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Near-Quantitative Formation of Imines in Water with Allosteric Control

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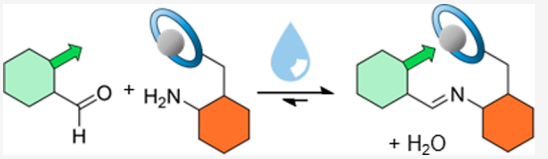
ABSTRACT: We demonstrate the near-quantitative formation of an imine bond in water under physiologically relevant conditions using a stoichiometric ratio of aldehyde and amine. To overcome the low hydrolytic stability of imines in water, a complementary metal coordination bond between the amine and aldehyde monomers is introduced to enhance the thermodynamic stability of the imine. Imine bond formation is substrate-selective, tolerates the presence of a variety of functional groups, and produces imines that are stable in a broad pH range. We also show that the presence of a second metal-ion complex in the building blocks provides a regulatory handle to 'switch on' imine-bond formation using ATP as an external allosteric effector.



INTRODUCTION

Imine bonds are a cornerstone of dynamic covalent chemistry.[1] Imines are typically formed easily and cleanly under mild conditions in primarily organic solvent mixtures via condensation between amines and aldehydes or ketones. Additionally, they undergo relatively fast exchange reactions or hydrolysis with a few side reactions. Furthermore, reduction of imines to the corresponding amines or oxidation to amides allows dynamically formed structures to be irreversibly "locked". These favorable properties have resulted in imines being used very broadly in the formation of supramolecular structures such as mechanically interlocked molecules and cages,[2−5] as a key element of dynamic combinatorial chemistry,[6−12] and even as functional groups in molecular machines.[13,14]

However, a major limitation of imines is their low hydrolytic stability, which limits their application under physiologically relevant conditions. Appreciable amounts of imine in water are only formed if imine formation is favored by cooperativity in multivalent structures,[15−17] by templation,[18,19] or by hydrophobic effects.[20−22] Gois et al. showed that a single imine bond could be formed in water with around 80% yields, exploiting aldehydes with a flanking boronic acid that stabilized the imine through an N−B interaction.[23] Inspired by the observation that the cofactor pyridoxal phosphate (PLP) forms a single imine bond in the catalytic pocket of enzymes, Lehn et al. demonstrated that stabilizing electrostatic interactions between functional groups flanking the amine and aldehyde allowed the formation of a single imine bond in water with similar yields.[24] Introducing a dynamic covalent capture approach, we had shown that electrostatic interactions between neighboring groups can also stabilize hydrazones, which are more stable analogs of imines.[25,26] Despite this progress, (near)quantitative formation of a single imine bond in water has remained elusive.

Here, we show the near-quantitative formation of imines in water under mild conditions using an amine and an aldehyde in a stoichiometric ratio. The dynamic covalent capture approach leverages a complementary, geometry-defined metal coordination bond between building blocks to preorganize the reactants and elevate their local concentration, thereby driving efficient imine formation in an aqueous buffer. Metal coordination furnishes thermodynamic stabilization, while the formed imine linkage provides kinetic locking.

Specifically, salicylaldehyde derivatives (**1**, Figure 1) condense with an aniline bearing a 1,4,7-triazacyclononane (TACN)·Zn(II) complex (**2**), which engages the phenolate in a cooperative metal coordination−covalent product (**3**). The resulting imines are stable in a broad pH-range and are tolerant to substituents: although electron-withdrawing groups favor reaction kinetics and equilibria, near-quantitative conversion is still achieved with electron-rich salicylaldehydes. The coordination bias also enforces selective imine formation in the presence of competing amines, and the resulting imines exhibit stability in water comparable to hydrazones.

We also show that the dual component strategy, combining imine-bond formation with flanking metal coordination, provides a regulatory tool to control imine bond formation by using an external modulator. An allosteric "switch" for imine

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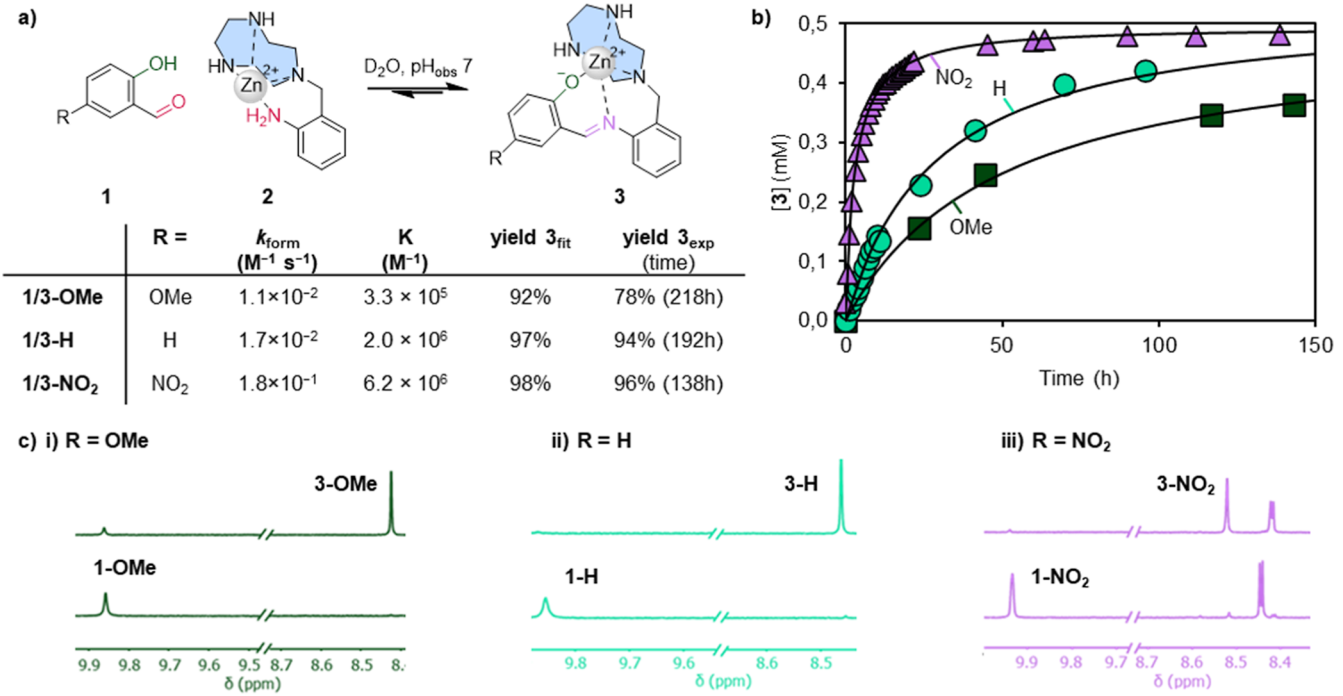

	R =	k_{form} (M^{-1} s^{-1})	K (M^{-1})	yield 3_{fit}	yield 3_{exp} (time)
1/3-OMe	OMe	1.1×10^{-2}	3.3×10^{5}	92%	78% (218h)
1/3-H	H	1.7×10^{-2}	2.0×10^{6}	97%	94% (192h)
1/3-NO_2	NO_2	1.8×10^{-1}	6.2×10^{6}	98%	96% (138h)

Figure 1. a) A strategy for the near-quantitative formation of an imine bond in water, using a secondary coordination bond to guide and stabilize formation. (b) The rate and equilibrium constants for imine formation (Supporting Information Section 2.1) are dependent on the electronics of the aldehyde building block. The reaction is substantially favored (both kinetically and thermodynamically) with more electron-deficient aldehydes. (c) Partial ^{1}H NMR of the reaction at time zero (bottom) and near thermodynamic equilibrium (upper) of the reaction between **2** and (i) **1-OMe** (218 h), (ii) **1-H** (192 h), and (iii) **1-NO_2** (138 h).

formation is introduced by adding a second TACN·Zn(II) unit on the salicylaldehyde moiety. This complex initially suppresses imine formation due to intramonomer coordination of the phenolate, which can, however, be disrupted by addition of adenosine triphosphate (ATP) to trigger imine formation, establishing the role of ATP as an allosteric effector. Subsequent enzymatic ATP hydrolysis re-establishes the repulsive interaction, triggering imine cleavage.

RESULTS AND DISCUSSION

Stabilization of the Imine Bond in Water

We reasoned that near-quantitative imine bond formation in water could be achieved by adding a stabilizing interaction between the amine and aldehyde with greater strength and specificity than the ion pairing used previously.[22,24] From our previous work, we knew that TACN·Zn(II) complexes form strong interactions with polyanions, such as ATP and ADP, in water.[27,28] We have exploited these interactions to stabilize a dynamic hydrazone bond in water between aldehyde and hydrazide monomers, each bearing a TACN·Zn(II) unit.[29,30] However, follow-up studies revealed that this approach was not sufficient to drive the formation of imines in water because of the much lower hydrolytic stability of imines compared to hydrazones.[31−33] Instead, we reasoned that direct coordination between a TACN·Zn(II) unit on one building block and an anion on the other might form a sufficiently strong interaction to stabilize an imine bond.

Taking previously used[29,30] aniline **2** equipped with a TACN·Zn(II) unit as one component, we used density functional theory (DFT) calculations for rationalizing the aldehyde scaffold; salicylaldehyde **1-H** was selected as a promising hit (see Supporting Information Section 2.1.2). Calculations suggested the presence of a geometrically well-defined Zn(II)-complex in the resulting imines **3-H** involving, in addition to the TACN-ligand, also the imine nitrogen and phenolate oxygen (Figure 1a and S18). We anticipated that, particularly, the presence of the additional phenolate O^-—Zn(II) interaction would favorably affect the kinetic and thermodynamic stability of the imine. Gratifyingly, we indeed observed the near-quantitative formation of imine **3-H** upon mixing equimolar amounts of **1-H** and **2** in D_2O ([**1-H**] = [**2**] = 0.5 mM, pH_{obs} = 7.0, [HEPES] = 10 mM, T = 25 °C) with an equilibrium constant, $K_{3\text{-}H}$, of 2.0×10^6 M^{-1} and a formation rate constant, k, of 1.7×10^{-2} M^{-1} s^{-1} (Figure 1).

Other salicylaldehyde derivatives were then studied to explore the effect of electronics on the condensation reaction. One derivative, **1-OMe**, was functionalized with an electron-donating OMe substituent to increase the electron density on the aromatic ring, which is expected to make the aldehyde less electrophilic and destabilize the phenolate. Conversely, derivative **1-NO_2**, containing an electron-withdrawing NO_2 substituent, was expected to have enhanced electrophilicity of the aldehyde and stabilize the phenolate. In both cases, imine formation approached near-quantitative yields with equilibrium constants ranging from 3.3×10^5 M^{-1} for **1-OMe** to 6.2×10^6 M^{-1} for **1-NO_2** (Figure 1a), although at different rates. Compared to **1-H**, imine formation was slower for electron-rich **1-OMe** (1.10×10^{-2} M^{-1} s^{-1}, k_{rel} = 0.64) but faster for the electron-poor derivative **1-NO_2** (1.83×10^{-1} M^{-1} s^{-1}, k_{rel} = 11). The substituent effect on both the rate of formation and equilibrium constant indicates that the degree of stabilization and activation is likely dependent on the degree of deprotonation of the phenol at pH_{obs} 7.0 (Figure 1b,c).

To obtain support for our DFT-based hypothesis that the formation of a geometrically well-defined Zn(II)-complex

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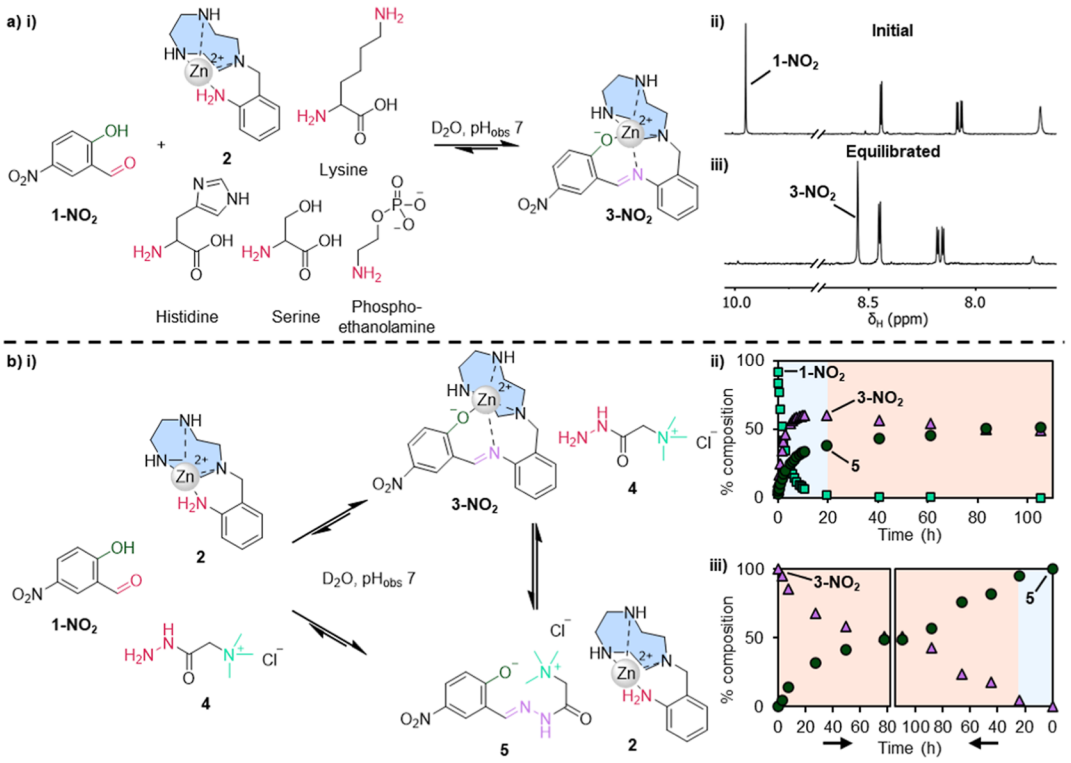

Figure 2. a) (i) Schematic representation of the imine bond formation using **1-NO$_2$** as an aldehyde building block and different amines. As shown by the partial ^{1}H NMR spectra (ii) before and (iii) after equilibration, only **3-NO$_2$** is observed due to the high selectivity for **2**, thanks to the cooperative stabilization provided by the coordination complex (for full spectra, see Figure S45). (b) (i) Schematic representation of the equilibria involved in the imine **3-NO$_2$** and hydrazone **5** exchange reactions (ii) Equilibration of **1-NO$_2$**, **2**, and **4** into **3** and **5** at 25 °C and then by heating at 70 °C. (iii) Competition experiments of **3-NO$_2$** with **4** (left) and of **5** with **2** (right); the orange area indicates that the sample has been heated at 70 °C, while the light-blue area indicates that the sample has been kept at 25 °C.

involving ortho-phenolic O$^-$−Zn(II) coordination is at the origin of imine formation, we carried out a series of negative controls (Supporting Information, Section 2). Imine formation was not observed at all when (a) the phenol was methylated, (b) $Zn(NO_3)_2$ was not added, (c) the TACN-unit was eliminated, and (d) the TACN·Zn(II)-complex was present in solution but not connected to the aniline. Furthermore, imine formation did not occur when the phenolic−OH or the methylene−TACN were in geometrically wrong positions (meta instead of ortho with respect to the aldehyde and amine, respectively). Final experimental evidence for the presence of a coordinatively saturated Zn(II)-complex in imine **3** came from adenosine triphosphate (ATP) affinity studies. The decreased affinity of ATP for imine **3-H** (1.0×10^2 M^{-1}) compared to aniline **2** (7.4×10^4 M^{-1}) can be attributed to the presence of the competing intramolecular phenolate O$^-$−Zn(II) interaction in **3-H** (Supporting Information Section S2.11).

Compatibility and Selectivity of Imine Bond Formation

In addition to the near-quantitative yield, the reaction presents other attractive features for application in a biological context.[34] Imine **3-OMe** with the lowest thermodynamic stability is stable across a wide range of pH between 4 and 10 (Supporting Information Section S2.2); below pH 4, imine hydrolysis is observed, which is attributed to the loss of affinity for Zn(II) by TACN upon protonation of the nitrogens. The robust pH tolerance of the imine, spanning a range that includes biologically relevant values, enables its use in a biological context as a pH-independent ligation strategy. The result also indicates that changes affecting Zn(II)-complexation, which provides the thermodynamic stability for the complex, act as a regulatory handle which can be used to control the extent of imine formation via the addition of external reagents.

Imine bond formation was found to be highly substrate-selective and tolerable with the presence of additional functional groups. Imine bond formation between **1-NO$_2$** and **2** was tested under competitive reaction environments. Alongside **2**, equimolar (0.5 mM) amounts of other amines (lysine, histidine, serine, and phosphoethanolamine) were added to the reaction mixture (Figure 2a(i)). The competitor compounds were chosen to represent a selection of common biologically relevant amines with a range of potential secondary interactions (e.g., electrostatic, coordination) that could develop with the phenolate or TACN·Zn(II) complex. After 36 h, ^{1}H NMR showed complete consumption of **1-NO$_2$** and exclusive formation of imine **3-NO$_2$** (Figure 2a(ii,iii)). The near-perfect selectivity is of particular interest for site-selective tagging via imine formation in biological contexts.[35,36]

To challenge imine bond formation even further, we carried out a competition experiment between amine **2** and Girard's reagent T (**4**) (Figure 2b(i)). Acyl hydrazides such as **4** condense with aldehydes to form acyl hydrazones, which are known to have a much higher thermodynamic stability than imines because of the delocalization of electron density via resonance. In addition, the thermodynamic stability of hydrazone **5** is expected to be even further enhanced because of potential stabilizing electrostatic interactions between the ammonium group and the phenolate oxygen in **5**. Surprisingly, when **1-NO$_2$**, **2**, and **4** were mixed in equimolar quantities (0.5

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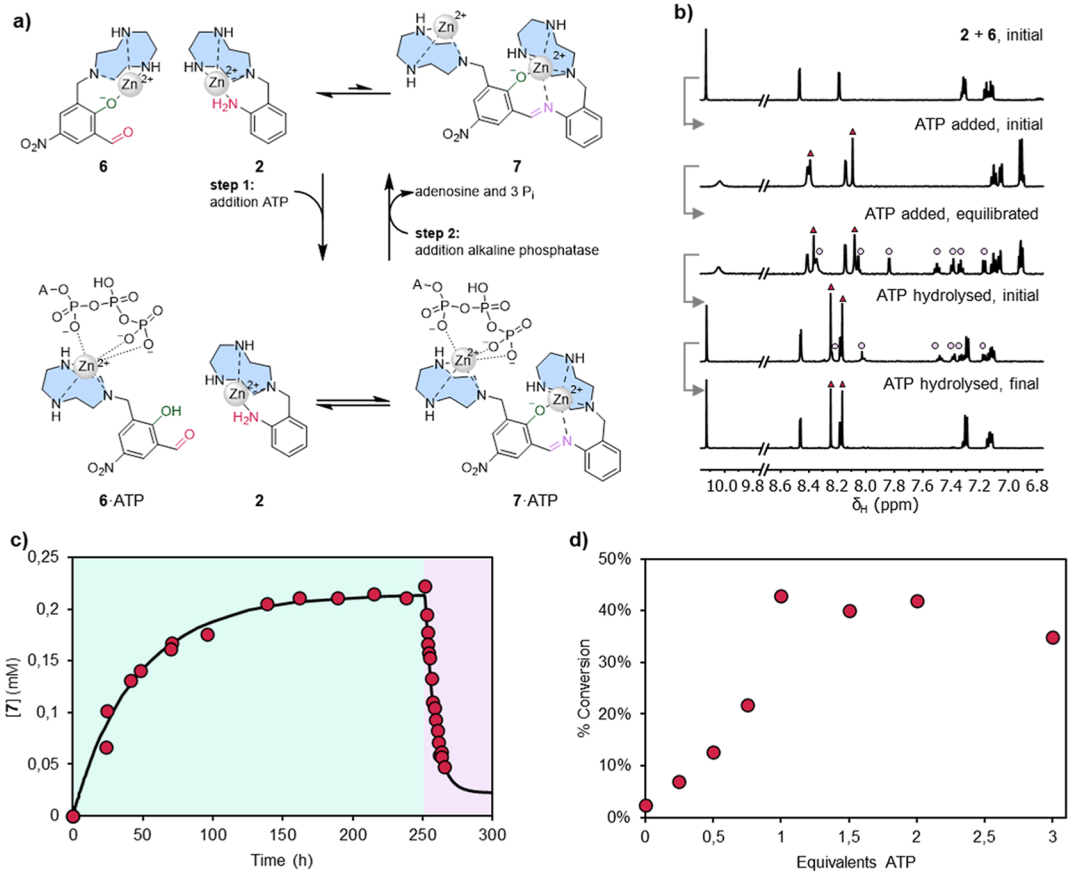

Figure 3. a) Schematic representation of the chemical reaction cycle presented in this paper: the addition of ATP binds the TACN·Zn(II) complex present in **6**; once the phenolate is no longer coordinated to Zn(II), imine **7** can be formed. The subsequent addition of alkaline phosphatase caused the hydrolysis of ATP. In the absence of ATP, the imine bond is destabilized and hydrolyzes slowly. (b) Partial ^{1}H NMR spectra of **2** and **6** in equilibrium with **7** at the different stages of the reaction, red triangles represent the H-2 and H-8 signals of adenine, while pink circles represent the aromatic imine peaks. (c) Graph representing the concentration of imine **7** with time in the presence (light blue) after hydrolysis of ATP (purple). (d) Concentration of imine **7** at the equilibrium after mixing **2** and **6** at a concentration of 0.5 mM each in the presence of different equivalents of ATP.

mM, in D_2O buffered at pH_{obs} 7.0), imine formation was still clearly kinetically favored, yielding 66% upon full depletion of the aldehyde (see Figure 2b(ii)). Upon heating to 70 °C for 4 days, the reaction equilibrated to give a 1:1 mixture of imine **3-NO$_2$** and hydrazone **5**, indicating that the equilibrium constant between the imine and hydrazone is approximately 1. The equilibrium state was confirmed by starting the reaction from different compositions: either by mixing **3-NO$_2$** with **4** or by mixing **1-NO$_2$** with **5**. All reactions reached the same 1:1 mixture after 4 days at 70 °C (Figure 2b(iii)).

The slow exchange (even at high temperatures) was somewhat surprising as imines tend to undergo rapid exchange with acyl hydrazides to form stable acyl hydrazones.[37] Our results indicate that TACN·Zn(II)-stabilized imines are both thermodynamically and kinetically very stable. We hypothesize that this is due to cooperativity between the coordination and covalent imine bond. The covalent imine bond provides higher kinetic stability than could be gained from the O$^-$–Zn(II) interaction, while the coordination complex provides thermodynamic stabilization.

Installing a Regulatory Handle to Activate and Deactivate Imine Bond Formation

A high thermodynamic and kinetic stability of an imine is a very attractive property for many applications. However, in the context of supramolecular chemistry, it would be desirable to restore the dynamicity to develop responsive systems. An ideal scenario would combine both features, realizing a system that can be switched between a 'dynamic' and 'locked' state under the control of an external input.[38] The key role of the coordination bond in stabilizing the imine suggests that disrupting that interaction would be a good strategy to achieve such a switchable system. However, in all derivatives of imine **3**, the intramolecular interaction proved to be too strong to allow disruption by external anions. Therefore, we developed an alternative strategy relying on a second competing intramolecular interaction to block imine formation. This was achieved by introducing a TACN·Zn(II) complex also in the aldehyde component, such as in **6** (Figure 3a). Intramolecular coordination of the phenolate-O$^-$ to the Zn(II)-metal ion in **6** renders the phenolate oxygen unavailable for coordination to the TACN·Zn(II) unit on **2**, which serves to stabilize the imine. As a result, imine **7** was formed only in trace amounts (2% yield) under the standard conditions. Accurate values for the rate and equilibrium constants could be determined only by studying the imine bond formation between **2** and **6** at higher concentrations (2 mM rather than 0.5 mM, Supporting Information Section S4.2).

In previous work, we have shown that the addition of ATP triggers bond formation between two TACN·Zn(II)-bearing

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monomers as a result of minimized electrostatic repulsion.[29,30] We reasoned that addition of ATP might compete with intramolecular binding in **6** and minimize the intermonomer repulsion, thus favoring the formation of imine **7**. Indeed, the addition of increasing amounts of ATP (0−1.5 mM) to a mixture of **2** and **6** increased the yield of imine formation, reaching a maximum of 43% after the addition of 1 equiv (Figure 3b−d).

The reversibility of the system was shown by adding 100 U ml^{-1} of the enzyme alkaline phosphatase to the equilibrated mixture with ATP (**7** at 40%). After addition of the enzyme, ATP was converted to phosphate and adenosine over ∼2 h (Figure 3c) and, following ATP hydrolysis, the concentration of **7** gradually decreased over a much longer period, progressing toward the concentration obtained after equilibration in the absence of a template (∼2%, Supporting Information Section S4.5). This indicates that the residual phosphate has little detectable templating effect that favors the imine formation, although it does slow the hydrolysis (Supporting Information Section S4.3). The data and fitted rates from individual experiments were combined to create a full kinetic model for the behavior of the system, which confirms the self-consistency of the experiments on this system (Supporting Information Section S4.5).

Finally, the change in the composition of the imine in response to the sequential addition of ATP (step 1) and enzyme (step 2) is reminiscent of the energy ratchet mechanism that is well known in the context of molecular machines and has recently been applied in the synthesis of small molecules.[39,40] As the role of ratchets is to transduce energy from an external source (here, ATP hydrolysis) and store it in a different degree of freedom, it is possible to quantify the stored nonequilibrium free energy in these cases, corresponding to the free energy released while moving from the nonequilibrium state back to equilibrium. For this system, the peak nonequilibrium free energy stored in the unfavorable imine following ATP hydrolysis is 1.9 kJ mol^{-1} (evaluated based on the concentration of 1 equiv = 0.5 mM) or 0.93 J L^{-1} (SI Section 4).

CONCLUSIONS

The use of a coordination bond between TACN·Zn(II) and a phenolate to create a metal-coordination-covalent complex that stabilizes the formation of imines in water provides new opportunities for the use of imines in biologically relevant reactions. A salicylaldehyde derivative could be engineered into a non-natural amino acid, which will enable highly site-selective tagging by a TACN·Zn(II) aniline (or vice versa).[41] This strategy has the particular advantage that imine formation occurs under very mild conditions, with no need for catalysts or toxic reagents. With further optimization, for example, picking the right electron-withdrawing salicylaldehyde derivative, the reaction may join the suite of available "click" reactions for selective bio-orthogonal bond formation.[42] An advantage of this approach over other chemistries used for bioconjugation is the possibility of versions of the reaction that are reversible upon the application of the correct chemical trigger. Here, we demonstrated that the equilibrium between the imine and building blocks can be shifted by ATP, and it can be envisaged that such self-regulated reactivity could have utility in autonomous targeting of drug release into specific tissues with, for example, enhanced extracellular ATP concentrations.

Furthermore, this work provides new tools for control over the molecular structure and reactivity in systems chemistry. Modulation of the imine bond by targeting the coordination bond can be considered to be a form of allosteric control.[43] While it remains at a very basic level in comparison with biochemical examples, a technique in which control over reactivity can be exerted over a chemical reaction through indirect means potentially provides a means of vastly expanding the systems chemists' toolbox. Designing this sort of functional allosteric control handle into chemical systems could lead to much finer or multiimpetus control over outcomes. This has utility in the creation of chemical Boolean logic, as signals dependent on one of, or all inputs, are analogous to OR or AND gates.[44] Additionally, the type and structure of the composite ionic−covalent bonds discussed here naturally lend themselves to use in responsive materials, catalysts, sensors, or dynamic polymers, especially in view of the ease with which salicylaldehyde and aniline derivatives can be functionalized with different catalytic or reactive units.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.5c20524.

Materials and instrumentation, experimental procedures, and additional supporting data (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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