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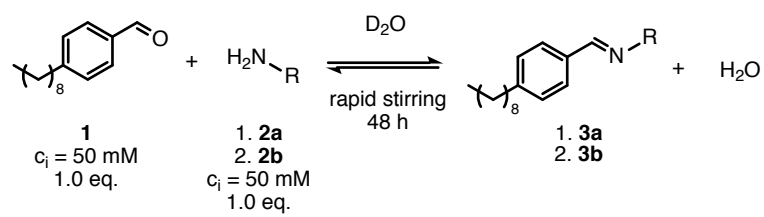
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Potential for Minimal Self-Replicating Systems in a Dynamic Combinatorial Library of
Equilibrating Imines

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Abstract

The presence of a self-replicator in a dynamic combinatorial library (DCL) offers function above and beyond libraries under thermodynamic control, moving towards out-of-equilibrium systems which mimic biological networks. In this work, we examine a previously reported DCL based on reversible imine formation to give amphiphilic structures. The amphiphilic imines were readily produced in organic solvents, and were found to aggregate to micelles in water as judged by diffusion-ordered NMR spectroscopy, dynamic light scattering and interferometric scattering microscopy. Unfortunately, the autocatalytic formation of products was not observed in water, and preformed imines slowly hydrolysed to aldehyde and amine components at neutral pH.

Introduction

Dynamic combinatorial libraries (DCLs) typically operate under thermodynamic control. The resultant equilibrium distribution of products from a complex mixture maximises the total thermodynamic stability of the system, which can be a result of assembly into stable interlocked structures,¹ binding to a template,² or the formation of supramolecular aggregates³ amongst other factors.⁴

Coupling a DCL with the amplification of components based on kinetic control gives rise to a more complex, emergent system, with the promise for increased functionality in comparison to systems under thermodynamic control.⁴ Biological systems operate far from equilibrium – many biological structures persist under dissipative conditions to perform function before their eventual decay into less functional materials. The construction of DCLs whose components can be transiently amplified by autocatalysis offers one approach towards mimicking these systems (Figure 1).⁵ In the first report of self-replication coupled to a dynamic combinatorial

library, the replication step acted as an irreversible kinetic trap, selecting between equilibrating components.⁵ Now, the autocatalytic behaviour of template replicators within DCLs is known where both the exchange between equilibrating components and the self-replication itself is, in theory, reversible.^{6–11} These systems have the potential to operate far from equilibrium, like biological systems, where the replicator persists under dissipative conditions.^{4,12}

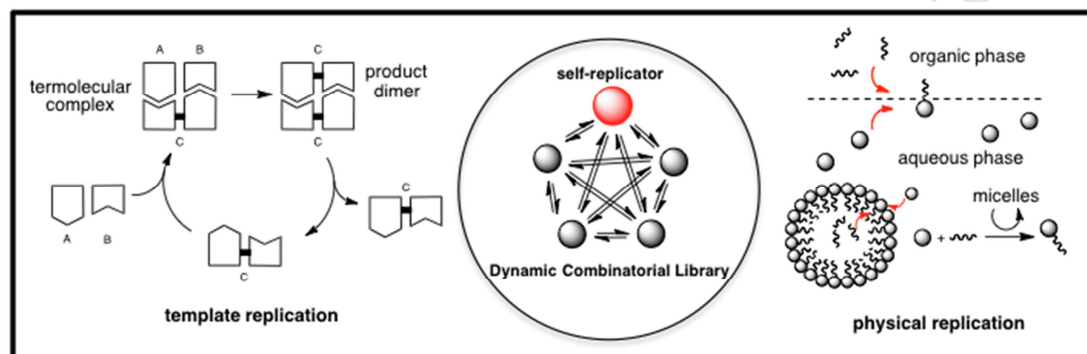


Figure 1 A dynamic combinatorial library (DCL) is characterised by the equilibration between library members by the exchange of building blocks. If a member of the library is a self-replicator, it may be amplified. Replication may occur via template or physical replication.¹³ Template replication: molecular recognition between product C and substrates A and B catalyses their reaction to form another molecule C. Physical replication: hydrophobic and hydrophilic components react slowly at the interface to form amphiphilic molecules. Above the critical aggregation concentration, aggregate structures are formed which allow increased mixing between phases and an increased rate of formation of amphiphilic products.

Aiming to overcome the strong product inhibition that can accompany template replication and reduce self-replicative efficiency, Nguyen et al. reported a system based on the self-replication of micellar assemblies (Figure 1).^{13–15} The synthesis of the amphiphilic components occurs by reversible imine bond formation between a hydrophobic aldehyde and a series of hydrophilic amines, and has the potential to create a DCL of equilibrating imines 3 (Figure 2). The amphiphilic imines are

reported to be stable in water on account of their aggregation into micellar structures and can self-reproduce via physical autocatalysis.^{13,16,17} The kinetics of the reaction between aldehyde 1 and amine 2c in isolation show sigmoidal growth of imine 3c, and additional seeding experiments also convincingly illustrate autocatalysis in this system.

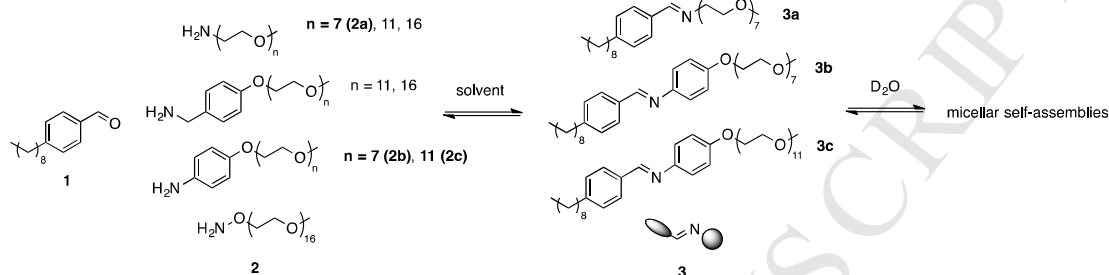


Figure 2 Reaction between aldehyde 1 and amines 2 in a DCL results in the formation of imines 3 which aggregate into supramolecular structures in D₂O. In isolation, the reaction between amine 2c and aldehyde 1 in D₂O is shown to be autocatalytic; micelles of product 3c catalyse the condensation between the hydrophobic and hydrophilic components.

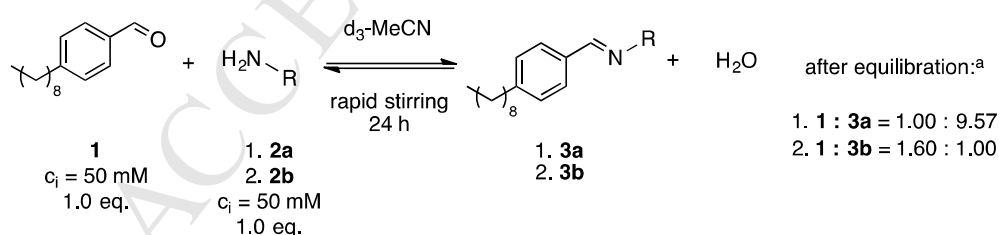
A competition experiment between amines 2a and 2c for the imine formation with aldehyde 1 illustrates self-replication within a (small) DCL. In this case, imine 3a is thermodynamically less stable than imine 3c in water, producing 3c autocatalytically at the expense of imine 3a. It should be noted that the autocatalytic replication of imine 3c does not push the system out-of-equilibrium; the ratio of imine 3c to imine 3a at the end of the autocatalytic period is equal to the thermodynamic distribution.

The high reported thermodynamic stability of the imine library in water and the construction of self-reproducing amphiphiles based on a reversible imine bond formation was of interest to our group.^{3,14,18–26} We have previously studied physical autocatalysis under kinetic control, in which the bond-forming step is an irreversible thiol-Michael reaction.¹⁶ The reaction was studied both in the bulk and at the single-particle level using interferometric scattering microscopy (iSCAT).²⁷ At this level the

autocatalytic self-reproduction of micellar structures can be observed and quantified at the nano-scale in real time, allowing direct observation of the reactive interface and local variations in reactivity which are not possible on observation of the bulk.²⁸ We were therefore interested in using the technique to observe an autocatalytic reaction under thermodynamic control, and decided to examine components of the imine-based system reported by Nguyen *et al.*¹⁴

Of the reported components we chose to examine amines 2a and 2b (Figure 2), allowing comparison between amines of different nucleophilicity with the same polyethylene glycol chain length. The amine components were synthesised in accordance with a later report,²⁴ and the aldehyde according to an independent publication.²⁹

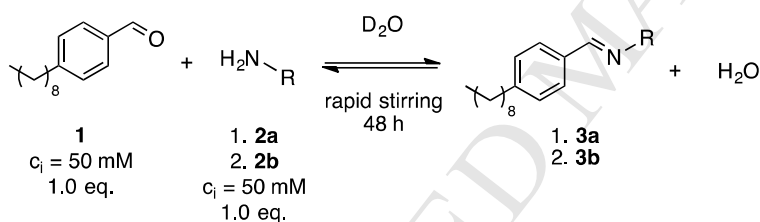
We first performed a reaction to determine the thermodynamic stability of the imines 3a and 3b in deuterated acetonitrile. In order to reach equilibrium, each amine was stirred rapidly with an equivalent of aldehyde at room temperature for 24 hours before taking a ¹H NMR spectrum (Scheme 1). Increased imine condensation was observed for amine 2a as expected on account of the increased nucleophilicity of the amine and concomitant increased stability of the imine bond, as expected and as previously reported.¹⁴



Scheme 1 Equilibrium formation of imines 3a and 3b in d_3 -MeCN. Both starting material components are fully soluble under these conditions. ^a Ratios determined by integration of aldehyde and imine peaks in ¹H NMR spectra of the equilibrated mixtures.

Using diffusion-ordered spectroscopy (DOSY) we confirmed that no aggregate structures were formed from imines 3a and 3b in d_3 -MeCN, as expected, since all components are fully soluble and there is therefore no phase separation. These experiments also showed that the free aldehyde and amine components at equilibrium diffuse independently of imines 3a and 3b (see SI).

Nguyen et al. subsequently measured the equilibrium composition of the system in aqueous conditions by dilution of the experiment performed in d_3 -MeCN with D_2O , before removal of d_3 -MeCN in vacuo to reach a final concentration of 50 mM in D_2O .¹⁴ It is unclear whether this mixture was allowed to equilibrate after the solvent exchange and hence whether it is truly an equilibrium measurement. We performed this measurement as detailed in Scheme 2 in D_2O , analogous to the equilibration conditions in d_3 -MeCN.



Scheme 2 Attempted formation of imines 3a and 3b in D_2O . Aldehyde 1 is poorly soluble in D_2O .

After rapid stirring of both systems for 48 hours, we saw no evidence of imine formation in D_2O . This is in contrast to the results obtained by the reported procedure, which involved a solvent switch of the pre-equilibrated mixture from d_3 -MeCN to D_2O followed by NMR spectroscopy of the mixtures to determine an equilibrium ratio under aqueous conditions. In the previous report, although relatively little condensation between amine 2a and aldehyde 1 was seen, for amine 2b nearly complete (> 95%) formation of 3b from aldehyde 1 was reported at equilibrium. The

aqueous stability of 3b in these experiments was attributed to its self-assembly into supramolecular aggregates, which provided protection against hydrolysis.

In order to test the thermodynamic stability of imines 3a and 3b they were synthesised under anhydrous conditions before dissolution in D₂O. Both imines showed extensive hydrolysis in an NMR tube over approximately 15 hours, and both showed complete hydrolysis to the starting materials after several days. Imine 3b showed more rapid decomposition to D₂O-soluble amine 2b and D₂O-insoluble aldehyde 1 (Figure 4, Figure S1), whilst imine 3a decomposed more slowly, with an apparent solubilisation of the forming aldehyde 1. It appeared clear that in our hands imines 3a and 3b were thermodynamically unstable under neutral aqueous conditions (Figure 4), excluding the possibility for their formation in situ from aldehyde and amine components.

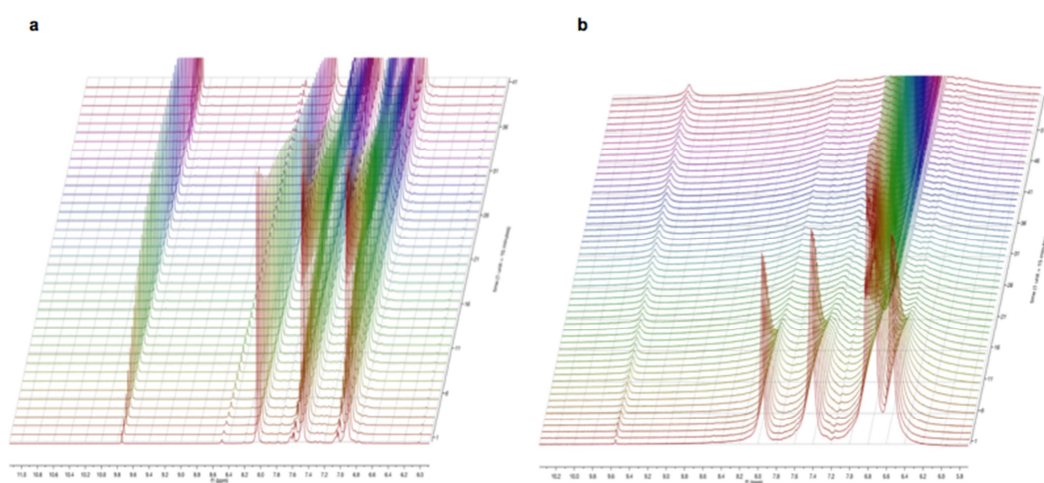


Figure 4 a) Imine 3a ([imine]₀ = 50 mM) showed significant hydrolysis to amine and aldehyde components over 15 h, ¹H NMR spectra recorded every 15 minutes. b) Imine 3b ([imine]₀ = 50 mM) showed very rapid hydrolysis over 15 hours to amine and aldehyde components, ¹H NMR spectra recorded every 15 minutes.

The previous publication reports the use of neutral aqueous solutions only. However, later reports from the same group also investigate the stability of these compounds

over a range of pH/pD values.^{24–26} We were therefore interested to see whether aqueous solutions at higher pD offered enhanced stability.

Solutions of imines 3a and 3b were prepared in D₂O at pH* 12 (pD 12.45)³⁰ and their thermodynamic stability was monitored by NMR, as above. Imine 3a showed no increase in stability, hydrolysing to aldehyde and amine components. However, imine 3b, in contrast to the experiment at neutral pD, showed no hydrolysis over at least 13 hours.

Encouraged by this result, we attempted to observe the formation of imine 3b from aldehyde and amine components at pD 12.45 over 20 hours in an experiment analogous to that in Scheme 2. However, only trace quantities of imine 3b were observed.

Failing to observe autocatalysis, it was important to determine whether imines 3a and 3b did, in fact, aggregate in aqueous solution. The aggregation properties of 3a and 3b were studied using DOSY as well as dynamic light scattering (DLS) in water.

DOSY of imine 3a was most facile since it was well solvated with sharp, well-resolved peaks in its ¹H NMR spectrum in D₂O. Imine 3a was found to diffuse with a hydrodynamic radius of 6.0 nm in D₂O, comparable to the value reported of 5 nm and consistent with the formation of a supramolecular aggregate structure (Table 1, entry 1). Fortuitously, a small quantity of aldehyde 1 was present in the sample since imine 3a was not purified following synthesis. DOSY revealed this aldehyde to be associated with the aggregate structure, diffusing at a rate of similar magnitude to the imine 3a and at a much decreased rate to the control experiment of the aldehyde alone in D₂O (Table 2, entries 1 and 4; Figure 3). The association of the hydrophobic component of the reaction with the micelle structure is a crucial requirement for the development of an autocatalytic system.

Hydrodynamic radii R_h /nm						
		This study		Literature values		
Entry	Species	DOSY	DLS	DOSY	DLS	SANS ^a
1	3a	6.0	-	5	-	-
2	3b	8.1	6.1	-	-	6 x 34 ^b

Table 1 Hydrodynamic radii recorded in D₂O, 50 mM. ^a Small angle neutron scattering ^b diameter x length: small angle neutron scattering found cylindrical micelles

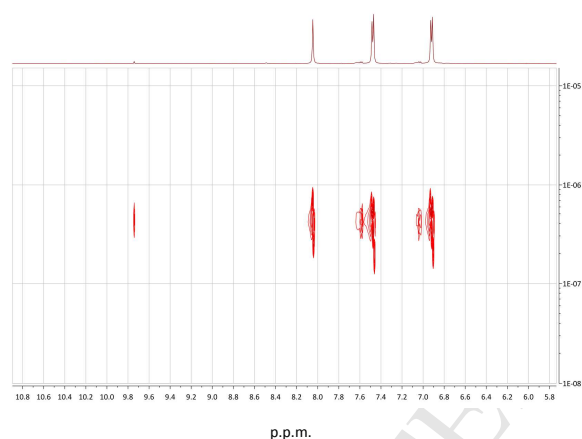


Figure 3 2D DOSY NMR showing uptake of aldehyde 1 into micelles of 3a (entry 4, Table 1). Imine 3a was dissolved in D₂O at a concentration of 50 mM.

		Diffusion coefficient $D/10^{-10} \text{ m}^2\text{s}^{-1}$				
Entry	Species present	1	2a	2b	3a	3b
1	1	1.41	-	-	-	-
2	2a	-	4.23	-	-	-
3	2b	-	-	3.70	-	-
4	3a	0.26	n.d. ^a	-	0.41	-
5	3b	n.d. ^b	-	n.d. ^a	-	0.30

Table 2 Diffusion coefficients established by DOSY NMR spectroscopy. All components were measured at a concentration of 50 mM in D₂O (assuming 100% purity). The presence of a small quantity of aldehyde 1 and amines 2a and 2b in experiments on 3a and 3b (entries 4 and 5) are a result of their synthesis without purification. ^a Could not determine D due to overlap of peaks ^b Aldehyde peak too small for accurate integration.

DOSY of imine 3b was less facile on account of the relatively broad peaks in the spectrum, but consistent with a hydrodynamic radius of 8.1 nm (Table 1, entry 2). Fortunately, however, we were also able to perform DLS experiments on this species, and observed micelles with a hydrodynamic radius of 6.1 nm. The previous publication reports aggregates too large to observe by DOSY spectroscopy, which may explain the broad peaks we observed; neutron scattering experiments revealed cylindrical micelles of 6 nm in diameter and 34 nm in length (Table 2).¹⁴ It is difficult to compare our DLS data with this measurement since our methods cannot give accurate information for non-spherical objects. We could be reasonably confident, however, that we also observed aggregation of imine 3b.

Finally, unable to observe any autocatalysis of these systems in the bulk, it was of interest to examine them by iSCAT microscopy. Our previous study of the thiol-Michael reaction found that performing biphasic autocatalytic reactions on the microliter scale on a glass coverslip significantly reduced the lag period before the onset of autocatalysis and the production of product. We were unable to observe aggregates of imine 3a by iSCAT, but experiments using imine 3b allowed us to observe the presence of supramolecular aggregates consistent with micelles binding to the coverslip, as shown in Figure 5. However, upon mixing aldehyde 1 and amine 2b on the glass coverslip, no micelle formation appeared to occur (Figure S2).

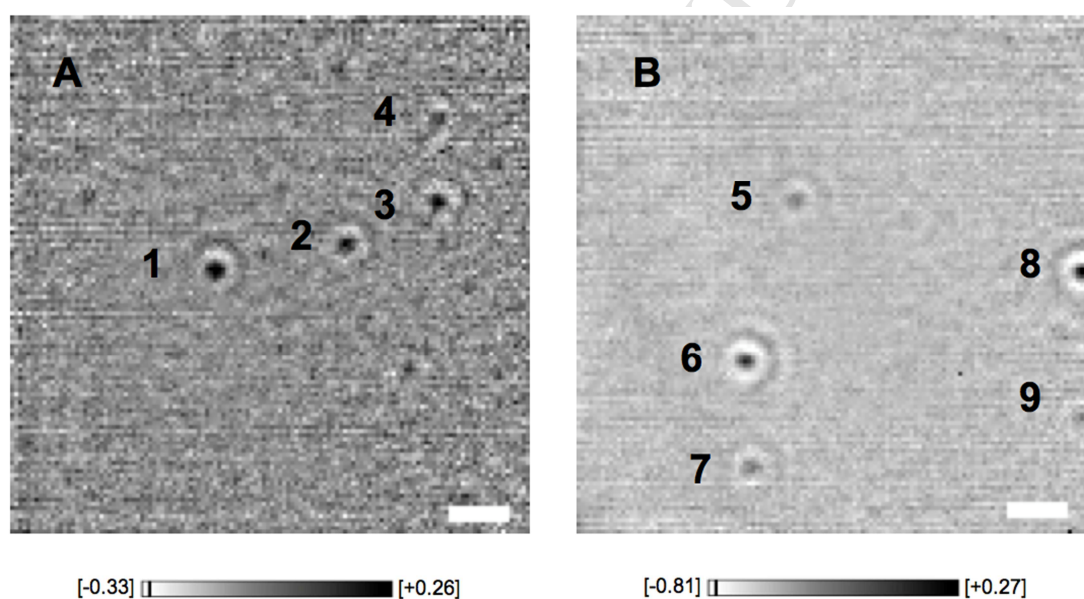


Figure 5 Images **A** and **B** are independent images of the binding of imine 3b (~ 1 mM in H_2O) to the microscope coverslip as aggregates (individual aggregates numbered within each image). Each image is the average of 100 frames. Scale bar = $1\ \mu\text{m}$. The bar below the images represent the contrast range of each image; the individual contrast of each aggregate provides a measure of its size.

In conclusion, Nguyen et al. reported the physical self-amplification of imine 3c, whose dominance in a DCL is based on its high thermodynamic stability in water. We

were interested in this system as an autocatalytic reaction in which the bond-forming step was reversible, and examined parts of their reported DCL system which we felt might be able to undergo physical autocatalysis.

Though imines 3a and 3b formed micellar structures in water according to a variety of techniques (DOSY, DLS, iSCAT), we found that these structures were thermodynamically unstable and hydrolysed fully to aldehyde and amine components in neutral aqueous solution. The only apparent difference between this work and the previous study of 3a and 3b is the way the aqueous samples were prepared. Since it was not clear to us that the reported solvent-switch from d_3 -MeCN to D_2O would measure the true equilibrium position, we instead allowed the aldehyde and amine components to equilibrate in D_2O , and, from the other direction, dissolved pre-formed imines in D_2O . Both procedures resulted in no detectable imine at equilibrium.

The enhancement in stability for imine 3b at pD 12.45 did not result in the observation of autocatalysis over the first 20 hours of the reaction. As the kinetics of formation of 3b were not reported by Nguyen *et al.* it is difficult to draw a direct comparison. However the strong dependence on pH/pD, variation in sample preparation, and subsequent reports of the group in which other parameters are varied such as stoichiometry,^{24–26} reveal the complex and numerous factors which may influence imine stability and the observation of autocatalysis.

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Financial interests

The authors declare no competing financial interests.

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