Nanopore-Based Instruments as Biosensors for Future Planetary Missions

Article *in* Astrobiology · March 2014

DOI: 10.1089/ast.2013.1120 · Source: PubMed

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ASTROBIOLOGY
Volume 14, Number 4, 2014

DOI: 10.1089/ast.2013.1120

Hypothesis Article

Nanopore-Based Instruments as Biosensors for Future Planetary Missions

Fabio Rezzonico

Abstract

Data from automated orbiters and landers have dashed humankind's hopes of finding complex life-forms elsewhere in the Solar System. The focus of exobiological research was thus forced to shift from the detection of life through simple visual imaging to complex biochemical experiments aimed at the detection of microbial activity. Searching for biosignatures over interplanetary distances is a formidable task and poses the dilemma of what are the proper experiments that can be performed on-site to maximize the chances of success if extraterrestrial life is present but not evident. Despite their astonishing morphological diversity, all known organisms on Earth share the same basic molecular architecture; thus the vast majority of our detection and identification techniques are b(i)ased on Terran biochemistry. There is, however, a distinct possibility that life may have emerged elsewhere by using other molecular building blocks, a fact that is likely to make the outcome of most of the current molecular biological and biochemical life-detection protocols difficult to interpret if not completely ineffective. Nanopore-based sensing devices allow the analysis of single molecules, including the sequence of informational biopolymers such as DNA or RNA, by measuring current changes across an electrically resistant membrane when the analyte flows through an embedded transmembrane protein or a solid-state nanopore. Under certain basic assumptions about their physical properties, this technology has the potential to discriminate and possibly analyze biopolymers, in particular genetic information carriers, without prior detailed knowledge of their fundamental chemistry and is sufficiently portable to be used for automated analysis in planetary exploration, all of which makes it the ideal candidate for the search for life signatures in remote watery environments such as Mars, Europa, or Enceladus. Key Words: Astrobiology—Biopolymers—Biosignatures—Nucleic acids—Life detection. Astrobiology 14, xxx–xxx.

1. Life-Detection Approaches

Life, a chemical system capable of Darwinian evolution, may in principle exist in a wide range of environments, including non-aqueous solvent systems, or be based on scaffolding elements alternative to carbon, the most cited example being silicon (Benner *et al.*, 2004). This paradigm shift poses a formidable challenge for the detection of extraterrestrial biological systems because we may not be able to recognize signatures of life elsewhere in the Solar System if, as appears to be the case, they are not self-evident. Even slight deviations or variations from our familiar biochemistry may be sufficient to impair our detection protocols and make tools that are routinely used to explore our microbial world totally inadequate.

Direct cultivation of microorganisms requires that the right combination of substrates, environmental parameters (temperature, pH, salinity, light, etc.), and possibly even microbial interactions is provided in order to allow growth. It was initially estimated that only 0.1–1.0% of the living bacteria present in soils can be cultured under standard laboratory conditions, a number that is even lower for aquatic environments (Staley and Konopka, 1985; National Research Council, 2007). Although these figures have improved with the introduction of novel cultivation methods (Connon and Giovannoni, 2002), the *great plate-count anomaly* still stands, and it is conceivable that culture media provided to test the growth of extraterrestrial microorganisms on-site may fail to provide the right substrates for their metabolism and growth, and lead to false-negative results.

Current physicochemical approaches to find biosignatures include the search for complex organic molecules, the analysis of chemical chirality, the quest for nonstandard isotope ratios, or the detection of metabolic activities. The only experiments carried out so far to look specifically for biological activity on another planet were performed by the Viking landers on Mars, and their results are still regarded as inconclusive after decades (Plaxco and Gross, 2006). Most of these approaches

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have been reproached to suffer from a lack of specificity owing to possible abiological routes leading to the same effects and insufficient sensitivity to find life in lean samples (Carr *et al.*, 2010).

To overcome these limitations, highly sensitive life-detection strategies centered on polymerase chain reaction (PCR) amplification of DNA have been proposed (Isenbarger *et al.*, 2008). These methodologies target extremely conserved genes and are based on the assumption that extraterrestrial life possibly shares a common ancestry with life on Earth due to dispersal of microbes between planets (panspermia) or delivery of analogous building blocks to habitable environments (pseudo-panspermia), which has biased the evolution of life toward the utilization of the same nucleic acids. If the dissemination event occurred before the evolution of present-day conserved genes, non-specific methods such as whole-genome amplification, which use random primers, would be required and eventually supplemented by a reverse-transcription step if such transfer dates back to the RNA-world era (Carr *et al.*, 2010). A whole-genome approach under development by a joint team of MIT and Harvard researchers (http://web.mit.edu/setg) is based on the Ion Torrent semiconductor technology, a sequencing technique that senses hydrogen ions released by nucleotide incorporation during DNA synthesis and enables massively parallel sequencing in a small optics-free CMOS chip format (Carr *et al.*, 2013). Doctor Craig Venter, founder of Synthetic Genomics and known for the creation of the first cell with a synthetic chromosome, went even further by postulating the Digital Biological Converter, a machine with the capacity to sequence a genome on Mars by using the SOLiD (Sequencing by Oligonucleotide Ligation and Detection) technology and beaming back DNA data for its artificial synthesis on Earth (Venter, 2012).

While meteoritic transfers are in principle possible between Earth and Mars, as demonstrated from a record of 132 martian meteorites (http://www.lpi.usra.edu/meteor), their probability strongly diminishes with the increasing distance among celestial bodies, as in the case between our planet and gas giant moons like Europa or Enceladus (Worth *et al.*, 2013). In any case, the major pitfall of the above sequencing-by-synthesis approaches is that they can become quickly useless if the target genetic information carrier does not share exactly the same fundamental components as nucleic acids on Earth; the four canonical nucleotides included in amplification mixtures that serve as substrates for DNA synthesis will provide inadequate building blocks for alien genetic information carriers if the latter are based on different biochemistries.

2. Possible Alternate Chemistries for Life and Nucleic Acids

Life on Earth shares a number of fundamental characteristics at the molecular level. A three-biopolymer system composed by polynucleotides (RNA and DNA), polypeptides (proteins), and polysaccharides (sugars) is at the basis of living cells and biological processes. With the exception of some groups of RNA viruses, the genetic information is universally stored in the DNA, while RNA carries out multiple roles in the expression and regulation of genes, in the translation of their genetic information into proteins, and, acting as ribozymes, as catalysts of specific biochemical reactions. Proteins act as structural elements of the cell and perform a number of functions within living organisms, including catalyzing metabolic reactions, transporting molecules, binding ligands, and transmitting signals to respond to stimuli. Polysaccharides are often quite heterogeneous, range in structure from linear to highly branched, and serve a double function of structural cell components and long-term energy storage molecules.

All these biopolymers are remarkably well suited for their particular functions, but it is clear that, if not their general architecture, at least some of their molecular building blocks could have emerged differently from the prebiotic soup. The number of amino acids that are encoded by the standard genetic code is 20, while two others (selenocysteine and pyrrolysine) are incorporated into proteins by unique biosynthetic mechanisms. Additional amino acids may result from posttranslational modification of proteins; thus the number of possible proteinogenic side chains is at least theoretically unlimited. Experiments with unusual amino acids have shown that it is possible to expand the natural repertoire by using a regular ribosome to incorporate novel amino acids into proteins (Hohsaka and Masahiko, 2002; Chin *et al.*, 2003). Similarly, alternative nucleic acids can conceivably be based on chemical backbones that differ from conventional sugars deoxyribose and ribose or with the phosphate internucleoside linkage replaced by other chemical units such as guanidinium or *N*-(2-aminoethyl)-glycine (Table 1). These so-called xeno-nucleic acids (XNA) were synthetically created under laboratory conditions and have shown their ability to form base pairs and double helix

Table 1. Overview of Some Noncanonical Nucleic Acids Synthesized So Far and Their Properties (Adapted from Schmidt, 2010)

Name	*Nucleotide*	*Backbone*	*Charge*	*Base pairs*	*Double helix*	*References*
HNA	hNTP	Hexose	Negative	A-T, G-C	Self, DNA, RNA	Hendrix *et al.*, 1997
TNA	tNTP	Threose	Negative	A-T, G-C	Self, DNA, RNA	Schoning *et al.*, 2000
GNA	gNTP	Glycol	Negative	A-T, G-C	Self, RNA	Zhang *et al.*, 2005
CeNA	ceNTP	Cyclohexene	Negative	A-T, G-C	RNA, DNA[a]	Wang *et al.*, 2000
LNA	lNTP	2′-4′ locked ribose	Negative	A-T, G-C	Self, DNA, RNA	Koshkin *et al.*, 1998
DNG	N-DNG	Guanidinium	Positive	A-**T**, **C**-G	DNA, DNA[a]	Dempcy *et al.*, 1994; Szabo and Bruice, 2004
PNA	N-AEG	*N*-(2-aminoethyl)-glycine	None	A-T, 7DG-C[b]	Self, DNA, RNA	Nielsen and Egholm, 1999

[a]Triple helix.
[b]7-Deazaguanine.

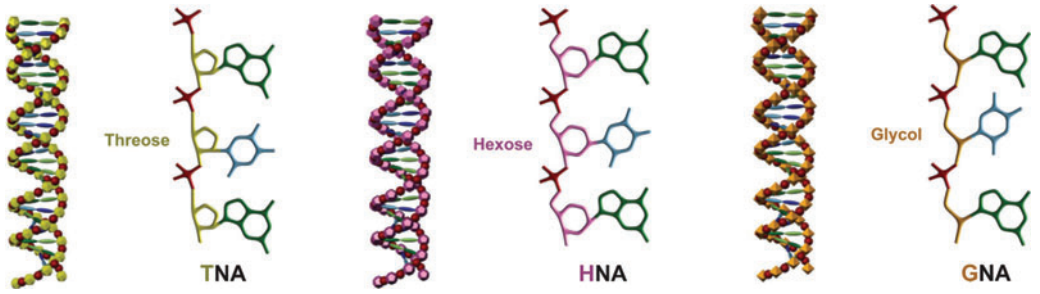

FIG. 1. XNA backbone motifs bearing canonical bases capable of forming regular homoduplexes. Reprinted with permission from Schmidt (2010), copyright 2010 Wiley Periodicals, Inc. Color images available online at www.liebertpub.com/ast

structures similarly to DNA, making them suitable for the storage of genetic information (Schmidt, 2010) (Fig. 1).

Further explored modifications of the DNA structure include enlarged or alternative genetic alphabets such as the Artificially Expanded Genetic Information System (AEGIS), where the four canonical nucleobases adenine-thymine and guanine-cytosine (forming the base pairs A-T and G-C, respectively) are replaced by different purine and pyrimidine residues that mediate as hydrogen bond donors and acceptors (Benner, 2004; Yang *et al.*, 2011) (Fig. 2), or even systems including non-natural hydrophobic nucleobases (Leconte *et al.*, 2008).

Polymerases have proven to accept nonstandard components of DNA only inefficiently (Horlacher *et al.*, 1995), and tools such as rapid sequencing methods are unavailable to most synthetic genetic systems (Yang *et al.*, 2011). Reversed-chirality sugars within nucleotides or different residues constituting the DNA backbone are not recognized by natural DNA polymerases at all, yet even the presence of single unconventional bases in putative extraterrestrial nucleic acids may be sufficient to stop the polymerization reaction due to the lack of matching substrates among the reaction components (Fig. 3), making both conventional PCR or any sequencing-by-synthesis approach futile.

3. Hypothetical Universal Blueprint for Genetic Information Carriers in Water

Despite the multiplicity of possible modifications that are in principle possible (*i.e.*, different backbone, alternative

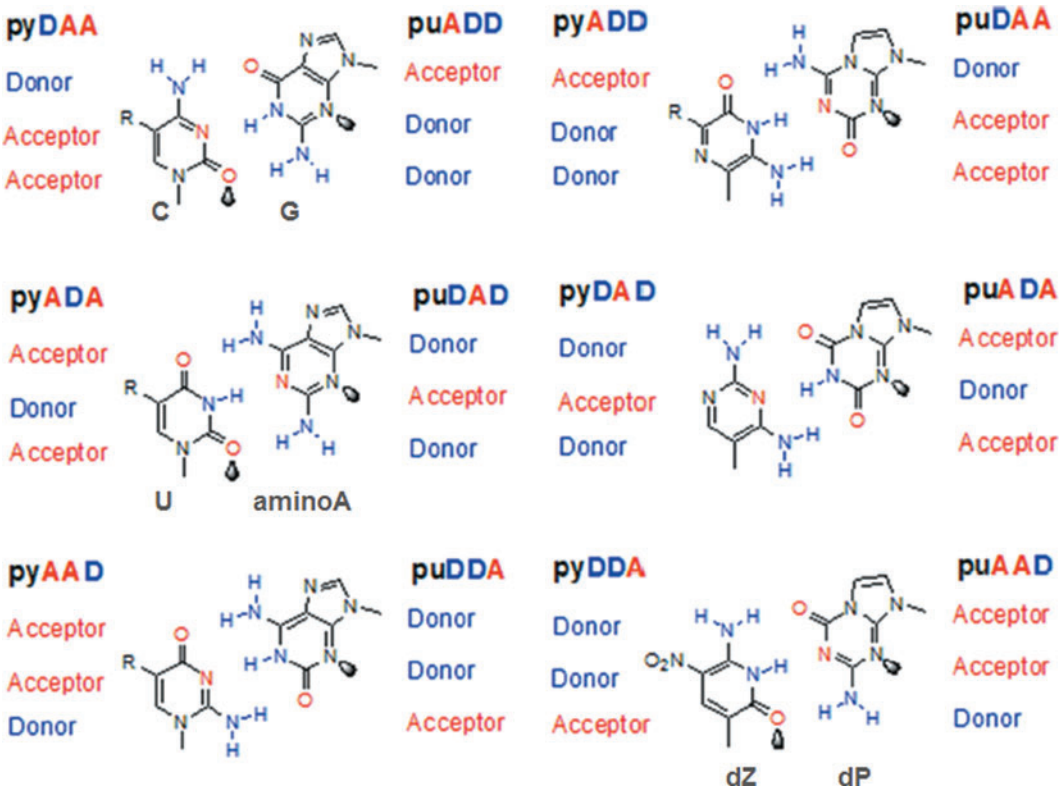

FIG. 2. The AEGIS that follows closely the geometry and hydrogen bonding architecture of natural nucleotides. The various hydrogen bonding patterns are named pu (purine-like) or py (pyrimidine-like), depending on the heterocyclic ring system, with hydrogen bond donor (D) and acceptor (A) groups listed starting in the major groove and ending in the minor groove. Reprinted with permission from Benner (2004), copyright 2004 American Chemical Society. Color images available online at www.liebertpub.com/ast

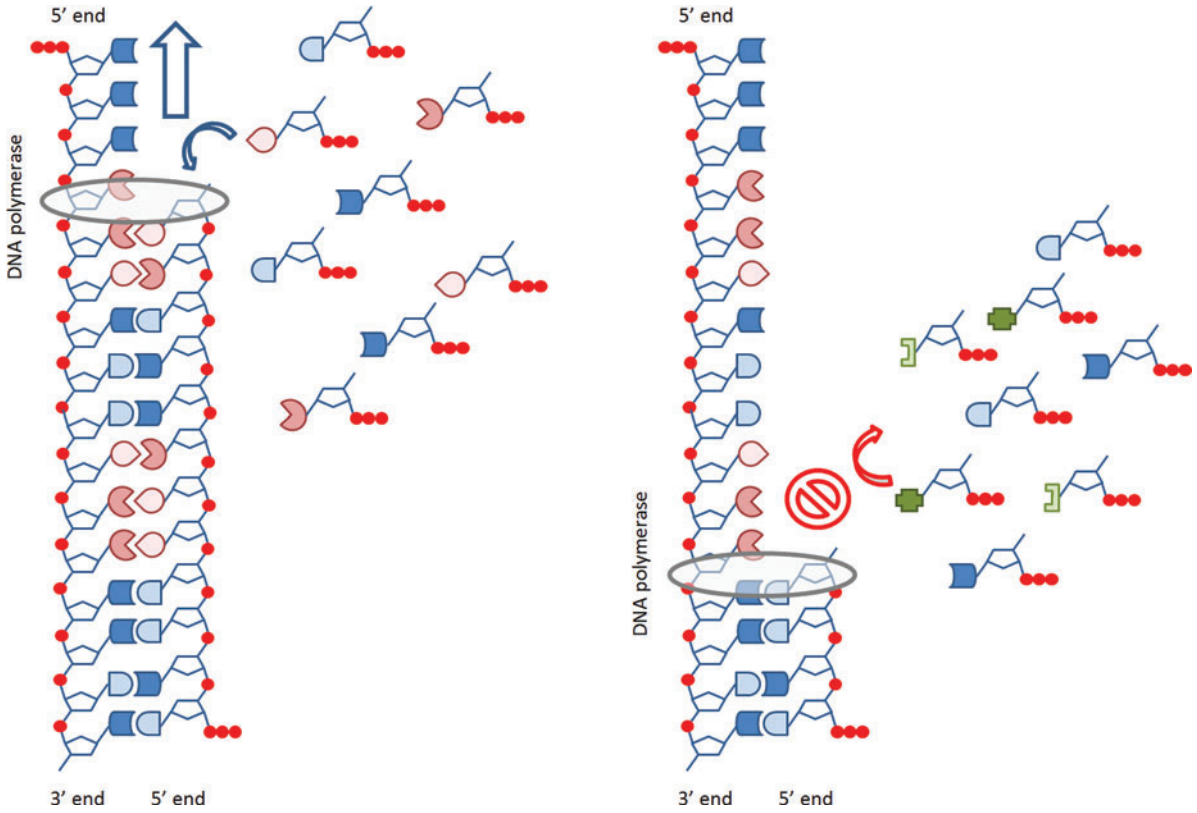

FIG. 3. Nucleotide incorporation through the DNA polymerase during a PCR reaction. The synthesis of a new DNA strand can only proceed if the enzyme can resort to the matching nucleotides as reaction substrates (left); in the case where these are not available, the polymerase breaks off, and the elongation terminates (right). Color images available online at www.liebertpub.com/ast

nucleobases, or combinations of both) and that can concur to make established molecular biological tools useless for most extraterrestrial genetic systems, genetic information carriers may still remain privileged targets for the detection of biomolecules and life signatures on other planets due to the fact that some of their elementary characteristics are conceivably universal because of physical and chemical constraints (Benner, 2004). It can safely be assumed that any molecule encoding genetic data will be organized in the form of a linear biological polymer that uses the sequence of its monomer subunits to store the hereditary information. In polar solvents like water, repeating charges carried by the backbone are the key for such a molecular system to support high-fidelity copying through inter-strand interactions, as they force the strands to contact away from the backbone (in the case of DNA at the Watson-Crick edge of the heterocycles) rather than in a random orientation. The charged backbone dominates the physical properties of DNA so that replacement of single nucleobases during Darwinian evolution has only a marginal effect on the physical behavior of the molecule and does not affect its replication properties; a flexible polyanion or polycation is more likely to adopt the extended conformation needed to function as a template than a neutral polymer. The repeating charges favor solubility in water and keep strands from folding, as happens, for instance, with proteins, whose backbone is electrically uncharged and in which a single amino acid change can dramatically affect their secondary structure and, by consequence, their functionality (Benner *et al.*, 2004).

4. Nanopore-Based Detection of Nucleic Acids

Nanopore-based sensing devices may take advantage of the fundamental constraints of genetic information carriers in watery systems to detect and possibly analyze them without further *a priori* assumptions about their chemical composition. A nanopore is a hole of a few nanometers in diameter that is enclosed in an insulating membrane between two chambers containing an electrolyte solution. The most commonly described biological nanopore is the heptameric protein α-hemolysin (αHL), which is constituted from a transmembrane β-barrel of about 5 nm in length that has a limiting aperture of about 1.5 nm at its narrowest point. This setup can be used to detect and analyze different types of molecules by measuring the ionic current flowing across the membrane as they are translocated electrophoretically through the αHL pore (Fig. 4). As the diameter of the individual DNA duplex can vary between 2.4 and 2.0 nm, depending on whether the double helix assumes the less common A-form or the biologically more relevant B-form, respectively, the central constriction allows only single-stranded (but not double-stranded) DNA to be driven through the nanopore. Due to their length, these biopolymers require extensive times to translocate across the membrane, which results in a characteristic signal composed by a population of hundreds to thousands of ionic current blockades that is clearly distinguishable from those produced by other types of molecules, as low-molecular-weight compounds cause only minor sporadic disruptions in the

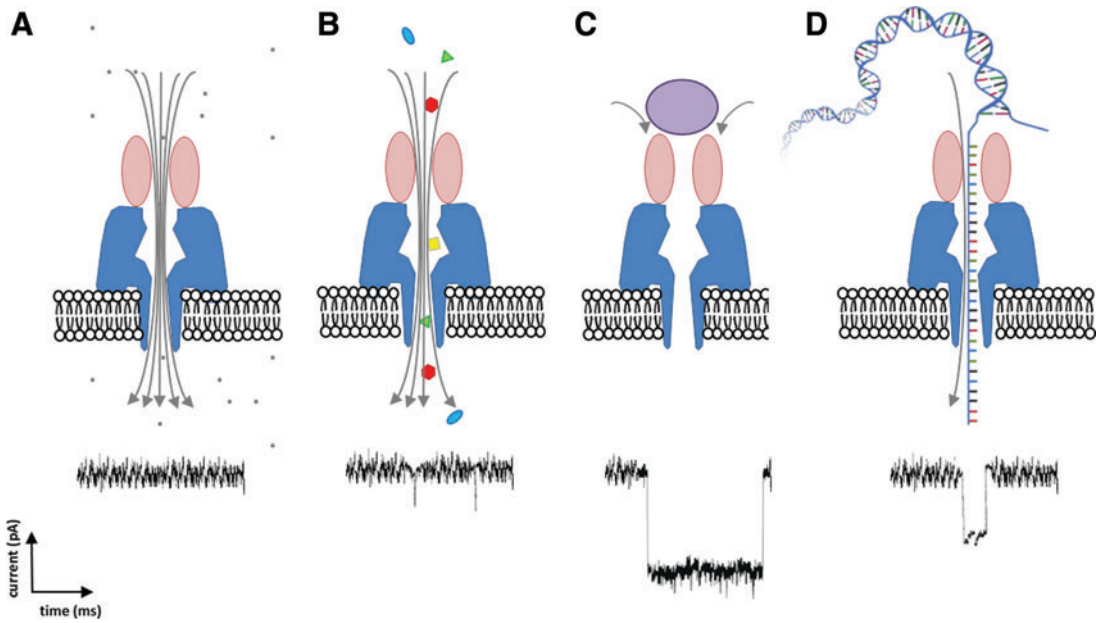

FIG. 4. A biological nanopore such as αHL can be used to detect and analyze target molecules by measuring the ionic current after setting a voltage across the electrically resistant membrane bilayer in which the protein is embedded. (**A**) When no analyte passes through the nanopore, the ionic current (represented by the gray arrows) can flow freely. (**B**) Small molecules translocate quickly through the nanopore, causing a reduction in current intensity of relatively short duration and small amplitude that can be approximately related to their size. (**C**) Bigger molecules that cannot enter the nanopore cause an almost complete blockade in the ionic flow, resulting in a sharp drop of the signal. (**D**) Single-stranded nucleic acids barely fit into the channel and require more time to translocate through the biological nanopore due to their length. This results, respectively, in a reduction of the current intensity that is both large and of extended duration. As the molecule is transferred through the nanopore, the ionic flow across the membrane is modulated by the transiting nucleotides into an electric signal that can be eventually decoded to reveal, at least in the case of DNA and RNA, the sequence of its monomers. Color images available online at www.liebertpub.com/ast

current flow, whereas larger molecular debris in a sample are simply excluded from the nanopore (Fig. 4). All four natural nucleobases can be identified both in DNA and RNA by measuring their differential effect on the current flowing across the membrane as the strand moves through the αHL pore (Stoddart *et al.*, 2009; Ayub *et al.*, 2013). This channel was used in the first experimental demonstration of nucleic acid detection and analysis and still remains a choice for third-generation DNA sequencing. Other bacterial proteins, such as porin A of *Mycobacterium smegmatis* (MspA), show improved single molecule sensitivity and extreme resolution in base recognition with respect to αHL and are able to resolve single base modifications such as the presence of an additional methyl group on cytosine (Laszlo *et al.*, 2013; Schreiber *et al.*, 2013). MspA is currently being investigated as a potential candidate for improved nucleic acid analysis with the use of biological nanopores (Manrao *et al.*, 2011).

A nonbiological alternative that has been explored for solid-state nanopores is graphene (Garaj *et al.*, 2010), one of the crystalline forms of carbon, where atoms are arranged in a one-atom-thick layer with a repeating hexagonal pattern. With respect to the biological alternatives, graphene has the advantage of a reduced membrane thickness that can be adjusted to closely match the distance between neighboring bases in a DNA strand, tolerating only one nucleotide at a time in the nanopore and thus simplifying measurements in single-nucleotide steps (Wells *et al.*, 2012). Furthermore, graphene permits a precise sculpting of the membrane surface, which enables the production of nanopores with a wide range of diameters (Song *et al.*, 2011), a fact that may allow not only for the targeting of single-stranded nucleic acids but also detection of double-stranded molecules (Garaj *et al.*, 2010). This may also allow discrimination of other biopolymers with alternate chemical and sterical properties such as widened DNA (yDNA) (Lu *et al.*, 2009) or expanded DNA (xDNA) (Lynch *et al.*, 2006), which are constituted by nucleobases that are modified by the addition of an extra benzene ring (Fig. 5).

A nanopore-based sensing device could be inserted in the payload of a planetary mission to Mars, Europa, or Enceladus for *in situ* detection of extraterrestrial biopolymers that serve as genetic information carriers. A multicell design could be implemented to evaluate molecules comparable to single-stranded DNA and modifications thereof by using different pore diameters. Opposed polarities could be alternatively applied to the two sides of the graphene membrane to test for the translocation of both negatively and positively charged biopolymers. This approach would allow the detection of a broad variety of genetic information carriers without virtually any further conjecture about the chemistry of their basic building blocks other than the electrically charged linear biopolymer blueprint (Benner *et al.*, 2004). Since there are practically no naturally occurring nonbiological polymers that have these characteristics, any signal coming from an extraterrestrial sample would be highly significant and would represent a compelling indication for the presence of present or past biological

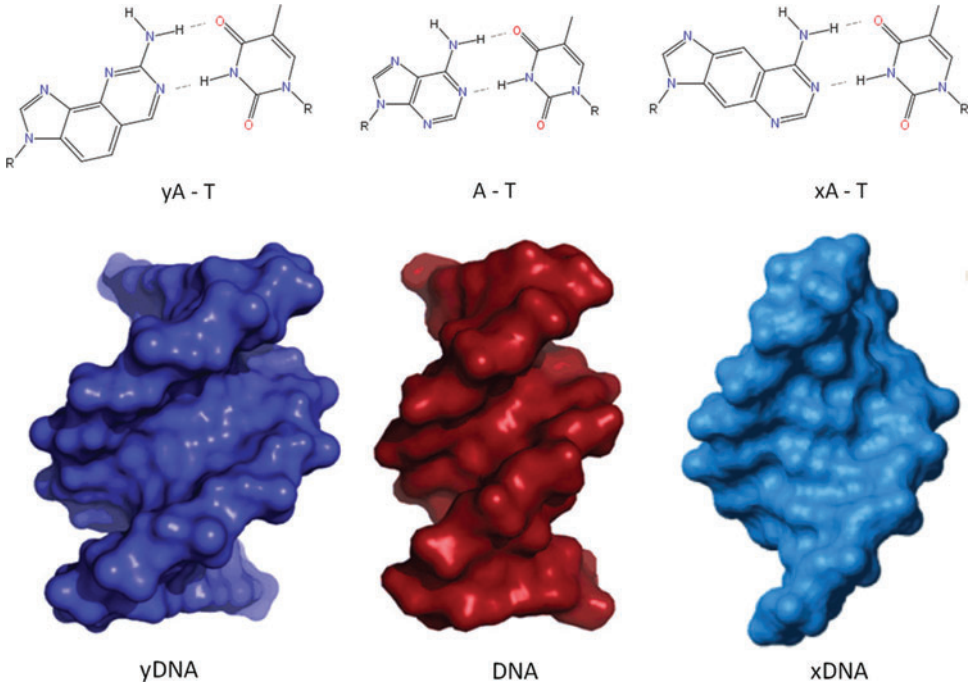

FIG. 5. Minor groove views of yDNA (left) and xDNA (right) compared to the B-form of conventional DNA (center). Structures of modified nucleobases are shown above the space-filling models at the example of widened and expanded adenine analogues and their base pairing with thymine. Adapted with permission from Lu *et al.* (2009) and Lynch *et al.* (2006), copyright 2006 American Chemical Society and 2009 Wiley Periodicals, Inc. Color images available online at www.liebertpub.com/ast

processes. Moreover, under ideal conditions, molecules identical to DNA or RNA would readily be identified and possibly sequenced on the basis of the protocols currently in development for third-generation sequencing of Terran microorganisms, whereas exotic biopolymers and unconventional nucleic acids could be detected, modeled with computer simulation (De Biase *et al.*, 2014), synthesized, and tested empirically in identical devices in laboratories back on Earth to interpret their structure.

5. Possible Issues

Despite having achieved sufficient portability, as in the case of the MinION device, which is currently distributed to early users by Oxford Nanopore Technologies Ltd, there are still several challenges to be solved before nanopore-based sensing instruments are fully mature to be embarked on a planetary mission to these watery worlds. One of the major analysis-related problems that, to date, has to be tackled is the high electrophoretic speed of nucleic acids, which makes low-noise measurements of the small electric current variations caused by the free movement of single-stranded DNA through the αHL nanopore very difficult (Bashir, 2013). A promising approach employed for regulating the translocation speed of nucleic acids is through the use of processive enzymes like polymerases or exonucleases coupled with the biological nanopore; this allows the controlled translocation of individual DNA strands at speeds suitable for accurate single-base reads in the order of tens of milliseconds (Cherf *et al.*, 2012) or the detection of the individual bases cleaved off from the polymer (Clarke *et al.*, 2009), respectively. However, due to their specificity to Terran nucleic acids, biological nanopores and processive enzymes would not be the best choice for the detection and analysis of alien genetic information carriers, but other approaches based on solid-state nanopores combined with purely physical speed-controlling factors such as temperature, salinity, and viscosity of the buffer, or the voltage applied across the nanopore, would be preferred (Peng *et al.*, 2011). A further option would be to attempt to couple these unknown target biopolymers with other molecules through nonspecific electrostatic forces like ionic bonds so as to slow their passage through the nanopore by reducing their overall charge or by simple steric hindrance (Kowalczyk *et al.*, 2012).

The extreme dilution of the target molecules in the collected samples will certainly be a factor negatively affecting the chances of detection of any biopolymer or genetic information carrier that may be present, but the same applies also to any methods based on nucleic acid synthesis as, obviously, at least one target molecule is needed in the reaction to obtain a genuine amplification signal. Though PCR is theoretically capable of detecting a single copy of a target DNA, a copy number per reaction of at least 10 is commonly specified as the lowest target level that can be reliably quantified when using PCR applications. Nevertheless, these figures apply in earthly laboratories, under extraordinarily favorable settings and with perfect knowledge of the matching template sequence, which would most probably not be the case when looking for extraterrestrial life signatures. Since in a nanopore device translocation and recognition of nucleic acid are recorded in real time on a molecule-by-molecule basis, even a single translocation event is in principle detectable with this technology, although the average capture rate is ultimately dependent on the analyte concentration, the pore shape and

cross sectional area, the applied electric voltage, and the temperature (Meller and Branton, 2002; Peng *et al.*, 2014).

Radiation protection and device stability during extended missions will also be issues; in order to arrive at a destination with a fully functional nanopore device, it will be essential to study how high-energy particles from the Sun combined with extreme vacuum and low temperatures of space will affect its components during a typical mission profile, and to provide the necessary shielding. In the case of a mission to Europa, surviving within Jupiter's radiation belts will be an additional concern. It can be argued, however, that the simple design of a solid-state nanopore device, combined with its reduced requirement for chemical and biochemical reagents with respect to biological nanopores or sequencing-by-synthesis approaches, will make this technique less vulnerable to radiation damages.

6. Conclusions

Current technology allows the sending of robotic chemistry laboratories to other Solar System bodies to search for life signatures. The jovian moon Europa, Saturn's moon Enceladus, and the everlasting Mars are the targets of choice if we consider life based on liquid water. In such environments, it is reasonable to assume that genetic information carriers would present themselves in the form of electrically charged linear biopolymers that are not necessarily identical to DNA or RNA but are consistent with the same general blueprint and may display chemical variations both in the backbone as well in the nucleobases.

The latter fact devalues any attempt to detect and eventually sequence these molecules by using replication or cleavage approaches, as most enzymes that perform these tasks are highly discriminating with regard to the type of templates and substrates accepted. In contrast, upcoming approaches that are not directly dependent on the chemical structure of the analyte, such as nanopore-based sensing devices, could maximize the chances of the detection of alternate genetic information carriers and maintain the possibility of detecting conventional nucleic acids fully intact, which makes these the methods of choice for future planetary probes. Conceivable mission concepts could possibly include sampling of surface or subsurface ice probes or, in the case of Europa and Enceladus, even direct in-orbit sampling of the watery material vented from the interior of the icy moons (Tsou *et al.*, 2012; Roth *et al.*, 2014).

Acknowledgments

The author wants to thank the members of the research group Environmental Genomics and Systems Biology at ZHAW Wädenswil (http://www.iunr.zhaw.ch/EGSB) for the fruitful discussions and their support; the authors of the artwork included in Figures 1, 2, and 5 for the kind permission to reproduce the images and for providing the original high-resolution files; two anonymous reviewers and the editorial team of *Astrobiology* for their help in improving this manuscript; and the crowd-researched project Objective Europa (http://www.objective-europa.com) for the inspiration that lead to the elaboration of this article.

Author Disclosure Statement

No competing financial interests exist.

Abbreviations

αHL, α-hemolysin; AEGIS, Artificially Expanded Genetic Information System; MspA, *Mycobacterium smegmatis;* PCR, polymerase chain reaction; xDNA, expanded DNA; XNA, xeno-nucleic acids; yDNA, widened DNA.

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Submitted 11 November 2013
Accepted 27 February 2014