the incorporation of unnatural amino acids in response to a four-base codon. Through rounds of positive and negative selection, mutant $tRNA_{XXXX}$s were identified that showed increased efficiencies for unnatural amino acid incorporation in response to a four-base codon, and a decreased propensity to be aminoacylated with natural amino acids. The resulting synethetase/tRNA pairs such as Mj TyrRS/$tRNA_{UCCU}$ and Mb PylRS/$tRNA_{UACU}$ were then used to incorporate two unique unnatural amino acids at two unique four-base codons, a first in living *E. coli*. Refining the system further using Mb PylRS/$tRNA_{UACU}$ and Mj TyrRS/$tRNA_{CUA}$ enabled efficiencies of up to 20% for the incorporation of two unique unnatural amino acids in the Ca^{2+}-binding protein calmodulin.

Chin and co-workers then identified mutually orthogonal reactions that could be used to doubly label the protein in a one-pot reaction (Fig. 1b). They selected norbornyl-lysine (NorK) and tetrazinyl-phenylalanine (TetPhe) unnatural amino acids, which had been used previously in protein labelling but had not been tested in each other's presence[6]. Electron donation from the aniline substituent on the TetPhe unnatural amino acid limits its reactivity with NorK ($k \sim 10^{-5}$ $M^{-1}s^{-1}$), preventing unwanted crosslinking. Independent labelling of NorK with a more activated tetrazine probe (**1**) and TetPhe with a bicyclononyne probe (**3**) was achieved with rapid rates for both reactions ($k \sim 1$ $M^{-1}s^{-1}$) without any cross-reactivity. Dual fluorophore labelling was then accomplished using calmodulin containing the NorK and TetPhe unnatural amino acids and two dyes (BODIPY-TMR-X-**3** and BODIPY-FL-**1**) containing the appropriate crosslinking groups (Fig. 1c). FRET from the green BODIPY-FL dye to the red BODIPY-TMR-X dye was used to monitor urea denaturation of this doubly labelled calmodulin construct. Finally, FRET was used to detect multiple distinct conformational changes in the calmodulin N-terminus on titration with four equivalents of Ca^{2+}.

The development of this system represents an important leap forward in protein labelling for FRET studies. The past twenty years have seen enormous improvements in the sensitivity, spatial and temporal resolution of fluorescence spectroscopy and microscopy, enabling relatively routine single-molecule or femtosecond spectroscopy and video-rate and/or super-resolution microscopy[7–9]. The ability to easily generate proteins containing multiple fluorescent labels is now often the limiting factor in such experiments. Alternative approaches such as fluorescent protein labels (for example green fluorescent protein), are easy to attach through genetic fusions, however, they can often be disruptive to protein dynamics and are usually limited to attachment at the protein termini[10]. In contrast, the methods described by Chin and co-workers give a great deal of freedom in the choice of the two synthetic fluorophores used to label a protein and also the labelling positions on the protein. The labels can be changed simply by changing the R groups on tetrazine **1** and bicyclononyne **3**, and their positions can be changed simply by making mutations in the gene that encodes the protein of interest. Furthermore, FRET is certainly not the only application for these double labelling methods. For example, the same reactions could be used to attach a photocrosslinker and a biotin purification handle for protein interactome studies. The work reported by Chin and co-workers represents an important combination of molecular biology and organic chemistry that should be dramatically enabling to the biochemistry community. ❐

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POLYMER MECHANOCHEMISTRY

Flex, release and repeat

Force-induced covalent bond changes in mechanophore-linked polymers typically require large, irreversible material deformation, limiting successive activation cycles. Now, repeated force-induced reactions have been achieved by incorporating flex-activated mechanophores into elastomeric networks.

Nancy R. Sottos

Traditionally, chemical reactions are driven by thermal, chemical or electrical means. However, application of mechanical force or pressure can also alter the pathway for chemical reactions. The default response of polymers to external stress is unselective homolytic bond scission, resulting in material failure[1]. The incorporation of force-sensitive functional groups known as mechanophores into polymer backbones, however, can lead to more selective and productive chemistry. Such mechanophores react when mechanically deformed, and have been developed with the aim of imparting damage sensing, self-healing and self-reinforcing properties to bulk polymers[2,3].

The activation of mechanophores is inherently tied to the mechanical behaviour of the host polymer. Although a critical amount of force is required to overcome the potential energy barrier for a desired reaction, force-induced activation must also occur prior to failure of the bulk polymer. Owing to this inherent competition between activation and failure, mechanochemical activity in bulk polymers has been induced primarily by bond elongation in polymers that respond to force by undergoing significant plastic (non-recoverable) deformation before failure. Although outright failure is avoided, the irreversible deformation of the bulk material means that the force required to activate bonds cannot be

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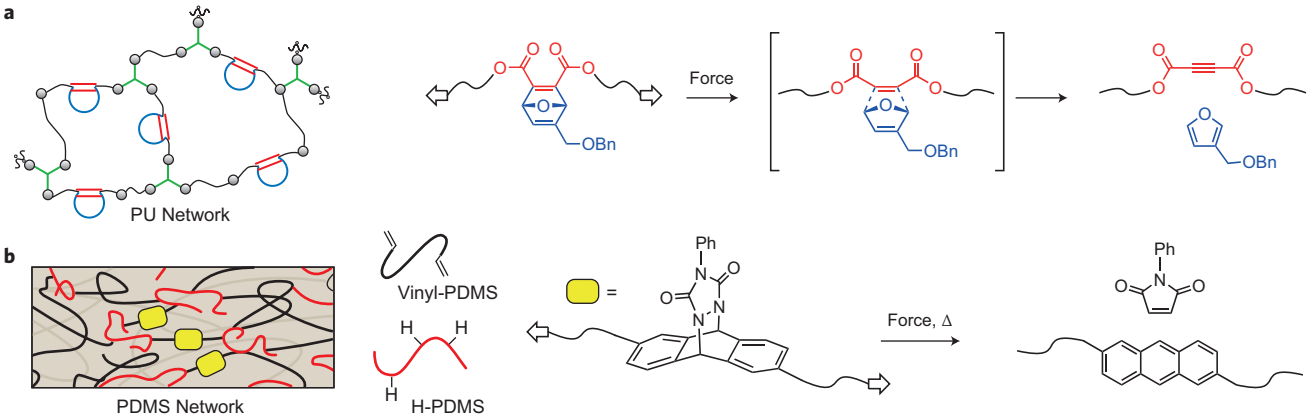

Figure 1 | Mechanisms for flex-activated small-molecule release in elastomeric networks. **a**, Retro-[4+2] cycloaddition of an oxanorbornadiene-based mechanophore in a segmented PU network[4]. **b**, Release of the dienophile from a Diels–Alder adduct of anthracene and phenyltriazolinedione crosslinked in PDMS[5]. Parts **a** and **b** reproduced with permission from refs 4 and 5 respectively, © 2014 ACS.

applied multiple times without inducing undesirable shape change or failure, and therefore repeated mechanochemical events are difficult to achieve.

Recently, two research groups have reported new platforms that aim to overcome the limitations of single activation in a mechanoresponsive polymer and enable the recurrent force-induced release of small molecules. Two different 'flex-activated' mechanophore motifs have been incorporated into elastomeric networks capable of successive loading cycles[4,5]. For flex activation, the mechanophores are cleverly linked into the polymer so that applied force leads to bond bending and subsequent scission of bonds that are not an integral part of the polymer backbone[6]. This departure from the more typical elongational bond activation associated with previous mechanophores[7] enables the force-induced release of a desired small molecule without impacting the integrity of the overall molecular architecture.

As they report in the *Journal of the American Chemical Society,* Michael Larsen and Andrew Boydston[4] have incorporated an oxanorbornadiene-based mechanophore into an elastomeric, segmented polyurethane (PU) network (Fig. 1a). When the bulk polymer is compressed, the oxanorbornadiene undergoes a retro-[4+2] cycloaddition to form an alkyne and release a small molecule that then diffuses out of the polymer matrix. This mechanophore was initially incorporated as a crosslinker in a poly(methyl acrylate) (PMA) matrix[6], but the compressive forces required for successful activation also caused failure of the PMA — the conversion of mechanophores was limited and successive loading cycles were not possible. By linking the same mechanophore in a PU matrix with higher strength and elasticity than PMA, Larsen and Boydston were able to achieve multiple actuation cycles. The retro-cycloaddition is activated at significantly lower stress levels in the PU system compared with the PMA system, although a plateau of about 7% actuation is reached after 15 compressions. This upper limit to mechanophore activation is attributed to random scission of chemical crosslinks and destruction of physical crosslinks by the break-up of hard domains within the PU matrix.

Writing in *ACS Macro Letters*, Stephen Craig and co-workers describe[5] how they have taken this concept one step further and integrated a phenyltriazolinedione-anthracene adduct as a crosslinker in a fully elastic polydimethysiloxane (PDMS) matrix (Fig. 1b). Application of a tensile force triggers a retro-Diels–Alder reaction through planarization of the anthracene component, releasing a small molecule (phenyltriazolinedione). In this case, an elevated temperature was also required to achieve a detectable level of reactivity. Although mechanochemical activation in this system was achieved with full shape recovery of the PDMS matrix and no plastic deformation, the effect of successive loading cycles on mechanophore conversion was not reported. Interestingly, repeated activation of a spiropyran mechanophore, which does not release a small molecule but rather switches between two differently coloured states on application and release of a force, was successfully demonstrated with full shape recovery of an identical PDMS matrix.

Force-induced release of a functional small molecule has the potential to trigger other chemical reactions and greatly expand the range of chemical outputs and responses for mechanochemically active polymers. Future applications, which could all benefit from repeatable activation, may include force-mediated delivery of drugs and other therapeutics, cascading damage detection schemes, and continuous remodelling or self-healing in materials at the molecular level. There is much work to be done, however, to achieve these far-reaching applications. Both of these initial investigations into flex-activated, small-molecule release in bulk elastomers report less than optimal conversions of the mechanophores, and this is an area in which we will undoubtedly see future improvements.

A key element to mechanochemical activation in bulk polymers is the efficient transfer of force to activate selected bonds in the mechanophore. This inherently depends on the molecular architecture of the mechanophore, the attachment of the mechanophore to the polymer chains, and ultimately the properties of the bulk polymer matrix. As both of these papers suggest, the mechanophore and host polymer are a coupled system. Synthesis of new mechanophore motifs is expanding, but achieving higher levels of activity may rely on the development of novel strategies to focus force from the polymer chains to the mechanophore or mechanisms to chemically amplify the initial force-triggered response.

In biological systems, mechano-transduction processes are found across many different length scales and involve highly efficient transformations of mechanical stimuli into productive chemical responses[8]. Force is routinely used to control vital processes by locally turning on or turning off chemical reactions. For example, individual cells possess the

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capability to sense mechanical signals from the extracellular matrix through receptors on the cell surface. These mechanical signals are propagated through the cytoplasm to the nucleus, which responds to the signal[9]. Cellular reactions to mechanical stimuli also occur through mechanically gated membrane ion channels, and the release of enzymes, calcium ions and transmitters[8]. Although a long way in the future, the recent advances in enabling repeated small-molecule release[4] and fully reversible activation[5] represent important steps towards developing biomimetic soft materials with reversible on/off responses to mechanical stress. ❐

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CYCLOPARAPHENYLENES

Closing the loop

The synthesis of [5]CPP, the smallest and by far the most strained member of the family of macrocycles known as cycloparaphenylenes, has been reported in quick succession by two different research groups. But how long will the new record holder retain its title?

Graham J. Bodwell

Homologous series of organic compounds, especially hydrocarbons, are particularly useful and interesting because they offer opportunities to learn something fundamental about how chemical and physical properties alter with incremental changes in structure. At their limits, they can also provide formidable synthetic challenges, thereby affording information about the scope of existing methodology and providing impetus to develop new chemistry. In the field of designed π systems, the homologous series that is currently attracting the most attention is the cycloparaphenylenes (CPPs; Fig. 1a). An [*n*]CPP is a macrocycle consisting of *n para*-linked phenylene units. The quest to construct ever-smaller members of this series has just reached another level with two independent, yet similar, syntheses of [5]CPP, now reported[1,2] by Shigeru Yamago and co-workers in the *Journal of the American Chemical Society* and Ramesh Jasti and colleagues in *Nature Chemistry*.

Although work aimed at the synthesis of CPPs can be traced as far back as 1934[3], it wasn't until 2008 that the syntheses of [9]-, [12]- and [18]CPP was finally reported[4]. This development, by Bertozzi and her team, sparked a period of frenetic activity in the field that continues unabated. What makes CPPs so interesting is that they are the shortest possible slices of armchair single-walled carbon nanotubes (SWCNTs; Fig. 1b) that retain six-membered (aromatic) rings. Smaller CPPs also map onto the surfaces of certain fullerenes — for example, the newly reported [5]CPP maps onto the surface of C_{60} (Fig. 1b).

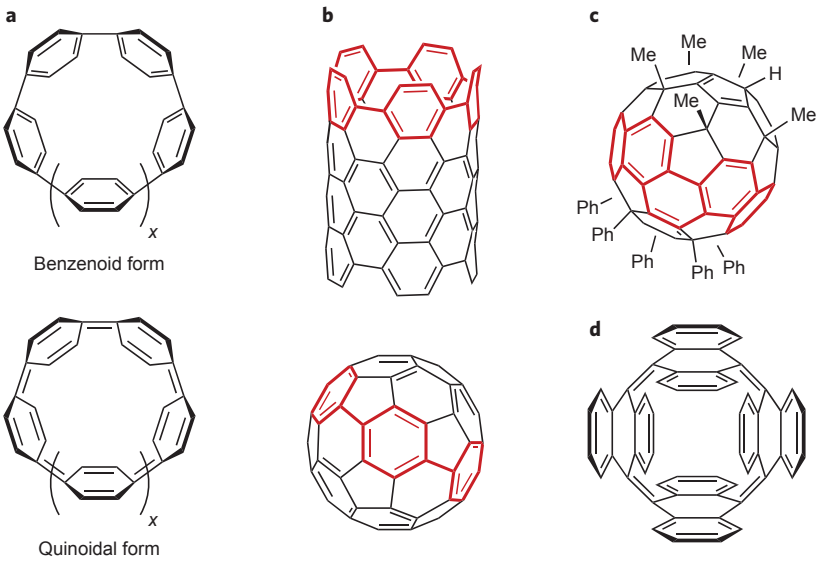

Figure 1 | Strained hydrocarbon hoops. **a**, The structure of an [*n*]CPP in its benzenoid and quinoidal forms ($n = x + 4$). **b**, The [5]CPP unit (red) can be found in a (5,5) SWCNT as well as C_{60}. **c**, Nakamura's C_{60}-derived doubly capped [10]cyclophenacene. **d**, Herges's picotube.

The enforced non-planarity of the benzene rings implies strain, which, in the context of hydrocarbon chemistry, has long been known to translate directly into challenging syntheses as well as unusual chemical and physical properties. Indeed, as the number of known CPPs has grown, unexpected trends in the electronic properties have been observed. For example, in stark contrast to the linear (acyclic) oligophenylenes, the HOMO–LUMO gap of the CPPs becomes smaller as the value of *n* decreases[1,2]. Host–guest chemistry involving the central cavity has also been reported[5], but perhaps the most appealing aspect of CPPs is their potential use as seeds for the controlled growth[6] of single-chirality SWCNTs.

Following the original report of [9]CPP by Bertozzi in 2008, the smallest known member of the CPP family successively dropped to [8]CPP in 2010[7],

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