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Buckling, Wrinkling, and Folding: Microstructure, Active Matter Behavior, and Geometric Modeling of Bacterial Biofilms

Biofilms—The Multicellular Form of Bacterial Life

Bacteria are usually seen as single cell organisms that can swim around in the environment or in our bodies. But bacteria have also invented multicellularity—in fact, they even prefer to live in structured communities termed *biofilms.* By definition, biofilms are large aggregates of bacterial cells held together by an extracellular matrix of self-produced biopolymers.[1] However, these communities are not just simple heaps of bacterial cells: rather, they can form complex three-dimensional patterns that become visible to the naked eye.[2] In the laboratory, these biofilms are called macrocolonies. They are usually grown over several days on agar plates until they reach a diameter of two to three centimeters (fig. 1).

Macrocolony biofilms of many bacterial species form intriguing folding patterns that are quite variable. These patterns depend on the genetic makeup and can thus differ for certain mutants, but they are also influenced by the actual growth conditions, for example, the chemical composition of the growth medium, the growth temperature, and the humidity of the agar support (fig. 1 shows macrocolonies of several *Escherichia coli* strains obtained at three different temperatures). Three-dimensional patterns arise by buckling and folding of entire areas of the macrocolonies. At first glance, the explanation for these surprising large-scale movements is quite simple. These cells stick together because of their extracellular matrix (fig. 2A). As a consequence, increasing numbers of growing cells cannot just pile up independently, but they behave like a growing tissue—in order to fill additional space, they have to buckle up and fold as a connected consortium.

The dynamic appearance of these folding and wrinkling patterns has been analyzed in time-lapse movies that for *E. coli* macrocolonies on nutrient-rich media usually cover seventy-two hours. To start biofilm growth, a tiny droplet containing *E. coli* bacteria is put onto an agar plate that often also contains the dye Congo red, which turns red upon binding to the extracellular matrix. Therefore, a growing macrocolony turns dark red as soon as the cells start to produce the fibrous matrix components, which

1 Hans-Curt Flemming and Stefan Wuertz, "Bacteria and Archaea on Earth and Their Abundance in Biofilms," *Nature Reviews Microbiology* 17 (2019): 247–60, https://doi.org/10.1038/s41579–019–0158–9.

2 Diego O. Serra, Anja M. Richter, and Regine Hengge, "Cellulose as an Architectural Element in Spatially Structured Escherichia coli Biofilms," *Journal of Bacteriology* 195 (2013): 5540–54, https://doi.org/10.1128/JB.00946–13.

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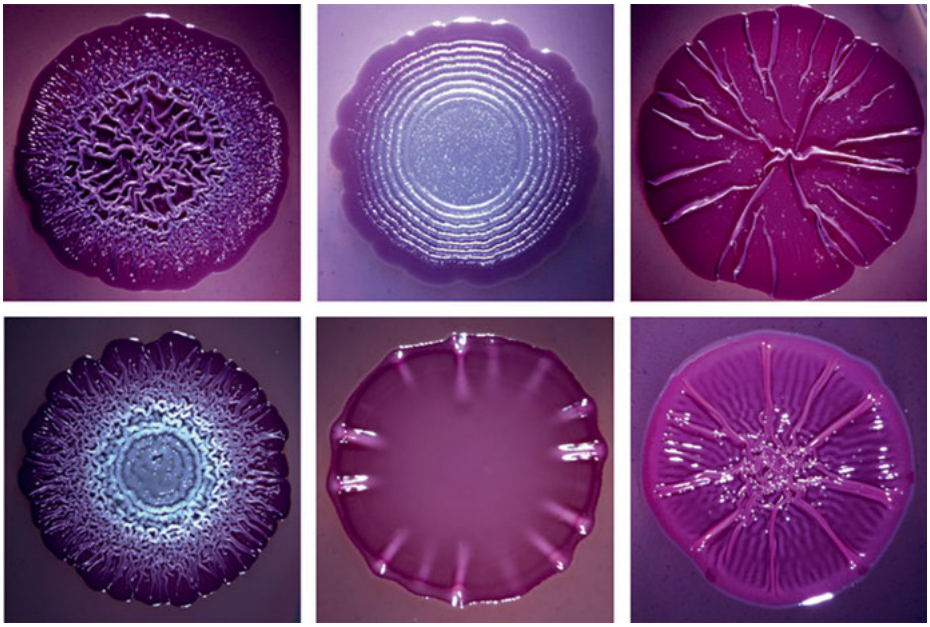

Fig. 1: Morphological diversity of bacterial macrocolony biofilms: biofilms of different strains of the human gut bacterium *Escherichia coli* were grown for three days at different temperatures (between 20° C and 37° C) on agar plates containing a complex mixture of nutrients as well as the dyes Congo red (which binds to the extracellular matrix) and Coomassie Brilliant Blue (which binds to various proteins). The diameter of the colony biofilms is approximately 25 mm.

happens after approximately twelve hours of growth. By twenty-four hours, small regular ripples begin to appear in a ring just behind the outer edges of the round macrocolony. Over time, these ripples become larger buckles that after forty hours further develop into radial ridges that propagate towards the colony center, where they finally intertwine and mix with smaller local wrinkles.[3]

The Interplay of Material, Form, and Function in Macrocolony Biofilms

Why are wrinkling macrocolony biofilms interesting to researchers at the Cluster of Excellence "Matters of Activity"? The answer is that these biofilms are a superb model to study fundamental relationships between material, form, and function.

3 Diego O. Serra and Regine Hengge, "Experimental Detection and Visualization of the Extracellular Matrix in Macrocolony Biofilms," in *C-di-GMP Signaling: Methods & Protocols—Methods in Molecular Biology*, ed. Karin Sauer (New York: Humana Press, 2017), 133–45.

Since biofilms are living matter, these relationships depend on an interplay of genes, which means an endogenous source of information that defines the entire repertoire of what is possible, and the environment, which determines which part of this genetic potential is actually realized and when and where this happens.

The biological *function* of biofilm formation becomes clear when we think of a biofilm as a "bacterial fortress." Within this fortress, bacteria are well protected against most physical or chemical stresses and also against other microbes that love to eat bacteria. In addition, biofilm formation provides the potential for homeostasis, which means bacteria can "design" their immediate extracellular space, which is internal to the biofilm, according to their specific requirements. The result is a strong reduction in maintenance energy, which means that multicellularity is thermodynamically favored and therefore can be considered an emerging property of cellular life itself.[4] A biofilm thus represents a microbial example of a space of "extended physiology," as it has been described in detail for the large-scale structures built by social insects such as termites, ants, or bees.[5]

In the following, however, the focus lies on the relationship between *material*—at the molecular and cellular scale—and macroscopic *form.* Thus, we have to take a closer look at the "building materials" of biofilms and how these arrange into a biofilm-internal microarchitecture. We also have to consider the origin of the tissue-like elasticity that allows buckling and folding without breakage. Furthermore, the question arises of what drives the macroscopic activity of the system, that is, the morphogenetic folding movements that generate the complex patterns of ridges and wrinkles. Finally, we address the geometry that emerges during the transition from a flat layer of proliferating cells to an elaborately folded and wrinkled macrocolony.

The Molecular Material Basis of the Extracellular Biofilm Matik

The building materials of a biofilm are bacterial cells and the extracellular matrix, with the latter basically consisting of a network of entangled fibers around the cells (fig. 2A). In the case of the *E. coli* biofilms that are used here as a model, there are just two types of fibers. On the one hand, these are "curli" fibers which consist of proteins in the very stable β-amyloid conformation (which is very similar to the structures

4 Regine Hengge, "Linking Bacterial Growth, Survival and Multicellularity—Small Signaling Molecules as Triggers and Drivers," *Current Opinion in Microbiology* 55 (2020): 57–66, https://doi.org/10.1016/j.mib.2020.02.007.

5 See J. Scott Turner, *The Extended Organism: The Physiology of Animal-Built Structures* (Cambridge, MA: Harvard University Press, 2000).

found in Alzheimer plaques).[6] When certain strains of *E. coli* produce only curli fibers as an extracellular matrix, these fibers are tightly packed around the producing cells like little baskets (fig. 2B). Curli fibers alone are non-elastic and break easily.[7] However, many *E. coli* strains also produce another matrix component, a chemically modified form of cellulose,[8] which forms long-range fibrillar connections and sheets (fig. 2C). Such a cellulose network is highly elastic, but it cannot support large structures because it does not hold on to the cells. By contrast, wildtype *E. coli* cells produce both fibers, curli fibers and cellulose fibrils, which together form a fibrous composite with superb material properties—it is elastic, it can stand large forces and it fully surrounds and holds cells at their place (fig. 2D).

A Matrix Architecture Much Larger than the Cells that Collectively Produce It

The images in figure 2 show the extracellular matrix arrangement at the surface of a macrocolony biofilm. However, the two matrix components can also be visualized inside a macrocolony biofilm by exploiting their binding of the green fluorescent dye Thioflavin-S (TS) added to the growth medium of the agar plates. After several days of growth, the macrocolonies can be shock-frozen, cut into thin vertical slices, fixed, and analyzed by fluorescence microscopy.[9]

At the rapidly growing outer edge of a macrocolony, which consists of densely packed cells everywhere, a simple two-layer structure can be observed, meaning no matrix at the bottom and the green fluorescent TS-stained matrix at the top. However, when taking a look into the older more central area of the macrocolony, a more complex architecture is revealed (fig. 3). Also here, a matrix is found in the top layer, but this matrix has developed into a complex architecture with several strata showing different patterns.[10] At the upper surface, a "dense brickwork"-like matrix arrangement consists of a composite of curli fibers and cellulose. Somewhat deeper, the matrix

6 Margery L. Evans and Matthew R. Chapman, "Curli Biogenesis: Order out of Disorder," *Biochimica et Biophysica Acta (BBA)—Molecular Cell Research* 1843, no. 8 (2014): 1551–58, https://doi.org/10.1016/j.bbamcr.2013.09.010.

7 Diego O. Serra, Anja M. Richter, Gisela Klauck, Franziska Mika, and Regine Hengge, "Microanatomy at Cellular Resolution and Spatial Order of Physiological Differentiation in a Bacterial Biofilm," *mBio* 4, no. 2 (2013): e00103–13, https://doi.org/10.1128/mBio.00103–13.

8 Wiriya Thongsomboon, Diego O. Serra, Alexandra Possling, Chris Hadjineophytou, Regine Hengge, and Lynette Cegelski. "Phosphoethanolamine Cellulose: A Naturally Produced Chemically Modified Cellulose," *Science* 359 (2018): 334–38, https://doi.org/10.1126/science.aao4096.

9 Serra and Hengge, "Experimental Detection and Visualization of the Extracellular Matrix in Macrocolony Biofilms."

10 Gisela Klauck, Diego O. Serra, Alexandra Possling, and Regine Hengge, "Spatial Organization of Different Sigma Factor Activities and c-di-GMP Signalling within the Three-Dimensional Landscape of a Bacterial Biofilm," *Open Biology* 8 (2018): 180066, https://doi.org/10.1098/rsob.180066.

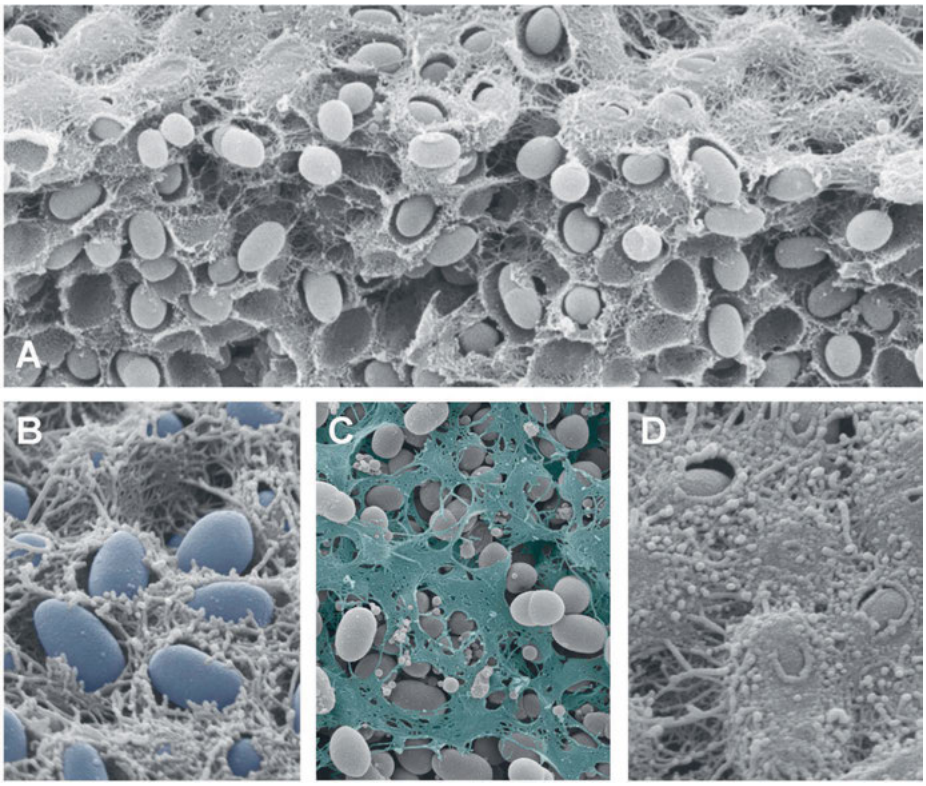

Fig. 2: The fibrous extracellular matrix of *E. coli* macrocolony biofilms as visualized by scanning electron microscopy (SEM). In (2A) the surface of a macrocolony of an *E. coli* strain, which produces a tight composite of amyloid curli fibers and cellulose, is shown. The macrocolony was broken open in order to show the fully matrix-covered colony surface (also at larger magnification in D) as well as the dense "brickwork-like" matrix arrangement within the biofilm upper layer. In (2B) an *E. coli* strain is shown (cells false colored in blue) that produces curli fibers only, whereas the *E. coli* strain in (2C) synthesizes only cellulose (false colored in green). Scale: the shorter diameter of the ovoid *E. coli* cells corresponds to approximately 1/1000 mm.

forms "vertical pillars," which mostly consist of long cellulose fibrils and sheaths, and a "loose horizontal network" consisting only of curli fibers can be detected further toward the bottom. In contrast to the upper dense brickwork layer, the vertical pillar and horizontal network zones also contain "dark areas" with matrix-free cell clusters right next to the matrix-producing cells, which means matrix production is quite heterogeneous. Finally, at the very bottom of the biofilm, there is a network of entangled flagella, which are a type of extracellular filaments that do not stain with the fluorescent dye TS, but that can be visualized by scanning electron microscopy (fig. 3).

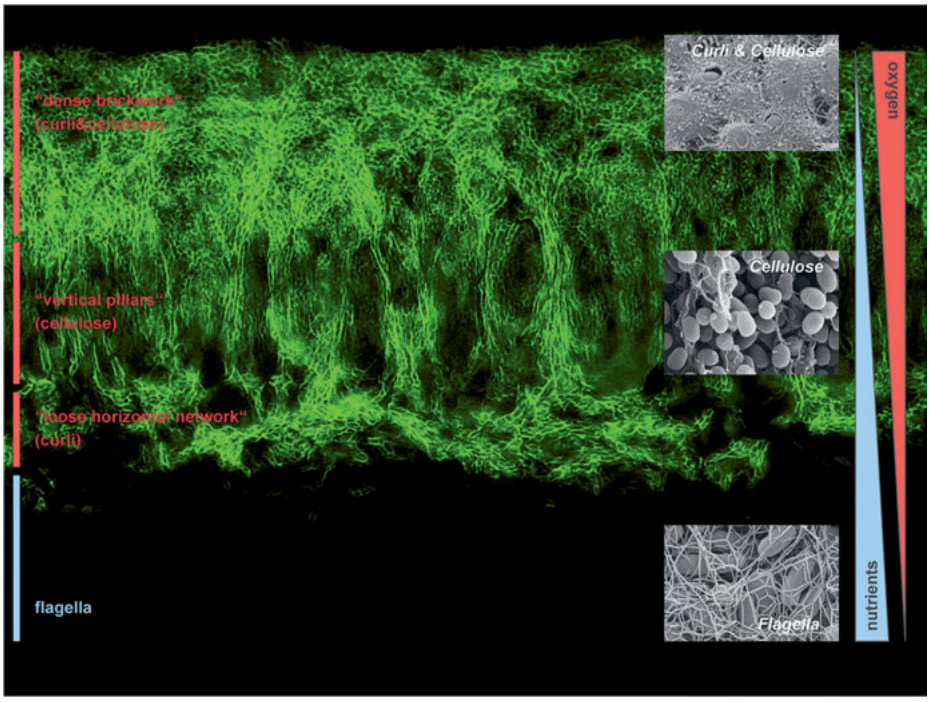

Fig. 3: The extracellular matrix architecture within *E. coli* macrocolony biofilms: in a vertical thin section through an *E. coli* macrocolony biofilm, the extracellular matrix was visualized by staining with the fluorescent dye Thioflavin-S (TS), which was present already during growth of the biofilm. TS tightly binds to both curli fibers and cellulose, thus allowing detection of these matrix fibers by fluorescence microscopy. The entire biofilm is densely packed with bacterial cells, but only matrix-embedded bacterial cells can be seen as small black dots surrounded by the green fluorescent matrix dye. Note that the entire matrix architecture, in which three morphologically distinct strata can be distinguished as indicated, is approximately 50-fold larger (on the vertical axis) than the cells that coordinately produce it. The insets show larger magnification scanning electron microscopic images of cells in the respective zones. The matrix-free cells in the dark bottom layer produce a network of entangled flagella, which do not bind the fluorescent dye, i. e., this entire bottom layer of cells remains dark.

The Elaborate Large Scale Matrix Architecture Matters for Biofilm Elasticity

Over the last twenty-five years, the intricate genetic control network that senses and transduces local environmental signals into the formation of this three-dimensional matrix architecture has been elucidated.[11] Steep chemical gradients, mainly of nutrients (from the agar support below the biofilm) and oxygen (from the air on top of

11 Hengge, "Linking Bacterial Growth, Survival and Multicellularity."

the biofilm), have turned out to serve as major signal inputs. Thus, only cells experiencing nutrient-limitation can synthesize matrix components, which explains why the matrix builds up in the upper layer of the macrocolony that is far away from the nutrient-providing agar support. Knowledge about the regulatory network underlying these processes also allows us to introduce mutations that knock out distinct regulatory genes and proteins. The result are changes both in the biofilm-internal matrix architecture and in the wrinkled macrocolony morphology, thus demonstrating that the macroscopic form is genetically controlled.

One of the most interesting mutations eliminated a regulatory protein called PdeR, which serves as an inhibitor of matrix production by sensing and degrading the intracellular biofilm-promoting signaling molecule c-di-GMP. In the absence of this key regulator, heterogeneity of matrix production is lost, whitch means *all* cells in the upper layer are now homogenously producing matrix components. The consequence of this change in matrix architecture is drastic: these biofilms are stiffer, which makes buckling more difficult and therefore a rarer event. Moreover, closer inspection by scanning electron microscopy showed deep breaks all over the surface of the macrocolonies—in other words, there is a large-scale elasticity problem. Therefore, the tissue-like elasticity does not only depend on the material properties of the matrix fibers, in particular on the highly elastic cellulose fibrils, but also on the much larger matrix architecture, in particular on the presence of the matrix-free cell clusters within the matrix layer.[12]

Spatial Self-organization into Two Distinct Cell Populations Generates the Matrix Architecture and Triggers Morphogenetic Movement

So, what is the exact role of these matrix-free cell clusters in biofilm buckling and elasticity? Introducing a green genetic marker—designed to light up in cells that grow and divide rapidly[13]—showed that these globular matrix-free cell clusters represent the fastest growing cellular subpopulation in the older central area of a macrocolony. By contrast, the massively matrix-producing cells in the same biofilm zone grow only slowly into the thin cellulose-sheathed vertical matrix pillars. So, there is a division of labor between two cell populations in different physiological states: one that uses the available resources for rapid growth and expansive proliferation, and another one that invests these resources in building the matrix architecture.

12 Diego O. Serra and Regine Hengge, "A C-di-GMP-Based Switch Controls Local Heterogeneity of Extracellular Matrix Synthesis Which Is Crucial for Integrity and Morphogenesis of Escherichia coli Macrocolony Biofilms," *Journal of Molecular Biology* 431 (2019): 4775–93, https://doi.org/10.1016/j.jmb.2019.04.001.
13 A reporter fusion of the gene for green fluorescent protein (Gfp) linked to the ribosomal *rrnB* gene (Klauck et al., "Spatial Organization of Different Sigma Factor Activities").

Most intriguingly, the rapidly proliferating matrix-free cell clusters were found to change their position while the biofilm buckles up and folds into the high ridges, that is, when the initially flat area goes through a transient bending event, which is associated with strong compression and stretching of distinct microzones of the biofilm. This indicates that by just growing rapidly, these proliferating cell clusters first *build up* local compression forces and therefore local instability, which triggers local buckling. In addition, during the actual bending event, these cells also contribute to *dissipating* these local forces by getting squeezed through the matrix network—this explains how these non-matrix-fixed cell clusters, which can be flexibly pushed around, prevent breakage of the biofilm at large during bending.

In summary, morphogenetic activity of macrocolony biofilms is driven by a division of labor between physiologically different subpopulations of cells, which self-organize in space to produce the large-scale elastic matrix architecture and to first generate and then dissipate the local forces that trigger buckling and folding during biofilm growth.[14]

Local Exponential Growth Drives the Buckling and Folding of Biofilms

When we look at a macrocolony from above, that is, quasi in 2D (as in fig. 1), its morphogenetic movements are not randomly distributed but seem to develop into a distinct geometry. At the outer biofilm edges, rapidly growing cells drive the radial expansion of the colony. The first buckling always starts somewhat behind these outer edges, which is where cells begin to produce matrix in the upper layer of the macrocolony. It turns out that this initial buckling can be described as a simple consequence of geometry. When a round bacterial colony is growing rapidly at its outer edge—which for real macrocolonies is an oversimplification as somewhat slower growth also occurs in specific zones inside the macrocolony as described above—, the macrocolony radius (r) increases over time (t). During this process, the colony circumference grows in a linear manner ($2\pi r$), while its area grows by the square (πr^2). However, the cells that drive this expansion, grow exponentially ($N=N_o e^{kt}$), which means, in absolute terms, cell numbers initially increase only slowly, then accelerate in growth and finally "explode." As a practical consequence, the growing area of a macrocolony biofilm can no longer be accommodated in a flat circular plane—there is no other possibility than buckling out into the third dimension. In geometrical terms, a surface increasing by exponential growth becomes hyperbolic, meaning it is necessarily "negatively" curved. In fact, nature makes ample use of hyperbolic surfaces—we find them for instance in marine flatworms, cabbage leaves, or fungi. The mechanisms behind generating many of these biological hyperbolic forms are the same, that is, local proliferation of cells—

14 Kim Nguyen and Regine Hengge, unpublished results.

with multiple cell divisions representing an exponential growth pattern—forces initially planar tissues to curve out of the flat plane.

Curvature and Hyperbolic Surfaces

We have used the terms *hyperbolic* and *curved* here to describe the geometry of the biofilm surface as it buckles as a result of the exponential proliferation of cells. Understanding these ideas in a more precise way enables us to consider the biofilm geometry in a more complete way. To talk about hyperbolic surfaces and the hyperbolic plane, we first need to discuss what it means for a surface to be *curved.*

There are numerous strategies to define curvature from a mathematical perspective. It is easiest to start with a curved one-dimensional line in the plane, before moving onto the more complicated case of a two-dimensional surface embedded in our three-dimensional world. There are two distinct perspectives that we can take here, looking at how the line curves over its whole length—the *total curvature*—and how the line curves at a particular point—the *pointwise curvature.* The total curvature of a line can be considered by constructing a series of lines parallel and with increasing distance to the original line. As the distance (t) from the original line increases, the length of the line changes linearly with a weighting that is the curvature: a straight line will have parallel lines of the same length, and a curved line will have parallel lines that increase in length with increasing t. This is a very intuitive way to capture the total curvature of a line. From the pointwise perspective, for each infinitesimally small segment of the line, we can imagine it wrapping around a circle of a given radius. This is known as the *osculating circle* of a line at a point. When the line is curving gently and the circle is big, the curve is said to have a large radius of curvature (R, the radius of the circle), but a small curvature given by 1/R. When the line curves more strongly and the circle is small, the curve has a small radius of curvature and hence a larger curvature. These definitions capture our intuitive notion of the curved line.

On a two-dimensional surface, things are a bit more complicated, as there are fundamentally different ways that a surface can curve. We can consider the same idea of total curvature of a surface as we did for a line, only now we look at parallel surface and their change in area as t increases. It turns out that this area changes quadratically with t, with a curvature component that changes linearly with t, and a curvature component that changes quadratically with t^2. The linear component is known as the *mean curvature*, and the quadratic component is the *Gaussian curvature.* These quantities can also be computed at a particular point on the surface: the curvature at this point is encapsulated by considering the directions where the surface curves the most, and where it curves the least (which may also involve curving in the opposite direction). More precisely, you could make a slice of your surface through the point (where the slice plane contains the surface at the point) and look at the curvature of the line along that slice. So, at a point on the surface, we can now consider the curvature of a line radiating from the point in each direction on the surface. If we consid-

er all of these curved lines radiating from a point, we can find the directions where the curvature is maximal and minimal (fig. 4). The curvature in these directions are the *principal curvatures* at a given point on the surface, and they are always perpendicular to each other.

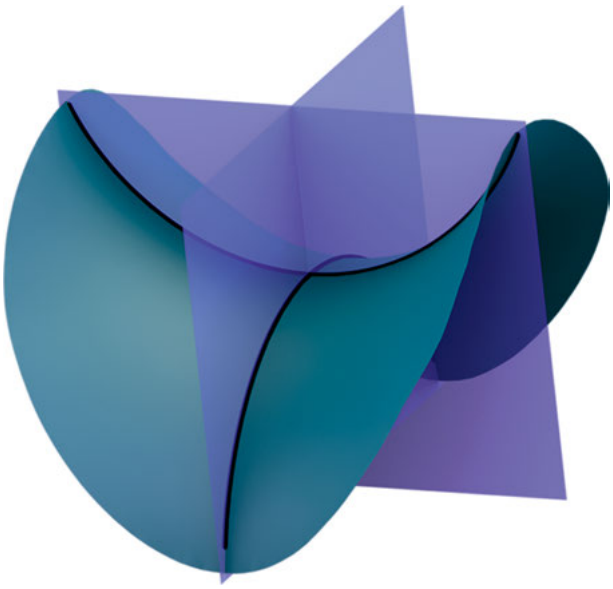

Fig. 4: Principal curvature lines on a hyperbolic saddle surface. A hyperbolic saddle surface is shown in green, along with the planes (purple) intersecting the surface along the principal curvature directions (shown in black) at a particular point on the surface. The two principal curvatures curve in opposite directions, and thus have the opposite sign, which means that their product—the Gaussian curvature—will be negative. All negative Gaussian curvature surfaces are termed hyperbolic surfaces.

The concepts of mean and Gaussian curvature at a point on the surface can be described using these two principal curvatures. The mean curvature is the average of these two principal curvatures, and refers to an extrinsic notion of curvature, related to how the surface is embedded in space. The Gaussian curvature is the product of the two principal curvatures and is an intrinsic property of the surface. In general, the sign of the Gaussian curvature tells us what kind of surface we have: when it is positive, we have something spherical, when it is zero, we have something flat, and when it is negative, we have a hyperbolic surface. The surface in figure 4 has the principal curvatures curving in opposite directions, so one is positive and one is negative, which is the characteristic shape of a hyperbolic surface. We now have a definition of a hyperbolic surface with which to think about the biofilms, namely that at each point on the surface, it has negative *Gaussian curvature.*

A Wrinkled Macrocolony Biofilm Can Be Described as a Finite Disc That Is Intrinsically Hyperbolic

One very natural way to explore such a hyperbolic surface is through a crochet model (fig. 5). The idea comes from the mathematician Daina Taimina, who first introduced the model in 1997.[15] The addition of stitches in a particular proportion with each round of the crochet dictates a pattern of local growth in the crocheted surface. Local growth can be described by what is known as the *metric* of the surface, which characterizes the distances between points. This metric is precisely what defines the *intrinsic* properties of the surface, so it is related to the Gaussian curvature of the surface. In this case, the exponential growth implies that the surface has negative Gaussian curvature, making the crocheted surface hyperbolic. The textile model was a breakthrough for people trying to understand hyperbolic geometry because it was suddenly possible to touch and experience a hyperbolic surface. The construction of a hyperbolic surface using the exponential growth of the stitch numbers mirrors the emergence of hyperbolic surfaces through cell proliferation in the biofilm.

Given a finite disc that is intrinsically hyperbolic, we can consider how this disc arranges itself extrinsically, in what shape it embeds itself in space, which is captured by the *mean curvature* of the surface. For a hyperbolic disc, there are many ways to create an extrinsic embedding, where the disc can take on a variety of undulations, folds and oscillations while satisfying the intrinsic curvature conditions. The different presentations of the hyperbolic crochet in figure 5 show some of the possibilities. In an unconstrained elastic disk, such as the crochet or various other physical systems, this extrinsic embedding will be found as an equilibrium between bending and stretching energies, where homogeneous local curvature can lead to embeddings with large scale folds and small-scale oscillations.[16]

In the case of the biofilm growth, an important geometric consideration is that the hyperbolic nature of the biofilm is fundamentally incompatible with the flat substrate on which it is growing. Thus, when the tissue-like biofilm grows, we actually have a growth process together with adhesion to a substrate. This now becomes a balance of growth within the film and the hyperbolic geometry of the disc, the elasticity of the material, and the adhesion properties of the film to the substrate.[17] The collective solutions to these conditions, as well as the idea of inhomogeneous growth patterns

15 Daina Taimina, *Crocheting Adventures with Hyperbolic Planes: Tactile Mathematics, Art and Craft for All to Explore* (Boca Raton: CRC Press, Taylor & Francis, 2018).

16 Yael Klein, Efi Efrati, and Eran Sharon, "Shaping of Elastic Sheets by Prescription of Non-Euclidean Metrics," *Science* 315, no. 5815 (2007): 1116–20, https://doi.org/10.1126/science.1135994.

17 Martine Ben Amar and Min Wu, "Patterns in Biofilms: From Contour Undulations to Fold Focussing." *EPL* 108 (2014): 38003, https://doi.org/10.1209/0295-5075/108/38003; Julien Dervaux and Martine Ben Amar, "Morphogenesis of Growing Soft Tissues," *Physical Review Letters* 101 (2008): 068101, https://doi.org/10.1103/PhysRevLett.101.068101.

Fig. 5: A crochet model of a hyperbolic plane. This particular model started from a small circle of ten stitches, with stitches added in a regular manner in each new round along the circular outer edge (four stitches over three stitches in the previous round). This represents an exponential growth of the number of stitches that leads to buckling in the outer area, which resembles the initial buckling of macrocolony biofilms. This similarity (in particular to the colony shown in the middle of the lower row in fig. 1) is best seen in the photograph in the upper left corner of the panel shown here. Note that all images show a single object, with the different forms illustrating the potential of the hyperbolic disc to take on a variety of undulations, folds and oscillations compatible with the intrinsic curvature conditions.

over the surface, has the potential to explain the rich variety of biofilm folding, buckling, and wrinkling patterns observed experimentally.

In conclusion, if we want to fully understand how macrocolony biofilms grow into their intriguing three-dimensional form, we should explore not only the spatial control of matrix gene expression in response to environmental and cellular signals and the material properties of the extracellular matrix components and large-scale architecture, but also the rules and possibilities of hyperbolic geometry.