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Adsorption of RNA on mineral surfaces and mineral precipitates

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Full Research Paper

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Abstract

The prebiotic significance of laboratory experiments that study the interactions between oligomeric RNA and mineral species is difficult to know. Natural exemplars of specific minerals can differ widely depending on their provenance. While laboratory-generated samples of synthetic minerals can have controlled compositions, they are often viewed as "unnatural". Here, we show how trends in the interaction of RNA with natural mineral specimens, synthetic mineral specimens, and co-precipitated pairs of synthetic minerals, can make a persuasive case that the observed interactions reflect the composition of the minerals themselves, rather than their being simply examples of large molecules associating nonspecifically with large surfaces. Using this approach, we have discovered Periodic Table trends in the binding of oligomeric RNA to alkaline earth carbonate minerals and alkaline earth sulfate minerals, where those trends are the same when measured in natural and synthetic minerals. They are also validated by comparison of co-precipitated synthetic minerals. We also show differential binding of RNA to polymorphic forms of calcium carbonate, and the stabilization of bound RNA on aragonite. These have relevance to the prebiotic stabilization of RNA, where such carbonate minerals are expected to have been abundant, as they appear to be today on Mars.

Introduction

It has been nearly 70 years since Bernal broadly conjectured on possible roles of rocks and minerals in the assembly of complex organic species relevant to the origin of life [1]. This theme has now been revisited multiple times [2,3]. Rocks and minerals have been proposed to have had multiple roles that might have been productive for the emergence of Darwinism on Earth. Classically, these roles have included:

(i) Concentration. Whether they are delivered by meteorite or created on Earth, prebiotic organic molecules are expected to be

dilute and, if concentrated, react unproductively with each other. Rock and mineral surfaces offer the opportunity to concentrate relevant species from dilute aqueous environments, perhaps without unproductive intermolecular reactions. Such adsorption as a concentration mechanism offers an alternative to evaporation in a desert environment. Mineral adsorption from a large ocean is an especially attractive alternative to desert evaporation for those who think that dry land was sparse on the early Earth [4].

(ii) Productive catalysis. Concentration is itself a way of "catalyzing" bimolecular reactions. However, rocks and minerals have also been considered as sources of conventional catalysis, where species on the surface of the mineral stabilize a transition state with respect to adsorbed ground state species [5].

More recently, and especially after the emergence of the "RNA first" hypothesis for the origin of Darwinism on Earth [6], rocks and minerals have been considered in other roles.

(iii) As inhibitors of reactions. One key problem obstructing the assembly of prebiotically productive organic species is the well-known propensity of organic molecules, especially those containing carbonyl groups such as carbohydrates, to react further to yield unproductive "tars". Mineral species, especially if they are slightly soluble into an aqueous environment, have been proposed to prevent classes of unproductive reactions [7,8].

(iv) Stabilizers. Many useful pre-biological polymers are subject to destruction by environmental forces, such as ultraviolet radiation and radioactivity. Adsorption of these onto mineral surfaces has been shown to slow that destruction [9], in some cases without greatly damaging the catalytic activity of those pre-biopolymers [10], in other cases with evolution [11].

As Hazen and Sverjensky remark [12], mineral environments are far more complex than the "Pyrex® prebiotic chemistry" that dominates the field. However, in addition to creating an opportunity, this complexity creates problems, both intrinsic and experimental. For every constructive reaction that might be catalyzed by a mineral, the potential exists for that mineral to catalyze a destructive reaction. Further, although a mineral (by definition) is a pure substance, real minerals invariably have non-canonical elements incorporated within them; these defects may easily be the reason why a natural mineral adsorbs organic molecules or has an interesting reactivity. Further, even with an ideally pure mineral, the catalysis of interest can occur in defects in its crystalline surface. All of these problems are difficult to manage in a controlled laboratory environment.

How are we to explore this new complexity as we accommodate those who "plead" for a role for mineralogy in models for the origin of life? Two approaches are possible. On one hand, we might build a collection of natural minerals, and then run experiments on them with biopolymers having prebiotic interest, such as RNA. Unfortunately, natural minerals vary in chemical composition from specimen to specimen, and certainly from locale to locale. This is obvious even to an amateur. For example, natural calcium phosphate (apatite), of possible prebiotic interest as a source of the phosphate essential to prebiotic RNA synthesis [13], has different colors that reflect inclusion of different atomic species that are not in the canonical formula of the mineral.

Alternatively, reagents that have the components of those minerals, with exacting levels of purity, might be mixed in the appropriate ratio to create a synthetic mineral as a precipitate. Experiments might then be run on these synthetic minerals to study their interaction with biopolymers of interest, such as RNA. This approach has the advantage of offering exactly the kind of "controlled experiments" that chemists like. However, it is frequently criticized as being "artificial".

Even if this problem were to be mitigated or ignored, general chemical physics intervenes. Solid phases with high surface areas, and precipitates in particular, are general adsorbents, especially for macromolecules. Therefore, it is difficult to know, if RNA (for example) adsorbs onto a surface, whether the adsorption is in any sense specific, or whether it is just a general manifestation of big molecules adsorbing to big surfaces.

Here, we introduce a general strategy that mitigates some of these problems. The experiments measure the adsorbance of radiolabeled RNA onto binary inorganic species that have been obtained in two ways. In one, the species is precipitated as a synthetic mineral via a double decomposition reaction between the two mineral components. In the second, the mineral itself is obtained from a natural source, and the experiment measures the percentage of radiolabeled RNA bound to the natural mineral. In a third approach, two precipitated minerals are combined, and the partition of radiolabel RNA between the two is measured.

This strategy then asks whether the trend in radiolabeled RNA adsorption is consistent across their various forms and presentations, especially within a set of minerals having a common anion (for example, all carbonates) but differing in their cationic components (for example, magnesium carbonate, calcium carbonate, strontium carbonate, and barium carbonate). Here, we may even seek a Periodic Table trend, where adsorption changes consistently in a series of minerals as one of their ele-

ments is replaced by another element in a row or column of the Periodic Table.

Underlying these experiments is the following rationale: If the same trends are observed both in precipitated synthetic minerals as well as in natural minerals, and if radiolabeled molecules are partitioned consistently between two mineral species precipitated together, the effects cannot easily be nonspecific as general adsorption of big biopolymers onto big surfaces.

We report here the first cases where this rationale has been applied for RNA over a range of minerals. Surprisingly, some of these showed Periodic Table trends, in both their precipitated synthetic and natural forms. Further, we speculate that these trends can be accounted for by the changing size of the mineral lattice resulting from different ionic radii of different elements in a Periodic Table series.

Results

Carbonates

We examined first various binary carbonate minerals with Group II (alkaline earth) cations. These are interesting not only because carbon dioxide is likely to have been an abundant component of an early Earth atmosphere, but also because alkaline earths form a well-known set of binary carbonates that include insoluble magnesium, calcium, strontium, and barium carbonates (magnesite, calcite, strontianite, and witherite, respectively).

The magnesite specimen was from Minas Gerias, Brazil; the calcite was a specimen of "Iceland spar". The strontianite was obtained from the Minerva Mine in Illinois , and the witherite was obtained from Cave in Rock, Illinois. The specimens were washed with hydrogen peroxide (30%) followed by water and then ethanol to remove potential organic surface contaminants. The samples were then dried in air while covered.

To flat surfaces of the cleaned mineral were added droplets of an aqueous (unbuffered) solution of 5'-^{32}P labeled 83-mer RNA (2 μL, 50 nM). This length was chosen because it is representative of lengths that Holliger, Joyce and others suggest is needed to initiate Darwinism [14,15]. Although shorter lengths have been recently shown to be able to assemble in longer molecules with replicase activity [16], these were not tested in this study. Data from Ferris' lab suggest that, for adsorption on montmorillonite clays, longer RNAs adsorb better than short RNAs [17]. This deserves to be addressed systematically in a separate study.

After adsorption, the mineral surface was washed several times with H_2O to remove unbound RNA. Then, the amount of RNA bound was calculated by subtraction of counts per minute in the washes.

The results are shown in Figure 1. Here, we were surprised to see a Periodic Table trend in these carbonates. Thus, while only a quarter of the radioactivity remained bound to the surface of the specimen of magnesite (with magnesium), ≈94% of the reactivity was bound to the surface of the specimen of witherite (with barium). The fraction bound to calcite (calcium) and strontianite (strontium) were intermediate, 47% and 83%. Thus, a Periodic Table trend is observed with the binding of RNA to the carbonates relatively Ba > Sr > Ca > Mg.

Following the dual-approach rationale, we then asked whether the same results could be qualitatively observed with precipitat-

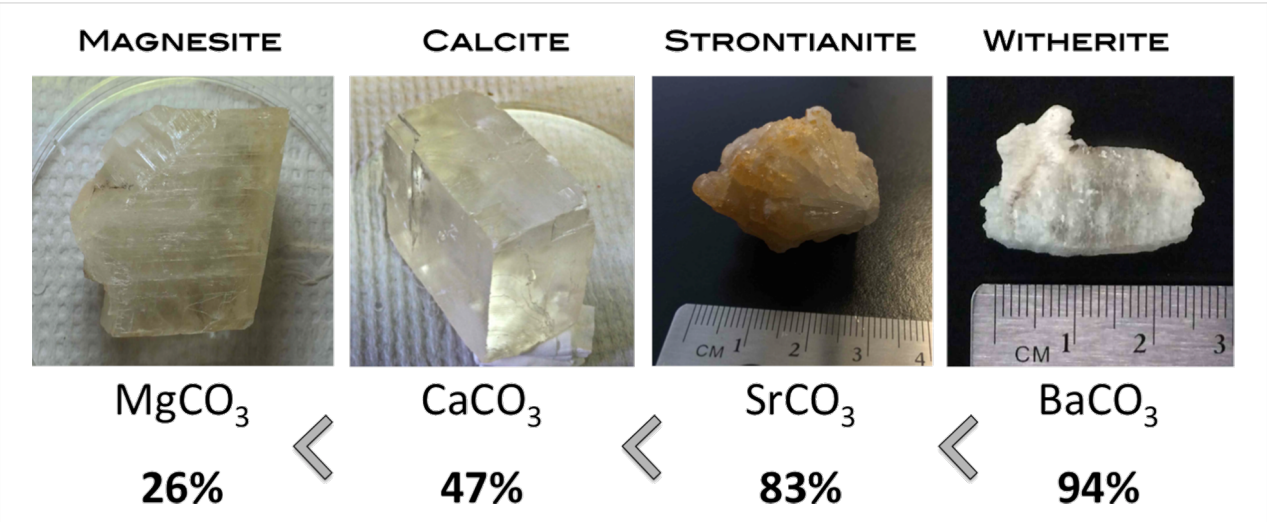


Figure 1: Adsorption of RNA on natural carbonate mineral samples.

ed synthetic minerals (Materials and Methods). These results are collected in Table 1. The same Periodic Table trend is observed with the precipitated synthetic carbonate minerals. Here, the percentage adsorption ranged from 95% to 77%, again with the ranking Ba > Sr > Ca > Mg. Again, the precise percentages have no easy interpretation (but see below). However, the fact that the same trend is observed with the precipitated synthetic minerals suggests that the trend with the natural minerals is not due to impurities in the natural species.

To complete the analysis, we then co-precipitated various pairs of synthetic carbonates by mixing the appropriate aqueous solutions of the alkali metal chlorides with an aqueous solution of sodium carbonate in a 1:1 ratio (Figure 2). These were then easily separated gravitationally, as the different carbonates have different densities ($CaCO_3$ 2.71 g/cm^3; $MgCO_3$ 2.96 g/cm^3; $SrCO_3$ 3.5 g/cm^3; $BaCO_3$ 4.29 g/cm^3). The partition of RNA between each pair was then observed by pre-equilibration of the radiolabeled 83-mer RNA in a column with the two minerals, followed by dissection of the column and counting various slices within it. The labeled RNA partitioned as seen in the synthetic minerals precipitated individually: Ba > Sr > Ca > Mg.

Sulfates

Binary alkaline earth sulfates are fewer in mineral form, since the first member of the Periodic Table series (magnesium sulfate, epsomite) is quite soluble in water. However, with precipitated synthetic minerals, the same trend was observed, with barium sulfate binding RNA more than strontium sulfate, which bound more RNA than calcium sulfate (Table 1). The corresponding trend was also observed in the specimens of the natural minerals, with baryte > celestine > gypsum (59% > 49% > 20%) (Table 2).

Constraints of natural mineralogy

However, and as a limitation of this approach, many minerals that might be made in the laboratory have no known natural correlates that we can examine in parallel. For example, in the synthetic borate minerals, the barium species also binds most tightly. However, to our knowledge, no natural strontium or barium borate mineral has been reported. The natural calcium borate mineral that we tested (colemanite) bound 31% of the RNA presented to it. Further, although the magnesium borate mineral is known naturally as boracite, we were unable to get a precipitate of the synthetic mineral by mixing magnesium chloride and sodium borate (Table 1).

Table 1: Adsorption of RNA on synthetic minerals formed by double-decomposition reactions.[a]

	$MgCl_2$	$CaCl_2$	$SrCl_2$	$BaCl_2$	$MnCl_2$	
$Na_2B_4O_7$	no PPT	86%	87%	95%	87%	
Na_2CO_3	magnesite 77%	calcite 86%	strontianite 90%	witherite 95%	rhodochrosite 89%	
Na_2PO_4[b]	64%	apatite 93%	strontium apatite 84%	barium apatite 32%	metaswitzerite 86%	
Na_2SO_4	no PPT	gypsum 2%	celestine 71%	baryte 88%	no PPT	
Na_3VO_4	magnesium coulsonite 78%	cavoite 92%	73%	85%	ansermetite 38%	
Na_2HAsO_4	6%	johnbaumite 73%	4%	gurimite 30%	61%	
NaF	no PPT	fluorite no PPT	72%	15%	no PPT	
	$FeCl_2$	$FeCl_3$	$CoCl_2$	$NiCl_2$	$CuCl_2$	$ZnCl_2$
$Na_2B_4O_7$	88%	no PPT	87%	94%	96%	93%
Na_2CO_3	siderite 65%	no PPT	cobalite 95%	94%	malachite 73%	smithsonite 93%
Na_2PO_4[b]	vivianite 68%	30%	pakhomovskyte 80%	75%	libethenite 84%	hopeite 6%
Na_2SO_4	22%	no PPT	no PPT	no PPT	no PPT	no PPT
Na_3VO_4	fervanite 2%	46%	2%	12%	73%	17%
Na_2HAsO_4	75%	10%	erythrite 12%	49%	lammerite 79%	adamite 62%
NaF	24%	29%	no PPT	no PPT	43%	no PPT

[a]No PPT: no precipitate observed. For some minerals, the name of the natural species is reported. [b]Na_3PO_4 + $NaHPO_4$.

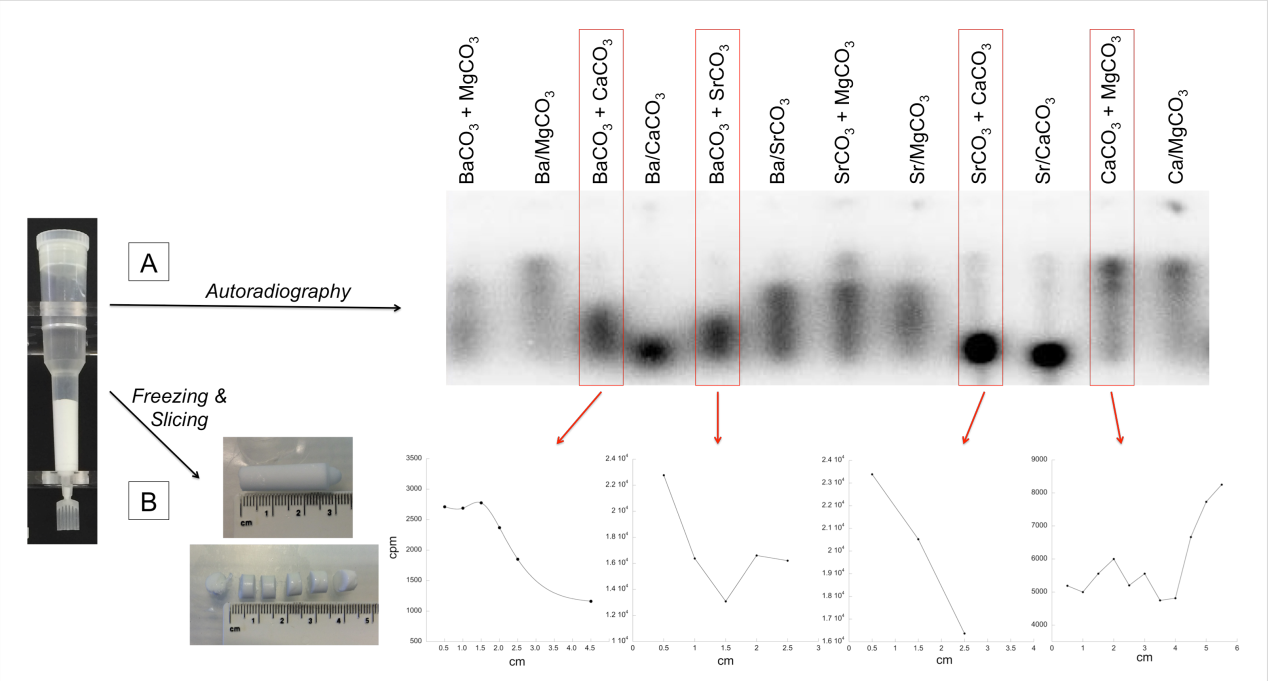


Figure 2: Co-precipitation experiments on carbonate minerals for RNA-binding competition. The precipitated column formed by two carbonates and containing radioactive RNA (left, see text for details) is exposed to a phosphorimager screen to observe RNA localization in the column (A). For each pair, carbonates are either prepared separately and then mixed together ("xCO_3 + yCO_3", first column per pair), or all the ingredients are co-precipitated ("$xyCO_3$", second column per pair). Subsequently (B), columns xCO_3 + yCO_3 are frozen, cut in slices, and the radioactivity in each slide plotted versus the column height in cm. Columns for the pair Ba–Mg and Sr–Ca could not be frozen and sliced due to their high lability; their autoradiograms were profiled instead for RNA localization (data not shown). Carbonate density: $CaCO_3$ 2.71 g/cm^3 < $MgCO_3$ 2.96 g/cm^3 < $SrCO_3$ 3.5 g/cm^3 < $BaCO_3$ 4.29 g/cm^3.

Differential adsorbance need not proceed uniformly across the Periodic Table

While a Periodic Table trend is easy to observe, there is no reason a priori why such a trend should exist. For example, one might speculate that RNA would adsorb better onto a surface if the pattern of anion and cation sites on that surface matches more closely the distances of the anionic sites (phosphates) on the RNA molecule. While one might expect different cations in a mineral would change the spacing of those sites, there is no reason why the heaviest cation would have sites that match RNA the best. Indeed, if this were the mechanism for different surfaces having different affinities for RNA, one might expect within a Periodic Table trend to have a mineral that maximally absorbs somewhere in the middle of the series, rather the end of the series.

We may, in fact, see this in these data. For example, among the precipitated phosphates, the calcium species bound more RNA (93%) than the magnesium phosphate (64%), the strontium phosphate (84%), and barium phosphate (32%). While the calcium phosphate is well-known in the natural world in various forms (apatite), and while calcium is known to be replaced in natural minerals by strontium and barium to give species known as "strontium apatite" and "barium apatite", the strontium and barium forms are very seldom found in nature, and are not available for this kind of study.

The same comments apply to vanadates and arsenates, which we examined because of their structural resemblance to phosphates [18,19]. Here, the synthetic alkaline earth minerals showing the best binding are calcium vanadate and calcium arsenate. The synthetic transition element arsenates and vanadates that bind RNA best are both with copper. However, natural minerals that incorporate these specific atomic constituents are quite rare. For example, the most common vanadate mineral in museums (vanadinite) has lead as its cation. Vanadinite and calcium phosphate have analogous crystal forms (as do the lead arsenate mimetite and the lead phosphate pyromorphite). Further, vanadinite adsorbed RNA well (72%). However, lead strikes us as being an unlikely element to have been involved in prebiotic chemistry (but see refs. [20-22]).

Adding complexity

The alkaline earth carbonates and sulfates make conveniently simple systems where the natural-synthetic combination analysis can be easily applied. Other classes of minerals are more difficult to manage for two classes of reasons.

Table 2: Adsorption of RNA on all the natural minerals tested in this study.

Family	Mineral	Adsorption
carbonates	magnesite, $MgCO_3$	26%
	calcite, $CaCO_3$	47%
	aragonite, $CaCO_3$	76%
	strontianite, $SrCO_3$	83%
	witherite, $BaCO_3$	94%
	rhodochrosite, $MnCO_3$	11%
	smithsonite, $ZnCO_3$	5%
sulfates	gypsum, $CaSO_4$	20%
	celestine, $SrSO_4$	49%
	baryte, $BaSO_4$	59%
phosphates & vanadates (apatite family)	apatite, $Ca_2(PO_4)_3Cl$	28%
	vanadinite, $Pb_5(V/AsO_4)_3Cl$	72%
	vivianite, $Fe_3(PO_4)_2$	12%
arsenates	erythrite, $Co_3(AsO_4)_2$	92%
	adamite, Zn_2AsO_4OH	2%
fluorites	purple fluorite, CaF_2	no adsorption
	green fluorite, CaF_2 + Fe or Sm inclusions	25%
borates	colemanite, $CaB_3O_4(OH)_3$	31%
silicates	opal, SiO_2	27%
	talc, $Mg_3Si_4O_{10}(OH)_2$	95%
	topaz, $Al_2SiO_4(F,OH)_2$	33%
	amazonite, $KAlSi_3O_8$	31%
	mica, $KAl_3Si_3O_{10}(OH)_2$	22%
	beryl, $Be_3Al_2Si_6O_{18}$	17%
	olivine, $(Mg,Fe)_2SiO_4$	12%
	obsidian, SiO_2+MgO+Fe_3O_4	8%
	danburite, $CaB_2(SiO_4)_2$	no adsorption
	tourmaline, $(Na,Ca)(Mg,Li,Al,Fe^{2+})_3Al_6(BO_3)_3Si_6O_{18}(OH)_4$	no adsorption
	agate, SiO_2	no adsorption
	herkimer Diamond, SiO_2	no adsorption
oxides	pyrite, FeS_2	95%
	hematite, Fe_2O_3	30%
	rutile, TiO_2	21%
	olivine, $(Mg,Fe)_2SiO_4$	12%
	magnetite, Fe_3O_4	no adsorption

First, the cation(s) in the mineral may be redox active. Here, in a precipitation to give a synthetic mineral, the presence of oxygen can lead to a precipitate having the cation in mixed oxidation states.

Second, we cannot conveniently add a buffer to control the pH in an experiment that precipitates synthetic minerals; it would add an unnatural component into the system. This means that different anions with different protonation states (for example, $H_2PO_4^-$, HPO_4^{2-}, and PO_4^{3-}) are, de facto, the buffering species in the precipitation experiments. Nevertheless, we collected data for a variety of natural species, including several that are not conveniently made by double decomposition reactions from water-dissolved salts. These are shown in Table 2.

For example, manganese carbonates (rhodochrosite) and zinc carbonate (smithsonite) were examined. Both adsorbed comparable amounts of RNA to their surfaces in the mineral specimens that were examined, 11% and 5% respectively. Comparable amounts of RNA adsorbed to each of the precipitated minerals (93% and 89%, respectively). However, it was difficult to

find a rationale to compare these numbers across the Periodic Table to numbers obtained with the alkaline earth carbonates.

Finally, several silicates were examined for their ability to adsorb RNA. Silicates, of course, are represented by a very large number of minerals, and this work examined only a very small fraction of these. We recently reported work examining the adsorption and stabilization of RNA on opal [23].

Polymorphism

Another layer of complexity comes from the fact that the same set of atoms can form different crystal forms. For example, calcium carbonate can precipitate as calcite, aragonite, or vaterite. Calcite crystallizes in a trigonal space group; aragonite has an orthorhombic structure [24,25]. Calcite is the more stable and consequently most common phase, while aragonite is less stable and less common, although it does occur in nature as a metastable phase [26]. Vaterite, also μ-$CaCO_3$, is a third metastable phase of $CaCO_3$. It occurs much less commonly in nature because it is the least thermodynamically stable. It generally and rapidly transforms itself into one of the other two forms [27]. Vaterite is mostly seen when biological systems intervened to precipitate calcium carbonate. In forming the minerals synthetically, calcite dominates $CaCO_3$ that precipitates upon mixing $CaCl_2$ and Na_2CO_3 in water at near-neutral pH and room temperature and pressure; absent contaminants [28], aragonite is not formed. We easily reproduced this general result, establishing the structure of the precipitated phases that we obtained by both staining with Feigl's stain (silver sulfate and manganese sulfate) [29] and by powder X-ray diffraction.

To complete our analysis of the $CaCO_3$ system, we obtained natural specimens of the mineral aragonite and calcite. Experiments consistently showed that aragonite adsorbed more radiolabeled RNA than calcite. To obtain a synthetic mineral by precipitation, we reasoned that if RNA prefers to bind to aragonite over calcite, then perhaps RNA would nucleate the formation of an aragonite precipitate over a calcite precipitate.

Initial results were auspicious. Feigl's stain suggested that $CaCO_3$ precipitated preferentially as aragonite in the presence of RNA, here isolated from *Aspergillus*. This was first observed when a solution of Na_2CO_3 (1 M) was mixed with a solution of $CaCl_2$ (1 M) in the presence of 160 ng rRNA, with control experiments identical except for the absence of RNA. Both precipitates were stained with Feigl's stain, with which aragonite is stained black, while calcite remains white (Figure 3). We then did powder X-ray diffraction to confirm the crystalline form of the precipitated calcium carbonate. Here, the results were variable, but the precipitate formed in

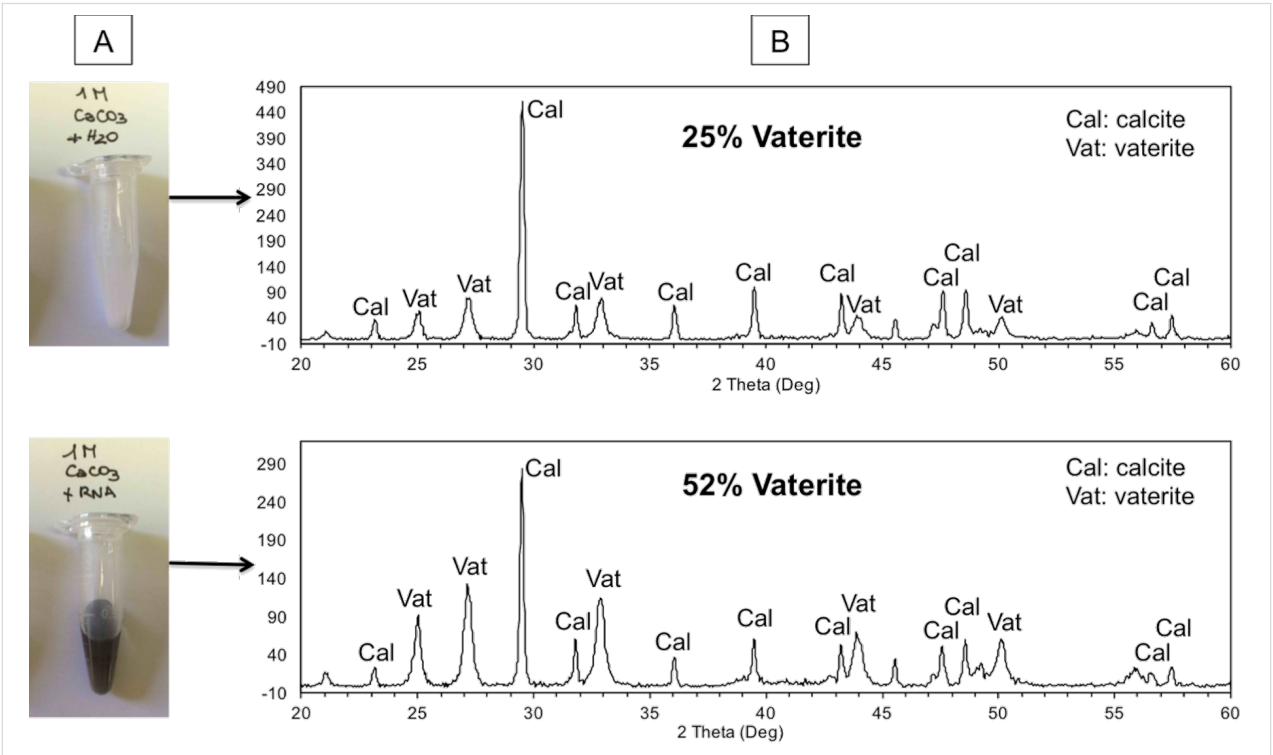


Figure 3: RNA-induced calcium carbonate polymorphism. A: Feigl's stain of $CaCO_3$ precipitate formed by double decomposition of 1 M $CaCl_2$ + 1 M Na_2CO_3 in the absence (upper tube) or presence (lower tube) of RNA. B: X-ray powder diffraction of samples prepared the same way showing a net increase in vaterite versus calcite.

the presence of RNA was often identified as being primarily vaterite. We do not have a molecular interpretation of these observations.

Stability of bound RNA

We then showed that RNA bound to aragonite was more stable than the same RNA in aqueous solution. For these experiments, the same 5'-^{32}P labeled 83-mer RNA (2 µL, 50 nM) was spotted on five pieces of natural aragonite, washed with H_2O to eliminate unbound RNA, placed dry in a thermoblock at increasing temperatures (25 °C, 37 °C, 55 °C, 75 °C, or 95 °C), and incubated for two hours. After incubation, RNA was eluted from aragonite with 1 M formic acid, purified, and loaded on denaturing PAGE with a set of control samples where RNA was treated the same way, but in aqueous phase (see Materials and Methods). Interestingly, ≈70% of the RNA bound to aragonite remained full-length after incubation at 95 °C for 2 hours. In contrast, RNA treated the same way but in aqueous solution (Figure 4, compare lanes 6 and 11) showed high levels of degradation, with no detectable full-length RNA left.

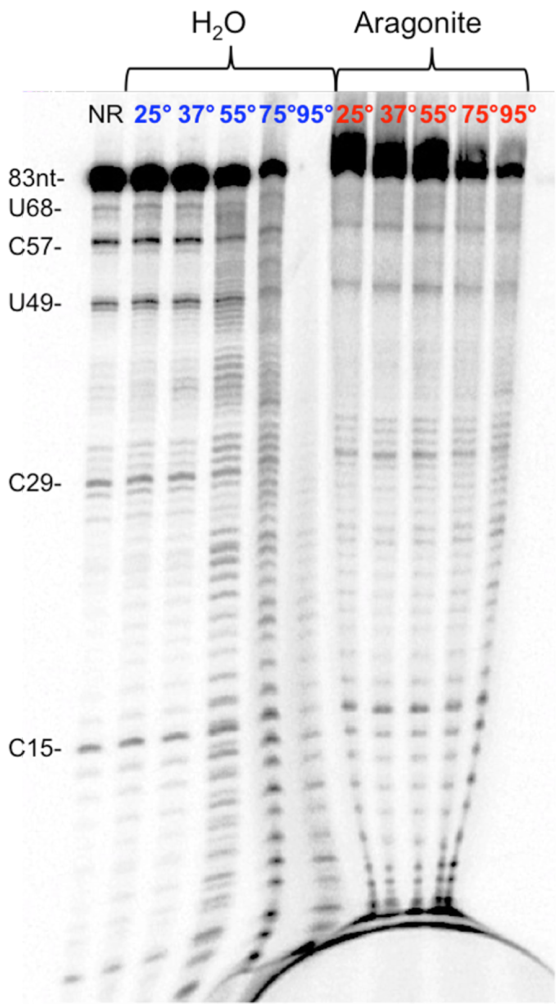


Figure 4: RNA adsorbed on aragonite is resistant to thermal degradation in aqueous solution. 18% denaturing PAGE of a 83-mer ssRNA incubated for 2 hours at 25, 37, 55, 75, and 95 °C, either free (left) or adsorbed to aragonite (right). Full length RNA and nucleotides in the sequence that are hot spots for degradation are indicated on the left.

Discussion

The results reported here show that where it is possible, a comparison of the natural minerals, the synthetically precipitated minerals, and co-precipitated mineral combinations can be used to drive the conclusion that the adsorbance data collected are relevant to the mineral species themselves, and do not merely reflect the adherence of large macromolecules to large surfaces. This comparative approach also allows us to avoid a difficult discussion about what "percentage adsorbance" actually means in molecular terms, where the actual surface areas involved are essentially unknowable.

It should be noted that precipitated minerals are not necessarily (or even generally) amorphous materials. However, the size of their crystals is generally smaller than the size of crystals of minerals collected in the field.

Where possible (for example, multiple fluorite specimens, large homogeneous surfaces of calcite and magnesite, etc.), replicas were done. However, the main point presented here is that the error is not the kind of "error" that can be analyzed by standard statistical methods. This requires that the error be "normally distributed". Here, the error problems come from systematic errors relating to the natural samples, as two different exemplars of the "same" mineral, or even two different portions of the same specimen, may in fact be of different composition and thus may give different results. They are not Boltzmann "normally" distributed, and adding standard deviations from multiple runs provides only the deceptive illusion of statistical support. In this work, we circumvented this problem by asking whether the trend in radiolabeled RNA adsorption is consistent across the various forms and presentations of a mineral.

The most obvious limitation of this comparative approach comes from nature herself. The rarity of minerals having different elemental compositions determines their availability for these experiments. Some elemental compositions are simply not found in nature at all.

Thus, an analysis that involves Group (vertical) comparison of transition metal minerals in the Periodic Table is not particularly sensible using this approach. For example, iron phosphate is a well-known mineral (vivianite) and it adsorbs RNA (≈12%,

Table 2). However, it does not make sense to seek the Periodic Table correlates of vivianite below iron. This would require us making and/or finding ruthenium phosphate and osmium phosphate, neither of which has been reported in mineralogy.

Likewise, horizontal comparisons across the Periodic Table are problematic, even within transition metals. For example, we found that RNA binds to natural titanium dioxide (rutile, 21% in our experiments) and iron(III) oxide (Fe_2O_3, hematite, 30% in our experiments), but not to magnetite (Fe_3O_4) [30]. However, the differences in redox states accessible to these different elements make direct comparison unlikely to be productive.

The most striking outcome of these results is the Periodic Table relationship in the adsorbance of RNA to alkaline earth minerals, both the carbonates and the sulfates. In both cases, the barium mineral bound more RNA than the strontium mineral, which bound more RNA than the calcium mineral, which bound more RNA than the magnesium mineral (when available).

Some evidence suggests that the crystalline surface is important in this trend. For example, like witherite and strontianite, aragonite adsorbs RNA better than calcite. Further, the crystal structures of witherite and strontianite belong in the same family as the crystal structure of aragonite. Likewise, vaterite resembles aragonite in its crystal structure more than either resembles calcite. Together, these results suggest, at least at the level of hypothesis, that the molecular structure of RNA is more compatible with the surface of an orthorhombic carbonate crystal (the "aragonite group") having cations arranged with pseudo-hexagonal symmetry, than with the trigonal crystals observed in calcite and magnesite.

Here, the adsorbance of RNA on aragonite, and the ability of aragonite to nucleate the growth of aragonite and/or vaterite over the thermodynamically more stable calcite has potential prebiotic significance, as does the stabilization of RNA on these carbonates. All of these minerals are likely to have been present on early Earth. They are also known on Mars [31]. Today, most calcium carbonate is the result of biological activity. Where that activity is not present today (perhaps, but perhaps not, on Mars), we might expect to find stabilized RNA formed abiologically.

These results must remain tentative until reproduced by other laboratories, of course. We remain concerned that specific properties of natural minerals may differ with different sources, different impurities, different levels of success in cleaning their surfaces, and a thousand other variables that might influence these results [32]. Mitigating this concern is the fact that the patterns of adsorbance were unchanged in these experiments whether or not the mineral was cleaned by treating with hydrogen peroxide or diluted acid.

However, as a cautionary note, we point to the results (Table 2) that were obtained with different specimens of calcium fluoride (fluorite). Fluorites in nature are known for their dramatic and often attractive color variation, including colorless, green, yellow and purple varieties. Often, these colors are graded across a single specimen, as different impurities responsible for the color are consumed from the environment as that specimen crystalizes. Here, we examined samples of both natural green and natural purple fluorite. The purple fluorite specimen was found to not adsorb RNA. In contrast, the green fluorite specimen adsorbed about 25% of the RNA. The green color is often attributed to small amounts of iron or samarium within the mineral lattice. This difference, although observed on only two different specimens from two different sources, is cautionary.

Materials and Methods

Radiolabeled RNA

For this study, a 83-nt long labeled RNA molecule was produced by in vitro transcription and isolated by gel electrophoresis. Its sequence was:

5'-^{32}P-CGCUGUACGCAACACAAGGCUUAUG-GUGUAUCCUCCUGGAUCACGUGUGGUACGUA-CUGUCCGAUUAUUUCUAAUCGGGAUAC-3'.

Data suggest that this RNA may fold into a rod-like, stem-loop structure including three bulges separated by four stems (data not shown, Biondi et al., in preparation).

Preparation of samples of synthetic minerals

Double decomposition reactions to obtain synthetic minerals by precipitation were prepared by mixing 100 μL each of 100 mM solutions; these gave the synthetic minerals used to collect the data given in Table 1. After precipitation (precipitation times varied from 5 min to a few days), pellets were produced by brief centrifugation, the supernatant was discarded, and 50 μL RNA ≈0.1–0.15 nM were added. RNA was incubated with the mineral for 5 min, after which samples were briefly centrifuged and the supernatant collected for scintillation counter reading. Each pellet was then washed twice with 500 μL H_2O, once briefly, and once overnight. All fractions were read at the scintillation counter by Cherenkov counting. Percent adsorption was calculated dividing the amount of radioactivity remained in the pellet by the sum of radioactivity in all the washes, multiplied by 100.

In another set of experiments, pellets were lyophilized and weighed prior to the addition of labeled RNA (100 fmol/mg of precipitated mineral). Unfortunately, this approach was not suc-

cessful for comparative purposes, due to two opposite effects. In some cases (especially with carbonates), the facility with which precipitate minerals redissolved in aqueous RNA solutions prevented any possibility for measurements. In other cases, the increased generic adsorption of aqueous solutions by dry surfaces allowed the powdered minerals to retain all the RNA added regardless of the interactions specific to the mineral (data not shown).

We also collected data for adsorption on precipitated minerals formed at different starting pHs, with values taken before mixing the salts, after the precipitate is formed (pH of the supernatant), and after the RNA is adsorbed (pH of the supernatant) (data not shown). The observations did not alter the conclusions of this paper and were thus omitted.

Adsorption of RNA on natural minerals

All natural minerals used where from the Benner collection (Table 3). Prior to RNA adsorption, minerals were washed with (in this order) tap water, ddH_2O, 30% H_2O_2, ddH_2O, EtOH 99%. Minerals were then air-dried for about 30 min in a sterile environment.

For each mineral, droplets (2 μL) containing ≈100 fmol of radiolabeled RNA were spotted on the surface and let adsorb for 45 min at room temperature. Macro-surface areas of the droplets were obtained with the program ImageJ (NIH). Subsequently, H_2O droplets of increasing sizes (10 μL to 100 μL) were used to wash the area were the RNA was spotted, until no radioactivity could be detected in the washes. All fractions were then read at the scintillation counter, along with 2 μL of the

Table 3: Listed are the origins of each mineral, in alphabetical order.

Mineral	Origin
adamite	Ojuela Mine, Mapimi, Durango, Mexico
agate	location unknown
amazonite	Crystal Peak district, Teller County, CO, USA
apatite	Liscombe Deposit, Wilberforce, Ontario, Canada
aragonite	Atlas Mountains, Morocco
baryte	Sulcis, Sardinia, Italy
beryl	Hunza Mine, Gilit, Pakistan
calcite	a specimen of "Iceland spar", Iceland
celestine	N'Chwaning Mine, Kuruman, South Africa
colemanite	Death Valley, Inyo County, CA, USA
danburite	San Sebastian Mine, Charcas, Mun. de Charcas, San Luis Potosí, Mexico
erythrite	unknown mine, Morocco
green fluorite	Cave in Rock, Hardin county, IL, USA
gypsum	Naica Mine, Chihuahua, Mexico
hematite	Mesabi Range, MN, USA
herkimer diamond	Quartz, Herkimer, NY, USA
magnesite	Minas Gerias, Brazil
magnetite	location unknown
mica	North Carolina, USA
obsidian	location unknown
olivine	(peridotite) Pakistan
opal	Queensland, Australia
ourple fluorite	Cave in Rock, Hardin county, IL, USA
pyrite	Madoc, Ontario, Canada
rhodochrosite	Perú
rutile	Minas Gerias, Brazil
smithsonite	Kelly Mine, NM, USA
strontianite	Winfield Quarry, Winfield Union County, PA, USA
talc	Canada Talc Mine, Madoc, Ontario Canada
topaz	Minas Gerias, Brazil
tourmaline	Minas Gerias, Brazil
vanadinite	Taouz, Er Rachida Province, Morocco
vivianite	Tomokoni mine, Machacamarca District, Potosí, Bolivia
witherite	Cave in Rock, Hardin county, IL, USA

radioactive RNA originally used. The amount of RNA adsorbed was calculated by subtracting the cpm in all the washes from the cpm of the original 2 μL. In the case of vanadinite, the mineral piece was small enough to fit directly into a scintillation vial, allowing the direct measurement of the radioactivity bound to the piece. This compared to the subtration method showed that the latter was accurate to within ±5%.

Competitive adsorption of RNA on two competing minerals

To obtain carbonate columns, either of two methods was used. In the first, each carbonate was prepared separately by mixing 1 mL of a 1 M aqueous solution of the chloride (x = Mg/Ca/Sr/Ba) with 1 mL of 1 M Na_2CO_3. Two of the carbonates were then combined into a 5 mL chromatographic column. In the second, the chloride salts of two competing metal species were mixed first (1 mL 1 M each) and then let react with 2 mL of 1 M Na_2CO_3; in this method, the two minerals co-precipitated, allowing the formation of ternary carbonates that contained two metals together (for example, dolomite is a well-known magnesium calcium carbonate).

With either method, after formation of a precipitate, ≈1 pmol of 5'-^{32}P-labeled RNA in 1 mL of H_2O was added to the mixture. The RNA was allowed to interact with the minerals by 40 min tumbling at room temperature (rt). After this time, each column was set upright in a undisturbed environment for about 15–20 hours to allow the different minerals with different densities to separate.

Autoradiography of the RNA in the columns was obtained by setting a phosphorimager screen (BioRad) tightly against the row of columns in their rack, with the aid of paper clips and weights, for 2 hours in a dark room. Screens were scanned with a Personal Molecular Imager (PMI) phosphorimager (BioRad) and analyzed with the software QuantityOne (BioRad).

After removing the supernatant, carbonate columns were then quickly frozen in liquid nitrogen, extruded from the plastic container by tapping, set against a sterile ruler, and quickly sliced into 0.5–1 cm slices. These were finally passed into clean tubes and radioactive counts in each slide were read at the scintillation counter with the Cherenkov method.

Feigl's staining and X-ray powder diffraction

Feigl's stain [29] is a solution of silver and manganese sulfates. The stain colors orthorhombic and hexagonal carbonates black, but does not stain trigonal carbonates, in the first 30–60 minutes. The reagent was made with 1% silver sulfate (w/v) and 12% manganese sulfate in H_2O. In the staining experiments, samples were obtained by double decomposition reaction by mixing aqueous solutions of Na_2CO_3 (200 μL, 1 M) and $CaCl_2$ (200 μL, 1 M). Samples were pelleted, supernatants discharged, and Feigl's stain (400 μL) was added with vortexing.

The mixtures were then incubated at room temperature, where development of gray color was monitored for up to 3 days. Samples that turned gray generally started developing color after about 20 minutes, while samples that were unstained (white) remained such for the duration of the monitoring period.

Temperature stability of RNA adsorbed onto aragonite surfaces

For these experiments, five small clusters of aragonite were obtained from an original crystal cluster with the use of a hammer. These were extensively washed with tap water, ddH_2O, 30% H_2O_2, ddH_2O, and then EtOH (99%) to remove all organic species. The specimens were then air-dried under cover for about 30 min.

Droplets (2 μL) containing ≈100 fmol of radiolabeled RNA were spotted on the surface of each crystal. The material was allowed to adsorb with liquid evaporation and by incubating the mineral pieces at 25, 37, 55, 75, or 95 °C in a sterile environment for 2 hours. In parallel, the same amounts of RNA were incubated at the same temperatures in 1.5 mL low-binding test tubes.

After incubation, RNA adsorbed to aragonite surfaces, or adhering to the tubes' plastic, was released by washing the surfaces with 100 mM aqueous formic acid (100 μL); the released RNA was recovered in new tubes. These samples were subjected to three cycles of evaporation and resuspension in ddH_2O to eliminate formic acid. The residue was then dissolved in 95% formamide gel loading buffer for denaturing PAGE analysis (18%, 7 M urea). Gels were dried for 30 min at 80 °C before being exposed to a phosphorimager screen for quantitative autoradiography.

Mineral identification with X-ray powder diffraction

Identification of synthetic minerals was conducted with a power X-ray diffractometer equipped with a copper target (X-Pert Powder; Philips Co.). All diffraction profiles were obtained at a step size of 0.01°, with a divergence and receiving slit of 1° and 0.3 mm, respectively.

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Conjecture and hypothesis: The importance of reality checks

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Commentary

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Abstract

In origins of life research, it is important to understand the difference between conjecture and hypothesis. This commentary explores the difference and recommends alternative hypotheses as a way to advance our understanding of how life can begin on the Earth and other habitable planets. As an example of how this approach can be used, two conditions have been proposed for sites conducive to the origin of life: hydrothermal vents in salty seawater, and fresh water hydrothermal fields associated with volcanic landmasses. These are considered as alternative hypotheses and the accumulating weight of evidence for each site is described and analyzed.

Introduction

The word conjecture is defined as an opinion based on incomplete information. The word can be taken to be slightly pejorative, but given that conjecture also involves imagination and creative effort, I will argue here that in scientific research there is a natural progression from conjecture to hypothesis to consensus. Conjecture is an idea, hypothesis is a conjecture that can be tested by experiment or observation, and consensus emerges when other interested colleagues agree that evidence supports a hypothesis that has explanatory value. This approach is clearly relevant to origins of life research which is still at a stage where multiple conjectures abound yet vast gaps in knowledge and understanding remain, mostly due to lack of significant funding for research in this area. The result is that only a few dozen laboratories are supported in the global scientific community, in contrast to thousands of scientists investigating health related research or chemistry and physics having applications in industry. Another reason is that the origin of life is best understood in interdisciplinary terms involving knowledge of astronomy, planetary science, biophysics, chemistry and biochemistry, molecular biology and evolution. Relatively few scientists have a taste for research that demands such broad knowledge to make significant advances. The historical development of origins research has been well described by Iris Fry [1] and Antonio Lazcano [2].

Discussion

Most scientists agree that hypothesis testing is an essential feature of research, and a typical proposal to a funding agency usually has a clearly stated hypothesis. However, there is a very human tendency for investigators to prefer positive results that support their idea. Karl Popper [3] had some good advice in this regard: Don't try to prove an idea is right. Instead, try to falsify it. Those rare ideas that cannot be falsified then emerge from the majority of ideas that fail the testing process. Günther Wächtershäuser [4] recently commented on how Popper's advice can be applied in origins of life research.

Hypothesis testing is an essential feature of good research, but its value can be increased by one additional step which was first clearly stated in 1964 by John Platt [5]. The title of Platt's article was *Strong Inference*, which he defines in the following way:

"Strong inference consists of applying the following steps to every problem in science, formally and explicitly and regularly.

1. Devising alternative hypotheses.
2. Devising crucial experiments ... with alternative possible outcomes, each of which will, as nearly as possible, exclude one or more of the hypotheses.
3. Carrying out the experiment so as to get a clean result."

Research approaches that incorporate alternative hypotheses avoid the tendency to prefer positive results, because both positive and negative results have value in inferring which of the two alternatives is better supported by accumulating evidence. The aim of this commentary is to describe how alternative hypotheses can be applied to understanding the origin of life, with the focus on a simple question: Did life begin in salty water in a marine environment, or did life begin in fresh water in a terrestrial setting? Although the question seems simple, there are significant ramifications of possible answers for life detection missions to other planetary objects in the solar system.

We can begin with two conjectures and then attempt to turn them into alternative hypotheses. The first conjecture follows from the discovery of hydrothermal vents and observations related to their properties:

- All life requires liquid water
- Most of the water on Earth is in the ocean.
- Hydrothermal vents emerging from the ocean floor are sources of chemical energy.
- Populations of chemotrophic microbial life thrive in hydrothermal vents.

Conjecture: life originated in hydrothermal vents and later adapted to fresh water on volcanic and continental land masses. In the absence of alternatives this idea has been accepted as a reasonable suggestion.

Is there an alternative? Here is another list of facts:

- A small fraction of the Earth's water is distilled from seawater and precipitates as fresh water on volcanic land masses.
- The water accumulates in hydrothermal fields that undergo cycles of evaporation and refilling.
- During evaporation, dilute solutes in the water become concentrated films on mineral surfaces.
- If the solutes can undergo chemical or physical interactions, they will do so in the concentrated films.
- The products will accumulate in the pools when water returns either in the form of precipitation or as fluctuations in water levels related to hot springs or geyser activity.

Conjecture: life originated in fresh water hydrothermal fields associated with volcanic land masses, then adapted to the salty seawater of the early ocean.

The current paradigm: Life began in the ocean in salty seawater

Now we can provide a few more details about two geophysical conditions that have been proposed as alternative sites conducive for the origin of life. Hydrothermal vents were discovered in 1977 [6] and were soon proposed to be a likely site for life to begin [7-10]. Hydrothermal vents referred to as black smokers are produced when seawater comes into contact with rocks heated by magma underlying mid-ocean ridges. The hot water dissolves mineral components of the rock and then emerges through the ocean floor where the mineral solutes come out of solution to form characteristic chimneys that emit a black smoke of precipitated metal sulfide particles.

A second type of hydrothermal vent was discovered in 2001 [11] that does not depend on volcanism. Instead they form when seawater reacts with mineral components of peridotite in the sea floor, a process called serpentinization. The reaction produces hydrogen and a strongly alkaline (pH 9–11) hot medium saturated with carbonate. When the warm fluid contacts cooler seawater, calcium carbonate and other minerals precipitate to form white chimney structures.

The hydrogen gas dissolved in the alkaline vent fluid is a potential source of reducing power. Certain microorganisms already

use hydrogen for this purpose, so the hydrothermal vent hypothesis proposes that on the prebiotic Earth hydrogen could potentially reduce carbon dioxide to organic compounds that are then incorporated into a primitive metabolism [12]. Lane and Martin [13] noted that the alkaline vent minerals have a porous structure that could serve as cellular compartments with mineral membranes as boundaries. The assumption that the membranes may separate a strongly alkaline medium from mildly acidic Hadean sea water across suggested that a primitive version of chemiosmotic energy transduction might be possible to supply chemical energy for primitive forms of life. Weiss et al. [14] used genomic analysis of vent microorganisms to test the possibility that the last universal common ancestor (LUCA) may have originated in hydrothermal vents.

The iron-sulfur chemistry proposed for hydrothermal vents was tested by Huber and Wächtershäuser [15,16] who simulated vent conditions with boiling mixtures of iron and nickel sulfides to which various reactants were added. They reported that acetic acid, amino acids and peptide bonds could be synthesized under these conditions, and claimed that "The results support the theory of a chemoautotrophic origin of life with a CO-driven, (Fe,Ni)S-dependent primordial metabolism."

More recently Herschy et al. [17] simulated hydrothermal vent conditions by injecting a solution of potassium phosphate, sodium silicate and sodium sulfide (pH 11) into a second solution of ferrous chloride, sodium bicarbonate and nickel chloride (pH 5). The aim was to determine whether carbon dioxide (present as 10 mM sodium bicarbonate) can be reduced under these conditions, and they were able to detect ≈50 μM formic acid. In a similar laboratory simulation of an alkaline hydrothermal vent, Burcar et al. [18] used mass spectrometry to detect a small yield of dimers produced from adenosine monophosphate circulating in the medium.

An alternative hypothesis: Life began in terrestrial fresh water

Although most of the Earth's water today is salty seawater, a small fraction (~1%) is present in the form of fresh water distilled by evaporation from the ocean and falling on continental land masses as precipitation. The Hadean Earth did not have continents but was likely to have volcanoes similar to those from the same era still visible on Mars. The volcanism associated with such islands suggests an alternative hydrothermal site we will refer to as hydrothermal fields. Iceland is an analogous site on today's Earth, with several active volcanoes and associated hydrothermal areas supplied by precipitation and dominated by hot springs and geyser activity. In contrast to the single rock-water interface of hydrothermal vents, hydrothermal fields have a more complex array of three interfaces in which minerals, water and atmosphere undergo continuous fluctuations of wetting and drying.

The fluctuating hydrothermal field hypothesis has been used as a model for polymerization reactions in which monomers like amino acids and mononucleotides form peptide and ester bonds of biologically relevant polymers. The idea that evaporation and heat can drive polymerization is obvious and was first proposed years ago [19]. Lahav and White [20] adopted the approach and demonstrated that peptide bonds could be produced using clay as a catalyst. The approach was largely abandoned with the advent of the RNA World scenario that suggested a way for life to begin in solution, rather than by evaporation to dryness. However, polymerization in an aqueous medium requires chemical activation of the monomers, and so far there is no obvious mechanism by which activation can occur. Recent studies have returned to evaporation as a way to drive polymerization reactions [21,22].

There are several advantages to using evaporation in this regard. First, simply concentrating potential reactants adds significant free energy to a system that can be used to drive condensation reactions [23]. Furthermore, if amphiphilic compounds are present they can organize and concentrate reactants within a two dimensional plane with the result that polymerization is enhanced [24,25].

The hydrothermal field hypothesis has been tested in laboratory simulations. For instance, peptide bonds have been produced [26,27] and cycles of drying and rehydration have been shown to drive polymerization of mononucleotides [22,28,29]. Because the resulting polymers can be encapsulated in lipid vesicles, it has been proposed that the resulting protocells are candidates for combinatorial selection and the first steps of evolution [30].

Conclusion

From the above discussion, alternative conjectures have been published and are available for critical analysis and commentary. How can we turn the two conjectures into John Platt's alternative hypotheses? The answer is simple. We follow Platt's advice to devise critical experiments that will add weight of evidence to either or both of the alternative conjectures which then become testable hypotheses. Here is a proposed list of conditions that seem to be essential prerequisites if cellular life is to originate in one of the two alternative conditions:

- There must be a source of organic compounds relevant to biological processes, such as amino acids, nucleobases, simple sugars and phosphate.

- The organic solutes are likely to be present as very dilute solutions, so there should be a process by which they can be sufficiently concentrated to undergo chemical reactions relevant to cellular life.
- Energy sources must be present in the environment to drive a primitive metabolism and polymerization.
- Products of reactions should accumulate within the site rather than dispersing into the bulk phase environment.
- Biologically relevant polymers are synthesized with chain lengths sufficient to act as catalysts or incorporate genetic information.
- If amphiphilic compounds are present in the mixture, the conditions will allow them to assemble into membranous compartments.
- A plausible physical mechanism can produce encapsulated polymers in the form of protocells and subject them to combinatorial selection.

These conditions can also be considered to be predictions, because each condition in the above list can be tested by observation, by theoretical analysis or in laboratory simulations. If any one of the predictions fails experimentally or is shown to be impossible, for instance by being inconsistent with thermodynamic principles, that alternative can be considered to be falsified. As evidence accumulates, we will be able to judge the relative plausibility and explanatory power of the competing ideas. Continued testing of the alternative hypotheses is essential, because neither has yet reached the level of consensus. In both cases, laboratory simulations will ideally be extended to a second important step, which is to visit the alternative sites and demonstrate that what happens in the laboratory can also occur in the actual conditions of hydrothermal vents or fields.

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How and why kinetics, thermodynamics, and chemistry induce the logic of biological evolution

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Full Research Paper

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Abstract

Thermodynamic stability, as expressed by the Second Law, generally constitutes the driving force for chemical assembly processes. Yet, somehow, within the living world most self-organisation processes appear to challenge this fundamental rule. Even though the Second Law remains an inescapable constraint, under energy-fuelled, far-from-equilibrium conditions, populations of chemical systems capable of exponential growth can manifest another kind of stability, dynamic kinetic stability (DKS). It is this stability kind based on time/persistence, rather than on free energy, that offers a basis for understanding the evolutionary process. Furthermore, a threshold distance from equilibrium, leading to irreversibility in the reproduction cycle, is needed to switch the directive for evolution from thermodynamic to DKS. The present report develops these lines of thought and argues against the validity of a thermodynamic approach in which the maximisation of the rate of energy dissipation/entropy production is considered to direct the evolutionary process. More generally, our analysis reaffirms the predominant role of kinetics in the self-organisation of life, which, in turn, allows an assessment of semi-quantitative constraints on systems and environments from which life could evolve.

Introduction

Although it is mostly understood in historic terms, the origin of life constitutes a well-established field of research in chemical science [1] even though identifying the actual pathway by which life emerged on the early Earth will likely forever remain out of reach. The corresponding historical events left no record owing to the instability of the chemical components of the first living organisms and the tool of phylogenetic analysis is also limited, due to what might be called a horizon of knowledge, one which has been associated with the theoretical concept of the last common ancestor [2]. Current living organisms on

Earth, in their extraordinary diversity, are unable to provide information on preceding stages of evolution that reach back beyond that horizon. And since the last common ancestor corresponds to an organism endowed with most of the essential functions present in current cells, phylogenetic studies are of little help when tackling the very early stages of life. The only alternative possibility is then to consider prebiotically available chemical pathways, as well as the constraints for chemical self-organisation, and to attempt to answer two questions: (1) Is there a driving force towards self-organisation of the kind observed in the living state? (2) If so, by what mechanistic means can a chemical system self-organise to yield the living state, consistent with the constraints of the Second Law. These considerations infer that an overall spontaneous decrease in entropy is statistically highly unlikely, and for macroscopic systems, effectively impossible. Accordingly, the emergence of life as the result of a single unlikely event is highly improbable [3-5]. Any alternative approach worthy of scientific investigation would therefore require the existence of a driving force for self-organisation, one necessarily associated with the production of entropy in the environment. The identification of such a driving force would make it possible to determine the parameters influencing change, even though no historical information regarding its early expression is available. Furthermore, identification of that driving force could serve as a logical bridge connecting the general rules governing change in the universe with Darwin's theory of evolution. Indeed, analysis of the thermodynamics of the processes considered to underlie life's emergence might assist in closing the conceptual gap that continues to separate the physical and life sciences [6,7]. But does this mean that the history of the early evolution of life could be deterministically reconstituted through identification of life's driving forces? The answer is certainly negative. The number of available chemical degrees of freedom is such that an almost infinite number of paths could potentially have been followed, so contingent events, historical by necessity, would also have had to play a cardinal role in determining the specific pathway that life processes happened to have taken. This statement does not preclude the possible occurrence of chemically predisposed pathways that could induce the selective formation of limited sets of building blocks potentially favourable toward that transition [8,9].

Much work has previously been devoted to the physicochemical characterisation of life. These attempts can be divided into two major approaches. Authors favouring a thermodynamic approach have emphasised the fact that life corresponds to dissipative processes taking place far from equilibrium [10], thereby explaining how self-organisation can arise without violation of the Second Law [11]. On the other hand, experimental molecular evolution [12] as well as theoretical developments [13,14] have supported a kinetically based view. Taking that kinetic approach, the concept of natural selection was able to be extended beyond biology so as to be applicable at the molecular level. Both views progressed separately in a context dominated by the RNA world hypothesis, though that hypothesis failed to eliminate the fundamental dilemma, as it led to conflicting so-called genetic and metabolic approaches to the origin of life [15].

Actually, as early as 1922, Lotka's pioneering work, through two consecutive articles published in the same issue of PNAS and entitled "Contribution to the energetics of evolution" [16] and "Natural selection as a physical principle" [17], respectively, considered both approaches to the problem in order to account for the specificity of life (though the issue of the origin was not mentioned). This simple fact demonstrates how intimately bound he considered the metabolic and genetic features of life to be. Any physicochemical description of the origin of life that seeks to identify the physical principles responsible for life's emergence should therefore take both considerations into account. Indeed, we believe it is through such a dual approach that a theoretical framework for describing the origin of life can be established, one able to help identify the driving forces responsible for self-organisation, as well as identify possible conditions able to support life's emergence and early development. Thus the present work, extending ideas described in some detail in a series of earlier publications, is aimed at outlining the central features of a physicochemical approach to the origin of life, one which emphasises its kinetic character – how the evolutionary process from its outset is kinetically rather than thermodynamically determined, and provides new information in support of that view.

Results and Discussion

From thermodynamic self-assembly to kinetic self-assembly

Organised supramolecular structures are commonly formed when favourable interactions lead to the assembly of different components [18]. The release of chemical binding energy, i.e., the realisation of potential energy by dissipation of heat into the environment, compensates for the decrease in entropy associated with the loss of degrees of freedom of the individual chemical components. The increase in thermodynamic stability therefore constitutes the driving force for self-organisation, as required by the Second Law (Figure 1A).

With regard to living organisms, the situation is more complex. On the one hand, association processes directly driven by the Second Law are common in living organisms (e.g., protein folding, the assembly of protein sub-units through molecular recognition, assembly of nucleic acid duplexes as well as that of phospholipids to form a bilayer membrane). On the other hand,

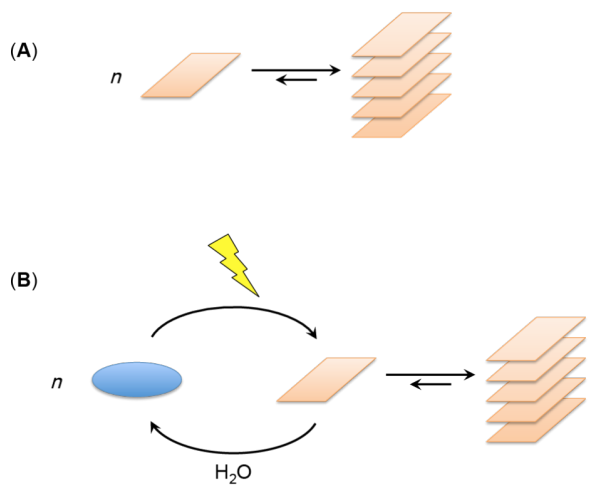


Figure 1: Self-assembly. (A) Macromolecular structures or patterns can form as the result of binding energy being released through the interaction of units which compensates for the decrease in entropy associated with self-organisation. (B) An example of dissipative self-assembly of reactants unable to react in the ground state but which can be activated to yield unstable reactive units (e.g., susceptible to hydrolysis). The supramolecular structure is dynamically stable as long as the system is held far from equilibrium through the energy-fuelled supply of reactive units.

even though the Second Law must always remain an inescapable constraint, a simple drift towards the equilibrium state is not sufficient to account for the evolutionary changes of life. More elaborate processes, in particular that of increasing complexity, are clearly involved. As an example of a higher degree of complexity, out-of-equilibrium self-assembly can be observed when reactants that have no affinity for self-assembly in themselves, can be converted upon activation into transient species which can interact, leading to macromolecular structures or patterns [18]. The kinetic stability of the organised structures in those cases is associated with energy dissipation from an activating agent able to convert some reactant into transient species able to undergo intermolecular association (Figure 1B). These structures therefore result from dissipative self-assembly for which fascinating examples have been provided in the recent literature [19-21]. In biology, one of the most typical examples of this kind of assembly processes can be found in the dynamics of the cytoskeleton. However, even if these processes can explain some particular features of living organisms, they are not sufficient by themselves to constitute a driving force towards the self-organisation of living systems and to explain how life itself could have emerged and evolved.

Life as a dissipative process emerging far from equilibrium

It has recently been claimed that thermodynamics could drive the self-organisation of life through an increase of energy dissipation rates [22,23], or, alternatively, in a continuing focus on the energy facet, that the evolutionary process takes place such that the total energy flux through the system is increased [16]. In yet another thermodynamic variant, it has also been suggested that the process leads to a maximisation of energy intensity [24]. Though Lotka introduced the maximisation concept, he was explicitly reluctant in making this proposal an absolute principle. This cautious approach has not been shared in more recent studies, in which a so-called "maximum entropy production" (MEP) principle, applicable within different fields of physical, biological and environmental sciences, has been introduced as an extension of the Second Law (see for example: [25-29]). That principle has also been seen as relevant when considering the origin and evolution of life problem (see for example: [30-35]). According to that proposal, a system that is held in a far from equilibrium situation should evolve towards an increase of energy dissipation and along a pathway in which the rate of dissipation, and thus of entropy production, is maximised. This approach, as well as closely related ones [22,23,36], expresses the view that the life phenomenon could therefore just be a consequence of a tendency of systems to maximise the dissipation of energy so that more complex systems, ones able to act as more effective dissipators, would be selected for. Also, it should be emphasised that though the MEP principle refers to the rate of entropy production, the basis for the "maximum entropy production" principle remains fundamentally thermodynamic, not kinetic, and, as will be discussed, that thermodynamic approach is opposed by more recent theoretical considerations, as expressed by Ross et al. [37] and our own analyses, described subsequently.

More detailed views on the role of thermodynamics in biology have been critical of the position that natural selection expresses the drive towards maximum entropy production/energy dissipation/flux of reactants, and have proposed a less simplistic relationship that takes into account the self-reproducing property of living entities [17,38-42]. That approach toward living organisms [1,4,6,7,43-54] also favours a kinetic approach rather than a thermodynamic one, since there is no direct relationship between Gibbs free energy of reactions and kinetic barriers [37]. Indeed, the most significant flaw in attempts to derive natural selection from thermodynamics is that the kinetic behaviour of complex systems can hardly be deduced from data governing free energy minima, data which ignores the free energy barrier heights separating reactants and products. Organic chemists are fully cognisant of the fact that kinetic barriers cannot usually be deduced from thermodynamic data. Indeed, there are many examples in which product formation is controlled by kinetics (reactions under kinetic control, corresponding to the situation in Figure 2), rather than by thermodynamic stabilities. In fact the presence of kinetic barriers is actually a requirement for the system to be held far from equilibrium [43,44] so that life can

only evolve from systems tightly bound, typically through covalent bonds [55,56]. Activated chemical species involved in these systems would not rapidly evolve at low temperature allowing the selection of efficient catalytic processes [50]. This observation therefore can explain the emergence of processes that lead to increased rates of transformation, and therefore energy dissipation. Thus one might say that the driving force for the emergence of life is related to the circumvention of kinetic barriers [42,43] rather than a consequence of the Second Law acting on a system held far from equilibrium.

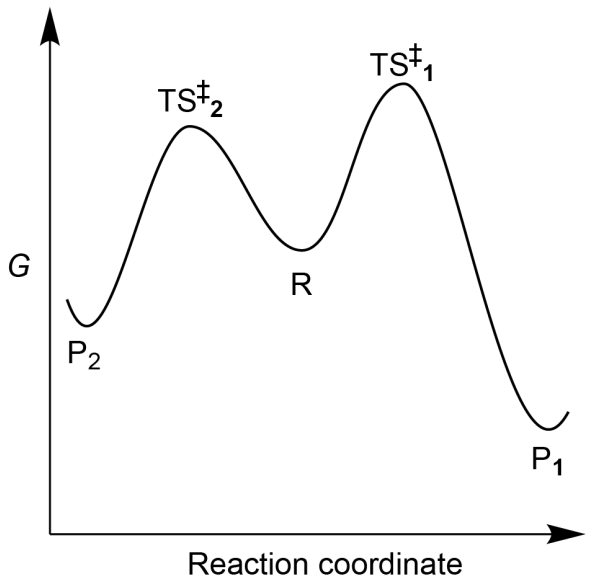


Figure 2: Kinetic control. In many chemical reactions leading to different products, the final composition is determined by the height of the kinetic barriers corresponding to transition states ($TS^{‡}_{1}$ and $TS^{‡}_{2}$) rather than by the relative free energies of reactant (**R**) and products (P_1 and P_2). Under kinetic control, P_2 would be favoured over P_1.

Based on detailed physicochemical analyses, the idea of a MEP principle has indeed attracted criticism [37,57] and specific examples that are inconsistent with a thermodynamic directive have been discussed [37]. In addition, the expectation that biological systems would evolve towards systems exhibiting maximum entropy production is contradicted by the high yield that is observed in the conversion of nutrients into cell components, as for instance during glucose metabolism. In this case entropy production only slightly exceeds the minimum required by the Second Law indicating that the cell has evolved to minimise entropy production [58], not to maximise it. That observation in itself clearly shows that the production of cellular components is more important to the cell than the dissipation of energy. Indeed, in further support of a kinetic approach to evolution we have proposed [51] that the driving force for evolution can be identified as an expression of a persistence principle – a tendency of systems to evolve towards states in which their ability to change is reduced until they eventually reach a stable/persistent state in which no further change takes place. Though that idea is usually expressed in isolated systems as the Second Law, it can manifest itself as a trend toward greater DKS for populations able to reproduce themselves under favourable conditions. Actually, the probabilistic drive towards equilibrium expressed by the Second Law is replaced by a new one based on the mathematics of exponential growth for systems able to reproduce themselves in far from equilibrium situations [51-54]. In sum, as Ross et al. have pointedly noted: "predictions based on MEP-like principles should not be considered scientifically founded" [37]. Indeed, to strengthen that conclusion we now offer a kinetic simulation for a self-reproducing chemical system which further questions the generality of the MEP principle reaffirming the importance of kinetic considerations for such systems.

Consider a chemical system in which a chemically activated reagent (resource **R**) is produced transiently (Figure 3). After a delay required for equilibration, a minute concentration of an autocatalyst **A**, growing at the expense of this resource (Figure 3), is added to the system (see Figure 4). Given numerical simulation of rate constants, k_2 and k_3, for which the autocatalyst is viable (see Supporting Information File 1), the

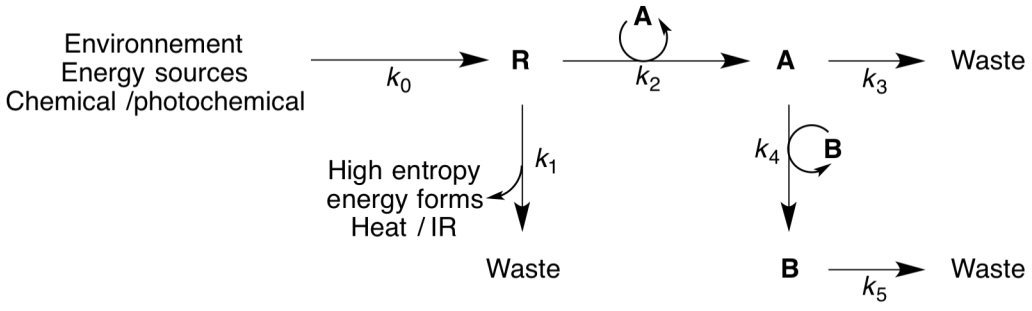


Figure 3: Evolution of an autocatalytic network involving a parasite. **R**: resource; **A**: autocatalyst; **B**: predator autocatalyst.

system is found to evolve irreversibly in the direction of increasing reactant flux corresponding to the autocatalytic dissipative process (catalysed by **A**) compared to its initial value. Changes in both the kinetic stability and reactant flux (reflecting entropy production through the dissipative process associated with autocatalytic step, k_2) take place until a new steady state is achieved (after a transient peak).

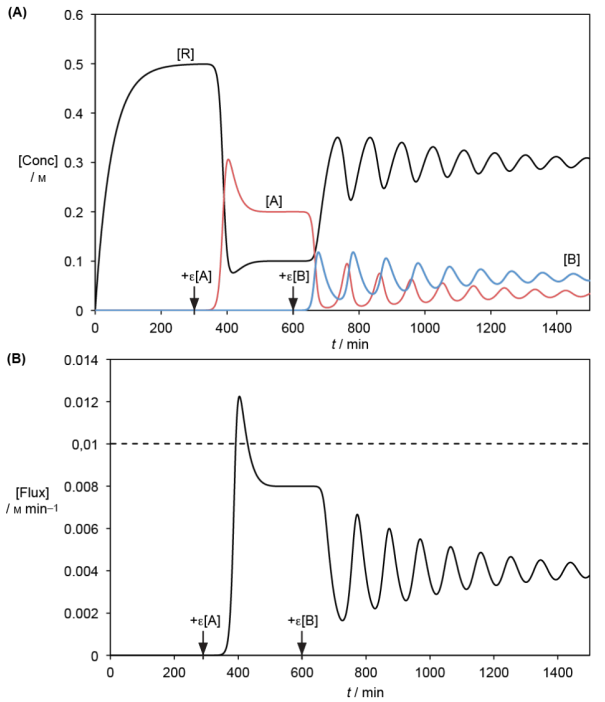


Figure 4: Numerical simulation of the system of Figure 3 (k_0 = 0.01 M min^{-1}, k_1 = 0.02 min^{-1}, k_2 = 0.4 M^{-1} min^{-1}, k_3 = 0.04 min^{-1}, k_4 = 1.2 M^{-1} min^{-1} and k_5 = 0.04 min^{-1}). (A) Evolution of the concentrations of resource (**R**), autocatalyst (**A**) and predator (**B**) species; (B) flux of product formation through the autocatalytic system from **A** and **B**. The initial concentrations [R] = [A] = [B] = 0 were selected. After [R] approaches a steady state ($[R]_{*1} = k_0/k_1$ = 0.5 M), at 300 min 10^{-6} M **A** is added leading to a new steady state ($[R]_{*2} = k_3/k_2$ = 0.1 M and $[A]_{*2} = (k_0 - k_1 \times k_3/k_2)/k_3$ = 0.2 M). A new regime is initiated by the addition of 10^{-6} M **B** at 600 min (steady state $[A]_{*3} = k_5/k_4$ = 0.033 M and $[R]_{*3} = k_0/(k_1 + k_2\, k_5/k_4)$ = 0.3 M). Simulation results were not changed using a twice-shorter interval of time between iterations (0.5 min instead of 1 min).

Consider now the case in which a minute concentration of a parasite autocatalyst **B** formed from **A** and behaving as a predator, is introduced into the system. Surprisingly, for certain sets of rate constants (see Supporting Information File 1), the parasite can persist, but its incorporation into the system leads to a decrease in the overall reactant flux towards dissipation associated with autocatalytic step, k_2. Note also that once the system with the parasite becomes stable (depending on the ratio of rate constants k_4/k_5; see Supporting Information File 1), it does not revert to the preceding state. The key point however: instead of the system evolving towards an increase in energy dissipation, parasite addition leads to a more complex state which is less dissipative, one displaying damped oscillations (so-called Lotka–Volterra behaviour). Kinetic stability and energy dissipation have evolved in opposite directions. Thus, through this simple kinetic simulation, one differing from natural selection between species variants (corresponding to concepts defined within the biological field), a more general view of evolution involving chemical autocatalysts is obtained. Once again we observe an instance in which the MEP principle is inapplicable, further reaffirming Ross's critical MEP assessment [37]. The level of energy dissipation (corresponding to the amount of activated reactant **R** converted into inactivated products through the autocatalytic path k_2) is influenced by contingent events, rather than by a general thermodynamic law. In fact, what the introduction of the predator into the system does do (leading to Lotka–Volterra oscillating behaviour, Figure 4), is to lead to an increase in the system's complexity. This aspect will be discussed subsequently.

Stability and complexity

Even though the Second Law drive towards equilibrium is brought about through the minimisation of the Gibbs free energy of the system, we learn from Figure 4 that the maximisation of free energy dissipation does not account for the direction of change. Indeed the system described in Figure 3 will never revert to its previous state in which **B** was absent and energy dissipation was higher. What the addition of **B** brings about is an increase in complexity, suggesting that it is not just stability/persistence which increases, and that whatever quantities are being optimised, they should also include a term related, whether directly or indirectly, to complexity. It is worth noting that the meaning of complexity considered here refers to the degree of organisation within the system, to the interconnections of its parts, and not just to the number and diversity of its components. This observation of increase in complexity supports the hypothesis that the evolution of reproducing systems is ruled by a Second Law analogue in which complexity plays a role similar to that of entropy during the evolution of non-replicative systems towards thermodynamic equilibrium [7,50-54]. Unfortunately, as complexity is notoriously difficult to both define and measure [59-61], quantification of such a Second Law analogue seems out of reach at present.

Thus though the evolution of a dynamic system based on entities able to self-reproduce is continuously governed by an increase of dynamic kinetic stability, predicting the result of long-term evolution becomes impossible, primarily because it depends on the particular path followed during the process. These complex systems can reach bifurcation points from which the system can evolve along different paths [10] rendering any

prediction of evolutionary paths impossible. Evolutionary possibilities invariably depend on earlier choices. Additionally, the boundaries of a necessarily open system cannot be defined so that events in the environment can influence the future of the system. However, the impossibility of measuring dynamic kinetic stability is precisely the source of unlimited possibilities for evolution, its open-ended character coupled with its divergent nature [47]. Indeed, provided that the environment provides energy in sufficient quantities and potential to sustain life, there should be no end to the evolutionary process as neither DKS, nor the complexity which accompanies it, appear to have an upper bound.

A free energy potential threshold as a requirement for the origin of life

Key conditions for observing physicochemical behaviour governed by dynamic kinetic stability is that the system is self-reproducing and able to undergo exponential growth [13,14,62,63]. These conditions further imply that the system is maintained in a far-from-equilibrium state and that the chemical autocatalytic process involved must be kinetically irreversible (i.e., the rate of the reverse reaction must be negligible on the timescale of reproduction/generation) [1,4]. The nature of this requirement may be understood more readily by analysing a well-known example of emergence of dissipative structures. One of the most studied is the emergence of convection when the bottom surface of a liquid layer is heated (Figure 5). It turns out that a low temperature gradient is insufficient for convection to be observed and the minimum gradient must exceed a threshold above which Raleigh–Bénard instability is observed (Figure 5). The result of convection is, of course, an increase of energy dissipation by the resulting nonlinear process, though its emergence depends on the action of gravity and the laws of fluid dynamics.

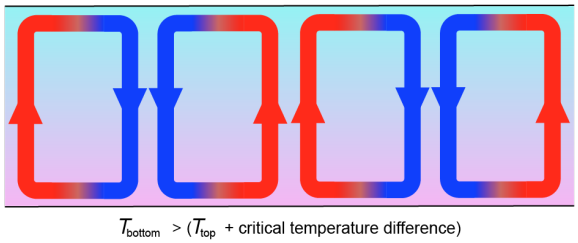


Figure 5: The Raleigh–Bénard instability. Convection takes place in a liquid layer provided that the temperature difference between the bottom and the top of the layer exceeds a threshold value.

Regarding the origin of life, we suggested that an analogous threshold is also present [4], which can be identified as a consequence for the need for kinetic irreversibility. Above that threshold (associated with a value of chemical free energy potential expressing a distance from equilibrium), kinetic selection among variants of autocatalysts becomes efficient [13,14,62,63], which reproduces similar behaviour to the one responsible for natural selection. The condition for irreversibility associated with this threshold, expressed as a reproduction/generation timescale shorter than that for the reverse process, has provided a means of semi-quantitatively assessing kinetic barriers [55,56]. This assessment was based on a relationship between time scale, kinetic barriers and temperatures, and taking into account the following hypotheses:

- a temperature as low as possible, but allowing the presence of water in its liquid state (higher temperature strongly increase the threshold),
- generation times of 1 second to 100 years.

The threshold can therefore be expressed as a minimum free energy potential corresponding to chemical quanta feeding the system in energy. Kinetic barriers needed for ensuring kinetic irreversibility correspond to a value of ca. 100 kJ mol^{-1} at 300 K. This value corresponds to a significant fraction of the free energy of covalent bonds (and then to the kinetic barriers commonly observed for their reactions), which is a strong indication that in a range of moderate temperatures (ca. 300 K), the chemistry of carbon – the element that most easily forms covalent bonds – should be preferentially involved in a self-organisation process based on the specificity of entities able to reproduce themselves. Moreover, the energy input allowing the irreversible formation of intermediates having a degree of activation equivalent to that of biochemical intermediates like ATP, requires a free energy potential exceeding a value of 150 kJ mol^{-1}, equivalent to that of visible light [4,55,56]. Therefore, it turns out that considering the kinetic conditions for dynamic kinetic stability leads to the definition of conditions for the origin of life that more or less correspond to the conditions for the development of life on the primitive Earth (organic chemistry, liquid water and visible or UV light). Here again, some recent experimental work has shown how photochemistry could lead to biochemical building blocks compatible with further developments towards the origin of life [8,9].

Evolvability and the origins of life

This discussion has not taken into account the ability of a system to evolve, which was not the goal of the present work, but is obviously a requirement for any possibility of evolution [64,65]. Extended possibilities for variation are indeed a requirement for systems to undergo open-ended evolution [66]. The storage of genetic information as a sequence in a polymer associated with template replication through base-pairing

constitutes an efficient system to ensure evolvability. It is that evolvability which allows selection toward life as we know it on Earth. However, as the proximity from equilibrium has been mentioned above as a limitation, the higher affinity of long strands compared to fragments is the source of another limitation (product inhibition). That limitation, discovered for template replication by von Kiedrowski [67], leads to sub-exponential growth. It turns out, at least at this time, that no isolated system able to reproduce itself, presents all of the qualities required for the emergence of life: i.e., the replication of nucleic acids through base-pairing is limited by parabolic growth and autocatalytic networks present limited possibilities of variability. This situation has led many researchers in this field to support a co-evolutionary approach in which several subsystems able to reproduce themselves could co-operate to initiate a possibility of natural selection [68,69]. It is worth noting that some years ago, the need for cooperation between sub-systems had already been suggested as a requirement for an autonomous self-reproducing system, through the pioneering work of Tibor Gánti [70]. If we consider that the process starting from inanimate matter to living organisms progressed through stages of increasing DKS, then the most important transitions very likely corresponded to the initiation of cooperative associations corresponding to both an increase in complexity of the system and its dynamic kinetic stability. The groundbreaking endosymbiotic theory put forward by Lynn Margulis [71] half a century ago to explain eukaryotic cell formation is in fact just a particularly striking example of a cooperative association in action. It is also important to emphasise that cooperation may either have involved a physical linkage between different components through direct binding or encapsulation, but that functional linkages in which reactants or products could be common to different systems would have been important as well.

Organic chemistry and the origin of life

The lines of thought developed here point towards a global approach to account for the emergence of life as a consequence of contingent events that occur in a context in which kinetic driving forces towards more efficient self-reproducing systems are constrained by thermodynamics, as well as by the properties of covalent bonds involving carbon. They support the essential role of organic chemistry in the origin of life process as a result of the kinetic barriers associated with covalent bonds. It is encouraging that recent experiments have demonstrated that complex kinetic behaviour can be observed in simple organics [72,73], and is not particular to inorganic systems or enzymatic reaction networks. Our approach, beginning with the hypothesis of an auto-organisational process based on the kinetic properties of self-reproducing entities, leads to a semiquantitative assessment of the environmental conditions required for a self-organisation process based on organic chemistry. It is instructive to note that this assessment is compatible with visible light as an energy source as well as moderate temperatures, both of which could be found at the surface of the early Earth. However, these considerations by themselves do not solve the question of the origin of life, or at least the point of initiation of an evolutionary process driven by an increase in DKS. The precise nature of the chemical species involved in that process remains unknown. Interestingly, however, recent investigations [74,75] have demonstrated that some kind of selective chemistry can simultaneously yield, via photochemical pathways, a wide range of precursors similar to those found in biochemistry (amino acids, nucleotides and lipid precursors).

Conclusion

This paper attempts to place life and its emergence within a general physicochemical context. Once it is appreciated that life emerged from inanimate beginnings in a well-defined process with an identifiable driving force, the Chinese wall that has somehow managed to separate the conceptual worlds of animate and inanimate, can finally be breached. The biological and physical worlds are intimately connected through process. There is a process, explicit and physicochemically defined, that under appropriate contingent conditions, leads from chemistry to biology such that these two worlds merge into one. So, though life is a complex chemical system exhibiting complex kinetic behaviour, that complex behaviour can be traced back to self-reproducing chemical systems maintained far from equilibrium and directed by kinetic driving forces. Chemical systems able to evolve in the direction of increased dynamic kinetic stability – toward life – need to be endowed with three essential properties. They must be able to reproduce themselves, their structure should be compatible with the possibility of variation, and they should be maintained in a dynamic far from equilibrium state through a continual energy supply. Selection is then the inevitable consequence. According to Darwinian theory, it is selection that drives evolution. However, natural selection is a very specific process which applies to only a part of the natural world, and is seemingly detached from traditional physicochemical behaviour. Neither the distance from equilibrium nor the maximisation of energy dissipation constitute driving forces for the emergence of life but they correspond to a condition for its development for the former and a manifestation associated with their behaviour for the latter. The actual driving force for life is associated with the power of exponential growth that is expressed by self-reproducing entities. Moreover, the hypothesis of an auto-organisational process based on the kinetic properties of these entities leads to a semiquantitative assessment of the environmental conditions required for a self-organisation process, one based on established organic chemical processes. It is intriguing to note that this assessment is compatible with

visible light as an energy source, and a moderate temperature, both of which would have been found on the surface of the early Earth.

This approach to biological systems that focuses on their emergence from chemical ones has some far reaching consequences. The "autonomy of biology" view of life [76], still deeply engrained within life science thinking, needs to be reassessed as it undermines attempts to understand biology's deeper essence. The very fact that chemistry almost certainly evolved over time into biology is the clearest statement that the physical and biological worlds are merely two regions of a physicochemical–biological continuum. It also means that biological understanding in its deeper sense must lie in physics and chemistry. The awkward reality for biologists – that biology's essence, secreted within those physicochemical origins lies largely outside the subject that purports to study it.

Finally, understanding life as a complex kinetic process allows conclusions to be drawn regarding the widely held view that life, its emergence and evolution, can be understood as a thermodynamic phenomenon. We believe that there is now clear evidence that argues against that thermodynamic viewpoint (though life processes are necessarily bound by thermodynamic constraints). The key points in support of a kinetic paradigm may be summarised as follows:

1. The cell, the fundamental unit of biology, has evolved from simpler chemical beginnings to minimise energy dissipation, not to maximise it. This is reflected in the extraordinary efficiency of the cell-reproduction apparatus which has evolved to maximise reproduction, not energy dissipation.

2. Whereas an evolutionary process toward increasing complexity is a widely observed phenomenon, the transition to that more complex state may lead to a reduction in energy dissipation, as expressed in a variety of experimental situations [37] as well as in the kinetic simulation described in this paper. The existence of clear exceptions to the energy dissipation view of life questions the validity of a general thermodynamic paradigm.

3. Kinetic pathways cannot, as a general rule, be deduced from thermodynamic factors. Any two thermodynamic states are potentially connected by an infinite number of kinetic pathways and extra-thermodynamic information is required to deduce which pathway is followed in any particular case. Given that all persistent replicative systems are in essence kinetic steady states, the evolutionary process based on that replicative essence must therefore also be kinetic in nature. Accordingly, any process governed primarily by kinetic factors is unlikely to be generally describable in thermodynamic terms.

A closing comment: in order to address the most general of life questions – for example, could life be based on an alternative chemistry, how could we identify such life forms – a more chemically explicit understanding of what life is, is necessary. Richard Feynman's famous aphorism: "what I cannot create, I do not understand" points the way forward. Given the precise physicochemical description of the life process presented here and in earlier publications, specific chemical steps toward the synthesis of simple protolife systems are now indicated [54]. This goal, if and when achieved, would go a long way toward answering the perennial 'what is life' question, as well as answering the ahistorical question, how was inanimate matter of whatever kind able to evolve into life.

Supporting Information

Supporting Information File 1

Conditions for exponential growth and steady states calculated for the system of Figure 3.
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-13-66-S1.pdf]

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BEILSTEIN JOURNAL OF ORGANIC CHEMISTRY

G-Protein coupled receptors: answers from simulations

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Review

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Abstract

Molecular-dynamics (MD) simulations are playing an increasingly important role in research into the modes of action of G-protein coupled receptors (GPCRs). In this field, MD simulations are unusually important as, because of the difficult experimental situation, they often offer the only opportunity to determine structural and mechanistic features in atomistic detail. Modern combinations of soft- and hardware have made MD simulations a powerful tool in GPCR research. This is important because GPCRs are targeted by approximately half of the drugs on the market, so that computer-aided drug design plays a major role in GPCR research.

Introduction

Evolution is a unique optimization mechanism. Firstly, it stops optimizing as soon as an acceptable solution is reached. There is no evolutionary pressure for elegance, simplicity or even effectiveness above the critical threshold. Secondly, because evolution always starts with what is already available, it reuses successful designs again and again in slightly modified forms. This is the case for the most common means of communicating across cell walls in eukaryotes, G-protein coupled receptors (GPCRs). GPCRs span the cell membrane and generally complex switching ligands from the extracellular medium in order to effect changes in the G-protein signaling system inside the cell. There are many variations on the scenario, some of which will be outlined below. Approximately 800 GPCRs are encoded in the human genome [1], earning them the label "*The Evolutionarily Triumphant G-Protein Coupled Receptor*" [2]. Their functions are myriad, from olfactory and visual receptors to pure signaling systems that govern cell function. Malfunction of GPCRs is prevalent in human diseases, so that approximately half (estimates vary between 30 and 60%) of marketed drugs target GPCRs. Furthermore, cancer cells can misuse existing GPCRs to ensure their own survival and prevalence [3]. It is therefore not surprising that GPCRs are the subject of a vast research effort that was recognized by the award of the 2012 Nobel Prize in Chemistry to Robert Lefkowitz [4] and Brian Kobilka [5].

The experimental research to date on GPCRs represents a landmark in scientific achievement because of the complexity and

experimental intractability of GPCRs themselves. Structural information that can be used as the basis for simulations is most important for the purposes of this review. Ultimately, structures at atomistic resolution are needed to decipher the intimate details of the modes of action of GPCRs. X-ray crystallographic studies on GPCRs are, however, fraught with difficulties [6]. The structure of rhodopsin, the first GPCR X-ray structure, was published in 2000 [7], was not followed by the second, the β2-adrenergic receptor, until 2007 [8]. Figure 1 shows the growth in the number of GPCR structures from 2000–2016. After a very slow start, structures for on average six new receptors per year have been becoming available in the last five years. Each of these structures is a significant experimental achievement, so that the low number of structures being published represents the output of a major worldwide research effort to obtain structures for receptors that unfortunately require considerable ingenuity (and luck) to obtain suitable crystals for X-ray crystallography [6].

Not only has the paucity of available structures hampered investigations, however, GPCRs can exist in active or inactive conformations and in binary complexes with ligands or intracellular binding partners (IBPs, G-proteins or β-arrestin) or in ternary complexes with a ligand and an IBP. Thus, many structures would be necessary in order to obtain a complete atomistic picture of the mode of action of the GPCR. A further problem is that we need structures that correspond to the receptors in their natural surroundings as they occur and function in nature. Proteins, especially membrane-bound ones, do not necessarily crystallize in their biologically active structures and the measures needed to obtain suitable GPCR crystals tend to increase the diversity between the natural environment and the crystal.

These measures are needed to overcome some inherent problems in crystallizing GPCRs. These problems may arise from flexible or non-polar regions of the GPCR, especially intracellular loop 3 (IL3), that do not form the rigid, specific interactions needed for crystallization. Such problems are sometimes overcome by truncating the flexible termini, complexing the GPCR with antibody fragments [9], or by replacing IL3 with a stable, polar fragment such as the T4 lysozyme [10,11] or other suitable protein fragments [12]. Other techniques used to obtain GPCR crystals include mutations to enhance the thermal stability [13], solubilization with custom detergents [14] or in conjunction with high-affinity ligands, which promote one stable conformation. These techniques are discussed in far more detail in reference [6].

One further problem in GPCR structure determination is to obtain crystals in which the GPCR is in the active conformation. The active conformation could be stabilized at low pH with detergents for opsin/rhodopsin [15,16] and the critical IL3 was resolved in both cases. However, the loop conformation

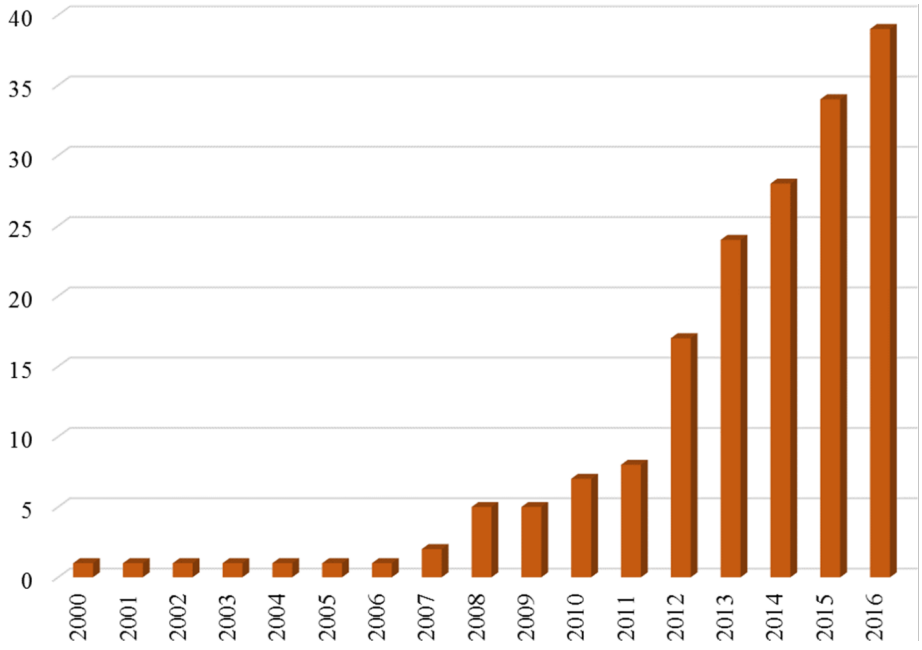


Figure 1: The cumulative number of different GPCRs for which X-ray structures were available in a given year. The data represent a total of 174 structures on 91 ligand–receptor complexes for 39 different receptors. The data are taken from http://gpcrdb.org/structure/statistics (2nd February 2017).

was stabilized by intermolecular contacts in the crystals, as was later shown spectroscopically [17]. This conformation is only stable in ternary complexes with G-proteins in the natural systems but G-proteins are not stable enough for crystallization. The solution to this problem has been to use the variable domains of camelid antibodies, which are generally designated protein nanobodies, as a surrogate for the G-protein [18]. This technique will be discussed in the context of the simulations below. Note, however, that the opsin/rhodopsin structures [15,16] used so-called high-affinity peptides to mimic the G-protein. It is likely that these proteins behave more like the native G-protein than the protein nanobodies.

Review

General GPCR structure

Figure 2 shows a schematic diagram of the general structure of GPCRs.

GPCRs consist of seven α-helices that span the membrane between the extra- and intracellular sides. The N-terminus is extracellular and the C-terminus intracellular. The helices are connected by three intracellular loops (IL1, H1-H2; IL2, H3-H4 and IL3, H5-H6) and three extracellular ones (EL1, H2-H3; EL2, H4-H5 and EL3, H6-H7). The extracellular loops are often involved in ligand recognition and binding, whereas the intracellular ones interact with the IBPs, usually a G-protein. The activation process involves switching of the binding on the intracellular side of the receptor, as outlined below.

Mechanism of G-protein signaling

Figure 3 and Figure 4 show modeled structures to illustrate the mechanisms of signaling in GPCRs. In the simulations discussed below, the G-protein is represented only by the α-subunit, which binds directly to the GPCR.

In the ligand-free state shown in Figure 3a, no agonist ligand is bound. The G-protein is bound to the intracellular side of the receptor. In this state, the GPCR exhibits its basal activity, which can range from completely inactive to significantly active. Figure 3b shows the fully activated complex, which requires both an agonist ligand and the G-protein. On activation, bound guanosine diphosphate (GDP) in the G-protein is replaced by the triphosphate (GTP) and the α-subunit separates from β/γ. The separated G-protein subunits migrate to effectors in the nearby membrane, where GTP is hydrolyzed to GDP and the signaling cascade initiated.

GPCRs are normally deactivated by β-arrestin, as shown in Figure 4. After activation and dissociation of the β/γ subunit, IL3 and the C-terminus of the GPCR are phosphorylated at serine and threonine residues (Figure 4a). This phosphorylation allows the recruitment of β-arrestin (Figure 4b), which can then form a strongly bound complex with the receptor (Figure 4c). This complex can lead to internalization of the receptor (its removal from the cell wall) or to an independent β-arrestin signaling pathway. This inactivation process is subject to very many variations, depending on the GPCR, and internalization

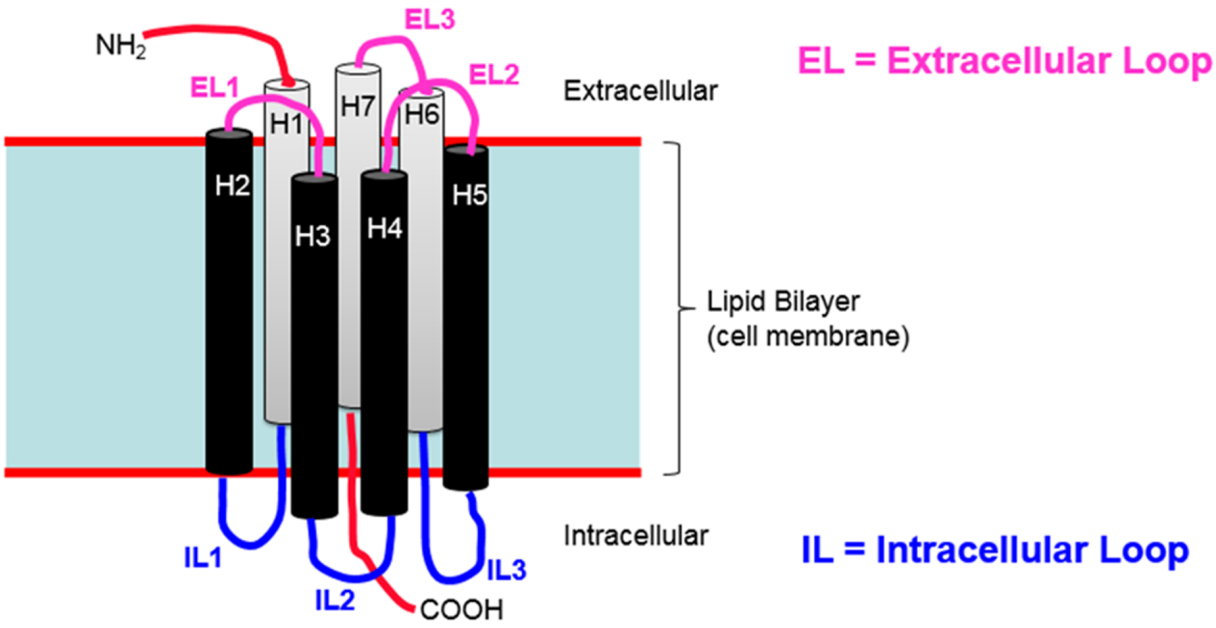


Figure 2: A schematic diagram of the general structure of GPCRs. Most GPCRs also contain an eighth helix, H8, at the C-terminus (not shown in the Figure).

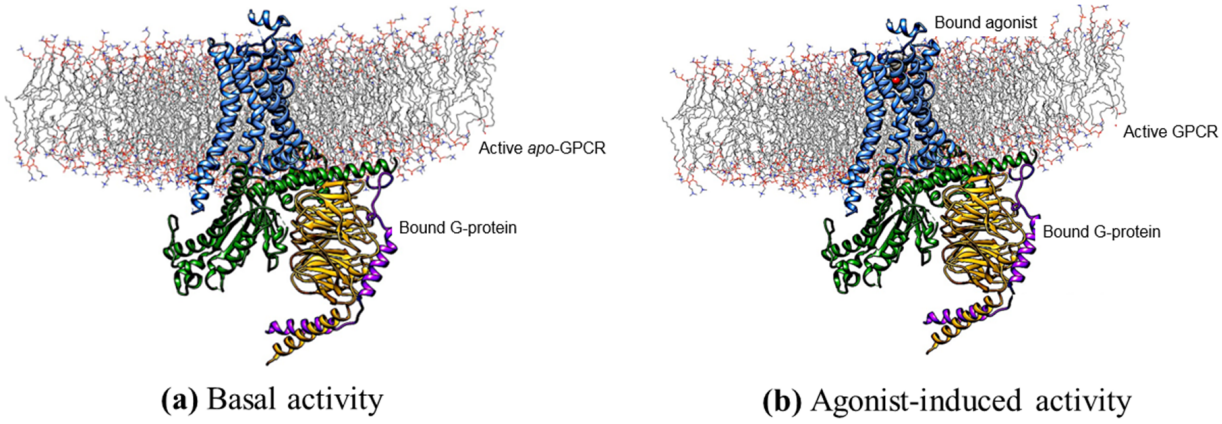


Figure 3: (a) The inactive state of a GPCR: No ligand is bound. The α-subunit of the G-protein is shown in green, β in yellow and γ in magenta. In this state, the GPCR exhibits basal activity. This figure assumes pre-association of the G-protein to the receptor. (b) Fully activated GPCR. Both an agonist ligand and the G-protein are required for full activation. The structures shown are based on homology models.

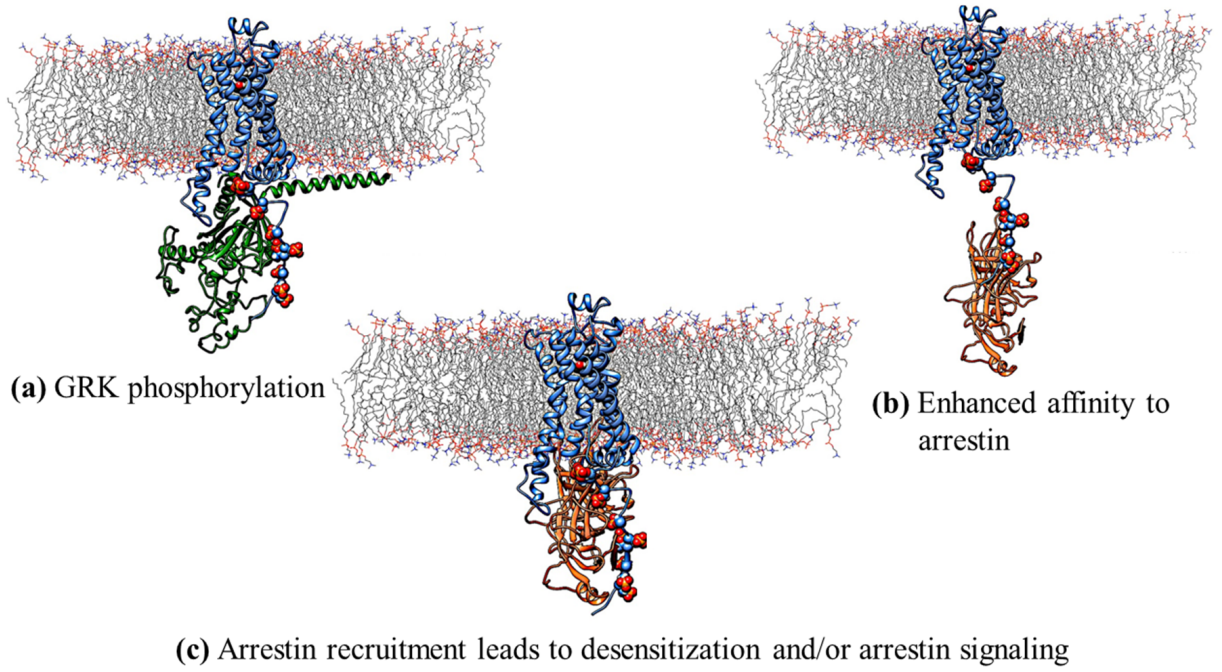


Figure 4: (a) After activation of the GPCR and dissociation of the β/γ subunits, IL3 and the C-terminus of the GPCR is phosphorylated by G-protein regulating kinases (GRKs). (b) Arrestin is recruited by the phosphorylated tail. (c) The fully bound arrestin can lead either to internalization of the receptor (inactivation) or to a separate arrestin signaling path. This Figure represents only one of many possibilities, which have been reviewed by Tobin [19].

may be reversible if the phosphorylated residues are hydrolyzed within the cell.

GPCR modeling and simulation

GPCRs are such important pharmaceutical targets that homology models of various receptors were constructed [20] almost as soon as the rhodopsin structure [7] became available. Even though it has played a major role in the determination of the mechanisms of action of GPCRs [21,22] and was the only structure available, in general, rhodopsin was not considered the ideal template for GPCR drug targets. This was because of both its relatively low similarity to medicinal targets [23] and its distance from them in the G-protein phylogenetic tree [1]. Later, when the β2-adrenergic receptor structure was published [8] it

was concluded that homology models would play an increasingly important role in computer-aided drug design (CADD) [24]. With hindsight, this conclusion was perhaps a little optimistic. However, the situation has changed considerably in the last five years. Not only are more (and more relevant) GPCR structures available, but modern protein force fields have attained a level of reliability that makes them truly predictive in most drug-design scenarios; it was found five years ago that "*the calculation error is comparable to the uncertainty in the experimental comparison*" [25]. The error in this case refers to the ability of the force field to reproduce peptide and protein conformations determined using calibrated Karplus equations in conjunction with NMR experiments. The more important development is, however, the power of modern hardware. For years, 10 ns simulations were state of the art. These simulations had little relevance for real biological systems, which are generally far slower. It became evident from studies on transcription factors that simulations often require a simulation induction period of several hundred nanoseconds to a microsecond before they undergo important conformational changes [26]. This is possibly because the starting structures are usually taken from X-ray crystal structures or homology model derived from them. As the force fields have been optimized to reproduce X-ray structures, they likely have a kinetic bias that hinders rearrangement from typical starting structures. Nonetheless, the simulations were able to predict whether the receptor was induced (i.e., allows transcription) or not [26], in contrast to X-ray structures in which the allosteric rearrangement was overlaid by crystal-packing effects.

Today's combinations of hard- and software allow routine simulations of several μs, which means that homology models can be equilibrated long enough for them to adopt what is probably a biologically relevant structure. These simulations are, however, still not long enough to investigate processes such as the binding or unbinding of GPCR ligands and IBPs. In these cases, we must resort to enhanced-sampling techniques, the most effective of which in our hands has proven to be metadynamics [27]. As outlined below, modern variations of metadynamics allow very effective use of massively parallel supercomputers in order to investigate ligand binding and unbinding and transitions between active and inactive receptor conformations. Indeed, the power of modern simulations is such that we must revisit the relationship between simulations and experiment, especially for GPCRs.

Experiment and simulations

The traditional, and very persistent, view of the relationship between experiments and simulations is that, if the latter cannot reproduce the former, the simulations are inaccurate. This may in many cases be true, although, subjectively at least, the accuracy of simulations is closely related to that of experiments in the same research area. That is not, however, the main point with GPCR-research. We are faced with an extremely difficult research area in which every result is valuable. Some experimental results are obtained under extremely difficult conditions and may not be reproducible. A major aspect of this discussion, however, has to do with the relationship between the biological system and the necessarily modified objects studied in experiments. GPCRs are flexible, sensitive proteins that, because of their biological function, react sensitively to perturbations. Given the reliability of protein force fields pointed out above, it should be clear that it is often possible to simulate systems that are closer to the biological situation than the crystals used to obtain X-ray structures. An early example of this is the fact that crystal-packing forces are large enough to change the induction state of the tetracycline repressor [26]. GPCR simulations often also show geometric rearrangements after several hundred nanoseconds, which suggests that the simulation is perhaps switching to a conformation closer to the biologically relevant one than the X-ray structure. Also, the simulations do not need the modifications outlined above for obtaining suitable crystals; they can be performed for the original biological system.

Thus, simulations can reasonably be expected in many cases to give a closer picture of the biological situation than some experiments. Another point is, however, important and does not result in competition between simulations and experiment; simulations can provide information that is so far not available from experiment. This is an important but still largely unrecognized aspect of GPCR research. Even the most skeptical about the accuracy and relevance of simulation can accept at least the role of simulations to point towards detailed mechanistic aspects that suggest specific experimental tests. Of course, the simulations must be validated as far as possible by comparison with experiment but without forgetting that the error limits for the experiments are often comparable to those of the simulations. For instance, free binding energies from simulations that agreed with experimental ones by, say, less than 0.5 kcal mol^{-1} would mean that not only the simulations but also the experiments are far more accurate than we expect.

In the following, GPCR simulations that provide atomistic details of GPCR activation mechanisms will be described. These are mostly from our own work but also include some landmark simulations from elsewhere.

Binary and ternary complexes

The ternary complex model [28] and experimental findings [29] suggest that both an agonist ligand and a bound G-protein are necessary in order to activate GPCRs. It is therefore significant

that the first molecular dynamics (MD) simulations of a ternary GPCR complex were reported only four years ago [30]. Such simulations are now commonplace and the comparison between binary ligand–receptor and ternary complexes has become a valuable tool in GPCR research.

Activation mechanism

The first simulations to demonstrate a binding pathway for ligands approaching a GPCR from the extracellular medium were reported for the β1- and β2-adrenergic receptors in 2011 [31]. Notably, these simulations were performed on Anton, a specially constructed computer for MD simulations [32], and were unconstrained, so that they simulated the ligand-binding process without enhanced sampling on a time scale of several μs. Later simulations of the same type revealed a mechanism for allosteric modulation for the muscarinic M2 receptor [33]. Most importantly, though, long unconstrained simulations were able to demonstrate the deactivation of an active conformation of the β2-adrenergic receptor (taken from the X-ray structure) [18] in binary ligand–receptor complexes. These unconstrained simulations lay the foundations for more targeted ones that use enhanced-sampling techniques to determine, for instance, the activation mechanism of the muscarinic M2 receptor [34].

Free energies of binding by metadynamics

Very long timescale MD simulations can be performed on specialized hardware such as Anton [25] but are less effective on more conventional massively parallel supercomputers because the simulations only scale up to a relatively limited number of CPUs or GPUs [35]. Luckily, of the many enhanced-sampling techniques [35], modern variations of metadynamics [27] can make very effective use of massively parallel hardware. Briefly, metadynamics enhances the sampling in MD simulations by adding small Gaussian destabilizing potentials at positions that the simulations has already visited enough. "Positions" need to be defined in terms of a small number of geometrical variables (the collective variables, CVs) that are relevant (e.g., as a reaction coordinate) for the process being studied. In this respect, the relatively fixed orientation of the GPCR in the membrane allows us to define a generally applicable CV perpendicular to the plane of the membrane [36]. This general CV describes the binding process of ligands approaching from the extracellular medium remarkably well. This in itself would not make the simulations effective on massively parallel supercomputers but the use of many replicas at the same time to enhance the sampling (multi-walker metadynamics) [37] further allows many simulations to be carried out in parallel, and thus makes excellent use of massively parallel hardware. The final enhancement to the simulations is to apply a so-called funnel constraint [38] that limits the sampling in the extracellular solution, where it is not necessary [36].

In our context, the most important advantage of metadynamics is that it gives a free-energy profile of the process being simulated [27]. This means that we can obtain complete free-energy profiles along the binding path for both ligands and IBPs [39]. This, in turn, allows us to validate the simulations by comparison with experimental free energies of binding obtained from measured binding constants. This comparison turned out to be an unqualified success; the simulated binding energies for 23 different binary and ternary complexes comprising five different receptors and 13 different ligands gave a root mean square deviation of 0.8 kcal mol^{-1} [36]. In contrast to other techniques used to predict binding energies, the simulations deliver an excellent agreement with the experiment (the correlation line has a slope of 0.99 and an intercept of zero, with $R^2 = 0.81$), rather than simply correlating well. Remarkably, the ligands span a wide range of efficacies; in 10 cases, they act as agonists, in 11 as antagonist and twice as partial agonists. One key to this success is that the simulations were able to identify the most stable binding site of several alternatives in each case.

Multiple binding sites

We have often observed that in quantitative structure–activity relationships (QSAR) for GPCRs, agonists give far better results than antagonists [40]. Metadynamics simulations on the vasopressin receptor [41] revealed the reason for this behavior. As also found previously in unbiased simulations of the β2-adrenergic receptor [31], ligands can occupy more than one binding site along the binding path. In the case of vasopressin, a cyclic peptide hormone, the simulations revealed three different sites, the conventional orthosteric one that activates the ligand, an "intermediate" and a "vestibule" site. Significantly, antagonists bind to one of the alternative sites more strongly than to the orthosteric one. Of pharmacological importance is the fact that antagonists bind to different sites in the two subtypes of the vasopressin receptor investigated, so that a general QSAR that encompasses agonists and antagonists for both receptors would need to consider all three sites [41].

Multiple binding sites along the binding path are common in GPCRs. The human chemokine receptor CXCR3, for instance, exhibits distinct alternative binding sites that can be occupied simultaneously by competing ligands, which explains contradictory experimental results obtained in competition experiments [42]. Multiple binding sites have also been found for the β2-adrenergic, muscarinic M2 and μ-opioid receptors [36,39].

Functional bias

For those GPCRs that can activate both G-protein and β-arrestin pathways, some ligands may exhibit a functional bias and activate one or other of the two alternative paths. Metadynamics simulations have proven to be able to determine the bias, or lack

of it, by considering the change in ligand-binding free energy between the binary ligand–receptor complex and the alternative ternary complexes with either the G-protein α-subunit or β-arrestin. We define two free-energy differences:

$$\Delta\Delta G_{(\text{G-protein})} = \Delta G_{(\text{ligand:[receptor:G-protein]})} - \Delta G_{(\text{ligand:receptor})}$$

$$\Delta\Delta G_{(\beta\text{-arrestin})} = \Delta G_{(\text{ligand:[receptor:}\beta\text{-arrestin]})} - \Delta G_{(\text{ligand:receptor})}$$

The ligand bias can be determined from these energies according to Table 1 [39].

Table 1: Scheme for determining the bias of GPCR ligands according to the calculated changes in ligand-binding free energies [39].

$\Delta\Delta G_{(\text{G-protein})}$	$\Delta\Delta G_{(\beta\text{-arrestin})}$	Ligand bias
negative	negative	unbiased agonist
negative	positive	G-protein biased agonist
positive	negative	arrestin biased agonist
positive	positive	unbiased reverse agonist
≈zero	≈zero	neutral antagonist

Thus, the simulations allow not only the calculation of the free energy of binding for unknown ligands but also the functional bias.

Conclusion

The simulations described are extremely compute-intensive; they have been performed on SuperMUC [43] with grants totaling 85 million CPU hours and using thousands of cores per simulation. However, considering the progress being made constantly in computer soft- and hardware, such simulations will become routine within a decade or less. Two take-home messages are important.

Firstly, the simulations can provide information not available (yet) from experiments. This is because the experiments are very difficult, because they must be performed in many cases on modified receptors and because atomistic details are available from very few experimental sources. Thus, simulations should be accepted as valuable tools in GPCR research.

Secondly, even given their very high computational cost, simulations may even now be a viable alternative to experiment for determining binding constants (= free energies of binding) and ligand bias. The simulations are predictive and can therefore be used in prospective computer-aided drug design.

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From chemical metabolism to life: the origin of the genetic coding process

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Review

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Abstract

Looking for origins is so much rooted in ideology that most studies reflect opinions that fail to explore the first realistic scenarios. To be sure, trying to understand the origins of life should be based on what we know of current chemistry in the solar system and beyond. There, amino acids and very small compounds such as carbon dioxide, dihydrogen or dinitrogen and their immediate derivatives are ubiquitous. Surface-based chemical metabolism using these basic chemicals is the most likely beginning in which amino acids, coenzymes and phosphate-based small carbon molecules were built up. Nucleotides, and of course RNAs, must have come to being much later. As a consequence, the key question to account for life is to understand how chemical metabolism that began with amino acids progressively shaped into a coding process involving RNAs. Here I explore the role of building up complementarity rules as the first information-based process that allowed for the genetic code to emerge, after RNAs were substituted to surfaces to carry over the basic metabolic pathways that drive the pursuit of life.

Introduction

"Man is the measure of all things" (Protagoras), making it difficult to get around an anthropocentric view of the reality that envelops us. Conjectures about the origins of life do not escape this unfortunate shortcoming. Even the quest for our own origin is far from settled: There is no Adam or Eve in the origin of mankind. If you doubt, just try to work out a single-step process that would account for a change from a set of 48 chromosomes (their number in apes) to 46 (their number in man) in a sexed species. Starting with accidental fusion of two chromosomes, a ratchet-like continuum of changes must have distanced us from our ape ancestors. In the same way, it is implausible that there was only one origin of life, as unfortunately many still try to

call forth. Thirty years ago, Freeman Dyson provided a convincing demonstration that, contrary to the widespread "adamist" view, which looks for a single origin to all things, there were at least two origins of life [1]. He established that before the emergence of replication processes (making exact copies), a metabolic system must have reproduced (making similar copies), progressively increasing the accuracy of its pathways before allowing a spin-off system to initiate replication. Here I try to pursue this track and go beyond standard views of what life is, and how it emerged, trying to find the simplest ways forward. I focus on one single question, that of the origin of the coding relationship that links the effectors of life functions (in material, molecular terms, the proteins) to the providers of the memory (the genetic program made of nucleic acids) used as a blueprint to propagate them across generations. To this aim, I take the stance of the engineer who, when designing new inventions, tries first to think them in terms of functions. This implies that I combine an abstract view of what life is with its concrete implementation on Earth as we know it. Choosing abstraction first is a way to postpone the restrictions imposed by the intrinsic properties of matter in order to avoid the trite but certainly inaccurate view of life as always made of animal-like creatures.

This presentation entails using the concept of function, a notoriously difficult one [2]. A main problem that lies behind the difficulty of defining what is a function is its relationships with evolution (how did this particular function come into being?), and this is what I discuss. A key idea behind the view I support is that beside the four currencies constituting our world (matter, energy, space and time) we must add a fifth one, information, taken as an authentic physical currency [3]. To make this idea concrete I see cells (and living organisms) as computers, but not those we use today, computers that would be able to generate a progeny of computers [4]. As in common computers, this means a machine and a separate program that is run by the machine. Here, I identify the program driving the life of the cell with its genetic program, chemically embodied in its genome based on nucleic acids and I study how the innards of the machine emerged first. I propose that what we currently know from the analysis of genomes (in particular the functions of the genes that belong to the operating system of life, that we named the "paleome" [5]) gives us hints to progress in our understanding of how life came to being. Finally, among the many functions required for the development of life, the processes that allow aged organisms to construct young ones are of key interest. These processes, in turn, give a direction to the very process of "life and evolution" via accumulation of information, in a ratchet-like manner. Combining "action" with "orientation" will help us to understand the concept of function and how functions keep emerging as life develops.

Review

Abstract requirements for the existence of life

A fiction

Following "The Black Cloud", published in 1957 and already based on a very abstract view of life, Fred Hoyle wrote another fictional work for the BBC, "A for Andromeda", with John Elliot (published in 1962 from the screenplay of a television series [6]). In this book he pictured the remote action, on Earth, of an intelligent civilisation located in the Great Nebula of Andromeda. This action was triggered by an unknown form of life, detected by astronomers as they scanned the universe for non-random signals. A group of British astronomers, in their analysis of the sky –in an effort reminiscent of the still ongoing SETI program [7]– points out an electromagnetic signal within the Andromeda galaxy that does not look random. The scientist who analyses the electromagnetic waves coming from heaven realises that this is not accidental, because the signal is clearly sent in a repeated form by what can only be a scheming intelligence. It takes some time to reconstruct the signal in its entirety because the daily Earth rotation hides it partially. The astronomer then understands that the signal is a message, and that this message has properties reminiscent of a computer program. To decipher its meaning, he runs it as an algorithm in a pioneering computer built thanks to funds from the Ministry of Defence in the mists of northern Scotland. After running first steps of the message in the computer, the astronomer understands that this algorithm is a kind of blueprint for the construction of a new computer. This new computer should combine the calculations run by many small pre-processing computers that must then be introduced into the main frame. The algorithm begins by asking questions about the chemical nature of living matter, and then proposes a scenario for the synthesis of living tissue. The ultimate purpose of the message is to take control of our earthly life.

This fiction is particularly revealing in that it stresses that, while matter is essential in the living objects we see, the key to life is not matter. The entity that is transported from distant stars is physical, yet immaterial (despite photons being its vehicle). It is a piece of information, serving as an invasive and guileful program, not the traditional little green man-like creatures. Life is seen as the physical implementation of a program. In Hoyle's novel, life is the program. An attractive feature of information, vividly prominent in this fiction, is that it is not simply an isolated, worthless independent entity. It may, and must, interact with other sources of information as well as with matter, a feature that someday will need to be included in theories of information. In Hoyle's novel human action is an intermediary for processing digital messages into material devices. While this touches a key point to understand what life is, it also illustrates a widespread confusion: Because it uses humans as an interme-

diate, this scenario mixes up the program with its implementation, which requires a specific source of information. Like a virus without a cell to infect, without a living human intermediate, the program would be ineffective, it would not be alive. As in many contemporary views of biology, this fiction is based on an animistic vision, which we might call “the animism of DNA”. This is summarised by the astronomer who discovered the extraterrestrial message: "If we are able to use the computer as a control device, and if we can build a chemical reactor that can act from its instructions as they appear –in fact, if we can make a DNA synthesizer– then I think we can start building live tissue”. Today, it is not difficult to find statements of this kind in connection with the study of the genome of living organisms, and, naturally in scenarios of the origin of life. This is based on the involuntary occultation of what is nevertheless an obvious fact: to run a program requires a machine! We know, certainly, that having a CD with a state-of-the-art operating system (OS) is useless if it is not placed in an actual computer, and that this (information-rich) computer must still be compatible with the OS. Naturally, of course, there is still another feature that is absent in the fiction: creation of a progeny. Yet, this is, as everybody will accept, a core function of life.

The key role of coding

In parallel with a remarkably prescient vision of cardinal features of life, we find in this book the misunderstandings –the most common ones– of what is today named “synthetic biology” as well as the beliefs spread by mass media, namely the mix-up of a program, the expression of the program, and the machine able to read and express the program (we consistently forget this machine). Just as for superficial minds there are ”genes of” everything (for instance of intelligence, diseases, obesity, and old age), in the novel written by Hoyle, the program is sufficient to establish and produce the final form of the organism whose manufacture it prescribes. It is as if the cooking recipe produced the meal, or rather, a musical score produced the symphony you are listening to. One of the reasons for this deep misunderstanding is that the concept of a program entails a central role for coding, a very deep and abstract concept that is rarely mastered in what is taught in current education systems (the widespread and very misleading use of “genetic code” as a replacement for genetic program is a case in point). The coding process (i.e., using a cypher) establishes a correspondence between the abstract world of information and its material implementation.

The idea here is that, because our world comprises information as one of its basic currencies, any entity can be described via the use of a symbolic representative, a text written in a finite alphabet (at the most abstract level digitised or, at the very root of coding, represented as a sequence of 0 and 1). The coding process is based on two properties: decomposition of any entity into a finite set of building blocks (amino acids for proteins, atoms for molecules, and protons, neutrons and electrons for atoms) and a correspondence, a code table, between a string of symbols and these building blocks (e.g., for the atomic composition of matter: N for nitrogen, Fe, for iron, C for carbon). Thus a chemical molecule is information-rich. *sn*-Glycerol-3-phosphate can be described, including an outline of its three-dimensional configuration, by a limited alphabet of symbols (e.g., the Simplified Molecular Input Line Entry Specification (SMILES) code table [8]), C([C@H](COP(=O)(O)O)O)O, while its mirror symmetry *sn*-glycerol-1-phosphate is summarised as C([C@@H](COP(=O)(O)O)O)O. That this coding is sufficient (if associated to a concrete machine) is visible in Figure 1, where I used these codes with an algorithm to generate the picture of the corresponding molecules. Remarkably, the link between the genetic program and the effectors of biological processes of the machine that runs the program, the proteins, is mediated by such a code, the genetic code, which establishes a correspondence between the nucleotide building blocks making nucleic acids that carry the program, and the amino acid building blocks making proteins.

This has very deep consequences that ask for a thorough and time-consuming study. The abstract process of coding has given Douglas Hofstadter the subject of a book more than six hundred pages long, “Gödel, Escher, Bach. An Eternal Golden Braid” [9], which despite its depth and length won the popular Pulitzer Prize in 1979. You should never, therefore, expect to understand what life is in two sentences. Even though, after a reasonable effort, you may understand that it is not a large blend of complicated concepts. Instead it comprises just a handful of essential albeit very deep concepts, among which the process of coding has a paramount position. Yet, amusingly, it appears that everybody may talk about biology, give their opinion on natural selection, evolution of the species, or the benefits or misdeeds of genetic engineering. And of course, because this talking does not explore the key questions, it is the most anecdotal characters, accidents and variations that are placed in the limelight, not the profound laws that govern life.

Once more, understanding biology requires a long and deep work, little compatible with the lazy tendencies of the moment. To understand that the key law of life is the coding that relates the memory of the genome to its expression, requires the understanding of the concept and consequences of recursion (i.e., the implementation of a procedure that calls upon itself to determine the subsequent sequence of events), extensively discussed by Hofstadter in his book (again, in some 600 pages). Among its major consequences is an apparently paradoxical property of life: all processes associated to life may be considered as deter-

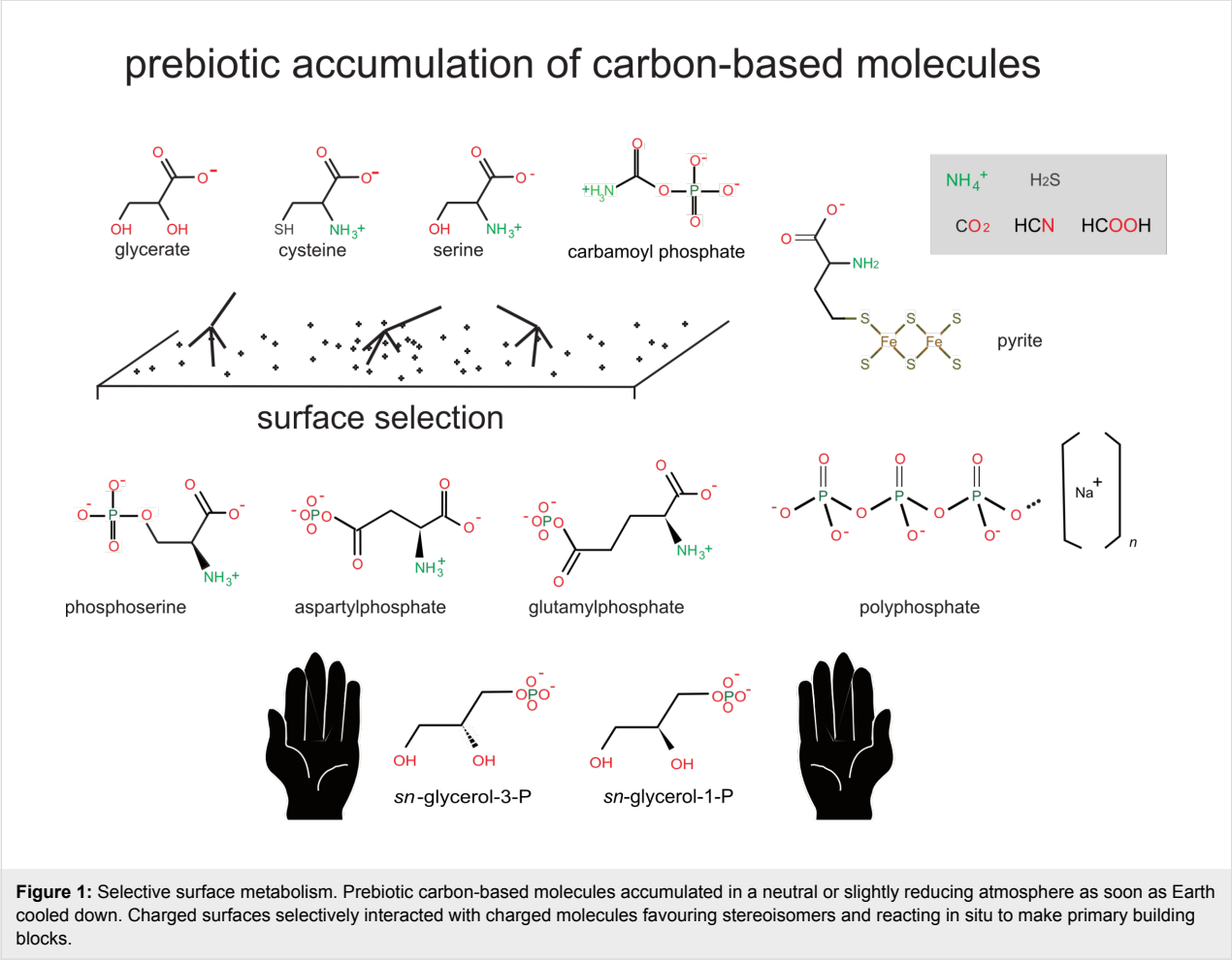


Figure 1: Selective surface metabolism. Prebiotic carbon-based molecules accumulated in a neutral or slightly reducing atmosphere as soon as Earth cooled down. Charged surfaces selectively interacted with charged molecules favouring stereoisomers and reacting in situ to make primary building blocks.

ministic, but they are not of a mechanical type, as it is, by construction of a recursion, impossible to predict their consequences in the long term (even knowing initial conditions). Living processes are both deterministic and unpredictable. This may read as an oxymoron, but here is a straightforward example using whole numbers (apparently so simple). Knowing the recursive algorithm that allows you to compute the digits of the number π, try to predict the value of the p-long sequence ($p \geq 1$) that follows the n-th digit of π (you may associate to this sequence to triggering a major earthquake for example, so that knowing it would matter). Because the only way is to run the algorithm until n is reached, this will not be possible if you choose n sufficiently large, even with the most powerful computers. Nothing is more deterministic than running this algorithm.

Once this is understood it becomes fairly obvious that cells have abstract properties highly reminiscent of the abstract design of what became our computers, the Turing machine [4]. Indeed this machine combines two separate entities, an authentic machine and a sequence of symbols that acts as a program, controlling the behaviour of the machine. The latter reads, writes and moves the program support (which must be material, but this requirement is not concretely discussed in the abstract formulation of the Turing machine) to reach its symbols. Importantly, exactly as in the living cell, where there is no specific instruction (no design) to tell it to start living, in Turing's description the information manipulated by the machine is purely declarative (i.e., the very presence of the program triggers the running of the machine), and not prescriptive (i.e., there is no need to tell the program to start running). This implies that, for a Turing machine, there is no conceptual split between data and program. Prescription would assume that an external principle prescribes, while there is absolutely no need for any external principle to trigger the onset of life (see the demonstration by Freeman Dyson [1]). Hence, the very word “program” is somewhat misleading. How do we make it concrete, using the building blocks that make cells? And above all, how could a coding process, associating molecules from widely distant chemical classes, proteins and nucleic acids, emerge without some sort of design? A brief scenario for the origin of cells will tell.

A short scenario for the material implementation of life

Once accepted that life results from the dynamic information processing of organised relationships between material entities, it becomes necessary to identify what those entities are and how they are combined together. Life, as we know it, stems from four well-identified operations: compartmentalisation, metabolism, manipulation and memorisation. The former two operations are performed mainly by small molecules (carbon-based and comprising a few tens of atoms), whereas the latter two are carried out by macromolecules (nucleic acids and proteins, made of a limited number of building blocks). To these operations we must add two essential laws, complementarity and its major consequence, coding (just brought up as key to life).

Making cells

Compartmentalisation

The atom of life is the cell, and a cell generate cells: "omnis cellula e cellula" [10]. The obvious function associated to this view is that the cell separates between an inside and an outside. In 1935, James Danielli proposed with Hugh Davson that this embodiment was achieved by formation of a bilayer made of amphiphilic lipid molecules [11]. This process is entropy-driven (life belongs to physics, it is not a fight against the second principle of thermodynamics), using the global distribution of water molecules as a driving force to order lipids into cell-like structures (of a considerable variety, even in bacteria [12]). Membranes also contain proteins as essential components. It took very long to understand the way proteins interact with membrane lipids, and our knowledge in the domain is still far from complete. There exist many models describing the operation (including ideas about the asymmetry of the bilayer, its local changes and lipid rafts). Work exploring the way proteins are inserted into membranes is a thriving domain of research [13].

Membranes serve a variety of functions such as transport, sensing, protection or supporting movement. They are also involved in energy production via vectorial transport of ions, generally protons. Transport and management of energy imply manipulation of the electro-chemical gradient built up between the inside and the outside of the cell (in particular with the fascinating nanomotor ATP synthase [14]). Membrane components age and waste away: This implies maintenance. Finally, there are specific needs to allow for division while the role of the membrane differs during states of growth and non-growth. The former implies a constructive function of the membrane. Proteins are the effectors of this function with the key operation of allowing protein insertion within the membrane.

Studies investigating spontaneous evolution of lipid vesicles showed that they split, fuse, get internalised and make complex internal networks [15]. Beside lipids, polypeptides form coacervates, which also allow for compartmentalisation [16]. A main difficulty to understand the process is that membrane proteins must fold within two-dimensional (2D) bilayers. This implies the management of construction and maintenance within a 2D structure, while the metabolism that develops in the cytoplasm and produces the building blocks for membranes and their proteins is expressed in three dimensions (3D). Matching the syntheses in both compartments is not a trivial matter because adequate tuning of the corresponding rates of synthesis depends on the volume that will be occupied by the synthesised compounds. Remarkably, rather than in prokaryotes, this hurdle is much easier to solve in eukaryotes with their endoplasmic reticulum, which is a kind of membrane structure folded within the cytoplasm as a Peano surface, thus solving the 2D/3D dilemma. It is therefore natural to assume that the first cells harboured an internal membrane network [17] coupled to peptide metabolism.

Finally, an essential feature of compartmentalisation is more subtle: A cell must give birth to another cell. This implies that its envelope is susceptible to growth and division. In summary, this early key function to life is inseparable from the existence of proteins, or, at least of chemical compounds related to proteins.

Metabolism

Life is not static. Dormancy, that we find in the microbial spore or the plant seed, is an intermediary state between life and death. But it will only be associated to life when an organised set of dynamic processes, metabolism, starts to unfold. As its Greek name implies metabolism is a (chemical) state of flux. It drives the construction of molecules from smaller parts (anabolism) and the breakdown of the larger ones into smaller parts (catabolism), building up the individual components of the living machine, and the energy needed to run it. Metabolism follows a logic that accounts for the reason why a narrow subset of atoms and molecules has been retained [18]. To make a long story short, the atoms of life must both be abundant in the universe and form stable covalent bonds at 300 K in water. In order to carry as much information as possible the building blocks of life must be able to polymerise and form macromolecules. Again, this can be driven in water by an entropy increase, if a selection process retains the macromolecules in a specific compartment. Surface metabolism at the origin of life is perhaps the simplest way to harness this ubiquitous property of thermodynamics. Samuel Granick, very early on, remarked the important role of transition metals in biological processes. He further noticed that extant metabolism was organised around common minerals on which biosynthetic chains developed extending his view to an experimental approach [19]. Later on Wächtershäuser refined this view and proposed that iron–sulfur

centres were the organising minerals [20]. Despite some efforts, we still lack experiments, however, that would trigger a convincing scenario for a mineral origin of metabolism. This lack of experimental substantiation may be due to the fact that our present reflection on surface metabolism is driven by carbon chemistry, while the question of nitrogen availability, as discussed below, may be a central limitation to prebiotic scenarios. As a chemical constraint that must be accounted for, the building blocks of proteins, amino acids, do not make a random collection at all. A subset is found repeatedly in outer space (e.g., glycine, the smallest amino acid is even found in comets [21], and meteorites contain alanine and aspartate as well as many other common proteinogenic amino acids [22]).

Many other scenarios for prebiotic chemistry have been proposed. Most rest on the popular view of a prebiotic soup, which allows for the use of active gaseous molecules such as HCN or H_2S, further activated by UV light [23]. Continuous synthesis of ribose would be a difficult challenge to solve, and first studies described a possible scenario with arabinose aminooxazoline instead. A solution for the synthesis of ribose aminooxazoline was recently proposed by the same author and his colleagues [24]. However, while these syntheses may operate under relatively mild conditions with compounds from volcanic emanations, they still need to be complemented by an entropy-driven process favouring polymerisation. Alternating dry and wet episodes might provide an efficacious mechanism, but this involves surfaces in a straightforward way. Furthermore, it is still essential to associate prebiotic processes with selective steps that would retain only compounds that will evolve further into biomaterials. Surfaces, again, are a natural way forward. In summary, the most likely compounds that make the very first metabolic pathways are charged compounds with one to three carbon atoms, amino acids and a variety of peptides or related compounds, certainly not RNA [25].

Phosphates, with their remarkable metastable state in water were selected as surface attachment groups and first units involved in energy exchanges [26]. Alternating drying steps followed by rains or floods resulted in the condensation of phosphate moieties on many primeval compounds. These include serine as serine phosphate and aspartate protected against cyclisation as aspartyl phosphate (Figure 1). This created a collection of charged metabolites that would stick to surfaces and come in contact with each other, promoting a variety of reactions. The first stages of reproductive surface metabolism were prone to produce charged variants of peptides. Among the minerals that would carry over the first (iso)peptide-based metabolic pathways one finds iron–sulfur clusters (pyrite) [20] and polyphosphates [27]. Obviously, selected peptides would be part of the first prebiotic building blocks and compounds, exhibiting a range of promiscuous catalytic activities. This includes hydrolytic self-degradation (proteolysis). Interestingly, rather than working against the ubiquitous presence of polypeptides during early steps of metabolism, this activity opened up a complementary function, that of resisting proteolysis. This created an essential selective step that enriched metabolic pathways with a limited subset of stable active peptides and derived compounds. Finally, surface selection is prone to favour specific spatial shapes. Symmetry is an unstable condition with symmetry breaking the rule [28]. It had to be broken in the choice of amino acids for building polymers, exactly as we have to drive either on the right or on the left to prevent collisions or traffic jams. Any accidental local enrichment of a particular shape would be symmetry-breaking. This contingent pick is a straightforward explanation of the ubiquitous presence of one family of stereoisomers, L-amino acids, in proteins.

Remarkably, most coenzymes –necessary effectors of metabolism, the existence of which is a prerequisite for any plausible scenario of origin– are today synthesised from simple carbon molecules and amino acids. Among those, 4′-phosphopantetheine (cysteine condensed with pantothenate, a derivative of valine synthesis, and a phosphate as a charged group) has the remarkable role of a swinging arm transporting a variety of thioester substrates between sulfhydryl catalytic sites (Figure 2). It could well have been involved in its own synthesis as well as that of diverse compounds involving acyl groups (lipids, essential for compartmentalisation [29]), a variety of (iso)peptides as in the synthesis of fatty acids today, non-ribosomal peptides and polyketides [30]. The involvement of thioesters in a primitive metabolism, predating the systematic input of phosphate has been documented by Segré and co-workers in a convincing way [31]. Other coenzymes, possibly generated by such a swinging-arm thioester-dependent catalysis, may have been precursors of nucleotides, the essential building blocks of nucleic acids. As a matter of fact, extant biosynthesis of nucleotides (built on purine and pyrimidine carbon–nitrogen aromatic heterocycles) is based on the incorporation of amino acids in the core of nucleotide precursors. Pyrimidine nucleotide biosynthesis uses aspartate and combines together ubiquitous molecules, water, carbon dioxide, ammonium and phosphate (forming carbamoyl phosphate, also a precursor of arginine, an amino acid absent from the very first steps of prebiotic metabolism), while purine biosynthesis combines glycine and aspartate, together with phosphorylated derivatives of ribose.

These pathways open up a major chemical challenge. Ribose is a very unstable metabolite. Any scenario that advances nucleotides (and even more RNA) at the origin of life should be able to account for a steady synthesis of this molecule. In passing, this also argues fairly strongly against an origin involv-

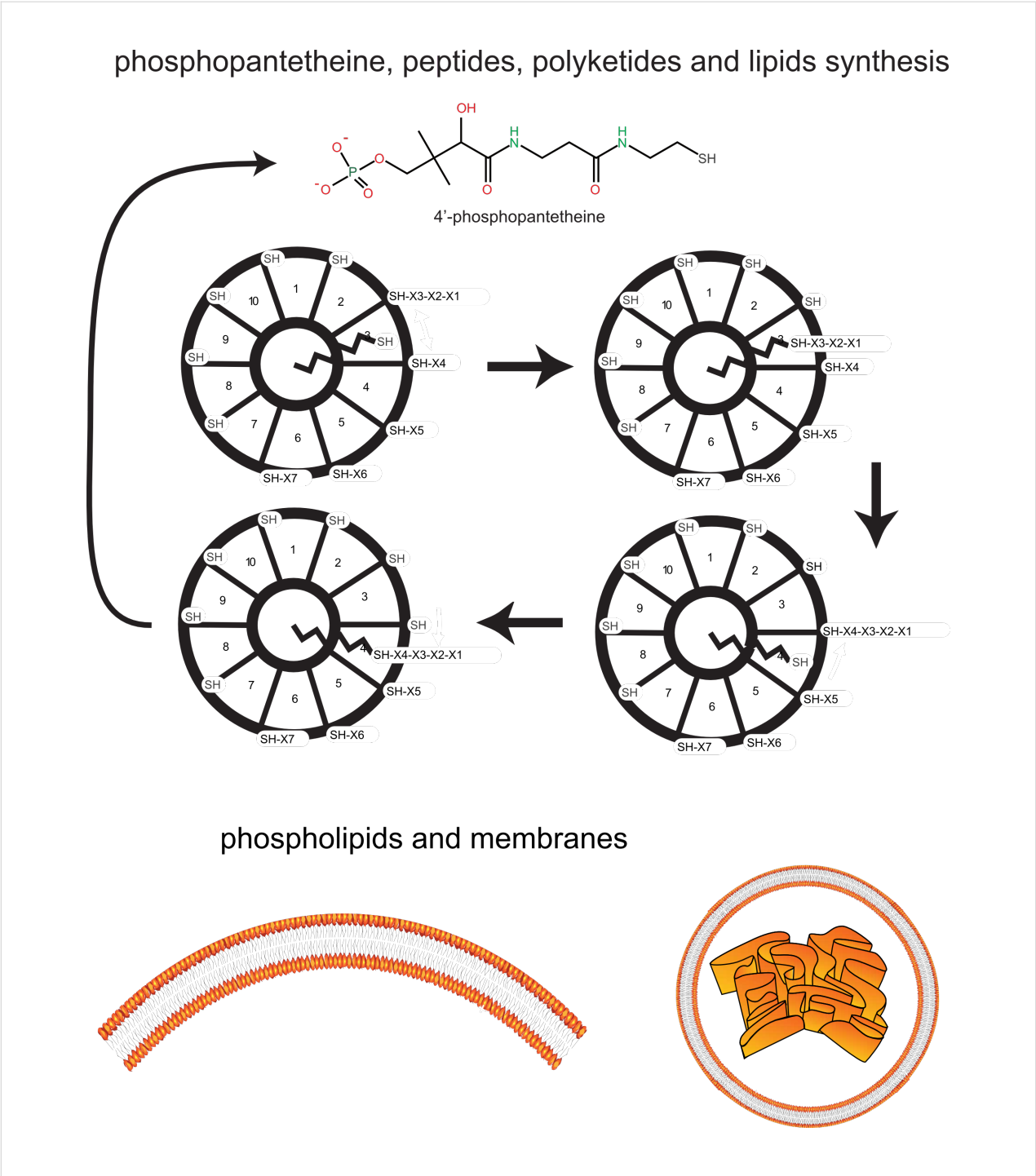


Figure 2: Building up membranes, peptides and co-enzymes. Thioester-based metabolism resulted in the synthesis of a variety of precursors of coenzymes (including 4′-phosphopantetheine as an isopeptide), lipids and peptides, via a swinging-arm catalytic engine.

ing hot temperatures, because heat considerably increases ribose instability [32]. Another argument for a late appearance of ribose is the following: Sugars involved in anabolism are essentially of the D-isomer type. This results from selective evolution involving competition with L-amino acids in early essential processes [33]. As a consequence, we can be fairly confident that ribose, and therefore nucleotides, appeared after an (iso)peptide-based metabolism was commonplace.

Cofactors such as pterins and riboflavin are ubiquitously present in living organisms. Precursors of these essential compounds may have been synthesised by a thioester swinging-arm path-

way and phosphorylated by polyphosphate. Remarkably, in living cells these pathways associate interconversions between purines and pyrimidines [34,35]. Furthermore, the nitrogen-rich intermediate 5-amino-6-(D-ribitylamino)uracil comprises building blocks that are commonly found under prebiotic conditions. Once phosphorylated (as discussed previously, via alternation of dry and wet conditions), this molecule would be reduced to a compound containing a 5-phosphoribosylamino group. Simple steps would finally condense formate in the presence of pyrophosphate, leading to phosphorylated guanosine, without requiring a prior synthesis of ribose. The only specific requirement would be that some catalysis allowed for a redox reaction (this is a general requirement of cell metabolism, involved in many metabolic steps, that is difficult, if not impossible, to fulfil using only RNA). As a consequence, primitive metabolic pathways would subsequently synthesise general precursors of nucleotides via phosphorolysis, allowing for both the synthesis of all nucleotides and the creation of a carbon metabolism derived from D-ribose-phosphate (Figure 3). This scenario is of course a bold conjecture but it illustrates how syntheses based on the activity of thioesters [36] and a surface-bound swinging arm may have produced a variety of metabolites.

Manipulation

In contrast to metabolism and compartmentalisation, manipulation and memorisation involve entities that are not small molecules but molecules made of thousands, millions, sometimes billions of atoms. These processes organise and rule the flow of information that is key to life. The synthesis of macromolecules requires an abundant supply of basic building blocks produced by metabolism. Up to this point, we have followed Dyson's reasoning. We have assumed that small-molecule metabolism progressively improved autocatalytic cycles producing and retaining a limited dictionary of building blocks enclosed in lipid vesicles made semi-permeable by peptides. These chemical reactions required functional catalytic power to handle substrates and reject products for further manipulation. The swinging-arm conjecture is a telling illustration of the way peptides and, later, proteins may be proficient in creating metabolic functions.

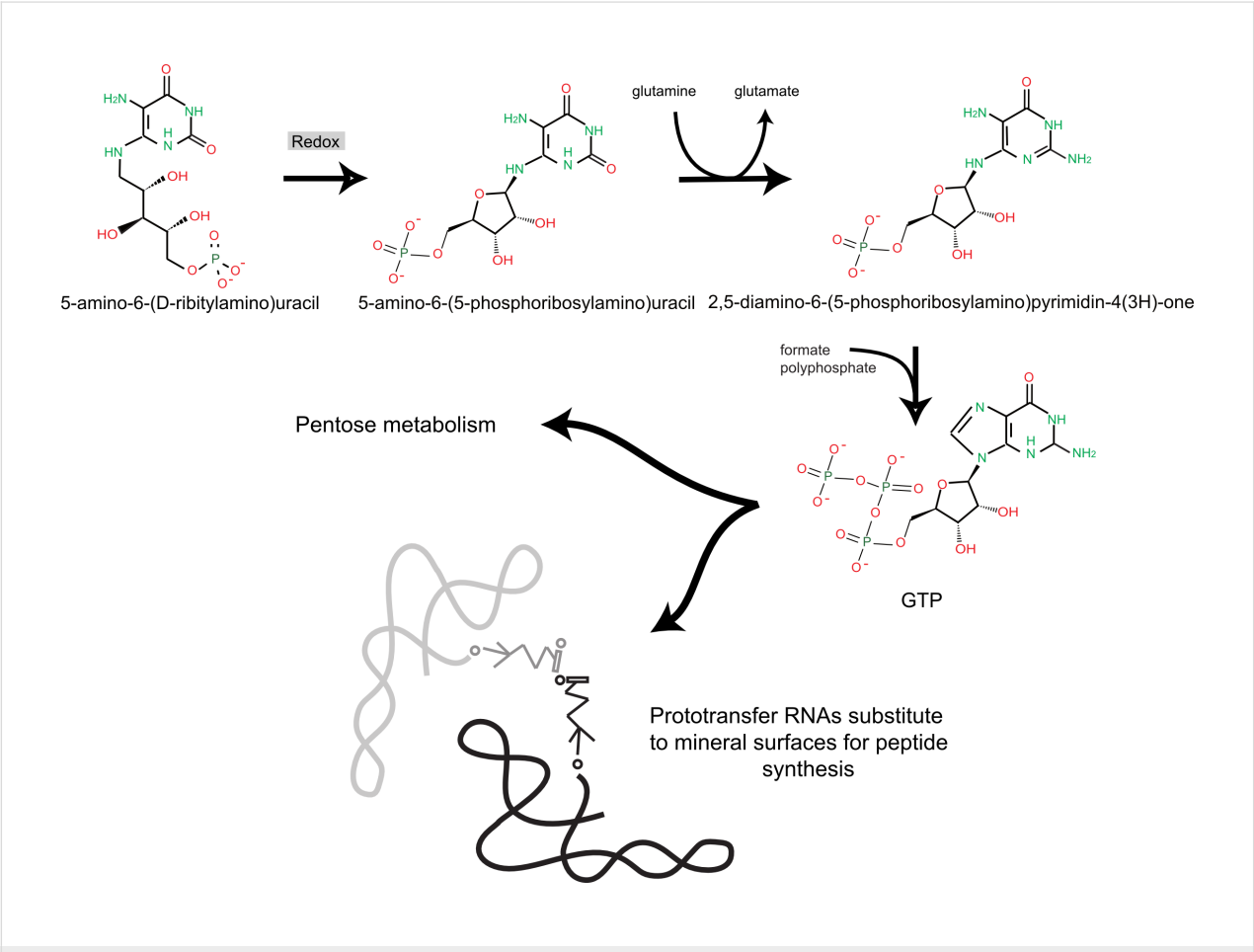


Figure 3: The RNA metabolism world. Among molecules built up by a swinging-arm thioester are pyrimidines coupled to reduced phosphocarbohydrates. This may lead to direct synthesis of nucleotides and later RNA metabolism coupled to ribose metabolism.

In order to fulfil their main functions, exploration of the environment and generation of a young progeny, living cells must display a huge variety of further actions that allow for the construction of cells, as well as transport across membranes, movement for exploration (including predation), protection against accidents and management of competition, but also repair, sensing and regulation. Almost all of these actions are operated by proteins in extant living organisms. A major question, therefore, was the understanding of the processes that made them come into being. Scenarios for the emergence of catalytic properties have been briefly outlined above as synthesis of peptides and coenzymes. Information management will be tackled later on when we consider the laws of complementarity and coding.

Memorisation

We now need to consider the process of memorisation that allows primitive cells to transmit to their progeny some of the information they collected as metabolism proceeded and evolved. A living organism is autonomous. To develop and survive it rests on the existence of some entity that is propagated from a generation to the subsequent one, a blueprint, a memory. This memory will perpetuate, as exactly as possible, the information that controls the birth and development of the organism, from generation to generation. An early level of memorisation is present in autocatalytic metabolic cycles, but, as noticed by Dyson, this is an unstable way to keep traces of past events [1]. A further memorisation step, at the origin of replication, must have followed the reproduction of metabolism. Concretely, in living cells the replicated substance of the blueprint memory of the cell is its genome, which is made of nucleic acids. Let us be guided by Dyson again and remark that, because this step is considerably more accurate than the fairly fuzzy reproduction of metabolic pathways, it must follow, not predate, the time when protein-based processes (the manipulation stage) emerged. In line with the Andromeda metaphor, in cells, the memory heritage is made of the chaining of nucleotides, summarised as a sequence of letters, similar to the words and sentences of an alphabetical text.

These processes, memorisation and manipulation, are tightly linked to two fundamental information managing laws, complementarity, accounting for the vertical transmission of memory, and coding, allowing for the correspondence between the carriers of the processes of memorisation and manipulation.

Managing information

Complementarity

In biology, complementarity is a feature of reality based on asymmetric shapes of molecules [37]. After discovering that only one 3D form of tartaric acid was present in the lees of wine Pasteur claimed “La dissymétrie, c’est la vie” (dissymmetry, this is life). Indeed, the carbon-based molecules of life are restricted to a subset of compounds with identical chemical structure but diverse 3D structures, selecting only a very limited panel of stereoisomers among those possible (for example, there are four isomers of the amino acid isoleucine, but only L-isoleucine is proteinogenic, while D-isoleucine and L- and D-*allo*-isoleucine are not). This ubiquitous dissymmetry is the basic level on which life manages information [38]. Asymmetry has an important consequence: It creates a set of highly stereospecific environments, leaving room for a particular complement, as described by Fisher in his lock-and-key image of enzyme catalysis [39], or in the widespread image of the antigen–antibody interactions during the immune response [40]. Complementarity illustrates a formal correspondence that may be used subsequently as a recognition signal. It manages information as signals in sensor–receptor interactions. This is exploited in living organisms in the way sensors monitor their environment. For example, there are receptors for taste with exquisite recognition of specific molecules, sugars for example. The sweet taste is triggered by a lock-and-key process in which sugars fit within a specific cavity of the receptor. This interaction can be mimicked by compounds that have nothing in common with sugars or with each other, such as the highly ”sweet” but completely unrelated proteins thaumatin and monellin [41]. Within cells, networks of protein interactions are mediated by rules following complementarity patterns that are yet to be discovered, but are central for the genetic or epigenetic build-up of functions after selective stabilisation [42].

A noteworthy case of the complementarity rule, possibly protein-related and associated to a duplication process, is widespread in eukaryotes. These cells consistently possess protein structures based on tubulin subunits, the centrioles, which undergo exact duplication in each generation. The process that drives this duplication of a protein structure is still a matter of speculation [43]. Centrioles are cylindrical protein complexes with a nine-fold symmetry that is broken with a very precise timing when cells prepare to produce a progeny. Following this symmetry-breaking event of yet unknown origin, a set of priming proteins attaches at a specific site to the outside of the parent centriole. It then progressively builds up, orthogonal to it, a pre-centriole which, once completed, will separate from the parent as a full blown centriole. This daughter organelle will then play the same role as that of the parent for organising chromosome distribution in the daughter cell. This structure is remarkable as it is apparently a protein-only structure that undergoes exact duplication. However, the parent structure is not used as a template for the daughter, as in nucleic acids, for example. In fact, the entity that is replicated is not a protein complex but an algorithm of construction. Hence, in this particular instance, replication is not a protein-replication system, nor

is it directly associated to nucleic acids used as templates. The algorithm that drives duplication of the centriole is a piece of information with delayed implementation, associated to a specific set of genes that are replicated when the cell reproduces.

Protein-network replication might have predated replication mediated by nucleic acids, via organisation of information mediated by the formation of protein complexes. However, direct peptide replication has not been observed in biology yet, although it has been demonstrated in artificial systems [44,45]. Complementarity is ubiquitous in protein interactions but varies extremely. The situation where complementarity is the most obvious feature of processes of life is that of interactions involving nucleic acids. In these molecules, complementarity, which leads to the famous double helical structure, is a straightforward consequence of steric rules between isosteric piles of pairs of purines and pyrimidines. This opened up the idea that a primitive coding process was at work during replication, with one strand of DNA entirely specifying the complementary strand. However, this first rule does not solve the riddle of the correspondence between the sequence of DNA and that of proteins, which requires a higher level of coding.

Coding

Coding is a case of organised complementarity used in a repetitive way. Because of its intrinsic asymmetry, any biological form creates, by default, the possibility for complementary interactions, opening up a recognition process similar to that using a code. A remarkable consequence is that this is an abstract way to create an association between matter and information, exactly as the integer "3" can be coded in a variety of languages (e.g., three, trois, τρια, Ξ). The one-to-one correspondence of complementary strands in nucleic acids was a straightforward coding process, but suggested that there could be a coding rule associating the DNA sequence with that of proteins in which amino acids are chained exactly as nucleotides are chained in nucleic acids.

In an astute analysis of the double-helix structure of DNA, George Gamow remarked that the possible diamond-shaped pockets in the 3D grooves of the double helix were of 20 different types, exactly matching the number of proteinogenic amino acids. Each pocket is defined by specific arrangements of the four nucleotide bases. This "diamond code" is made of 20 overlapping triplets suggesting that each amino acid in the corresponding polypeptide sequence is determined by a group of three bases in the corresponding section of the nucleic-acid chain [46]. However, the overlap between the sides of the consecutive pockets imposed an overlap in the corresponding coding nucleotides, telling that some sequences of amino acids should never be observed in proteins of biological origin. Yet, proteins in data libraries displayed such "impossible" sequences. This demonstrated that while one needs at least three bases to encode 20 amino acids (doublets of four nucleotides would code at most for 16 amino acids), the code is unlikely to be overlapping.

Later on, two major discoveries changed the picture and established the modern way to see how proteins are translated from their gene. It was found that the process required two code-dependent "rewriting" steps, a first step using the minimal one-to-one complementarity code between DNA and RNA nucleotides (transcription), followed by a machinery that operates in a way quite similar to that reading the tape of the Turing machine (translation). Nanomachines, the ribosomes, read contiguous (not overlapping) triplets of nucleotides (codons) in succession. To this aim they use specific transfer RNAs (tRNAs) loaded with amino acids, which use a possibly degenerate RNA complementary code (using the triplets that form anticodons) to establish the correspondence between the codons and each of the 20 amino acids.

Some triplets (for example, the four codons ACN code for threonine) are ambiguous, imposing that they are sometimes deciphered by different tRNAs. The consequence is that there are always more specific tRNAs than amino acids [47], although less than the 61 codons specifying the amino acids (three codons are used to mark the end of the gene coding region that has to be translated into a polypeptide) because the codon–anticodon interaction can use a relaxed complementarity rule. This situation led to a further coding requirement between a specific tRNA and its cognate amino acid (anticodon–amino acid correspondence). As in the case of complementarity in protein complexes there is no general rule establishing this correspondence. It is more or less ad hoc, obviously the result of historical events that governed the origin of the translation process [48]. Perhaps, if we follow a reasoning similar to that of Gamow, it emerged via direct interaction between each amino acid and a cognate anticodon [49].

This observation establishes that a more or less contingent sequence of events is at the origin of the way the genetic code emerged. It was based on the concomitant presence of amino acids and RNAs elicited by the local constraints of chemistry and geology. This simultaneity channelled information into the formation of the first living organisms.

From substrates to templates, RNAs at the origin of the genetic code

Among the many codes still to be discovered, the genetic code is at the heart of life. Having set the stage, we now can try to understand how such an abstract operation as that of the corre-

spondence between the sequence of DNA and that of proteins could have come to being. The most straightforward process would have been a direct interaction between amino acids and nucleic acids, as imagined by George Gamow. However, in the absence of any design, things could not develop in this intelligent way, but unfolded more slowly. The actual emergence of the genetic code required a succession of small steps involving progressive improvement of peptide-based metabolism. As expected from a stepwise development, this process created a fair number of anecdotal features that were consequences of purely historical events. This clarifies why the implementation of general abstract laws, such as those driving recursive gene expression, was systematically plagued with "illogical" (for the planning mind of an engineer) tracks, making biology fairly difficult to grasp for the unprepared mind.

The RNA-metabolism world

The reproduction of progressively more efficient metabolic pathways preceded the replication of nucleic acids (perhaps in parallel with the replication of proteins, as we saw with the centriole example). A key question is now to understand how both processes could be linked together, associating proteins and nucleic acids. What we discussed above can be summarised with the words of Monnard: "(1) The synthesis of RNA monomers is difficult; (2) efficient pathways for monomer polymerization into functional RNAs and their subsequent, sequence-specific replication remain elusive; and (3) the evolution of the RNA function towards cellular metabolism in isolation is questionable in view of the chemical mixtures expected on the early Earth" [50]. We have left our scenario of the origins of the first cells at a moment when peptides and nucleotides were present simultaneously in cell structures likely to associate an outside envelope and a variety of internal membranes supporting metabolism. Subjected to an alternation of dry and wet conditions ribonucleotides began to polymerise [51]. However, if not associated with other molecules, this polymerisation involved both free hydroxyl groups of ribose, resulting in a mixture of 2′,5′- and 3′,5′-phosphodiester bonds. By contrast, when peptides are present in the mixture, polymerisation is essentially happens through 3′,5′-phosphodiester bonds [52], stressing again the importance of peptides at the onset of prebiotic nucleic-acid chemistry.

At this point, RNA molecules with 3′,5′-bonds were formed. They are flexible molecules that explore the formation of double-stranded regions based on a relaxed complementarity code (A–U and G–C or G–U), forming stems and loops. This situation has long been investigated [53]. It is the basis of a considerable number of works about RNAs involved in a large number of functions, including catalytic activities (ribozymes). It can therefore be expected that primeval metabolism was developed at this point as a mixture of peptide- and RNA-mediated catalytic activities, within protocells. Because of their structures, RNA molecules could easily become substrates for metabolic reactions [54], progressively substituting the mineral surfaces that had been present at the onset of metabolism [34]. This defined the stage of the RNA-metabolism world.

Notably, the involvement of these RNA molecules in pre-translation metabolic processes is still prominent in a variety of metabolic reactions where tRNA molecules are definitely uncalled-for. This is the case for the pathway to an essential cofactor of electron transfers, heme (synthesis of aminolevulinate [55]), and above all, of membrane components such as aminoacyl phospholipids [56]. This is also consistent with the observation that some non-ribosomal syntheses of (iso)peptides are performed by enzymes highly related to class-I transfer RNA synthetases [57], in keeping with a simultaneous development of non-ribosomal protein synthesis and RNAs. Furthermore a variety of activated aminoacyl tRNAs are modified by homeotopic (or pre-translational) modification [34,58], reminiscent of what could be a role of tRNA as support of group transfer in early metabolic pathways. This includes asparaginyl, glutaminyl and selenocysteyl tRNA, as well as formylated methionyl tRNA for the initiation of translation.

All these observations can be considered as archives of past metabolism [54,59], with tRNA ancestors as key support molecules. Interestingly these processes must have started with molecules shorter than the ca. 76 nucleotide-long extant tRNA, which still display a variety of forms [60]. As a case in point, Hopfield remarked that tRNAs are probably the result of an early duplication, and that they could have been involved in the selection of amino acids interacting with the region that now forms the anticodon loop [49]. An interesting time line for the origin and evolution of tRNA has been proposed recently [61]. With ribozymes involved in the catalysis of peptide-bond formation, and primal tRNAs as handles carrying amino acids used in the process, an alternative or complement to the swinging-arm peptide synthesis would have evolved in parallel, with RNA-dependent peptide synthesis progressively taking the lead. At this point RNA molecules are substrates involved in metabolic pathways and in catalysis. In parallel, the complementarity law allowed for fuzzy pairing between RNA molecules (in particular G could pair with U in addition to pair with C). Ongoing polymerisation of ribonucleotides resulted in the emergence of a new function. Polypeptide synthesis used RNA substrates carrying amino acids and RNA ribozymes (the forerunner of the ribosomal RNA peptide centre) for peptide formation. Subsequently, another class of RNA molecules complementary to part of the tRNA ancestors carrying amino acids created a positive interference in this process that improved the

formation of peptides. This class of RNAs behaved as templates to order the amino acid residues of the peptides into a well-defined sequence.

The RNA-genome world

Accumulation of these latter "peptide sequence-specifying" templates of RNA sequences matching the peptide sequence via a coding process, asked for the synthesis of their exact copies, i.e., replication. This operation evolved from natural RNA catalytic activity [62] and progressively improved its autocatalytic reproduction by using increasingly more accurate complementarity rules (i.e., limiting the fuzzy complementarity rule used in specific peptide synthesis to standard A–U and G–C pairs). While this would perhaps also have been possible in a pure RNA environment, it was assisted by the same class of co-evolving molecules, the peptides that had favoured the formation of 3′,5′ over 2′,5′ bonds. Furthermore, peptides were also necessary for the machinery to help separating replicated strands, allowing for a further round of replication [63]. It can therefore be expected that RNA replicases evolved rapidly, in parallel with non-RNA-directed peptide synthesis.

In summary, these primitive enzymes associated an RNA molecule capable of catalysing peptide-bond formation (the ancestor of the ribosomal RNA peptidyl transferase centre) and the resulting protein functioning as an RNA-dependent RNA polymerase [64]. Today, and this is further evidence of early roles of tRNAs in RNA metabolism, viral RNA replicases still initiate replication using tRNA-like structure as primers, involving these molecules in yet another non translation-related function. Together with the previously discussed view of ancestor tRNAs as handles carrying over metabolic pathways, this supports the idea that these structures are archives of past RNA replication processes [65]. These replicases had to evolve in parallel with the synthesis and replication of ribosomal RNA. A variety of models involving ribozymes and introns of the group-1 family have been proposed to account for this parallel requirement [66]. This view of the RNA-genome world summarises in fact the widespread accepted view of the RNA world, which, in the absence of the idea of an RNA-metabolism world, obscures all the metabolic steps that would have been necessary for stable synthesis of the nucleotides essential for building up RNA [67].

The first cells and their descent

In the same way as coacervates can multiply compartments within a single entity [16], phospholipid vesicles form a variety of cell structures, involving vesicle engulfment [17]. It is therefore quite plausible that the RNA-metabolism and RNA-genome worlds were combined together within a single cellular entity, replete with membrane structures (Figure 1), that displayed a general tendency for a primitive form of phagocytosis [68]. While it is routine to think that smaller means less complex and more primitive, comparing the huge Electronic Numerical Integrator And Computer (ENIAC, 1945) with your cell phone tells you that this is a widely mistaken assumption. The saving of space, matter and energy tends to evolve toward miniaturisation, and highly evolved forms are often much smaller than their ancestors. This makes it likely that these primitive multicompartment cells were considerably bigger than most of the extant bacterial cells (although the variety of forms and sizes they can display is huge [69]), which are certainly highly evolved living organisms (in any event their progeny will survive on Earth for a time much longer than animals and plants will do).

Emergence of DNA and chromosomes

In the proposed scenario, the correspondence between proteins and RNA has been established, on a one-to-one basis. RNA templates specifying proteins, together with catalytic RNAs and transfer RNAs are also replicated by RNA replicases in an RNA-genome compartment. The machinery is still quite inaccurate and rapidly exploring a variety of sequence variants. The coordination between synthesis of internal (cytoplasmic) and membrane proteins is maintained by a network of membranes filling the cytoplasm of the cell. This is (at geological time scales) a rapidly evolving situation, fairly unstable because of the lability of the ribose moiety of nucleotides. This opened up the possibility of a selectively favourable metabolic pathway where ribose would be replaced by a much stabler counterpart, namely deoxyribose. This pathway, which is unlikely to be catalysed by ribozymes, is today performed by a family of enzymes, ribonucleotide reductases [70], followed by synthesis of the corresponding nucleic acid, DNA.

The emergence of deoxyribonucleotides extended the range of cell evolution with several new functions. In particular, RNA replicases had to evolve into two activities, DNA replicases and DNA-dependent RNA polymerases (transcription), because translation was RNA-based. This makes it likely that a process resulting in the concatenation of genes developed at the same time. Indeed, the correspondence of one nucleic acid gene with one protein introduced a competition between genes. This was unlikely to sustain stable reproduction of the cells because of an inevitable quantitative mismatch between the different wielding activities of the proteins (and RNAs). Resolving this issue required some regulation allowing for their concerted transcription. A strong selection pressure that allowed for the concomitant presence of genes in a cell led to fuse them together, forming primitive multigenic chromosomes. However, this resulted in a need for identification of gene starts (promoters) as well as of control elements. Located in the promoter region these elements did not need to be transcribed, although they

were replicated. The simplest way to account for their emergence is that they evolved from a combinatorial assortment of sequences of a common origin, allowing for the recognition by transcription factors that evolved in parallel. The consequence was that primitive chromosomes contained elements that were approximately repeated, thus allowing for the combinatorial association of transcription factors upstream of genes. This is the situation still witnessed in extant chromosomes of eukaryotes, but generally not in prokaryotes.

While it is important to ensure the propagation of a consistent set of genes, despite their likely huge difference in requirement as effectors of the cell metabolism, the formation of chromosomes required that the DNA replication is asymmetric, continuous on the leading strand and discontinuous on the complementary strand. It also required a machinery priming replication. Extant DNA polymerases still keep the memory of the fact that RNA preceded DNA in the initiation of replication as it remains triggered by RNA primers. The lack of homology of some of DNA polymerase constituents in the different domains of life suggests that their origin is fuzzy, with concomitant processes operating first simultaneously before the emergence of different lineages of species [71].

Like many chemical processes, replication is error-prone. This tends to produce a considerable number of mutations, leading to inactive products and sometimes “hopeful monsters” [72]. The lack of intrinsic accuracy led to proofreading and repair systems. Proofreading was ensured by the reversibility of strand elongation in the presence of pyrophosphate (and metabolic compartmentalisation of pyrophosphatase) and 3′-5′ exonuclease activity associated to the polymerases. There was also proofreading against the necessarily widespread accidental input of abundant ribonucleotides into DNA, as well as a need for mismatch repair [73]. The latter process required that the parent strand could be told from the daughter strand. As luck would have it, cytosine is unstable, as it tautomerises easily and is subsequently deaminated into uracil. This functional pressure resulted in the discovery of thymine, a DNA-specific, isosteric analog of uracil (discovered at least twice [74]). Indeed, uracil DNA glycosylase would take care of cytosine-related mutations, while the presence of uracil during replication would be used as a marker of the newly synthesised strand (when dUTP was used instead of dTTP) allowing the proofreading machinery to identify the correct strand when enabling mismatch repair.

Finally, the linear chromosomes must be synthesised in parallel with general metabolism, which developed in a 3D structure. This results in the need to make them longer than required by their strict protein-coding capacity. Another way out appears to have been via limitation of the availability of their nucleotide building blocks. Indeed, in all organisms that make de novo DNA synthesis, deoxyribonucleotides are synthesised using NDPs, not NTPs [75]. Because the concentration of NDPs is 10–100 times lower than that of NTPs the overall rate of DNA synthesis is maintained at a considerably lower level than most cytoplasmic components.

Escaping phagocytosis

The first cells must have associated together the progeny of an RNA-metabolism world (the ancestor of the cytoplasm with its internal membrane network) and an RNA-genome world (the ancestor of the nucleus). These protokaryotic cells explored the environment by developing engulfment processes. I have discussed elsewhere the consequence of phagocytosis: It immediately created a complementary function, that of evading phagocytosis [76]. This could be performed by at least two means, the formation of a complex engulfement-resistant envelope, or the formation of a proteolipidic cell membrane unable to fuse with that of the phagocyte. The former led to Bacteria, while the latter led to Archaea, with their membranes based on *sn*-glycerol-1-phosphate in the place of *sn*-glycerol-3-phosphate [33]. Bilayers made of mixtures of these molecules can form and are stable [77], but this is far from enough to permit functional proteolipid membrane fusion. To be sure, proteins embedded in lipid bilayers interact specifically with them [78], which constrains their ability to recognise other structures (asymmetry imposes mirror convergent evolution, see for an example the evolution of methionine sulfoxide reductases [79]). The consequence is that Archaea have envelope structures that drastically differ from those of Bacteria, and this is likely the reason for their lack of pathogenicity [80]. These escape routes allowed cells to begin to evolve toward miniaturisation, further evolving the process that had led to the formation of chromosomes, now grouping together genes with common functional associations and co-transcribing them together as operons [81]. These processes would be reflected in a stepwise evolution of the structure of proteins, as indeed observed by Caetano-Anollés and co-workers [82].

In parallel, the genome length got streamlined (remember again that DNA is linear, while size reduction goes with the third power of overall breadth), leading to a considerably dominant proportion of protein-coding genes. Furthermore, regulatory regions that were contiguous started overlapping, resulting in a progressive decrease of repeated regions. This is consistent with a common observation of genome sequences: At first sight, the genomes of eukaryotes look repeated (and therefore more primitive, with low algorithmic complexity [4]) whereas those of prokaryotes (Bacteria and Archaea alike) look random (with ”hidden” algorithmic complexity). This was however at a cost:

Superposition of control regions misses the rich combinatorial possibilities of contiguous control regions, which could be used to make multicellular organisms. Modern eukaryotes came to being when protokaryotes finally succeeded in engulfing some miniaturised bacteria that were later kept as symbionts, evolving into present day organelles (mitochondria and chloroplasts). This process is still ongoing, and visible in widespread symbionts co-evolving with a large number of organisms, often multicellular [83]. While eukaryotes maintained their somewhat repetitive control regions, using them to drive the fate of a rich dictionary of differentiated cells, prokaryotes (Bacteria and Archaea) could only display a very limited range of cell differentiation, remaining essentially unicellular while retaining a still very rich family of shapes [69].

Conclusion

At this point we have a scenario of the origin of the first cells. Admittedly, it is somewhat heterodox (many still consider bacteria as "primitive"), but consistent with what we know about the evolution of biological (and engineering) functions. This scenario is based on the accumulation of specific functional constraints, beginning with selection by surfaces of a subset of charged chemical compounds that react together, creating building blocks for future macromolecules, as well as coenzymes essential for catalysis. Among these molecules are the first nucleotides, which begin to polymerise, substitute charged surfaces as substrates for metabolism then, via nucleic-base complementarity, explore the role of template for coded peptide synthesis. In parallel, a rich network of membrane structures is emerging, allowing the cell to create electrochemical gradients that are used to drive exchanges between the cell and the environment, as well as processes of growth, fusion, fission and engulfement (Figure 1). After emergence of RNA replication, cells evolve via combining an RNA-metabolism world and an RNA-genome world [25,76].

Rapidly, these cells are stable enough to survive for a significant amount of time. This is enough to create a new challenge, that of ageing. Indeed all metabolites and macromolecules will change over time, simply because of their spatial and physicochemical constitution. We have already observed a consequence of this inevitable burden in the recruitment of deoxyribose and thymine, leading to DNA as a memory, compensating for instability of ribose and cytosine. In proteins, ageing is manifest in the ubiquitous spontaneous cyclisation of aspartate and asparagine residues [84]. The consequence is that cells progressively become bags of products of different age. In general (but not always, as the positive consequences of time-dependent maturation tells us) aged compounds will lack proper functional capacities. The cell will progressively become senescent and then die.

A way out is to create a progeny. However it is essential that this progeny is chiefly composed of young compounds. This creates a remarkable challenge: How can the cell keep old compounds in the parent, while the daughter cell will essentially be composed of young compounds? This question is reminiscent of the question tackled by James Clerk Maxwell when discussing his "Theory of Heat" [85]. How could we separate moving gas molecules according to their speed, if we could have an enclosure split into two compartments by a thin wall with an opening trap that could be opened or closed at will? Maxwell proposed that an intelligent being (later named Maxwell's demon) could measure the speed of incoming molecules and either open or close the trap, according to their measured speed. This process retained all fast molecules on one side (making it hot) and the slow one on the other side (making it cold). If this were possible, this would allow one to create a steam machine, and hence a perpetual movement, as it appeared that it could be possible to use such a demon without energy. This was discussed for decades until a fairly final demonstration by Rolf Landauer followed by Charles Bennett showed that acquiring memory (computing) indeed does not require energy, but that erasing memory will, so that the process is indeed energy consuming, precluding perpetual movement [86,87]. Apart from the trap mechanics, many other processes would settle the conundrum. Besides separating things according to their age into two compartments, another way would be, for example, to evolve specific devices (other types of Maxwell's demons) that patrol within the cell compartment, consistently interacting with molecules there (via a selective process of complementarity), and destroying those that have aged, then using ATP or GTP hydrolysis to reset their memory for another fruitful interaction. This latter way of coping with altered components of the cell has been shown to be consistent with the law that illustrates the probability of death in most living organisms, Gompertz law [88].

This requirement, making a young progeny, asks that cells provide the code for objects, likely proteins, operating as Maxwell's demons [89]. Notably, if living organisms code for Maxwell's demons that select and maintain cells in a way that accumulates information, these demons have highly specific families of targets, or are located spatially at precise sites. They cannot have any global grand design. Because these demons are only local they cannot directly organise the whole of a multicellular organism in a single step. This may explain why many organisms undergo metamorphoses, with specific stages, each one essential to promote the smooth unfolding of the next stage. This also explains why the final outcome of their activity is akin to tinkering, as François Jacob put it (making "kludges" might be a more appropriate word), and leads to the extraordinary diversity of life forms (that often look gratuitous). In some situ-

ations, however, physical constraints may restore some order in the outcome (for example spheres, tubes, the pythagorean/platonic regular polyedra of viral capsids, and more complex structures, such as phyllotaxis, the way leaves are distributed along a stem, or flowers within a composite inflorescence [90]). Yet these constraints, contrary to the great expectations of laypersons looking for evidence of design in living organisms, do not say much about what life is. They just provide borders within which information-rich physical systems (information gathering and utilising systems as named by Gell-Mann [91]) can explore reality.

The central feature of what is life is that of a specific way to manage information. The main problem with this general function is that it must be performed via a material set-up, putting together matter and energy in order to manipulate information. The consequence is that we must consider several quite different levels of description. There is a completely abstract level, that only considers the fate of information (this is the idea of Maxwell's demons in biology), and there is a series of more concrete levels that involve the machine, with its idiosyncrasies, that reads the genetic program. The latter involve the necessary constraints operating on matter and its coupling to energy in the set-up of life as we know it on Earth. This is where engineering has to be called for. All this is fairly similar to what happens when engineers construct computers. At the abstract level we have the Turing machine, so abstract that nothing is said about the innards of the machine. We have physical constraints operating on the global behaviour of the machine (management of heat in particular) and we have the many kludges of the explicit manifestation of a personal computer.

The main question we have to tackle, then, is to articulate the way we link a conceptual view of what life is, to experiments meant to make it in concrete terms. A large fraction of the design of what is a cell is now understood. This is what came out in the *Mycoplasma mycoides* JCVI Syn3.0 construct [92], after one has gone further than the original paper, with identification of much of the "unknown" functions [93]. There, we find a set-up of the Turing machine, with a concrete implementation of the reading heads of the program, the ribosomes. While the concept is well understood, there is not much latitude to modify what has been selected during the 3.5 billion years of evolution. It seems difficult, if not plainly impossible, to "re-invent" a ribosome, but we can study variations upon this theme. The same is true for replication and for a first level of cell division. However there remains an enigma that is amenable to experiments. How, in these constructs, is a young progeny created? It would be extremely interesting to see how the colonies formed with the JCVI Syn3.0 construct can be reproduced over many generations, perhaps by streaking them on plates of constant composition, to see whether this is at all possible (i.e., see whether or not there is a finite number of possible generations) and, if so, to see how the system evolved. We should remember that the first example of a similar construct, with a much smaller genome, and not of a living cell but of a bacteriophage (T7) evolved via erasing at least one third of the human construct [94].

If this experiment does not result in a progressive degeneracy of the genome, as doomed to happen if Muller's ratchet operates and drives an error catastrophe [95,96], exploring the genome after many generation will allow us to decipher how key functions in a minimal genome could lead to emerging novel functions. Functional analysis tells us that there is always an open door to a novel function [97], provided an existing structure is promiscous enough to allow that function to operate. Losing a function such as the protease that is required to maturate protein L27 in the ribosome of the Syn3.0 construct [93] might well recruit another endopeptidase for that particular function, for example. However, with this streamlined genome there is not much room left to trap the contextual information related to the process of evolution. This is exactly where gene duplication may come in [98], knowing in particular that selection pressure tends to increase the length of the chromosome to match the three-dimensional metabolism of the cytoplasm, as we have seen. A way out would be a spontaneous duplication of some or all of the genome, creating room for innovation. This apparently neutral process would in fact create novel information by allowing the cell to create a novel asymmetry, typical of what is needed for creation of information. We can suspect that a large number of sequences interpreted by many authors as "useless" in genomes [99] are in fact a way for those to prepare for the future.

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Towards open-ended evolution in self-replicating molecular systems

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Review

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Abstract

In this review we discuss systems of self-replicating molecules in the context of the origin of life and the synthesis of de novo life. One of the important aspects of life is the ability to reproduce and evolve continuously. In this review we consider some of the prerequisites for obtaining unbounded evolution of self-replicating molecules and describe some recent advances in this field. While evolution experiments involving self-replicating molecules have shown promising results, true open-ended evolution has not been realized so far. A full understanding of the requirements for open-ended evolution would provide a better understanding of how life could have emerged from molecular building blocks and what is needed to create a minimal form of life in the laboratory.

Introduction

Mankind has always pondered upon its own existence and has sought to understand the origin of life. This led us to trace back our roots, from the great apes to a last universal common ancestor, a simple cellular lifeform from which all other present-day organisms have descended. Ultimately this leads us to one of the great questions in science; how can life emerge from inanimate matter? And even more interestingly, can we achieve such a process in the lab and create life from scratch?

There are many different theories surrounding the origin of life and several attempts have been made to realize the synthesis of de novo life. All theories involve the presence of molecules that can create copies of themselves at some stage. It remains unclear whether such molecules were already important at the very early stages of the origin of life or whether life started with large autocatalytic networks [1] and specific molecules that store genetic information only appeared later. These self-replicating molecules carry hereditary information in the form of their molecular structure that can be passed on to successive generations. If mutations occur during the replication process, genetic information can change from one generation to the next. Natural selection can act on these variations, favoring those

varieties that are beneficial for the stability and reproduction of the replicator. Under the right conditions, such Darwinian type evolution can eventually lead to diversification and complexification of the molecules in the system.

This review aims to provide an insight into the historical background and recent developments in the field of in vitro evolution of self-replicating molecules. To do so, we will first cover a few important principles of Darwinian evolution and will show how these concepts apply to the case of molecular self-replication. This is followed by a description of some self-replicating systems and their properties, starting from the very first report on self-replication to more elaborate systems. Finally, some recent experiments concerning in vitro evolution of self-replicating molecules and networks will be discussed. We argue that, although systems that show intriguing evolutionary capabilities have been devised, there is still a long way to go before a system that is capable of true undirected or open-ended evolution has been realized. Worryingly, the phenomenon of open-ended evolution in itself is currently not well-defined nor understood. If we are to create life in the lab, a thorough knowledge of this concept and its prerequisites is probably essential.

Review

1 Requirements for Darwinian evolution

One of the most remarkable and key features of life is the fact that it has a strong tendency (or, at least ability) to diversify and increase in complexity. Whereas life once must have started out as a comparatively simple and primitive form, it has diversified into a vast variety of species ranging from aquatic to airborne ones. The principles governing this diversification in biological systems were already described by Darwin in his famous work On the Origin of Species, but are still not understood in full detail [2]. It was only in the 1960's that Spiegelman extended the scope of Darwinian evolution to chemical systems by studying the evolution of RNA-complexes [3]. In these experiments RNA was replicated using enzymes "borrowed" from contemporary biology. The outcome of the selection experiments was the shortening of the RNA sequence, as shorter sequences could be replicated faster. It was soon realized that a better understanding about how evolution acts on the molecular level would not only provide valuable insights into the origin of life and the emergence of species, but it could also pave the way towards the realization of synthetic life.

In biology, Darwinian evolution in a chemical system can be considered to be the result of an interplay of the three different processes that are summarized in Figure 1 [4]. These concepts can, in principle, be extended to what we will consider as Darwinian evolution in chemical systems. First the parent molecule, or replicator, is replicated to yield a large number of copies. This can for instance be achieved via an autocatalytic cycle, as will be discussed below. Mutation involves the emergence of a difference between the parent template and its copies. The accuracy of the replication process of DNA is generally safeguarded by sophisticated enzymes, but systems that lack such machinery are more prone to occasional errors during replication. Mutations, however, might actually be advantageous for the replicator if the newly formed copies are more stable, replicate more efficiently or prevail under a change of environment. If such advantageous mutations arise, competition between different replicators might occur, leading to a process of natural selection and survival of the fittest replicator. We consider replication, mutation and selection to be necessary and sufficient conditions for Darwinian evolution.

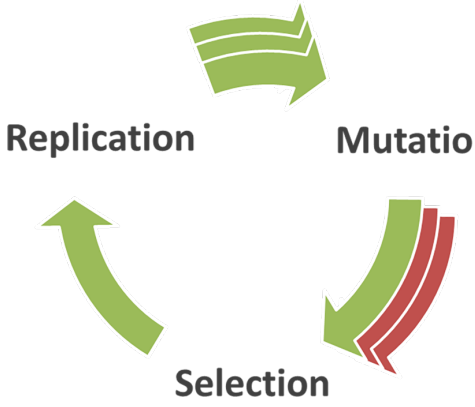


Figure 1: Three processes involved in Darwinian evolution. Species must be replicated to obtain a large population. During the replication process mutations can occur, on which natural or artificial selection can then take place.

1.1 Replication

During replication a large number of copies of the replicator is produced via an autocatalytic process. In animals or other lifeforms it is quite clear that reproduction leads to a transfer of genetic information from the parent to the offspring. Not only are the vital structures of the organism transferred but also some peculiarities like a specific eye color or a hereditary disease is passed on to the next generation. The transfer of information in replicating molecules may be less obvious, but if one considers a polymer with a specific sequence of subunits, it is clear that some form of genetic information is transferred if the copies have an identical sequence to the parent molecule.

The survival of a particular molecular structure under a set of environmental conditions depends on both the rate of replication and the rate of decomposition of this replicator. If a replicator decomposes at a higher rate than that it is produced, that particular replicator may become extinct. If, on the other hand,

the sequence and structure of the molecule is such that the replication rate exceeds its destruction rate, the replicator is sufficiently adapted to its environment and will persist under the given conditions.

1.2 Mutation

The environmental conditions, to which a set of replicators is exposed however, may not be in a steady-state. Instead, the environment may be continuously changing. Consider for instance changes in temperature, acidity, light intensity and humidity to which a system will inevitably be subjected. A replicator that is very well adapted to a certain environment, might not persist at a later time when the environmental conditions have changed. In fact, if it were not for the presence of small mutations in the genetic information, a species would be very fragile. If the number of mutations is only small compared to the length of the molecule, hereditary information is still largely preserved. Moreover, the mutants may be better adapted to the new environmental conditions than their predecessors. If the mutants indeed have a higher rate of accumulation than their parents, they will eventually overtake their parents and will become the new dominant species. In practice a replicator generally has to exhibit exponential growth in order to dominate over a weaker replicator [5-7].

Eigen et al. noted, however, that the case is somewhat more complicated than a single type of mutant replicator overtaking another replicator. They introduced the concept of quasi-species, as an analogue to conventional species in biology [8]. A quasi-species consists of a master sequence with a dynamic distribution of closely related mutants. This concept captures the fact that for relatively high mutation rates not a single fittest replicator, but rather a distribution of closely related mutants survives. The mutants in this distribution around a master sequence all replicate at a different rate and are cross-catalytic, which leads to the production of further mutants. Selection in these systems thus does not act on the level of individual mutants, but rather on the entire quasi-species [8-10]. Such quasi-species behavior was recently reported in an in vitro evolution experiment with replicating RNA species [11].

There is of course a constraint on the number of mutations that can occur without losing too much hereditary information from the parent molecules. In the same work, Eigen showed that unless mutation rates were significantly diminished, the increase in the length of the genome would unavoidably lead to a catastrophic loss of hereditary information. That is, the replication process of a long molecule requires a much higher fidelity than that of a smaller molecule. If the rate at which errors in the replication occur exceeds a certain error threshold, the genetic information will disintegrate and the species will go extinct [8,12]. In fact, the reason that viruses are so good in adapting to different environments and always seem to be one step ahead of the defense mechanisms of the host is because the replication process of the viral genome operates very close to the error threshold, allowing for as many mutations as possible without the loss of genetic information [12,13].

1.3 Selection

In biology natural selection operates on the phenotype, i.e., the observable traits of a species. An individual that is better adapted to its environment is more likely to survive then one that is less adapted. This higher survival rate will lead to a larger amount of offspring for that type of individual, favoring their presence in the population. The phenomenon of natural selection can also operate at the molecular level. This requires experiments to be run under conditions where replication and replicator destruction occur in parallel. Such conditions were employed in only a small subset of the work on self-replicating molecules where the emphasis has mostly been on replication in the absence of destruction. Which replicators then end up being selected depends on their rates of replication relative to their rate of destruction, or, as proposed by Pross, their dynamic kinetic stability [14]. Selection in the Darwinian sense requires extinction of the weaker replicators, so that only the fitter ones remain. There are some detailed kinetic considerations that lead to specific mechanistic requirements for the replication process. Szathmary and Lifson showed that in a scenario where different replicators compete for common building blocks, extinction of the weakest replicators occurs only if the kinetic order of the replicator in the replication process is at least equal to the order of the replicator in the destruction process [5,6]. As for most plausible mechanisms the destruction process is first order in replicator, this implies that the replication process must also be at the least first order in replicator; i.e., replicators need to be able to grow exponentially in order to exhibit Darwinian evolution in the most common scenarios. This consideration has spurred many efforts to develop exponential replicators, which are far from trivial to produce (vide infra). But even with exponential replicators, Darwinian evolution does not necessarily lead to complexification and the spontaneous emergence of new function, as the Spiegelman experiments made painfully clear [3].

Yet, in order to obtain a form of life from a molecular system, it must be able to grow increasingly complex and diverse. Systems that undergo such undirected diversification may in the end give rise to ecosystems full of complex organisms or structures [15]. Note that these organisms then would all be part of an evolving ecosystem, and it has been argued that a proper and complete description of life should therefore not only be at the individual level but also at the level of entire ecosystems [16].

1.4 Dynamic kinetic stability

Pross has introduced the useful concept of dynamic kinetic stability for describing the fate of systems in which replication and selection occur concurrently [14]. The idea is that the stability of a self-replicator in a system in which replication and destruction processes occur simultaneously is not determined by the thermodynamic stability of the replicator, nor by the rate of formation of the replicator alone, but by the balance between the rate of formation and the rate of destruction of the replicator. As either replication or destruction (or both) are typically coupled to other chemical reactions that convert high-energy reactants to low-energy products, replication in a replication/destruction regime should normally be chemically fueled. Such fueling, in principle, allows complexification of the replicator, without defying the second law of thermodynamics, as the system as a whole still evolves towards increasing entropy. With replicator complexification having been made feasible, it then only depends on evolutionary possibilities and benefits whether complexification also actually occurs.

In order for the considerations of dynamic kinetic stability to apply and in order for Darwinian evolution to occur, it is essential that replicators are subjected to a replication–destruction regime. Unfortunately, until now, very few systems reported in the literature are (see below).

1.5 Open-ended evolution

As mentioned before, the Darwinian triad of replication, selection, and mutation in itself is not sufficient to drive the complexification of a chemical system. But what determines whether a (chemical) system is capable of growing in complexity or is condemned to remain at a low level of complexity? This is a question that is not only relevant in evolutionary chemistry, but also has far-reaching consequences for the development of artificial life in computer models. As Moreno and Ruiz-Mirazo point out, in order for a system to fully evolve it should not only exhibit structural variety, but also some form of functional variety [17]. In the context of this review, we consider such function as any property of the replicator that benefits the dynamic kinetic stability of the system as a whole. A term that is widely used to describe the emergence of novel functionality is that of open-ended evolution. Although a clear consensus about a definition of open-ended evolution is lacking in literature, we will adopt the definition provided by Taylor here. Open-endedness means *the capability of components in a system to develop new forms continuously* [18]. From this definition it follows that a self-replicating system should be able to explore a huge number of possible mutants, otherwise the system will either get trapped in a stationary optimum situation or will recycle already explored forms of the replicator [19,20]. Both of these situations cannot lead to the continuous development of new forms of replicators and are thus detrimental to the open-endedness of the system. Another requirement is that the total structural space available to the system should exceed by many orders of magnitude the actual structural space that the system occupies at any one time, or as Maynard-Smith and Szathmáry put it; the replicators should possess unlimited heredity [21]. It is also important to note that newly evolved replicators are not necessarily more advanced or better than the original replicator. It is the mere development of novelty that is the vital aspect of open-ended evolution, causing it to be an undirected process that does not necessarily entail progress [18].

It is however not that trivial that a replicator can give rise to such a large number of new forms. As Crutchfield and Schuster pointed out, the dichotomy of genotype and phenotype is a powerful mechanism to obtain such a vast number of possible mutants [22]. Since mutations act on the genotype only and selection pressure exclusively acts on the phenotype, the two mechanisms are partially decoupled. If this were not the case only those mutations that are favored by selection will occur, strongly decreasing the possible number and randomness of new forms of the replicator.

It is apparent that open-ended evolution plays an important role in the emergence of novelty from simple replicators and that Darwinian evolution alone is an insufficient requirement for true unbounded evolution in a chemical system. This undirectional evolutionary process is therefore considered to be of importance in the transition from inanimate matter to life. The exact principles governing open-ended evolution are however not yet fully understood and it is not clear what the precise requirements for a system are in order for it to be capable of open-endedness [23].

In the following section we will discuss some basic principles of self-replication, followed by a discussion on recent developments towards the realization of open-ended evolution in chemistry.

2 Replicating systems

The most instructive and intuitive self-replicating system to consider is probably that of DNA. A DNA molecule consists of two strands of nucleotides that are intertwined to form a double helix. During the replication process of DNA, each of these strands can act as a template for the formation of a complementary strand. In this way an exact copy of the original structure of DNA is formed and the DNA has successfully become replicated. The replication of DNA however is a complex process mediated by enzymes such as DNA polymerase and topoisomerase. To better understand the origin of life and as a possible

first step in the synthesis of de novo life it would be very interesting indeed to achieve molecular replication without the need of such enzymes, since enzymes themselves must be products of an evolutionary process and can thus not explain the emergence of living systems from basic chemical building blocks. The following section will treat a representative selection of self-replicating systems, for a more comprehensive overview, see: Philp and Vidonne [24], Von Kiedrowski and Bag [25] and Bissette and Fletcher [26].

2.1 Minimal self-replicating system

The simplest form of a self-replicating system is that in which the replicator acts as a catalyst for its own formation from a set of basic building blocks. This fundamental form of a self-replicating system is depicted in Figure 2 and is called a minimal self-replicating system. An essential requirement for a minimal replicating system is that molecules **A** and **B** are complementary to template **T** so that they are able to bind to it via noncovalent interactions.

Figure 2 shows three different channels in a minimal replicating system. Building blocks **A** and **B** can react via the bimolecular reaction pathway, to form the template molecule **T**. In the second pathway – binary complex formation – **A** and **B** bind together reversibly to form a complex [**A·B**]. This complex may undergo a covalent reaction if **A** and **B** experience an increased effective molarity, leading to an inactive template $\mathbf{T_{inactive}}$ which is folded back onto itself. The third pathway in the minimal replication system is the autocatalytic cycle. In this cycle, the building blocks **A** and **B** bind reversibly to the complementary recognition sites on the template molecule **T**. This arrangement brings molecules **A** and **B** in close proximity, leading to an increased effective molarity and enhanced rate of bond formation. When **A** and **B** ligate to each other, a [**T·T**] complex is formed, which can then dissociate to yield two identical **T** molecules. The autocatalytic cycle thus leads to a replication of the original template molecule. The final, and often overlooked, pathway was identified by Reinhoudt et al. following a fierce discussion between Rebek and Menger about the mechanisms involved in the self-replication in their systems [27]. In this pathway (not depicted in the diagram) one of the building blocks, say **A**, binds to the template molecule. In certain systems this can lead to the activation of **A**, such that **B** can then react with **A** directly from solution.

Initially, when there are virtually no template molecules in the mixture but only building blocks **A** and **B**, the bimolecular and binary complex pathways leading to the formation of **T** and $\mathbf{T_{inactive}}$ will be dominant. Clearly, the inactive template cannot lead to autocatalysis and therefore hinders the self-replication process. Upon formation of **T**, the autocatalytic pathway will become increasingly important, in principle allowing for exponential growth of the template. A requirement for effective autocatalysis, however, is the dissociation of the [**T·T**] complex into two individual template molecules. If this complex does not dissociate, the newly formed template molecule cannot lead to further enhancement of the reaction rate, effectively arresting the autocatalytic cycle. Such product inhibition is an important limiting factor in many synthetic replicator systems and prevents them from attaining exponential growth.

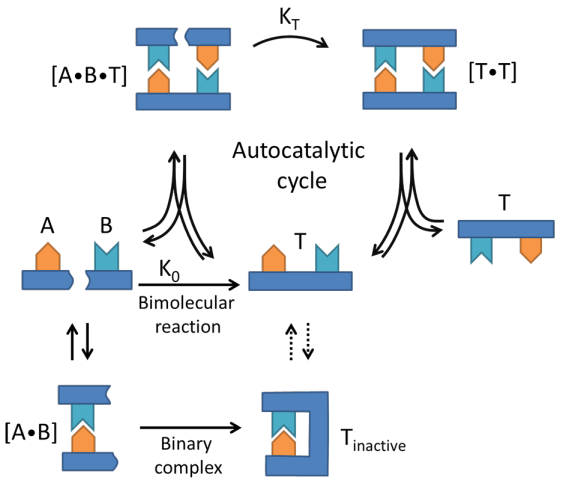


Figure 2: Minimal system for self-replication. Building blocks **A** and **B** can react to form either template **T** or its inactive counterpart $\mathbf{T_{inactive}}$. The formation of template **T** can direct **A** and **B** to a configuration in which they are in close proximity, accelerating the reaction between **A** and **B** leading to the formation of the [**T·T**] complex. Dissociation of this complex completes the replication cycle of the initial template molecule.

2.2 Reciprocal self-replicating system

A more complicated situation arises when the template molecules under consideration are no longer self-complementary, but instead are complimentary to a second template molecule. The replication of DNA is a prime example of such a reciprocal self-replicating system. One strand of the double helix acts as a template for the formation of the other complementary strand and vice versa. Figure 3 shows a schematic representation of a reciprocal replicating system. It consists of two catalytic cycles which both lead to the same template duplex [$\mathbf{T_{CD}}$·$\mathbf{T_{EF}}$]. Instead of only two building blocks the reciprocal system has four basic building blocks labeled **C**, **D**, **E** and **F**. Building blocks **C** and **D**, can react to form the template $\mathbf{T_{CD}}$ which catalyzes the formation of the complementary template $\mathbf{T_{EF}}$ from building blocks **E** and **F**. Similarly the $\mathbf{T_{EF}}$ template can promote the formation of the $\mathbf{T_{CD}}$ template.

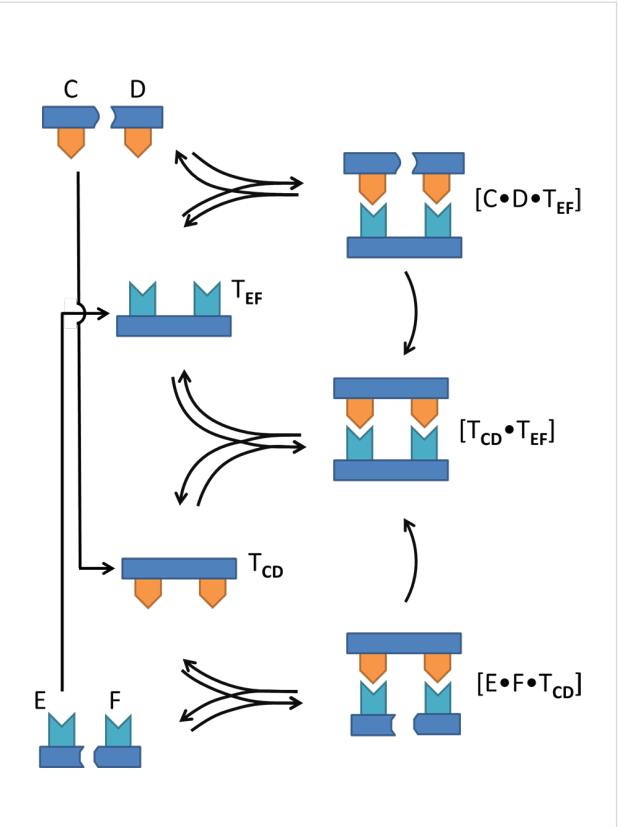


Figure 3: A cross-catalytic replication scheme in which the formation of one template stimulates the formation of a different, complementary template.

2.3 Reaction kinetics and its implications

When considering a mixture containing only building blocks **A** and **B** in the minimal replicator model (Figure 2), the formation of template molecules **T** can initially only take place via the bimolecular reaction pathway. The bimolecular reaction is a relatively slow reaction, since it involves the unassisted formation of a covalent bond between the two reactants. However, if a sufficiently large amount of the template molecules is formed, the autocatalytic cycle will play an increasingly dominant role. Because the catalytic cycle leads to a doubling of the template molecules after each run, an exponential increase in the concentration of the reaction product would be expected. Naturally, this exponential increase cannot continue indefinitely and will slow down as the concentration of available building blocks decreases. In summary, this would mean that for an idealized minimal self-replicating system the concentration of the reaction product **T** would show an S-like or sigmoidal shape.

A system in which product inhibition occurs, will not show exponential growth (for exponential growth the kinetic order in replicator $r = 1$) but only sub exponential growth. In many cases $r = 1/2$ and the system is said to obey the square root law of autocatalytic systems [28].

By seeding mixtures with different amounts of preformed templates **T** and measuring the initial rate of template formation, a plot of log(d[**T**]/dt) versus log[**T**] can be constructed. From the slope of this plot the reaction order r of the system can be determined [28]. It should however be considered that if the uncatalyzed bimolecular pathway ($r = 0$) also contributes to the formation of **T**, the measured reaction order r reflects a weighted average of the catalyzed and uncatalyzed pathways and can therefore have a value smaller than 1, even for cases where the autocatalytic pathway itself would have a reaction order $r = 1$. In such situations computational simulations of the system can provide additional information on the replication processes that are involved [29].

2.4 Achieving exponential replication

Pioneering work in the field of non-enzymatic self-replication has been performed by the group of von Kiedrowksi, who was the first to report on a template-directed self-replicating oligonucleotide (Figure 4) [30]. To achieve template-directed self-

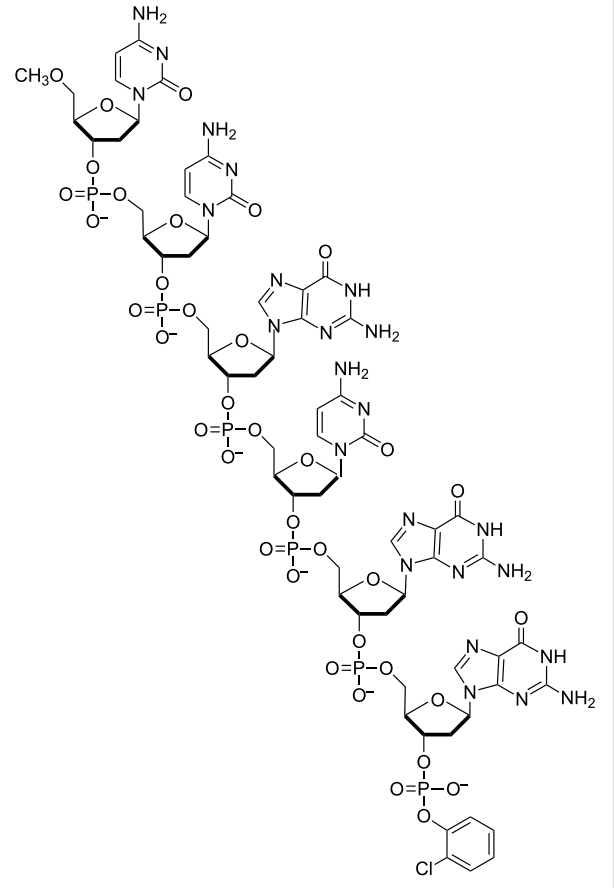


Figure 4: The first oligonucleotide capable of template directed self-replication without the need of enzymes. The depicted hexamer template **T** is formed from two trimer building blocks and catalyzes its own formation. Self-reproduction of this molecule was shown to result in parabolic growth of the template concentration [30].

replication without the aid of enzymes, they used two trinucleotides. Upon activation, these trinucleotides can condense to form a hexamer template molecule **T**, depicted in Figure 4, which catalyzes its own formation. The autocatalytic nature of the reaction was proven by adding small amounts of preformed template molecules to the reaction mixture. Kinetic analysis revealed that the system exhibits parabolic replication (p = 1/2). Exponential growth in this system is not obtained due to the high thermodynamic stability of the [**T**·**T**] dimer, leading to product inhibition. Although the efficiency of the reported autocatalytic cycle is rather low, it still was a clear demonstration of a template-directed self-replicating system and von Kiedrowski did not fail to recognize the potential of natural selection in such systems.

Later research focused on overcoming the product inhibition problem in order to obtain exponential instead of parabolic growth of the replicators. A successful approach to overcoming product inhibition involves the immobilization of the template molecules by fixing them onto a solid support. This approach was partially inspired by the notion that surfaces of minerals might have played a major role in catalyzing the formation of biopolymers [31,32]. Von Kiedrowski et al. were able to demonstrate exponential growth of oligonucleotides using a method that they gave the eloquent anagram; SPREAD (Surface-Promoted Replication and Exponential Amplification of DNA analogues) [33]. In the SPREAD technique, depicted in Figure 5, an oligonucleotide template strand is immobilized via an irreversible interaction with a solid support. A complementary strand is then produced via the template-directed binding of free nucleotides from the solution. The copied strand is released from the template and is in turn itself immobilized on a solid support, thereby preventing product inhibition via the formation of stable template dimers. As von Kiedrowski and coworkers rightfully notice, this system allows for evolutionary processes to take place. Moreover, such immobilized systems are proposed to be even capable of amplification of mutations. The introduction of mutations can lead to a weaker base pairing between the template molecule and its copy, thus increasing the efficiency of the separation of this particular template duplex.

Considering the proposed abundance of amino acids, it is natural to assume the presence of peptides and oligopeptides under prebiotic conditions. However, initially only very short peptides were produced in experiments under such conditions, raising doubts over their potential role as a precursor of life. When forming α-helices however, longer polypeptides can be stabilized by the formation of coiled-coil motifs as in Figure 6. If every a and d position of each individual helix is occupied by a hydrophobic amino acid, the helices can intertwine and bury their hydrophobic side groups into each other. This hydrophobic interaction that drives the formation of coiled-coil motifs can be further enhanced by electrostatic interactions between amino acids residing on the c and g positions of the α-helices.

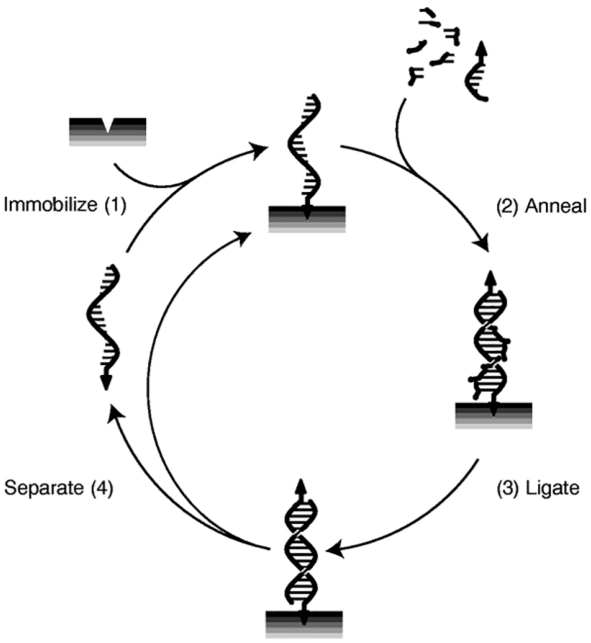


Figure 5: Replication involving the SPREAD technique which prevents product inhibition. (1) A template molecule is immobilized on a solid support and (2, 3) a complementary copy is produced by template-directed replication. Finally the copied strand is (4) released from the template and is in turn immobilized [33].

Ghadiri et al. showed that such coiled-coil peptides are capable of self-replication [35]. As depicted in Figure 7, helical polypeptides can act as a template for shorter peptide fragments by means of molecular recognition. The peptide building blocks again are ligated, resulting in the formation of a template duplex with a coiled-coil motif. When separated from the original template, a copy of the template is obtained. Initially these repli-

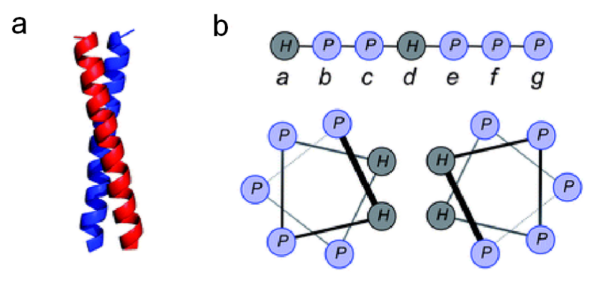


Figure 6: Figure showing (**a**) a coiled coil motif due to hydrophobic interactions between hydrophobic amino acids in the individual helices. (**b**) Helical-wheel diagram showing how the hydrophobic amino acids situated on the a and d sites can interact with each other to form the coiled coil [34].

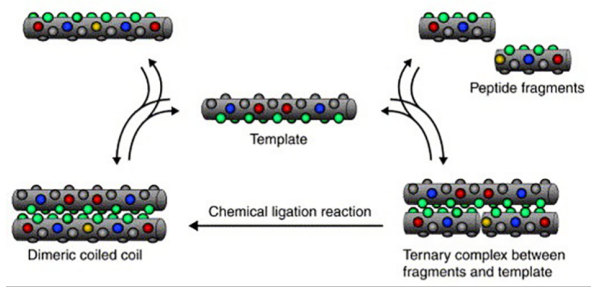


Figure 7: Self-replication of a helical peptide. Molecular recognition leads to the formation of a stable coiled coil structure from smaller peptide fragments. Depending on the length and stability of the coiled coil structure, the template-duplex dissociates in the original template and a copy [37].

cating systems were reported to show only parabolic growth, because of the very high stability of the coiled-coil structure. This problem was later addressed by Issac and coworkers by reducing the length of the template molecule, which led to a decreased stability of the template duplex. Using this approach they obtained near exponential growth of the template concentration of $p = 0{,}91$ [36,37]. The above examples all illustrate that, while not trivial, it is indeed possible to obtain self-replicating behavior in the absence of enzymes. While this marks a significant contribution to our understanding of the early stages of the transition from chemistry to biology, it does not directly explain the emergence of the RNA and DNA dominated world as we know it, which would probably have required open-ended evolution.

3 Evolutionary dynamics of replicators

3.1 Enzyme mediated replication

Iconic early experiments aiming to achieve Darwinian type evolution in a chemical system were performed by Spiegelman et al. in 1967 [3]. RNA replicase and a small input of genomic RNA were successfully isolated from the bacteriophage Qβ. The RNA molecules in this system are replicated by an RNA replicase enzyme. By successive rounds of amplification and selection, selection pressure was introduced to the system by favoring fast reproducing entities of the genomic RNA. Since shorter sequences are being replicated at a higher rate than longer sequences, shortened mutants are favored over longer sequences. This eventually led to a strong decrease in the genome size of the RNA molecules. However, this result is not as trivial as it may seem at first sight, since it is of vital importance that the mutant species do not lose their ability to be replicated, indicating that only specific parts of the genome that are not needed for recognition by the polymerase were deleted. Although the RNA molecules involved are not self-replicating but are replicated by the RNA replicase, the study still marks a starting point in the field of in vitro evolution. Later, Braun et al. managed to apply a selection pressure that favors the replication of long DNA sequences over short strands by creating heat gradients in pores that act as a thermal trap [38]. The thermal traps selectively retain longer DNA sequences, thereby effectively overcoming the inherent advantage of the replication of short sequences.

3.2 Dynamics of self-replicators

Ashkenasy recently reported a peptide based synthetic autocatalytic network that shows two significantly distinct steady states depending on the history of the system [39]. Depending on the initial concentration of replicator molecules provided to the system, the system will reach either a low or a high steady state replicator concentration. Switching between these two states can be achieved by applying external stimuli in the form of heat or the addition of decomposing agents. The switchable behavior and memory of such a self-replicating system constitute an exciting step, moving systems of self-replicating molecules away from equilibrium, with potential impact on evolutionary behavior [40].

Another interesting dynamic emergent property of self-replicating systems was demonstrated by Philp and coworkers [41]. They showed how self-replicating molecules can create a reaction-diffusion front when seeded to a homogeneous mixture of building blocks. Dynamically evolving out-of-equilibrium environments like these could enable interesting behavior of replicators that is not achievable in homogenous reaction mixtures. It will be very exciting to observe the evolutionary behavior of mixtures of replicators in such spatially resolved environments.

3.3 RNA self-replication

Owing to the importance of RNA in viral species and in the origin of life, evolution experiments are most often performed using RNA molecules or closely related derivatives. In fact, it has become possible to perform natural selection on oligonucleotides by iterative amplification and selection processes using a technique called systematic evolution of ligands by exponential enrichment, or SELEX. In SELEX, a library of DNA and RNA sequences is exposed to a certain target. In multiple selection rounds the binding species are selected and amplified, while the non-binding DNA and RNA molecules are disposed of. In this way molecules are evolved based on their ability to bind to a specific target [42,43].

However, these in vitro evolution experiments all exploit RNA-based enzymes (ribozymes) or proteins in their replication process to obtain exponential growth and are consequently not self-replicating. Efforts have been made to obtain in vitro evolution of RNA in the absence of any enzymes. Unfortunately, the demonstration of multiple cycles of non-enzymatic RNA repli-

cation in a test tube is troubled by the fact that the RNA duplex that is formed upon replication is quite stable and can have dissociation temperatures as high as 90 °C [44]. Without an enzyme that separates the newly created strands, this stability would lead to product inhibition, halting the self-replication process.

3.4 Cross-catalyzing RNA replicators

Joyce and Lincoln showed, however, that a system of two RNA enzymes can catalyze each other's synthesis from a mixture of four different building blocks via template-directed reciprocal replication [45]. The RNA ligase molecule **E** can bind two oligonucleotide building blocks **A'** and **B'** and promote their ligation to form the ligase **E'**. The newly formed ligase **E'** can then in turn promote the formation of **E**, as depicted in Figure 8a. But this cross-catalytic reaction typically occurs at a very slow rate. In order to enhance this rate, enzymatic in vitro evolution of the RNA molecules was performed in order to obtain a set of fast replicating species.

It was found from in vitro evolution experiments that the introduction of **G·U** base pairs close to the site of ligation leads to enhanced cross-catalytic activity. Figure 8b shows the sequence and secondary structure of the **A·B·E'** complex. The site of ligation is indicated by the curved arrow and the **G·U** pairs that are depicted in a solid box induce a wobble in the sequence that results in the enhanced catalytic activity. When this wobble is installed in both enzymes of the cross-catalytic set, exponen-

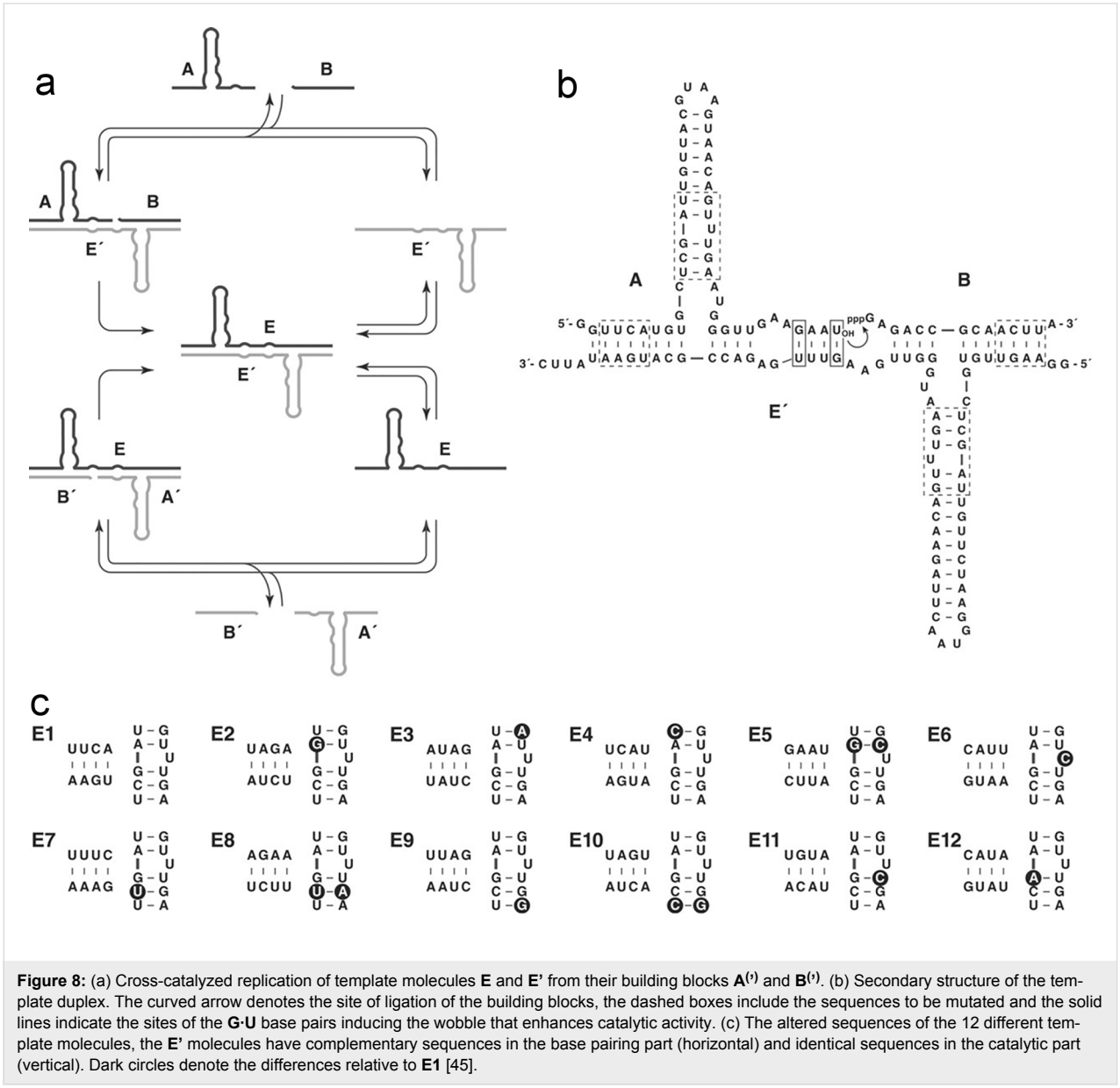


Figure 8: (a) Cross-catalyzed replication of template molecules **E** and **E'** from their building blocks **A**$^{(\prime)}$ and **B**$^{(\prime)}$. (b) Secondary structure of the template duplex. The curved arrow denotes the site of ligation of the building blocks, the dashed boxes include the sequences to be mutated and the solid lines indicate the sites of the **G·U** base pairs inducing the wobble that enhances catalytic activity. (c) The altered sequences of the 12 different template molecules, the **E'** molecules have complementary sequences in the base pairing part (horizontal) and identical sequences in the catalytic part (vertical). Dark circles denote the differences relative to **E1** [45].

tial growth of the system can be achieved over multiple cycles. With an exponential replicator in hand, Darwinian evolution in a cross-catalytic system lies within reach. To study this, Joyce and his team prepared 12 pairs of cross-catalytic enzymes and their corresponding building blocks, that have alteration in parts of the sequence denoted by the dashed line in Figure 8b. The different pairs are denoted as **E1** to **E12** and are shown in Figure 8c. It is important to note that mutations between enzymes are such that the stability of the ligase duplex due to base pairing is not altered, but only the catalytic activity and replication rate are affected. All these enzymes were shown to cross-replicate, with the **E1** pair showing the highest rate of replication.

A serial transfer experiment was performed on a mixture that contained the 12 different enzyme pairs and their corresponding 48 building blocks (**A1**, **A1'**, **B1**, and **B1'** for pair **E1**, for example). In such a serial transfer experiment a small percentage, in this case 5%, is transferred to a new reaction mixture after a replication round took place. This effectively eliminates the slow replicators that are only present in small quantities in the mixture so that they tend to go extinct. The transferred replicators, however, are presented with a fresh batch of building blocks and can continue to replicate. By doing this for multiple rounds, large amplification factors can be achieved. For this experiment it is important to realize that **A1** does not necessarily have to be ligated to **B1**, but that it can ligate to any of the other **B**-type building blocks, although they may be mismatched to the template. This freedom of recombination leads to 132 possible combinations of building blocks. After 20 successive transfers a 1025-fold amplification was reached. A sample of 100 of these clones contained only 7 non-recombinant clones, whereas the rest were all ligated to building blocks that were not their original partners. Figure 9 shows the distribution of different **E** (dark columns) and **E'** (light columns) enzymes in the final sample. This result shows how fitter replicators can come to dominate the population after several rounds of amplification. Fitness of the molecules depends in this case on their ability to perform cross-catalytic replication with other molecules.

This study by Joyce et al. demonstrates how selection pressure can lead to certain replicators dominating a population in a cross-catalytic replication process. However, the environmental conditions in this experiment are static and the system lacks open-endedness because the number of building blocks that is provided to the system restricts the total diversity of the newly formed species, in this case 12 × 12 different possible replicators. This will cause the system to reach a steady state in which no novel forms of the replicator can be explored anymore.

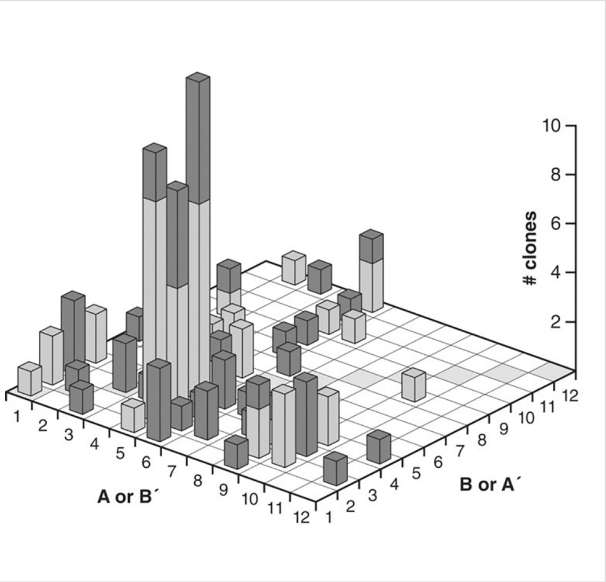


Figure 9: Distribution of the species present in the reaction mixture after 20 serial transfers. **E** and **E'** molecules are represented by the dark and light shaded bars, respectively. Note how certain species have come to dominate the population, particularly **A**5**B**3. Moreover, only 7 molecules were found to be paired to their original partner (corresponding to the shaded diagonal) [45].

3.5 Cooperative catalytic system

The concept of such a cross-replicating system can be readily extended to higher order systems, involving three, four or even more components. Eventually, one could envision an entire network of cross-replicating molecules. Lehman et al. showed that a mixture of relatively short RNA segments can self-assemble to form self-replicating ribozymes [46]. These ribozymes in turn gave rise to spontaneous formation of cooperative networks that were shown to grow faster than the autocatalytic replication rate of the individual ribozymes. Moreover, cooperative systems are generally more stable towards parasites then autocatalytic self-replicators and are, in principle, able to gain in complexity [46,47].

In the study a ribozyme of around 200 nucleotides called *Azoarcus* was used. This ribozyme is made from four different RNA strands (W, X, Y and Z) that can self-assemble covalently in an autocatalytic manner, as depicted in Figure 10a. The effectiveness of this self-replication process depends on the ability of the internal guide strand (IGS) to recognize its target. To form a cooperative set, the *Azoarcus* ribozyme was fragmented in two different pieces in three different ways, creating three different pairs I1, I2 and I3 which are shown encircled in Figure 10b. Furthermore, the target and IGS sequences were altered such that autocatalytic self-replication is minimized. The sequence was, however, chosen such that the IGS of one pair is matched to the target sites of the next pair. In this way one ribozyme, say **E1**, can catalyze the formation of the next

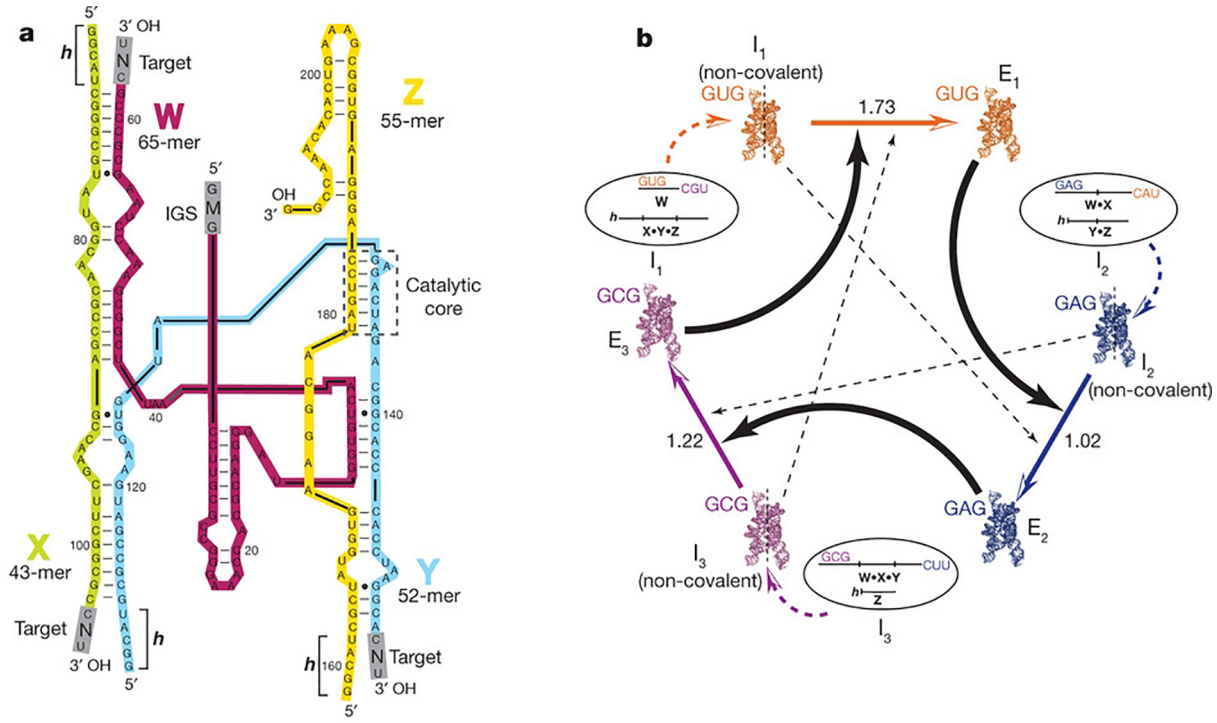


Figure 10: (a) Secondary structure of the *Azoarcus* ribozyme consisting of four different strands of RNA, W, X, Y, and Z. Self-replication is mediated by recognition of the target sites by the IGS (grey boxes), leading to ligation of the strands. The dashed line indicates the catalytic core of the resulting ribozyme. (b) Cooperative replicating system. The formation of the covalent ribozyme **E3** from the non-covalent **I3** complex is catalyzed by **E2** ribozyme. The formation of this **E2** is in turn catalyzed by **E1**, which is catalyzed by **E3**, resulting in a cyclic dependence. Numbers above the arrows denote the advantage of cooperativity [46].

ribozyme, **E2**, from its non-covalently bound building blocks **I2**. This ribozyme can in turn catalyze the formation of **E3** from its building block and finally, to close the cycle, **E3** can catalyze the formation of the **E1** ribozyme. This cooperative system is depicted in Figure 10b and it was observed that a mixture containing all three pairs resulted in a much higher yield of full-length RNA (a factor 125) than obtained from the sum of the isolated pairs, proving that the system replicates in a cooperative manner.

Interestingly, it was shown that in isolation the autocatalytic replicators (with the IGS programmed to recognize itself) replicated faster than the cross-catalytic system, whereas in a mixture of all different components the cooperative network grows faster than the selfishly replicating molecules. However, this result was obtained using deliberately designed pairs with specific targets. Behavior becomes a lot more fascinating when one of the nucleotides of the IGS (M) and target sites (N) is randomized, creating a mixture of 48 matched and unmatched pairs in total, as schematically depicted in Figure 11a. After incubation of all six sets of Figure 11a for several hours, all of these 48 possible sequences were indeed found in the mixture. Initially the replication is dominated by autocatalytic cycles in which N and M are complimentary. This initial rise of the autocatalytic replicators is depicted in Figure 11b by the dashed line with crosses, the contribution of the two-membered cycles is depicted by the dashed lines with dots (depicted value ×10). At later times a transition to the more complex three-membered cycles was observed as witnessed by the rise of the solid line (×10.000). After 8 hours, it was observed that replication occurs increasingly via cooperative cycles and that all genotypes contribute increasingly with time. This result shows how an initially autocatalytic cycle can give rise to increasingly complex systems of cooperative replication over time. Interestingly, the overall replication efficiency of the randomized multi-component network exceeded that of the engineered 3-component network in Figure 10b.

To better mimic prebiotic conditions in which iterations over multiple generations would have occurred, a serial transfer experiment using the same set of replicators was also performed. In this experiment an aliquot of the reaction mixture is transferred to a new flask with building blocks every hour, so that the more stable and fast replicating molecules and networks are favored. Again a transition from autocatalytic cycles to more complex systems was observed.

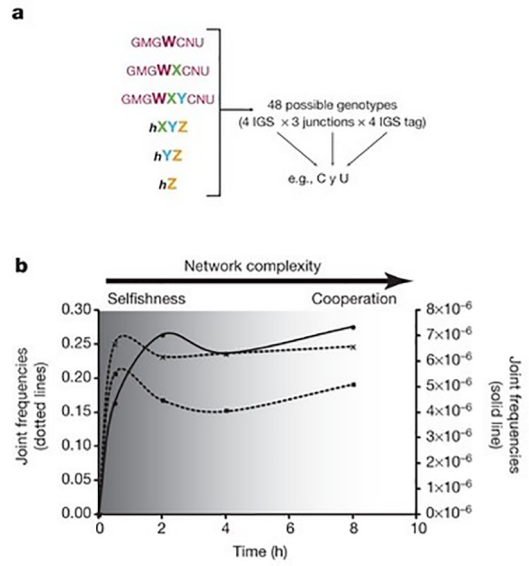


Figure 11: (a) The different combinations of IGS strands, tags and break junction give rise to a total of 48 different pairs. (b) Graph showing the frequency of autocatalytic replicators (dashed, crosses), the two-membered cycles (dashed, dots; ×10) and the three-membered cycles (solid; ×10.000) over time. Note the emergence of the more complex three-membered cycles at later times [46].

Such cooperative systems are capable of complexification and natural selection and can therefore be of importance in bridging the gap between replication of simple short RNA molecules from nucleotide building blocks and the formation of more complex ribozymes. The observed cooperative behavior relies on recognition strands and tags, so that it will only play a role for the assembly of intermediate-sized oligonucleotides. Small oligonucleotides would likely still replicate more efficiently via auto or cross catalytic cycles. At a certain length scale the formation of cooperative systems becomes favorable and these mechanisms might take over the replication process, allowing for complexification and diversification of the system. However, since the replication of each member is dependent on one or more other members of the system, the members should all be in close proximity to each other in order to obtain a stable system. This requires high concentrations of the reaction mixture, which is of course readily achieved in the laboratory but is probably less likely under prebiotic conditions. In order to increase the concentration of replicators locally, a specialized compartmentalization should act in concert with the cooperative replication system. How such compartmentalization might occur is another topic entirely and beyond the scope of this review, but it is proposed that compartmentalization can actually aid in the evolution of replicating molecules [48-50].

3.6 Diversification of self-replicators

Other types of molecules than RNA that are capable of self-replication and information storage are showing interesting results in the study of open-ended evolution and the synthesis of life as well [51]. Recently, we have demonstrated a self-replicating system involving peptides capable of diversification using a systems chemistry approach [52]. Following the discovery of an exponentially growing self-replicating system [53], we used two building blocks, **1** and **2**, to form a dynamic combinational library (DCL) of self-replicating molecules. These building blocks consist of an aromatic core that is functionalized with two thiol groups and a peptide chain (Figure 12a). Building block **1** and **2** are very closely related to each other and differ only in a single amino acid of the peptide chain. These peptide building blocks can then be oxidized to form macrocycles of different sizes as depicted in Figure 12b. The design of the peptide chains is such that self-assembly of the chains into parallel β-sheets is promoted, which in turn leads to the formation of stacks of macrocycles as shown in Figure 12c. Growth of these stacks occurs exclusively via the ends of the fibers and it is therefore not surprising that the reaction rate is strongly dependent on the amount of fibers present in the mixture. As soon as a fiber reaches a critical length it can fragment when mechanically agitated. When fragmentation occurs, the number of available fiber ends is doubled, leading to an exponential self-replication.

In previous work it was already shown that the less hydrophobic building block **2** tends to form larger octameric macrocycles than the more hydrophobic building block **1** which forms hexamers [54]. This is reasonable, since a weaker hydrophobic interaction provided by **2** would need more individual interactions in order to achieve the same stability as a more hydrophobic counterpart **1**.

By using a mixture containing two different building blocks instead of one, the replicators can potentially undergo mutation by incorporating a different building block into their structures. A mixture with equal concentrations of both building blocks was prepared and monitored over the course of 35 days. Initially a complex mixture of four different trimers and five different tetramers was observed. After some days, however, a set of hexamers which was enriched in building block **1** arose in the mixture (set I) as shown by the red line in Figure 13. As the emergence of set I depletes the mixture from building block **1** the environmental conditions are essentially changed up to the point where a second set of hexamers arises which is rich in building block **2**.

It was shown that set I is the ancestor of set II. When macrocycles that are rich in building block **2** are exposed at the fiber

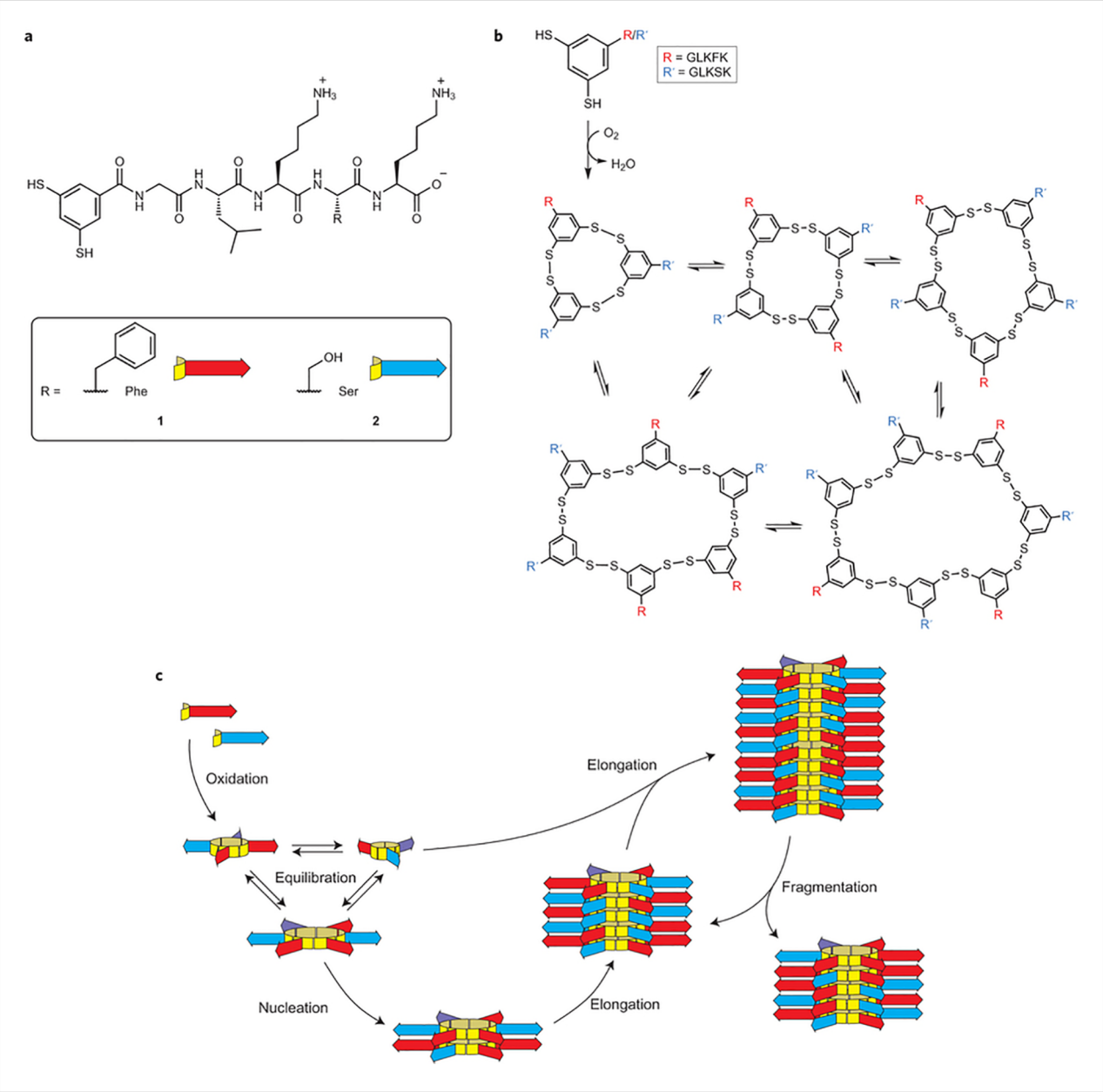


Figure 12: Figure depicting (a) building blocks consisting of a peptide attached to an aromatic ring. Building blocks **1** and **2** differ only in the nature of the penultimate amino acid. (b) The building blocks can form macrocycles of different sizes upon oxidation, which can exchange building blocks with each other. (c) Schematic representation showing how building blocks oxidize to form macrocycles that in turn form stacks due to β-sheet formation. Stacks grow from their ends and fragment upon agitation, leading to more fiber ends and faster growth [52].

ends of set I, they act as a template for the formation of members of set II. Indeed, significant amounts of set II members only form when a seed of set I is present that contains **2**-enriched members. Set I is therefore able to transfer information about its macrocyclic size to set II. This process bears a crude resemblance to how species originate in biology.

Conclusion

Self-replicating molecules have been remarkably hard to develop and after 30 years of research there are still only a handful of efficient self-replicators. Achieving Darwinian evolution with these systems has proven even more challenging. The evolutionary potential of many self-replicating molecules is limited due to the fact that it is difficult to achieve exponential growth of the replicator. Factors limiting the efficiency of the self-replication process are the presence of non-autocatalytic pathways and product inhibition. Methods aiming to minimize the effect of product inhibition, like the SPREAD technique and the destabilization of template-duplexes, have successfully been developed to allow for exponential growth of some simple

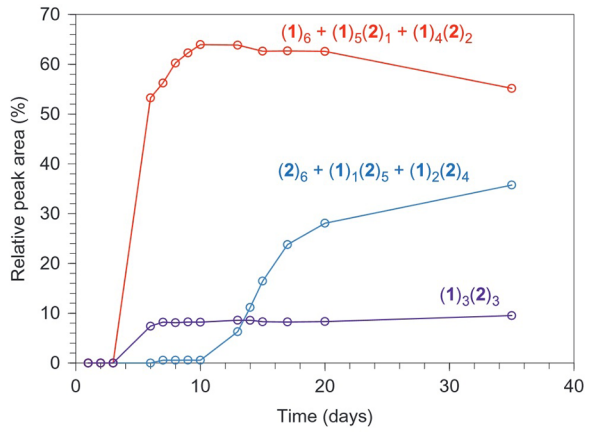


Figure 13: Plot showing the relative concentrations of set I (red), set II (blue) and the link between them; $(1)_3(2)_3$ (purple). Note how at first only set I is present in the mixture, while at some point this set gives rise to the descendant set II. [52]

replicators. Also mechanical forces may be utilized to break up larger assemblies of self-replicating molecules and liberate the assembly edges or fiber ends that promote replication.

The most impressive progress with respect to Darwinian evolution has been achieved with RNA-based cross-replicators. In serial transfer experiments changes in replicator populations were observed that were not immediately predictable and that favored the most efficient replicators or networks of cooperating replicators. What these systems have not (yet) shown is the emergence of new functions that contribute to the dynamic kinetic stability of the replicators.

The true challenge of any in vitro evolution experiment lies in the realization of a system that has the capability to undergo open-ended evolution. Such systems can diversify and increase in complexity and invent new functions indefinitely. Until now, chemical systems that show evolutionary behavior have involved relatively simple replicators that only had access to a very limited structural space of possible mutations. This rapidly causes the system to be incapable of exploring new structures and the development of novelty will stagnate. An additional limitation of simple replicators is the strong relation between their genotype and phenotype. This lack of dichotomy causes the mechanisms of mutation and natural selection to couple to each another, hampering the evolvability of the systems. It is far from trivial to design a system that is simple enough to be capable of exponential replication and has a large structural space of mutations at the same time. Yet a push in this direction is probably needed, expanding the structural space available for existing replicators to explore, enabling them to discover new functions, one of which might eventually be the decoupling between genotype and phenotype, which would allow the system to explore a dramatically larger structural and functional space.

Besides these issues concerning the design of replicators, it is still not studied in detail how the environment of the replicators can interact with the evolutionary process. Can environmental conditions like acidity or temperature, for instance, be an incentive towards the development of novel functionalities in the replicators? And how is the notion of death introduced in an experiment in which the researcher does not actively intervene with the system through, for example, serial dilution? In any true open-ended system replicators interact with the environment on their own account and are not steered by the experimenter to a significant extent.

Thus, the challenge is now to design systems of self- or cross-replicating molecules that can access and evolve into a vast structural and functional space and facilitate, by appropriate design of building blocks and experimental conditions, the invention of new functions and thereby achieve open-ended evolution.

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BEILSTEIN JOURNAL OF ORGANIC CHEMISTRY

Biomimetic molecular design tools that learn, evolve, and adapt

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Review

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Abstract

A dominant hallmark of living systems is their ability to adapt to changes in the environment by learning and evolving. Nature does this so superbly that intensive research efforts are now attempting to mimic biological processes. Initially this biomimicry involved developing synthetic methods to generate complex bioactive natural products. Recent work is attempting to understand how molecular machines operate so their principles can be copied, and learning how to employ biomimetic evolution and learning methods to solve complex problems in science, medicine and engineering. Automation, robotics, artificial intelligence, and evolutionary algorithms are now converging to generate what might broadly be called in silico-based adaptive evolution of materials. These methods are being applied to organic chemistry to systematize reactions, create synthesis robots to carry out unit operations, and to devise closed loop flow self-optimizing chemical synthesis systems. Most scientific innovations and technologies pass through the well-known "S curve", with slow beginning, an almost exponential growth in capability, and a stable applications period. Adaptive, evolving, machine learning-based molecular design and optimization methods are approaching the period of very rapid growth and their impact is already being described as potentially disruptive. This paper describes new developments in biomimetic adaptive, evolving, learning computational molecular design methods and their potential impacts in chemistry, engineering, and medicine.

Introduction

There is still not a clear understanding of how 'life' emerges from 'non-life'. One definition of life (NASA) is "A self-sustaining chemical system capable of Darwinian evolution" [1]. Clearly all living things in our world are complex and extremely organized. They are, or contain components that are self-organized, requiring input of energy and matter from the

environment and using it to sustain self-organized states, enabling for growth and reproduction. Living creatures must maintain their internal states (homeostasis) but, conspicuously, must also respond to their surroundings, fostering a reaction-like motion, recoil and, in advanced forms, learning (feature recognition). As life is by definition reproductive, a mechanism for copying is also essential for indefinite existence, and for evolution to act through mutation and natural selection on a population of related individuals.

Increasingly, some of these essential operations and characteristics of living entities can now be simulated in silico and in the laboratory. We are now experiencing another type of evolution, driven by human intellect, that is modifying the way life evolves now and in the future. Figure 1 illustrates how modification and adaptation of organisms, initially arising from natural processes, is now being supplanted increasingly by intentional, precision genetic manipulations, and in the future by a greatly increased understanding of what constitutes a living system, spawning in silico, artificial intelligence processes [1].

Living versus synthetic systems

Living systems adapt to changes in the environment by learning and evolving. Nature achieves this so effectively that much contemporary research now aims to understand and mimic biological processes. Historically, biomimicry in chemistry involved learning from Nature by exploiting and synthesizing bioactive natural products as drugs, for example (Figure 2). Contemporary research aims to elucidate how molecular machines self-assemble, and to discover the mechanisms by which they operate, thereby providing a template for the rational, intentional design of useful molecular machines at the nanoscale [2].

Intensive experimental effort has been applied to the deliberate reengineering of biosynthetic pathways for natural product synthesis which, when combined with directed evolution, can generate libraries of potentially bioactive organic molecules with significant diversity and high chemical complexity [4].

Concurrently, biomimetic computational evolution, feature identification, and learning methods are being developed to solve complex problems in science, medicine and engineering. Many of these new and very useful metaheuristic methods, such as ant colony optimization, agent-based, evolutionary [5,6], and particle swarm algorithms, are indeed inspired by solutions that Nature has evolved to solve difficult problems [7]. We are also beginning to understand how to create artificial self-organized systems (reliant on the continuous input of matter and energy) that are ubiquitous in the natural world rather than the self-assembled systems that have been a major feature of contemporary nanotechnology [8-10]. Computational adaptive, evolving, self-learning design and optimization methods are approaching an era of very rapid growth, and their impact is already being seen as potentially disruptive. Their application to chemistry, particularly synthetic chemistry, is still at an embryonic stage but they have the potential to generate rapid paradigm changes in the short to medium term.

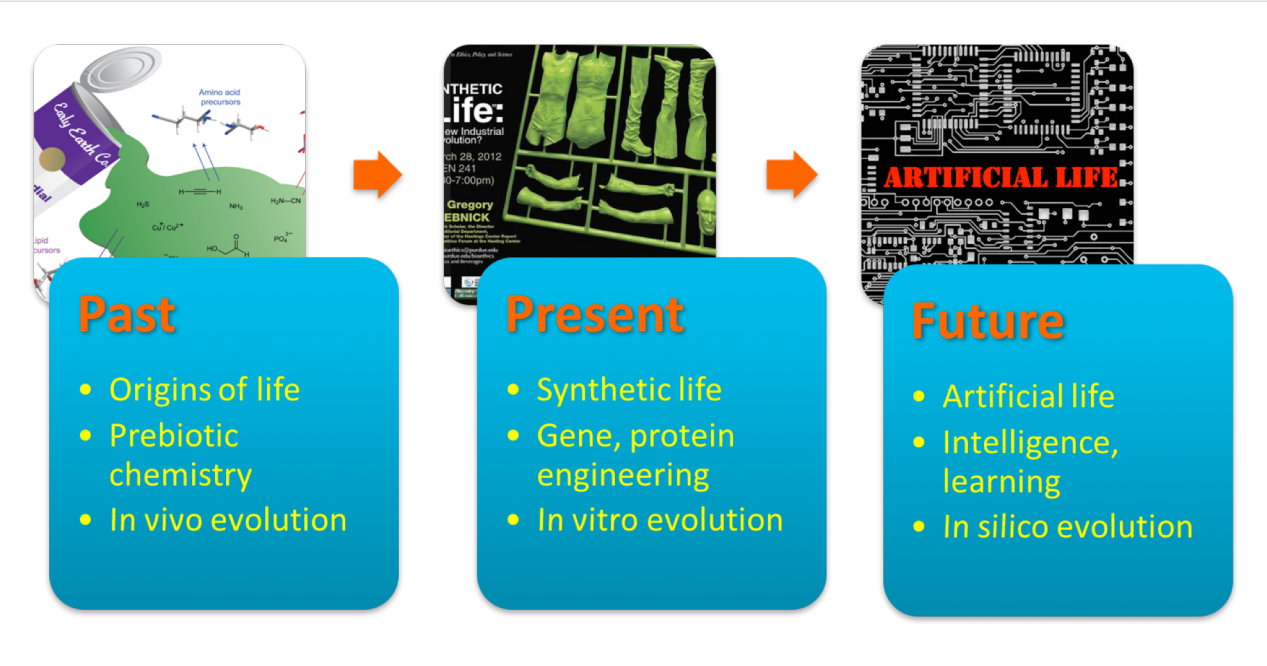


Figure 1: Hypothesized evolution of 'life' and 'intelligence'.

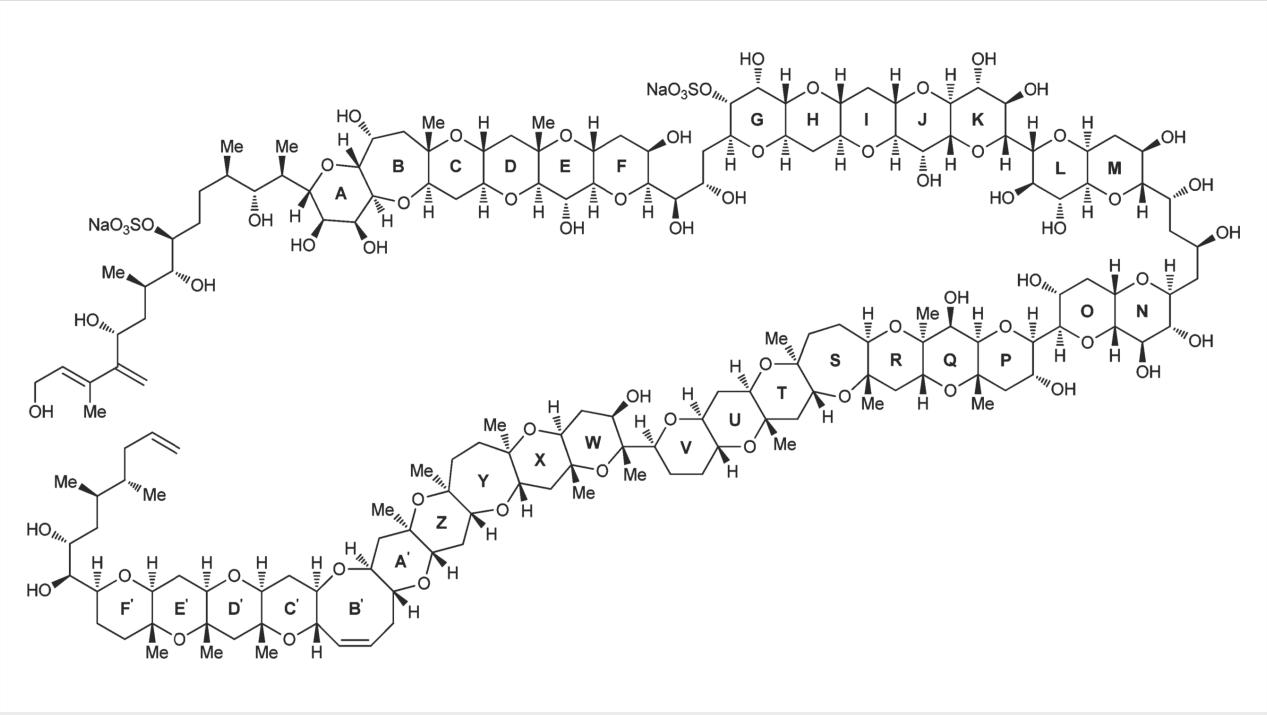


Figure 2: Structure of maitotoxin, one of the most complex natural products ever tackled by total synthesis. Reprinted with permission from [3]; copyright 2014 American Chemical Society.

This perspective paper provides a brief overview of these methods for chemists who may wish to understand their current and future impact. It introduces the most common type of algorithm, machine learning. A discussion of a very useful machine-learning algorithm, the neural network follows, and problems that often arise in their use, and solutions to these difficulties described. A new type of deep learning neural network algorithm is then discussed and its performance compared to traditional 'shallow' neural networks is described in the context of mathematical theorem governing the performance of neural networks. The paper then discusses another very important concept in life and in silico learning, feature selection. Biomimetic in silico evolutionary methods and their synergy with high throughput materials synthesis technologies (materials defined very broadly) are then briefly described. Finally, all of these concepts are combined in the discussion of new adaptive, learning in silico evolutionary methods for the discovery of new bioactive molecules and materials, with examples.

Review

Open questions in artificial intelligence (AI)

Before describing these AI methods and how they can be used in chemistry, biology and elsewhere, it is instructive to consider some of the "big picture" questions of the AI field. Among the many open questions relating to artificial intelligence, the most pertinent to this paper relate to how life is connected to mind, machines, and culture [11]:

- Demonstrating emergence of intelligence and mind in an artificial living system.
- Evaluating the influence of machines on the next major evolutionary transition of life.
- Establishing ethical principles for artificial life.

Development of advanced computational AI methods is likely to cause social disruption in the next two decades but they should bring unprecedented benefits, such as improved medical diagnostics, and cheaper more efficient services [12]. These benefits are not without risk, as most strongly disruptive technologies have demonstrated to date. Apart from possible social and employment upheaval, some technology leaders have cautioned about other major detrimental outcomes if AI systems are developed and implemented without sufficient thought and constraints [13,14]. Like all powerful scientific discoveries and technologies, care must be taken to ensure that their very considerable benefits are captured, and their possible misuse minimized.

Machine learning and artificial intelligence

Among the myriad of AI methods developed to date, one of the most useful and topical methods is machine learning. Machine learning algorithms are a family of computational methods that find relationships between objects (e.g., molecules, materials, people) and a useful property of these objects (e.g., biological activity, melting point, hardness, credit worthiness etc.). They

include artificial neural networks, decision trees and several other types of biologically inspired computational algorithms. They have been applied to most areas of science and technology and have made important contributions to chemistry and related molecular and biological sciences. For example, they have recently been applied to predicting the feasibility of chemical reactions by learning relationships between the molecular properties of the reaction partners and the outcomes of the reactions in a large database [15]. Another recent example is the robot scientists Adam and Eve that automate drug development via cycles of quantitative structure–activity relationship (QSAR) learning and biological testing (Figure 3) [16-18]. Eve's selection of compounds was more cost efficient than standard drug screening, and the robotic scientist has identified several new drugs active against tropical disease parasites [19].

Neural networks are the machine learning algorithm most widely used in chemistry and related research areas such as drug and materials discovery. Consequently, the following discussion relates to these highly useful algorithms, and the potentially paradigm shifting new variants called deep learning. We provide a brief summary of these types of machine learning algorithms to assist those organic chemists who are not familiar with them.

Traditional backpropagation algorithm

A common machine learning algorithm is the backpropagation neural network. This is a mathematical object usually consisting of three layers, each of which contains a variable number of nodes (see Figure 4). A mathematical representation of an object (such as a molecule) is applied to the input layer nodes. The representations are distributed via a set of weights to the hidden layer nodes where nonlinear computation is performed. The inputs to each hidden layer node are summed and transformed by a nonlinear transfer function in the hidden layer node. The output of these nodes is transmitted to the output layer node (there can be more than one) where the weights are summed and used to generate the output. Initially the weights are set to random numbers. During training, the difference between the predicted outputs from the neural network and the measured properties of the molecules used to train the network generates errors. These errors are propagated backwards using

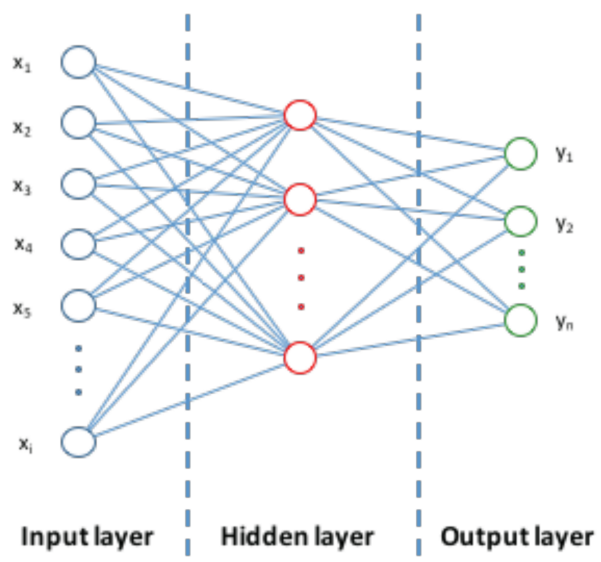


Figure 4: Traditional backpropagation neural network machine learning algorithm.

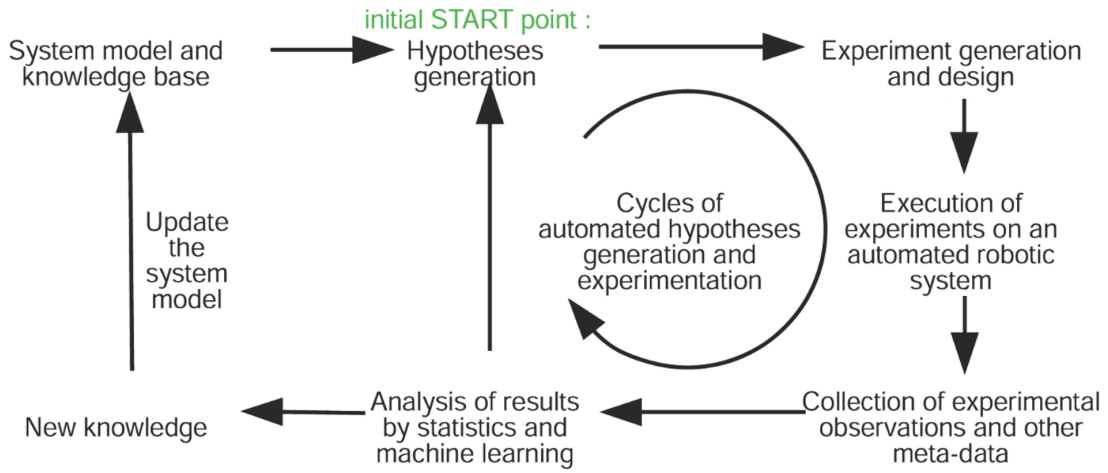


Figure 3: Hypothesis-driven closed-loop learning rationale for Adam and Eve. Hypothesis-driven experimentation cycles can autonomously generate new knowledge. Creative Commons Attribution License, http://creativecommons.org/licenses/by/2.0, from the work of Sparkes et al. [20]; copyright the authors of [20].

the chain rule to modify the weights so as to minimize the errors in the predicted property values generated by the neural network. The training stops when the predictions of the neural network do not improve. While these types of neural network work very well they do have some problems, some of which are common to any regression method (e.g., overfitting) and some specific to neural networks (overtraining, difficulty in choosing the best neural network architecture). While traditional back-propagation neural networks like those described above are undoubtedly useful, their shortcomings can be almost entirely eliminated by the additional of an additional operation called regularization, essentially applying a penalty to models that are more complex (nonlinear). A balance is struck between the accuracy and complexity of the model, thus minimizing overfitting, optimizing the predictive power of models, and identifying the most salient molecular properties that control the property being modelled.

Bayesian regularized neural networks

Applying regularization to neural networks, or any other types of regression, involves defining a new cost function, the parameter that is minimized when the regression algorithm operates. A cost function M listed below describes this balance, with the α and β parameters adjusting the relative importance of the errors in the model predictions (β parameter) and the size of the neural network weights (a measure of model complexity, α parameter).

$$M(w) = \beta \sum_{i=1}^{N_D} [y_i - f(X_i)]^2 + \alpha \sum_{j=1}^{N_W} w_j^2$$

where N_D is the number of data points and N_W is the number of neural network weights (w_j).

Unregularized models use cost functions containing only the first (error) term, corresponding to the normal least squares criterion. In applying any type of regularization, it is essential to identify the best values for the α and β parameters, often by trial and error. It has been shown that Bayesian statistics can be used to find the optimal values of α and β to generate models with the best prediction performance. Detailed discussion is beyond the scope of this paper but are available elsewhere [21-23].

Deep learning

Very recently, LeCun, Bengio and Hinton described a different type of neural network AI method called deep learning [24]. Unlike shallow neural networks with three layers and few hidden layer nodes, deep neural networks have several hidden layers with thousands of nodes in each layer (see for example Figure 5). They are not trained in the same way as traditional neural networks because the very large number of adjustable weights they contain would lead to training difficulties and overfitting, seriously compromising their ability to predict. Instead they make use of sparsity-inducing methods that involve a 'linear rectifier' transfer function in the hidden layer nodes, and implementation of random weight drop outs. The linear rectifier function returns zero if the sum of the input weights is below a given threshold (zero for example), and returns a multiple of the sum of the input weights if this is above the threshold. Random weight dropout involves randomly selecting weights or hidden layer nodes, setting them identically to zero for one or more training cycles. Both of these methods effectively 'switch off' relatively large parts of the deep neural network, this reducing the number of fitted parameters (network weights) and minimizing overfitting.

While deep learning is attracting much attention in fields like image and voice recognition, it may not be superior to three

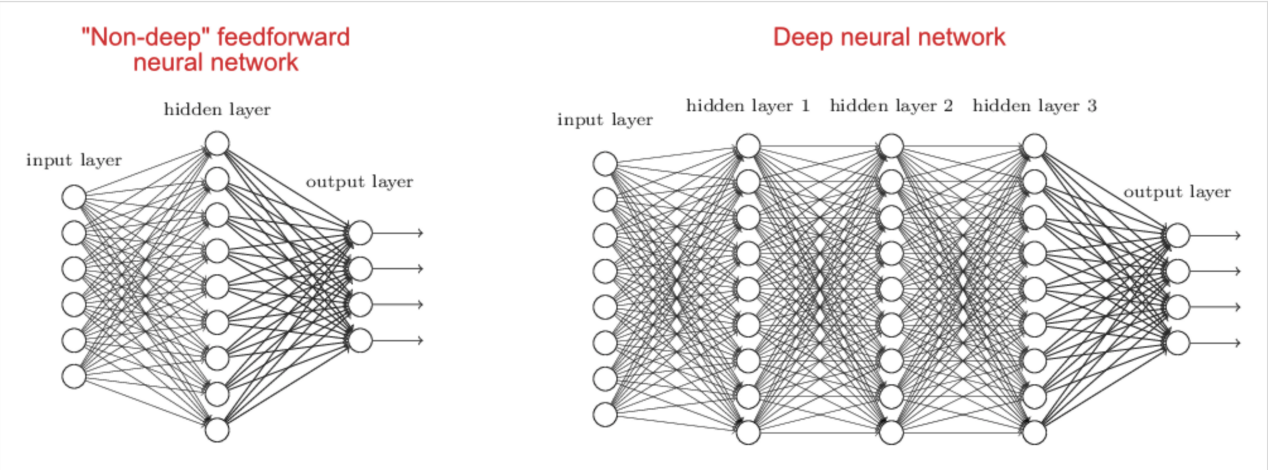


Figure 5: Comparison of architectures of shallow (non-deep) and deep neural networks. Adapted with permission by Michael Nielsen from http://neuralnetworksanddeeplearning.com/chap5.html.

layer ‘shallow’ neural networks for modelling chemical, molecular and biological properties. An important mathematical theorem, the Universal Approximation Theorem states that a feed-forward network with a single hidden layer containing a finite number of neurons can approximate any continuous function under mild assumptions on the activation function. Consequently, although deep learning methods are currently attracting much interest in some emerging technologies, they may not offer any advantages over shallow neural networks for chemical problems. A recent publication has shown how deep and shallow neural networks exhibit similar performance in predicting the activities of drug-like molecules against important pharmaceutical targets [25].

Table 1 summarizes the prediction performance of deep neural networks (DNN) and (shallow) Bayesian regularized neural networks (BNN) for very large sets of organic drug-like molecules screened against fifteen protein targets [25]. Good predictions have low RMS errors (RMSE) or standard error of prediction (SEP) values. Table 1 clearly shows that, on average deep and shallow neural networks have broadly similar prediction performance. Conspicuously, the very significant advantages of regularized machine learning methods can be further enhanced when processes to identify the most important features in a conceptual landscape are also employed.

Sparse feature detection in vivo

Detection of important features in the environment is critical for the long-term sustainability of life. For example, the roughly 100 million photoreceptors in a human retina cannot not directly transmit a picture to the brain due to the limited capacity of the optic nerve (there are 100 times more photoreceptor cells than ganglion cells). The retina carries out extensive signal analysis and feature detection on the image and sends this processed, compressed image along the optic nerve to the brain. This is achieved by the way the ganglion cells' receptive fields are organized, detecting contrast and edges. This allows a much smaller amount of information to be sent to the brain for subsequent analysis and response. We can learn from biology and teach computational analysis methods to identify features in data in an analogous way. This facilitates the development of models with higher predictive performance and the identification of the factors that have the most influence over the property being modelled, leading to clearer interpretation of the structure–activity relationships represented by the model. This capability is particularly useful in phenomena described by many parameters (high dimensionality) and those sampled by very large numbers of observations (Big Data).

Sparse feature selection in silico

An increasing number of experiments are employing large scale, high throughput ‘omics’ technologies to probe deep scientific questions [26]. Examples include gene expression microarray technologies, rapid development of glycomics technologies, large-scale use of proteomics, and the proliferation of mathematical descriptions of molecules and more complex materials. Analogous to biological feature detection, informatics methods attempt to use mathematical methods to identify the

Table 1: Comparison of large drug data set standard errors of prediction (SEP) from deep (DNN) and shallow (BNN) neural networks [25].

Data set	Size of data set		Test set SEP	
	Training	Test	DNN	BNN
CYP P450 3A4 inhibition pIC_{50}[a]	37241	12338	0.48	0.50
Binding to cannabinoid receptor 1 pIC_{50}	8716	2907	1.25	1.14
Inhibition of dipeptidyl peptidase 4 pIC_{50}	6148	2045	1.30	1.27
Inhibition of HIV integrase pIC_{50}	1815	598	0.44	0.46
Inhibition of HIV protease pIC_{50}	3212	1072	1.66	1.04
LogD measured by HPLC method	37388	12406	0.51	0.53
Metabolism – % remaining after 30 min microsomal incubation	1569	523	21.78	23.89
Inhibition of neurokinin1 receptor pIC_{50}	9965	3335	0.76	0.72
Inhibition of orexin 1 receptor pK_i[b]	5351	1769	0.73	0.79
Inhibition of orexin 2 receptor pK_i M	11151	3707	0.95	1.08
Transport by P-glycoprotein log(BA/AB)	6399	2093	0.36	0.40
Log(bound/unbound) to human plasma protein	8651	2899	0.56	0.58
Log(rat bioavailability) at 2 mg/kg	6105	1707	0.54	0.49
Time dependent Cyp 3A4 inhibition[c]	4165	1382	0.40	0.39
Human thrombin inhibition pIC_{50}	5059	1698	2.04	1.53

[a]$pIC_{50} = -\log(IC_{50})$ M; [b]$pK_i = -\log(K_i)$ M; [c]log(IC_{50} without NADPH/IC_{50} with NADPH).

most relevant features in these data sets so that interpretation of experiments is easier, and predictions of outcomes in new experiments are more reliable (see for example Saeys et al. [27]).

In our research we have adapted an elegant sparse feature selection method, initially reported by Figueiredo [28]. It employs a sparsity-inducing Laplacian prior that can be used in conjunction with linear regression and neural networks to prune the irrelevant features from models and less relevant weights from neural networks, resulting in models with optimal predictivity and interpretability [28]. Although mathematically too complex to describe here, the sparsity-inducing Laplacian prior has the very useful property of removing uninformative features and neural network weights by setting them to zero [21,29]. These, and related feature selection methods provide a valuable adjunct to molecular and materials modelling methods based on structure–activity/property regression and neural networks models. Such machine learning-based models have been used successfully in pharmaceutical discovery for several decades. More recently, they have been applied to modelling materials other than small, discrete, organic molecules, with considerable success. Many types of materials are considerably more complex than small organic molecules (e.g., with size and weight distributions, diverse shapes, variable degree of crosslinking, different degrees of porosity, processing-dependence of final properties etc.) and the size of 'materials space' is consequently much larger than that of 'drug-like' space. This recognition has accelerated the development of very high throughput synthesis and characterization methods for materials, and spawned the application of evolutionary algorithms to explore materials space more quickly and effectively than other methods. When coupled with learning algorithms, in silico evolutionary adaptation is possible, as we now describe.

Evolving materials for the future

The development and application of evolutionary methods for the design and discovery of novel technologies, materials, and molecules has its origin in two seemingly unrelated historical figures.

Charles Darwin and Josiah Wedgwood

Many are not aware that, arguably, one of the first 'combinatorial' materials scientists was Josiah Wedgwood. His ultimate products were the ceramics used in the eponymous fine china. He developed a rigorous and systematic way of understanding the relationships between the properties of the clays used, the manufacturing process variables, and the performance of the final ceramics. Figure 6 shows a tray of jasper tiles from a typical "high throughput" experiment.

It is also not well known that Charles Darwin, the 'father of evolution" was related to Josiah Wedgwood, who financed some of Darwin's expeditions. Fittingly, there has been a recent synergistic convergence of the concepts of natural selection and evolution with high-throughput synthesis and testing of molecules and more complex materials in the past decade. Recognition of the enormous, essentially infinite, size of materials space ($\approx 10^{100}$) has driven to the development of evolutionary methods for molecular and materials discovery. Evolutionary algorithms mimic the processes of natural selection, and they are efficient ways of exploring extremely large materials spaces. Although accelerated synthesis and testing methods for bioactive molecules (drugs and agrochemicals) and materials are invaluable for accelerating drug and materials research, they cannot alone solve the problem of the size of materials space. Exhaustive searches are intractable and will always be so (even making and testing a billion materials per second would not make an impact on the total number of materials that could theoretically be synthesized). A synergistic combination of these accelerated experimental technologies with evolutionary algorithms provides a potentially disruptive change in the way molecules and materials are designed. Recent reviews describe the application of evolutionary approaches to drug and materials discovery [5,6].

High-throughput experimentation

The pharmaceutical industry developed high-throughput chemical synthesis and screening technologies in the late 20th century. Materials scientists have recently begun adapting these technologies to the synthesis and characterization of materials. Figure 7 shows a new high-throughput-materials synthesis and characterization facility at CSIRO Manufacturing in Melbourne Australia. This can generate and test hundreds of polymers, nanomaterials, catalysts, or metal organic frameworks in a day.

Clearly, certain types of chemistries (benzodiazepines, click reactions, etc.) are amenable to large chemical library synthesis, and peptides and oligonucleotides can also be synthesized efficiently using automated methods, it is not yet possible to carry out chemical syntheses in a general sense using these technologies. However, several groups are making significant breakthroughs in generalizing and expanding the automated synthesis of organic compounds. Rzepa, and Murray-Rust among others, have begun systematizing chemistry using a type of chemical mark-up language (a machine-readable language designed to describe the central concepts in chemistry) and chemical ontologies (a formal naming and definition of the types, properties, and interrelationships of chemical entities) [31-34]. One aim to transform every type of chemical synthesis into a precisely defined language that can be used by instruments and synthesis robots to carry out all of the unit operations required in chemical synthesis and analysis. The ultimate aim is to



Figure 6: Tray of Josiah Wedgwood's jasper trials from 1773 (copyright Wedgwood Museum; all rights reserved). Each ceramic sample is marked with labels that correspond to an entry in Wedgwood's 'Experiment Book' or relate to firing instructions, e.g., 'TTBO' for 'tip-top of biscuit oven'. Used with permission from the Wedgwood Museum. Also see the summary of Josiah Wedgwood's work by Sammut [30].



Figure 7: A high-throughput-materials synthesis and characterization facility RAMP, (Rapid Automated Materials and Processing) https://www.csiro.au/en/Research/MF/Areas/Chemicals-and-fibres/RAMP.

develop a technology that will allow a machine to carry out the same chemical reaction in the same way with the same yield and purity, regardless of where it is performed. Cronin's group recently reported how to employ 3D-printed chemical reaction ware (Figure 8) to carry out chemical synthesis and analysis under computer control [35].

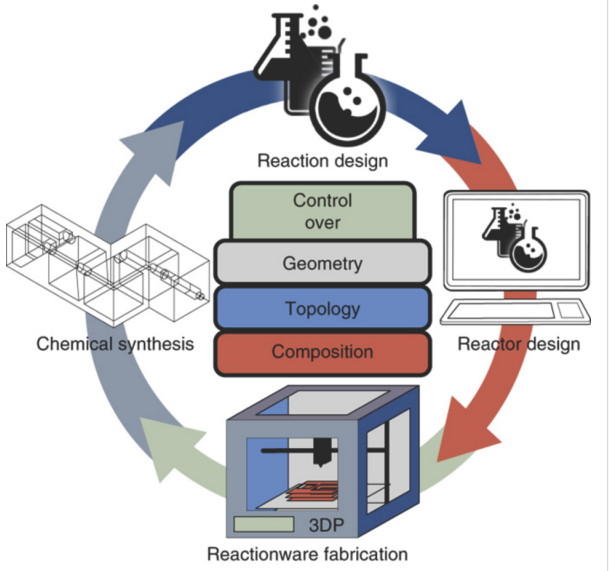


Figure 8: An interactive procedure to design and 3D print bespoke reaction ware to optimization yield and purity of a chemical synthesis. Reprinted with permission from [36]; copyright 2016 Macmillan Publishers Ltd.

Another very recent and important step towards general automated chemical synthesis was reported in Science in 2015 (Figure 9) [37]. This platform provided a proof of concept of a general and broadly accessible automated solution to the problems of small-molecule synthesis. These technologies have now made practical the autonomous evolution of materials, where the design-synthesis-testing cycle is run by algorithmic evolutionary control and implemented robotically.

In order to achieve autonomous algorithmic control, it is necessary to translate the essential operations of evolution by natural selection into mathematical form. The basic components of evolutionary algorithms are summarized below to assist organic chemists who are not familiar with them.

Representing materials mathematically (materials 'genome')

To model or evolve molecules or materials, it is necessary to convert key compositional, structural, synthesis, or processing properties into a numerical 'genome'. These must encapsulate salient features of the molecule or material that influence the property being modelled, mutated and optimised in an evolutionary process. For example, the components in a molecule (or material) can be represented as a binary string.

00010100010101000101010011110100

where 0 = fragment (e.g., CH_3) not present in the structure and 1 = fragment present in the structure (perhaps multiple times).

There are many other ways of generating these molecular representations, commonly called descriptors. Compositional descriptors have been successfully used to model and evolve materials like catalysts and phosphors. These are vectors of real numbers encoding composition (Figure 10). These strings represent a material or molecular 'genome', that can be used to predict the materials property or that can be operated on by mutation.

Mutation operators

Once materials or molecules have been converted into mathematical entities, several types of mutation operators can be

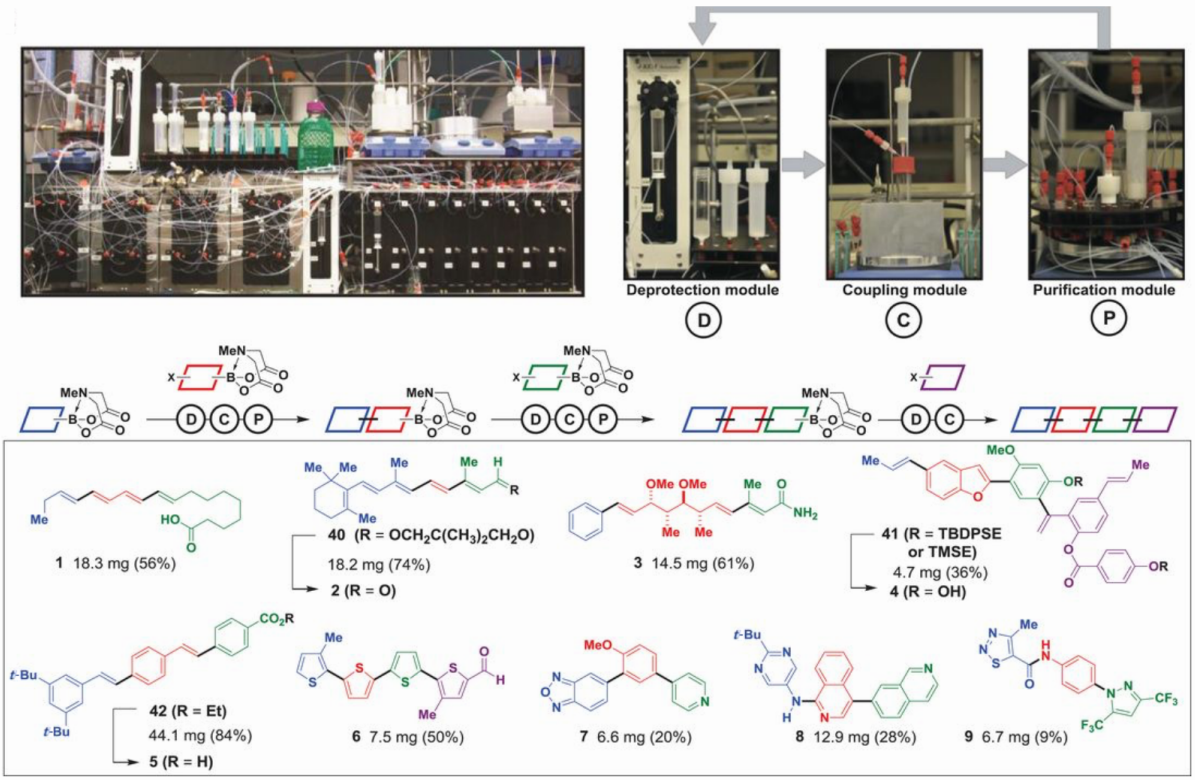


Figure 9: (Top) Photograph of a small-molecule synthesizer comprised of three modules for deprotection, coupling, and purification steps. (Bottom) Natural products, materials, pharmaceuticals, and biological probes synthesized by automated synthesis by iterative coupling of different building blocks (colors). TBDPSE, *tert*-butyldiphenylsilylethyl; TMSE, trimethylsilylethyl. Adapted with permission from [37]; copyright 2015 American Association for the Advancement of Science.

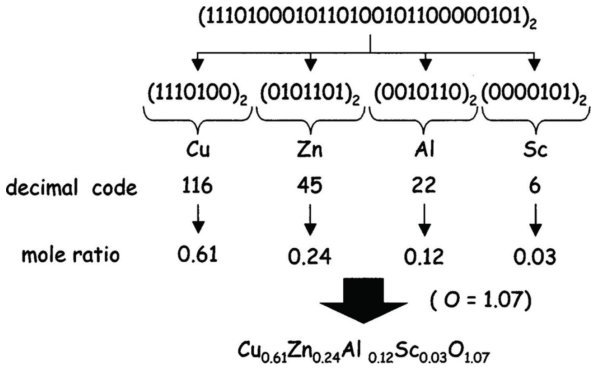


Figure 10: An example of a composition-based descriptor vector that could be used to model or evolve materials properties like phosphor brightness and colour, or catalyst efficiency. Adapted with permission from [38]; copyright 2003 American Chemical Society.

applied to the materials genome. The simplest and most commonly used are the point mutation and crossover operators. Point mutation involves altering a single element in the string representing the genome of a material or molecule. For example, a bit string genome might have a single bit flipped into the alternate state. Alternatively, a compositional genome could have the amount of one of the components increased or decreased. Crossover operators take genomes from two materials, select an arbitrary point to split them, and the fragments swapped between the two (Figure 11).

Fitness functions and the evolutionary cycle

Once the materials have been represented mathematically in a genome, and the mutation operators defined, a fitness function must be defined. The fitness function is a method (experimental or computational) of determining the suitability of molecules or materials in the population of entities being evolved. The fitness is usually some useful property, or a combination of properties, that needs to be improved. Examples include, phosphor brightness, drug binding efficacy, toxicity, catalytic efficiency, ability of the material to support the growth of cells, efficiency of gas adsorption, and many others.

The relationship between the materials genome and the fitness can be presented as a surface, commonly called the fitness landscape (Figure 12). The object of an evolutionary process is to

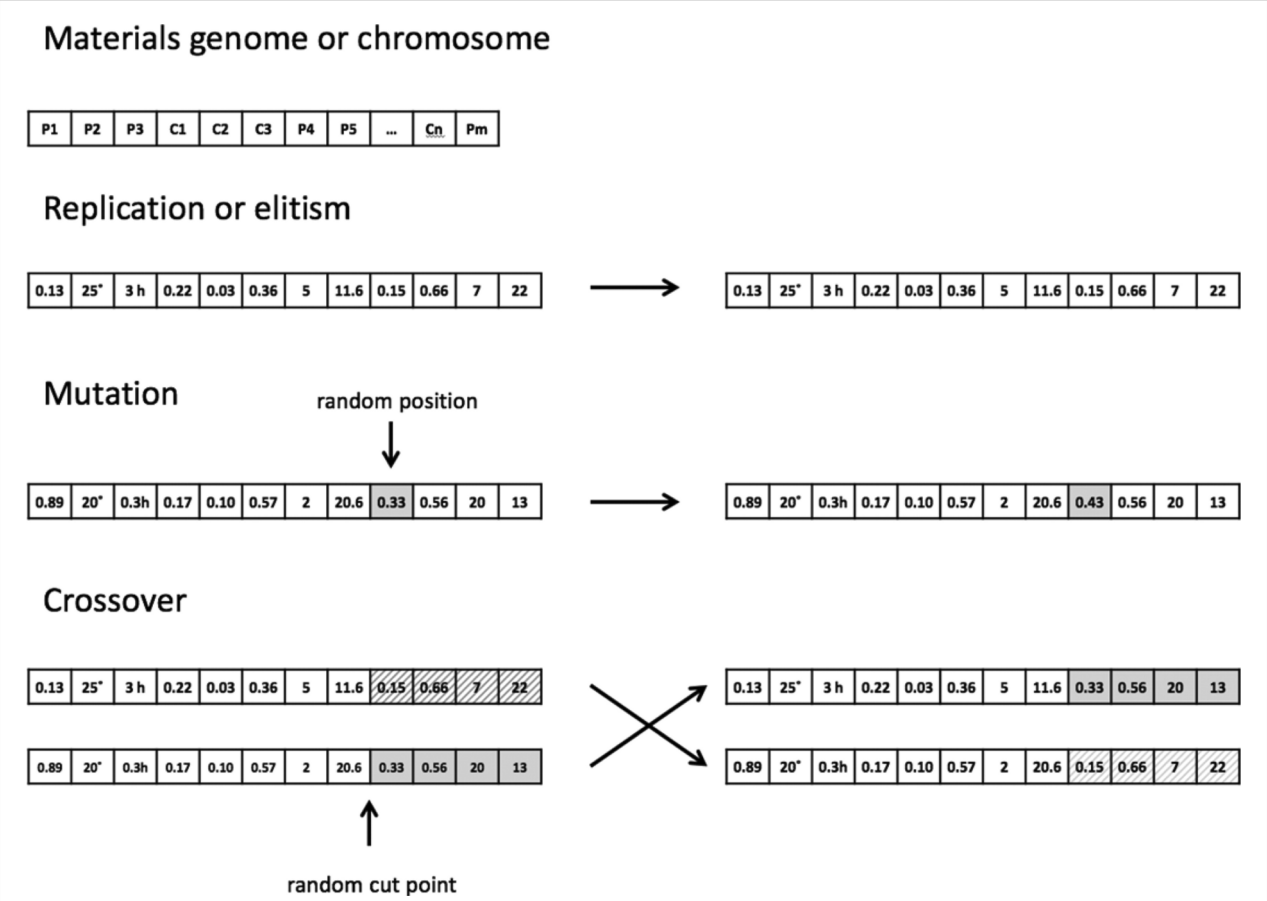


Figure 11: Example of a simple elitism (copy unchanged), crossover, and point mutation operations acting on the genomes of two materials. Reprinted with permission from [6]; copyright 2016 American Chemical Society.

find the peaks (or valleys, if a property is to be minimized instead of maximized) on the fitness landscape. The complexity lies in the fact the almost all fitness landscapes are multidimensional, often highly so. Applying mathematical evolutionary algorithms to the system allows vast, multidimensional fitness landscapes to be searched efficiently.

Once an initial population of molecules or materials is created, and the mutational operators and fitness function(s) have been defined, an iterative cycle is traversed where the fitness of the population is measured and the best (fittest) entities are mutated and bred to generate the next generation. This generation proceeds through the same process of selection, mutation, and breeding for several more cycles. The process stops when members of the population exceed some performance criterion or when no further improvement occurs. Evolutionary algorithms are very efficient at searching large materials spaces to find excellent (although not optimal) solutions, just as natural selection does with biological populations. Table 2 shows how extremely large search spaces (up to 10^{22}) can be traversed to find good solutions using a modest number of experiments.

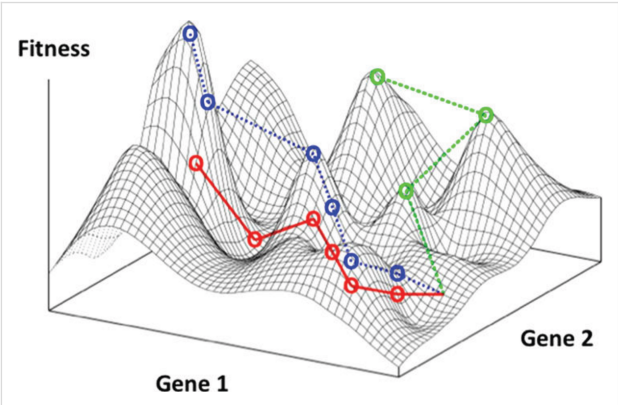


Figure 12: A simple example of a two-dimensional fitness functions. The lines represent different evolutionary trajectories on the landscape that lead to different local optima. Real fitness landscapes are dependent on many more dimensions (multiple materials 'genes' in the genome). Reprinted with permission from [39]; copyright Randal S. Olson.

Two recent reviews have summarised how evolutionary methods have been used to discover and optimize drug leads [5], and materials [6].

Table 2: Examples of evolutionary optimization experiments showing the number of control variables (parameters or dimensions), fitness or objective function (mainly catalysis) and the number of experiments used to sample the theoretical experimental space. From Moore et al. [40].

Variables	Objective	Number of experiments	Size of space
6	binding to stromelysin	300	6.4×10^7
8	propane → propene	328	NA
4	inhibition of thrombin	400	1.6×10^5
8	propane → CO_2	150	NA
8	propane → propene	280	NA
13	propane → propene	60	NA
23	$NH_3 + CH_4 \rightarrow HCN$	644	NA
9	$CO \rightarrow CO_2$	189	NA
4	$CO + CO_2 + H_2 \rightarrow CH_3OH$	115	2.7×10^9
5	$3CO + 3H_2 \rightarrow C_2H_6O + CO_2$	160	2.4×10^{11}
6	$CO + CO_2 + H_2 \rightarrow CH_3OH$	235	4.7×10^9
10	*n*-pentane isomerization	72	1.44×10^4
7	propane → aldehydes	80	NA
8	isobutane → methacrolein	90	10^9
8	membrane permeability	192	9×10^{21}
4	cyclohexene epoxidation	114	NA
3	protein inhibition	160	10^{16}
6	red luminescence	216	NA
7	green luminescence	540	10^{14}
6	colour chromaticity	168	NA
8	red luminescence	270	NA
7	red luminescence	1080	NA

Evolution coupled with learning

As with natural biological systems, evolutionary processes like natural selection (and the in silico analogue) can couple synergistically with learning. This is a part of adaptation (generically named complex adaptive systems). The Baldwin effect describes the influence of learned behaviour on evolution. In 1987 Hinton and Nowlan used computer simulation to show that learning accelerates evolution and associated it with the Baldwin effect. In practice, machine learning models of fitness functions can significantly accelerate the rate of optimization of evolutionary processes in silico [41-43].

Examples of applications of AI methods, feature selection, evolution of materials

The following brief examples show how these new in silico feature selection, machine learning, and adaptive evolution have been applied to chemical problems.

Sparse feature selection: how strontium ion controls mesenchymal stem cells (MSCs)

Bioglass materials containing strontium ions have been shown to reduce bone loss and fractures by stimulating mesenchymal stem cells (MSCs) to differentiate down the osteogenic (bone forming) pathway. The mechanism by which this occurs was far from clear. A broad gene expression microarray experiment was performed on MSCs exposed to different levels of strontium and other minerals from the bioglass. Computational sparse feature selection methods identified around ten genes from the tens of thousands on the microarray chips used to determine how gene expression changed in MSCs in response to strontium levels [44]. These genes suggested the sterol and fatty acid biosynthetic pathways were activated in the MSCs, and subsequent experiments validated the model predictions of increased levels of proteins in these pathways and the formation of lipid rafts on the cell membranes. In silico sparse feature selection thus revealed a hitherto unknown mechanism for osteogenesis that may be exploited to stimulate bone growth in grafts or in patients suffering age-related bone loss.

Machine learning and evolutionary design: pathogen-resistant polymers

Antimicrobial drugs and materials are becoming extremely important due to the rise in nosocomial infections and drug resistant pathogens, and the increased use of implantable and indwelling medical devices. Much research is now focusing on developing materials that resist bacterial attachment and growth as an alternative to new antibacterial agents to which the development of resistance is inevitable. Artificial intelligence methods such as machine learning have proven very effective in predicting the propensity of pathogens to colonize polymer

coatings, for example. Hook et al. generated large libraries of copolymers using robotic methods, and exposed these to three common hospital pathogens to try to identify low adhesion materials for coating medical devices [45]. These data were used to generate a sparse machine learning model for each pathogen (Figure 13) that predicted pathogen attachment and described the relationship between polymer surface chemistry and attachment [46]. The pathogen attachment performance of the polymers determined experimentally and predicted by the machine learning models was used as a fitness function to evolve several populations of polymers with deceasing pathogen affinities. Subsequently, machine learning methods were used to generate a multipathogen model that could quantitatively predict the likely attachment of several pathogens simultaneously [47]. The research showed that models to predict attachment of an even broader range of pathogens would be possible, accelerating discovery of new materials with superior performance in medical devices.

Adaptive evolutionary design of porous materials for hydrogen storage and CO_2 capture and reduction

Porous materials, such as metal organic frameworks (MOFs), covalent organic frameworks (COFs) and zeolitic imidazolate frameworks (ZIFs) are attracting much interest because of the large numbers of bespoke materials that can be designed and synthesized using these self-assembly paradigms. They are being developed to tackle two major and interrelated environmental challenges facing the planet, the rise in CO_2 levels in the atmosphere due to burning of fossil fuels, and the storage of hydrogen for zero carbon emission transport. Millions of hypothetical porous materials have been designed, and it is infeasible to try to synthesize and test all of them to find more effective gas-adsorbing materials. Computational prediction of the performance of these materials is feasible using compute intensive Grand Canonical Monte Carlo calculations. However, these are intractable for libraries of millions of porous materials. Thornton et al. recently showed how a combined artificial intelligence-based modelling paradigm could be combined with evolutionary algorithms to discover materials with superior gas-adsorption properties in a more timely and resource efficient way than by experiments or GCMC calculations alone (Figure 14) [48].

Perspectives, and the Future

Evolutionary methods have been shown to be effective in materials discovery, helping with the "curse of dimensionality". They are complementary to the new high throughput materials synthesis, characterization, and testing technologies – e.g., RAMP, flow chemistry, high-throughput beam lines, combinatorial chemistry. They suggest that an automatic, closed loop system could be developed where the fittest materials synthesized in a given generation are used to design the next generation of improved materials. Early progress in this area has been made – for example, a closed loop flow synthesis method has been developed that automatically optimizes the yield and selectivity of the products [49]. Use of evolutionary and machine learning in silico methods as well as robotic synthesis and characterization methods could explore large materials spaces and accelerate discovery of novel, useful materials. The

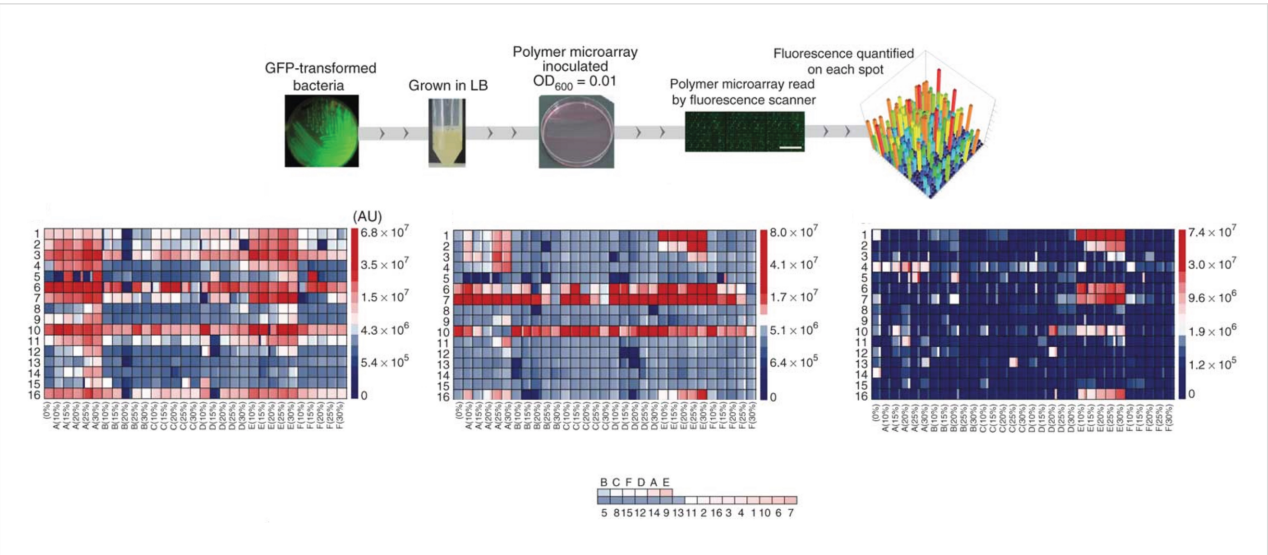


Figure 13: Robotic synthesis and testing of populations of pathogen-resistant polymers evolved by a combination of machine learning and evolution. The top panel shows a summary of the experiments ≈500 polymer spots are generated in an array and exposed to GFP transformed pathogenic bacteria. The lower panel shows how the average pathogen attachment decreases markedly (less red, more blue) between the first (left) and third (right) generations of polymers. Adapted with permission from [45]; copyright 2012 Macmillan Publishers Limited.

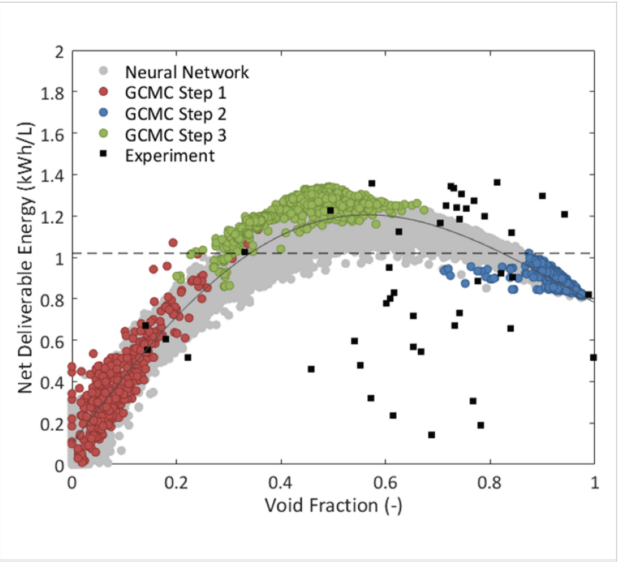


Figure 14: Net deliverable energy as a function of porous material void fraction at 77 K cycling between 100 and 1 bar. Predictions include the GCMC-simulated sample sets for three rounds of evolution (colours), and the final neural network model for the complete genome (grey). Experimental data from the literature is shown as black squares. Adapted with permission from [48]; copyright 2017 American Chemical Society.

progress in the field of artificial intelligence and machine learning is rapid and it is difficult to make clear predictions about where this will lead. However, it is also already obvious that a synergistic combination of robotics and automation with machine learning and evolutionary algorithms will lead to a step change in the ability to discover, design, and optimize molecules and more complex materials with useful properties thought to be inaccessible in the past. If evolutionary methods can be efficiently coupled with AI so that systems for the discovery of new materials become adaptive learning systems, the implications for the progress of science and technology (and employment) are massive and unpredictable. Such developments are already occurring in other fields, with AI systems making more accurate diagnoses than medical experts [50], an AI system taking a position on a company Board of Directors [51], autonomous cars [52] and the mooted replacement of many jobs by AI systems [53]. Perhaps the predictions of the 'singularity' (the point in time where machine learning matches that of humans) by between 2029 and 2045 are not so unrealistic.

Acknowledgements

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Framing major prebiotic transitions as stages of protocell development: three challenges for origins-of-life research

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Commentary

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Abstract

Conceiving the process of biogenesis as the evolutionary development of highly dynamic and integrated protocell populations provides the most appropriate framework to address the difficult problem of how prebiotic chemistry bridged the gap to full-fledged living organisms on the early Earth. In this contribution we briefly discuss the implications of taking dynamic, functionally integrated protocell systems (rather than complex reaction networks in bulk solution, sets of artificially evolvable replicating molecules, or even these same replicating molecules encapsulated in passive compartments) as the proper units of prebiotic evolution. We highlight, in particular, how the organisational features of those chemically active and reactive protocells, at different stages of the process, would strongly influence their corresponding evolutionary capacities. As a result of our analysis, we suggest three experimental challenges aimed at constructing protocell systems made of a diversity of functionally coupled components and, thereby, at characterizing more precisely the type of prebiotic evolutionary dynamics that such protocells could engage in.

Introduction

Living beings on Earth, even in their simplest prokaryote versions, are extremely complex systems, made of a great diversity of molecular components in continuous transformation and interaction. At the base level, each cell is sustained by means of an impressive biopolymer apparatus, which essentially consists of proteins and nucleic acids carrying out complementary tasks to orchestrate an intricate and heterogeneous dynamic organisation with surprising robustness. In addition, this organisation always involves an endogenously synthesized boundary that protects those components/processes from the surrounding milieu and, not less importantly, provides a selective interface that couples them to that external environment. Indeed, all known organisms (genetically-instructed cellular metabolisms) intrinsically depend, both in material and energetic terms, upon

a variety of processes that take place across their boundaries – lipid membranes in/on which highly sophisticated mechanisms of transport and energy transduction reside, making possible the maintenance of the system, as a whole, in open, far-from-equilibrium conditions. In a metaphoric sense, a cell is a special type of "nano-factory", whose molecular machinery conducts chemical syntheses from simpler precursors and uses the products of that complex chemical activity for its continuous reinforcement, managing to re-fabricate the complete synthesis machinery itself.

The problem of origins of life consists in finding a plausible sequence of transitions from abiotic physical and chemical processes towards this level of molecular and organisational complexity, unparalleled by any other phenomena that we observe in the natural world. Therefore, facing this challenge always involves making a strong set of simplifying assumptions, both in terms of the molecular and the organisational features of life as we currently know them. The simplifications tried so far have met with limited success, probably because they represent oversimplifications. From a historical perspective, one can say that the extraordinary success of molecular biology led a whole generation of origin-of-life researchers to believe that the initial steps towards life could be performed by molecules of a single kind (not embedded in a wider chemical organisation). Then, for years, a strong debate was established in the field about, precisely, what kind of molecule (often, what kind of biopolymer) came first, analysing either the abiotic pathway of synthesis that could have brought it about, or the reactive processes that it could have provoked (i.e., the replication or catalytic processes it hypothetically took part in). Fortunately, following the advent of systems biology at the turn of the century, an increasing awareness about the irreducibility of living phenomena to a specific type of molecular mechanism is extending throughout a new generation of scientists, including those interested in the problem of origins [1,2].

In this context, we would like to bring to the fore a fundamental but clearly underappreciated aspect of biological phenomenology: namely, the diversity of components and phase heterogeneity it involves. Aqueous solution chemistry is important for life, but one should not forget that all living organisms require additional physicochemical processes that take place in environments where water is excluded, to different extents. Luckily, we are not alone in the recognition of this basic biological feature: researchers exploring 'molecular crowding' [3-5] share the view and criticize, on similar grounds, a significant part of the biochemical knowledge inherited from last century. Membrane biophysicists have also repeatedly complained about the traditional imbalance in biochemistry between the attention given to soluble enzymes over membrane proteins, whose physiological tasks have equal relevance, but are carried out at interfaces or in conditions that are radically different from bulk water (see, e.g., [6]). Even stronger claims about the intrinsic 'vectorial' character of metabolism have been made by several authors coming from the field of bioenergetics, who underline the role of chemiosmotic mechanisms for the sustainability of any type of cell [7,8]. Furthermore, this more encompassing approach to life is fully congruent with other insights coming from investigations on reaction–diffusion processes in biology, which have revealed, since the pioneering work of Turing [9], the enormous potential of coupling chemistry with the constrained spatial diffusion of the molecules involved [10,11]. Therefore, given the cellular nature of all life known on our planet, and given the importance of compartmentalized chemistries for understanding many biological phenomena, it may be productive to try origin-of-life simplifications that do not completely erase this aspect at the beginning. The combination of diverse chemical reactions with self-organization and self-assembly processes in heterogeneous, multi-phase conditions could actually be crucial at those first stages: this is the main assumption that most of us working in the 'protocell camp' make [12-16].

Under this general hypothesis, one can distinguish two major avenues of research. According to the first, organic compartments of different types (micelles or other colloidal structures) would initially play the role of harbouring surfaces or hydrophobic domains, on which several prebiotic compounds might be adsorbed, in such a way that their chemical reactivity is promoted, leading to more intricate transformation networks and molecular species of various kinds. Several models have been suggested in this direction, from the classical coacervates of Oparin's [17] and more recent versions of it [18], to the obcell theory of Cavalier Smith's [19], based on Blobel's ideas [20], later also revisited by Griffiths [21]. These proposals do not especially favour vesicle compartments, because the encapsulation of the incipient chemistries within a distinct, aqueous micro-environment is not taken to be so relevant at that stage. Quite the contrary, they actually consider that complex biomolecular machinery could be developed outside, to be somehow internalized at subsequent stages [21]. So their main concern is to show how soft hydrophobic clusters and interfaces might have been helpful as aggregating agents, fostering reactions of prebiotic relevance that would be thermodynamically unfeasible in open water solution. In this regard, the former proposals are not very different from other scenarios that have suggested 'harder' mineral surfaces as the local settings on which prebiotic chemistry could initially thrive [22-26].

Nevertheless, without denying the important role that all these (hard and soft) surface- or interface-chemistry scenarios could play in order to discover reaction pathways to diverse organic

compounds, the majority of 'compartment-first' approaches have focused on a second research objective: capturing cell-like behaviours by means of vesicle model systems. Compartmentalization could initially be tried with a two-phase system (e.g., droplets or micro-emulsions) but liposome research techniques, developed during the twentieth century, allowed the in vitro exploration of many – both structural and dynamic – properties of supramolecular assemblies that involve, at least, three-phases (water-membrane-water) and show a striking resemblance to biomembranes, despite their much simpler composition and functional capacities (see [15] for a review). In particular, fatty acid vesicles have become the standard protocell model, not just because of their prebiotic plausibility [27,28], but also because of their remarkable stability as compartments [29,30]; their rapid self-assembly kinetics and amenability to be grown and multiplied under lab conditions [31]; their rich inherent dynamics [32]; and the competition–selection experiments they make possible, if mixed with different liposome populations [33-36]. Thus, the interest of working with these model systems stems from the fact that they provide a very natural connection to real cells, which is attractive both for research groups investigating the chemical roots of biological *organisation* and for others trying to determine the first steps of biological *evolution*.

Discussion

This commentary is aimed at providing a global vision of how these two fundamental aspects of biological phenomenology (the organisational and evolutionary aspects) can be brought together by means of a general scheme of prebiotic transitions that puts 'protocells' at the very centre, as the prime axis of the process of biogenesis (see Figure 1). Furthermore, we will defend the view that in order to reconstruct this process a strict 'bottom-up' approach should be pursued, starting with chemi-

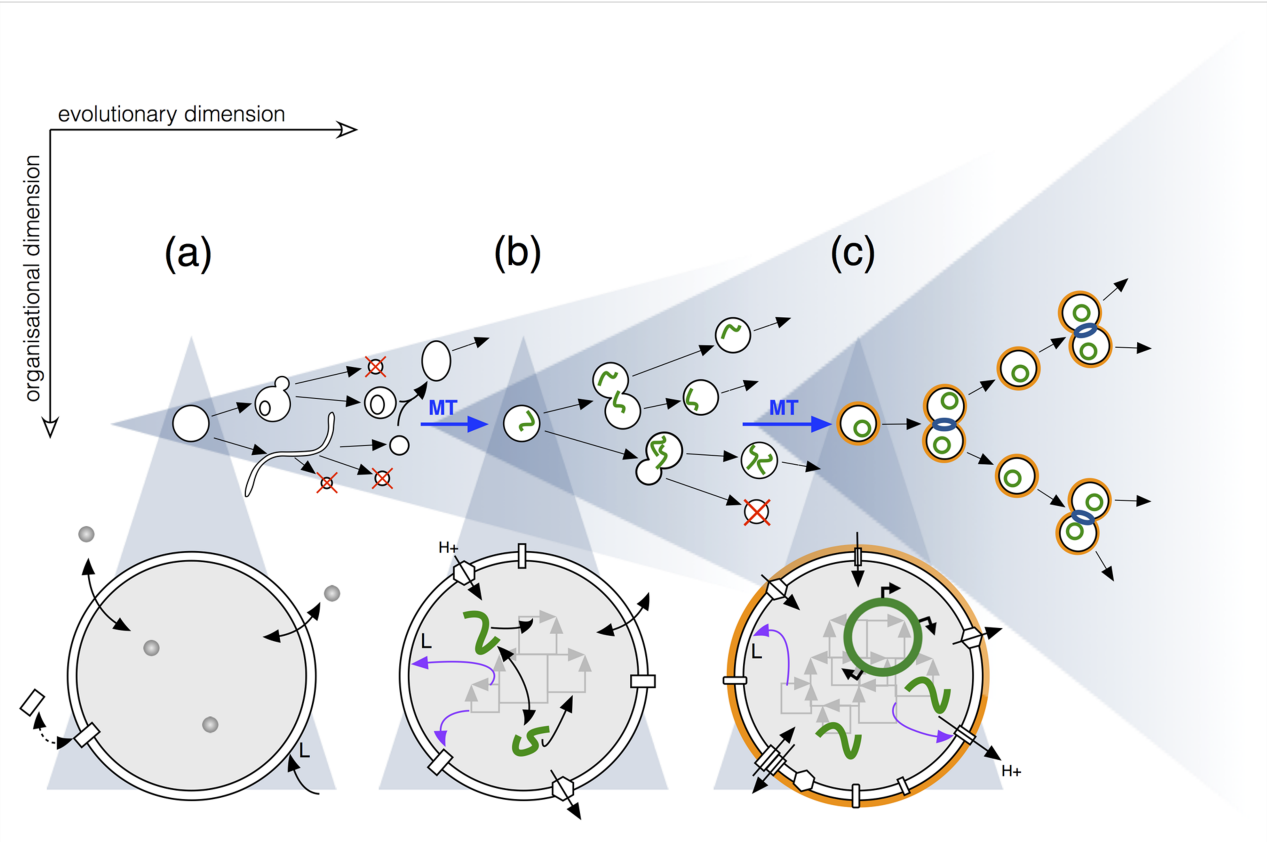


Figure 1: Protocells as the main units of prebiotic evolution: three hypothetical stages of development toward LUCA, with the correlation between protocell organisation and evolutionary potential depicted at each stage. Adapted from [43]. (a) Self-assembled (poly-disperse and likely multilamellar) fatty acid vesicles first start to grow and divide in an unregulated and error-prone way, relying extensively on environmental conditions and external stimuli. (b) After a major prebiotic transition (blue arrow 'MT'), the first self-producing protocells appeared, able to endogenously synthesise membrane lipids and other membrane components. These protocells, hypothetically making use of the first 'energy transduction mechanisms' (leading to precursor 'energy currencies' – based on thioesters [62] and/or pH gradients [53], for instance) and a metabolism that incorporated oligonucleotides and oligopeptides (to become RNA and proteins only at a later stage), could activate growth and – still not fully reliable – division cycles more independently of the environment. (c) After a further major prebiotic transition, protocells would reach complexity levels analogous to LUCA's. Metabolism at that stage already operated on the basis of a 'genotype–phenotype' decoupling, with the development of DNA and coding, to enable an open-ended search for new functionalities. The invention of the cell wall and complex protein machinery controlling cell division made reproduction cycles much more coordinated and reliable.

cal precursors of biomolecules, rather than with fully functional biomolecules. Whereas the encapsulation of biopolymers (DNA, RNA, proteins) or cell extracts in self-assembling vesicles of different composition [37-39] constitutes an important proof of principle that biochemistry can be carried out within strongly simplified compartments, these experiments tell us very little about the actual process of origins. A major challenge that must be tackled in order to move the field of origins of life forward would be to couple simple chemistry to prebiotic vesicle dynamics: chemical reactions provide the power for endogenous synthesis and vesicles the adequate scaffolding for the functional integration of what is synthesized. We will proceed briefly with the issue of functional integration below, but the main point to highlight here is that both for reactive processes to become proto-metabolic and for vesicles to become proto-cellular, their mutual, dynamic engagement could well be an early, unavoidable requirement [40].

As Szostak [41] has also noted, the longer we postpone the appearance of chemical encapsulated systems, the more intractable the problem of compartmentalization will surely become. Indeed, if reaction networks could develop their catalytic efficiency in compartment-free scenarios, their eventual encapsulation within lipid vesicles would most probably drive them to self-suffocation, simply because they would run too fast in relation to the (passive) accessibility of nutrients to the internal milieu [42]. The management of osmotic imbalances would be another obvious difficulty, if incipient reaction networks suddenly became incorporated inside semi-permeable membranes [43]. For these reasons, an early 'co-evolutionary' scenario in which membranes and internal chemistries develop 'hand-in-hand', tightly linked and supportive to each other, makes more sense (see also [44]). Hence our first corollary, expressed in terms of a challenge for the field:

Challenge 1: coupling chemistry with vesicle dynamics. *A special effort should be made to discover simple reaction networks whose products include amphiphiles or surfactant molecules that can be spontaneously absorbed by pre-existing vesicles, modifying their basic properties (e.g., their stability, the permeability/fluidity of their membranes) and displacing them, as a result, from their primary quasi-equilibrium states (e.g., inducing their growth and potential reproduction). In turn, vesicle dynamics should prove supportive of – or at least compatible with – that chemistry.*

Cell physiology shows us that endogenous synthesis is a necessary condition to consider a molecular component functional in the most basic biological sense: that is, functional with regard to the (proto-metabolic) organisation that it belongs to. According to this organisational conception, more extensively argued for in [45,46], a component is functional in so far as it contributes in a specific, distinctive way to the overall maintenance of the far-from-equilibrium system that brought it about. Thus, a molecule, taken in isolation, should not be ascribed a function (however, tempted one may be to attribute one to it). Autonomous functionality (orthogonal to the engineering conception of functionality – linked, one way or another, to external human goals) ought to be understood as a relational property to be established and characterized in the context of a dynamic, self-maintaining/self-producing system, in which a diversity of components and processes of interaction come together. In fact, it is most likely that several different types of components/processes were involved in the constitution of the most basic systems with functional parts (in this naturalized, autonomous sense). Determining the minimal number and the specific nature of these prebiotic components/processes (i.e., that 'irreducible core' required for functional emergence) remains an open empirical question [46]. One needs to try different combinations of precursors, taking part in various reactive and self-assembling processes, and study their mutual compatibility and overall integration dynamics. We will refer to this as the problem of minimal functional integration in a prebiotic context: namely, the quest to determine the experimental conditions under which the simplest – but at the same time sufficiently robust – systems with autonomous functional components could develop. Arguably, this might be the most urgent question that the field of origins of life should tackle in the near future (also possibly related to what Sutherland [47] calls, in a recent review, the first 'major system innovation').

Compartmentalized chemistry, fortunately, is very rich in terms of possibilities for coupling different types of processes and, thereby, its careful exploration is bound to lead us towards proper proto-cellular and proto-metabolic systems ('a-to-b' transition in Figure 1). In addition to direct reaction couplings and negative and positive feedback loops (autocatalytic cycles) that can take place within the internal water pool, the presence of closed lipid bilayers strongly restricts the free diffusion of the various soluble species involved, allows the selective passage of precursors and excludes water in limited areas in which an alternative reaction domain is offered (especially for hydrophobic species to interact, or for water-producing reactions to proceed). In recent years, evidence is accumulating to support various potential functions that these self-assembling supramolecular structures could have as reactor promoters and regulators [48-50], i.e., beyond their traditionally ascribed role as selectively permeable enclosures that keep concentrations above critical threshold values. One could mention here, for instance, their catalytic effects on diverse reactive processes (like peptide formation – [51,52]), or the dynamic changes they could provoke in the conditions under which the chemistry takes place (e.g.,

their capacity to generate pH gradients during growth [53] or the 'osmotic couplings' they may induce among internal molecular species via volume changes [54]).

In any case, all these projected or hypothetical functions would only turn real if vesicle compartments effectively contributed to maintain internal chemistries which, in turn, produced a reinforcing effect on the compartments (on their dynamic robustness and/or on their capacity for growth and reproduction). The degree of molecular inter-specificity and functional integration achieved in a first protocellular scenario may be modest, but it is important that both kinetic control and spatial control mechanisms are included in the equation from the beginning, so that they can complement each other in their development. For an interesting bottom-up synthetic-biology example of how this can be approached, see [55].

Challenge 2: finding conditions and mechanisms for minimal functional integration. *A focused search for the specific experimental conditions and the set of molecular interaction mechanisms (physicochemical couplings) that lead to minimal functional systems should be pushed forward. The proto-cellular scenario proposed in this commentary makes explicit the need to combine, at least, kinetic and spatial control mechanisms in order to achieve this goal – which would certainly be a major breakthrough, even if the robustness of those initial functional systems proved relatively modest with regard to extant cells.*

Only through time and selection pressure may those initial elementary functions become more refined and intermolecularly specific, leading to stronger modes of functional integration. But in order to walk that pathway, natural selection (NS) and evolutionary dynamics must come to the picture, too. Obviously, it is not legitimate to assume that the exquisite molecular machinery currently responsible for matter transport or energy transduction in cells (for example, ATP-synthases), even if they constitute a common feature across all living domains [7], could be present at the first stages of biogenesis. Such complex membrane mechanisms were, no doubt, latecomers – highly optimized products of evolution. However, any plausible evolutionary explanation of their emergence should begin with simpler lipid compartments and with less efficient, precursor (transport/transduction) mechanisms embedded in them.

Competition–selection experiments carried out among different vesicle populations [33-35] have shown that interactions at that global collective level may be highly relevant from very early stages, long before macromolecular structures, like proteins or nucleic acids, took control of metabolic dynamics. In fact, although the mainstream way to experimentally investigate protocells and their evolutionary capacity has been to take a 'semi-synthetic' approach (encapsulating populations of RNA or DNA polymers inside lipid compartments [56-58] or in droplets [59]), we will here propose a more strict 'bottom-up' strategy to face this issue, as well. So to speak, everything must come 'in the same package': i.e., a deep conceptual shift must also take place to account for the origins of natural selection and proper Darwinian evolution (as explained in more detail in [60]). Instead of using compartmentalization simply as a way to segregate populations of nucleic acids (with the aim to avoid problems like parasitism [61]), the idea here is that integrated protocells constitute the actual units of evolutionary change from the very beginning of the process. Thus, the various stages of vesicle/protocell development should be envisioned in close correlation with differences in the potential for evolution of the populations involved, as schematically shown in Figure 1. In other words, the organisational and evolutionary dimensions of biological phenomena must start unfolding and interweaving very early, feeding on each other, in a scenario where complex biopolymers would be produced by – and incorporated in the workings of – those 'proto-organisms' much later. This crudely opens (or re-opens) the question of when should the evolutionary process be called Darwinian (i.e., when NS actually emerges as an operational mechanism), but we consider that the debate ought to take place through an adequate characterization of 'pre-Darwinian' competitive/selective dynamics, which remain largely unexplored.

The main advantage of a scheme of transitions like the one portrayed in Figure 1, looking at it from an evolutionary perspective, is that the individuals that lead the process are protocellular systems whose phenotypic space is intrinsically wider than that associated to replicating molecular entities (as in traditional approaches to the origins of life – reviewed in [46] – or in more recent theoretical proposals, like those pointing to the concept of dynamic kinetic stability [63] – see comments below). Protocells constitute 'scaffolds' in which a high diversity of functional components may be hooked (including those very replicating molecules but possibly many other simpler ones), leading to multiple state variables and dynamic behaviours for each unit of selection. This endows those systems with the potential to become real Darwinian entities, i.e., organisms (or 'proto-organisms', as we called them above) on which natural selection effectively operates [60]. Moreover, major evolutionary bottlenecks in this scenario should not be reduced to a single variable or property but, instead, ought to be related, at least, to the capacity of such systems to: (i) maintain robust dynamics of self-production that underlie their far-from-equilibrium (individual) organization and (ii) reproduce reliably to spread that type of organization in the population. In practice,

this entails becoming autonomous from an energetic point of view (hence the importance of setting up the first energy transduction mechanisms [40]) and achieving regularity in the actual process of protocell division, as well as developing mechanisms of heredity (i.e., control of trans-generational variation) [60,64].

A possible – though still tentative – narrative would proceed as follows: initially, (Figure 1a) fatty acid vesicles could self-maintain and grow through the acquisition of external lipid molecules, or by fusing with neighbours, and then divide through a number of pathways, including budding (internal and external) and filamentous intermediates [65]. These growth and division pathways would be largely at the mercy of prevailing environmental conditions and often would lead to a decrease in the mean size of the offspring. Then (Figure 1b) protocells would develop an inner chemistry helping them activate growth and division cycles more independently of environmental factors (first autonomous proto-metabolisms) and avoiding the tendency to decrease in volume at each generation. Nevertheless, such division would be still stochastic, producing a significant amount of non-viable progeny, in a context in which protocell fusion and mixing would still be rife [43]. At later stages (Figure 1c) protocells getting closer to LUCA (the 'last universal common ancestor' species) would emerge, with metabolism running now on the basis of more complex (code-mediated) 'genotype-phenotype' mappings among functional, subsystem components/modules, all surrounded by an increasingly sophisticated, effective and selective boundary (which would include, at some point, the additional protection of a primitive cell wall). Under these conditions, (i) the space for exploration of new functionalities would widen enormously (getting progressively closer to open-endedness) and (ii) reproduction cycles would become much more reliable, by means of a more elaborate protein machinery specifically devoted to control division processes.

Challenge 3: characterizing the evolutionary dynamics of pre-Darwinian protocells. *Rather than focusing on the reaction kinetics and evolutionary dynamics of populations of naked nucleic acid molecules (the core idea underlying the 'RNA world' hypothesis), or even compartmentalised chemistries run by poly-nucleotides (e.g., the 'ribocell' model), protocell systems with molecular components of much lower molecular complexity should be investigated as units of pre-Darwinian evolution. The overarching question then becomes: how can far-from-equilibrium chemical assemblies that involve low-molecular-weight species be launched in the lab, so that they manage to divide with regularity, explore an ample range of – sufficiently robust – phenotypes, and have potential to set up mechanisms for increasingly reliable heredity?*

It is easy to draw cones, arrows, dead ends, bifurcations and bottlenecks, like we do in Figure 1. Real breakthroughs require the development of experimental strategies and specific protocell models that justify the assumptions and ideas projected through such graphs – or force us to reconsider them. The task is not trivial, though; and not only because the current gap between chemistry and biology is still overwhelming, but also because the devil hides in the details. Prebiotic transitions are particularly tricky due to the fact that the chemical systems involved must work against the natural tendency towards thermodynamic equilibrium (i.e., they must find ways to pay the 'cost of irreversibility' as Pascal and colleagues [63] express it). But in order to understand what might be underlying or 'driving' those transitions towards higher complexity levels (i.e., the blue arrows signalling the 'MT's in Figure 1), one should beware of reductionist or oversimplified explanations. First, as we suggested along the previous lines, a combination of evolutionary and organizational principles should be sought. However, this combination should not be simply conceived on a one-dimensional axis (e.g., in terms of the relative weights of 'self-organization' vs. 'natural selection' forces, as it has been so commonly done in the past [66,67]). Second, also related to the latter comment, both the actual form of those principles (the main variables and relationships involved) as well as the way they get intertwined should still await the results of ongoing research avenues in the field of systems chemistry [1,2]. For instance, although kinetic control mechanisms must play a central part in the explanation, dynamic kinetic stability [63] is not *the* answer (because replication is not all what matters for evolution, chemical or biological). It is probably too early to draw conclusions and try to make generalizations when we still lack the relevant empirical results (e.g., on the initial set of coupling mechanisms that could transform external sources of free energy into a system's own means – and sustain, in this way, the first forms of autonomous functionality [46]).

Elucidating the molecular, organisational and evolutionary innovations leading to the major transitions in the process of origins of life will surely require the effort of many research groups in the future. To our eyes, at least, the bottlenecks represented in Figure 1 do not look simple to overcome: we should be aware that the problem is not just developing and coordinating new mechanisms of molecular control, but also implies more complex processes of functional re-organisation and re-integration by the individuals involved, in the context of a constant interaction with other individuals in the population. On these lines, we would like to end this commentary highlighting that 'protocell population dynamics', so necessary for the progressive unfolding of phylogenetic (i.e., reliable trans-generational) pathways, are also bound to have other, more immediate proto-ecological implications that could turn very relevant

in order to understand those bottleneck transitions. For example, the generation of competitive relationships among different kinds of protocells, could lead to primitive food-webs and diverse modes of selective pressure, and could also be accompanied by other types of symbiotic or collaborative interactions that probably played non-trivial roles in that sense. In fact, those collective dynamics could trigger (through protocell fusion and recombination of complementary components) functional (re-)integration events beyond the minimal compartmentalized chemistries that were under primary focus here. Still regretting Harold Morowitz's recent passing, we consider that his intuition that «sustained life is a property of an ecological system rather than a single organism or species» [68] should guide future scientific attempts to bring light into the fascinating riddle of biogenesis.

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Grip on complexity in chemical reaction networks

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Review

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Abstract

A new discipline of “systems chemistry” is emerging, which aims to capture the complexity observed in natural systems within a synthetic chemical framework. Living systems rely on complex networks of chemical reactions to control the concentration of molecules in space and time. Despite the enormous complexity in biological networks, it is possible to identify network motifs that lead to functional outputs such as bistability or oscillations. To truly understand how living systems function, we need a complete understanding of how chemical reaction networks (CRNs) create function. We propose the development of a bottom-up approach to design and construct CRNs where we can follow the influence of single chemical entities on the properties of the network as a whole. Ultimately, this approach should allow us to not only understand such complex networks but also to guide and control their behavior.

Review

Introduction

Natural phenomena, such as the earth’s climate, ecosystems, animal group behavior, our brain, and living cells, are all systems that display dynamic behavior marked by an apparent complexity [1-5]. Some of the remarkable properties of complex systems lie in their robustness (i.e., error tolerance), resilience (i.e., restoration ability) and adaptive capacity (i.e., to compete or to cooperate for resources) in response to changes in environmental conditions [6-11]. Such system-level functions represent the prerequisite in natural phenomena to prevent abrupt climate shifts or the sudden diminishing of populations in ecosystems and are, arguably, the key properties supporting complex systems to transition from non-living to living [12-14]. Understanding the principles enabling transitions between dynamically distinct but stable states will unravel the predictability and perhaps the possibility to influence the dynamics of change, but science has yet to find an answer to this complexity [15].

One of the ultimate aims for systems like a living cell, is to understand how the interplay between molecular level events and network topology determines the behavior that emerges from complex networks of chemical reactions [16]. Vast meta-

bolic and genetic networks of chemical reactions allow living cells to sense their environment, react to stimuli, and use nutrients for cell growth and division. In the past decades, complexity science has made tremendous progress in developing mathematical tools that capture the key properties underlying such networks [17-19]. Our in-depth knowledge of actual systems, however, is often insufficient to precisely predict when, and by how much, systems respond to changes in the environment. This is especially true when those changes induce systems beyond a critical value, where the resulting abrupt shifts or phase transitions become unpredictable [20-22]. The analysis of the structure and the dynamics of a complex web of intricate interactions is a risk in removing the link between molecular structure and function and network behavior. Hence, we need new approaches that allow guidance and ultimately control of unanticipated behavior of complex molecular systems.

Networks are daunting in complexity but do exhibit structural patterns [23]. The reduction of a network into wiring diagrams enables accurate modelling and has revealed fundamental features that would otherwise be too difficult to comprehend [24]. It is generally accepted that complex molecular networks, like electrical circuits, are constructed from simpler modules (network motifs) and control the regulatory functions as well as the system level behavior of larger networks [25]. In fact, simple motifs with a few positive and negative feedback loops create functionality, such as bistable switching, adaptation and oscillations [26]. Such building blocks can be reconstructed, and this has sparked enormous activity in the fields of synthetic and systems biology as well as metabolic engineering [27].

We must now learn how to apply retrosynthesis to network motifs, and we believe chemistry offers a unique opportunity to the design of chemical reaction networks (CRNs) [28-30]. A major challenge for systems chemistry is to translate the design principles of biological systems into a practical "programming language" and learn how to create functionality using chemical reactions. Early work has resulted in numerous exciting examples, ranging from functional out-of-equilibrium systems that can perform logic operations, to dissipative self-assembling structures, creating new forms of smart materials [31-35]. Yet, we are severely limited by too few examples of systems which are both extensive enough to exhibit dynamics, and at the same time, simple enough to be tunable [36].

In this perspective, we will attempt to lay out a general strategy for the design and implementation of CRNs that operate under out-of-equilibrium conditions and show complex behavior. We believe that new approaches are needed to build molecular networks, firmly rooted in (synthetic) chemistry but incorporating mathematical modelling and borrowing principles from chemical engineering [37]. Isolating the influence of molecular structure on network function and dynamics will reveal the rules governing CRNs, as well as the complexity in systems like the cell.

Minimal chemical reaction networks

Network motifs assembled from feedback loops

Much of our inspiration for constructing CRNs comes from the living cell [38-41]. The biochemical network that governs the dynamic properties of physiological responses such as growth, division and death, can be depicted as a wiring diagram [42,43]. Despite the large number of possible connections, certain patterns of interconnections, so-called "network motifs", are relatively common [23]. Hence, underneath the complexity, local regulation based on minimal systems comprises a fairly simple set of basic events (i.e., activation and inhibition).

Minimal network motifs have the advantage of being simple enough (i.e., analytically solvable) and are therefore well-suited for approaches viewed in the framework of rates of chemical reactions. Figure 1a shows more detail on how a simple phosphorylation and dephosphorylation system can influence the rates of its own formation creating either a positive and/or a negative feedback loop [24,26] Through feedback loops, even simple systems composed from minimal motifs can display dynamic behavior. A minimum of one positive feedback structurally promotes bistability in networks [44] but additional interactions linking of the activation and inhibition provide the necessary nonlinearity to stabilize the on/off state [45-48]. Figure 1b depicts several examples of motifs that are considered responsible for the regulatory functions that generate discontinuous bistable dynamics or oscillations [49].

Network motifs are dynamic building blocks

Network motifs, like bistable switches and oscillators, form the basic building blocks of dynamic behavior. A common approach to understand the underlying biological phenomena uses a mathematical model that consists of coupled nonlinear ordinary differential equations (ODEs) [50-53]. Feedback loops, in fact, are simply interactions based upon elementary mono- and bimolecular chemical reactions that are subject to the same chemical laws as classical reactions [54]. As such, the motifs summarized in Figure 1b can be translated into stoichiometric reaction schemes. Under the assumption of spatially homogeneous conditions, the dynamics can be fully described by the rate equations in the subsequent column [49,55].

The practical realization of dynamic properties in such reaction schemes is daunting in part because it also requires the systems to operate far from equilibrium [56,57]. A venture beyond the confines of equilibrium, however, does not require deeper

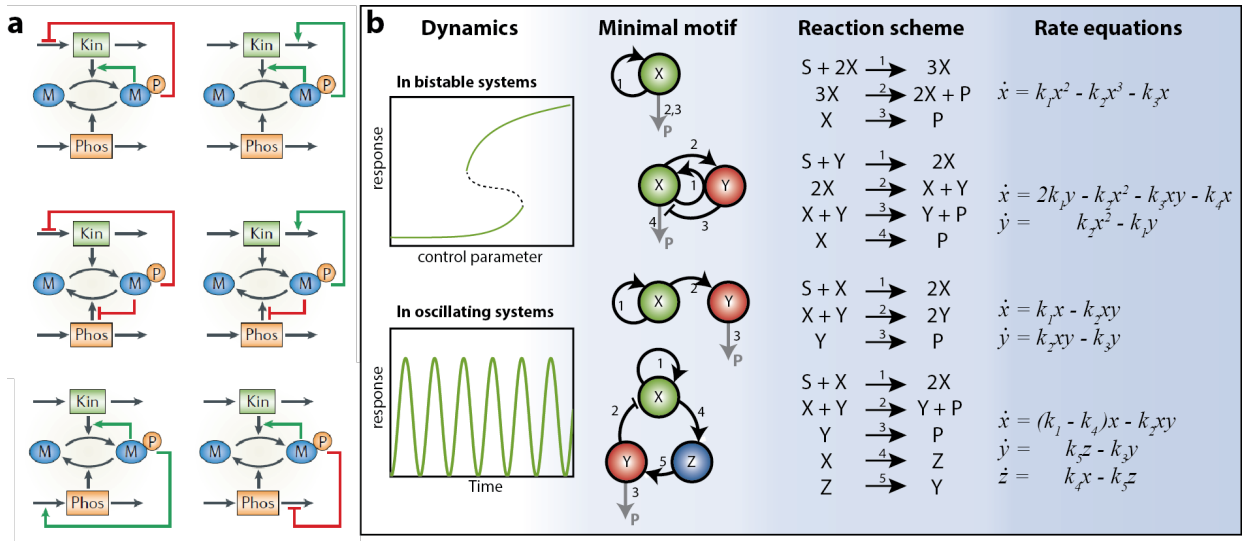


Figure 1: Network motifs. (a) Examples of network motifs composed from different feedback loops. Each design can turn a universal signaling cycle into a bistable switch and relaxation oscillator. Adapted with permission from [24], copyright 2006 Nature Publishing Group. (b) Minimal network motifs that describe the dynamics of minimal bistable or oscillatory systems using mass-action kinetics [49].

understanding of the thermodynamic laws in nonequilibrium systems. Open systems allow environmental conditions to influence the accessibility or stability of the final state, marking the key difference between systems *in* and *out* of equilibrium [58]. Although their behavior is only predictable by a full understanding of the exact ensemble of rate equations, the steady state solutions satisfy the same algebraic equation that controls equilibrium state solutions: $0 = dx/dt = dy/dt (= dz/dt)$ [59]. To keep the chemical system from reaching equilibrium (i.e., in a thermodynamically open system), the implementation of CRNs often suffices with the assumption of an excess of a source (S) combined with a product (P) that acts as a sink. In such dissipative conditions, reactions do not necessarily settle for the state with the highest entropy but instead are drawn towards a steady state.

From network motifs to dissipative systems

Classical example of a chemical dissipative system

Network motifs can guide the design of CRNs, but first, we need to develop an intuition for the components that make up a network motif. The Belousov–Zhabotinsky (BZ) oscillating reaction is arguably the best-known chemical network (Figure 2a) [60]. As a prototypical out-of-equilibrium system, the BZ reaction provided the fundamental and experimental basis for nonlinear chemistry [61-64]. Studies as diverse as synchronization in coupled systems, oscillatory Turing patterns, and spatio-temporal chaos show that the rich dynamics depend solely on how energy dissipates from the system, initiated by local instabilities [65-67].

We must learn how to apply retrosynthesis to chemical reaction networks such as the BZ reaction. The reaction scheme in Figure 2b shows that the BZ network comprises five reactions that can be translated into three inorganic processes in acidic conditions: (1) autocatalytic production of $HOBr_2$ (X) in the presence of Br^- (Y), (2) oxidation of the cerium catalyst, $Ce^{3+} \rightarrow Ce^{4+}$ (Z), and (3) the regeneration of Br^- and Ce^{3+} fueled by the oxidation of malonic acid (MA) [68,69]. Translation from the reaction scheme or equations (back) to the network motif, however, is far from intuitive. Hence, despite its beauty and obvious potential for making exciting discoveries, the BZ reaction (and similar classical chemical systems [70-73]) lack bottom-up design opportunities. Furthermore, the incorporation of a wider range of (organic) chemical reactions is challenging due to the aggressive nature of the medium and reactants.

Chemical dissipative systems based on tunable organic structures

The more recent work is focused on building chemical dissipative networks from organic structures. This has resulted in numerous exciting examples, ranging from functional out-of-equilibrium systems that can perform logic operations to dissipative self-assembling structures, creating new forms of smart materials (Figure 3a–d) [31-35,74-77]. The underlying principle of compartmentalization, dynamic combinatorial chemistry, and hydrogelation also appears in different types of networks [78-84]. Chemical networks can be readily made from tunable organic structures, holding considerable potential in the chemi-

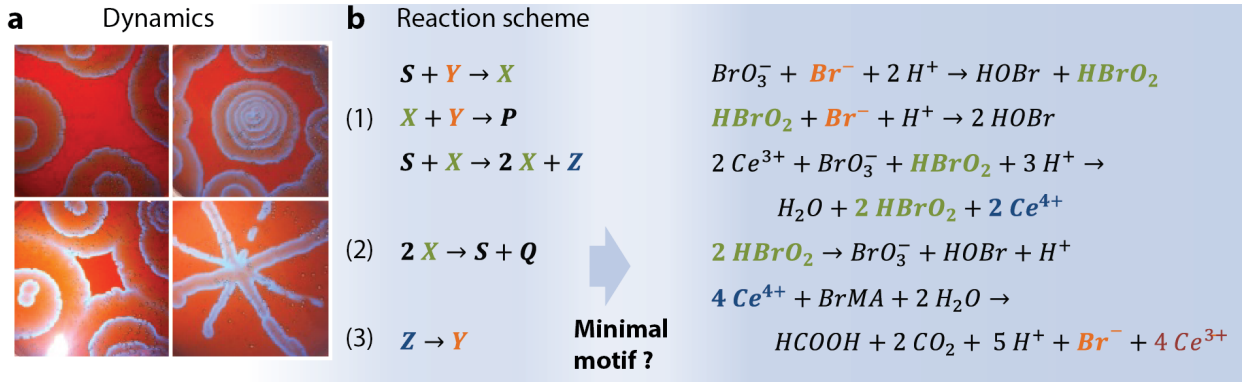


Figure 2: Belousov–Zhabotinsky (BZ) reaction. (a) Classical example of pattern formation in the BZ reaction when perturbed with a silver thread. Adapted with permission from [60], copyright 1970 Nature Publishing Group. (b) The multitude of reactions in the BZ reaction can be reduced to the Oregonator, a three-variable scheme (with key species X = $HBrO_2$, Y = Br^-, and Z = Ce^{4+}) [68,69].

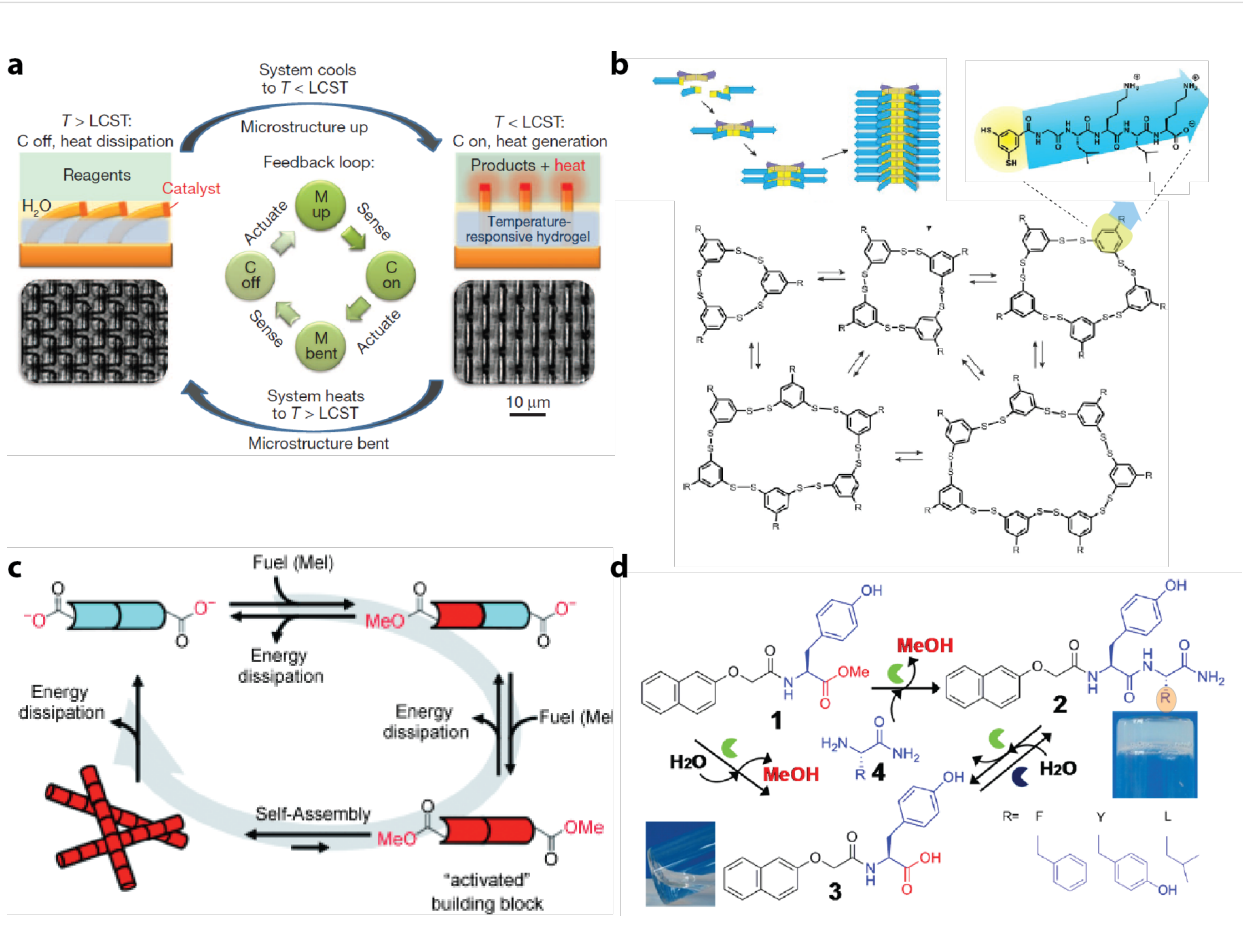


Figure 3: Examples of synthetic dissipative systems. (a) Feedback cycle of a bilayer network composed of the mechanical action of a temperature-responsive gel coupled with various exothermic reactions. Reprinted with permission from [34], copyright 2012 Nature Publishing Group. (b) Small dynamic combinatorial library made from dithiol building blocks. Adapted with permission from [75], copyright 2013 American Chemical Society. (c) Self-assembly fibrous structures fueled by molecular gelators. Reprinted with permission from [76], copyright 2010 Wiley-VCH Verlag GmbH & Co. (d) Biocatalytic self-assembly in the presence of chymotrypsin (green) forming hydrogelators that can be modified by the choice of amino acids depicted in the bottom right side. Reprinted with permission from [77], copyright 2013 American Chemical Society.

cal sciences for the development of a new approach to the construction of out-of-equilibrium molecular networks.

A remaining key challenge encountered in the experimental realization of robust steady-state output in such systems is to balance the reaction rates between various feedback loops in the network. Despite the advances made, the behavior in reactions while approaching equilibrium (Figure 3c,d) is transient and not a reflection of a dynamic steady state. Hence, the bottom-up construction of chemical reaction networks requires more than convenient chemical components. A general methodology is needed that integrates the thorough appreciation of reaction rates in the design of chemical networks.

Learning from the design principles applied in synthetic biology

Genetic and small DNA-oligonucleotide networks provide an ideal test bed to address the basic principles of designing (bio)chemical complex systems [85-87]. Figure 4a shows the successful translation of an earlier discussed network motif into a molecular predator (P)–prey (N) network [88,89]. The information concerning the predator and prey growth and degrada-

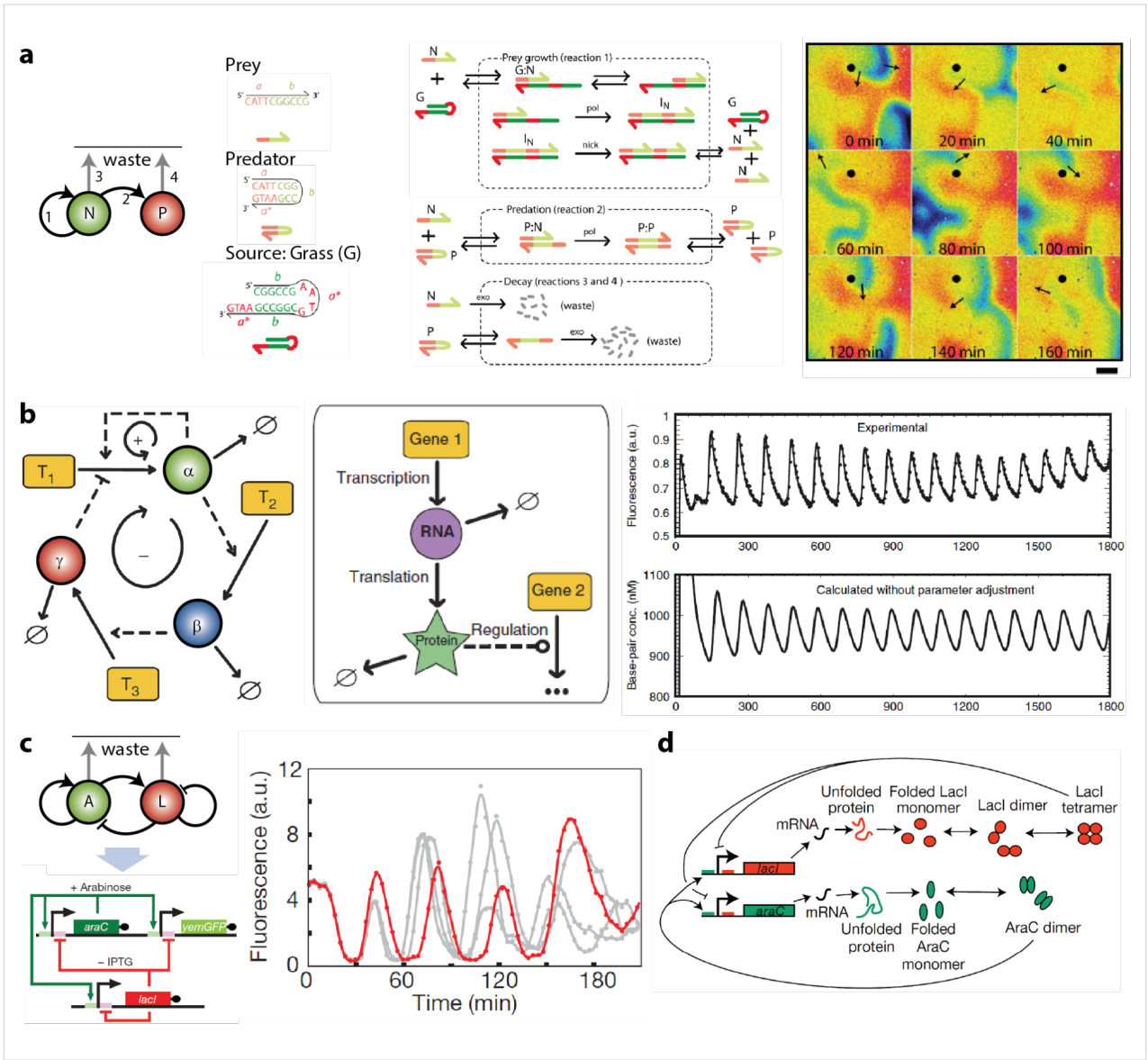


Figure 4: Design principles applied in synthetic biology. (a) Network topology, mechanism, and the clockwise-rotating spiral of the prey in the molecular predator–prey network. Adapted with permission from [88], copyright 2013 American Chemical Society. (b) The gene regulation pathway and of an oscillator based on a positive and delayed negative feedback motif, with experimentally observed oscillations shown to be in good agreement with the simulations. Adapted with permission from [90], copyright 2011 the authors. (c) Oscillations in the dual-feedback motif. (d) Illustration of the explicit intermediate processes required for accurate simulations in the mathematical modelling of genetic reaction networks. Adapted with permission from [91], copyright 2008 Nature Publishing Group.

tion is stored in single-stranded DNA (ssDNA). Importantly, the reaction scheme and rate equations could be approximated based on the predictability in the thermodynamics of DNA binding. In presence of an excess of the source ssDNA (denoted by G for "Grass"), traveling waves of a predator–prey molecular network (similar to the spatio-temporal patterns in the BZ reaction) were obtained. In stark contrast to the BZ reaction, however, the use of DNA or DNA-enzyme-based in vitro systems are amenable to rational design.

Other approaches in synthetic biology use gene regulatory networks. Gene regulations provide both conceptual simplicity and modularity to design networks exhibiting oscillatory behavior. Within this framework, Figure 4b shows an in vitro implementation of an oscillator comprising a positive and a delayed negative feedback loop [90]. The canonical gene regulation pathway uses the information encoded in DNA templates T_1–T_3. Similarly, a genetic oscillator can be engineered in *Escherichia coli* [85-87]. Figure 4c shows the network composed of AraC, LacI and yemGFP genes [91]. The additional yemGFP gene serves as a read-out component and is not depicted in the regulatory network motif.

Together, the examples in Figure 4 demonstrate that complex dynamics could be achieved by transcription and translation processes. Dissipation arises from an approximated constant supply of nucleotides, amino acids, and enzymes among other cellular machinery (see Figure 4d) [92]. Arguably, the ability to rationally assemble test tube CRNs lags behind that for in vivo systems due to difficulties faced in mimicking such cellular composition [93]. Consequently, in molecular "circuits" based on DNA as building blocks, certain reaction rates are often not known, cannot be known, or cannot be tuned easily.

Blueprint for the construction of chemical complex systems

A chemical approach, in contrast to synthetic biology, might involve the construction of a network of individual reactions that are well-characterized where the key kinetic parameters can all be experimentally verified. We recently showed that a chemical reaction network can be designed using enzymatic reactions combined with the tuning of the reaction rates in (small) molecules [94]. The initial point of the design was to select a network motif for which the steady state output is known. Our network combines a positive and a delayed negative feedback loop (Figure 5a) that is built around a key enzyme E_1*. In the reaction network, trypsin (Tr) catalyzes its own formation from the precursor trypsinogen (Tg). Opposed to this positive feedback, Tr is inhibited by the negative feedback that is composed of three sequential steps (Figure 5b). In the activation step, Tr converts a pro-inhibitor into an intermediate inhibitor (Int-Inh), which consists of a glutamine (Gln) residue attached to a potent inhibitor for Tr. Another enzyme, aminopeptidase N (Ap), controls the release of the inhibitor moiety by cleaving off Gln in the delay step. In the final step, Tr recognition of the active inhibitor (Inh) closes the negative feedback loop.

The network displays complex behavior in an open system. In contrast to earlier examples, we used a continuous flow of the reactants (Tg, Ap, and Pro-I) to create out-of-equilibrium conditions (Figure 5c) [95]. A poly(dimethylsiloxane) (PDMS)-based microfluidic continuous stirred tank reactor (CSTR) was conveniently prepared in which the flow (i.e., the reciprocal of the residence time defined by the ratio of the outflux and the reactor volume) maintained out-of-equilibrium conditions for the system (Figure 5d). The response of the system is determined by the concentration of the time course of Tr. Figure 5e demonstrates that the CRN is capable of producing sustained oscillations.

We further processed the oscillating enzyme activity by coupling the multiple reactor modules, each with a specific chemical reaction. Figure 6 shows the feed forward designs that use an enzyme or a substrate with a high affinity to Tr in a subsequent CSTR. Figure 6a demonstrates that the initial oscillating signal can be used to create an identical timing in a subsequent enzymatic reaction. Depending on the feed concentration of chymotrypsinogen (ChTg), the initial oscillations are amplified. Similarly, the design is used to create an analog-to-digital output by introducing a trypsin inhibitor (soybean trypsin inhbitor (STI)) in the second CSTR (Figure 6b). The STI effectively thresholds the local minima in the initial oscillations, converting the initial signal into a switch-like output, creating a binary signal. Finally, we used oppositely charged polyelectrolytes to form complex coacervates in Figure 6c. Coacervates are formed in the second CSTR only in the absence of Tr, as Tr catalyzes the lysine-functionalized polycation. This demonstrates that the relatively long oscillation periods enable the construction of more complex systems capable of dynamical self-assembly. In this case, it is a dynamic self-assembly that is exactly out-of-phase with the initial oscillations.

Correlating the molecular structure to network behavior

This design strategy enables the chemist to exploit the full power of chemical synthesis. Figure 7a depicts the synthetic sites at which the pro-inhibitor can be altered (R^1–R^4). In general, this allows us to create a "Swiss army knife" out of the source molecule that controls the negative feedback [30]. The possibility to make small synthetic variations provides the controllability to influence the precise rates in feedback loops. Essentially, this flexibly helped us enormously at the stage of

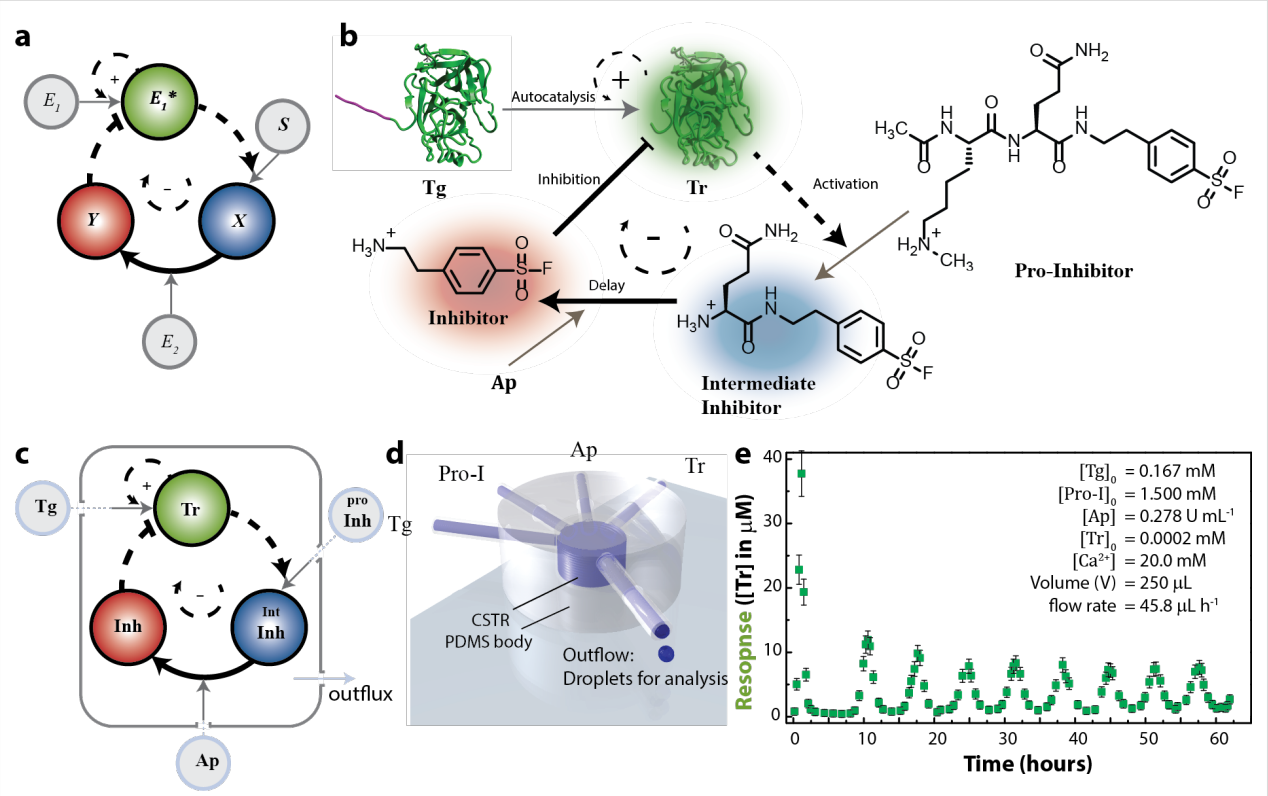


Figure 5: A retrosynthetic design strategy to implement an oscillating enzymatic reaction network [94]. (a) Schematic network layout of the enzymatic oscillator based on a delayed negative feedback motif. (b) Detailed reaction diagram of the CRN. (c) Illustration of the central network motif based on autocatalytic production and delayed inhibition of an enzyme that was kept out-of-equilibrium. (d) Schematic representation of the flow reactor that is used to implement flow. (e) The output of the reactor was measured using an enzymatic activity in an offline analysis. The response shows oscillations in Tr activity (with experimental conditions in inset). Adapted with permission from [94], copyright 2015 Nature Publishing Group.

(1) retrosynthetic screening, as well as (2) in studies correlating the molecular interactions to the behavior in networks at both regulatory as well as at the systems level.

As the networks are "synthesized", it is in principle possible to fully know all the components and reactions in the network. We unambiguously determined the state of the system by measuring the variation in the intermediate inhibitor and the inhibitor in addition to Tr [94]. Such a molecular level understanding of networks ultimately allows us to ask questions about the relationship between individual molecules or reactions and the robustness or resilience of the network that cannot otherwise be asked in other systems [97,98].

Mathematical modelling

Our network is inherently nonlinear, and like most artificial complex systems, analytically unsolvable. The construction of the network combined the design of small molecules with a mathematical simulation of the complete network. Nonlinear mathematical problems that comprise more than three variables are typically difficult, if not impossible, to solve without the reduction of variables [99]. To avoid loss in chemical information, we implemented the full set of rate equations in MATLAB and COPASI that could simulate the trajectory of the individual species by the stepwise numerical integration in time. Importantly, all rate constants were determined from kinetic studies in isolated individual reactions, allowing accurate simulations to test specific details of the experiments.

We used the model to vary the rate constant that is induced by the changes to the molecular structure. First we show in Figure 7b that the tuning of R^1 alters the steady state behavior of the CRN under identical conditions. The qualitative changes in the final state shown here are called bifurcations and show that the subtle changes in the small molecules influence the out-of-equilibrium behavior of the CRN. This analysis is expanded in Figure 7c to find the range of intrinsic (initial concentration of Ap and Pro-I), as well as a global parameters (flow rates), that we can start the experiments with. The grey volume shows the parameter space in which sustained oscillations can be found (i.e., the oscillatory regime). Typically, the CRN is robust to variations in the screened parameters but that there are differences in the size of the oscillatory regime when, for example, the feed concentration of the Pro-I ($[x]_0$) is changed. Repeating

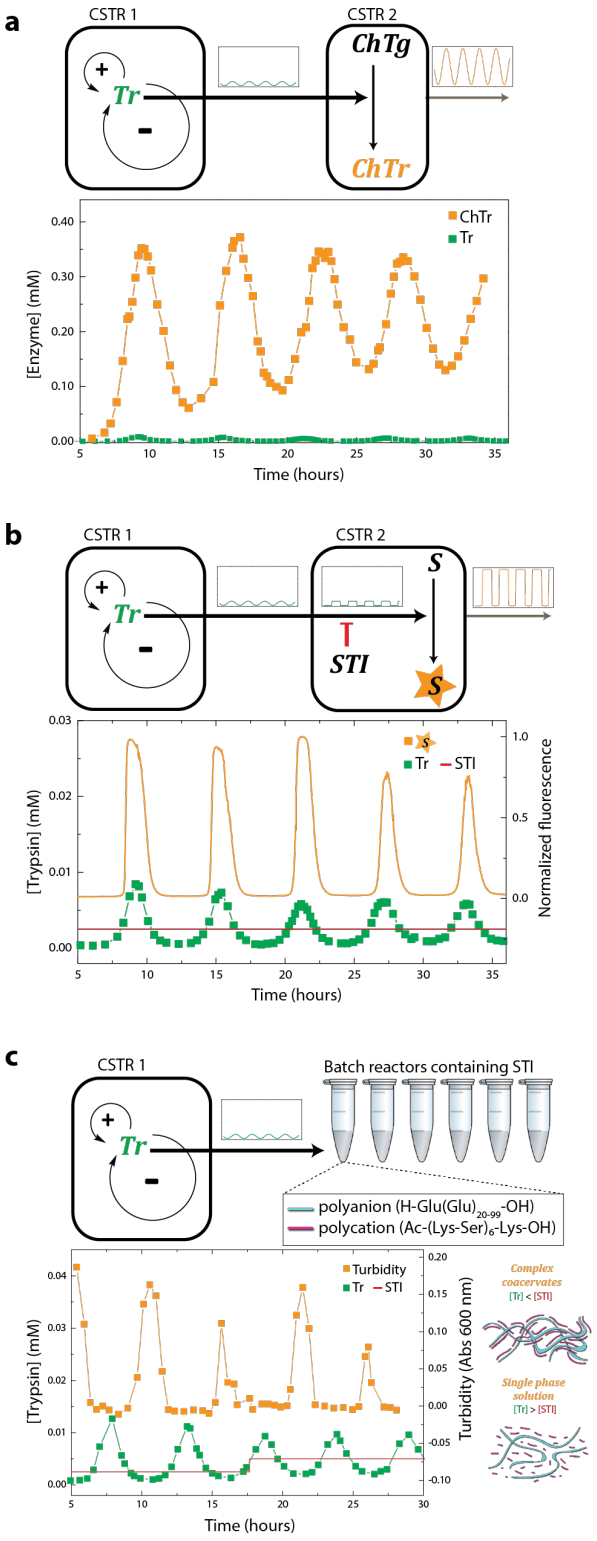


Figure 6: Functions obtained by linking multiple network modules in microfluidic flow reactors (depicted as CSTR 1 and 2). In each case, the oscillating catalyst concentration [Tr] from CSTR 1 is coupled to another CSTR that contains reagents producing (a) an amplification, (b) analog-to-digital conversion, or (c) a periodic control over equilibrium systems. The respective processes are: trypsin-catalyzed conversion of chymotrypsinogen (ChTg) to chymotrypsin (ChTr), trypsin-catalyzed conversion of a rhodamine substrate (S) to a fluorescent product (P) in the presence of a strong inhibitor (soybean trypsin inhibitor, STI), and trypsin-catalyzed fragmentation of a polycation (in purple) in the presence of a polyanion (in cyan). Adapted with permission from [94], copyright 2015 Nature Publishing Group.

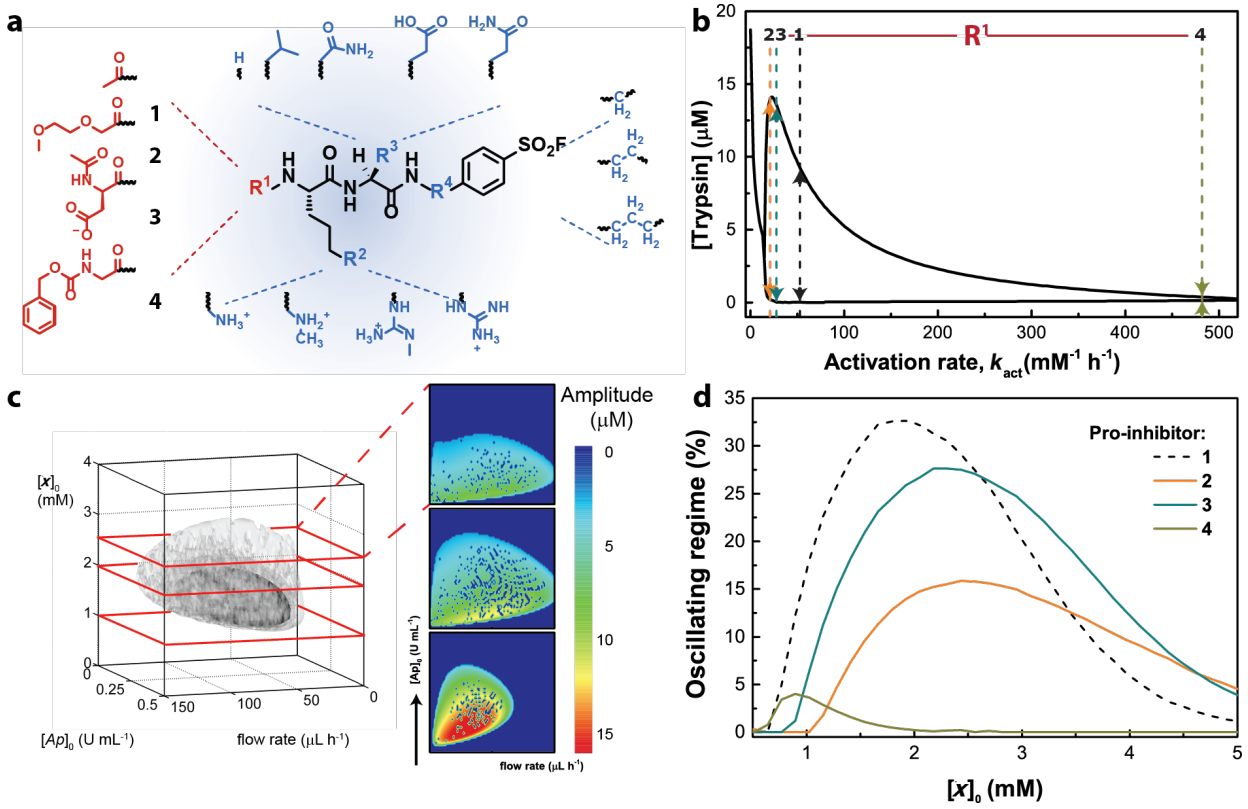


Figure 7: Influence of molecular structure on the properties of CRNs. (a) Molecular "Swiss army knives" showing the opportunities for tunability in different enzymatic reaction networks. (b) Bifurcation diagram with the control parameter activation rate of pro-inhibitors that were modified at position R^1 (in red). (c) Phase plot analysis showing regimes of stable steady states are stable limit cycles, with the latter depicted as a grey volume. (d) This was used to predict the performance of the pro-inhibitor under flow conditions. Adapted with permission from [96], copyright 2015 American Chemical Society.

this analysis for the different substituents depicted in Figure 7a reveals that both the size of the oscillatory regime as well as the optimal $[x]_0$ for **1**–**4** differs significantly (Figure 7d) [96]. Hence, the use of mathematical modelling is an imperative tool that allows guidance to the appropriate conditions to produce sustained oscillations with **1**–**4**.

Conclusion

Natural phenomena are enormously complex networks. Nonetheless, such systems remain an ensemble of smaller networks of molecules. Historically, our (dis)ability to comprehend the apparent complexity pushes science to develop theories to solve problems which were thought to be analytically unsolvable (e.g., classical or quantum mechanics) [100]. The development of the field of complex systems science will most likely follow a similar pattern, where we will get a grip on systems of increasing complexity. In this development, the rapid progress of computational methods will most probably allow us to tackle ever-larger complex systems.

This perspective, however, urges an approach using a synthetic strategy based on the stepwise build-up of complex molecular systems. We envision the development of a toolbox that allows us to go beyond describing and understanding systems, extending to the rational design of function arising from a collection of molecular network motifs. In this respect, we believe that the complexity of future complex and functional molecular systems is by no means restricted to the network motifs and the organic chemistry we have introduced here. We conveniently made use of the specificity as well as the high turnover numbers in enzymatic reactions as a starting point to test the implementation of our design strategy. A more recent example of a chemical network capable of auto-amplification using thiols and thioesters (Figure 8) provides the ultimate proof that complex molecular systems can be designed "from scratch" [101].

Future "synthetic moves"

We hope that the method developed here allows researchers, and especially chemists, to address important features of self-

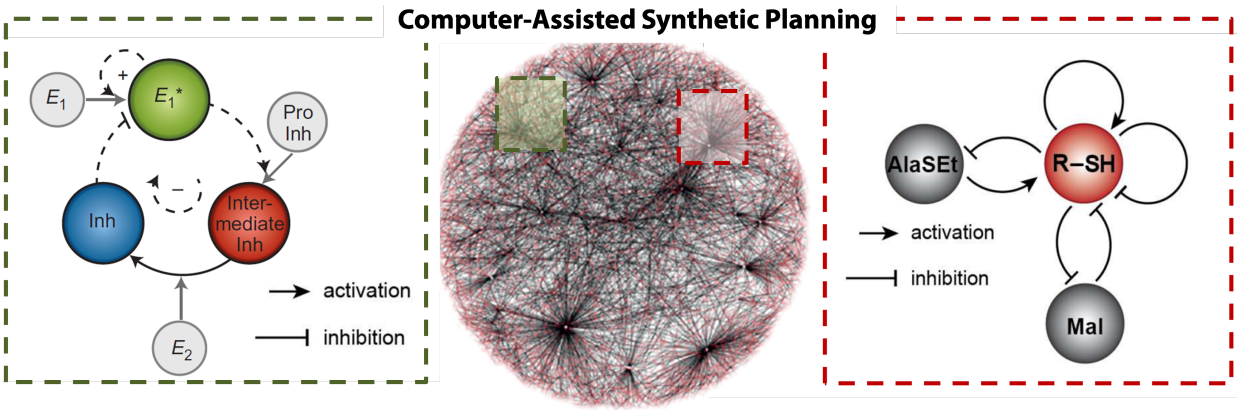


Figure 8: Network motifs as building blocks for the step-wise build-up of complexity. Chemical reaction networks are central to the future synthetic moves in systems chemistry. Adapted with permission from [94] copyright 2015 Nature Publishing Group, [101] copyright 2016 Nature Publishing Group, and [102] copyright 2016 Wiley-VCH Verlag GmbH & Co.

organization in complex systems. We briefly showed how the tuning of molecular structures allows one to explore the robustness of CRNs. From this perspective, other intriguing questions that still remain to be answered on the transition from non-living to living systems are: which molecules should we select from the vast pool of molecules available? Which structures allow networks to gain greater robustness and resilience? How do these systems find their steady state behavior? What trajectories do these systems take when they transition from one state to another? We fully expect these questions could move our focus from "how to build a complex system?" to "how can they emerge in a competitive or a fluctuating environment?" to "how could we employ control over a network in the presence of other networks?".

The interactions among individual components in CRNs can change over time and space [103-107], enabling regulatory functions to emerge that are dynamic and have limited predictability. The major challenge for systems chemistry is to translate the design principles of living systems into a practical "programming language". Computer-assisted approaches will undoubtedly aid the future plan for "synthetic moves" for complex systems [102]. Altogether, the syntheses in the context of complexity could provide a truly molecular-level insight into how chemical reactions create functionality, and ultimately, how molecules create life.

Acknowledgements

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Chemical systems, chemical contiguity and the emergence of life

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Review

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Abstract

Charting the emergence of living cells from inanimate matter remains an intensely challenging scientific problem. The complexity of the biochemical machinery of cells with its exquisite intricacies hints at cells being the product of a long evolutionary process. Research on the emergence of life has long been focusing on specific, well-defined problems related to one aspect of cellular make-up, such as the formation of membranes or the build-up of information/catalytic apparatus. This approach is being gradually replaced by a more “systemic” approach that privileges processes inherent to complex chemical systems over specific isolated functional apparatuses. We will summarize the recent advances in system chemistry and show that chemical systems in the geochemical context imply a form of chemical contiguity in the syntheses of the various molecules that precede modern biomolecules.

Review

Introduction

Research in the origins of life field or abiogenesis (emergence of life from non-life) attempts to answer a question that has fascinated humanity for millennia: Where do we come from? Whereas early attempts were more metaphysical in nature, insights into the nature of living systems with the discovery of cells as the basic unit of life and more recent advances in the understanding of the inner workings of its biochemistry have transformed the question into a scientific, empirical endeavor with two complementary goals. One is to explain of the emergence of contemporary cells through historical reconstruction,

i.e., the construction of chemical models called protocells [1] (Figure 1); the other is to mimic cellular architectures to create artificial cell-like entities in relation with various applications that range from medicine to environmental remediation, over chemical/biological manufacturing [2].

The main challenge in the historical reconstruction is the scarcity of, occasionally even contradictory, information about i) the early Earth, both in terms of environmental conditions and chemical inventory, and ii) the putative transitions that must have been involved to convert a dynamic, molecularly diverse chemical environment into a coherent, interconnected network of chemical processes, leading ultimately to contemporary biochemistry. Even when a deconstructive (top-down) approach, i.e., the attempt to simplify the current biochemistry towards a simpler origin, is used, the fact that contemporary biomolecules and biochemical molecular assemblies, and their precursors themselves are likely optimized products of a long evolutionary process [4] renders this endeavor quite difficult. Hence, researchers in the field have tended to pursue alternative approaches in relation to the emergence of specific biomolecules and biochemical assemblies. The pursuance of such, normally parallel, approaches has led to the development of hypotheses either called by their chemical embodiment, such the lipid- [5], PAH- (polycyclic aromatic hydrocarbons) [6], and RNA-worlds [7], or designated by a general concept such as the metabolism- and gene-first scenarios [8]. This multi-faceted approach (Figure 2), whilst suffering somewhat from a lack of effective integration or cohesion, has nonetheless permitted the accumulation of essential insights in the characteristics of various biomolecules, e.g., the catalytic activity of RNAs and their evolution potential [9-11], as well as processes that were

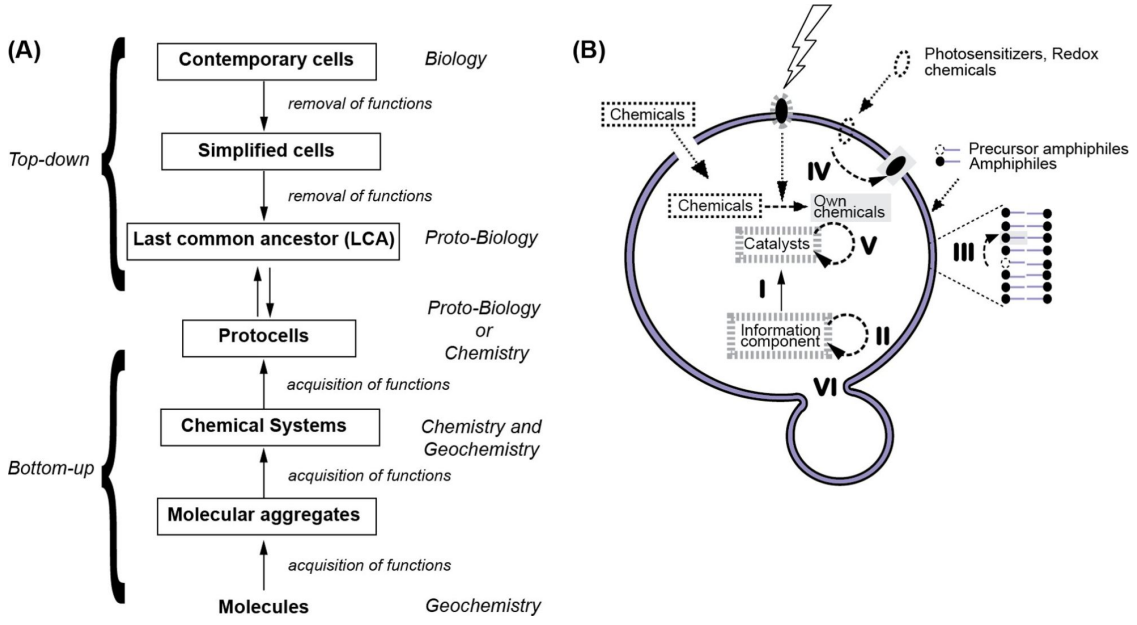


Figure 1: (A) Possible approaches to the historical reconstruction. Two complementary approaches exist: top-down and bottom-up. In the former, the idea is to simplify the cellular architecture and cellular biochemistry by removing redundant or dispensable functions. These are functions that can be either replaced by providing chemicals or taken over by simpler chemicals easily synthesized by, e.g., "non-coded" protein catalysts, or performed, perhaps less efficiently, by other catalysts in the cells. The process should be repeated until a very simple putative "protocell" stage (vide infra) is attained. This is likely a point in time at which biology did not yet exist, but instead pure chemistry defined the protocellular reaction network. The latter approach is based on the use of molecule sets that can self-assemble into chemical aggregates and systems that will then be able to perform an increasingly more complex chemistry. These systems are precursors of protocells that preceded the emergence of ancestral cells. (B) Putative representation of a protocell (adapted from [3]). Independently of the type of chemicals involved, e.g., pure RNA catalysts/"genetic" information or peptide/RNA, a protocell should contain three components: a compartment, a catalytic and energy harvesting machinery, and an information system. These components should work in an interconnected fashion to achieve the prolonged activity necessary for the protocell evolution. The interconnectedness in the systems is visible if one considers the various arrows between molecules/components: The catalytic machinery is defined and controlled by the information component (I) and the compartment (via encapsulation), whose molecular species are in turn produced by the catalytic machinery (II: information replication, III: amphiphile production, IV: energy harvesting and chemical replication, and V: catalyst amplification, which can lead to VI: replication process of the whole protocell). The compartment will also define the access of the protocell to environmental resources and, in part, the energy harvesting capabilities. It will also be instrumental in the replication (VI). It might also permit an interface-driven multiphase chemistry (see text below). Molecular precursors (i.e., resources to build protocell chemicals) are highlighted by black dotted structures or frames. Original chemicals of the protocell are highlighted by thick grey dotted frames. Products of the catalytic machinery are placed over a grey background. The involvement of catalysts is depicted by dashed arrows, that of information components with a plain arrow, and that of the compartment (expect the encapsulation) by dotted arrows. Note that the energy-related aspect would be involved in all chemical syntheses but, for the sake of clarity, is only shown once.

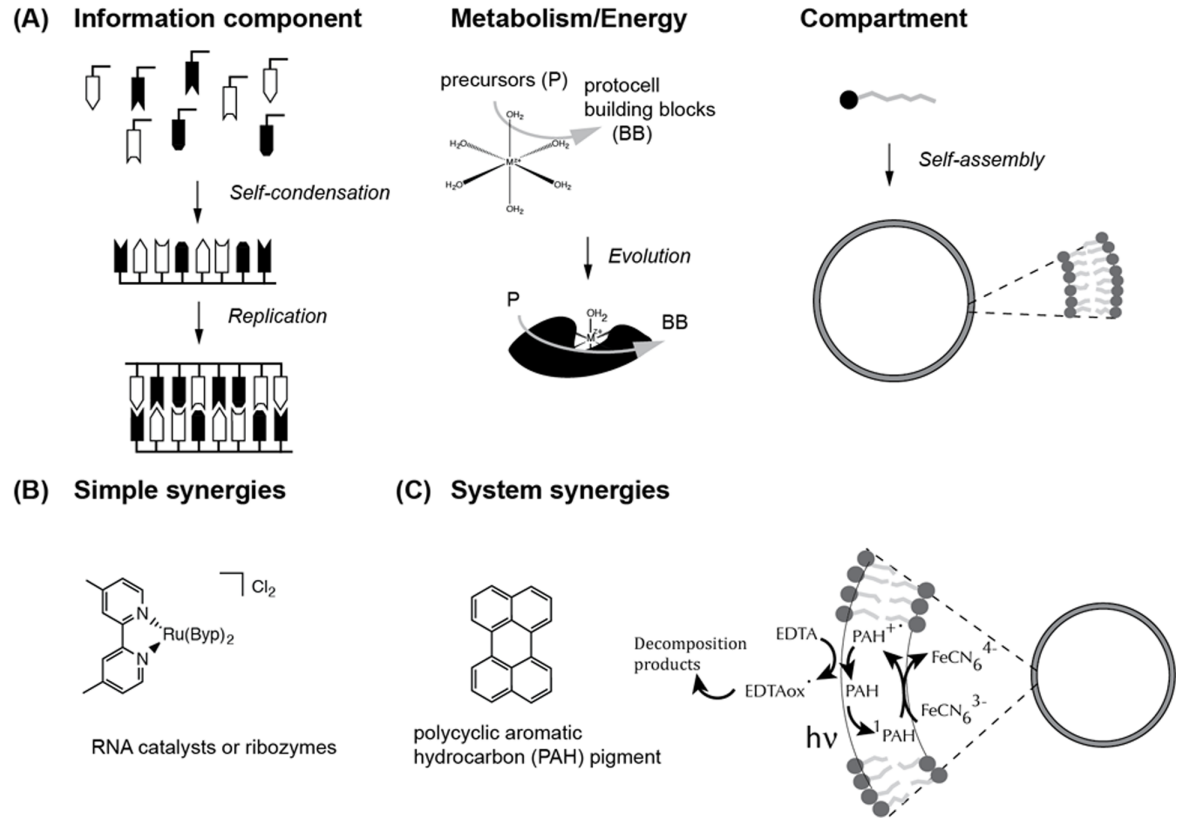


Figure 2: The bottom-up approach research strategies. (A) Each protocell component (vide infra) can be investigated in "isolation" to better understand the various processes pertaining to its synthesis/formation: information polymer, functional catalysts or self-assembly/stability of the compartment. (B) A higher degree of complexity can be attained by using chemicals that by themselves already link to two component functions: for instance, metal complexes that can harvest light (here a ruthenium tris(bipyridine) and catalyse reactions, or polymers such RNA ribozymes that are both genotype (information component) and phenotype (catalyst). (C) The systems approach offers insight into the increased level of cooperativity necessary to grasp the complexity of living interactions. The creation of chemical gradients, for example, requires the presence of a compartment and an energy harvesting system. In the case of PAHs, which are sparingly soluble in water, the compartment boundary not only allows for a distinction between two aqueous volumes, it also increases the availability of the PAH molecules by providing a specific hydrophobic environment for their solubilization, thereby improving the energy conversion.

essential for their syntheses, such as Fischer–Tropsch-like reactions [12], non-enzymatic RNA [13] or peptide polymerization [14]. Moreover, it has also allowed for the determination of environmental conditions conducive to the self-assembly of several cellular-like components, such as bilayer membranes [15] and simple energy systems [16], or dynamic processes, such as growth and division [17,18] and potential evolution [19]. However, the experimental set-ups during these investigations have often been optimized to yield the best possible outcome rather than allow for chemical diversity and integration to "evolve" as a function of time, energy and molecular inputs.

This modular research mechanism, where themes are explored in relative isolation has clear limitations when these various "prebiotic" molecular systems are to be consolidated in a single protocell model. Moreover, situations emerge where one line of experimental enquiry becomes at odds with another feature that is equally integral to the whole. An example of this involves the selection of RNAs for catalytic activity, which often requires the presence of high ion concentrations that are disruptive for the formation of primitive membrane models. Membranes composed of putatively prebiotic amphiphiles, such as single hydrocarbon chain species [20,21] may have been exemplars of such membrane components. Furthermore, experimental conditions are sometimes implausible from the geochemical perspective. Finally, the evolutionary continuity of the systems, which should be paramount to explain the emergence of protocellular systems and evolution towards true cells, is often neglected in these experiments.

This short, necessarily selective, overview clearly underscores the necessity of new approaches, a fact that has led many researchers to propose the concept of chemical systems [22,23].

That is, the origin(s) of life, which is(are) hallmarked by the appearances of emergent properties (capacity of self-maintenance, self-replication and evolution under external constraints), should be investigated using a systemic approach where the functionalities in a chemical mixture are derived from the multiple interactions or "interconnected work" that exists between the various chemical processes. This approach has the advantage of allowing for the emergence of chemical interconnections between the various biomolecular classes, which should explain the deep interconnection between cellular subsystems, and implies the fact that the various molecular systems in cells might have co-evolved in relation to a specific geochemical environment. It also encompasses an important, often neglected, dimension: the fact that mixtures of disparate molecular classes imply a certain chemical contiguity in their syntheses. From the point of view of chemical research, a systems approach has, however, one obvious drawback: One should not expect the usual high reaction yields and chemical purity for the products. This fact highlights a fundamental difference in granularity of vision between traditional synthetic chemistry and systems chemistry in a prebiotic context. Whilst yield, purity, and conversion rates are key drivers of synthetic chemistry, those drivers for prebiotic systems chemistry appear to be less important than integration, contiguity, auto-catalysis and periodicity.

In this short article, we will first attempt at defining chemical systems and chemical contiguity. Then, using recent reports on chemical systems, we will highlight the potential of the "chemical system" approach for the investigation of the origin of precellular systems and protocells.

What are chemical systems?

Chemical systems are defined here as chemical mixtures comprising a network or set of interacting molecules. That is, system-dependent behavior and the system processes cannot be ascribed to any of the components acting in isolation. For instance, the catalysis by a metal complex in a bulk medium is inherently dependent on the nature of the chemicals (catalyst and substrates). However, if the catalysis is only possible in the presence of a third substance, not per-se involved in the catalytic process, but nevertheless necessary for it because it acts to organize the reactants, then one observes a chemical system. In a mathematical sense, chemical systems are sets or a collection of distinct objects/molecules, considered as an object in their own right.

Using this rather inclusive definition, a chemical system can be composed, in its simplest manifestation, of very few molecules also incorporating elements of their geochemical environment. At first glance, this definition seems too broad in terms of system composition. But the important aspect of the definition should in all cases remain the emergent properties, namely interconnectedness of the system and how the system behaves, rather than the contingent chemical composition of the system processes.

What is chemical contiguity?

The notion of chemical systems also implies the existence of chemical contiguity. Many aspects of cellular biochemistry, e.g., in bioenergetics, glycolysis, the Krebs cycle or the intricate peptide formation systems, pre-suppose a form of chemical contiguity in their emergence. The Oxford English Dictionary defines contiguity as "the condition of touching or being in contact whether physical or non-physical". In the chemical context employed here contiguity is seen as a connected gradient of physico-chemical conditions through which the different components of a chemical system (or "set" as above) can be synthesized and achieve their connectivity.

System chemistry and chemical contiguity in the geochemical context

Geochemistry in conjunction with extra-terrestrial delivery of compounds must have defined not only the types of molecules that were present on the early earth, but also the molecular composition of early chemical systems and by extension that of protocells and contemporary cells. Furthermore, the environmental conditions must have defined the potential reactivity of these compounds. While these statements are agreed upon, the exact environmental parameters, i.e., chemical composition, temperature or availability of light energy, and the global geological make-up, for instance, a water-immersed mineral-[24] continent-island [25] or ice-covered earth [26] remain highly debated because of the lack of direct evidence. Interestingly, the experimental studies that attempt to link environmental conditions and chemical processes deemed essential for the emergence of life show that whatever the actual conditions, one can in many cases demonstrate that these diverse environments can foster comparable processes. In most cases, the type of chemistry envisioned can be categorized as heterogeneous catalysis [27] and ultimately periodic. There are reports of chemical synthetic continuity in aqueous solutions, but under conditions that seem to be unlikely in the geochemical context [28].

Thus, short of proposing a global, environmentally anchored solution to the syntheses of all molecules necessary for life to emerge [29], distinct geochemical environments could have not only produced specific chemicals, but could also have contributed to their evolution at different stages.

For instance, the idea of RNA polymers as information components, precursors of a genetic system, can be partially realized: Monomers can be efficiently polymerized in salt eutectic [30] and ice/water systems [31,32] or on mineral surfaces [33] or likely in porous mineral formations, i.e., formations that are presenting embedded channels or cavities within the minerals, where their accumulation has been suggested possible [34]. However, caution should be exercised over in extrapolating what is a computational study [34] to experimental scenarios. Moreover, the same environments are likely conducive to the function and evolution of these RNA polymers towards higher catalysis. In this case, direct evidence only exists for the eutectic phase in water/ice [35-37], but computer modelling [38] and preliminary wet-chemistry experiments, which show a selective accumulation of long oligomers [39], already hint at the possibility of similar processes taking place in mineral formations. In the same environments, short peptides, which are potential functional catalysts, can also be synthesized from simple amino acids [40]. Indeed, dipeptides can catalyse RNA oligomer formation in the eutectic phase of water/ice [41], underscoring another possible chemical contiguity within the geochemical context.

The ubiquity of polyphosphate in bioenergetics, but also of phosphate in cellular sensing and, in general, in the composition of some essential synthetic cellular products also suggest a common origin for the involvement of phosphate, that is, a form of synthetic contiguity [28]. This ubiquity of phosphoesters, mostly as phosphorus (P) in +5 oxidation state, is puzzling to some extent as this element is today a limiting nutrient for life [42]. But the prebiotic availability of P is now being far better understood [43-45]. In addition, the reactivity of phosphate and polyphosphate is low in aqueous media in the absence of catalysts, which affords a barrier to these species having been instrumental in the origins of life [7]. However, the reactivity of pyrophosphites (P with a +3 oxidation state) [46,47] is large enough to concomitantly permit phosphorylation reactions to activate small chemicals, as such as amino acids and permit their oligomerization, as well as to synthesize other compounds essential to life, such as amphiphiles, the proposed building blocks of prebiotic compartments, which can then self-assemble into vesicles under the same experimental conditions [48]. Pyrophosphites could thus be considered a common precursor energy currency for prebiotic catalysis, the activity of which is likely to be broader than these two chemical examples.

Mineral surfaces and porous matrices can also induce the formation of chemical systems of potential interest in the context of the origins of life. Several research groups have demonstrated their abilities to induce formation of evolved protocell systems. For instance, they have been shown to be capable of accumulating small molecules on their charged surfaces (electrostatic interactions) [49] or within pores and brines by thermophoresis and convection processes [50]. In the case of amphiphiles, these phenomena lead to the formation of compartments by self-assembly, which can encapsulate other solutes, e.g., RNA [17,51]. The accumulation ability of porous minerals allows for the amphiphile concentration to surpass their critical vesicle concentration to effect self-assembly [51]. Thus, mineral surfaces and porous formations could have been excellent media to foster the emergence of "self-contained", dispersed chemical systems.

Furthermore, mineral surfaces can serve as supports for chemical systems to undergo organization. The polymerization of nucleic acid monomers has been achieved in this manner: When amphiphile vesicles or liposomes are dried in the presence of solutes on a silicate support, a system of stacked lipid bilayers with intercalated solutes is formed [52]. In this arrangement, the nucleotides are optimally spaced to react and form nucleic acid oligomers [53-55]. The presence of the mineral support is crucial here as it permits the preservation of the amphiphile bilayer structure during drying, thereby promoting the conversion of an "unreactive" organization (free floating vesicles and free monomers) into reactive chemical systems (stacks of alternating amphiphile bilayers and monomer layers). In stark contrast to the polymerization of RNA on montmorillonite, the absence of strong direct interactions between the mineral surfaces and the molecular species does neither reduce the chemical availability of the reaction products, nor preclude the "re"-dispersion of the lipid phases into dispersed aggregates with encapsulated catalysis products [52].

Chemical systems and chemical contiguity in the dispersed state

The chemical systems aspect during the emergence of cell-like entities can also be highlighted once the chemical systems become dispersed; i.e., once a stage in chemical evolution is reached where self-propagating, chemically simple compartmentalized systems have emerged [56]. As mentioned earlier, the expectations when approaching the question of life origins from a chemical system point of view are related to the emergence of properties that are systemic in nature. The different properties can occur at various levels: i) Systems are able to segregate chemicals, thereby explaining why a class of molecules or specific molecules have been selected or discarded during chemical evolution; ii) systems are able to allow for the physical organization of molecules into functional catalytic/information networks; iii) systems foster evolutionary processes by maintaining chemicals in close proximity, that is, at physical distances permitting their further reactivity, while allowing for reaction wastes to be disposed of, and finally

iv) systems could have conditioned the proliferation of functional systems.

Chemical selection

The investigation of synthetic pathways to biochemically relevant molecules has clearly underlined the need for some form of selection. Indeed, molecules of interest (nucleobases [57], sugars [58], amphiphiles [59]) are usually synthesized as minor products within a larger collection of derivatives even in the case of polymeric products, e.g., RNA analogues are formed with varying phosphodiester-bond regioselectivity [32]. The time frame in which this selection occurs is still uncertain, as are the "processes" that led to the selection. While the selection of fatty acids is undisputed as they are the main constituents of the hydrophobic core of modern membranes, their involvement in forming protocell compartments as the only type of amphiphiles can be disputed. Indeed, other amphiphiles or co-surfactants, if available via prebiotic syntheses [20,60-62], could have also contributed to the formation of primitive amphiphile-based structures, by allowing structure stabilization under prebiotic conditions, e.g., high ionic strength or temperature or stringent pH values.

Selective association of chemicals with fatty acid vesicles demonstrates that chemical systems, even simple ones, could have spawned such a selection by conditioning the interactions between their molecular constituents. For instance, canonical nucleobases interact more extensively with the vesicles structures than some of their derivatives and even stabilize them [63]. The same observation was made for ribose over other sugars. Moreover, when the permeability of fatty acid vesicle bilayers towards sugars was examined, ribose was determined to have the highest diffusion rates among aldopentoses or hexoses [64], a fact that could also explain its selection for the backbone of nucleic acids.

Catalysis support

The promotion of some complex catalyses was also shown to occur more readily in the presence of molecular assemblies, that is, in the context of a chemical system. Such effects could be either directly linked to the insertion into/association with the chemical system structure or to the encapsulation of a reaction "machinery" within it.

Interface-linked catalysis: The oligomerization of peptides from amino acids with condensing agents has been demonstrated to occur in the presence of phospholipid vesicles [65-67]. In these studies, the polymerization of hydrophobic amino acids was enhanced (in terms of yield and product length in monomer units), whereas that of hydrophilic, charged amino acids depended on the types of lipid headgroups used, i.e., whether ionic interactions could occur between amphiphile and amino acid. The authors surmised that the product length (up 29 monomer units compared to 9 in aqueous set-ups) was possible due to solubilization of the products within the hydrophobic core of the vesicle bilayers. Recent investigations with potentially prebiotic fatty acid structures have confirmed these observations [68]. In this case, the catalytic enhancement could be directly related to the protonation state of the acid function of the amphiphile head-groups.

Several studies also underscore the strength of the chemical systems approach in fostering complex catalysis and energy harvesting functions through association with the interface of chemical systems. For instance, the activity of an RNA polymerase ribozyme was improved when the various RNA compounds of the system (the ribozyme, the template/primer) were derivatized with amphiphilic moieties and co-associated within micelle structures [69]. Although no catalysis was demonstrated yet, amino acid and peptide-derivatized fatty acids (synthesized via a prebiotically plausible route) have been shown to associate with fatty acid vesicles. Vesicles with arginine-derivatized fatty acids could even electrostatically recruit RNA from the surrounding medium [70]. Such vesicles with associated ribozymes could eventually prove to be novel functional chemical systems.

The production of fatty acids from non-amphiphilic picolylesters performed using a photochemical reaction involving a ruthenium tris(bipyridine), functioning as photosensitizer and redox catalyst, and a nucleobase, 8-oxoguanine, serving as recyclable electron donor to trigger the redox cleavage of the precursor molecule, [71] was also found to be enhanced by the presence of pre-formed fatty acid vesicles. In aqueous media, both parts of the photochemical catalyst needed to be covalently linked (i.e., the intramolecular electron transfer was necessary for efficient conversion of the precursor), whereas when independently associated onto compartments they could work with the same efficiency via an intermolecular electron transfer [72]. Thus, the existence of chemical systems that incorporate boundaries with differing hydrophilicities and hydrophobicities could have enabled complex chemistries to emerge.

Energy harvesting from primary sources (light, geothermal, or chemical energy) and its conversion into chemical energy, such as proton and electron gradients or molecular energy currencies, is ubiquitous within contemporary biological cells. Thus, the emergence of such functions seems to be conditioned by the existence of chemical systems. Compartment models with their high molecular permeability [73] have long been considered an obstacle to the early emergence of energy harvesting apparatus.

However, recent studies [16,61,74] have substantiated their potential early existence. Indeed, a class of photosensitive chemicals, the polycyclic aromatic hydrocarbons, PAHs (Figure 2), are capable of spontaneously inserting into amphiphile structures, even medium-length fatty acid vesicles (fatty acids with a hydrocarbon chain length of 8–12 carbon atoms), where they can drive the formation of proton [75] or electron gradients [16]. In the case of photo-induced electron transport over membranes, the differentiated permeability of small anionic solutes with high charge density, such as potassium ferricyanide, and EDTA used as an external sacrificial reductant was key to the reduction of the ferricyanide to ferrocyanide. Thus, simple compartments can harbor a directional charge transfer, induced by light harvesting.

By contrast, even though the formation of proton gradients upon the irradiation of bilayers into which PAHs have been incorporated has been reported [75], their dissipation is rapid. That is, utilization of the energy gradient should be directly linked to its formation. The build-up of the proton gradient underlines the importance of having a compartmentalization system. Indeed, the proton release upon irradiation of PAHs is not directional. Thus, only 50% of the protons generated will enter the lumen of the structures, the remainder being lost to the surroundings. However, the ensuing local concentration can result in transient pH gradients as large as three pH units, which could be large enough to couple a proton gradient to a reaction network (presumably as long as its dynamic stability is on a similar scale or longer to reaction rates).

Interestingly, while the presence of amphiphile structures acts to solubilize the highly hydrophobic PAHs, hence their light harvesting activity, the inserted PAH molecules in turn contributed to stabilizing the aggregates and reducing the bilayer permeability to additional small solutes [74]. That is, feedback interactions between system components significantly increase the probability of coupled functionality, in this case coupling of a light harvesting apparatus to chemical energy gradient formation.

Volume-enclosed catalysis: Compartmentalization of an aqueous volume within defined, preferably semi-permeable boundaries, was recognized very early on as paramount for the emergence of life [76]. Following the elucidation of the cellular membrane architecture, amphiphile vesicles or liposomes, became the main type of compartment models for the study of the origins of life, although other systems could also serve the very same purpose [77-81]. Besides the chemical continuity arguments, amphiphile bilayers offer a very fine-tuned permeability to solutes and allow for the insertion of chemical species in their hydrophobic cores, thereby enabling a multiphase chemistry.

This protocell development has focused on two types of processes required for self-maintenance and self-reproduction: the synthesis of protocell building blocks, such as amphiphiles and catalytic and information biopolymers, and the processes linked to protocell replication (see section “iii) Support of functional systems proliferation”) occasionally linked to uptake and conversion of energy from a primary source, such as light. From the evolutionary point of view, syntheses of catalytic and information biopolymers seemed to be central to the origin of life because of ubiquitous presence in every aspect of the cellular metabolism, hence their involvement in early stages of life emergence seemed to be necessary. In particular, the synthesis of RNA, because of the ability of RNA to catalyse reactions as well as encode the cellular information (each RNA in principle represents both a genotype and phenotype), was often singled out as the “only” approach to solve the famous “chicken–egg” dilemma [4,7]. However, as advocated here and elsewhere [4,7], the complexity of de novo RNA synthesis and its functional interconnection with other biopolymers in the cellular context question its early, single-handed role.

The polymerization of short RNA chains and peptides has been investigated within aqueous vesicle lumens as well as water/oil emulsions, and coacervates. Two types of catalysts, metal ions [21,82] and enzymes [77,83-85], have been utilized, the latter catalyst type to remedy the absence of true “prebiotic” catalysts, such short peptides and RNA enzymes. Nevertheless, all these experiments highlight crucial aspects for the development of protocellular „metabolism“.

Inspired by the non-enzymatic, template-directed RNA polymerization in bulk aqueous solutions [7] (the synthesis of a RNA using a primer/template system and magnesium ions as catalysts), the Szostak group [21,82] has demonstrated that RNA could be synthesized within mixed vesicles composed of several types of “prebiotic” fatty acids and co-surfactants. That is, the vesicles could have retained the primer/template system while activated monomers crossed the vesicle bilayers by passive diffusion. Similarly, amino acids could be dimerized within vesicles [86]. In related experiments, Chen et al. [87] established that an inorganic catalyst itself, magnesium ions, could be delivered to non-functional hammerhead ribozymes with consequent induction of activity (self-cleavage). The enzymatic reactions were conducted within vesicles formed by long chained fatty acids, such as octadecenoic acid (oleic acid) using polynucleotide phosphorylase (PNPase, whose activity under normal conditions leads to RNA degradation, but in the presence of ribonucleotide diphosphates, NDPs, can polymerize

random RNA strands) [83] and Q-beta replicase [88]. In the PNPase experiments, the selective permeability of simple membranes was sufficient to permit an internalized synthetic or catalytic activity albeit at low yield and rate levels. However, both highlighted a different aspect of the compartmentalization: The use of aqueous metal ions could jeopardize the integrity of the compartment [20], and the compatibility of protein catalysts, presumably products of a long evolution, with the compartment building blocks could be problematic. Indeed, the use of decanoic acid vesicles completely inhibited the PNPase activity (unpublished observations), a clear support for a co-evolution of the various components of protocellular systems. The metal-sensitivity issue could be partially resolved using mixed amphiphile membranes [20] or trapping of the metal ions via complexation [21].

Uptake and transduction of energy (light, geothermal, or chemical energy) is essential to permit the emergence of truly (semi-)autonomous protocells [89] and as mentioned above requires a form of compartmentalization. The direct linking of the energy harvesting with chemical conversions, although likely one of the first forms of energy transduction, had limited applicability considering that the formation of a carbon–carbon bond is a two-electron process and that current biochemistry is hallmarked by energy storage and timely-defined consumption. It is therefore apposite to ponder on the question of the emergence of energy storage in the form of high-energy currency molecular systems. Some experimental evidence exists to support scenarios involving membranes as a central participant in energy harvesting and conversion into usable chemical energy, by creation of high-energy bonds in P compounds or other molecules. So far the energy harvesting in protocell models composed of fatty acid vesicles has, to the best of our knowledge, not been attempted yet. There is perhaps one notable exception [90], which, however, does not produce a phosphodiester bond. This might be due to the fact that the bioenergetics of P is intimately linked to the presence of sophisticated protein machinery for the harvesting of light itself, and its conversion to a proton gradient, as well as its dissipation by the formation of ATP. The question as to whether, and if so what, alternative molecular assemblies could have been developed as primitive energy currency systems remains open and a topic of considerable debate.

However, experiments have been carried out to reconstitute photosynthetic machinery in phospholipid liposomes [91,92] and polymersomes [93]. In these experiments, the use of photosensitizer triads or bacteriorhodopsin has allowed for the conversion of light energy into a proton gradient, which in turn could be utilized to power an ATP synthase to produce ATP from inorganic phosphate and ADP. In these systems, the "artificial" photosynthesis attained transduction levels that were comparable to those observed in cells, but in a completely artificial compartment. That such a complex dynamic system can be realized in artificial membranes is remarkable. The correct orientation of the various compounds was easily determined chemically, e.g., by derivatization of the triad photo-sensitizer with a charged group that defined which side of the molecule could insert into the hydrophobic core of the membranes [92]. However, a correct addition sequence during system preparation was still necessary and it speaks against a separate evolution of the system parts. In the case of fatty acid experiments [90], fatty acid vesicles were formed on/around titanium oxide particles and the irradiation of the photosensitizer powered the reduction of NAD^+ to NADH using a mediator, rhenium bipyridine (a molecule similar to the ruthenium complex in Figure 2B).

A concomitant development (complexity increase) of membranes and light/energy harvesting/conversion systems can thus be seen as a prerequisite in the evolution of the ancestral bioenergetics en route to the sophisticated organisation of the contemporary one.

Support of functional systems proliferation

To achieve a "life"-like status, protocells should have been able not only to maintain themselves, but also to reproduce and change (evolve). The reproduction phase involves replication of all its internal content (metabolic networks and information component) within a chemical system while its compartment boundaries grow. This growth–reproduction phase is then subsequently followed by a division–reproduction event leading to the formation of two "daughter" systems.

The propensity of amphiphiles to integrate pre-existing structures [94,95] has been experimentally exploited either by adding more amphiphiles at a pace that prevents the de novo formation of novel structures [17] or by adding amphiphile precursors that had to be converted within the structures into amphiphilic molecules themselves [83,96,97]. However, two features that are potentially detrimental to the reproduction of functional protocells were recognized: a) Even in the presence of a metabolic model, the reproduction of the internal "metabolic" network and compartment boundaries must be linked to avoid the production of non-functional systems [98]; b) the spontaneous division of the growing systems was found difficult to achieve in a predictable way. Early experiments used extrusion methods (i.e., structures were physically pressed through filters with very small pores, a procedure that leads to structure re-sizing, thereby to the production of smaller, more numerous structures) as a way to model a division process mediated by external stresses [17]. Alternatively, the agitation of grown vesicles was sufficient to induce division [18].

To address the first issue, the idea of linking the growth and division of the compartment boundaries to the internal metabolic activity, was explored in various ways. Assuming that an efficient, internal reaction network would change the osmotic balance across the bilayers, Chen et al. [99] demonstrated that vesicles experiencing a stronger osmotic pressure across their bilayers were able to scavenge amphiphiles from other vesicles in an isotonic state. That is, they can grow at the expense of "non-functional" (isotonic) systems. This result whilst interesting seems to be difficult to envision in a natural setting as the difference in ionic strength needed to observe this result was quite large and the vesicle boundary permeability is known to be high. However, an internal chemical production can achieve similar results [86,100]. The formation of a hydrophobic dipeptide [86] for example led to growth of functional protocells at the expense of non-functional ones.

The division of vesicles could also be linked to an internal chemical reaction. In this case [101], the irradiation of membrane-located photosensitizers stimulated the formation of disulfide bonds in small hydrophilic molecules in the vesicle lumen, which then migrated subsequently into the boundaries provoking changes in the membrane packing and, ultimately, division.

Relevance of chemical systems and chemical contiguity to the emergence of life

During the last fifty years, research on the emergence of life has focused mainly on exploring mechanisms for obtaining biochemicals and related functions under prebiotically plausible conditions. These chemicals were then considered indispensable for the emergence and evolution of cellular life, and were extensively studied using simple chemical reactions or selection schemes to evolve them and enable novel functions. Many insights were gained and have allowed for a better understanding of living systems or their components to emerge, even allowing for new aspects of biochemistry to be revealed, such as for example, the discovery of riboswitch activity in bacteria after their selection in the laboratory [102].

However, the knowledge gained has also highlighted some clear issues about this approach, in particular the question of compatibility between the various, required biochemicals, their plausibility within a prebiotic context and their capacity to remain active outside of the cellular environments [4]. Today, it seems clear that a change of paradigm is warranted, thus the idea of chemical systems and its corollary, chemical contiguity, which must be explored in relation to early earth geochemistry. Although this approach is not new per se (one can correctly argue that Oparin's coarcervates were already chemical systems) [77], more recent "conscious" developments of this approach have already yielded some noteworthy successes, which augur rather well for the future of the field. Indeed, the integration of the various components of presumptive pre-cellular entities within single chemical models have led to the discovery of new dynamic couplings between chemicals within a chemical system that might explain how and why certain molecules or functions were selected during chemical evolution from a large inventory of molecules or possible chemical reactivities.

It is certain that some examples used as illustrations in this article are too artificial to have played any role in the actual evolution on the early Earth or are even altogether wrong. However, they underscore the potential of the chemical system approach to facilitate the study of the emergence of life and also document the work at hand. Its power lies in the variability of the concept that allows us to envision ever more complex systems, even consortia of them, which could have coalesced into protocells and later on ancestral cells (Figure 3). The main obstacle to that realization remains the fact that "dirty", suboptimal systems are difficult to understand with the rigor expected from chemistry.

Conclusion

While it is obvious that the abiotic chemistry must have delivered the molecules needed for the emergence of cells or their precursors, the question about the transition between that abiotic chemistry and biochemistry remains unanswered. Many scenarios that often are referred to as "world" hypotheses have been proposed to explain that transition or its various stages, e.g., the lipid-, metabolism- or RNA-world, which in general tend to emphasize an aspect of the question that is directly related to the research field of their proponents. Each of these different, reductionist views is a natural one in the context of the Western scientific method. However, by electing to use a different granularity of vision, as by focusing on the system and what the system does, we can begin to explore connectivity of processes and how that integrates to system functionality. We expect these facets to be emergent in a molecular sense. Whilst they depend upon the specific chemical components used, it is how those chemicals integrate that leads to the function rather than any isolated property of the individual molecules themselves.

One of the chief historical features of the above origins hypotheses is their mutual exclusivity in respect of which chemical elements came first. However, a consensus is slowly building that co-emergence and co-evolution of the cellular functions must have started at an early stage. This hypothesis has resulted in a heightened focus on chemical systems in the field concerning the "Origin of Life". Indeed, the study of complex molecular aggregates, which is now called "system chemistry"

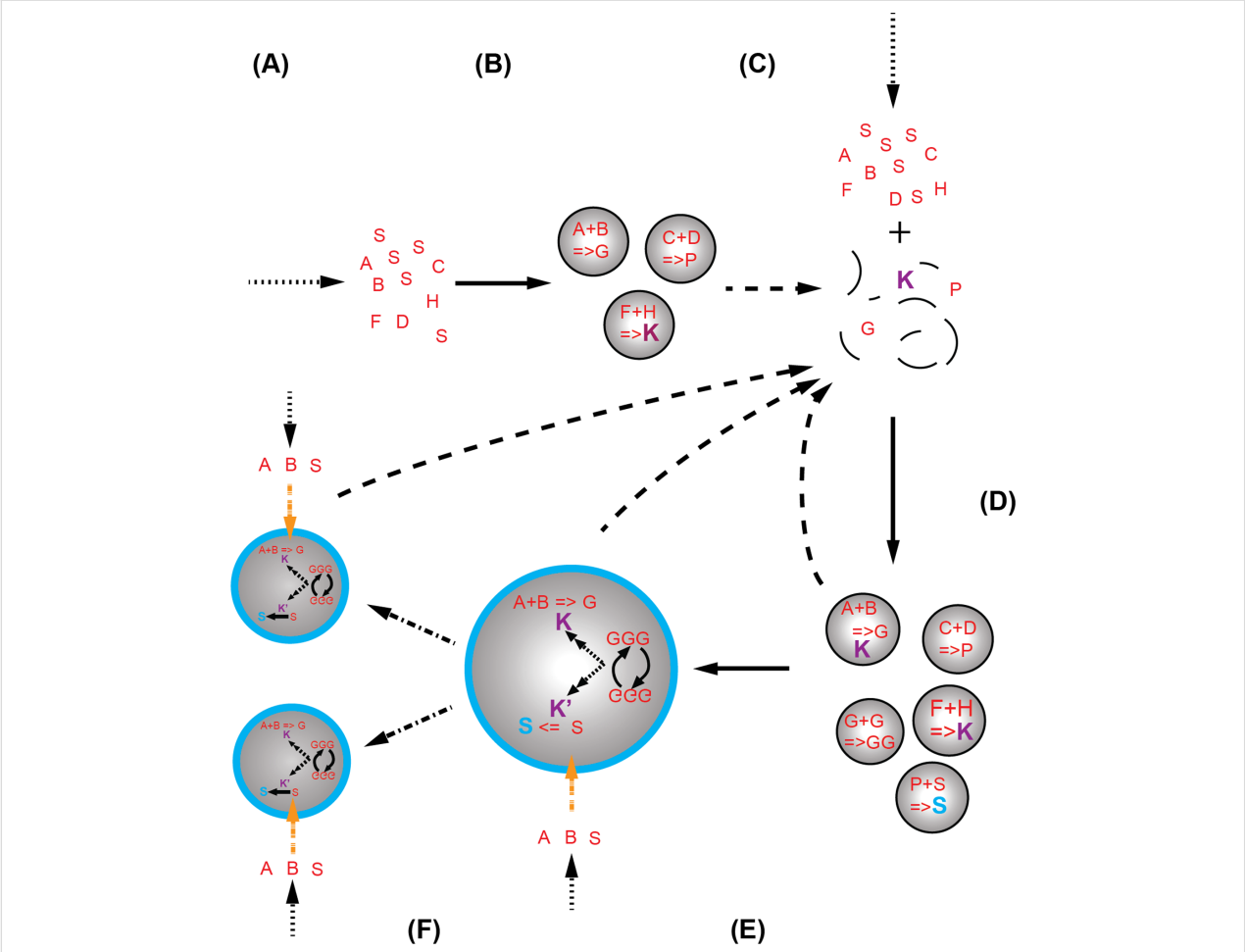


Figure 3: A putative scenario for the evolution of chemical systems towards protocells. (A) Prebiotic chemistry in the geochemical environment delivers an inventory of molecules (dotted arrow), some of which are amphiphiles (red S). When the aggregate critical concentration of the amphiphiles is reached, perhaps via trapping within a mineral pore structure, system compartments spontaneously form (plain arrow) and in the process co-locate chemicals, which could be either on the surface or within the system volume (B). The co-location allows a different chemistry (reactions are represented by letters with a "=>") to take place as now both hydrophilic and hydrophobic environments contiguously co-exist. Upon subsequent disruption of the compartments (C) due to chemico-physical fluctuations (pH value, ionic strength, pressure or temperature) the prebiotic molecular inventory (dashed arrow) is enriched in a new set of basic building blocks, some of which "K" might be catalysts for the syntheses of building blocks of the system. Once the environmental conditions become again conducive to self-assembly, new chemical systems form (D). Some of them will have capability to produce further chemical complexity (new products or catalysed reactions). Cycles of formation/disruption will occur until (E) system compartments with improved stability (here highlighted by the blue boundaries composed of blue S, i.e., new amphiphiles) appear. These compartments will then gradually increase their internal catalytic network (dotted–dashed arrow) and gain some element of information processing capability, thus forming primitive protocells (Figure 1B). At that stage, they might still require chemical input from the environment (orange dotted arrow). However, they likely only take up certain chemicals selectively due to boundary permeability. These systems with increased half-life will perhaps also be disrupted cyclically until they are capable to self-replicate and adapt to environmental fluctuations (F). Once stable over long time periods, these systems would be clearly the first complete embodiment of a protocell (Figure 1B). Plain arrows relate to a self-assembly process, dotted arrows the prebiotic synthesis of chemicals, dashed arrows the disruption of a chemical system, the orange dashed arrows the selective permeability towards chemicals of the chemical system boundaries and the dotted–dashed arrows the replication process.

[103], seems to be consistent with the emergence of cellular complexity. Moreover, it has the potential to inherently satisfy the concept of evolutionary continuity. Obviously, an unambiguous demonstration is still necessary.

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A recursive microfluidic platform to explore the emergence of chemical evolution

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Full Research Paper

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Abstract

We propose that a chemically agnostic approach to explore the origin of life, using an automated recursive platform based on droplet microfluidics, could be used to induce artificial chemical evolution by iterations of growth, speciation, selection, and propagation. To explore this, we set about designing an open source prototype of a fully automated evolution machine, comprising seven modules. These modules are a droplet generator, droplet transfer, passive and active size sorting, splitter, incubation chamber, reservoir, and injectors, all run together via a LabVIEW^{TM} program integration system. Together we aim for the system to be used to drive cycles of droplet birth, selection, fusion, and propagation. As a proof of principle, in addition to the working individual modules, we present data showing the osmotic exchange of glycylglycine containing and pure aqueous droplets, showing that the fittest droplets exhibit higher osomolarity relative to their neighbours, and increase in size compared to their neighbours. This demonstrates the ability of our platform to explore some different physicochemical conditions, combining the efficiency and unbiased nature of automation with our ability to select droplets as functional units based on simple criteria.

Introduction

The transition from an inanimate inorganic world, principally consisting of minerals, gases and small organic compounds, to the living world with the first life forms remains one of the greatest mysteries in science [1]. In the early 20th century, Alexander Oparin and John Haldane proposed that the first minimal living systems on Earth formed via a series of chemical steps of increasing sophistication and functionality. In subsequent decades, knowledge of the materials and environments that would have been available on the early, prebiotic Earth has expanded dramatically [1-5]. This has enabled the

reduction of the potential chemical and geochemical landscape for abiogenesis from a vast parameter space, but has also led scientists to propose hypotheses on the origin of life under very constrained conditions [6].

Many heated debates in the field of prebiotic chemistry have raged over which precise historical environment(s) gave rise to the first lifeforms. However, it is unlikely that this question can ever be answered with reasonable certainty [7]. Therefore, the puzzle most ripe for scientific inquiry is not how did life first arise, but what kind of processes can facilitate the origin of life? Identification of processes that produce complex, autocatalytic chemical networks [8] from simple inputs via gradual, step-wise complexification could go some way towards answering the latter question. This approach engenders a "chemically agnostic" perspective, in which strict adherence to the chemical repertoire found in currently extant biochemistry is not required [9]. Indeed, the simplest biological units can be considered as nothing more than complex autocatalytic networks that reproduce, with more or less the same stoichiometry, all functionally active components of their heterogeneous chemical mixtures. Such systems could easily exist outside the boundaries of known biology, and perhaps may not even require a template-driven genetic polymer to reproduce [10].

However, irrespective of their chemistry, it is likely that any artificial or alternative life-forms would need at least the following attributes:

i) Compartmentalisation: a means of discretising individual living units and enabling controlled selective exchange between these units and their external environment.

ii) Metabolism: chemical reaction networks that extract energy from the environment in a useable form.

iii) Heritance: reliable transmission of functional information from one generation to the next.

iv) Evolution: a means of undergoing an evolutionary selection process, driven by errors or variations in the heritance process.

Attempts to facilitate the emergence of adaptive evolution in artificial systems have been fraught with difficulties. A lack of clear, tangible criteria for identifying this process when it occurs has hindered efforts to create artificial life. The hallmark of evolution is adaptation in response to selection pressure and environmental change. Evolutionary biologists often track this process using biochemical signatures such as genome sequence. However, this would be difficult in artificial or otherwise alternative life, especially if there is no conventional, template-directed genetic system. Thus, the first step is to establish a suitable metric for identifying and measuring their capacity for evolution.

We propose that, for any given population of discrete living or proto-living units, the average fitness (w_i) of the population will be evaluated as a function of time, environmental change (Δe_i) and population size. Fitness will be determined and thresholded by intensity of an observable, quantitative trait (z). Only those units with a fitness exceeding a pre-determined threshold (f) will be permitted to reproduce and pass on information to the next generation. Repeating this process in an iterative manner, allowing only the fittest members of each generation to affect the chemical composition of subsequent generations, will lead to adaptive evolution.

$$\Delta z = \frac{1}{w} Cov\left(w_i, z_i\right) + \frac{1}{w} E\left(w_i \Delta z_i \Delta e_i\right) \quad (1)$$

Where Cov = covariance and E = sample mean.

Equation 1 is a modified Price equation [11] with the change in the environment Δe_i factored into the second term which is nominally $E(w_i \Delta z_i)$. Evolution in any given system will be confirmed via a successive change in Δz over time.

Evolvability is a pre-requisite for life, but it is not sufficient for a system to be deemed living or life-like. Therefore, our group is also developing a metric for evaluating the complexity of chemical species produced in artificially evolving systems. This complexity measurement will be thresholded using existing biological systems and by comparison with the starting inputs into our evolutionary platform. An artificial living system would be capable not only of evolution, but also of producing species with a greater complexity than would be expected to arise from any non-biological system [12]. Thus, the transition from an evolving but non-living chemical system to an evolving living system will be marked by production of species of comparable complexity to those found exclusively in biology, as depicted in Figure 1.

Droplet compartmentalisation

In our previous work, we described the assembly of a custom-made 3D printed robotic platform that uses artificial evolution to select for desired behaviours in chemical systems [13]. In this case, the macroscopic behaviour of oil droplets was studied. We used a genetic algorithm to generate a series of droplets, each with a different set of chemical compositions, which were evaluated according to various fitness functions based on observable traits, such as motility, vibration and division. The chemical mixtures that produced the fittest droplets in each genera-

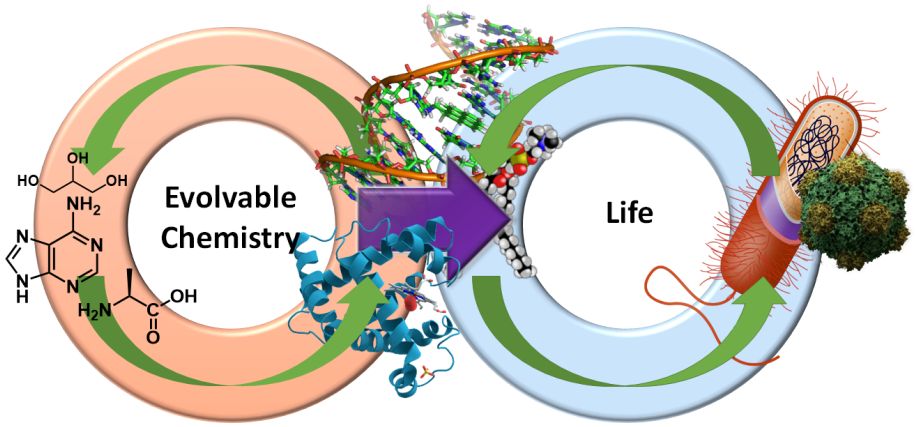


Figure 1: Evolution of life from non-living, complex chemistry via chemical evolution of complex chemical composites towards increasing complexity. A transition to biological evolution occurs when composites become sufficiently complex to transition from chemical to biological units. Green arrows indicate continuous adaptation and complexification under selection pressure; the purple arrow indicates the transition from evolving chemical composites to evolving living units after exceeding a complexity threshold.

tion of experiments were allowed to influence the compositions of the next generation, while the rest were discarded. As this process was repeated iteratively over successive generations, the fitness value of the population was increased (Figure 2).

Droplets provide a means of creating discrete, compartmentalised units, defining the "self" or units of evolution. These defined units can then be subject to conventional selection processes. The work described above was carried out in microlitre

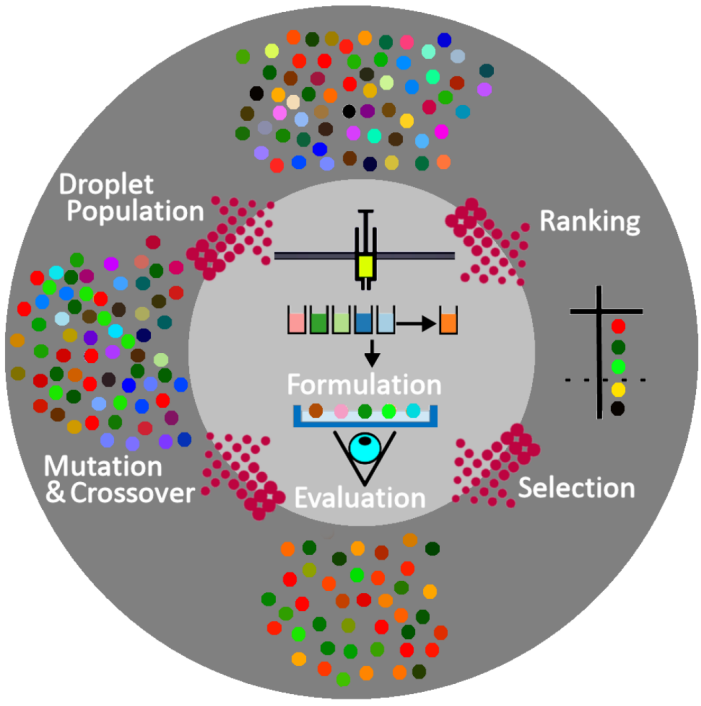


Figure 2: Schematic describing the evolutionary process. The inner circle represents the robotic process and outer circle represents the computational algorithm. A random selection of the droplet formulations are used as the starting 'Droplet Population'. These droplets are generated in the 'Formulation' step. Droplet behaviours are then recorded using a camera, and then undergo image analysis against a user desired property (e.g., colour) in the 'Evaluation' step. The droplets are ranked in terms of desired property (e.g., movement, division), and the least good rejected in the 'Ranking' step allowing a new population to be 'Selected'. Meanwhile the accepted formulations are used as a basis to create a new 'Droplet Population' after random 'Mutation' and 'Crossover'. This figure was reproduced from our earlier article [13], copyright 2014 Macmillan Publishers Limited.

scale droplets. However, a few recent examples in the literature report the utilisation of pico- and nanolitre microfluidic water-in-oil droplets and liposomes as artificial cell analogues [14,15]. Aqueous, single emulsion microdroplets can be produced at kilohertz frequencies, and provide compartmentalisation on a similar length scale to biological cells. Soft interface interactions at liquid–liquid boundaries in microdroplets can also have a catalytic effect via the adsorption of otherwise unstable molecules [16], similar to catalysis reported at liquid–mineral interfaces [17].

Microfluidic platform for artificial evolution in droplets

Here, we propose a system for facilitating chemical evolution in populations of co-incubating aqueous, single emulsion microfluidic droplets.

Each microdroplet can be considered an autonomous microreactor, loaded with a self-propagating chemical reaction network. However, it has been observed, both in our own work and in the literature that limited exchange of material can occur between neighbouring water-in-oil microdroplets (see Figure 3). The rate of diffusion of molecules between microdroplets is inversely proportional to their molecular weight, with the result that microdroplets containing higher molecular weight species exhibit greater osmotic pressure, and thus physically grow in size at the expense of their neighbours via osmotic effects [18-20]. This is particularly the case when microdroplets contain reactions that convert relatively simple, low molecular weight starting materials into larger, more complex products. Such a set-up is amenable to inducing competition and evolutionary selection pressure within populations of microdroplets, using physical droplet growth as a fitness metric. The quicker droplets can produce larger, more complex products, the more likely they are to grow. Size sorting can then be applied to select for the fittest, fastest growing droplets and ensure only these droplets are recirculated in the next iteration of reaction and selection.

Results and Discussion

To test the ability of aqueous droplets to grow at the expense of each other we undertook some experiments to explore osmotic exchange between microdroplets. A mixed but monodisperse population of 50 mM glycylglycine droplets and pure water droplets was used as a model for this process. Due to their greater osmotic pressure, the glycylglycine droplets grew at the expense of the water only droplets (Figure 4 and Figure 5). This effect was not observed for unmixed droplet populations containing only glycylglycine or pure water. Using LabVIEW™ image analysis, the osmotic exchange process can be tracked in real time by measuring average droplet size and polydispersity (Figure 5).

Various microdroplet size sorting techniques [21,22] can be used to enforce a positive selection pressure for increase in droplet size. By doing this iteratively, over multiple generations and ensuring a continuous (but not unlimited) supply of fresh feed-stocks, it will be possible to observe the emergence

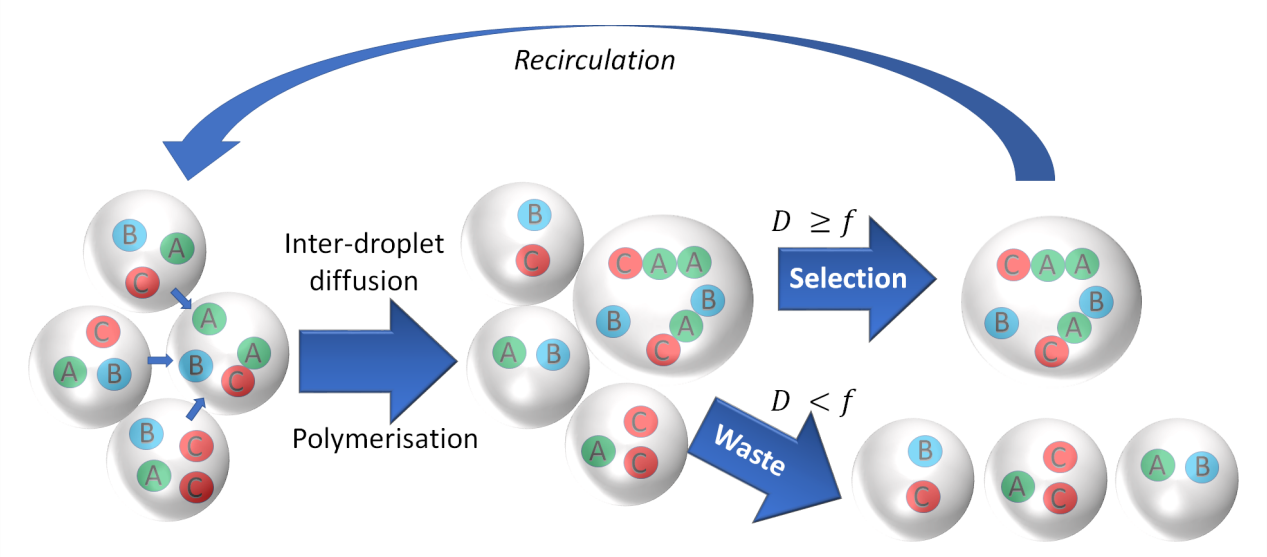


Figure 3: Recursive size-based selection and recirculation of droplets. Monodisperse droplets loaded with complex autocatalytic chemical networks are incubated in the microfluidic device. Those droplets which facilitate the fastest production of high molecular weight polymers from simple precursors exhibit an increase in osmolarity and subsequently grow in size at the expense of neighbouring droplets. Size sorting is then used to select for droplets with a diameter (*D*) above a size threshold for droplets to be used in the next generation (*f*). Those droplets are recirculated into the next generation, replenished with fresh feedstock, and the process is repeated in an iterative fashion.

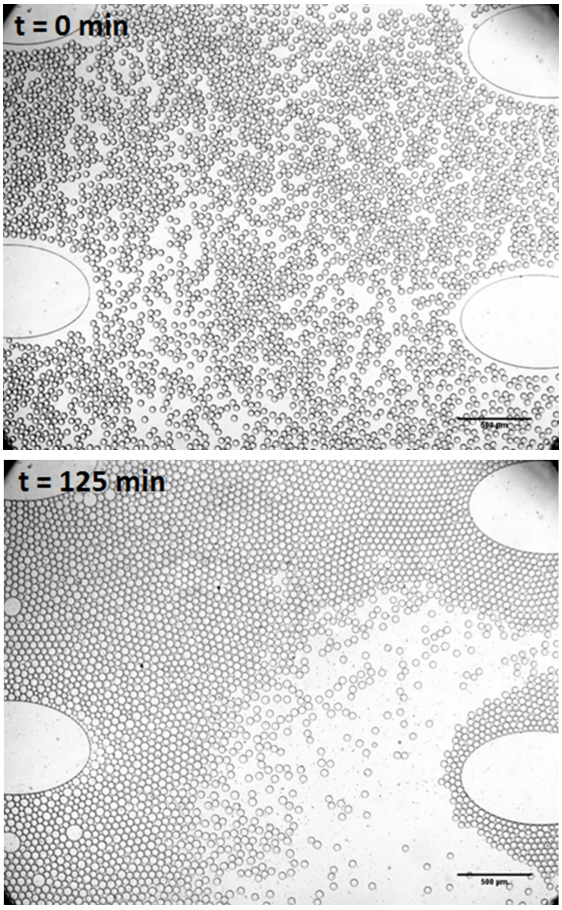


Figure 4: Osmotic exchange and coarsening of co-incubating aqueous microdroplets. 50 mM glycylglycine and pure H_2O droplets were co-incubated in the same chamber. Upper panel: mixed but monodisperse droplet population at t = 0 min; lower panel: t = 125 min.

of adaptive evolution. Differential fitness can then be induced in droplets when they are forced to compete for the same feedstocks [23]. Successive increases in the rate of droplet growth could be indicative of evolutionary processes in response to the continuous selection pressure. In parallel, the chemical composition of microdroplets will be analysed after each iteration.

In principle, this device should be able to carry out multiple cycles of automated droplet generation, manipulation and selection, as shown in the process diagram in Figure 6. Passive and active size sorting methods will be used for selection of droplets in sub-populations and as individuals, respectively. For active sorting, real-time image processing will be used to screen individual droplets as they pass through a microfluidic channel. If the droplets exceed a pre-defined size threshold for fitness, an air-actuated polydimethylsiloxane (PDMS) valve will be activated and the droplets will be isolated and put through a new round of growth and selection. Passive sorting techniques (such as pinched flow fractionation) [22] have been used to sort droplets into groups (or sub-populations). This process can also be monitored in real-time, but this is not a requirement for the droplet sorting and selection to proceed. Also, unlike active sorting, passive sorting is not reliant upon automation, and is therefore technically less complex. In both systems, droplets below a critical size threshold for fitness are discarded.

Our aim is to design and fabricate a complete device containing a droplet generator, an incubation chamber, a droplet size sorter, a droplet fuser, and a droplet splitter; see Figure 7 for the device template. Microfluidic droplet generators will produce the droplet populations that will then be co-incubated in different envi-

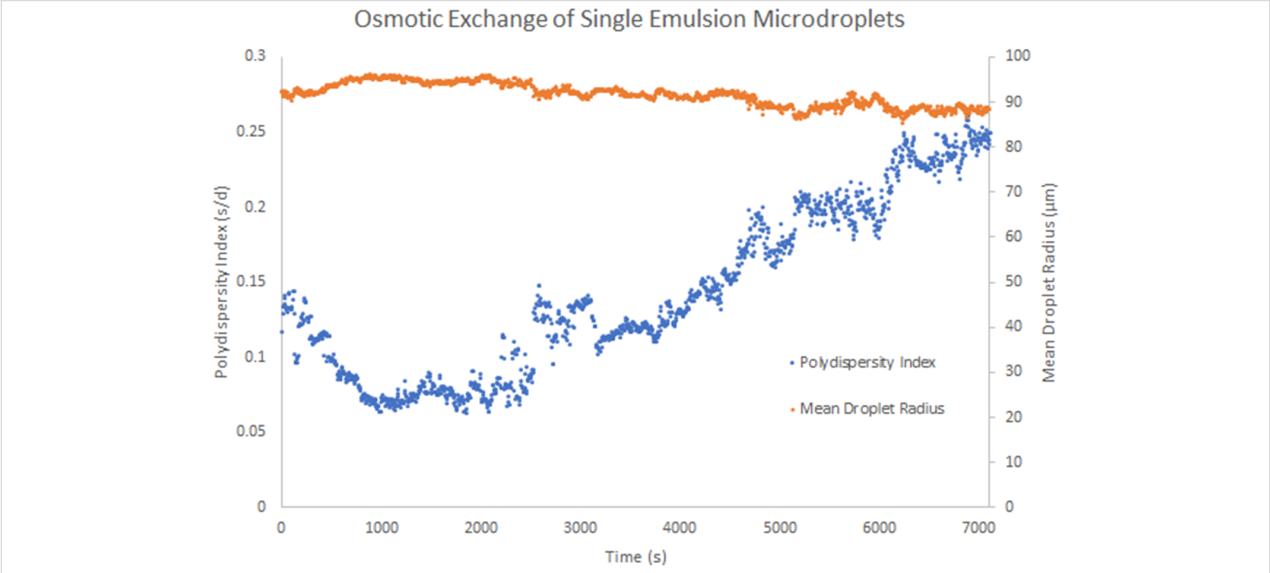


Figure 5: Real-time, LabVIEW™ tracking of osmosis-driven coarsening of 50 mM glycylglycine and pure water droplets. Increase in droplet polydispersity is monitored using LabVIEW™ image analysis. s = standard deviation of droplet radius, d = mean radius.

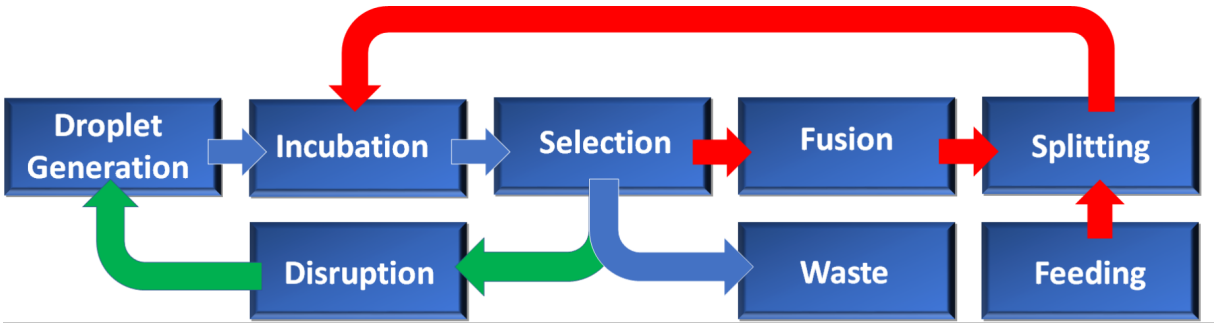


Figure 6: Process of the automated microfluidic platform, in which recursive evolution is applied at both individual droplet and sub-population level. Blue arrows indicate processes common to all devices; green arrows indicate processes unique to sub-population selection; red arrows indicate processes unique to individual droplet selection.

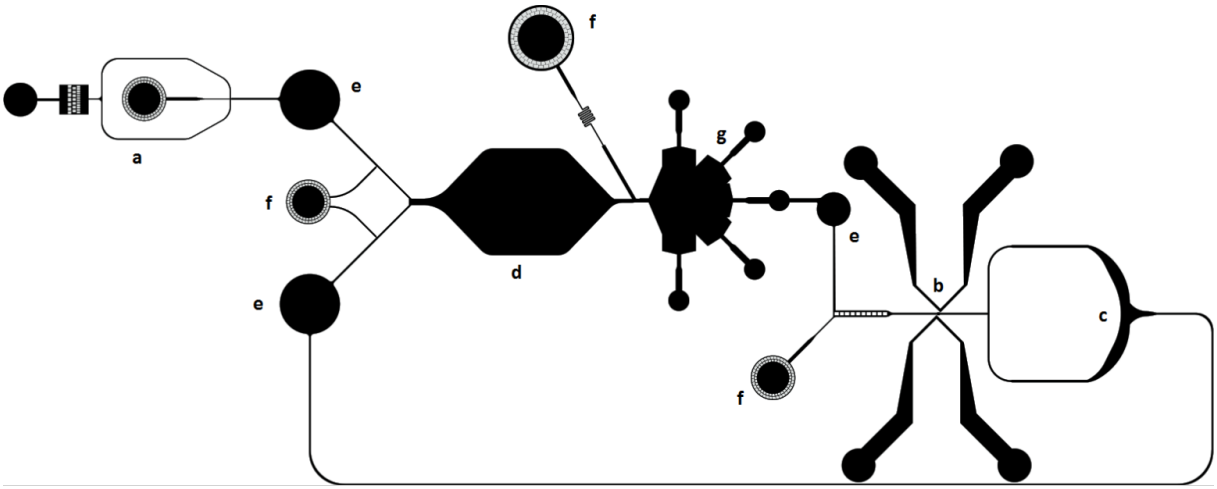


Figure 7: The proposed device for droplet selection and evolution. The device is comprised of the following modules: a) droplet generator; b) droplet fuser; c) droplet splitter; d) incubation chamber or delay line; e) droplet packing reservoirs; f) oil injectors; g) droplet size sorter.

ronments (e.g., pH, salt, temperature, surface chemistry, chemical inputs). Droplets that are able to grow sufficiently will be re-circulated with fresh feedstocks for further cycles of incubation and selection, whilst droplets that get smaller will be discarded. Thus, a continuous selection pressure for droplet growth will be enforced in a recursive manner.

While operating such a device with many interconnected (but independently operating) modules can be challenging, we control timing and feedback issues using interspersed packing reservoirs (Figure 7e) and actuated mechanical valves. The packing reservoirs represent 3-dimensional structures that take advantage of the tendency of aqueous droplets to float in the surrounding fluorinated oil, and require an external outlet below the device (to allow for excess oil drainage) connected to an automated valve. This has been done successfully in our lab using syringe pumps, but could be controlled through other automated means. The addition of air pressure-actuated valves throughout the device should also help to control the timing of droplet movement, and experimentation will determine at which points in the device these valves are necessary. The incubation chamber (Figure 7d) represents a means of visualising a monolayer of droplets over time, which could be useful if we are looking to monitor the droplet coarsening process over time. However, this module could be replaced by a delay line or an off-chip incubation receptacle if the experimental parameters are not conducive to long-term on-chip incubation. Finally, successful operation of the device will depend on automated movement of the microscope stage to focus on the different modules, along with collecting visual data for the purposes of tuning rates of flow for the individual modules to carry out their functions.

Also, to test if the platform is feasible, we have made several working versions of the modules (Figure 8) which include a

droplet sorter. Droplet synchroniser, droplet fuser, and droplet splitter modules are required for replenishment of microdroplets with fresh feedstocks. However, in the future droplet chemistry could be adjusted so as to allow spontaneous droplet division, thus imparting a greater degree of autonomy (and thus "aliveness") in the system.

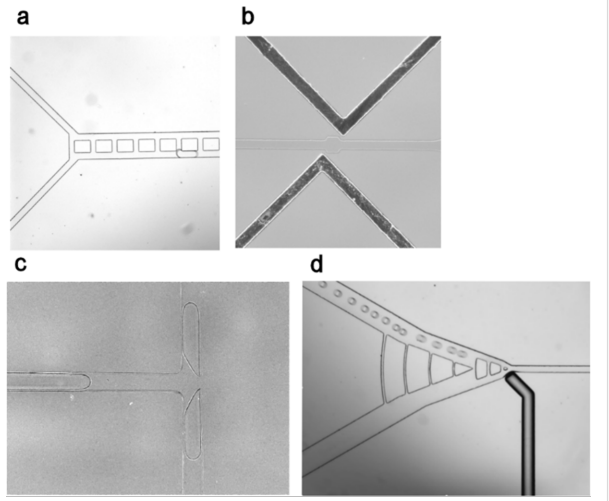


Figure 8: Photographic images of individual microfluidic modules, fabricated our laboratory in PDMS from standard soft lithography masters: a) droplet synchroniser for b) droplet fuser; c) droplet splitter; d) droplet sorter with air pressure-activated mechanical valve.

Conclusion

We have presented a new conceptual approach, and platform design, to search for chemical systems within an automated microfluidic platform that allows the creation of a population of individuals, the application of selection pressure, selection, combination, then splitting of the members of the population. We have produced each of the modules individually in our laboratory, but integration into a single device will be a bigger challenge. However, the exploration of osmotically driven droplet growth has been successful and this is an important step in producing populations of droplets with different chemical constituents capable of guest exchange. This will be done by recirculating droplets that meet our fitness criteria and combining them with new droplets from our variable input system. The evolutionary capacity of droplet units will be evaluated by the modified Price equation (Equation 1), with change in droplet size being equivalent to Δz. In this way, we can search for emergent physical properties of compartmentalised systems in an unbiased and fully automated manner.

We have already designed, fabricated and tested several of the individual modules in single-layer PDMS devices that comprise the platform. The chemical inputs, selection pressure, and population size will be varied as a function of cycle number. As the fitness of the population approaches a threshold we will investigate the populations for evidence of the emergence of life like properties 'evolved' within the device. With this approach, we suggest that such a platform may allow compartmentalised chemical units to undergo a process like evolution at the chemical level.

Supporting Information

Supporting Information File 1

Additional material.
[http://www.beilstein-journals.org/bjoc/content/supplementary/1860-5397-13-164-S1.pdf]

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