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Engineering of protein nanopores for sequencing, chemical or protein sensing and disease diagnosis

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Biological systems contain highly-ordered structures performing diverse functions. The elegant structures of biomachines have inspired the development of nanopores as single molecule sensors. Over the years, the utility of nanopores for detecting a wide variety of analytes have rapidly emerged for sensing, sequencing and diagnostic applications. Several protein channels with diverse shapes and sizes, such as motor channels from bacteriophage Phi29, SPP1, T3, and T4, as well as α-hemolysin, MspA, aerolysin, FluA, OmpF/G, CsgG, ClyA, have been continually investigated and developed as nanopores. Herein, we focus on advances in biological nanopores for single molecule sensing and DNA sequencing from a protein engineering standpoint for changing pore sizes, altering charge distributions, enhancing sensitivity, improving stability, and imparting new detection capabilities.

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Introduction

The intriguing design and elegant architectures of biological machineries have inspired the development of nanopores. The proof-of-concept was established in 1996 using a membrane-embedded α-hemolysin channel [1•]. Single-stranded DNA was electrically driven through α-hemolysin and the resulting translocation was verified by qRT-PCR. Over the last decades, nanopore has demonstrated potentials for sensing a wide range of analytes, and is well positioned to bring a revolution to medical diagnostics and DNA/RNA sequencing. Compared with other technologies, nanopore offers [2,3]: single molecule detection with high specificity and sensitivity; is label-free; is amplification-free; offers real time recognition; requires low sample volumes and minimal sample processing; rapid electrical detection method; high-throughput in nature; multiplexing capabilities; and requires no special expertise for operation.

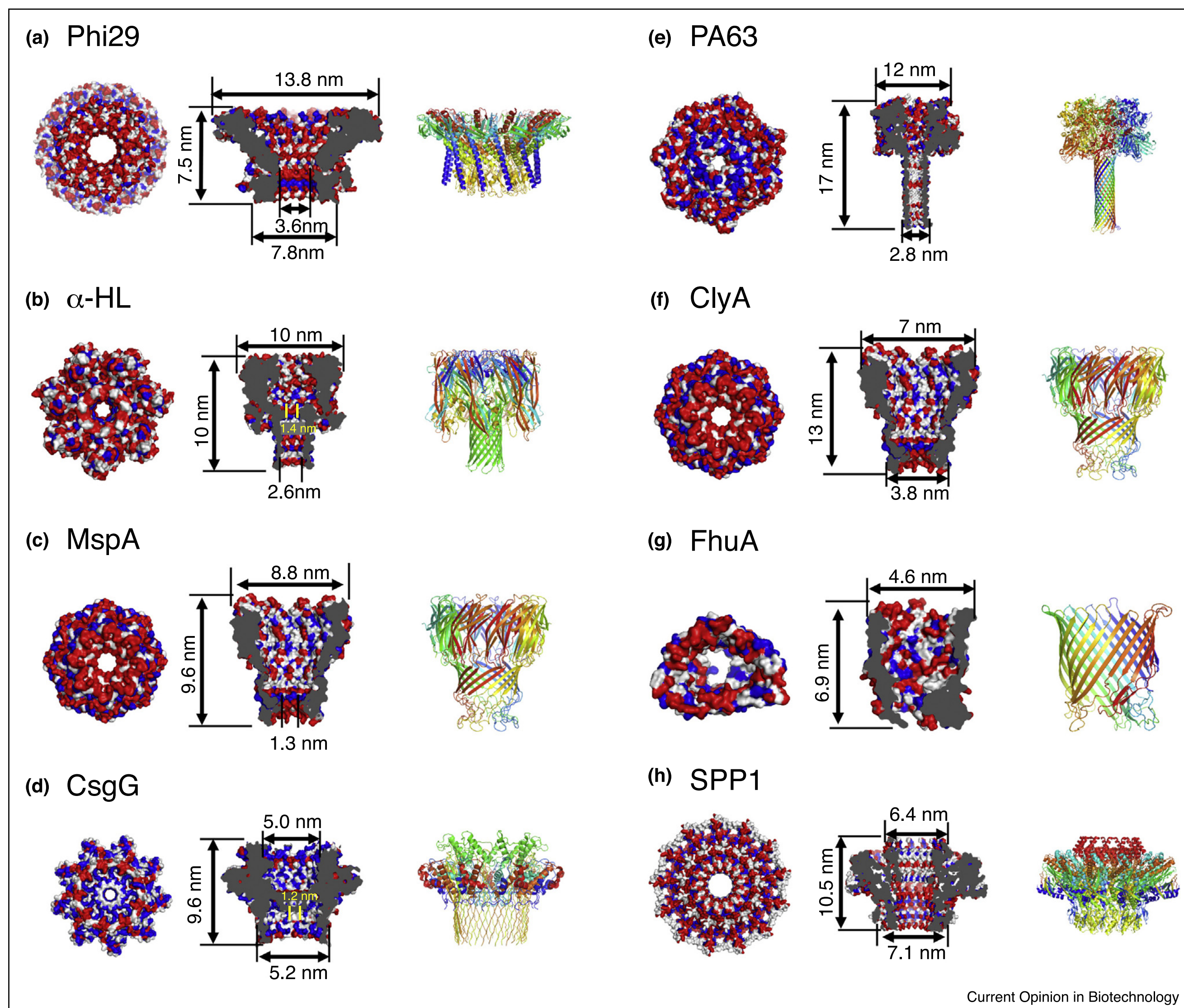
To date, several protein channels, such as α-hemolysin [4], MspA [5•], aerolysin [6], FluA [7], Omp F/G [8,9], CsgG [10], ClyA [11], PA_{63} [12] and, viral connectors phi29 [13•], SPP1 [14], T3 [14] and T4 [14] (Figure 1) have been successfully developed as nanopore for detection of small molecules, polymers, polypeptides, and DNA/RNA [2,15]. Protein engineering, such as site directed mutagenesis, insertion, and deletion of amino acids, and introduction of functional modules have been extensively employed to tune nanopore properties [16]. Different analytes require unique nanopores with different shape, size, and hydrophilic/hydrophobic properties in order to be detected with high sensitivity and specificity. Herein, we summarize progress in engineering protein nanopores for sensing and sequencing applications. Due to space limitations, we omitted discussion on DNA-based biological pores and synthetic pores, which have been reviewed elsewhere [17,18].

General strategies for engineering protein nanopore or channels

Among various channel forming proteins [16,19], only a handful are fit to serve as nanopores (Figure 1). A major limitation is the lack of crystal structures to guide engineering at specific sites. We also have limited knowledge of how the structure of the channel relates to its function. Generally, to be a good candidate, wild-type or engineered channel needs to meet at least four criteria: (1) robust with channel forming capability after mutagenesis; (2) appropriate geometry including the entrance, vestibule, and constriction; (3) proper charge and hydrophilic/hydrophobic surface; and (4) stable current with controllable voltage gating.

Several engineering approaches have been employed to change the intrinsic properties of nanopores for increasing the stability, changing the oligomeric state, introducing

Figure 1



Structure of commonly used biological nanopores. Bottom, cross-section and side views of: **(a)** phi29 connector (PDB: 1H5W), **(b)** α-hemolysin (PDB: 3ANZ), **(c)** MspA (PDB: 1UUN), **(d)** CsgG (PDB: 4UV3), **(e)** PA_{63} (PDB: 1V36), **(f)** ClyA (PDB: 2WCD), **(g)** FhuA (PDB: 1BY5), and **(h)** SPP1 connector (PDB: 2JES).

recognition sites, enhancing sensitivity, facilitate membrane insertion, and imparting selectivity for target analyte (Figure 2). The first approach is based on site-directed mutagenesis that substitutes specific amino acids at certain locations in the primary sequence. The availability of a wide range of unnatural amino acids with unique side chains and functional properties makes it possible to expand the scope of nanopore reengineering. Furthermore, mutations can result in conformational changes of the channel, making it possible to tune the channel size and to enhance the sensing scope and sensitivity. The second approach is to attach specific tags and enzymes (such as His-tag, Strep tag, or TAT as well as proteins like DNA polymerase, helicase and exonuclease) either through fusion protein expression or *in vitro* conjugation to the anticipated sites of the pore. The third approach is to introduce adapter ligands either covalently or non-covalently into the pore for substrate binding. The fourth approach involves more significant truncation or insertion of several amino acids to alter the chain segment aggressively.

Engineering protein nanopores or channels for DNA and RNA sequencing

α-Hemolysin

The idea of using nanopores for sequencing of an intact DNA strand was proposed three decades ago by David Deamer and Daniel Branton. Since the publication of the

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