Strategies Underlying Bacterial Survival on Surfaces

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IMA AVALOS VIZCARRA

Dipl. Biol. t. o., University of Stuttgart

born on 11.05.1983

citizen of Germany and Perú

accepted on the recommendation of

Prof. Dr. Dr. h. c. Viola Vogel, examiner
Prof. Dr. Martin Ackermann, co-examiner
Prof. Dr. Bradley Nelson, co-examiner

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Most bacteria adhering to surfaces within the human body contribute to healthy body functions, while some bacteria exploit their host for their own purposes, which defines them as pathogens. Natural mechanisms to prevent the survival of these pathogens include specialized host cells that search pathogens, adhere to them, and ingest them for clearance. Engineered strategies against pathogens aim to prevent adhesion of bacteria or their growth by antibacterial drugs. Since the interactions between the host and the pathogen are in a dynamic and sensitive equilibrium, the factors that maintain and shift this equilibrium need to be understood to actively devise new strategies against pathogens. So far, bacteria still manage to escape the many approaches directed against them despite careful and rational design of antibacterial drugs.

The aim of this thesis is to gain insight on specific bacterial strategies that help bacteria to survive despite natural and man-made anti-bacterial approaches and which thus have the potential to shift the sensitive equilibrium between host and bacterium. To identify if bacteria could defeat previously described, highly efficient antibacterial drugs, antibacterial surfaces which kill bacteria upon contact were
produced and tested, and an improved in situ method to monitor bacterial survival on surfaces was introduced. It was found that these surfaces were indeed highly efficient in killing bacteria but that they would fail under conditions inside the host, since host fluids could mask the antibacterial drugs that were immobilized on the surface (Chapter 2). The results from this study indicated that host protein adsorption is an important aspect of bacterial surface interactions which can benefit bacterial survival.

Antibiotics are another set of powerful antibacterial drugs that prevent bacteria from multiplying by the bacterial cell division process but bacteria can adapt to escape their effects as well. Many approaches have been described of how bacteria actively counteract the effects of antibiotics. Here we could identify how bacteria managed to exploit a side effect of antibiotics to accelerate their colonization of surfaces: It was found that bacteria could turn the detrimental block of cell division which led to their filamentation into a kinetic advantage when they colonized surfaces that featured adhesive spots in a nonadhesive background. Bacteria continued to grow without completing their cell division, and could thereby bridge the nonadhesive surfaces faster than bacteria that had not been treated with antibiotics (Chapter 3). This study identified unwanted consequences of the use of antibiotics that had not been described before regarding their impact on bacterial surface colonization.

Among the biological antibacterial strategies, macrophages are specialized cells of the innate immune system that recognize foreign bodies such as bacteria and internalize them for intracellular degradation. By closely investigating the impact of adhesion efficiency on bacterial survival, it was found that the adhesin type 1 fimbriae of *Escherichia coli* sped up the adhesion to and internalization by macrophages which had an important consequence for its survival: *E. coli* could escape the effect of antibiotics by accelerating the entry into the intracellular environment, despite a simultaneous higher risk of being degraded intracellularly. Particularly for pathogenic bacteria with additional virulence factors and survival strategies, the accelerated adhesion and internalization can be a good opportunity to increase their survival chances in cases when they would otherwise be killed by
extracellular antibiotics.

The defensive act of foreign body internalization for clearance by macrophages is a long-known but still incompletely understood process. To better understand the antibacterial approaches of the host, a recently developed, highly precise magnetic trap was used to study the dynamic macrophage response to foreign bodies. Using magnetic micrometer-sized particles as prey for macrophages, the dynamic mechanical manipulation in terms of pulling, pushing and rotational motions that macrophages exert during the pick-up of their prey could be observed and recorded with high resolution (Chapter 5). The observations made during this study led to the question if particularly the rotation during particle pick-up is important in the interaction between foreign bodies such as pathogens and the host immune system. The high spatio-temporal resolution of the method in particular allowed to investigate for the first time if a ”wiggling-out” strategy could be employed by the model systems for pathogenic microorganisms which would help the prey to evade uptake by immune cells (Chapter 6). Such strategies could also emerge as new applications in modulating immune response or strategies for improved drug delivery by turning mechanical constraints into a constructive therapeutic use.
La plupart des bactéries adhérant aux surfaces internes du corps humain contribuent au bon fonctionnement de l’organisme, d’autres, dites pathogènes, exploitent leur hôte à leurs propres fins. Les mécanismes naturels pour empêcher la survie de ces bactéries pathogènes, comme certaines cellules-hôtes spécialisées, recherchent ces éléments pathogènes et y adhèrent afin de les ingérer complètement. Les stratégies développées pour lutter contre ces pathogènes consistent à empêcher, soit l’adhésion des bactéries, soit leur croissance à l’aide de médicaments antibactériens. Etant donné l’équilibre dynamique et fragile des interactions entre l’hôte et les agents pathogènes, les facteurs responsables du maintien ou de la perturbation de cet équilibre doivent être compris afin de mettre sur pied des nouvelles stratégies de lutte contre ces éléments pathogènes. Jusqu’à présent, les bactéries arrivent toujours à échapper aux nombreuses approches faites à leur encontre, et ce, malgré une conception prudente et rationnel des médicaments antibactériens.
Le but de cette thèse est de mieux comprendre les stratégies bactériennes spécifiques qui permettent aux bactéries de survivre malgré les démarches antibactériennes, naturelles et artificielles, ayant la capacité de perturber l’équilibre s’étant créé entre l’hôte et la bactérie. Afin de déterminer si les bactéries réussiront à résister aux très efficaces médicaments antibactériens décrits précédemment, des surfaces antibactériennes, ayant la propriété de tuer les bactéries au moindre contact, ont été fabriquées et testées. Un procédé in situ amélioré permettant l’observation de la survie des bactéries a également été mis en place. Il a été constaté que ces surfaces étaient en effet très efficaces pour tuer ces bactéries mais pourraient échouer, sous certaines conditions, à l’intérieur de l’hôte en raison des fluides corporels, masquant les médicaments antibactériens déposés sur ces surfaces (Chapitre 2). Les résultats de cette étude montrent que l’absorption par l’hôte de protéine est un aspect important des interactions bactérie-surface, pouvant aider à la survie bactérienne.

Les antibiotiques sont un autre type de puissant médicament antibactérien, qui empêchent les bactéries de se multiplier via la division cellulaire, mais les bactéries peuvent également s’adapter afin d’éviter ces effets. Différentes démarches ont été décrites, sur les méthodes utilisées par les bactéries éviter les effets des antibiotiques. Ici, nous avons pu identifier comment les bactéries arrivent à exploiter les effets secondaires des antibiotiques afin d’accélérer la colonisation des surfaces: Il a été constaté que les bactéries peuvent tourner à leur avantage les effets indésirable de la division cellulaire qui provoquent leur filamentation, en un atout cinétique, lorsqu’elles colonisent des surfaces contenant des zones adhésive dans un milieu non adhésif. Les bactéries poursuivent leur croissance malgré une division cellulaire incomplète, et par conséquent peuvent combler les zones non-adhésives plus rapidement que des bactéries ayant été traité à l’aide d’antibiotiques (Chapitre 3). Cette étude identifie les conséquences indésirables de l’utilisation des antibiotiques, non-décrits précédemment, sur la colonisation bactérienne des surfaces.

Parmi les stratégies antibactériennes biologiques, les macrophages sont des cellules spécialisées du système immunitaire inné qui reconnaissent les corps étrangers, tels que les bactéries, et les internalisent afin de réaliser une dégradation
intracellulaire. En étudiant de près l’impact de l’efficacité de l’adhésion sur la survivie bactérienne, il a été constaté que l’adhésine type 1 fimbriae d’Escherichia coli a accéléré l’adhésion et l’internalisation par les macrophages, ce qui a eu d’importantes conséquences sur sa survivie: E. coli pouvait échapper à l’effet des antibiotiques en accélérant sa pénétration dans l’environnement intracellulaire, et ce, malgré un risque très élevé d’être dégradé simultanément au niveau intracellulaire. En particulier pour les bactéries pathogènes avec des facteurs de virulence et des stratégies de survie supplémentaires, l’adhésion et l’internalisation accélérée peuvent être une bonne technique pour augmenter leurs chances de survie, notamment dans le cas où elles auraient autrement été tuées par les antibiotiques extracellulaires.

L’acte défensif d’internalisation d’un corps étranger pour sa dégradation par des macrophages est un procédé connu de longue date, mais reste partiellement incompris. Afin d’améliorer notre compréhension sur les approches antibactériennes de l’hôte, un piège magnétique, récemment développé et très précis, a été utilisé pour étudier la réponse dynamique des macrophages au contact de corps étranger. En utilisant des particules magnétiques, de l’échelle du micromètre, comme proie pour les macrophages, la manipulation mécanique dynamique, comme l’action de tirer, pousser ou mettre en rotation, que les macrophages exercent lorsqu’ils se saisissent de leurs proies a pu être observé et enregistré en haute résolution (Chapitre 5). Les observations réalisées durant cette étude ont abouti à la question de savoir si les mouvements de rotation durant la saisie de la particule est un aspect important dans l’interaction entre le corps étranger, tel que les pathogènes, et le système immunitaire de l’hôte. La haute résolution spatio-temporelle, de cette méthode en particulier, a permis d’examiner pour la première fois si la stratégie du « tortillement » est une stratégie envisageable pour des microorganismes pathogènes, qui permettront aux proies d’échapper aux cellules immunitaires (Chapitre 6). De telles stratégies pourrait aussi aboutir sur de nouvelles applications dans la modulation de la réponse immunitaire ou dans des stratégies pour l’amélioration de l’administration de médicaments en utilisant ces contraintes mécanique dans un but thérapeutique constructif.
1.1 Bacteria know us inside out: the human body as substrate for bacterial life

Bacteria are single-celled organisms on the scale of micrometers that live on the surfaces of our skin, organs and tissues [2, 3, 4]. The majority of bacteria in the human body collaborate with the metabolism and are not perceived as threat by our built-in defense mechanisms, the innate immune system [5, 6]. Pathogenic bacteria on the other hand have means to infect, invade, and exploit their host and survive within host cells [7, 8, 9, 10]. To reach a well shielded intracellular location, these pathogens devise sophisticated strategies without alarming the immune response, e.g. by adhering to host proteins and thus covering themselves to prevent recognition of their own surface markers [11]. To increase our understanding of
the interactions between bacteria and their adhesion substrates includes also the
dynamic interactions with the immune system and can yield important points of
attack for antibacterial approaches [12].

Bacterial life on surfaces and thus adhesion to surfaces plays a key role during a
bacterial infection of technical surfaces such as implants [13]. This is especially
relevant in the clinical context, since many implant materials provide unintentional
adhesion sites for bacteria by the physico-chemical properties of these materials
[14]. Surface charge, hydrophobicity, or topography as well as the environment
of the material facilitate adhesion of bacteria [15, 16, 17, 18]. Antibacterial ap-
proaches have focused on designing surfaces which can kill bacteria by disrupting
their membrane integrity [19, 20, 21, 22] or preventing the adhesion of bacteria
[23, 24]. However, adhesion sites can also be generated by defects in engineered
surface coatings that would otherwise prevent bacteria from adhesion [25, 26, 27]
or by the interaction with host fluids that change the surface properties [28, 29].

1.1.1 Bacteria colonize host cells and tissues and some can
survive within their hosts

Adhesion as such can be seen as a double-edged sword for bacteria: on the one
hand, it is an opportunity for bacteria to achieve larger retention times in the body
and profit from host nutrients. On the other hand, adhesion is a commitment to a
fixed location towards which a localized and amplified immune response can be
directed that may clear bacteria efficiently [30, 31]. It was found that many bacteria
circumvent recognition by the host by having evolved mechanisms to invade the
living surfaces of their host. This includes hiding within cells or building protective
shields around themselves after the surface colonization has been accomplished,
such that they are not directly exposed to the immune system of the host anymore
and evade the risk of clearance [32].

Just as adhesion can have ambiguous benefits for bacteria, the bacterium itself can
be helpful or harmful and many bacteria can be both. Bacterial traits that enable
them to exert pathogenic behavior are called virulence factors. One example of
a bacterium that can switch between the sides of good and bad, is Escherichia
coli (from here on: E.coli). Having originally been isolated as part of the healthy digestive system of the gastrointestinal tract [33], it was later found that variants of the same bacterium are also involved in minor and major health threats [34]. Pathogenic strains of E.coli can cause infections of the gastrointestinal tract, the urinary tract, the bloodstream and the central nervous system that range from acute over chronic to lethal[34, 35]. Switches from helpful to harmful behavior have been described for many other bacteria as well [36, 37]. In general, such large differences in virulence of an apparently identical organism can be explained by

- genomic plasticity, i.e. differences in the functionalities that are encoded by the genes, e.g. via acquisition of genes that encode pathogenic functions. The genomic differences can e.g. be used to diagnose clearly pathogenic strains of one bacterial species

- differences in how the genetic information is used, i.e. the expression levels of the same genetic information, a phenomenon that is summarized under the term phenotypic heterogeneity of isogenic individuals. Phenotypic heterogeneity can depend on the environment of bacteria and result in transiently pathogenic bacteria that are difficult to track and diagnose

Based on its dual role for its host, E.coli continues as an important model system of a highly diverse and adaptable bacterium with large relevance for health. In particular, the adhesive properties of pathogenic and non-pathogenic E.coli strains were shown to impact a large range of diseases from acute infection to autoimmune diseases. Insights from studies on E.coli can serve both specific questions on disease mechanisms as well as parallels to the behavior and strategies of other bacterial organisms.

The strategies and virulence factors that pathogens employ to attack and invade their host can also be used by the immune system to recognize which bacteria mediate pathogenic and destructive functions [38, 39]. Specialized cells of the immune system recognize foreign bodies like pathogenic bacteria, e.g. by the exact adhesins that they produce to invade host cells (Figure 1.1). following the recognition, the immune cell internalizes the pathogen for intracellular degradation, i.e. clearance from the body. A particular health risk occurs when pathogens manage to reach and persist in niches within the immune system since they then become inaccessi-
ble to their predators as well as therapeutic approaches by drugs. This favors the emergence of chronic and recurring infections. Adhesins are at the direct interface between pathogens and the host and can serve both the attacker and defender during host pathogen interactions.

Next to strategies of evading the immune system, many bacterial pathogens identify molecular targets on host cells by which they can trigger internalization into the host cells [11, 41]. Such internalization can be a good opportunity for pathogens. Particularly attractive host cells are those that are not specialized on recognizing and killing bacteria, e.g. tissue cells of skin (epithelial cells) [42]. Pathogens that are internalized into e.g. epithelial cells via unsuspicious uptake routes have a lower risk of detection and clearance [42, 43]. One example for such an unsuspicious uptake strategy was found for pathogenic *Shigella* strains. *Shigella* produces proteins that it injects locally into the host cell and that bind to proteins used by the cell for anchoring themselves in the tissue, e.g. vinculin. When *Shigella* injects these proteins into the host cell, their binding to vinculin leads to changes in the cytoskeleton.
from which local membrane protrusions result. The bacterium is then engulfed by these membrane protrusions which allows its entry into the cell [44]. Intracellular location can furthermore protect bacteria from drugs that do not penetrate host cell membranes well during therapeutic approaches. Examples for such drugs are the antibiotics gentamicin, phosphomycin, or vancomycin [45, 46, 47]. Finally, if the pathogen achieved to reach the cytosol of a host cell, it gains access to the nutrients and resources of the cell [48, 49]. This makes the intracellular niche an attractive habitat for bacterial pathogens - as long as they can reach it without alarming the immune system.

1.1.2 The immune system responds to bacterial surface colonization with predatory immune cells

The clearance of potentially harmful material or organisms by specialized predatory immune cells (phagocytes) is an important task of maintaining the hosts’ integrity and thus health [50, 51]. The immune system of warm-blooded mammals such as humans consists of specialized organs and cell types that screen for foreign material and organisms. Subsequently, the foreign material is ingested (phagocytosed) and degraded intracellularly, in membrane-enclosed entities called phagolysosomes. Phagolysosomes contain harsh chemicals such as reactive oxygen and nitrogen species as well as acids [52]. Macrophages are one type of phagocytes that specialized on the recognition, uptake and intracellular degradation of their prey using the phagolysosomal pathway. Macrophages have been described as acting in the first line of defense of the body which they do by recognizing and ingesting their prey for intracellular degradation.

The recognition of pathogens by phagocytes such as macrophages is mediated by cell surface receptors that identify molecular targets on the surface of bacteria and trigger the engulfment into a membrane vesicle that matures into a phagolysosome. The phagolysosomal environment is characterized by acids, and aggressive chemicals such as reactive nitrogen and oxygen species which cause corrosive damage on the bacteria components. The adhesion of bacteria to macrophages is therefore expected to be a risk for their survival chances [53]. However, some pathogens
including strains of *E.coli* turn risk into opportunity and specifically target predatory cells such as macrophages. These bacteria have identified steps in the processes of phagocytosis and phagolysosome maturation that they exploit to escape the killing by macrophages [48, 54]. For instance, the uropathogenic *E.coli* strain J96 was shown to adhere to glycosylated macrophage cell surface receptors and cause a less aggressive response of macrophages, more specifically less reactive oxygen species were released by the macrophages and the bacteria-containing vesicles were less acidic than those containing bacteria that had been recognized via macrophage Fc receptors. As a result, *E.coli* J96 showed increased survival inside macrophages [54].

### 1.1.3 The adhesin type 1 fimbriae mediates bacterial colonization of mannosylated surfaces

Bacterial adhesins can be categorized based on their structure, i.e. whether they occur as monomeric proteins on the surface of bacteria or as multimodular protein complexes that can extend far out from the bacterial surface. The latter category includes the pilus-like adhesins that comprise the so far best studied bacterial adhesins, e.g. type 1 fimbriae, P-pili, type IV pili, and curli, which are implicated in host pathogen interactions and facilitate internalization into host cells [11] (Figure 1.2). Of particular medical interest are such adhesins that are found in both harmless and pathogenic bacteria as they might be suited for evasion of immune defense by mimicking harmless bacteria until the pathogen carrying them has achieved an intracellular localization as evasion of the host immune response [55].

Among the many adhesins that bacteria employ to gain access to host cells, type 1 fimbriae have been described as specifically matched for the infection and persistent colonization of the urinary tract [7]. Type 1 fimbriae are extracellular, membrane-anchored appendages that protrude from the outer membrane of *E.coli* like hairs. The shaft of type 1 fimbriae consists of many repeating units of the FimA protein, ending with the so called fimbrial tip which mediates the specific adhesion to the sugar mannose [57]. The adhesive unit of type 1 fimbriae is the terminal FimH protein that sits in the fimbriae tip and is a sugar-binding protein (lectin) that specifically recognizes the sugar mannose (Figure 1.1c, 1.1d). Mannose is a frequent
Figure 1.2: Overview of bacterial adhesins that target host cells and the specific case of the type 1 fimbrial adhesin of *E. coli*. a) The interactions between bacterial adhesins and host cell surface receptors are indicated by arrows. Adapted from [11] b) The type 1 fimbrial tip contains the lectin domain of the protein FimH that recognizes the type 1 fimbriae ligand mannose on host cell proteins. Adapted from [56] c) Schematic overview of the structural changes occurring in the FimH protein when force is applied. Adapted from [56].
component of the sugar chains that belong to many mammalian cell surface proteins (glycosylation). Accordingly, several host proteins to which fimbriae can adhere have been described, both on epithelial as well as immune cells.[11, 58, 59].

Type 1 fimbriae are a classical example of the adaptation of pathogens to a specific host environment. The environment of the urinary tract is inherently protected against adhesion of bacteria since a more or less continuous flow of urine washes away bacteria that pass by. Uropathogenic bacteria have evolved a molecular design principle that allows them to persist against this wash-off: a shear force-induced change in the three-dimensional arrangement of the FimH protein (Figure 1.1c, 1.1d). More exactly, an auto-inhibition by a regulatory domain of the FimH protein restricts the binding pocket of the lectin domain, keeping it in a more open, three-dimensional arrangement (conformation) of the ligand binding pocket. This results in a loose, easy to revert binding to its ligand mannose. When shear force acts upon the fimbriae, the FimH protein is stretched and the auto-inhibitory domain of the protein releases the lectin domain. As a result, a conformational change occurs in the lectin binding pocket that is better suited to fit its ligand, thus resulting in a stable, high affinity binding of mannose as long as shear force is exerted (Figure 1.1d).

Uropathogenic *E.coli* enters host cells in the uroepithelium after fimbria-mediated adhesion and can survive and replicate within as well as spread to deeper layers of the tissue. This spreading is enabled by some of the host cells committing cell death (apoptosis and necrosis) as a reaction to the bacterial assault. While such destructive reactions increase the risk of alarming the immune system, additional adhesion sites in the lower, uninfected levels of the epithelium are simultaneously generated. Uropathogenic *E.coli* then uses these additional adhesion sites to repeat the cycle of host cell infection and spreading [60, 61]. In this way, type 1 fimbriae mediated adhesion enables acute as well as chronic urinary tract infections [11].
1.1.4 Geometrical constraints and forces play an important role during the clearance of bacteria by their predators

The uptake of bacteria by phagocytes requires internalization, i.e. engulfment by the immune cell membrane. This engulfment is a mechanical and actively driven process of membrane protrusion around the target. Membrane protrusions are initiated and driven by the cellular cytoskeleton that orchestrates pushing and contractile movements by microfilaments of the actin protein, tubular structure made from microtubule proteins and pulling movements by motor proteins such as myosin, dynein and kinesin. Phagocytosis is an inherently mechanical process that underlies fine tuning and modulation by many force generating proteins [62].

1.1.5 The frustration of phagocytosis

Phagocytosis is a mechanobiological process and accordingly it underlies geometrical, mechanical as well as biochemical regulation. One of the geometrical constraints is the size of an object which can readily be observed from a phenomenon in the context of medical implants. In the so called foreign body reaction, phagocytes attack the surface of an implant which can exceed the size of single cells by a factor of thousand or more [63, 64, 65]. In this case it is observed that several immune cells fuse in the attempt of engulfing the whole implant and ultimately form a tissue capsule around the implant [65]. However, a complete internalization by the cells is not successful which is referred to as frustrated phagocytosis. Frustrated phagocytosis occurs also on the level of single phagocytic cells and in their interaction with pathogens. Here, an important constraint is the aspect ratio of a phagocytosis target since the engulfment of an elongated object requires a large amount of membrane material to be pushed around the object. Accordingly, the aspect ratio was found to be exploited by pathogenic bacteria to prevent their killing [66]. Particularly rod-shaped bacteria were found to modulate their elongation such that they reach very high aspect ratios when they grow into filamentous structures [67]. Yet another well defined constraint in phagocytosis is the shape, particularly in terms of curvature of an object. It was observed that changes from convex to
concave curvature on the same phagocytosis target can lead to such high tension of the phagocyte membrane that phagocytosis is aborted [68]. These results emphasize how the interaction of phagocytes with their prey can be modulated by geometrical and force-dependent factors.

1.2 Scope of this work

The work presented in this thesis is based on an innocently sounding insight:

Adhesion has a pivotal role in bacterial pathogenicity

To strengthen this statement with a thought experiment, let us imagine that a highly pathogenic bacterium enters the body of a human being, e.g. by sitting on food that is consumed by that human. If this bacterium or even several hundred of such bacteria could not find a single spot in the body where they could stick to, they would simply be transported out of the body again through the digestive system. Accordingly, bacteria could only cause disease and spread infection when they could adhere to host cell tissues and destroy the integrity of the host, e.g. by invasion. Only then could they settle in a niche, replicate, and spread to new habitats (where they again have to adhere to get a foothold).

Next to the site of adhesion, it is also important to consider the environment in which adhesion takes place. For instance, the human body itself offers a very complex, multifactorial environment that is often summarized under the term physiological conditions. Physiological conditions comprise the complete biochemical conditions that can be found in the body, e.g. the pH of body fluids or their composition, i.e. proteins, sugars, lipids and their molecular building blocks. Physical parameters such as temperature, viscosity, and shear flow are also factors in the physiological setting and can ultimately modulate the interactions with bacteria.

Consequently, the above considerations can also be used constructively by looking into therapeutic approaches once the underlying principles have been understood.
Figure 1.3: Scope of this work. Bacteria surface interactions from technical to biological surfaces. The contributions from the single chapters are indicated in magenta.
One example of such an approach is the effective treatment of urinary tract infection by mannose. Having understood that type 1 fimbriae mediate specific adhesion to the sugar mannose which is immobilized on host cells in their glycosylation (Fig. 1.2.c 1.2d), soluble mannose was used as competitive inhibitor as a useful therapeutic approach against urinary tract infections in the early stages [69]. Along this line of thought, the work described in this thesis builds upon existing technologies and projects that have been in the lab to follow up on the larger research question:

Can we use the lessons learned from bacterial survival on surfaces for future therapeutic approaches?

Here, "surface" is an overarching theme ranging from technical, engineered surfaces to the natural, dynamic membrane of host cells. The investigations on bacterial survival on surfaces started with a model for technical surfaces. Antibacterial surfaces were produced that kill bacteria upon contact by piercing their membrane, thereby mediating a so called bioactivity. To benchmark such surfaces, it was important to devise reliable means for reading out the viability kinetics of bacteria. Determining if a bacterium is dead is by no means a straightforward task because bacteria have been shown to persist in dormant states with very low metabolic activity that easily confounds activity readouts [70, 71]. A very prominent readout that has been largely exploited to determine the viability of a bacterium is the integrity of its membrane. Another very reliable sign of life with importance for bacterial pathogenicity is the ability of bacteria to replicate which occurs preferentially under favorable conditions. Due to the importance of adhesion in bacterial survival, it was important to develop assays of bacterial viability for their use on surfaces (Chapter 2). To test the impact of physiological conditions, the highly efficient antibacterial surfaces were put to the practical test by priming them with the body fluid of blood serum. Serum contains high concentrations of proteins. It was observed that the previously deathly surfaces could be turned into harmless, bacteria-friendly surfaces on which bacteria could replicate and grow as they were not in direct contact with the bioactive components anymore. Since these results were based on antimicrobial agents that were surface-immobilized and could be masked, in a second study, bacterial replication on surfaces was inves-
igated under the antimicrobial setting of soluble, bacteria-killing components such as antibiotics. In this study, surface-adherent bacteria were exposed to an antibiotic that does not allow them to replicate anymore. Interestingly, one bacterial survival strategy when hindered to replicate is to continue growth without the last step of cell division, which leads to an elongated, filamentous morphology in the case of the rod-shaped *E. coli*. It was found that such a morphology can give a kinetic advantage to surface-adherent bacteria for the colonization of surfaces particularly when these are only partly colonizable (Chapter 3).

Since adhesion plays an essential role in pathogenesis, we next asked if adhesion could impact bacterial survival during the battle with immune cells which are their opponents by default (Chapter 4). In this study, it was found that it was important to separate the quantity of binding from the quality of binding as the number of bacteria per macrophage (bacterial burden) could impact the bacterial survival chances days after infection. By furthermore identifying a role for kinetic constraints, mechanistic insight on bacterial survival strategies in relation to adhesion efficiency was gained. Although the assays performed in these studies were performed under *in vitro* conditions that only partly represent the situation in the body, the results on the kinetics could explain how highly pathogenic bacteria infect host cells even at very low infection doses by modulating their efficiency of adhesion to host cells.

One additional factor that comes into play when bacteria adhere to living matter such as host cells, is the ability of these living systems to react to the bacterial adhesion. In particular immune cells such as macrophages are trained to not only react biochemically (e.g. by releasing alarming substances like cytokines) but also to react mechanically by internalizing bacteria for intracellular degradation. To obtain a more comprehensive picture of the dynamic mechanical interaction of immune cells and their prey, a magnetic microparticle manipulation technique was used where magnetic particles served as model system for bacteria. The precise micromanipulation approach by a magnetic trap allowed to finely resolve spatial characteristics during the early phagocytosis events (Chapter 5). This approach also allowed investigation of a resistive force, here applied through the magnetic system, which mimics e.g. the resistive force of surface-adherent bacteria.

Inspired by this study and the knowledge that morphological parameters such as the aspect ratio of an object are important determinants of phagocytosis [72], this collaboration was extended to test a more dynamic scenario where a particle
could first be prevented from being rotated by the macrophage and secondly exert additional motion to potentially resist phagocytosis, mimicking e.g. a bacterium that exerts a momentum by swimming (Chapter 6). The results from this study suggest that rotational motion can be used to interfere with phagocytosis efficiency - which is both interesting as pathogen evasion strategy as well as for drug delivery approaches.

Summary: Factors that bacteria can exploit to colonize surfaces and survive

- shielding of antibacterial surfaces by host proteins (Chapter 2)
- exploiting the side effects of antibiotics (Chapter 3)
- accelerating escape to intracellular niche inside immune cells and escape the effect of antibiotics (Chapter 4)
- exerting forces to resist phagocytosis (Chapter 5 and 6)
CHAPTER 2

Fluorescence-based in situ assay to probe the viability and growth kinetics of surface-adhering and suspended recombinant bacteria

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Ima Avalos Vizcarra, Philippe Emge, Philipp Miermeister, Mamta Chabria, Rupert Konradi, Viola Vogel, Jens Möller. IAV, JM, MC, RK and VV designed research, PE provided eGFP expressing bacteria, PM developed semiautomatic image analysis software, IAV and JM performed research, IAV, PE, PM and JM analyzed data, IAV, PE, RK, W and JM wrote the paper. All authors read and approved the final manuscript.
2.1 Abstract

Bacterial adhesion and biofilm growth can cause severe biomaterial-related infections and failure of medical implants. To assess the antifouling properties of engineered coatings, advanced approaches are needed for in situ monitoring of bacterial viability and growth kinetics as the bacteria colonize a surface. Here, we present an optimized protocol for optical real-time quantification of bacterial viability. To stain living bacteria, we replaced the commonly used fluorescent dye SYTO®9 with endogenously expressed eGFP, as SYTO®9 inhibited bacterial growth. With the addition of nontoxic concentrations of propidium iodide (PI) to the culture medium, the fraction of live and dead bacteria could be continuously monitored by fluorescence microscopy as demonstrated here using GFP expressing Escherichia coli as model organism. The viability of bacteria was thereby monitored on untreated and bioactive dimethyloctadecyl[3-trimethoxysilyl]propyl]ammonium chloride (DMOAC)-coated glass substrates over several hours. Pre-adsorption of the antimicrobial surfaces with serum proteins, which mimics typical protein adsorption to biomaterial surfaces upon contact with host body fluids, completely blocked the antimicrobial activity of the DMOAC surfaces as we observed the recovery of bacterial growth. Hence, this optimized eGFP/PI viability assay provides a protocol for unperturbed in situ monitoring of bacterial viability and colonization on engineered biomaterial surfaces with single-bacteria sensitivity under physiologically relevant conditions.

Keywords: Antimicrobial surfaces; Optical viability monitoring; Green fluorescent protein (GFP); SYTO®9; Propidium iodide (PI)

2.2 Introduction

Clinically relevant nosocomial infections are frequently caused by adherent bacteria and the subsequent biofilm formation within tissues or on biomaterial surfaces [73]. Surface biofouling commonly starts with the adhesion of individual bacteria that subsequently grow into mature biofilms. To prevent bacterial adhesion and growth...
already during the pre-biofilm phase, two main surface engineering strategies have been developed so far: non-fouling “stealth” surface coatings that inhibit adhesion of proteins and bacteria [23, 74, 75] and bioactive materials, which upon bacterial contact or release of the active molecules interfere with bacterial viability [18, 19, 76, 77, 78, 79].

To compare the antimicrobial properties of surface coatings and to study the kinetics of bacterial surface colonization, assays are needed that allow for in situ monitoring of bacterial adhesion and viability. The gold standard for bacterial viability tests has long been quantification of colony forming units (CFU) by plating bacterial suspensions that were incubated with the test surface on nutrient agar [80]. Counting bacterial colonies, which result from plating suspended viable and cultivatable bacteria, however, does not account for the inherent phenotypic heterogeneity and the ability of the bacteria to persist in dormant states [81, 82]. Furthermore, plating assays lack the ability to measure the colonization and viability kinetics directly on the test surface and might not be representative for the surface-attached bacterial population.

An alternative to determine bacterial viability is to probe for the bacterial membrane integrity that is maintained by energy-dependent processes in living bacteria and is lost upon bacterial death [82]. Membrane integrity can be tested optically by using a combination of membrane permeable and impermeable fluorescent dyes that selectively enter live and dead bacteria (Figure 2.1a) [70, 78, 82, 83, 84]. While being broadly employed as endpoint staining assays to determine the viability of single bacteria and bacterial colonies directly on the test surface, these assays are not optimized for real-time in situ bacterial viability monitoring. Particularly when DNA intercalating dyes like SYTO®9 and propidium iodide (PI) are used [85], the impact of the potentially toxic stains on bacterial physiology has to be considered to avoid false negative results [86]. Furthermore, since both stains target DNA, the competitive displacement of the SYTO®9 (live stain) by the high affinity PI (dead stain) upon membrane breakdown can affect the staining reliability [87]. To eliminate the competitive displacement of the two DNA stains and the demand for prolonged incubation times caused by the passive diffusion of the SYTO®9 live stain through the bacterial membrane, it was suggested to replace SYTO®9 with green fluorescent protein (GFP) expressed by the bacteria as demonstrated previously for flow cytometry applications (Figure 2.1b) [88].
Figure 2.1: Bacterial viability assays in comparison. (a) Conventional endpoint dual staining bacterial viability assay and (b) eGFP/PI assay optimized for in situ bacterial viability monitoring. The dual staining assay commonly employs two DNA stains, SYTO®9 (green triangle) and propidium iodide (PI, red rectangle) that both intercalate into the bacterial DNA. SYTO®9 diffuses passively into living bacteria whereas PI cannot pass intact bacterial membranes and only enters permeabilized dead bacteria. The use of constitutive eGFP expression rather than SYTO®9 provides direct detection of viable bacteria without the addition of a fluorescent dye and circumvents the competitive displacement of SYTO®9 by PI that can result in a dual staining of dead bacteria.
Although flow cytometry has been used to measure the viability of GFP expressing bacteria adsorbed to polystyrene beads functionalized with antimicrobial coatings [89], it cannot be applied for continuous in situ bacterial viability monitoring on planar surfaces.

Building upon those observations, we present an optimized protocol to probe the viability and growth kinetics of surface-adhering and suspended bacteria using non-toxic concentrations of propidium iodide and Escherichia coli that express the fluorescent protein GFP. Beyond calibrating the assay and monitoring E.coli surface colonization kinetics on bare glass substrates, we demonstrate that this assay is applicable to monitor the inactivation kinetics of E.coli in contact with antimicrobial surface coatings, using dimethyloctadecyl[3-(trimethoxysilyl)propyl]ammonium chloride (DMOAC) coated glass surfaces as model substrate. To kill bacteria, the quaternary ammonium chloride complexes of surface-bound DMOAC have to directly interact with the bacterial membrane [90]. We previously showed that bacterial fimbriae strongly influence the unspecific adhesion of E.coli to engineered surfaces [75]. Type 1 fimbriae (7 nm diameter, several 100 µmeter length) protrude from the bacterial membrane thereby preventing the bulk bacterial body from direct interaction with the underlying material surface. To ensure a physical contact of the bacterial membrane with the material surface, we used here the non-fimbriated K-12 derivative AAEC191A E.coli strain. In addition, we highlight the effect of serum protein adsorption on the bactericidal properties of antimicrobial surfaces. We incubated the DMOAC surfaces with fetal bovine serum (FBS) to mimic the physiological situation where serum proteins adsorb to engineered biomaterials upon contact with host body fluids.

2.3 Results

2.3.1 Impact of SYTO®9 and propidium iodide concentrations on the growth of suspended E.coli

To determine toxicity levels of the fluorescent DNA stains, we probed the effect of SYTO®9 and propidium iodide (PI) on E.coli growth. We supplemented
bacterial batch cultures in M9 growth medium with varying dye concentrations (0, 3, 30 µM PI, 6 µM SYTO®9) and monitored bacterial growth at 37°C by quantifying the increase in the turbidity of the solution at 600 nm (Figure 2.2a). 6 µM SYTO®9 in combination with 30 µM PI, as recommended in the conventional and commercial dual staining assay [91], completely inhibited E.coli growth. Supplementing the E.coli suspensions with 6 µM SYTO®9 alone showed the same growth inhibition, while 30 µM PI itself did not inhibit growth completely but did reduce the growth rate compared to the pure M9 medium. This indicates that 6 µM SYTO®9 causes major changes in bacterial physiology. The impaired growth rate upon addition of PI was eliminated when we decreased the PI concentration in the bacterial growth medium tenfold, i.e. from 30 µM to 3 µM (Figure 2.2a). To confirm that the viable E.coli in the medium supplemented with 3 µM PI were able to replicate, we compared the turbidity increase of a 50% live / 50% isopropanol killed bacterial mixture to cultures containing 100% live and 100% isopropanol treated E.coli (Figure 2.2a).

Figure 2.2: Impact of SYTO®9 and propidium iodide (PI) concentration on E.coli growth rate and the detection efficiency of dead bacteria in M9 bacterial medium. (a) Inhibition of bacterial growth in the presence of 6 µM SYTO®9 and 30 µM PI. PI alone showed a dose-dependent growth inhibition. At a concentration of 30 µM E.coli growth was inhibited, which was not detected when the PI concentration was reduced tenfold from 30 to 3 µM. Replication of E.coli in 3 µM PI containing M9 medium was confirmed by growth rate measurements from a starting culture of 50% live / 50% dead E.coli (inset) (b) PI fluorescence of E.coli cultivated in M9 medium containing different PI concentrations. 3 µM PI sufficiently stained dead bacteria in a 50% live / 50% dead E.coli mixture, while no significant background signal increase was detected for 3 µM PI compared to the background for a 100% live bacterial solution. In contrast, supplementing the medium with 30 µM PI resulted in a significant increase of PI stained bacteria from a 100% live starting culture indicating that this high concentration of the DNA stain interferes with bacterial viability. Background fluorescence of PI supplemented M9 medium was subtracted for each of the three PI concentrations, respectively. Error bars represent the standard error of the mean.
Within 4 hours, the turbidity increase for the mixed 50% live / 50% dead starting culture did not reach the same level as for the 100% live culture. Those results are consistent with the expected exponential growth rate of viable bacterial batch cultures and thus show that the bacteria replicated normally in 3 µM PI containing medium. To determine if the reduced PI concentration was sufficient to detect dead bacteria in solution, we incubated bacterial batch cultures starting from either 100% live or 50% live / 50% killed bacteria in M9 medium containing 3 and 30 µM PI respectively. The PI fluorescence at 630 nm was subsequently measured by fluorescence spectroscopy over 3 hours (Figure 2.2b). Supplementing the growth medium with 3 µM PI adequately stained the isopropanol treated bacteria in the 1:1 mixture of live and dead E.coli but did not result in a significant increase of the background fluorescence of the 100% live starting culture. In contrast, supplementing the medium with 30 µM PI significantly increased the PI fluorescence from the 100% live starting culture (Figure 2.2b), which was consistent with the impaired growth rate under those conditions (Figure 2.2a), indicating that 30 µM but not 3 µM PI is toxic to E.coli bacteria.

### 2.3.2 Viability and growth rate of surface-adhering E.coli is strongly reduced upon long-term incubation in culture medium supplemented with SYTO®9

For viability and growth kinetic studies of surface-adherent bacteria, E.coli that unspecifically adhered to bare glass surfaces were incubated in M9 growth medium that contained either 3 µM PI or a mixture of 6 µM SYTO®9 and 30 µM PI (Figure 2.3a). For SYTO®9 containing medium, E.coli replication and surface colonization was completely blocked, as determined by time lapse video microscopy (Figure 2.3a, Additional file 1: Figure S1).

In addition to inhibiting bacterial growth, the viability of surface-adhering E.coli (AAEC191A), decreased for incubation times longer than 1.5 hours, as detected by two-channel fluorescence microscopy. In contrast, no decrease of viability or impaired growth was observed for endogenously eGFP expressing E.coli (AAEC191A pHis-GFP) counterstained with 3 µM PI (Figure 2.3a), which is in agreement
Figure 2.3: In situ monitoring of the growth and viability of surface-adhering E.coli using the eGFP / 3 µM PI assay. (a) Time series of surface-adhering E.coli on bare glass substrates. The dual staining assay (6 µM SYTO®9 / 30 µM PI) decreases bacterial viability on untreated glass substrates after incubation times longer than 1 hour as E.coli (AAEC191A) incubated with 6 µM SYTO®9 containing medium failed to replicate (inset). In contrast, eGFP-expressing E.coli (AAEC191A pHis-GFP) that were incubated with 3 µM PI were able to replicate and grow on the glass surface. (b) Viability of E.coli AAEC191A pHis-GFP on antimicrobial DMOAC-coated glass surfaces as monitored by eGFP/PI fluorescence microscopy. Pre-exposure of the DMOAC surfaces to fetal bovine serum (FBS) completely blocked the antimicrobial activity. Microscopy images show the overlay of the SYTO®9 / eGFP and PI fluorescence channels, i.e. differentiating live (green) from dead bacteria (red). 3 independent fields of view from different experiments were analyzed containing a total of 125 – 250 surface attached bacteria for each condition. Error bars represent the standard deviation. Scale bar 20 µm.
with the results from the batch culture experiments (Figure 2.2). In controls we confirmed that GFP expression itself did not perturb *E. coli* adhesion and growth (Figure 2.4a,b). Furthermore, the fraction of GFP fluorescent *E. coli* (87%) was not significantly different (\(\alpha = 0.05\)) for eGFP expression from the IPTG inducible pHis plasmid under the control of lac promoter (AAEC191A pHis-GFP) and under the control of a constitutive rpsm promoter (AAEC191A rpsm-GFP) (Figure 2.4c,d). To evaluate whether the eGFP/3 \(\mu\)M PI assay is suited for in situ monitoring of bacterial viability and growth on a bioactive model substrate, eGFP-expressing *E. coli* (AAEC191A pHis-GFP) were incubated on antimicrobial dimethyloctadecyl[3-(trimethoxysilyl)propyl]ammonium chloride (DMOAC) coated glass surfaces [90, 92] (Figure 2.3b). Homogeneous DMOAC coating with a dry adlayer thickness of 2.2 nm was confirmed by variable-angle spectroscopic ellipsometry. To allow for a direct contact of the bacterial membrane with the surface-immobilized membrane-active DMOAC molecules, non-fimbriated eGFP expressing *E. coli* (AAEC191A pHis-GFP) were used [75]. As detected by 3 \(\mu\)M PI staining, all adherent bacteria on the DMOAC surface were killed within 30 minutes of surface incubation and no measurable bacterial growth occurred (Figure 2.3b, Additional file 1: Figure S2, Additional file 2: Movie S1).

### 2.3.3 Pre-incubation of bioactive DMOAC surfaces with serum proteins completely blocked the antimicrobial activity and restored bacterial growth on the surface

To investigate if unspecific protein adsorption would interfere with the bactericidal activity of the DMOAC surfaces, the DMOAC surfaces were pre-incubated with fetal bovine serum (FBS) prior to bacterial incubation. Preconditioning of the antimicrobial surface with serum provides a model system for the rapid protein adsorption on biomaterial surfaces upon contact with host body fluids, notably blood, that can significantly impact the specific and unspecific binding of bacteria to the engineered material [28, 79]. Serum protein adsorption increased the dry adlayer thickness from 2.2 nm for pure DMOAC surfaces to 4.5 nm, as measured by ellipsometry. Using the optimized in situ eGFP/PI assay, we found that serum pre-incubation not only delayed but completely eliminated the bactericidal effect...
of the DMOAC surfaces on adherent *E.coli* (Figure 2.3b, Additional file 3: Movie S2). The bacteria survived and were able to divide on the protein-coated DMOAC surfaces. Division times of the surface-attached *E.coli* were comparable to those on bare control glass surfaces without bactericidal activity (Figure 2.3a).

### 2.4 Discussion

A fluorescence based assay is introduced here that is well suited for the in situ monitoring of the viability and growth kinetics of surface-adhering and suspended bacteria. While we used *E.coli* as model organism, this assay should be applicable to other bacterial species as well if (i) the commonly used live DNA stains, such as SYTO®9, are replaced by endogenous eGFP expression and (ii) if the concentrations of propidium iodide (PI) needed to detect dead bacteria is reduced to non-toxic levels (3 µM for *E.coli*). While most available viability assays are restricted to suspended bacteria (i.e. CFU assay) or optical endpoint determinations (i.e. SYTO®9/PI LIVE/DEAD BacLight™ viability kit, CTC assays), we show by fluorescence time-lapse microscopy and turbidity measurements, that reducing the PI concentration to 3 µM readily stained dead *E.coli* on bioactive DMOAC surfaces without disturbing bacterial growth (Figure 2.2, 2.3). Our assay allows viability monitoring of single bacteria and emerging bacterial colonies. We should note though that the assay is not directly transferable to the study of mature biofilms without additional calibrations since the metabolic and genetic profile might change during biofilm mode of growth [93, 94, 95], and the synthesis of extracellular polymeric substances (EPS) [96, 97] might influence the bacterial GFP expression as well as the passive diffusion of PI through the biofilm matrix. Furthermore, detection of single bacteria within a dense three-dimensional biofilm matrix by epifluorescence microscopy might be challenging. However, since bacterial surface colonization starts with the adhesion of individual bacteria, the presented assay provides a versatile new tool for high spatial and temporal evaluation of bacterial viability on engineered surface coatings. The assay thus adds to the previously reported eGFP/PI flow cytometry assay that was limited to viability determination of suspended bacteria [88] and to the eGFP/PI endpoint viability study of groundwater *E.coli* [98]. Evaluating bacterial viability on the test surface omits the extraction
of the adherent bacteria as required for solution based assays, e.g. CFU counts. Therefore, testing the bacterial viability on the substrate might increase the reliability of the assay since extraction is commonly achieved by ultrasonication or harsh washing procedures, both of which can harm bacteria. Furthermore, all CFU assays require prolonged incubation times for colony growth and are thus not applicable for real-time viability monitoring. As an alternative to extraction, an agar sandwich assay has been suggested to determine the viability of surface-adherent bacteria [99]. This method, however, is prone to errors, since each transferred bacterium will grow into a colony that in turn might overlap with colonies nearby. As an alternative direct optical viability assay of surface-adherent bacteria, the respiratory potential of bacteria can be monitored using 5-cyano-2,3-ditolyltetrazolium chloride (CTC) [70]. The drawback however is that the CTC stain cannot be used for real-time monitoring since CTC disrupts the respiratory chain and is toxic to bacteria. This makes the CTC assay only suitable for end point determinations. We compared the performance of our eGFP/PI assay to the well-established SYTO®9/PI end-point dual staining assay and found identical detection efficiencies of dead E.coli (Additional file 1: Figure S2). The SYTO®9/PI assay itself has been extensively compared to the above-mentioned viability tests [70, 86, 88, 91, 100] and showed comparative results to the solution based CFU assay as well as to other microscopy based endpoint viability protocols including the CTC assay. The added advantage of our assay is the ability to monitor the viability of adherent bacteria in real-time. For each bacterial strain and species, one needs to optimize the PI concentrations to keep the bacteria viable, as done here for Escherichia coli (E.coli) K-12 MG1655. E.coli K-12 derivatives have been widely used as model strains in surface adhesion and biofilm studies [75, 95, 101, 102, 103]. We expressed eGFP from the pHis plasmid under the control of the IPTG inducible tac promoter to replace the growth inhibiting DNA stain SYTO®9 as live bacterial marker. To exclude negative effects of GFP expression as well as IPTG and antibiotic addition on bacterial viability and adhesion, we compared the adhesion properties and growth kinetics of the K-12 AAEC191A background strain [104] to a constitutive eGFP expressing strain (rpsm-GFP) and the IPTG inducible pHis-GFP strain (Figure 2.4a,b). No significant difference in adhesion and growth was observed. Furthermore, no significant difference in the fraction of GFP-expressing bacteria was found for the eGFP expression under the constitutive and inducible promoter used in this study (Figure 2.4c,d).
However, even non-toxic gene products like GFP can have detrimental effects on bacteria when overexpressed [105], since overexpression of an introduced gene requires a lot of resources and thus might disorganize the bacterial metabolism. Therefore, the inducible IPTG-based expression system, as compared to constitutive expression systems, allows for a control of GFP expression and guarantees a balance between fluorescent and healthy bacteria (Figure 4c,d). Since eGFP is a very stable protein [106, 107] new GFP variants with reduced half-lives, e.g. GFP (LVA), have been suggested to study transient gene expression in bacteria [108]. The enzymatic degradation of unstable GFP(LVA) however, requires a metabolically active viable bacterium (Additional file 1: Figure S3). Thus, the use of GFP(LVA) variants does not improve our assay to limit false-positive detection of dead bacteria. For live cell imaging, GFP expression in bacteria is used extensively and expression systems are available for many different bacterial species, including clinically relevant strains of Salmonella, Streptococcus, Listeria monocytogenes, Pseudomonas aeruginosa, Staphylococcus aureus and Escherichia coli O157:H7 [106, 109, 110, 111, 112]. Plasmid based gene expression is a well-established and long-used method in microbiology. Handling of plasmids is usually easy and versatile and genes, promoters or selection markers can quickly be exchanged and adapted to needs. Compared to plasmids, chromosomal insertions are more complicated and cannot be adapted as easily. Depending on the insertion method used, the gene of interest is inserted in the chromosome at a random location and might disrupt an important chromosomal gene. As for plasmids, selection markers like antibiotic resistances are commonly used for chromosomal insertions, too [113]. Furthermore, chromosomal insertions usually result in the insertion of a single copy of the gene into the chromosome. The pHis plasmid used in this study carries a ColE1-like replicator and occurs at nearly 20 copies per bacterium [114], each providing the gene of interest. This results in very stable and usually higher expression levels of the gene of interest (here eGFP) than with chromosomal insertions. Plasmids with ColE1-like promoters are stably inherited even without the presence of the corresponding selection agent [115, 116], long-term experiments with GFP expression from a plasmid rather than from a chromosomal insertion are feasible for more than a few hours. For other bacterial species and expression systems, the IPTG level and the concentration of the antibiotic selection marker should be re-evaluated to assure a stable GFP expression without disturbance of bacterial growth. Finally, we applied our viability
assay to highlight the impact of protein adsorption on the antimicrobial activity of engineered DMOAC surfaces. Upon incubation of the DMOAC surfaces with protein-rich fetal bovine serum, bacterial growth on the otherwise bactericidal surface was possible, indicating that the protein layer on top blocked the bioactive quaternary ammonium groups of the DMOAC coating (Figure 3b). The growth rate of the surface-attached *E. coli* on the control glass and serum-coated DMOAC substrates were identical, illustrating that the design rules for antimicrobial coatings primarily have to be tuned to prevent both, bacterial and protein adsorption since additional bioactive modifications can be lost when the biomaterial gets in contact with protein-rich (host) fluids.
Figure 2.4: Influence of GFP expression on the adhesion and fluorescence of *E.coli* K-12 derivative strains. (a) The number of *E.coli* adhering to bare glass substrates was analyzed for the non-fimbriated empty strain *E.coli* AAEC191A, strain AAEC191A pHis-GFP that expresses GFP from pHis plasmid upon IPTG induction and strain AAEC191A rpsm-GFP carrying a plasmid to express GFP from the constitutive rpsm promoter. Per strain, bacteria from 20 fields of view (each 200x200 µm) were analyzed. Mean and standard deviation are shown and a two-independent sample two-sided t-test (\( \alpha = 0.05 \)) was performed. For the *E.coli* strains tested, the number of adherent bacteria was not significantly different (n.s.) with and without GFP expression. Population variances were not significantly different as tested by a two-sided F-test (\( \alpha = 0.05 \)). (b) Growth curves of *E.coli* K-12 derivative strains with and without plasmids for GFP expression. Turbidity of bacterial suspensions in 96 well plates was measured at 600 nm. Mean and standard deviation of a triplicate measurement are shown. (c,d) The fraction of adherent, GFP- fluorescent *E.coli* was analyzed. The empty strain (AAEC191A, \( n = 2468 \)) was not fluorescent. GFP expression from both inducible and constitutive promoters yielded similar fractions of fluorescent *E.coli* (87.3% and 87.2%, respectively). A total of 2852 bacteria carrying the inducible gfp gene (pHis-GFP) and a total of 2036 bacteria carrying the constitutive gfp gene (rpsm-GFP) were analyzed. Shown are means and standard deviations. A two-independent sample two-sided t-test (\( \alpha = 0.05 \)) was performed and the fraction of fluorescent bacteria was not significantly different (n.s.) for the different GFP expressing *E.coli* strains. Population variances were not significantly different as tested by a two-sided F-test (\( \alpha = 0.05 \)).
2.5 Conclusion

In conclusion, we show that the eGFP/PI assay is suited to study the antimicrobial properties of (bio-)material surface coatings under physiological conditions in real time and with single-bacterium sensitivity. This was so far not possible with the widely used solution based assays (i.e. CFU) or endpoint dual staining protocols (i.e. LIVE/DEAD BacLight™ viability kit, CTC). Possible applications for the assay include studies of bacterial fitness and pathogenicity on biomaterial surfaces using live cell imaging of bacteria as additional readout. While we calibrated and illustrated the advantages of the assay for E. coli, other PI concentrations might have to be employed to optimize the kinetic viability monitoring of other bacterial species. While conventional bacteria viability assays allow for fast endpoint checks without requiring genetic modifications, the eGFP/PI assay presented here constitutes a viability test procedure that requires only one sample and its replicates per time series and is particularly suited for kinetic studies.

2.6 Material and Methods

Bacteria  Non-fimbriated E. coli AAEC191A bacteria, a derivative of E. coli K-12 MG1655 containing a deletion in the entire fim cluster [?] was provided by Prof. E. Sokurenko, University of Washington, Seattle, USA. For GFP expression, chemocompetent AAEC191A E. coli were transformed with eGFP pHis plasmid under the control of the tac promoter (AAEC191A pHis-GFP). To obtain E. coli that express eGFP under the control of the constitutive rpsm promoter (AAEC191A rpsm-GFP), E. coli AAEC191A were transformed with the rpsm-GFP plasmid that was extracted from the original fusion library strain MG1655 rpsm-GFP [117] by Qiaprep Spin Miniprep kit (Qiagen 27106). Transformed bacteria were selected by cultivation on LB agar plates supplemented with either 100 µg/ml ampicillin (pHis-GFP) or 50 µg/ml kanamycin (rpsm-GFP). Bacterial precultures were inoculated from glycerol stocks into LB medium (5 g/l yeast extract, 10 g/l tryptone, 10 g/l NaCl) containing appropriate antibiotics. To induce GFP expression in AAEC191A pHis-GFP E. coli, 0.1 mM Isopropyl- β-D-thiogalactopyranoside (IPTG, Applichem A1008) was
added. LB precultures were grown overnight at 37°C under continuous shaking at 180 rpm (Infors Unitron HT). To ensure defined culture conditions for the bacterial growth and viability assays, bacteria from the overnight culture were centrifuged at 1700 g, washed three times and subcultured in 20 ml minimal M9 medium (1x M9 salts (Sigma-Aldrich M6030), 10 mM Mg 2 SO 4 (Sigma-Aldrich 63126), 10 g/l Glucose (Sigma-Aldrich G8270), 0.5 mM CaCl 2 (Sigma-Aldrich C5080), 1x MEM vitamins (Gibco 11120), 1x MEM amino acids (Gibco 11130)) supplemented with the appropriate antibiotics and 0.1 mM IPTG for AAEC191A pHis-GFP E.coli. Bacteria were subcultured at 37°C, 180 rpm until exponential growth phase (OD 600 = 0.3-0.8). Bacteria were harvested by centrifugation at 1700 g followed by three washing steps. Immediately before the experiment, bacteria were resuspended in M9 medium that contained the strain- specific antibiotics, IPTG as well as 0, 3, 30 µM PI (Sigma-Aldrich, 81845) and 6 µM SYTO®9 (Invitrogen L13152), respectively.

**Growth curve measurements**  Growth curves of suspended bacteria were recorded by turbidity measurements at 600 nm in 96 well plates (Tecan Infinity 200 Pro plate reader). Kinetic measurements were performed every 15 minutes at 37°C and continuous shaking. Bacteria were inoculated to an initial turbidity of 0.01 at 600 nm in M9 medium containing appropriate antibiotics, PI and SYTO®9.

**Viability assay**  For kinetic viability measurements under physiological conditions, bacteria were cultivated in an ibidi® glass bottom flow chamber (ibidi, 80168) within a temperature- controlled microscope incubator to guarantee constant nutrient supply and optimal growth conditions at 37°C. Bioactive surfaces were prepared according to published protocols [90]. Briefly, glass cover slides, that later resemble the bottom slide of the flow chamber, were exposed to air plasma for 15 seconds (Harrick Plasma, PDC-32G) followed by dipping into a 5% (v/v) aqueous DMOAC (Sigma-Aldrich) solution for 1 second, and drying at 105°C overnight. To test the effect of protein pre-incubation on the antimicrobial activity of the DMOAC coatings, slides were incubated in undiluted fetal bovine serum (FBS, Thermo Scientific SH30071.02) for 1 h prior to the assembly of the flow chamber. Bare glass cover slides were attached to the ibidi® chambers as control surfaces. The bacterial
suspension (OD 600 0.05) in M9 medium containing different concentrations of PI and SYTO®9, was directly added to the flow chamber and immediately transferred to an epifluorescence microscope (Nikon TE2000-E) for in situ viability monitoring. Adhesion of bacteria to the glass bottom slide was allowed for 5 minutes before the flow chamber was gently washed with 5 ml M9 medium (flow rate 0.01 ml/min) to remove non-adherent bacteria. As control staining at defined time points, the BacLight™ viability kit (Invitrogen, L13152) was used according to the supplier instructions.

**Ellipsometry** The adsorbed dry film thickness of DMOAC and DMOAC + FBS layers on silicone wafers was measured by variable-angle spectroscopic ellipsometry (VASE) using the M2000F variable-angle spectroscopic ellipsometer (J.A. Woollam Co., Inc.). The measurement was performed at 70°C relative to the surface normal under ambient conditions. Ellipsometry data were fitted using a cauchy model with parameters for organic layers

\[
n(\lambda) = A_n \lambda + \frac{B_n}{\lambda^2} + \frac{C_n}{\lambda^4}
\]

with \(A_n = 1.45\), \(B_n = 0.01\), \(C_n = 0.0\) to obtain dry thickness of adlayers.

**Image segmentation and quantification** To limit the viability analysis to fluorescent *E. coli* and to eliminate bias in the data analysis based on GFP fluorescence intensity, fluorescence images were thresholded and segmented using the morphological strel algorithm of the image processing toolbox of MATLAB® software (MATLAB, MathWorks; version R2010b) that combines image erosion and dilation operations. The algorithm was included into a semiautomatic image processing workflow that allows for manual adjustment of the thresholding levels of the entire time series as well as individual time frames. Binary masks were generated from the thresholded images and surface-adherent bacteria were counted automatically. The summing of binary masks from consecutive time points allowed for correction of fluorescence signal loss caused by GFP bleaching, washout and degradation of the stained DNA. To prevent false-positive results, binary masks
that were not positive for the GFP channel before, were excluded from the PI positive counts to limit the analysis to bacteria that were viable initially. Elimination of x,y drift of time series data was achieved by the register virtual stack slices and transform virtual stack slices plugins of Fiji that were incorporated into a MATLAB® routine using the MIJ java package for bi-directional communication between MATLAB® and ImageJ by D. Sage. The MATLAB® file for the analysis workflow is available in the Additional file 1.

2.7 Acknowledgements

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Bacterial filamentation accelerates colonization of adhesive spots embedded in biopassive surfaces

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Jens Möller, Philippe Emge, Ima Avalos Vizcarra, Philip Kollmannsberger, and Viola Vogel  JM, PE, IAV and VV designed the experiments. PE and IAV performed experiments; PK developed the mathematical model and performed simulations. PE, PK, JM, and IAV analyzed data. All authors contributed to the writing and the discussion of the manuscript.
3.1 Abstract

Sessile bacteria adhere to engineered surfaces and host tissues and pose a substantial clinical and economical risk when growing into biofilms. Most engineered and biological interfaces are of chemically heterogeneous nature and provide adhesive islands for bacterial attachment and growth. To mimic either defects in a surface coating of biomedical implants or heterogeneities within mucosal layers (Peyer’s patches), we embedded micrometre-sized adhesive islands in a poly(ethylene glycol) biopassive background. We show experimentally and computationally that filamentation of Escherichia coli can significantly accelerate the bacterial surface colonization under physiological flow conditions. Filamentation can thus provide an advantage to a bacterial population to bridge non-adhesive distances exceeding 5 µm. Bacterial filamentation, caused by blocking of bacterial division, is common among bacterial species and can be triggered by environmental conditions or antibiotic treatment. While great awareness exists that the build-up of antibiotic resistance serves as intrinsic survival strategy, we show here that antibiotic treatment can actually promote surface colonization by triggering filamentation, which in turn prevents daughter cells from being washed away. Our combined microfabrication and computational approaches provide quantitative insights into mechanisms that enable biofouling of biopassive surfaces with embedded adhesive spots, even for spot distances that are multiples of the bacterial length.

3.2 Introduction

Bacterial attachment is the first step in the colonization of surfaces and biofilm growth [118]. As the preferred mode of microbial life, biofilms consist of surface attached and densely packed populations of bacteria, held together by a self-produced matrix [119]. Biofilms growing on medical devices and biomedical implants are generally accepted to be the major cause of bacterial infections in clinical environments [120], causing tremendous clinical and economical complications. Commonly, bacterial infections are treated by antibiotic therapies, but due to an increasing number of antibiotic-resistant bacterial strains, pharmaceutical agents become
ineffective and new ways to prevent bacteria borne infections need to be explored [121, 122, 123]. One alternative strategy to deal with bacteria-related infections is to interfere with the initial adhesion of planktonic (free floating) bacteria to biomedical and technical surfaces by rendering the surfaces biopassive [124, 125, 126]. Upon exposure to the environment however, biopassive coatings can be subjected to degradation and corrosion [127]. Defects within the antimicrobial coating in turn can provide adhesion sites for bacteria serving as nucleation points for the formation of confluent surface biofilms. Bacteria not only adhere to and colonize engineered materials. To invade the host, pathogens adhere to host tissues by specific surface receptor-ligand interactions. The mammalian intestine is a preferred area of infection as it provides a large epithelial surface that can be colonized by potentially pathogenic microorganisms [128]. Within the intestine epithelium, Peyer’s patches, i.e. groups of lymphoid follices, provide a transport system for antigens as an essential part in the immune defence. Several pathogenic bacteria bind preferentially to cell types found in Peyer’s patches [129] as the non-adhesive mucous layer is thin compared to the surrounding tissue, making Peyer’s patches a preferred site for invasion of pathogens into the host tissue [129]. Peyer’s patches within the intestine epithelium therefore have similar properties like the adhesive defects in antimicrobial coatings described above: they present preferred sites for bacterial attachment within a nonadhesive surface. To form a confluent bacterial layer as a requisite for biofilm development, the adherent bacteria have to bridge the non-adhesive areas on those heterogeneously adhesive interfaces. (E.coli), one of the best studied intestinal microorganisms, are rod-shaped bacteria with a length of 2-4 µm [130]. However, the morphology and in particular the aspect ratio of E.coli can largely change upon exposure to antibiotics and non-optimal growth conditions [131]. Filamentation occurs when cell division is blocked while growth continues, resulting in filaments with high aspect ratios of several tens of micrometres. As a survival strategy, filamentation can slow down the phagocytic uptake of bacteria by immune cells [132], promoting their survival within host tissues [54, 133]. Since bacterial filaments are commonly observed in patients being treated with antibiotics [134], it has been proposed that filamentation might be a defence mechanism to temporarily withstand treatment with β-lactam antibiotics, e.g. cephalexin [135, 136]. Cephalexin belongs to the family of β-lactams and targets FtsI, a penicillin binding protein of the divisome complex that is
required for bacterial division. It is among the largest selling antibiotics worldwide and is applied against otitis media and urinary tract infections. Since the exposure to antibiotics or the local growth conditions that promote filamentation can change quickly in natural environments, we ask here whether filamentation offers a selective advantage to colonize heterogeneously adhesive surfaces. Using *E. coli* as model organism, we hypothesize that filamentous bacteria bridge non-adhesive regions between adhesive spots faster than non-filamentous bacteria. To test our hypothesis we performed live cell microscopy and analysed the kinetics of *E. coli* adhesion and growth on heterogeneous surfaces. To allow for firm adhesion of *E. coli* under physiological flow conditions [23, 56], we used a photoresist lift-off process to micropattern the glycoprotein Ribonuclease B (RNaseB) to which *E. coli* bind by their type 1 fimbriae. Adhesive RNaseB islands of 10 µm in diameter were spaced at distances reaching many multiples of the bacterial length. Unspecific adhesion was blocked by passivation with PLL-g-PEG [104, 126]. *E. coli* filamentation was induced by cephalixin. Based on the experimentally derived parameters of bacterial adhesion and growth, we implemented a Monte Carlo simulation of bacterial surface colonization to confirm the experimental data and obtained additional predictions for the impact of filamentation, flow and adhesive spot distances on the bacterial surface colonization kinetics. This study provides novel mechanistic insights into the initial stages of surface biofouling and it highlights the selective advantage of bacterial shape adaptation to colonize heterogeneously adhesive substrates.

### 3.3 Results

#### 3.3.1 Increasing distances of adhesive spots inhibit the formation of a confluent layer of non-filamentous *E. coli*

To quantify the surface colonization kinetics of non-filamentous *E. coli* on heterogeneously adhesive surfaces, we prepared mannosylated RNaseB glycoprotein patterns within a stable biopassive PLL-g-PEG layer [126, 137] by adaptation of a combined photolithography and molecular assembly process (Figure 3.1) [138]. The lift off process rendered the glass surfaces with a distinct pattern of circular
RNaseB spots as it was visualized by fluorescence microscopy. No fluorescent signal was detected within the biopassive PLL-g-PEG coated areas (Figure 3.1, iv). The RNaseB pattern promoted firm adhesion of type 1 fimbriated \textit{E.coli} under physiological flow conditions [56] and no bacterial adhesion to the passivated areas was observed (Figure 3.1, v).

To analyse the effect of surface patterning on the kinetics of bacterial surface colonization, \textit{E.coli} were incubated on adhesive RNaseB spots of 10 µm in diameter separated by distances of 5, 10 and 20 µm. Surface-attached \textit{E.coli} were grown under physiological relevant shear stress (1 ml/min flow rate; 0.6 pN/µm² wall shear stress) [139, 140, 141]. The surface colonization kinetics on the different patterns was quantified from time-lapse movies for a region of interest that included multiple adhesive spots within the direction of medium flow (Figure 3.2b). Starting from single adherent \textit{E.coli}, bacterial microcolonies grew in size by cell division until the edge of an adhesive spot was reached. An almost confluent bacterial layer was observed after 4 hours for a spot distance of 5 µm (Figure 3.2a, top). With increased spot distances of 10 and 20 µm, the bacterial surface coverage decreased and characteristic colonization patterns were observed (Figure 3.2a, middle and bottom). \textit{E.coli} microcolonies on unpatterned adhesive surfaces merged into a confluent bacterial layer after 4 hours (Figure 3.2b). Non-filamentous \textit{E.coli} colonized surfaces with 5 and 10 µm spot distances are very similar to unpatterned adhesive RNaseB surfaces. No confluent layer had formed on surfaces with 20 µm spot distances (Figure 3.2c). Since the initial number of adherent \textit{E}. coli per field of view was higher for surfaces with higher adhesive spot densities, we corrected the colonization kinetics analysis accordingly. By image thresholding of the phase contrast time-lapse movies, the fractions of pixels covered by bacteria were determined. This analysis was very robust for low to moderate bacterial surface coverage. At higher surface coverage though, light halos around the bacteria occurred such that the phase contrast imaging and thresholding was not an accurate measurement of surface coverage any longer. We estimated that a pixel threshold of 58% corresponds to a confluent \textit{E}. coli monolayer (Figure 3.2c).
Figure 3.1: Patterning surfaces with bacteria-adhesive islands within a biopassive surface coating. (i) S1818 photoresist structures (red) were fabricated by photolithography on Nb$_2$O$_5$ coated glass cover slides. (ii) Incubation of photoresist-patterned substrate with PLL-g[3.5]-PEG(2). (iii) Photoresist lift-off by N-Methyl-2-pyrrolidone. (iv) Backfill of non-PEGylated regions with the mannose-exposing glycoprotein RNaseB, labelled with Alexa Fluor 488 for visualization. (v) *E. coli* expose the adhesin FimH at the tip of type 1 fimbriae and adhere to the RNaseB pattern by specific FimH-mannose interactions but not to the biopassive PLL-g-PEG coated regions. The corresponding images of the photoresist pattern before the lift-off process and Alexa Fluor 488 labelled RNaseB patterns are shown (circular 10 µm spots with 20 µm spot-to-spot distance). Bacteria adhere selectively to the adhesive spots under fluid flow conditions as shown here for a flow rate of 1 mL/min.
Figure 3.2: *E. coli* colonization kinetics on micropatterned surfaces under physiological flow. (a) Phase contrast images of *E. coli* on RNaseB spots with different distances after 4 h growth at 37 °C with a flow rate of 1 mL/min (0.6 pN/µm² shear stress). Distances of the adhesive spots and flow direction are indicated. Dashed circles visualize the location of the adhesive spots. (b) Growth kinetics of *E. coli* on patterned and unpatterned surfaces. The bacteria-covered area was determined by thresholding phase contrast images. The region of interest was defined by a row of adhesive spots in the flow direction (red false coloured). Exemplary image sequences show the bacterial growth kinetics on a 10 µm circular pattern with 20 µm spot-to-spot distance (top panel) and on an unpatterned surface (bottom panel). Only a part of the actual analysed area is shown. (c) Area fractions of bacterial surface coverage. Standard errors of the mean are shown (n=5). A confluent *E. coli* monolayer on the unpatterned surface was estimated to correspond to an area fraction of 0.58 (indicated in the graph by a grey bar) based on the thresholding method of the phase contrast images.
3.3.2 E.coli filamentation accelerates bridging of non-adhesive areas

While bacterial filamentation can be induced by various environmental factors including low temperature and high pressure [134, 142, 143], we induced E.coli filamentation here by inhibiting cell septation by the antibiotic cephalexin (20 µg/mL) (Figure 3.3a, top panel) [134]. The filaments started to lyse 2 hours after continued exposure to cephalexin, which defined the time window of our investigation. The time for a bacterial filament to double in length was 25.4±1.6 minutes (n=10). The filament growth rate was not significantly different from the length doubling time of non-filamentous E.coli (27.6±3.4 min, n=10).

To study the impact of bacterial filamentation on the bridging of non-adhesive regions, we compared the bridging of non-adhesive spot-to-spot distances of 5, 10 or 20 µm for filamentous and non-filamentous E.coli. Non-filamentous E.coli divided and grew on the adhesive patterns until the edge of the spots was reached (Figure 3.3a). Once the spot edge was reached, we observed that daughter cells that grew into the passivated area were frequently washed away by the fluid flow after the division was completed (Figure 3.3a, bottom panel). This behaviour led to a characteristic “shark-tooth” pattern in the bridge advancement (Figure 3.3a, 3b, blue lines and arrow 1 and 2). When an adhesive spot became crowded after 2-2.5 hours, daughter cells were no longer washed away and the bridge advancement accelerated until the full distance of 20 µm to the downstream patch was bridged (Figure 3.3a).

Analysing the bridge advancement for different pairs of adhesive spots revealed substantial heterogeneity in the colonization kinetics of non-filamentous E.coli (Figure 3.3b, blue lines showing 3 representative measurements). In contrast, filaments were able to grow and extend across the nonadhesive areas beyond the pattern edges (Figure 3.3b). As the filaments grew, no “shark-tooth” patterns in the advancement kinetics of the filamentous bacterial bridge were seen since no daughter cells were washed away (Figure 3.3b, red lines and Figure 3.3a, top panel).

For 20 µm spot-to-spot distances, filamentous E.coli bridged the non-adhesive regions 3 times faster than non-filamentous bacteria (Figure 3.3b). For spot distances of 5 and 10 µm, fast bridging between the neighbouring adhesive spots was observed for filamentous and non-filamentous E.coli (Figure 3.3c,d).
Figure 3.3: E.coli colonization kinetics on micropatterned surfaces under physiological flow. (a) Phase contrast images of E.coli on RNaseB spots with different distances after 4 h growth at 37°C with a flow rate of 1 mL/min (0.6 pN/µm² shear stress). Distances of the adhesive spots and flow direction are indicated. Dashed circles visualize the location of the adhesive spots. (b) Growth kinetics of E.coli on patterned and unpatterned surfaces. The bacteria-covered area was determined by thresholding phase contrast images. The region of interest was defined by a row of adhesive spots in the flow direction (red false coloured). Exemplary image sequences show the bacterial growth kinetics on a 10 µm circular pattern with 20 µm spot-to-spot distance (top panel) and on an unpatterned surface (bottom panel). Only a part of the actual analysed area is shown. (c) Area fractions of bacterial surface coverage. Standard errors of the mean are shown (n=5). A confluent E.coli monolayer on the unpatterned surface was estimated to correspond to an area fraction of 0.58 (indicated in the graph by a grey bar) based on the thresholding method of the phase contrast images.
filamentous *E. coli*, the time to bridge the non-adhesive regions changed proportionally to the spot distance as no daughter cells are washed off (Figure 3.3e). For non-filamentous *E. coli*, the bridging time did not scale with the spot distance. Non-filamentous *E. coli* bridged the 5 µm distance non-proportionally faster than the 10 and 20 µm (Figure 3.3e). The bridging time, i.e. the time that filamentous and non-filamentous *E. coli* needed to fully bridge the distance between two adhesive spots, was statistically significantly different at all spot distances, however, at 10 and 20 µm spot distance the difference was highly significant (p<0.001) (Figure 3.3e).

3.3.3 Filament bridges disintegrate when removing cephalexin from the medium

To mimic the effect of fluctuating growth conditions, we asked how the removal of antibiotics from the cell medium might affect the further colonization. In the presence of cephalexin, *E. coli* filaments extended beyond the pattern edge and once the non-adhesive region was bridged, the filament tip adhered to the adjacent downstream adhesive spot (Figure 3.4a). Being fixed at both ends, further growth resulted in buckling of the filament as indicated by the filament being out of focus. Bacterial division resumed when cephalexin was reduced to sub-inhibitory concentrations (lower than 10 µg/mL), which leads to a rapid fragmentation of the filaments. After cephalexin withdrawal, the time-lapse series shows how the bacterium at the filament tip that contacted the downstream adhesive spot (Figure 3.4a, 2:12 h, arrow) remained attached on the surface and started to divide (Figure 3.4a, 2:12-2:54 h). The non-adhesive parts of the fragmented filament are washed away (Figure 3.4a, 2:54 h). The growth of the filaments was not restricted by the medium flow direction (Figure 3.4). We observed filaments that grew both in and against the flow direction (Figure 3.4a, 1:42 h-1:51 h). A second filament grew against the flow direction towards the downstream patch but detached after 2 h and was washed away by the fluid flow.
Figure 3.4: *E. coli* filaments grow in the presence of antibiotics and disintegrate after cephalexin removal, leaving behind their off-spring on the adhesive RNaseB spots. (a) Time series of cephalexin-induced filament growth on patterned surface under fluid flow (1 mL/min; 0.6 pN/µm²). filamentous *E. coli* bridged passivated areas between adhesive spots (dashed circles) reaching downstream spots to which they can adhere. Continued growth of the filament between adhesive spots resulted in buckling of the filament bending it out of the focal plane. After gradually purging with cephalexin-free medium, cell division was resumed and the filaments fragmented. Only bacteria at the filament tip that were already in contact with the adhesive spots were able to hold on and further divide. A second filament is seen to grow against the flow direction (1:42-1:52 h) before it detached and was removed by fluid flow after 2 h. (b) Time series of filament growth on adhesive unstructured surfaces. Filaments can also be seen to grow at angles to the flow direction. Once cell division was restored, the filament fragmented into single bacteria over its entire length. The time point where the cephalexin was diluted below inhibitory concentration is indicated. Arrows indicate cell division events.
On a homogenously adhesive area, the filaments divided into progeny over the entire length of the filament after the exchange to cephalexin-free medium and grew into a microcolony on the surface (Figure 3.4).

### 3.3.4 A computational model of bacterial surface colonization predicts a kinetic advantage of filaments when colonizing passivated surfaces that contain adhesive spots

The results obtained by time-lapse microscopy showed that bacterial filamentation accelerated the bridging of neighbouring adhesive spots (Figure 3.3). To evaluate if the kinetic advantage of bridging at early stages results in increased surface coverage at later times, a 2D computational model of bacterial growth was developed that accounted for filamentous and non-filamentous bacterial growth on micropatterned adhesive surfaces. Four basic model assumptions were made that were derived from our experimental data: (i) All bacteria grow with a constant doubling time $T_d$ of 26 minutes. This parameter was derived from our finding that the elongation rates did not differ significantly between filamentous and non-filamentous bacteria (Figure S4.8).(ii) Filamentation of bacteria occurs with a probability $P_{\text{filament}}$, while existing filaments can divide with a probability $P_{\text{divide}}$. These two parameters were based on our experimental observation that cephalexin induced a homogenously filamenting population which could be switched back to non-filamentous growth when cephalexin was diluted out (Figure 3.4). (iii) Bacteria at the edge of the colony are washed off with a probability $P_{\text{wash-off}}$ that depends on the magnitude of flow and the orientation of the bacteria relative to the flow direction. The two parameters magnitude and orientation are represented by a flow factor $F$ and an anisotropy factor (see supplementary information). (iv) Wash-off can only occur over non-adhesive regions. This model assumption is valid since bacteria firmly adhered to the adhesive RNaseB spots (Figure 3.3b).

We applied our model to adhesive spot distances of 5, 10 and 20 µm as we probed experimentally. Three representative curves of filamenting (red) and non-filamenting (blue) conditions for a spot distance of 20 µm are shown (Figure 3.5a). Our model predicts a 3 times faster bridging for filamentous compared to non-filamentous conditions, which is in good agreement with our experimental observations (Figure...
3.3a). Since the model reproduces the experimental results for 5, 10 and 20 µm distances, we applied it to larger distances of 40 and 80 µm (Figure 3.5b). The model predicts an increasing difference in bridging time between filamentous and non-filamentous bacteria for larger spot distances (Figure 3.5b). To gain insight into the effect of the flow factor F on the bridging time, we varied the flow factor from 30 to 300 (Figure 3.5c). Higher flow factors resulted in an increased bridging time of a 20 µm distance for non-filamentous bacteria (Figure 3.5c, blue bars). Interestingly, the bridging time of filamentous bacteria was not influenced by an increase in the flow factor (Figure 3.5c, red bars).

In our model, \( P_{\text{filament}} \) defines the probability of a dividing bacterium to form a filament and was set to 0.5 for all simulations mimicking filamenting conditions.

Figure 3.5: Monte Carlo simulations of bacterial bridging of non-adhesive regions. (a) Bridging kinetics of non-adhesive regions for filamentous (red) and non-filamentous conditions (blue). The fraction of the bridged distance (\( d_{\text{bridged}}/d_{\text{gap}} \)) is plotted over time. The distance between two adhesive spots is bridged three times faster if filamentation is allowed. (b) Predicted bridging time for spot distances of 5, 10, 20, 40 and 80 µm for filamentous (red) and non-filamentous (blue) conditions. (c) Predicted bridging time of filamentous and non-filamentous bacteria for 20 µm distance at different flow magnitudes. (d) Predicted bridging time of 20 µm distances for different probabilities of filamentation (\( P_{\text{filament}} \)). For (b), (c) and (d) means and standard deviations of \( n=10 \) simulations are shown.
This probability reflects the amount of filaments in a bacterial population. To determine the influence of a variation in the filamentation probability, the parameter $P_{filament}$ was set to 0.01, 0.03 and 0.1 and compared with non-filamenting conditions ($P_{filament} = 0$) and the filamenting conditions used before ($P_{filament} = 0.5$) (Figure 3.5d). The model predicts that a probability for filamentation of 0.03 is sufficient to reproduce the effect of filamentation on bridging times that we observed experimentally (Figure 3.3). Higher probability of filamentation did not decrease the bridging time any further (Figure 3.5d).

To investigate the effect of bridging time on surface colonization, we extended the simulations towards later time points and larger spot arrays. We modelled a 400 times 300 µm surface area with 10 µm adhesive spot diameter and 20 µm spot distances (Figure 3.2a and b). Bacterial adhesion on five adhesive spots at the upstream side of the surface was defined as initial condition (Figure 3.6a, 0 h). The model parameters were set to $P_{filament} = 0.5$ (filamenting condition), $P_{filament} = 0$ (non-filamenting conditions) and flow factor $F = 100$ (Figure 3.6b). As suggested from the previous comparison of filamentous and non-filamentous *E.coli* surface colonization (Figure 3.3), the filamenting conditions accelerated the surface coverage (Figure 3.6a).

Finally, and analogous to the experimental setting where we removed cephalaxin from the medium, the filament division in the model was resumed after the first 5 doubling periods by setting the parameters $P_{filament}$ to 0 and $P_{divide}$ to 0.5 (Figure 3.6). The model again predicts a kinetic advantage of the filaments over non-filamentous bacteria. This kinetic advantage resulted from the ability of the filaments to bridge the distances to downstream adhesive spots that served as nucleation sites for further surface colonization. By lateral cell-cell contacts, non-filamentous bacteria bridged the 20 µm distance between adhesive spots at later time points (Figure 3.6a). Those differences in the early colonization kinetics are enhanced at later time points (Figure 3.6b). We investigated the kinetic advantage of filamentation not only in terms of colonized surface area but also as a function of the number of adhesive spots that were colonized (Figure 3.6c).

Although the filamenting conditions were restricted to the first 5 doubling periods, this was sufficient to colonize two times as many spots than with non-filamenting conditions.
Figure 3.6: Filamentation accelerates the bacterial surface colonization of heterogeneous surfaces as shown by Monte Carlo Simulations. (a) Time series of the simulated bacterial colonization of a surface with adhesive spots (green, 10 µm diameter, 20 µm distance) embedded in a non-adhesive surface (white) for non-filamenting (upper panel, blue) and filamenting (lower panel, red) conditions. Until 2:09 h, filamentation was allowed resulting in the formation of filaments (red) that bridge the non-adhesive regions (lower panel, 1:50 h, red filaments). After 2:09 h, the fragmentation of the filaments was resumed by setting P_filament to zero thereby mimicking for example the withdrawal of antibiotics. Further division events resulted in non-filamenting offspring shown in blue (lower panel, 3:40 h). The switch from filamenting (P_filament = 0.5) to no filamentation (P_filament = 0) is indicated. (b) Simulated bacterial surface coverage expressed as fraction of the entire area (400 x 300 µm) for initial filamenting conditions (red, P_filament = 0.5) during the first five doubling times. Shown are mean and standard deviation of 3 simulations. (c) The number of adhesive spots (green in Figure 3.6a) that are colonized is plotted over time. The arrow shows the switch from filamenting (red) to non-filamenting (blue) conditions. Shown are means and standard deviations of 3 simulations. The arrows indicate the switch from filamenting to non-filamenting conditions.
3.4 Discussion

Filamentation is a common trait of various bacteria, including Escherichia coli, Pseudomonas aeruginosa and Salmonella enterica, and occurs in many natural and industrial habitats [18, 22]. Here we have shown that filamentation of *E. coli* can accelerate the colonization of non-adhesive surfaces that expose microscale adhesive spots (Figure 3.2), mimicking for example defects in a surface coating or heterogeneities within mucosal layers. Non-filamentous *E. coli* were typically washed off by the medium flow when they grew beyond the border of the adhesive spots (Figure 3.3a, b). In contrast, no offspring is washed away during filament growth. As a result, filamentous *E. coli* bridged nonadhesive distances that are greater than the length of an *E. coli* bacterium shortly before division (4-8 µm) significantly faster (Figure 3.3) although the time needed to double in length is very similar for both phenotypes (25.4 ± 1.6 minutes and 27.6 ± 3.4 minutes, respectively) (Figure S4.8).

As the microcolonies grew, we observed that non-filamentous *E. coli* were also able to bridge large non-adhesive distances (Figure 3.2a, b). Since the PLL-g-PEG chemistry used here to passivate the inter-spot areas completely suppressed bacterial binding under low and high flow for at least 9 hours (Figure S4.7), the bridging of the non-adhesive regions might be possible only by lateral cell-cell interactions. This interpretation is in agreement with the observation that bacteria growing on adhesive surfaces arrange to maximize lateral cell-cell contacts. In the presence of adjacent neighbours within a microcolony, bacteria reached into the non-adhesive area (Figure 3.3a, bottom panel and 3b, blue lines). Stabilized by sufficient cell-cell interactions (Figure 3.3b, 2 h), bridging of the non-adhesive regions can occur gradually.

We implemented a 2D Monte Carlo simulation based on four rules derived from our experimental data. We found good agreement of bridging rates between simulations (Figure 3.5) and experimental data (Figure 3.3). The model furthermore allowed us to predict the impact of filamentation on the surface colonization speed and bridging times for conditions not addressed experimentally. Even for adhesive spot distances of 40 and 80 µm, filamentation continued to accelerate the surface colonization (Figure 3.5b). An higher flow factor F prolonged the bridging time for the non-filamenting system which reflects the influence of increased flow rates and wall
shear stresses. Interestingly, changing the flow factor $F$ had no effect under filamenting conditions (Figure 3.5c). This implies that the prevention of offspring wash-off is the mechanism leading to the observed accelerated bridging between adhesive spots by filaments. Our data suggests that short periods of filament-promoting conditions are sufficient to create a kinetic advantage over non-filamenting bacterial populations (Figure 3.6b,c). We further varied the probability for filamentation in the model. This reflects the fraction of bacteria in a population that grow into filaments. Our model predicts a threshold probability of 0.03 at which the full kinetic advantage of filamentation is reached (Figure 3.5d). This suggests that it is sufficient if only a fraction of a bacterial population grows in filaments to have a colonization advantage over non-filamenting bacteria. An increase of the fraction of filamenting bacteria beyond this threshold does not further accelerate the surface colonization.

During the treatment of infections with antibiotics, little attention has been given to the fact that antibiotic doses that are too small to instantaneously kill bacteria might induce their filamentation. Previously, we showed that filamentation of $E. coli$ slows their uptake by macrophages [19]. Macrophages have to reach the terminal ends of the filaments before they can form a phagocytic cup and internalize them. Otherwise, they are often observed to contact filaments, even pull on them, but the filamentation in those cases prevents phagocytosis thus impairing an immune response. Here we illustrate a second mechanism where filamentation might have an adverse effect in the fight against biofilms.

We show that the antibiotic cephalexin can drastically accelerate the bridging of non-adhesive areas (Figure 3.3). Our results imply that antibiotics that induce filamentation can lead to an accelerated colonization of a heterogeneously adhesive surface, which was not considered in the literature so far and should be taken into account to tailor the dosage of antibiotics. We show that environmental changes that promote a switch in the bacterial phenotype towards filamentation can accelerate bridging of non-adhesive areas (Figure 3.3). We demonstrated that filaments can attach to downstream adhesive regions leaving offspring there, which itself divide and grow into a colony, if the surrounding conditions allow division (Figure 3.4a). This may also suggest that heterogeneous surfaces in body tissues like Peyer’s patches in the intestine or implant surfaces can be colonized faster if an infection is treated with
a filament inducing antibiotic which thus might be detrimental for the host organism.

3.5 Conclusion

Using *E.coli* as bacterial model organism, our study highlights the profound kinetic advantage of bacterial filamentation to accelerate the colonization of heterogeneously adhesive surfaces. Our combined experimental and computational approaches show for early and late time points that filamentation can accelerate the surface colonization. Filamentation increased the rate of bridging non-adhesive areas and can thereby accelerate biofouling of passivated surfaces. While great awareness exists that the build-up of antibiotic resistance serves as intrinsic survival strategy, the local underdosing of antibiotics can switch bacteria into a filamentous state which can accelerate the formation of difficult-to-treat biofilms on heterogeneously adhesive surfaces.

3.6 Material and Methods

**Bacteria**  *E.coli* AAEC191A (Δfim null mutant) harbouring plasmid pSH2 [104, 144] expressing a naturally occurring uropathogenic FimH variant were grown overnight at 37°C in an orbital shaking incubator in lysogeny broth (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) with 10 µg/mL chloramphenicol to maintain plasmid pSH2. For flow chamber experiments, the culture was washed twice in phosphate-buffered saline (PBS, pH 7.4) and adjusted to OD600=1.

**Preparation of adhesive glycoprotein patterns and unpatterned adhesive surfaces**  RNaseB glycoprotein patterns with a diameter of 10 µm, spaced by 5, 10, and 20 µm were prepared using a combined photolithography and lift off process (Figure 3.1). The MAPL protocol [145] was adapted to pattern proteins onto glass substrates. Photoresist patterns on glass cover slides were created using
positive S1818 photoresist. Briefly, undiluted S1818 photoresist (Microposit) was
spin coated on the cover glasses in a two-step spin process with 2000 rpm for
5 seconds (ramp step of 500 rpm/s) and 4000 rpm for 90 seconds (ramp step of
800 rpm/s), respectively, followed by soft baking for 60 seconds at 100°C and
UV light exposure (Karl Süss MA6 mask aligner) with an energy dose of 150
mW/cm² at 405 nm. Exposed resist was developed in undiluted MF319 developer
(Microposit) for 60 seconds. The resist micropatterns were rendered biopassive by 1
hour incubation at room temperature with 100 µg/mL PLL-g[3.5]-PEG(2) (SuSoS
AG) in PBS. For enhanced stability of the polymer layer, the glass surface was
coated with 21 nm niobium oxide prior to photolithography. The photoresist was
removed by chemical lift off with N-Methyl-2-pyrrolidone (NMP, Sigma 494496),
leaving PLL-g-PEG coated regions on the surface. The non-PEGylated areas were
backfilled by 30 minutes incubation at room temperature with 100 µg/mL Alexa
Fluor 488 labelled RNaseB. The terminalmannoses of the N-linked glycans present
on RNaseB serve as receptor for the bacterial type 1 fimbriae tip adhesin FimH.
The biopassive properties of PLL-g-PEG prevent the adsorption of RNaseB to
previously passivated areas. The unpatterned adhesive surfaces were prepared by
incubating glass slides 30 minutes at room temperature with 100 µg/mL unlabelled
RNaseB.

Parallel plate flow chamber experiments The flow chamber comprised of
a vacuum mediated assembly of an acrylic top, a silicone separating gasket and
a glass cover slide. A channel of 2.5 mm width was cut into the silicone gasket.
The channel height was defined by the gasket thickness of 0.254 mm. The channel
dimensions and the volumetric flow rate of 1 mL/min defined a shear stress of
0.6 pN/µm² applied to the surface-bound E.coli. The flow cell setup was placed
within a microscope incubation box heated to 37°C. A custom-made bubble trap
that was incor-porated upstream of the flow chamber inlet reduced the probability
of air bubbles running into the flow chamber. LB medium supplemented with
10 µg/mL chloramphenicol was circulated through the flow chamber setup for at
least 1 hour prior to each experiment to equilibrate the system. To seed the glass
surface with bacteria, the flow was stopped, the tubing clamped and disconnected
at the flow chamber inlet port. A syringe with the inoculation culture was used
to inject the bacteria directly into the inlet port of the flow chamber. The bacteria
were allowed to adhere to the surface for 1 minute. The tubing was reconnected
and the flow resumed. Unbound bacteria were washed off by the medium flow.
Bacterial filamentation was induced by supplementing the LB medium with 20
$\mu$g/mL cephalexin. To restore bacterial division, the medium was exchanged with
cephalexin-free LB medium. The medium change led to gradual dilution of the
cephalexin in the setup over time.

**Microscopy** Flow chamber assays were performed on an inverted live cell micro-
scope (Nikon TE2000-E) equipped with an electron multiplying charge-coupled
device (EM-CCD) camera (Hamamatsu 9100-02). Time-lapse movies of multi-
ple stage positions were acquired every 3 minutes for up to 4 hours with a 40x
phase contrast objective. Fluorescence im-
ages were acquired with a fluorescein
isothiocyanate (FITC) bandpass filter set (Chroma 49002).

**Data analysis** Surface colonization kinetics:
To determine the colonized area fraction, phase contrast images were thresholded
within a rectangular region of interest including multiple adhesive spots in the
direction of flow. To include bacterial growth perpendicular to the flow, the region
of interest was 2 times wider than the spot width and centred over the spots (Figure
3.2b). The area fraction is calculated as the ratio of thresholded pixels by the total
number of pixels in the region of interest. To assess differences in the initial surface
coverage, individual plots were fitted and the parameters obtained for generation
times were corrected.

Bacterial bridging of passivated areas between adhesive spots:
The position of the adhesive spots was determined from fluorescent images of Alexa
Fluor 488 labelled RNaseB. A position mask of the adhesive islands was created
and overlaid with the phase contrast images to visualize the adhesive spots in the
images and time-lapse movies. Bridge advancement was measured from phase
contrast images in between two neighbouring spots in flow direction. Bacteria
growing beyond the border of an upstream spot towards an adjacent downstream
spot contributed to the bridge advancement. Pairs of adhesive spots (n=10) were
randomly chosen and the bridge advancement was determined by measuring the
distance that the bacteria advanced from the border of the upstream spot towards
the downstream spot. The maximum distance of bridge advancement was the edge-to-edge distance between two spots (5, 10 or 20 µm, respectively). Bridging time was defined as the time point when bacteria from an upstream adhesive spot connected to the next downstream spot. Analysis of *E.coli* filaments was restricted to the time interval where filamentation occurs and before bacteria lysed.

**Bacterial length doubling time measurements:**
For filamentous *E.coli*, the time a filament needed to double its length was considered to be an adequate measure of growth and was determined from the pole-to-pole length of a filament. The filament length (n=10) was measured every 3 minutes for 2 hours. The data was plotted and fitted with theoretical growth curves to obtain a length doubling time. To compare the length doubling time of filaments with non-filamentous *E.coli*, the pole-to-pole length of all progeny of non-filamentous *E.coli* (n=10) was summed up at each time point and a length doubling time was determined likewise. Data analysis was performed using ImageJ software.

**Computational modelling of surface colonization kinetics**
2D Monte Carlo simulations of a model describing the bacterial growth on adhesive spots were performed using MATLAB (MathWorks, version R2010b). The surface was modelled as a lattice in which bacteria occupy one lattice site, corresponding to an area of 1 µm². The parameters of the model were the doubling time $T_d$ of filamentous and non-filamentous *E.coli* as derived from experimental data, a probability $P_{filament}$ for growth without septation, resulting in filaments, a probability $P_{divide}$ to account for septation (as observed in undisturbed bacterial growth as well as for filaments when removing cephalexin antibiotics), and a probability $P_{wash-off}$ for bacteria being washed off from the edge of the colony over biopassivated surface areas. The wash-off probability depends on the magnitude of flow, modelled by a flow factor $F$, and the position of the bacteria relative to the flow direction, described by an anisotropy factor $A$. A more detailed description of the model and implementation is found in the supplementary information.

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CHAPTER 4

How bacteria evade antibiotics while surviving inside macrophages

note on August 18, 2015: An advanced version of the work presented in this chapter is currently under peer-review with Scientific Reports (nature publishing group)

Ima Avalos Vizcarra, Vahid Hosseini, Philip Kollmannsberger, Stefanie Meier, Stefan S. Weber, Markus Arnoldini, Martin Ackermann, Viola Vogel VV and IAV designed research, IAV, SM, and VH designed additional experiments, performed research, and analyzed data, PK contributed mathematical description of the exponential model as alternative to the Michaelis Menten kinetics, MArnoldini suggested and contributed bacterial MG1655 strains and plasmids, SW provided experimental procedures and analysis procedures for the design of
research. IAV, VH, PK, MAckermann, and VV wrote the manuscript and all authors read and approved of the manuscript.
4.1 Abstract

Phagocytosed bacteria are not necessarily killed, but have a chance to survive inside macrophages thereby evading antibiotic treatment. The impact of fimbriae-mediated binding in this process has not been studied quantitatively. Survival and single cell infection assays in combination with a two-step kinetic model to derive rate constants of bacterial binding and uptake by macrophages now reveal that the presence of type I fimbriae significantly increased the binding affinity, and the internalization rate of bacteria into macrophages. Fimbriae were necessary for efficient internalization at low bacteria doses and the uptake probability was reduced by a soluble mannose-analogue as fimbriae-specific inhibitor. upon exposure to bacteria, the macrophages became more adhesive to the substrates which might help them to generate the forces necessary to pick up surface-bound bacteria. These findings could explain how expression of fimbriae modulates virulence to yield high infection probabilities at very low pathogen doses, and how bacteria can subvert being killed by antibiotics.

4.2 Introduction

While it is commonly thought that phagocytosis of microbes by immune cells leads to their intracellular degradation, survival within host cells is a potent mechanism of bacterial virulence [8, 146, 147, 148]. Internalization of bacteria into eukaryotic host cells can be triggered by either the host cells, e.g. when immune cells recognize and actively phagocytose infecting bacteria, or by the bacteria, e.g. via virulence factors such as secretion systems[11, 34]. Although immune cells have optimized their ability to recognize and kill bacteria, some pathogenic bacteria specifically adapted to cells of the immune system and achieve intracellular survival. Immune cells can thus also be infected by bacteria and their failure to clear bacteria can lead to systemic infections since most immune cells are highly migratory and tissue-
invasive [48, 146]. Intracellular survival strategies of pathogens include escape from the phagosome into the cytosol, and adaptation to their hostile surroundings by changing either the biochemistry of their compartment or their own physiology [8, 148, 149]. To evade intracellular degradation, many bacterial species have developed strategies to actively enter host cells and adapt to the life within [11, 148, 149].

Bacterial binding to host cells precedes internalization and is a key factor in the progression of bacterial infection caused by intracellular pathogens [11, 48, 150]. Among the large variety of bacterial adhesion factors that promote internalization into host cells, type 1 fimbriae mediate non-covalent interactions to mannosylated surfaces and cellular receptors with force activated catch bonds [56, 151, 152] that stabilize binding to host cells. This shear-force enhanced binding of type 1 fimbriae was found to be essential for the virulence of uropathogenic E.coli strains by enabling attachment to host cells that enables internalization and intracellular survival [147, 153, 154]. Type 1 fimbriae also mediate binding and increased survival of E.coli to macrophages, which are predators of pathogenic E.coli [25, 54]. Since it was shown that virulence of some E.coli strains correlates with expression levels of type 1 fimbriae [34, 146, 155], but commensal E.coli also express fimbriae[156], the understanding of direct dependencies between bacterial adhesion and bacterial survival after internalization into phagocytes is of great medical relevance.

Phagocytes such as macrophages and neutrophils are immune cells specialized in the recognition and removal of foreign particles which involves uptake and intracellular degradation by aggressive chemicals, acids and enzymes. Many bacteria have thus evolved strategies to prevent recognition and phagocytosis by immune cells [11, 157]. It has been hypothesized that bacteria exploit adhesion factors like type 1 fimbriae for internalization into epithelial cells in which they can survive, but should avoid binding to phagocytes that are their predators [11, 53]. However, type 1 fimbriae-specific receptors of phagocytes have been identified that trigger fimbriae-specific internalization. Surprisingly, fimbriae-dependent uptake was found to result in higher survival chances of bacteria, suggesting that fimbriae-mediated internalization by macrophages helps E.coli to avoid clearance by the innate immune system [54, 158, 159, 160]. These studies have compared the effects
of different pathogenic strains which often vary in the expression of other virulence factors. Different pathogens may vary in their efficiency to bind to macrophages and a quantification of how adhesion efficiency affects survival inside macrophages is still lacking [53]. In particular, the question remains open how the expression of fimbriae influences *E. coli* survival as well as the macrophage response to bacterial infection. Asking the question how fimbriation affects bacterial infection of macrophages and survival inside macrophages has additional clinical significance, since vital macrophages have been reported to also release living pathogens which then continued to progress through their normal lifecycles [161].

To quantify how adhesion by fimbriated *E. coli*, independent of other virulence factors impacts intracellular survival, we first assessed bacterial survival chances for three different non-pathogenic strains of *E. coli*: a wild type strain (wt), which exhibits intrinsic stochastic fluctuations in fimbriae expression [156], a knock-out strain for type 1 fimbriae expression (Δfim) as well as a fimbriae overexpression strain (fim↑), which is isogenic with Δfim but overexpresses fimbriae from the plasmid pSH2 [144, 162]. The pSH2 plasmid contains the whole fimbriae gene cluster and is a well-established expression system for investigations on overexpression of type 1 fimbriae [144, 162]. Secondly, we asked if intracellular survival correlated with the number of bacteria that are internalized by macrophages, i.e. the bacterial burden on macrophages. To answer this question, we established a dose-response curve for bacterial binding to macrophages and modulated the bacterial burden with fimbriae-specific inhibitors and cytoskeletal inhibitors that prevent phagocytosis. The bacteria to macrophage ratios (which have often been referred to as multiplicities of infection (MOI) under the assumption of a linear relationship between bacterial concentration and host infection efficiency) were systematically varied in a range from 0.5 to 32. We furthermore asked if we could identify a mechanistic model underlying bacterial binding to macrophages that could quantify the binding parameters in dependence of fimbriae expression. Finally, we asked if bacterial burden influenced the macrophage phenotypic response which was quantified regarding macrophage viability, proliferation and surface adhesion.
4.3 Results

Intracellular survival was assessed with the gentamicin protection assay. The adhesion efficiency, i.e. the bacteria-bound macrophage population, was quantified by flow cytometry. In all experiments, macrophages were allowed to adhere to 24 well plates for at least 2 hours before adding bacteria at the specified ratios (Figure 4.1a). Serum-free media was used for 0.5 hours during binding, whereas all other cultivation was performed in media supplemented with serum to prevent nutrient limitation of macrophages.

4.3.1 More intracellular survivors were observed when E.coli overexpressed type 1 fimbriae

To investigate the impact of fimbriation on the survival chances of E.coli - independently of other virulence factors – the intracellular survival was quantified in gentamicin protection assays for up to 48 hours post infection (Figure 4.1a). The exposure to the antibiotic gentamicin ensured that all bacteria that had not been internalized by the phagocytes were rendered incapable of replication. Bacterial survivors were quantified by the ability to replicate on agar plates after macrophage lysis (Figure 4.1a, b). The first interesting observation from the assay of intracellular survival was that the total numbers of colony forming units extracted from macrophages were 6-fold increased for fim↑ as opposed to ∆fim and 3-fold increased to wt, while using the same bacteria to macrophage ratio of 10 for all three strains (Figure 4.1c). Intracellular survival of E.coli in macrophages was increased for bacteria overexpressing type 1 fimbriae, which is in agreement with previous studies [54, 158, 160]. However, it was not possible to differentiate whether the presence of fimbriae yielded overall higher numbers of infected macrophages with low bacterial burden or just increased the bacterial burden and fraction of survivors in a small macrophage subpopulation. Furthermore, a pronounced increase in intracellular viable fim↑ bacteria was observed 2 and 4 hours post infection, indicative of inefficient clearance of these bacteria and their replication inside macrophages (Figure 4.1d). After 4 hours, a decrease in intracellular survivors was observed also...
for fim↑, although the fraction of intracellular fim↑ survivors was still significantly higher than for wt and Δfim and the bacteria survived for more than 2 days despite continued exposure to gentamicin (Figure 4.1d).

4.3.2 Initial adhesion of E.coli to macrophages was strongly increased when type 1 fimbriae were expressed

While we observed increased numbers of intracellular survivors for fim↑ (Figure 4.1d), the gentamicin assay limited us to quantify whole populations of macrophages and intracellular bacteria. We therefore aimed at resolving bacterial burden on the single cell level of the macrophage population to investigate if subpopulations could explain the observed differences in the macrophage-mediated clearance of bacteria. To quantify numbers of infected macrophages on the single cell level, we investigated how type 1 fimbriae affected bacterial binding to macrophages using GFP-expressing E.coli of the fim↑, wt and Δfim strains, and non-fluorescent macrophages. Non-fluorescent macrophages that bind or internalize GFP-expressing bacteria can then be detected by their fluorescence signal using flow cytometry (Figure 4.2a). To quantify the bacterial burden on macrophages, the ratio of GFP-expressing bacteria to macrophages was systematically varied from 0.5 to 32 and the relative amount of GFP-positive macrophages, i.e. bacteria-bound macrophages in the macrophage population was quantified by flow cytometry (Figure 4.2a).
Figure 4.1: *E. coli* overexpressing type 1 fimbriae yielded more intracellular survivors in macrophages. (a) Schematic overview of the timeline and experimental procedure of the gentamicin assay for measurement of intracellular survivors employed in this study. Gentamicin was applied in a 15 minute pulse to 100µg/ml for instantaneous effect on extracellular bacteria and then reduced to 10µg/ml for long term co-cultivation. (b) composite image of 3 agar plates from macrophage lysates after incubation with fim↑, wt fim and Δfim, respectively. Macrophages were lysed with 0.1% Triton X-100 0.5 hours post infection and the lysate plated on LB agar plates for quantification of intracellular survivors. (c) Total colony forming units recovered from gentamicin protection assays 0.5 hours post infection showed increased uptake of fim↑ compared to wt fim and Δfim bacteria. RAW macrophages were seeded to 10^3 cells/cm² and incubated with the three different *E. coli* strains, respectively, at a bacteria to macrophage ratio of 10. Box plot whiskers indicate the S.D., variance of population means was analyzed using a one way ANOVA and post-hoc Tukey test. Groups for statistical significance are given by upper case letters. Grouping indicated that at the 0.01 level, the population means were significantly different from each other, whereas for the population means of members of one group were not significantly different at the 0.01 level. (d) Fold changes of colony forming units, normalized to 0.5 hours post infection at the specified time points indicated higher survival chances of fim↑ bacteria compared to wt fim and Δfim. Error bars are S.D. fim↑, fimbriae overexpression strain; wt, fimbriae wild type strain; Δfim, fimbriae knockout strain; h, hours.
Figure 4.2: Bacterial burden of macrophages depended on fimbriae expression and impacted intracellular survival of *E. coli* in macrophages. (a) Schematic overview of the timeline and experimental procedure of the flow cytometric assay for measurement of the efficiency of bacterial adhesion to macrophages 0.5 hours post infection. (b) The amounts of bacteria-bound macrophages for *E. coli* fim↑, wt fim and Δfim strains for bacteria to macrophage ratios from 0.5 to 32 showed the dose response behavior and binding efficiencies of bacterial binding to macrophages. Error bars show the S.D. (c) Inhibitors that impacted bacterial adhesion also impacted survival of *E. coli* in macrophages, as indicated by the effect 2% of alpha-methyl mannose pyranoside (αMM), a competitive inhibitor of type 1 fimbriae (error bars show the S.D.), and (d) actin-depolymerizing Latrunculin B (LatB), on adhesion (error bars show the S.D.). 1 µM LatB was employed to block uptake of bacteria by macrophages. (e) 3D reconstruction of surface adherent macrophages after 0.5 hours incubation with the *E. coli* fim↑ strain at a ratio of 10. (f) Representative 3D reconstruction of macrophages after 0.5 hours incubation with the *E. coli* Δfim strain at a ratio of 10. GFP-expressing bacteria are colored in green, actin-binding phalloidin is colored in grey. Images were chosen randomly from a set of 25 images. (g) Distribution of the number of bacteria per macrophage for 25 depended on the bacterial strain employed. Bacteria per single macrophages were counted from 25 randomly chosen microscopic images. Higher numbers of bound bacteria for macrophages incubated with fim↑ and wt fim bacteria at the same ratio of 10. Variance of population means was analyzed using a one way ANOVA and post-hoc Tukey test. Groups for statistical significance are given by upper case letters. Different grouping indicated that at the 0.01 level, the population means were significantly different from each other, whereas for the population means of members of one group were not significantly different at the 0.01 level. (h) Intracellular survival was assessed in the presence of LatB and αMM 2 hours post infection, indicating the dependence of survival chances on internalization by macrophages and fimbriae-mediated binding. Normalized intracellular survival was obtained by relating cfu 2 hours post infection to cfu 0.5 hours post infection. Variance of population means was analyzed using a one way ANOVA and post-hoc Tukey test. Groups for statistical significance are given by upper case letters. Different grouping indicated that at the 0.01 level, the population means were significantly different from each other, whereas for the population means of members of one group were not significantly different at the 0.01 level. cfu, colony forming units; fim↑, fimbriae overexpression strain; wt, fimbriae wild type strain; Δfim, fimbriae knockout strain; a.u., arbitrary units; GFP, green fluorescent protein; αMM, alpha-methyl mannosepyranoside.
When macrophages were incubated with ∆fim bacteria, the percentage of GFP-positive macrophages was strongly reduced compared to fim↑ after the same incubation time of 0.5 hours and the same bacteria to macrophage ratio of 10 (Figure 4.2b). The wt strain showed intermediate binding efficiency between fim↑ and ∆fim (Fig 2b), consistent with stochastically distributed fimbriae expression in bacterial wild type populations[156]. While these results showed that the efficiency with which bacteria bind to and are taken up by macrophages was strongly increased when fimbriae were expressed, they did not allow a conclusion on the bacterial burden per macrophage. Since bacterial burden of macrophages could be an important factor in increased intracellular survival of bacteria, we quantified macrophage-bound bacteria using confocal fluorescence microscopy 0.5 hours post infection and a bacteria-to-macrophage ratio of 10 (Figure 4.2g, randomly selected images shown in e and f). The microscopic analysis showed that for a bacteria-to-macrophage ratio of 10, fim↑ bound with higher numbers per macrophage than ∆fim (Figure 4.2e, f, g). We showed that higher bacteria to macrophage ratios results in a larger fraction of macrophages carrying a bacterial burden, and also an increased bacterial burden per macrophage. Comparison between experiments with the differently fimbriated bacteria showed the same, i.e. that expression of fimbriae led to a larger infected fraction, and increased bacterial burden per macrophage.

Since the efficiency of macrophage infection as read out by the GFP positive macrophage population showed a clear dependency of type 1 fimbriae expression and adhesion to macrophages (Figure 4.2b, c, d), we next aimed at modulating the adhesion efficiency of bacteria to macrophages so that we could later use modulated adhesion efficiency to investigate its effect on intracellular survival. Fimbriae mediate binding to glycosylated host cell receptors by the FimH protein which is a mannose-specific lectin. To modulate fimbriae-mediated binding, a competitive inhibitor for the FimH lectin, the mannose analogue alpha-methyl-pyranoside (αMM), was added to the medium[25, 144, 151]. Indeed, the increased binding of the wt and fim↑ strain was due to the presence of fimbriae, as αMM strongly reduced binding of the ∆fim strain as well as of the wt strain (Figure 4.2c). Consequently, αMM had no effect on binding of ∆fim bacteria, as expected from its specificity for blocking fimbriae-mediated macrophage binding (Figure 4.2c).
a second approach, Latrunculin B (LatB), a potent inhibitor of actin polymerization, was used to inhibit actin-dependent internalization of bacteria[163]. Stable, fimbriae-mediated adhesion without internalization still occurred independently of the depolymerization of filamentous actin which LatB causes (Figure 4.2d). Incubation with LatB led to a decrease of the bacteria-associated macrophage population in all cases (Figure 4.2d).

To identify if intracellular survival and binding efficiencies were linked functionally, we next asked if modulating bacterial binding would have consequences on intracellular survival as well, using the gentamicin protection assays under the influence of inhibitors. We tested if the inhibitors could modulate intracellular survival chances in a similar manner as they modulated binding behavior, finding that incubation with LatB did not yield any colony forming units in gentamicin assays (Figure 4.2h). This finding is consistent with the assumption of the assay that only intracellular bacteria can survive antibiotic treatment, while bacterial binding to the outer plasma membrane of macrophages alone is not sufficient to protect bacteria from the effect of antibacterial drugs. However, interfering with the fimbriae-mediated binding of bacteria to macrophages via the monomannose analogue αMM - which competes for the FimH binding pocket[151] - reduced intracellular colony forming units of fim↑ and wt fim, but not of ∆fim (Figure 4.2h). Taken together, incubation with αMM reduced intracellular colony forming units of fim↑ bacteria to those levels of ∆fim, consistent with the ability of αMM to block fimbriae-mediated enhanced infection of macrophages (Figure 4.2c). Most significantly, this suggests that increased survival correlated with higher bacterial burden on macrophages (Figure 4.2h).

4.3.3 Increased bacterial burden can be fitted to Michaelis Menten kinetics allowing quantification of infection doses and the influence of inhibitors

To further increase our mechanistic understanding of the bacterial binding process by type 1 fimbriae, we evaluated models that could quantify the key features of modulated adhesion efficiencies. The best fit was obtained using a model inspired by Michaelis Menten kinetics (Equation 1), a well-established model for saturating
reactions such as enzyme catalyzed reactions and ligand-receptor interactions. The model describes a binding process with a first step under an equilibrium assumption of binding and a second irreversible and thus rate-limiting step. By adapting the Michaelis Menten kinetics to bacteria-macrophage binding (Figure 4.3a), bacterial binding can be described by two sequential steps: first, a reversible step of initial binding with an on-rate and an off-rate that leads to an equilibrium of a bacteria-macrophage complex that can dissociate again. The second step is an irreversible and thus rate-limiting step leading to stable binding of bacteria and their internalization (Figure 4.3a). The overall uptake rate $R(x)$ of bacteria by macrophages is described as function of the percentage of macrophage population that can bind bacteria $M_{max}$, the ratio of bacteria to macrophages $x$, and the Michaelis Menten constant $K_S$ which is the ratio of the off-rate and on-rate for the first reversible binding step:

$$R(x) = \frac{M_{max}x}{K_S + x}$$

(4.1)

An increase in $\frac{1}{K_S}$ is a quantitative measure for the affinity of bacteria to macrophages as it corresponds to a decreased off-rate for the initial binding equilibrium (Figure 4.3a). The value of $M_{max}$ is proportional to the rate constant of the second, rate limiting step leading to internalization and thus determines the infection efficiency by the maximum possible number of infected macrophages. The parameters $M_{max}$ and the binding constant obtained from the fits followed a similar trend as the experimentally determined binding efficiencies for both bacterial strains (Figure 4.3b-e).

In summary, the Michaelis Menten-like model explains how for the same amounts of bacteria which surround macrophages, different infection efficiencies can be observed. According to this kinetic model, type 1 fimbriae lead to a highly decreased off-rate towards the bound state. This in turn increases the likelihood of internalization via the second, rate-limiting step (Figure 4.3a). Thus, the expression of type 1 fimbriae shifted the initial equilibrium adhesion strongly to the side of stable binding by decreasing the off-rate. While the experiments here were conducted without flow, the presence of flow is expected to additionally decrease the off-rate since the FimH-mannose complex can form force-activated catch bonds[151].
Figure 4.3: Quantifying the internalization of *E.coli* by macrophages with Michaelis Menten-like kinetics allows inferring infection doses for macrophage populations. a) Formalized binding sequence between macrophages and bacteria according to Michaelis Menten kinetic model. b) Experimentally derived data and Michaelis Menten fit for fim↑. c) Experimentally derived data and Michaelis Menten fit for Δfim. Inset graph shows full range for comparability with graph in b. d) The Michaelis Menten model predictions of the inverse of binding constant as obtained from the Michaelis Menten fit, indicated the kinetic rate constants for macrophage binding of *E.coli* fim↑, wt, and Δfim strains. (e) Michaelis Menten model predictions of the $M_{max}$ parameter indicate the maximal relative amount of macrophages that can bind *E.coli* fim↑, wt, and Δfim. Error bars are S.D.; $K_S$, Michaelis Menten constant; $k_{on}$, reversible binding on-rate; $k_{off}$, reversible binding off-rate; $k_2$, irreversible binding rate; $M_{max}$, maximum fraction of bacteria-bound macrophages;
4.3.4 Macrophages change their phenotype in an ungraded response to bacteria independent of the bacterial burden

Finally, we asked whether the increased bacterial survival was a consequence of differences in macrophage viability, which could e.g. be caused by toxic effects of fimbriae or the associated higher exposure to bacteria. The viability of macrophages was assessed by staining with the calcein dye which stains only cells with intact membranes (Figure 4.4a) and additional staining of dead cells with propidium iodide which can enter only cells with lysed or permeable membranes. This assay yielded two important insights: First, the viability of macrophages incubated with fim↑ for 24 hours was not significantly different from the viability of macrophages incubated with Δfim for 24 hours (Figure 4.4b). Second, the number of macrophages per field of view was larger, but their cell spreading area, i.e. the surface area that cells are attached to, was smaller for macrophages in the control. This indicated that macrophages that had not been incubated with bacteria proliferated more and interacted less with the surface. Therefore, the macrophage proliferation during 48 hours of incubation with and without bacteria was investigated by counting live cells over time, showing that proliferation was inhibited when macrophages were incubated with bacteria (Figure 4.4c).
Figure 4.4: Macrophages react in an ungraded, all-or-none behavior to *E. coli*, independent of bacterial fimbriae expression and the associated bacterial burden. (a) Viability of macrophages is stable during exposure to *E. coli* and independent of fimbriae expression. Viability was assessed by membrane permeability assays using calcein and propidium iodide, and shows no significant decrease of macrophage viability after 0.5, 24 and 48 hours post infection of incubation with differently fimbriated bacteria. (b) The proliferation of macrophages is inhibited by incubation with bacteria. Macrophage proliferation was assessed by counting calcein-stained cells per field of view after 0.5, 24, and 48, hours post infection, shows that incubation with bacteria resulted in irreversible inhibition of macrophage proliferation. Analysis of variance between the samples was calculated via a one-way ANOVA and post-hoc Tukey test at a significance level of 0.05 (c) Macrophage surface interaction as a marker for macrophage activation was assessed by immunostaining for vinculin and counting of focal adhesion complexes per macrophage, showing a pronounced increase in focal adhesion complexes after incubation with bacteria. Representative images of thresholded images from vinculin immunostainings 24 hours post infection are shown on the left, identified focal adhesion circumference is shown as magenta-colored outline in the zoom-in insets. Quantification of focal adhesions from single cells is shown as bar plot on the right. Comparison of means was done by a student’s t-test. ** denotes a p<0.01. (d) Cell surface areas as a measure of macrophage activation and assessed by thresholding fluorescent micrographs of macrophages stained with calcein live dye 24 hours post infection. Cells in the control were not challenged with bacteria; supernatant refers to macrophages incubated with bacteria-conditioned media. Analysis of variance was done by a one-way ANOVA and post-hoc Tukey comparison of means. *** denotes statistically significant differences at p<0.001, error bars are S.D. of three biological replicates, i.e. measured on independent days and S.D. of the mean of 9 images in panel e; n.s., not significant.
Differences in macrophage phenotypes have been described previously and depend on their role in wound healing and inflammation. With macrophages undergoing a transition from naïve, suspended cells in the blood stream to firmly adherent, proinflammatory cells in sites of infection, differences in surface adhesion of macrophages are expected [164, 165, 166]. Furthermore, it was recently found that increasing the formation of long-lived surface contacts by so called focal adhesions had an impact on intracellular trafficking and survival of pathogens [30]. To probe whether the tensile state by which macrophages are anchored to the substrate is affected upon exposure to differently fimbriated bacteria, we performed immunostainings of the protein vinculin which is recruited to stretched talin within focal adhesions [167]. Based on the observations of increased cell size (Figure 4.4a), the area of adherent macrophages was also quantified (Fig 4e). We found that macrophages in contact with bacteria contained more focal adhesion complexes per macrophage, indicative of stronger surface interaction. The increase in focal adhesion complexes was independent of whether the bacteria they had been exposed to were fimbriated or not (Figure 4.4e). Consistent with this, the spreading area of macrophages doubled 24 hours post infection, independent of the bacterial strain that had been employed (Figure 4.4d). The number of focal adhesions, surface spreading area and macrophage viability were thus independent of the bacterial burden. This indicates that the expression of fimbriae and differences in bacterial burden did not affect the overall macrophage phenotype, while the sole presence of bacteria did so in an ungraded response.

4.4 Discussion

While bacterial adhesion via type 1 fimbriae help bacteria to invade host cells [34, 147, 153, 168], the same adhesins also pose a risk for bacteria when they contribute to the recognition and clearance by phagocytic cells [25, 159]. Most remarkably, fimbriation not only promotes the adhesion to phagocytes, but also the bacterial survival in macrophages [146, 154, 158, 169]. To shed insights into the mechanisms why the presence of type 1 fimbriae correlates with their survival rate, we used non-pathogenic E. coli strain and only modulated the type 1 fimbriae expression levels. This comparison revealed a link between adhesion efficiency (Figure 4.2b), bacterial burden (Figure 4.2g) and survival (Figure
4.2h) in macrophages. Our data now suggest that highly fimbriated bacteria yield efficient infection of macrophages even at very low infection doses (Figure 4.2b, c, d). To clearly distinguish correlations from causes, our data suggest that intracellular survival mechanisms of bacteria should generally be investigated by correlating only the same amounts of infected macrophages which do not necessarily correspond to the same multiplicities of infection for different strains. From the quantification of the macrophage burden, we conclude that fimbriation per se does not increase the chance of a phagocytosed bacterium to survive (Fig 2h). Instead, fimbriation increases the number of E.coli bacteria within a macrophage when compared to the same exposure to unfimbriated bacteria (Figure 4.2e, f, g). Upregulated fimbriation accelerates entry into the intracellular niche. In addition to the increased survival, we also observed increased initial replication inside macrophages which enhanced the kinetic survival advantage of bacteria in the intracellular niche (Figure 4.1d). Seeing that a short incubation time of 0.5 hours was sufficient to result in large survival differences between fimbriated and non-fimbriated bacteria, we suggest that accelerating stable binding and internalization ensures faster removal of bacteria from an antibiotics-containing environment, and is thus a favorable asset for intracellular pathogens, in agreement with previous studies [170, 171, 172].

The physiological significance of our findings is multifold. First, type 1 fimbriae are required virulence factors in urinary tract infections and intracellular biofilm formation [147, 150, 153, 154]. Moreover, harmless, commensal E.coli strains also express fimbriae[156] although the switching rates to the on-state of expression were found to be higher in pathogenic than in commensal strains, indicating higher expression levels of fimbriae in pathogenic strains[10, 153, 155]. Consistently, we found that fimbriae-overexpressing bacteria increased infection efficiency of macrophages even at very low infection doses of bacteria, making upregulation of fimbriae-expression a striking possibility of virulence tuning. We identified the kinetic mechanism how fimbriation increases the bacterial burden on host cells, and consequently the internalization rate, which correlated with increased absolute numbers of surviving bacteria. Under the assay conditions that we used here, the increased survival suggested that high bacterial burden can reduce the macrophages’ efficiency to clear bacteria which gained an additional head start for entering the intracellular niche.
Second, survival in macrophages without inducing macrophage apoptosis\cite{34, 146} (Figure 4.1c, 4.4b) can be a powerful strategy of bacterial pathogens to use these migratory and tissue-invasive host cells as transport vehicles, resulting in overcoming of epithelial barriers, invasion of deep tissue, and spreading inside the host. Such spreading can lead to the establishment of persistent and chronic infections\cite{34, 146, 150}. This is especially relevant when infection with bacteria does not lead directly to host cell death as observed for many clinical isolates.

Third, and perhaps most importantly in the context of bacterial survival, internalized bacteria are shielded from treatments with antibiotics that do not pass the cell membrane which was noticed already in the 60s\cite{173} and broadly exploited to distinguish surface-bound from internalized bacteria \cite{174}. While the antibiotic gentamicin that we used in this study was used in the purpose of the assay and not therapeutically, correlating bacterial binding efficiency and survival has also significant implications for medical drug applications: a kinetic advantage for entry into the intracellular niche is an intriguing survival benefit for antibiotic-sensitive bacteria, such as we have used here\cite{170, 171, 172}. The duration as well as the recurrence of antibiotic exposure have a substantial impact on the survival and persistence of virulent bacteria in antibiotics-containing environments \cite{1, 47}. Since killing of extracellular and unrestrictedly growing bacteria by antibiotics and antibacterial drugs occurs fast, usually in the course of one to few hours \cite{29, 175, 176}, escaping from an antibiotics-containing environment is a benefit for bacteria that are under the selective pressure of antibacterial drugs \cite{47, 149, 171}.

### 4.5 Material and Methods

**Macrophages.** The immortalized monocyte-derived murine cell line RAW264.7 was used, which has been widely employed in the study of host-pathogen interactions as it provides a stable genetic background and constant expression of macrophage surface markers. RAW264.7 macrophage-like cells were cultivated in RPMI (Gibco 1640) supplemented with 10% FBS, 2mM Glutamine (Gibco 25030), and 25 mM HEPES (Biowest L0180).
**Bacteria.** Bacterial strains employed were the K12 derivate AAEC191 which is chromosomally knocked out for type 1 fimbriae expression, the same strain reconstituted for fimbriae overexpression with the constitutively expressed plasmid pSH2 encoding the complete fim operon and K12 derivative MG1655 as *E.coli* wt. Constitutive GFP expression was achieved by transforming all strains with the rpsM-GFP vector from a genomic fusion library. *E.coli* strains were grown in LB media and diluted in serum-free cell culture medium prior to experiments after overnight growth to an optical density of 0.1.

**Bacterial survival and macrophage viability assays.** All experiments were based on a 30 minute incubation of surface adherent macrophages with bacteria in which phagocytosis was allowed to occur unperturbed. Intracellular bacterial survival was assessed by standard gentamicin protection assays[54, 158, 169]. Briefly, $10^5$ RAW264.7 cells per well were seeded in tissue culture treated 24-well-plates (TPP 92424) in serum-free media (Sigma 5921) supplemented with 25 mM HEPES, and 2 mM L-Glutamine. Bacteria transformed with the rpsM-GFP promoter fusion construct[117] were added in the respective bacteria to macrophage ratio. Synchronization of infection occurred by centrifugation for 1 minute at 395 g and subsequent incubation at 37°C for 30 minutes. Media was then replaced for 15 minutes to serum free media supplemented with 100 µ/ml gentamicin (Sigma 48760-1G-F) and after that to media supplemented additionally with 10% FBS and 10 µ/g/ml gentamicin. Cultivation was continued until each indicated time point when cells were lysed with 0.1% sodium deoxycholate and cell lysates plated on agar plates for quantification of colony forming units (cfu). Viability of RAW264.7 cells was determined using the live/dead viability kit (life technologies, u.S.) according to manufacturer’s specifications. Briefly, the calcein live dye stains all cells and the propidium iodide dead cells. Fluorescent images were acquired by exciting at 488 nm and thresholding of fluorescent images to determine cell outlines and counting of live and dead cells was performed using Fiji software[177].

**Phagocytosis efficiency assays.** The binding assays were performed in serum-free media to exclude potentially convolving effects from serum components. The assay was conducted in the same manner as the gentamicin assay up to the incubation for 30 minutes. Non-adherent bacteria were then removed by three washing
steps with ice-cold phosphate buffered saline pH 7.4 (PBS) supplemented with 5mM Ethylenediaminetetraacetate (EDTA, Fluka 03677) after which cells were detached by vigorous pipetting with PBS-EDTA. Flow cytometric analysis was carried out on a BD LRS Fortessa and quantified using Flowjo V10 software (Treestar). The assay was repeated on 3 independent days with two biological replicates and two technical duplicates on each day.

**Live cell imaging.** Automated live cell imaging of adhesion of *E.coli* to macrophages was performed in 3-15 minute intervals using a 40x objective and Nikon TE2000 and Leica SP5 inverted microscopes in accordance with the experimental procedures of the gentamicin protection assay (Supplementary Figure 1). Briefly, RAW264.7 macrophages were seeded on fibronectin coated cover glasses and custom made Polydimethylsiloxane (PDMS). Bacteria transformed with the rpsM-GFP promoter fusion construct [117] were added to the respective bacteria to macrophage ratio and incubated at 37°C for 30 minutes. Media was then replaced for 15 minutes to serum free media supplemented with 100µ/ml gentamicin (Sigma 48760-1G-F) and after that to media supplemented additionally with 10% FBS and 10 µ/ml gentamicin for the cultivation during image acquisition.

**Statistical analysis.** Descriptive statistics of mean values, standard deviation (S.D.) and 1-way ANOVA tests of single cell and population data were performed using Origin 9.0 software (OriginLab). Statistical significance was estimated using the Tukey and Bonferroni Tests at the specified thresholds of 0.01. Predictive statistics for fitting was performed using Origin 9.0 software and the built-in Michaelis Menten formula as well as custom-build functions for a one-step irreversible model.

### 4.6 Acknowledgements

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A versatile 3D magnetic manipulation system to capture translational and rotational modes as macrophages capture their prey

The work presented in this chapter is in preparation for submission.

Simone Schuerle*, Jens Möller*, Ima Avalos Vizcarra*, Mahmut Selman Sakar, Berna Özkale, Fajer Mushtaq, Salvador Pané, Viola Vogel, and Bradley Nelson. SS IAV JM VV, and BN designed research, SS IAV JM MSS performed research, SS and IAV analyzed data, BÖ, FM, and SP fabricated magnetic probes, SS IAV JM VV, MSS and BN wrote the paper. All authors read and approved of the manuscript.
*equally contributing first authors
5.1 Abstract

A magnetic tweezer system coupled to a light microscope is introduced that consists of eight electromagnets (NanoMag) allowing for translational and rotational control of arbitrarily shaped magnetic microparticles in 3D. The NanoMag enables twisting experiments where the orientation of the probe can be controlled independently of the applied force. In dynamic force spectroscopy measurements, the particle displacement is used as feedback parameter to create an artificial potential well with online adjustable trap stiffness. Designed to study mechanobiological processes at various length scales ranging from single cells to engineered tissues and organs, we illustrated its power by quantifying the forces of macrophages capturing magnetic microparticles that mimic the size and shape of microorganisms. By exploiting dynamic force clamping, the kinetics of pushing, rotational and sideways attack during macrophage-particle encounters preceding phagocytosis are revealed. Pulling on objects using sideways and rotational modes rather than pulling straight may allow macrophages to actively increase the number as well as the mechanical stability of adhesive bonds formed with the object, and to change the loading rate and angle under which the clusters of bonds are zipped open.

5.2 Introduction

Cells sense mechanical cues in their microenvironment and convert those physical signals into biochemical responses (mechanotransduction) to co-regulate cell and tissue structure and function [178][179][180]. Subtle differences in the mechanical properties of the microenvironment have distinct effects on cell migration, differentiation, and morphology [181][182][183][184][185]. Today, microfabricated substrates and cantilever-based techniques including AFM and MEMS sensors are widely used to apply and sense physiologically relevant pico- to microNewton forces in biological specimens [179][186][187][188][189][190]. Cantilever-based tools, however, are restricted to the outer surface of the specimen and are limited in the direction of force application and sensing [191][192]. To overcome these limitations, contact-free force spectroscopy techniques to optically or magnetically trap micro- and nanoparticles in the vicinity or within cells and tissues have been developed [193][194][195][196][197][198]. While forces in the range of femto to nanoNewtons can be transduced, most contact-free techniques are limited in the rota-
tional and translational 3D control of arbitrarily shaped probes [199, 200, 201, 202]. In contrast to spherical probes, the use of non-spherical probes offers the possibility to apply and record a defined torque. Precise control of the applied torque is of particular interest in force spectroscopy of non-covalent interactions, as the mechanical strength of molecular bonds and of cells with materials is not only determined by the total number of bonds, but also the geometry by which the bonds are opened, e.g. in a shear or zipper mode. Even thermodynamically stable clusters of bonds can be opened easily if their bonds are broken in series, one-by-one [203][204][205]. To study the influence of torque on biological, non-covalent interactions, magnetic twisting cytometry with spherical probes has been used extensively [197][206][207][208]. However, 3D rotational tracking using rotationally symmetric particles remains challenging. Rather than active steering, previous approaches required the labeling of the probe with a tracer particle to indirectly read-out the angular position [208][209][210]. Alternatively, optical wrenches in combination with cylindrical quartz probes have been used for direct rotational read-out but they did not allow to decouple torque and force, were limited to a small selection of appropriate probes, and underwent photo damage and sample heating [211][212][213]. Due to the limitations of current techniques, little information is available on how cells generate torque and exploit the full 3D space to probe their environments, interact with neighboring cells, pull on objects or phagocytose particles. Here we introduce the NanoMag magnetic tweezer system that allows quantifying the translational and rotational dynamics of cells and microtissues upon binding and force application to trapped particles. Compared to current state-of-the-art, the NanoMag provides an unconstrained 3D control of the position and orientation of arbitrarily shaped particles, which has not been demonstrated by other contact-free optical or magnetic force spectroscopy techniques [193][195][214][215]. The system enables twisting experiments in 3D under constant force and torque as well as experiments exploring how particle shape in addition to particle size affects the cellular response[216][131][217]. With its large working distance, the NanoMag can be used for mechanobiological studies at various length scales ranging from single cells to engineered tissues and organs integrated in lab-on-a-chip devices and bioreactors. To illustrate the power of the NanoMag, we quantified the cellular forces of macrophages maneuvering on a surface to capture biofunctionalized spherical and rod-like magnetic particles that mimic bacteria in both size and shape. To
monitor the early stages preceding phagocytosis, we positioned the particles near the macrophage’s leading edge using a closed-loop magnetic micromanipulation strategy and tracked the particles by real-time computer vision algorithms. Through vision-based feedback control, the system applies magnetic restoring forces to keep the particle in the user-defined target position. This fast feedback control strategy allows for different modes of force spectroscopy such as dynamic force clamp as well as constant force and constant velocity measurements. Unique to magnetic traps, in comparison to optical traps, is the ability to apply a torque independent of the force and to dynamically adjust the trap stiffness, a feature that we routinely exploited in our study.

5.3 Results and Discussion

5.3.1 NanoMag Characteristics and Features

The NanoMag design consists of eight electromagnets arranged in two inclined sets from the z-axis (Figure 5.1a). This configuration provides a large working space between the coils and the sample plane, making it compatible with standard cell culture conditions and correlative force and live-cell imaging on conventional inverted (confocal) microscopes. The currents through each coil are controlled individually and defined through an actuation matrix, which allows for full control of the translational forces and rotating torques applied in 3D on arbitrarily shaped objects [218][219].
Figure 5.1: Precise positioning of magnetic particles in 2D or 3D by automated tracking through computer vision-based closed-loop control. a) Schematic side view of the NanoMag system. The eight electromagnetic coils are located above the sample stage of an inverted microscope. b) The magnetic particle is tracked by using a Canny edge detection algorithm. Orange outlines indicate the position of the detected particle edges. The particle can be placed in a user-defined target position (indicated by a grey circle). When the target is reached (indicated by a green circle), the position is stabilized by automated feedback control. c) Automated feedback control compensates for Brownian motion and fluidic disturbances. The trajectory of a freely moving particle and the motion under feedback control is shown, each with best least square circular fit of their x-y trajectories. d) The control algorithm was used to precisely maneuver a M-450 Dynabead® particle along a user-defined trajectory (Eth) in a fully automated fashion. The particle was actuated in closed-loop control by applying a constant field of 10 mT and gradients in the order of 0.5-1.5 T/m. Their magnitude was calculated online and depends on the tracked position feedback and resulting current distance to the target.
The actuation matrix is determined through an in situ calibration with a 3D Hall sensor and allows the real-time calculation of the required currents to generate the desired magnetic fields and gradients at update rates of up to 100 Hz. Higher frequencies of up to 2 kHz can be achieved, however only at low magnetic fields at low magnetic fields due to the inductivity of the ferromagnetic core. The resulting fields and gradients are homogenously distributed inside a central spherical workspace of 10 mm diameter, as predicted in FEM simulations and verified by in situ measurements [220]. By carefully selecting the appropriate size, shape and magnetic properties of the probe, the applicable forces, torques and angular and translational stiffness can be tuned on demand. With the magnetic probes used in this work, forces in the range from tens of fN up to 100 pN can be exerted. To increase the force range for a given probe, conical core extensions that reduce the working distance to 500 µm can be inserted to increase the magnetic field strength up to 10-fold.

To study how a macrophage approaches its prey, we positioned a magnetic microparticle at a user-defined target position near the macrophage leading edge using closed-loop control (Figure 5.1). While the spherical particles used here were commercially available Dynabeads® (M-450, Life Technologies) with 4.5 µm in diameter, we fabricated other custom-designed structures with diameters ranging from 1-5 µm and lengths between 5 and 12 µm using a combined two-photon photopolymerization and electrodeposition technology [221]. To trigger particle recognition and binding, the magnetic probes were selectively functionalized with biologically relevant ligands, including anti Escherichia coli (E.coli) antibodies as well as purified E.coli type 1 fimbriae. Once the target was reached, we selected one of two possible recording strategies. First, by applying a particle-tracking mode without applying an external force, we studied the unperturbed interaction between macrophages and the probes. We quantified the macrophage and particle kinetics upon binding. Second, by magnetically “holding” the particle (force-clamp), we studied how macrophages picked up particles that were kept within the magnetic trap at different magnetic trap stiffness’s as we modulated the applied magnetic forces under vision-based closed-loop control.
5.3.2 Implementation of a Dynamic Force-Clamp Concept in Magnetic Trapping

For real-time optical detection of the microparticle position, we employed a modified shape detection algorithm to stably track the particle with sub-pixel resolution, i.e. below 100 nm for our optical setup at update rates between 15 and 30 Hz. After bead localization, a circular target with a radius of 2 µm was defined that corresponds to less than half the size of the probe. The algorithm considers the spatial target position as reached if the centroid of the trapped particle is localized within this region. Once reached the grey threshold patch turns green as shown in Figure 5.1b. The trapped particle stabilized within the trap by a mean deviation of 220 nm at room temperature. Without cell contact, the main disturbance of the particle position was due to Brownian motion (Figure 5.1c). The force clamp was active with a trap stiffness set to 0.66x10^{-4} pN/nm and we successfully picked up a bead and moved it in a fully automated fashion along predefined trajectories, as shown here by writing Eth (Figure 5.1d). Three dimensional path control with the NanoMag has been demonstrated previously by implementing a depth-from-defocus algorithm to extract the position information of the object along z from 2D images [220].

When an external force is exerted that resulted in a displacement of the particle from the target position, for example upon cell contact, the opposing magnetic force is determined by

\[ F = K_s d(k) \]

where \( K_s \) denotes the spring constant and

\[ d(k) = p_t - p(k) \]

defines the 2D in-plane displacement vector at discrete time k that spans between the target position \( p_t \) and the object position \( p(k) \). The displacement \( d(k) \) is detected by an edge detection algorithm in every frame and serves as controller input. The controller determines the magnitude of the magnetic force needed to correct for the offset. As for an ideal oscillator, the applied force increases linearly with increasing displacement. An equilibrium between the cellular force (\( F_{cell} \)) and the counteracting magnetic force (\( F_{mag} \)) is reached when the bead stops moving (Figure 5.1b). Without further bead displacement in the trap, Brownian motion is negligible and the applied magnetic force reflects the force exerted by the cell,
i.e. \( F_{\text{mag}} = F_{\text{cell}} \). The applied magnetic field gradients along \( x \) and \( y \) are the direct output of the implemented proportional-integral (PI) controller. Accordingly, the control output as function of time \( u(k) \)

\[
u(k) = K_P e(k) + K_I \left( T_S \sum_{n=0}^{k} e(n) \right)
\]

consists of two terms, where \( K_P \) and \( K_I \) are the proportional and integral gain of the magnetic trap, respectively, \( u(k) \) is the control output, \( e(k) \) is the current position error, here equal to \( d(k) \), and \( T_S \) denotes the sampling time which is equal to one over the frame rate of the camera. In our experiments \( T_S \) was \( 1/15 \) s and \( 1/30 \) s. The error signal \( e(k) \) served as input for the controller to automatically update the magnetic field gradients magnitude and direction to compensate for this error and to move the particle back into its target position. With this control scheme and the ability of the NanoMag to generated fields and gradients in 3D, we created an artificial potential well that stabilized any selectively tracked object in 2 or 3D. The magnitude of the magnetic gradients is proportional to the resulting magnetic force and therefore increases linearly with the magnitude of the positional error \( e(k) \). Depending on the magnetic moment of the particles and the viscosity of the fluid, higher or lower magnetic forces are required to overcome the drag forces and to replace the bead in its target position. This can be adjusted though the proportional input term \( K_P \). This proportional gain defines the magnitude of the magnetic force for a certain position error and thus, reflects the spring constant of the trap. In case of a constant deviation in the system, such as fluidic drift, the integral part \( K_I \) can be applied for compensation. In most cases, we did not observe fluidic drift, and the system was reduced to a simple P-controller, with \( K_I = 0 \). Therefore, the displacement \( d(k) \), which is equal to the signal error \( e(k) \), is related by \( u(k) = K_P d(k) \). Stable trapping was achieved for \( K_P = 300 \) and increased up to \( K_P = 800 \) during the experiment resulting in a spring constant of \( k_{\text{min}} = 0.66 \times 10^{-4} \) pN/nm and \( k_{\text{max}} = 1.76 \times 10^{-4} \) pN/nm for an individual M-450 spherical magnetic bead (Figure 5.1d).
5.3.3 3D positioning of magnetic particles in the vicinity of macrophages to record the macrophage movement once it touched the particle

Activated macrophages exhibit a polarized cell shape with highly anisotropic mechanical properties [222], whereby filopodia at the leading edge in concert with lamellipodia explore the local microenvironment [25][223]. Using the NanoMag, we picked freely floating anti-\textit{E.coli} IgG functionalized microbeads and placed them at a user-defined target position at the leading edge of the cells (Figure 5.2a). Once the target position was reached, the magnetic control was turned off and we recorded the particle trajectory upon macrophage contact until the macrophages formed a phagocytic cup around the beads (Figure 5.2b). Until binding, small oscillations of the particle position indicated that the particle is free floating and not adherent to the substrate. Soon after macrophages touched a bead, we repeatedly observed that the bead was initially pushed aside (Figure 5.2b, \( t_1 - t_2 \)) before it was pulled towards the cell body. Interestingly, the macrophage typically did not pull the bead directly towards the cell body but moved it inward in a characteristic c-shape trajectory (Figure 5.2b, \( t_3 - t_4 \)). After the initial push with velocities of up to 1 \( \mu \text{m/s} \), the cell pulled on the bead with a mean velocity of approximately 180 nm/s as measured along the curved trajectory starting at \( t = 30 \text{ s} \) (Figure 2c). Similar trajectories were recorded for beads coated with other ligands (\textit{E.coli} type 1 fimbriae mannose-sensitive adhesion FimH), for experiments with mannose inhibitor and for experiments with and without counteracting external magnetic force.

5.3.4 Force-clamp measurements revealed unexpected pushing and rotational dynamics of macrophage-bound microbeads

To monitor the dynamic force-full macrophage-particle interaction, we continuously applied magnetic forces to maintain the particle target position. Upon deviation from the target the macrophage worked against the dynamically applied counteracting magnetic force that linearly increased with the distance of the particle from the target position. This force was opposed to the cell and altered the cytoskeletal extension and retraction processes. When a force plateau evolved the applied magnetic forces were equal to the cellular forces. The maximum achievable force for a single M-450 Dynabead\textregistered{} was 2 pN with a spring constant of \( k_{\text{max}} = 1.76 \times 10^{-4} \text{ pN/nm} \). This
Figure 5.2: NanoMag experiments provide precise local cues for single cells and allow to quantify the pick-up kinetics, such as the trajectory and velocity, of a particle moved by a macrophage in real-time. a) To trigger Fc receptor mediated phagocytosis, a freely suspended IgG functionalized magnetic Dynabead® was placed in close proximity to the leading edge of a surface-adherent macrophage. Once the bead reached its target position, the magnetic control was switched off to observe undisturbed cellular pick-up kinetics. b) Trajectories of a macrophage leading edge (blue and red lines) and a captured spherical particle (colored trace) without force-clamp. Initial push to pulling event resulted in a characteristic c-shape trajectory prior to particle uptake (t1-t4). c) Kinetics of the particle distance to the projected cell center of mass during the pick-up starting at the first physical contact with the cell membrane. To determine the cell centroid, the cell outline was automatically traced from phase contrast images using an “Active Contours”-based tracking algorithm. The velocity of the bead during the particle uptake is overlaid and shows a peak velocity of 1 μm/s upon macrophage push. A mean velocity of 180 nm/s is recorded as the bead is pulled inwards.
is in good agreement with the specifications of other magnetic tweezers used for single cell and molecule manipulation. When cells interact with their environment, they typically exert traction as well as pushing forces. Typically, the first adhesive interaction between a protruding lamellipodium and the bead resulted in a displacement of the bead away from the cell. The bead position was updated at the same frequency as the camera frame rate (here 15 to 30 Hz) and used as direct input to the P-controller. At the same control rate, the magnetic force was updated and linearly increased with the distance of the bead to the target position. As an example, the experiment outlined in Figure 5.3 a-d depicts important stages of the phagocytic pick-up process, the trajectory of the bead, and the monitored forces during those stages. Directional changes of the forces during such push-pull sequences mirror the force vector applied by the cell, as revealed by the force component graphs (Figure 5.3d). During the events shown in Figure 5.3, the bead was displaced along positive y starting 30 s after the initial bead positioning (Figure 5.3a, t₁), leading to an increase in the counteracting force F_y (Figure 5.3d). The velocity of the bead increased during this push up to 1.8 μm/s (Figure 5.3d) at t = 43 s, followed by a slight push along negative x at t₂ = 50 s, thus, increasing the +F_x force component. The direction of the motion was then reverted, as the bead was pushed sideways along positive x. At a displacement of approximately 4 μm and an opposing force of 0.53 pN at t₃ = 69 s, the bead direction changed again and was pulled towards the cell and the bead velocity decreased to a mean velocity of 550 nm/s. As depicted in Figure 3b, this push-pull motion does not occur along a linear trajectory. Instead it follows a c-shaped path between t = 60 s (yellow) and t = 80 s (orange/red), which is evident in the bead trajectory and can be also followed in the force component plot shown in Figure 5.3c.

For this particular event, the opposing force decreased first as the bead was pulled through the predefined target location, and thus, the positional error signal e(k) decreased until t = 80 s. Then, with decreasing distance to the cell membrane, the magnetic restoring force increased again. An increasing number of membrane ruffles formed around the bead, and, at t = 115 s with an opposing force of 1.25 pN, the bead was briefly restored to its original target position, and was then instantly pulled back by the cell. The restoring force was increased again, and we ultimately switched off the force clamp to allow particle internalization. In general, we were
Figure 5.3: In force-clamp mode, the NanoMag allows quantification of cellular forces and associated particle dynamics. a)-d) Force-clamp experiment with an anti-E. coli antibody coated M-450 Dynabead®. a) The spherical particle was placed in close proximity to the cell (green target position at $t_1$). Membrane protrusions developed towards the trapped bead pushing the bead away from the cell body for 20 seconds with a counteracting force reaching up to 0.35 pN ($t_2$). Subsequently, the bead was pushed sideways (to the left) and pulled inwards following a characteristic C-shape trajectory ($t_3$). The counteracting magnetic force decreased as the bead was pulled inward. b) Bead trajectory during the first 80s after contact c-d) Absolute force and force component graphs of pickup sequence. e)-h) Experiment with an E.coli type 1 fimbriae mannose-sensitive adhesion FimH coated M-450 Dynabead®. e) Representative phase contrast images. g) Absolute force over the entire contact sequence. The time intervals of the pushing events are shown. Trajectories of side-/downward and side-/upward pushing at different time sequences with corresponding images are depicted in f) and h) for two pushing peaks indicated with (I) and (II) in the force plot.
unable to pull off particles that were fully engulfed by the macrophage phagocytic cup, as the maximum force that can be applied to the Dynabeads is limited to 2 pN in the standard NanoMag configuration, unless the conical extensions were applied. By reaching forces in the range of tens of pN, adhering particles could be detached from the cell surface in the early stages of phagocytosis. During the force clamp experiments, we observed several sideward and upward pushing events without subsequent particle internalization (Figure 5.3e-h). Those events originated either from very dynamic lamellipodia or thick, long filopodia-like spikes and lasted for 10-30 s at forces as high as 1.3 pN. Interestingly, we did not observe such behavior in the experiments without external force-clamp (Figure 5.2), which suggests that the mechanical resistance induced by the counteracting force inhibited uptake.

To study if instantaneously increasing counteracting forces affect particle pick-up, we increased the stiffness of the magnetic trap during the force clamp experiment. For a FimH functionalized bead, we recorded slight pushes to the side within the first 35 seconds after the initial bead positioning (Figure 5.4). Subsequently, the cell pulled the bead inwards along a straight line while increasing the trap stiffness dynamically increased the opposing force. The first increase was induced at $t_2 = 45$ s which was followed immediately by a formation of a thick membrane ruffle (see Figure 5.4a, $t_3 = 59s$).

Further stiffening was applied by changing the gain of the PI controller online until the bead was completely surrounded by membrane ruffles. Similar behavior was observed in other experiments where a FimH-coated bead was presented to the macrophage.

### 5.3.5 Controlling the orientation of rod-shaped particles reveals rotational movement during particle pick-up

In addition to particle size, particle shape is an important design parameter to modulate the cellular response [216][226]. To efficiently clear pathogens, shape recognition of dead cells or of foreign particles plays a crucial role for cells of the immune system [226][227]. The precise 3D control of non-spherical magnetic particles, however, poses control challenges and has so far mainly been demonstrated using nanowires (NWs) maneuvered in 2D by magnetic [194], electrical [228], and optical tweezers [229]. Control of NWs in 3D has recently been reported using multiple beam optical traps but the manipulation control was
Figure 5.4: Dynamic adjustment of the magnetic trap stiffness during live cell experiments (dynamic force clamp). a) Representative time frames of a macrophage during particle pushing of a spherical magnetic particle that was functionalized with *E.coli* type 1 fimbriae. After initial contact within the first 10 seconds, a lever arm-like membrane protrusion formed towards the bead to pull it towards the cell body. This resulted in a slight side push of the particle (t1). The particle was then pulled with a lever arm towards the cell body (t2). As the cell moved towards the particle in parallel, the cell occupied the initial target region. Thus the target position was adjusted and shifted away from the cell, increasing the applied force instantly. Upon this resistance, followed by further stiffening of the trap, a strong membrane ruffle formation was observed (t3). b) Bead trajectory during macrophage encounter. c) Applied absolute forces during macrophage-particle encounter monitored in dynamic force-clamp mode. Push and pull events of the particle are indicated. d) The x and y components of the forces during particle pushing are depicted for the time interval between 10 and 35 seconds where the initial macrophage encounter occurred. The slight push along negative y at t1 = 27 s is opposed with restoring force of 0.12 pN along positive y.
limited and the systems have not been applied to manipulate cells \cite{202,230,231}. For any arbitrarily shaped object exposed to an external magnetic field, the long axis is the preferential magnetization axis of the object that aligns with the field axis. With the ability of the NanoMag system to generate fields and gradients in 3D, the non-spherical particles can be freely manipulated and presented to surface-adhering cells and the mechanics of cell-particle interaction can be studied as a function of particle orientation (Figure 5.5a-c). As a proof-of-concept, we positioned custom-made CoNi-alloy microrods in the vicinity of the macrophages. Once the target was reached, gradient-based actuation was applied for precise translational positioning. We further introduced particles of other shapes such as ellipses and bowling pin-like microobjects, which were maneuvered towards single cells demonstrating the capability to perform shape-dependency studies under externally applied forces.

We then used microrods (r =1.5 µm, l= 7 µm) to study the effect of the probe orientation on the phagocytic uptake process, as a microrod was placed parallel to the direction of macrophage lamellipodia protrusion (Figure 5.5b).
Figure 5.5: Rod-shaped microparticles in combination with force-clamp allow dynamic control and quantification of torques applied by macrophages during particle pick-up. a) 3D rotation of non-functionalized CoNi microrod. In close vicinity to the cell, the microrod position and orientation were precisely controlled with respect to the cell. Schematic side views of the associated particle orientation are depicted. The distribution of the input current for each of the eight electromagnets is shown in the upper left corner with the length of the bars being proportional to the magnitude of the current, whereas red and blue represent positive and negative signals respectively. b) Representative sequence of a macrophage-microrod encounter. The microrod was initially placed parallel to the active lamellipodia protrusion. Upon lamellipodia binding, the macrophage rotated the rod by 90° parallel to the x-y plane. An active contour model-based vision algorithm was applied to trace the circumference of the cell (red) and the nucleus (green). The turquoise and green trace show the particle and nucleus trajectory. c) Plot of the angle α during particle pick up with unlocked particle orientation. The angle α spans between the long axis of the microrod and the x-axis of the image frame and is depicted positive when the long axis of the object lies in the first quadrant, and negative if positioned in the second quadrant. d) Presentation of cylindrical probe in an initially locked orientation by the application of an external magnetic torque T. Upon release from the orientation perpendicular to the cell leading edge, particle uptake occurred. e) Corresponding plot of the angle during particle encounter with locked orientation and subsequent particle reorientation by the macrophage under unlocked conditions.
The magnetic field was then gradually decreased to maintain the orientation and avoid relaxation induced by the torque release. Membrane ruffles formed underneath the rod, which was then transported towards the cell body. An increased vacuole activity, including fusion and motility, was observed along the pillar edges, which is most prominent in the transition from time stamp $t_1 = 0 \text{s}$ to $t_2 = 162 \text{s}$ (Figure 5.5b). The membrane was constantly remodeled and a co-planar dependency of the cellular long axis with the pillar long axis was detected, as evident in all time frames. The microrod was rotated by about $90^\circ$ (Figure 5.5c) and internalization starting at one pole was observed, as indicated by focal plane changes across the cell body and shortening of the projected microrod length. We observed this co-planar dependency in the uptake process repeatedly, even when the probe was initially placed perpendicular to the cell axis supporting earlier findings that the shape in addition to target size influences the kinetics and efficiency of particle uptake [226][227][216].

Next, we investigated the effect of locking the angular orientation of the microrod on the uptake process. With an estimated magnetic moment per microrod of 1.2 pAm$^2$ at 3 mT an angular trapping stiffness in the order of $6 \times 10^4$ pN nm/rad was applied during the experiment depicted in Figure 5d-e. The magnetic torque was applied such that the long axis of the particle was orientated perpendicular to the cellular axis ($-40^\circ$). The rod was shuffled towards the cell body at a mean angle of $-36^\circ \pm 6$ through the formation of large lamellipodial protrusion that formed underneath the rod. Upon release from the locked orientation at $t=t_2$, the rod was instantaneously rotated on the cell membrane and was eventually aligned with the long axis of the cell (Figure 5.5d, $t_3$). An increased transport velocity and standard deviation of the angular orientation of factor of two were measured compared to the locked condition (Figure 5.5d-e,$t_1$ -$t_2$ vs. $t_2$ -$t_4$). At higher field magnitudes reaching an angular trapping stiffness in the order of and $3.5 \times 10^6$ pN nm/rad, the cells were unable to rotate the rods, which was also apparent in significantly lower standard deviations of the angular orientation and has been observed repeatedly. Depending on the experiment, the angular trapping stiffness can be adjusted and e.g. significantly lowered by choosing smaller probes or probes with lower mass magnetization properties.
5.4 Conclusion

To illustrate the power of the NanoMag system in a proof-of-concept study, we recorded the forces that macrophages exert when they approach and capture their prey. We presented trapped prey-mimicking biofunctionalized spherical and rod-shaped magnetic microparticles to the macrophages and automatically tracked the kinetics of the cell-induced particle displacement and variations in cell shape (Figure 5.1). We repeatedly recorded a push-pull behavior with pushing forces on the order of 1.5 pN after the initial macrophage-particle contact was formed (Figure 5.3 and 5.4). Upon adhesion, reinforcement of the contact region could be triggered by dynamic adjustment of the trap stiffness. We observed that macrophages typically approached spherical beads at an angle and pulled on the beads exploiting a C-shaped trajectory (Figure 5.2 and 5.3). Such rotational characteristics of the pick-up kinetics became even more distinct in experiments with rod-shaped particles (Figure 5.5). To position arbitrarily shaped magnetic microparticles in a user-defined target, the NanoMag exploits optical displacement measurements of as feedback parameters to create and steer an artificial potential well through 3D space. The forces to maintain the pre-selected target position are applied at frequencies that are determined by the image feedback rate and, thus, the camera frame rate. Depending on the NanoMag configuration and the magnetic properties of the probe, forces in the femto- to nanoNewton range can be applied. By introducing uniaxial symmetric, non-spherical particles, the NanoMag can control and track the rotational orientation of the probes independent of force application (Figure 5.5d). Engineered probes with application-specific dimensions and magnetic properties allow for controllable out of xy-plane twisting at precisely tuned angular trapping stiffness. The force and displacement traces recorded in this study may suggest that macrophages use the advantage of pulling on objects at an angle to actively increase the number and mechanical stability of adhesive bonds formed and exploit that higher forces are needed to break clusters of bonds in a shear versus zipper geometry [232][233]. In summary, the NanoMag system provides a new platform for a wide range of applications in mechanobiology as it combines versatile 3D rotational and translational control of arbitrarily shaped soft-magnetic probes with feedback controlled force application and automated trap stiffness adjustments. The system allows to hold on to a pre-selected particle at a defined angle or to measure the dynamic force development at a predefined (angular) position with a
precision of a few 100 nm. Those features are applicable at a multi-length scale, ranging from single cells, microtissues, to in vivo studies and will enable further force spectroscopy studies on e.g. how loading rate and angle of force application influence the thermodynamic and mechanical stability of non-covalent clusters of bonds.

5.5 Material and Methods

Experimental Setup The system consists of eight stationary electromagnets with ferromagnetic cores, and generates arbitrary magnetic fields and field gradients up to 50 mT and 5 T/m at frequencies up to 2 kHz. With a working distance of 2 cm, a fairly large workspace is provided that allows for manipulation at different size scales and geometric constraints. The in situ calibration procedure allows for online calculation of the required current inputs required for the desired control outputs, which are either set manually or automated through vision-feedback based control. For live cell experiments, the NanoMag setup was equipped with an incubation chamber to maintain a constant temperature of 37°C and atmosphere of 5% CO2. The NanoMag is incorporated into the Olympus IX 81 inverted microscope equipped with a fluorescence light source, a Pointgrey Grasshopper GRAS-50S5C 15 fps Firewire camera or a Basler sca-1400 30 fps Firewire camera, respectively. The highest possible magnification is achieved with a 100x Olympus LCPlanPF objective at an effective pixel size of 68.12 nm. We worked with a 40x objective at 1.6x additional magnification resulting in a resolution of 1392 pixel x 1040 pixel at a pixel size of 100.78 nm.

Cell culture and bead functionalization Mouse macrophage-like cells (J774A.1) were cultivated in DMEM media (Sigma D5921) supplemented with 10L018), 50 µg/ml kanamycin, and 2.5 mM L-Glutamine. 24 hours prior to magnetic measurements, J774A.1 cells were seeded to 20000 cells/cm² in custom-made polydimethylsiloxane (PDMS) (Dow Corning Sylgard 184) wells with fibronectin-coated glass bottoms. To prime macrophages into M1 classically activated cells, IFN-γ was added 24 hours and E.coli lipopolysaccharide (LPS) (Sigma L4391) 6 hours before the measurements. Magnetic microspheres (Invitrogen Dynabeads M-450) were functionalized via covalent coupling of the Tosyl group on the Dyn-
abeads to amino residues within purified *E.coli* type 1 fimbriae, and goat and mouse IgG against LPS (abcam ab35654), respectively. Custom-made CoNi microrods were added to macrophages without prior functionalization.

### 5.6 Acknowledgements

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Wiggling and arm-twisting between magnetic microparticles and macrophages: Approaches to study how the prey can escape

The work presented in this chapter is ongoing as a follow-up study of the work described in Chapter 5. This work is performed in collaboration between:

Ima Avalos Vizcarra, Simone Schürle, André Lindo, Salvador Pané, Ingmar Schön, Philip Kolmannsberger, Selman Sakar, Bradley Nelson, and Viola Vogel.   IAV, SS, and VV and designed research, SS and BN developed the magnetic trap, IAV and SS performed experiments, IAV, SS and IS analyzed data, AL and SP designed and fabricated magnetic microparticles.
6.1 Motivation

When phagocytes capture non-spherical microscopic prey such as rod-shaped and filamentous bacteria, they preferably start to ingest their prey from one of the poles. To ease the uptake, they apply striking rotational maneuvers to adapt the orientation of the foreign object. With the ability to mimic such prey in shape and motion, we asked how the orientation and motion of the non-spherical phagocytic target might influence the pick-up dynamics by macrophages to reveal strategies of attack on the particles, learn the underlying mechanics and identify levels of frustration. We determined repeatable first contact orientations and applied orientation clamps in 2D and 3D with varying stiffness. In a next step, we applied a dynamic stepping torque to generate a wiggling motion such as swimming rod-shaped bacteria employ. Exploiting potential torque thresholds may also aid drug delivery strategies in therapeutic approaches using non-spherical microparticles.

6.2 Introduction

Phagocytes, specialized cells of the innate immune system of multicellular organisms, act against potentially harmful intruders by recognizing and ingesting them for intracellular degradation[234][235][236]. This protective ingestion of pathogens and foreign objects is an active and inherently mechanical process, driven by the phagocyte’s force-generating cytoskeleton[237][238][239] which allows building membrane protrusions that engulf the foreign body. Pathogenic microorganisms have in turn developed strategies to exploit or modulate cytoskeletal mechanics of host cells for their own survival purposes [240][32][241]. Understanding the strategies that pathogens use to manipulate host cell mechanics can benefit both therapeutic approaches as well as fundamental insights on the cytoskeletal dynamics of immune cells[236].

The cytoskeleton, consisting of actin-based microfilaments, microtubules, intermediate filaments and motor proteins is a key player in pathogen entry as well as the uptake by host cells. Studies that have employed readouts on the cytoskeletal mechanics have yielded important insights on the forces and mechanical aspects that need to be considered in the study of host-pathogen interactions: Size and shape of an object are important, albeit static factors which can limit the
phagocytes’ ability to ingest particles[242][67]. In particular, changes in curvature and aspect ratio of objects can result in abortion of engulfment, a process that is called frustration of phagocytosis. Inducing such frustration of phagocytosis by limiting the phagocyte’s ability of engulfment was described as a powerful evasion strategy of pathogens [68][66]. Since the cytoskeletal dynamics are highly dynamic with response times on the minute scale, recent advances in live cell imaging have furthermore inspired engineering approaches to quantify the mechanics in phagocytosis[243][223] with the resulting finding that rotational motions play an important role during the interaction of macrophages with their targets[244].

Many studies of phagocyte dynamics have focused on microbeads or living spherical targets such as baker’s yeast[68][244]. Spherical objects provide symmetrical points of attack for membrane attachment and engulfment and can be easily manipulated by optical tweezers or AFM tips which has resulted in a common use of spherical microbeads for single cell studies [245][246][247]. While studies with spherical objects provide valuable insight into the mechanics of phagocytosis, macrophages also engulf non-spherical objects, e.g. many bacterial species such as strains of Salmonella, Escherichia and Listeria, which resemble rod-like and even filamentous shapes. Rod-shaped phagocytosis targets differ from spherical objects in that the points of attack on rods were distributed preferentially on the poles of the rods which had a large impact on the success and efficiency of phagocytosis[67][66]. Studying cylindrical particles particularly allows resolution of rotational activity of macrophages which can be followed by particle trajectories during uptake[223][244]. The rotation of spherical and rod-shaped phagocytosis targets has emerged as an important variable in phagocytosis by macrophages[244].

Here we take rotational analysis one step further and reverse the situation for the phagocyte by presenting a non-spherical, elongated object that can be trapped to probe the cellular torque. While many studies have employed manipulation of particles to actively impose forces on cells and monitor their reaction [238][239][245], we asked here which decisions and attack strategies macrophages employ. We presented macrophages with rod-shaped magnetic particles in either a parallel or perpendicular orientation to the major axis of macrophages. We thereby locked the orientation of the magnetic rod in an 8-coil magnetic trap whose stiffness could be dynamically adjusted to apply and modulate
torques precisely. Since many bacteria are able to swim or wiggle [248][249], we also employed the dynamically adjustable magnetic trap to mimic a wiggling motion after the model system of flagellated, motile bacteria. Swimming bacteria can apply a resistive counterforce to phagocytosing macrophages and could potentially delay or destabilize membrane engulfment [250][251][252][219].

6.3 Results and Discussion

Trapping of the orientation (orientation clamp) but not the translation of magnetic microparticles was achieved by a magnetic trap with eight separately controllable electromagnetic coils [219]. Using this magnetic trap, rod-shaped magnetic particles with surface-adsorbed mouse immunoglobulin G were placed in the close vicinity of macrophage membrane protrusions and time lapse imaging of particle dynamics during the interaction with macrophages was performed with an imaging rate of 15-30 Hz. Freely rotatable particles were used to mimic phagocytic targets that can be picked up without mechanical restraints. The orientation was then clamped using the magnetic trap to mimic phagocytic targets that resist phagocytosis, first investigating the impact of static orientation clamp and then adding a rotational component to generate a wiggling motion to mimic a bacterium wiggling against the phagocytes’ membrane.

6.3.1 Macrophages rotated their prey during pick-up

Since it was found that macrophages attack rod-shaped particles and bacteria at the poles rather than from the center of mass [67][131][66], we asked here if a particle orientation perpendicular to the cell axes would prompt the macrophage to rotate it. Using the NanoMag system allowed us to apply a torque to rod-shaped magnetic particles and lock them initially in a pre-defined orientation without locking the translational motion of the rod, and thus its absolute position. By slowly reducing the magnetic trap stiffness to 0, a particle reorientation by relaxation was reduced so that a freely rotatable particle could be presented to the macrophage with a defined initial orientation (Figure 6.1). Large changes in angular orientation could be observed consistently from experiments with freely rotatable particles (Figure
We asked if the application of a torque changed the ability of macrophages to pick up particles. We were interested in finding out if a macrophage would enhance its effort of particle uptake if an opposing force in the form of a particle orientation clamp was applied. Towards this end, we specifically investigated particle dynamics such as velocity, trajectory, angular deviation and velocity, and torque thresholds to quantify particle handling by macrophages in a range of applied torques and target orientations and in combination with fluorescent readout of the cellular cytoskeleton dynamics. To investigate cytoskeletal dynamics, we used the LifeAct marker that binds to the F-actin component of the force-generating actin filaments.

6.3.2 Orientation clamp impaired the rotation of phagocytic targets by macrophages

We started the investigation by applying a defined orientation to particles that were oriented perpendicular to the cell axis. In this setting, only the orientation of the particle was locked while the position was left free, i.e. the cell could apply translational motion like pulling and pushing of the particle. We followed the particle position and orientation over the duration of macrophage-particle interaction and analyzed the standard deviation of the particle angle. We assumed that variations in the particle angle were mainly induced by the macrophage. To test this assumption, we compared the standard deviation of freely rotatable particles with those that had been orientation-clamped (Figure 6.2). We observed consistent interaction of the macrophages with the magnetic prey in terms of translational as well as rotational manipulation. The standard deviation of the angle was much larger but also spread over a larger range when particles were freely rotatable (Figure 6.2), indicating that the applied torques were at the upper range of what a macrophage can apply to rotate it.

Since we were interested in investigating rotational aspects, we compared the angular velocities of freely rotatable and orientation-clamped magnetic particles during the pick up by macrophages (Figure 6.2d) which showed heterogenous results, and did thereby not directly allow to derive a trend from the data. A more thorough
Figure 6.1: Macrophages rotated rod-shaped particles that were presented to them in a perpendicular orientation. a) and b) Scanning electron micrographs of macrophages interacting with freely rotatable magnetic microparticles indicated parallel alignment of the particles towards the cell axis. c) Overlay of timeseries from live cell imaging of macrophages interacting with freely rotatable magnetic microparticles indicated a rotation by the macrophage during pick-up. d) The change of particle angle over time is given as the difference to the initial particle angle at the first timepoint of the observation. Angular deviation from three randomly picked experiments are shown that correspond to the timeseries in c (the respective images in c were highlighted by the same color of the respective curve in d).
comparison of means and analysis of variances would be required to conclude on a trend in this parameter.
Figure 6.2: The interactions between macrophages and magnetic bacteria-mimicking particles are limited by the application of torque. a) One example of a macrophage picking up a freely rotatable particle. The change of particle angle over time is given as the difference to the initial particle angle and was calculated by subtracting the angle of the first time point from the angle of each subsequent time point. b) One example of a macrophage picking up an orientation clamped particle. The change of particle angle over time is given as the difference to the initial particle angle and was calculated by subtracting the angle of the first time point from the angle of each subsequent time point. c) Overview of 12 experiments in a large range of cell-opposing torques. Single data points represent time intervals with a defined torque. Standard deviation of the particle angle was reduced with increased applied torque d) Box plots of the angular velocities of particles during 12 independent experiments with varying torques. Experiments without applied torque are highlighted by blue boxes in the plot.
6.3.3 Monitoring of LifeAct suggested actin recruitment to orientation-clamped particles

Using LifeAct, which binds to actin of the macrophage and can be used to visualize cytoskeletal mechanics, we were interested in following actin recruitment in the vicinity of particles to visualize the underlying structural mechanics. (Figure 6.3e) At the same time we were interested in defining force thresholds of macrophages during potential rotational movement of rod-shaped particles. Towards this end, we monitored the LifeAct signal under the area that was covered by the particle to investigate potential recruitment. We observed a continuous increase in fluorescene intensity towards of of the poles of the orientation clamped rod, which could indicate that macrophage actin was recruited to this site to oppose the applied torque. By monitoring the particle dynamics, we observed that by reducing the torque via reduced trap stiffness, larger angular deviation occurred (Figure 6.3b).

6.3.4 Applying wiggling rotations to magnetic particles may help microparticles to evade phagocytosis by macrophages

While it has been described that bacterial motility can enhance phagocytosis by macrophages, we argued that from a mechanical point of view, motility can induce also resistive forces that counteract membrane engulfment. To test this assumption, we applied rotation to the magnetic particles to generate a wiggling motion as it can be observed by motile rod-shaped bacteria such as E.coli but without the associated directional momentum. Again, we monitored the intensity of LifeAct in the region of the macrophage that was in contact with the magnetic particle (Figure 6.4). The intensity profiles of the region of interest appeared fluctuating over the selected region of interest with local, sporadic maxima that seemed not correlated with the location of the microparticle. However, these observations still require repeated observation to allow extrapolation to a general trend.

6.3.5 Outlook

To fulfill their health-protective clearing task, phagocytes need to recognize, pick-up and ingest foreign bodies of various sizes and shapes which was successfully exploited to identify optima and limitations of the phagocytic process [68, 242, 254].
Figure 6.3: A Macrophage rotated perpendicular oriented particles during phagocytosis attempts and recruited actin to one of the particle pole. 

a) The trajectory of the particle over time is plotted over the cell outline, indicating the particle path at the specified time points. 
b) The changes of the angle of the particle indicate rotations of the particle by the macrophage in dependence of the applied torque. 
c) The velocity of the particle over time indicates handling dynamics of the particle by the cell. 
d) Plot of the applied magnetic field B oscillations. First, the interaction was studied in static conditions by solely applying a magnetic torque to lock the particle orientation. Wiggling motion was then induced to the particle in later stages of the interactions by applying sinusoidal or stepping signals in the range of 0.2-3 Hz. 
e) The LiveAct signal under the particle covered area indicates local actin recruitment and reinforcement of macrophage actin to the particle.
Figure 6.4: A wiggling motion resulted in fluctuating LifeAct "wave formation". The fluorescence intensity of LifeAct transfected macrophages was monitored over time while a stepping wiggling motion was applied to a magnetic microparticle on top of the cell. The intensity profiles of the fluorescence intensity appeared fluctuating without obvious preference to a particular location of the cell.
Particularly the limitations of phagocytosis have helped to identify pathogen survival strategies [25, 67, 133] and mechanical bottlenecks in therapeutic approaches when phagocytes try to ingest medical implants and drug carriers [242]. While a requirement of rotation in phagocytosis has been studied before, it was so far not possible to use non-spherical objects which can be actively controlled and manipulated during the interaction with macrophages. Here we show for the first time that rod-shaped particles could be locked in orientation by applying defined torques, but without interfering with their translation, which is important in evaluating the individual contribution of rotation in phagocytosis. We find that macrophages preferentially rotated their rod-shaped prey during pick-up and that this rotation was consistently downregulated by applied torques mainly in the range of $10^4$-$10^5$ pN nm/rad. Even for strongly impacted rotation by the applied torque, macrophages still picked up their prey using lamellipodial membrane protrusions and transported it rearwards, with velocities ranging between 0.02 and 0.8 $\mu$m/s. This observed range of particle velocities is in general agreement with previously reported velocities of actin retrograde flow in migrating cells, although the velocities that we observed here extend the range in both directions which was reported to lie within 0.08 and 0.2 $\mu$m/s [238]. To conclude on swimming and wiggling as a bacterial escape strategy from phagocytosis, it will be necessary to investigate lower torques than have so far been applied in this study. Literature values of torques exerted by motile bacteria range from 100-1000 pN nm/rad [249, 255] which is 1-2 magnitudes lower than what we have applied. It will therefore be important to perform additional magnetic trap experiments to make conclusions about the impact of wiggling on frustration of phagocytosis. Reaching the required range of lower torques will be possible by decreasing e.g. the dimensions or magnetic material used for the microparticles as well as decreasing the stiffness of the magnetic trap. Future work on quantifying the absolute internalization efficiency of the magnetic particles by macrophages is required. These quantifications will be important to conclude on frustration levels of phagocytosis after handling orientation-clamped particles versus freely rotatable ones. The link between particle torque and impaired handling by macrophages which we observed has potential implications for drug delivery if a link to frustrated phagocytosis can be established. Since therapeutic approaches can fail when the employed drug carriers are cleared by phagocytes, frustration of phagocytosis could increase specificity of drug applications which
need to be applied very locally. Such frustration of phagocytosis could be achieved by restraining the access to the pole of a particle by applying torque and wiggling.
Outlook

The work presented in this thesis shows a large focus on the consequences of bacterial adhesion for bacterial survival on surfaces. Growth and morphological aspects were discovered that play a role in bacteria-surface interactions and can impact bacterial survival. Some of the studies have also investigated the response of macrophages to *E.coli* adhesion and microparticles that attach to the macrophage membrane. For instance, it was observed that macrophages undergo pronounced changes in cell size and adhesive behavior after being exposed to bacteria (Chapter 4), a finding which has not been presented in this context before. Many phenotypic effects, which take place on the timescale of hours to days, are induced by much faster intracellular signaling pathways that are triggered upon the stimulation of macrophage membrane receptors. These membrane receptors transmit adhesion events and mechanical stimulation to the intracellular side of the membrane and transform them into chemical signals such as phosphorylation which in turn produce further signal propagation throughout the cell. One of the next steps in following up the results presented in this thesis is therefore to include the intracellular dynamics that occur in macrophage phagocytosis of bacteria and
foreign bodies.

7.1 The dynamic macrophage response during phagocytosis of foreign bodies

One protein that is involved in the transduction of signals that occur outside the cell and influence the expression of genes, is the protein nuclear factor kappa B (NF-κB). NF-κB controls the expression of genes that promote as well as suppress inflammatory responses from infections to cancer [256]. Recent findings suggest that temporal patterns of nuclear NF-κB retention rather than its absolute concentrations may be key to this differential control [257] and it is still not fully understood how one transcription factor can control several opposing functionalities.

7.1.1 NF-κB fulfills essential functions in macrophage survival and pro-inflammatory response in infections

The activity of NF-κB is regulated by a negative feedback loop. In unstimulated cells, NF-κB is found in the cytosol in an “inactive” form and it can induce expression of its targets genes after translocating from cytoplasm into the cell nucleus, i.e. by overcoming the barrier of the nuclear envelope [258]. Once NF-κB has translocated to the nucleus, it is in its “active” form. Translocation to the nucleus is enabled exclusively by a nuclear localization sequence (NLS) - an amino acid sequence that is highly conserved among eukaryotes and which also has a high affinity for the inhibitors of NF-κB. The NLS is therefore shielded and prevents NF-κB from entering the nucleus as long as inhibitors are present and able to bind the NLS. Several inhibitors of NF-κB have been identified that bind the NF-κB . To release the NLS and make it accessible to the import receptors of the nucleus, the inhibitor needs to dissociate from NF-κB, which is only possible after phosphorylation of a specific tyrosine residue of the inhibitor. The phosphorylation of its inhibitors and activation of NF-κB itself is controlled by a large network of biochemical ligands. Therefore, signal-specific temporal dynamics of NF-κB activation may be a means to control the specific activation of a subset of the many different target genes of
NF-κB.
Actuating stimuli of NF-κB vary from cell-secreted factors such as TNF, IL-1, glutamate to pathogen secreted factors such as LPS, and bacterial metabolic products and PAMPs on pathogens themselves. NF-κB plays a central role in the signal transduction that is induced by the recognition of bacterial pathogens. Activation of NF-κB occurs in a pulse-like manner on the timescale of minutes\[259\].

7.1.2 Been there, done that - could sustained oscillations in NF-κB encode decision making during phagocytosis?

Degradation of bacteria may require a different intracellular procedure as well as increased material requirements in terms of membrane and production of the phagolysosomal components than phagocytosing small molecules like soluble bacterial factors. Whether additional information is encoded and signal-specific patterns are contained in the dynamic oscillations of NF-κB is not comprehensively understood \[257\]. Concentration-dependencies of the input signal and intrinsic noise in signal propagation have been found to play an important role \[259\]. However, many immune scenarios do not only involve stimulation by soluble factors, but also localized stimuli, e.g. when immune cells bind single bacteria in a wound or infection site and much needs to be learned about the comparison of receptor triggering of immune cells caused by soluble vs. object-tethered ligands.

It is therefore planned to investigate:
- NF-κB activation and mechanical stimulation of macrophages. Differences between stimulation with soluble small molecules and a combined phagocytic stimulation with particles which requires more membrane could be expected from the different timescale on which different phagocytic events take place.
- Long term oscillations of NF-κB activation
- FimH specific signalling since fimbriae are found on both pathogenic and non-pathogenic E.coli but implicated in increased survival of E.coli in macrophages. Therefore, monitoring the dynamic macrophage response after fimbriae-mediated adhesion and uptake in comparison to non-fimbriated bacteria as well as particles may shed further light on the role of how adhesion of bacteria is perceived by the macrophage.
Figure 7.1: The dynamic NF-κB response of single macrophage cells to the presence of *E. coli*. A non-surface-attached *E. coli* (indicated in the image by a yellow colored arrow) approaches a cluster of macrophages and attaches to one of the macrophages. In all the macrophages that were observed, the localization of the NF-κB changes from cytosolic to nuclear (one example for a macrophage during this activation is indicated by a magenta-colored arrow in the time series images).
7.1.3 Following the fate of phagocytosed intracellular E.coli in real-time and in color

To learn more about the bacterial survival inside macrophages, discussions with Philip Kollmannsberger about the options for following intracellular bacteria during live cell experiments (using the fluorescence readout described in section 8.1 of this thesis) led to the development of a MATLAB analysis script by him which reads timeseries of fluorescence images and applies automated thresholding and background correction to identify fluorescent bacteria in the images. A user interface enables the manual selection of single bacteria in each of the images of the respective timepoints to plot intensity and bacterial trajectory. This tool allows tracking and fluorescence quantification of intracellular bacteria (Figure 7.2) which is important during studies of intracellular trafficking, kinetics of intracellular degradation as well as bleaching studies. It would be very interesting to apply this tool to the specific question of ligand-dependent trafficking of phagocytosed bacteria as was found to occur e.g. FimH-dependent. [54].

Figure 7.2: Tracking x-y-trajectories as well as fluorescence intensity of phagocytosed pkatGp-GFP expressing E.coli inside macrophages. (a) Trajectories of three single bacteria inside a macrophage are depicted. (b) The intensity of the same three single bacteria which also express pkatG-GFP bacteria is plotted, indicating that the promoter of the catalaseG gene was activated in phagocytosed bacteria.
7.2 Studies of disease-related macrophage systems

Terminally kidney-insufficient patients who require regular dialysis treatment receive during their treatment an incubation of glucose solution of the peritoneum. After 2-3 hours, the solution is released again from the patients peritoneum, and usually discarded. The peritoneal solution contains high levels of a pure macrophage population (peritoneal macrophages) which due to their good accessability as well as their high activity, have been used extensively to study host pathogen interactions. It would be interesting to include such patients samples in considerations on macrophage activity and bacterial survival as well as the efficiency of clearing bacteria from infected macrophages. The requirements for such a collaboration between the Unispital Zurich and ETH Zurich would be to obtain an ethics approval such that the patient samples could be used for research purposes as well as control samples from healthy patients could be obtained. The advantages of such a model system would be that a direct comparability to the studies with mouse macrophages could be obtained as well as a further insight into links between metabolic disorders such as diabetes type 2 and the effectiveness of innate immune response.

7.3 The next step in surface colonization: bacteria form biofilms

The work discussed in this thesis centers around the understanding of the early processes of bacterial surface colonization. While surface-adherent bacteria continue replication on surfaces, some bacteria have the ability to form threedimensional communities of many bacterial cells that are called biofilms. One particularly interesting aspect of biofilms is their spatial organisation which includes jelly-like extrabacterial substances with which bacteria stick to each other and the surfaces and inside which channel systems can form. This extra-bacterial structure is called the biofilm matrix since bacteria are embedded in it similar to the extracellular matrix of tissues. If such biofilms form inside the body, e.g. on implant surfaces, the single bacteria in it are thus not easily accessible to immune cells such as macrophages anymore. Having seen how important mechanical considerations in the phagocytosis of surface-attached bacteria are, one can only wonder how immune cells would try to ingest bacteria that are embedded in a biofilm matrix. Much
could be learned from high resolution images of the interface between macrophages and biofilm surfaces. For instance it would be very interesting to compare the NF-κB response of macrophages to biofilms to first evaluate how the response of macrophages towards this phagocytic target is different from that to single bacteria. Additionally, it would be important to evaluate the phenotypic response of the macrophage, e.g. in terms of adhesion to the biofilm by analyzing focal adhesions. Since it was found that macrophages can exert forces in the nano Newton range to handle their targets, it would be interesting if they manage to pull bacteria from a biofilm. Of particular interest is the type of NF-κB response as macrophages tackle bacteria in a biofilm. Towards this end, a systematic study of the NF-κB response to bacteria as compared to soluble factors like LPS and antibodies would be required.

7.4 Future applications

In the following, ideas are outlined that were inspired by the results from the before described chapters and encouraging discussions with Simone Schürle, André Lindo and Jon Hansen.

7.4.1 Emergence of improved drug delivery approaches

Using precise manipulation of magnetic particles, it could be envisioned that specific microparticles with release kinetics of pharmaceutically active drugs could be developed and steered to specific locations. One possible challenge in this scenario are phagocytes which try to clear these foreign bodies. As a consequence, the drug delivery shuttles could be taken up by immune cells and not reach their ultimate goal. Therefore, it might be a promising approach to apply resistive forces, translational and rotational control and potentially also wiggling motions which might keep these particles in place so that they can fulfill their release functions. Depending on the material properties, the stability and consequences of degradation during release may of course also pose a health risk. In a first sequence of experiments, it was observed that untreated cobalt nickel particles are stable in water but show distinct signs of degradation under physiological conditions as provided by DMEM cell culture medium. Deposition of silicon dioxide layers in combination with gold-bottom (performed by André Lindo and Berna Özkale) showed however,
that the optically observed degradation of particles was circumvented by the silicon dioxide coating.

7.4.2 Using microfabrication and -manipulation to make biofilms accessible to macrophages and therapeutic approaches

In a future approach it will be important to also investigate methods to interfere with already formed biofilms and devise strategies to assist the immune system in its battle against these persistent reservoirs of potentially pathogenic bacteria. From the collaboration on fabrication of magnetic microparticles, discussions with André Lindo from the Nelson group have emerged to use microparticles to tackle biofilms. Particularly for the cases where macrophages do not manage to reach bacteria within the biofilm, a technical approach using magnetically actuated particles that can potentially break up the biofilm are envisioned. Alternatively to completely breaking up the biofilm, it may also be helpful to make it more accessible to antibacterial drugs or bioactive components that could be delivered into the biofilm.

7.4.3 Using microfluidics to gain a deeper understanding of macrophage-biofilm interactions

Furthermore, advanced microfluidic devices from the research group of Savas Tay for the continued study of macrophages and bacteria co-cultures are being used and the studies are extended by Jon Hansen to include bacteria in biofilms and macrophage interactions with the surface of biofilms. These studies will gain important and clinically relevant understanding on the processes that are involved in macrophage-biofilm interactions.
8.1 Monitoring bacterial oxidative stress response of surface-adherent and phagocytosed E.coli

Phagolysosomes of phagocytic cells contain reactive oxygen species that have the potential to cause serious damage to membranes, proteins and the genetic material of bacteria. The presence of reactive oxygen species like hydrogen peroxide (H₂O₂) triggers a bacterial stress response when elevated concentrations of H₂O₂ occur. Already 0.5 μM cause damages to the genetic material of bacteria. Harmful consequences arise not directly from the direct H₂O₂ exposure, but subsequent reactions by contact of the peroxide with metal impurities. In E.coli, the OxyR regulon is induced at concentration of 100 nM H₂O₂. One of the genes regulated by OxyR is the gene for katalaseG (katG), which disproportionates H₂O₂ into H₂O and O₂. This reaction protects E.coli from the harmful consequences of reactive oxygen species.

After a macrophage internalized E.coli into a nascent phagosome, many chemical processes occur inside the maturing compartment, in particular acidification of the lumen and fusing with other membrane organelles, such as endosomes, late endo-
somes and lysosomes, by which the compartment matures into a phagolysosome [52]. Phagolysosomes contain many different reactants toxic to E. coli, such as proteases, antimicrobial peptides, lysozymes, reactive oxygen and nitrogen species. In particular, the enzyme phagocytic oxidase (Phox) is assembled on the membrane of nascent phagosomes and pumps electrons into the phagocytic compartment which result in the formation of superoxide ($O_2^-$). The superoxide $O_2^-$ can spontaneously degrade into hydrogen peroxide and $O_2$ in a pH- and concentration dependent manner. The release of reactive oxygen species such as the superoxide radical and hydrogen peroxide by phagocytic cells is called oxidative burst. Bacteria can protect themselves against this oxidative burst by their oxidative stress response.

To obtain E. coli that express eGFP under the control of the katG promoter (AAEC191A pkatG-GFP), E. coli AAEC191A were transformed with the katG-GFP plasmid that was extracted from the original fusion library strain MG1655 katG-GFP [117] by Qiaprep Spin Miniprep kit (Qiagen 27106). Transformed bacteria were selected by cultivation on LB agar plates supplemented with either 100 µg/ml ampicillin (pHis-GFP) or 50 µg/ml kanamycin (rpm-GFP). The plasmids from this library were also transformed into the AAEC strain in which the fim gene cluster is knocked out as well as the same strain re-constituted for fimbriae expression by the pSH2 plasmid that contains the complete fim gene cluster. This makes a comparison of oxidative stress of fimbriated and non-fimbriated bacteria possible. Using this tool, the differences in intracellular localization can be investigated which is important information when differentiating intracellular routes e.g. via the phagolysosome versus the autophagy pathway [169].
Figure 8.1: Expression of the catalaseG promoter-GFP fusion can be used to monitor oxidative stress response of E.coli to reactive oxygen species. (a) Time series of brightfield and fluorescence micrographs of surface-attached E.coli harboring the pkatG-GFP plasmid which contains a copy of the promoter region of the katG gene encoding katalase G, a hydrogen peroxide scavenging enzyme that is expressed upon exposure to reactive oxygen species. The pkatG-GFP plasmid contains the promoter of the katG gene fused to the GFP gene. (b) Fluorescence quantification of surface attached pkatG-GFP expressing E.coli that were exposed to hydrogen peroxide concentrations ranging from 0-200 µM. (c) Time series of micrographs showing the uptake of E.coli by a macrophage and intracellular activation of the katG-GFP promoter fusion. Error bars are S.D.
8.2 Using microfluidics and microcontact printing to pattern two different proteins side by side without passivation

In collaboration with Vahid Hosseini, the idea of extending the protein patterning assay by [145] to pattern two proteins without passivation next to each other came up. Since without biopassivation of the surface, there is a high risk for the mixture of the two proteins, we had to devise means of spatially separating the two proteins during the patterning. To this end, a combined approach of microcontact printing and microfluidics was employed. This assay may also be interesting for studying interactions of one cell with different ligands, e.g. two different proteins on the surface.
Figure 8.2: Patterning of two different proteins on glass surfaces to pattern e.g. bacteria (shown in panel c) with specific ligand preference (here *E. coli* adhering to mannosylated RNAseB (shown in panel a) versus Fibronectin (shown in panel b)) on non-passivated surfaces.
8.3 Using microfluidics to monitor microbial longevity and gene expression

The research field of microfluidics emerged in the 1980’s and has since been extensively used for technologies ranging from ink jet printing to cell cultivation and diagnostic approaches. Due to the high resolution of structures that are used for microfluidic devices, it is possible to restrain and manipulate individual cells and bacteria (refs Wang et al, Sung Sik and others). In a similar approach using microfluidics, I could also apply microfluidics to learn more about the lifespan of baker’s yeast, a eukaryotic microbial organism that was extensively used as model organisms for longevity studies and the impact of metabolism on ageing (Figure 8.4c).
Figure 8.3: The cultivation of microbial cells from bacteria to yeast in microscopic cultivation chambers and channels (microfluidics) allows to monitor microbial life and gene expression under stable and dynamically changing environmental conditions for the duration of many generations of microbial life. (a) Schematic of the typical steps involved in producing micrometer sized structures and fluidic devices for the cultivation of microbial cells. Adapted from [260] (b) Overview of the workflow for producing a microfluidic device, adapted from [260] (c) Monitoring lifespan of single yeast cells cultivated in microfluidic channels. Adapted from [261]. (d) Monitoring bacterial gene expression during cultivation in microfluidic channels. Adapted from [1]
Appendix (General Methods)

In this chapter, step-by-step protocols that have been used in the above studies as well as unpublished methodologies are collected. The aim of this chapter is the sole documentation of employed methods for future reproduction purposes.
9.1 Generating bioactive surfaces that kill E.coli upon surface contact (used in Chapter 2)

This protocol is based on publications [90, 92] and the suggestion of using quaternary ammonium compounds as antibacterial agent from Rupert Konradi. The protocol was further adapted by me for use in live cell imaging of surface attached bacteria using the eGFP/PI assay (see section 9.2).

- Ultrasonicate glass slides in 100% (v/v) isopropyl alcohol, then dH2O (surface cleaning) for 5 mins each
- Prepare 5% DMOAC solution from 72% stock: 5% x 100 ml / 72% = 6.94 ml DMOAC stock in 93 ml dH2O
- Dry glass slides with air gun
- Incubate glass slides at room temperature for 30 mins
- Dip slides into 5% DMOAC (=antibacterial Si-QAC, Dow Corning 5700, DOW 5700)
- Dry slides O/N at 105°C
- keep in sterile petri dishes until used

9.2 Resealable custom-made PDMS wells for live cell imaging and subsequent sample fixation (used in Chapters 5 and 6)

This protocol was inspired from a procedure by Jens Möller for making PDMS wells which are covalently to glass surfaces. It has been extended by the use of ibidi technology to generate chambers that can be detached from the glass substrate after fixation. In combination with the use of photolithography patterned glass surfaces (see below protocol), these extensions facilitate correlative live cell and SEM imaging.
• Mix PDMS and curing base (Sylgard 184, Dow corning) in a 10:1 w/w ratio. Stir well for 1 minute. Pour PDMS mixture into a 10-15 mm glass petri dish to a thickness of 2-10mm (depending on the amount of media and duration of cultivation that is required for your purpose, consider nutrient limitations and number of required media exchanges). Centrifuge at 200 g for 1 minute in a 50 ml Falcon tube to remove air bubbles from mixing.

• Cure for a minimum of 60 minutes in a convection oven. Superfluous mixture of PDMS and curing base can be stored in 50 ml Falcon tubes at -20°C without solidifying.

• Peel off cured PDMS from the petri dish, lay with bottom side upwards and cut into blocks that will fit on the cover slides you will use for imaging later, e.g. 25x75mm. Use a 12 mm tissue puncher to punch a hole into the PDMS block.

• Cut a stripe from VHB 3M doublesided tape with a similar length of your PDMS block. Punch the tape with the same tissue puncher that you used for the PDMS.

• Stick the tape on the cover slide, ensure surface contact by tapping with tweezers. Remove the protective layer and align the PDMS block and the tape by the circles that resulted from punching the holes.

• Use tweezers to tap around the hole to ensure contact between tape and PDMS, otherwise device may become leaky.

• UV sterilize the device and wash with sterile PBS before adding Fibronectin solution of a concentration of 0.05mg/ml (e.g. in PBS). Incubate the Fibronectin solution for 30 minutes in the well, wash three times with sterile PBS and pre-warmed media before seeding cells to the desired density (e.g. 50000 cells/cm²).

• Culture and fix cells in the well, remove the PDMS and double sided tape from the glass surface by lifting it off with thin tweezer tips. Perform this under liquid, e.g. in a filled petri dish so as to not let the sample dry out.
9.3 Using photolithography to mark coverglasses for correlative live cell-SEM studies (used in Chapter 6)

- produce SU8200.5 from SU8 2002 by mixing 725 ul SU82002 and 275 ul cyclopentanone (or 7.25 ml and 2.75 ml). Clean bottle for dilution with Acetone/IPA/H2O (each 5 min plus ultrasonication in waterbath) and dry at 200°C for 12 hours. Let bottle cool down to room temperature before filling in the dilution..

- Let resist mixture stand over night at room temperature to ensure mixing of the solid contents and the solvent.

- clean silicon wafer (e.g. 3”) in Acetone/IPA/H2O (each 5 min plus ultrasonication in waterbath)

- bake: 200°C for 600 s (essential to bake for long time to remove all water molecules from surface)

- spin SU8 2002 using a two-step protocol (no bubbles in the resist! Provide continuous stream of resist in the middle of the wafer, not drops, use SU8 spinner bowl)
  - 1. speed: 500rpm/s, acceleration for 5s, duration:5s
  - 2. speed: 3000rpm/s, acceleration for 8s, duration:30s

- soft bake: 60 sec at 95°C

- expose: 60-80 mJ/cm², Program 1, e.g.: 11-15 sec at a lamp intensity of 5.3 mW/cm² at 365 nm wavelength

- three step post exposure bake:
  - 1. 60 sec at 65°C
  - 2. 180 sec at 95°C
  - 3. 60 sec at 65°C

- develop in PGMEA (a.k.a. mrdev600) for 60 sec under mild agitation, wash with IPA, H2O, airdry, check under Mike, measure height with Dektek profilometer
9.4 Modified photolithography protocol for generation of wafers for the microfluidic device "mothermachine" (used in [1])

This protocol is part of the modified mothermachine microfluidic chip fabrication protocol (the original protocol is described in the supplemental information of [262] and the modified version was applied in [1]. Helpful cleanroom discussions with Jörg Albuschies, Phillip Kuhn, and Daan Kiviet are gratefully acknowledged.

First layer on wafer: SU8 2002

- clean silicon wafer (e.g. 3") in Acetone/IPA/H2O (each 5 min plus ultrasonication in waterbath)
- bake: 200°C for 600 s (essential to bake for long time to remove all water molecules from surface)
- spin SU8 2002 using a two-step protocol (no bubbles in the resist! Provide continuous stream of resist in the middle of the wafer, not drops, use SU8 spinner bowl)
  - 1. speed: 500rpm/s, acceleration for 5s, duration:5s
  - 2. speed: 3000rpm/s, acceleration for 8s, duration:30s
- soft bake: 60 sec at 95°C
- expose: 60-80 mJ/cm², Program 1, e.g.: 11-15 sec at a lamp intensity of 5.3 mW/cm² at 365 nm wavelength
- three step post exposure bake:
  - 1. 60 sec at 65°C
  - 2. 180 sec at 95°C
  - 3. 60 sec at 65°C
• develop in PGMEA (a.k.a. mrdev600) for 60 sec under mild agitation, wash with IPA, H2O, air dry, check under Mike, measure height with Dektek profilometer

• hard bake: 200 sec at 200°C

Second layer on wafer: SU8 3025

• spin (pour resist directly from aliquoted bottle with large opening b/c of high viscosity)

  • 1. speed: 500rpm/s, acceleration for 5s, duration:10s
  • 2. speed: 3000rpm/s, acceleration for 12s, duration:30s

• edge removal: 5 ml syringe with PGMEA, fine needle, small angle on spincoater

• soft bake: 600-900 sec at 95°C

• align sample to first layer (if using SU8 2025 instead of 3025, alignment markers need to be taped before spincoating to be distinguished(remove tape directly after spincoating!), only mixture of 2000 and 3000 resist series allows alignment without further measures because of differences in refractive indices)

• expose: 150-250 mJ/cm² (measure lamp intensity at 365 nm and calculate required exposure time) post exposure bake:
  – 1. 60 sec at 65°C
  – 2. 300 sec at 95°C
  – 3. 60 sec at 65°C

• develop: 5-7 mins in PGMEA, wash in IPA, air dry, check under Mike and profilometer

• hard bake 200 sec at 200°C
Gratitude is the key to happiness’ is a message that has deep roots in many cultures and was also confirmed by modern psychological research [263, 264]. In the interest of happiness therefore I do not feel bad if my acknowledgements are a little more extensive.

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Curriculum vitae Ima Avalos
*11.05.1983

**Education**
PhD studies, ETH Zurich (February 2010-present), Thesis title “Strategies Underlying Bacterial Survival on Surfaces”

- teaching at ETH Zurich (D-MATL and D-HEST):
  supervised students during their thesis projects
  contributed practical and theoretical learning material for material science classes

- learning at ETH Zurich:
  courses in project supervision and management, creativity and rhetorics

Diploma of Technical Biology, University of Stuttgart (2003-2008), graduated with distinction and a grade of 1.0 (excellent, on a scale of 1 to 6 with 1 being the best)

- Diploma thesis at the Institute of Systems Dynamics (ISYS), University of Stuttgart (2008), using Boolean modeling to simulate biological signal networks (published)

- Semester thesis at the Massachusetts Institute of Technology (MIT, 2008), genetic screens on yeast oxidative protein folding and toxicity of protein fragments (6 month stay abroad in Cambridge, US)

Study of Molecular Biotechnology, University of Bielefeld (2002), continued at University of Stuttgart

**Professional experience**
January 2009-February 2010, ETH Zurich
Scientific employee and PhD student at the Institute of Molecular Systems Biology (D-BIOL)
acquired expertise in a new research area (longevity and microfluidics, published)

July 2006-September 2007, University of Stuttgart
Student research assistant at the Institutes of Technical Biology, Bioengineering, and Biology.
Activities and Interests

- Board member of AVETH and member of Hochschulversammlung, ETH Zurich
  organized and contributed to events and career fairs, public relations, mission statements, revisions of regulations and strategic planning

- Initiator of a peer-coaching concept for doctoral students and PostDocs, ETH Zurich
  organized and moderated meetings, prepared a workshop on peer coaching for scientific staff

- Board member of the association of scientific staff of D-HEST, ETH Zurich
  represented interests of scientific staff at the department conferences, contributed to revisions of regulations, statements, strategic planning of the department

- Board member of the Fachschaft of Technical Biologists, University of Stuttgart
  initiated the InterQuer AG, an association for integration of international and new arriving students

Awards and Honors

Jury member of the idea contest 2013 (VS Stab and Standortentwicklung), ETH Zurich
Award for exceptional student activities, University of Stuttgart
Travel Fellowship award for the participation in the Federation of the European Biochemical Societies (FEBS) Advanced Lecture Course on Systems Biology 2009
Stipend from the International Bureau for support of international students, University of Stuttgart

Publications in peer-reviewed journals


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