in 1D, 2D and 3D. Radial profiles are shown at \(t = 0.005\). The initial half-width of the Gaussian patch was \(\sigma_0 = 0.02\). In the remainder of this work, 3D simulations are discussed. Inset: the resource patch reaches the boundary of the simulation domain \((r = 1)\) after \(t \approx 0.02\), which was adopted as the temporal horizon for all simulations.
Because patch utilizations via chemotaxis occurs over short time scales, typically several minutes for marine microorganisms \(^{32,33}\), we focus on simulations over short temporal horizons. The latter was chosen based on a consideration of the boundary conditions. The inset in Fig. 4-1 shows that the diffusing patch begins reaching the boundary of the domain \((r = 1)\) at \(t \approx 0.02\). Thus, we chose to run all simulations up to \(t = 0.02\). For example, when the nutrient is made of small molecules \((D_A = 10^{-9} \text{ m}^2 \text{ s}^{-1})\) diffusing in an \(L = 10 \text{ mm}\) domain, this corresponds to a dimensional time of \(0.02L^2/D_A = 33 \text{ min}\). For large molecules \((D_A = 10^{-10} \text{ m}^2 \text{ s}^{-1})\), the simulation horizon is 5.5 hours.

We begin by studying a two-level food chain \((A,B)\), to gain insight into the processes involved in the response to a resource patch. The key idea is exemplified in Fig. 4-2, where three fundamental scenarios are considered: a uniform distribution of resource \(A\) (‘uniform’; green line); a patch of resource \(A\) with a non-motile consumer \(B\) (‘passive’; blue line); and a patch of resource \(A\) with a motile consumer \(B\) (‘active’; red line).

Consumers are initially distributed uniformly \((B = 3/(4\pi))\) in all cases. The passive case corresponds to \(\kappa_B = \chi_B = 0\) (no motility), whereas for the active case we chose \(\kappa_B = 1, \chi_B = 100\) (for all cases, \(\eta_B = 1\)). In a uniform environment, nutrient availability and consumption occur uniformly, hence motility is irrelevant: there are no cues that make the consumer distribution depart from uniform. Consumers therefore grow and deplete the resource uniformly. Because the temporal horizon is short, the growth is small \((i.e., B \text{ remains } \approx 3/(4\pi); \text{ Fig. 4-2a, green solid line})\).
Figure 4-2 Three possible microbial food chains: uniform, passive and active. (a) Shown is the radial concentration of resources (A, dashed lines) and consumers (B, solid lines) at time $t = 0.02$ for three cases, uniform (green), passive (blue), and active (red). In a uniform food chain, the resource is uniform over space, hence consumers remain uniformly distributed (with a value close to their initial value of $3/(4\pi)$). In a passive food chain ($\kappa_B = \chi_B = 0$), a resource patch triggers a consumer patch, but the latter is small because it is caused purely by growth and the duration of the process is short. In an active food chain, consumers respond to a resource patch using chemotactic motility (here: $\kappa_B = 1$, $\chi_B = 100$), markedly enhancing accumulation. For all cases, $\eta_B = 1$. Note that the green dashed line is indistinguishable from the blue dashed line. (b) These different responses can significantly affect resource uptake, as shown by the spatially integrated, instantaneous transfer ($\int Abdv$). Uptake is considerably larger and faster when the consumers respond actively to the resource patch.

The passive and active scenarios share a heterogeneous resource distribution in the form of a patch, but only the active scenario incorporates a chemotactic response of the consumers. A resource patch can trigger an accumulation (hence, a patch) of consumers by one of two mechanisms: local growth (passive case) or recruitment of surrounding organisms by motility (active case). When the time scale of patch diffusion and organism response is short compared to the organisms' doubling time, as is the case for most microscale solute patches in the ocean, active accumulation by motility (Fig. 4-2a, red solid line) is considerably stronger than local
growth (Fig. 4-2a, blue solid line). By moving into the center of the patch, active consumers are exposed to higher resource concentrations than passive ones, enhancing uptake. This results in a faster depletion of the resource (compare the red and blue dashed lines in Fig. 4-2a). Although the time over which an active response is superior to a passive one is finite, because resource gradients are eroded by diffusion and ultimately vanish, we will see that typical response time scales nonetheless afford a marked uptake advantage to active responders.

It is essential to distinguish between rates and amounts. In the absence of additional factors (e.g., changes in the resource’s bioavailability or the existence of a lower threshold for uptake), the entire resource will ultimately be consumed both in the passive and in the active case. Hence, the primary effect of an active response will be on the rate at which the resource is consumed, which ultimately determines the rate at which resources are transferred further up the food chain and can potentially affect the amounts transferred (see Discussion). Equivalently, one can determine the amount of resources transferred instantaneously or on average over a given finite time. The instantaneous measure is obtained by computing the instantaneous integrated uptake over the domain, \( \int ABdV \), since resource consumption is proportional to the product \( AB \) (Eqs. 1,2). The average measure is then simply the amount of resource transferred over a finite time, here \( t = 0.02 \). Figure 4-1b shows that an active response can significantly enhance instantaneous and average uptake: for the case in Fig. 4-1b, both measures of trophic transfer are about an order of magnitude larger for the active food chain than both the passive and uniform food chains. This is a direct result of the strong motility-mediated accumulation of the active consumers within the resource patch (Fig. 4-1a).
Figure 4-3 Spatiotemporal dynamics of a two-level food chain. Color maps represent the distribution of consumers (B) and resource uptake (AB) in space (r) and time (t), for (a,b) a passive food chain and (c,d) an active food chain (for definitions of ‘passive’ and ‘active’, see Fig. 2). For comparison, in a uniform food chain (not shown) consumers (B) are uniformly distributed with $B \approx 3/(4\pi) = 0.239$ (growth is weak, hence the value is nearly constant). Different consumer responses generate not only different consumer distributions (a,c), but also different uptake landscapes (b,d). Parameters are the same as in Fig. 4-2.

The comparison between active and passive scenarios is presented in more detail in Fig.4-3, which shows spatiotemporal maps of consumer distribution (panels a,c) and trophic transfer (panels b,d). Accumulation is considerably stronger for active consumers (panel c) compared to passive ones (panel a), and the spatial distribution of active consumers follows the diffusion of
the resource patch (diffusion-like spreading in panel c). Consequently, trophic transfer, which is mainly limited to early times and organisms initially in the patch for the passive case (panel b), is not only very strong at early times in the center of the patch for the active case, but lasts longer in the center of the patch and extends to surrounding regions (panel d), as motile organisms follow gradients of the diffusing resource by chemotaxis.

The critical parameter determining the strength with which a consumer is attracted to a resource is the chemotactic sensitivity. In Fig. 4-4 we examine the effect of increasing the chemotactic sensitivity, $\chi_B$, on trophic transfer for a two-level food chain, by computing the time course of the increase in consumer biomass, $\Delta B_T$.

Trophic transfer increases with $\chi_B$, as stronger chemotaxis affords earlier accumulation in the resource patch. This increase is dramatic between $\chi_B = 100$ and 1000, when $\Delta B_T$ at $t = 0.02$ jumps from 42% at $\chi_B = 100$ to 95% at $\chi_B = 1000$. A further increase in $\chi_B$ saturates transfer, and further investment by the organisms in improved sensitivity is thus unnecessary. The comparison with the passive and uniform food chains is insightful. $\Delta B_T$ is larger in all three active food chains than in the uniform food chain (for which $\Delta B_T = 5\%$ at $t = 0.02$): motility and patchiness thus markedly enhance transfer rates. Unsurprisingly, chemotaxis ($\chi_B > 0$) is also beneficial compared to random motility alone ($\chi_B = 0$; 'diffusive' food chain in Fig. 4-4). On the other hand, Fig. 4-4 surprisingly shows that chemotactic organisms can perform worst than non-motile ones. For example, $\Delta B_T$ is lower for the food chain with $\chi_B = 100$ (42% at $t = 0.02$) than for the passive food chain (57% at $t = 0.02$). As expected, organisms with stronger chemotaxis ($\chi_B = 1000$) outperform ($\Delta B_T = 95\%$ at $t = 0.02$) passive ones. This indicates that chemotactic motility is only beneficial when the chemotactic sensitivity exceeds a minimum threshold.
Figure 4-4 Effect of chemotactic sensitivity on trophic transfer. The increase in consumer biomass, $\Delta B_T$, is shown for a two-level food chain (A, B), for six cases: uniform (no resource patch), passive (no consumer motility), purely diffusive (motility, but no chemotaxis; $\chi_B = 0$) and three cases of chemotactic ($\chi_B = 100, 1000$ or $10000$) food chains. For all cases $\kappa_B = 10, \eta_B = 10$, except for the passive case for which $\kappa_B = 0, \eta_B = 10$. Numbers on the right indicate the increase in consumer biomass at $t = 0.02$. Notice that the trophic transfer is larger in a passive food chain than in an active one with $\chi_B = 100$ (but less than the $\chi_B = 1000$ case).

To shed light on this apparently counterintuitive result, we examined the effect of random motility, $\kappa_B$. Fig. 4-5 shows that the relative performance of chemotactic and non-motile (i.e., passive) consumers depends on the magnitude of the random swimming component. The passive strategy is superior only when random motility is large ($\kappa_B = 10$ in Fig. 4-5), while chemotaxis is superior when random motility is small ($\kappa_B = 1$ in Fig. 4-5). Furthermore, trophic transfer of non-motile cells depends on the biomass transfer rate, $\eta_B$: whereas for $\eta_B = 10$ the passive strategy is
superior to one with moderate chemotaxis ($\chi_B = 100$), as seen in Fig. 4-4, the opposite occurs for $\eta_B = 1$ (not shown). In summary, chemotaxis confers a competitive advantage to motile species over non-motile ones only if (i) the directional component of movement is suitably larger than the random component, and (ii) accumulation, rather than local growth, is the dominant mechanism for trophic transfer. Motile but weakly chemotactic cells ($\chi_B = 100$ in Fig. 4-4), therefore, accumulate poorly in the resource patch and tend to diffuse rapidly out of it, diminishing trophic transfer by local growth.

**Figure 4-5** Effect of random motility on trophic transfer. The increase in consumer biomass, $\Delta B_T$, is shown for three two-level (A, B) food chains: passive (no consumer motility) and chemotactic ($\chi_B = 100$, $\eta_B = 10$), the latter with $\kappa_B = 1$ or 10. For $\kappa_B = 10$, the figure further shows the effect of the saturation gradient $\delta$: the upper and lower boundaries of the shaded region correspond to $\delta = 10^{-1}$ to $10^4$. 
Although primarily determined by the chemotactic sensitivity, the chemotactic ability of our model organisms is also affected by the threshold resource gradient, $g$. The lower $g$, the higher the resource transfer (Fig. 4-5, shaded region), because cells respond with the full chemotactic velocity even to weaker gradients. In reality, there is a trade-off between the ability to respond to weaker gradients and the robustness of weak signals to noise $^{34}$, preventing organisms from responding to exceedingly weak gradients. This was not pursued here, and we chose instead to focus on the case $\delta = 10^2$ for all simulations.

To quantify the effect of random and directed (i.e. chemotactic) motility on transfer, we carried out simulations for a grid of values of $\kappa_B$ and $\chi_B$. A summary of the resource transfer $\Delta B_T$ at $t = 0.02$ is shown in Fig. 4-6, both for $\eta_B = 1$ (Fig. 4-6a) and $\eta_B = 10$ (Fig. 4-6b).

**Figure 4-6** Trophic transfer for different chemotactic food chains. Shown is the increase in consumer biomass, $\Delta B_T$, at time $t = 0.02$ for a two-level food chain (A, B), two values of the biomass transfer rate, (a) $\eta_B = 1$ and (b) $\eta_B = 10$, and a matrix of motility values, $\kappa_B$ and $\chi_B$. The case $\chi_B = \kappa_B = 0$ corresponds to a passive food chain, while cases with $\chi_B = 0$ correspond to ‘diffusive’ consumers (motile but non-chemotactic).
The opposing effects of chemotaxis and random motility are evident: an increase in \( \chi_B \) enhances trophic transfer, whereas an increase in \( \kappa_B \) reduces it. Both parameters ultimately depend on swimming speed, showing that an increase in swimming speed alone does not necessarily increase resource uptake. Instead, it is the relative importance of directed versus random motility that primarily affect transfer rates. The results for \( \eta_B = 10 \) confirm that this conclusion is largely independent of the biomass transfer rate and reiterate the existence of a chemotactic sensitivity threshold when \( \eta_B \) is large.

Quantitatively, we find that strongly chemotactic consumers can increase their biomass by up to 30% within the temporal horizon \( t = 0.02 \) for small biomass transfer rates (\( \eta_B = 1 \)) or, equivalently (given our assumption of perfect consumption, \( \gamma_B = 1 \), Eq. 1), that they consume 30% of the nutrients within \( t = 0.02 \). For imperfect consumption, numbers can be easily rescaled by \( \gamma_B \), so that a \( \gamma_B = 0.5 \) consumption efficiency corresponds here to a maximal biomass increase of 15% from a patch. For large biomass transfer rates (\( \eta_B = 10 \)), even moderately chemotactic consumers can consume the entire patch within \( t = 0.02 \), hence obtain a marked biomass boost from single patches.

To what extent, then, does chemotaxis affect the rates of trophic transfer? To quantify this, we computed the ratio \( RC \) between the time to transfer 50% of the resources to the consumers in the passive case, versus the same time computed for the active case. When \( RC > 1 \), the active food chain transfers resources faster than the passive one. Fig. 4-7 shows \( RC \) for increasing values of \( \chi_B \), for \( \kappa_B = 10 \) and \( \eta_B = 10 \). For weak chemotaxis (\( \chi_B \leq 100 \)) transfer is slower in the active case, consistent with our findings above (Fig. 4-4). In contrast, moderate to strong chemotaxis (\( \chi_B = 1000–10000 \)) can enhance transfer rates by over an order of magnitude, relative to a passive food chain.
**Figure 4-7** Increase in the trophic transfer rate due to chemotactic motility, for a two-level (A, B) food chain with \( \kappa_B = 10 \) and \( \eta_B = 10 \). \( R_C \) represents the ratio of times at which 50% of the resource (A) has been taken up by the consumers (B), for a passive (i.e., non-motile) food chain relative to an active (i.e., chemotactic) one. When \( R_C = 1 \) (dotted line) the transfer rate is equal to that of a passive food chain. Biomass can be transferred over ten times faster due to chemotactic motility.

As shown in Fig. 4-3, the mode of response to a resource patch affects not only the rate of resource transfer, but also the spatial distribution of consumers. This, in turn, affects resource transfer at the next level up in the food chain. We used the correlation metrics \( P_{BB} \) and \( P_{AB} \) (see Methods) to quantify this heterogeneity: \( P_{BB} \) measures the heterogeneity of consumers, while \( P_{AB} \) measures the co-localization of resources and consumers. Fig. 4-8 presents the temporal dynamics of the correlation metrics. Fig. 4-8a shows that, at low biomass transfer rates (\( \eta_B = 1 \)), moderate chemotaxis (\( \chi_B = 1000 \)) can markedly enhance consumer heterogeneity compared to the case of a food chain where heterogeneity arises only via local growth (passive case in Fig. 4-
8a), by a factor of nearly 200 at $t = 0.02$. Only when biomass transfer rates are high ($\eta_B = 10$) can local growth result in stronger patchiness than chemotaxis. The covariance $P_{AB}$, on the other hand, is larger for a chemotactic population ($\chi_B = 1000$) compared to a non-motile one for both $\eta_B = 1$ and 10 (Fig. 4-8b).

Figure 4-8 The degree of patchiness in the consumer distribution depends on the consumer’s response. Shown are (a) the patchiness index, $P_{BB}$, and (b) the resource-consumer correlation index, $P_{AB}$, for a passive (i.e., non motile) and an active ($\chi_B = 1000$, $\kappa_B = 1$) two-level food chain, for two values of the biomass transfer coefficient, $\eta_B = 1$ and $\eta_B = 10$. Note that the patchiness in the consumer distribution, $P_{BB}$, can be larger for the passive case when biomass transfer is rapid ($\eta_B = 10$; local growth), but the consumer-resource correlation $P_{AB}$ (and thus trophic transfer) is always larger for the active case (except in the earliest stages).

This superior co-localization with the resources is what allows chemotactic consumers to obtain the advantage in resource transfer, shown in Fig. 4-6. A grid of simulations (Fig. 4-10) shows similar trends for consumer patchiness $P_{BB}$ at $t = 0.02$ than resource transfer $\Delta B_T$ (Fig. 4-6). As expected, directed motility enhances consumer patchiness, random motility reduces it. However, in contrast to resource transfer (Fig. 4-6), consumer patchiness is reduced at higher values of $\eta_B$ (Fig. 4-9b), because of higher local growth effects.
Figure 4-9 Increase in consumer patchiness due to chemotactic motility. Shown is the patchiness index, $P_{BB}$, at time $t = 0.02$ for a two-level food chain (A, B), two values of the biomass transfer rate, (a) $\eta_B = 1$ and (b) $\eta_B = 10$, and a matrix of motility values, $\kappa_B$ and $\chi_B$. The case $\chi_B = \kappa_B = 0$ corresponds to a passive food chain, while cases with $\chi_B = 0$ correspond to 'diffusive' consumers (motile but non-chemotactic).

How are these dynamics affected by an increase in the length of the food chain? In Fig. 4-10 we examine a three-level food chain, by showing the increase in biomass of the top consumer, $\Delta C_T$, for the active, passive and uniform scenarios. $\Delta C_T$ is expressed in % of the initial biomass of C, hence it can reach up to 200% (when all resources, A and B, have been transferred to C). Consistent with the two-level food chain, a heterogeneous resource distribution results in considerably faster transfer compared to a uniform one. A strong chemotactic response by both consumers ($\chi_B = \chi_C = 1000$) at intermediate values of random motility ($\kappa_B = \kappa_C = 10$) results in a three-fold larger resource transfer to the top consumer compared to an entirely passive food chain ($\chi_B = \chi_C = \kappa_B = \kappa_C = 0$), even when transfer rates are high ($\eta_B = \eta_C = 10$). Interestingly, at
early times (up to $t = 0.004$) the chemotactic response is inferior to the passive response, a result of the fact that predation by C limits uptake of A by B in the central, nutrient-rich region, slowing overall trophic transfer. At larger times ($0.004 < t < 0.02$), however, this is reversed and the chemotactic food chain channels a significantly larger fraction of the resources to the top consumer within $t = 0.02$. This conclusion is even stronger than that for the two-level food chain (Fig. 4-4), for which the advantage of a chemotactic response with $\chi_B = 1000$ was less than twice that of the passive food chain in terms of nutrient transfer to the top consumer.

**Figure 4-10** Effect of chemotaxis on trophic transfer in a three-level (A, B, C) food chain. The increase in the biomass of the top consumer, $\Delta C_T$, is shown for three consumer responses: uniform (no resource patch), passive (no motility of the consumers, B and C), and chemotactic ($\chi_B = \chi_C = 1000$). For all cases $\kappa_B = \kappa_C = 10$, $\eta_B = \eta_C = 10$, except for the passive case for which $\kappa_B = \kappa_C = 0$, $\eta_B = \eta_C = 10$. Numbers on the right indicate the increase in the biomass of the top consumer (C) at $t = 0.02$. Biomass transfer is most rapid in the active food chain and least rapid in the uniform food chain.
Organisms occupying different levels of the food chain likely have different levels of motility and chemotaxis. In Fig. 4-10 we assumed the same motility parameters for both consumers (B and C), but when this assumption is relaxed a rich set of possible outcomes emerges. Fig. 4-11 shows the temporal dynamics of the change in biomass for all three trophic levels ($\Delta A_T$, $\Delta B_T$, $\Delta C_T$), along with the spatiotemporal distribution of the two top consumers (B,C) and their co-localization (the product BC). This was repeated for four combinations of the chemotactic response of the two consumers: (i) weak chemotactic response by both ($\chi_B = \chi_C = 100$); (ii) stronger response of the lower consumer ($\chi_B = 1000; \chi_C = 100$); (iii) stronger response of the top consumer ($\chi_B = 100; \chi_C = 1000$); strong response by both ($\chi_B = \chi_C = 1000$). For all cases, $\kappa_B = \kappa_C = \eta_B = \eta_C = 10$.

The main conclusion that emerges is that the trophic transfer is large (167%; Fig. 4-11, panel m) only when both consumers exhibit strong chemotaxis (panels m-p). When B responds weakly (panels a-d and i-l), the resource A is depleted slowly (panels a, i) and B grows slowly and weakly (panels b, j). When the ensuing response of the top predator is also weak (panels a-d), there is significant delay in the transfer of biomass to C (panel c) and the overall trophic transfer is very small (17%). A strong response of the top predator to a weakly responding lower predator (panels i-l) creates a strong accumulation of top predators (panel k), but localized to a narrow region in the center of the patch. Ultimately, the overall transfer is still small (31%). On the other hand, when the lower consumer responds strongly but the top one does not (panels e-h), B thrives (panels e,f) but C cannot take advantage of it (panel g) and the overall transfer is again small (22%). Comparison of panels e-h with i-l reveals that an increase in the chemotaxis of the top predator has a stronger effect on resource transfer compared to an increase in the chemotaxis of the lower predator.
Figure 4-11 Effect on trophic transfer of a differential chemotactic response by different trophic levels, for a three-level food chain. Curves (panels a, e, i, m) show the time course of biomass change for all three trophic levels (A, B, C). Numbers on the right of each panel show the increase in the biomass of the top consumer at $t = 0.02$. Color maps represent the spatiotemporal distribution of the lower consumer, B (panels b, f, j, n), the top consumer, C (panels c, g, k, o), and the co-localization between B and C, BC (panels d, h, l, p). The four rows correspond to four consumer responses. Row 1: $\chi_B = \chi_C = 100$. Row 2: $\chi_B = 1000$, $\chi_C = 100$. Row 3: $\chi_B = 100$, $\chi_C = 1000$. Row 4: $\chi_B = \chi_C = 1000$. For all cases, $\kappa_B = \kappa_C = \eta_B = \eta_C = 10$. 

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On the other hand, when both predators exhibit a strong chemotactic response (panels m-p) resource transfer is greatly magnified. The resource A is rapidly depleted by B (panel m), which exhibits fast growth (panel m), only to be curbed by C. Consumption by the top predator remains focused near the center after a mild broadening, resulting in a vase-shaped spatiotemporal profile (panels n-p). In this regime, the top predator gains rapid access to nearly the entire resource biomass and further takes advantage of the chemotaxis of B towards A.

From the point of view of the top predator, strong chemotaxis is clearly beneficial in all cases. Interestingly, from the point of view of the lower predator (B), a strong chemotactic response is advantageous when the predator is weakly chemotactic (compare panels e-h to panels a-d), whereas in the presence of a strongly chemotactic top predator a weak chemotactic response is preferable, to avoid ‘concentrating’ the resources for the top predator (compare panels i-l to panels m-p). This represents an additional trade-off for chemotaxis: not only does the directional component of motility have to be sufficient to overcome the diffusive tendency imparted by random motility, but the strength of the chemotactic response must be commensurate with the chemotactic abilities of one’s predator.

### 4.4 Discussion and Conclusions

In this study, we have developed a spatially explicit mathematical model of a food chain with multiple trophic levels with the purpose of quantifying the extent to which the active response of organisms to resource patches increases resource transfer and propagates patchiness in the ocean. We found that chemotaxis can enhance the rate at which resources are transferred to the top consumer by up to tenfold. On the other hand, chemotaxis does not automatically confer motile species a competitive advantage over non-motile ones. A competitive advantage only occurs
when the directional component of movement (chemotaxis) outweighs the randomness intrinsically associated with organism movement, particularly at small scales. Furthermore, optimal strength of chemotaxis depends on the chemotaxis of one’s predator – creating a complex optimization problem, in a trophic cascade of chemotaxis.

When background resource availability is low, a consumer waiting for prey (passive case) is less likely to find resources than one actively foraging. On the other hand, active foraging incurs an energetic cost, since a faster response implies a great power expenditure for motility. This power increases with the square of the swimming speed and further affects the choice of the optimum movement behavior of an organism. Hence, one might expect motile behavior to reflect a trade-off between benefits (higher nutrient gain) and costs (power expenditure). We are currently working on this.

Predation dynamics become more complex with an increase in the food chain length. Already with 3 levels (Fig. 4-12) we found a range of outcomes when the relative magnitude of chemotaxis was varied among the intermediate and top predator.
Figure 4-12 Trophic dynamics in a four-level chemotactic food web. The time course of the change in biomass of all four trophic levels (A, B, C, D) is shown for $\kappa_B = 10$, $\chi_B = 1000$, $\eta_B = 10$, $\kappa_C = 0.1$, $\chi_C = 100$, $\eta_C = 10$, $\kappa_D = 10$, $\chi_D = 100$, $\eta_D = 10$. The case of a fully passive food chain (all consumers B, C and D are non-motile) is shown for comparison, limited to the biomass change of the top predator (D). While population dynamics become more complex in the presence of multiple trophic levels, an active response can still result in a significant enhancement in trophic transfer rates.
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APPENDIX A

Synergistic Effects of Chlorine and Antifouling Surfaces

The experiments on surface biofouling described in this paper, which resulted from a collaboration with Prof. Karen Gleason’s group in Chemical Engineering at MIT and was published in *Advanced Materials* in 2013, were conducted by me, taking advantage of the approaches and expertise developed in this thesis. Specifically, I contributed Figures 3 and 4 (and associated text and Methods sections) in this paper. This publication is included here with permission of all the authors.
Synergistic Prevention of Biofouling in Seawater Desalination by Zwitterionic Surfaces and Low-Level Chlorination

Rong Yang, Hongchul Jang, Roman Stocker,* and Karen K. Gleason*

Water scarcity affects one in three people in the world. With nearly 98% of the world's available water supply being seawater or brackish water, desalination has become an important means to address the scarcity of freshwater resources. Thin film composite (TFC) reverse osmosis (RO) membranes enable the removal of salt ions from seawater at room temperature by applying pressure to the seawater feed. TFC-RO has quickly become the dominating desalination method since its commercialization in the 1980s and is now used in nearly all RO desalination plants. TFC-RO is considered to have the greatest water permeability with high salt rejection rate. The bottleneck for TFC-RO to produce freshwater via seawater desalination at a comparable price to natural freshwater is severe membrane fouling, which impairs water permeation and salt rejection and thus reduces freshwater yield. Currently, marine biota and in particular bacteria are removed from the feed by pretreatment, the most energy-intensive (responsible for >36% of total plant energy consumption) and chemical-intensive step in a desalination plant and one that poses environmental risks to marine organisms when treated water is discharged back into the ocean. Fouling-resistant RO membranes would bring major improvements in energy usage, process reliability and lower the environmental impact of seawater desalination.

Zwitterions are a type of molecular structures with ultra-low fouling properties, demonstrated in applications ranging from bio-assays to artificial tissues, originating from the extreme hydrophilicity induced by electrostatic interaction with water molecules, which makes the replacement of surface-bound water molecules by foulants enthalpically unfavorable. However, the zwitterionic coatings fabricated so far are not sufficient in long-term antifouling applications due to the limited stability in real-world environments.

The major challenge in the surface modification of TFC-RO membranes is to implement antifouling chemistries without compromising salt rejection and high water flux. The limiting step for the transport of water and salt across membranes is the extremely thin (~100 to 200 nm) polyamide selective layer. Surface modification methods involving solvents or exposure to high temperatures can generate or enlarge the undesirable pinhole defects. Surface modification layers produce an additional resistance to water permeability. We have shown previously that the coatings on RO membranes should be 30 nm or thinner and thicknesses >100 nm are undesirable because they cause ~40% reduction in the water flux.

Recently, we showed that anti-biofouling coatings of various compositions can be grafted and directly deposited on commercial TFC-RO membranes via an all-dry process, called initiated chemical vapor deposition (iCVD). The low-temperature solvent-free processing leaves the delicate polyamide intact and thus maintains the high salt rejection. Water flux is maintained by utilizing ultrathin (30 nm) iCVD layers. However, these acrylate-based films do not resist the degradation by chlorine, the most prevalent disinfection agent in water treatment.

We report here a novel pyridine-based zwitterionic surface chemistry that displays significantly improved resistance against a variety of molecular foulants and improved tolerance to chlorine exposure as compared to acrylate-based analogues. The chlorine-resistant surface provides a new perspective for achieving long-term antifouling. The pyridine-based zwitterionic surfaces demonstrate a synergy with drinking-water-level chlorination (5 ppm), resulting in exceedingly high antifouling performance. Synergistic effects have often been observed in the interactions between pairs of molecules such as pairs of drugs or toxins, or pairs of surface properties, such as surface energy and roughness. However, to our knowledge, synergistic effects have not been specifically identified between a functional surface and a solution species. The chlorine-resistant antifouling surfaces are derived from ultrathin iCVD poly(4-vinylpyridine) (P4VP) and its copolymers. The vapor deposition allows the synthesis of insoluble cross-linked coatings as thin films directly on a surface in a single step. Enhanced durability results from cross-linking co-monomers and in situ grafting. The in situ reaction with 1,3-propanesultone (PS) vapors produces pyridine-based sulfobetaine zwitterionic functional groups, having a balanced surface charge. The iCVD synthesis is carried out at low surface temperature (20 °C) to produce robustly adhered, smooth, ultrathin layers (30 nm) directly on even delicate substrates, such as TFC-RO membranes without damaging them. Accelerated testing against marine...
Figure 1. Antifouling zwitterionic coatings applied onto commercial RO membranes via iCVD. ab) Cross-sectional SEM image of (a) bare and (b) iCVD coated RO membrane. Panel (a) shows the porous supportive polysulfone layer (colored in orange) beneath the nonporous, 200-nm-thick, selective polyamide layer of the RO membrane. In (b), the smooth top layer is the iCVD zwitterionic coating, which is grafted to the selective layer. c) AFM scan of coated membrane and (inset) bare membrane. Both surfaces are exceptionally smooth, with ~1 nm RMS roughness. d) N(1s) XPS high resolution scan of the iCVD P4VP as-deposited (blue) and derivatized by PS (red), demonstrating full conversion of pyridine to zwitterion. e) Salt rejection of bare and coated membranes. The comparable values of salt rejection indicate that the coating leaves the thin selective layer of the delicate RO membranes intact. f) Water flux through bare and coated membranes. Membranes coated with 30-nm functionalized copolymer maintain 86% of the original water flux. Error bars (ef) represent the standard deviations obtained with 3 parallel tests.

bacteria in multichannel microfluidic devices shows 100-fold reduction in biofouling on the coated surface compared to bare glass. The unique resistance of the pyridine-based films against degradation by chlorine allows a new synergistic approach to antifouling, which substantially enhances longer-term fouling resistance compared to either surface modification or chlorination alone, and has the potential to reduce or eliminate pretreatment of seawater, the most energy- and chemical-intensive step in desalination plants, and thus to reduce the cost of freshwater production and its collateral toxicity to marine biota. This approach can facilitate the rational design of the next generation of RO membranes and of antifouling strategies for desalination plants, and find additional utility on the hulls of ships and for submerged marine structures.

Ultrathin (30 to 300 nm) iCVD coatings are successfully grafted and deposited directly onto commercial TFC-RO membranes (Figure 1b), followed by the vapor phase derivatization (Supporting Information, Figure S1). The all-dry-processed coating conforms to the geometry of the underlying substrate (Figure 1b,c), because surface tension and dewetting are avoided. The root-mean-square (RMS) roughness of bare and coated RO membranes is 1.1±0.3 nm (Figure 1c, inset) and

Table 1. Comparison of the important characteristics of surface modification techniques for zwitterionic antifouling chemistries.

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Ultrathin (30 to 300 nm) iCVD coatings are successfully grafted and deposited directly onto commercial TFC-RO membranes (Figure 1b), followed by the vapor phase derivatization (Supporting Information, Figure S1). The all-dry-processed coating conforms to the geometry of the underlying substrate (Figure 1b,c), because surface tension and dewetting are avoided. The root-mean-square (RMS) roughness of bare and coated RO membranes is 1.1±0.3 nm (Figure 1c, inset) and
Advances in the monomers are retained in the iCVD films. Successful water flux. Taken together, these results cross-link density. have similar water flux as hioiopolymer density. Although water flux is reduced silicon wafer, which is monitored via in situ interferometry. This substrate-independent method allows simultaneous deposition on multiple substrates. This feature is used to simultaneously deposit on RO membranes and on a silicon wafer in order to achieve precise control of coating thickness. Water flux nearly intact (Supporting Information Figure S1). This high water flux is achieved with a solvent-free scheme.

The salt rejection of the surface-modified RO membranes is unaltered, confirming the nature of the solvent-free process (Figure 1c). This substrate-independent method allows simultaneous deposition on multiple substrates. This feature is used to simultaneously deposit on RO membranes and on a silicon wafer in order to achieve precise control of coating thickness. Water flux nearly intact (Supporting Information Figure S1). This high water flux is achieved with a solvent-free scheme.

In the spectra of functionalized P4VP (red) and copolymer 1 (orange) (Figure 2a), we observe new peaks at 1670 cm\(^{-1}\) and 1036 cm\(^{-1}\). These are attributed to the symmetric stretching of the N-N-C and C-O-C bonds, respectively. For the iCVD polymers PDVB (black), copolymer 2 (wine), copolymer 1 (magenta) and P4VP (blue), there is a decreasing trend in the area under the 716 cm\(^{-1}\) peak, a measure of the number of m-substituted aromatic rings in the DVB repeat units (Figure 2a). This is utilized to calculate compositions of the iCVD copolymers, which are confirmed by XPS surveys. The composition of the copolymers can be tuned simply by varying the flow rate ratios of 4VP and DVB monomers (Supporting Information, Figure S1). In the spectra of P4VP and copolymers, the strong peak at 1600 cm\(^{-1}\) is attributed to the C-C and C-N stretching vibrations in the pyridine ring (Figure 2c). This peak intensity increases with more P4VP repeat units (Supporting Information, Figure S1). FTIR spectra collected after the PS derivatization (Figures 2a and S1) confirm the formation of the pyridine-based sulfobetaine (Figure 2d) via ring-opening of PS, as evident by the appearance of a peak at 1036 cm\(^{-1}\) in the spectra of functionalized P4VP (red) and copolymer 1 (orange) (Figure 2a). This peak is attributed to the symmetric stretching of the SO\(_2\) group.

Therefore, pyridine-based zwitterionic structures designed to resist oxidative damages are successfully synthesized using the solvent-free scheme.

To evaluate the chlorine resistance of the iCVD films, we subject the functionalized homopolymer P4VP, copolymer 1 and copolymer 2 to treatment with a 1000 ppm solution of sodium hypochlorite and we acquire FTIR spectra after different treatment durations. From the spectra, we measure the areas under the 1600 cm\(^{-1}\) peak (Figure 2a) to quantify the functional retention of the zwitterionic structure: the strong peak intensity renders the quantification more accurate. The excellent chlorine resistance of copolymer 1 (4% DVB) is evident from the negligible changes in its spectrum after 2 (green) and 24 (gray) hours of chlorine treatment (Figure 2a). In contrast, homopolymer P4VP is rendered soluble by a 10-hour exposure, as shown by the absence of functional peaks in the FTIR spectrum (Supporting Information, Figure S3). Importantly, the addition of 4% DVB cross-linker produces a major increase in the resistance to chlorine, whereas additions beyond 4% result in minor additional resistance (Figure 2e). After 10000 ppm h exposure to chlorine, -94% and -99% pyridine functionalities remain in functionalized copolymers 1 (4% DVB) and 2 (17% DVB), respectively. Functionalized copolymer 1 is thus most desirable because it resists chlorine very effectively while leaving the water flux nearly intact (Supporting Information, Figure S2).

These observations are corroborated by dynamic contact angle measurements on functionalized P4VP and copolymer 1 before and after chlorine treatment, which yields a comprehensive evaluation of the effects of chlorine on the coatings, because the dynamic contact angles of coated surfaces are affected by coating chemistry, surface roughness, swelling, and surface chain reorganization. For the functionalized P4VP, before chlorine treatment we measure advancing and receding contact angles of 31° and 20°, whereas after 2000 h chlorine exposure these values become 48° and 18°, respectively (Supporting Information, Figure S4). These considerable changes in dynamic contact angles reflect the poor chlorine resistance of the functionalized P4VP films. In contrast, the advancing and receding contact angles of the functionalized copolymer 1 are 51° and 24°, respectively. In spite of the higher...
We demonstrate the anti-biofouling properties of the new surface chemistry both with dissolved foulants and with marine bacteria. Quantification of the surface adsorption of 1 mg ml\(^{-1}\) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) is conducted via quartz crystal microbalance with dissipation monitoring (QCM-D). BSA is a widely used test protein for antifouling studies.\(^{3,21}\) Analogous tests are carried out with a representative polysaccharide, 1 mg ml\(^{-1}\) sodium alginate, the major component of extracellular materials that lead to membrane biofouling.\(^{22}\) QCM-D tests reveal no adsorption of either foulant over 200 minutes on the functionalized copolymer 1 surface (Supporting Information, Figure S5). The thickness of the coating does not have an impact on the fouling resistance (Supporting Information, Figure S6), because the derivatization with PS is diffusion-limited and the zwitterionic moieties are only present in the top few nanometers.\(^{23}\) The consistent fouling resistance under low (PBS buffer) and high (2 M NaCl added to PBS buffer; corresponding to ~117,000 ppm NaCl) salt concentrations implies that the functionalized copolymer 1 surface is charge-neutral (Figure S6).

The good fouling resistance against dissolved chemicals leads us to test the surfaces against fouling by marine bacteria. We use both natural seawater samples and a culture of Vibrio cyclitrophicus, a species broadly representative of bacteria prevalent in coastal waters, from where seawater for desalination typically originates. The dynamics of bacterial attachment are studied in a microfluidic flow system and imaged with an inverted microscope equipped with a CCD camera.\(^{24}\) Images are extracted from full movies (Supporting Information, Movies) and quantified by image analysis. Microchannels of 600 × 100 μm rectangular cross-section are fabricated out of polydimethylsiloxane (PDMS) using standard soft lithography techniques\(^{25}\) and mounted on a microscope glass slide that has been coated with a ~300-nm-thick film of functionalized copolymer 1. Fresh seawater is harvested and used on the same day as the feed solution for the microfluidic fouling tests, without any pretreatment, through continuous injection at a rate of 2 ml min\(^{-1}\) (corresponding to a mean flow of...
velocity of $v = 560 \mu m \cdot s^{-1}$. Fabrication of multiple (2-4) microchannels on the same chip allows parallel, simultaneous experiments and thus a direct comparison of different treatments and the minimization of confounding factors. Because experiments lasting up to 100 hours reveal no discernable surface attachment (Supporting Information, Figure S7), Supporting Information, Movies 1 and 2), irrespective of surface conditions, we run accelerated fouling experiments with concentrated cultures of V. cyclitrophicus, grown overnight in artificial seawater and concentrated to an optical density $OD_{600} = 0.2$, $2 \times 10^6$ cells mL$^{-1}$ corresponding to early exponential phase. This bacterial concentration is 200 times that of typical seawater.

In the accelerated tests, the Zwitterionic coatings show much greater resistance to bacterial attachment than bare glass (Figure 3a,b,c). Supporting Information, Figure S8; Supporting Information, Movie 3). Feeding is quantified by time-lapse imaging of the surface, followed by image analyses to determine the number of attached cells and the percent surface coverage by bacteria. As the variables are time-dependent, the behavior at 5 hours and 12 hours will be discussed, but general conclusions apply also to the data at other times. Despite the intrinsic fouling resistance of glass surfaces, the number of attached cells on bare glass increases steadily over time, and exponentially after 50 minutes. After 5 hours,
the cell count over a 0.16-mm² area of the bare glass surface reaches ~7500 (Figure 1a), whereas it remains close to zero on the coated surface (Figure 1b). Defining a relative fouling index, $F_1$, as the fraction of surface coverage for the coated surface compared to the bare glass control, we find that $F_1$ decreases drastically over time for functionalized copolymer 1 and drops to ~0.01 after 5 hours (Supporting Information, Figure S8). This result demonstrates the exceptional fouling resistance of iCVD zwitterionic coatings, in particular in view of the fact that smooth, bare glass is already a rather good anti-fouling surface.105

The surfaces' antifouling effects are further boosted by low-level chlorination, resulting in a new synergistic approach against fouling made possible by functionalized copolymer 1's good resistance to chlorine (Figure 2a-c-f). We run additional, accelerated microfluidic tests, where the suspension of V. cyclitrophicus is amended with 5 ppm of sodium hypochlorite (Figure 3c-d-g-h; Supporting Information, Movie 4), a concentration comparable to the residual chlorine level in the USA national drinking water standards.110 To quantify the effect of chlorination we define a second fouling index, $F_2$, computed as the fraction of surface coverage in the presence of chlorination, compared to that in the absence of chlorination, for the case of a bare glass surface. Although chlorination overall reduces surface fouling, signs of fouling on bare glass in the presence of 5 ppm chlorine emerge after 5 hours ($F_2 = 0.45$; Figure 3a) and after 12 hours fouling is severe ($F_2 = 0.58$; Figure 3b). Therefore, chlorination at a level of 5 ppm is less effective than the zwitterionic coating in preventing bacterial attachment. However, the synergistic effect of the zwitterionic coating and chlorination dramatically increases fouling resistance over each treatment in isolation (Figure 3d-h). After 12-hour exposure to the V. cyclitrophicus suspension, the surface coverage is 33.3 ± 1.7% on bare glass in the presence of 5 ppm chlorine ($F_2 = 0.58$), 14.1 ± 1.4% on the coated surface without chlorine ($F_2 = 0.14$), and only 1.5 ± 0.4% on the coated surface in the presence of 5 ppm chlorine. The percent surface coverage in the synergistic treatment is 0.02% of that of a bare glass surface without chlorine, four-fold smaller than the prediction $(F_1 \times F_2)$ obtained if the effect was simply multiplicative.

To quantify the synergistic effect of the two antifouling strategies, we compute an anti-fouling synergistic index, $S$ (Figure 4a, inset). Synergistic indices have been used among others to describe the effects of multi-strategy anti-tumor treatments, where $S < 1$ indicates a synergistic effect in killing tumor cells by the different strategies in the treatment.131,132 Here we define $S$ as

$$S = \frac{F_1 \times F_2}{\text{Surface coverage}_{\text{control}}}$$

The temporal dynamics of $S$ (Figure 4a, inset) reveal values of $S < 1$ after ~400 minutes, and a subsequent steady decrease to ~0.1 after 900 minutes. No signs of saturation in the decrease are observed, demonstrating the long-term nature of the synergies. Values of $S$ over the first 5 hours are not reported because the surface chemistry alone reduces fouling to non-detectable levels.

Figure 4. Synergistic prevention of bacterial fouling by the combination treatment. a) Surface coverage by V. cyclitrophicus bacteria under different conditions. The synergistic treatment — integrating iCVD zwitterionic coating with low-level (5 ppm) chlorination — shows exceptional long-term anti-fouling activity even under accelerated biofouling conditions (i.e., dense bacterial suspensions), whereas the control conditions show immediate loss of fouling resistance. b) Viability of V. cyclitrophicus upon addition of chlorine at different concentrations. 1 ppm chlorine does not significantly impact bacterial growth, whereas 5 ppm chlorine reduces the optical density by 42%, but does not kill bacteria. Killing by chlorine is thus not the dominant factor in the success of the synergistic treatment. c) Mean swimming speed of V. cyclitrophicus, obtained by tracking of individual cells. Addition of up to 5 ppm chlorine does not significantly change the bacteria's swimming speed, suggesting that prevention of attachment is not due to a reduction of encounter rates with surfaces.
levels (i.e., $F_1 = 0$) during this time and thus the quantification of $S$ is not meaningful.

In the attempt to reveal the mechanism underpinning the synergistic effect, the cell-surface interaction is investigated by observing a single bacterium for its proliferation and motility on the surface for the different treatments (Figure 3i-q; Supporting Information, Movie 5). After 85 minutes, replication has occurred under all conditions (Figure 3i-n), at a mildly lower rate in the presence of 5 ppm chlorine (Figure 3m-n), suggesting that the low dose of chlorine has only small effects on cell growth. This hypothesis is supported by direct viability tests (Figure 4b), showing that the growth of V. cytychophicas (measured as the optical density of cell cultures) is negligibly affected by addition of 1 ppm chlorine and exhibits a 42% reduction with 5 ppm chlorine addition. Furthermore, tracking of individual cells shows that motility is not significantly affected by 1 ppm or 5 ppm chlorination (Figure 4c). Although growth in batch culture might differ from growth on a microchannel surface, taken together these results (Figures 4b and 4a) demonstrate that the observed antifouling and synergistic effect of chlorine are not based on killing of the bacteria. Instead, the primary difference among the three single-cell cases (Figure 3i-q) resides in the dependence of cell removal from the surface chemistry (Figure 3o-q), whereas bacteria remain largely attached to the bare glass surface; they are easily removed from the coated surface by ambient fluid flow, independent of the presence of chlorine. In particular, bacterial removal from the CVD zwitterionic coating occurs readily even under the low-laminar flow conditions within the microchannel (Reynolds number ~0.1).

We have demonstrated the ability of ultrathin, chlorine-resistant (CVD zwitterion copolymers to act as antifouling coatings and, based on their resistance to chlorine, we have proposed a novel, multi-strategy approach to antifouling, which hinges on the synergy between surface chemistry and chlorine resistance. The zwitterionic coating prevents the attachment of V. cytychophicas almost 100 times more effectively than glass after 5 hours (Figures 4b and 4a; Supporting Information, Figure S8), while chlorination, with concentrations as low as the regulated chlorine residue in drinking water, is able to enhance the long-term fouling resistance of the zwitterionic coating by 9.4-fold after 12 hours (Figures 3m-l and 4a), with no signs of saturation.

A key advantage of the zwitterionic coatings reported here is the substrate-independence of the vapor application process, which makes these coatings easily applicable to a broad range of surfaces. In particular, these coatings may be applied on the latest salt-rejecting layers, which resist exposure to chlorine, providing a path towards solving the desalination industry's bottleneck of the susceptibility of TFC-RO membranes to oxidative damage by chlorine. The surface treatment is benign, easily scalable, and compatible with the infrastructure in membrane industry, which gives rise to a stable, non-toxic and inexpensive ultrathin coating. The good fouling resistance and chlorine resistance of this coating can help eliminate the most energy- and chemical-intensive step (pretreatment of seawater) in a RO desalination plant and reduce the environmental impacts of brine discharge. This approach therefore promises to lower the price of freshwater in water-scarce countries, where desalination may serve as the only viable means to provide the water supply necessary to sustain agriculture, support personal consumption, and promote economic development.

Experimental Section

Film Deposition and Derivatization: All CVD films were deposited in a custom-built vacuum reactor (Sharon Vacuum), as previously described. All the chemicals were used as purchased without further purification. Silica (5) wafers (Wacker World, test grade) were coated with P4VP or the copolymer of 4VP and DVB without pre-treatment. Prior to deposition, commercial RO membranes (Toray Membrane System, TFC-HR) were cleaned with filtered nitrogen, and then treated with oxygen plasma for 1 minute and then placed in the reactor chamber. The glass slides were treated with trichlorovinylsilane (Aldrich, 97%), as described previously. CVD depositions, 4VP (Aldrich, 95%) and DVB (Aldrich, 80%) monomers were heated up to 50 °C and 65 °C in glass jars, respectively and delivered into the reactor using mass flow controllers (1150 MFC, MKS Instruments). Argon patch flow was metered into the reactor through a mass flow controller (1479 MFC, MKS Instruments) and the flow rate was varied to keep the residence time constant. Systematic variation of the flow rate ratios of the two monomers was performed to yield high-zwitterionic-percentage, yet chlorine-resistant films of poly(4-ethylpyridine-co-divinylbenzene) (PVD). Films were deposited at a flatment temperature of 250 °C and a stage temperature of 20 °C. Total pressure in the vacuum chamber was maintained at 0.8 Torr for all depositions.

In situ interferometry with a 633 nm HeNe laser source (DS Linphase) was used to monitor the film growth and deposit desired thicknesses on Si substrates. A more accurate film thickness on the Si wafer substrates was measured post-deposition using a J.A. Woollam M-2000 spectroscopic ellipsometer at three different incidence angles (65°, 70°, 75°) using 190 wavelengths from 315 to 718 nm. The data were fit using a Cauchy-Urbach model. After deposition, the PVD-coated substrates were derivatized as reported previously.

Permeation and Salt Rejection Tests: Tests of the coated/bare membranes were performed using a commercial dead-end membrane filtration unit (Sterlitech Corporation) with a nitrogen cylinder to supply feed pressure, which was kept at 700 psi for all tests. The flow rates of the permeate were determined using a 100 ml metered flask. For the salt rejection tests, 3500 ppm sodium chloride dissolved in deionized water was used as feed solution. A conductivity meter (CDH-152, Omega Engineering Inc.) was used to measure the conductivities of the feed and permeate to calculate the salt rejection.

Chlorine Resistance Tests: Samples subject to chlorine resistance tests were soaked in deionized water for 2 hours, to remove the surface absorbed PS molecules and loosely attached oligomers of 4VP. Samples were dried with nitrogen gas and soaked in aqueous solution of sodium hypochlorite with the concentration of 1000 ppm for various treatment durations. FTIR spectra and dynamic contact angle measurements were taken before and after with chlorine solutions.

Bacterial Adhesion Tests: V. cytychophicas was used as the model microorganism. Bacteria cells from freeze stocks were inoculated and grown overnight in artificial seawater at 30 °C to an optical density (OD) of 1 while agitated on a shaker (150 rpm). Cells were suspended in fresh artificial seawater and incubated at 37 °C on a shaker (180 rpm) until the optical density reached 0.2. The bacterial solution was then injected into the microfluidic channels at a constant flow rate of 2 μl min⁻¹, which corresponds to an average flow velocity of 560 μm s⁻¹. During the combination treatment, chlorine was directly added to the vessel containing the media with bacteria to a final concentration of 5 ppm. Note that in this case the images (Figure 3) were acquired at a certain time (1 hour) captured bacteria that have been exposed to chlorine for 1 hour.
Supporting Information

Supporting information is available from the Wiley Online Library or from the author.

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APPENDIX B

Spatial Control of Cell Attachment by Mucin Coatings

The heterogeneously coated surfaces described in this paper, which resulted from a collaboration with Prof. Katharina Ribbeck’s group in Biological Engineering at MIT and was published in *Biomicromolecules* in 2013, were prepared by me, taking advantage of the approaches and expertise developed in this thesis. Specifically, I fabricated microchannels with an arrangement of spatially localized hydrophobic surfaces, which formed the basis for the experiments on confined attachment presented in this paper. This publication is included here with permission of all the authors.
Cell Patterning with Mucin Biopolymers

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Supporting Information

ABSTRACT: The precise spatial control of cell adhesion to surfaces is an endeavor that has enabled discoveries in cell biology and new possibilities in tissue engineering. The generation of cell-repellent surfaces currently requires advanced chemistry techniques and could be simplified. Here we show that mucins, glycoproteins of high structural and chemical complexity, spontaneously adsorb on hydrophobic substrates to form coatings that prevent the surface adhesion of mammalian epithelial cells, fibroblasts, and myoblasts. These mucin coatings can be patterned with micrometer precision using a microfluidic device, and are stable enough to support myoblast differentiation over seven days. Moreover, our data indicate that the cell-repellent effect is dependent on mucin-associated glycans because their removal results in a loss of effective cell-repulsion. Last, we show that a critical surface density of mucins, which is required to achieve cell-repulsion, is efficiently obtained on hydrophobic surfaces, but not on hydrophilic glass surfaces. However, this limitation can be overcome by coating glass with hydrophobic fluorosilane. We conclude that mucin biopolymers are attractive candidates to control cell adhesion on surfaces.

INTRODUCTION

Adhesion of mammalian cells to synthetic materials is the first, necessary step in many cellular processes including migration, differentiation, and activation of the foreign body reaction, a process that leads to the rejection of implanted materials.1 Controlling the adhesion of mammalian cells to solid surfaces is thus of relevance for biomaterials science, as it can improve overall biocompatibility of implants and facilitate tissue formation in tissue engineering applications. As an example, spatial control of pericardium myoblast adhesion can accelerate the formation of contractile muscle tissue and facilitate the study of cell alignment2 and differentiation.3 Tuning cell adhesion to surfaces is also becoming increasingly important for cell biologists. Controlling cell shape and positioning on substrates has facilitated the study of tissue formation and organization, cell–cell interactions4 and has enabled well-controlled coculture experiments.5

One important aspect of cell patterning is the coating of substrates with cell-repellent molecules in spatially confined ways. Effective cell repulsion can be achieved with a number of different synthetic molecules including polyethylene glycol (PEG) and zwitterionic molecules.6 Additionally, surface-immobilized biopolymers such as hyaluronic acid, alginate acid or carboxymethyl-dextran, can also be repulsive toward cells. However, these molecules typically do not spontaneously adsorb on surfaces with sufficient density to exert effective cell repulsion. Common strategies to overcome this limitation are the addition of surface-anchoring groups to the cytophobic molecules,7–11 or the chemical activation of the surface to retain the cytophobic molecules to the surface for extended periods of time. These efforts can be time-consuming and costly. Surface grafting in particular is limited to few substrates such as gold or silicon and often requires the use of organic solvents, acids, and other hazardous chemicals.12 Hence, it is desirable to develop more efficient and biocompatible strategies to control the adhesion of mammalian cells to surfaces.

Mucin biopolymers constitute the main gel-forming components of the mucus barrier13 and are interesting candidates for regulating mammalian cell adhesion. Secreted mucins are densely glycosylated, thread-like molecules with random coil configurations,14–16 which can reach molecular weights of up to tens of MDa. The diversity of chemical groups provided by the protein backbone and glycan side chains enable mucins to adsorb to a number of different substrates, including polystyrene, gold, and silica. On polystyrene, which is used for flasks, multiwell plates, and tubes, mucins adsorb through their exposed hydrophobic protein core portions while protruding their hydrophilic and glycosylated domains into the aqueous solvent.17 This configuration results in highly hydrophilic18–20 and hydrated monolayers, with around 95% of the adsorbed mass being water.18 Of note is that mucin coatings are able to decrease the adhesion of certain bacteria,21–25 neutrophils, and fibroblasts,26–27 but their use in mammalian cells patterning has not been explored. Here, we investigate the potential of mucin-based coatings to pattern the attachment of mammalian cells.

UV- and photolithography-based patterning techniques such as micro stamping28,29 and local etching30–32 or activation33 of cell-repellent polymer coatings are routinely used by cell biologist and tissue engineers. An even more accessible and low cost alternative to UV and photolithography is Micro Molding.
in capillaries, which is based on placing a mold (often polydimethylsiloxane (PDMS)) with geometrically defined open channels on a surface. Cell-repellent molecules are distributed through the channels, adsorb to the surface, and leave behind patterned cell-repellent coating once the mold is removed. Here we combine this relatively simple patterning technique with the use of mucin biopolymers to explore novel protocols for cell patterning.

Our work shows that mucin can readily adsorb to hydrophobic substrates and prevent cells from adhering to, and spreading on, these substrates. Using a simple microfluidic device to pattern mucin coatings, precise control of cell positioning and attachment can be achieved. Moreover, the mucin pattern can be maintained over 14 days in serum-containing media and is stable enough to spatially confine myoblasts during their differentiation into myoblast over a 7-day culture. Last, the repulsive effect toward cells is likely mediated by mucin-associated glycans because their removal eliminates the cell-repellent effect. Although cell-repellent mucin coatings could not be generated on hydrophilic glass surfaces, modifying the glass surface with a hydrophilic fluorosilane layer before generating the mucin coating recovered the effect. This makes the technology interesting for a wide variety of materials and applications.

**MATERIALS AND METHODS**

**Materials and Reagents.** Commercial bovine submaxillary mucin (BSM, Sigma-Aldrich, lot 0397003) and pig gastric mucin (PGM, Sigma-Aldrich, lot 116K7560) were dialyzed against water (Spectra/ Por dialysis membrane; 100 kDa molecular weight cutoff; Spectrum Labs) to remove impurities before lyophilization for storage. Skim milk (Becton Dickinson), fibronectin (Sigma-Aldrich), bovine serum albumin (BSA, Sigma-Aldrich) and lysozyme (Sigma-Aldrich) were used as received. Pig gastric mucin was purified in lab as reported. 

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**Generating Micropatterned Mucin Coatings.** The PDMS molds were fabricated using SU-8 (MicroChem, MA, USA) using conventional photolithography. The masters used for patterning had recessing features, which resulted in PDMS replicas with the opposite sense. To assist in removal of cured PDMS from the masters, the SU-8 masters were silanized by exposure to the vapor of 1,3,5-triethoxy-1,3,5-trimethylbenzene, CH₃(OC₂H₅)₂Cl, overnight. To cure the PDMS prepolymer, a mixture of 1:1:1 silicon elastomer and the curing agent was poured on the master and placed at 65 °C for 2 h. The PDMS prepolymer was then peeled from the silicon masters.

**Characterization of Apo-Mucin Coatings by QCM-D.** Quartz crystal microbalance with dissipation monitoring was used (QCM-D, Biotector, Sweden) to characterize the apomucins. The crystals used were purchased coated with polystyrene (QCM-D, Qsense, Sweden). All QCM-D experiments were carried out in no-flow conditions. The crystal vibration was followed at its fundamental frequency (about 5 MHz) and the six overtones (15, 25, 35, 45, 55 and 65 MHz). Changes in the resonance frequencies, and in dissipation of the vibration once the excitation was stopped, were followed at the
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seven frequencies. Shifts in frequency are related to changes in adsorbed mass (due to adsorbed polymer, water and ions), whereas changes in dissipation are influenced by the mechanical properties of the adsorbed layer. The mucin coatings are highly hydrated, hence, the dissipation values are significant, and the Voigt model, which relates frequency to the adsorbed mass through a linear relationship does not apply. The Voigt based model was used to accurately estimate the hydrated thickness. The density of the adsorbed layer was fixed at 1050 kg m$^{-3}$, which lies between that of pure water (1000 kg m$^{-3}$) and pure protein (1150 kg m$^{-3}$). This approximation was validated for a related system and was reported to have minimal impact on the final modeled thickness.

Fluorosilane Coating. Glass microscope slides were coated with fluorosilane as reported. In brief, microscope slides (VWR Scientific) were sonicated in a 4 vol % Micro-90 cleaning solutions (International Product) for 15 min, which was followed by two subsequent steps of sonication in Milli-Q water. After being dried with compressed air, glass slides were placed and sealed in a Teflon canister with one drop of 1H,1H,2H,2H-perfluoro-decyl-trichloro-silane (Sigma-Aldrich). The Teflon canisters were in a sealed glovebox under dry nitrogen atmosphere during the fluoroalkyl silane treatment, and then placed in a beaker overnight at 110 °C.

Microscopy. For imaging, the coatings were submerged in PBS (pH 7.4), using an Observer Z1 inverted fluorescent microscope (Zeiss, Oberkochen, Germany) and 100 × 0.3 NA objective (Zeiss, Germany). Image analysis was performed with built-in plug-ins of the ImageJ software.

Quantification of Mucin Adsorption. To measure the quantity of adsorbed BSM on polystyrene, glass and fluorosilane coated glass surfaces, a 50 μL drop of fluorescently labeled mucins (lmg/mL in PBS) was placed onto each surface and the mucin was allowed to adsorb for 30 minutes. The slides were rinsed and kept in PBS for observation. Each surface was prepared in duplicate, and 10 images were taken per condition. The fluorescence intensity was averaged over all images taken per surface.

RESULTS AND DISCUSSION

Mucin Coatings Prevent Adhesion of Several Cell Types. To test whether mucins are suitable substrates to regulate cell adhesion in vitro, we generated mucin coatings by placing a 0.1% mucin solution in contact with a polystyrene surface. In addition, those cells that attached to the mucin coatings were typically rounder in shape than the cells on the uncoated surface. A simultaneous calcein and EthD-1 staining suggests that the cells on the mucin coatings were still alive (Figure 1A), and that the mucin coatings were not toxic toward the cells.

A quantification of adherent cells showed that both BSM and native in-lab purified PGM coatings reduced adhesion of all three cell types by nearly 90%, compared to polystyrene (Figure 1B). In contrast, the common BSA layer albumin (BSA) and skim milk (Figure 1B) did not significantly reduce cell adhesion. These data show that BSM and in-lab purified PGM biopolymers are both excellent candidate molecules to create cell-repelling coatings. Of note is that commercial PGM (from Sigma-Aldrich) did not exert the cell-repulsive effect that was observed with the other mucin types. We speculate that the industrial purification procedure may result in an altered biochemistry of the PGM, which compromises their function. Indeed, as opposed to in-lab purified PGM, Sigma PGM have lost their ability to efficiently gelate at low pH.

Spatially Defined Micropatterned Mucin Coatings with Durable Cell-Repulsion. Based on these robust antiahesive properties, we tested the potential of mucin coatings for cell patterning applications. For this purpose, we adapted micromolding in capillaries technology to generate BSM coatings. In brief, we used a microfluidic channel that contained chemical removable posts, which blocked mucin adhesion to the polystyrene surface at designated regions (Figure 2A). The mucins were flown into the channel where they adhered to the unmasked regions of the polystyrene bottom. Then the posts were removed to uncover the uncoated surface. With this technique, patterns of various shapes and dimensions (between tens and hundreds of micrometers) could be generated in less than 30 min. Figure 2B shows different geometries of mucin coatings in which fluorescently labeled mucins were used for visualization by epifluorescent microscopy (Figure 2B). When cultured with cells, the mucin-coated areas remained distinctly cell-free, while the mucin-free areas were densely populated by all three cell types tested (Figure 2C,D,E). The sharp transition between cell-covered and uncovered zones suggests that the cell-repulsive effect of the patterned mucin coatings has high spatial accuracy. From a practical perspective it is interesting to note that the coatings can be dried and rehydrated without losing their cytophobic effect (see Supporting Information 2). This is particularly
Figure 2. Mucin coatings for cell patterning. (A) Mucin coatings were patterned using a microfluidic device with posts masking defined areas of the surface. (B) Patterns of mucin-free areas (in black) can be generated in different sizes and shapes. When seeded on the patterned surfaces, the epithelial cells (C), fibroblasts (D) and myoblasts (E) accumulated in the uncoated regions, avoiding the mucin coatings. Scale bars: 250 µm.

Figure 3. Mucin coatings and their cytophobic effect are robust. (A) Mucin patterns immersed in cell culture media for 14 days showed no alteration in their shape or fluorescence intensity. C2C12 myoblasts that were cultured on the coatings for 7 days expressed troponin T, which is a marker for early myogenic differentiation. (B) Actin staining reveals that cells are confined within the pattern. A Troponin T/Actin overlay is shown in (B'). Scale bars: 250 µm.

important if these surfaces are to be sterilized and stored before use.

To test the stability and functionality of mucin coatings over time, we monitored patterned fluorescent mucin coatings over 14 days in cell-free culture media. The patterns showed no obvious signs of deterioration; both the fluorescence intensity and the sharp edges of the patterns remained stable (Figure 3A, A' and A''). To test for loss of cytophobicity over time, C2C12 myoblast cells were seeded on the patterned coatings and monitored over 7 days. Over this period, the cells remained confined within the mucin-free regions (Figure 3B'), which suggests that the cell-repulsion effect was maintained despite the tendency of C2C12 cells to migrate and secrete extracellular matrix and proteases. Immunofluorescence for Troponin T, an early marker for myotube differentiation (Figure 3B'), and actin (Figure 3B') showed that troponin T-positive myotubes emerged after 7 days. These data suggest that in standard cell culture conditions, mucin coatings preserve their cytophobic effect toward C2C12 cells, while allowing for basic cellular differentiation.

Mucin-Linked Glycans Contribute to Cell-Repulsion. To better understand the molecular origin of mucin-mediated cell-repulsion, we focused our attention on the features that distinguish mucins from other proteins such as albumin that do not generate cell-repellent coatings. Mucins are largely linear molecules that are densely coated with O-linked glycans. We hypothesized that mucin-associated
poly(ethylene glycol) (Figure 5A). Reduced mucin adsorption on glass was correlated with a lack of cell repulsion compared to the coatings on polyethylene (Figure 5B). This finding is in accordance with previous studies, which show that mucin adsorbs in thick and strongly bound layers on hydrophobic substrates, while layers can be sparser on hydrophilic substrates. Since glass is more hydrophilic than polyethylene, surface chemistry could be the main reason for the inefficient mucin adsorption. If this is the case, it should then be possible to restore efficient mucin adsorption by rendering the glass surface hydrophilic. We coated the glass with fluorocarbons using plasma treatment and found that mucin adsorption increased (Figure 5A). Moreover, the presence of the fluorocarbons coating restored the cytophobic effect, with cells excluded from mucin coated areas (Figure 5B).

## CONCLUSIONS

We have shown that mucins, the basic constituents of the mucus barrier, are excellent biological substrates for the generation of spatially precise and patterned coatings to control cell adhesion and differentiation. Expanding on earlier reports on mucins' ability to repel mammalian cells, we have presented an analysis of the cytophobic effect of mucins against a range of different cell lines, their use in microfluidic-based cell patterning, and the molecular mechanism responsible for the cytophobic effect. Mucin coatings present a fast, accessible, cheap, and effective way of controlling cell adhesion, and provides great flexibility when coupled to patterning techniques. From cell biology to biomaterial and tissue engineering studies, these coatings could be used to control cell adhesion with high spatial resolution. Importantly, robustness against drying will allow these coatings to be stored and sterilized. Beyond these technological applications, these results are intriguing from a basic science perspective as they lead to interesting questions regarding cell-mucus interaction in vivo. For example, our results suggest that interaction between mammalian cells and the natural mucus matrix could be independent of adhesion, and thus very different from connective tissue extracellular matrices.

## ASSOCIATED CONTENT

### Supporting Information

Supporting Information for this manuscript includes mucin (both PGM and BSM) adsorption kinetics on polyethylene surface as measured by QCM-D (SI 1), measurement of cell repulsion effect of the BSM coating after a dehydration/rehydration cycle (SI 2), and a table containing the modeled viscoelastic parameters of the QCM-D data fitting (SI 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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