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Commentary

Repurposing bacterial toxins for intracellular delivery of therapeutic proteins

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ABSTRACT

Despite enormous efforts, achieving efficacious levels of proteins inside mammalian cells remains one of the greatest challenges in biologics-based drug discovery and development. The inability of proteins to readily cross biological membranes precludes access to the wealth of intracellular targets and applications that lie within mammalian cells. Existing methods of delivery commonly suffer from an inability to target specific cells and tissues, poor endosomal escape, and limited *in vivo* efficacy. The aim of the present commentary is to highlight the potential of certain classes of bacterial toxins, which naturally deliver a large protein into the cytosolic compartment of target cells after binding a host cell-surface receptor with high affinity, as robust protein delivery platforms. We review the progress made in recent years toward demonstrating the utility of these systems at delivering a wide variety of protein cargo, with special attention paid to three distinct toxin-based platforms. We contend that with recent advances in protein deimmunization strategies, bacterial toxins are poised to introduce biologics into the inner sanctum of cells and treat a wealth of heretofore untreatable diseases with a new generation of therapeutics.

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1. Introduction

In the past 30 years, since the approval of recombinant human insulin, protein-based therapeutics have grown in prominence and are now among the fastest-growing class of drugs [1]. In 2015, 13 new biologics were approved by the FDA, representing 29% of all approved drugs [2]. In the same year, seven of the top 10 best selling drugs were biologics, led by AbbVie's Humira, which brought in $14 billion (USD) in sales [3]. Therapeutic proteins and peptides comprise ~85% of the approximately 300 biologics that are currently approved for clinical use [4]. Despite this dramatic expansion in large-molecule therapeutics, the full potential of proteins as drugs is constrained by their inability to cross biological membranes and enter cells. As such, approximately 60% of all human proteins – and the entire human genome – remain inaccessible to protein-based drugs [5]. Intracellular targets with a clear therapeutic rationale, including protein-protein interactions, large protein complexes, and important targets that are notoriously difficult to target with small molecules such as p53 and RAS [6,7], are best suited to biologics-based therapies. Moreover, enzymes with tremendous therapeutic potential including genome editing machinery such as CRISPR/Cas9 or TALENs must be delivered as nucleic acids or electroporated into cells, complicating their use as therapeutics.

To solve this issue, several classes of protein carriers have been explored to circumvent the barrier posed by the plasma membrane, however, to date, no single platform has been identified that is simultaneously efficient, safe, versatile and specific. We contend that bacterial toxins, which bind specific cells and can efficiently transport a broad spectrum of proteins to the cytosol have great promise as platforms for delivering protein cargo into specific cells *in vitro* and *in vivo*. Notably, the one class of biologics that enter cells as part of their mechanism is immunotoxins, which use the cell-penetrating machinery of bacterial toxins. The field of protein delivery has greatly matured in the past several years, and novel protein engineering techniques combined with improved methods for definitively detecting cargo in the cytosol have set the stage for bacterial toxins to emerge as a major platform to deliver the next generation of biologics.

2. Existing delivery platforms

Cell-penetrating peptides (CPPs) have been used to deliver proteins of various sizes, as well as diverse nucleic acids and even liposomes and nanoparticles [8,9]. As a group, CPPs generally show varying efficacy based on the CPP used, the cell type to be targeted, and even buffer conditions [10]. The mechanism by which CPPs enter cells has not been fully elucidated, however there is evidence that CPPs can either directly cross the plasma membrane, or gain entry via endocytosis and subsequent escape from endosomes [11]. One or both of these mechanisms may be used depending on concentration, temperature, and the choice of counter ion [11]. Engineered supercharged proteins such as +36 GFP are highly positively charged and appear to transport protein and nucleic acid cargo into cells by a mechanism that is similar to CPPs [12]. While generally effective *in vitro*, CPPs suffer from a lack of cell/tissue specificity, which contributes to variable efficacy *in vivo* [13,14].

Virus-like particles (VLPs) are a loosely-defined group of self-assembling structures based on viral proteins. VLPs of diverse structures can be assembled from various mammalian, plant, microbial and insect viruses, and are replication deficient, as they lack any genetic material [15]. VLPs are able to encapsulate protein cargo fused with viral proteins, or express them on their surface as is often done in the case of VLP-based vaccines [16]. It has been demonstrated that certain enveloped VLPs (HSV-1, HIV-1, etc.) deliver their encapsidated cargo to the cytosol by direct membrane fusion, whereas other non-enveloped viruses have complex endosomal escape mechanisms that are not completely understood [17,18]. While VLPs are perfectly suited for vaccine development, their strong induction of both humoral and cellular immune responses likely restricts their *in vivo* use [15].

Liposomes and nanoparticles are broadly similar carrier systems that have been explored for delivery. Liposomes and nanoparticles typically do not have built-in mechanisms for endosomal escape, although attempts have been made to decorate the surface of nanoparticles or liposomes with CPPs or fusogenic peptides/lipids to increase cytosolic delivery [19,20]. While both classes can be targeted to specific cells by displaying antibodies or other targeting moieties on their surface, achieving high surface densities can be challenging, and even when successful can result in faster plasma clearance [21,22]. *In vivo* toxicity of nanoparticles has been described depending on the nanomaterials involved; liposomes generally do not show overt toxicity in animal models but can be genotoxic even at low doses, as well as being immunogenic to varying degrees based on the specific lipids used [23,24].

Each of the above protein delivery systems possess one or more of the ideal attributes, however, no single system has the full complement of the features that would be desired in an idealized delivery platform. Bacterial toxins, which have evolved the ability to bind host cell receptors, and subsequently escape from endosomes to the cytosol of mammalian cells, are replete with features that make them attractive candidates for delivery vectors. Anthrax toxin (*B. anthracis*), exotoxin A (*P. aeruginosa)* and diphtheria toxin (*C. diphtheriae*) are three different bacterial toxins that use three distinct strategies to deliver their catalytic cargo into cells. The modular structure of these toxins, with three exchangeable functional domains, makes them particularly amenable to protein engineering. The major perceived limitation of using toxins in humans is the immune response, however recent work discussed herein suggests that this is manageable and no longer a hurdle to development.

3. Toxin cell entry mechanisms: three distinct strategies

Bacterial exotoxins are secreted proteins that are designed to specifically bind, enter, and damage host tissue. The so-called "AB class" of toxins are of interest to the field of protein delivery, as they are autonomous delivery vectors; that is, AB toxins possess all the components necessary to gain access to the cytosol independent of any other bacterial machinery. Members of this class all minimally consist of an enzymatic A moiety and a B moiety that both binds a cell-surface receptor and mediates entry into cells. The A and B fragments are typically linked by a furin-cleavable linker and a disulfide bond [25]. AB toxins bind to a cell surface receptor, which triggers endocytosis, transporting the toxin into the early endosome for sorting. From here, some toxins are trafficked to the endoplasmic reticulum (ER) where they undergo retrograde translocation, while others escape directly from the endosome (Fig. 1).

Among the toxins that can escape directly from endosomes, two distinct classes of AB toxins – exemplified by anthrax toxin and diphtheria toxin – are observed. In the case of anthrax toxin-like platforms, the A and B fragments are independent entities that associate non-covalently upon oligomerization of the B-fragments, whereas for diphtheria toxin-like platforms, the A and B fragments are encoded on the same polypeptide. The translocation pores created by these two types of toxins are also distinct. Whereas the anthrax toxin pore spans the endosomal membrane as a rigid β-barrel, diphtheria toxin creates flexible α-helical pores across the membrane. Importantly, the properties of these two