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Synthetic Toxicology: Where Engineering Meets Biology and Toxicology

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This article examines the implications of synthetic biology (SB) for toxicological sciences. Starting with a working definition of SB, we describe its current subfields, namely, DNA synthesis, the engineering of DNA-based biological circuits, minimal genome research, attempts to construct protocells and synthetic cells, and efforts to diversify the biochemistry of life through xenobiology. Based on the most important techniques, tools, and expected applications in SB, we describe the ramifications of SB for toxicology under the label of synthetic toxicology. We differentiate between cases where SB offers opportunities for toxicology and where SB poses challenges for toxicology. Among the opportunities, we identified the assistance of SB to construct novel toxicity testing platforms, define new toxicity-pathway assays, explore the potential of SB to improve *in vivo* biotransformation of toxins, present novel biosensors developed by SB for environmental toxicology, discuss cell-free protein synthesis of toxins, reflect on the contribution to toxic use reduction, and the democratization of toxicology through do-it-yourself biology. Among the identified challenges for toxicology, we identify synthetic toxins and novel xenobiotics, biosecurity and dual-use considerations, the potential bridging of toxic substances and infectious agents, and do-it-yourself toxin production.

Key Words: **synthetic biology; toxicology; protocells; xenobiology; do-it-yourself biology.**

Synthetic biology (SB) is an emerging science and engineering field that applies engineering principles to biology. SB has become one of the most dynamic new fields of biology, with the potential to revolutionize the way we do biotechnology today. By applying the toolbox of engineering disciplines to biology, bioengineers aim to identify a whole set of potential applications ranging very widely across scientific and engineering disciplines. The prediction is that novel chemicals, devices, systems, and eventually new species will be generated as SB develops from a new and emerging science and technology to a mature field. Some of the potential benefits of SB, such as the development of low-cost drugs or the production of chemicals and renewable energy carriers by engineered bacteria, are projected to be enormous. There are, however, also potential and perceived risks related to deliberate misuse or accidental damage. Some of the tools and applications derived from SB are of particular interest for toxicological sciences. This review article summarizes current developments in SB, their possible applications in toxicological sciences, and addresses opportunities and challenges to toxicology.

The first part of the article, "SB: What Is in a Name?" section, provides an overview of the different SB research subfields. The second part, "Synthetic Toxicology" section, explores the ramifications of SB for toxicological sciences, focusing both on the supportive character of SB and on new risks and challenges for toxicology. Special attention is devoted to recent achievements of SB in life sciences and design innovations, which may be extended to new applications in toxicological studies. Some of these novel biological systems and products, however, will pose new challenges to toxicologists and established risk assessment practices. Furthermore, the advance of SB will raise concerns over safety and security because of the increased accessibility of the technology to individuals and institutions with less well-equipped facilities and biosafety training.

SB: WHAT IS IN A NAME?

Although the term "SB" was already used about 100 years ago (Leduc, 1912), the contemporary version is a relatively young field at the intersection of biology, engineering, chemistry, and information technology. Not atypical for an emerging science and engineering field, a variety of definitions are circulating in the scientific community, and no one definition would receive total support by the researchers involved in SB

activities. The probably least contested definition is that found at the SB community webpage (http://syntheticbiology.org/):

> Synthetic Biology is: A) the design and construction of new biological parts, devices, and systems, and; B) the re-design of existing, natural biological systems for useful purposes.
>
> Synthetic biologists are currently working to

- specify and populate a set of standard parts that have well-defined performance characteristics and can be used (and re-used) to build biological systems,
- develop and incorporate design methods and tools into an integrated engineering environment,
- reverse engineer and re-design preexisting biological parts and devices in order to expand the set of functions that we can access and program,
- reverse engineer and re-design a 'simple' natural bacterium,
- minimize the genome of natural bacteria and build so-called protocells in the lab, to define the minimal requirements of living entities, and
- construct orthogonal biological systems, such as a genetic code with an enlarged alphabet of base pairs.

The lack of a well-accepted definition, however, does not seem to stop the community from going ahead and doing SB, naturally leading to a quite diverse area of science and engineering. Activities that fall under SB are currently performed in several fields. For a number of reasons (e.g., different school of thoughts), the diverse activities are not always presented by SB scientists. By and large, however, the following activities are usually subsumed under SB (Bedau and Parke, 2009; Benner and Sismour, 2005; Deplazes, 2009; Luisi, 2007; O'Malley *et al.*, 2008; Schmidt *et al.*, 2009; Torgersen *et al.*, 2010):

- DNA synthesis (or synthetic genomics),
- Engineering DNA-based biological circuits (based on genetic engineering but using real engineering principles),
- Defining the minimal genome (or minimal cell),
- Building protocells (or synthetic cells),
- Xenobiology (aka chemical SB).

The following sections and Table 1 explain the five subfields in greater detail.

Statements From the U.S. Presidential Commission for the Study of Bioethical Issues' First Meeting on SB on 8–9 Juy 2010 in Washington, DC. (emphasis added by the authors)

James J Wagner (vice chair of the U.S. Presidential Commission for the Study of Bioethical Issues): *"Connected to that, the question comes on my head, if we can't get this answered, let's give it two minutes to see if it converges. But I do wonder if one of the contributions the committee could . . . be able to give a better definition of what we understand synthetic biology to be. I went back over my notes I have five definitions we were offered and they are not necessarily complimentary . . .. I hate to have all these scientists leave the room without my having asked: have I missed the definition for synthetic biology?"*

Drew Endy (Synthetic Biologist): "*You haven't got them all."*

The Presidential Commission for the Study of Bioethical Issues (2010). http://www.tvworldwide.com/events/bioethics/100708/. Accessed December 2, 2010

DNA Synthesis

The technical basis of SB is provided by DNA synthesis: developments in the rapid sequencing of genomes are mirrored by the increased capabilities to chemically synthesize DNA that is longer in size and cheaper than before. The pace and proliferation in which the increase in DNA synthesis capacity is growing has been compared with Moore's law for integrated circuits (Carlson, 2003). Over recent years, scientists have been able to chemically synthesize the genomes of different viruses without the need of a natural template (Cello *et al.*, 2002; Tumpey *et al.*, 2005; Wimmer *et al.*, 2009). Scientists at the J. Craig Venter Institute developed the first-ever complete *de novo* synthesis of a whole bacterial genome (*Mycoplasma genitalium* with 580,000 bp); they recently repeated the effort with *Mycoplasma mycoides* (over 1 million bp), which was also successfully "booted up" (in other words, the cell was made to accept and use the transplant genome as its own) in a natural bacterium (Gibson *et al.*, 2010). Likewise, artificial small eukaryotic chromosomes, so-called mini-chromosomes (or artificial chromosomes), have been constructed. They open up perspectives into engineering or fundamentally altering eukaryotic multicellular organisms (Carlson *et al.*, 2007; Macnab and Whitehouse, 2009).

Engineering DNA-Based Biological Circuits

Advanced genetic constructs are used in metabolic engineering; the increase in DNA synthesis capacities, however, is much larger than that of current engineering abilities. Attempts to redesign meaningfully metabolic pathways or fine-tuned genetic circuits and systems are limited to a complexity involving no more than about 10–15 genes today. Nevertheless, substantial progress has been made, e.g., in finding novel ways of producing biofuels from cellulose or other raw materials. The most celebrated achievement is the design of a metabolic pathway to produce a precursor of the antimalaria compound artimisinin, a substance naturally found in the wormwood plant that could not be produced by microorganisms previously. The design and construction of this

TABLE 1
Complexity Levels (1–4) and "Unfamiliarity" Levels (A–E) in SB

	A: DNA synthesis	B: Genetic circuits[a]	C: Minimal genomes	D: Protocells	E: Xenobiology
1: Biochemistry	—	—	—	Standard or alternative biochemistry (Rasmussen *et al.*, 2004)	Alternative biochemistry (XNA; Declercq *et al.*, 2002; Herdewijn and Marliere, 2009; Nielsen and Egholm, 1999; Schoning *et al.*, 2000; unnatural bases, Henry and Romesberg, 2003; Yang *et al.*, 2006, 2007; and amino acid, Hartman *et al.*, 2007)
2: Genes/parts	Synthetic genes (Carlson, 2003, 2009; May, 2009)	Genes and bioparts, bioparts (Canton *et al.*, 2008; Endy, 2005; Smolke, 2009)	—	Engineered phospholipids (Murtas, 2009; Rasmussen *et al.*, 2004; Szostak *et al.*, 2001)	Changing the codon assignment of genes (Budisa, 2004; Luisi, 2007)
3: Biological systems	Artificial chromosomes (Carlson *et al.*, 2007; Macnab and Whitehouse, 2009), synthetic viruses (Cello *et al.*, 2002; Tumpey *et al.*, 2005; Wimmer *et al.*, 2009)	Enhanced metabolic engineering[b], bioparts and devices (Elowitz and Leibler, 2000; Lu *et al.*, 2009; Ro *et al.*, 2006; Tigges *et al.*, 2010)	—	Cellular vesicles lacking key features of life (Hanczyc and Szostak, 2004; Mansy *et al.*, 2008; Mansy and Szostak, 2008)	Development of novel polymerase and ribosomes (Ichida *et al.*, 2005; Loakes and Holliger, 2009; Neumann *et al.*, 2010)
4: Organelles, single-cell organisms[c]	Whole-genome synthesis (Carr and Church, 2009; Gibson *et al.*, 2008, 2010; Lartigue *et al.*, 2007)	—	Top-down SB reducing existing organisms' genomes (Hutchison *et al.*, 1999; Mushegian, 1999; Posfai *et al.*, 2006)	Real synthetic cells, bottom-up SB manufacturing whole cells (Rasmussen, 2009)	Xeno-organisms, chemically modified organisms (Marliere, 2009; Schmidt, 2010)

Note. XNA, xeno-nucleic acids.

[a]The area of metabolic engineering might constitute a separate column; however, if it largely follows established practices of genetic engineering (though more ambitious), it would clearly have to be considered ''familiar''; more advanced forms would perhaps fall under column B (genetic circuits).

[b]Such as the synthetic artimisinin project.

[c]The list is far from exhaustive—e.g., with regard to complexity, in the more distant future, we could also think of engineered tissues and organs, of altered multicellular and higher organisms or even of entirely synthetic ecosystems composed of fully synthetic organisms.

precursor, however, representing the state of the art of enhanced metabolic engineering, was a tedious process that took about 150 person years. This explains the call for rationalization and reduction in design complexity. One possible solution could be to create so-called standard biological parts (aka biobricks, a trademark created to promote the underlying approach, whereas "biopart" is the technical expression). This would represent a toolbox of well-characterized, prefabricated, standardized, and modularized genetic compounds (such as sequences of DNA) for engineering biological systems. Such standard biological parts can be freely combined and built into larger "devices" that fulfill certain defined functions. The hope is that when these bioparts are reintroduced into "systems" or larger genetic circuits, they will work as designed. So far, however, few properly working devices have been created, and only few parts have been thoroughly characterized. To date, typical applications have been rather simple, e.g., a chemical oscillator, banana scent–producing *Escherichia coli*, light-sensitive bacteria, etc. (Carlson, 2010; Elowitz and Leibler, 2000; Gardner *et al.*, 2000; Greber and Fussenegger, 2007; Levskaya *et al.*, 2005; Stricker *et al.*, 2008). Conceptually, the parallel to electronic circuits made of simple interchangeable building blocks is no coincidence, and some ideas in electronics are emulated into the world of molecular genetics. The bioparts lie at the heart of the annual international genetic engineered machine (iGEM) competition, attracting thousands of students from across the globe. The team spirit and enthusiasm of the participants are in large part responsible for the popularization and media coverage of the bioparts approach in SB.

Minimal Genome

One of the major problems to understand life is life's enormous complexity. The minimal genome research tries to reduce this complexity in order to define a minimal form of life that should be the first life form that can truly be understood (Itaya, 1995; Koonin, 2000; Mushegian, 1999). The goal is to create a cellular platform or "chassis," in other words a simple cell with a so-called minimal genome that has the least possible number of genes able to survive under (specific) laboratory conditions (Danchin, 2009; Deplazes and Huppenbauer, 2009). The minimal genome also has the advantage of serving as a cellular platform for engineered biological circuits and reducing possible interactions between the chassis and the crafted genetic circuits introduced. The construction of minimal genomes starts from already small natural genomes (e.g., *Mycoplasma*) by eliminating "unnecessary" genes even further in a top-down approach (Hutchison *et al*., 1999). The resulting truncated organism is dependent on very stable laboratory conditions (supply of essential substances, no pathogens, etc.) to survive. Minimal genomes will help to reduce the inherent complexity of life to the lowest possible level. That approach will yield the best organism model to reach a complete understanding of (simple) living systems.

Protocells and Synthetic Cells

The minimal genome is contrasted by the bottom-up approach to construct so-called protocells or synthetic cells. This is another attempt to construct minimal versions of life such as synthetic cell-like vesicles, but in this case, they are assembled from nonliving chemical components (Rasmussen, 2009; Walde, 2010). Constituents are membrane-like structures to separate the inner from the outer world as well as those that enable a simple metabolism and procreation by fission such as the emergence of "daughter cells" (Hanczyc and Szostak, 2004; Szostak *et al*., 2001). Attempts at constructing metabolically active protocells have been conducted for several years, independently of the other subfields in SB, focusing on various life functions such as metabolism instead of genetics. Recent advances include attempts at combining different scientific approaches with the aim to create artificial cell-like devices that propagate genetic information such as those containing genome-like sequences that are copied before fission (Fig. 1).

Xenobiology

The SB subfields described above apply a biochemical similar to that of natural life forms. For practical reasons, however, metabolic engineering aims at separating the newly introduced, constructed pathways from the naturally occurring ones; it seeks to introduce a certain degree of "orthogonality" while using the same or very similar building blocks. Alternatively, the biochemistry of synthetic life could be entirely different. Fully orthogonal systems are biochemical pathways that cannot interfere with naturally occurring ones at all (Schmidt, 2010). They arise from chemical SB where the very basics of life biochemistry are changed in order to create biological systems that are truly different both in metabolism and on the genetic information level. Examples include altered or nonnaturally occurring bases within the DNA and comprise the idea of entirely different genetic information storage molecules, so-called xeno-nucleic acids that cannot interact with naturally occurring DNA (Fig. 2) (Benner and Sismour, 2005; Herdewijn and Marliere, 2009; Marliere, 2009; Yang *et al*., 2006, 2007). Another example is the use of nonnatural building blocks such as noncanonical amino acids. More visionary ideas even toy with the idea to replace carbon with silicon in essential biomolecules. Other visions encompass possible life forms using not only nonnatural elements but also architectures entirely different from the genome-ribosome-protein architecture of "life as we know it" (Schmidt, 2010).

SYNTHETIC TOXICOLOGY

Recent advances in SB have drawn the attention of toxicologists, although they have so far not embraced SB. Given the diversity and interdisciplinarity of SB, however, toxicologists can expect to encounter different aspects of SB in their future work. In most cases, SB will support their work ("SB as an Opportunity for Toxicology" section), but we can also foresee cases in which SB will pose new challenges to toxicologists (see "SB as a Challenge for Toxicology" section). Both sections

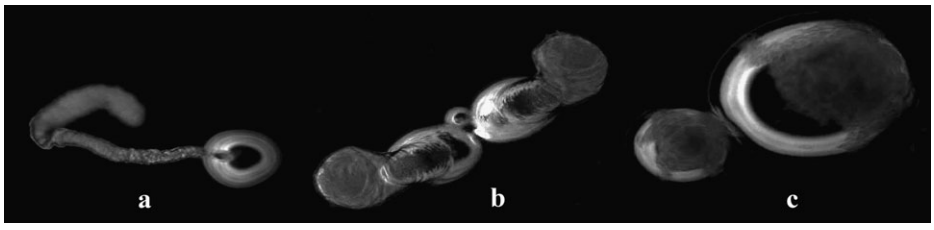

FIG. 1. Protocells exhibit interactive and possibly even ''social'' behavior appearing to share a chemical ''language'' that appears as ''fingers'' (A) produce material at the interface of adjacent agents (B) and exhibit life-like properties including movement and the shedding of skins (C). (Photographs by Rachel Armstrong, 2010, www.rachelarmstrong.me.)

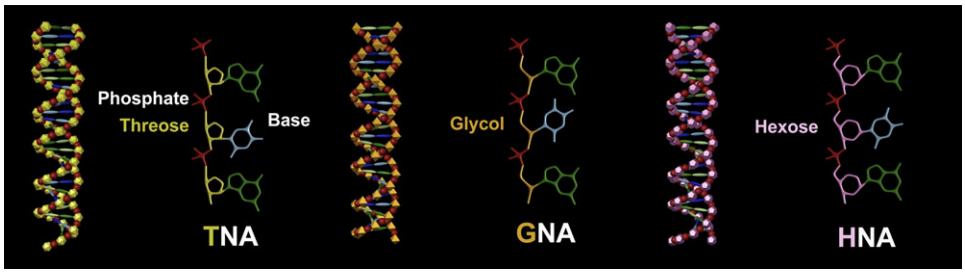

FIG. 2. Several non-DNA non-RNA xeno nucleotides (xeno-nucleic acids [XNA]) can form Watson-Crick–type double helices. These XNAs can be used as alternative information-storing biopolymers. TNA, Threose nucleic acid; GNA, glycol nucleic acid; HNA, hexitol nucleic acid (Illustrations by Simone Fuchs, www.simfuchs.com).

review past work in SB and related areas and provide a preview of what the toxicology community may expect in the upcoming years (see Table 2 for an overview).

SB as an Opportunity for Toxicology

The advances in SB provide unique opportunities for improved approaches to toxicological assessment. These include discoveries of more relevant biomarkers, biosensors for monitoring biomarkers, and better technologies to transform toxicity testing from a system based on animal models to *in vitro* models that evaluate changes in human physiology. The development of molecular biology, genomic, and proteomic technologies has already improved our understanding of the molecular details of cell and tissue functions; applying these insights with SB may help transform this knowledge to toxicological practice (Krewski *et al.*, 2009). SB has the potential to define new cellular mechanisms to replace traditional biomarkers of cellular integrity, cell and tissue homeostasis, and morphological alterations that result from cell toxicity. The discussion below focuses on the potential contributions of SB to improve toxicological assessments and support the work of toxicologists.

SB in Novel Toxicity Testing Platforms

The demand for new toxicological tests is growing, for instance, to provide timely and relevant information for new chemicals, particularly for pharmaceuticals. Based on current technology, the attrition of new drugs in development because of toxicity is high and remains a significant factor in the overall research and development productivity. Toxicological tests with more discriminatory power might eliminate suboptimal

TABLE 2
Overview of Opportunities and Challenges of SB to Toxicology (see "SYNTHETIC TOXICOLOGY" section for details)

	DNA synthesis	DNA-based biological circuits	Minimal genome	Protocells	Xenobiology
Description of subfield	Synthesis of oligos, genes, chromosomes, and complete genomes	Designing genetic circuits, e.g., from standardized biological parts, devices, and systems	Finding the smallest possible genome that can ''run'' a cell, to be used as a chassis, life with reduced complexity	To construct viable approximations of cells and their precursors, to understand biology and the origin of life	Using atypical/ noncanonical biochemical systems and molecules for biological processes, creating a parallel/ orthogonal biological world
Opportunities for toxicology	Supporting fabrication of DNA-based biological circuits	Novel toxicity testing platforms, toxicity-pathway assay, *in vivo* biotransformation of toxins, synthetic biosensors, cell-free protein synthesis, toxic use reduction, do-it-yourself biology	Novel toxicity testing platforms, *in vivo* biotransformation of toxins, synthetic biosensors, toxic use reduction, do-it-yourself biology	Novel toxicity testing platforms, *in vivo* biotransformation of toxins, synthetic biosensors, toxic use reduction, do-it-yourself biology	*in vivo* biotransformation of toxins, synthetic biosensors, toxic use reduction
Challenges for toxicology	Synthetic toxins and novel xenobiotics, biosecurity considerations	Synthetic toxins and novel xenobiotics, biosecurity considerations, do-it-yourself toxin production	N/A	Bridging toxic substances and infectious agents	Synthetic toxins and novel xenobiotics

compounds earlier in development, reducing costs in new drug development. Moreover, traditional toxicity testing strategies based on animal studies often do not reflect human-specific metabolic and toxic effects. Finally, most toxicity biomarkers are associated with drugs that cause significant organ damage when applied at high dose over short duration, whereas there are no biomarkers for damage caused by drugs at low doses over long durations.

Studies based on animal models have long been the "gold standard" of toxicological evaluations. Animal models, particularly rodent models, have been used extensively for chronic toxicity tests of subacute repeated-dose studies (28 day) and chronic repeated-dose studies (3–9 months) usually by oral feeding. The endpoints for repeat dose testing involve clinical observations, blood analysis, whole-body gross necropsy, and microscopic examination of all organs and tissues (histopathology). The target organs/systems evaluated may include liver, kidney, lung, neural (central nervous) system, reproductive organs, the hematopoietic system, the immune system, and the endocrine system.

Several *in vitro* toxicological testing platforms are available (Bhogal *et al*., 2005). The liver is a major organ for drug metabolism and biotransformation. *In vitro* models of the liver are an important platform to obtain knowledge on drug metabolism, mechanisms of action, and potential adverse effects. The current *in vitro* models include primary human hepatocytes, liver slices, perfused liver, collagen sandwich, and bioreactor test systems based on human liver cell lines. The use of primary hepatocytes is one *in vitro* model to study the impacts of drugs on liver functions such as oxidative energy metabolism, carbohydrate metabolism, lipid metabolism, nitrogen metabolism, bile conjugation, and xenobiotic metabolism. Primary cultures are augmented by several human liver cell lines such as HepG2, Hep3B, or HepLiu. Liver perfused models allow short-term studies on liver function but are unsuitable for chronic toxicity tests (Grosse-Siestrup *et al*., 2001). Much like the liver slice systems, they are applicable only to short-term toxicity studies (Drahushuk *et al*., 1996). The current hepatocyte bioreactor test system is configured by stable hepatocytes cultivated on packed bed systems, which offer a tuneable cellular microenvironment and uniform perfusion. The bioreactor containing perfused hepatocytes proved better than conventional cultures in cell viability and liver-specific function (Powers *et al*., 2002). Supplementary substrates to the cell cultures, however, artificially influence the liver-specific function, and the immortality of these cell lines may have altered some of its specificities (Cederbaum *et al*., 2001). This calls for developing better *in vitro* models for toxicological assays. Here, SB may provide solutions. An alternative strategy to immortalize human hepatocytes has been proposed by Cascio using Cre/loxP site-specific recombination (Cascio, 2001). Another approach generates a reversible immortalized porcine hepatocyte cell by retroviral transfer and site-specific recombination (Meng *et al*., 2010). Based on advances in tissue engineering, a Lung-on-a-Chip has been created (Huh *et al*., 2010). It contains two chambers separated by a porous membrane lined with human lung cells on one side and cells from capillary blood vessels on the other, mimicking the function of the human lung. This approach can be applied to create Liver-on-a-Chip to engineer better devices to predict the effects of toxins and to identify better biomarkers for liver toxicity.

Besides the liver, the kidney is a major target organ of drug toxicity. Most nephrotic damage remains undetectable for a long time. The current clinical biomarkers such as a drop in glomerular filtration rate come into play later when significant injury to renal tubules is already occurred. This calls for developing early toxicity biomarkers for the kidneys. A synthetic kidney or Kidney-on-a-Chip composed of engineered cells with a built-in reporting system would be an excellent platform for toxicological studies, particularly those on dose response and time course. In addition, toxicant-induced kidney damage is related to the damaged cell membranes, which induces/increases glomerular permeability. Such toxic effects on cell membranes can be studied using the protocell models mentioned in the following sections.

The cosmetics industry also needs alternatives to animal studies, especially because Europe issued a testing ban on finished cosmetic products (since 2004) and on ingredients or combinations of ingredients (since 2009). An additional marketing ban has been in force since 11 March 2009 for all human health effects with the exception of repeated-dose toxicity, reproductive toxicity, and toxicokinetics. For these specific health effects, the marketing ban will apply step by step as soon as alternative methods are validated and adopted in European Union legislation with due regard to the Organization for Economic Cooperation and Development (OECD) validation process. The maximum cutoff date is 10 years after entry into force of the Directive, which is due on 11 March 2013, irrespective of the availability of alternative nonanimal tests (EC, 2004, 2010). A step toward alternative testing methods has been made, e.g., with the synthetic skin "Episkin" (Gregoire *et al*., 2008; Lelievre *et al*., 2007; Spielmann *et al*., 2007). Although current SB techniques have not yet yielded synthetic tissues or organs (most research focuses on single-celled organisms and biological subsystems), further developments can be expected in the field of tissue and organ engineering, creating new toxicity testing methods.

Toxicity-Pathway Assays and Targeted Testing

Synthetic cells or protocells provide a useful platform to explore toxicological testing that can mimic and quantify cellular responses to toxins. Protocells can be designed to perform the desired cell function within a stabilized internal cytoplasm-like environment containing a number of well-defined components or pathways. A vivid example is provided by the protocells constructed to study the energy conversion in cells (Xu *et al*., 2010). A similar approach can be extended to construct protocells for toxicological testing. The basic

protocells are composed by lipid bilayer membranes and membrane proteins. These provide a unique platform to study the interaction of membrane receptors with toxins and the dynamics of toxin uptake (Zepik *et al.*, 2008). More advanced protocells could be constructed, which contain genomes encoding genes of those pathways that will plausibly be affected by the respective toxic substance. In a further step, toxicity-pathway assays could be carried out in these types of protocells. Compared with toxicity tests on the primary isolated cells or cell lines, the protocell-based tests will be more robust to handle than the conventional cell culture and easier to cultivate, further reducing costs.

Using protocells to elucidate cellular responses to toxin-induced cellular damage suggests the possibility of new biomarkers based on molecular responses to functional perturbations and cellular damage. It would be possible to monitor specific molecular responses in these protocell models based on our improved understanding of the molecular basis of various chemicals. The opportunity for continuously monitoring protocells with built-in reporter systems would help better predict the effects on humans. Responses monitored directly in the cell (particularly protocells that have certain characteristics of human cells engineered into them) should provide more relevant biomarkers. This would eliminate much of the current uncertainty in extrapolating from laboratory models to humans.

Thus, SB might offer the opportunity for custom-made toxicological testing systems that would yield better biomarkers and a broader application of molecular approaches to monitor functional disturbances.

SB In Vivo Biotransformation of Toxins

Some toxin-related disorders are caused by abnormal concentrations of chemical substances circulating in the body, such as the level of uric acid in the blood stream. If the level exceeds 6.8 mg/dl, then uric acid becomes toxic to organs: it forms crystals that cause kidney stones and gout. Cells containing gene circuits that help control the uric acid concentration have been tested in mice (Kemmer *et al.*, 2010). A biological network of genes called uric acid-responsive expression network has been constructed, which contained an uric acid sensor and control elements on uric acid processing. If the sensory circuit detects a high uric acid concentration, the control elements are activated and a corresponding level of urate oxydase (the enzyme that controls the uric acid level) will be released to maintain the normal level. This case study highlights what SB can do to metabolize toxic substances. A similar approach can be applied to construct genetic circuits in cells for arsenic detoxification. Arsenic contamination is widespread, and this toxic metalloid is mutagenic to organs such as liver, kidney, skin, and lung. Several arsenic detoxification pathways have been newly identified. One involves the Gram-negative nitrogen-fixing legume symbiont *Sinorhizobium meliloti* (Yang *et al.*, 2005). The *S. meliloti ars* operon consists of three genes, *arsR* (transcriptional regulator), *aqpS* (aquaglyceroporin), and *arsC* (arsenate reductase). This operon encodes a unique detoxification pathway wherein reduction of As (V) by ArsC is coupled to downhill transport of As (III) through the AqpS channel. Another pathway was identified in *Crambe abyssinica* (an oilseed crop). This revealed a novel defense mechanism of plants and the regulation of genes and gene networks in response to arsenate toxicity. It illustrated that, under arsenate stress, changes took place in gene regulations of glutathione-*S*-transferases, antioxidants, sulfur metabolism, heat shock proteins, metal transporters, and enzymes in the ubiquitination pathway of protein degradation (Paulose *et al.*, 2010). This investigation provided novel insight into the effective plant defense against arsenate. If cells harboring these pathways could be generated by SB, coupled with arsenic detection pathways, then alternative strategies for arsenic detoxification *in vivo* and *in vitro* could be achieved.

Another point of interest is aptamers. Nucleic acid aptamers are usually DNA or RNA molecules that have been engineered from random pools based on their ability to bind selected molecules. Aptamers have been generated to bind specifically to nucleic acids, proteins, organic compounds, and cells of interest. They are commonly generated by repeated rounds of *in vitro* selection termed systematic evolution of ligands by exponential enrichment to bind to various targeted molecules (Tuerk and Gold, 1990). These tailor-made molecules generated by *in vitro* selection can bind targets with high affinity and specificity, generating many potential uses in biotechnological and clinical applications (Guo *et al.*, 2008). They offer molecular recognition properties similar to commonly used biomolecules, namely, antibodies, yet with the advantage that their design can be easily tailored, that they can produced by chemical synthesis, and that they have a long shelf life. This makes them highly interesting in the field of therapeutics and target identification. Several aptamers that target coagulation factors have been investigated (Rusconi *et al.*, 2000, 2002). They are duplex RNAs with specific affinity to coagulation factor IXa and the von Willebrand factor and can therefore prevent clot formation. They will, however, result in complications such as bleeding. Recent studies demonstrated that side effects of drugs could be reversed by administering a simple aptamer-based antidote. This greatly enhances the potential value of aptamers as therapeutic agents. Customized antidote oligonuleotides can reverse the activity by specifically binding to the particular aptamers but with limitations because of the additional cost (Dyke *et al.*, 2006). More universal antidotes to control aptamer activity have been identified (Oney *et al.*, 2009). These oligonucleotide-based molecules can sequester the aptamers in sequence-independent manners. This type of oligonucleotide-based molecules can be designed to function as universal antidotes for other molecules of interest, for instance, as antidotes to toxic substances. They are therefore better than the current antibody-based ones.

Synthetic Biosensors for Environmental Toxicology

Even the most advanced toxicity tests fail to provide timely information to support real-time environmental monitoring. Environmental protection agencies are increasingly calling for better strategies to detect hazardous agents promptly. Current testing strategies on environmental toxicity are based on tests involving laboratory animals treated with high doses of the toxin agents that are usually orders of magnitude higher than those in the field. Moreover, clinical observations in laboratory animals may not be relevant to human and other wild species. The fast pace of introduction of new substances urgently calls for achieving toxicological knowledge on a broad coverage of chemicals. Traditional toxicity tests performed on animal models may not meet these demands. More robust, inexpensive, and quick cell-based tests for assessing the toxic effects of environmental chemicals are necessary. Novel environmental biosensors can be developed by SB, providing cheap on-site monitoring. The SB-derived biosensors consist of a receptor component to capture a target molecule and a signal transduction component to convert the target-receptor binding event into measurable signals, such as fluorescence, chemoilluminescence, colorimetric, electrochemical, and magnetic responses to produce a signal proportional to detect molecules of interest (de las Heras *et al*., 2008, 2010; Rodriguez-Mozaz *et al*., 2004). This approach will provide a robust, cost-effective, and quantitative method to rapidly and selectively detect and monitor chemical and biological agents in the environment. In conjunction with advanced detection technologies, biosensors are becoming important tools for environmental monitoring: they can identify environmental toxic substances ranging from heavy metals, insecticides, genotoxins, and phenols to persistent organic pollutants. These biosensors have a wide range of applications besides environmental monitoring. Examples include medical diagnostics, food safety, and military applications (e.g., detection of landmines). Compared with the standard analytical methods that directly monitor environmental pollutants in the field, biosensors offer distinct advantages such as implementation in real time with minimal sample preparation.

Several types of biosensors have been investigated for environmental monitoring (Monk and Walt, 2004). Among them, the nucleic acid–based biosensors and the whole-cell biosensors are of particular interest for SB. The nucleic acid–based biosensors employ oligonucleotides as sensing elements, with a known sequence of bases or a complex structure of DNA or RNA. They have been used to detect environmental pollution and toxins. These DNA/RNA elements play the role as the receptors of specific biological and/or chemical agents, such as target proteins, metals, or organic compounds. The DNA biosensors are based on the affinity of the target molecules (particularly those of mutagenic and carcinogenic activity) to the immobilized DNA or on the *in situ* hybridization of the target DNA to the immobilized ones (Grubor *et al*., 2004; Oliveira *et al*., 2007). The RNA sensors are biological parts that detect signals (Win *et al*., 2009). One type of RNA device is based on the integration of a distinct information transmission function. A second type of device design links the sensor and actuator parts through a separate transmitter part. RNA sensors can detect diverse signals such as temperature and molecular ligands through various binding events, including hybridization and tertiary interactions. The binding event encoded in an RNA sensor is generally transduced to an actionable event such that RNA sensors are typically coupled to other RNA parts.

The whole-cell biosensors, particularly microbial ones, are analytical devices that couple whole cells (microorganisms) with a transducer (Harms *et al*., 2006). Earlier microbial biosensors were designed to detect a substrate or an inhibitor of the microbial pathways of respiratory and metabolic functions. More biosensors are developed based on fusing reporter elements (such as *lux*, *gfp*, or *lacZ*) to an inducible gene promoter to detect targets of interest. This type of biosensors can be applied to assess biological effects such as biological oxygen demand, DNA damage, toxicity, ecotoxicity, or estrogenicity (Kobayashi *et al*., 2004). Four types of synthetic biosensors have been proposed by Khalil and Collins (2010).

- The first type is a transcriptional biosensor that is built by linking environment-responsive promoters to engineered gene circuits for programed transcriptional changes. A transcriptional gate was designed to sense and report only the simultaneous presence of environmental signals (e.g., salicylate and arabinose).
- The second type is a translational biosensor that is typically built by linking RNA aptamer domains to RNA regulatory domains. The target molecule will be recognized and bound by the aptamer stem of the RNA biosensor. This leads to a conformational change in the molecule and thus inhibits the translation of an output reporter.
- The third type is a posttranslational biosensor that is protein based. Signal transduction pathways are complex; yet, these pathways are essentially hierarchical schemes based on sensitive elements and downstream transducer modules. Thus, the protein receptor of a pathway and the associated cascade can be used for signal detection.
- Finally, the fourth type is a hybrid type of sensor, the combination of a posttranslational circuit with a transcriptional one.

Biosensors show dual functions that can complement both laboratory-based and field analytical methods for environmental monitoring. A wide range of biosensors have been reported for potential environmental applications. Using an SB approach, a strain of *E. coli* was engineered to equip with the ability to hunt down the herbicide atrazine and metabolize it (Sinha *et al*., 2010). Atrazine is an environmental pollutant that can be harmful to wildlife. The key to the transformation is the combination of a synthetic switch that allows the bacteria to

chase the chemical and a gene taken from another species of bacteria for breaking down atrazine. RNA molecules have been used to develop an atrazine-binding molecule called a riboswitch, a piece of RNA that binds to a small molecule and changes shape in doing so. This then alters the gene expression. In the second step, the switch-carrying bacteria have been equipped with an atrazine-degrading gene isolated from a different bacterium species. The resulting bacteria demonstrate their seek-and-destroy function by forming rings in petri dishes covered with atrazine as they move toward the atrazine and clear it from the plate. Another example of an SB-derived biosensor is the bacterial arsenic biosensor developed as an iGEM project (The iGEM Competition, see: www.igem.org) (Team Edinburgh iGEM, 2006). The team proposed to develop a device that responds to a range of arsenic concentrations and produces a change in pH that can be calibrated in relation with the arsenic concentration. The rationale was to use a pH change as a signal for the presence of arsenic. Compared with previous arsenic detector designs, the signal was easily detectable using a pH meter. It was also relatively easy to make the bacterial biosensors available to detect arsenic in rural areas. Applying the engineered approaches, a B cell–based biosensor has been developed for the rapid identification of pathogens (Rider *et al*., 2003). These biosensors rely on a laboratory-developed technology called Cellular Analysis and Notification of Antigen Risks and Yields. These biosensors contain built-in circuits for the specific antibody expression pathway as a detector and the photon expression (light emission) as a reporter. A similar approach can be applied to design B cell–based biosensors for the rapid detection of toxic substances. One such effort involves developing an aptamer-based electrochemical biosensor for the Botulinum neurotoxin (BoNT) (Wei and Ho, 2009). Conformational change resulting from the interaction between the aptamer probe and the BoNT is reported by an enzymatic amplification (limit of detection 40 pg/ml). Similar aptamer-based biosensors can also be applied to detect other toxins such as ricin or endotoxins.

Some of the biosensors mentioned above can be taken to the field and be used as a handheld device for on-site testing. Some could in theory be released into the environment, e.g., as biosensors for landmines by detecting volatile components of the explosive (de las Heras *et al*., 2008). Although microorganisms have been released into the environment for benign purposes for many years now, there are few if any success stories to tell. In addition to the technical challenges regarding the evolutionary stability of the engineered constructs, general biosafety concerns are paramount (Cases and de Lorenzo, 2005; de Lorenzo, 2009). This also holds true for the deployment of microorganisms for bioremediation purposes (Schmidt *et al*., 2010). SB, however, may also hold an answer to this long-discussed challenge, particularly through the use of orthogonal xenobiological systems that cannot exchange genetic information with wild-type organisms (Herdewijn and Marliere, 2009; Marliere, 2009; Schmidt, 2010).

Cell-Free Protein Synthesis of Toxins

Improved cell-free protein synthesis is currently being tackled by SB, with the perspective to offer a unique opportunity to expand the capabilities of natural biological systems for useful purposes. Cell-free protein synthesis systems will reduce complexity, remove structural barriers, and do not require maintaining cell viability (Voloshin and Swartz, 2005). The molecules of interest (of natural or nonnatural origin) are synthesized in a chemical environment that can be controlled, actively monitored, and rapidly sampled. An *E. coli* cell-free platform known as the Cytomim system has been designed to activate natural metabolism (Calhoun and Swartz, 2007). In this synthetic system, the central catabolism, oxidative phosphorylation, and protein synthesis have been coactivated in a single reaction system. Previously, such complex systems were not known to be simultaneously activated without living cells (Jewett *et al*., 2008). This novel cell-free protein synthesis system can serve as a fertile platform for a broad range of potential cell-free SB projects (Jewett *et al*., 2008; Zuo *et al*., 2009). It may be used to produce protein therapeutics, toxins, and other biochemicals that are difficult to make *in vivo* because of their toxicity, complexity, or unusual cofactor requirements. In addition to cell-free production systems, SB approaches can also increase the production rate of toxic products in living cells. For example, the biofuel production of butanol, meant to replace ethanol as a better suited biofuel, is currently hampered by the toxicity of butanol to butanol-producing microorganisms at high concentration. Equipping the cells with an increased butanol toxicity tolerance would make the production economically feasible (Schmidt *et al*., 2010). A similar problem encountered in the production of lantibiotics is currently being tackled by a European Science Foundation (ESF)–funded research consortium (ESF, 2010).

Toxic Use Reduction through SB

One approach to pollution prevention is designed to reduce the use and generation of toxic substances in various production processes. Toxicant reduction emphasizes the more preventive aspects and highlights alternative production processes that need less input from toxic chemicals. SB could help identify and improve alternative production processes that require less or no toxic chemicals while still remain economically competitive. In Europe, e.g., attention to toxic use reduction is incorporated in the new regulatory framework (adopted 2007) for the Registration, Evaluation, and Authorization of Chemicals (EC, 2006).

SB could significantly influence the chemical market, particularly in the areas of fine chemicals, bioplastics, and chemical processes (World Economic Forum, 2010). The bulk chemicals industry will also considerably benefit from the SB-based technology. Adoption by industry, however, can be slow; although when new practices are finally adopted, changes

will have a significant impact over the long term. In the fine chemicals industry, the incentives for investment relate to the economic potential of the end product (in contrast to the situation in bulk chemical manufacturing). The payoffs could have environmental impacts, although these may be limited to a more efficient use of energy because the core manufacturing practice (still) relies on petrochemicals. SB-based techniques also have the potential to help avoid the use of recalcitrant molecules in the production process. Nonetheless, investment in SB-based processes in bulk chemicals will probably also have positive effect on the manufacturing systems used to operate plants, such as the use of biodiesel and less overall chemical waste. Because of the scale of these processes, small changes may have significant positive environmental effects (Schmidt *et al.*, 2010).

Do-It-Yourself Biology

One aim of SB is "to make biology easier to engineer." This explains why more and more people are entering the biotechnology field despite no traditional education in biology or genetics (some probably without any higher education). SB gave rise to a new kind of hacker culture, the so-called biohackers or do-it-yourself biologists. Biohackery means designing and manufacturing biological systems in an open way but with minimal regulatory oversight or enforcement. Although the number of biohackers with real biotech skills might be quite limited, a community of motivated young citizen scientists has formed (see www.diybio.org). The young crowd of enthusiastic biohackers may well follow the example of the "Homebrew Computer Club" from the mid-1970's, and a true biohacker community might spark a wave of innovation unseen in cooperative research programs (Schmidt, 2008). Helping a large number of people to construct biological systems and biochemical substances, however, also creates an inherent safety risk. This challenge has been recognized by the DIYbio community, and a variety of safety improvement programs are under consideration (Fig. 3). In addition, DIYbio could provide average citizens with a new set of tools to detect toxins themselves, as the following newspaper report testifies:

> Meredith Patterson is not your typical genetic scientist. Her laboratory is based in the dining room of her San Francisco apartment. She uses a plastic salad spinner as a centrifuge and Ziploc plastic bags as airtight containers for her samples. But the genetically modified organism (GMO) she is attempting to create on a budget of less than $500 could provide a breakthrough in food safety. The 31-year-old ex-computer programmer and now biohacker is working on modifying jellyfish genes and adding them to yoghurt to detect the toxic chemical

FIG. 3. Private biotech laboratory in a Californian garage. The people who built this laboratory are semi-wealthy Silicon valley engineers who got interested in biotechnology. The project that they are pursuing is technically well beyond anything discussed by the www.DIYBio.org community. The exact location of this garage has not been revealed because of fear of police raids, as garage biologists may still be perceived as potential bioterrorists by the U.S. government. (Photograph by Rob Carlson, www.synthesis.cc.)

melamine, which was found in baby milk in China last year after causing a number of deaths, and kidney damage to thousands of infants. Her idea is to engineer yoghurt so that in the presence of the toxin it turns fluorescent green, warning the producer that the food is contaminated. If her experiment is successful, she will release the design into the public domain. (Bloom, 2009) see also Ledford, (2010), Whalen, (2009), and Wohlsen, (2008).

If this approach is successful, then we will soon find a number of "how-to" descriptions regarding biosensors and other useful biotech tools. This will automatically lead to a democratization of biotechnology, which will also affect toxicological analysis. The future may hold a dispersed crowd of people conducting toxicological tests, e.g., testing their food or samples from the environment. Such a crowd sourcing approach could increase the availability and decrease the costs of toxicological analysis, improving the utility of toxicological testing as such (Ekins and Williams, 2010; Howe, 2006; Oprea *et al*., 2009).

SB as a Challenge for Toxicology

Most new developments in SB will probably make life easier for toxicologists, as discussed above. Some aspects, however, could cause headaches and a series of new challenges to toxicologists. We will walk through them step by step.

Synthetic Toxins and Novel Xenobiotics

In SB, researchers have started to modify and exchange some of the elementary biochemical building blocks of life. The focus of their efforts has been to come up with alternative biomolecules to sustain living processes. This research focuses on chemically modifying amino acids, proteins, or nucleic acids. One area of research is the identification of proteins with amino acid sequences that produce stable tertiary structures but that are not known to occur in nature. A thought experiment helps to understand the implications of such nonnatural proteins for toxicology. Proteins are long molecular chains made up of the 20 amino acids, with the shortest known protein, "TC5b," having only 20 amino acids (Neidigh *et al*., 2002) or even as few as 10 (Honda *et al*., 2004); the longest one, titin, counts 34,350 amino acids. Estimating a conservative average amino acid number of 500 results in an unimaginable 20^{500} theoretically possible proteins, i.e., approximately 10^{650} (Luisi, 2007). This number is not only clearly considerably higher than the number of known proteins, up to 6.5 million according to Price *et al*. (2008), but is also higher than the estimated number of atoms in the observable universe (10^{80}). Moreover, an additional enlargement of the protein hyperspace is achieved by incorporating so-called noncanonical amino acids in "code engineering" and using quadruplet codons based on four instead of three bases to code for an amino acid (Budisa, 2004; Cropp *et al*., 2007; Neumann *et al*., 2010; Wang *et al*., 2006, 2009; Xie and Schultz, 2005). Code engineering is an attempt to reengineer the genetic code, in other words to assign noncanonical amino acids to the 64 possible base triplets, allowing for the incorporation of more than the typical (canonical) 20 amino acids. For example, researchers have genetically incorporated more than 40 different types of nonnatural amino acids in *E. coli*; similar results have been achieved in yeast and mammalian cells (Wang *et al*., 2009). As the number of nonnatural amino acids, not used by organisms, goes into the hundreds, code engineering offers striking new opportunities for the development of unnatural proteins with novel functions. Theoretically, up to 63 (4^3 minus the stop codon) different amino acids could be used (at least one must be used as stop codon) through code engineering, although the full use of codons would lead to nonrobust error-prone codes. The potential number of amino acids increases to 255 (4^4 minus the stop codon) when using quadruplet (4) instead of triplet (3) codons. Accordingly, a hypothetical 255^{500} proteins with different amino acid sequences are possible with code engineering and quadruplet codons (Moroder and Budisa, 2010). Clearly, neither 20^{500} much less 255^{500} proteins could ever be realized in the lifetime of the universe (and many of them would be unstable when folding). Nonetheless, this space of proteins probably contains remedies for every disease as well as a whole new set of toxins with hitherto unknown properties (For example, the ESF-funded project SB to obtain novel antibiotics and optimized production systems (2010–2013) produces over 1000 novel lantibiotics, a very effective type of antibiotics, through a combinatorial SB approach. see http://www.esf.org/activities/eurocores/running-programmes/eurosynbio/collaborative-research-projects.html.). With our current knowledge, we can only speculate about the location of these remedies and toxins in the vast protein hyperspace; identifying some of them will be a profound computational and theoretical challenge for the future. There is little evidence to believe that synthetic and unnatural chemicals are more or less toxic than natural ones (Ames *et al*., 1990): the challenges lie mainly in the sheer number of tests that will have to be done. Toxicologists will need to assess the toxicity of a flood of novel proteins made up of canonical and noncanonical amino acids (and other combinatorial biochemicals). Given the potential number of new and unnatural biomolecules, toxicology faces a substantial challenge regarding the scale-up of current testing schemes.

Another aspect of newly constructed biochemical structures relates to artificial antibodies. Antibodies are a type of protein used by the immune system to recognize and neutralize non-self molecules. In most cases, these proteins play important roles in combating infections caused by pathogens. In recent years, antibody-based treatments for cancers were of great therapeutic interest because of their specific affinities to antigens presented on the abnormal cells. Because of the ability of antibodies to recognize a specific target molecule,

they are in high demand for applications in medicine and biology. Numerous investigations have been conducted to produce antibodies. To date, antibodies have been produced from vaccinated animals, in recombinant forms from fermentations, or in humanized forms achieved by the latest techniques. All these protein-based antibodies, however, are expensive to produce and have a limited life span. A novel, interdisciplinary approach was developed to make artificial antibodies from polymers. These antibodies may be just as effective as the native ones. Artificial antibodies made from polymers rather than amino acids promise to be cheap and long lasting (Hoshino *et al*., 2008). This approach, called molecular imprinting (Ansell *et al*., 1996), involves taking a target molecule and placing it in a solution containing the polymer building blocks. These building blocks will grow around that target, which will configure to target-shaped modes. Once solidified, the target molecules will be leached out, leaving only the moulded polymers. These can function with antibody-like affinity and are termed artificial antibodies. This direct approach to generate polymer-based artificial antibodies to molecules of interest has been shown in proof-of-principle by creating antibodies to melittin, a toxin which is an active component of bee venom (Hoshino *et al*., 2009, 2010). The efficacy of these synthetic artificial antibodies specific to melittin was tested *in vivo* on animal models. Mice that received artificial antibodies were partially protected against a lethal dose injection of melittin, demonstrating their ability to capture the toxin *in vivo*. Applications of artificial antibodies in medical science are wide, ranging from antidotes for toxins, novel agents against infections, to substitutes for current antibody-based cancer treatment. These polymers have some advantages over the native ones, e.g., lower susceptibility to proteolysis degradation. This might make them suitable for therapeutic use in parts of the body where normal antibodies break down quickly, such as the digestive tract. On the other hand, these polymers may be difficult to clear by the natural filtering systems of the blood and may trigger unwanted immune responses. More investigations are needed to address these concerns. No doubt, combined with the innovations from SB, better building blocks with improved biological properties will be designed to make imprinting molecules more compatible for clinical applications. Besides the applications mentioned above, the longer life span of the polymers versus proteins simplifies their use in portable devices in the field for environmental monitoring and chemical detections (Boopathi *et al*., 2006). Finally, because the artificial antibodies are generated from polymers by synthesis totally *in vitro*, they can be produced in large quantities in low cost to be used in affinity chromatography to purify molecules of interest, particularly those which are nonimmunogenic.

As mentioned in the previous section, aptamers exhibit significant advantages relative to protein therapeutics in terms of size, synthetic accessibility, and modification by medicinal chemistry (Bouchard *et al*., 2010; Keefe *et al*., 2010; Zhou and Rossi, 2010). Since the recent U.S. Food and Drug Adminstration approval of the first aptamer drug Macugen (an antivascular endothelial growth factor aptamer) to treat age-related macular degeneration, more research has been conducted on developing therapeutic aptamers for a wide range of diseases (Ng *et al*., 2006). Currently, a few therapeutic aptamers are in clinical development. These include ARC1779 targeting the von Willebrand factor (Diener *et al*., 2009), REG1 targeting Factor IXa (Chan *et al*., 2008), and AS1411 targeting nucloelin (Bates *et al*., 2009). Some aptamers are under investigation, among them a new class of drugs against human immunodeficiency virus (aptamers against gp120 and trans-activation responsive element) (Joubert *et al*., 2010; Watrin *et al*., 2009) and prostate cancer (RNA aptamers against PSMA, prostate-specific membrane antigen) (Dassie *et al*., 2009). When unmodified oligonucleotide aptamers are administrated systemically as potential therapeutics, they encounter several hurdles: metabolic instability, rapid renal filtration and elimination, and rapid biodistribution from the plasma compartment into the tissues. Thus, postselection modifications through chemical synthesis are needed to make aptamers suitable for therapeutic application (Bouchard *et al*., 2010). A key feature of modified therapeutic aptamers is that the molecules can be tailored to improve the pharmacokinetic profile. The optimizations aim to improve affinity and metabolic stability. One of these modifications is controlling the conjugation of the aptamers to various sizes of polyethylene glycol (PEG). The aptamers conjugated with PEG have increased the molecular mass and extended the half-life by slowing renal filtration and elimination. Toxicology studies reveal that modified therapeutic aptamers can be improved with better efficacy and safety profile than the unmodified one, which has the previously reported side effects on immune stimulation and complement activation (Keefe *et al*., 2010). In some applications, however, a prolonged half-life of the drugs may cause unintended side effects. As mentioned in an earlier section, aptamers targeting coagulation factors remain free in the circulation system for a prolonged period if no endogenous counterparts are present. Therefore, antidotes are needed to control the aptamer activity. This may hold true for other therapeutic aptamers as well: antidotes are needed to limit the possible unintended effects of unnatural molecules. Although they are similar to antisense oligonucleotides (ASOs), which have been investigated extensively, aptamers differ from ASOs in structure, chemical composition, and mechanisms of action. Only limited information is available on the toxicological properties of aptamers. It will be a new challenge to design appropriate assays to address their toxicological effects. Bouchard suggested considering certain potential toxicological effects of aptamers (Bouchard *et al*., 2010). Three involve common toxicities of oligonucleotides as drugs. The first is the polyanion effect resulting from nonspecific off-target protein interactions that may affect normal cell functions when administering the oligo at high dose. The second is the effect

on the innate immune system because of the sequence of the oligos. The third is the accumulation of oligos in certain tissues and cells. Moreover, considerations should be given to the possible side effects specific to aptamers because of their chemical compositions, preferable administration route, and cellular destination in the plasma.

Yet another aspect of potentially problematic, newly constructed biochemical structures relates to artificial chromosomes. The applications of gene therapy to correct an inherited or acquired gene defect or to introduce a new function have stimulated research on novel DNA delivery system (Verdier and Descotes, 1999). Earlier studies have raised safety concerns on viral vector–based delivery systems because of their toxicological profiles. The commonly used viral gene deliver systems are based on adenoviruses, adenovirus-associated viruses, herpes simplex viruses, and lentiviruses (Raty *et al*., 2008). If administrated systemically, some will induce toxic shock responses and cytotoxic effects. Some will integrate into the chromosomes and may trigger unknown regulatory effects. There were several reports of severe clinical complications because of toxic side effects after receiving viral vectors (Couzin and Kaiser, 2005; Fox, 2003a,b; Kohn *et al*., 2003; Marwick, 2003; van der Eb *et al*., 1998). Partly in response to this problem, several nonviral DNA delivery systems are currently in development. One of these systems is based on artificial chromosomes. There are three major types of artificial chromosomes: bacteria artificial chromosomes, yeast artificial chromosomes, and mammalian artificial chromosomes (MAC). Generally, artificial chromosomes are ideal for gene delivery because they have the potential to carry very large fragments of foreign DNA containing all the natural genetic elements in correct spatial orientation. Human artificial chromosomes (HAC) are a subgroup of MAC and are synthetic molecules (see ''DNA Synthesis'' section on DNA synthesis). They can perform a desired function in human cells, potentially enabling long-term gene expression in human cells (Monaco and Moralli, 2006). There are two traditional approaches to generating HAC. One approach generates HAC in the mini-chromosome forms developed by truncation of normal human chromosomes; this is mediated by inserting human telomeres into the chromosomal arms. The other approach is ''bottom up''. It assembles the known functional elements of human chromosomes, including human telomeres, alpha satellite DNA, and genomic fragment containing replication origins. Now, owing to the rapid development of DNA synthesis techniques accompanying the development of SB, larger HACs can be synthesized *de novo*. Together with more knowledge on genetic regulation obtained from studies on synthetic systems, HAC applications will extend to the field of medicine. To date, HACs have been used successfully in cell culture models for gene transfer and expression (Katoh *et al*., 2010; Kawahara *et al*., 2007). HACs may also serve as a better delivery system for treatments based on genetic approaches. One example is HAC-induced phur-ipotent stem cells (Ren *et al*., 2006). Further clinical applications of HACs in gene therapy, however, need to be investigated. Comparing HACs to the viral vectors for gene therapy, they show low toxicity, low immunogenicity, and lack of infectious or mutagenic potentials. However, when administered in large quantity and over the long term to reach therapeutic concentrations, the physiological consequences of the artificial DNA remain unclear. It will be a new challenge to design proper toxicological tests to evaluate the safety of these artificial chromosomes.

Biosecurity Considerations

According to the World Health Organization (WHO, 2004), biosafety is the prevention of ''unintentional'' exposure to pathogens and toxins or their accidental release, whereas biosecurity is the prevention of loss, theft, misuse, diversion, or ''intentional'' release of pathogens and toxins. The consolidation of the research field SB came soon after the 11 September 2001 event and the United States anthrax letters, setting the stage for a thorough examination of this new technology from the biosecurity point of view. Increasing concerns in the United States that research in the life sciences might be misused for bioterrorist or biowarfare purposes were fueled by a number of experiments that triggered substantial debate. In particular, three experiments gave rise to such debates (Kelle, 2007):

- Unintentionally enhancing the virulence of the mousepox virus by inserting an interleukin-4 gene into the mousepox genome (Jackson *et al*., 2001). Although this experiment unexpectedly created a killer mousepox virus, subsequent work by another scientist has intentionally carried these experiments one step further by increasing the lethality of the mousepox virus and by conducting similar manipulations in the cowpox virus (Buller, 2003; MacKenzie, 2003; Steinbruner and Harris, 2003).
- Synthesis of the poliovirus and 1918 Spanish flu genome from chemically synthesized oligonucleotides that were linked together and then transfected into cells. This created an infectious virus from scratch, combining knowledge of the viral DNA with assembly of the correct chemical compounds (Cello *et al*., 2002; Tumpey *et al*., 2005).
- Transfer of the virulence factor of variola major (which causes smallpox) into the vaccinia virus, which is of much lower virulence and usually used for vaccinations against smallpox (Rosengard *et al*., 2002).

These experiments drew the attention of the security community to synthetic genomics and SB. Since then, U.S. security institutions, think tanks, and others have examined SB in detail. These include the National Security Advisory Board on Biotechnology (NSABB), the Strategic Assessment Group of the National Academy of Sciences (CIA, 2003), the FBI (arresting innocent biotech artist Steve Kurtz for having biotech equipment in his house, preemptive investigation of, and attempts to cooperate with, the SB Do-It-Yourself DIYbio

community), the Commission on the Prevention of Weapons of Mass Destruction, Proliferation, and Terrorism (Graham *et al.*, 2008), and the National Academies.

In 2004, one of the lead scientists in SB put forward "A Synthetic Biohazard Non-proliferation Proposal" (Church, 2004) to address some of the biosecurity concerns of SB. Since then, the debate on the biosecurity implications of SB has made some progress, again most notably in the United States (Kelle, 2007), including the following reports:

- Fink Committee: the work of the Committee on Research Standards and Practices to Prevent the Destructive Application of Biotechnology, of the U.S. National Academies of Sciences, chaired by Gerald R. Fink on seven categories of problematic experiments (Fink Committee, 2004).
- Lemon-Relman Committee: Globalization, biosecurity, and the future of the life sciences, report by the Committee on Advances in Technology and the Prevention of Their Application to Next Generation Biowarfare Threats, Washington, DC (Lemon-Relman Committee, 2006).
- Declaration of the Second International Meeting on SB (Synthetic Biology 2.0, 2006).
- J Craig Venter Institute, Massachusetts Institute of Technology, Center for Strategic and International Studies report: synthetic genomics: options for governance. (Garfinkel *et al.*, 2007).
- NSABB SB working group produced several reports, among them NSABB: addressing biosecurity concerns related to SB (NSABB, 2010).
- Controlling dangerous pathogens project: controlling dangerous pathogens. A prototype protective oversight system (Steinbruner *et al.*, 2007).

In recent years, most of the biosecurity debate, however, gravitated around the risks stemming from DNA synthesis. Several papers and draft guidelines have been produced, both from industry (United States and Europe) and the U.S. National Institute of Health. They stress two main points of assessment: the customer and the ordered DNA sequence (Bernauer *et al.*, 2008; Bugl *et al.*, 2007; IASB, 2009; IGSC, 2009; USDHHS, 2009).

Although most concerns gravitate around novel infectious biological material, the concerns also include toxins. Of particular interest to the toxicology community are two of the seven experiments of concern outlined by the Fink Committee (highlighted in bold). According to Fink Committee (2004), a scientific experiment raises concern if it

- demonstrates how to render an animal or human vaccine ineffective;
- confers resistance to antibiotics or antiviral agents that are used therapeutically to control diseases in humans, animals, or crops;
- enhances the virulence of a plant, animal, or human pathogen or renders a nonpathogen virulent;
- increases transmissibility of a pathogen within or between species;
- alters the host range of a plant, animal, or human pathogen, including making nonzoonotics into zoonotic agents;
- enables **the evasion of diagnostic or detection modalities, e.g., by microencapsulation**
- enables the **weaponization of a biological agent or toxin**, including environmental stabilization of pathogens.

Using SB to evade diagnostic and detection modalities "of toxins" is highly relevant for toxicologists as it impedes their ability to detect and measure the concentration of a given toxin in a sample. Evasion of detection, e.g., is a by-product of current (non-SB) insecticide paints. The formulation of these paints includes microcapsules that allow the gradual release of the active ingredients. The paint is a suspension of microcapsules that range from one to several hundred micrometers in size. Successful results of this paint formulation have been reported for the control of mosquitoes and cockroaches (Amelotti *et al.*, 2009). SB could well create novel forms of microcapsules using, e.g., protocells (Bedau and Parke, 2009) or so-called virosomes (Pevion, 2010; Stegmann *et al.*, 2010); these would release biochemical substances (toxins) either automatically over a long period of time or when triggered by an external signal.

Biosecurity or dual-use–related work is certainly of interest to toxicology because it may considerably impede well-tested measurement and assessment tools. Awareness of the dual-use characteristics of biotechnological research among the life science community (and probably in the toxicological science community as well), however, is rather limited (Kelle, 2007).

Bridging Toxic Substances and Infectious Agents?

As mentioned above, an alternative to creating novel organisms through the "top-down" approach to SB involves creating them from the bottom up; this involves assembling them from nonliving components. The products of this approach are called "protocells" (Bedau *et al.*, 2009).

Creating or constructing living entities from nonliving substances (simple chemicals) somehow contradicts the law of biogenesis: *omne vivum ex ovo*, Latin for "every living thing comes from an egg," but this is exactly what scientists in the protocell community intend to do: bridging nonliving and living matter (Rasmussen, 2009; Rasmussen *et al.*, 2003). Living cells are seen as complex wet machines that can, at least in principle, be (re)constructed from scratch. There is of course evidence that life did develop from nonliving chemicals at least once in early earth history about 4 billion years ago (Szostak, 2009; Szostak *et al.*, 2001). Protocells are life-like entities that have some but not all properties of life (such as compartimentalization, growth, metabolism, self-replication, reproduction, interaction with the environment, evolutionary changes, and

autopoesis). The idea is that the protocells will, step by step, increasingly gain the characteristics of life, ultimately mimicking natural life forms. In this case, the resulting entity would be called a "synthetic cell." Accordingly, protocells are precursors to the hypothetical synthetic cells. Interestingly, although we have a good notion about what constitutes a life form and what not, protocells challenge the way we draw an exact border between the two realms (Deplazes and Huppenbauer, 2009). In a way, the distinction between the inanimate and animate world, especially the ability to self-replicate, is also reflected in the definitions of toxic substances and infectious agents. Infectious agents can potentially be transmitted from one person or species to another by a replicating agent as opposed to toxic substances that cannot replicate. Note, however, that there are special forms of replicating agents such as viruses, which are not able to replicate without their hosts. In some cases even relatively simple biomolecules such as prions can be considered to be infectious agents because they have the ability to self-replicate.

Protocells could theoretically be constructed in such a way that they have most, but not all, of the characteristics of life. Some, e.g., see future applications of protocells as reef builders under the city of Venice or as surfaces on houses that capture carbon (Armstrong, 2009). Among the many possible applications for protocells are some that involve (unintended) infections or the production of toxins (Bedau *et al*., 2009). Protocells could function as a type of smart chemical complex: they release chemical substances only under specific environmental conditions or only after a set of stimuli such as only if exposed to a magnetic field and to UV light. This makes it extremely difficult to predict the toxic effect of protocells because the toxins are encapsulated (or not produced) as long as the specific environmental condition does not occur. In many ways, protocells challenge the distinction between living and nonliving entities and therefore also blur the distinction between chemicals (toxic substances) and living entities.

Do-It-Yourself Toxin Production

As described in "Do-It-Yourself Biology" section, "Do-it-yourself biology," tools of SB will make it easier for nonprofessionals to use biotechnology. Although most so-called biohackers are inherently benign and curious persons, certain biosafety risks have to be acknowledged. SB, especially the use of standard biological parts and biofabs (BIOFAB, 2010), might enable users to produce toxic substances, either intentionally (e.g., as insect repellent) or unintentionally (as an unintended outcome of a biotech experiment in their kitchen).

Although little evidence is available that this has happened or will happen, such concerns are not totally absurd, as synthetic biologist Drew Endy mentioned (FORA.tv, 2008):

> I have had one example of a project which did concern me was . . . not part of iGEM but in a laboratory class and she (a student) decided that it is a shame if you will if not worse that people who had wrinkles had to go for repeated injections at the plastic surgeons office of a bacterial toxin, botox, and wouldn't it be better if you'd got a skin cream, that was a living skin cream that would make small amounts of that toxin on your face. So she set off to design that, and we thought that that was a bad idea, and had a conversation about that. (Ultimately, the student understood that it was not a good idea to try to produce that cream, because of security concerns.)

Other problematic production systems could involve illegal drugs or chemical weapons that, in principle, could be generated by future biohackers (Schmidt, 2008). The security community, at least in the United States, is aware of these facts and has started outreach activities with the DIYbio community (Ledford, 2010; You, 2010).

CONCLUSION AND OUTLOOK

SB offers the ability to study cellular regulation and pathways by (re)designing bioparts, devices, and systems. Currently, most of the research in SB is focused on developing basic tools and applications to produce biofuels or other chemicals of industrial interest via a standardized modular version of metabolic engineering. Engineered microbes are being programed to transform biomass into chemicals of interest. The same techniques can be used as templates to build more complex biological systems. Thus, although SB is still in its infancy, we can expect that its applications will be extended to the field of medicine, agriculture, and environmental biotechnology. In our opinion, toxicological sciences will benefit from the developments in SB in many ways, as illustrated above. We can expect novel toxicity testing platforms and toxicity assays to be developed. Biosensors derived from SB are already able to detect toxic substance in the environment promptly and on site—a tremendous improvement on current environmental monitoring practices. In addition, some biosensors could be deployed as bioremediation reactors with built-in biocircuits to process the toxins. Similar strategies may also find use in medical applications. The potential applications of SB in industrial chemistry will almost certainly lead to a more sustainable production system that uses renewable resources and avoids problematic intermediate toxic and recalcitrant compounds. SB will also encourage citizens to become citizen scientists, enabling them to plug and play with do-it-yourself biotech toolboxes. These toolboxes will enable them to brew their home-based laboratory, e.g., to carry out analytical tests they are interested in or produce the desired substances. Many scientists in the field expect that SB will also yield other innovations that we cannot imagine today. However, SB will

also bring new challenges, new risks, and new uncertainties. Although most of the work in SB is reminiscent of traditional biotechnology, some will generate real novelty. This refers to biological systems that are very different from natural ones, such as proteins containing unnatural amino acids, xenobiological systems in general, artificial antibodies, or artificial human chromosomes. Although these molecules are developed using natural templates as inspiration, safety and toxicity issues will need to be considered. For example, what properties of these molecules will determine their *in vivo* compatibility, metabolism, cytotoxicity, and degradation? SB also carries a dual-use risk, namely, the potential of misuse for bioweapons and bioterrorism, something that toxicological sciences have to consider as well.

In summary, SB will extend the toolbox of engineers and scientists, including toxicological scientists. In that respect, toxicologists may embrace the next wave of biotech innovations. In terms of new challenges, SB will generate hitherto unknown and unnatural molecules that need to be tested for their toxicity to natural and future synthetic biological systems. SB, however, creates even more headaches for those scientists who have to deal with the safety of self-replicating synthetic organisms: toxicologists merely have the difficult job to assess the toxicity of a whole new set of (not self-replicating) synthetic biomolecules. Accordingly, toxicologists will be mostly spared from the grand challenge of SB, namely, to assess the fate of synthetic organisms after their release into the environment. Still, SB will not mean business as usual for toxicologists—they will have to adapt to its many ramifications. But, as the Chinese proverb says: ''Be not afraid of growing slowly, be afraid only of standing still.''

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