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Four Simultaneously Dynamic Covalent Reactions. Experimental Proof of Orthogonality.

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ABSTRACT: Dynamic covalent reactions are widely used in dynamic combinatorial chemistry. Most of these reactions are run under differing reaction conditions and exhibit cross-reactivity when components of multiple reactions are present in one reaction vessel. Herein, we report the study of four dynamic covalent reactions that react reversibly under identical reaction conditions and do not exhibit any cross-reactivity. Dynamic behavior was shown via ^{1}H-NMR based exchange experiments. Computational deconvolution of ^{1}H-NMR spectra containing the components for more than one of the orthogonal reactions allowed for a semi-quantitative analysis of the complex mixtures formed, showing that the reactions proceed independently of each other. Therefore, it is possible to use all four reactions in one pot in a simultaneous, yet orthogonal fashion. This opens up possibilities for the pre-programmed formation of complex thermodynamic assemblies.

Introduction

Dynamic covalent reactions (DCRs) are widely used in dynamic combinatorial chemistry (DCC).[1–4] Applications of DCC include receptor[2,3] and drug discovery.[1,5] DCC is an important tool to screen for multivalent recognition systems by target-driven amplification of the best binder.[6] Especially in aqueous systems, such as in biological settings, the binding and recognition of specific target molecules by supramolecular interactions is challenging due to competing solvation.[6] In terms of thermodynamic and kinetic stability, dynamic covalent interactions are in between irreversible covalent reactions and supramolecular interactions, therefore making them potential alternatives for guest binding. The dual nature of dynamic covalent reactions (reversible or permanent depending on conditions) allows the system to equilibrate to the most thermodynamically stable state, while at the same time allowing for analysis and isolation of the product that is formed.[1,7]

While a large number of DCRs are known, most of them require different reaction conditions from each other or are not orthogonal to each other; for instance, disulfide exchange occurs simultaneously with thioester exchange.[7–10] Only a limited number of examples using more than one type of DCR in an orthogonal fashion have been studied.[7] Otto and coworkers showed that disulfide exchange and hydrazone exchange can be operated either simultaneously or one reaction at a time, depending on the pH.[11] Other known pairs of orthogonal DCRs are boronic ester and imine exchange, boronic ester and hydrazone exchange, disulfide and imine exchange, as well as imine exchange and olefin methathesis.[12–18]

To our knowledge, the largest number of orthogonal dynamic covalent reactions used in one experiment is three.[19–22] For instance, Matile and coworkers recently published a series of studies in which they used disulfide exchange under basic conditions, hydrazone exchange under acidic conditions, and boronic ester exchange under neutral conditions.[19,20,22] Thus, the reactions they used did not proceed simultaneously. Instead, they controlled the pH of the solution to selectively turn on only one reaction at a time.

In a recent paper Bonifazi and coworkers reported, for the first time, the use of three simultaneous, orthogonal dynamic covalent reactions for the assembly of a multicomponent architecture.[21] Bonifazi used the same three dynamic covalent reactions as Matile – disulfide exchange, hydrazone exchange, and boronic ester exchange. However, they used non-aqueous reaction conditions (THF with a catalytic amount of *m*-phenylene diamine), as well as modified versions of the reacting partners to speed up the exchange, in order to allow the reactions to proceed simultaneously. They used this set of reactions to decorate a pre-programmed α-helical peptide bearing receptor sites with chromophores containing the corresponding reaction partners.

In the current study, we set out to expand the number of dynamic covalent reactions that can be used simultaneously in the same flask without exhibiting cross-reactivity. In reversible covalent and supramolecular chemistry, reactions that do not interfere with each other and do not exhibit cross-reactivity have been termed orthogonal. This is in contrast to the definition of orthogonality as used in protecting group chemistry, where each orthogonal group can be removed in any order depending on the conditions without altering the others.[23] Therefore, the reactions studied in this paper can be called simultaneous, yet orthogonal.

The simultaneous use of multiple orthogonal dynamic covalent reactions is expected to aid in the design of increasingly complex templated structures with preprogrammed structural features.[7] We also set out to devise a method to prove orthogonality and exchange of reaction components, and latched onto a ^{1}H-NMR spectroscopy modeling technique, as discussed herein.

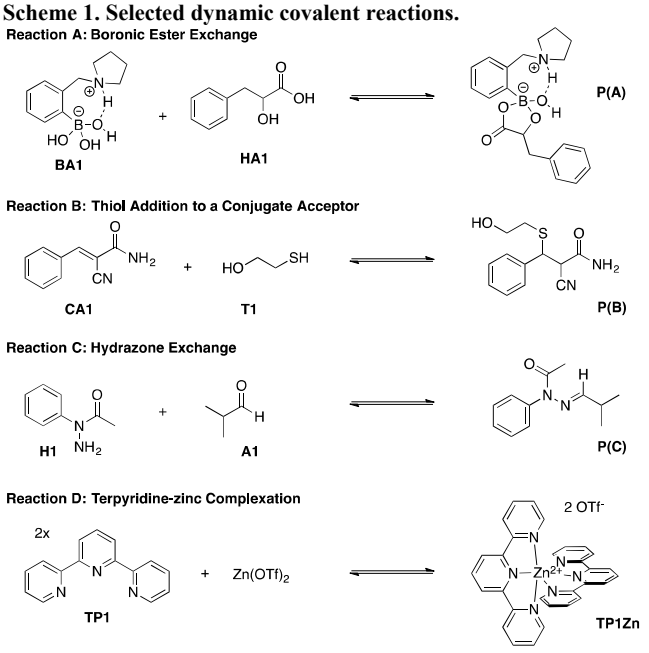
Design Criteria

Four reactions were chosen for the current study of reversibility and orthogonality. Each of the four had been previously reported to be reversible, but the reactions conditions reported were not identical to each other, nor had rigorous tests of orthogonality been performed. For the first (**Reaction A**; **Scheme 1**) we chose the reaction of α-hydroxy acids with

boronic acids to form boronic esters. Boronic acids are well known to react reversibly and selectively with 1,2- and 1,3-diols, as well as α-hydroxy acids under neutral conditions.[24–28] Aromatic boronic acids containing an aminomethyl functionality in the 2-position have been shown to exhibit particularly favorable kinetic and thermodynamic properties.[29–32] The two boronic acids **BA1** and **BA2** as well as α-hydroxy acids **HA1** and **HA2** (**Chart 1**) chosen for our exchange studies were previously reported by our group.[28,33]

Reaction B is the reversible addition of thiols to a conjugate acceptor (**Reaction B**; **Scheme 1**). This reaction occurs more readily under basic conditions. However, a couple of examples have been reported that occur reversibly under close to neutral conditions.[34,35] The comparatively fast exchange kinetics of the addition of thiols to benzalcyanoacetamides had been previously studied by our group.[34] Benzalcyanoacetamide **CA1** was chosen as the first conjugate acceptor. As a second exchange partner, we chose ethacrynic acid (**CA2**), which has been shown to be suitable for dynamic combinatorial chemistry.[35] Due to their low volatility, 2-mercaptoethanol (**T1**) and 4-mercaptobenzoic acid (**T2**) were selected as the thiols (**Chart 1**).

Scheme 1. Selected dynamic covalent reactions.



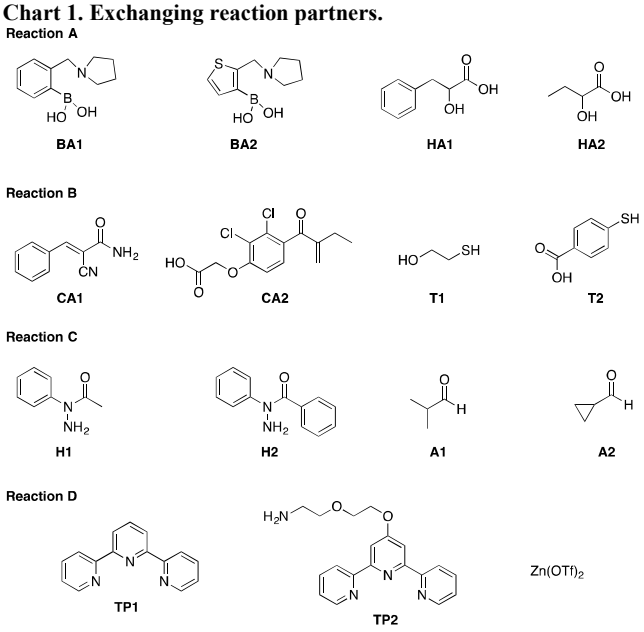
Reaction C is the addition of a hydrazine to an aldehyde to form a hydrazone. Hydrazones are typically inert under neutral conditions and require an acidic pH to hydrolyze and exchange.[36,37] Huc and coworkers reported that hydrazones formed from hydrazines bearing electron-withdrawing groups are sufficiently activated for the reaction to be reversible even at neutral pH.[38] Hydrazine **H1** was directly taken from Huc's report. Replacing the methyl group with a phenyl group gave a similarly reactive second exchange partner (**H2**). Several different aldehydes were screened for reversible hydrazone formation with the selected hydrazines. It was found that aliphatic aldehydes equilibrated more readily than aromatic aldehydes.[39] Isobutyraldehyde (**A1**) and cyclopropane carboxaldehyde (**A2**) were selected for our study (**Chart 1**).

Finally, as a last orthogonal reaction (**Reaction D**), complexation of two terpyridine ligands to a zinc(II) metal center was chosen. Terpyridine complex formation is not typically employed in DCC, although Lehn has recently used it as an orthogonal dynamic reaction pair in conjunction with imine formation.[40] While some people might argue whether the bond formed between the nitrogen atoms of the terpyridine and the zinc can be considered covalent, IUPAC defines coordination as the formation of a covalent bond where both electrons in the bond come from the same molecular entity.[41] The complex formation of terpyridines to zinc(II) has long been known to be reversible and exchange has been shown to take place.[42] Hence, terpyridine complexation can be considered a covalent, dynamic reaction. Parent 2,2':6',2"-Terpyridine (**TP1**) was chosen as the first reacting partner. As a second exchange partner, **TP2** (**Chart 1**) was selected, after other, commercially available substituted terpyridines were observed to form insoluble zinc(II) complexes in our reaction medium.

To confirm reversibility and orthogonality of the selected reactions, the exchange of individual reaction components was followed by ^{1}H-NMR spectroscopy. To test for orthogonality, the reaction partners required for two or more of the individual reactions were added to a single vessel and the resulting ^{1}H-NMR spectrum was compared to that of the individual reactions.

To get a more quantitative analysis of the mixture composition of the complex spectra, computational deconvolution methods based on a pure variable approach were employed. The goal of this analysis was twofold: The first goal was to compare the extent of product formation in the individual reactions to that in the orthogonality experiments. The second goal was to extract the spectra of the products from the spectra of the individual reaction mixtures and finally reconstruct the orthogonality experiment spectra from the calculated concentrations and the individual component spectra. The R^2 values between the reconstructed and experimental spectra were taken as a measure of how well the spectra were modeled by the expected number of components. We devised this approach as a rigorous test of orthogonality.

Chart 1. Exchanging reaction partners.



Results and Discussion

Reversibility and Exchange

The reversibility of each individual reaction was tested *via* NMR exchange experiments. In all studies, two of the reaction partners were allowed to react with each other in 3:1 CD_3OD/aqueous HEPES buffer (pH 7.4). After reaching equilibrium, a second reaction partner was introduced. The exchange process was followed by ^{1}H-NMR spectroscopy. In all cases, decrease of the preformed product concentration was observed, concomitant with formation of the product containing the newly introduced compound. Further, as described below, it was shown that for each exchanging system, the same product distribution was obtained independent of the order of additions of the components. The formation of the predicted products was confirmed by ESI-mass spectrometry. However, due to the reversible nature of the reactions and the complexity of the mixtures, not all products were observed when mixtures of more than one reaction were submitted to mass spectroscopic analysis.

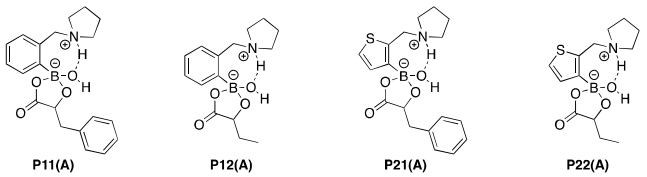
A: Boronic Ester Exchange

As expected, the boronic ester exchange reactions reached equilibrium in less than 30 minutes in a 3:1 mixture of CD_3OD and water at pH 7.4 (HEPES buffer). The product ^{1}H-NMR spectra were complex due to the presence of two stereocenters in the products, which leads to the formation of two diastereomers and makes the methylene protons of the boronic acid diastereotopic. Due to the complex nature of the ^{1}H-NMR spectra and extensive overlap of product and starting material peaks, it was not possible to calculate a percentage of product formation from integration of isolated ^{1}H-NMR peaks.

Figure 1 shows characteristic peaks in the methyl region of the ^{1}H-NMR spectra following the exchange of the boronic acid components in the presence of **HA2**. **Figure 1a** shows the spectrum of the reaction of **BA1** and **HA2** after equilibration, while **Figure 1b** shows the spectrum of the reaction of **BA2** and **HA2**. The triplets corresponding to the methyl peaks of **HA2** and the corresponding peaks in the products (**Chart 2**) are labeled in the spectra. Both **P12** and **P22** can exist in two diastereomers. After the spectra were measured, one equivalent of the corresponding other boronic acid was added. The spectra in **Figure 1c** and **d** were taken after allowing for the equilibration after addition of the second boronic acid. Both spectra look essentially identical, independent of the order of addition, showing the reversibility of boronic ester formation under the reaction conditions.

In a similar fashion, boronic acids were exchanged in the presence of **HA1**, and the α-hydroxy acids were exchanged in the presence of either boronic acid (see Supporting Information for ^{1}H-NMR spectra).

Chart 2: Products formed in boronic ester exchange experiments.



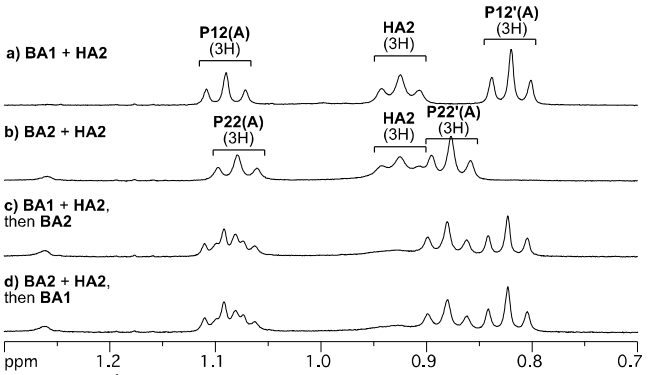
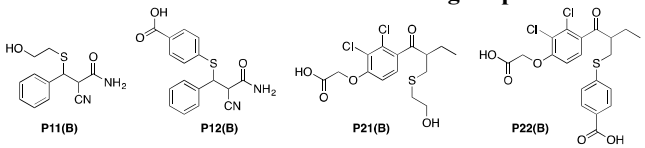


Figure 1. ^{1}H-NMR (400 MHz) spectra of boronic acid exchange in the presence of **HA2**. Characteristic methyl peaks of **HA2** and the products **P12** and **P22** are labeled. Reaction conditions: 3:1 CD_3OD/HEPES (1 M, pH = 7.4). All reaction components: 10 mM. Temperature: 25 ˚C. a) and b) The reactions were allowed to equilibrate for 1 day. c) and d) The corresponding other boronic acid was added to a and b, and the reactions were allowed to equilibrate for 1 day.

B: Thiol and Conjugate Acceptor Exchange

The thiol-conjugate acceptor exchange reactions were run in the same solvent mixture that was used for boronic ester exchange (3:1 CD_3OD/HEPES buffer) in sealed NMR tubes under N_2 to avoid oxidation of the thiols. In each NMR tube, two components (one thiol and one conjugate acceptor) were mixed together. At different time points, ^{1}H-NMR spectra were recorded and concentrations of the components were calculated from isolated peaks relative to the total concentration of each conjugate acceptor (**Figure 2**). **Chart 3** shows the structures of the products that are formed from the four possible combinations of thiol and conjugate acceptor.

Chart 3. Products formed in thiol exchange experiments.



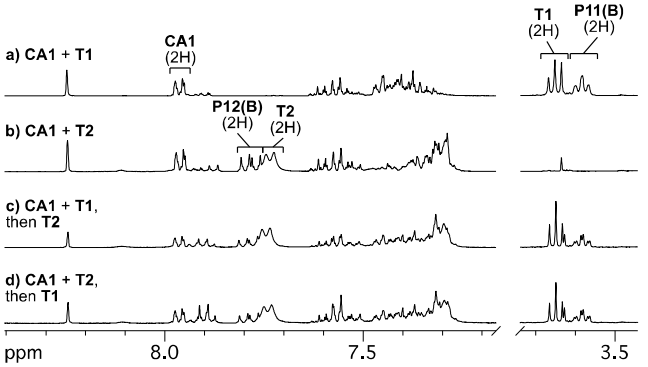


Figure 2. ^{1}H-NMR spectra of thiol exchange experiments with **CA1**. Reaction conditions: 3:1 CD_3OD/HEPES (100 mM, pH = 7.4). All reaction components: 10 mM. Temperature: 25 ˚C. Peaks that are labeled were used to calculate relative concentrations of starting materials and products. a) and b) The reaction was allowed to equilibrate for 7 days. c) and d) The corresponding other thiol was added to a and b, and the reaction was allowed to equilibrate for 9 days.

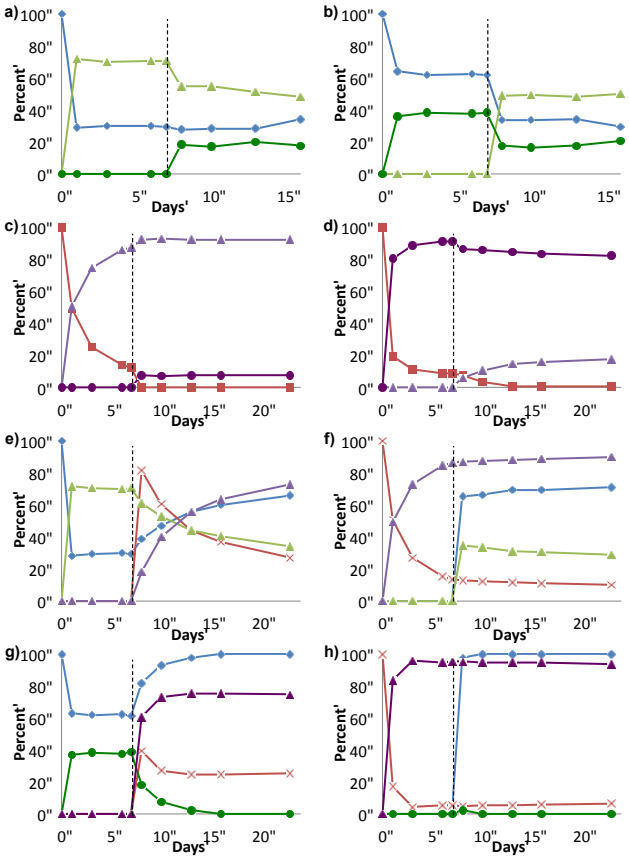


Figure 3. Exchange reaction of thiols and conjugate acceptors. The exchanging components were added after 7 days (dotted lines). Percentages are calculated relative to the starting concentration of the corresponding conjugate acceptor. ◆ = **CA1**; ■ = **CA2**; ▲ = **P11**; ● = **P12**; ▲ = **P21**; ● = **P22.** a) **CA1** + **T1**, then **T2**. b) **CA1** + **T2**, then **T1**. c) **CA2** + **T1**, then **T2**. d) **CA2** + **T2**, then **T1**. e) **CA1** + **T1**, then **CA2**. f) **CA2** + **T1**, then **CA1**. g) **CA1** + **T2**, then **CA2**. h) **CA2** + **T2**, then **CA1**.

Starting material and product concentrations relative to the total concentration of each conjugate acceptor are plotted in **Figure 3**. The addition of thiols **T1** and **T2** to **CA1** reached equilibrium in less than a day (**Figure 3a**, **b**, **f**, and **g**). However, their addition to **CA2** took several days to reach equilibrium (**Figure 3c**, **d**, **e**, and **h**). This difference in kinetics is not surprising, considering that **CA2** is lacking a second electron-withdrawing group attached to the α-carbon of the conjugate acceptor. All reactions were allowed to equilibrate at room temperature for 7 days before the exchanging component was added. After addition of the second thiol to the NMR tubes containing **CA1**, exchange was complete after less than one day. As can be seen in **Figure 3a** and **b**, the relative percentages of the two products are almost the same, independent of the order of addition. Comparison of the ^{1}H-NMR spectra (**Figure 2c** and **d**) shows some minor differences, most likely due to thiol oxidation in spite of precautions that were taken, but they look very similar. However, addition of a second thiol to the tubes containing **CA2** only led to minimal exchange, and even after one week the reactions were still far from equilibrium (**Figure 3c** and **d**). Addition of **CA2** to NMR tubes containing **CA1** and either thiol led to slow exchange of the conjugate acceptor, taking several days to approach equilibrium (**Figure 3e** and **g**). However, addition of **CA1** to tubes containing **CA2** and either thiol did not lead to exchange of the conjugate acceptor, again confirming that the thiol addition to **CA2** appears to be very slowly reversible under the reaction conditions studied (**Figure 3f** and **h**). Due to the near irreversibility of the reaction with **CA2**, the product distribution of **Figure 3e** is different from **Figure 3f** and the product distribution of **Figure 3g** is different from **Figure 3h**. The differences can also be seen when comparing the resulting ^{1}H-NMR spectra (see supplementary material). Thus, while previous literature reports reversibility of **CA2**, we find it to be very slow under our reaction conditions. Importantly, however, **CA1** readily exchanges thiols. The lack of exchange with **CA2** has no bearing on whether thiol conjugate additions are orthogonal to the other three reactions, as described below.

C: Hydrazone Exchange

Hydrazone exchange reactions were initially attempted using 4-carboxybenzaldehyde as the second aldehyde, since this aldehyde had been reported to reversibly react with **H1** under similar conditions.[38] However, it appears that the reaction of **H1** with aromatic aldehydes occurs irreversibly under the conditions we used. After screening several different aldehydes, cyclopropanecarboxaldehyde (**A2**) was chosen. Unlike other aliphatic aldehydes we screened, aldehyde **A2** preferentially exists in the aldehyde form under the reactions conditions, which simplifies NMR analysis. As described above for the boronic ester and thiol exchange, experiments were set up to follow the exchange of components. In each NMR tube, two components (one hydrazide and one aldehyde) were mixed together. At different time points, ^{1}H-NMR spectra (see supporting information) were recorded, and concentrations of the components were calculated from isolated peaks relative to the concentration of each aldehyde (**Figure 4**). **Chart 4** shows the possible products arising from the four possible combinations of hydrazides and aldehydes. The rates of hydrazone formation with aldehydes **A1** and **A2** and both hydrazines are comparable, with the equilibrium being reached after approximately three days at room temperature (**Figure 5**).

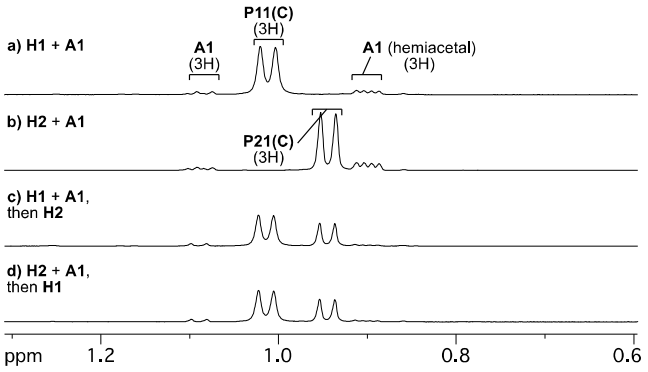


Figure 4. ^{1}H-NMR (400 MHz) of hydrazine exchange in presence of **A1**. Reaction conditions: 3:1 CD_3OD/HEPES (100 mM, pH = 7.4). All reaction components: 10 mM. Temperature: 25 °C. a) and b) The reaction was allowed to equilibrate for 7 days. c) and d) The corresponding other hydrazine was added to a) and b), and the reaction was allowed to equilibrate for 14 days.

Chart 4. Products formed in hydrazone exchange experiments.

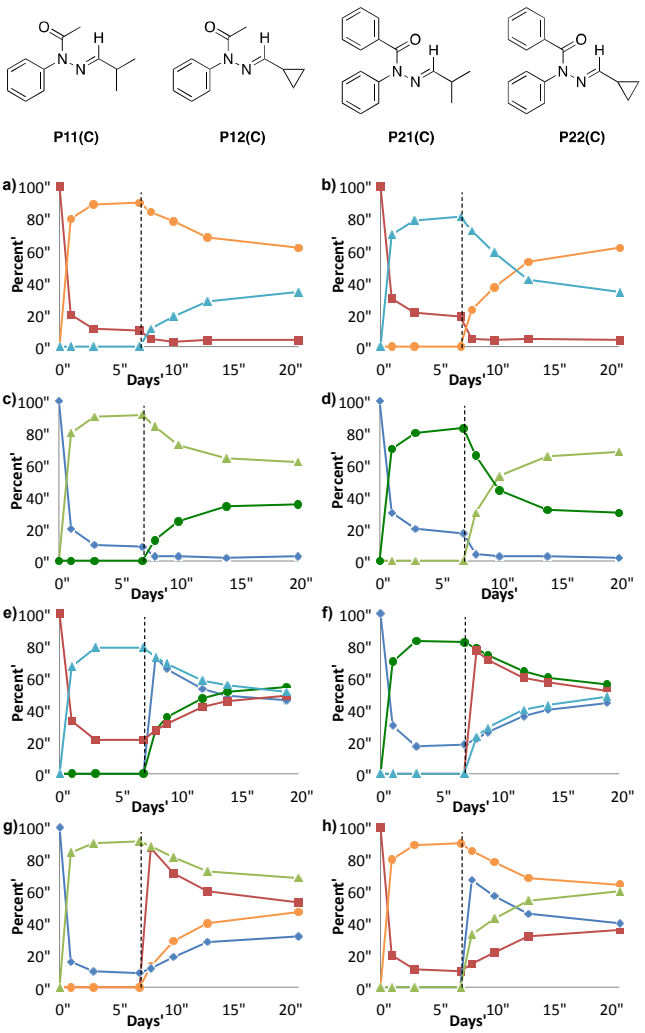


Figure 5. Exchange reaction of hydrazides and aldehydes. The exchanging components were added after 7 days (dotted lines). Percentages are calculated relative to the starting concentration of the corresponding aldehyde. ■ = **A1**; ◆ = **A2**; ● = **P11**; ▲ = **P12**; ▲ = **P21**; ● = **P22.** a) **H1** + **A2**, then **H2**. b) **H2** + **A2**, then **H1**. c) **H1** + **A1**, then **H2**. d) **H2** + **A1**, then **H1**. e) **H2** + **A1**, then **A2**. f) **H2** + **A2**, then **A1**. g) **H1** + **A2**, then **A1**. h) **H1** + **A1**, then **A2**.

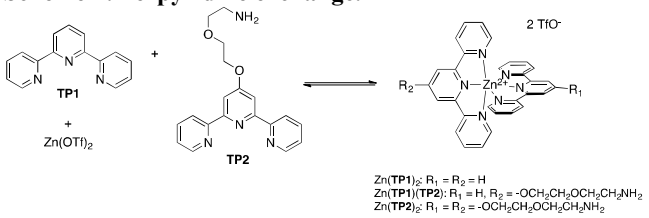
After 7 days at room temperature, the second component was added. In all cases, exchange took place. Hydrazide exchange in the presence of **A1** took approximately two weeks to reach equilibrium after addition of the second hydrazide (**Figure 5a** and **b**). After this time, the product distributions are the same independent of the order of addition, and the NMR spectra look virtually identical (**Figure 4**), with the product formed from **H1** being slightly preferred over the product formed from **H2**. The hydrazide exchange in the presence of **A2** is slightly faster than in the presence of **A1**, taking about one week to reach equilibrium (**Figure 5c** and **d**). As with **A1**, the product formed from **H1** was slightly preferred over the product formed from **H2**. Aldehyde exchange in the presence of **H2** also took approximately two weeks to reach equilibrium. After equilibrium was reached, the products containing each aldehyde (**P21(C)** and **P22(C)**) were present in a 1:1 ratio (**Figure 5e** and **f**). Aldehyde exchange in the presence of **H1** did not reach equilibrium even after two weeks (**Figure 5g** and **h**). This difference in rates correlates with the higher stability of the products formed from **H1** relative to those formed from **H2**.

Compared to the other reactions studied, the hydrazone exchange reaction is the slowest. Huc reported that aldehyde exchange using **H1** reached equilibrium in less than one hour in water at pH 8, and even shorter times at a lower pH.[38] In contrast, we used a large percentage of CD_3OD, which appears to slow down the reaction. We note that the exchange can be accelerated by increasing the proportion of water used as solvent, or by adding catalytic amounts of aniline or other organocatalysts such as Kool's 2-aminophenol catalysts,[11,22] but the speed of the reaction has no bearing on whether it is orthogonal to the others we explored, as described below.

D: Terpyridine Exchange

The final reaction that was studied was the ligand exchange of terpyridines complexed to zinc(II). The spectra of the free ligands **TP1** and **TP2** are shown in **Figure 6a** and **b**. Formation of the zinc complexes was completed in less than half an hour (**Figure 6c** and **d**). After addition of the second terpyridine, ligand exchange was complete in less than five minutes to give an equilibrium mixture of Zn(**TP1**)$_2$, Zn(**TP1**)(**TP2**), and Zn(**TP2**)$_2$ (**Scheme 2**; **Figure 6e** and **f**). The mixture of products obtained is independent of the order of addition of the terpyridine ligands.

Scheme 2: Terpyridine exchange.



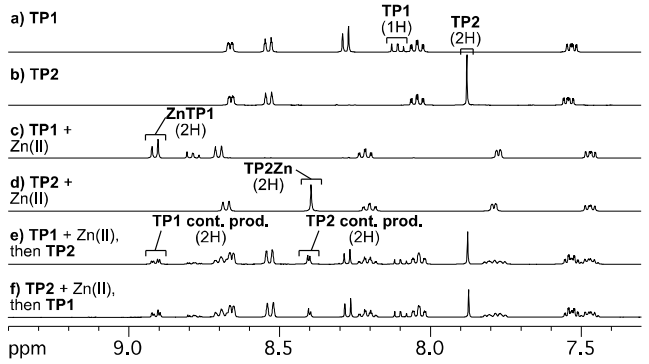


Figure 6. ^{1}H-NMR (400 MHz) of terpyridine exchange. Reaction conditions: 3:1 CD_3OD/HEPES (100 mM, pH = 7.4). [Zn(OTf)$_2$] = 5 mM. All other reaction components: 10 mM. Temperature: 25 °C. a) and b) Reference spectra of the terpyridines without zinc. c) and d) The reaction was allowed to equilibrate for 30-60 min. c) and d) The corresponding other terpyridine was added to a) and b), and the reaction was allowed to equilibrate for 5 min.

In addition, since terpyridine exchange has not previously been demonstrated in presence of the remaining functionalities present in the other exchanging molecules, one equivalent of **TP2** was added to the reaction mixture containing all 8 components. Again, terpyridine exchange was complete in less than five minutes. No changes are seen in the ^{1}H-NMR peaks corresponding to the remaining reactions, indicating that the terpyridine exchange occurs independently of the other three reactions.

Orthogonality

To test the orthogonality of all four reactions, reaction partners of two of the reactions were added to the same flask. After one day at room temperature, the resulting reaction mixtures were analyzed by ^{1}H-NMR spectroscopy and the resulting spectra were compared to those of each individual reaction run by itself. For all combinations of two reactions, the resulting ^{1}H-NMR spectra did not show any extra peaks (see supporting information) and looked essentially identical to the sum of the two spectra of the independently run reactions, indicating that the reactions occurred independently of each other and can therefore be considered orthogonal.

Even when all eight components necessary for the four reversible reactions (**BA1**, **HA1**, **CA1**, **T1**, **H1**, **A1**, **TP1**, and $Zn(OTf)_2$) were added to the same vial, the resulting ^{1}H-NMR spectrum did not show any indication of cross-reactions occurring (**Figure 7**). However, when a slight amount of extra $Zn(OTf)_2$ was added, extra peaks were observed in all reactions (see supplementary information). This is presumably due to the additional zinc coordinating to other starting materials. Therefore it is necessary to use equal or less than 0.5 equivalents of zinc relative to the total amount of terpyridine used if orthogonality is a concern.

To exclude the possibility of irreversible side reactions if zinc is added before addition of the terpyridines a control experiment was done where all reaction components of all four reactions except for the zinc were added and allowed to react for one day. The resulting ^{1}H-NMR spectrum does not show the extra peaks that were seen when 0.6 equivalents of terpyridine were added, however, a small change in the chemical shift of **T1** was observed (see supplementary information). Upon addition of **TP1**, the **T1** chemical shift returned to its original value, and the resulting NMR spectrum looked identical to the one obtained when all components were added at the same time. We conclude that the extra peaks are most likely a result of reversible metal complexation of other reaction components if less than two equivalents terpyridine are present.

Computational Analysis

One goal of our study was to develop a general method to confirm orthogonality of DCRs. In an attempt to further analyze and rigorously confirm the orthogonality of the four reactions, deconvolution attempts using SIMPLISMA[43,44] were undertaken. SIMPLISMA was developed in 1991 by Windig and coworkers. SIMPLISMA (Simple-to-use interactive self-modeling mixture analysis) is a software tool used to extract information about the components of a mixture, such as the pure component spectra and their concentrations in the mixture, from the spectra of mixtures if pure component spectra are unavailable. SIMPLISMA is based on a pure variable approach. A pure variable is a chemical shift at which the intensity only depends on the concentration of one component. This pure variable is then used to calculate the relative concentration of the component in each spectrum. This information is then used to resolve the spectra of all pure components mathematically.[43] SIMPLISMA has been most widely applied to IR and UV-Vis spectroscopy, but recent reports have shown its applicability for ^{1}H-NMR spectroscopy.[45]

For our studies, a simple, non-interactive version of SIMPLISMA taken from an article by Windig *et al*. was used.[44] To prepare the ^{1}H-NMR spectra for analysis, the regions containing buffer and solvent peaks were deleted. This was necessary due to the high intensity and varying chemical shift of the solvent and buffer peaks. In addition, bucketing was applied to the spectra before importing them into MATLAB. Buckets are small, regular spectral intervals over which an integral is calculated. These integrals are then used in place of the intensity at the ppm value for which the integral was calculated. Bucketing has the advantage of correcting for minor variations in peak shapes between spectra, as well as reducing the number of data points and therefore decreasing the computational time.[45] Monakhova and coworkers had found the best quantitative results for a bucket width of 0.04 ppm.[45] However, we found that the differences in obtained concentrations were minimal for bucket widths between 0.04 and 0.005 ppm, so we chose to use a bucket width of 0.005 ppm to retain structural information, such as splitting patterns. In order to successfully resolve the components, the number of linearly independent spectra needs to be bigger than the number of components. To achieve this, additional experiments were conducted where the relative concentrations of starting materials were varied (see **Table 1**, (a) and (b)). In addition to the spectra of each reaction by itself, the orthogonality experiments and reference spectra of the starting materials were used as input spectra.

SIMPLISMA was able to resolve all components successfully for three of the orthogonal pairs (Reactions **A** + **C**, **B** + **C**, and **C** + **D, Scheme 1**) For the remaining combinations, the algorithm was unable to find the correct pure variables for all components, or selected several resonances corresponding to the same compound. To circumvent this problem, instead of using SIMPLISMA to find the pure variables, the pure variables were chosen manually by visual inspection of the NMR spectra. Those pure variables were then used to calculate the component spectra and relative concentrations employing the same algorithm that SIMPLISMA uses. Since our goal was to extract the component spectra and calculate the concentrations of all components present, and not to automatically find the pure components, this work-around did not affect the applicability of the deconvolution method to our problem.

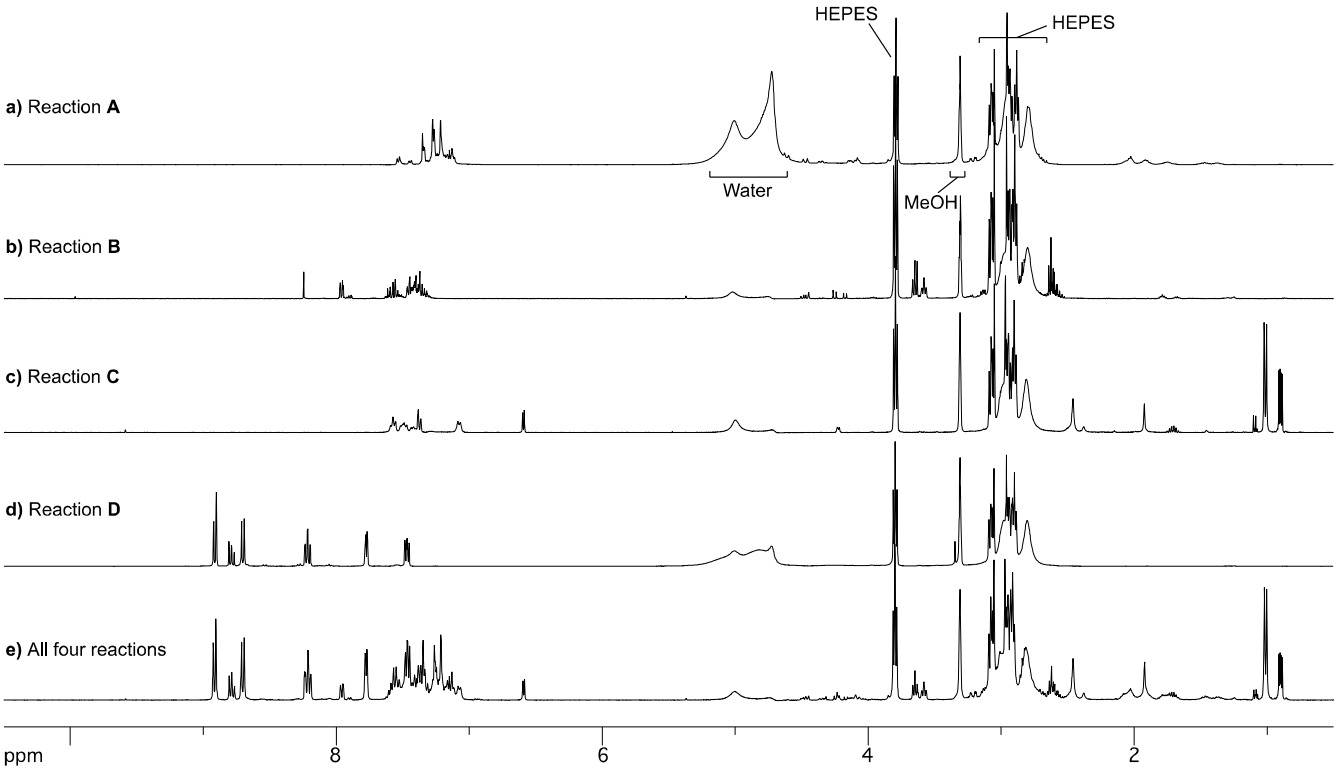


Figure 7. NMR experiment showing orthogonality if all components of all four reactions are present in the same reaction vessel. Reaction conditions: 3:1 CD_3OD/HEPES (100 mM, pH = 7.4). [Zn(OTf)2] = 5 mM. All other reaction components: 10 mM. Temperature: 25 ˚C. a)-d) Reference spectra of reactions A-D after 1 day equilibration time. e) All components of reactions A-D were added to the same flask and allowed to equilibrate for 1 day.

The resolved product spectra from the deconvolution of each reaction by itself, as well as the starting material spectra were used to calculate concentrations of each component in all acquired spectra (see supporting information). The known concentrations of the starting materials in the reference spectra were used as a standard. From the calculated concentrations, relative concentrations (percentages of product formation; **Table 1**) were calculated. When comparing the resulting concentrations in the isolated reactions to those in the spectrum containing all four reactions (highlighted cells in **Table 1**), similar numbers were obtained, indicating that the equilibria are not perturbed by the presence of the additional compounds. For instance, considering the results for **reaction A** by itself and in combination with other reactions (Entries 1, 6, 9, 12, and 24), the values for the relative concentrations of **BA1**, **HA1**, and **P(A)** are comparable. In all cases, more than 90% of product is formed, which is consistent with the ^{11}B-NMR (See supplementary material). The calculated **HA1** concentrations are slightly higher than expected, which is likely to be an artifact due to imperfect separation of the **HA1**, **BA1** and **P(A)** spectra due to extensive spectral overlap in the aromatic region. For **reaction B**, **CA1** and **P(B)** concentrations are consistent between entry 2 (reaction **B** by itself) and entry 24 (all four reactions in one). A possible explanation for the difference in **T1** concentration is that the **T1** NMR peaks are partially overlapping with the buffer peaks, therefore getting an accurate value is difficult. The outlier is entry 18 (**B** + **D**). The reason for the higher product concentration [**P(B)**] in this entry is that this experiment was allowed to equilibrate for a longer time (two days instead of one). Similarly, for **reaction C**, relative concentrations of **H1**, **A1**, and **P(C)**, are consistent throughout (entries 3, 9, 15, and 24), with the exception of entry 21, which was also recorded after an equilibration time of two days. As expected, **reaction D** is completely on the side of the product for all entries.

To further confirm that the spectra are sufficiently well modeled with the number of components, the spectra were reconstructed from the calculated relative concentrations and the input spectra. The R^2-values for the difference between those spectra and the measured spectra were calculated (**Table 1**). When using only one input spectrum for **TP1Zn**, the R^2 values for spectra containing **TP1Zn** were consistently lower. Upon closer inspection of the ^{1}H-NMR spectra, it was visible that the chemical shifts of **TP1Zn** vary slightly between spectra. After adding a second component representing **TP1Zn**, all R^2 values were around 0.9 or higher, indicating that the spectra are sufficiently well modeled and additional components are unnecessary.

When comparing the results obtained by NMR deconvolution to those obtained using integration of single peaks (not possible for **reaction A**), the relative product concentrations obtained using the deconvolution method are accurate, but consistently slightly lower than the concentrations obtained by integration of single NMR peaks. This is likely due to imperfect separation of the product and starting material spectra (see supplementary information). However, trends in the relative product concentrations are well captured, as is visible when comparing the percent of product formation with varying concentrations of starting material.

Table 1. Relative concentrations (in percent) of components in each spectrum.

Rxn #	Label	BA1	HA1	P(A)	CA1	T1	P(B)	H1	A1	P(C)	TP1	TP1Zn	R^2
1	**A**	9	35	91									0.96
2	**B**				45	53	55						0.94
3	**C (1d)**							42	39	58			1.00
4	**C (7d)**							17	15	83			1.00
5	**D**										0	100	1.00
6	**A + B**	9	20	91	48	47	52						0.95
7	**A + B**[a]	10	30	90	51	33	49						0.96
8	**A + B**[b]	8	24	92	68	48	32						0.97
9	**A + C**	9	20	91				46	28	54			0.96
10	**A + C**[a]	13	29	87				28	37	72			0.99
11	**A + C**[b]	8	24	92				60	64	40			0.97
12	**A + D**	7	22	93							0	100	0.90
13	**A + D**[a]	14	16	86							-2	102	0.97
14	**A+ D**[b]	9	20	91							-5	105	0.92
15	**B + C**				43	49	57	47	28	53			0.99
16	**B + C**[a]				60	53	40	25	35	75			1.00
17	**B + C**[b]				39	39	61	52	57	48			0.99
18	**B + D**				28	51	72				7	93	0.98
19	**B + D**[a]				69	52	31				3	97	1.00
20	**B + D**[b]				39	37	61				-2	102	0.96
21	**C + D**							21	45	79	12	88	0.98
22	**C + D**[a]							54	53	46	-3	103	0.92
23	**C + D**[b]							24	32	76	0	100	0.89
24	**All 4**	5	22	95	41	31	59	40	34	60	0	100	0.91

A = **BA1** + **HA1**. **B** = **CA1** + **T1**. **C** = **H1** + **A1**. **D** = **TP1** + $Zn(OTf)_2$.
[a]5 mM in starting concentrations of first reaction (e.g. reaction A ([**BA1**]$_0$ and [**HA1**]$_0$) in (**A** + **B**)), 15 mM for second reaction (7.5 mM for $Zn(OTf)_2$) (e.g. reaction **B** ([**BA1**]$_0$ and [**HA1**]$_0$) in (**A** + **B**)).
[b]15 mM in starting concentrations of first reaction, 5 mM for second reaction (2.5 mM for $Zn(OTf)_2$). All other starting concentrations are 10 mM (5 mM for $Zn(OTf)_2$). **P(A)** = Product of reaction **A**, etc. (see **Scheme 1**) Product percent is relative to **BA1** for reaction **A**, **CA1** for reaction **B**, **H1** for reaction **C**, and **TP1** for reaction **D**.

Conclusion

In conclusion, we have shown that the four reactions studied, boronic ester exchange, thiol addition to conjugate acceptor **CA1**, hydrazone exchange, and zinc complexation of terpyridines, are reversible and orthogonal in a mixture of methanol and water at close to neutral pH. In addition, we demonstrated an analytical protocol that should be widely applicable to confirm that dynamic covalent reactions can operate in a simultaneous and orthogonal fashion. Additional work will be necessary to speed up the hydrazone exchange and thiol/conjugate acceptor exchange to make their rates of formation and exchange more practical for applications in dynamic combinatorial chemistry.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures, ^{1}H- and ^{11}B-NMR spectra, mass spectra, extracted product ^{1}H-NMR spectra, calculation of component concentrations (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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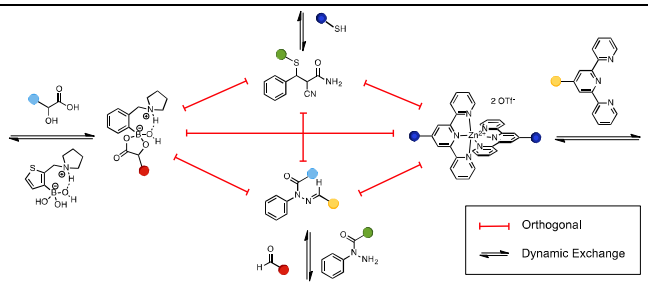
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