CHEMICAL BIOLOGY

A machine to manage modifications

An automated platform mass-produces RNAs incorporating a wide variety of precisely positioned chemical alterations.

Nuclear magnetic resonance (NMR) spectroscopy can give researchers a detailed glimpse of small RNA structures, but it quickly runs into trouble with larger RNA molecules. Part of the problem is that RNA is composed of just four chemical building blocks—in contrast to the 20 found in proteins—that are relatively similar in structure, yielding heavily overlapping NMR signals that can be difficult or impossible to interpret.

A technology for introducing chemically labeled nucleotides at selected RNA positions now promises to simplify NMR analysis of RNA structure as well as a host of other applications that rely on functional modification of RNA strands. A team led by US National Cancer Institute researcher Yun-Xing Wang and University of Texas Health Science Center professor Rui Sousa turned to a natural solution: the bacteriophage-derived T7 RNA polymerase enzyme. "It's a single-chain polymerase that proceeds at about 300 nucleotides per second," says Wang, "so it is far more efficient than the current state-of-the-art chemical synthesis." Position-selective labeling of RNA (PLOR) works by stalling transcription on a DNA template right before T7 reaches the intended labeling site. This is done by 'starving' the enzyme of the necessary nucleotide at specific points in transcription and then providing the labeled form of that nucleotide to reinitiate synthesis. T7 is coupled to microparticles, such that RNA synthesis can be tightly controlled by simply switching out the nucleotide solution surrounding the beads over the course of transcription, and the entire process is automated to run under computer control.

As a demonstration, Wang and colleagues introduced isotopically labeled nucleotides at

STRUCTURAL BIOLOGY

CRYO-EM GOES HIGH-RESOLUTION

The highest-resolution structure solved by cryo-electron microscopy to date reveals what it takes to reach the resolution realm of X-ray crystallography.

Recent, rapid technical advances to microscopes, detectors and image processing have substantially improved the resolution of cryo-electron microscopy (cryo-EM), causing the broader biology community to sit up and take notice of this once niche technique. An increasing number of near-atomic-resolution structures of biologically interesting protein complexes solved by cryo-EM are being reported in high-profile journals. But these advances notwithstanding, the cryo-EM community has been unable to penetrate the 3-Å resolution barrier, despite predictions showing that there is no theoretical limit to reaching atomic (~2-Å) resolution.

X-ray crystallography is routinely used to solve protein structures below 2.3-Å resolution, which allows visualization of fine details such as hydrogen bonding, salt bridges and ordered water molecules. The ability to attain such resolution with cryo-EM—which uses samples frozen in a thin layer of ice rather than crystallization and is particularly suitable for studying large protein complexes—is certain to open new doors in structural biology.

In recent work, Sriram Subramaniam of the US National Cancer Institute and colleagues reported the highest-resolution structure solved by cryo-EM to date, of a complex between *Escherichia coli* β-galactosidase and an inhibitor (phenylethyl β-D-thiogalactopyranoside) (Bartesaghi *et al.*, 2015). The reported resolution: 2.2 Å.

Just last year, Subramaniam's group reported a 3.2-Å structure of *E. coli* β-galactosidase, a fairly ordinary enzyme of about 460 kDa whose structure had been solved by crystallography, allowing the researchers to vet the cryo-EM structure. Reaching 3.2 Å was commendable, but Subramaniam was eager to explore what his group might do to break through the 3-Å barrier. "There are so many steps to go from

individual positions or multiple discrete sites within specific loop and stem structures in riboA71, a well-characterized 71-nucleotide 'riboswitch' that undergoes a major structural rearrangement upon binding adenine. PLOR made it possible to clearly resolve NMR signatures confirming known interactions between nucleotides, and it unambiguously revealed the presence of multiple distinct conformations that would have been hopelessly muddled with labeling strategies that tag the entire RNA molecule. The researchers subsequently used PLOR to modify riboA71 with the donor and acceptor fluorophore tags needed for fluorescence resonance energy transfer (FRET) experiments. FRET showed the conformational changes of individual riboA71 molecules and revealed that the riboswitch can assume its 'bound' conformation even in the absence of adenine. "People thought this might be the case before, but there was never any evidence," says Wang.

The first-generation, proof-of-concept PLOR instrument is suitable for production-scale work, with the capacity for churning out milligrams of precisely modified RNA, but Wang sees ample room for improvement—for example, the solution-switching process could be made more efficient by scaling down to a microfluidics-based device. Sousa has also identified some 'supercharged' T7 mutants that could facilitate faster production of longer RNAs. "These mutants are more tolerant and robust," says Wang, "and could offer much greater efficiency."

Because T7 interacts primarily with the base component of nucleotides, PLOR should be able to accommodate a wide range of other chemical modifications without severely hindering transcription. And although NMR applications initially led Wang's team to devise this solution, he also sees great potential in modifying RNAs for crystallographic analysis as well as a variety of nonstructural biology applications. "For example, you could introduce single fluorophores at sites that allow RNA aptamers to be used as sensors," he says, "and people have already made aptamers to detect drugs like cocaine or cancer markers."

Michael Eisenstein

RESEARCH PAPERS

Liu, Y. *et al.* Synthesis and applications of RNAs with position-selective labelling and mosaic composition. *Nature* doi:10.1038/nature14352 (4 May 2015).

purified protein to final structure," he notes. "I just felt we needed to look at each step carefully and make sure we're doing the best we could."

For their most recent work, Subramaniam's team used the same equipment and methods as in 2014, but performed an exhaustive study of different conditions for specimen preparation, imaging and data processing steps. The outcome of their work is a potentially generalizable set of imaging condition and image processing workflows. Once sample preparation is also optimized, these developments offer the hope of routine structure determination at ~2-Å resolution, at least for structurally homogeneous proteins. They obtained the best resolution when the specimen ice thickness was just right: thin enough to obtain strong signals but thick enough to obtain particles in different orientations (important for image processing). Despite the great recent cryo-EM advances, "we have not paid a lot of attention to specimen preparation," says Subramaniam. "Nothing has changed in almost three decades of doing it the same way." He hopes that his group's work will inspire the development of new methods for preparing better cryo-EM specimens.

Subramaniam's study comes on the heels of another recent 3-Å barrier-breaking report from Holger Stark and colleagues at the Max Planck Institute for Biophysical Chemistry. Stark's team reported a cryo-EM structure of the *E. coli* 70S ribosome in complex with the elongation factor Tu at a resolution of 2.65–2.9 Å using a microscope equipped with a spherical aberration corrector (Fischer *et al.*, 2015). These two studies together solidly demonstrate that resolutions that were formerly the realm of X-ray crystallography are now possible with cryo-EM.

Allison Doerr

RESEARCH PAPERS

Bartesaghi, A. *et al.* 2.2 Å resolution cryo-EM structure of β-galactosidase in complex with a cell-permeant inhibitor. *Science* **348**, 1147–1151 (2015).

Fischer, N. *et al.* Structure of the *E. coli* ribosome–EF-Tu complex at <3 Å resolution by C_s-corrected cryo-EM. *Nature* **520**, 567–570 (2015).