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Signal transduction in living systems is the conversion of information into a chemical change and the principal process by which cells communicate. This process enables phenomena such as time-keeping and signal amplification. In nature, these functions are encoded in non-equilibrium (bio)chemical reaction networks (CRNs) controlled by enzymes. While these catalytically controlled processes are an integral part of biocatalytic pathways, man-made analogs are rare. Here, we incorporate catalysis in an artificial fuel driven out-of-equilibrium CRN. The study entails the design of an organocatalytically controlled fuel driven esterification CRN, where the forward (ester formation) and backward reaction (ester hydrolysis) are controlled by varying the ratio of two different organocatalysts: pyridine and imidazole. This catalytic regulation enables full control over ester yield and lifetime. The fuel-driven strategy is subsequently used in the design of a responsive polymer system, where transient polymer conformation and aggregation can be controlled through variation of fuel and catalysts levels. Altogether, we show how organocatalysis is an important tool to exert control over a man-made fuel driven system and induce a change in a macromolecular superstructure, as ubiquitously found in natural non-equilibrium systems.

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Organocatalytic control over a fuel-driven transient esterification network

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Abstract
Signal transduction in living systems is the conversion of information into a chemical change and the principal process by which cells communicate. This process enables phenomena such as time-keeping and signal amplification. In nature, these functions are encoded in non-equilibrium (bio)chemical reaction networks (CRNs) controlled by enzymes. While these catalytically controlled processes are an integral part of biocatalytic pathways, man-made analogs are rare. Here, we incorporate catalysis in an artificial fuel driven out-of-equilibrium CRN. The study entails the design of an organocatalytically controlled fuel driven esterification CRN, where the forward (ester formation) and backward reaction (ester hydrolysis) are controlled by varying the ratio of two different organocatalysts: pyridine and imidazole. This catalytic regulation enables full control over ester yield and lifetime. The fuel-driven strategy is subsequently used in the design of a responsive polymer system, where transient polymer conformation and aggregation can be controlled through variation of fuel and catalysts levels. Altogether, we show how organocatalysis is an important tool to exert control over a man-made fuel driven system and induce a change in a macromolecular superstructure, as ubiquitously found in natural non-equilibrium systems.
Introduction

Signal transduction in living systems is the conversion of information into a chemical change and the primary process by which living cells are able to communicate over micrometre distances. Cells harbour very specific and sensitive signal-transducing mechanisms, which are regulated by membrane-bound protein receptors that respond to external signals such as antigens, light, hormones, pathogens, hypoxia, pheromones, amongst others. ¹ This rudimentary communication enables functions such as time keeping and signal-amplification, which are encoded in non-equilibrium (bio)chemical reaction networks (CRNs) regulated by enzymes. ²-⁴ As such, out-of-equilibrium systems are an integral part of biological pathways and most complex living systems need the constant input of a chemical fuel to sustain themselves. The fuel is typically a high-energy bond molecule, e.g. adenosine triphosphate (ATP), guanosine triphosphate (GTP) or acyl-coenzyme A (acyl-CoA). For example, the latter fuel together with two enzymes plays a key role in histone (de)acetylation, which regulates gene activity and is recognized as a very important process in epigenetics. ⁵-⁷ Overall, catalysis is crucial in non-equilibrium biological CRNs and can turn ON and OFF the complete process. Inspired by catalytically controlled natural processes, we set out to design a man-made catalytically controlled out-of-equilibrium network. Successful fuel-driven CRNs have been designed before with direct and indirect chemical fuels, providing spatiotemporal control over material formation and exhibiting non-linear behaviour, such as stochastic collapse or oscillations ⁸-¹⁸. Yet, the incorporation of catalysis as a control switch in a non-equilibrium biological CRN has not been investigated. A few recent examples of cooperative catalysis exist, but those typically involve the building blocks as catalysts ¹⁹-²⁰. In the current work however, we use two external catalysts in parallel to control the reaction kinetics of the CRN. Instead of enzymatic or metal-based catalysis we turned to organocatalysis. Compared to enzymes and metal-based catalysts, organocatalysts are simple, frequently less toxic, easily accessible, less substrate specific and favour networks over single transformations ²¹-²². Small molecule organocatalysts are used for a myriad of synthesis reactions and can be used in aqueous environment ²². Here, we show how two organocatalysts can be used together to control the yield and lifetime of a transiently stable product in a fuel-driven esterification CRN. First, we explain the system design and characteristics. Then, we show the performance of the system with a small molecule model CRN and investigate the reaction kinetics. Finally, we apply the same organocatalysed fuel-driven strategy to control the shape of an amino acid functionalized polymer system by transient (de)acetylation, showing temporal regulation over polymer chain conformation and aggregation behaviour.
Results and discussion

Choice of fuel-driven CRN and reaction conditions

We designed a CRN, where the forward reaction (ester formation) can be accelerated by pyridine and the backward reaction by imidazole (ester hydrolysis) (Scheme 1). Acetic anhydride 2 is used as a direct chemical fuel, which reacts with the starting material (p-nitrophenol(ate) 1) generating the building blocks for the transient state (p-nitrophenyl ester 3) and acetic acid as waste 4 (Scheme 1).

Scheme 1: Fuel-driven out-of-equilibrium esterification CRN, where the formation and degradation pathways can be controlled by different nucleophilic catalysts: the rate of ester formation is accelerated by pyridine and the backward ester hydrolysis reaction by imidazole.

Pyridine is a versatile tertiary amine organocatalyst, used in a plethora of everyday synthesis reactions. In particular, O-acetylation is typically carried out with DMAP or pyridine as organocatalyst.23 Here, pyridine acts as nucleophilic catalyst (Lewis base – with nucleophilicity index of $N$ 11.05 in water24), creating a reactive intermediate with acetic anhydride 2: the acetyl-pyridinium species. The $pK_a$ of pyridine is 5.2 meaning that at neutral pH the free base species is dominant.25 In contrast, imidazole is known as an effective catalyst for the hydrolysis of activated esters such as $p$-nitrophenyl acetate26-28. The imidazole catalytic cycle proceeds through an acetyl-imidazole reactive intermediate and the catalytic mechanism of imidazole depends on the leaving group strength of imidazole versus the –OR group.29 Esters with weak leaving groups are subject to general base catalysis, whereas for activated esters with better leaving groups (here: nitro-phenolates) the imidazole catalysis exhibits a nucleophilic substitution mechanism.26-28, Additionally, the nucleophilic catalysis of imidazole ($pK_a$ 6.9) is pH-dependent and favours higher pH, since more free base is present.30-31 Imidazole has a higher basicity than pyridine, yet the nucleophilicity is lower ($N$ 9.63 in water32). Overall, imidazole has a preference for less reactive acylation agents (activated esters) compared to pyridine, which is a better catalyst for highly reactive acylation agents (anhydrides)33-34. The latter is exploited in this fuel-driven CRN to control the temporal acetylation of $p$-nitrophenol(ate) 1. Experiments are performed at near neutral pH 7.5 in a strongly buffered system to
take care of the acid waste. At pH 7.5 the phenolate is dominant over the phenol form, and the uncatalysed ester and anhydride hydrolysis are less prevalent than at more acidic or basic conditions.

**Organocatalysed fuel-driven esterification CRN**

In this CRN, \( p \)-nitrophenol(ate) 1 is acetylated with acetic anhydride 2 to generate ester 3, which is hydrolytically unstable and over time the phenol(ate) 1 is regenerated along with forming acetic acid waste product 4. Pyridine and imidazole are used as organocatalysts to accelerate the ester formation and hydrolysis. We varied the organocatalysts and fuel concentrations to achieve yield and lifetime variations in the transient \( p \)-nitrophenyl ester 3 (Figure 1 – shows the conversion of 1 monitored by UV-VIS at 400 nm). While a blank reaction without organocatalysts resulted in ester lifetimes of \(~68\) h and a max. yield of \(55\%\) after \(25\) min. (SI Figure S7), increasing the pyridine concentration accelerated the forward reaction and augmented the overall ester yield (Figure 1A). Supply of more pyridine than anhydride 2 (0.5 mM) however did not give any effect in yield or kinetics as shown in Figure 1A. This observation supports the nucleophilic catalytic mechanism for pyridine, reacting directly with the anhydride and forming the reactive acetyl-pyridinium intermediate. Similarly, the ester degradation could be very precisely controlled with imidazole (Figure 1B). When imidazole is absent, the ester is stable for over \(120\) h (with a yield close to 100% after \(25\) min), whereas increasing the imidazole concentration leads to lower ester yields and shorter lifetimes, varying from 3 to \(120\) h. The amount of fuel provides another way to control the ester formation and lifetime. Logically, addition of more fuel results in higher ester yields and lifetimes as shown in Figure 1C. On top of that, multiple fuel cycles could be performed sequentially (Figure 1D). Fresh fuel was added for three consecutive times and the reaction cycle was completely repeatable and robust.
Figure 1: Fuel-driven esterification network controlled by organocatalysts pyridine and imidazole in MOPS buffer (100 mM, pH 7.5) with 5% acetonitrile. The conversion of p-nitrophenol(ate) 1 is monitored by UV-VIS, following the absorbance at 400 nm over time. (A). Pyridine variation: 0.1 mM p-nitrophenol 1, 0.5 mM acetic anhydride 2, 0.25 mM imidazole and varying 0-1.0 mM pyridine. (B). Imidazole variation: 0.1 mM p-nitrophenol 1, 0.5 mM acetic anhydride 2, 0.1 mM pyridine and varying 0-1.0 mM imidazole. (C). Acetic anhydride 2 variation: 0.1 mM p-nitrophenol 1, varying 0.25-0.75 mM acetic anhydride 2, 0.25 mM imidazole and 0.1 mM pyridine. (D). Three consecutive fuel cycles: 0.1 mM p-nitrophenol 1, 0.5 mM acetic anhydride 2, 0.25 mM imidazole and 0.1 mM pyridine. The second and third cycles were initiated by addition of a new batch of fuel 2.

HPLC-MS data confirmed the formation and degradation of 3 in the fuel cycle (SI Figures S9-13). Additionally, monitoring the colour change of the fuel cycle over time (SI Figure S14) proves the successful formation (yellow to colourless solution) and subsequent degradation of the ester (colourless to yellow solution), while the pH is stable (SI Figure S15). Nonetheless, the drawbacks of this system are the rapid hydrolysis of the acetic anhydride 2 fuel in water (hydrolysis rate constant 0.1575 min⁻¹ 3), resulting that after 30 min only the backward reaction remains. Also, the generation of twice as much acetic acid as waste limits the amount or number of fuel additions and calls for a strongly buffered system. Therefore, other activated carboxylic acids (i.e. vinyl acetate, isopropenyl acetate) were also tested. Yet, they were not efficient (> 500 fuel equivalents were required) and besides did not dissolve well in the water phase (SI Figure S8 and Table S1).

In order to understand the underlying reaction mechanisms and the role of the catalysts in the rate accelerations, a kinetic model was developed and numerically solved with Matlab software (see SI for full
explanation, reaction equations and mechanism). In this simplified model it was assumed that both pyridine and imidazole catalyse the ester formation, hydrolysis and anhydride hydrolysis (Figure 2A). The preference for imidazole for ester hydrolysis and pyridine for ester formation is expressed in the value of the rate constants. The imidazole catalysed ester hydrolysis rate constant was determined experimentally and all other constants were taken from the literature (both used as initial guesses) or optimized by the kinetic model with experimental data input. In Figure 2 (and SI Figures S38-40) the experimental data are compared with the modelled data. The model predicts the experimental data accurately for varying fuel (Figure 2B) and organocatalyst concentrations (Figure 2C). Also in extreme situations where only one of the catalysts is present, the model prediction with the simplified CRN matches with the experiments (Figure 2D). Only for high imidazole concentrations the model starts to deviate from the experimental data (Figure 2C and SI Figure S39F). We expect that this deviation is due to the presence of the acetyl-imidazole species, which is not consumed immediately, and hence the experimental data show a more gradual decay than the model. In this simplified model, it was assumed that the reactive intermediates are unstable and hence were not taken into account. Within these limits, transient nitrophenol acetylation is accurately described by the model, corroborating the proposed simplified catalytic CRN. Also, it allows prediction of ester yield and lifetime based on the catalyst input.

Figure 2: Experimental and model data comparison for varying organocatalysts and fuel concentrations, showing the conversion of p-nitrophenolate \(1\) (start: 0.1 mM) in MOPS buffer (100 mM, pH 7.5) with 5% acetonitrile: (A). Simplified chemical reaction network with pyridine and imidazole catalysing ester formation, hydrolysis and anhydride hydrolysis and blank reactions. (B).
0.25/0.5/0.75 mM acetic anhydride, 0.25 mM imidazole and 0.1 mM pyridine. (C). 0.5 mM acetic anhydride, 0.1/0.25/0.5 mM imidazole and 0.1 mM pyridine. (D). Extreme situations: 0.5 mM acetic anhydride, 0/0.25 mM imidazole and 0/0.1 mM pyridine. The presented experimental data are equal to Figure 1. Details of the kinetic model are provided in the SI.

**Fuel-driven responsive polymer system**

Having established a CRN in which the formation and degradation pathways can be controlled by two organocatalysts, we then applied this CRN to a macromolecular system. The importance of catalytic control in fuel-driven macromolecular systems is also witnessed in chromatin condensation, a process that regulates the accessibility of DNA for transcription. The DNA (un)binding to histone proteins due to fuel-driven transient (de)acetylation of lysines in the amino-terminal tails of histone proteins is regulated by two enzymes and changes the nucleosome superstructure. 

In analogy, with catalytically controlled fuel-driven acetylation of a polymer, we aim to control transient charge density and in that way polymer chain conformations, such as shape and hydrodynamic radius (Figure 3A). In our system, a nitrophenol amino acid analogue, 3-nitro-L-tyrosine, was grafted onto a 130 kDa poly(acrylic acid) (PAA) backbone via peptide coupling, giving PAANY (poly(acrylic acid) 3-nitro-L-tyrosine) with 25% NY coverage (see SI for synthetic details and characterization). PAA of relatively high molecular weight is known to have a shape transition from random coil to compact globule upon pH change due to (de)protonation of the acetate groups. Based on previous reports on transient polymer assembly, we hypothesized that such coil to globule shape transitions can be induced by transient acylation leading to a decrease of charge and reduced repulsion, and an increase in hydrophobic surface. Successful fuel cycles of the PAANY polymer with pyridine and imidazole catalysis were confirmed by UV-VIS experiments by following the absorbance of 3-nitro-L-tyrosine at 420 nm (Figure 3C). Next, the polymer size transition in a fuel cycle was investigated with dynamic light scattering (DLS) (Figure 3B,D). The size change in z-average diameter was monitored, which includes both single chain polymers (~10 nm) and polymer aggregates (~100 nm) (Figure 3B and SI Figure S20-21). The aggregate formation is most likely caused by borate interacting with carboxylate groups on the PAA backbone and carboxylate and phenolate anions on the NY functionalities. Boric acid or borate anions are known to form polymer hybrid structures due to hydrogen bonding. At neutral pH boric acid can even react with carboxylate groups to form a boric anhydride crosslink. The aggregate formation in borate is not specific to functionalized PAA, because it is also observed for unmodified PAA in borate buffer (SI Figure S19).
Figure 3: Fuel-driven responsive polymer system controlled by organocatalysts pyridine and imidazole in borate buffer (200 mM, pH 8.0). (A) Schematic overview of transient acetylation of PAANY (poly(acrylic acid) 3-nitro-L-tyrosine) to PAANY-acyl by acetic anhydride (left) and the random coil to compact globule transition (top right). (B) DLS size distributions by intensity over time for 0.30 mM PAANY (0.24 mg/mL), 6 mM acetic anhydride 2, 0.75 mM imidazole and 0.30 mM pyridine. (C) The conversion of PAANY is monitored by UV-VIS, following the absorbance at 420 nm over time. Conditions: 0.15 mM PAANY (0.12 mg/mL), 3 mM acetic anhydride 2, 0-0.375 mM imidazole and 0-0.15 mM pyridine. (D) Polymer size change monitored over time based on z-average size (nm) measured with DLS. (E) Intensity ratio of single polymer chain vs aggregate as a function of time calculated from the peak areas of the unimer and the aggregate. Conditions: 0.30 mM PAANY (0.24 mg/mL), 6 mM acetic anhydride 2, 0-0.75 mM imidazole and 0-0.30 mM pyridine. See SI Figures S20-21 for corresponding DLS size distributions.

With pyridine present, upon addition of fuel 2 the hydrodynamic radius quickly reduces by more than 50% after which it slowly comes back to almost the original size (Figure 3D and S20-21). We expect that the radius does not fully come back to the original value due to a slight drop in pH (from 8.0 to 7.7). In detail, the z-average diameter starts at 49 nm, goes to 22 nm (at t = 2 min) and back to 44 nm after 2 h, while the number average goes from 8.6 ± 2.6 nm to 5.9 ± 1.6 nm (at t = 2 min) back to 7.4 ± 2.0 nm. Concomitantly, the ratio between the two peaks (peak area) in the DLS size intensity distribution changes (Figure 3B and SI Figures S20A,B). At the start the aggregate peak around 100 nm is more prevalent than the single polymer chain (unimer) peak around 10 nm, whereas after fuel addition (at t = 2 min) the unimer
peak has a larger contribution, which changes again at the end of the fuel cycle. Based on this finding, it becomes clear that the fuel-driven acetylation changes the portion of polymers present as single chains (unimers) and in an aggregated form. The unimer to aggregate ratio as a function of time based on the peak areas is depicted in Figure 3E. Similar to the change in the z-average diameter over time, we can identify a transient shift towards the unimer upon fuel addition and acetylation in the presence of pyridine. Hence, the transient acetylation shifts the unimeraggregate equilibrium and after 2 h the equilibrium is restored again. This equilibrium shift could indicate that the non-equilibrium compact polymer structure (when acetylated) has a preference for the unimer over the aggregate, whereas the equilibrium random coil structure is rather in the aggregated form. However, when only imidazole or no catalysts (blank reaction) are present, no significant size change and no aggregate to unimer shift is observed (Figure 3D, E and SI Figures S20-21C,D). By comparing the DLS size data with the UV-Vis conversion (Figure 3C) for the measurements with imidazole and the blank it becomes clear that the compact polymer structure expands to a coil after 2 h, with about 70% of conversion as a threshold value, meaning about 60% of phenolate (negative charge) needs to be acetylated to induce a polymer conformational change. Based on this threshold criterion and by looking at the UV-VIS conversion result (Figure 3C), the polymer sample with only pyridine should collapse after 16 h, but in Figure 3D, we observe a faster decay. We hypothesize the behaviour of the polymer chain in solution is more complex and the conversion kinetics and the polymer shape transition have a non-linear relationship. Similar to the small molecule CRN, consecutive fuel cycles could be performed with PAANY by addition of a new batch of fuel (Figure 4).

Figure 4: Two consecutive fuel cycles for the fuel-driven responsive polymer system controlled by organocatalysts pyridine and imidazole in borate buffer (300 mM, pH 8.0): (A). Polymer size change monitored over time based on z-average size (nm) measured with DLS. (B). DLS size distributions by intensity over time. Conditions: 0.30 mM PAANY (0.24 mg/mL), 6/12 mM acetic anhydride 2, 0-0.75 mM imidazole and 0-0.30 mM pyridine. The second cycle was initiated by addition of fresh fuel 2.

The z-average diameter change (Figure 4A) and the size distributions (Figure 4B) show similar trends as with the single fuel cycle, having a sharp size change directly after fuel addition and shift in the intensity distribution to ~10 nm. Because of the addition of more anhydride and hence more acid waste production, the pH drops from 8.0 to 7.5, even though a buffer of 300 mM was used to mitigate this pH drop. Hence, the final size of the polymer is 34 nm and does not come back to the original value (47 nm). Moreover, the true hydrodynamic radius of the single polymer chain was confirmed by DOSY NMR diffusion coefficient measurements 40 using the Stokes Einstein relation, giving similar sizes in the nanometre range.
(~8 nm) as with DLS (SI Figure S23) and in good agreement with earlier literature examples on PAA polymers \(^{41-43}\). The polymer in borate buffer was also imaged with cryo-EM, revealing globular structures with an average diameter of 9.7 ± 1.6 nm along with larger clusters with an average largest dimension of 23.9 ± 7.1 nm (Figure 5 and S41). The 10 nm structures are in line with the DLS and DOSY single polymer diameter, yet the diameter of the polymer aggregates in cryo-EM appears somewhat lower than the 100 nm observed with DLS. There, it might be that the polymer aggregate diameter from DLS is overestimated, as DLS measures fractal dimensions.

**Figure 5**: Cryo-EM measurement of PAANY in borate buffer (200 mM, pH 8.0): (A). Representative cryo-EM image of PAANY, where the dark blue circles represent spherical polymer structures and the light blue circles the polymer clusters. (B). Histograms of PAANY unimers (top) with average diameter of 9.7 ± 1.6 nm and PAANY aggregates (bottom) with average diameter of 23.9 ± 7.1 nm, both calculated from multiple cryo-EM images.

Altogether, transient polymer conformational and aggregation changes of PAANY were achieved with this fuel-driven esterification CRN for which the presence of the pyridine catalyst turned out to be critical. Yet, the exact behaviour of the functionalized polyelectrolyte in response to organocatalytic action is more complex than anticipated.

**Conclusions**

In this work, we have shown how two organocatalysts can be incorporated in a fuel-driven esterification CRN and individually regulate product yield and lifetime. In this CRN, p-nitrophenol(ate) is acetylated with acetic anhydride as a chemical fuel, and pyridine and imidazole are used as organocatalysts to accelerate the forward and backward reaction, respectively. Variation of the chemical fuel and organocatalysts concentrations enabled full control over ester yield and lifetime. A kinetic model was developed, which corroborated the experimental results. As a proof of principle, the same organocatalytic control was applied on an amino acid functionalized polymer system, leading to control over polymer chain conformation in time. Overall, we have demonstrated that organocatalysis is a powerful tool to regulate reaction kinetics of a non-equilibrium CRN and with that temporally control material properties by inducing a change in a macromolecular superstructure, reminiscent of living systems.
Associated content

Supporting information

The Supporting Information is available free of charge at DOI:
Experimental details, supplemental figures referred to in the main text obtained with different techniques UV-VIS, HPLC-MS, NMR, FTIR, DLS, pH monitoring and the description and fitting procedure of the kinetic model in Matlab (PDF)

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Notes

The authors declare no competing financial interest.

Author Contributions

M.P.v.d.H and C-L.W. carried out the experiments and analyzed the results. M.M. assisted and advised with the synthetic procedures. E.M. provided suggestions on experiments, analysis and improvements. M.P.v.d.H and R.E. designed the experiments. M.P.v.d.H. wrote the manuscript. R.E. conceived and directed the overall research project and revised the manuscript. All authors commented on the work and the manuscript.

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TOC graphic
Supporting information

Organocatalytic control over a fuel-driven transient esterification network

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Experimental details

1.1 General materials and methods

Chemicals were purchased in the highest available purity and used without further purification unless reported otherwise. \( p \)-Nitrophenol 1, acetic anhydride 2, pyridine anhydrous, imidazole, isopropenyl acetate, vinyl acetate, poly(acrylic acid) (PAA \( \sim 130 \) kDa), \( N,N \)-dimethyl formamide (DMF) anhydrous, dimethyl sulfoxide (DMSO), \( N \)-(3-Dimethylaminopropyl)-\( N' \)-ethylcarbodiimide hydrochloride (EDC), acetonitrile, \( N,N \)-diisopropylethylamine (DIPEA), were purchased from Sigma-Aldrich. \( p \)-Nitrophenyl acetate 3a was from TCI Europe. 3-nitro-L-tyrosine ethyl ester (NY-ethyl ester) was from Chem Impex International. 1-Hydroxybenzotriazole hydrate (HOBT) was from Acros Organics. Solid salts were used for the preparation of aqueous buffers: sodium tetraborate decahydrate (Borax), boric acid, sodium hydroxide were from Sigma Aldrich and 3-(N-Morpholino)propanesulfonic acid (MOPS) from Alfa Aesar. Unless stated otherwise, all preparations and analyses were performed at room temperature (RT) (~21 °C) and atmospheric pressure. Nuclear Magnetic Resonance (NMR) experiments were performed using Agilent-400 MR DD2 (400 MHz for \( ^1 \)H and 100.5 MHz for \( ^{13} \)C) at 25 °C using residual deuterated solvent signals as internal standard. To suppress the water peak, PRESAT configuration (suppress one highest peak) was used. Prior to every diffusion-ordered spectroscopy (DOSY) NMR measurement, scouting experiments were performed to obtain the longest t1 relaxation and get an optimal diffusion gradient and delay. DOSY spectra were analysed with MestReNova software. UV-Vis spectroscopic experiments were carried out using Analytik Jena Specord 250 spectrophotometer; quartz cuvette with a 1 cm path length, volume of 3 mL, at RT. Liquid Chromatography–Mass Spectrometry (LC–MS) was performed on a Shimadzu Liquid Chromatograph Mass Spectrometer 2010, LC-8A pump with a diode array detector SPD-M20. Negative and/or positive mode Electro Spray Ionization Mass Spectrometry (ESI-MS) was used for the peak assignment. Fourier Transform Infrared Spectroscopy (FTIR) spectroscopy was performed with NicoletTM 6700 FT-IR Spectrometer from Thermo Electron Corporation equipped with OMNIC Software using the ATR method. Spectra were recorded at wavenumber range 4000-4000 cm\(^{-1}\) with 4 cm\(^{-1}\) resolution. Prior to each experiment a background of the ZnSe crystal was measured. Dynamic light scattering (DLS) measurements were recorded with a Malvern Zetasizer Nano equipped with a 633 nm laser, collecting the optical data with a 90° scattering angle; quartz cuvette with 1 cm path length, volume of 3 mL, at 20 °C. Cryo-EM (electron cryo-microscopy) images were obtained with a JEOL JEM3200-FSC, operated at 300kV with Gatan camera K2-Summit operated in counting mode with 10s exposure time, dose fractionated 0.2s aligned by SerialEM. Zero-Loss filtered with a 20eV slit. Samples were prepared with a Leica plunger EM GP, 21 °C, 98% RH, 8 seconds blotting time. The final images were analysed with ImageJ software.

1.2 Polymer functionalization: 3-nitro-L-tyrosine on poly(acrylic acid) PAANY

![Polymer functionalization diagram](image)

Poly(acrylic acid) PAA (\( M_n \), 130-230 kDa (reported by Sigma-Aldrich), \( D = 2.3 \) (GPC in water)) was functionalized with 3-nitro-L-tyrosine (NY)-ethyl ester via peptide coupling with EDC/HOBT and DIPEA in DMF. For 25% coverage: PAA (50 mg) was mixed with NY-ethyl ester (50 mg, 0.25 molar eq.), EDC (50 mg, 0.5 molar eq.), HOBT (102.3 mg, 0.625 molar eq.) and DIPEA (138 µL, 1.125 molar eq.) in DMF (15 mL). The reaction mixture was stirred for 24 h at RT. Afterwards, the mixture was concentrated under reduced pressure and the final product precipitated in a
DMSO/ water mixture, giving PAANY-ethyl ester (yield: 79%). To obtain the hydrolysed polymer PAANY, stock solutions were left overnight in the respective buffer solution and the hydrolysis was confirmed by $^1$H NMR (Figure S18). $^1$H NMR (400 MHz, DMF): δ = 12.61 (s, 1H, -OH), 10.93 (s, 1H, -COOH), 7.88 (s, 1H, -ArH-), 7.57 (s, 1H, -ArH-), 7.17 (s, 1H, -ArH), 4.64 (s, 1H, -CHα-), 4.11 (s, 2H, -CH2-), 3.12 (s, 2H, -CHβ-), 2.00-1.50 (PAA backbone), 1.17 (s, 3H, -CH3). N.B. the multiplicity of 3-nitro-l-tyrosine in the NMR spectrum is lost because the peaks are too broad on the polymer (appearance as broad singlets). After hydrolysis the peaks of the ethyl ester at 4.11 and 1.17 ppm have disappeared. FTIR (ATR, cm$^{-1}$): ν 1730 (C=O stretch acid backbone), 1680 (C=O stretch amide), 1350 (OH bend phenol), 1050 (C-O-C stretch ester).

1.3 UV-Vis assay

Stock solutions were prepared in MOPS buffer (pH 7.5, 100 mM), borate buffer (pH 8.0, 200 mM) or acetonitrile (for acetic anhydride 2 only – to avoid significant hydrolysis in the stock solution). Unless stated otherwise, the fuel cycle was performed with 0.1 mM p-nitrophenol 1, 0.5 mM acetic anhydride 2, 0-1 mM of pyridine, and 0-1 mM of imidazole in MOPS buffer (pH 7.5, 100 mM), in quartz cuvettes, path length of 1 cm (total reaction volume of 3 mL) at RT. The stock solutions of the reactants were always added in the following order: p-nitrophenol 1, pyridine, imidazole and acetic anhydride 2. Teflon caps were used to close the cuvette. The cuvette was turned upside down to mix the solution. The reactant peak was followed using slow time scan, measuring wavelength 400 nm. The pH was measured before and after the reaction. The conversion was calculated with the extinction coefficients (see SI for calibration lines) and Lambert-Beer law:

$$A = εIC$$

where $A$ is the absorbance, $ε$ the extinction coefficient, $l$ the path length of the cuvette and $C$ the concentration. The experiments with PAANY were performed similarly, only following the decrease/increase at 420 nm from NY. Because we anticipated problems with concentrated sulfonate buffers with macromolecules (such as precipitation), we used borate buffer (pH 8.0, 200 mM). Experiments were performed at pH 8.0 to have a higher percentage of phenolate (negative charge) compared to phenol and co-solvent was avoided, since it could affect the polyelectrolyte behaviour in solution.

1.4 DLS measurements

Stock solutions were prepared in borate buffer (pH 8.0, 200 mM) and filtered with syringe filters (0.2 µm) before use. Unless stated otherwise, the fuel cycle was performed with 0.30 mM PAANY (0.24 mg/mL), 6 mM acetic anhydride 2, 0-30 mM of pyridine, and 0-0.75 mM of imidazole in borate buffer (pH 8.0, 200 mM), in quartz cuvettes, 1 cm path length (3 mL reaction volume) at 20 °C. The stock solutions of the reactants were always added in the following order: PAANY, pyridine, imidazole and acetic anhydride 2. Cuvettes were closed with Teflon caps and turned upside down to mix the solution. A fuel cycle was measured in continuous mode with 1000 measurements consisting of 11 runs each 3 min. An equilibration time of 2 min was applied for each measurement. Calculated size changes (%) are based on z-average diameters (nm). A DLS size calibration was performed to find the polymer concentration with minimal size fluctuation (see SI: DLS size calibration). The pH was always measured before and after the reaction.
1.5 Kinetic modelling

A kinetic model for the esterification CRN was written in Matlab 2018b. The rate constant of imidazole catalysed ester hydrolysis was determined by varying the concentration of imidazole in the hydrolysis of p-nitrophenyl acetate 3 with UV-VIS, following the appearance of the hydrolysis product 1 at 400 nm. The other rate constants were taken from the literature or obtained from the Matlab model fitting (see SI: Kinetic model for fuel-driven esterification CRN).

2 UV-VIS spectroscopy

2.1 Calibration lines

![Absorbance vs Wavelength](image1)

Figure S1: Extinction coefficient for p-nitrophenol (1) in MOPS buffer (100 mM, pH 7.5) at 400 nm: 16.74 mM⁻¹cm⁻¹. (A). UV-Vis absorbance spectra of p-nitrophenol (1) at different concentrations. (B). Absorbance at 400 nm of p-nitrophenol (1) at different concentrations.

![Absorbance vs Concentration](image2)

![Absorbance vs Wavelength](image3)

Figure S2: Extinction coefficient for 3-nitro-L-tyrosine (NY) in Borate buffer (200 mM, pH 8.0) at 420 nm: 5.03 mM⁻¹cm⁻¹. (A). UV-Vis absorbance spectra of 3-nitro-L-tyrosine (NY) at different concentrations. (B). Absorbance at 420 nm of 3-nitro-L-tyrosine (NY) at different concentrations.
2.2 Absorbance plots

Figure S3: Absorbance vs wavelength for fuel-driven esterification network controlled by organocatalysts pyridine and imidazole in MOPS buffer (100 mM, pH 7.5) with 5% acetonitrile. Monitoring the conversion of p-nitrophenol(ate): 0.1 mM \( p \)-nitrophenol 1, 0.5 mM acetic anhydride 2 (stock in acetonitrile), 0.25 mM imidazole and 0.1 mM pyridine. (A) First 10 min of reaction, showing the decrease in 400 nm and increase for 270 nm – from dark to light blue (N.B. the first sample was measured after 10-30 s, hence part of the phenolate was already consumed). (B). From 10 min to 16.5 h, showing the increase in 400 nm and decrease for 270 nm - from dark to light blue. The isobestic point for this reaction is located at 305 nm. N.B Acetyl-imidazole disappearance is shown at 245 nm and acetyl-pyridinium at 272 nm \(^1\). \(^2\).

Figure S4: Absorbance vs wavelength for fuel-driven responsive polymer system controlled by organocatalysts pyridine and imidazole in borate buffer (200 mM, pH 8.0). Monitoring the conversion of PAANY by UV-VIS: 0.15 mM PAANY (0.12 mg/mL), 1.5 mM acetic anhydride 2, 0.375 mM imidazole and 0.15 mM pyridine. (A) First 5 min of reaction, showing the decrease in 420 nm (N.B. the first sample was measured after 10-30 s, hence part of the phenolate was already consumed). (B). From 5 min to 3 h, showing the increase in 420 nm.
3 Fuel cycle error analysis

![Figure S5](image)

Figure S5: Error analysis of fuel-driven esterification network controlled by organocatalysts pyridine and imidazole in MOPS buffer (100 mM, pH 7.5) with 5% acetonitrile: (A). Monitoring the conversion of $p$-nitrophenolate: 0.1 mM $p$-nitrophenol 1, 0.5 mM acetic anhydride 2 (stock in acetonitrile), 0.1 mM pyridine and 0.1 mM imidazole, given in triplicate with average and the average standard deviation is 2.8% (n=3). (B). Monitoring the conversion of $p$-nitrophenolate: 0.1 mM $p$-nitrophenol 1, 0.5 mM acetic anhydride 2 (stock in acetonitrile), 0.1 mM pyridine and 0.2 mM imidazole, given in quadruplicate with average and the average standard deviation is 10.5% (n=4). (C). Monitoring the conversion of $p$-nitrophenolate: 0.1 mM $p$-nitrophenol 1, 0.5 mM acetic anhydride 2 (stock in acetonitrile), 0.1 mM pyridine and 0.3 mM imidazole, given in triplicate with average and the average standard deviation is 6.5% (n=3).
Figure S6: Three consecutive fuel cycles: 0.1 mM $p$-nitrophenol 1, 0.5 mM acetic anhydride 2 (stock in acetonitrile), 0.25 mM imidazole and 0.1 mM pyridine. The second and third cycles were initiated by addition of a new batch of fuel 2.

4 Blank reaction without catalysts

Figure S7: Blank reaction for fuel-driven esterification network in MOPS buffer (100 mM, pH 7.5) with 5% acetonitrile. Monitoring the conversion of $p$-nitrophenol(ate): 0.1 mM $p$-nitrophenol 1 with 0.5 mM acetic anhydride 2 (stock in acetonitrile).
5 Different acyl donors

Figure S8: Fuel-driven esterification network with varying concentration of organocatalysts, monitoring the conversion of p-nitrophenol(ate): (A). 450-900 molar eq. isopropenyl acetate. (B). 1000 molar eq. vinyl acetate. 0.1 mM p-nitrophenol 1 in MOPS buffer (100 mM, pH 7.5), pyridine (0.1-1 mM) and imidazole (0.1-0.25 mM).

Table S1: Overview of tested acyl donors as fuels for esterification of p-nitrophenol(ate) (1).

| Fuel                | Structure         | Waste product(s) | UV-VIS            | Remarks                                                                 |
|---------------------|-------------------|------------------|-------------------|-------------------------------------------------------------------------|
| Acetic anhydride    | ![Acetic anhydride](image) | 2 OOH            | 5 molar eq. of fuel to drive the esterification | Acetic anhydride completely hydrolysed after 30 minutes. Then only backward reactions remain. |
| Isopropenyl acetate | ![Isopropenyl acetate](image) | 450-900 molar eq. of fuel (45-90 mM) needed to drive the reaction | - Heterogeneous mixture (fuel hardly dissolves in water and creates little bubbles). - Due to high concentration of fuel pH drops to 7.46 at the end of the reaction. |
| Vinyl acetate       | ![Vinyl acetate](image) | 1000 molar eq. of fuel (100 mM) not sufficient | | |
Figure S9: HPLC-MS: Fuel-driven esterification network controlled by organocatalysts pyridine and imidazole in MOPS buffer (100 mM, pH 7.5) with 5% acetonitrile. Conditions: 0.1 mM \textit{p}-nitrophenol 1, 0.5 mM acetic anhydride 2 (stock in acetonitrile), 0.1 mM imidazole and 0.1 mM pyridine. Sample was measured 10 min after addition of all compounds. \textit{p}-Nitrophenylacetate elutes at \textasciitilde2.05 min, shows an absorbance maximum \textasciitilde270 nm and 138 as m/z value (\textit{p}-nitrophenyl esters give base peaks of the corresponding phenolate \textsuperscript{3+4}).
Figure S10: HPLC-MS: Fuel-driven esterification network controlled by organocatalysts pyridine and imidazole in MOPS buffer (100 mM, pH 7.5) with 5% acetonitrile. Conditions: 0.1 mM p-nitrophenol 1, 0.5 mM acetic anhydride 2 (stock in acetonitrile), 0.1 mM imidazole and 0.1 mM pyridine. Sample was measured 18h after addition of all compounds. p-Nitrophenol(ate) elutes at ~1.3 min, shows an absorbance maximum ~400 nm and 138 as m/z value.
Figure S11: HPLC-MS: Blank reaction for fuel-driven esterification network in MOPS buffer (100 mM, pH 7.5) with 5% acetonitrile. Conditions: 0.1 mM p-nitrophenol and 0.5 mM acetic anhydride (stock in acetonitrile). Sample was measured 25 min after addition of all compounds. p-Nitrophenylacetate elutes at ~2.05 min, shows an absorbance maximum ~270 nm and 138 as m/z value (p-nitrophenyl esters give base peaks of the corresponding phenolate $^3$-4).
Figure S12: HPLC-MS: \(p\)-Nitrophenylacetate reference compound in MOPS buffer (100 mM, pH 7.5) with 5% acetonitrile elutes at \(~2.05\) min, shows an absorbance maximum \(~270\) nm and \(138\) as m/z value (\(p\)-nitrophenyl esters give base peaks of the corresponding phenolate \(^3\)\(^-\)\(^4\)).
Figure S13: HPLC-MS: p-Nitrophenol(ate) reference compound in MOPS buffer (100 mM, pH 7.5) elutes at ~1.3 min, shows an absorbance maximum ~400 nm and 138 as m/z value.
Monitoring esterification network by color progress
Figure S14: Fuel-driven esterification network controlled by organocatalysts pyridine and imidazole in MOPS buffer (100 mM, pH 7.5) with 5% acetonitrile, monitoring reaction by colour progress; yellow to transparent and back to yellow again (Conditions: 0.1 mM p-nitrophenol 1, 0.5 mM acetic anhydride 2 (stock in acetonitrile), 0.1 mM imidazole and 0.1 mM pyridine).
8  pH monitoring of fuel-driven esterification network

Figure S15: pH monitoring of reaction with organocatalysts, blank reaction and anhydride stock in MOPS buffer pH 7.5 100 mM.

9  FTIR PAANY

Figure S16: FTIR spectra comparison between PAANY-ethyl ester, PAA, NY-ethyl ester and NY. The following peaks can be identified: C=O stretching mode 1730 cm\(^{-1}\) from the ester (in PAANY-ethyl ester and NY-ethyl ester), C=O stretching mode 1700 cm\(^{-1}\) from the acid backbone (in PAANY-ethyl ester and PAA), C=O stretching mode 1680 cm\(^{-1}\) from the amide bond (in PAANY-ethyl ester, NY-ethyl ester and NY), O-H bending mode 1350 cm\(^{-1}\) from the phenol (in PAANY-ethyl ester, NY-ethyl ester and NY) and the C-O-C stretching mode 1050 cm\(^{-1}\) from the ester (in PAANY-ethyl ester and NY-ethyl ester).
Figure S17: $^1$H NMR PAANY-ethyl ester in DMF-d7.

Figure S18: $^1$H NMR PAANY-ethyl ester hydrolysis in borate buffer (200 mM, pH 8.0), showing the appearance of sharp ethanol peaks around 0.95 ppm (triplet) and 3.35 ppm (quartet) highlighted in the blue frames and the disappearance of the broader polymer ethyl ester peaks (0.8 ppm and 3.8 ppm) highlighted in the green frames.
11 DLS size distributions

Figure S19: (A) DLS size distribution by intensity of unmodified PAA (0.24 mg/mL) in borate buffer (200 mM, pH 8.0), showing two peaks: a small unimer and larger aggregate peak. (B) DLS size distribution by intensity of blank reaction with unmodified PAA in borate buffer (200 mM, pH 8.0), showing no size change over time. Conditions: PAA (0.24 mg/mL), 6 mM acetic anhydride, 0.75 mM imidazole and 0.30 mM pyridine.

Figure S20: DLS size distributions by intensity, showing the change in size for PAANY in borate buffer (200 mM, pH 8.0). (A). 0.30 mM PAANY (0.24 mg/mL), 6 mM acetic anhydride, 0.75 mM imidazole and 0.30 mM pyridine. (B). 0.30 mM PAANY (0.24 mg/mL), 6 mM acetic anhydride and 0.30 mM pyridine. (C). 0.30 mM PAANY (0.24 mg/mL) and 6 mM acetic anhydride. (D). 0.30 mM PAANY (0.24 mg/mL), 6 mM acetic anhydride and 0.75 mM imidazole.
Figure S21: DLS size distributions by number, showing the change in size for PAANY (~10 nm) in borate buffer (200 mM, pH 8.0). (A) 0.30 mM PAANY (0.24 mg/mL), 6 mM acetic anhydride 2, 0.75 mM imidazole and 0.30 mM pyridine. (B) 0.30 mM PAANY (0.24 mg/mL), 6 mM acetic anhydride 2 and 0.30 mM pyridine. (C) 0.30 mM PAANY (0.24 mg/mL) and 6 mM acetic anhydride 2. (D) 0.30 mM PAANY (0.24 mg/mL), 6 mM acetic anhydride 2 and 0.75 mM imidazole.

12 DLS size calibration

Figure S22: DLS size calibration for various PAANY concentrations in borate buffer (200 mM, pH 8.0). With higher polymer concentrations the size fluctuations become smaller. The reported diameter is the z-average diameter, which takes into account PAANY (~10 nm) and buffer/polymer aggregates (~100 nm).
13 DOSY diffusion coefficient and size calculation

By measuring the DOSY spectrum of the polymer with NMR and extracting the diffusion coefficient, we can confirm the particle size via the Stokes-Einstein equation:

\[ D = \frac{k_B T}{3\pi \mu d} \]

, where \( D \) is the diffusion coefficient, \( k_B \) the Boltzmann constant, \( T \) the temperature, \( \mu \) the viscosity of the bulk medium and \( d \) the solute diameter (polymer).

Figure S23: DOSY NMR spectrum of PAANY (0.5 mM – 0.4 mg/mL) in borate buffer (pH 8.0, 200 mM).
14 Kinetic model for fuel-driven esterification CRN

In this section the development of the numerical model for the reaction kinetics of the esterification CRN written in Matlab 2018b is discussed. First we deal with the kinetics of the blank reaction (uncatalysed), then the pyridine catalysis, followed by imidazole catalysis and eventually all individual reaction schemes are combined to model the entire fuel cycle (Scheme S1) with varying catalysts and fuel concentrations. In all cases, first an overview of the reaction pathway is given, followed by the rate equations (system of ODEs) and the experimental data fitting, showing the concentration profiles of the different species over time for the modelled and experimental data. We end with a note on how the model was optimized for the various experimental conditions.

Scheme S1: Full reaction pathway overview, including organocatalysed and blank (uncatalysed) reactions. Both pyridine and imidazole catalyse the ester formation, hydrolysis and anhydride hydrolysis.
14.1 Blank reaction

\[ \ce{O=O} + \ce{\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot\cdot}\]
Experimental data fitting

Using $k_0 \ 0.0001 \ \text{min}^{-1}$ (blank hydrolysis – determined experimentally), the literature value for $k_3 \ 0.1575 \ \text{min}^{-1}$ for the hydrolysis of acetic anhydride 2 and $k_1 \ 0.25 \ \text{mM}^{-1}\text{min}^{-1}$ as initial guesses. $k_1$ was determined by fitting the data of the blank reaction, giving $k_1 \ 0.3750 \ \text{mM}^{-1}\text{min}^{-1}$, $k_3 \ 0.2219 \ \text{min}^{-1}$ and $k_0 \ 0.0001 \ \text{min}^{-1}$.

Figure S24: Concentration profiles of different species (phenolate 1, ester 3, acetic anhydride 2 and acetic acid 4) over time for the blank reaction (uncatalysed): experimental data (dots) and model (lines) with new $k$-values $k_1 \ 0.375 \ \text{mM}^{-1}\text{min}^{-1}$, $k_3 \ 0.2219 \ \text{min}^{-1}$ and $k_0 \ 0.0001 \ \text{min}^{-1}$. 
14.2 Pyridine catalysis and blank reaction

Next, when only the blank reaction takes place and the pyridine catalysis, the following equations apply:

Formation and degradation of the ester (3)

\[
\frac{d[3]}{dt} = k_1[1][2] + k_{cat2}[Py][1][2] - k_0[3] - k_{cat5}[Py][3]
\]

Formation of the acid (4)

\[
\frac{d[4]}{dt} = k_1[1][2] + k_{cat2}[Py][1][2] + 2k_3[2] + k_0[3] + k_{cat5}[Py][3] + 2k_{cat6}[Py][2]
\]

Formation and degradation of the phenolate (1)

\[-\frac{d[1]}{dt} = k_1[1][2] + k_{cat2}[Py][1][2] - k_0[3] - k_{cat5}[Py][3]\]
Degradation of the anhydride (2)

\[- \frac{d[2]}{dt} = k_1[1][2] + k_{cat2}[Py][2] + k_5[2] + k_{cat6}[Py][2] \]

Experimental data fitting

The previous k-values from the blank reaction were again used to determine \( k_{cat2} \) 35.0 mM\(^{-2}\)min\(^{-1} \), \( k_{cat5} \) 0.002 mM\(^{-1}\)min\(^{-1} \) and \( k_{cat6} \) 1.525 mM\(^{-1}\)min\(^{-1} \).

Figure S25: Concentration profiles of different species (phenolate 1, ester 3, acetic anhydride 2 and acetic acid 4) over time for the pyridine (0.1 mM) catalysed ester formation: Experimental data (dots) and model (line) with new k-values \( k_{cat2} \) 35.0 mM\(^{-2}\)min\(^{-1} \), \( k_{cat5} \) 0.002 mM\(^{-1}\)min\(^{-1} \) and \( k_{cat6} \) 1.525 mM\(^{-1}\)min\(^{-1} \).
14.3 Imidazole catalysis and blank reaction

When only the blank reaction takes place and the imidazole catalysis, the following equations apply:

**Formation and degradation of the ester (3)**

\[
\frac{d[3]}{dt} = k_1 [1][2] + k_{cat3}[Im][1][2] - k_0 [3] - k_{cat1}[Im][3]
\]

**Formation of the acid (4)**

\[
\frac{d[4]}{dt} = k_1 [1][2] + k_{cat3}[Im][1][2] + 2k_3 [2] + k_0 [3] + k_{cat1}[Im][3] + 2k_{cat4}[Im][2]
\]

**Formation and degradation of the phenolate (1)**
Degradation of the anhydride (2)

\[- \frac{d[1]}{dt} = k_1[1][2] + k_{\text{cat}3}[Im][1][2] - k_0[3] - k_{\text{cat}1}[Im][3]\]

\[- \frac{d[2]}{dt} = k_1[1][2] + k_{\text{cat}3}[Im][1][2] + k_3[2] + k_{\text{cat}4}[Im][2]\]

Experimental data fitting

$k_{\text{cat1}}$ and $k_0$ were determined experimentally ($k_{\text{cat1}} 0.475 \text{ M}^{-1}\text{s}^{-1}$ and $k_0 -4.0\cdot10^{-5} \text{ s}^{-1}$) (Table S2 comparison with literature). Yet, the k-values were optimized with Matlab to fit the experimental data better (N.B. data for 5 mM imidazole were used as experimental values). Lower and upper bounds for the k-values were opposed with the fmincon constrained optimization function using a least squared cost function. The new k-values are: $k_{\text{cat1}} 0.0254 \text{ mM}^{-1}\text{min}^{-1} (0.423 \text{ M}^{-1}\text{s}^{-1})$ and $k_0 0.0001 \text{ min}^{-1} (1.67\cdot10^{-6} \text{ s}^{-1})$.

Table S2: Rate constants comparison against literature values.

|                  | This work     | Bruice$^6$    | Bruice$^6$    | Lombardo$^7$ |
|------------------|---------------|---------------|---------------|--------------|
| $k_{\text{cat1}} (\text{M}^{-1}\text{s}^{-1})$ | 0.475 ± 0.0034 | 0.211 ± 0.0035 | 0.328 ± 0.0152 | 0.130        |
| $k_0 (\text{s}^{-1})$     | $-4.0\cdot10^{-5}$ | 9.0 ·10$^{-6}$ | 7.33 ·10$^{-5}$ | 4.35 ·10$^{-5}$ |
| Buffer            | MOPS pH 7.5   | Phosphate pH 7.9-8.0 | Phosphate pH 7.9-8.0 | Imidazole in 200 mM |
|                   | 100 mM        | 5.4 mM        | 200 mM        | 0.1 M KCl    |
| Temperature       | 21 °C (294 K) | 25 °C (298 K) | 30 °C (298 K) | 25 °C (298 K) |
| Method            | Abs 400 nm    | Abs 400 nm    | Abs 400 nm    | Abs 400 nm   |
| Remarks           | Corrected for imidazole free base species | Corrected for imidazole free base species | Corrected for imidazole free base species | Pure imidazole buffers used |
Figure S26: Optimization of k-values with fmincon: (A). Experimental data (dots) and model (line) with experimentally obtained k-values ($k_{\text{cat}} 0.475 \text{ M}^{-1}\text{s}^{-1}$ and $k_0 -4.0 \times 10^{-5} \text{ s}^{-1}$). (B). Experimental data (dots) and model (line) with new k-values $k_{\text{cat}} 0.0254 \text{ mM}^{-1}\text{min}^{-1}$ and $k_0 0.00010 \text{ min}^{-1}$.

Then, the previous k-values were again used to determine $k_{\text{cat}3} 0.124 \text{ mM}^2\text{min}^{-1}$ and $k_{\text{cat}4} 0.0565 \text{ mM}^{-1}\text{min}^{-1}$.

Figure S27: Concentration profiles of different species (phenolate 1, ester 3, acetic anhydride 2 and acetic acid 4) over time for the imidazole (0.25 mM) catalysed ester formation: Experimental data (dots) and model (line) with new k-values $k_{\text{cat}3} 0.124 \text{ mM}^2\text{min}^{-1}$ and $k_{\text{cat}4} 0.0565 \text{ mM}^{-1}\text{min}^{-1}$.
14.4 Pyridine and imidazole catalysed reaction cycle

Rate equations

When the blank reaction, pyridine and imidazole catalysis take place the following equations apply:

Formation and degradation of the ester (3)

\[
\frac{d[3]}{dt} = k_1[1][2] + k_{cat2}[Py][1][2] - k_0[3] - k_{cat5}[Py][3] + k_{cat3}[Im][1][2] - k_{cat1}[1][3]
\]

Formation of the acid (4)

\[
\frac{d[4]}{dt} = k_1[1][2] + k_{cat2}[Py][1][2] + 2k_3[2] + k_0[3] + k_{cat5}[Py][3] + 2k_{cat6}[Py][2] + k_{cat1}[Im][3] + k_{cat3}[Im][1][2] + 2k_{cat4}[Im][2]
\]

Formation and degradation of the phenolate (1)

\[
-\frac{d[1]}{dt} = k_1[1][2] + k_{cat2}[Py][1][2] - k_0[3] - k_{cat5}[Py][3] + k_{cat3}[Im][1][2] - k_{cat1}[Im][3]
\]
Degradation of the anhydride (2)

\[- \frac{d[2]}{dt} = k_1[1][2] + k_{cat2}[Py][1][2] + k_3[2] + k_{cat6}[Py][2] + k_{cat3}[Im][1][2] + k_{cat4}[Im][2]\]

Experimental data fitting

All the simulated and experimentally determined k-values were again used to fit the esterification cycle with different pyridine, imidazole and acetic anhydride concentrations. In the next figures the fits are provided together with the specific k-values (optimized if needed).

![Figure S28: Concentration profiles of different species (phenolate 1, ester 3, acetic anhydride 2 and acetic acid 4) over time for the ester formation with pyridine (0.1 mM) and imidazole (0.1 mM): Experimental data (black dots) and model (lines).](image)

| k-values               | Value                  |
|-----------------------|------------------------|
| \(k_0\)               | 0.0002 min\(^{-1}\)    |
| \(k_1\)               | 0.485 mM\(^{-1}\) min\(^{-1}\) |
| \(k_3\)               | 0.118 min\(^{-1}\)    |
| \(k_{cat1}\)          | 0.0147 mM\(^{-1}\) min\(^{-1}\) |
| \(k_{cat2}\)          | 26.2377 mM\(^2\) min\(^{-1}\) |
| \(k_{cat3}\)          | 0.0873 mM\(^{-1}\) min\(^{-1}\) |
| \(k_{cat4}\)          | 0.2258 mM\(^{-1}\) min\(^{-1}\) |
| \(k_{cat5}\)          | 0.0046 mM\(^{-1}\) min\(^{-1}\) |
| \(k_{cat6}\)          | 2.266 mM\(^{-1}\) min\(^{-1}\) |
Increasing imidazole concentration (0.1 mM $\rightarrow$ 0.2 mM $\rightarrow$ 0.25 mM $\rightarrow$ 0.3 mM $\rightarrow$ 0.5 mM $\rightarrow$ 1 mM)

Figure S29: Concentration profiles of different species (phenolate 1, ester 3, acetic anhydride 2 and acetic acid 4) over time for the ester formation with pyridine (0.1 mM) and imidazole (0.2 mM): Experimental data (black dots) and model (lines).

| k-values                  |
|---------------------------|
| $k_0$ | 0.0001 min$^{-1}$  |
| $k_1$ | 0.1993 mM$^{-1}$min$^{-1}$  |
| $k_3$ | 0.1257 min$^{-1}$  |
| $k_{cat1}$ | 0.0151 mM$^{-1}$min$^{-1}$  |
| $k_{cat2}$ | 16.5306 mM$^{-2}$min$^{-1}$  |
| $k_{cat3}$ | 0.0376 mM$^{-2}$min$^{-1}$  |
| $k_{cat4}$ | 0.0565 mM$^{-1}$min$^{-1}$  |
| $k_{cat5}$ | 0.0046 mM$^{-2}$min$^{-1}$  |
| $k_{cat6}$ | 3.36620 mM$^{-2}$min$^{-1}$  |

Figure S30: Concentration profiles of different species (phenolate 1, ester 3, acetic anhydride 2 and acetic acid 4) over time for the ester formation with pyridine (0.1 mM) and imidazole (0.25 mM): Experimental data (black dots) and model (lines).

| k-values                  |
|---------------------------|
| $k_0$ | 0.0001 min$^{-1}$  |
| $k_1$ | 0.1875 mM$^{-1}$min$^{-1}$  |
| $k_3$ | 0.2762 min$^{-1}$  |
| $k_{cat1}$ | 0.0199 mM$^{-1}$min$^{-1}$  |
| $k_{cat2}$ | 16.52 mM$^{-2}$min$^{-1}$  |
| $k_{cat3}$ | 0.0311 mM$^{-2}$min$^{-1}$  |
| $k_{cat4}$ | 0.1321 mM$^{-1}$min$^{-1}$  |
| $k_{cat5}$ | 0.0046 mM$^{-2}$min$^{-1}$  |
| $k_{cat6}$ | 3.0617 mM$^{-2}$min$^{-1}$  |
Figure S31: Concentration profiles of different species (phenolate 1, ester 3, acetic anhydride 2 and acetic acid 4) over time for the ester formation with pyridine (0.1 mM) and imidazole (0.3 mM): Experimental data (black dots) and model (lines).

$k$-values

| $k$   | Units            |
|-------|-----------------|
| $k_0$ | 0.0002 min${}^{-1}$ |
| $k_1$ | 0.1883 mM$^{-1}$min${}^{-1}$ |
| $k_3$ | 0.302 min$^{-1}$ |
| $k_{cat1}$ | 0.0127 mM$^{-1}$min${}^{-1}$ |
| $k_{cat2}$ | 10.1817 mM$^{-2}$min${}^{-1}$ |
| $k_{cat3}$ | 0.0329 mM$^{-2}$min${}^{-1}$ |
| $k_{cat4}$ | 0.1744 mM$^{-1}$min${}^{-1}$ |
| $k_{cat5}$ | 0.0032 mM$^{-1}$min${}^{-1}$ |
| $k_{cat6}$ | 5.2003 mM$^{-1}$min${}^{-1}$ |

Figure S32: Concentration profiles of different species (phenolate 1, ester 3, acetic anhydride 2 and acetic acid 4) over time for the ester formation with pyridine (0.1 mM) and imidazole (0.5 mM): Experimental data (black dots) and model (lines).

$k$-values

| $k$   | Units            |
|-------|-----------------|
| $k_0$ | 0.0001 min${}^{-1}$ |
| $k_1$ | 0.1875 mM$^{-1}$min${}^{-1}$ |
| $k_3$ | 0.4438 min$^{-1}$ |
| $k_{cat1}$ | 0.0295 mM$^{-1}$min${}^{-1}$ |
| $k_{cat2}$ | 8.75 mM$^{-2}$min${}^{-1}$ |
| $k_{cat3}$ | 0.031 mM$^{-2}$min${}^{-1}$ |
| $k_{cat4}$ | 0.2258 mM$^{-1}$min${}^{-1}$ |
| $k_{cat5}$ | 0.0013 mM$^{-1}$min${}^{-1}$ |
| $k_{cat6}$ | 6.1 mM$^{-1}$min${}^{-1}$ |
Figure S33: Concentration profiles of different species (phenolate 1, ester 3, acetic anhydride 2 and acetic acid 4) over time for the ester formation with pyridine (0.1 mM) and imidazole (1 mM): Experimental data (black dots) and model (lines).

Increasing pyridine concentration (0.1 mM $\rightarrow$ 0.5 mM $\rightarrow$ 1 mM)

Figure S34: Concentration profiles of different species (phenolate 1, ester 3, acetic anhydride 2 and acetic acid 4) over time for the ester formation with pyridine (0.5 mM) and imidazole (0.25 mM): Experimental data (black dots) and model (lines).
Figure S35: Concentration profiles of different species (phenolate 1, ester 3, acetic anhydride 2 and acetic acid 4) over time for the ester formation with pyridine (1 mM) and imidazole (0.25 mM): Experimental data (black dots) and model (lines).

**Decreasing anhydride concentration (0.5 mM → 0.25 mM)**

Figure S36: Concentration profiles of different species (phenolate 1, ester 3, acetic anhydride 2 and acetic acid 4) over time for the ester formation with pyridine (0.1 mM), imidazole (0.25 mM) and acetic anhydride (0.25 mM): Experimental data (black dots) and model (lines).
Increasing anhydride concentration (0.5 mM → 0.75 mM)

Figure S37: Concentration profiles of different species (phenolate 1, ester 3, acetic anhydride 2 and acetic acid 4) over time for the ester formation with pyridine (0.1 mM), imidazole (0.25 mM) and acetic anhydride (0.75 mM): Experimental data (black dots) and model (lines).

| $k$-values       |
|------------------|
| $k_0$            | 0.0001 min$^{-1}$ |
| $k_1$            | 0.3313 mM$^{-1}$min$^{-1}$ |
| $k_3$            | 0.1116 min$^{-1}$ |
| $k_{cat1}$       | 0.0191 mM$^{-1}$min$^{-1}$ |
| $k_{cat2}$       | 11.2411 mM$^{-2}$min$^{-1}$ |
| $k_{cat3}$       | 0.053 mM$^{-2}$min$^{-1}$ |
| $k_{cat4}$       | 0.0571 mM$^{-1}$min$^{-1}$ |
| $k_{cat5}$       | 0.0012 mM$^{-1}$min$^{-1}$ |
| $k_{cat6}$       | 2.268 mM$^{-1}$min$^{-1}$ |
14.4.1 Pyridine variation: experimental data versus model

Figure S38: Experimental and model data comparison for varying pyridine 1c concentrations, showing the conversion of p-nitrophenolate 1 (start: 0.1 mM) in MOPS buffer (100 mM, pH 7.5) with 5% acetonitrile: (A). 0.5 mM acetic anhydride, 0.25 mM imidazole and 0 mM pyridine. (B). 0.5 mM acetic anhydride, 0.25 mM imidazole and 0.1 mM pyridine. (C). 0.5 mM acetic anhydride, 0.25 mM imidazole and 0.5 mM pyridine. (D). 0.5 mM acetic anhydride, 0.25 mM imidazole and 1 mM pyridine. The presented experimental data are equal to Figure 1A.
14.4.2 Imidazole variation: experimental data versus model

Figure S39: Experimental and model data comparison for varying imidazole 1d concentrations, showing the conversion of \( p \)-nitrophenolate 1 (start: 0.1 mM) in MOPS buffer (100 mM, pH 7.5) with 5% acetonitrile: (A) 0.5 mM acetic anhydride, 0.1 mM imidazole and 0.1 mM pyridine. (B) 0.5 mM acetic anhydride, 0.2 mM imidazole and 0.1 mM pyridine. (C) 0.5 mM acetic anhydride, 0.25 mM imidazole and 0.1 mM pyridine. (D) 0.5 mM acetic anhydride, 0.3 mM imidazole and 0.1 mM pyridine. (E) 0.5 mM acetic anhydride, 0.5 mM imidazole and 0.1 mM pyridine. (F) 0.5 mM acetic anhydride, 1 mM imidazole and 0.1 mM pyridine. The presented experimental data are equal to Figure 1B.
14.4.3 Acetic anhydride variation: experimental data versus model

Figure S40: Experimental and model data comparison for varying acetic anhydride 2 concentrations and imidazole, showing the conversion of $p$-nitrophenol(ate) 1 (start: 0.1 mM) in MOPS buffer (100 mM, pH 7.5) with 5% acetonitrile: (A). 0.75 mM acetic anhydride 2, 0.25 mM imidazole and 0.1 mM pyridine. (B). 0.5 mM acetic anhydride 2, 0.25 mM imidazole and 0.1 mM pyridine. (C). 0.25 mM acetic anhydride 2, 0.25 mM imidazole and 0.1 mM pyridine. (D). 0.5 mM acetic anhydride 2, 0 mM imidazole and 0.1 mM pyridine. The presented experimental data are equal to Figure 1C.
14.4.4 Explanation for model optimization and deviations

The k-values were allowed to be optimised by Matlab using the constrained optimization function (fmincon) with a least squared cost function (opposing lower and upper bounds for the k-values: \( k_{\text{min}} = 0.5 \cdot k_0 \) and \( k_{\text{max}} = 2 \cdot k_0 \)). Yet even with the optimization, these k-values show some deviation (Table S3):

Table S3: Final reaction rate constants as determined from experimental data and subsequent Matlab fitting.

| k-value | Units | Reaction | Average | St. Dev. | Final     |
|---------|-------|----------|---------|----------|-----------|
| \( k_0 \) | \( (\cdot 10^4) \text{ min}^{-1} \) | Ester hydrolysis uncatalysed | 1.23 | 0.44 | 1.23 ± 0.44 |
| \( k_1 \) | \( (\cdot 10^1) \text{ mM}^{-1}\text{min}^{-1} \) | Ester formation uncatalysed | 3.57 | 2.03 | 3.57 ± 2.03 |
| \( k_3 \) | \( (\cdot 10^1) \text{ min}^{-1} \) | Anhydride hydrolysis uncatalysed | 3.00 | 1.33 | 3.00 ± 1.33 |
| \( k_{\text{cat1}} \) | \( (\cdot 10^2) \text{ mM}^{-1}\text{min}^{-1} \) | Ester hydrolysis imidazole catalysed | 2.23 | 1.06 | 2.23 ± 1.06 |
| \( k_{\text{cat2}} \) | \( (\cdot 10^1) \text{ mM}^{-2}\text{min}^{-1} \) | Ester formation pyridine catalysed | 1.88 | 1.04 | 1.88 ± 1.04 |
| \( k_{\text{cat3}} \) | \( (\cdot 10^2) \text{ mM}^{-2}\text{min}^{-1} \) | Ester formation imidazole catalysed | 6.41 | 4.15 | 6.41 ± 4.15 |
| \( k_{\text{cat4}} \) | \( (\cdot 10^1) \text{ mM}^{-1}\text{min}^{-1} \) | Anhydride hydrolysis imidazole catalysed | 1.66 | 0.76 | 1.66 ± 0.76 |
| \( k_{\text{cat5}} \) | \( (\cdot 10^3) \text{ mM}^{-1}\text{min}^{-1} \) | Ester hydrolysis pyridine catalysed | 2.68 | 1.64 | 2.68 ± 1.64 |
| \( k_{\text{cat6}} \) | \( \text{mM}^{-1}\text{min}^{-1} \) | Anhydride hydrolysis pyridine catalysed | 4.03 | 1.68 | 4.03 ± 1.68 |

The k-values with most variation are related to the anhydride species and a deviation is anticipated, since the acetic anhydride hydrolysis is very rapid \(^5\) and already occurs before the first sample has been measured. Besides, the mechanism for imidazole and pyridine catalysis is in fact more complicated than was assumed in this simplified model and deals with a pre-equilibrium of reactive intermediate formation (acetyl-pyridinium and acetyl-imidazole), acetate ion inhibition and contributions from nucleophilic and general acid/ base catalysis \(^2,8\). Finally, the reactive intermediates for imidazole and pyridine catalysis can also be interconverted, complicating the model.
Figure S41: Representative Cryo-EM images of PAANY in borate buffer (200 mM, pH 8.0) at different magnifications. Small spherical structures can be identified with an average diameter of 10 nm along with larger clusters with an average diameter of 24 nm.
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