A New Technology for the Synthesis of Long DNA Strings

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Abstract:

We present a new theoretical technical solution application of nanopore sequencing (Oxford Nanopore Technologies, <https://www.nanoporetech.com/>), which has primarily been used to read molecules. We describe how to apply nanopore sequencing for the production of a new type of device synthesizing of molecules according to our requirements of a chosen database that has been prepared in advance. This technology can implement the programmed synthesis of long DNA strings, ideally entire chromosomes. We propose a device using nanopores and a system of nanotubes that can synthesize molecules using individual nucleotides, existing single-stranded DNA (ssDNA) ligase or modified forms of ssDNA ligase and the energy provided by ATP. The second strand is synthesized by Taq polymerase, which is located below the nanopore. The synthesis of long strings is performed using many nanopores positioned linearly. The strings are collected in wells below the nanopore, after which the strings are ligated to form a long single string (or to form part or all of a chromosome) by double-stranded DNA (dsDNA) ligase. There are many options for successful implementation, particularly in the field of genetic engineering

Keywords: Oxford Nanopore Technologies, Nanopore, Programmed Synthesis, Long Dna Strings, Single-Stranded DNA Ligase

Introduction:

Synthetic DNA can currently be prepared as short strings. Oligonucleotides (up to approximately 200 nucleotide residues) are chemically synthesized as short nucleic acid fragments with a defined chemical structure. The building blocks are gradually coupled to the oligonucleotide chain. The number of errors generated sets the practical limits of the length of the chain being synthesized (Beaucage and Iyer 1992). The products of oligonucleotide synthesis are isolated by high-performance liquid chromatography (HPLC) to obtain the desired oligonucleotides in high purity. Typically, synthetic oligonucleotides are single-stranded DNA or RNA molecules of approximately 15–25 bases in length. For example, the One-Step Isothermal DNA Assembly method currently allows for the efficient assembly of DNA constructs using fragments of up to several hundred kilobases (Rodrigues and Bayer 2013). T.S. Some companies offer the

commercial synthesis of long sequences. For example, GenScript's GenBrick™ synthesis service provides 100% accurate 8-15-kb DNA sequences. However, the method still requires the construction of short oligonucleotides, and the final length remains limited. Eukaryotic chromosomes are much larger. However, the synthesis of very long DNA molecules is necessary and desirable because it is more effective to construct an entirely new chromosome rather than to use targeted integration to insert a gene into a preexisting chromosome. The original chromosome in the nucleus could be replaced with a newly created chromosome using this technology. Eventually, all chromosomes could be prepared according to our needs, and these new chromosomes could form a new nucleus that could then replace the original nucleus in the cell. The preparation of a new bacterial genome to develop or extend various bacterial substrates for the needs of industry or medicine is also necessary and desirable. Pro-gene editing includes the addition, disruption or

alteration of existing technology using the CRISPR/Cas system. The latter system has dramatically transformed our ability to edit the genomes of diverse organisms (Mali et al 2013). This editing was first proven as a genome engineering/editing method in 2012 (Jinek et al 2012). This technology is revolutionary and has been validated. It is necessary not only to develop the technology but also to create new possibilities that are based not only on the modification of an existing genome but also on the accelerated preparation of a new genome without complex modifications. Currently, the nanopore sequencing products from Oxford Nanopore Technologies (<https://www.nanoporetech.com/>) have been used to analyze DNA, RNA, proteins and small molecules, which are then used in personalized medicine or scientific research (Mikheyev et al 2014, Check and Hayden 2012). Oxford Nanopore Technologies Ltd. is developing a disruptive, proprietary technology platform for the direct electronic analysis of single molecules. Oxford Nanopore is based at the Oxford Science Park outside Oxford, United Kingdom, and has satellite offices in Cambridge (UK), New York and Boston. An electric current is used to read the shape and size of the nanopores that comprise 1-nanometer-sized holes. It is important to create a hyper-sensitive ionic environment using a conduction fluid in this nanopore system.

The pores, comprising a ring of seven α -hemolysin membrane proteins, are the same as those that the infectious bacterium *Staphylococcus aureus* pushes into the membranes of other cells to create damaging holes. Branton's team from Harvard University in 1996 proposed that the identity of each of the four DNA bases, while traversing the hole, may be revealed by distinctive changes in ion flow, which can be read as electrical signals (Sanderson 2008). The nanopore could also be constructed of materials other than proteins. Nabsys, which was formed in 2004 by Xinsheng Sean Ling, a physicist at Brown University (Providence, RI, USA), is also pursuing nanopore sequencing. The method is based on a silicon chip that is dotted with synthetic nanopores. Through these pores pass 100,000-base fragments of genomic DNA that have six-base probes attached to them at intervals.⁷ Studies of double- and single-stranded DNA transport through nanopores have also been conducted using ultrathin (2-to 7-nm thick) freestanding hafnium oxide (HfO₂) membranes. The HfO₂ nanopores exhibit favorable physicochemical interactions with nucleic acids that can be leveraged to slow down DNA in a nanopore (Larkin et al 2013). In this study, we aimed to develop these unique nanopore systems, not only to identify molecules but also to directly synthesize long DNA strings and, ideally, entire chromosomes.

2 Technology description

Currently, the implemented system is based on proteins. We assume that in the near future, the system will be based on synthetic materials. We will describe the possible functions of basic functional feature of the device, the protein-based system. The device (Fig 1A) is constructed using nanopore technology and comprises a membrane (lipid bilayer), an α -hemolysin nanopore, or better an MspA pore protein derived from *Mycobacterium smegmatis* (Manrao et al 2011). A molecule of ssDNA ligase (for example, CircLigase or a modified version of the molecule)

Fig 1A. a – membrane, b – α -hemolysin nanopore, single nucleotide according to the program, c – ssDNA ligase, d – protein as a funnel, directing the nucleotide to the active center of ssDNA ligase, e – nanotube controlled by a computer with an electromagnetic field, f – bottom view of the nanotube.

lies above the membrane. This ligase must connect individual nucleotides. Extensive research into the production of artificial enzymes will enable the construction of enzymes according to specific requirements (see Taylor et al 2015, Selvakumar et al 2015).

From the wells in the device, individual nucleotides will be distributed to the active center of the enzyme that will synthesize the new string. All of these activities will be managed by software installed on a connected computer. It is necessary to harmonize the software and discharge of specific molecules. The nucleotides will be placed in the wells. Tubes that narrow gradually to 1 nm in width will lead to each nanopore in the device. Thus, part of the device would contain one well for adenine, another well for thymine, etc. However, for each nanopore, the discharge of individual nucleotides must be precisely managed. The narrowing and opening would be constructed from a material such as carbon nanotubes. For each base (A, T, G, C), there would be three nanopores. The narrowing nanotube would lead to the well with the relevant nucleotides. Next, two nanotubes would be placed along the sides, functioning as electrodes. Among nanotubes-electrodes would be nanotubes containing nucleotides (A or T, etc.). The field would hold the nucleotides within the middle nanotubes. Nucleotides would be attracted to one electrode as in electrophoresis. The diameter of these two nanotubes would remain the same along their length, and the tubes would lead to electrodes that are connected to the computer. The two nanotubes would function as anode and cathode and, during influx, would maintain the molecules in an electromagnetic field (Fig 2). Using a short pulse, the field could be stopped, and a specific nucleotide would remain at the active center of the synthetic enzyme because of the second electromagnetic field. Another field on membrane would drag the nucleotides to the α -hemolysin or MspA pore nanopore when they were not held inside the carbon nanotubes (Fig 1A). The second electromagnetic field on membrane would be continuous in the system and would pull DNA molecules through the membrane

nanopore. Alternatively, individual and desired nucleotides would be squeezed out by the pressure of photons (using a laser) or electrons. In this case, the nanotube would need to be constructed of a material such as silicon rather than carbon. Thus, individual intermittent electrical pulses would always open only one of four nanotubes—A, T, G, C—and discharge the required nucleotide. There would not be needed the system of three carbon nanotubes. There would be any need for holding the electromagnetic field of each A, T, G, C nanotubes base. Instead, we consider carbon nanotubes with a diameter of approximately 1 nanometer and a length that can be many millions of times longer. It is important to electrically isolate the carbon nanotubes from the surrounding environment and from each other. All four nanotubes (A, T, G, C) must be directed into the active center. The lateral nanotubes that are used as electrodes could be slightly shorter. However, we can imagine the system where these four nanotubes would also be used for the influx and would be made from protein (i.e., an α -hemolysin nanopore). However, the system comprising tubes and electrodes to supply four bases would be too large. Therefore, the system may be complemented by a circular protein that would act as a funnel and direct nucleotides to the active center of the ssDNA ligase (Fig 1A). Upon tuning of this instrumentation, the design for fast nucleotide coupling must follow.

The appropriate nucleotide would be delivered to the active center of the enzyme (a ssDNA ligase, such as CircLigase or a modified type of the ligase), which would lie above the α -hemolysin nanopore. In the active center, the enzyme would connect the nucleotides to form the ssDNA string that passes through the nanopore to the well below it. The ssDNA ligase would connect the nucleotides in the presence of the ATP supplied

Fig 1B. g – DNA polymerase III, h – free nucleotide for the synthesis of a parallel DNA string and RNA primer, i – polymerase I, j – primase, k – RNA primer, l – ligase

to the surrounding environment. DNA polymerase and a mixture of nucleotides would be placed below the nanopore membrane in the well for the synthesis of the complementary strand (Fig 1B). If it would not be possible to create a suitable ssDNA ligase, the whole system could work with RNA.

It would be probably easier to find the suitable RNA ligase.

Fig 2. Construction of an injection nanotube. a – middle gradually expanding nanotube, b – nanotube as electrodes, c – electrical insulation cover from the polymer

Then, the newly formed RNA would be classically converted to DNA by reverse transcriptase classically. The second strand can be synthesized in various options. A primer is required, as would (according to the currently proposed system) needed enzyme proteins. We prefer to conduct the synthesis in an analogous manner to that which occurs in a living system (an organism). Thus, we propose used enzyme primase, RNA nucleotides, DNA polymerase III, DNA polymerase I and the appropriate ligase. The end replication problem could be practically solved by using the sticky end to connect strings along the neighboring wells. It is also possible to use some other type way of to synthesize the second string. It is necessary to tune the efficiency of the system in practice. The most suitable enzymes will have to be selected and tested, or new enzymes, which do not exist yet, can be created. In the proposed scheme, tubular cyclodextrin will probably not be necessary as it is for sequencing.

In the final version of the device, the structural element described in Fig. 1 should be stacked into mutually adjacent series. When the testing and tuning of the system containing a small number of nanopores is finished, a series of thousands of cells would be created. The cells would be managed using new software. Currently, this is not an unsolvable problem. Long strings would be synthesized using many nanopores positioned linearly (Fig 3). Long strings would be collected in wells below the nanopore and then connected into joined to form a very long string (possibly to an entire chromosome). On one Wells side of the wells would have, on one side, be a closeable hole with containing dsDNA ligase; the holes must be positioned linearly in each well. The neighboring well again has would have an α -hemolysin nanopore with a dsDNA ligase. The end of the new string is closer to the dsDNA ligase on one side of the well (e.g., because of the using a short electromagnetic field perpendicular to the continuous field;

Fig 3. a – Above the nanopore with ssDNA ligase are 4 nanotubes (e.g., carbon nanotube), b – upper well, c – bottom well, d – the bottom well contains polymerase that synthesizes dsDNA, e – link with another well and with α -hemolysin nanopore and connected dsDNA ligase.

the continuous field would be off at that moment). Of course, the whole process would connect into one long string could be executed until when all ssDNAs synthesis are terminated, via ssDNA ligase. When long strings are required in the wells, the following strings from next wells are would be connected, and dsDNA ligase would links join these strings into one a long linear string. These dsDNA ligases would connect only dsDNA strings, not the free nucleotides in the environment (for the synthesis of dsDNA).

Next, it is possible to further use the dsDNA, for example, to package DNA into chromatin (histones and chromatin assembly factors) with chromosome proteins or, for prokaryotic DNA, to insert it into a previously inactivated cell.

An alternative solution uses the same principle with other materials. Nanopores will be constructed using hafnium dioxide (HfO₂) (see Fahrenkopf et al 2012, Larkin et al 2013) or synthetic silicone (Zhang et al 2015) in the future. Similarly, the nanotube used to supply nucleotides could be constructed from materials other than carbon. It is also desirable to make functional enzymes (ssDNA ligase, dsDNA ligase, DNA polymerase) from suitable polymers—for example, silicone-based material or silicone with a stable center of resistant normal live proteins.

3 Conclusions

The successful implementation of technique for synthesizing long DNA strings may dramatically change genetic engineering. The construction of the first prototype is on current technical capabilities. The proposed solution combines the controlled injection of individual, clearly identified nucleotides and a protein nanopore with a system of separated wells and enzymes (ssDNA ligase, dsDNA ligase, and polymerase). It is possible to realize the system in practice using mainly current technologies. This idea for synthesizing long DNA strings is based on the development of such technologies in the near future using functional enzymes that are constructed from durable materials, e.g., materials based on silicone. There are many potential applications, particularly in the field of genetic engineering; for example, to produce and alter entire genomes, to transfer organisms and to clone extinct species

for which the genome is known (e.g., thylacine, mammoth).

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