Clinical Chemistry

Transport of Full-Length Proteins through a Nanopore: One Step Closer to Single-Molecule Proteomics

Article:
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Clin Chem 2024; 70(2): 462–3. https://doi.org/10.1093/clinchem/hvad201

Guest: Dr. Ruben Luo from Stanford University and the Clinical Chemistry and Immunology Laboratory at Stanford Health Care.

Bob Barrett: This is a podcast from *Clinical Chemistry*, a production of the Association for Diagnostics & Laboratory Medicine. I'm Bob Barrett. Proteins are complex chains of amino acids that are involved in innumerable processes in the human body, many of which are routinely measured in clinical laboratories to evaluate states of health and disease. One drawback of currently available immunoassays is that they recognize proteins on the basis of their three dimensional shape, but fail to distinguish between different forms of the same protein with similar shape but slight modifications that may result in very different biological functions. What insights are we missing by lumping all protein variants into a single aggregate measurement and how might patient care be improved if we could sequence single protein molecules?

A News & Views article appearing in the February 2024 issue of *Clinical Chemistry* highlights two recent studies describing advances in nanopore protein sequencing and possible implications for protein measurement in clinical laboratories. In this podcast, we are excited to chat with the News & Views article's senior author. Dr. Ruben Luo is an assistant professor of pathology at Stanford University and the associate director of the Clinical Chemistry and Immunology Laboratory at Stanford Health Care. His research focuses on using advanced analytical technologies to discover and characterize novel clinical diagnostic markers. So, Dr. Luo, let's get really basic. What are proteins and why are they important in clinical diagnostics?

Ruben Luo: Well, proteins are large, complex molecules that play critical roles in the living organisms. They do most of the work in cells, and they are required for the structure, function, and the regulation of the body's tissues and organs. At a molecular level, a protein is a polymer made up of many small units called amino acids, which are attached to one another in long chains. There are 20 different types of amino acids that can be combined to make a protein. Proteins are very important in laboratory medicine because they constitute a major group of clinical diagnostic markers. Clinical laboratories test protein markers all the time for the diagnosis of many different diseases. For example, transferrin, which

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plays the essential role of transporting iron in human body, is a marker to diagnose iron metabolism disorders.

Bob Barrett: So why are we interested in seeing just a single molecule of protein? What's the advantage of single-molecule proteomics?

Ruben Luo: Well, that's a great question. As we know, today most existing clinical testing methods for protein markers are primarily based on superficial molecular features of proteins, such as specific reactivity for enzymatic assays or epitope binding for amino acids. Besides quantified proteins, these technologies provide little information about structural characteristics. Furthermore, the test results from all these conventional technologies represent an ensemble average of numerous molecules measured at once, which may not accurately reflect the possible heterogeneity of protein structural modifications, which are called proteoforms.

Therefore, the ultimate goal of protein measurement would be the ability to observe the complete primary structure of every single protein molecule along with its proteoforms. In addition, the term proteomics refers to the large-scale study of proteins. With the help of informatics, proteomics can integrate information from multiple different proteins to reveal underlying biopathways and disease mechanisms. Thus, proteomics, especially single-molecule proteomics, has a great potential in clinical diagnosis because it provides in depth information of the human body.

Bob Barrett: Let's talk about nanopore sequencing. How do these technologies work to measure single protein molecules?

Ruben Luo: Well, nanopore sequencing technologies utilize a biological or solid-state membrane where a nanopore is located. The nanopore is surrounded by electrolyte solution and a bias voltage is applied across it. So protein sequencing through a nanopore requires a denatured protein strand to pass the narrow nanopore in a predictable orientation, one amino acid at a time. The sequential conduction of individual residuals is associated with signature changes in the electric current across the nanopore, which can then be used to derive the complete amino acid sequence as well as post translation modifications of an intact single protein molecule. This is how nanopore protein sequencing works.

Bob Barrett: And what innovation in nanopore protein sequencing did you report on in your *Clinical Chemistry* article?

Ruben Luo: Well, the article is about the recent advancements in nanopore sequencing technologies that made a groundbreaking step towards single protein molecule measurement, as reported in the *Nature Biotechnology* paper

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by Professor Meni Wanunu's team at Northeastern University. The paper showcases a novel enzyme-free approach to thread an intact protein through a nanopore. Prior to this study, the development of nanopore protein sequencing often requires using the enzymes unfoldase and translocase to denature and guide protein molecules through the nanopore. However, this study showed that a high concentration guanidinium chloride buffer could both denature the protein and create a sufficiently strong electroosmotic force to drive it through an alpha-hemolysin nanopore. Thus, no enzyme is required.

The unidirectional transport was facilitated by the coating of guanidine ions near the mouth of the nanopore, which generated a local net positive charge to facilitate protein capture. As a proof of concept, they demonstrated successful denaturation and single molecule fingerprinting of an alpha helix rich maltose binding protein and stable beta barrel structured green fluorescent protein. In addition, a supervised machine learning model was implemented to differentiate mixtures of tact forms of these two proteins with over 90% accuracy.

Bob Barrett: And finally, Dr. Luo, how is this innovation likely to impact the field of nanopore protein sequencing?

Ruben Luo: Well, I think this innovative technique opens new avenues for enzyme-free nanopore protein sequencing. Particularly, there is another *Nature Biotechnology* paper by Professor Hagan Bayley's team at the University of Oxford, showing that guanidinium chloride-based electroosmotic flow through an engineered alpha-hemolysin nanopore could differentiate post-translational modifications on the protein. So remember, the post-translational modifications are another dimension of the protein structure information.

And in addition, there is a more recent *Nature Methods* paper reporting a new type of nanopore that is able to fully differentiate all 20 amino acids as well as some modified amino acids. These related works further enhanced the utility of this innovation and altogether laid a cornerstone in the maturation of nanopore protein sequencing. Although a substantial amount of progress is still needed to reach the final goal, we anticipate that it will not be long until structural characteristics of an intact protein molecule can be fully determined reliably using nanopore protein sequencing. So we foresee that the power of de novo protein sequencing and the digital quantitation by this technology will revolutionize the landscape of proteomics and clinical testing of protein markers.

Bob Barrett: That was Dr. Ruben Luo from Stanford Healthcare in Palo Alto, California. He served as senior author for a News & Views article describing nanopore protein sequencing in the

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February 2024 issue of *Clinical Chemistry*, and he's been our guest in this podcast on that topic. I'm Bob Barrett. Thanks for listening.

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