Structure-Function Relationships in Dynamic Combinatorial Libraries

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Chapter 1

Advances on Complex Molecular Systems

1.1 Introduction

Science has always been inspired by nature. What makes nature so attractive is the perfect harmony between its constituents. From a scientific point of view, the key to understand nature is to resolve how it works and evolves over time. Scientists have always been fascinated by the assembly of natural structures and tried to mimic these assemblies to construct different materials. Especially after Darwin's proposal of the evolution of species from a common ancestor, understanding nature in terms of how life emerged became a more crucial and challenging task. Considering that all living systems have evolved to give enormous diversity from a quite limited variety of resources, it is very impressive that all have different emergent functions as a result of evolution. However, this fascination also brought up debates on how life as we know emerged on earth. Many scientists claim that RNA emerged first since it unites two vital functions in the same molecule: the abilities to store information and to catalyze certain chemical reactions. But over time, theories explaining the origins of life moved beyond RNA. It has been argued that RNA is too large to exist in a prebiotic world and is a relatively poor catalyst. This notion has prompted the search for simpler molecular entities that could lead to the emergence of life. These entities are mostly peptides and nucleobases which are the main constituents of functional proteins and nucleic acids which take part in almost all cellular processes.

In this chapter, we will first give examples on how small structural variations affect protein function. Then we will briefly describe commonly believed and verified hypotheses, covering biological and biochemical evolution and recently developed studies in chemistry to understand origins of life on earth focused on complex molecular systems.

1.2 Structure-to-Function in Biology

In biology, even the smallest molecular details can have profound effects on the ultimate function in life. Take, for example, the difference between methyl and ethyl alcohol: one being a toxic agent and causing blindness while the other is used as mild narcotic. Although sequential similarity is generally considered as a good indicator of functional similarity in proteins, there have been theoretical[1] and experimental[2,3] studies with homologous, or nearly homologous, proteins possessing different or even opposite functions. For example, in 2015, Amir et al. provided a molecular explanation for two structurally homologous proteins exerting opposite functions with different interaction phases.[4] They performed their study in a class of apoptosis stimulating proteins (ASPP) and showed that, while one of them (ASPP2) induces apoptosis, the other (iASPP) inhibits it (Figure 1.1).

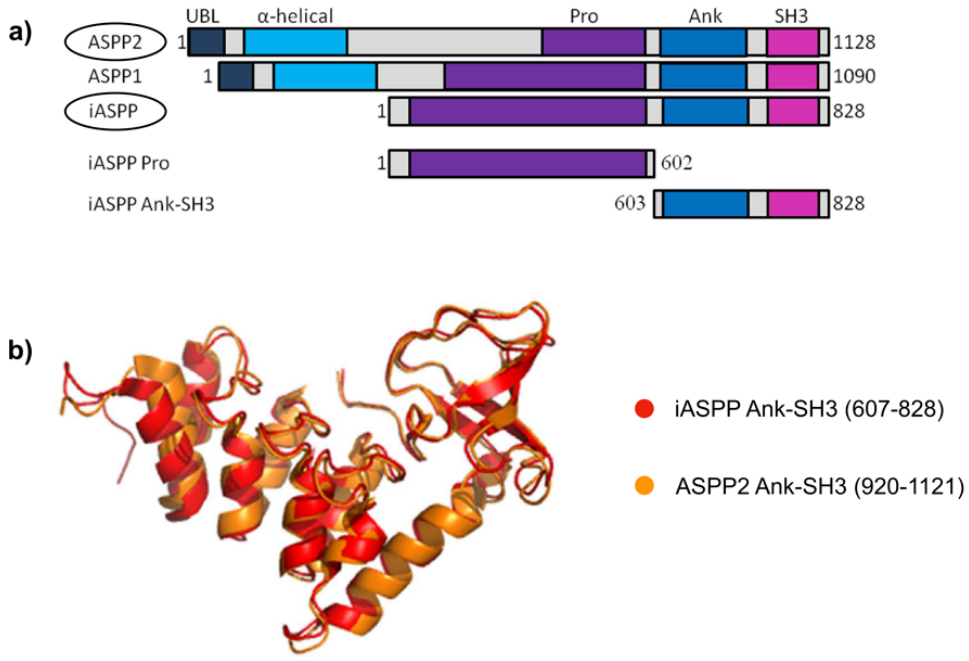

Figure 1.1: a) All the ASPP proteins contain a proline domain (Pro), four ankyrin repeats (Ank) and an SH3 domain. ASPP2 and ASPP1 also contain a putative α-helical domain at their N-termini. The N-terminal part of ASPP2 has the structure of a β-Grasp ubiquitin-like fold (UBL). It has been stated that the N-terminal domain that is unique to ASPP2 is not responsible for the opposite activity in apoptosis regulation. b) Overlapped crystal structures of ASPP2 Ank-SH3 (orange) and iASPP Ank-SH3 (red). The alignment shows the structural similarity between the two protein parts. Figure adapted from reference.[4]

From an evolutionary point of view, the main information carriers, i.e. the ones that determine function, are the genes. Spontaneous changes in genes direct the ultimate diversity in living organisms when the information they carry is translated into proteins. These proteins are responsible for many cellular activities, like catalyzing certain reactions, transporting small molecules etc. They perform their functions with the help of regulatory molecules which are either other enzymes or smaller molecules such as amino acids or nucleotides. While some of these regulators can turn proteins on and off, some others regulate the enzyme activity.[5] Considering that many structural mutations occur spontaneously in nature, even a small change, such as a single base of a gene, results in a single amino acid being changed in the encoded protein, may have a dramatic impact on evolution. In order to understand

how today's complex biochemical machineries evolved, one approach is to search for the first/simplest molecule or system that is able to carry out the basic functions of a living system.

1.3 What is Life?

'What is life?' is a question that is much more complicated than one might think, to the point that attempts to define 'life' are threatening to hinder the actual search of life's origins. Since the last century, scientists have been trying to reach consensus on how to define life but there is still no clear definition. A full understanding of living systems would possibly require resolving how the first living entity arose on earth.

What is called 'living' might differ for each biological species. As Carl Sagan states in the book chapter *Definitions of life*:[6] 'Man tends to define life in terms of the familiar. But the fundamental truths may not be familiar'. Therefore, he introduced five different approaches to define life: 1) physiological: a system capable of eating, reproducing, breathing, growing etc; 2) metabolic: a living system that exchanges materials with surroundings but still has a certain boundary; 3) biochemical: systems that contain hereditary information coded in nucleic acids and metabolism in the form of certain chemical reactions catalyzed and controlled by enzymes; 4) genetic: systems that are able to reproduce and evolve as a result of natural selection and 5) thermodynamic: systems that are open, meaning being dependent on a flow of energy. All these definitions address important aspects of life, but each also misses elements when viewed from a different perspective.

Combining the transition from non-living to living with insights from biological evolution, provides a lot of research questions to explore. After theories related to origins of life proposed by Darwin[7] and Oparin,[8] the first iconic experiment that supported these theories was published in 1953 by Stanley Miller and aimed at mimicking the early atmosphere of earth.[9] With electrical sparks in a gas mixture, they were able to produce some of the vital building blocks of life, such as glycine and alanine. Nearly 50 years after this experiment, re-analysis of the residues from Miller's samples actually showed that all 22 amino acids were present.[10] Although geoscientists argue that the early atmosphere may not have had such a reducing power,[11] this key experiment still supports the idea that localized prebiotic synthesis may have been powerful enough to synthesize the simple building blocks that form the basis of living systems.

Life on earth, as we know it, comprises three main functions: replication, metabolism and compartment formation. Although it is extremely difficult to functionally integrate these aspects in a single system, there have been many attempts to create minimal cells: synthetic cellular structures that contain important features of 'living'

cells in biology.[12–14] Recent examples will be discussed in the following subchapters.

1.4 Steps Towards Synthetic 'Living' Systems

Life that we know today is a highly complex and dynamically evolving system. In a commonly believed prebiotic scenario, life could have started from simple organic molecules that were stable in the harsh conditions of early earth which then formed larger biomolecules. After this stage, those biomolecules may have started to interact with each other, thus fulfilling different functions. When membrane-like species started to form, less stable biopolymers could have been isolated in such environments allowing them to participate in biochemical evolution.[15] Depending on such a scenario, different scientific areas approach evolution by investigating different life-like functions such as replication, metabolic activity or formation of cell-like compartments. While chemists have mostly focused on information carrying molecules potentially capable of undergoing evolution, the synthesis of replicating minimal cells has been one of the major focusses in synthetic biology.

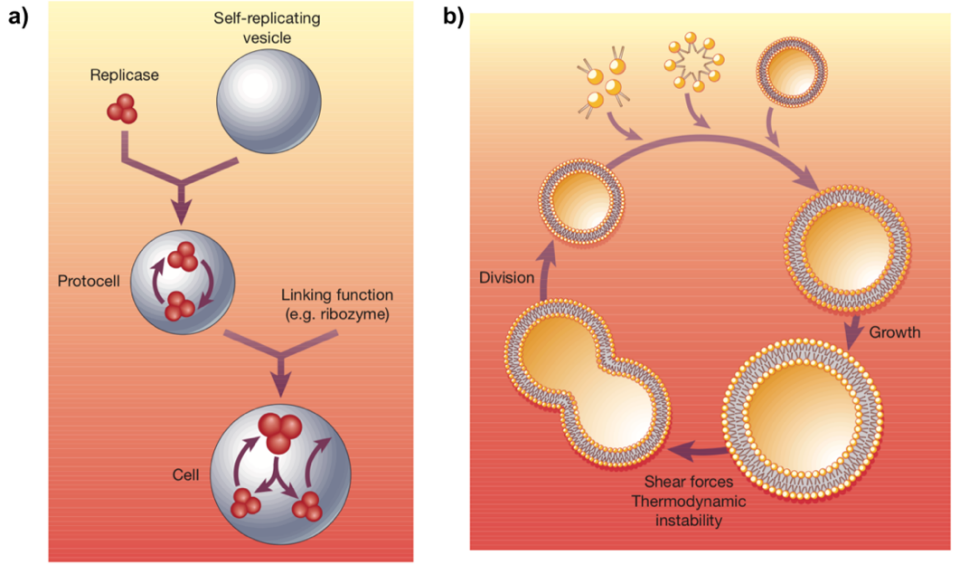

Figure 1.2: a) A possible pathway a synthetic cell by merging a replicase with a self-replicating vesicle. b) Proposal of vesicle growth from a bilayer forming precursors followed by division, either spontaneously, or by an external stimulus. Figure adapted from reference.[16]

Even the simplest compartmentalized living entities that we know are comprised of a drastic number of expressed proteins and catalytic reactions. Therefore, it is critical to define what is minimal, in order to define what is an artificial cell and which functions/reactions must be carried out. In their review published in 2001, Szostak and co-workers describe their designed protocells as compartmentalized entities comprised of a replicating RNA replicase in a replicating membrane vesicle.[16] Although in that form the protocell is still incapable of autonomous reproduction, they claim that coupling it with ribozymes that can synthesize lipids for membrane-growth might allow the 'living' protocell to act as an autonomously replicating system. Figure 1.2 summarizes two proposed pathways towards the synthesis of life.

1.5 Systems Chemistry

In nature, every biophysical process happens through interactions between different (classes of) molecules. While lipid-like bio(macro)molecules keep the cellular materials isolated and achieve chemical transport, different proteins inside the cell are responsible for keeping the cell alive. Each vital function is achieved through ordered assembly of molecules and their co-operation with each other. Learning and mimicking the nature would require to study its constituents with a systems approach.

Systems chemistry is a relatively new field in chemistry that deals with dynamically complex systems that are comprised of structurally relatively simple molecules. The term was first used in a study by von Kiedrowski in 2005 where auto- and cross-catalysis was achieved in a Diels-Alder reaction.[17] Since then, this new concept has been developed by utilizing oscillating reactions,[18] self-assembling materials,[19,20] dynamic combinatorial chemistry,[21] out-of-equilibrium systems[22,23] etc. Complex chemical networks are one of the major focusses of systems chemistry together with (Darwinian) approaches to the origins of life and aspects of material science.

1.6 Dynamic Combinatorial Chemistry (DCC)

Dynamic combinatorial chemistry is a useful tool to develop new, and possibly not so easy to synthesize, molecules with the help of reversible covalent linkages.[24] The most widely used dynamic covalent reactions in DCC are disulfide, acetal, imine and hydrazone exchange.[21] Building on concepts in supramolecular chemistry, DCC gives access to adaptive systems through templated synthesis and intra- and intermolecular assembly processes.[25] Since dynamic combinatorial libraries (DCLs) are generally under thermodynamic control, library distribution can easily be altered by external templates or self-templating.[26,27] Figure 1.3 shows an overview of template-induced

processes in DCLs.

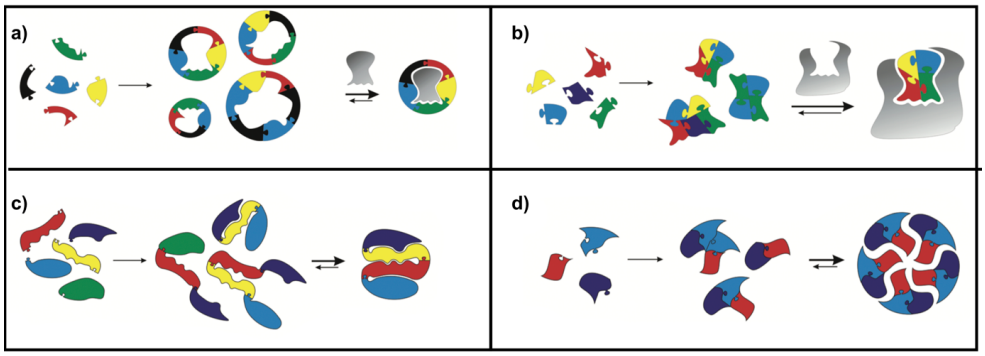

Figure 1.3: Cartoon representation for different template induced processes in DCC mediated by a) an external guest, b) an external host or by self-templating that is either c) intramolecular or d) intermolecular. Figure adapted from references.[26,27]

1.7 Out-of-equilibrium Systems

Nature is a highly dynamic system and possesses many functional characteristics which have inspired scientists for a very long time. Some of these functions are the result of thermodynamically stable assemblies, like lipid bilayer membranes, while more complex functions require constant input of matter and energy. It is useful to classify the different thermodynamic regimes in nature and in supramolecular chemistry.[28] Early examples of supramolecular chemistry mostly involved thermodynamically stable assemblies, like complex interlocked molecules or supramolecular polymers.[29–31] More recently, attention has shifted to kinetically controlled systems. Such systems are either in a kinetically trapped state from which they may or may not transform into the thermodynamically stable state (Figure 1.4b) or in a dynamic high-energy state that can only be sustained with a constant input of energy (Figure 1.4c).

For example, kinetically controlled self-assembly processes can give rise to different nanostructures depending on external stimuli. Ulijn and co-workers reported such a system with short peptide building blocks.[32] In the study, they used Fmoc-dipeptide precursors with different substituents in the R positions (tyrosine, phenyl alanine, leucine or valine) for self-assembly and a hydrolytic enzyme (subtilisin) that hydrolyzes methyl ester precursors to self-assembling peptide derivatives (Figure 1.5). Nucleation and early growth happen at site of enzyme action as evident from AFM images which show enzymes as bright spots at the end of newly-formed fibers. Since

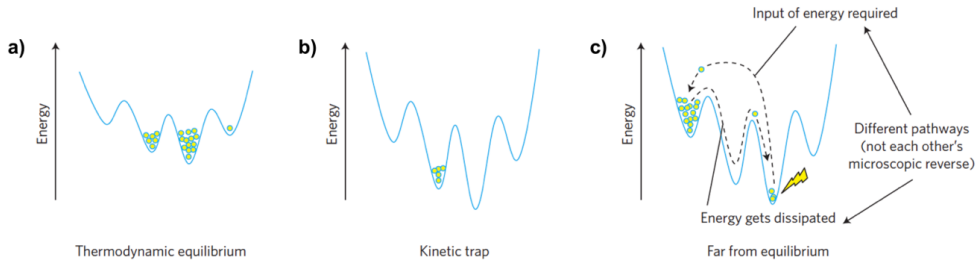

Figure 1.4: Thermodynamic regimes observed in chemical systems. a) Thermodynamic equilibrium: final state is determined by the Boltzmann distribution and irrelevant of the pathway. b) Kinetic control: final distribution depends on the synthetic pathway. c) Far from equilibrium systems in which continuous energy supply is required to persist. Final distribution reflects the balance between continuous synthesis and degradation. Figure adapted from reference.[28]

the self-assembly occurs as a result of enzyme activity (hydrolysis), the rate of growth and higher order chirality in some cases can be altered by varying the amount of enzyme.

In addition to self-assembly of small-scale building blocks, supramolecular polymers of larger molecules can also be formed under kinetic control. Transformations under different experimental conditions can result in helicity inversions,[30] or differ-

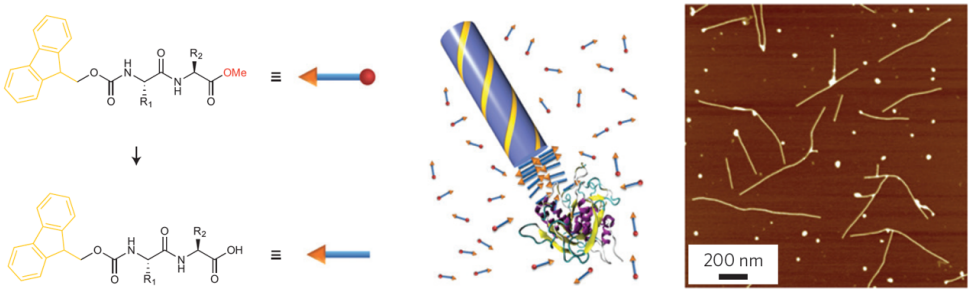

Figure 1.5: Chemical structure of Fmoc-dipeptides that hydrolytic enzymes convert from a methyl ester (top) into a gelating carboxylic acid (bottom). Supramolecular assemblies (blue/yellow tube) are formed from these building blocks in solution. The assemblies constitute the framework for the gel in which enzymes (coloured ribbon structure) are embedded. The enzymes appear as bright spots at the fibre ends in the atomic force microscope image (right). Different enzyme concentrations yield different gel strengths. Figure adapted from reference.[32]

ent polymorphic self-assembling structures.[33] More recently, out-of-equilibrium self-assembly has been combined with another process popular in material science: living supramolecular polymerization. Living polymerization provides better control and uniformity (lower polydispersity) to supramolecular polymers in which chain growth occurs only with an initiator.[34] The first example was reported by Takeuchi and co-workers in 2014.[35] They showed that self-assembling structures that are produced from a porphyrin derivative follow two different aggregation pathways depending on kinetics, resulting in nanoparticles or fibers. While the nanoparticles are formed as kinetically stable products, they transform into fibers upon addition of a small aliquot from the solution containing nanofibers (initiators).

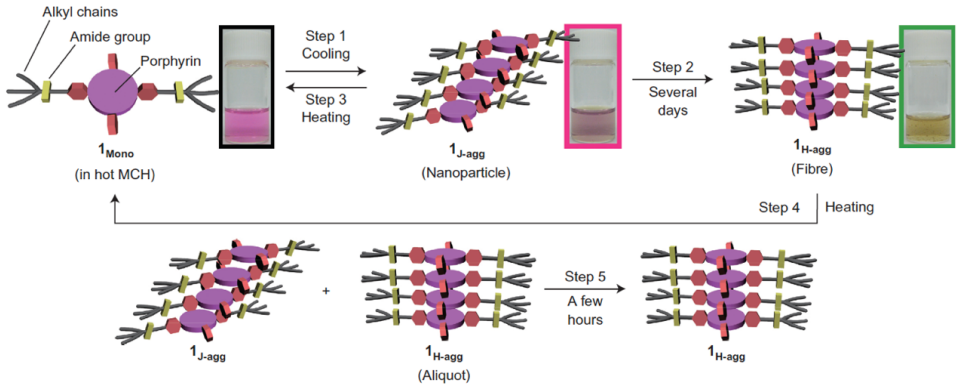

Figure 1.6: Cartoon representation of formation and transformation mechanism of supramolecular polymers made from porphyrin based monomers ($\mathbf{1_{mono}}$). Monomers in hot methylcyclohexane solution self-assemble into nanoparticles ($\mathbf{1_{j\text{-}agg}}$) upon cooling. When this nanoparticle solution is left unagitated, nanoparticles are transformed into fibers ($\mathbf{1_{H\text{-}agg}}$). Both steps are reversible and it was reported that the transformation of nanoparticles into fibers was significantly faster in the presence of a small amount of fiber as initiator. Figure adapted from reference.[35]

Out-of-equilibrium systems are not only developed in material science but are also highly relevant for understanding the origins of life in terms of replication, speciation and adaptation. These topics will be discussed in the following sections.

1.8 Self-Replication

As we briefly discussed earlier in this chapter, self-replication is one of the prerequisites to call something 'living'. No matter how the first living entities formed on

earth, evolvable life would not be possible if those entities were not capable of making copies of themselves. In biology, genetic information is copied by replication of DNA during cell division, which is a process highly dependent on complex enzymes. Short RNA primers are required for the synthesis of DNA which lends some support for the RNA world hypothesis. These biologically vital molecules inspire scientists not only to construct new molecular networks that mimic biology but also to search for non-enzymatic replicating systems. Considering the fact that life on earth may have evolved from molecules that are much simpler than RNA, peptides and nucleobases would be the best candidates as they are the constituents of proteins and RNA, respectively. The minimal autocatalytic cycle features a template-directed synthesis of the autocatalytically active molecule.[36] According to this model, molecules **A** and **B** first react to form either binary complex $\mathbf{T_{inactive}}$ or template molecule **T** (Figure 1.7a). When the template forms, it enters to the autocatalytic cycle by interacting with precursor molecules **A** and **B** which then react to form a dimer of the template molecule. Only if this dimer ([**T.T**]) is capable of dissociating into two distinct template molecules (**T**), the autocatalytic cycle can be completed. Figure 1.7b and Figure 1.7c show possible kinetic profiles in a such minimal system depending on the reaction dynamics.[37,38]

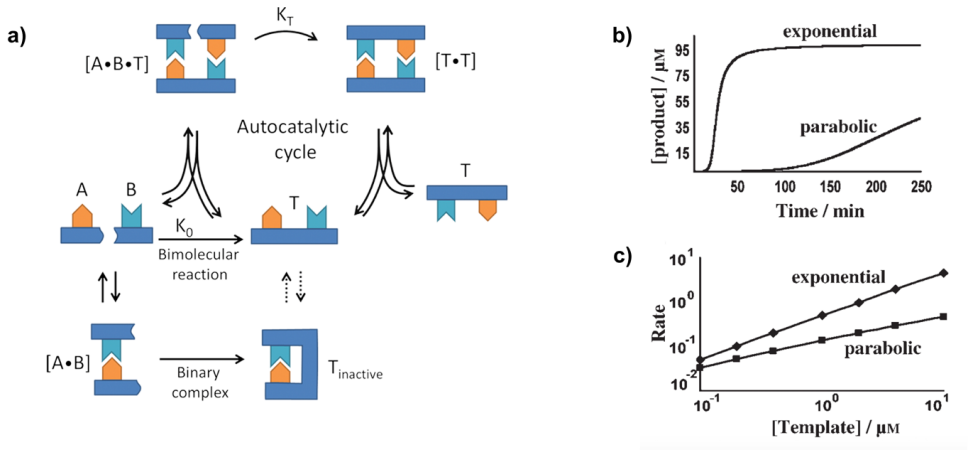

Figure 1.7: a) Cartoon representation of a minimal self-replicating cycle b) and c) possible kinetic profiles for the autocatalytic growth. Figure adapted from references.[36–38]

The first non-enzymatic replicating system was reported by von Kiedrowski in 1986.[39] In his study, he used two trinucleotides to form a hexamer with a palindromic sequence. Therefore, the hexamer could template its own production. Following their pioneering contribution on self-replicating molecules, the same group, in 2014,

reported one of the first examples of template-directed synthesis of self-replicating peptide nucleic acids (PNAs) (Figure 1.8).[40] In this study, they set up a reaction network using water soluble carbodiimide (EDC) as a coupling agent to mediate imidazole catalyzed PNA ligation. Kinetic modelling studies verified the parabolic growth characteristics of the replicator.

In addition to von Kiedrowski's studies, there have been many contributions to synthetic self-replicating systems.[41–43] Apart from nucleic acid-based design of self-replicators, peptides are another important class of molecules in this area. The first

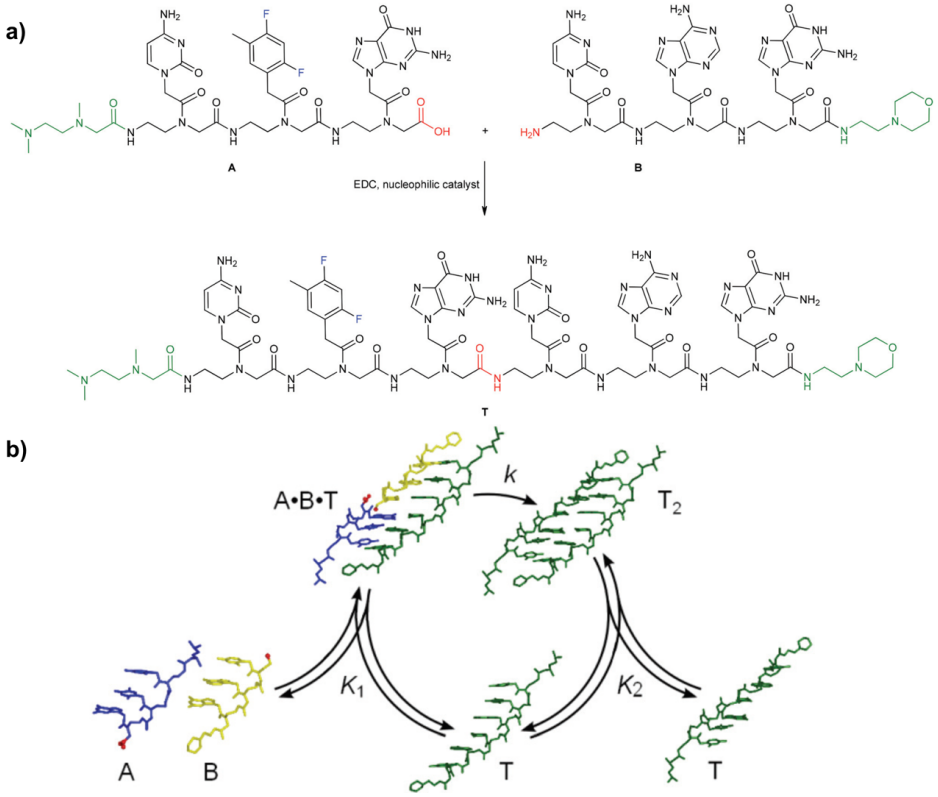

Figure 1.8: a) Chemical structures of building blocks for the self-replicating system based on PNA. Trimeric building blocks **A** and **B** react to give self-complementary hexa-PNA (**T**) upon condensation. Green: solubility enhancer; red: ligation site; blue: fluorine label; b) minimal model for the self-replication. Hexa-PNA (**T**) catalyzes its own formation from building blocks **A** and **B** with an autocatalytic ligation reaction. Figure adapted from reference.[40]

self-replicating peptide was studied by Ghadiri's group and dates back to 1996.[44] In their study, they reported that a 32-residue α-helical peptide acts autocatalytically and templates its own synthesis from 15- and 17- residue fragments by amide bond condensation. Considering that nucleic acids can provide easier complementary interactions than peptides, their peptide-based study is the first that overcomes this inherent challenge. Additionally, achieving self-replication in an α-helical design is important since many naturally occurring proteins fold into α-helical coiled coils. After their discovery, Ghadiri's research group reported many more replicators based on peptides.[45–47] In addition, other scientists also made prominent contributions to peptide based self-replication including Chmielewski[48,49] and Ashkenasy.[50,51]

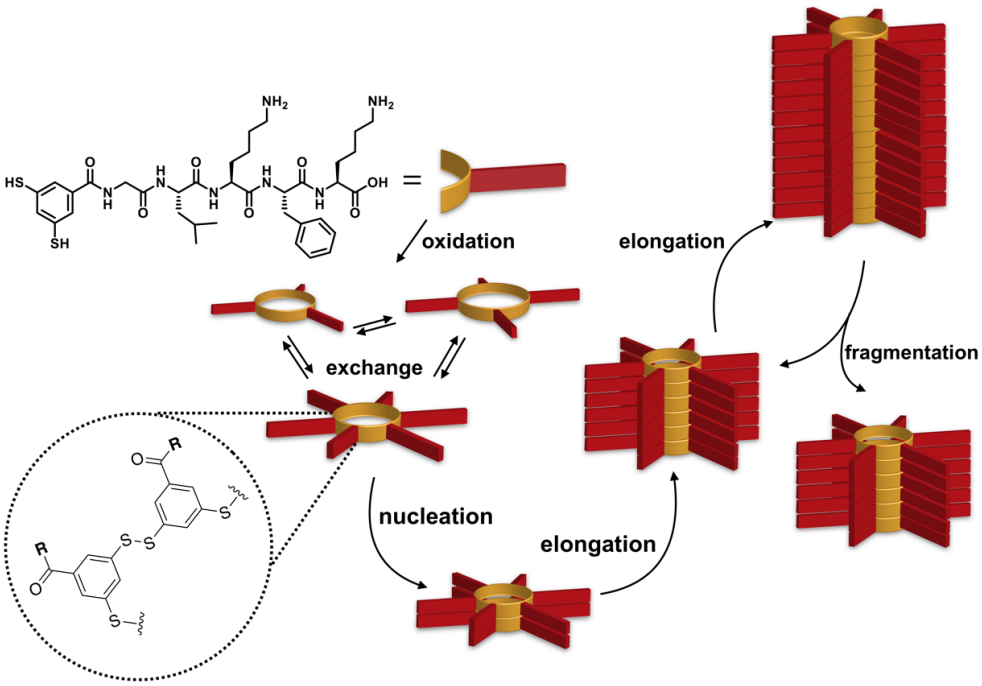

Figure 1.9: General replication mechanism discovered by the Otto research group. First, dithiol-bearing building blocks are oxidized and give a dynamic disulfide exchange pool that contains differently sized macrocycles. When one of the species is capable of forming small stacks (self-nucleation), the equilibrium shifts towards more of this product and the small stacks are elongated into fibers. Upon mechanical agitation, fibers break into shorter fragments increasing the number of growing fiber ends, giving rise to an autocatalytic cycle.

It has been argued and shown that molecules that are shorter and simpler than the

first synthetic peptide replicators can also self-replicate, driven by self-organization into ordered structures. Dynamic combinatorial chemistry served as a powerful tool to access these complex systems from simpler molecular entities. Our group made an important contribution following the discovery of a mechano-sensitive self-replicator in 2010.[52] Self-replicators emerged spontaneously from DCLs prepared from building blocks bearing aromatic dithiol unit for disulfide exchange and a short peptide tail (Figure 1.9). The peptide contains alternating hydrophilic and hydrophobic residues which promote self-assembly by means of β-sheet formation. After this discovery, the self-assembly of the self-replicator has been studied both theoretically and experimentally.[53,54] Furthermore, dynamic multi-building block systems and auto- and cross-catalysis in complex molecular networks have been studied. Some representative examples will be discussed in the following chapter and throughout this thesis.

1.9 Diversification of Replicators and Molecular Ecosystems

The interplay between self-replicating molecules is an important topic in understanding the origins of life on earth. Until recently, studies were only focusing on single replicators in isolated environments. However, scientists now start to study co-operation and competition in multi-building block systems. Some early examples have been reported by Lehman,[55,56] Ashkenasy[57,58] and Philp.[59–62] In one of the studies, in 2017, Philp reported a molecular network in which self-replicating templates were produced from two maleimides and two nitrones with different sizes (Figure 1.10a).[63] When these molecules react via 1,3-dipolar cycloaddition reactions, they produce four differently sized replicator templates. In isolation, three out of four of the templates can trigger their own formation from the constituents (SNSM, SNLM and LNLM). However, when the network is constructed from all four individual molecules without any template instruction, all four replicators are produced with significant percentages. Among these template molecules, only two of them can cross-catalyze the formation of each other in a cross-catalytic cycle (SNLM → LNSM and LNSM → SNLM). When one of the instructing templates is added, specific auto- and cross-catalytic behavior is observed, in each case in a predictable way (Figure 1.10b). The study clearly exemplifies how system-level responses can be generated from simple molecules.

In addition to these studies mainly based on RNA, α-helical peptides and other synthetic molecules, our group made an important contribution to dynamic molecular networks specifically based on self-replicating peptides. Diversification of replicators can be achieved by using multiple building block systems. In 2016, Sadownik et. al.

reported such a system in which two sets of co-existing replicators compete for two common building blocks.[64] As one set of replicators is the ancestor of the second set, the system shows behavior that resembles the process by which new species form in biology.

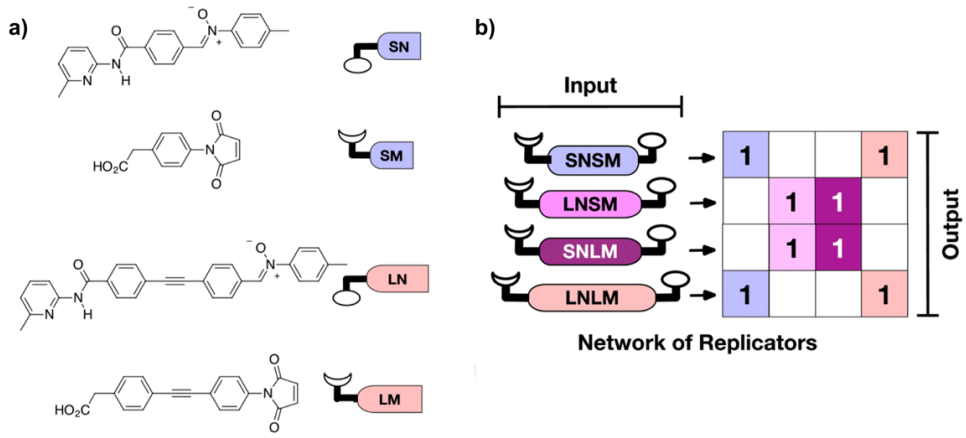

Figure 1.10: System developed by the Philp research group; a) Chemical structures of the replicator precursors, b) four replicator molecules and diagram summarizing the auto- and cross-catalytic behavior in a single input system. Figure adapted from reference.[63]

As evolution is probably not possible for species existing in isolation, it is relevant to investigate how co-existing self-replicators affect each other. Recently we reported the emergence of a new self-replicator ($\mathbf{1}_6$) based on a threonine containing building block that was directed by an pre-existing one ($\mathbf{4}_8$) (Figure 1.11).[65] The formation of the 6-ring replicator occurred only when seeded with an 8-ring replicator but not upon seeding with any other 6-ring replicator. This system features an important ingredient for evolution: replicator mutation.

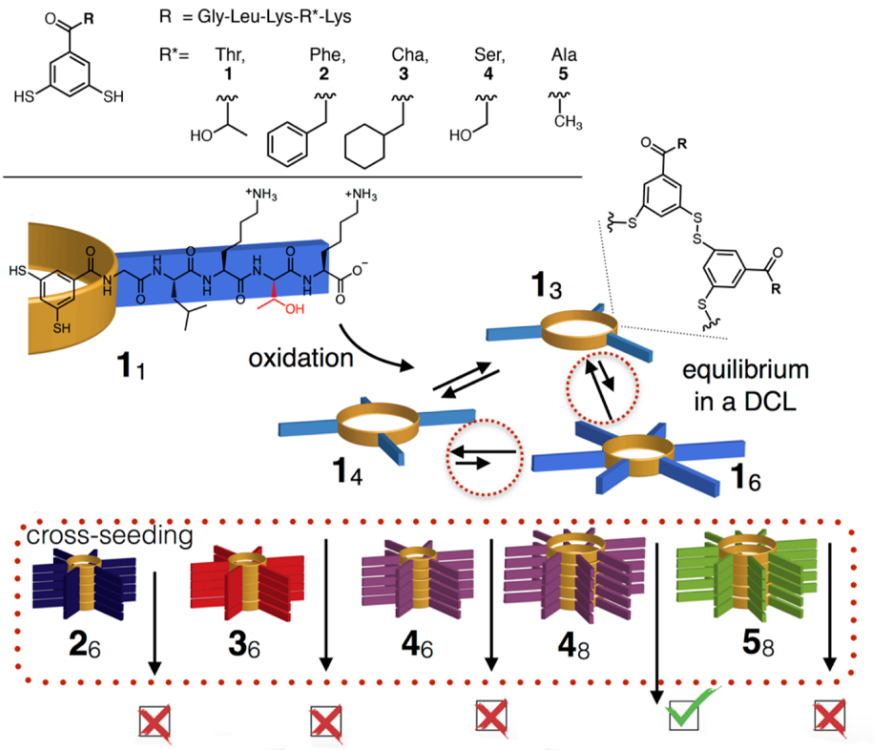

Figure 1.11: Building block structures and different replicators that showed selective cross-catalysis. Figure adapted from reference.[65]

1.10 Content and Outline of This Thesis

Research into the origins of life is an important topic in systems chemistry. Since Darwinian evolution possibly started right after the emergence of first replicating species, the development of multi-replicator systems would be the next step to synthesize life de-novo. In this thesis, we take advantage of dynamic combinatorial chemistry to construct such systems from peptide based building blocks and investigated how small structural variations in these building blocks affect self-replication and the resulting self-assembly processes.

In **Chapter 2** we used structurally closely related building blocks to control composition and length of self-synthesizing supramolecular assemblies and synthesized

supramolecular block-copolymers with varying composition and unprecedented low polydispersity indices. We modified one of the building blocks by introducing halogen atoms to one of the amino acid's side chain and attempted the direct visualization of the block-copolymers by electron microscopy.

In **Chapter 3** we investigated self-replicating behavior using building blocks with small structural variations in the peptide backbone. Without effecting the H-bonding propensity of the peptide chain, we were able to obtain differently sized self-replicators. In addition, we also showed how the environment affects the size of self-replicators in a single building block system. Lastly, we checked the history dependence in multi-building block DCLs made from structurally closely related peptide building blocks that all form differently sized self-assemblies.

Chapter 4 describes the emergence of a parasitic replicator which owes its existence to another structurally related replicator in the same environment. Unidirectional parasitic behavior resulted from the disassembly of the pre-existing one in the cross-catalytic process. We were able to alter the composition of the parasitic replicator in two ways: changing the amount of the cross-seed or repeating cross-seeding events until the parasite becomes homogeneous and contains no trace of the initial replicator.

To achieve diversification in a dynamic system, in **Chapter 5** we constructed a continuous flow set-up that infuses nutrients required for replication with constantly changing composition and outflows material to keep the population size constant. We tried to produce a continuous series of cross-catalytic replicators of which the final one should not be capable of cross-catalyzing formation of the original ancestor.

Finally, **Chapter 6** provides an overview and evaluation of the impact of our studies in the context of dynamic combinatorial chemistry in particular and systems chemistry and the origin of life / synthesis of de-novo life in general.

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