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# Surface-promoted replication and exponential amplification of DNA analogues

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**Self-replicating chemical systems have been designed and studied to identify the minimal requirements for molecular replication[1], to translate the principle into synthetic supramolecular systems[2] and to derive a better understanding of the scope and limitations of self-organization processes[3] that are believed to be relevant to the origin of life on Earth[4]. Current implementations make use of oligonucleotide analogues[5–12], peptides[13–17], and other molecules[18–24] as templates and are based either on autocatalytic, cross-catalytic, or collectively catalytic pathways for template formation. A common problem of these systems is product inhibition, leading to parabolic instead of exponential amplification[25]. The latter is the dynamic prerequisite for selection in the darwinian sense[26,27]. We here describe an iterative, stepwise procedure for chemical replication which permits an exponential increase in the concentration of oligonucleotide analogues. The procedure employs the surface of a solid support and is called SPREAD (surface-promoted replication and exponential amplification of DNA analogues). Copies are synthesized from precursor fragments by chemical ligation on immobilized templates, and then liberated and immobilized to become new templates. The process is repeated iteratively. The role of the support is to separate complementary templates which would form stable duplexes in solution. SPREAD combines the advantages of solid-phase chemistry with chemical replication, and can be further developed for the non-enzymatic and enzymatic amplification of RNA, peptides and other templates as well as for studies of *in vitro* evolution and competition in artificial chemical systems. Similar processes may also have played a role in the origin of life on Earth, because the earliest replication systems may have proliferated by spreading on mineral surfaces[28–33].**

Stepwise 'feeding' procedures were previously employed in two different chemical systems that were reported as models of potentially prebiotic processes[10,34,35]. Li and Nicolaou achieved chemical replication of duplex DNA composed of palindromic (symmetrical) homopyrimidine and homopurine strands[10]. The homopyrimidine strand was synthesized from its precursor fragments via triple helix ligation, and then served as a template for the chemical ligation of the precursors of the homopurine strand. Thus, stepwise feeding with homopyrimidine and homopurine fragments prevented fragment complexation and therefore allowed switching between the respective triplex and duplex ligation intermediates. Ferris *et al.* have demonstrated the synthesis of long oligonucleotide- and peptide-like materials on the surface of mineral supports[34,35]. In these systems, stepwise feeding enabled the replenishment of activated precursors, and thus overcame the length-limiting effect of precursor hydrolysis. The conjunction of the above approaches, stepwise chemical replication and solid-phase chemistry, forms the basis of our procedure (Fig. 1).

For a demonstration of SPREAD (Fig. 2), two complementary 14-meric templates, **X** and **Y**, as well as four template fragments, $\mathbf{A}^{\mathbf{x}}$, $\mathbf{B}^{\mathbf{x}}$, $\mathbf{A}^{\mathbf{y}}$ and $\mathbf{B}^{\mathbf{y}}$, were synthesized using standard phosphoramidite chemistry. A thiol-modified support was obtained from

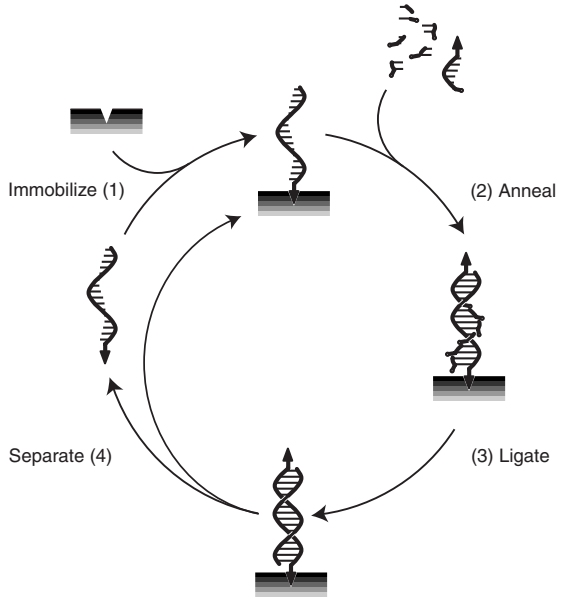


**Figure 1** General scheme of the SPREAD procedure. (1) A template is immobilized by an irreversible reaction with the surface of a solid support. (2) The template binds complementary fragments from solution. (3) The fragments are linked together by chemical ligation. (4) The copy is released, and re-immobilized at another part of the solid support to become a template for the next cycle of steps. Irreversible immobilization of template molecules is thus a means to overcome product inhibition.

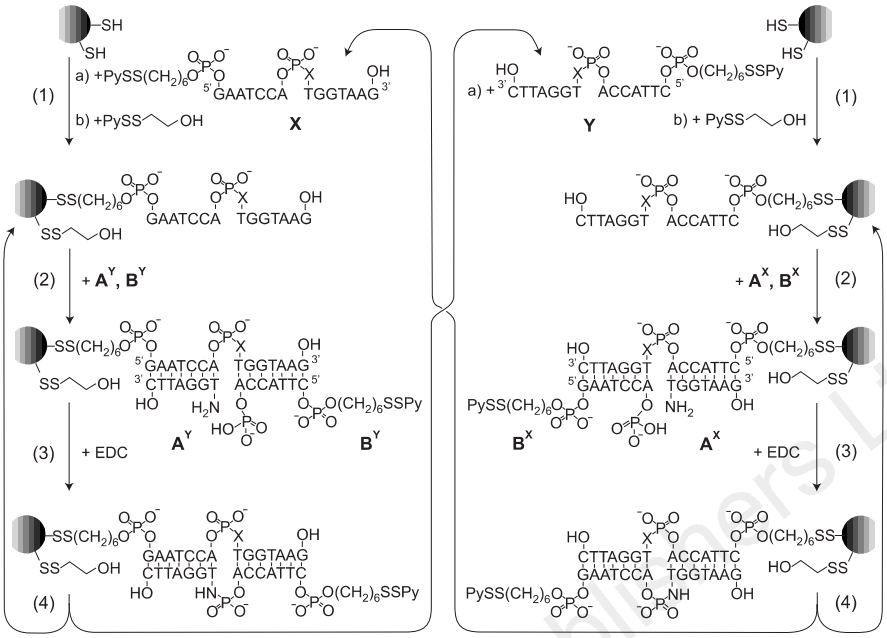


**Figure 2** Oligonucleotide analogues and reactions employed in the experiment. The individual steps of procedure (1)–(4) were performed separately for the complementary templates **X** and **Y**, with each template in a separate tube. PySS denotes a 2-pyridyl-disulphide moiety that is cleaved during immobilization (1a) and capping (1b) to give 2-thiopyridone. The backbone modification X at the central inter-nucleotide link of **X** and **Y** is X = N-H, except for the first immobilization where X = O. $\mathbf{A^x}$, $\mathbf{B^x}$, $\mathbf{A^y}$ and $\mathbf{B^y}$ denote the corresponding template fragments. The hybridization step (2) gives rise to a termolecular complex from the immobilized templates and the respective fragments $\mathbf{A^x}$, $\mathbf{B^x}$, $\mathbf{A^y}$ and $\mathbf{B^y}$. In the presence of the water-soluble carbodiimide EDC, chemical ligation (3) leads to a 3′-5′ phosphoramidate linkage between an adjacent 5′-amino and a 3′-phosphate group. Each resulting double-stranded complex is denatured (4) yielding the templated support, as well as a PySS-modified copy that is immobilized on fresh SH support.

commercially available 2-pyridyldisulphide-activated agarose (Sepharose 6B, Pharmacia) by reduction with dithiothreitol (DTT). Using disulphide exchange reactions[36], the templates **X** and **Y** were separately immobilized on two batches of SH support to give the templated supports **X0** and **Y0**. The efficiency of the immobilization step was determined by the HPLC analysis of the supernatant (Fig. 3a, b). The remaining thiol groups of the supports were capped by reaction with *S*-(2-thiopyridyl)-2-mercaptoethanol. Fragments $\mathbf{A^x}$, $\mathbf{B^x}$ and $\mathbf{A^y}$, $\mathbf{B^y}$ were then hybridized on the immobilized templates, **Y0** and **X0**, respectively. To determine the hybridization efficiency a fraction of the support was reduced by DTT and analysed by HPLC (Fig. 3c). Chemical ligation[37,38] was achieved by replacing the hybridization buffer with a ligation buffer containing *N*-ethyl-*N*′-(dimethylaminopropyl)-carbodiimide hydrochloride (EDC) as the condensing agent. To determine the efficiency of ligation, product formation was monitored by the HPLC analysis of the mixture of products obtained after cleaving the disulphide bonds of an aliquot of the templated supports using DTT as the reducing agent (Fig. 3d). The copies were then liberated by rinsing the supports with 0.1 M NaOH, analysed by HPLC, and re-immobilized on two new batches of the SH support to yield the templated supports **X1** (copy from the templated support **Y0**) and **Y1** (copy from the templated support **X0**). For the next generation, the whole cycle of steps was repeated with each of the four batches **X0**, **Y0**, **X1** and **Y1** to give **Y2**, **X2**, **Y3** and **X3**, respectively. An additional round generated the templated supports **X4**..**X7**, **Y4**..**Y7** (Fig. 3e, f).

Figure 4 summarizes the pathway of each individual support together with the amount of material obtained after each copying cycle. Generally, the yield of an replication cycle $p$ need not necessarily reach $p = 1$ in order to enable an exponential mode of amplification. For the case of palindromic templates, where $\mathbf{X} = \mathbf{Y}$, $\mathbf{A^x} = \mathbf{A^y}$, $\mathbf{B^x} = \mathbf{B^y}$, the amount of material $x_n$ obtained from an initial amount $x_0$ after $n$ replication cycles is given by:

$$x_n = x_0(1 + p)^n \tag{1}$$

allowing an exponential increase for the condition $p > 0$. For the case of non-palindromic templates underlying our experiments, each round of replication consists of two copying cycles with the individual yields $p_x$ and $p_y$ for the synthesis of **X** and **Y**, respectively. Here, the amount of immobilized templates **X** and **Y** follows the iterative progression:

$$x_{n+1} = x_n + p_x y_n \quad \text{and} \quad y_{n+1} = y_n + p_y x_n \tag{2a, b}$$

requiring both $p_x > 0$ and $p_y > 0$ for an exponential increase. Equation (2a, b) gives;

$$\begin{aligned} x_n &= \frac{1}{2}\left(x_0 + y_0\sqrt{\frac{p_x}{p_y}}\right)(1 + \sqrt{p_x p_y})^n \\ &\quad + \frac{1}{2}\left(x_0 - y_0\sqrt{\frac{p_x}{p_y}}\right)(1 - \sqrt{p_x p_y})^n \\ y_n &= \frac{1}{2}\left(y_0 + x_0\sqrt{\frac{p_y}{p_x}}\right)(1 + \sqrt{p_y p_x})^n \\ &\quad + \frac{1}{2}\left(y_0 - x_0\sqrt{\frac{p_y}{p_x}}\right)(1 - \sqrt{p_y p_x})^n \end{aligned} \tag{3a, b}$$

where $x_0$ and $y_0$ denote the initial amount of **X** and **Y**, respectively. A comparison of experimental and theoretical values for $x_n$ and $y_n$, as given in Fig. 4 legend, confirms that the data shown in Fig. 4 are consistent with an exponential mode of amplification for the template materials.

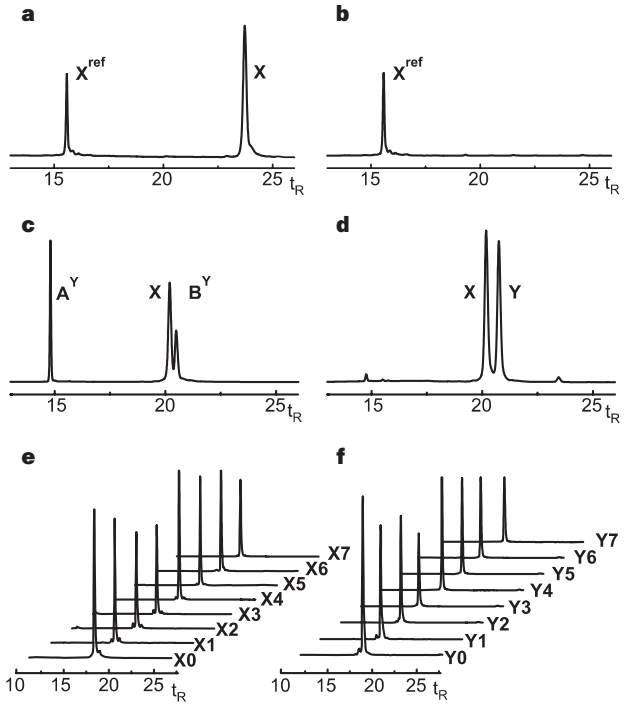


**Figure 3** HPLC analysis of products under denaturing conditions obtained in the consecutive steps of a SPREAD cycle. All HPLC samples without reduction buffer (RB) were brought to a concentration of 100 mM DTT before analysis (except **a**) to ensure reproducibility of HPLC quantification. $t_R$ is the retention time in minutes. **a**, Reaction mixture containing **X** and GAATCCATGGTAAG (**X**$^{ref}$) as internal standard before immobilization. **b**, Supernatant after immobilization. **c**, Reaction mixture after hybridization of immobilized **X** with **A**$^{y}$ and **B**$^{y}$ and treatment of the support with RB. **d**, Reaction mixture after chemical ligation and reductive cleavage from the support using RB. **e**, **f**, HPLC analysis of the set of 16 samples obtained from the reductive cleavage of immobilized templates that were generated by three SPREAD cycles. For the symbols to the right of the HPLC profiles, see Fig. 4. We note that the small amounts of impurities visible in the front profiles are consecutively removed in the course of SPREAD amplification.

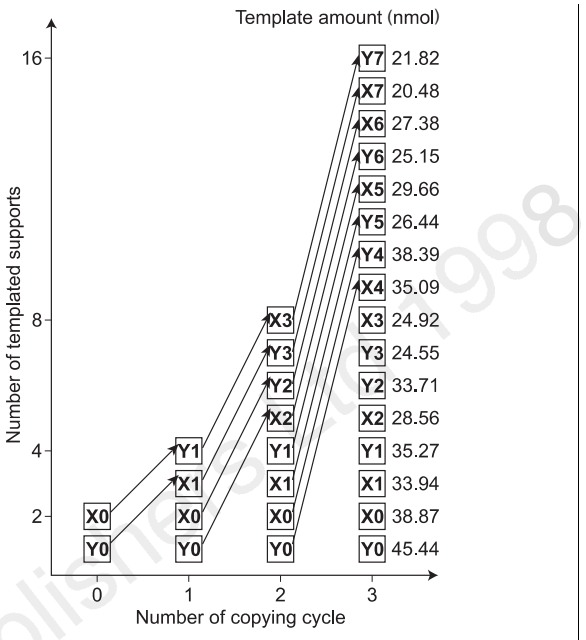


**Figure 4** Pathway of template transfers in the course of three cycles of SPREAD amplification. Boxes and lettering symbolize reaction tubes and templated supports, respectively. Arrows indicate which copy is generated from which template. The amount of template material shown was quantified by HPLC analysis after cleavage of the disulphide links by reduction with DTT. From these data, 14 individual yields were calculated. The average yields and standard errors are: $p_x = 0.763 \pm 0.071$ and $p_y = 0.855 \pm 0.079$. The number of nanomoles of each template at cycle $n$, compared with its theoretical value (in brackets) as calculated from equation (3a, b), is as follows: $x_0 = 38.87$ (38.87), $y_0 = 45.44$ (45.44), $x_1 = 72.81$ (73.54), $y_1 = 80.71$ (78.68), $x_2 = 126.29$ (133.58), $y_2 = 138.97$ (141.57), $x_3 = 238.90$ (241.61), $y_3 = 249.77$ (255.80).

The SPREAD procedure demonstrates exponential amplification in chemical replication. Solid-phase chemistry is a prerequisite for a practicable automation of the procedure, as basically pipetting and filtration steps are involved. Exponential amplification opens the opportunity for directed molecular evolution: in this context, the procedure has the potential for controllable mutation frequency, as copy liberation in step (4) (see Fig. 1) should depend on the thermodynamic stability of the immobilized duplexes and thus mutants should elute faster than perfect copies. Protocols based on the cycling of temperature (or other environmental parameters) as well as on solution chemistry are perhaps less applicable for chemical systems based on short templates. Fast negative temperature jumps as well as a fast condensation chemistry were needed to prevent the re-equilibration of the oligonucleotide complexes involved. These conditions would then necessarily limit the fidelity of template copying, because the thermodynamic control of molecular recognition sets the threshold for copying fidelity in the absence of an energy-dissipating 'proof-reading' mechanism. We thus conclude that SPREAD may be of considerable value for the design of evolutionary chemical systems. Other templates, surfaces, and template–surface links may be employed, enzymatic variants may be explored, and different steps may be combined to arrive at a higher level of process integration and autonomy. Protocols are conceivable in which related templates are immobilized close to each other, thus giving the concept of quasispecies[3] a spatial dimension. Furthermore, protocols in which two or more different classes of template molecules—such as oligonucleotides and peptides—are involved may contribute to the selection and discovery of more complex, cooperative systems. In the short term, the procedure is ready to be applied to the replication of peptides[13], pRNA[11], and enantiomeric forms of RNA[39]. In the long run, the search for autonomous variants of SPREAD may lead to a self-sustaining chemical system capable of undergoing darwinian evolution, compatible with Joyce's definition of life[40]. □

## Methods

**Materials.** The synthesis of **X**, **Y**, **A**$^{x}$, **A**$^{y}$ and **B**$^{y}$ will be reported elsewhere. Reduction buffer (RB): 100 mM DTT, 40 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA, pH 7.9; immobilization buffer (IB): 370 mM sodium acetate/acetic acid, 0.5 M NaCl, 1 mM EDTA, pH 4.4, degassed with argon for oxygen liberation; capping buffer (CB): 60 mM *S*-(2-thiopyridyl)-2-mercaptoethanol, 40 mM MES, 0.5 M NaCl, 1 mM EDTA, pH 6.5; hybridization buffer (HB): 100 mM MES, 40 mM $MgCl_2$, 1 M NaCl, 1 mM 2,2′-dipyridyldisulphide, pH 6.1; ligation buffer (LB): HB with 0.2 M EDC, fresh before chemical ligation.

**General methods.** Unless otherwise specified, all supported reactions were performed at 25 °C in Mikro-Spin centrifuge filters (0.5 ml, cellulose acetate with 0.45-μm pores, Roth) stacked into Eppendorf tubes (1.5 ml). Each of a series of 16 filters was loaded with ~50 mg of wet Sepharose 6B (washed according to the manufacturers' guidelines) and then centrifuged (3 min at 4,000*g*) to remove the supernatant. Before an immobilization step, the beads in

400 μl RB were vortexed for 1 h to generate the thiol form of Sepharose 6B. RB was then replaced by IB, repeating buffer addition, sonification (10 s), and centrifugation six times.

**Immobilization.** A suspension of SH Sepharose in 400 μl IB containing 20–40 nmol of the respective template was agitated by vortexing at 1,000 r.p.m. for 1 h.

**Capping.** 100 μl CB was added to the above suspension. After agitation for 1 h, the beads were filtered by centrifugation, resuspended in 400 μl CB and vortexed for 1 h at 25 °C. CB was then replaced by a solution of 200 mM *S*-(2-thiopyridyl)-2-mercaptoethanol in ethanol. After vortexing for 2 h at 70 °C, the beads were washed with ethanol (5× with 200 μl) and 1 M NaCl (5× with 200 μl).

**Hybridization.** The beads were resuspended in 250 μl HB containing the complementary heptamers at 400 μM concentration. The temperature was raised to 85 °C, decreased to 4 °C within 1 h, and kept there for 2 h. Excess heptamers were removed by washing the beads with three 200-μl portions of 1 M NaCl at 4 °C.

**Chemical ligation.** The beads were resuspended in 375 μl LB, vortexed for 40 h at 4 °C, and washed with 200 μl 1 M NaCl at 4 °C three times.

**Denaturation.** A Mikro-Spin filter with the respective beads was inserted into an Eppendorf tube containing 150 μl 1 M acetate buffer (pH 4.4). The beads were resuspended and gently agitated in 50 μl 0.1 M NaOH, and the supernatant was transferred by centrifugation (7,000*g*, 30 s) into an Eppendorf tube. After repeating this procedure three times, the resulting solution (400 μl) was ready for the next cycle. The beads were recycled by washing with four portions of HB.

**HPLC.** All HPLC samples without reduction buffer (RB) were brought to a concentration of 100 mM DTT before analysis to ensure reproducibility. All separation were performed on a RP C-18 column (250/4, Nucleosil 120-5 AB, Macherey and Nagel). Eluates: 0.1 M triethylammonium acetate (pH 7)/MeCN 1% (A) and MeCN (B). Analytical measurements were taken at 50 °C by using a gradient of 2% to 6% A in 2 min, 6% to 25% A in 30 min, and a flow rate of 1 ml $min^{-1}$. The eluate was monitored simultaneously at 254 nm and 273 nm. Equipment (Kontron): two pumps 422, autosampler 465, diode array detector 440, Kroma 2000 was used as data acquisition system.

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# Iron acquisition by photosynthetic marine phytoplankton from ingested bacteria

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**Iron is unique among biologically essential trace metals in having a higher particulate than dissolved concentration in ocean surface waters[1]. Uptake of dissolved iron is generally considered to be the norm for phytoplankton, as even the smallest iron-bearing particles are unavailable for transport into cells[2,3]. But the oceanic dissolved fraction is so small, and the particulate fraction so inert[2], that phytoplankton production is limited by a dearth of available iron in some regions[4]. Here we use incubation experiments to show that *Ochromonas* sp., a common photosynthetic flagellate from the Pacific Ocean, can obtain iron directly in particulate form, by ingesting bacteria. Iron acquisition is highly efficient; *Ochromonas* assimilates 30% of the ingested ration, acquiring a high intracellular iron concentration and maintaining a significantly faster growth rate than when iron is provided in the dissolved phase. Phytoplankton capable of such phagotrophy (so-called mixotrophic species) may thus be able to assimilate iron in both particulate and dissolved forms in the ocean. Moreover, when iron availability is limited, the iron 'cost' of growth is diminished because *Ochromonas* derives a greater fraction of its energy from the bacteria. Analysis of standing**