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A Method to Quench Carbodiimide-Fueled Self-Assembly

--Manuscript Draft--

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Abstract:	In chemically fueled self-assembly, the activation and deactivation of molecules for self-assembly is coupled to a reaction cycle. In biological examples, these reactions are typically fast, such that the building blocks remain activated for mere seconds. In contrast, synthetic reaction cycles are slower for self-assembly , i.e., with half-lives on the order of minutes. In search of life-like, dynamic behavior in synthetic systems, several groups explore faster reaction cycles that form transient labile building blocks with half-lives of tens of seconds. These cycles show exciting properties, but brought about a new challenge, i.e. , accurately analyzing the fast cycle is impossible with classical techniques. We thus introduce the notion of quenching chemical reaction cycles for self-assembly. As a model, we use the fast carbodiimide-fueled chemical reaction cycle and demonstrate a method that quenches all reactions immediately. We show its accuracy and demonstrate the application for several reaction cycles and a range of dissipative assemblies. Finally, we offer preliminary design rules to quench other chemically fueled reaction cycles.
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COMMENTS TO AUTHOR:

Reviewer 1: The manuscript by Schnitter and Boekhoven reports a recipe to rapidly quench all the on-going reactions in the media when carboxylic acids are exposed with high energy coupling agents such as EDC. This method makes the HPLC analyses fast and more reliable. The quenched mixture can be kept overnight before the analysis. The results are interesting and useful specifically to the field of carbodiimide fueled self-assembly. I suggest accepting this work after the authors address the following issues.

We thank you for the comments and are glad that you find the method useful.

1) All the O-acyl urea that react with benzylamine might not necessarily have formed the anhydride. Hence, the yield of amide should not just reflect the yield of anhydride. Please clarify.

The reviewer brings up a valid point that we have not addressed in our initial work. We added a short statement at the end of page 3. Briefly, the concentration O-acyl urea is never observed without the quench (from which we conclude its concentration will be low. The concentration is low, because it is rapidly converted to the anhydride. Indeed, the O-acyl urea is calculated to be below 5% of the anhydride concentration according to our kinetic models.

2) Even at pH 9, there will be some deprotection of Fmoc. It will be good to include data on this.

This is probably a safe thing to do, just to be sure. We added the data to the manuscript (Figure S9, S10). As we suspected, at pH 9, no Fmoc hydrolysis takes place.

3) The use of the adjective 'versatility or versatile' in numerous places is not appropriate according to me, as this technique applies to a very specific chemistry and also within that, not all base labile substrates can be used.

We removed the statements.

4) The statement in the introduction "….typically by solvolysis or by reaction with an abundant species in solution." does not cover all works on EDC. For instance, the work of Das et al (cited as ref 24) reports hydrolysis via cooperative catalysis from assembled state.

Even in that case, the reaction occurs with an abundant species in the system (H_2O), yet catalyzed by local catalyst. Interestingly, it is the exact same reaction cycle (EDC + H2O => EDU).

5) Please mention the pH when the reactions were performed using pentanoate(2). Also, please suggest why the EDC concentration is kept low.

We added the pH value (6.0). The EDC concentration is at 10 mM and the precursor concentration at 300 mM to avoid the formation of the N-acylurea. The details are worked out in the original work (briefly, you need a very high concentration acid to avoid the O-acyl urea form “surviving” too long. This is avoided by working with a high concentration of acid, or by working with a dicarboxylate. We have clarified this in the text now.

6) Some editorial errors remain such as in page 3, line 26, first column, it should be "stop" and not "stops". Page 4, line 11-12, 2nd column "…the concentration EDC at the". Page5, line 56 "We measured the concentration anhydride.." Page 6, line 45 second column.."concentration anhydride can …" .

Thank you for picking those up. They have been corrected.

7) Figure S7 y axis should be [amide] rather than [anhydride].

That has now been corrected.

Reviewer 2: Professor Job Boekhoven and coworkers showed previously that removing negative charges from dicarboxylate dianions (by EDC induced anhydride formation) decreases their solubility, leading to precipitation. A competing reaction, hydrolysis of the anhydride, regenerates the initial state (solution of carboxylates in water). This is apparently a simple extension of what the corresponding author did as a student, reference 16, where he removed the negative charges from carboxylates by an esterification reaction (hydrolysis that followed produced the initial carboxylate). In the current paper, it is shown that using a competing reaction, the aggregates can be stabilized against fast hydrolysis. Thanks to this, Boekhoven et al. can image the aggregates, but this is simply a technical trick on something that they published before and as such, it is far below the level of papers routinely appearing at Angewandte Chemie. Furthermore, there is zero new chemistry in this paper. Unfortunately, I cannot recommend the acceptance.

Thank you for your opinion. We are disappointed that we did not receive constructive feedback. We try to address some of your critisism, but, in all honesty, I think a 153-word report is not sufficient to review a manuscript (it's the shortest report, I've received in my career).

"In the current paper, it is shown that using a competing reaction, the aggregates can be stabilized against fast hydrolysis." I am afraid you may have misunderstood the goal of the manuscript entirely. We quench the reaction in order to stabilize the transient anhydride. The "aggregates" we observe, are in the "unquenched" state. The goal is to quench the reaction cycle because it offers more accurate, faster, and easier analysis. That is important because the cycle is now used by other labs worldwide.

"This is apparently a simple extension of what the corresponding author did as a student, reference 16, where he removed the negative charges from carboxylates by an esterification reaction (hydrolysis that followed produced the initial carboxylate)". I believe we may have found the culprit. I honestly fail to understand why that should be an argument to reject a manuscript 10 years later, but it sounds like a very strong biased opinion. Me, and 20+ other labs worldwide are working on this chemically fueled dissipative self-assembly. We believe in this direction for supramolecular chemistry is the right one. Should these other labs also get their work rejected, because it is conceptually similar to my Ph.D. work? Or is this a bias specifically against me?

Reviewer 3: In this manuscript, Boekhoven and Schnitter report on a quenching method that allows out-of-equilibrium systems fueled by EDC to be analyzed much more conveniently. They show that benzylamine efficiently stops the reactions and converts both anhydrides and the excess EDC to stable products. These can then be analyzed by, for example, HPLC.

This is, in my opinion, an important development for the field of abiotic chemically fueled systems that is appropriate for Angewandte Chemie. The chemistry here is fairly simple but the implications are significant (precisely because it is simple). I have personally tried to think of a way to do this but did not come up with a solution, mostly because I did not realize that the change in pH would so effectively prevent further reaction between the EDC and the carboxylic acid. This method will absolutely be applied by other groups working in this area.

We thank you for the comments and are glad to see you will use the method. We believe in sharing useful methods and it is for scientists like you that we developed and published the method.

I have only some minor comments:

- Could the authors comment on the potential for competition with anhydride hydrolysis? This is accelerated at higher pH. I could see this being a concern for more electrophilic and/or acyclic anhydrides.

We assumed that the reaction with the benzyl amine (amidation) would outcompete reaction with the water (hydrolysis). To ensure this was correct, we carried out an experiment in which we injected an aqueous solution of 20 mM of the (pre-synthesized) anhydride into the quenching solution, and found no significant hydrolysis (we observed 20 mM amide). Thus, the experiment (Figure S3) shows that the amide formation outcompetes the hydrolysis reaction as we observed full conversion of the applied anhydride. We added a remark to the updated manuscript on page 3 halfway column 1.

- The authors note at the end of the manuscript that the data obtained with this new method matches known data for the "C5C5" system. I think that comparing with data obtained by other methods is an important validation for the quenching system. I would encourage the authors to include previously determined results throughout the manuscript wherever applicable, and perhaps to include the earlier data on some of the plots where it can be directly compared.

We agree and have now included previously obtained data of the dissipative self-assembly of precursor **2**, **3** and **4**. We compare the old data to data obtained from the described quenching method (Fig. S13, S14, S15).

- The anhydride of 1 is unsymmetrical and could open in either direction. Were both products observed, or just the one in Fig. 2D?

The evidence of both products is mostly apparent for precursor 4 since we detect the evidence of a split peak. Since for the concentration determination it is not necessary to discriminate between both products, we put no further effort in separation by HPLC. We addressed this issue at page 3, column 1, and added a chromatogram (Fig. S17)

- p4 "...can also be adapted to anhydrides containing weaker nucleophilic centers." Should this be "electrophilic" centers?

We agree and, we changed “nucleophilic” to “electrophilic” in the updated manuscript.

- I don't think "C5C5" (p 5) has been defined in this paper.

Correct. We have included it in the updated manuscript at page 4, column 2.

The authors mention expanding this work to fluorescent amines. I would also like to suggest that they may wish to consider trying this with some different carbodiimides as well in future work. As I am sure they are aware, EDC has a fairly complex pH-dependent speciation (J. Am. Chem. Soc. 1978, 100, 7420) and is likely cyclized and not a bare carbodiimide in these reactions. The fully quaternized analogue of EDC is commercially available and it would be interesting to see whether the quench is still effective.

We thank the reviewer for the valuable input. When it comes to other carbodiimide derivatives, EDC has been by far the most successful we tried in our previous work (ref19a). We will test further derivatives in the future!

Reviewer 4: The manuscript describes a method to improve the quantitative determination of species concentrations in dynamic systems controlled by reaction cycles. The research is motivated by the observation that the trend towards faster reaction cycles in this research area is compromising the accuracy in determining the composition of the system. In particular, this regards reaction cycles that involve anhydrides as the transiently stable species, which have recently gained in popularity. Overall, the protocol appears interesting for researchers working with this kind of systems, but, in my opinion, the manuscript does not appear to reveal any new insights or advances that would appeal to a broader audience. For that reason, I do not think that Angewandte Chemie is the appropriate platform to communicate these results. It may be more suitable for Chemistry - A European Journal or ChemSystemsChem.

We thank the reviewer for their time and the helpful comments.

We appreciate your comment regarding the interest for a broader audience. We agree that we failed to put this work in a broader context.

Carbodiimide-driven cycles were our response to slow chemical reaction cycles. In the last years, many other reaction cycles have been developed. Stephen Fletcher works with redox-driven cycles which are relatively fast. Hermans and coworkers also using the redox-fueled PDI cycle with very fast kinetics (Nature Nanotech. 2018). Similarly, Sijbren Otto, a pioneer in the field of autocatalytic cycles, recently started working with chemically fueled cycles based on oxidizing and reducing agents (ChemRxiv 2020). Many other supramolecular chemists are now also using chemically fueled cycles. These cycles will suffer, currently or in future work, from similar problems as we initially did: the kinetics of their cycles is so fast, it becomes hard to keep up with HPLC or NMR. A quench for their cycle would solve many of the problems they have been facing.

Our manuscript, introduces the notion of quenching chemically fueled assembly. That is of great value to the broader audience working on non-equilibrium assemblies. Even though it was developed for carbodiimide-based cycles, I can foresee that it will serve as inspiration and that it will be redesigned by others to quench other reaction cycles. Our work lays the foundation for these future developments, and helps scientist already working with the carbodiimide-fueled cycles. However, that was not clear from the initially submitted work.

Action points: Therefore, we added a few statements to put our work in broader context. More importantly, towards the end of the manuscript we added general design rules for quenching chemically-fueled reactions cycles and our thought processes in desiring ours. We also added critical experiments that should be carried out to test the validity of the quench.

There are also some technical issues that need to be clarified:

1. Both the reaction of the O-acylisourea intermediate and the anhydride will yield the same product upon reaction with benzylamine. In case the intermediate has some stability, it implies that the concentration of amide cannot be directly correlated to anhydride concentration.

This comments is in line with the first comment of reviewer one. The reviewer brings up a valid point that we have not addressed in our initial work. We added a short statement at the end of page 3. Briefly, the concentration O-acyl urea is never observed without the quench (from which we conclude its concentration will be low. The concentration is low, because it is rapidly converted to the anhydride. Indeed, the O-acyl urea is calculated to be below 5% of the anhydride concentration according to our kinetic models.

2. The authors mention that in these systems, the O-acylisourea intermediate can react with a second nucleophile to give an intra- or intermolecular anhydride. In cases where a mixture of intra- and intermolecular products are formed, the method cannot distinguish between these, because the reaction with benzylamine will yield the same product.

This case is true for the dicarboxylic precursors 1, 3 and 4. Nevertheless, we did not observe the formation of dimers which would be apparent in an additional signal when the quench is not applied. We addressed this remark at page 2, column 1.

3. Opening of asymmetric anhydrides (which include the intramolecular anhydrides formed from 1, 3 and 4) yields two products. This is never discussed in the manuscript, but is a relevant issue as it may complicate HPLC peak assignment and quantification - especially for more complex systems. Indeed, for a manuscript that reports on a protocol, it surprises me that not a single HPLC-chromatogram is given in the manuscript or supporting

information. Demonstration of peak broadening would also lend additional support for on-column anhydride degradation.

We now addressed this comment at page 3, column 1. Because the two isomers are so similar, they have almost the same retention time. Only for precursor 4, we have evidence did we manage to “see” the two derivatives in the HPLC chromatogram as a split peak (Fig. S20). We assume that both compounds have the same extinction coefficient. Therefore, in the concentration determination, it is not necessary to discriminate between both products, i.e., we integrate the entire peak area.

4. Experimental data that demonstrate the (long-term) stability of the Fmoc-group in the quenching mixture should be provided.

In line with the comment of reviewer 1, we have now added that data (Figure S9, S10)

5. The introduction can be shortened.

We have removed some excessive text on the biological examples of DSA.

Reviewer 4: By adding a section dedicated to general design rules for quenching reaction cycles, the authors have made a significant effort to contextualize the results in a larger framework. This has certainly added value to the manuscript. Also my technical comments and the issues raised by the other (positive) reviewers have been addressed in a satisfactory way. For these reasons, I recommend the manuscript for publication.

Apart from the fact that reviewer 2, who advises against publication, clearly has not understood the work described in the manuscript, the used argumentation is far below the level that can be expected from a reviewer for Angewandte Chemie.

Thank you for the positive response.

Reviewer 5: Boekhoven's group have been extensively working on carbodiimide fuelled transient assembly. In the previous work, they have used HPLC to monitor the reaction kinetics and in the present work the group have reported a method to quench the reaction during the cycle to monitor it more effectively.
Being a court-of-appeal referee, I had a critical look at the manuscript and also the reports from other reviewers. I think, the manuscript lacks enough novelty to be published in Angewandte due to the following reasons:

Thank you for the response.

The major comments are:
1. The manuscript lacks novelty, it's an extensively reported fuelled assembly by Boekhoven and group.
2. The idea of using quenching chemistry to delay the assembly kinetics is not an entirely new one. Introducing inhibitor to the chemical reaction network based on imine chemistry, has been demonstrated in this field.
3. The only novelty and objective of the current work is the method to monitor the kinetics. But it has major issue.
4. Although authors claim in a different way, I feel that the strategy is very specific and cannot be applied to diverse chemical fuelled reaction systems. Although authors do suggest a way to create new quenching method, the current reported method cannot be extended.
5. The method utilizes a quenching reaction which has been extensively studied before and is a well-known peptide coupling reaction and hence again a novelty issue. (Bioconjugate chemistry 6, no. 1 (1995): 123-130).
6. The method is cumbersome and requires preparative methods same as previously performed by authors in their previous works.
7. The method cannot be used for immediate monitoring, unlike a spectroscopy method whch can be monitored in situ.
8. The method consumes the reaction mixture and hence the components are consumed.
9. The method cannot distinguish between anhydride and O-acyl urea which results in error of 5 % which is huge for a paper aiming to propose an analytic method for detection.
10. The method basically uses detection of benzyl ring at 254 nm during HPLC.
11. The manuscript lacks of proper citation!

A better method is use of a signalling molecules like fluorophores/chromophores are more sensitive, easier to perform and in situ method rather than a quenching method especially where self-assembly is involved. (Nat.

Commun., 2015, 6, 7790-7798, Nature Nanotechnology 2018, 13, 1021-1027, Nat. Commun. 2019, 10, 450)
Another major issue: Authors have used kinetic modelling to fit their kinetics of same molecule and reaction cycle in their previous work, where they mentioned it fits properly to the experimental data (E.g. Nat. Commun. 2018, 9, 2044.). However, herein authors comment that the monitoring was not correct and effective method and thus, the kinetic fitting doesn't match (Figure 2). But, it does match the new data using the current method. There is a major concern here regarding reliability of these kinetic models to study the reaction kinetics.
The experimental comments are:
12. What is the pH of benzyl amine in distilled water? How is pH 12 of benzyl amine maintained?

The high concentrated benzylamine quenching solution had a pH of 12. It acted as a buffer when added to the reaction cycle and the pH did not drop significantly due to the high concentraiton.

13. In Fig 2A-C and in figure 4A,F,G the term should be without/with "quencher" not "quench"

We updated it.

14. As mentioned by the authors, "The protecting groups are labile under basic conditions, which resulted in partial deprotection by our quenching chemistry. We showed that the deprotection can be reduced to an insignificant amount by using a less concentrated quenching solution, such that the pH does not exceed 9 (Figure S9, S10)." The authors must compare the kinetics of reaction by quenching with different concentrations of benzyl amine (different pH) to see the effect of the side reaction.

In this work, we were mainly interested to stop the deprotection completely. In the described method the benzylamine concentration was sufficiently high to quench both EDC and the anhydride. There is no need to further increase the benzylamine concentration which, we agree, would at some time promote the deprotection reaction.

15. Why is storage after quenching required? Compare the kinetic graph with monitoring immediately and after 10 hours. Do they differ? Comment.

Storage is required for determining the EDC concentration exclusively by the formation of guanidine. But, as shown by the Fmoc protected precursor, the EDC concentration can also be determined right after the quench.

16. How was the pH maintained in Fig S5? Mention in the caption.

We added the buffer conditions in the caption (0.2 M MES). The activation reaction depends on the protonation of EDC in an acidic media. We agree that within the first 3 datapoints a change in pH is possible. Nevertheless, the proton concentration was sufficiently high to buffer the proton consumption by the activation reaction (We found highest activation at lowest pH).

17. Unlike Fig. 2 which shows a huge difference between with and without quenching, Fig. S13 show a very insignificant different in maximum concentration of the components. Why Is it so? This suggests it's not always the issue of stability of the anhydride. Comment. Also, join the dots for clearer elucidation of the cycles in Fig. S13-15.

The difference is obvious for anhydrides with high deactivation rates. Since precursor **2** is somewhat more stable against hydrolysis, the difference between the methods is less obvious. We have updated the manuscript where we thought it was reasonably improving the manuscript.

Reviewer 6: I have reviewed the revised version of the manuscript and carefully read the comments provided by the 4 reviewers and the response of the authors. I am more inclined to agree with reviewers 1 and 2 as this manuscript reports an interesting advance in the field of abiotic chemically fueled systems that is appropriate for Angewandte Chemie.

I can see that the authors have already improved their manuscript compared to the original submission so I do not have any specific suggestion. Only one comment: the title in my opinion could be of higher impact. I am not sure that the current title really delivers the message to the readers. I would probably revise it to make it more general and to better highlight what are the implications of the proposed strategy for the field of dissipative self-assembly. This said, it seems like no other reviewers has my opinion so the authors (together with the editor) can decide the best course of action with regards to the title.

Minor point: pag. 2 line 25. I think that there is a typo ("cross reference to HERMANS Chem Commun"). Please check.

Thank you for the positive response. We have updated the manuscript.

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ARTICLE

A Method to Quench Carbodiimide-Fueled Self-Assembly

Fabian Schnitter,[a] and Job Boekhoven*[a],[b]

Abstract: In chemically fueled self-assembly, the activation and deactivation of molecules for self-assembly is coupled to a reaction cycle. In biological examples, these reactions are typically fast, such that the building blocks remain activated for mere seconds. In contrast, synthetic reaction cycles are slower for self-assembly, *i.e.,* with half-lives on the order of minutes. In search of life-like, dynamic behavior in synthetic systems, several groups explore faster reaction cycles that form transient labile building blocks with half-lives of tens of seconds. These cycles show exciting properties, but brought about a new challenge, *i.e.*, accurately analyzing the fast cycle is impossible with classical techniques. We thus introduce the notion of quenching chemical reaction cycles for self-assembly. As a model, we use the fast carbodiimide-fueled chemical reaction cycle and demonstrate a method that quenches all reactions immediately. We show its accuracy and demonstrate the application for several reaction cycles and a range of dissipative assemblies. Finally, we offer preliminary design rules to quench other chemically fueled reaction cycles.

Introduction

In molecular self-assembly, molecules are designed to interact with each other via non-covalent interactions.[1] The range of molecular assemblies that consequently form is enormous, ranging from anisotropic fibers,[2] tapes,[3] and tubes,[4] to isotropic assemblies like (block-copolymer)[5] micelles,[6] vesicles[7] and colloids.[8] The field has slowly evolved from rather serendipitous findings to well-defined design rules allowing for a range of applications, so-called supramolecular materials.[9] Inspired by biology, the field has recently focused on the assembly of molecules, driven out of equilibrium by energy-consuming chemical reaction cycles.[10] In these chemically-fueled assemblies, the assembly and disassembly of molecules is coupled to a chemical reaction cycle.[11] The reaction cycle comprises at least two reactions: a building block activation and deactivation reaction (Figure 1A). A precursor molecule is converted into a building block for self-assembly at the expense of a high energy reagent (chemical fuel) in the activation. In the deactivation, the building block is reverted to the precursor spontaneously, *e.g.,* by solvolysis or by reaction with an abundant species in solution. The sum of the reaction thus means that a precursor is temporarily converted into a self-assembling building block driven by the conversion of a molecule of fuel.

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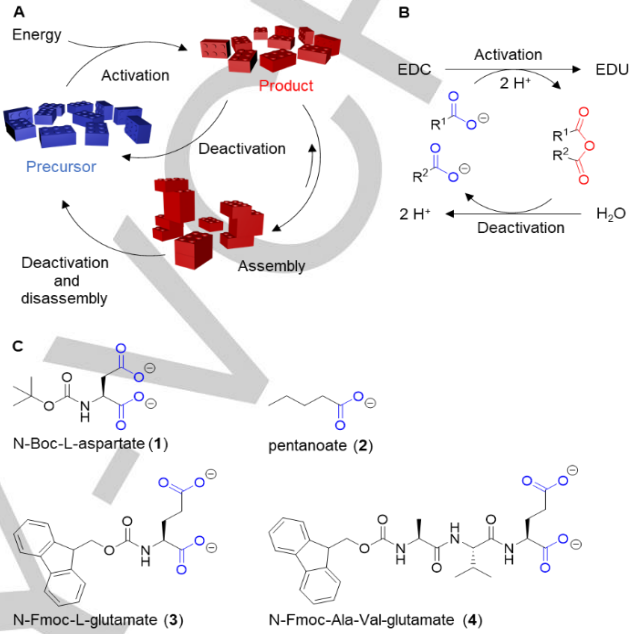

Figure 1. Chemical reaction cycle that fuels self-assembly. A) A generalized reaction scheme for chemically fueled self-assembly. B) The formation of a transient anhydride product from a carboxylate precursor is driven by the hydrolysis of EDC as carbodiimide fuel. C) Precursors used in this work as precursors for the chemical reaction cycle.

The consequence of the fuel-driven self-assembly is that the assembly and disassembly rates are now, in part, regulated by the rates in the chemical reaction cycle. For example, the cytoskeletal microtubules and actin filaments are tightly regulated by the hydrolysis of GTP and ATP, respectively.[12] Because of the kinetic control over self-assembly, their corresponding behavior is rich and includes dynamic instabilities,[13] as well as the treadmilling of actin filaments. It is important to note that the reaction rates of deactivation in these biological examples are fast. For example, tubulin-bound GTP has a half-life of less than a second.[14]

Inspired by the dynamic behavior of biological chemically fueled assemblies, researchers have developed synthetic counterparts. Exciting behaviors of these assemblies have been described, which include solutions that spontaneously oscillate between different types of assemblies,[15] molecular fibers that undergo dynamic instabilities,[16] and molecular assemblies that can select molecules from a pool of reactants.[17] One of the challenges in the synthetic approach towards dynamic materials is the lack of fast chemical reaction cycles. For example, the early work by van Esch and Eelkema used the carboxylic acid alkylation and its hydrolysis as a chemical reaction cycle.[18] At a pH of 10, the product had a half-life of roughly 0.3 hours.[16] To accelerate the rates of activation and deactivation, our group and the group of Hartley recently introduced the activation of carboxylates into

transient anhydrides driven by the hydrolysis of carbodiimide-based condensing agents like 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Figure 1B).[19] These anhydrides are prone to hydrolysis resulting in a half-life ranging from several minutes to tens of seconds, depending on the nature of the anhydride. We indeed observed that such systems had high dynamics with rapid assembly and disassembly.[20] The carbodiimide fuel has been further explored by us and others to yield symmetric,[19c, 21] and asymmetric anhydrides,[19a, 20, 22] activated esters like N-hydroxysuccinimide esters,[23] and phenol esters.[24] Due to the fast reaction cycle with tunable kinetics, the carbodiimide driven cycle has successfully been used for the self-assembly of peptide-based gels,[19a] catalytic nanofibers,[25] vesicles,[20], coacervates,[26] and others. However, as the hydrolysis rate of the product increased, we encountered a new challenge: the techniques we used for quantification (HPLC and NMR) are not able to accurately measure concentration because of the rapid deactivation of the compound of interest. For example, when a labile anhydride retains on the HPLC column, we observed significant amounts were hydrolyzed. Similarly, the acquisition times of NMR were too long to acquire reliable spectra of the compounds related to the reaction cycle. Other research groups have also introduced new chemical reaction cycles with fast kinetics. For example, Hermans recently introduced a redox-chemistry driven reaction cycle that operates with half-lives of minutes.[27] Likewise, Fletcher uses redox coupled (autocatalytic) reaction cycles that operate on minute timescales. These fast reaction cycles potentially also suffer from the inaccurate and complicated kinetic analysis by HPLC or NMR as they are further accelerated in the future.

In this work, we thus introduce the notion of quenching chemical reaction cycles to perform kinetic analysis. We describe a simple method to accurately analyze several carbodiimide-fueled chemical reaction cycles. We used a reagent that can almost immediately stop all four reactions taking place in the reaction cycle. We demonstrate that quenching chemistry can be applied to several types of anhydrides and works in the presence or absence of assemblies. Due to the effectiveness of the chemistry, the quenched samples can either be stored for up to several weeks or directly be analyzed. Finally, we establish design rules for quenching chemistry for chemical reaction cycles. Our method opens the door to further accelerate chemical reaction cycles and explore rapid dynamic behavior while retaining a quantitively understanding of the reactions involved.

Results and Discussion

Chemically fueled reaction cycles that can drive self-assembly comprise at least two reactions: a chemical reaction that activates building blocks for self-assembly and one that deactivates them (Figure 1A). In the carbodiimide driven reaction cycle, the precursor in the cycle carries at least one carboxylate (Figure 1B). In the first reaction, the precursor's carboxylate reacts with a carbodiimide based condensing agent to form the corresponding O-acyl urea intermediate (Figure S1). In the second reaction, the O-acyl urea reacts with a second nucleophile, *i.e.*, an intra- or inter-molecular carboxylate yielding, respectively, an intra- or inter-molecular anhydride. Regarding asymmetric precursor molecules like **1** the ring closure is favored compared to dimer formation because of the near proximity of the second carboxylic group. In the aqueous environment, the anhydride product is rapidly hydrolyzed to the corresponding precursor, which constitutes the deactivation reaction. Finally, the O-acyl urea can hydrolyze directly to the precursor without forming the anhydride (Figure S1). In summary, the overall reaction cycle forms a transient anhydride driven by the hydrolysis of a carbodiimide condensing agent into its corresponding urea.

In this study, we use 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) as a condensing agent because of its high solubility in water, but other work has shown that this cycle can also be driven by other carbodiimide derivatives. In the first part of these studies, we use N-Boc-L-aspartate (**1**) as a precursor molecule (Figure 1C). Its rapid deactivation makes it a great test compound for our quenching chemistry. In order to accurately determine the rate constant of deactivation for the reaction cycle in Scheme 2A, we synthesized N-Boc-L-aspartic anhydride (see synthesis section in SI). The powder was dissolved in acetonitrile and further diluted in 200 mM aqueous MES buffer at pH 6. After dilution, the decreasing concentration of anhydride was monitored by HPLC. We were surprised to find that the anhydride concentration of 20 mM had decreased from 20 to 7.5 mM after the first 30 seconds in the reaction cycle (Figure 2B). From there on, the concentration decayed with first-order kinetics. We could not fit the unconventional decay properly using first-order hydrolysis kinetics. Thus, we reasoned that the peculiar kinetics was not due to hydrolysis of the anhydride in the reaction mixture, but rather due to hydrolysis in the mobile phase of the HPLC. If additional hydrolysis in the mobile phase occurred, the concentration anhydride measured would be drastically lower than at given times in the reaction mixture. To test this hypothesis, we injected N-Boc-L-aspartic anhydride dissolved in dry acetonitrile into the HPLC. Even though 20 mM of the anhydride was injected, only 11 mM anhydride was detected, pointing to a loss of over 40% of the initially injected anhydride.

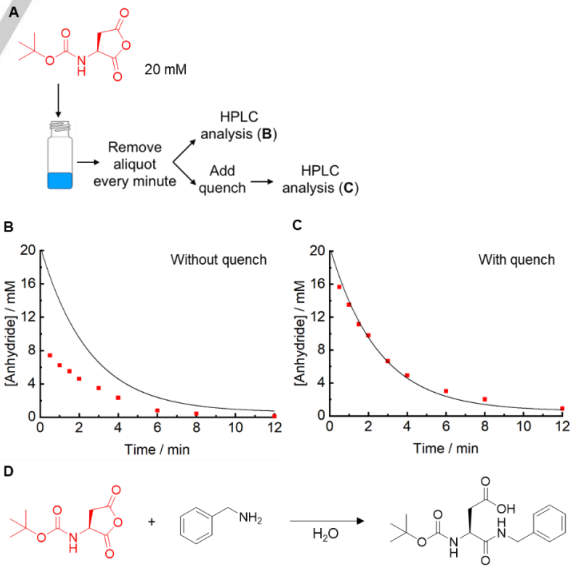

Figure 2. Does the anhydride hydrolyze during the HPLC analysis? A) The hydrolysis reaction of the activated precursor **1**. We injected 20 mM N-Boc-L-aspartic anhydride in water at pH 6. B-C) The concentration anhydride against time as measured by B) directly injecting the reaction mixture, and C) injecting a quenched reaction mixture into HPLC. The black line represents a first-order decay profile as the best fit. D) The quenching solution converts the anhydride of **1** into a stable N-benzyl amide.

Moreover, when we increased the anhydride retention time, by changing the HPLC-method, we observed less anhydride (Figure S2) confirming that the labile anhydride rapidly hydrolyzes during the analysis resulting in inaccurate data. Given that the acquisition times for 1H-NMR are typically in the range of several minutes, it is expected that following the hydrolysis of the anhydride by 1H-NMR would not yield much more accurate data. We thus set out to introduce a quenching method that rapidly stops all the reactions in our reaction cycle. We were seeking a method that (i) reacts with the anhydride rapidly to a stable product, (ii) stops the activation reaction, and (iii) reacts with the fuel to stop any side reactions. We used benzylamine as a reagent in our method because: (i) it rapidly reacts with the anhydride forming the stable amide. It will also react with the O-acyl urea forming the same amide. (ii) Due to its basicity, it will immediately increase the pH of the reaction mixture, thus inhibiting the pH-sensitive activation. Moreover, (iii) we found that benzylamine slowly reacts with EDC to the non-reactive guanidine moiety, stopping any further side reaction. Finally, the products (guanidine-derivative and N-benzyl amides) will carry the aromatic benzyl ring, which vastly facilitates HPLC-detection at 254 nm.

We repeated the above-described experiment (20 mM N-Boc-L-aspartic anhydride) and removed aliquots from the reaction mixture at given time points, which we injected in a quenching solution (400 mM benzylamine in water at pH 12). After the quench, we determined the concentration of the stable amide as a measure for the anhydride concentration at the time benzylamine was added (Figure 2C-D). The opening of asymmetric anhydrides formed by precursors like **1** yields two products, which is the benzylamine added either at the sidechain or the backbone of the amino acid. When determining the anhydride concentration, it is not necessary to discriminate between both products and thus the whole signal area assigned to the corresponding benzyl amide is considered for calculation. This method yielded much higher quality data that could be fitted with first-order decay. Another advantage of the quenching method became apparent. Without the quenching chemistry, each data point in the time series required the preparation of a new sample. For example, when the 15 minute HPLC method was finished with determining the concentration anhydride in the first data point (minute 1), all anhydride had hydrolyzed, and a new sample had to be started to measure the concentration in a second data point. With the quenching method, each time interval, an aliquot could be injected into the benzylamine solution. That solution could be stored and analyzed later. Finally, it is worth mentioning that the reaction of the anhydride with the benzylamine is fast, and all anhydride is converted within a minute after addition (Figure S3). Since full conversion was detected, we conclude that the amide formation outcompetes the accelerated hydrolysis reaction at an elevated pH. With this simple quenching method, we determined the half-life of the anhydride to be two minutes under these conditions.

Besides quenching the deactivation reaction, all other reactions in the cycle have to be quenched too. To test the effects of benzylamine on the activation reaction, we reacted N-Boc-L-aspartate (**1**) in aqueous buffer at pH 6 with EDC. After 1 minute, the entire solution was quenched by the addition of benzylamine. We measured that the quenched sample contained 6 mM of N-Boc-aspartate (**1**), 3 mM of amide (Figure 3A-B), and 36 mM of EDC (Figure 3C-D). We were interested in the activity of the remaining EDC in the quenched solution, *i.e.*, is EDC in the presence of benzylamine able to form new anhydride and thereby compromise our measurements? To test the reactivity of the remaining EDC, we followed the concentration of **1**, its corresponding N-benzyl amide, and EDC over the course of 10 hours at room temperature. We were surprised to find that the EDC was fully consumed within 10 hours, but no conversion of new anhydride or amide was apparent (Figure 3A-B). We explain the lack of the conversion of the acid to the anhydride by a sudden increase in pH upon quenching the reaction mixture (Figure S4). The O-acyl urea formation is highly pH-dependent, with the highest reactivity around pH 2 - 4, depending on the carboxylic acid (Figure S5). At the pH of the quenching solutions, the activation reaction was completely halted. In contrast, we found that EDC reacted with benzylamine to form its guanidine-derivative (Figure 3C-D). The HPLC data shows that this transformation was completed within 10 hours. Because this reaction did not produce further side products, the concentration EDC at the time of the quench could be determined accurately by measuring the remaining concentration of EDC and the concentration of the guanidine derivative.

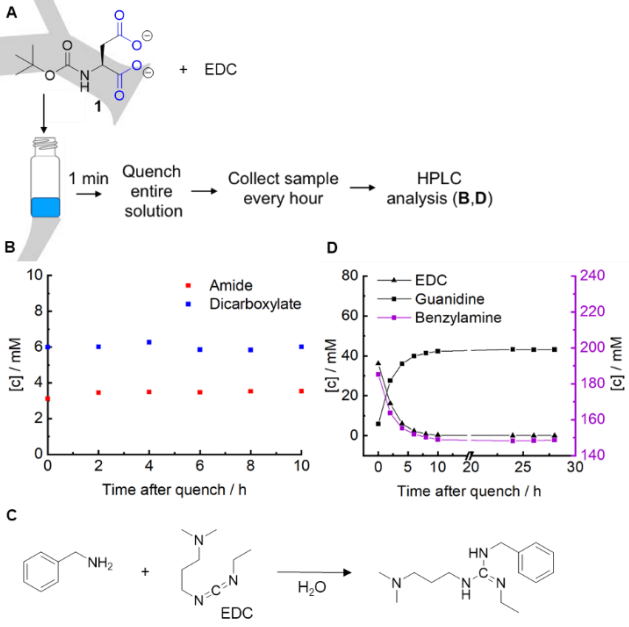

Figure 3. Does the quench stop our activation reaction? A) The activation reaction converts the dicarboxylate of **1** into its corresponding anhydride by the consumption of EDC. B) The concentration of dicarboxylate and N-benzyl amide against time after quenching the activation reaction. C) The reaction of EDC with benzylamine to form a stable guanidine-derivative. D) The concentration of EDC, benzylamine, and the guanidine-derivative against time as measured by HPLC. The markers represent HPLC data, and the lines are added to guide the eye.

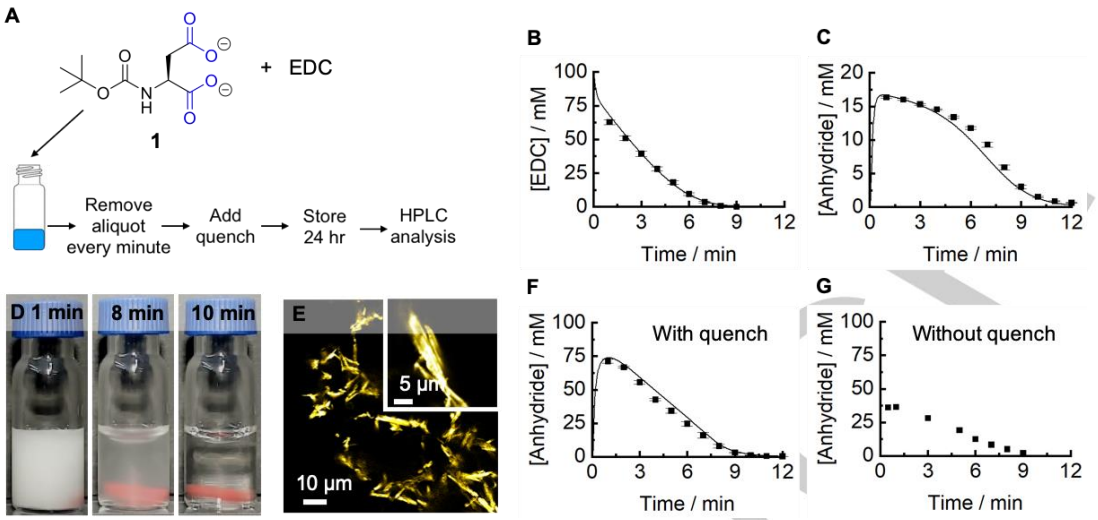

Figure 4. Analysis of a carbodiimide fueled reaction cycle with N-Boc-L-aspartate (**1**) as a precursor. A) Schematic overview of our quenching experiment. The concentration of B) EDC and C) anhydride against time as measured by HPLC. Initial concentration was 100 mM in EDC and 20 mM in **1**. The markers represent HPLC data, and the lines are predicted values from our kinetic model. The error bars depict the standard deviation from the average (n = 3). D) Time-lapse photography of a solution of 100 mM EDC and 100 mM N-Boc-L-aspartate (**1**). E) Fluorescence microscopy micrographs of the turbid solution. F) The concentration anhydride against time as measured by injecting quenched aliquots into HPLC. G) The concentration anhydride against time as measured by directly injecting the reaction mixture into HPLC.

Next, we set out to test the quenching method on the full chemical reaction cycle using EDC as fuel and **1** as a precursor (Figure 4A). To analyze the kinetics of the chemical reaction cycle, we added 100 mM EDC to 20 mM precursor dissolved in 200 mM MES buffer at pH 3.5 and, every minute, quenched an aliquot of the mixture. The quenched samples were stored overnight and analyzed by HPLC (Figure 4A-C). One downside of our method is that it cannot discriminate between the intermediate O-acyl urea or the anhydride product, *i.e.,* both will react to form the benzylamide. Nevertheless, due to the fast conversion of the O-acyl urea to the anhydride, only very low concentrations of the O-acyl urea are present in the reaction mixture. For example, our model calculates the concentration O-acyl urea to 0.63 mM in comparison to 17 mM of anhydride within the first minute. Thus, less than 5% of the amide formed stems from the O-acyl urea.

The assay showed that in the reaction mixture, the EDC concentration dropped rapidly in the first minute, after which it decayed linearly. As the EDC was being consumed, the anhydride concentration rapidly increased to 16 mM, while the precursor concentration dropped to 4 mM. Because a large fraction of the precursor was converted, the anhydride concentration settled to a pseudo-steady state. The pseudo-steady state implies that activation and deactivation occur at almost equal rates. This state was sustained for about 5 minutes, which is the time until most EDC had reacted. After that, the concentration anhydride decayed until it had all hydrolyzed. When we used a simple kinetic model, we could accurately predict the concentrations of EDC, precursor, and anhydride. From the kinetic model, we calculated an anhydride half-life ($\ln(2) / k_{hydrolysis}$) of 50 seconds. This value differs from the above mentioned 2 minutes because of the increased water content. It is worth mentioning that we found no evidence of self-assembly when we mixed 20 mM precursor with 100 mM EDC.

We tested if the supramolecular structures formed by the activated precursor molecules interfere with our quenching method. Specifically, we were interested to see whether the benzylamine quench was sufficiently fast to simultaneously dissolve and amidate the anhydrides in an assembly. In contrast to the experiment above, we used 100 mM of N-Boc-L-aspartate (**1**) precursor with 100 mM of EDC. The higher precursor concentration allows for the formation of more anhydride than the experiment above. We found that the solution almost immediately turned turbid (Figure 4D). The increased turbidity was present for roughly eight minutes, after which the solutions turned clear. Fluorescence microscopy of these turbid solutions revealed the presence of micron-sized rectangular crystals, which explains the increased turbidity (Figure 4E). We measured the concentration anhydride and fuel with the method described above. It is worth noting that the turbid solution turned transparent immediately upon injecting it in the benzylamine quenching solution, pointing to a rapid dissolution of the anhydride. We found that all fuel was consumed within two minutes (Figure S6), after which the anhydride decayed linearly (Figure 4F). We explain the linear decay with a self-protection mechanism that we described in earlier work.[22b] In the self-protection mechanism, the hydrolysis reaction only takes place on the fraction of anhydride that remains in solution after self-assembly. Thus, when the concentration anhydride passes its solubility, the hydrolysis converts from first-order kinetics to zero-order kinetics. We incorporated the self-protection mechanism into our kinetic model and could accurately predict the HPLC data. Importantly, the same rate constants were used in the model as described above, which is a good indication that the benzylamine quench is effective in samples that contain assemblies or not.

Finally, we aim to show the generality of our quenching method, both for different types of activated carboxylate precursors and different types of assemblies. First, we tested the precursor pentanoate (**2**), which forms the non-cyclic pentanoic anhydride (C_5C_5) when activated by EDC. Several research groups, including ours, have used symmetric anhydride formed carbodiimide-fueled chemical reaction cycles to drive dissipative self-assembly.[19b, 21a, 23a] Given the slightly different reactivities of

these anhydrides, we were first interested in whether our quenching method would yield the N-benzyl amide. We prepared a solution of 10 mM pentanoic anhydride in acetonitrile and quenched the mixture with benzylamine. The approximately 10 mM of amide measured (Figure S7) serves as strong evidence that the quenching method can also be adapted to anhydrides containing weaker electrophilic centers. To test if the method also works on the fueled reaction cycle with pentanoate (**2**) as the precursor, we fueled 300 mM of **2** at pH 6.0 with 10 mM of EDC and tested the evolution of the anhydride and EDC concentration (Figure 5A, S8). These conditions reflect earlier work we carried out on this precursor.[19c] Even though the solution forms droplets (Figure 5B-C), we found that the quenching method yielded reliable data.

Fmoc-protecting groups are frequently used in the design of self-assembling peptides,[28] and have also been used in the carbodiimide-driven reaction cycles.[19a, 22b] We thus tested our quenching method on peptide precursors that contained this group, *i.e.*, N-Fmoc-L-glutamate (**3**) and N-Fmoc-Ala-Val-glutamate (**4**). It should be noted that these protecting groups are labile under basic conditions, which resulted in partial deprotection by our quenching chemistry. We showed that the deprotection can be reduced to an insignificant amount by using a less concentrated quenching solution, such that the pH does not exceed 9 (Figure S9, S10).

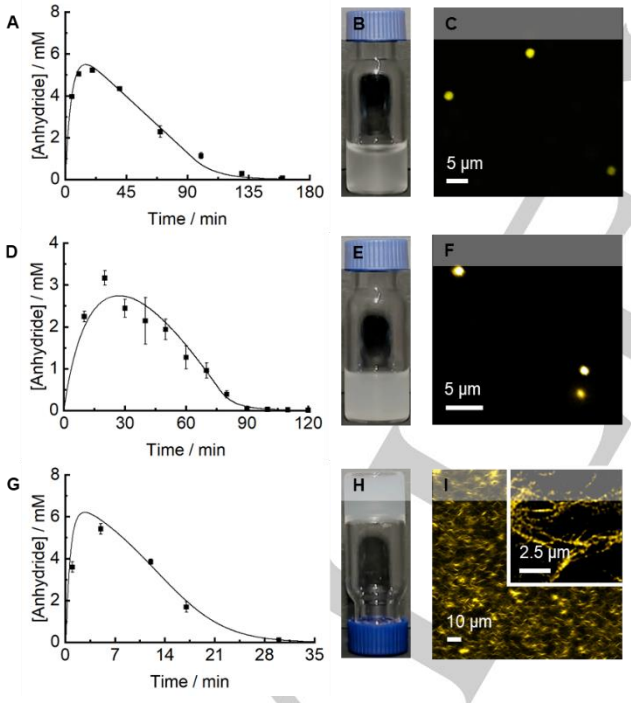

Figure 5. Analysis of different self-assembling reaction networks by our quenching method. A-C) Dissipative self-assembly of 300 mM pentanoate (**2**) fueled with 10 mM EDC. A) The concentration anhydride against time as measured by HPLC. The markers represent HPLC data, the lines are predicted values from our kinetic model. The error bars depict the standard deviation from the average (n = 3). B) Photograph of a turbid solution. C) Fluorescence microscopy micrograph of the turbid solution. D-F) Dissipative self-assembly of 10 mM N-Fmoc-L-glutamate (**3**) fueled with 10 mM EDC. D) The concentration anhydride against time as measured by HPLC. E) Photograph of a turbid solution. F) Fluorescence microscopy of the turbid solution. G-I) Dissipative self-assembly of 10 mM N-Fmoc-Ala-Val-glutamate (**4**) fueled with 50 mM EDC. H) Photograph of a self-sustaining hydrogel. I) Fluorescence microscopy of the hydrogel.

Nevertheless, the benzylamine remained in a 20-fold excess relative to the maximum anhydride concentration. In addition to that we stored the quenched samples at 8 °C. With the adjusted method, we measured the kinetics of the reaction cycle when 10 mM N-Fmoc-L-glutamate (**3**) was fueled with 10 mM EDC at pH 6.0 (Figure 5D, S11). Moreover, **3** self-assembled into colloids in response to the fuel, further demonstrating the versatility of our method (Figure 5E-F). To further demonstrate the generality of our method, we measured the kinetics of the chemical reaction cycle with N-Fmoc-Ala-Val-glutamate (**4**) as a precursor at pH 6.0. Upon EDC fueled activation, the peptide precursor can self-assemble into fibers, which subsequently yields a hydrogel (Figure 5H-I).[19a] We were particularly interested if the increased viscosity of the material interfered with our quenching method. We used a wide-bore pipette to remove samples and analyzed those by HPLC after applying our quenching method (Figure 5G, S12). The kinetic data showed close correspondence to the predictions by our kinetic model.

Why should one use the benzylamine quenching method? The strongest argument in favor of the benzylamine quench is that it vastly improves the data quality. This argument is particularly valid for labile anhydrides that hydrolyze during the analysis. Even for somewhat more stable anhydrides (*e.g.*, C_5C_5, $t_{1/2}$ = 12 minutes), we found improvement in data quality when applying the quenching method (Figure S13). Furthermore, a strong argument for the quenching method is that it vastly facilitates data acquisition. Without the quench, the rate of data acquisition was determined by the length of the analysis method. Specifically, we can inject one sample in the HPLC every ~20 minutes. Thus, our data acquisition is limited to ~0.05 data points.min^{-1}. For ^{1}H-NMR that is somewhat faster, providing one acquisition every ~2 minutes (0.5 data points.min^{-1}). Due to the quenching method, the limiting factor of data acquisition frequency is the pipetting frequency. For example, with our pipetting robot, the highest data acquisition frequency is now 6 data points.min^{-1} (Figure S16). That means that samples can be collected in real-time and analyzed overnight, which significantly accelerates data throughput. Finally, an argument in favor of the benzylamine quenching method is that it further facilitates the detection of the anhydride product by converting an anhydride into the N-benzyl amide. The benzyl ring vastly facilitates the detection of the product by HPLC because of its absorbance at 254 nm.

The arguments in favor of using quenching chemistry above apply to other chemically fueled reaction cycles, too.[21a, 24, 29] To aid the broader community of scientists exploring the field of chemically fueled self-assembly, we set out some preliminary design rules for such quenching chemistry.

Firstly, quenching chemistry should be identified that immediately stops both the activation and deactivation reaction. If the activation is partly deactivated, the concentration product is overestimated. Likewise, the product is underestimated in case of an insufficient quench of the deactivation. To test whether the deactivation quench is quantitative and sufficiently fast, the pure product should be synthesized and added to the quenching solution (See the experiment in Fig S3, S7). In the case of instantaneous quenching, the product concentration matches the amount added. Similarly, to test if the activation reaction is completely quenched, a solution of the precursor and fuel should be quenched and analyzed immediately after, and several hours after addition of the quench. If the product concentrations are

similar, the activation is completely stopped (See the experiment in Fig 3B).

Secondly, the quenching chemistry of the activation and deactivation should be orthogonal to each other. Finding such chemistry can be challenging. We thus describe our thought process when designing the benzylamine quench. The deactivation is a pseudo-first-order reaction (hydrolysis). That means that, realistically, there is only one reagent that can be quenched, *i.e.,* the anhydride. That severely limits the possible chemistry, and we therefore first designed chemistry for quenching the deactivation, *i.e.,* reaction with benzylamine. The activation is a second-order reaction between fuel and precursor. That offers two possibilities to quench the reaction, *i.e.*, we decrease the reactivity of the precursor or the fuel. We thus tested possible chemistries while keeping in mind the orthogonality to the benzylamine quench of the deactivation. We chose to decrease the reactivity of the fuel by increasing the pH, which is orthogonal.

Finally, to quench reactions, there are several possible chemical strategies. One can add a reactive species that yields a stable product (benzylamine in the case of the anhydride) or one can change the environmental conditions to quench reactions (change the pH or temperature).

Conclusion

We introduced a method to quench a carbodiimide-driven reaction cycle. This reaction cycle is frequently used by us and others to drive the dissipative self-assembly of molecules or nanoparticles. We found that the quenching method can successfully stop all reactions in the cycle. Moreover, we described that the quench works both for reaction cycles that yield assemblies or not. Finally, we showed that the quench can be applied to a diverse set of anhydrides. We trust that the method will be further used to more accurately, more conveniently, and more reliably analyze carbodiimide-driven chemical reaction cycles. Moreover, we believe that our work also lays the groundwork for the development of quenching methods for other reaction cycles that are used to fuel dissipative assemblies. In future work, we aim to further facilitate the quench using fluorogenic amines. In such an assay, the concentration anhydride can be read-out using plate reader fluorescent assays. Finally, we foresee that the described quenching method will help with the further acceleration of the reaction cycles and the search for new dynamic behavior of chemically fueled assemblies.

Acknowledgments

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Keywords: Chemically fueled self-assembly • Reaction cycles • Dissipative self-assembly

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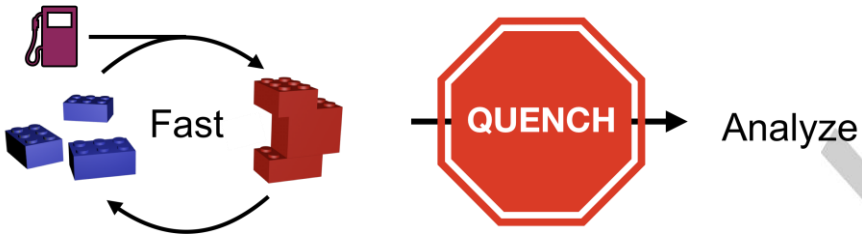
Counting bricks in the wall:

Chemically fueled assemblies are regulated by a chemical reaction cycle. A fast reaction cycle was recently introduced that shows exciting, dynamic self-assembly behavior. However, analysis of its kinetic properties is challenging due to its speed. Thus, we introduce a simple, powerful method to quench all reactions in the reaction cycle. We show the accuracy and application for several reaction cycles and a range of molecular assemblies.



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A Method to Quench Carbodiimide-Fueled Self-Assembly

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Abstract: In chemically fueled self-assembly, the activation and deactivation of molecules for self-assembly is coupled to a reaction cycle. In biological examples, these reactions are typically fast, such that the building blocks remain activated for mere seconds. In contrast, synthetic reaction cycles are slower for self-assembly, *i.e.,* with half-lives on the order of minutes. In search of life-like, dynamic behavior in synthetic systems, several groups explore faster reaction cycles that form transient labile building blocks with half-lives of tens of seconds. These cycles show exciting properties, but brought about a new challenge, *i.e.*, accurately analyzing the fast cycle is impossible with classical techniques. We thus introduce the notion of quenching chemical reaction cycles for self-assembly. As a model, we use the fast carbodiimide-fueled chemical reaction cycle and demonstrate a method that quenches all reactions immediately. We show its accuracy and demonstrate the application for several reaction cycles and a range of dissipative assemblies. Finally, we offer preliminary design rules to quench other chemically fueled reaction cycles.

Introduction

In molecular self-assembly, molecules are designed to interact with each other via non-covalent interactions.[1] The range of molecular assemblies that consequently form is enormous, ranging from anisotropic fibers,[2] tapes,[3] and tubes,[4] to isotropic assemblies like (block-copolymer)[5] micelles,[6] vesicles[7] and colloids.[8] The field has slowly evolved from rather serendipitous findings to well-defined design rules allowing for a range of applications, so-called supramolecular materials.[9] Inspired by biology, the field has recently focused on the assembly of molecules, driven out of equilibrium by energy-consuming chemical reaction cycles.[10] In these chemically-fueled assemblies, the assembly and disassembly of molecules is coupled to a chemical reaction cycle.[11] The reaction cycle comprises at least two reactions: a building block activation and deactivation reaction (Figure 1A). A precursor molecule is converted into a building block for self-assembly at the expense of a high energy reagent (chemical fuel) in the activation. In the deactivation, the building block is reverted to the precursor spontaneously, *e.g.,* by solvolysis or by reaction with an abundant species in solution. The sum of the reaction thus means that a precursor is temporarily converted into a self-assembling building block driven by the conversion of a molecule of fuel.

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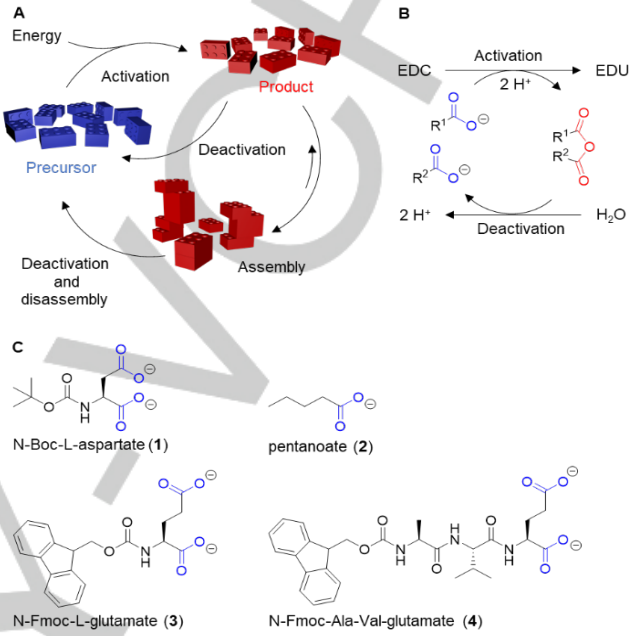

Figure 1. Chemical reaction cycle that fuels self-assembly. A) A generalized reaction scheme for chemically fueled self-assembly. B) The formation of a transient anhydride product from a carboxylate precursor is driven by the hydrolysis of EDC as carbodiimide fuel. C) Precursors used in this work as precursors for the chemical reaction cycle.

The consequence of the fuel-driven self-assembly is that the assembly and disassembly rates are now, in part, regulated by the rates in the chemical reaction cycle. For example, the cytoskeletal microtubules and actin filaments are tightly regulated by the hydrolysis of GTP and ATP, respectively.[12] Because of the kinetic control over self-assembly, their corresponding behavior is rich and includes dynamic instabilities,[13] as well as the treadmilling of actin filaments. It is important to note that the reaction rates of deactivation in these biological examples are fast. For example, tubulin-bound GTP has a half-life of less than a second.[14]

Inspired by the dynamic behavior of biological chemically fueled assemblies, researchers have developed synthetic counterparts. Exciting behaviors of these assemblies have been described, which include solutions that spontaneously oscillate between different types of assemblies,[15] molecular fibers that undergo dynamic instabilities,[16] and molecular assemblies that can select molecules from a pool of reactants.[17] One of the challenges in the synthetic approach towards dynamic materials is the lack of fast chemical reaction cycles. For example, the early work by van Esch and Eelkema used the carboxylic acid alkylation and its hydrolysis as a chemical reaction cycle.[18] At a pH of 10, the product had a half-life of roughly 0.3 hours.[16] To accelerate the rates of activation and deactivation, our group and the group of Hartley recently introduced the activation of carboxylates into

transient anhydrides driven by the hydrolysis of carbodiimide-based condensing agents like 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Figure 1B).[19] These anhydrides are prone to hydrolysis resulting in a half-life ranging from several minutes to tens of seconds, depending on the nature of the anhydride. We indeed observed that such systems had high dynamics with rapid assembly and disassembly.[20] The carbodiimide fuel has been further explored by us and others to yield symmetric,[19c, 21] and asymmetric anhydrides,[19a, 20, 22] activated esters like N-hydroxysuccinimide esters,[23] and phenol esters.[24] Due to the fast reaction cycle with tunable kinetics, the carbodiimide driven cycle has successfully been used for the self-assembly of peptide-based gels,[19a] catalytic nanofibers,[25] vesicles,[20], coacervates,[26] and others. However, as the hydrolysis rate of the product increased, we encountered a new challenge: the techniques we used for quantification (HPLC and NMR) are not able to accurately measure concentration because of the rapid deactivation of the compound of interest. For example, when a labile anhydride retains on the HPLC column, we observed significant amounts were hydrolyzed. Similarly, the acquisition times of NMR were too long to acquire reliable spectra of the compounds related to the reaction cycle. Other research groups have also introduced new chemical reaction cycles with fast kinetics. For example, Hermans recently introduced a redox-chemistry driven reaction cycle that operates with half-lives of minutes.[27] Likewise, Fletcher uses redox coupled (autocatalytic) reaction cycles that operate on minute timescales. These fast reaction cycles potentially also suffer from the inaccurate and complicated kinetic analysis by HPLC or NMR as they are further accelerated in the future.

In this work, we thus introduce the notion of quenching chemical reaction cycles to perform kinetic analysis. We describe a simple method to accurately analyze several carbodiimide-fueled chemical reaction cycles. We used a reagent that can almost immediately stop all four reactions taking place in the reaction cycle. We demonstrate that quenching chemistry can be applied to several types of anhydrides and works in the presence or absence of assemblies. Due to the effectiveness of the chemistry, the quenched samples can either be stored for up to several weeks or directly be analyzed. Finally, we establish design rules for quenching chemistry for chemical reaction cycles. Our method opens the door to further accelerate chemical reaction cycles and explore rapid dynamic behavior while retaining a quantitively understanding of the reactions involved.

Results and Discussion

Chemically fueled reaction cycles that can drive self-assembly comprise at least two reactions: a chemical reaction that activates building blocks for self-assembly and one that deactivates them (Figure 1A). In the carbodiimide driven reaction cycle, the precursor in the cycle carries at least one carboxylate (Figure 1B). In the first reaction, the precursor's carboxylate reacts with a carbodiimide based condensing agent to form the corresponding O-acyl urea intermediate (Figure S1). In the second reaction, the O-acyl urea reacts with a second nucleophile, *i.e.*, an intra- or inter-molecular carboxylate yielding, respectively, an intra- or inter-molecular anhydride. Regarding asymmetric precursor molecules like **1** the ring closure is favored compared to dimer formation because of the near proximity of the second carboxylic group. In the aqueous environment, the anhydride product is rapidly hydrolyzed to the corresponding precursor, which constitutes the deactivation reaction. Finally, the O-acyl urea can hydrolyze directly to the precursor without forming the anhydride (Figure S1). In summary, the overall reaction cycle forms a transient anhydride driven by the hydrolysis of a carbodiimide condensing agent into its corresponding urea.

In this study, we use 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) as a condensing agent because of its high solubility in water, but other work has shown that this cycle can also be driven by other carbodiimide derivatives. In the first part of these studies, we use N-Boc-L-aspartate (**1**) as a precursor molecule (Figure 1C). Its rapid deactivation makes it a great test compound for our quenching chemistry. In order to accurately determine the rate constant of deactivation for the reaction cycle in Scheme 2A, we synthesized N-Boc-L-aspartic anhydride (see synthesis section in SI). The powder was dissolved in acetonitrile and further diluted in 200 mM aqueous MES buffer at pH 6. After dilution, the decreasing concentration of anhydride was monitored by HPLC. We were surprised to find that the anhydride concentration of 20 mM had decreased from 20 to 7.5 mM after the first 30 seconds in the reaction cycle (Figure 2B). From there on, the concentration decayed with first-order kinetics. We could not fit the unconventional decay properly using first-order hydrolysis kinetics. Thus, we reasoned that the peculiar kinetics was not due to hydrolysis of the anhydride in the reaction mixture, but rather due to hydrolysis in the mobile phase of the HPLC. If additional hydrolysis in the mobile phase occurred, the concentration anhydride measured would be drastically lower than at given times in the reaction mixture. To test this hypothesis, we injected N-Boc-L-aspartic anhydride dissolved in dry acetonitrile into the HPLC. Even though 20 mM of the anhydride was injected, only 11 mM anhydride was detected, pointing to a loss of over 40% of the initially injected anhydride.

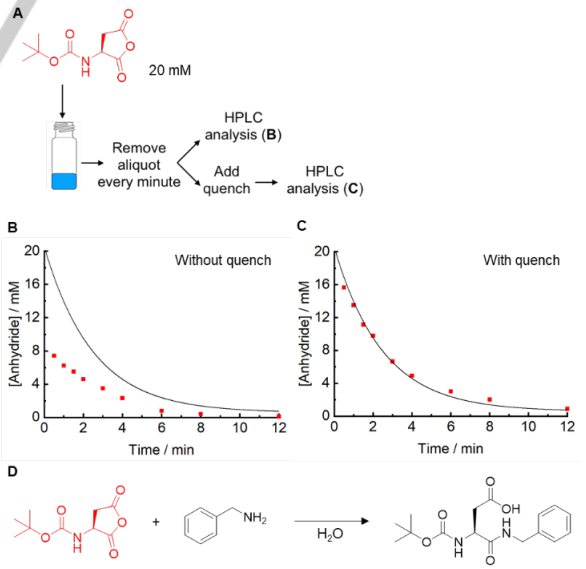

Figure 2. Does the anhydride hydrolyze during the HPLC analysis? A) The hydrolysis reaction of the activated precursor **1**. We injected 20 mM N-Boc-L-aspartic anhydride in water at pH 6. B-C) The concentration anhydride against time as measured by B) directly injecting the reaction mixture, and C) injecting a quenched reaction mixture into HPLC. The black line represents a first-order decay profile as the best fit. D) The quenching solution converts the anhydride of **1** into a stable N-benzyl amide.

Moreover, when we increased the anhydride retention time, by changing the HPLC-method, we observed less anhydride (Figure S2) confirming that the labile anhydride rapidly hydrolyzes during the analysis resulting in inaccurate data. Given that the acquisition times for 1H-NMR are typically in the range of several minutes, it is expected that following the hydrolysis of the anhydride by 1H-NMR would not yield much more accurate data. We thus set out to introduce a quenching method that rapidly stops all the reactions in our reaction cycle. We were seeking a method that (i) reacts with the anhydride rapidly to a stable product, (ii) stops the activation reaction, and (iii) reacts with the fuel to stop any side reactions. We used benzylamine as a reagent in our method because: (i) it rapidly reacts with the anhydride forming the stable amide. It will also react with the O-acyl urea forming the same amide. (ii) Due to its basicity, it will immediately increase the pH of the reaction mixture, thus inhibiting the pH-sensitive activation. Moreover, (iii) we found that benzylamine slowly reacts with EDC to the non-reactive guanidine moiety, stopping any further side reaction. Finally, the products (guanidine-derivative and N-benzyl amides) will carry the aromatic benzyl ring, which vastly facilitates HPLC-detection at 254 nm.

We repeated the above-described experiment (20 mM N-Boc-L-aspartic anhydride) and removed aliquots from the reaction mixture at given time points, which we injected in a quenching solution (400 mM benzylamine in water at pH 12). After the quench, we determined the concentration of the stable amide as a measure for the anhydride concentration at the time benzylamine was added (Figure 2C-D). The opening of asymmetric anhydrides formed by precursors like **1** yields two products, which is the benzylamine added either at the sidechain or the backbone of the amino acid. When determining the anhydride concentration, it is not necessary to discriminate between both products and thus the whole signal area assigned to the corresponding benzyl amide is considered for calculation. This method yielded much higher quality data that could be fitted with first-order decay. Another advantage of the quenching method became apparent. Without the quenching chemistry, each data point in the time series required the preparation of a new sample. For example, when the 15 minute HPLC method was finished with determining the concentration anhydride in the first data point (minute 1), all anhydride had hydrolyzed, and a new sample had to be started to measure the concentration in a second data point. With the quenching method, each time interval, an aliquot could be injected into the benzylamine solution. That solution could be stored and analyzed later. Finally, it is worth mentioning that the reaction of the anhydride with the benzylamine is fast, and all anhydride is converted within a minute after addition (Figure S3). Since full conversion was detected, we conclude that the amide formation outcompetes the accelerated hydrolysis reaction at an elevated pH. With this simple quenching method, we determined the half-life of the anhydride to be two minutes under these conditions.

Besides quenching the deactivation reaction, all other reactions in the cycle have to be quenched too. To test the effects of benzylamine on the activation reaction, we reacted N-Boc-L-aspartate (**1**) in aqueous buffer at pH 6 with EDC. After 1 minute, the entire solution was quenched by the addition of benzylamine. We measured that the quenched sample contained 6 mM of N-Boc-aspartate (**1**), 3 mM of amide (Figure 3A-B), and 36 mM of EDC (Figure 3C-D). We were interested in the activity of the remaining EDC in the quenched solution, *i.e.*, is EDC in the presence of benzylamine able to form new anhydride and thereby compromise our measurements? To test the reactivity of the remaining EDC, we followed the concentration of **1**, its corresponding N-benzyl amide, and EDC over the course of 10 hours at room temperature. We were surprised to find that the EDC was fully consumed within 10 hours, but no conversion of new anhydride or amide was apparent (Figure 3A-B). We explain the lack of the conversion of the acid to the anhydride by a sudden increase in pH upon quenching the reaction mixture (Figure S4). The O-acyl urea formation is highly pH-dependent, with the highest reactivity around pH 2 - 4, depending on the carboxylic acid (Figure S5). At the pH of the quenching solutions, the activation reaction was completely halted. In contrast, we found that EDC reacted with benzylamine to form its guanidine-derivative (Figure 3C-D). The HPLC data shows that this transformation was completed within 10 hours. Because this reaction did not produce further side products, the concentration EDC at the time of the quench could be determined accurately by measuring the remaining concentration of EDC and the concentration of the guanidine derivative.

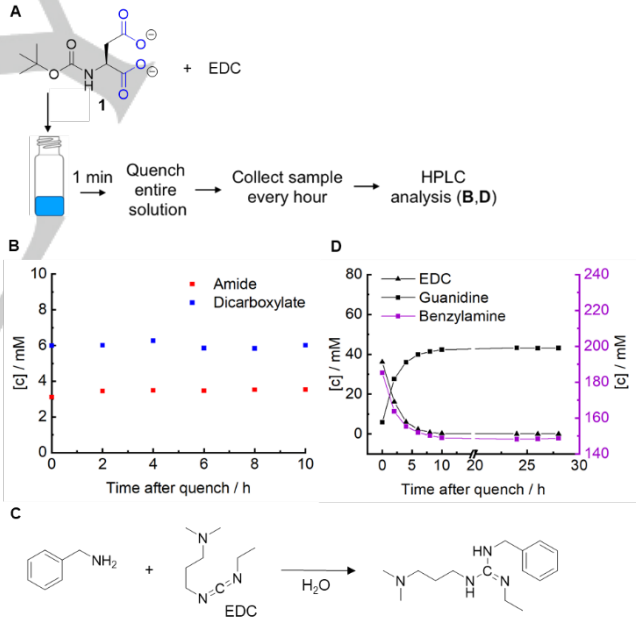

Figure 3. Does the quench stop our activation reaction? A) The activation reaction converts the dicarboxylate of **1** into its corresponding anhydride by the consumption of EDC. B) The concentration of dicarboxylate and N-benzyl amide against time after quenching the activation reaction. C) The reaction of EDC with benzylamine to form a stable guanidine-derivative. D) The concentration of EDC, benzylamine, and the guanidine-derivative against time as measured by HPLC. The markers represent HPLC data, and the lines are added to guide the eye.

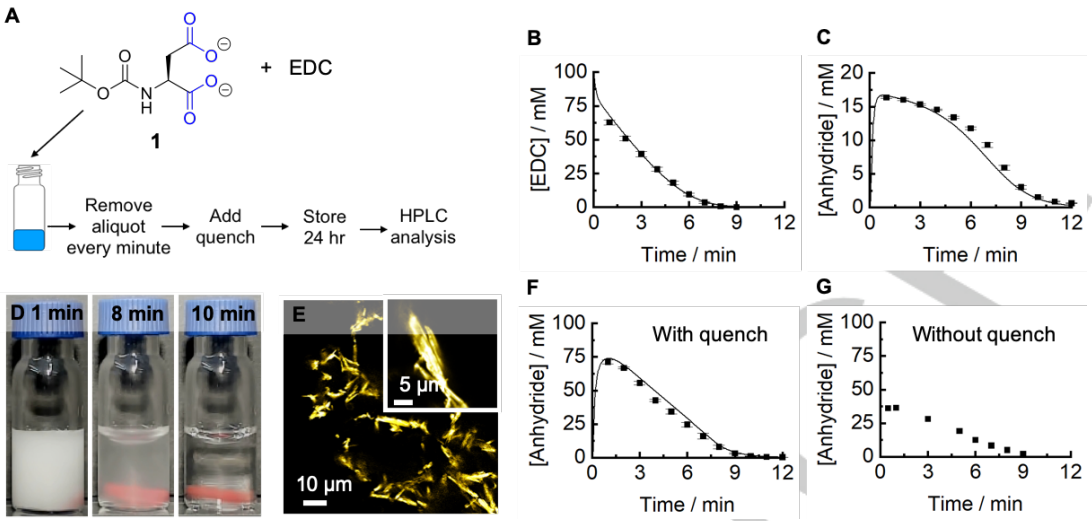

Figure 4. Analysis of a carbodiimide fueled reaction cycle with N-Boc-L-aspartate (**1**) as a precursor. A) Schematic overview of our quenching experiment. The concentration of B) EDC and C) anhydride against time as measured by HPLC. Initial concentration was 100 mM in EDC and 20 mM in **1**. The markers represent HPLC data, and the lines are predicted values from our kinetic model. The error bars depict the standard deviation from the average (n = 3). D) Time-lapse photography of a solution of 100 mM EDC and 100 mM N-Boc-L-aspartate (**1**). E) Fluorescence microscopy micrographs of the turbid solution. F) The concentration anhydride against time as measured by injecting quenched aliquots into HPLC. G) The concentration anhydride against time as measured by directly injecting the reaction mixture into HPLC.

Next, we set out to test the quenching method on the full chemical reaction cycle using EDC as fuel and **1** as a precursor (Figure 4A). To analyze the kinetics of the chemical reaction cycle, we added 100 mM EDC to 20 mM precursor dissolved in 200 mM MES buffer at pH 3.5 and, every minute, quenched an aliquot of the mixture. The quenched samples were stored overnight and analyzed by HPLC (Figure 4A-C). One downside of our method is that it cannot discriminate between the intermediate O-acyl urea or the anhydride product, *i.e.,* both will react to form the benzylamide. Nevertheless, due to the fast conversion of the O-acyl urea to the anhydride, only very low concentrations of the O-acyl urea are present in the reaction mixture. For example, our model calculates the concentration O-acyl urea to 0.63 mM in comparison to 17 mM of anhydride within the first minute. Thus, less than 5% of the amide formed stems from the O-acyl urea.

The assay showed that in the reaction mixture, the EDC concentration dropped rapidly in the first minute, after which it decayed linearly. As the EDC was being consumed, the anhydride concentration rapidly increased to 16 mM, while the precursor concentration dropped to 4 mM. Because a large fraction of the precursor was converted, the anhydride concentration settled to a pseudo-steady state. The pseudo-steady state implies that activation and deactivation occur at almost equal rates. This state was sustained for about 5 minutes, which is the time until most EDC had reacted. After that, the concentration anhydride decayed until it had all hydrolyzed. When we used a simple kinetic model, we could accurately predict the concentrations of EDC, precursor, and anhydride. From the kinetic model, we calculated an anhydride half-life ($\ln(2) / k_{hydrolysis}$) of 50 seconds. This value differs from the above mentioned 2 minutes because of the increased water content. It is worth mentioning that we found no evidence of self-assembly when we mixed 20 mM precursor with 100 mM EDC.

We tested if the supramolecular structures formed by the activated precursor molecules interfere with our quenching method. Specifically, we were interested to see whether the benzylamine quench was sufficiently fast to simultaneously dissolve and amidate the anhydrides in an assembly. In contrast to the experiment above, we used 100 mM of N-Boc-L-aspartate (**1**) precursor with 100 mM of EDC. The higher precursor concentration allows for the formation of more anhydride than the experiment above. We found that the solution almost immediately turned turbid (Figure 4D). The increased turbidity was present for roughly eight minutes, after which the solutions turned clear. Fluorescence microscopy of these turbid solutions revealed the presence of micron-sized rectangular crystals, which explains the increased turbidity (Figure 4E). We measured the concentration anhydride and fuel with the method described above. It is worth noting that the turbid solution turned transparent immediately upon injecting it in the benzylamine quenching solution, pointing to a rapid dissolution of the anhydride. We found that all fuel was consumed within two minutes (Figure S6), after which the anhydride decayed linearly (Figure 4F). We explain the linear decay with a self-protection mechanism that we described in earlier work.[22b] In the self-protection mechanism, the hydrolysis reaction only takes place on the fraction of anhydride that remains in solution after self-assembly. Thus, when the concentration anhydride passes its solubility, the hydrolysis converts from first-order kinetics to zero-order kinetics. We incorporated the self-protection mechanism into our kinetic model and could accurately predict the HPLC data. Importantly, the same rate constants were used in the model as described above, which is a good indication that the benzylamine quench is effective in samples that contain assemblies or not.

Finally, we aim to show the generality of our quenching method, both for different types of activated carboxylate precursors and different types of assemblies. First, we tested the precursor pentanoate (**2**), which forms the non-cyclic pentanoic anhydride (C_5C_5) when activated by EDC. Several research groups, including ours, have used symmetric anhydride formed carbodiimide-fueled chemical reaction cycles to drive dissipative self-assembly.[19b, 21a, 23a] Given the slightly different reactivities of

these anhydrides, we were first interested in whether our quenching method would yield the N-benzyl amide. We prepared a solution of 10 mM pentanoic anhydride in acetonitrile and quenched the mixture with benzylamine. The approximately 10 mM of amide measured (Figure S7) serves as strong evidence that the quenching method can also be adapted to anhydrides containing weaker electrophilic centers. To test if the method also works on the fueled reaction cycle with pentanoate (**2**) as the precursor, we fueled 300 mM of **2** at pH 6.0 with 10 mM of EDC and tested the evolution of the anhydride and EDC concentration (Figure 5A, S8). These conditions reflect earlier work we carried out on this precursor.[19c] Even though the solution forms droplets (Figure 5B-C), we found that the quenching method yielded reliable data.

Fmoc-protecting groups are frequently used in the design of self-assembling peptides,[28] and have also been used in the carbodiimide-driven reaction cycles.[19a, 22b] We thus tested our quenching method on peptide precursors that contained this group, *i.e.*, N-Fmoc-L-glutamate (**3**) and N-Fmoc-Ala-Val-glutamate (**4**). It should be noted that these protecting groups are labile under basic conditions, which resulted in partial deprotection by our quenching chemistry. We showed that the deprotection can be reduced to an insignificant amount by using a less concentrated quenching solution, such that the pH does not exceed 9 (Figure S9, S10).

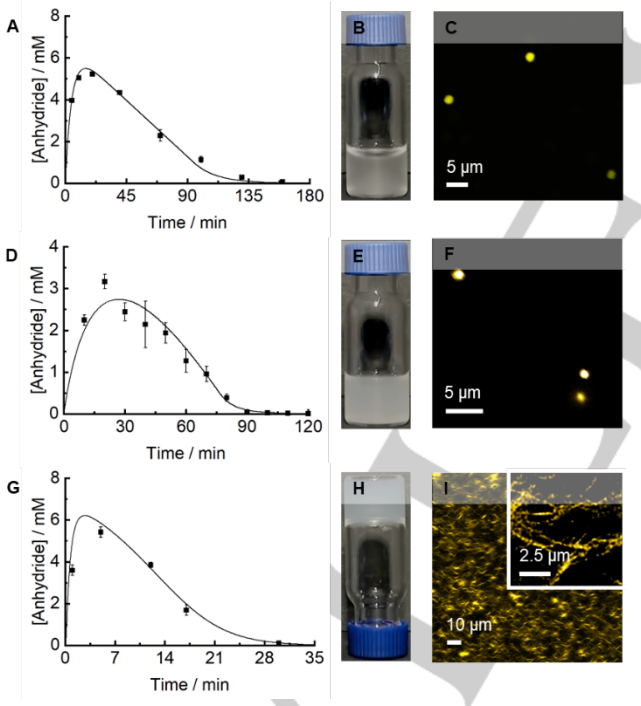

Figure 5. Analysis of different self-assembling reaction networks by our quenching method. A-C) Dissipative self-assembly of 300 mM pentanoate (**2**) fueled with 10 mM EDC. A) The concentration anhydride against time as measured by HPLC. The markers represent HPLC data, the lines are predicted values from our kinetic model. The error bars depict the standard deviation from the average (n = 3). B) Photograph of a turbid solution. C) Fluorescence microscopy micrograph of the turbid solution. D-F) Dissipative self-assembly of 10 mM N-Fmoc-L-glutamate (**3**) fueled with 10 mM EDC. D) The concentration anhydride against time as measured by HPLC. E) Photograph of a turbid solution. F) Fluorescence microscopy of the turbid solution. G-I) Dissipative self-assembly of 10 mM N-Fmoc-Ala-Val-glutamate (**4**) fueled with 50 mM EDC. H) Photograph of a self-sustaining hydrogel. I) Fluorescence microscopy of the hydrogel.

Nevertheless, the benzylamine remained in a 20-fold excess relative to the maximum anhydride concentration. In addition to that we stored the quenched samples at 8 °C. With the adjusted method, we measured the kinetics of the reaction cycle when 10 mM N-Fmoc-L-glutamate (**3**) was fueled with 10 mM EDC at pH 6.0 (Figure 5D, S11). Moreover, **3** self-assembled into colloids in response to the fuel, further demonstrating the versatility of our method (Figure 5E-F). To further demonstrate the generality of our method, we measured the kinetics of the chemical reaction cycle with N-Fmoc-Ala-Val-glutamate (**4**) as a precursor at pH 6.0. Upon EDC fueled activation, the peptide precursor can self-assemble into fibers, which subsequently yields a hydrogel (Figure 5H-I).[19a] We were particularly interested if the increased viscosity of the material interfered with our quenching method. We used a wide-bore pipette to remove samples and analyzed those by HPLC after applying our quenching method (Figure 5G, S12). The kinetic data showed close correspondence to the predictions by our kinetic model.

Why should one use the benzylamine quenching method? The strongest argument in favor of the benzylamine quench is that it vastly improves the data quality. This argument is particularly valid for labile anhydrides that hydrolyze during the analysis. Even for somewhat more stable anhydrides (*e.g.*, C_5C_5, $t_{1/2}$ = 12 minutes), we found improvement in data quality when applying the quenching method (Figure S13). Furthermore, a strong argument for the quenching method is that it vastly facilitates data acquisition. Without the quench, the rate of data acquisition was determined by the length of the analysis method. Specifically, we can inject one sample in the HPLC every ~20 minutes. Thus, our data acquisition is limited to ~0.05 data points.min^{-1}. For ^{1}H-NMR that is somewhat faster, providing one acquisition every ~2 minutes (0.5 data points.min^{-1}). Due to the quenching method, the limiting factor of data acquisition frequency is the pipetting frequency. For example, with our pipetting robot, the highest data acquisition frequency is now 6 data points.min^{-1} (Figure S16). That means that samples can be collected in real-time and analyzed overnight, which significantly accelerates data throughput. Finally, an argument in favor of the benzylamine quenching method is that it further facilitates the detection of the anhydride product by converting an anhydride into the N-benzyl amide. The benzyl ring vastly facilitates the detection of the product by HPLC because of its absorbance at 254 nm.

The arguments in favor of using quenching chemistry above apply to other chemically fueled reaction cycles, too.[21a, 24, 29] To aid the broader community of scientists exploring the field of chemically fueled self-assembly, we set out some preliminary design rules for such quenching chemistry.

Firstly, quenching chemistry should be identified that immediately stops both the activation and deactivation reaction. If the activation is partly deactivated, the concentration product is overestimated. Likewise, the product is underestimated in case of an insufficient quench of the deactivation. To test whether the deactivation quench is quantitative and sufficiently fast, the pure product should be synthesized and added to the quenching solution (See the experiment in Fig S3, S7). In the case of instantaneous quenching, the product concentration matches the amount added. Similarly, to test if the activation reaction is completely quenched, a solution of the precursor and fuel should be quenched and analyzed immediately after, and several hours after addition of the quench. If the product concentrations are

similar, the activation is completely stopped (See the experiment in Fig 3B).

Secondly, the quenching chemistry of the activation and deactivation should be orthogonal to each other. Finding such chemistry can be challenging. We thus describe our thought process when designing the benzylamine quench. The deactivation is a pseudo-first-order reaction (hydrolysis). That means that, realistically, there is only one reagent that can be quenched, *i.e.,* the anhydride. That severely limits the possible chemistry, and we therefore first designed chemistry for quenching the deactivation, *i.e.,* reaction with benzylamine. The activation is a second-order reaction between fuel and precursor. That offers two possibilities to quench the reaction, *i.e.*, we decrease the reactivity of the precursor or the fuel. We thus tested possible chemistries while keeping in mind the orthogonality to the benzylamine quench of the deactivation. We chose to decrease the reactivity of the fuel by increasing the pH, which is orthogonal.

Finally, to quench reactions, there are several possible chemical strategies. One can add a reactive species that yields a stable product (benzylamine in the case of the anhydride) or one can change the environmental conditions to quench reactions (change the pH or temperature).

Conclusion

We introduced a method to quench a carbodiimide-driven reaction cycle. This reaction cycle is frequently used by us and others to drive the dissipative self-assembly of molecules or nanoparticles. We found that the quenching method can successfully stop all reactions in the cycle. Moreover, we described that the quench works both for reaction cycles that yield assemblies or not. Finally, we showed that the quench can be applied to a diverse set of anhydrides. We trust that the method will be further used to more accurately, more conveniently, and more reliably analyze carbodiimide-driven chemical reaction cycles. Moreover, we believe that our work also lays the groundwork for the development of quenching methods for other reaction cycles that are used to fuel dissipative assemblies. In future work, we aim to further facilitate the quench using fluorogenic amines. In such an assay, the concentration anhydride can be read-out using plate reader fluorescent assays. Finally, we foresee that the described quenching method will help with the further acceleration of the reaction cycles and the search for new dynamic behavior of chemically fueled assemblies.

Acknowledgments

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Keywords: Chemically fueled self-assembly • Reaction cycles • Dissipative self-assembly

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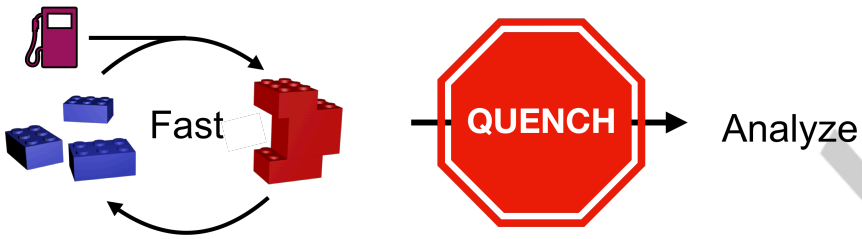

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