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Directed Evolution: A Historical Exploration into an Evolutionary Experimental System of Nanobiotechnology, 1965–2006

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Abstract This study explores the history of nanotechnology from the perspective of protein engineering, which differs from the history of nanotechnology that has arisen from mechanical and materials engineering; it also demonstrates points of convergence between the two. Focusing on directed evolution—an experimental system of molecular biomimetics that mimics nature as an inspiration for material design—this study follows the emergence of an evolutionary experimental system from the 1960s to the present, by detailing the material culture, practices, and techniques involved. Directed evolution, as an aspect of nanobiotechnology, is also distinct from the dominant biotechnologies of the 20th century. The experimental systems of directed evolution produce new ways of thinking about molecular diversity that could affect concepts concerning both biology and life.

Keywords Directed evolution · RNA world · Scanning probe microscopy · Molecular biomimetics · Nanotechnology · Molecular diversity

Introduction

A hallmark of nanotechnology (NT) is that it has been difficult to define as a distinct field, due to the multiple techniques, diverse materials, and histories involved. NT has generally been defined as the control of materials on a scale of 1–100 nm.[1] Beyond this simple definition, the term is ambiguous vis-à-vis material identity. However, the history of NT has been written exclusively with regard to inorganic

[1] National Nanotechnology Initiative. What is Nanotechnology? http://www.nano.gov/html/facts/whatIsNano.html. Accessed August 30, 2008.

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materials, rather than organic and biomaterials. A so-called 'standard' history of NT starts from mechanical and materials engineering (Baird and Shew 2004, p. 145).[2] It documents successes in the semiconductor industries, the invention of the scanning probe microscope (SPM) (see Baird and Shew 2004; Mody 2004), and the discovery of the carbon nanotube.

Presumably, the historical imbalance between inorganic materials and organic materials in the history of NT exists because the main figures of NT—such as Richard Feymann and K. Eric Drexler—worked in the areas of electrical, mechanical, or materials engineering. However, for Drexler (1986, p. 14), protein engineering was the first generation of NT. He distinguishes between 'protein machines' and what he called 'second-generation nanomachines', claiming that the latter are tougher than proteins and 'will do all that proteins can do, and more'. Drexler created a wide variety of images of nanomaterials out of inorganic materials, following the nature of proteins. The theory of 'grey goo' (Joy 2000), which portrays nanomaterials as out-of-control, self-replicating nanobots, is based on Drexler's logic of resemblance between proteins and nanomaterials. The 'grey goo' theory is currently treated as a sort of nano-hype by many scientists such as Richard Smalley at Rice University; however, the analogy between proteins and nanomaterials—which touches on such topics as the nature of self-assembly—is still prevalent in the discourses of NT.

Ironically, after Drexler's hypothesis, the history of protein engineering had been excluded from the history of NT until a technological convergence of NT, biotechnology (BT), and information technology (IT) emerged. The technological convergence on the nanoscale paves the way for the writing of a new genealogy of nano-materials as portrayed below by Angela Belcher at MIT—who first exploited viruses' ability to self-assemble, and to produce batteries in the field of molecular biomimetics:

> My dream is to have a material that's genetically controllable and genetically tunable. I'd like to have a DNA sequence that codes for the production of any kind of material you want … *You want a solar cell, here's the DNA sequence for it. You want a battery, here's the DNA sequence for it* (my emphasis).[3]

This study traces a 'hidden' history of NT, from the perspective of protein engineering (Fig. 1).[4] To do so, this study focuses on the history of directed evolution (DE)—an experimental system that mimics natural evolution at the

[2] The idea of 'standard' and 'hidden' histories of NT is indebted to historians of nanotechnology, such as David Baird, Ashley Shew, Cyrus Mody, and Patrick McCray. Mody and McCray have focused on hidden histories of NT related to material science and electrical engineering, such as molecular electronics and molecular beam epitaxy. See McCray (2007) and Choi and Mody (forthcoming). To see why hidden histories matter in the context of nanotechnology, see Mody (forthcoming). In this study, Mody is critical of the analogy between NT and BT because, as he claims, there is NT other than BT. I agree with his idea, but further criticize this analogy in a slightly different way, because the material culture of directed evolution as one of the nanobiotechnologies differs from those of dominant biotechnologies in the second half of the 20th century.

[3] Angela Belcher: Harnessing viral power: Letting nature do the work. http://alum.mit.edu/ne/opendoor/200507/belcher.html. Accessed 30 August 2008.

[4] One study of NT from the vantage of biology is Lenoir and Giannella (2006).

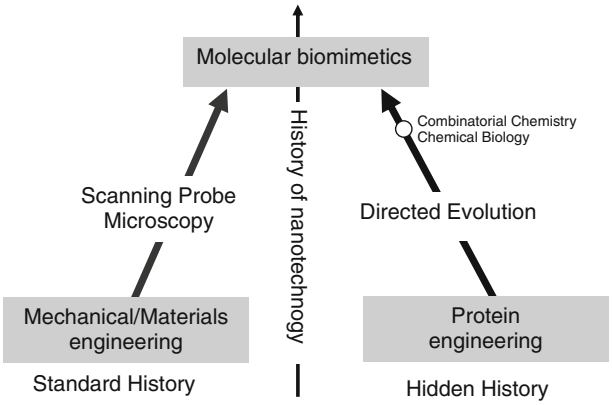

Fig. 1 A Standard and hidden history of nanotechnology

molecular level in a test tube while intervening to direct and speed the achievement of outcomes. DE is also called laboratory evolution, molecular evolution, evolution in vitro, and evolution in a test tube. DE is known as a key technique of both chemical biology and combinatorial chemistry; it is also a technique of molecular biomimetics that mimics nature as an inspiration for material design. As one of the research fields of nanobiotechnology, molecular biomimetics was regarded by the U.S. National Nanotechnology Initiative Leader Mihail Roco as the fourth generation of NT.[5] The contemporary DE experimental system comprises two techniques: random mutagenesis and high-throughput screening. DE creates a 'combinatorial library', a diverse pool of variants that are generated as randomly and as numerously as possible in a test tube; it then screens proteins with the desired properties that fit researchers' specific goals.

This study also asserts that the history of DE is not merely a parallel story; rather, certain developments in material culture—such as the marriage of DE and SPM—demonstrate points of convergence between the hidden and standard depictions of the history of NT. The invention of SPM in the 1980s was a pivotal moment in the standard history of NT, because it facilitated the ability to see images at the atomic level, on the surfaces of materials (Baird and Shew 2004). In the field of molecular biomimetics, SPM encounters DE in analyzing the interaction between electrodes and proteins produced via DE. I envision this encounter to be at the intersection of two contrasting histories of NT; this intersection is also an example of the so-called convergence between BT and NT.

Lastly, this study shows that DE, as one of nanobiotechnologies, differs from biotechnologies that were dominant in the second half of the 20th century. To do so, I will explore two themes in which DE and other biotechnologies differ: (1) the techniques and concepts they come to embody and (2) the material entities and

[5] More exactly, Roco defines the fourth generation of nanotechnology as 'molecular nanosystems with heterogeneous molecules, based on biomimetics and new design'. Roco, M. The future of National Nanotechnology Initiative. http://www.Nano.Gov/html/res/roco_aiche_48slides.pdf. 31 August 2008.

concepts they come to embody. First, I will articulate the difference between the random mutagenesis of DE and the site-directed mutagenesis of the dominant biotechnologies of the 20th century. Secondly, I will address how DE generates new material entities, such as catalytic RNA and metalloproteins, and provides new insights into the notion of life as broadly perceived in the 20th century (Keller 2002).

To start, I will provide the theoretical framework and methodology used in this study. This is followed by a description of the four periods of experimental systems, including a description of the relevant material culture, concepts, and techniques as they emerged.

Theoretical Framework

This study is based on science studies of instruments, experimental systems, and material cultures (e.g., Hacking 1983; Rheinberger 1997; Rabinow 1996; Kohler 1994). The term 'experimental system' in this study is borrowed from the biology historian Hans-Jorg Rheinberger (1997); it describes a heterogeneous ensemble of all the techniques, instruments, research materials, scientific/technical knowledge, and human practices that generate material culture. The experimental system creates not only natural order, but also social order. That is to say, the system co-produces new material entities, as well as the concepts they embody (Rheinberger 1997, p. 28). The experimental system also enables scientists to form new communities and institutions around research materials and instruments for particular experimental systems (Kohler 1994; Mody 2006).

Rheinberger (1997) argues that experimental systems are not closed, but open, with a series of unprecedented events. Experimental systems 'can be seen as clusters of materials and practices that evolve through drift (conjunctures), fusion (hybrids), and divergence (bifurcations)' (Rheinberger 1997, p. 138). Rheinberger uses the term 'differential reproduction' between experimental systems, to account for the *evolutionary* relationship between old and new experimental systems, within a historical context. In examining this idea, this study pays attention to the evolution of 'selection pressure' used in DE. All experimental systems of DE employ some sort of selection pressure, but its material practice has evolved.

I also use Rheinberger's (1997) idea of 'styles of experimental reasoning' (p. 138). Similar to Fleck's (1979) notion of 'thought style', which is related to a specific scientific community, Rheinberger created this term, which is linked to the material culture of experimental systems. I define *molecular diversity* as a kind of 'style of experimental reasoning' pertaining to DE; it is the most fundamental and peculiar thought in the contemporary DE experimental system. It means that the more diverse the variants within a combinatorial library of mutants made for DE, the easier it is to find proteins with specific, desired properties. The degree of molecular diversity, then, refers to the frequency of potential solutions in the search for target proteins. As will be explained later, molecular diversity enables DE to distinguish it from the other prevailing BTs of the second half of the 20th century. I will trace how this concept evolved from the DE experiments of the 1970s.

Thirdly, this study explores how material entities (i.e., what Rheinberger calls 'epistemic things') and their concepts are co-produced with experimental systems (Rheinberger 1997, p. 78). DE generates new material entities such as catalytic RNA and metalloproteins (i.e., metal-binding proteins)—thereby prompting the debates which have taken place since the 1960s regarding the *origin of life*, and since the late 1990s regarding a *newly emergent form of life* (Fisher 1999; Keller 2002; Rose 2007) in the field of molecular biomimetics. With regard to the origin of life, I will address the RNA world hypothesis that RNA was the first genetic material to exist before DNA emerged. With regard to new, emergent forms of life, I will deal with metalloproteins, which are used as materials in nanobioelectronics, in the field of molecular biomimetics. Metalloproteins are products of a so-called convergence between BT and NT.

Methods

This study explores how the concepts, techniques, and experimental systems of DE have evolved in the context of nanobiotechnology, from the 1960s to the 2000s. The data analyzed includes 220 scientific articles and five books about DE that have been published between 1965 and 2006. I first analyzed review articles in *Web of Science* and then searched for primary articles that marked pivotal moments in the history of DE. Additionally, Alan Porter and Alex Stephens from the Technology Policy and Assessment Center at Georgia Tech provided me with a bibliometric analysis of DE (see Fig. 2). The rate of publication in this area has rapidly increased since the late 1990s, and this analysis helps predict when DE experienced significant innovations. Moreover, to check the reliability of my interpretation of DE and to

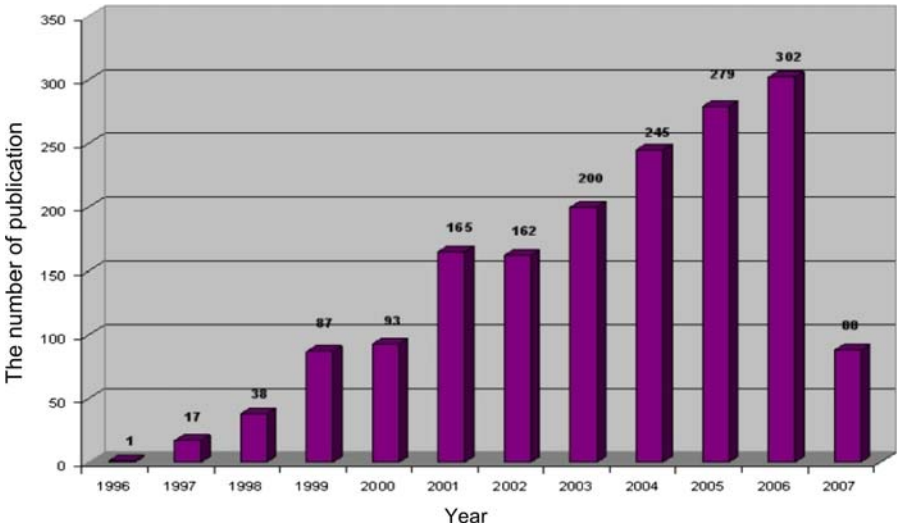

Fig. 2 The rate of publication on directed evolution

find additional information beyond mere content analysis, I also undertook personal communication via semi-structured phone interviews and open-ended interviews with some scientists at the University of Wisconsin-Madison and New York University.

I separate the history of DE into four periods: (1) the first DE experiment with the idea of the RNA world hypothesis, in the 1960s; (2) a DE experiment with the idea of 'competition' and 'mutant spectrum' as precursors to molecular diversity in the 1970s and the early 1980s; (3) a contemporary DE experimental system with molecular diversity, since the late 1980s; and (4) the emergence of a novel form of life called metalloproteins, as well as the combination of DE with SPM in the field of molecular biomimetics since the late 1990s. My distinction of these periods relies on the emergence and transformation of important concepts (like molecular diversity and life) and techniques (e.g., related to selection pressure) that are related to DE.

Directed Evolution in the 1960s: The Emergence of the RNA World Hypothesis

The earliest trials that can be characterized as DE originated from the 'serial transfer experiment'—also known as the 'serial dilution experiment'—carried out by Sol Spiegelman and coworkers at the University of Illinois in the 1960s. In 1965, their experiment was designed to test the success of an in vitro replication reaction of RNA in terms of whether or not the reaction successfully generated identical copies of original RNAs. Spiegelman's group prepared infectious viral RNAs called Qβ-RNA (RNA bacteriophage) as the original RNAs, as well as a series of tubes with the same amount of reaction mixture with both Qβ replicase and radioactive materials.[6]

To begin with, Qβ-RNA was inserted into the first test tube and incubated for an adequate period, to synthesize new RNAs (see Spiegelman et al. 1965, p. 924). After the first synthesis, a certain amount from the first test tube—which contained synthesized RNAs and original RNAs—was transferred to a second test tube and allowed to incubate again for a second synthesis of pre-synthesized RNAs, and so on. This experiment was continued until the original RNAs of the first tube had been diluted to an insignificant level. Finally, Spiegelman's group estimated the similarity between the synthesized RNAs and the original Qβ-RNA.[7] They found that the newly synthesized RNAs were as fully 'competent' as the original RNAs in the generation of more copies in the further synthesis of RNAs (Spiegelman et al. 1965, p. 926).

[6] The radioactive materials were used to distinguish original Qβ-RNAs from any new RNAs that might be synthesized by Qβ replicase in test tubes. The synthesized RNAs would be radioactive.

[7] Spiegelman et al. claimed that if the number of infectious units in all the tubes corresponded to the amount of radioactive RNA found, this evidence could prove that the newly synthesized RNAs are identical copies of the original infectious RNAs (Qβ-RNA) (Spiegelman et al. 1965, p. 925).

This experiment in 1965 was not originally designed to present one case of evolutionary experimental systems; rather, it aimed to prove whether or not the newly synthesized RNAs are replicas of original RNAs. In 1967 and 1971, Spiegelman et al. defined the serial transfer experiment as an 'extracellular Darwinian experiment' (Mills et al. 1967) that examined 'precellular evolution' (Spiegelman 1971). Let me compare the purposes of the 1965 experiment with those of the 1967 experiment:

> It is the purpose of the present paper to describe experiments demonstrating that the RNA produced by replicase is fully competent to program the production of complete virus particles. The data establish that the reaction being studied is indeed generating self-propagating replicas of the input RNA. (Spiegelman et al. 1965, p. 920)

> The primary purpose of the present paper was to demonstrate the potentialities of the replicase system for examining the extracellular evolution of a self-replicating nucleic acid molecule. (Mills et al. 1967, p. 223)

Both the 1965 experiment and the 1967 experiment deliberately continued to reduce the incubation intervals between transfers (see Spiegelman et al. 1965, p. 924); however, in the 1965 experiment, Spiegelman did not define this modification as a kind of 'selection pressure' (i.e., selecting for a specific type of RNA), because this experiment aimed to demonstrate the production of identical copies of added viral RNAs. In other words, he did not define his experiment as a kind of evolutionary experimental system; to do so, his experimental system would necessarily have required a selection process.

In contrast, the 1967 experiment defined the modification of incubation intervals between transfers as a kind of selection pressure for RNA variants that could replicate more quickly than the original RNAs (Mills et al. 1967, p. 219). By shortening these intervals, they could select RNAs with a shorter chain than the original RNAs. The smaller the chain, the shorter the time required for the complete dilution of original RNAs.

Spiegelman likely defined the serial transfer experiment as a form of an evolutionary experimental system in 1967 and in 1971, because RNA evolution was hypothesized by Carl Woese (1967), Leslie Orgel (1968), and Francis Crick (1968) during that period. A so-called 'extracellular Darwinian experiment' or 'precellular evolution' is the RNA evolution that was presumed to occur before DNA and proteins appeared and created a cell. Spiegelman at Columbia University worked with Leslie Orgel at the Salk Institute, co-publishing a paper about the evolutionary experiment in 1970 (Saffhill et al. 1970). Spiegelman also worked with Walter Gilbert, who coined the term 'RNA world' in 1986. According to Spiegelman's 1965 article, Spiegelman had used *E. coli* (*Q13*) that had been isolated in Gilbert's laboratory. In short, Spiegelman et al.'s 1967 serial transfer experiment (Mills et al. 1967) could be considered the first DE experiment, and it was built on the RNA world hypothesis (see Spiegelman 1971, p. 221). I will address the RNA world hypothesis again in the description of DE in the 1990s.

Directed Evolution in the 1970s and 1980s: Precursors to Molecular Diversity

This section traces the source of the idea of *molecular diversity*, which is crucial to the contemporary DE system. I compare two kinds of evolutionary experimental systems, from the early 1970s to 1985. The first is site-directed mutagenesis, in which one deliberately directs the mutations of specific sites of proteins. The second is a modification of the 1967 serial transfer experiment, in which one inhibits the self-replication system of RNAs under a new selection pressure.

Site-Directed Mutagenesis

Brian Hall (1978) at the University of Connecticut was among early leaders who applied site-directed mutagenesis in an evolutionary DE experimental system in the 1970s. In 1978, Hall studied a certain protein enzyme called the *ebg β-galactosidase* system of *E. coli*, in order to create new synthetic enzymes that were better than the original *ebg* enzymes, in terms of lactose utilization. To achieve this goal, Hall mutated two sites—the structural gene and the regulatory gene—of *ebg β-galactosidase*.[8] Hall called his experiment 'directed evolution'.

His experiment is historically interesting, because both random mutagenesis and site-directed mutagenesis were defined as DE techniques. Site-directed mutagenesis was a dominant mutation method in the second half of the 20th century, even after techniques for random mutagenesis (or random gene recombination) appeared in the 1980s–1990s. Recombinant DNA and genetic manipulation techniques that were prevalent in the second half of the 20th century were geared toward site-directed mutagenesis and recombination. Hall's site-directed mutagenesis, however, did not employ the idea of *molecular diversity* in protein design; his site-directed mutagenesis was intended to mutate the specific sites of proteins deliberately selected by researchers, in order to improve the functionality of proteins in a particular direction. Whereas random mutagenesis does not identify or predetermine genotypes, site-directed mutagenesis is a kind of rational design that requires a priori knowledge of the relationship between the genotypes that researchers target to mutate and the phenotypes that they want to achieve. Hall presumed the role of structural genes and regulatory genes prior to his experiment. Therefore, Hall's experiment has nothing to do with the contemporary experiment of DE, even if he used the same term.

The Inhibition of Self-Replication System of RNAs: The Mechanism of Competition

In the 1970s–1980s, Spiegelman's group at Columbia University and Manfred Eigen's group at the Max Planck Institute for Biophysical Chemistry conducted serial transfer experiments, which were distinct from the 1967 experiment of

[8] The first site was a structural gene called the '*ebgA*', a mutation that was presumed to increase activity toward lactose. The second site was a regulatory gene called the '*ebgR*', a mutation that would presumably increase the gene expression of *ebgA* (Hall 1978, p. 674).

Spiegelman et al. For instance, in 1970, Spiegelman's group at Columbia University and Orgel's group at the Salk Institute prepared a class of four RNA variants and inhibited the self-replication experiment of a specific RNA in a large amount of ethidium bromide that could affect the tertiary structure of RNA (see Saffhill et al. 1970). Ethidium bromide was used as a kind of selection pressure, as some RNAs possess structures that can minimize interaction with ethidium bromide.[9] This inhibition signifies that the 1967 experiment and the 1970 experiment employed distinct selection pressures. The former experiment installed a selection pressure for fast RNA growth, while the latter installed one to inhibit the production of specific RNA among diverse variants.

It is also quite interesting that Spiegelman et al. refers to the concept of 'competition' among RNA variants, claiming that '[e]thidium bromide inhibits initiation of RNA synthesis either by intercalation in the initiator sequence or by competition on the enzyme' (Saffhill et al. 1970, p. 538). The idea of 'competition' resulted from their idea of 'molecular evolution'. Later, I will connect the idea of *competition* with the idea of *molecular diversity*, a concept that is crucial to the contemporary DE experimental system.

In the 1980s, Manfred Eigen et al. continued to inherit Spiegelman's experiments. In 1984, Eigen and Gardiner hypothesized a controlled 'molecular evolution' experiment. They established an experimental system that could lead to the mechanism they called 'competition', among different self-replicating RNAs. They observed the formation of double-strand RNAs from initially formed single-strand RNAs. After a long replication period, the concentration of the single-strand RNAs achieved a steady state, while that of the double-strand RNAs continued to grow. The formation of double-strand RNAs could occur when the RNA concentration was high enough and when a selection pressure was imposed by changing salt concentrations or by using intercalating agents such as ethidium bromide (Eigen and Gardiner 1984, p. 975). Under such selection pressures, Eigen's group could create a kind of competition between single- and double-strand RNAs in the self-replication experiment. They portrayed the mechanism of competition as follows:

> The possibility of double strand formation has important implications for the outcome of *the competition that ensues when more than one template is present in the incubation mixture*. If double strand formation occurs only between the complementary partners, then it is possible for a coexistence to arise where both competitors share the enzyme and generate double strand in a constant ratio. If double strand can form between the two competitors also, then the stronger competitor drives the concentration of the weaker one to zero. (Eigen and Gardiner 1984, p. 971)(my emphasis)

In contrast to Hall's experiment, both Spiegelman et al.'s 1970 experiment and Eigen et al.'s 1984 experiment touched upon the idea of 'competition'. Eigen and Gardiner installed in their respective experimental systems the idea of 'molecular

[9] In this experiment, ethidium bromide inhibited the replication of a specific RNA variant called 'V2' from a class of RNA mutants.

evolution' with 'a broad variety of different forms of competition and selection' (Eigen and Gardiner 1984, p. 975). To use the idea of 'competition', their experimental systems required at least more than one type of RNA in the test tubes. Accordingly, the mechanism of competition within a diverse pool of mutants under a specific selection pressure enables scientists to select a target protein with certain desired properties. This is the very essence of *molecular diversity*.

In fact, Eigen and Gardiner hypothesized a theoretical model for the contemporary DE system. They proposed the ideas of 'mutant spectrum' and 'mutant distance':

> [T]he alternative method of designing a molecular evolution machine is to establish the value landscape of the mutant spectrum rather than looking for advantageous mutants. In order to do this practically one must know the mutation distances within the mutant spectrum. (Eigen and Gardiner 1984, p. 977)

The terms 'mutant spectrum' and 'mutant distance' would be respectively tantamount to 'combinatorial libraries' and 'sequence space'—the terms that are used in the contemporary DE experimental system. The width of sequence space relates to the existence of diverse mutants. In this sense, the terms 'mutant spectrum' and 'mutation distance' designate the production of molecular diversity in libraries of mutants.

It must be re-emphasized that although Hall's and Spiegelman's experiments were both regarded as touching on DE, the fundamental ideas of the contemporary DE system were established in the RNA synthesis of Spiegelman's and Eigen's groups, rather than the protein synthesis of Hall's group. The ideas of Eigen's group have materialized in protein synthesis experiments since the late 1980s, when random mutagenesis and high-throughput screening were developed.

The Contemporary Experimental System of Directed Evolution

The contemporary DE experimental system consists of two techniques: (1) random mutagenesis or random gene recombination and (2) high-throughput screening. As methods of random mutagenesis and gene recombination, error-prone PCR and DNA shuffling can turn what Eigen and Gardiner (1984) hypothesized—i.e., a 'mutant spectrum' (or, a combinatorial library of mutants)—into a reality. With the development of high-throughput screening, *selection pressure* evolved from both the use of ethidium bromide and the modification of time intervals in serial transfer experiments into various display techniques such as phage display, ribosome display, yeast display (cell surface display), and flow cytometry. While the role of ethidium bromide as a selection pressure was to inhibit the self-replication of undesired RNAs, the role of various display techniques in the contemporary DE experimental system is to select desired proteins.

I would say that the evolution of this selection pressure within the history of DE can serve as evidence of what Rheinberger (1997) calls the 'differential reproduction' of DE experimental systems, within its historical context. In other

words, each DE experimental system reproduces the idea of selection pressure, but their material practices are different. The DE experimental system has not been closed, but rather has continued to evolve.

In following its history, I will address concepts co-generated by the contemporary DE system. The techniques of error-prone PCR and DNA shuffling were co-produced with the idea of *molecular diversity*. High-throughput screening, particularly in conjunction with flow cytometry, co-generated the idea of the *velocity* of evolution. I will also address how DE has created new material entities such as catalytic RNA and metalloproteins, thereby generating concepts about the *origin of life* and a *newly emergent form of life*.

Random Mutagenesis and Gene Recombination: The Creation of Molecular Diversity

The concepts and tools of random mutagenesis and random gene recombination have continued to be developed since 1985. Random mutagenesis includes cassette mutagenesis (Wells et al. 1985) and error-prone polymerization chain reaction (error-prone PCR). DNA shuffling is a method of random gene recombination. Error-prone PCR and DNA shuffling were invented in the late 1980s and the early 1990s; both techniques are indebted to the development of regular PCR, invented in 1983 by Kary Mullis et al. of the Cetus Corporation (Rabinow 1996).

Error-prone PCR was first proposed by Leung et al. in 1989 and was later optimized by Cadwell et al. (1994). Error-prone PCR intentionally creates random mistakes in the PCR cycle; as with regular PCR, error-prone PCR also uses *Taq* DNA polymerase, but it utilizes *Taq*'s lack of proof-reading ability in a specific range of nucleotide, per pass of the polymerase.[10] This technique was not imported from other fields, but rather invented exclusively for DE. As Leung et al. argue, they developed this technique to generate what they called 'random mutant libraries' (Leung et al. 1989, p. 11). They were well aware of the limitations of site-directed mutagenesis and the benefits of random mutagenesis:

> The current methods for site-directed mutagenesis are highly efficient and quite effective when some idea of where to make the specific mutations is available. However, in cases in which the structure and function of the protein or DNA region of interest are not well defined, it is often difficult to predict which mutations to make in order to bring about a desired change. Random mutagenesis of the DNA region of interest coupled with a screening system is therefore generally the method of choice for the study of a gene or its regulatory functions. (Leung et al. 1989, p. 11)

One of the most significant breakthroughs in DE was made in 1994, with the invention of DNA shuffling by Willem Stemmer at the Affymax Research Institute. DNA shuffling is a method of in vitro recombining homologous mutants that

[10] The proof-reading ability of DNA synthesis by *Taq* DNA polymerase is also reduced in the presence of Mn^{2+} (Leung et al. 1989, p. 12).

naturally evolved from a progeny or random mutants created by error-prone PCR.[11] This technique was designed to create, quite deliberately, a combinatorial library (Stemmer 1994a). Like error-prone PCR, DNA shuffling was invented for exclusive use in DE studies. In defining DNA shuffling, Stemmer uses the term 'molecular evolution' (Stemmer 1994a) and the phrase 'rapid evolution of proteins in vitro' (Stemmer 1994b).

DNA family shuffling (i.e., DNA family breeding) is the most powerful method of in vitro random gene recombination. This technique synthesizes proteins from multiple parenting and cross-species interchanges in which it conducts several 'crossovers' (Crameri et al. 1998, p. 290) by recombining multiple homologous genes that originate from a progeny.

Also noteworthy is the fact that Stemmer (1994a, p. 10751) uses the sexual terms 'genetic mating', 'breeding', 'sexual recombination', and 'sexuality' in defining DNA shuffling. DNA family shuffling, called 'sexual PCR', installs the notion of what Frances Arnold (2001, p. 255) calls 'molecular sex' in its experimental system, to eliminate deleterious mutations and accumulate beneficial mutations. In DNA family shuffling, the notion of molecular sex is co-produced via a 'pool-wise' recombination, rather than a 'pair-wise' recombination. A pool-wise recombination refers to the recombination of multiple parental genes, while a pair-wise recombination refers to the recombination of two parental genes (Tobin et al. 2000, p. 422).

The benefit of molecular sex is related to the *quality*, rather than the *degree*, of molecular diversity. Stemmer (1994b, p. 390) claims that DNA shuffling can bring about more 'meaningful' diversity than random mutations produced by error-prone PCR, because DNA shuffling can reduce deleterious mutations produced by error-prone PCR. In defining single-gene shuffling as 'asexual evolution' (Arnold 1998, p. 126), while referring to DNA family shuffling as 'sexual evolution', Frances Arnold at the California Institute of Technology claims that sexual evolution is more useful than asexual evolution in producing more beneficial mutations.[12]

Molecular diversity is one of the most significant concepts co-produced by the contemporary DE experimental system. (For instance, see the use of the term 'molecular diversity' in Kauffman and Ellington 1999, p. 256.) DE is built upon a belief in a large number of mutants. In explaining what it means to 'think combinatorially', Bartlett and Joyce (1999, p. 253) highlight 'a trust in the power of large numbers, even above one's own intellect and intuition'. Such belief in molecular diversity is what Rheinberger (1997, p. 138) calls the 'styles of experimental reasoning' that DE produces.

[11] This method first fragments the genes of progeny with the restriction of an enzyme called 'DNaseI' into a pool of random DNA fragments. The DNA fragments are assembled into a full-length gene through repeated cycles of annealing in the presence of *Taq* DNA polymerase. This method then selects the best recombinations. The selected pool of improved recombinants becomes the starting point for a subsequent round of DNA shuffling (Stemmer 1994b).

[12] Arnold (1998, p. 127) notes that 'sex provides some significant benefits in natural evolution (to make up for its obvious costs?)'. Some of the benefits can be captured in the test tube. By recombining parental genes to produce libraries of different mutation combinations, we can quickly accumulate the beneficial mutations, while removing any deleterious ones. Using the 'DNA shuffling' method … we have been adding a little sex to our evolutionary design strategy'.

Site-directed mutagenesis does not employ molecular diversity in protein design. With site-directed mutagenesis, scientists deliberately direct some sort of human intervention toward a specific genotype; meanwhile, through error-prone PCR and DNA shuffling, scientists are almost blind toward genotype. In contrast to site-directed mutagenesis, DE scientists are little aware of what kinds of genetic information are evolving, even though they can check the genotype of a target protein in retrospect, after the selection process is complete. Because the genotype results from random mutations and the random recombination of multiple parental genes via cross-species interchanges, DE provides scientists with very limited knowledge of the extent to which each gene—or several genes—affects the phenotype.

The notion of molecular diversity therefore affects the concept of life. Because of such blindness toward genotype, the contemporary DE experimental system may provide new insights into the concept of genetic reductionism, which was prevalent in the 20th century. This blindness of DE toward genotype does not mean that the gene does not affect phenotype; rather, the implication is that it is difficult to explain the influence of one or several genes on a phenotype of proteins synthesized by way of DE. This coincides with the observation of Nicholas Rose (2007), vis-à-vis the decline of genetic reductionism in contemporary biomedicine in the 21st century. This decline is related to the completion of the Human Genome Project in 2003, as well as the concomitant growth of proteomics since then. The Human Genome Project proved that the function of proteins is more complex than modern genetics researchers had expected. The advent of proteomics has allowed scientists to pay greater attention to the function of proteins rather than the function of a gene itself. The development of DE coincides with the growth of proteomics; in fact, this technique is used to identify the unknown functionality of de novo proteins in the field of proteomics.

High-Throughput Screening: The Acceleration of Evolution

The development of high-throughput screening has been crucial to the acceleration of DE. Until various display techniques were invented after the late 1980s, screening techniques had depended on low-throughput screening techniques such as gas chromatography and electrophoresis. The role of these various display techniques is to express random mutants produced by error-prone PCR and DNA shuffling on the surface of filamentous phages or yeast cells. The selection process happens when the peptide-displayed phages or cells contact specific substrates such as metals, carbon nanotubes, or any other organic or inorganic materials that researchers select. If the phages have an affinity with these substrates, they are selected; otherwise, the phages or cells lacking binding affinity to these substrates will wash way. Selected phages or cells are then eluted from substrates and amplified by *E. coli*.

George P. Smith (1985) at the University of Missouri invented phage display in 1985, a technique for displaying foreign gene sequences over the surfaces of filamentous phages by inserting foreign gene fragments inside the phage. The fused

phages are used to screen the antibodies that are able to attach to these phages. Smith (1985, p. 228) suggests this as a DE technique, claiming that this technique 'might be used in isolating desired clones from a library of random inserts in a fusion-phage vector'. Even though he did not note the idea of evolution in his first 1985 article on phage display, it is evident that the idea of DE as an experimental system was implicitly embedded in Smith's invention of phage display from the outset. Since then, while collaborating with DE scientists such as Peter Schultz, Smith has situated phage display as a DE technique; Smith's 1997 article, written in collaboration with Valery A. Petrenko, is a good indication of this. Smith and Petrenko (1997) compare natural selection to in vitro evolution in explaining phage display.

The invention of phage display in 1985 was followed by other screening techniques, such as ribosome display in 1990, SELEX in 1991, and yeast display (yeast surface display or cell surface display) in 1997 (Boder and Wittrup 1997).[13] A significant breakthrough in the optimization of high-throughput techniques has occurred since then, in particular, when these techniques were combined with flow cytometry. A flow cytometer is an 'instrument that can measure physical, as well as multi-color fluorescence properties of cells flowing in a stream' (Chapman 2000, p. 3). It is an instrument used to accelerate the speed and improve the accuracy of high-throughput screening.[14]

George Georgiou et al. at the University of Texas-Austin are pioneers in flow cytometry for high-throughput screening (1997). In 1997, this group first used the fluorescence-activated cell sorter (FACS) produced by Becton Dickinson Inc. FACS enables scientists to analyze 'cells for fluorescence in real time and with high-throughput screening using multi parameter flow cytometry' (Olsen et al. 2000, p. 1071). Just as the SPM paved the way for NT by providing an atom-by-atom image on the nanoscale (Baird and Shew 2004), FACS enabled scientists to observe cell-by-cell and quantitatively optimize the screening process (Daugherty et al. 2000, p. 211). In addition to the invention of DNA shuffling, the combination of high-throughput screening with flow cytometry marked a pivotal moment in the history of DE.

Why were high-throughput screening techniques combined with flow cytometry in the late 1990s, even though flow cytometry has been used since the 1970s?[15] It

[13] Yeast display (i.e., cell surface display) can express proteins on the surface of a microorganism. Yeast display uses the fusion of proteins and *agglutinin*, a protein involved in cell adhesion. This protein can be tightly bound to a cell wall (Georgiou et al. 1997, p. 30). In 2001, yeast display was sold to Abbott Laboratories, which commercialized in 2002 a monoclonal antibody called Humira, which uses phage display.

[14] Flow cytometers consist of sorters and analyzers. They collect and analyze data on cells, but can also sort cells with desired properties. The development of sorters was indebted to the development of optics and lasers. Contemporary commercial high-speed cell sorters use one or two lasers and can generate between five and eight fluorescence signals from each cell (Ashcroft et al. 2000). The development of analyzers has resulted from the development of IT. Flow cytometers are equipped with computers that can automatically quantify the number of cells screened. .

[15] See Keating and Cambrosio (1994) for an early history of flow cytometry. Leonard Herzenberg at Stanford invented flow cytometry in 1969, using a mercury arc lamp; a second version followed in 1971 that used an argon ion laser to detect cells tagged with fluorescent markers. With funding from the National Institutes of Health, Herzenberg and the Stanford engineers commercialized their instruments through Becton Dickinson Inc. in 1975.

can be explained from two perspectives. First, the application of flow cytometry to the screening of combinatorial libraries was indebted to great progress in the expression system for the display of proteins—in particular, the development of cell surface display (i.e., yeast display) in the late 1990s.[16] In fact, phage display is not used in the FACS for high-throughput screening, because bacteriophages are too small.

Secondly, the combination of flow cytometry with high-throughput screening is related to the commercialization of high-speed cell sorters in the 1990s. According to Ashcroft et al. (2000), there were two significant events in relation to commercial model high-speed flow cytometers. The first commercial high-speed cell sorter came onto the market in 1994, and the second one became available in 1999. New commercial cell sorters can measure more cells and at a higher speed than pre-1994 conventional cell sorters. There are currently three types of cell sorters on the market: Vantage SE, Altra, and MoFlo (Ashcroft et al. 2000).[17] According to Daugherty et al. (2000, p. 214), the MoFlo from Cytomation Inc. can 'sort large populations at rates as high as 75,000 cells per second'. The speed of the cell sorters determines the speed of DE.

The acceleration of DE experimentation, as afforded by the development of high-throughput screening techniques, coincided with the institutionalization of chemical biology or combinatorial chemistry, as well as the commercialization of DE in the late 1990s. Data illustrating this shift is shown in Fig. 2. This emergence of chemical biology or combinatorial chemistry along with the commercialization of DE has caused an explosive growth in DE publications. At the same time, the notion of molecular diversity has also become institutionalized and commercialized. In 1995, for example, Springer Netherlands began to publish a journal of combinatorial chemistry entitled *Molecular Diversity*, and Stemmer established a startup company named 'Maxygen', based on the concept of 'maximizing genetic diversity'.[18] The institutionalization and commercialization of DE means that the DE experimental system helps create social order. As Rheinberger (1997) says, an experimental system is concurrently local and social. The next section will also deal with the shaping of a community, related to the DE experimental system from the vantage of the RNA world hypothesis.

[16] Cell surface display, in conjunction with flow cytometry, begins with the incubation of peptide-displayed libraries with a florescence substance called FRET. FRET is able to emit blue light and red light, depending on the binding of this substrate with the peptides displayed on the cell surface—that is to say, if FRET binds a target peptide, it emits red light; otherwise, it emits blue light. Therefore, flow cytometers can sort and quantify screened cells (see Daugherty et al. 2000, p. 213).

[17] For an introduction of commercial flow cytometer, see Chapman (2000). Three companies, Becton Dickinson (San Jose, CA, USA) and Beckman Coulter (Fullerton, CA, USA) and Cytomation (Fort Collins, CO, USA) dominate the commercial flow cytometer market (Chapman 2000, p. 4; Battye et al. 2000) Moreover, according to Ashcroft et al. (2000, p. 14), technological origins of commercial flow cytometers can be traced to 'groups in Europe, Australia and the USA. Europe had Radiobiological Institute (Netherlands) and the Max Planck Institutes (Germany), while biological groups in the US National laboratories, (Livermore, Los has seen a steady advance in the performance Alamos and Oak Ridge) plus groups at Stanford and Rochester were the US proponents. In Australia, researchers were at the Tumour Biology branch of the Ludwig Institute'.

[18] Available at http://www.maxygen.com/newsview.php?listid=91. Accessed on August 31, 2008.

Origin of Life: The RNA World Hypothesis Revisited

DE has created new material entities such as catalytic RNA and metalloproteins, and has thereby provoked two discussions of life that each entity comes to embody. On the one hand, it is about *the origin of life*; on the other hand, it is about a *newly emergent form of life*. This section will discuss the former issue, while the next section will discuss the latter.

The RNA world hypothesis, which materialized in the 1960s as the first in-vitro evolution in a test tube, had been nearly forgotten for a decade—that is, until Thomas Cech discovered ribozyme, or RNA enzyme, in 1981 and won a Nobel Prize in 1989 (see Cech et al. 1981; Cech 1987). The traditional functions of RNA are to act not only as a messenger, but also as a structural scaffold. The discovery of ribozyme meant that RNA could also act as a catalyst. This discovery strengthened the RNA world hypothesis—namely, that RNA evolution occurred prior to the emergence of DNA and proteins. In fact, according to Cech's recent DVD lecture entitled 'Double Life of RNA' at the Howard Hughes Medical Institute—of which he is currently president—Cech was not aware of the RNA world hypothesis before he discovered ribozymes. He noted that following his discovery of RNA enzymes, he realized that many scientists had been waiting for this scientific achievement. In particular, Francis Crick (1993)—who hypothesized the RNA world in the 1960s—praised Cech's discovery of ribozymes:

> There are speculations in the 1960s that RNA catalysts existed at that stage in evolution, that RNA was the sole genetic material, and that the standard Watson-Crick pairing was the basis of genetic replication. None of these early authors was smart enough to suggest that relics of these hypothetical catalytic RNA might still be around today. Indeed, it was speculated that the original ribosomes might have been made solely of RNA but not that their main catalytic activity might still be performed today by RNA alone, as recent evidence seems to suggest. (Crick 1993, p. xi).

In 1986, Walter Gilbert (1986, p. 618) coined the term 'RNA world', where RNA enzymes could serve to catalyze the synthesis of themselves, without the help of protein enzymes. Gilbert claims that the RNA world came to an end after more efficient protein enzymes replaced RNA enzymes. Cech's discovery of ribozymes drew significant attention from scientific communities to the RNA world hypothesis; and in 1993, Gesteland et al. (1993) edited a book called *The RNA World.* About 20 groups comprising approximately 400 people worldwide are investigating the RNA world hypothesis (Lewis 1997). They have established a community called the 'International Society for the Study of the Origin of Life', and the NASA Specialized Center of Research and Training/Exobiology La Jolla Consortium, located in southern California, has funded six major laboratories (Lewis 1997).

The discovery of ribozymes has stimulated DE scientists such as Jerald Joyce at Scripps Institute and Jack W. Szostak, a professor of molecular biology at Massachusetts General Hospital (Joyce 1991; Bartel and Szostak 1993). Joyce is one of the main figures to continue to assert the RNA world hypothesis. When he

gave a lecture about the RNA world at the 36th American Society for Cell Biology Annual Meeting in December 1996 in San Francisco, 2,000 people attended (Lewis 1997). Joyce has used DE to create new catalytic RNAs, in order to facilitate RNA-based evolution (see Joyce 2006, p. 223). Peter Radetsky suggests a relationship between Thomas Cech's discovery and Jerald Joyce's experiment:

> For Joyce, the discovery [by Tomas Cech] was a revelation. … 'What Scripps is about', he says, 'is the *never-never land between biology and chemistry*—collaborations between the two just aren't very usual'. It was a land he was about to visit. Using Cech's original ribozyme as a template, he produced 10 trillion versions of it, each with slight differences, and geared up to do what he called *directed evolution* (my emphasis).[19]

In Joyce's DE experiment, molecular diversity in combinatorial libraries is closely connected to the velocity of evolution. The degree of molecular diversity compensates for the evolution timeframe. Joyce claims that, 'We can't do nearly as many generations as nature has done. …Nature has had a 4-billion-year head start. So we compensate by having a very, very large population' (as cited in Radetsky, see footnote 19).

Molecular Biomimetics and Novel Life Forms

Since the late 1990s, DE has been combined with bioelectrochemistry in molecular biomimetics—which, as the name suggests, mimics nature as an inspiration for material design. At the same time, DE began to appear in the literature of nanobiotechnology. Molecular biomimetics resulted from a so-called convergence of NT and BT (or biology) (see Roco 2003; Sarikaya et al. 2003). This convergence blurred the boundaries of the material identities between inorganic materials and biomaterials. This convergence created a new form of life called metalloproteins (i.e., metal-binding proteins).[20] Although inorganic metal-binding proteins are also naturally occurring in the wild, DE optimizes these proteins and creates de novo proteins that are capable of electronic transfer, for use in batteries and bio-sensing applications (i.e., lab-on-a-chip) (see O'Connell and Guilbault 2001). The production of metal-binding proteins led to the generation of enormously influential and useful interfaces between gene sequences and metals.

One of the earliest DE experiments in the field of molecular biomimetics was carried out by Stanley Brown (1997), who isolated gold- and chromium-binding

[19] Radetsky, P. Speeding through evolution - Gerald Joyce's directed-evolution experiments. *Discover* (May 1994). http://findarticles.com/p/articles/mi_m1511/is_n5_v15/ai_15341763. Accessed 31 August 2008.

[20] Redox proteins and enzymes are metalloproteins. Redox proteins for bioelectronic devices are capable of electronic transfer and self-assembly at the nanoscale; they should be immobilized on an electrode surface and have multi-redox centers. According to Gilardi and Fantuzzi (2001), redox proteins 'are 'wired up' in efficient electron-transfer chains, are 'assembled' in artificial multidomain structures (molecular Lego), [and] are 'linked' to surfaces in nanodevices for biosensing and nanobiotechnological applications'.

peptides from a bacterial combinatorial library with a population of approximately five million polypeptides. Since then, extensive work has been accomplished. Angela Belcher's group at MIT and Mehmet Sarikaya's group at the University of Washington in Seattle are among the academic leaders who have used DE to produce genetically engineered proteins for inorganics (GEPIs) (see Seeman and Belcher 2002; Sarikaya et al. 2003). They have identified specific peptide sequences with high affinities for semiconductors (ZnS, CdS, or PbS), metals, and carbon nanotubes (e.g., Flynn et al. 2003). Phages are used as templates and linkers between inorganic materials (see Merzlyak and Lee 2006; Kriplani and Kay 2005). Such linkers—known as molecular erectors—are also used to build microassays suitable for modern genomics, pharmacogenetics, and proteomics; they are also used as building blocks for materials suitable for use in nanobioelectronic, nanophotonics, and nanomagnetics.

The emerging field of molecular biomimetics is built on the convergence between 'the physical sciences (atomic-scale microscopy), chemistry (electrochemistry and electron transfer) and biology (enzymology, protein design and molecular biology)' (Gilardi and Fantuzzi 2001, p. 468). Biosensor research—such as that touching on the 'lab-on-a-chip'—is one case. The most common component of biosensors is the enzyme electrode. DNA shuffling is used in the optimization of enzymes such as cytochrome P450 and de novo proteins, in the 'molecular Lego' approach to creating bioelectronic devices.[21]

SPM, which paved the way for NT in the 1980s and 1990s, is used to analyze metalloproteins such as cytochrome P450 variants, Azurin (blue copper proteins), metallothionein, and rubredoxin (see Davis et al. 2000). Davis' group reengineered these metalloproteins to create special attachments to gold, graphite, and carbon electrodes. Scientists tested the interactions between metalloproteins and electrodes with such SPM apparatus as the scanning tunneling microscope and the atomic force microscope. Accordingly, protein engineering is interfacing with bioelectrochemistry. Gilardi and Fantuzzi (2001) claim:

> *Protein engineering* has started to provide a valuable contribution towards the design of the surface and the functional properties of metalloproteins. This, combined with the controlled chemical modification of electrodes, is able to achieve a designed pseudo-biological recognition between electrode and protein. Moreover, the characterization of these interactions using high-resolution X-ray photoelectron spectroscopy and *scanning probe microscopy* provides an attractive and powerful way to combine structural and functional information on the biological element with the voltammetric response of the modified electrode. (Gilardi 2001, p. 497)(my emphasis)

[21] Gilardi and Fantuzzi (2001) notes, 'The aim of molecular Lego is to generate artificial redox chains by assembling genes of well characterized redox proteins and enzymes as prototypes for engineering systems that can be exploited by bioelectrochemistry. … *The DNA shuffling* of introns and exons generates multidomain proteins assembled from building blocks. The molecular Lego approach selects key domains, or building blocks, to assemble artificial redox chains with the desired properties, ultimately capable of communicating with electrode surfaces' (my emphasis).

The convergence of SPM with DE, along with the emergence of metalloproteins, shows that the standard history of NT is merging with the history of NT from protein engineering. Such a convergence allows the history of DE to become one of the histories of NT. Many scientists have treated protein engineering not as an NT field, but exclusively as a BT field. However, a new field of molecular biomimetics, which Mihail Roco calls the 'fourth generation of NT', is in pursuit of the integration of inorganic materials and biomaterials, in a molecular 'Lego' approach. Protein engineering in the wake of molecular biomimetics is currently in the realm of NT. In 1996, B.C. Crandall (1996) defined DE as one of the frontiers of NT, and NT scientist Richard Smalley (2002) definition of 'wet' NT suggests that it is the field of BT to which DE belongs.

Conclusion

This study explored how DE has evolved from protein engineering, through chemical biology or combinatorial chemistry, to nanobiotechnology. NT is not at all built on a specific material identity, but it does cut across the boundaries that otherwise separate technical disciplines. Therefore, it would be incorrect to place NT solely in the realm of inorganic materials, because NT also uses biomaterials. However, it is also erroneous to portray NT as being exclusively within the frame of the dominant biotechnologies of the second half of the 20th century. It is important to pay attention to the changes that take place in BT and molecular biology at large in the 21st century; needless to say, the material culture of DE will not be identical to that of the dominant biotechnologies of the 20th century.

The differences in material culture between DE and other BT, as noted earlier, result from differences in the techniques between site-directed mutagenesis and random mutagenesis. In contrast to the dominant biotechnologies, DE uses the techniques of random mutagenesis (i.e., error-prone PCR) or random gene recombination (i.e., DNA shuffling), thereby harnessing molecular diversity to its experimental system. This is not to suggest that site-directed mutagenesis is outdated; on the contrary, site-directed mutagenesis is still a prevailing method in molecular biology and BT. Nonetheless, the predominant method of mutagenesis used in molecular biomimetics is not site-directed but random mutagenesis (or random gene recombination), because the functionalities of proteins with metals and semiconductors have only rarely been known.

DE also co-produces new material entities and concepts of life that had not been sufficiently addressed by the molecular biology of the 20th century. By generating catalytic RNAs, DE provokes a new discussion about the origin of life in terms of the RNA world hypothesis. DE also creates a newly emergent form of life called metalloproteins and redox proteins that did not exist in the previous century. Finally, DE transforms molecular biology in conjunction with chemistry, physics, material science, and electrical engineering, in the fields of chemical biology, combinatorial chemistry, and molecular biomimetics. Indeed, if one is to grasp fully the complete history of NT, it is important to follow the distinctions between various streams of development, even as they continue to occur.

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