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The Nanostructure for Next Generation of Proteomic Analysis

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Abstract

Proteomic analysis is a powerful tool and is popularly applied in a range of studies in post-genome era. Utilizing advanced mass-spectrometry and bioinformatics analysis, target proteins can be rapidly detected and identified. The formidable mass-spectrometer is the most advantageous and also the most disadvantageous for proteomic analysis. For its physical dimensions and peripheral facilities, the mass-spectrometer is hard to be miniaturized and become a real portable device to be widely used in daily life. Nanotechnology has an unbeatable progression beyond Moore's law and nanofabrication has been recruited in medical sensor and instrument device fabrication. A nanofabricated nanopore device has been developed, commercialized and exploited in genomic DNA sequencing. In recent years, nanopillar sieve-spectrometry also has burgeoned and has evolved in biological macromolecules separation. In this article, we propose a concept that integrated nanopillar sieve-spectrometry and solid-state thin-film nanopore sequencing technology for the next generation of proteomic detection and analysis as an alternative deployment for mass-spectrometer. Limitation, criteria and challenges are discussed from a range of aspects of technology approach, scientific progression and market demand.

Introduction

It is nearly sixty years, since the ideas and concepts of "nanoscience and nanotechnology" was first time proposed 1959. The era of nanotechnology did not come to earth till the development of the first scanning tunneling microscope that can observe an individual atom in 1981 [1]. For a long time, research and development of nanotechnology focused on physics and electronics, especially on fabrication processes and physical dimensions of a semiconductor. Up to date, the 6 ηm fabrication process has been achieved in the advanced semiconductor manufacturing process. This means that a circuit or structure with 6 ηm width wires can be fabricated, processed and constructed on a semiconductor wafer. In the modern world, semiconductors are utilized in most electronic products. Nanotechnology has deepened into everyone's daily life, for example, globally average; everyone owns more than one mobile device.

In biological science, nanoscience and nanotechnology still emphasize on discoveries of fundamental science, such as biomolecular structure or interaction behavior of biomolecules. The development of molecular and cellular biology shift biology research to cellular or even the molecule level. Based on discoveries of cellular and molecular biology, several biological techniques with high accuracy have been developed, such as DNA ligation, recombinant protein, enzyme linked immuno sorbent assay (ELISA), and enzyme immune assay (EIA). Although these techniques have been widely applied in medicine or additives production, diagnosis or biological material detection, their application is constrained in professional laboratories, for their dependence on huge and high power instruments.

It is fifteen years since the completion of the human genome project. The emergence of bioinformatics, personal genomic and comparison proteomic analysis brings a new hope of human beings welfare in the prevention of genetic diseases and cancer diagnosis. These techniques provide powerful tools to comprehensively scan genome or to compare gene expression at different age/stages. The collected data are further analyzed and information can be retrieved from cloud database.

Extra-long DNA sequencing techniques are the core and bottleneck of genomic analysis. Capillary electrophoresis-based DNA sequencing method is time consuming and takes years to complete scanning a person's genome information. DNA microarray-based sequencing technology can rapidly scan several hundred thousand genome types of genes. DNA microarray-based method not only can carry out quantitative gene expression assay, but also the process can be completed

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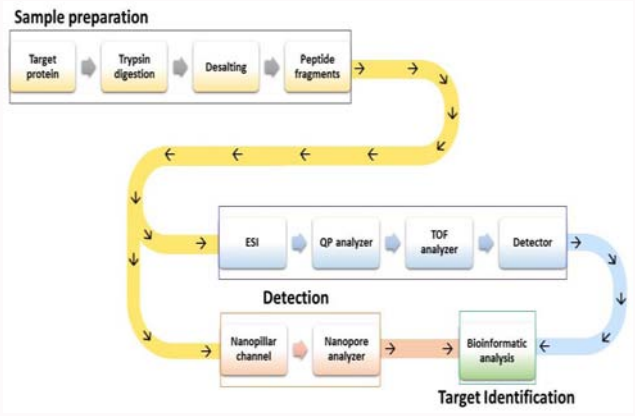

Figure 1: Nanostructure and next generation of proteomic analysis.
A proteomic identification process includes three major steps: sample preparation, detection, and target identification. The interesting target proteins are isolated or purified and trypsinized to small fragments. The digested peptides are further desalted. In a conventional proteomic process, MS/MS-ToF-Mass or MALDI-ToF are recruited to detected peptide molecular weight and charge to mass ratio. Sequences of the peptides can be retrieved by searching against genome database, then the possible target proteins can be identified. Nanostructure could replace the time-consuming mass-spectrometry process. A gradient nanopillar sieving matrix can separate and sort the trypsinized peptides by their molecular weight or 3-D structural conformations. The sorted peptides are driven through a nanopore by electrophoresis push-pull, and sequences of the peptides are directly read. Combinations of the sequences can be searched against to find out the possible ID of the target proteins.

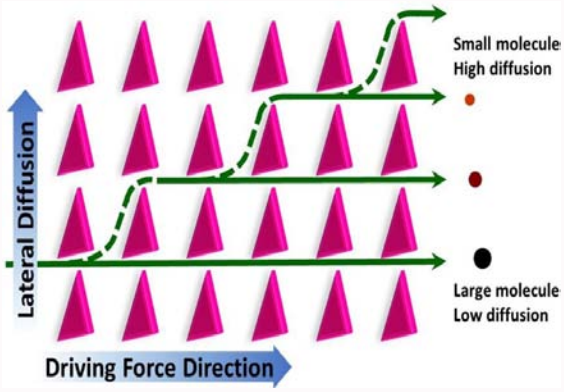

Figure 2: Separation principle for nanopillar sieving matrix.
The nanopillar array is set on the flow channel and the flow stream is driven by electrophoresis. The mixture of macromolecules is injected into the left end of the channel and the molecules are driven into the pillar array and traveling along the direction. For the lateral diffusion, each molecule has a limited chance to be deflected while flowing along the flow direction. The diffusive motion will be repeated because of asymmetric design of the pillar array.

and shorted to weeks and even several days [2]. Compared with conventional capillary electrophoresis based sequencing, DNA microarray method makes a big improvement on both speed and accuracy. Either capillary electrophoresis or DNA microarray is heavily dependent on professional technicians and the processes have to be performed in well-conditioned laboratories with professional equipment. On the other hand, sample preparation is never easy and plays as a key element to genomic DNA sequencing performance.

Application of nanostructure in DNA sequencing analysis

The cost of maintaining a professional laboratory is relatively high and it is one of the reasons that personal genome sequencing is not popular or used as a regular diagnostic tool. To reduce the cost and accelerate the speed of personal whole genome sequencing is the major goal of DNA sequencing technique research and development. Oxford Nanopore Technologies, a British nanotech company, announced the first commercialized portable DNA sequencing device, MinION, 2012. The device is composed of a nanostructure array and a biological lipid bilayer membrane is formed on the open of the chamber. Then recombinant human α-hemolysin is added and a biological nanopore is spontaneously assembled. While a single strand DNA passing through the nanopore, sequence information of the DNA can be retrieved in real time. The chambers of the devices are constructed as an array through a nano-fabrication process and multiple sequencing signals can be chronically sensed in real time and this will be an advantageous compared to current sequencers [3].

Compared with conventional genome sequencing techniques and devices, the nanopore sequencer demonstrates speed, promise of longer reads and a lower cost, and also is fueled excitement about new sequencing applications that are unattainable by current technologies [4]. The MinION is capable of reaching a reading speed at 300 bases per-second per-channel. Read length can potentially exceed 100 kb [5]. The device has been applied in genome sequencing of a range of organisms [6].

A solid-state nanopore upon a nanostructure also can be approached through a nano-fabrication process. The most straightforward is to stab a nanopore by recruiting electron beam technology [7]. Although electron beam can stab pores with a high accuracy and homogeneity, for its low speed and time consuming, the process usually is only applied in prototyping but not suitable for final product mass production. As mentioned above, a 6 ηm nanofabrication process has been developed and is utilized in semiconductor fabrication. Non-biological nanopore structure can be approached by the advanced 6 ηm nano-fabrication process in a well-controlled condition. It is believed that the solid-state non-biological nanopore could have a better performance than a biological nanopore, for its higher stability, strength, and homogeneity [8].

Possibility of applying nano-structure in proteomics analysis

Proteomics is a multiple-discipline subject that focuses on large-scale protein expression and profiles studies in biological samples [9]. Applied proteomics is an interesting and challenged topic not only for fundamental studies and research but also for practical application in rapid medical diagnosis and industrial utilities. How can nanostructure be used to facilitate proteomic technique progression? To answer this question, we have to take a brief look at how proteomic analysis works.

Proteomic analysis process can be roughly separated into as three stages as shown in Figure 1. The first stage is sample preparation, and the interested targets from the purified biological specimen, SDS-PAGE or 2D electrophoresis are extracted and trypsin digested to be peptides. The digested peptides are further purified to desalt and for mass-spectrometry analysis. The second stage, the molecular weight and electron-mass ration of peptides are detected. The third stage, the collected data at the 2nd stage, based on the detected mass and electron-mass ratio, a bioinformatics analysis algorithm is performed to identify amino acid residue sequences of the peptides and the possible protein IDs are searched against and retrieved from genome database [10,11]. The 3rd stage is very pure *in-silico* biology and has no

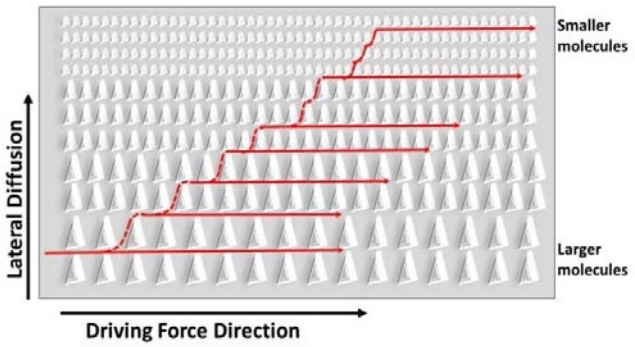

Figure 3: Gradient nanopillar sieving matrix.
Electrophoresis is the major driving force to push peptides to flow through sieving nanopillar matrix. An asymmetric gradient nanopillar array could improve the resolution and sensitivity of separation caused by lateral diffusion.

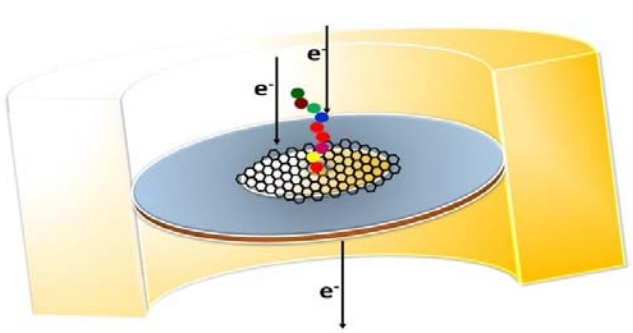

Figure 4: A nanopore structure and translocation of peptide.
A schematic diagram shows the cross-section of a vertical nanopore structure and the translocation of bio-molecule. A solid-state nanopore is formed in the center of a graphene film. The translocation of an unfolded and linear bio-molecule through a nanopore structure is driven by electrophoresis. Electric current is reduced while bio-molecule is passing the nanopore and sequencing information of the bio-molecule can be retrieved by electrical detection. A very different point from mass-spectrometric technology, in a nanopillar and nanopore system, peptide fragments are sieved and detected in an electro fluidic environment but not in a vacuum one.

requirement of physical structure for wet analysis.

Nanofluidic for enzyme digestion and sample preparation

In the 1st stage, lab-on-chip has been a common concept to simplify the enzyme digestion process and to prevent any possible contamination during operation by integrating micro- or nano-fluidic channels [12]. Enzyme-digestion chip can be widely applied in a range of biology, biochemistry or analytic chemistry research. The purpose of the chip is not necessarily specific for proteomic analysis. Design of the chip system is depended on the demand and application scenarios [13-15]. A reagent system is usually developed along with the chip system to ensure optimal performance.

In the 2nd stage, mass-spectrometry detection and analysis are both the most advantageous and the most disadvantageous of proteomics. The most advantageous and biggest benefits of mass-spectrometer are its powerful abilities in accurately measuring molecular weight down to just one electron difference. The disadvantage of mass-spectrometers is their physic size. Even the most advanced desktop mass-spectrometer is still a huge box. For its appropriate function, some peripheral facilities are required, such as on extra vacuum pump, gas cooling system, and stable power supplier and these facilities prevent a mass-spectrometer from being small and portable. On the other hand, the flying tube and ion mirror also constrain the physic size of a mass-spectrometer recruited in proteomic analysis.

Nanopillar sieving technology

We have to think alternatively if we want to make a real portable device for proteomic detection and analysis. Two issues have to be considered, one is how to sieve digested peptides and how to individually sense these peptides. Nanopillar sieving matrix might be one of the choices. Based on rectification of Brownian motion, a biological macromolecule separation and fractionation method that utilization of micro-/nano- pillar is proposed [16]. Based on a semiconductor lithography process, a periodic micro pillar array sieve is built on a silicon wafer. Electrophoresis is the driving force to form a fine stream of molecules. While flowing through the oblong obstacles, the molecules are deflected and each molecule follows a different trajectory, oblique to the flow (Figure 2 and 3) [16-18]. Han and Craighead have designed and demonstrated a nanofluidic channel device that can successfully and efficiently separate long DNA molecules with a range from 5000 to 160,000 base pairs into bands in 15mm-long channels after passing through 170 × 300 μm^2 of micro pillar array [19].

The regular sieving structure is constrained in separating of long DNA molecules. It is still a challenge to separate short DNA fragments and small macromolecules. For physiologically relevant macromolecules, such as proteins, are relatively much smaller than long DNA, to separate the smaller macromolecules is significantly important in research and it also represents industrialization and commercialization opportunities. To achieve separation and sort of smaller macromolecules in a nanofabricated device, a two-dimensional periodic nanofluidic filter array (Anisotropic Nanofilter Array; ANA) is proposed [20]. The structural anisotropy can be designed into the ANA and guides biomolecules of different size or different charge to follow distinct trajectories and leads to separation and sorting. Electrostatic separation also can be implemented on ANA along with other separation methods, and proteins of a range from 11 kDa to 400 kDa can be separated within a few minutes [20]. For its efficiency, nanopillar protein sieving chip has been designed and demonstrated for quality control of biologics production [21].

Up to date, the bottom limitation of nanopillar protein sieving technology is about 11 kDa, and it is equivalent to a protein approximately composed of 90 to 110 amino acid residues. The length is too long for proteomic analysis. An ideal peptide length for proteomic analysis is usually no longer than 20 amino acid residues. There is a big room for nanopillar sieving technology to improve sensitivity and resolution.

Nanopore sequencing analysis of peptide

Analysis of peptide sequence is always a challenge because peptides cannot be directly replicated and amplified as DNA via a polymerase chain reaction and labeled building blocks. For a long time, complicated processes of Edman degradation and carboxypeptidase digestion are golden rules for peptide sequence analysis till modern proteomic tools appeared. Any method that can directly decode and retrieve protein sequencing information will be a great scientific achievement.

As mentioned above, the biological nanopore has been applied in DNA sequencing and the products have been marketed for years. The

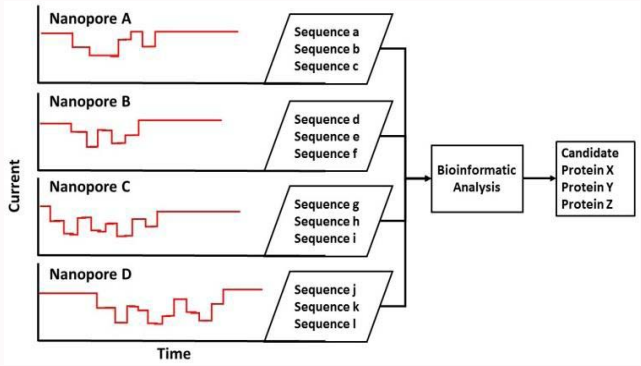

Figure 5: Peptide sequencing information retrieved from nanopore and analysis.
For the difference of side chain, the different amino acid residue has different block effect on the electric current. The electric current signal can be as an index to identify amino acid residue and the sequencing information of peptide can be directly retrieved. A nanopore array can be integrated with nanopillar sieving matrix. The sieved peptides can be immediately detected and sequencing information can be red. All information will be analyzed by blasting against genome database.

biological nanopore formed by α-hemolysin also has been modified to adapt as a sensor element for detecting protein analytes at the single-molecule level [22]. Combining the modified biological nanopore with a protein processing enzyme can unfold target proteins and push them passing the nanopore [23]. These results suggest that a biological nanopore might be possibly applied for directly protein sequencing analysis, but the nanopore has two challenges. One is its durability during analysis progression and the other one is its difficulty to manufacture in nanofabrication process.

The nanofabricated solid-state nanopore exhibits high strength and durability but its sensitivity is limited by thickness, for the constraint of material and fabrication process [8]. To detect proteins is very different from detecting DNA. There are two big challenges that have to be conquered for analyzing protein sequences by a nanopore system. One is protein structure has to be denatured and unfolded to release the protein as a linear form. The secondary structure could be formed in a peptide as short as only having three residues. Both tertiary and secondary structures of a peptide must be unfolded to allow the denatured peptide to thread through the pore. Another one is the complexity of its building blocks. Proteins employ amino acids as building blocks, but DNA employs nucleotides as building blocks. There are twenty amino acids that are commonly used for building proteins. The twenty amino acids have different side chain properties, including charge, size, length and, hydrophilicity. It must be confirmed that the peptide can continuity pass the nanopore with a consistent rate and have not stagnated during translocation. The peptides also have to achieve processive unidirectional translocation [24].

To conquer this challenge, one of the strategies is to form the nanopore on an extra-thin film to improve sensitivity and signal specificity. It has been demonstrated that a solid-state nanopore can be formed on a graphene film and successfully read DNA sequencing signals at a high sensitivity [25]. The simulation investigations that applies all-atom molecular dynamics model presents the feasibility of graphene nanopores used for protein sequencing (Figure 4) [24].

Conclusion

We propose a possible scenario for the application of nanostructure in proteomic analysis (Figure 5). The enzyme digested peptides are separated, sieved and sorted through a nanopillar sieving device and then the peptides are chronically sequenced with a solid-state nanopore array. The collected peptide sequence information are further searched against a genome database and the desired target is identified. A new bioinformatics algorithm may need to be developed because it is difficult to differentiate the sequences from C- or N-terminals.

In this article, we propose an alternative and innovative concept for proteomic analysis. In this concept, a nanopillar sieve-spectrometry and solid-state nanopore sensing technology are recruited as fundamental supports. The required technologies and knowledge are still on their way to maturation, but we believe that a nanopillar sieving matrix and solid-state nanopore will be integrated as a portable device in a few years. The integrated device might replace the role of mass-spectrometry in detection and identification of proteins. The cost of proteomic analysis will dramatically drop and be more acceptable by a regular medical diagnosis. The most benefit of the new technology is that proteomic analysis could be performed at any place at anytime. The prediction is based on following aspects and observations. The 3 ηm nanofabrication process will be achieved in three years or less. Fabrication of thin-film, such as graphene, on a functionalized nanostructure is a mature technology. The mobile device is more and more powerful and the bandwidth and speed of telecommunication growth are far beyond prediction. It is not difficult to access knowledge, technical support and consulting through a cloud service.

Another driving force is market demands either from research laboratories or precision healthcare. An economical, portable and powerful device will accelerate scientific progression and discovery, and diagnostic process can be rapidly and accurately completed. The integration of nanopillar sieve-spectrometer and solid-state nanopore will be a choice for next generation of proteomic analysis. This will be a disruptive innovation and new business model will be established.

We would like to emphasize the challenges for developing the technology. The first, current nanopillar sieving technology exists with a bottom resolution at 11 kDa. For proteomic analysis, the resolution should be improved and reach a level of 200 Da to ensure that the peptides with one or two residues difference in length can be separated. The peptides of a range of 1 to 3 kDa should be clearly separated as each individual. The second is the tertiary and secondary structure of protein. For nanopore sequencing analysis, both tertiary and secondary structures of peptide must be unfolded to allow the denatured peptide to thread through the pore. The third, the peptide must be able to continuity pass the nanopore with a consistent rate following a processive unidirectional translocation.

Applying nanofabricated devices in proteomic analysis, especially in sensing and analysis of targets, is a disruptive innovation. The complex, huge and expensive equipment might be replaced by a small portable device. The applications of proteomic analysis could be possibly exponentially expanded and deepen into our daily life thanks to a miniature and portable proteomic analyzer.

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