disease). This means that, if the toxin-encoding genes were ever transferred by conjugation to other bacterial species, no toxin could be made. Second, to ensure that any antibiotic-sensitive *V. cholerae* recipients do not die, the authors included the gene that encodes the antidote on the mobilizable gene set. This antidote is made in all antibiotic-sensitive *V. cholerae* but not in antibiotic-resistant *V. cholerae*, because in the latter bacteria, antidote production is turned off by a repressor protein, SetR, which is encoded by, and also regulates, the antibiotic-resistance genetic element *SXT* in these bacteria.

However, simply having the antidote present might not be enough to prevent the killing of bacteria that are not intended to be targeted. The CcdB toxin is highly potent and acts rapidly, killing bacteria by causing extensive damage to their genetic material — it acts by inhibiting an enzyme called gyrase, locking it to DNA, which results in DNA breaks. The authors therefore built in a delay mechanism — generating a ticking time-bomb that becomes deadly only after some time.

To do this, they inserted a genetic module that encodes a special protein known as an intein into the toxin gene. Expression of the modified toxin gene produces a non-functional toxin, from which the intein protein excises itself over time through a process called splicing, thereby generating the functional toxin. The effect is to delay the deadly action of the toxin, allowing time for bacteria that receive it to respond. The time it takes for the toxin to mature allows a bacterium that is antibiotic-sensitive to produce enough antidote to survive. If the bacterium is antibiotic-resistant, however, no antidote is produced and, after the toxin has matured, the cell will die.

López-Igual *et al.* went on to show that their approach is not limited to a single type of toxin protein, but that others — namely, HigB2 (which targets a type of enzyme called an mRNase), RelE4 (which inhibits protein synthesis) and ParE2 (another gyrase inhibitor) — are also functional after intein-mediated splicing. Finally, they tested their method in three natural *V. cholerae* habitats: water, zebrafish larvae and crustacean larvae. They found that their approach could eradicate antibiotic-resistant *V. cholerae* in all three habitats. The specific regulators they used work only for *V. cholerae*, but the system could easily be adapted to target different bacteria.

Other studies[2,3] have used bacterium-infecting viruses as well as conjugation methods to deliver DNA- or RNA-digesting enzymes called nucleases to target drug-resistant bacteria. How does López-Igual and colleagues' approach compare with these? One advantage of their system is that fewer bacteria that can resist the internal threat evolve, with around one such 'escape mutant' per 10^7 bacteria that receive the toxin, which is a level of escape mutants that is least a hundred times lower than is found with a virus-based approach[2,3].

Nevertheless, this escape rate is not low enough to prevent the development of resistance. One factor to consider is the typical population sizes of the bacteria being targeted[4]. If the population is larger than the size predicted to generate an escape mutant, then resistant bacteria will already be present. People who have cholera produce around 10^8 *V. cholerae* bacteria per gram of faecal material, and water reservoirs associated with an outbreak of the disease would probably host an even higher number of bacteria[5]. In such large populations, it is probable that thousands of mutant cells would be resistant to the toxin, and would be unaffected by the killing system described here. Further research should attempt to work out the mechanisms behind this resistance, and to find ways to optimize the system.

"The authors' aim is to offer a highly targeted alternative to standard broad-brush antibiotics."

A problem with conjugation approaches in general is that they are inefficient, with only a few cells out of a hundred actually receiving the genes. Two things will probably be necessary to generate a functional therapy: first, the use of several toxins and/or delivery systems, to try to limit the number of escape mutants; and second, improvements in the efficiency of gene transfer. Although López-Igual and colleagues' system will probably not immediately solve the problem of antibiotic-resistant cholera infections, it might make an important contribution to the arsenal of alternative treatments for critically ill people.

One final question is perhaps more profound. The severe watery diarrhoea that is characteristic of cholera causes an estimated 100,000 deaths every year[6]. With this grim statistic in mind, why target just the antibiotic-resistant bacteria when you could try to kill them all instead? A possible benefit could be a lower selection pressure on *V. cholerae* to become resistant to this new killing method. However, one would need to know in advance that the infection is indeed antibiotic-resistant, or it might be necessary also to use antibiotics in parallel to treat the infection. Perhaps the system described by López-Igual *et al.* should be viewed as an intriguing proof-of-concept of how selective antibiotic alternatives could be used in the future: it could easily be modified to target all *V. cholerae*, whether or not they are antibiotic-resistant. ■

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1. López-Igual, R. *et al. Nature Biotechnol.* https://doi.org/10.1038/s41587-019-0105-3 (2019).
2. Bikard, D. *et al. Nature Biotechnol.* **32**, 1146–1150 (2014).
3. Citorik, R. J., Mimee, M. & Lu, T. K. *Nature Biotechnol.* **32**, 1141–1145 (2014).
4. Hughes, D. & Andersson, D. I. *Annu. Rev. Microbiol.* **71**, 579–596 (2017).
5. Nelson, E. J. *et al. Nature Rev. Microbiol.* **7**, 693–702 (2009).
6. Ali, M., Nelson, A. R., Lopez, A. L. & Sack, D. A. *PLoS. Negl. Trop. Dis.* **9**, e0003832 (2015).

This article was published online on 17 June 2019.

PHYSICAL CHEMISTRY

Crystallization tracked atom by atom

Atoms of a metal alloy have been tracked as they form crystal nuclei — the first ordered clusters of atoms or molecules produced during crystallization. The findings might help to develop a general nucleation theory. SEE LETTER P.500

PETER G. VEKILOV

Nucleation is the earliest stage of crystallization, in which atoms or molecules dispersed in a crystallization medium first come together to form ordered clusters known as nuclei. Crystal nucleation underpins a vast range of phenomena, from the solidification of rocks from molten magma to the hardening of biological tissues through the formation of various minerals, and the protein fibrillation and crystallization associated with a plethora of diseases. In many instances, nucleus formation represents the rate-limiting stage of crystallization and determines the main properties of a crystal population, including the type, number and size distribution of the crystals that form. On page 500, Zhou *et al.*[1] report features of crystal nucleation that not only clash with several assumptions of classical nucleation theory, but also go beyond more recent non-classical models.

Crystal nucleation occurs in a medium (a solution, melt or vapour) that is supersaturated with respect to a crystal. In other words, the concentration of the material dissolved in

the medium is higher than that at which the medium can maintain an equilibrium with the crystal phase of the dissolved material. However, nucleation has to overcome an energy barrier owing to the formation of new crystal surfaces in the 'old' supersaturated phase[2]. The nucleation barrier can be overcome by means of fluctuations that bring the local concentration and structure of the dissolved material close to those of the developing crystal[2].

These basic tenets of nucleation, together with a kinetic model developed in the 1930s and 1940s[3–5], constitute what is now known as classical nucleation theory (CNT). The main assumptions underpinning CNT are that a sharp interface separates the old and the new phases (Fig. 1a), and that the nucleation barrier is proportional to the surface area of the nucleus, with a coefficient of proportionality that is independent of nucleus size. Numerous systems have been found to follow CNT faithfully, mostly at relatively small supersaturations[6,7].

But in the past 15 years, elaborate experiments have accumulated evidence that many nucleation processes can behave quite differently from what is assumed by CNT. The discrepancies are particularly dire for crystal nucleation from dilute media, such as solutions and vapours. The deviant behaviours can be categorized into three groups. The first is two-step nucleation (Fig. 1b), in which nucleation is preceded and facilitated by the formation of disordered precursors, mostly dense liquids[8–10]. This model refutes the classical assumption that fluctuations of concentration in the old phase coordinate with structure fluctuations to produce an ordered crystal nucleus[11].

The second category is barrier-free nucleation (Fig. 1c), in which the nucleation barrier drops below the kinetic energy carried by atoms or molecules at a given temperature, even at moderate supersaturations, as the nucleus size shrinks to one atom or molecule[12]. Tiny nuclei are too small to produce a defined interface with the surrounding medium, and therefore require special theoretical treatments to describe their formation[13]. The third category is for nuclei that have adopted the structure of the emerging crystal phase, but have shapes that do not minimize the nucleation barrier (Fig. 1d); such 'non-equilibrium' nucleus shapes can form if the kinetics of the processes that restructure the emerging new phase are slow[14,15].

Studying nucleation processes is a challenge, because it is devilishly difficult to image the structure of nuclei. Methods that have sufficiently high resolution to detect individual atoms or molecules have minuscule areas of view, and therefore struggle to find nuclei — which form in extremely small numbers and constantly move when in low-viscosity environments. Zhou *et al.* overcame this problem by studying crystal nucleation in nanoparticles of an iron–platinum alloy.

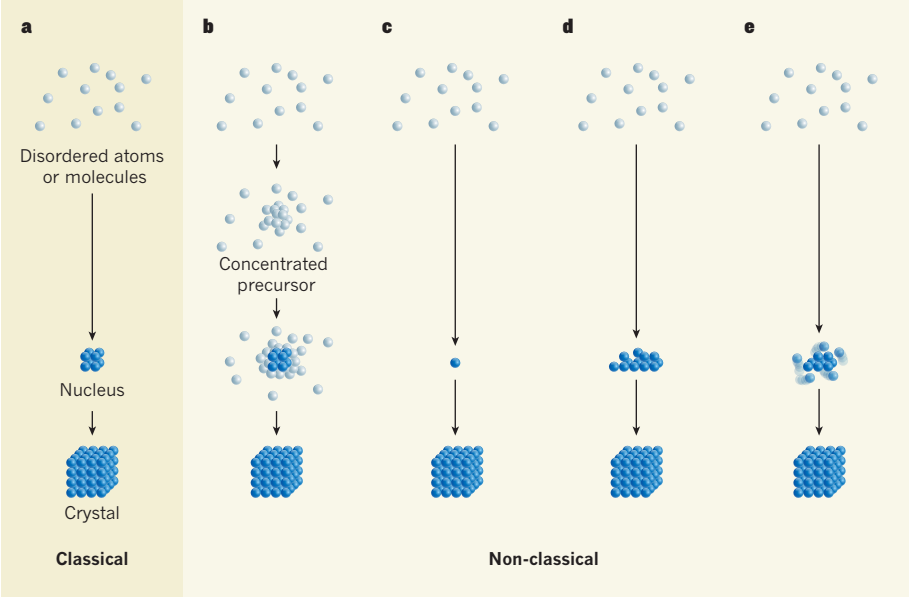

Figure 1 | Models of crystal nucleation. **a**, The initial stages of crystallization involve the formation of nuclei — clusters of atoms or molecules that then grow into crystals. In classical nucleation theory (CNT), nuclei form at a particular shape and size, with sharp edges. Their constituents have the exact arrangement of the final crystal. Disordered constituents, pale blue; ordered constituents, dark blue. **b**, Some non-classical models propose that the constituents initially form a completely disordered precursor that is more concentrated than the starting phase, within which a nucleus emerges in a separate step. **c**, Other non-classical theories account for situations in which just one atom (or very few atoms) acts as a nucleus. **d**, A third category of non-classical theory suggests that nuclei adopt the lattice of the emerging crystal, but not the ideal 'equilibrium' shape expected in CNT. **e**, Zhou *et al.*[1] report observations of nuclei that have diffuse edges and adopt non-equilibrium shapes that vary with time.

The atoms in the alloy have a disordered structure that, when heated, undergoes a solid-to-solid transition that can produce a tetragonal lattice. The tetragonal nuclei that emerge during this transition are immobile, and can therefore be studied using an imaging method called atomic electron tomography (AET), which can visualize all the atoms in a nanoparticle. The authors used AET to trace the positions of individual metal atoms as they lined up into crystal nuclei in the iron–platinum alloy. They appropriately describe this amazing experimental capability as 4D atomic resolution.

Zhou and colleagues noticed three nucleation behaviours (Fig. 1e) that go beyond both CNT and current non-classical models. First, they observed that the order parameter of a nucleus, which in this case quantifies how closely the atoms in the nucleus adopt a tetragonal arrangement, is not uniform. Instead, it varies with the distance from the nucleus core (which consists of one to a few atoms that have the maximum order parameter). As a result, the interface between the nucleus and the surrounding phase is not sharp, but diffuse, and its structure varies in time as the size of the nucleus increases. Second, the researchers observed that the overall shape of the nucleus is neither spherical nor tetragonal — the two shapes that would minimize the energy of the nucleus, assuming that the energy is proportional to the nucleus surface area (as is assumed in CNT). And third, the nucleus size and shape fluctuate broadly, which Zhou and co-workers interpret as evidence that the critical size of the nucleus (the size that has the highest energy) is not fixed, but varies in line with the order parameter.

Researchers have previously generally assumed that diffuse interfaces can form[16], albeit with some different features from those actually observed by Zhou and colleagues. However, the lack of methods that have sufficiently high spatial and temporal resolution to monitor crystal nucleation in real time has led to ad hoc assumptions about the structures of interfaces, and therefore also about their consequences for the nucleation barrier and the nucleation rate.

A phase transformation in a metal alloy is a convenient model system for the first application of 4D AET. However, the burning problems in the field of nucleation relate to the formation of biological minerals and disease-associated aggregates of proteins and small molecules, the production of pharmaceuticals and fine chemicals, and to other processes in which materials are synthesized in solution. A desirable future development would therefore be for AET to be implemented in liquids, used in combination with methods that could constrain the positions of emerging nuclei (for example, by using nanometre-scale pores, or laser systems known as optical traps). Zhou and colleagues' findings will also spur the development of a general theory that accounts for diffuse and dynamic interfaces, and could thus

predict the magnitude of nucleation barriers and the rate of formation of crystal nuclei.

Leo Tolstoy's novel *Anna Karenina* begins with the immortal words "All happy families are alike; each unhappy family is unhappy in its own way". The main message of Zhou and colleagues' paper can be summarized in a similar way: all nuclei that adopt an equilibrium shape are alike; every non-equilibrium-structured nucleus has its own shape. Moreover, the researchers demonstrate that non-equilibrium nuclei shapes not only are diverse, but also vary in time, and therefore probably enforce disparate nucleation pathways. ■

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1. Zhou, J. *et al. Nature* **570**, 500–503 (2019).
2. Gibbs, J. W. *Trans. Connect. Acad. Sci.* **3**, 108–248 (1876).
3. Volmer, M. & Schultze, W. *Z. Phys. Chem.* **156A**, 1–22 (1931).
4. Becker, R. & Döring, W. *Ann. Phys.* **416**, 719–752 (1935).
5. Zel'dovich, Y. B. *Acta Physicochim. URSS* **18**, 1–22 (1943).
6. Turnbull, D. *J. Chem. Phys.* **20**, 411–424 (1952).
7. Auer, S. & Frenkel, D. *Nature* **409**, 1020–1023 (2001).
8. Galkin, O., Chen, K., Nagel, R. L., Hirsch, R. E. & Vekilov, P. G. *Proc. Natl Acad. Sci. USA* **99**, 8479–8483 (2002).
9. Yamazaki, T. *et al. Proc. Natl Acad. Sci. USA* **114**, 2154–2159 (2017).
10. Ma, X. *et al. Nature Mater.* **16**, 767–774 (2017).
11. Vekilov, P. G. *Nanoscale* **2**, 2346–2357 (2010).
12. Kashchiev, D. *J. Chem. Phys.* **118**, 1837–1851 (2003).
13. Kashchiev, D. *Nucleation. Basic Theory with Applications* (Butterworth Heinemann, 2000).
14. Gasser, U., Weeks, E., Schofield, A., Pusey, P. & Weitz, D. *Science* **292**, 258–262 (2001).
15. Yau, S.-T. & Vekilov, P. G. *Nature* **406**, 494–497 (2000).
16. Cahn, J. W. & Hilliard, J. E. *J. Chem. Phys.* **28**, 258–267 (1958).

SYNTHETIC BIOLOGY

Universal control in biochemical circuits

A module for implementing robust feedback control in synthetic cellular networks has been reported. Its design is first proved mathematically to be universal for all networks, and then implemented in living cells. SEE LETTER P.533

NOAH OLSMAN & JOHAN PAULSSON

Anyone who has lived without central heating and cooling has had to learn the right combinations of opening windows, turning on radiators or adjusting blinds to get the temperature just right. Modern thermostats eliminate all that: you set them once and the built-in controllers do the rest, regardless of changes in the weather or the type of home. The temperature might still vary a little, but as long as the heaters and coolers are designed correctly, it should vary around the set point, rather than merely taking the edge off the cold or heat.

On page 533, Aoki and colleagues report[1] an analogous system for chemical reactions in living cells. Specifically, they design a reaction module in which two components sequester each other, and show that adding this to almost any network can force the output of the system to maintain a precise value that is proportional to an input signal, in a way that is robust to both external disturbances and uncertainty in the internal parameters — a behaviour known as robust perfect adaptation.

The results are striking for two reasons. First, most self-corrective biochemical circuits merely dampen the effects of external changes, rather than compensate for them perfectly. For example, by auto-repressing their own production, proteins can make their abundances less responsive to parameter changes than they would otherwise be, but still respond to some extent (Fig. 1a). Such systems are therefore known as homeostatic regulators because they maintain similar (homeo), rather than the same (homo), protein levels. Second, the impact of adding extra reactions to a biomolecular network usually depends on context. For instance, adding a repression step could create a positive or a negative feedback loop, depending on the rest of the network. Most systems have therefore been modelled and engineered on a case-by-case basis, and it has been hard even to imagine that any universal synthetic control could be found.

The approach taken by Aoki and co-workers is as striking as their results. Anyone working with synthetic biologists will eventually hear them quote the last words that physicist Richard Feynman wrote on his blackboard: "What I cannot create, I do not understand." However, Feynman was referring to mathematical derivations rather than to the building of real-world systems such as biological networks, and Aoki and colleagues' paper is one of the few in synthetic biology that truly lives up to the quote. The authors started by deriving exact mathematical rules that apply to broad classes of chemical-reaction system, and only then proceeded to physically build systems that illustrate the rule.

Although the authors' results pertain to

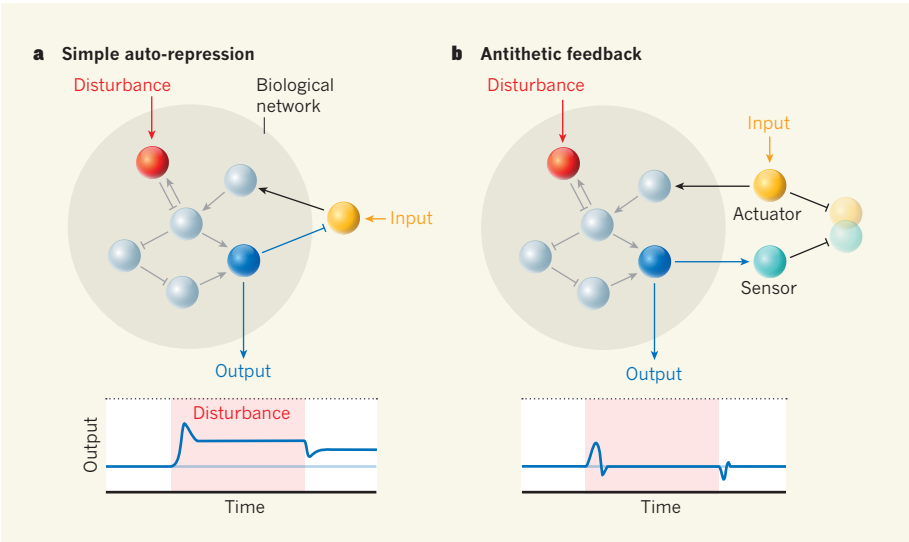

Figure 1 | Two modes of feedback regulation in biological networks. a, Auto-repression is a simple form of regulation for biological networks. In this general scheme, biomolecules (small spheres) in a network interact with each other, stimulated by an external input signal that acts on another molecule (yellow sphere). The molecule represented by the blue sphere inhibits the molecule acted on by the input, and produces a measurable output. When an external disturbance acts on part of the network (red sphere), altering the amplitude of the output, the network architecture partly compensates for the change, but does not precisely return the output to its original value. **b**, Aoki *et al.*[1] report an antithetic feedback module in which the input acts on an actuator molecule, the output acts on a sensor molecule, and the actuator and sensor molecules combine to cancel each other out. This architecture compensates for disturbances, and guarantees that the output returns to precisely its original value.