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Methods for protein delivery into cells:

from current approaches to future perspectives

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Abstract

The manipulation of cultured mammalian cells by the delivery of exogenous macromolecules is one of the cornerstones of experimental cell biology. Although the transfection of cells with DNA expressions constructs that encode proteins is routine and simple to perform, the direct delivery of proteins into cells has a number of advantages. For example proteins can be chemically modified, assembled into defined complexes and subject to biophysical analyses prior to their delivery into cells. Here we review new approaches to the injection and electroporation of proteins into cultured cells. In particular we focus on how recent developments in nanoscale injection probes and localized electroporation devices enable proteins to be delivered whilst minimizing cellular damage. Moreover, we discuss how nanopore sensing may ultimately enable the quantification of protein delivery at single molecule resolution.

Abbreviations

AFM: atomic force microscopy

CRISPR/Cas9: Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR-associated protein 9

NES: nanostraws electroporation system

NanoEP: nanopore-electroporation

RPS: resistive pulse sensing

SICM: scanning ion conductance microscope

STIM1: stromal interaction molecule 1

Introduction

The controlled manipulation of cells by the intracellular delivery of exogenous macromolecules is a cornerstone of modern cell biology. Transfection of cultured mammalian cells with DNA expression constructs that encode proteins is routine in cell biology and enables a protein of interest to be studied in a cellular context [1, 2]. Whilst, convenient and simple to perform, transfection of cells with DNA expression constructs relies on the cell to produce the protein and as such it has its limitations. For example, it is difficult to control the amount of protein being expressed and the protein may fail to fold in the cellular environment, a problem exacerbated if the protein is expressed at high levels [3]. One alternative to using DNA expression constructs is the direct protein delivery into cells [4-6]. Unlike the use of DNA constructs, which typically require many hours before the expression of proteins can be observed, the direct delivery of proteins can induce more immediate phenotypic changes [7, 8]. Crucially, proteins can also be manipulated and characterized before they are delivered into cells. This could include chemical modification to add functional probes, the assembly of defined protein complexes and the analysis of protein structure using biophysical techniques, thus a direct correlation between a protein's structure and its cellular function can be determined [4-6]. Protein delivery also has many potential applications for therapeutics, although a discussion of this is beyond the scope of this article [4-6].

Challenges for protein delivery into cells

In order for proteins to be delivered into cells, they must first traverse the plasma membrane. However, the plasma membrane is a highly selective permeability barrier across which the vast majority of proteins cannot pass unaided [5, 9]. Conversely, permeabilizing the plasma membrane, with solvents like ethanol or detergents such as saponin, allows the free movement of molecules into cells, but lacks specificity

and can result in cell death [10, 11]. Thus, the challenge of protein delivery is how to get a specific protein across the plasma membrane to the desired intracellular location without harming the cell. A variety of methods have been developed to deliver proteins into cells [4, 5]. For example, proteins can be packaged inside carriers such as liposomes, which interact with the cell plasma membrane and then release the proteins into the cells [12]. Proteins can also be covalently conjugated to a cell-penetrating peptide which either directly penetrates the cell or is taken up by endocytosis when it interacts with the cell plasma membrane [13]. Whilst these methods can be easily adopted, there are concerns about the toxicity of the carriers [14], and whether cell penetrating peptides alter the structure and function of proteins. Furthermore, delivery via these methods can result in many of the proteins being encapsulated within endosomes and degraded in lysosomes [15]. This mini-review will discuss electroporation and injection, both of which disrupt the plasma membrane in order to facilitate the delivery of proteins and other molecules, directly into cells. In particular, we will focus on how nanoscale injection probes and localized electroporation devices can be used to deliver proteins into cells and how these techniques may ultimately enable the quantitative delivery of proteins into cells.

Injection of cells: from microscale to nanoscale probes

Injection involves the mechanical penetration of the plasma membrane with a needle-like probe through which molecules can enter the cell (

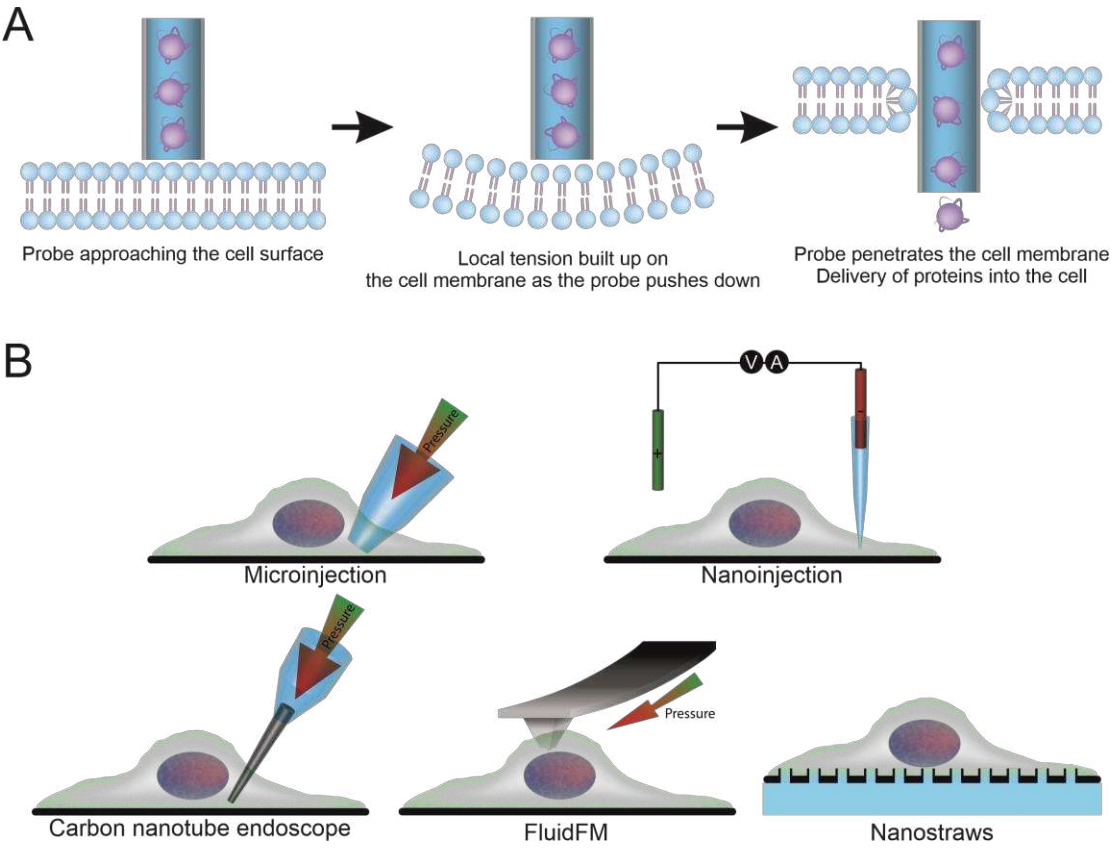

Figure 1A). Microinjection uses a conical micron-sized glass micropipette and a pressure controller, which controls delivery of material into the cell (Figure 1B) [16]. Positioning of the micropipette is controlled by a micromanipulator, which enables injection of cells at a defined site. For example, microinjection has been used to deliver antibodies and clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein 9 (CRISPR/Cas9) RNA-protein complexes, which are used for gene editing, into the nuclei of cells [17-21]. However, there are significant drawbacks for microinjection. Most notably post-injection cell survival rate is on average ~50%, although this can be improved by automating the injection process [22, 23]. One reason for the impact of microinjection on cells may be the large size of

the micropipette's tip relative to the cell. Indeed, injection of cells with a 1 µm diameter micropipette results in the significant perturbation of cells, with the majority of the actin filaments being disrupted and the cells develop a deformed morphology [24].

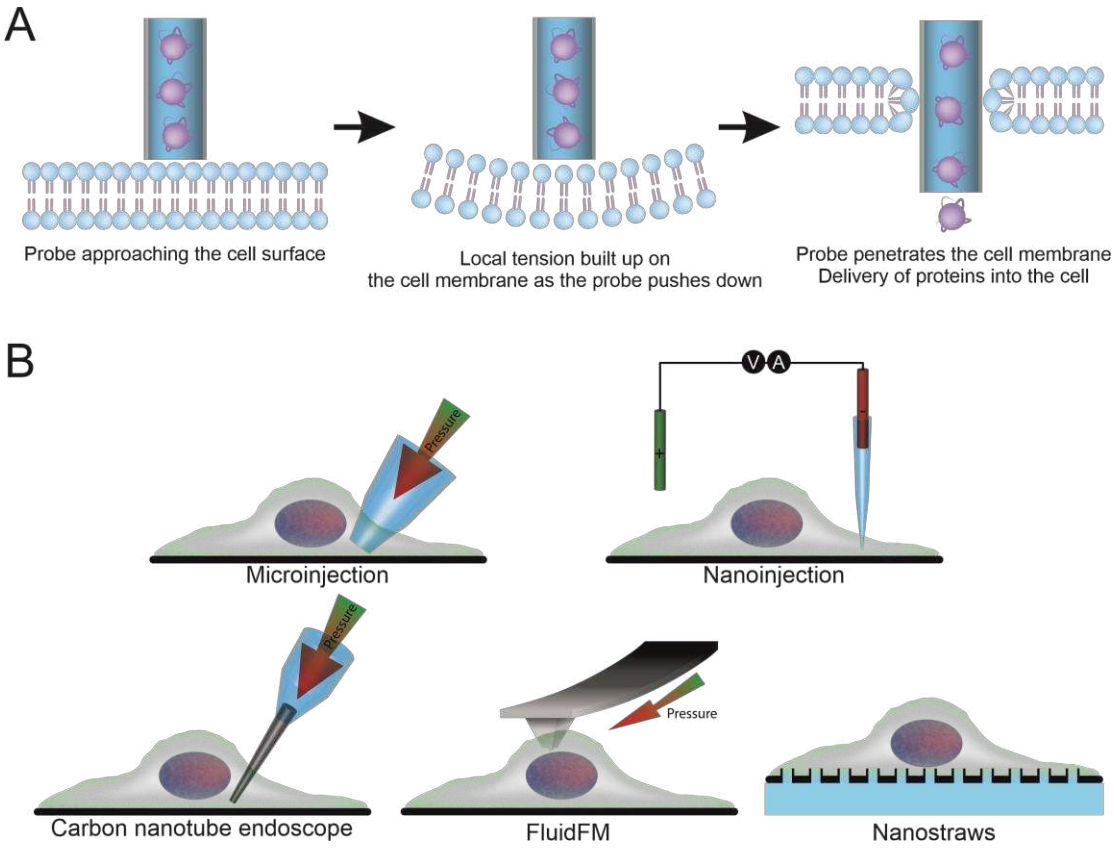

Figure 1. Injection of cells. (A) The mechanism of injection. The probe approaches the cell surface, it pushes down the cell membrane, leads to a build up of local tension, eventually penetrating the cell membrane, allows extracellular proteins and other molecules to access the intracellular space. (B) Schematic illustrations of the different methods for the injection of cells.

One strategy to minimize the cellular impact of the injection process is to reduce the size of the probe to the nanoscale (Figure 1B), which has been shown to improve the cell viability after injection [24-28]. One such probe-based device is the nanopipette, which is essentially a scaled down micropipette with a nanometer scale pore, a

nanopore, at its tip [28-32]. Nanopipettes can be easily fabricated from quartz capillaries with a laser puller and the pore size can be precisely adjusted within the 10 - 300 nm range. Nanopipettes can be integrated with nanomanipulators in a scanning ion conductance microscope (SICM), where the vertical position of the nanopipette with respect to surface can be controlled with nanometer precision [33, 34]. SICM has been successfully used for the high-resolution (20 nm) topographical imaging of living cells in culture [33-36]. The resultant topographical map can be then used to position the nanopipette anywhere on the cell membrane and precisely insert the nanopipette tip into the cell [28, 30, 37]. Because the nanopipettes are fitted with electrodes, the delivery of molecules into cells can be triggered by the application of a voltage of suitable polarity while achieving a high cell survival rate compared to micropipettes [28-30, 32]. Antibodies have been delivered into cells using this nanopipette based technique, demonstrating that it can be used for proteins [30].

Other nanoscale probe based devices have the potential to be used for protein delivery into cells. Carbon nanotube endoscopes are micropipettes with carbon nanotubes, with pore sizes of 50 – 200 nm, fitted onto their tips [24, 25]. Using a pressure controller to regulate delivery, quantum dots and fluorescent dyes can be delivered into cells with minimum disruption to the cytoskeleton and cell morphology [24, 25]. An alternative solution is based on the FluidFM platform, which is an adaptation of atomic force microscopy (AFM) in which the injection probe is a hollow AFM cantilever that can be manufactured with a pore diameter ranging from 10 nm to 10 µm 26, 38, 39]. Upon penetration of the plasma membrane, injection is pressure controlled and has been used to deliver DNA plasmids, fluorescent dyes and vaccinia virus [26, 38, 39].

Without automation, the injection of cells with nanoscale probes has a low throughput, as the user can only inject one cell at a time. One solution is to use

arrays of nanoscale probes to perform simultaneous injection of hundreds of cells at once [27, 40]. Nanostraws and carbon nanotubes are arrays of hollow elongated nanostructures which protrude from a polycarbonate or alumina membrane surface (Figure 1B) [27, 40]. They typically have a diameter of hundreds of nanometers or less and lengths on the micrometer scale and a density that can range from 0.01 to 1 straw per μm^2 [27]. Cells are grown on top of the nanostraws, which are connected to a fluidic reservoir that allows the delivery via fluidic pressure and concentration gradient diffusion of fluorescent dyes, membrane impermeable probes and DNA plasmids into the cells [27, 40, 41]. As such the platform has clear potential for use in the delivery of proteins into cells. Furthermore, nanostraws cause minimal perturbation of cells, as evidenced by the lack of any significant change in the expression of genes associated with stress responses [27].

Bulk and localized electroporation

In electroporation, a strong electric field temporarily permeabilizes the plasma membrane allowing the entrance into the cells of exogenous molecules present in the bath solution [42] (Figure 2A). Bulk electroporation is a well-established technique and typically involves the use of an electroporation cuvette in which there are parallel electrodes (Figure 2B). The cells are suspended in conductive buffer inside the cuvette and upon application of voltage an intense electric field is generated [5, 42]. The electric field combined with thermal fluctuations in the plasma membrane leads to the formation of membrane pores from 0.5 to 50 nm in diameter that allow proteins and other molecules to access the intracellular space [5, 43]. With a pulse time from microseconds to a second, the delivery window for proteins is very short as the membrane pores immediately shrink and reseal when the electric field is off [44]. Nonetheless, various proteins including antibodies, CRISPR/Cas9 RNA-

protein complexes and α-synuclein have been delivered into cells by bulk electroporation [45-49].

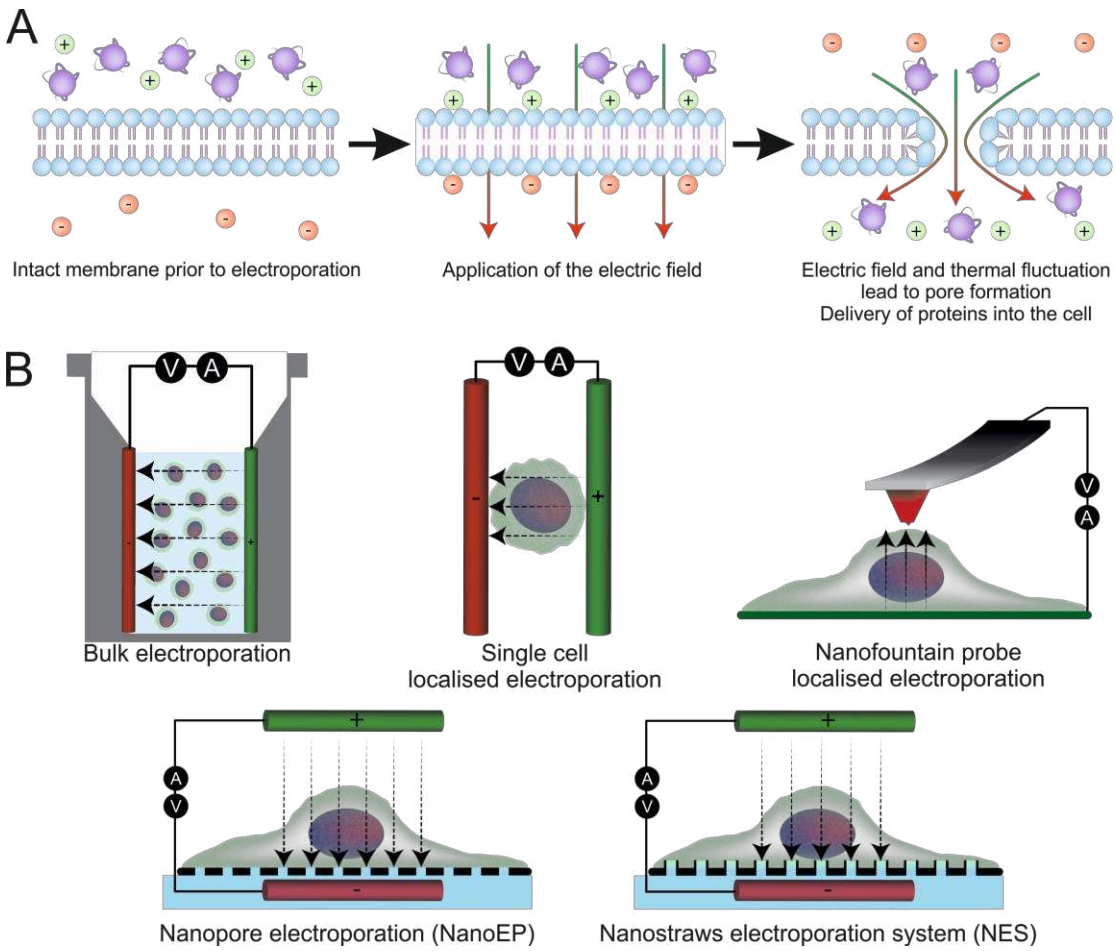

Figure 2. Electroporation of cells. (A) The mechanism of electroporation. An electric field is applied across the cell's plasma membrane, the electric field and membrane thermal fluctuation lead to the formation of transient pores, allowing access into the intracellular space of extracellular proteins and other molecules. (B) Schematic illustrations of the different electroporation methods.

Due to the large gap between the electrodes in a cuvette, a high voltage input has to be used in order to generate an electric field of sufficient strength to electroporate the cells [42]. This high input voltage leads to an increased temperature and pH changes

in the area close to the electrodes [50, 51]. Furthermore, the intense electric field causes lipid peroxidation and the generation of reactive oxygen species leading to the damage of proteins and DNA [52, 53]. As a consequence many cells die immediately after the procedure, whereas surviving cells have reduced viability and proliferative potential [54]. Developments in micro and nanofabrication technologies have enabled the production of localised electroporation devices in which there is much closer proximity between the cells and the electrodes than can be achieved in a cuvette. This allows the application of lower voltage without reducing the electric field strength. This approach minimizes heating and pH changes [55, 56]; as a consequence cell viability after electroporation and transfection efficiency are both increased [57-62].

A variety of methods have been developed that perform localized electroporation (Figure 2B). One way this can be achieved is by incorporating a microchip containing electrodes inside a microfluidic channel, in which the cells to flow through the channel whilst simultaneously being subjected to localized electroporation [63, 64]. This method has been shown to enable fluorescent dyes and siRNAs to be delivered into single cells as they pass through the electric field [63, 64]. Alternatively, a probe can be used to perform localized electroporation. The nanofountain system uses a hollow cantilever-based probe that is similar to that used in FluidFM [65]. In this approach the nanofountain probe is positioned in close proximity to the plasma membrane, then application of an electric field allows delivery of molecules, including the proteins bovine serum albumin, and CRISPR/Cas9 RNA-protein complexes, into cells [61, 65].

Array based methods can also be used to perform the localized electroporation whilst enabling the simultaneous electroporation of many cells. In the nanopore-electroporation (NanoEP) platform cells are deposited onto a polycarbonate

membrane that it contains 100 nm diameter nanopores with density of 0.2 pores per μm^2. The pores are connected to a liquid reservoir and cargoes can then delivered into numerous cells via localised electroporation via establishing an electric field [58]. NanoEP has been used to deliver a variety of cargoes, including mRNAs, DNA plasmids, CRISPR/Cas9 RNA-protein complexes and functional stromal interaction molecule 1 (STIM1) proteins into the cell cytoplasm [58]. The nanostraws electroporation system (NES) has essentially the same set-up as the nanostraws system described above, but the addition of electrodes in the liquid reservoir underneath the straws and in the cell culture medium reservoir allows localised electroporation to be performed [59, 66]. This increases the delivery efficiency over that of fluidic delivery and has been used to deliver proteins, CRISPR/Cas9 RNA-protein complexes and STIM1, into cells [59, 66].

Quantifying delivery with nanopore sensing

One key challenge that remains for protein delivery into cells is how to quantify the number of molecules that have been delivered. Current approaches are limited because they cannot quantify directly the number of molecules delivered. For example the fluid volume injected or duration of injection can be used to estimate the number of molecules delivered into a cell. Alternatively, fluorescence intensity can be used to estimate the number of fluorescently tagged molecules delivered into a cell. In contrast, nanopore sensing has the potential to perform the direct quantification of protein delivery at a single molecule level. Although, yet to be used for protein delivery into cells, a number of studies suggest clear potential for this approach.

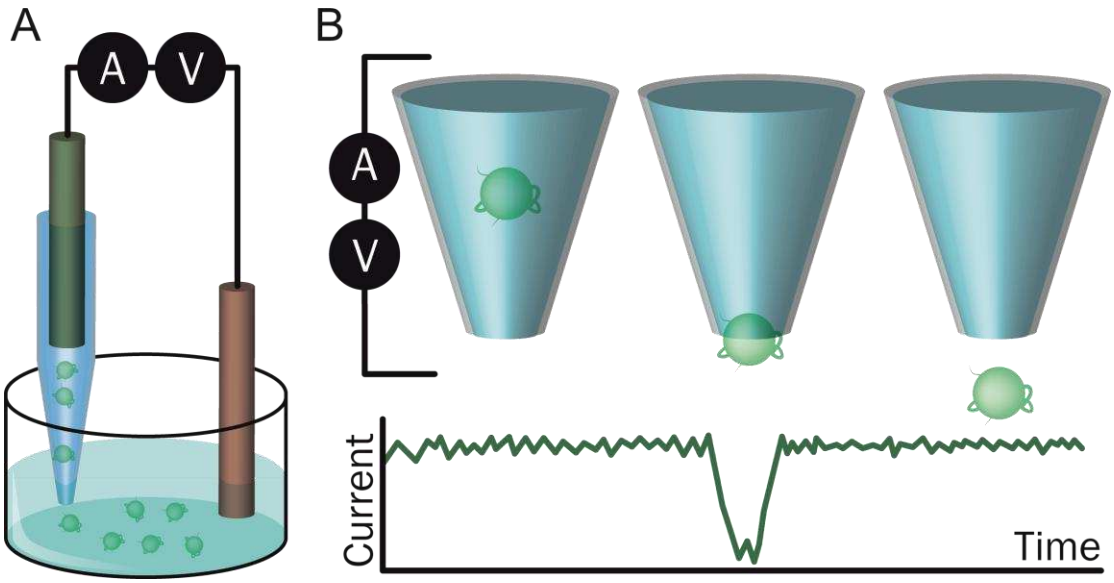

Figure 3. Nanopore sensing. (A) The schematic of the set-up of a single molecule nanopore sensor using the nanopipette as the example. An electric circuit is established by immersing two electrodes into the conductive electrolyte. The electrodes are separated by a nanopore, which in this example is at the nanopipette's tip. Molecules are driven towards the polar opposite electrode by the electric field. (B) Illustration of the generation of a resistive pulse sensing (RPS) signal. When a molecule passes through the nanopore there is an increase in resistance. This is detected as a drop in the current, with the duration of the drop corresponding to the time the molecule spends passing through the pore. Together these two parameters form the RPS signal.

Nanopore sensing requires that two electrodes in a conductive medium are separated by a nanopore (Figure 3A) [67]. Upon application of a voltage, molecules will move through the nanopore causing the temporary displacement of ions and disruption of the ion flow, leading to a change in the circuit resistance and formation of a resistive pulse-sensing (RPS) signal [67]. By counting the RPS signals the number of molecules that have passed through the nanopore can be quantified (Figure 3B) [67]. The detection of proteins does present a challenge however. Using various proteins including bovine serum albumin, β-galactosidase and streptavidin, it has been demonstrated that proteins pass through nanopores at a high speed, and

as a consequence they typically have a poor RPS signal to noise ratio when compared to nucleic acids [68]. Detection efficiency for proteins detection can be improved by reducing the diameter of the nanopore so it is comparable to the size of proteins which are typically less than 20 nm, and thus a prominent change in the ionic current can be observed [67, 69]. Furthermore, increasing solution viscosity has been shown to slow translocation speed and increase the RPS signal of DNA and can be adopted for protein sensing [70, 71]. Crucially, the cytoplasm of cells is a crowded and viscous environment [72, 73], thus nanopore sensing of molecules, including proteins, should be enhanced when they are delivered into cells by nanopore based devices.

To date two studies have demonstrated that nanopore sensing can quantify cellular delivery. The first used an optical tweezer to immobilize the cell on top of a 2 nm nanopore for the quantitative delivery of DNA plasmids [74]. In the other study, a NES-like system with 200 nm hollow electrodes was able to detect multiple RPS signals during the delivery of gold nanorods into cells [75].

Summary

In summary, the delivery of proteins directly into cells has a number of advantages over the expression of proteins using transfected DNA constructs. Bulk electroporation and microinjection are commonly used methods for delivery of proteins into cells. However, both methods can cause significant disruption to the cell, which can cause the death of many cells. By using nanoscale devices for injection and by performing localised electroporation, it is possible to reduce the harmful effects of delivering proteins into cells. Moreover, by applying nanopore

sensing to protein delivery, it may be possible to perform quantitative delivery of proteins into cells.

Perspectives

(i) Importance of the field

The delivery of proteins into cells is used to manipulate cells and enables the proteins to be studied in a cellular context.

(ii) Summary of current thinking

Two commonly used approaches are injection and electroporation, both of which disrupt the plasma membrane to facilitate the entry of proteins into cells. Microinjection and bulk electroporation of cells are highly invasive procedures and result in significant cellular damage and death. Nanoscale injection devices and localized electroporation can be used to deliver proteins into cells without causing significant cellular damage and death.

(iii) Future directions

Nanopore sensing has the potential to enable the number of proteins delivered into a cell to be quantified at single molecule resolution.

Author contribution

All authors wrote and corrected the manuscript.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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