Signal Transduction in a Covalent Post-Assembly Modification Cascade

Ben S. Pilgrim†, Derrick A. Roberts†, Thorsten G. Lohr†, Tanya K. Ronson† and Jonathan R. Nitschke†

†Department of Chemistry, The University of Cambridge, Lensfield Road, Cambridge CB2 1EW, United Kingdom. †These authors contributed equally to this work. *e-mail: jrn34@cam.ac.uk.

Nature Chemistry, 2017, Published Online - 21st August 2017
DOI:10.1038/nchem.2839

Full Text: http://www.nature.com/nchem/journal/vaop/ncurrent/full/nchem.2839.html

PDF: http://rdcu.be/vaKZ
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Ben S. Pilgrim†, Derrick A. Roberts††, Thorsten G. Lohr1, Tanya K. Ronson1 and Jonathan R. Nitschke††

1Department of Chemistry, The University of Cambridge, Lensfield Road, Cambridge CB2 1EW, United Kingdom. †These authors contributed equally to this work. *e-mail: jrn34@cam.ac.uk.

Natural reaction cascades control the movement of biomolecules between cellular compartments. Inspired by these systems, we report a synthetic reaction cascade employing post-assembly modification reactions to direct the partitioning of supramolecular complexes between phases. The system is comprised of a self-assembled tetrazine-edged FeLsL12 cube and a maleimide-functionalized FeL6 tetrahedron. Norbornadiene (NBD) functions as the stimulus that triggers the cascade, beginning with the inverse electron-demand Diels-Alder reaction of NBD with the tetrazine moieties of the cube. This reaction generates cyclopentadiene as a transient by-product, acting as a relay signal that subsequently undergoes a Diels-Alder reaction with the maleimide-functionalized tetrahedron. Cyclooctyne can selectively inhibit the cascade by outcompeting NBD as the initial trigger. Triggering the cascade with 2-octadecylNBD leads to selective alkylation of the tetrahedron upon cascade completion. The increased lipophilicity of the C18-tagged tetrahedron drives this complex into a non-polar phase, allowing its isolation from the initially inseparable mixture of complexes.

Reaction cascades allow information to be relayed1 from an initial stimulus to an eventual output across relatively long distances within biological systems.2 Such cascades frequently involve sequences of selective covalent bond forming reactions that act on self-assembled biomolecules, such as the post-translational modification (PTM) of proteins3 or epigenetic modification of DNA.4 These cascades, which can be mediated by the action of fleeting signal molecules such as nitric oxide,5 enable cells to respond dynamically to their environment in response to changing biochemical demands. Inspired by these natural cascades, organic chemists have developed cascade reactions for performing multiple bond-forming processes within a single reaction flask, thereby simplifying synthetic routes to complicated molecules. Recently, some of these cascades have featured supramolecular elements such as container molecules, which act as ‘nanoreactors’6, 7 to facilitate particular steps in the cascade.8-11 There have also been efforts to include features critical to biological functions, such as inhibition or feedback loops,12 to develop more complex supramolecular systems that exhibit nuanced responses to transient chemical species. To date, however, none of these supramolecular cascades have harnessed covalent post-assembly modification (PAM) of the supramolecular components as a mechanism for signal transduction.

Covalent PAM reactions must satisfy several prerequisites before they can be successfully employed for the structural alteration of a discrete supramolecular complex: they must be chemoselective, afford near-quantitative yields and proceed under mild conditions to avoid damaging the complex. Due to these stringent requirements, ‘click’ reactions, such as the strain-promoted Huisgen alkyne–azide cycloaddition13-14 or inverse electron-demand Diels-Alder (IEDDA) cycloaddition,15, 16 have been particularly useful as PAM reactions. To date, covalent PAM of metal-organic complexes17 has been used to alter the product distribution in systems of self-assembled complexes18-20 modify solubility properties,21 stabilize metastable structures,22 change the nature of a capsule’s cavity to control guest binding,23, 24 generate complex molecular topologies25-28 and graft molecules of interest onto self-assembled structures.29 Despite this
versatility, PAM in these previous examples has been exclusively carried out either on individual complexes or via orthogonal reactions on different complexes within the same system, rather than via the interlinked steps of a cascade. By coupling together multiple PAM reactions into a single sequence, here we demonstrate signal transduction in a supramolecular system to control the movement of a nanometre-sized complex across a phase boundary. This sequence evokes the cellular signalling pathways that drive the translocation of biomolecules across biological membranes.

**Results and Discussion**

The cascade system developed herein features tetrazine-edged Fe$^{II}_8$L$_{12}$ cube 1a and maleimide-functionalized Fe$^{II}_4$L$_6$ tetrahedron 3, which undergo a tandem sequence of IEDDA and normal electron-demand Diels-Alder (DA) reactions connected via a transient relay signal. The cascade is triggered by the addition of 2-octadecylnorbornadiene (C$_{18}$-NBD), which undergoes IEDDA reactions on its least-hindered side with the tetrazines of cube 1a to give pyridazine-edged cube 2 and 1-octadecyclooctapentadiene (C$_{18}$-CPD) as a metastable intermediate (Fig. 1). The C$_{18}$-CPD by-product of the first reaction step subsequently acts as a relay signal for the second DA reaction step, in which the maleimide units of tetrahedron 3 are converted into the C$_{18}$-norbornene moieties of tetrahedron 4d. In the absence of a C$_{18}$-NBD trigger, the initial state of the cascade system is stable due to the orthogonal Diels-Alder reactivities of cube 1a and tetrahedron 3. The alkyl chains appended to complex 4d following the cascade render it sufficiently lipophilic to undergo spontaneous phase transfer from a polar organic phase (CH$_3$CN/CHCl$_3$ 9:1) to a non-polar organic phase (cyclopentane).

Cube 1a is derived from subcomponent A, whose design combines two strategies: the use of a ligand whose twin 4-substituted 2-formylpyridine moieties are arrayed in the correct geometry to generate an M$_8$L$_{12}$ cubic framework, and the incorporation of 3,6-disubstituted tetrazine spacers into ligand frameworks to enable PAM by IEDDA reactions. Cube 1a was thus prepared (see Methods) by the subcomponent self-assembly of dialdehyde A, which was synthesized in a protecting-group-free three-step sequence (Supplementary Fig. 1),$^{32-34}$ para-tert-butylaniline and iron(II) bis(trifluoromethane)sulfonimide, (Fe(N Tf$_2$)$_2$), in CH$_3$CN at 333 K for 16 h (Fig. 1). After purification, ESI-MS of the reaction mixture confirmed the formation of an Fe$^{II}_8$L$_{12}$ complex in solution (Supplementary Fig. 12). $^1$H NMR analysis of the reaction mixture revealed the formation of a single product having only one signal for each unique ligand environment, consistent with the expected cubic geometry (Supplementary Fig. 10). The high solubility of 1a in typical non-solvents precluded its crystallization. Fortunately, the lower solubility of cube 1b, which was prepared using para-toluidine in place of 4-tert-butylaniline, proved conducive to the growth of single crystals of this complex, obtained by slow vapour diffusion of iPr$_2$O into a solution of 1b[NTf$_2$], containing KAsF$_6$ (~100 equiv.). X-ray diffraction analysis confirmed complex 1b to be an achiral Fe$^{II}_8$L$_{12}$ cube of idealized $T_h$ point group symmetry in the solid state,$^{30}$ with adjacent vertices of alternating $A$ and $A$ handedness (Fig. 2a) (Supplementary Figs 94-96).
Figure 1 | Overview of syntheses and post-assembly modification cascade. Complexes 1a and 3 are assembled from dialdehydes A and B respectively. The PAM cascade proceeds via inverse electron-demand Diels-Alder and subsequent normal electron-demand Diels-Alder reactions to convert 1a and 3 into complexes 2 and 4(a-d) respectively.

Tetrahedron 3, functionalized with pendant maleimide groups, was designed as a suitable reaction partner for the alkylated-CPD relay signal released from the IEDDA reaction in the first step, which required the synthesis of novel maleimide-functionalized aniline C. This aniline features an ethylene spacer to reduce steric congestion at the vertices of the tetrahedron. Aniline C was prepared in three steps from commercially-available starting materials (Supplementary Fig. 26). Tetrahedron 3 was subsequently prepared (see Methods) by subcomponent self-assembly of dialdehyde B, aniline C, and iron(II) bis(trifluoromethane)sulfonimide, (Fe(NTf₂)₂) in CH₃CN at 333 K for 16 h (Fig. 1). Slow vapour diffusion of iPr₂O into a CH₃CN solution of 3 containing Bu₄NPF₆ (~100 equiv.) yielded single crystals suitable for X-ray diffraction analysis, unambiguously confirming the T-symmetric framework of 3 in the solid state (Fig. 2b), echoing the results of solution ESI-MS and ¹H NMR analyses (Supplementary Figs 35 and 38).
Figure 2 | Depictions (same scale) of the X-ray crystal structures. a. Cube 1b. b. The PF$_6^-$ adduct of tetrahedron 3. c. The AsF$_6^-$ adduct of modified tetrahedron 4a. Key: grey = carbon, white = hydrogen, red = oxygen, blue = nitrogen, orange = iron, light green = fluorine. Disorder and non-encapsulated anions have been omitted for clarity. Iron atoms are connected with orange lines to illustrate the overall geometry of each complex.

Before investigating the PAM cascade with an alkylated-NBD, we first tested its viability in a model system using commercially-available, unsubstituted NBD. In this model system, the relay signal CPD could also be easily prepared by cracking dicyclopentadiene, allowing the IEDDA and DA steps to be examined in isolation to afford pure samples of both product complexes 2 and 4a for characterization purposes.

The reaction of cube 1a with NBD (16 equiv. per cube) proceeded cleanly to furnish dodecafunctionalized pyridazine-edged cube 2 as the only supramolecular product, alongside the CPD by-product, within 4 h, as verified by $^1$H NMR and ESI-MS analyses (Supplementary Figs 41 and 42). At intermediate time points, desymmetrization of the cage signals was observed; however, the characteristic signals from the vertex protons on the complex persisted and no signals from free subcomponents appeared, suggesting that the ligands did not dissociate from the cage during this reaction sequence.$^{16}$ To test the reactivity of tetrahedron 3 towards the CPD relay signal in isolation, freshly distilled CPD (16 equiv. per tetrahedron) was added to a CD$_3$CN solution of 3, leading to the clean formation of norbornene-grafted 4a, as confirmed by both ESI-MS and $^1$H NMR analyses (Supplementary Figs 44 and 47). Slow vapour diffusion of $i$Pr$_2$O into a CD$_3$CN solution of 4a containing KAsF$_6$ (~100 equiv.) yielded single crystals suitable for X-ray diffraction, the analysis of which confirmed the $T$-symmetric framework of 4a in the solid state and that DA reactions had
taken place with *endo* selectivity (Fig. 2c). Both complexes 3 and 4a contained a single encapsulated anion (PF$_6^-$ or AsF$_6^-$) in the crystal structure, consistent with the behaviours of other tetrahedra constructed from dialdehyde B. In batches of tetrahedron 3 synthesized with 1.0 equiv. of NMe$_4$PF$_6$, the encapsulation of PF$_6^-$ in the solution phase was verified by $^{19}$F NMR (Supplementary Fig. 37). Samples of 4a derived from the PAM of such batches of 3 also exhibited PF$_6^-$ encapsulation in solution (Supplementary Fig. 46), confirming that PAM does not disrupt the guest binding behaviour of the tetrahedral framework.

The full IEDDA-DA PAM cascade in this model system was initiated by the addition of NBD (30 equiv. per 1a) to a mixture of cube 1a and tetrahedron 3 in CD$_3$CN at 293 K. *In situ* $^1$H NMR analysis of the reaction (Fig. 3a) showed that the IEDDA pathway proceeded rapidly upon addition of NBD to the system, resulting in a decrease in the signals corresponding to 1a and concomitant appearance of cube 2 and CPD. As the concentration of CPD grew, the secondary DA reaction proceeded, resulting in clean conversion of 3 to 4a. The sharpness of the signals from 2 and 4a at the end of the reaction indicated the absence of ligand scrambling, further supporting our inference that the complexes remain intact during the PAM cascade.

We note that CPD is a metastable intermediate and will dimerise in the absence of an alternative reactive partner. It is produced here only in a small quantity, akin to the transient nitric oxide signals employed by biological systems. It would not be straightforward to extract the CPD from a system containing only it and cube 2, and then use it to modify either aniline C pre-self-assembly or tetrahedron 3 post-self-assembly. This observation highlights a particularly useful feature of a cascade approach to PAM, since the *in situ* formation of CPD overcomes the challenges of synthesising and isolating alkyl-substituted CPDs (*vide infra*). As expected, when NBD was added to tetrahedron 3 in isolation, or CPD added to cube 1a in isolation, no reaction was observed due to the sequence dependence of the cascade.
A hallmark of biological systems is their ability to respond to different signals in specific ways, with certain signals overriding others to inhibit a process. Cyclooctyne was shown to function as an inhibitor within the context of our PAM cascade system: when a solution of NBD (18 equiv. per cube) and cyclooctyne (12 equiv. per cube) was added to an equimolar mixture of cube 1a and tetrahedron 3 in CD$_3$CN, the more reactive cyclooctyne underwent IEDDA preferentially with complex 1a, affording cyclooctylpyridizine-edged complex 5 as the only observed product within minutes of addition (Fig. 4) (Supplementary Figs 58-62). Because NBD was prevented from reacting with 1a, no relay CPD signal was produced, thus inhibiting the cascade and showing how a more reactive signal can override a less reactive one.
Monitoring the inhibited post-assembly modification cascade. $^1$H NMR (500 MHz, 298 K, CD$_3$CN) spectra from in situ monitoring of the inhibited cascade (carried out at 293 K) at various time points are shown. Signals are marked as follows: tetrazine cube $1a$ (solid blue squares), pyridazine cube $5$ (hollow pink squares), maleimide tetrahedron $3$ (red triangles), NBD (grey stars).

To translate the effects of the microscopic PAM cascade to the macroscopic regime, we sought to couple PAM to a phase transition whereby tetrahedron $4$ would be rendered sufficiently lipophilic to spontaneously move into a non-polar solvent upon completion of the cascade. Three NBDs of increasing alkyl chain length—2-hexyl-NBD (C$_6$-NBD), 2-decyl-NBD (C$_{10}$-NBD) and 2-octadecyl-NBD (C$_{18}$-NBD)—were synthesized (Supplementary Fig. 64) as triggers for the cascade. The cascade reactions were performed in either CH$_3$CN (for C$_6$-NBD) or a 9:1 mixture of CH$_3$CN:CHCl$_3$ (for C$_6$-NBD, C$_{10}$-NBD and C$_{18}$-NBD) as the polar ‘reaction’ phase, with the CHCl$_3$ added to help solubilize the more lipophilic alkyl-NBDs. Cyclopentane was selected as the non-polar ‘destination’ phase for extracting the alkylated tetrahedra, because it is immiscible with the aforementioned polar phases and neither cage $1a$ nor $3$ was observed to partition into cyclopentane from either CH$_3$CN or 9:1 CH$_3$CN/CHCl$_3$. The cascade was initiated by the addition of the appropriate NBD to a mixture of $1a$ and $3$ (see Methods). After the reaction was judged complete by $^1$H NMR analysis, an equal volume of cyclopentane was added, the mixture shaken in a vortex mixer to enable phase transfer; the phases were then separated by centrifugation.

Addition of C$_6$-NBD to the system of $1$ and $3a$ in CD$_3$CN (or in a 9:1 CD$_3$CN/CHCl$_3$ mixture) resulted in clean conversions after 20-24 h at 293 K to cube $2$ and tetrahedron $4b$ respectively. The cyclopentane phase remained colourless following attempted extraction, however, indicating that tetrahedron $4b$ failed to
partition into cyclopentane. With C_{10}-NBD in 9:1 CD_{3}CN/CDCl_{3}, clean conversions to cube 2 and tetrahedron 4c were also observed by $^1$H NMR, but tetrahedron 4c also failed to partition into cyclopentane following attempted extraction. However, with C_{18}-NBD in 9:1 CD_{3}CN/CDCl_{3}, clean conversions of 1 and 3a to cube 2 and tetrahedron 4d were followed by successful extraction into cyclopentane, as evidenced by the deep purple colour of the cyclopentane layer (Fig. 5c).

This coloured cyclopentane layer was decanted and a further extraction performed with fresh cyclopentane. The second cyclopentane extract was much paler in colour, indicating almost all tetrahedron 4d underwent phase transfer upon the first extraction. The cyclopentane was not noticeably coloured following attempted extraction for the third time. Analysis of the remaining CD_{3}CN/CDCl_{3} layer from the cascade by $^1$H NMR showed exclusively the presence of cube 2 (Supplementary Fig. 87). The first two cyclopentane extracts were combined, concentrated and the residue dissolved in 1:1 CD_{3}CN/CDCl_{3}, whereby $^1$H NMR analysis revealed the presence of tetrahedron 4d only, confirming that this complex retained its structural integrity following successful transfer into a non-polar solvent phase. In cases where tetrahedron 3 contained encapsulated PF_{6}^{-} prior to initiation of the reaction cascade, $^{19}$F NMR analysis of tetrahedron 4d isolated after this phase transition indicated PF_{6}^{-} was still encapsulated (Supplementary Fig. 91). This experiment thus demonstrates the possibility of signal transduction in a synthetic system over large distances, whereby tetrahedron 4d crosses a macroscopic phase boundary with the cargo present in tetrahedron 3 before PAM also present in tetrahedron 4d after PAM and subsequent phase switching.
Figure 5 | Signal transduction leading to phase segregation of the two product complexes. a. No transitioning of the starting complexes 1a and 3 to the non-polar phase occurred, rendering them inseparable before application of the NBD signal. b. The C_6-NBD or C_10-NBD signals did not render the tetrahedra 4b or 4c sufficiently lipophilic to induce transfer into a non-polar phase. c. The C_{18}-NBD signal-induced transfer of tetrahedron 4d into a non-polar phase upon completion of the PAM cascade.

Summary and Outlook

Natural PTM cascade reactions underpin the ability of cells to dynamically regulate their activities in response to external stimuli. We propose that covalent PAM could serve an analogous role in supramolecular chemistry, enabling the construction of stimuli-responsive chemical systems\textsuperscript{37-39} that emulate the functional diversity of their biological counterparts. The two-step PAM cascade presented herein, for which the functional response is the transition of a large supramolecular structure across a phase boundary, demonstrates this proposition. This cascade system, which responds to a triggering signal (NBD), employs a relay shuttle (CPD) and can be interrupted by an inhibitor (cyclooctyne), functionally mirrors many of the key features of the complex signal transduction pathways of biology, but in an entirely abiological context. Such systems have clear potential to transport cargo across phase boundaries\textsuperscript{40} in response to chemical signals. Such a capability may be of use in designing new chemical separations\textsuperscript{41} inspired by the scale\textsuperscript{42-45} and the structural\textsuperscript{46-48} and functional complexity\textsuperscript{49,50} of natural signal transduction pathways.
Methods

Self-assembly of cube 1a

A suspension of tetrazine dialdehyde A (13.8 mg, 47.1 µmol) and 4-tert-butylaniline (14.1 mg, 94.2 µmol) in dry CH₃CN (2.4 mL) was deoxygenated by bubbling nitrogen gas through it for 15 min. Iron(II) bis(trifluoromethylsulfonyl)imide (22.1 mg, 31.4 µmol) was added, after which the solution turned dark blue-green. The resulting mixture was heated to 333 K for 16 h, then cooled to room temperature, filtered through a glass fibre filter (0.7 µm pore size) and then evaporated to dryness under a stream of nitrogen gas. The solid was redisolved in CH₂Cl₂/CH₃CN (95:5) and purified by size exclusion chromatography (Biobeads SX-1), eluting with CH₂Cl₂/CH₃CN (95:5). The main blue-green band was collected, the solvent removed under a stream of nitrogen gas to furnish cube 1a (41.9 mg, 3.61 µmol, 92%) as a fine dark-blue powder.

Self-assembly of tetrahedron 3

A suspension of dialdehyde B (10.8 mg, 50.9 µmol) and aniline C (22.0 mg, 102 µmol) in dry CH₃CN (2.5 mL) was deoxygenated by bubbling nitrogen gas through it for 15 min. Iron(II) bis(trifluoromethylsulfonyl)imide (24.0 mg, 33.9 µmol) was added, after which the solution turned purple. The resulting mixture was heated at 333 K for 16 h, then cooled to room temperature, filtered through a glass fibre filter (0.7 µm pore size) and then evaporated to dryness under a stream of nitrogen gas. iPr₂O (20 mL) was then added, the residue re-suspended and then centrifuged (5 min, 3000 rpm) and the iPr₂O decanted. This procedure was repeated with fresh iPr₂O. The residue was then dried in vacuo to furnish tetrahedron 3 (42.7 mg, 6.98 µmol, 82%) as a fine purple powder.

Post-assembly modification cascade and subsequent phase separation

A 2 mL microcentrifuge tube was charged with a solution of cube 1a (2.0 mg, 0.17 µmol, 1.1 equiv.) and tetrahedron 3 (1.0 mg, 0.16 µmol, 1.0 equiv.) in CD₃CN (0.9 mL). A solution of C₁₈-NBD (2.7 mg, 7.9 µmol, 50 equiv.) in CDCl₃ (0.1 mL) was added, the reaction mixture homogenized by vortex mixing for 30 s, and the solution was transferred to an NMR tube for in situ ¹H NMR monitoring. The reaction was performed at 293 K and reached completion after 22 h. The reaction mixture was transferred to a 2 mL microcentrifuge tube, cyclopentane (1 mL) was added, the mixture homogenized by vortex mixing for 30 s, and then the layers separated by centrifugation (9000 rpm, 30-60 sec). Partitioning of the alkylated tetrahedron 4d into the less dense cyclopentane layer was assessed by visual inspection, as evidenced by the characteristic purple colour of the cyclopentane layer (Supplementary Fig. 89). Tetrahedron 4d was extracted completely from the CD₃CN/CDCl₃ cascade medium by an additional 1 mL extraction into cyclopentane. Complete separation of cube 2 and tetrahedron 4d into polar and non-polar organic phases respectively, was verified by ¹H NMR analysis of the isolated phases. ¹H NMR samples were prepared by evaporating each isolated phase to dryness with a stream of nitrogen gas and then redissolving the residue in an appropriate solvent. The remnants of the more polar CD₃CN/CDCl₃ phase were redissolved in CD₃CN which revealed the exclusive presence of cube 2. The remnants of what had transferred to the cyclopentane phase were redissolved in 1:1 CD₃CN/CDCl₃, which revealed the exclusive presence of tetrahedron 4d (Supplementary Fig. 87).

Data Availability

Crystallographic data for the structures reported in this paper have been deposited at the Cambridge Crystallographic Data Centre, under the deposition numbers 1520129-1520131. Copies of these data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif. All other data supporting the findings
of this study are available within the Article and its Supplementary Information, or from the corresponding author upon reasonable request.
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**Acknowledgements**

B.S.P. acknowledges the Herchel Smith Research Fellowship, the Royal Commission for the Exhibition of 1851 Research Fellowship and the Fellowship from Corpus Christi College, Cambridge. D.A.R. acknowledges support from the Gates Cambridge Trust. This work was also supported by the UK Engineering and Physical Sciences Research Council (EPSRC, EP/M008258/1). The authors thank Diamond Light Source (UK) for synchrotron beamtime on I19 (MT11397), the NMR facility at the University of Cambridge Chemistry Department, and the EPSRC UK National Mass Spectrometry Facility at Swansea University.

**Author Contributions**

B.S.P., D.A.R. and J.R.N. conceived the initial ideas for the project. B.S.P. and D.A.R. designed the complexes. B.S.P., D.A.R. and T.G.L. performed the synthetic work. B.S.P. and D.A.R. characterized the compounds, performed the cascades and grew the single crystals. T.K.R. collected diffraction data, and solved and refined the X-ray crystal structures. B.S.P. prepared the initial draft of the paper and all authors contributed to the final draft of the paper.

**Additional information**

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to J.R.N.

**Competition financial interests**

The authors declare no competing financial interests.