Guiding Transient Peptide Assemblies with Structural Elements Around Abiotic Phosphate Fuels

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ABSTRACT: Biochemical energy carriers, such as triphosphates (ATP, GTP) drive selective processes, by incorporating chemical information (Adenine *vs.* Guanine) in their structure. These recognition elements match with complex machineries through a variety of non-covalent interactions, enabling specific targeting of functions. In contrast, most approaches in non-equilibrium systems do not consider the structure of the fuel as a critical element to control the processes. Herein, we show that the amino acid side chains (A, F, Nap) in the structure of activated aminoacyl phosphate esters can direct assembly and reactivity in the context of non-equilibrium structure formation. We focus on the ways in which the activated amino acids guide structure formation and how structures and reactivity cross regulate when constructing different assemblies. Through the chemical functionalization of energy-rich aminoacyl phosphate esters, we are able to control the coupling yield to esters and thioesters upon adding dipeptides containing tyrosine or cysteine amino acid residues. The structural elements around the phosphate esters guide the lifetime of the structures formed and their supramolecular assemblies, which can further be influenced by the structure and reactivity of dipeptide substrates. Moreover, we demonstrate that an aminoacyl phosphate ester incorporating a tyrosine residue (Y) can autonomously generate a pool of high energy molecules, where dynamic oligomerization and de-oligomerization of esters occurs in a single step process. These findings suggest that activated amino acids with varying reactivity and energy contents can pave the way for designing and fabricating structured fuels.

INTRODUCTION

Biochemical phosphates activate specific tasks by incorporating structural and recognition elements in their chemical structure.[1] Adenosine triphosphate (ATP) powers motor proteins,[2] while guanosine triphosphate (GTP) orthogonally activates microtubule assembly.[3] The triphosphate region in these structures reduces the propensity towards hydrolysis,[4] while the nucleoside region enables selective binding of biocatalysts.[5] This accurate match is further assisted through an array of non-covalent interactions, giving rise to selective targeting of functions.[6] In addition to phosphoric anhydrides, biology has chosen various other phosphate-containing molecules, like creatine or carbamoyl phosphate to drive diverse metabolic processes[7] - thus demonstrating its ability to accommodate structural diversity. This structural variation in turn, enables the activation of specific biological functions without any interference. Biochemical phosphates have inspired chemists to move from traditional systems which function under thermodynamic control,[8-9] to chemical reaction networks,[10-11] where kinetic effects[12-13] and non-equilibrium pathways[14-15] become important. Thus, biotic phosphates[16] have been used in the form of ATP [2, 17-18] (as a signal or as a co-assembling molecule) or DNA strands[19] to drive non-equilibrium processes. Some of these strategies utilize amino acid residues featuring hydroxyl groups, which can in turn be employed to access supramolecular assemblies via phosphorylation and de-phosphorylation cycles.[20] Moreover, acyl phosphates[21] and phosphoramidates[22] have recently been applied towards dissipative assemblies aided by carbodiimides.[23] Despite the great progress on the construction of chemically driven assemblies,[24-30] currently, most approaches do not consider the structure of the fuel as a critical element to control the processes. Current strategies mainly focus on variations in the chemical structure of the substrates to tune the formation of supramolecular assemblies.[31-32] The discovery of fuels, capable of incorporating structural and recognition

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elements in their structure remains challenging.[1] The reason that remains challenging is associated with stability of activated molecules in different environments, the presence of water which tends to hydrolyze labile intermediates, the management of side reactions and the use of enzymes, which often block recognition targets.

Inspired by biochemical phosphates, herein we design abiotic phosphates capable of incorporating structural elements in their structure. We demonstrate that the amino acid side chains (**A**, **F**, **Nap**) in the structure of aminoacyl phosphate esters direct the lifetime, the mechanical properties and the structural organization of esters and thioesters upon adding tyrosine or cysteine-containing peptides. Transferring energy and reactivity from the molecularly dissolved acyl phosphates, self-assembly protects the newly formed peptide derivatives from hydrolysis. This process allows for the transient formation of various supramolecular structures, such as fibers, tapes and sunflower-like fibrillar assemblies (Figure 1a). Furthermore, the incorporation of a tyrosine residue (**Y**) around the phosphate esters leads to autonomous oligomerization and de-oligomerization of esters in one-pot (Figure 1b). Our findings suggest that structured abiotic phosphates can be utilized to guide assembly and induce reactivity changes in the context of non-equilibrium structure formation.

RESULTS AND DISCUSSION

Design of phosphate-driven peptide assemblies. We previously demonstrated that aminoacyl phosphate esters can be utilized for the spontaneous and selective oligomerization of peptide bonds in water, where the hydrophobicity in the structure of monomers dictates the length of the oligomerization and the composition in different phases.[33] Herein, we focus on designing activated molecules, which can impact non-equilibrium processes. Thus, we design systems considering the ways in which activated molecules can: 1) incorporate structural elements in their structure but are not capable of pre-organization, 2) guide structure formation upon transferring energy and reactivity into other species and 3) interact with various substrates (peptide nucleophiles), which can further alter the pathway and contribute to the assembly process up coupling. For the latter point, we investigate how the exact nature of the chemical bonds involved influence reactivity, and most importantly how structures and reactivity cross regulate.

In order to satisfy these criteria, we use aminoacyl phosphate esters, where the phosphate esters are capable of solubilizing hydrophobic amino acid derivatives in an aqueous environment. Around the phosphate esters, we incorporate aliphatic (alanine), aromatic natural (phenylalanine), and non-natural amino acids (naphthylalanine). The amino acids are capped in the N-terminus with a Cbz (carboxybenzyl) group that has previously been utilized to assist the formation of supramolecular assemblies.[34] In order to generate labile bonds,[35] we use dipeptides consisted of tyrosine (-OH) or cysteine residues (-SH) to favor the formation of esters and thioesters respectively. These sequences are capped at the N-terminus with an acetyl group to prevent amide bond formation. On the C-terminus, various amino acids are used to impact the assembly and lifetime of the structures formed upon coupling.

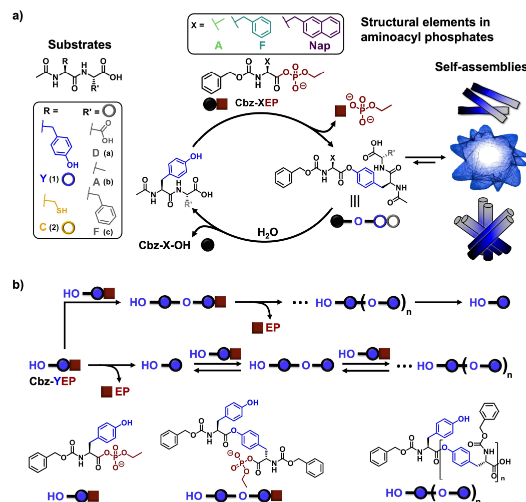

Figure 1. a) Schematic representation of the construction of transient peptide assemblies from the reaction between **Cbz**- phosphate esters (**Cbz-XEP**) and dipeptide substrates featuring tyrosine (**Ac-YX-OH**) or cysteine residues (**Ac-CX-OH**). Formation of various assemblies is directed upon the formation of transient esters or thioesters. Filled color spheres represent the amino acids in the structure of aminoacyl phosphate esters and open spheres represent the amino acids in the structure of the dipeptide substrates. **b)** Oligomerization and de-oligomerization of sequences featuring esters bonds resulting from **Cbz-YEP**, highlighting the different pathways of elongation and the chemical structures involved.

Guiding transient peptide assemblies from amino acyl phosphate esters. We started by investigating the hydrolysis of the aminoacyl phosphate esters in different pH using **Cbz-NapEP**, where **Nap** represents naphthylalanine and **EP** represents ethyl phosphate. Utilizing Ultraperformance liquid chromatography (UPLC), we determined that the half-life of the activated phosphate is 4 min in bicarbonate buffer (0.2 M pH 10.0). Upon hydrolysis, **Cbz-Nap-OH** and **EP** are formed. When the pH was reduced and borate buffer was used (0.6 M, pH 9.1), the half-life of **Cbz-NapEP** increased to 25 min (Supporting Figure S1). Throughout the reactions, the samples retain their macroscopic transparency, regardless of the pH used, indicating that the aminoacyl phosphate ester (**Cbz-NapEP)** and the free carboxylate amino acid derivative (**Cbz-Nap-OH**) remain in solution and do not aggregate.

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Next, we incorporated in the systems dipeptide substrates containing either tyrosine (**Ac-YX-OH**) or cysteine residues (**Ac-CX-OH**) with the aim of investigating the formation of assemblies upon coupling to esters and thioesters respectively (Figure 2a). We use the single letter code for the amino acids throughout our work. In the dipeptide sequences, X represents different amino acids, such as **D** (aspartic acid), **A** (alanine) and **F** (phenylalanine). We prepared samples by mixing different amino acyl phosphate esters (10 mM) with **Ac-YF-OH** (**1c,** 20 mM) in bicarbonate buffer (0.2 M pH 10.0). We use pH 10.0 considering the pKa of tyrosine.[36] Analysis of the samples using UPLC-MS indicate that the conversion to esters varies and increases with the hydrophobicity of the phosphate esters. Specifically, we observed the formation of 2.5 mM of the alanine ester, while the phenylalanine and naphthylalanine esters resulted in the formation of 6 and 7 mM respectively (Figure 2b and Supporting Figure S2). These findings suggest that the hydrophobic nature of the amino acid side chains within the structures of the aminoacyl phosphate esters plays an important role in influencing the yield of the coupling. The tendency of the formed esters to aggregate impacts the yield of the reaction.

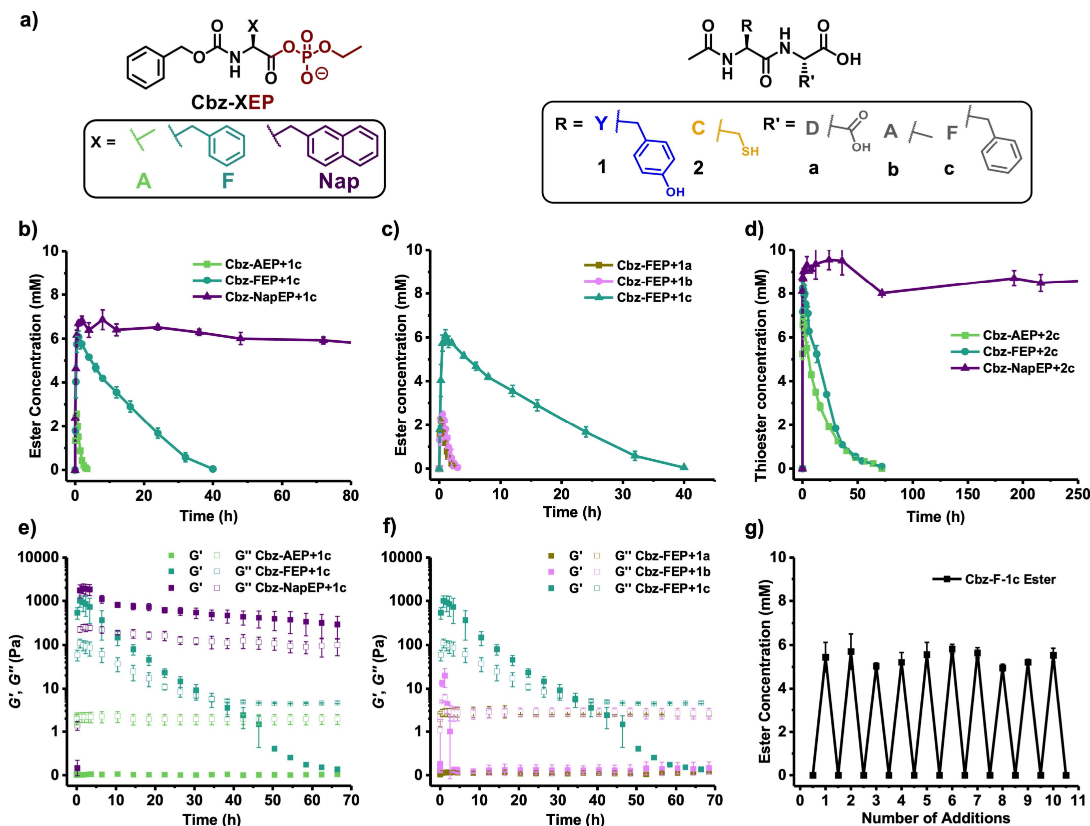

Figure 2. a) Chemical structures of aminoacyl phosphate esters and dipeptide substrates to drive non-equilibrium processes. Time-dependent ester formation from the reaction between **b)** 10 mM **Cbz-XEP** (X = **A**, **F** and **Nap**) and 20 mM **Ac-YF-OH** (**1c)** in bicarbonate buffer (0.2 M pH 10.0) and **c)** 10 mM **Cbz-FEP** and 20 mM **Ac-YX-OH** (X = **D**, **A** and **F**) (**1a-1c**) in bicarbonate buffer (0.2 M pH 10.0). In Figure 2c, the result of ester formation between **Cbz-FEP** and **Ac-YF-OH** is reproduced from Figure 2b for comparison. **d)** Time-dependent thioester formation of the reaction between 10 mM **Cbz-XEP** (X = **A**, **F** and **Nap**) and 10 mM **2c** (0.6 M pH 9.1). Storage and loss modulus as a function of time for the systems containing **e)** 10 mM **Cbz-XEP** and 20 mM **Ac-YF-OH** (**1c**) and **f)** 10 mM **Cbz-FEP** and 20 mM **1a-1c**. The solid squares represent the storage modulus (*G′*) and the open squares the loss modulus (*G″*). In Figure 2f, the result of the mechanical properties of **Cbz-FEP** with **Ac-YF-OH** is reproduced from Figure 2e for comparison. **g)** Ten consecutive additions of 10 mM **Cbz-FEP** with 20 mM **1c**. Conversion was determined 30 min after adding **Cbz-FEP**. NaOH was added to maintain the pH. In all graphs error bars represent the standard deviation of three experiments.

By replacing **Ac-YF-OH** (**1c**) with **Ac-YD-OH** (**1a**) or **Ac-YA-OH** (**1b**), the yield of the coupling was reduced, as more hydrophilic amino acids were incorporated into the transient ester structures (Figure 2c). For the cysteine containing peptides (**2a-2c**), we observed faster kinetics towards thioester formation, which is associated with the reactivity of the thiols (Figure 2d). These samples were prepared in borate buffer pH 9.1 considering the pKa of acetyl cysteine.[37] Moreover, in these conditions, the activated phosphates have longer half-lives compared to pH 10.0, thus could be consumed towards coupling rather than hydrolysis. The amount of thioesters formed was also

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dependent on the hydrophobicity of the amino acid side chains. Notably almost quantitative thioester formation was observed for the naphthylalanine derivative (9.5 mM) in presence of **2c**. High conversion was also noticed for the other peptide derivatives (**2a**, **2b**). As previously observed for the esters, the lifetime of thioesters was enhanced for the peptides containing hydrophobic amino acid residues. Overall, upon coupling, the lifetime of the structures formed can be tuned by chemical design (amino acid residues within the phosphate esters and dipeptides), resulting in lifetimes ranging from minutes to hours and even extending to days and months. The UPLC-MS analysis of all systems and their kinetic profiles are presented in Supporting Information (Supporting Figures S3-S42). In libraries containing the cysteine peptides and after thioester formation, we observed side reactions involving de-acetylation products (< 5%) (Supporting Figure S43). Time-dependent turbidity measurements (monitoring the absorbance at 600 nm) confirmed the phase transitions form soluble aminoacyl phosphates to the aggregated esters or thioesters, followed by the formation of transparent solutions upon hydrolysis (Supporting Figures S44 and S45). The macroscopic transitions coincided with the formation of supramolecular assemblies with tunable mechanical properties. Using rheology, we found that the stiffness was enhanced for systems containing hydrophobic peptides, (Figure 2e, f and Supporting Figure S46) which were capable of forming hydrogels ($G' > G''$). Following the reactions over time, we noticed that the stiffness was reduced and the systems behaved as solutions. The less hydrophobic peptide esters behaved like a weak gel or like a liquid ($G'' > G'$). The chemical structures and the macroscopic behavior of all systems containing the aminoacyl phosphate esters and the dipeptide substrates are available in the Supporting Information (Supporting Tables 1 and 2).

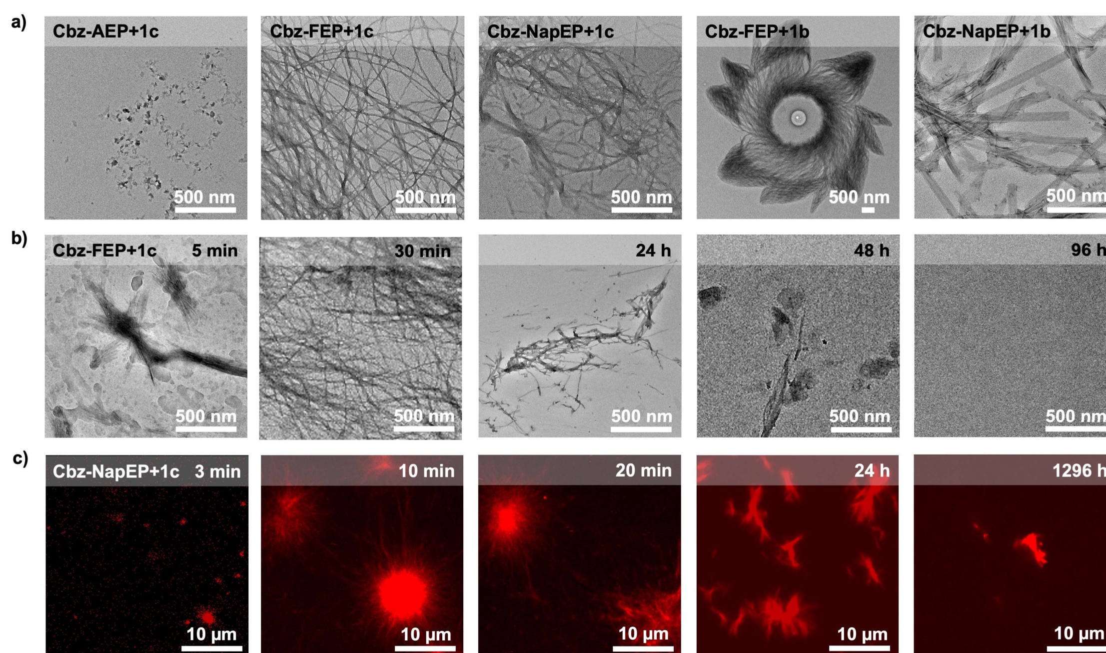

Figure 3. a) TEM images showing the formation of assemblies from the reactions: From left to right: 10 mM **Cbz-XEP** (X = **A**, **F**, **Nap**) with 20 mM **Ac-YF-OH** (**1c**)**,** followed by 10 mM **Cbz-FEP** with 20 mM **Ac-YA-OH** (**1b**) and 10 mM **Cbz-NapEP** with 20 mM **1b** in bicarbonate buffer (0.2 M pH 10.0). **b)** Time-dependent TEM images of 10 mM **Cbz-FEP** with 20 mM **1c** and **c)** Time-dependent confocal microscopy images of 10 mM **Cbz-NapEP** with 20 mM **1c** in bicarbonate buffer (0.2 M pH 10.0).

We moreover tested the possibility of reactivating the systems containing the esters and the thioesters, which were formed in a high yield. We focus on the systems containing **Cbz-FEP** and **1c**. UPLC analysis revealed that we are able to maintain high conversion to esters and sustain the macroscopic formation of hydrogels across 10 consecutive additions (Figure 2g). For the systems containing the cysteine peptides, we conducted similar experiments with **2c**. In order to prevent the construction of disulfides, which hinder thioester formation, we used TCEP (tris(2-carboxyethyl)phosphine) as a reducing agent. Our results demonstrate that thioesters could be formed for up to 5 additions (Supporting Figure S47). Overall, these findings suggest that hydrogels can form and break across various additions, where the background reaction does not affect gelation, which is a common issue on chemically-driven assemblies.[24] However, the number of additions we can perform is dependent on the properties of the nucleophiles, which can further participate in other reactions (such as disulfide formation) and impact the chemical systems. Our objective of incorporating structural elements (**A**, **F** and **Nap**) around abiotic phosphates and explore how amino acid residues can guide the transient formation of structures, motivated us to investigate the construction of assemblies

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using Transmission Electron Microscopy (TEM). Systems incorporating phenylalanine residues either into the structure of amino acyl phosphate esters (**Cbz-FEP**) or into the structures of dipeptide substrates (**1c**, **2c**) assembled into an entangled fibrillar network (Figure 3a). Over time, the fibers reduced both in length and density, as a result of the competing reaction involving ester or thioester hydrolysis (Figure 3b). Less hydrophobic residues around the phosphate esters (**Cbz-AEP**) lead to ill-defined aggregates in presence of **1c**. Notably, for the systems containing **Cbz-FEP** and **1b**, sunflower-like fibrillar assemblies were visualized (Figure 3a). For the most hydrophobic derivatives composed of naphthylalanine residues, fibers and tape-like assemblies were observed. Confocal microscopy images for the systems containing **NapEP** and **1c** (Figure 3c) revealed droplets at the early stages of the process. Over time, droplets reconfigured into fibers, which decreased in length and density. Additional TEM and confocal images for the systems containing amino acyl phosphate esters and dipeptides are available in the Supporting Information (Supporting Figures S48-S52), which further support the formation and destruction of assemblies from different mixtures.

Incorporating a tyrosine (Y) residue in the structure of aminoacyl phosphate esters. Having established the construction of chemically-driven esters and thioesters from aminoacyl phosphate esters using various dipeptide substrates, we then moved to systems where an aminoacyl phosphate ester can incorporate into its structure a reactive nucleophile and guide the formation of assemblies without the need of a substrate. Thus, we have synthesized the tyrosine derivative (**Cbz-YEP**), where the amino acid side chain (**Y**) around the phosphate esters can give rise to dynamic oligomerization and de-oligomerization of ester bonds in one pot (Figure 4a). Analysis of a 20 mM library using UPLC-MS, revealed at the early stages of the process the hydrolysis product (**Cbz-Y**), accompanied with the formation of a dimer featuring the phosphate moiety at the C-terminus (**$(Cbz\text{-}Y)_2$EP**). Over time longer oligomers were formed (Supporting Figures S39 and S40). In these libraries, self-ligation generates a pool of high energy molecules, where different pathways and nucleophiles can alter the dynamics and contribute to the self-assembly process. Throughout the time course of the reaction, we did not observe any re-phosphorylation. Nevertheless, in these systems, the continuous formation and hydrolysis of ester bonds maintain the dynamic nature of the process. Building libraries at different concentrations of **Cbz-YEP**, we observed that oligomerization is favored at higher monomer concentration. The phosphate esters allow us to solubilize the amino acid derivatives at high concentrations, which is challenging to achieve with other type of activating strategies.[38] Notably, at a concentration of 40 mM, the formation of oligomers up to a tetramer (**$(Cbz\text{-}Y)_4$**) was noticed (Figure 4b), which was also the dominant product of the library. The length of the oligomers in these libraries determines their supramolecular state. While the dimer (**$(Cbz\text{-}Y)_2$**) could be hydrolyzed over time to the single amino acid derivative (**Cbz-Y**), kinetically trapped assemblies were identified for the trimer (**$(Cbz\text{-}Y)_3$**) and tetramer (**$(Cbz\text{-}Y)_4$**) respectively (Figure 4c), where dense aggregates were macroscopically observed. Using confocal microscopy, we noticed that during the initial stages of the process poorly defined aggregates appeared. Subsequently these aggregates evolved into dense fibrillar assemblies, which, in turn partially disassembled (Figure 4d). We observed the formation of precipitates, primarily associated with the construction of longer oligomers. We confirmed the microscopic changes by monitoring the turbidity of the samples, which further support the ongoing formation and subsequent hydrolysis of esters (Supporting Figure S53).

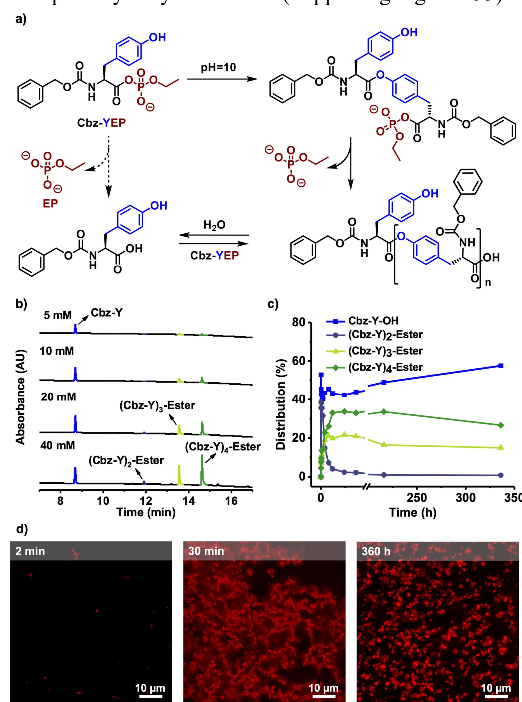

Figure 4. a) Chemical structures of species involved in formation and hydrolysis of esters using **Cbz-YEP**. **b)** Concentration-dependent UPLC chromatograms of **Cbz-YEP** in bicarbonate buffer (0.2 M pH 10.0). UPLC injections were performed after 12 h. **c)** Distribution of ester oligomers, resulting from 20 mM **Cbz-YEP** in bicarbonate buffer (0.2 M pH 10.0). **d)** Confocal images at different time points of the reaction resulting from 20 mM **Cbz-YEP** in bicarbonate buffer (0.2 M pH 10.0).

CONCLUSIONS

In this work, we focus on modifying the structure of high energy molecules to impact non-equilibrium process. Inspired by biochemical phosphates, we demonstrate the concept of installing structural elements (**A**, **F**, **Nap**) into the structure of abiotic aminoacyl phosphate esters to guide the lifetime and the structural organization of different types of assemblies, such as esters, thioesters and oligomers featuring ester bonds. The ability to regulate the yield of transient coupling, the lifetime of the resulting structures and consequently their supramolecular assemblies depends on the chemical structure of the phosphate esters and the reactivity (as well as the structure) of the peptide substrates. By incorporating a tyrosine residue (**Y**) around the aminoacyl phosphate esters, a pool of high energy

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molecules is autonomously generated. This process achieves dynamic formation and destruction of oligomers featuring ester bonds from a single building block and in a one-step process. Although it is currently challenging to predict which aminoacyl phosphate ester will induce a specific type of assembly[39] when transferred into other species, design rules are starting to emerge. These rules are associated with the hydrophobicity of the amino acid side chains within the structure of high energy molecules and substrates, where a delicate balance of supramolecular interactions can enhance or disrupt the construction of assemblies. We focus on understanding the chemical design space for the structural behavior of aminoacyl phosphate esters by exploring variations in the amino acid side chains, at the N-terminus of the sequences and in the phosphate esters. Such structural variations can in turn direct different pathways when transferring energy and reactivity for the formation and destruction of assemblies. This concept can also be extended to polymeric systems.[40] Designing structured fuels and coupling them to dynamic phosphorous chemistry[41] represents an unexplored opportunity to impact non equilibrium assemblies, by developing phosphate-driven systems chemistry.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. The Supporting Information contains a Materials and Methods description and additional UPLC chromatograms, LC-MS analysis, Transmission Electron Microscopy, confocal images and peptide library characterization using Rheology and turbidity measurements.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author Contributions

‡ M.D.P. and K.D. contributed equally to this work.

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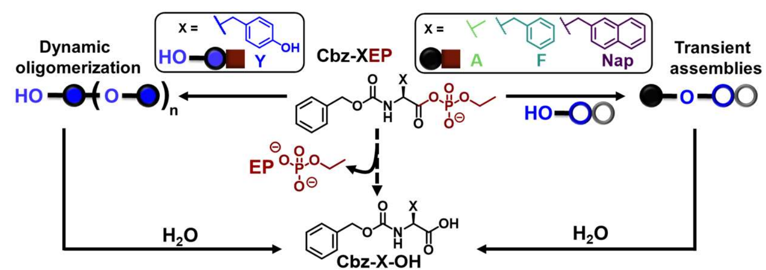

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