Synthetic life
(continuation of "The molecular origins of life" SoSe 2021)

WiSe 2021/22
Zbigniew Pianowski
Overview of the course

artificial ribozymes and aptamers for efficient catalysis and recognition (SELEX, DNAzymes, foldamers);

unnatural base pairing – expansion of the genetic alphabet;

Artificial genetic polymers and oligonucleotide analogues (XNA);

biosynthetic incorporation of unnatural aminoacids (UAAs) into proteins;

enzyme engineering – production of enzymes with unknown or unnatural properties, ab initio protein design, directed evolution, theozymes;

Artificial lipid vesicles as models for protocell multiplication;

design of artificial organisms
CHAPTER 1

OLIGONUCLEOTIDES

The less common side of RNA
Non-standard functions of RNA

**Ribozymes** – *Ribonucleic acid enzymes*

**Riboswitches** - RNA-based genetic regulatory elements

**Aptamers** - oligonucleotide or peptide molecules that bind to a specific target molecule.

**Viroids** - subviral pathogens – short (246 to 467 nt), circular, single-stranded, and non-coding RNA without a protein coat

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**Hammerhead ribozyme**

**SELEX – aptamer selection**

**TPP riboswitch**
Living relics of the RNA world:
Ribozymes, riboswitches, viroids, co-enzymes, structure of the ribosome

proteins play a structural role, but DO NOT CATALYZE THE ACYL TRANSFER PROCESS
The RNA world
RNA-dependent RNA polymerase ribozyme – Replicase

Replicase - problem

The replicase most likely needs to be long (> 200 nt) for the efficient replication – How could such long functional RNA be spontaneously generated?

Possible solution – autocatalytic networks

No component can replicate without all the others

D. P. Horning, G. F. Joyce Proc. Natl. Acad. Sci. USA (PNAS) 2016, 113 (35), 9786-9791
CHAPTER 1

OLIGONUCLEOTIDES

Topic 3 – modified canonical nucleobases
Canonical nucleobases and Watson-Crick pairing in DNA
Restriction modification system

„Immune system” of bacteria and archaea against attacking viruses

K. Vasu, V. Nagaraja

K. Ishikawa et al. DOI: 10.1093/dnares/dsq027
**EcoI – a typical restriction enzyme**

**Products of restriction enzymes**

Structure of the homodimeric restriction enzyme EcoRI (cyan and green cartoon diagram) bound to double stranded DNA (brown tubes). Two catalytic magnesium ions (one from each monomer) are shown as magenta spheres and are adjacent to the cleaved sites in the DNA made by the enzyme (depicted as gaps in the DNA backbone).
Non-canonical fluorescent nucleobases

Sanger sequencing

Sequencing Gel

DNAzyme

TACGTA
ATGCATTAGGGCCTGGCTCTTT

TACGTAA
ATGCATTAGGGCCTGGCTCTTT

TACGTAAATCCCGGA
ATGCATTAGGGCCTGGCTCTTT

TACGTAAATCCCGGACCGGA
ATGCATTAGGGCCTGGCTCTTT
CyDNA – synthesis and replication of highly fluorescently-labelled DNA

DNA Polymerase is evolutionarily optimized by mutagenesis to accept fluorescent Cy3 or Cy5 nucleotides instead of simple dCTP.

Pyrococcus furiosus – an extremophilic Archaeon from marine sediments
Optimal life temperature 100°C

P. Holliger et al. J. Am. Chem. Soc. 2010, 132, 5096-5104
Why are A, C, G and T the letters of genetic alphabet.
Prebiotic synthesis of nucleobases
Formose reaction in presence of borates

Pentose formation in the presence of borate

With borate (left)
Without borate (right)
Colemanite (background)

By NMR, the ribose borate complex 8 has the structure shown; cyclic structures for other pentoses are speculative.

A. Ricardo, M. A. Carrigan, A. N. Olcott, S. A. Benner
Science 2004, 303, 196
Cyanosulfidic chemistry

The aldol chemistry of sugars and cyanide chemistry of nucleobases can be combined at earlier stages than glycosylation.
Cyanosulfidic chemistry

M. W. Powner, B. Gerland, J. D. Sutherland, Nature 2009, 459, 239–242
Photochemistry of beta-ribocytidine-2’,3’-cyclic phosphate 1. Under conditions of irradiation that destroy most other pyrimidine nucleosides and nucleotides, 1 undergoes partial hydrolysis and slight nucleobase loss. Ura, N1-linked uracil; Cyt–H, cytosine; Ura–H, uracil.

M. W. Powner, B. Gerland, J. D. Sutherland, *Nature* 2009, **459**, 239–242


CHAPTER 1

OLIGONUCLEOTIDES

Topic 4 – expansion of the genetic alphabet
Expansion of the genetic alphabet

Expansion of the genetic alphabet and code by creating an unnatural base pair (UBP) as a third pair.

The creation of a UBP (i.e., X–Y) that functions in replication, transcription, and translation as a third base pair with the natural A–T(U) and G–C pairs allows the storage and retrieval of the expanded genetic information in vitro and in vivo, enabling a variety of applications using biopolymers with increased functionalities.

M. Kimoto, I. Hirao Chem. Soc. Rev. 2020, 49, 7602-7626
Tautomeric instability in non-canonical nucleobases

Figure 3.20 Chromosome 1 from hamster cells exposed to bromodeoxyuridine. (A) Normal chromosome. (B–E) Aberrant chromosomes. (From T.C. Hsu and C.E. Somers, Proc. Natl. Acad. Sci. USA 47: 396–403, 1961. With permission from the MD Anderson Cancer Center.)
DAP – one tautomer forms a base pair with guanine

iso-C/iso-G
- specificity (the enol tautomer of iso-G, stabilized by aromatization, complementary to thymine)
- the 2-amino group of iso-C hydrolyses easily to uracil
Natural and non-natural base pairs that function in polymerase reactions
Unnatural base pair (UBP) design rules:

Design

- Distance of 10.7-11.0 Å between the glycosidic bonds of the pair
- no mispairing with natural bases
- Reasonable hydrophobicity
- Chemical stability (phosphoramide chemistry)
- Recognition by DNA and RNA polymerases (fidelity, efficiency)

Chemical synthesis
Phosphoramide reagents for DNA chemical synthesis

Polymerase reaction tests
Single-nucleotide insertion

Polymerase reaction tests
Single-nucleotide insertion

Primer extension

Non-natural nucleobases compatible with polymerases


Steven Benner (1989-95): the artificially expanded genetic information system (AEGIS) including the isoG–isoC and X–κ pairs

Benner and Prudent (2004): new quantitative PCR (qPCR) methods, such as Plexor, using the isoG–isoC pair

Benner (2007): Z-P pair - the P base by removing the hydrogen at position 1 of G to exclude the keto–enol tautomerism, by introducing the nitro group into Z, the chemical stability of the nucleoside was improved;

With the same strategy, they also developed the B–S and X–K pairs from isoG–isoC and X–κ pairs, respectively
AEGIS – Artificially Expanded Genetic Information System

Watson–Crick pairing rules:
(a) size complementarity - large purines pair with small pyrimidines
(b) hydrogen-bonding complementarity  (hydrogen-bond acceptors, A, pair with hydrogen-bond donors, D).

Rearranging donor and acceptor groups on the nucleobases, while not changing the geometry of the Watson–Crick pair, creates an artificially expanded genetic information system (AEGIS). AEGIS components add information density to DNA strands built from them.

Artificial Gene Synthesis

Artificial gene synthesis (DNA printing) - method in synthetic biology to create artificial genes in the laboratory:

- currently based on solid-phase DNA synthesis,
- the user does not have to begin with preexisting DNA sequences.
- Therefore, it is possible to make a completely synthetic double-stranded DNA molecule with no apparent limits on either nucleotide sequence or size.

Applications:
- recombinant DNA technology including heterologous gene expression, vaccine development, gene therapy and molecular engineering.
- The synthesis of nucleic acid sequences can be more economical than classical cloning and mutagenesis procedures
- the ability to safely obtain genes for vaccine research without the need to grow the full pathogens.
- to optimize protein expression in a particular host, or to remove non-functional DNA segments
- For DNA digital data storage and computing
- For synthetic biological circuits

Hajissa et al. Parasites & Vectors (2015) 8:315
Self-assembly of whole genes and DNA nanostructures

Limitations of DNA puzzle assembly: unequal A:T vs. G:C strength, insufficient ACGT information density, higher-order structures

**Self-assembly of whole genes and DNA nanostructures**

Solution: an orthogonal pair from the AEGIS system, that can be removed from the product, yielding native DNA structures.

Conversion occurs when polymerases are forced to mismatch a standard nucleotide opposite an AEGIS nucleotide by

(a) not being provided the complementary AEGIS triphosphate and

(b) exploiting a chemical feature of the AEGIS nucleotide that directs a specific mismatch.

B in its major tautomeric form pairs with S; in its minor tautomeric form, B pairs with standard T. Assembly of the target gene/DNA nanostructure is followed by conversion of the S:B pairs to T:A pairs after two cycles of PCR: B → A via an intermediate B:T mispairing, S → T (intermediate S:B followed by a second B:T mispairing).

Self-assembly of whole genes and DNA nanostructures

The technology tested by assembly of the kanamycin-resistance gene and growing the bacteria in the environment containing kanamycin after assembly and conversion of that gene.

AEGIS – Artificially Expanded Genetic Information System

First Generation AEGIS

E. Biondi, S. Benner Biomedicines 2018, 6, 53; doi:10.3390/biomedicines6020053
AEGIS – Artificially Expanded Genetic Information System

AEGIS – Artificially Expanded Genetic Information System

AEGIS – Artificially Expanded Genetic Information System

AEGIS – Permanent orthogonal nucleobases surviving PCR

Error rate 0,2% per a PCR cycle – both removal and incorporation of Z and P → the artificial genetic system capable to evolve.

Pol: Deep Vent – 2 Z/P, Taq/Phu – 3-4 Z/P
dZTP (deprotonated) at higher pH pairs slightly with G → loss of some Z, but also gain of some new Z mutants.

ACGTZP-DNA crystal structures

18-mers: 2+2 Z:P pairs → B-DNA
6 consecutive Z:P → A-DNA
0,1 nm wider, but otherwise alike G:C pairs

Hachimoji DNA and RNA – a genetic system with eight (Jap.- Hachi) letters

Plots of experimental versus predicted (A) free energy changes ($\Delta G^\circ_{37}$) and (B) melting temperatures $T_m$ for 94 SBZP-containing hachimoji DNA duplexes.

S. Hoshika et al., Science 2019, 363, 884-887
Hachimoji DNA and RNA

Crystal structures of hachimoji DNA.

(A) The host-guest complex with two N-terminal fragments from Moloney murine leukemia virus reverse transcriptase bound to a 16-mer PP hachimoji DNA; Z:P pairs are green and S:B pairs are magenta.

(B) Hachimoji DNA structures PB (green), PC (red), and PP (blue) are superimposed with GC DNA (gray).

(C) Structure of hachimoji DNA with self-complementary duplex 5’-CTTATPBTASZATAAG (“PB”).

(D) Structure of hachimoji DNA with self-complementary duplex 5’-CTTAPCBTASGZTAAG (“PC”).

(E) Structure of hachimoji DNA with self-complementary duplex with six consecutive nonstandard 5’-CTTATPPSBZZATAAG (PP) components.

(F to I) Examples of largest differences in detailed structures. The Z:P pair from the PB structure (F) is more buckled than the corresponding G:C pair (G). The S:B pair from the PB structure (H) exhibits a propeller angle similar to that in the corresponding G:C pair (I).

S. Hoshika et al., Science 2019, 363, 884-887
**Hachimoji RNA aptamer**

T7 RNA polymerase incorporates ZTP, PTP, and BTP, but not STP opposite to dP, dZ, dS, and dB, respectively. A mutant of T7 RNA Pol (Y639F H784A P266L, “FAL”) incorporated also STP – full DNA→RNA conversion possible

The hachimoji variant of the spinach fluorescent RNA aptamer. In its standard form, spinach folds and binds 3,5-difluoro-4-hydroxybenzylidene imidazolinone, which fluoresces green when bound.

(B) Control with fluorophore only, lacking RNA.
(C) Hachimoji spinach with the sequence shown in (A).
(D) Native spinach aptamer with fluorophore.
(E) Fluorophore and spinach aptamer containing Z at position 50, replacing the A:U pair at positions 53:29 with G:C to restore the triple observed in the crystal structure. This places the quenching Z chromophore near the fluorophore;

S. Hoshika *et al.*, *Science* 2019, 363, 884-887
AEGIS – Artificially Expanded Genetic Information System

An xNA biopolymer having functionalized AEGIS components may allow SELEX to yield aptamers better than the standard DNA and RNA biopolymers.

enrichment of breast cancer cell (MDA-MB-231) binders with an optimum obtained after 11th round binding of chemically resynthesized ZAP-2012 aptamer obtained using AEGIS Cell–SELEX to MDA-MB-231 breast cancer cells

Where the Z or P are separately replaced with standard nucleotides, binding affinity is reduced.

Where the Z and P are both replaced with standard nucleotides, binding affinity is lost (not shown).

Nucleotides Z and P were added to a library of oligonucleotides used in a laboratory *in vitro* evolution (LIVE) experiment; the GACTZP library was challenged to deliver molecules that bind selectively to liver cancer cells, but not to untransformed liver cells. Unlike in classical *in vitro* selection, low levels of mutation allow this system to evolve to create binding molecules not necessarily present in the original library. Over a dozen binding species were recovered. The best had multiple Z and/or P in their sequences.

The aptamer-nanotrain assembly, charged with doxorubicin, selectively kills liver cancer cells in culture, as the selectivity of the aptamer binding directs doxorubicin into the aptamer-targeted cells. The assembly does not kill untransformed cells that the aptamer does not bind.

This architecture, built with an expanded genetic alphabet, is reminiscent of antibodies conjugated to drugs, which presumably act by this mechanism as well, but with the antibody replaced by an aptamer.

ACGTZP-aptamers against a specific protein

Laboratory in vitro evolution (LIVE) might deliver DNA aptamers that bind proteins expressed on the surface of cells.

Here, cell engineering was used to place glypican 3 (GPC3), a possible marker for liver cancer theranostics, on the surface of a liver cell line.

Libraries were then built from a six-letter genetic alphabet. With counterselection against non-engineered cells, eight AEGIS-containing aptamers were recovered. Five bound selectively to GPC3-overexpressing cells.

**LG5** $K_d = 6$ nM (without Z – no binding)

Steric exclusion and hydrophobic non-natural base pairs
Steric exclusion and hydrophobic non-natural base pairs

Hirao (2001): the steric hindrance concept to hydrogen-bonded UBPs to exclude the mispairing with natural bases → a series of hydrogen-bonded UBPs: x–y and s–y pairs

large residues at position 6 of x and s sterically and/or electrostatically clash with the 4-keto group of T, but not with hydrogen of the y base.

x–y and s–y pairs function in transcription with T7 RNA polymerase (T7 transcription), and the y substrate is incorporated (>96% selectivity s–y) site-specifically into RNA transcripts opposite x or s in DNA templates.

Problem: the y base cannot exclude the mispairing with A. Therefore, these UBPs cannot be used in replication.
Unnatural aminoacid incorporation using a noncanonical base pair

Non-template strand: 5'-d(ATAATACGACTCACTATAGGG)
Template strand: 3'-d(TATTATGCTGAGTGATATCCCTTGCCTGCTNCACGCC)
N = s, x, A, or G

1. T7 RNA polymerase
2. 1 mM NTPs, [α-32P]ATP, GMP
3. Full-length product (19-mer)
4. RNase T2 digestion
5. 2D-TLC

32P-labeled nucleotides
Gp* x 2, Ap* x 1, N*p* x 1

Unnatural amino acid incorporation using a noncanonical base pair

The coupled transcription–translation system using the nonstandard codon–anticodon interaction for the site-specific incorporation of 3-chlorotyrosine into the Ras protein.

Unnatural amino acid incorporation using a noncanonical base pair

(A) Construction of tRNA_{CUS}. (B) Acidic-gel electrophoresis of the products after aminoacylation of *S. cerevisiae* tRNA and tRNA_{CUS} with tyrosine and the 3′-substituted analogs, in the absence or presence of 20% DMSO and 0.25% Tween-20. The upper bands are the aminoacylated tRNAs, and the lower bands are the noncharged tRNAs.

A) Autoradiogram of *in vitro* transcription–translation products labeled with l-[\(^{14}\)C]leucine. The conditions are noted at the bottom of each lane.

B) LC patterns of the products digested by Lys-C. Chart a shows the products obtained from the reaction in the presence of the DNA template containing the CTs sequence, yTP, and CITyr-tRNA\(_{Cys}\); chart b shows those obtained in the presence of the standard DNA template and Tyr; chart c shows those obtained in the presence of the DNA template containing CTs but in the absence of yTP and tRNA\(_{Cys}\); and chart d shows those obtained in the presence of the DNA template containing CTs and yTP but in the absence of tRNA\(_{Cys}\).

C) Tandem mass spectrum of the F-cY32 fragment. The partial sequence, VDEcYD, of F-cY32 was confirmed from the ion series

Steric exclusion and hydrophobic non-natural base pairs


Hirao (2003): the Q–Pa pair to avoid steric clashes. Hirao (2007): Pa could be used as a pairing partner of the s base, and the s substrate was incorporated specifically into RNA opposite Pa in templates by T7 transcription. Hirao (2004): The s–z pair increased the s incorporation selectivity opposite z in T7 transcription, as compared to that opposite y in the s–y pair.
Steric exclusion and hydrophobic non-natural base pairs

Hirao (2006) the hydrophobic Ds base (improved s). The Ds–Pa pair exhibited high incorporation efficiency in replication. **Problems**: the non-cognate Ds–Ds and A–Pa pairings (solved by using γ-amido-triphosphates of Ds and A, which significantly reduced their mispairing with Ds and Pa - ~99.9% selectivity per cycle in PCR).

Hirao (2007): the Ds–Px pair - electrostatic clash with the 1-nitrogen of A (no A–Px pairing), The additional propynyl group reduces the Ds–Ds self-pairing. The Ds–Px pair exhibits high fidelity and high efficiency in PCR (>99.9% per cycle).
Fluorescent probing for RNA molecules by an unnatural base-pair system

(B) Incorporation of the fluorescent s base into GNRA hairpins. (left) The secondary structure of the RNA hairpin with a GAAA loop. The second A and third A in the loop are shown in blue and red, respectively. (right) The 3D structure of the GAAA-loop hairpin

The „s” incorporation sites in yeast tRNA$^{\text{Phe}}$

The fluorescent intensity of „s” in RNA molecules changes according to the structural environment. The site-specific s labeling of RNA hairpins and tRNA molecules provided characteristic fluorescent profiles, depending on the labeling sites, temperature and Mg$^{2+}$ concentration.

(A) The secondary structure of the original tRNA transcript. The positions substituted with s are circled. The broken lines show base–base interactions for the 3D structure.

(B–E) The deep-colored bases were substituted with s, which stacks with the light-colored bases, and the yellow spheres represent Mg$^{2+}$.

Steric exclusion and hydrophobic non-natural base pairs

The Ds–Pa pair for faithful replication in combination with γ-amidotriphosphates.

(A) The substrates, dDsTP and dATP, are incorporated opposite Pa and T in the templates, respectively. However, dDsTP and dATP are also misincorporated opposite Ds and Pa, respectively. The Ds incorporation opposite Ds inhibits further primer extension.

(B) The γ-amido-triphosphates, dDsTPγ and dATPγ, reduce the Ds–Ds and A–Pa mispairings in replication.

(C) The combination of the usual triphosphates (dG/C/T/PaTP) and the γ-amido-triphosphates (dDs/ATPγ) enables the faithful PCR amplification of 6-letter DNA.

M. Kimoto, I. Hirao Chem. Soc. Rev. 2020, 49, 7602-7626
Replication and transcription of the hydrophobic UBP

(a) The unnatural Ds-Pa and natural base pairs (Pa: R = H, Pa': R = C≡C–CH₃). (b) The unnatural base pair system that functions in PCR amplification, primer extension, DNA sequencing and T7 transcription. Original DNA templates were prepared by chemical synthesis and ligation, and were amplified by PCR with unmodified dNTPs (dPaTP, dGTP, dCTP and dTTP) and modified dNTP₉s (γ-amidotriphosphates, dDsTP₉ and dATP₉). RNA molecules containing Pa or modified Pa bases at specific positions were transcribed from DNA templates containing Ds in the template strands, by T7 RNA polymerase with PaTP (or modified PaTP) and the natural NTPs (left). RNA molecules containing Ds were transcribed from DNA templates containing Pa in the template strands, with DsTP and the natural NTPs (right).

(a) The double stranded DNA fragment (150-mer, DNA1) was prepared by primer extension using chemically synthesized DNA fragments (91-mer and 81-mer) containing Ds and Pa. (b) Agarose-gel analysis of original DNA fragments (0 cycle) and PCR products after 5 and 10 cycles of amplification. For DNA1, PCR was performed with 0.04 unit/μl Vent DNA polymerase and the following cycle conditions: 0.5 min at 94 °C, 0.5 min at 45 °C and 4 min at 65 °C. For DNAcont1 consisting only of the natural bases, PCR was performed with 0.01 unit/μl Vent DNA polymerase with the following cycle conditions: 0.5 min at 94 °C, 0.5 min at 45 °C and 1 min at 72 °C. (c–f) DNA sequencing, in the presence (c,e) or absence (d,f) of dPa`TP, of the original DNA1 (c,d) and PCR-amplified DNA1 after 10 cycles using the unnatural base pair system (e,f).
Ds-Px noncanonical base pair – High-affinity DNA aptamers

Targets:
VEGF-165 and IFN-γ

Characterizations and binding affinities of anti–VEGF-165 aptamer (VGd1-2Ds-47) and anti–IFN-γ aptamer (IFd1-3Ds-49)

Ds-Pa – need for deep sequencing in the amplification process

Ds-Pa – deep sequencing

NDsN2-49 5’ NN Ds NN 3’
NDsN3-49 5’ NNN Ds NNN 3’

Replacement PCR

Deep sequencing
Alignment of all the sequences

Natural-base substrates (dNTPs: N = A, G, C or T)
Intermediate substrates (dXTP: X = Pa’, Pa or Px)

Figure 3B

Deep sequencing

Figure 3C

Ds-Pa – deep sequencing

Referring to the encyclopedia data allows for simple and fast determination of the Ds positions.

Comparison of the replacement patterns between two conditions enables the Ds positions to be distinguished from other natural-base positions.
Ds-Pa aptamer selection using deep sequencing

M. Kimoto, I. Hirao Chem. Soc. Rev. 2020, 49, 7602-7626
**Ds-Pa aptamer selection using deep sequencing**

Secondary structures of the optimized Ds-DNA aptamers targeting VEGF165, IFNγ, and vWF.

Generation of a molecular affinity ruler using anti-IFNγ-Ds-DNA aptamer variants, replacing the Ds base with the natural A base.


A semi-synthetic organism with an expanded genetic alphabet

Nucleoside phosphorylation: a biosynthetic pathway to provide unnatural substrates (dX/dYTP) in a cell, using their unnatural nucleosides (dX/dY) as a source.

In Step 2, the nucleoside kinase from D. melanogaster (DmdNK) was explored for its ability to phosphorylate the unnatural nucleosides by three teams in vitro. Benner’s team confirmed that the kinase in Step 4 was available for their Z and P bases in vitro. All UB triphosphates are, at least, accepted by the Klenow fragment of E. coli Pol I in vitro.

M. Kimoto, I. Hirao Chem. Soc. Rev. 2020, 49, 7602-7626
A semi-synthetic organism with an expanded genetic alphabet

Homologous recombination strategy using the yeast strain TK369, to integrate dsDNA with the Ds–Px pair.

DmdNK – nucleoside kinase
hENT - human equilibrium nucleoside transporter

The Ds and Px nucleosides supplied in the media. The long dsDNA with the Ds–Px pair was prepared by fusion/overlapping PCR,

Analysis: colony PCR with dDsTP and dPxTP, then modified Sanger sequencing.

M. Kimoto, I. Hirao Chem. Soc. Rev. 2020, 49, 7602-7626
Romesberg (1999): PICS–PICS, but the polymerase reaction is paused at the PICS–PICS pairing position due to self-stacking. 
Romesberg (2008): the MMO2–5SICS pair - the methyl group of 5SICS prevented the disfavored 5SICS–5SICS self-pairing. 
Further optimized into the NaM–5SICS pair (>99% fidelity in PCR and transcription).
Romesberg (2014) the NaM–TPT3 pair (systematic in vitro replication screening) ➔ semi-synthetic organism (SSO) of E. coli with six-letter DNA.
Optimizations: the CNMO–TPT3 pair to increase the UBP retention on a plasmid within the SSO, the NaM–TAT1 pair for efficient transcription and translation in the SSO.
Using these UBP systems, they reported protein synthesis with the site-specific incorporation of unnatural amino acids into proteins in the SSO.
A semi-synthetic organism with an expanded genetic alphabet

The first creation of an SSO, using the NaM–TPT3 and NaM–5SCIS pairs and E. coli C41(DE3) as the host strain.

The plasmid containing the NaM–TPT3 pair was prepared by PCR and transformed into E. coli C41(DE3) with a PtNTT2 overexpression system. The transformants were cultured in inorganic phosphate-rich growth media in the presence of the NaM and 5SCIS triphosphates.

The propagated plasmids were isolated and subjected to the analysis of the NaM–5SCIS pair retention by a biotin-shift assay.

A semi-synthetic organism with an expanded genetic alphabet

**a**, Chemical structure of the d5SICS–dNaM UBP compared to the natural dG–dC base pair. **b**, Composition analysis of d5SICS and dNaM in the media (top) and cytoplasmic (bottom) fractions of cells expressing PtNT2 after 30 min incubation; dA shown for comparison. 3P, 2P, 1P and 0P correspond to triphosphate, diphosphate, monophosphate and nucleoside, respectively; [3P] is the intracellular concentration of triphosphate.

Malyshev, Denis A.; Romesberg, Floyd E. *et al.* Nature 2014, 509, 385–388
A semi-synthetic organism with an expanded genetic alphabet

The SSO replicates DNA containing the dNaM-dTPT3 UBP (blue and red, respectively), transcribes mRNA and tRNA with complementary codons and anticodons containing NaM or TPT3, uses an orthogonal synthetase to charge the tRNA with an ncAA, and uses the charged tRNA to translate the mRNA into proteins containing ncAAs.

Y. Zhang, F. Romesberg, Biochemistry 2018, 57, 15, 2177–2178
A semi-synthetic organism with an expanded genetic alphabet

Researchers added a synthetic base pair (nicknamed X and Y; shown in blue) to DNA to encode new amino acids, which cells then incorporated into the fluorescent protein GFP.

Six nucleotides
Three base pairs

216 possible codons could code for up to 172 amino acids

Exploiting unnatural amino acids could allow for the development of proteins with new characteristics.

A semi-synthetic organism with an expanded genetic alphabet

M. Kimoto, I. Hirao Chem. Soc. Rev. 2020, 49, 7602-7626
A semi-synthetic organism with an expanded genetic alphabet

The in vivo transcription of DNA containing dNaM and dTPT3 into mRNAs with two different unnatural codons and tRNAs with cognate unnatural anticodons, and their efficient decoding at the ribosome to direct the site-specific incorporation of natural or non-canonical amino acids into superfolder green fluorescent protein. The resulting semi-synthetic organism both encodes and retrieves increased information and should serve as a platform for the creation of new life forms and functions.

A semi-synthetic organism with an expanded genetic alphabet

A semi-synthetic organism with an expanded genetic alphabet

- An unnatural base pair (UBP) would increase the information storage potential of DNA
- and semisynthetic organisms (SSOs) that stably harbor this expanded alphabet would thereby have the potential to store and retrieve increased information,
- *Escherichia coli* grown in the presence of the unnatural nucleoside triphosphates dNaMTP and dSSICSTP, and provided with the means to import them via expression of a plasmid-borne nucleoside triphosphate transporter, replicates DNA containing a single dNaM-dSSICS UBP,
- to fortify and vivify the nascent SSO, a more chemically optimized UBP dTPT3 was used, and the power of the bacterial immune response was harnessed by using Cas9 to eliminate DNA that had lost the UBP.
- The optimized SSO grows robustly, constitutively imports the unnatural triphosphates, and is able to indefinitely retain multiple UBPs in virtually any sequence context. This SSO is thus a form of life that can stably store genetic information using a six-letter, three-base-pair alphabet

Romesberg, Floyd E. *et al.* *PNAS* 2017, 114, 1317-1322
A semi-synthetic organism with an expanded genetic alphabet

Romesberg, Floyd E. et al. PNAS 2017, 114, 1317-1322
A semi-synthetic organism with an expanded genetic alphabet

Romesberg, Floyd E. *et al.* *PNAS* 2017, 114, 1317-1322
UBPs are not well retained in all sequences, limiting the information that can be encoded, and are invariably lost upon extended growth. The contributions of the *E. coli* DNA replication and repair machinery to the propagation of DNA containing dNaM-dTPT3 have been explored and showed that replication by DNA polymerase III, supplemented with the activity of polymerase II and methyl-directed mismatch repair contribute to retention of the UBP and that recombinational repair of stalled forks is responsible for the majority of its loss.

Transcription and reverse transcription in vitro and in SSO

Transcription and reverse transcription in vitro and in SSO

Transcription fidelity measured by T-RT assay of (A) mRNA and (B) tRNA made by in vitro transcription using T7 RNA polymerase.

Transcription fidelity measured by T-RT assay of (A) mRNA and (B) tRNA extracted from SSO in vivo translation experiments

Expanded genetic alphabet - 2019

Romesberg, Floyd E. et al.
Unnatural nucleobases - overview