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Research review paper

Microbial carbohydrate-binding toxins – From etiology to biotechnological application

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ABSTRACT

Glycan-recognizing toxins play a significant role in the etiology of many diseases afflicting humanity. The carbohydrate recognition domains of these toxins play essential roles in the virulence of many microbial organisms with multiple modes of action, from promoting pore formation to facilitating the entry of toxic enzymatic subunits into the host cell. Carbohydrate-binding domains with an affinity for specific glycan-based receptors can also be exploited for various applications, including detecting glycobiomarkers, as drug delivery systems, and new generation biopharmaceutical products and devices (e.g. glycoselective capture of tumor-derived exosomes). Therefore, understanding how to efficiently express and purify recombinant toxins and their carbohydrate-binding domains can enable opportunities for the formulation of innovative biopharmaceuticals that can improve human health. Here, we provide an overview of carbohydrate-binding toxins in the context of biotechnological innovation. We review 1) structural characteristics concerning the toxins' mode of action; 2) applications and therapeutic design with a particular emphasis on exploiting carbohydrate-binding toxins for production of anti-tumor biopharmaceuticals; discuss 3) possible ways to manufacture those molecules at a bioreactor scale using microbial expression systems, and 4) their purification using their affinity for glycans.

1. Background

Applied glycoscience is a broad, interdisciplinary field in which the interactions of carbohydrates with proteins are used to provide innovative food, biomaterial, and biopharmaceutical solutions to critical social challenges. Glycoproteins often exhibit disease-associated changes in their glycosylation, for example, during cancerogenesis, and as a result, can act as biomarkers for medical diagnostics (Atsushi et al., 2019). Some bacterial toxins have evolved to recognize specific glycoproteins and glycolipids on the cell surface (Springer and Gagneux, 2013), as the first step for delivering their toxic payload. These toxins are often stable proteins that are amenable to protein engineering and thus present a perfect opportunity for exploitation in innovative applications such as novel diagnostic tools (Palomar et al., 2017), recognition of glycobiomarkers (Khatib et al., 2003), therapeutics and platforms for vaccine and adjuvant design (Antonio-Herrera et al., 2018).

The growing interest in applications of carbohydrate-binding toxins has led to a need for efficient manufacturing processes (Corolleur et al., 2020) for scalable production of carbohydrate-binding proteins to support the above-mentioned therapeutic approaches. Microbial-derived proteins are most effectively manufactured using a microbial expression system, typically *Escherichia coli* (*E. coli*) (Castiñeiras et al., 2018 and Rosano and Ceccarelli, 2014).

In this review, we will focus on the following key topics:

i) Introduction to carbohydrate-binding toxins
ii) Status of toxin-based biopharmaceuticals currently in clinical studies or available on the market
iii) Use of toxins in developing tumor- and vaccine-directed pharmacological agents

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iv) Choosing the best production regime for making relevant quantities of the desired toxins
v) Purification strategies that exploit the toxins' affinity towards different carbohydrates

To enable the production of carbohydrate-binding toxins in *E. coli*, upstream and downstream modules that provide suitable expression and purification strategies need to be coherent. This necessitates the development of a scalable system for the production of toxins that allows scale up from shake flask, through production in lab-scale bioreactors, to beyond pilot-scale (>100 L). Complementary to this, downstream purification must facilitate straightforward batch to batch reproducibility. Issues with manufacturability of these toxins are often related to their inherent properties, thereby being partly or fully toxic to the host organism (in this case, *E. coli)* (Saida et al., 2006), which can cause partial or even full cell lysis and tends to be a challenge for the entire manufacturing line.

2. Classification of microbial carbohydrate-binding toxins

2.1. Extracellular toxins

The classification of microbial carbohydrate-binding toxins is closely related to their structure, mode of action, and the location where the toxin acts (Chaudhuri and Chatterjee, 2009). Some bacterial toxins interact with a receptor to either form a pore in the membrane (extracellular toxins) or enter the cell by endocytosis (intracellular toxins) (Fig. 1).

Only a few extracellular toxins specialized in membrane damage express carbohydrate-binding domains that recognize a glycosylated receptor prior to inducing pore formation at the plasma membrane. Such carbohydrate-binding toxins are understudied and thus often neglected in novel applications. They are capable of pore-induced cell killing due to disruption of cell membrane integrity. Typically, they 1) recognize specific N-glycan receptors, 2) undergo proteolytic cleavage that enables oligomerization, 3) insert their β-barrel (stem) into the membrane to complete pore formation, and 4) deliver sub-components to the target cells or induce colloid-osmotic lysis (Olson and Gouaux, 2005). *Vibrio cholerae* cytolysin/hemolysin (VCC) and its ortholog *Vibrio vulnificus* hemolysin (VVH) are two of the few pore-forming toxins with carbohydrate-binding domains. VCC/VVH make erythrocyte membranes permeable to sodium, which results in swelling and rupture of mammalian erythrocytes (Mukherjee et al., 2016). VCC/VVH is initially expressed as the pro-toxin with its N-terminal pro-domain acting as a chaperone. The pro-toxin interacts with its N-glycan receptor on the erythrocyte membrane. After the pro-domain is cleaved off, it forms a β-barrel by the conformational lockdown of its cytolysin domain. To complete pore formation, it inserts itself into the membrane. The main difference between these two *Vibrio* hemolysins is that the VCC molecule recognizes complex N-glycans, preferably with heptasaccharide *N*-acetylglucosamine4Mannose3 (GlcNAc4Man3) NGA2 core. In contrast, the VVH preference is for GalNAc and LacNAc (Kaus et al., 2014; Kashimoto et al., 2017, and Levan et al., 2013).

Pore formation by membrane-damaging toxins is a property that has been successfully integrated into diverse biotechnological applications (Fig. 1). For example, the transmembrane delivery system is used to open the pore on command after interaction with ions, proteins, or other triggers (Bayley, 1995). They have also been used as stochastic sensors for single-molecule detection of ions passing through the membrane and as DNA/RNA translocation systems to detect unlabelled polynucleotides (Russo et al., 1997; Braha et al., 2000; He et al., 2013, and Gu et al., 2000). Pore-forming toxins that are biochemically triggered by ions, proteins, or other molecules can function as transmembrane channels and selectively deliver target molecules, such as carbohydrates or nucleotides, across the membrane. Depending on the application, these delivery systems can be integrated into a cell membrane or synthetic membrane systems, such as vesicles. Most applications of pore-forming toxins have been based on the famous α-hemolysin nanopore commonly used for DNA sequencing (Wang and Gu, 2015). However, the VCC/VVH molecules show potential in the design of future glycan-based

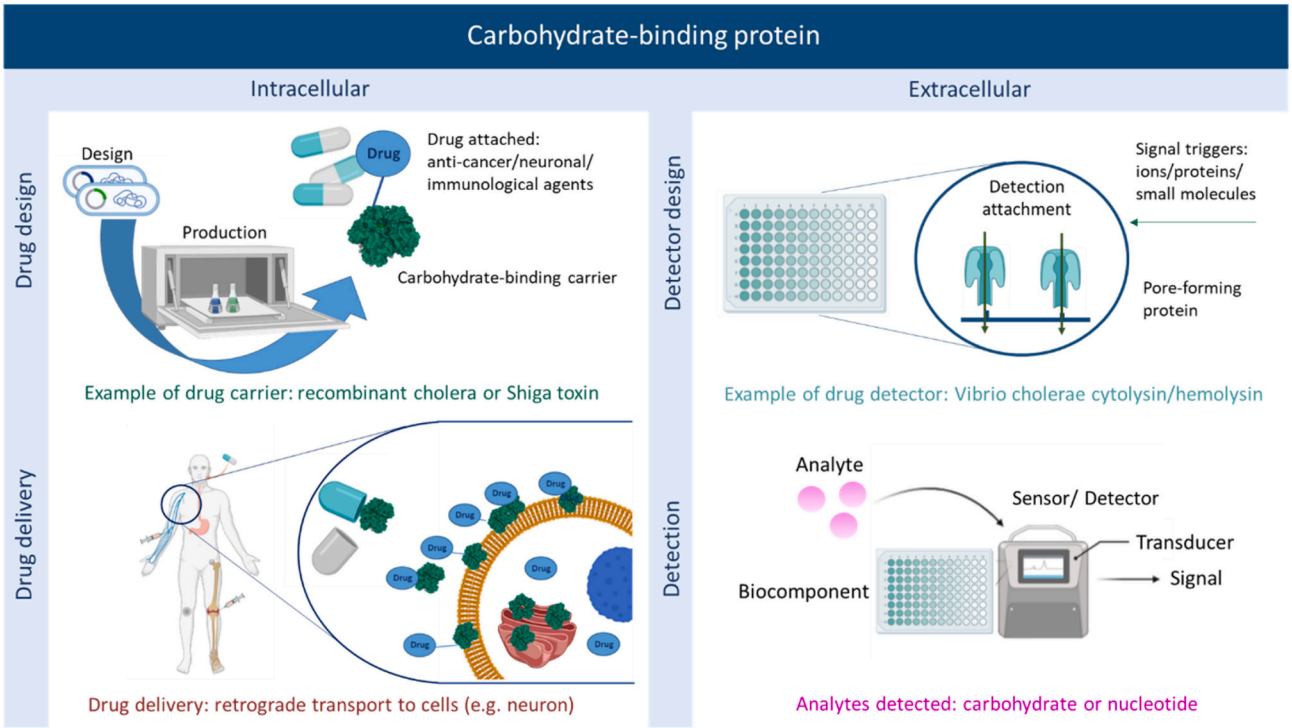

Fig. 1. Applications of different extracellular and intracellular carbohydrate-binding bacterial toxins. Intracellular toxins carry a drug (anti-cancer, neuronal and immunological agents) into cells (e.g. neuron, cancer or immune cells) used as a re-engineered drug-delivery platform. Pore-forming extracellular toxins triggered by signal (ions, protein or small molecules) activate transmembrane delivery of analyte (carbohydrate or nucleotide) used as sensor/detector system.

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2.2. Intracellular toxins

Intracellular acting toxins are mostly represented by the AB-toxins composed of an A-domain that facilitates an enzymatic reaction inside the cytosol and a B-domain responsible for recognizing species residing on the target membrane. A significant family of AB-toxins has five copies of the B-subunit protomer, making the AB_5 toxins (Beddoe et al., 2010; Fig. 2). While most AB-toxins require a carbohydrate-binding receptor domain for cell entry, some of them, like diphtheria toxin, rely mostly on protein-protein interactions for cell binding (Falnes and Sandvig, 2000). The mode of AB-toxin intracellular trafficking can vary from penetration of cellular membranes in endosomes (e.g., botulinum toxins; Lacy and Stevens, 1998; Fig. 2), to retrograde transport to the ER where translocation of the A-subunit to the cytosol occurs. From a functional perspective, intracellular bacterial toxins can be divided into ADP-ribosyl transferases and non-ribosylating toxins subgroups.

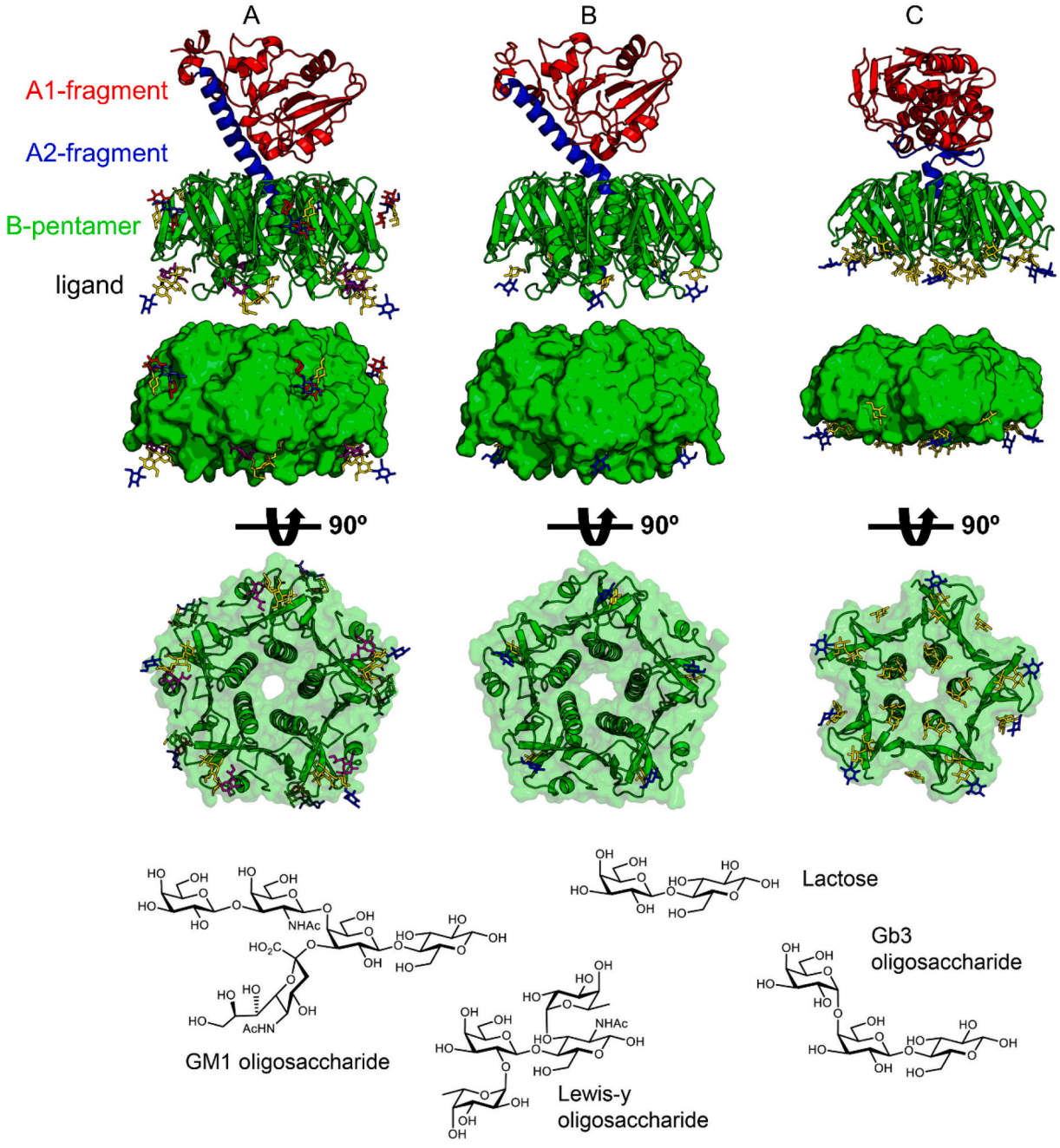

Fig. 2. Crystallographic structures of intracellular toxins, with ligand coloured according to Symbol Nomenclature for Glycans (SNFG). A) CTx holotoxin (PDB: 1S5E, 3CHB, 5ELC) with A1- (red) and A2- (blue) domains in complex with the pentameric B-subunit (green) interacting with GM1 pentasaccharide (PDB: 3CHB) at the bottom of CTxB as the primary binding site and with $Lewis^y$ (PDB: 5ELC) at the side of B pentamer as the secondary binding site B) LTx-I holotoxin (PDB: 1LTI, 1LTT) composed of A1- (red) and A2- (blue) domains in complex with the pentameric B-subunit (green) interacting with lactose (PDB: 1LTT) C) STx holotoxin (PDB: 1DM0, 1BOS) with A1- (red) and A2- (blue) domains in complex with the pentameric B-subunit (green), with 15 binding pockets occupied with Gb3 analogue 8-(methoxycarbonyl)octyl trisaccharide (Pk-MCO) (PDB: 1BOS) which obtains the same carbohydrate terminus with Gb3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2.1. ADP-ribosyl transferases

Cholera toxins from *V. cholerae* (CTx) are the classic examples of the AB_5 family. They are composed of a toxic A-subunit and a non-toxic and carbohydrate-binding pentameric B-subunit (CTxB) (Pierce et al., 1971). CTxB can bind to ganglioside GM1 (Galβ1 → 3GalNAcβ1 → 4(Neu5-Acα2 → 3) Galβ1 → 4Glcβ1 → ceramide) to induce endocytosis. Based on crystal structure analyses, the major contacts between CTxB and GM1 involve the ganglioside terminal galactose (Fig. 2). In addition to the ganglioside GM1 receptor, it is also known that fucose can bind to CTxB (Mertz et al., 1996), and it has been shown that fucosylation on *O*-linked glycans can mediate CTxB internalization and intoxication to human colonic epithelial cells (Wands et al., 2015); Cervin *et., al* 2018 indicated the ability of CTxB binding to $Lewis^x$ and $Lewis^y$, types of fucosylated histo-blood-group-related antigen (HBGAs), inducing intoxication in cells and mices with GM1 lacking context. It was not long before the co-crystallization structure between CTxB and $Lewix^x$, L-fucose, and glucosyl-GM1os was solved to high resolution (1.5–2.0 Å) and evidenced the existence of secondary binding sites in which $Lewis^x$ exhibits a weaker binding than $Lewis^y$, and the interaction was mediated by fucose (Fig. 2). indeed, ganglioside GM1 is not essential for CTx-mediated toxicity, and intoxication can occur via single GM1 binding site or non-GM1 receptors (Jobling et al., 2012; Cervin et al., 2018).

The A-subunit is composed of an A1-domain with enzymatic activity and an A2-linker peptide (Fig. 2). The wedge-shaped A1-domain is tethered to the A2-linker on the B-pentamer face distal to the GM1-binding sites. The A2-linker also contains a C-terminal KDEL sequence that directs the toxin to the endoplasmic reticulum (ER). Protein-disulfide isomerase (PDI) reduces the disulfide bond between Cys 187 and Cys 199 to release the A1-domain. The A1-domain unfolds and is retro-translocated via the Sec61 complex to the cytosol. The A1-domain catalyzes the transfer of ADP-ribose from NAD+ to $G_{s\alpha}$, thus triggering adenylate cyclase (AC) to increase intracellular cAMP levels. Consequently, cyclic AMP-dependent ion channels allow the influx of intestinal sodium and water into the gut lumen, causing diarrhea (Zhang et al., 1995 and Vanden Broeck et al., 2007).

Enterotoxigenic *Escherichia coli* (ETEC) strains produce a heat-labile toxin (LTx) that is analogous to CTx (Fig. 2). Heat-labile enterotoxins are classified into two groups: LTx-I and LTx-II (Pickett et al., 1986). LTx-I is expressed by *E. coli* strains isolated from humans (LTxh) and piglets (LTxp), respectively (Geary et al., 1982), and both molecules share 80% sequence identity with CTx (Sixma et al., 1992). Although some immunological differences were found between LTxh and LTxp (Honda et al., 1981), they are both similar to CTx in terms of their ligands (Holmgren, 1973; Holmner et al., 2007; Mandal et al., 2012; Heggelund et al., 2016), structure (Dallas and Falkow, 1980), functions (Spangler, 1992), and immunological cross-reactivity (Gyles and Barnum, 1969; Gyles, 1974). LTx-II was originally found in *E. coli* SA 53 isolated from water buffalo in Thailand, and rarely infects humans (Seriwatana et al., 1988). It also has two sub-types (a and b), neither of which can be neutralized by hyperimmune antisera against CTx or LTx-I (Green et al., 1983). LTx-I binds to GM1 ganglioside, but it also recognizes lactose (Galβ1-4Glc) (Shida et al., 1994) and LacNAc structures (Heggelund et al., 2019). LTx-IIa mainly binds to G_{D1a} and G_{T1b} and LTx-IIb interacts promiscuously with G_{d1a}, G_{T1b}, G_{Q1b}, GM1, G_{D2}, G_{M2}, and G_{M3} (Holmgren, 1973). LTx-IIb interaction is predominantly dependent on N-Acetylneuraminic acid (Neu5Ac) which accounts for lower specificity for different gangliosides (Zalem et al., 2016). The LTx-I and LTx-II catalytic A1-subunits share a 63% sequence identity (Pickett et al., 1987), but the LTx-I B-subunit has no detectable sequence similarity with LTx-II B-subunit (van den Akker et al., 1996). This explains why LTx-II is specific for different ligands from LTx-I, which heavily influences the versatility of purification methods among members in the LTx family.

Bordetella pertussis toxin (PTx) share common folds to LTx and CTx in the A1-domain region (Stein et al., 1994). Unlike other AB-toxin class members, PTx shows no distinction between A1- and A2-domain in the catalytic region of A-subunit. PTx exotoxin is composed of 6 subunits, called *S1-S5* with *S4*-subunit complexed as a duplicate. The active site remains homologous to other ADP-ribosylating bacterial toxins (e.g., LTx). Carbohydrate-binding oligomeric B-subunit (*S2-S5*-subunits) has a heterogeneous domain composition and resembles the symmetrical B-subunit of the CTx and STx (Fig. 3), but it interacts differently with the A-subunit (*S1*-subunit). In PTx, the A-B interaction is promoted by the carboxyl terminus of the A-subunit, and it only penetrates halfway through the oligomeric B-subunit. This interaction is supported by the A-subunit orientation towards the flat surface of B-subunits that rests against it. Some pathogenic extra-intestinal *E. coli* species were also reported to express pertussis-like toxins, called *Ec*Plt (Littler et al., 2017). The toxins share similarities with typhoid, and other ADP-ribosyl transferases toxins (ArtAB) observed in *Salmonella* species (Tamamura et al., 2017). The fundamental similarity is the B-subunit's avidity for branched glycans, mainly with *N*-acetylneuraminic acid (Neu5Ac) termini.

2.2.2. Non-ADP-ribosyl transferase AB_5 toxins

The existence of Shiga toxin (STx) produced by *Shigella dysenteriae* (*S. dysenteriae*) was first reported in 1903 (Conradi, 1903). It also belongs to the AB_5 family of bacterial toxins and shares a similar overall architecture with CTx and LTx (Fig. 2). The STxB pentamer (STxB) is composed of five identical fragments and it has up to 15 binding sites primarily for the glycosphingolipid Gb3 (Galα1 → 4Galβ1 → 4Glcβ1 → ceramide) (Paton and Paton, 2010). STxB binding to and clustering of Gb3 results in negative membrane curvature and leads to the formation of tubular membrane invaginations as the initial step of its cellular uptake (Römer et al., 2007; Römer et al., 2010; Windschiegl et al., 2009). The STx in many ways resembles CTx, with an enzymatic A-subunit composed of A1- and A2-domains which remain attached by a disulfide bridge following proteolytic cleavage (cell entry process). In the case of STx, the A-subunit is cleaved by furin in the Golgi apparatus (Fraser et al., 1994). The STx intracellular journey continues from the *trans*-Golgi network to the ER, where the A-subunit disulfide bond is reduced, and A1 is freed to undergo retro-translocation to the cytosol (Melton-Celsa, 2014 and Johannes and Römer, 2010). The A1-domain is thought to be protected from ubiquitin-mediated protein degradation in the proteasome because it has few lysine residues (Hazes and Read, 1997). The A1-domain has an N-glycosidase activity that cleaves an adenine base from ribosomal RNA (rRNA) and thus inhibits elongation factor-dependent aminoacyl tRNA binding, which effectively inhibits the host cell protein synthesis and proliferation. Through understanding its mechanism of action, STx has become a powerful tool to determine cellular processes and intracellular trafficking (Johannes and Römer, 2010).

Apart from STx, several *E. coli* and *Vibrio cholerae* strains also produce so-called Shiga-like toxins (SLTx), which share structural and immunological similarity with STx (O'Brien et al., 1984a). The *E. coli* O157:H7 strain was known to carry two toxin-converting bacteriophages – 933 J and 933 W (O'Brien et al., 1984b) — and the toxins produced accordingly were referred to as Shiga-like toxin 1 (SLTx-1) and Shiga-like toxin 2 (SLTx-2) (Strockbine et al., 1988). SLTx-1 and SLTx-2 exhibit only 58% similarity in nucleotide and 56% in amino acid sequences (Jackson et al., 1987), STx and SLTx1 are identical except for threonine at position 45 in the A-subunit (Strockbine et al., 1988). The homopentameric B-subunit expressed by SLTx-1 (SLTx-1B) recognizes Gb3 Pk-trisaccharide and Gb4 P-tetrasaccharide (GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glc → ceramide) with and without the presence of the ceramide lipid moiety. The homopentameric B-subunit expressed by SLTx-2 (SLTx-2B) does not recognize P or Pk oligosaccharide alone but can bind to the full form of Gb3 or Gb4 glycolipids (Gallegos et al., 2012). This is important for developing diverse treatment strategies effective against both *Shigella* or *E. coli* producing

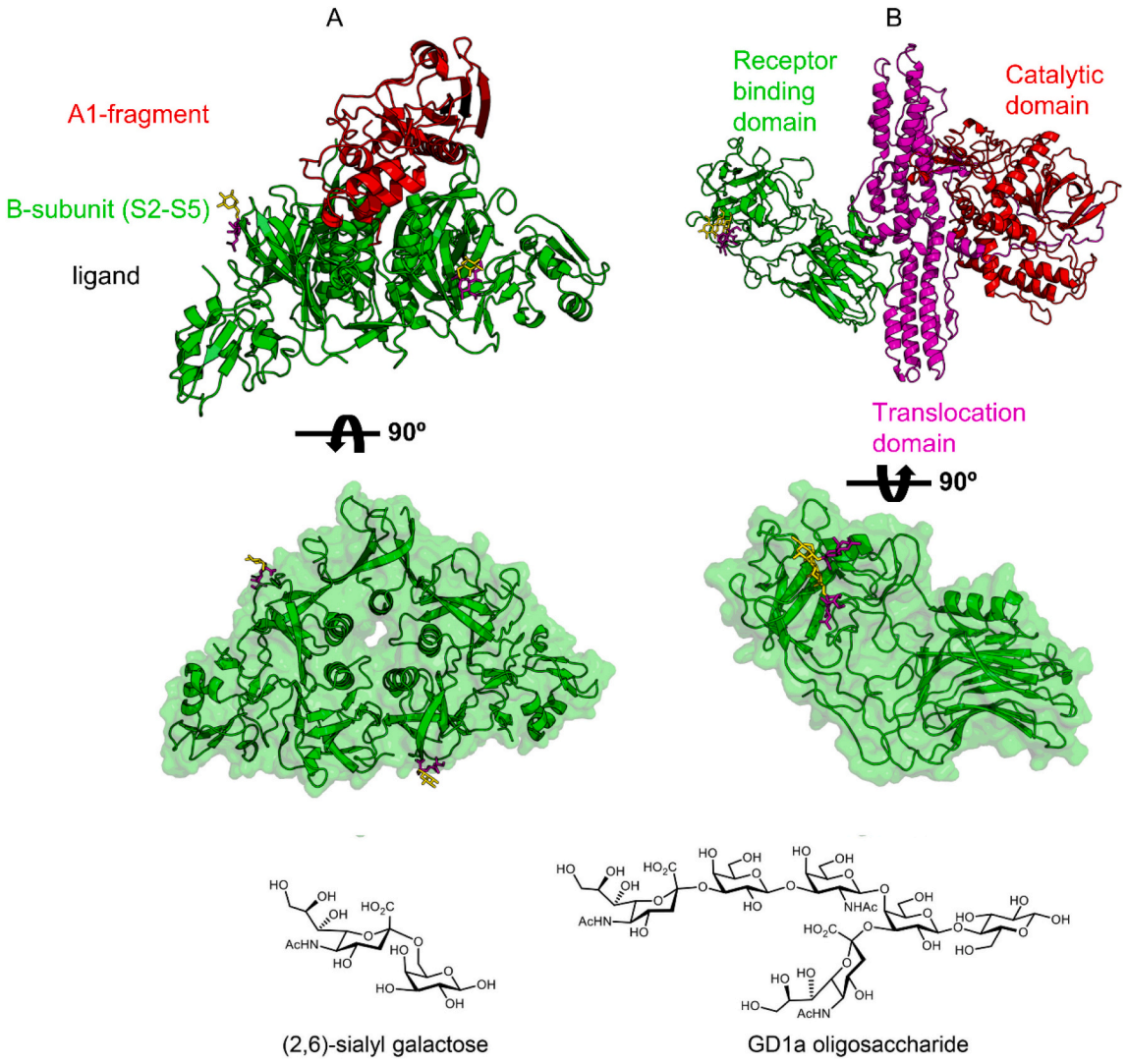

Fig. 3. Crystallographic structures of intracellular toxins, with ligand coloured according to SNFG rule. A) PTx holotoxin (PDB: 1PTO) with A- (red) domain in complex with pentameric B-subunit (green). The binding sites in S2 and S3 for the terminal sialic acid-galactose (orange) are presented. B) BoNT/A neurotoxin (PDB: 3BTA, 5TPC) with catalytic domain (red), receptor-binding domain (green) co-crystallized with GD1a (PDB: 5TPC), and translocation domain (magenta). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

STx or SLTx, respectively. Many potential treatments are still in clinical research ranging from toxin-based vaccines to whole-cell vaccines (Ashkenazi and Cohen, 2013 and Mühlen and Dersch, 2020).

Other examples of non-ribosylating intracellular toxins are the *Botulinum* neurotoxins (BoNT) with seven distinct serotypes named A, B, C, D, E, F, and G that inhibit acetylcholine release from nerve terminals. The detailed mode of action can be very distinct for different serotypes. BoNT forms a multimeric complex with the neurotoxin-associated proteins (NAPs), which are essentially non-toxic. Each BoNT serotype expresses co-diverged NAPs complementary to its binding pockets. The types A, B, E, and F are toxic in both humans and animals, while types C and D are predominantly effective in animals. Their intoxication process involves 1) the interaction of the toxin's ganglioside and protein-binding domain with receptors on the presynaptic membrane of motor neurons; 2) the entry of neurotoxin by receptor-mediated endocytosis; 3) the pore formation by translocation domain in the acidic endosome to allow the release of the toxic subunit to the cytosol of the neuron; 4) the proteolysis of specific SNARE proteins required for neurotransmitter release (Nuemket et al., 2011).

The BoNT targets a peripheral nerve terminal and cleaves SNARE proteins responsible for the fusion between synaptic vesicles and presynaptic membranes. This results in complete inhibition of acetylcholine release. A crucial role of HA in the BoNT complex is to bind to glycans on the neuron cells and enable intracellular transport of the toxin (Amatsu et al., 2013). The HA contains Ganglioside Binding Sites (GBS) that vary between different BoNT serotypes. BoNT also recognizes different isoforms of synaptic vesicle protein 2 (SV2). N-glycosylation site (N573Q) of SV2 mediate the entry of BoNT/A and BoNT/E through the cell membrane (Dong et al., 2008; Yao et al., 2016).

Type A (BoNT/A) has been the most widely studied toxin, and its genetic subtype BoNT/A1 is one of the few BoNT subtypes employed for pharmaceutical purposes (Mazuet et al., 2010). Clinical research confirmed use of BoNT/A affects walking speed in post-stroke spinal equinovarus foot. A type of BoNT/A, called incobotulinumtoxin A (Xeomin®), shows therapeutic potential in fighting against cervical dystonia, blepharospasm, axillary hyperhidrosis, hemifacial spasm, re-innervation synkinesis, and hypersalivation (Dressler, 2012).

These bacterial toxins still represent a cause of significant infectious diseases worldwide, associated with their ingestion in water or various types of food (Karmali, 2004). They can give rise to life-threatening symptoms such as diarrhea and hemolytic uremic syndrome (HUS), which leads to kidney failure (Tarr et al., 2005).

Despite the toxic properties and the health threat they represent, these toxins constitute promising tools for studying cellular processes and transport mechanisms. The growing knowledge on the role of the toxin domains in binding receptors and penetrating the cellular membrane indicate that they can lead to new products with biomedical applications.

3. From biomedical applications to therapeutics

Pathogen-derived toxins, such as STx, SLTx, and CTx, present a unique opportunity for clinical intervention. Indeed, the ability to target glycosylated molecules in a selective and specific way has inspired their use in diverse pharmaceutical fields, like cancer diagnosis and therapy, drug delivery, and microarrays. For example, toxin-based therapies have been developed by exploiting the A-subunit's cytotoxic activity. Except for CTx, the above-mentioned members of the AB-toxins family induce potent protein synthesis inhibition, resulting ultimately in apoptosis and cell cycle arrest in human cells (Odumosu et al., 2010). Such cytotoxic agents can be used to induce cell death in cancer cells (Guan et al., 2017; Luginbuehl et al., 2018). On the other hand, the non-toxic receptor-binding B-subunit has been investigated for selective drug delivery. The high specificity in glycan recognition and the unique intracellular pathways have led to several successful intracellular delivery approaches, which we will discuss in this chapter.

3.1. Toxins and toxin-drug conjugates for targeting cancer cells

It has been known for decades that dysregulation in glycosylation patterns constitute a hallmark of cancer cells (Munkley and Elliott, 2016). Cancer-associated glycans arise from 1) incomplete synthesis and generation of truncated structures, 2) de novo synthesis of neoantigens, 3) the overexpression or complete absence of specific glycans (Rodrigues et al., 2018). These alterations are often associated with advanced progression of solid tumors, development of metastasis, and more aggressive phenotypes. Indeed, the aberrant expression of glycosylation pathways plays a crucial role in tumor growth and aggressiveness by interfering with cell adhesion, migration, proliferation. Given their impact on disease progression and dissemination, these structural modifications on the surface of malignant cells are considered attractive therapeutic targets and biomarkers (Tuccillo et al., 2014).

Interestingly, several tumor-associated antigens are altered glycosphingolipids (Hakomori and Zhang, 1997). Among them, the glycosphingolipid Gb3 has been reported to be overexpressed in many types of human cancer, including Burkitt's lymphoma (Wiels et al., 1981), breast (LaCasse et al., 1999; Johansson et al., 2009), ovarian (Arab et al., 1997), colorectal (Kovbasnjuk et al., 2005; Falguières et al., 2008), and pancreatic cancers (Maak et al., 2011).

3.1.1. Application of holotoxins

The different expression levels in healthy and tumor states render Gb3 an attractive target, and STx a promising tool for targeted cancer therapy. STx has been used as a cytotoxic agent or a carrier for cytotoxic compounds to cancer cells (Odumosu et al., 2010). As mentioned above, when the A-subunit is delivered into the cytosol, inhibition of protein synthesis results in cell cycle arrest, and cell death. This mechanism can 1) kill cells in a very effective manner, 2) work with low doses of toxin, 3) overcome multi-drug resistance of many cancer cell lines. Based on this approach, the STx-1-induced cytotoxicity has been monitored in several murine cancer models. Remarkably, in mouse xenograft models of human renal carcinoma (Ishitoya et al., 2004) and human astrocytoma (Arab et al., 1999), the treatment with intratumoral injection of STx-1 induced complete regression of the tumor, with apoptosis in both cancer cells. More recent studies reported cell death induced by STx-1 holotoxin in glioma Gb3-expressing cells through the activation of the apoptotic pathway and ultimate DNA fragmentation (Johansson et al., 2006). However, there is a significant concern in using the holotoxin, related to the life-threatening side effects associated with STx-1/STx-2 infections (Paton and Paton, 1998). Kidneys from mice treated with STx revealed acute necrosis of tubular epithelial cells, paediatric renal tubular epithelial cells stimulated with STx, and in situ Terminal dUTP Nick End Labelling (TUNEL) assay staining of renal biopsy from HUS patients indicated dramatic apoptotic changes (Karpman et al., 1998; Te Loo et al., 2001).

CTx was also applied to target carcinomas and trigger apoptosis in cancer cells. Early studies in targeting cancer proved that CTx was able to inhibit cell growth in human lung cancer cells (Allam et al., 1997). The toxin was used to trigger deprivation of essential growth factors, showing success in interfering with the intracellular calcium signal and inducing, therefore, apoptosis in malignant cells.

In more recent studies, CTx was used to suppress carcinogenesis and inflammation-sustained tumor progression in a colon cancer mouse model (Doulberis et al., 2015). Cholera toxin was administered orally in a single, low, non-pathogenic dose to suppress the inflammation sustaining the tumor growth and induce a potent anti-inflammatory response to inhibit tumor survival and progression.

3.1.2. Application of non-toxic B-subunit

In this direction, the non-toxic B-subunit seems to represent a more promising option. STx-1B possesses principal characteristics that are interesting as a delivery tool, such as the specificity in binding Gb3, stability to proteases, fast intracellular uptake, and ability to cross tissue barriers (Chan and Ng, 2016). Some cytotoxic compounds have been coupled to STx-1B for specific targeting of tumors in the last decade. Two relevant studies showed the excellent potential of STx-1B for the intracellular transport of drugs. First, the B-subunit was covalently coupled to a prodrug based on SN38, an active metabolite of the topoisomerase I inhibitor irinotecan (CPT11), representing the standard treatment for carcinoma (El Alaoui et al., 2007; Distler et al., 2009; Geyer et al., 2016). The STx-1B-SN38 follows a retrograde route, which leads to targeted growth inhibition in colorectal carcinoma cells. This is mediated by the release of the prodrug in the endoplasmic reticulum of cells with high Gb3 expression levels. Along with this study, pancreatic cells were also successfully targeted by the same complex STx-1B-SN38 (Maak et al., 2011). STx-1B revealed a fast uptake and transport to the Golgi apparatus in pancreatic cancer cells, where Gb3 is highly expressed. Most interestingly, the complex STx-1B-SN38 showed 100-fold increased cytotoxicity when compared to the standard treatment with irinotecan. A variety of conjugates and linkers were designed to deliver more potent cytotoxic drugs to optimize the use of the B-subunit as a therapeutic carrier. This includes investigating STx-1B-induced cytotoxicity when the B-subunit is conjugated to Menomethylauristatin E and F, potent derivatives of dolostatin 10, an inhibitor of tubulin polymerization in dividing cells that leads to apoptosis (Batisse et al., 2015). In the study, STx-1B conjugated to the cytotoxic MMAE, and MMAF was able to inhibit cancer cell growth, thus confirming a high potential for the selective elimination of Gb3-positive tumors (Florent et al., 2012).

The non-toxic B-subunit of CTx has also been investigated for drug delivery to glioma cells (Guan et al., 2017). By targeting its receptor GM1 expressed in the blood-brain barrier (BBB), the CTxB was investigated for new anti-glioma chemotherapy strategies. CTxB nanoparticles loaded with paclitaxel showed efficient penetration of BBB in vitro models and successful induction of apoptosis of intracranial glioma cells in vivo, with a significant prolongation of survival in mice models.

These studies' efficacy and the evaluation in further animal experiments highly suggest that these holotoxins or their carbohydrate-binding subunits could be promising innovative tools for the enhanced delivery of chemotherapeutic drugs in selected carcinomas. Thus, the importance of massive production and scale-up in the expression of these proteins for more efficiency and full availability.

3.2. Carbohydrate-binding toxins as imaging tools that recognize glycobiomarkers

3.2.1. Tumor imaging with STxB

Non-invasive tumor imaging has been achieved by linking contrast agents to STx-1B. The naturally evolved transport properties of STx-1B, the high number of Gb3-positive tumor cell-binding sites, and its trafficking via the retrograde route can be diverted for tumor cell delivery of other exogenous compounds, such as contrast agents for tumor imaging. STx-1B accumulation in digestive Gb_3-expressing adenocarcinomas was observed in animals using fibered confocal fluorescence endoscopy and positron emission tomography (PET) imaging (Janssen et al., 2006; Luginbuehl et al., 2018).

Fluorescent STx-1B-Cy5 was used in two different human colorectal carcinoma models and administered systematically to xenografted nude mice. The study provided insights on its accumulation in Gb3 positive tumor area using confocal microscopy and its elimination after persisting several days in the systemic circulation (Tavitian et al., 2008). Additional analysis of the biodistribution of STx-1B by PET showed accumulation in the tumor area and slow elimination by renal excretion, proving again that this protein has great potential as a vector for diagnosis and targeted antitumoral therapy.

3.2.2. CTxB as a neuronal tracer

CTxB is a commonly used and sensitive retrograde neuronal tracer. The abundance of GM1 in the nervous system has provided this toxin's potential to be used in tracing and targeting neurons, especially when visualized immunochemically (Fort et al., 1989; Lima and Coimbra, 1989; Luppi et al., 1987, 1990). When compared to the well-established CTxB-horseradish peroxidase (HRP) conjugates for histochemical detection, CTxB conjugated with fluorophores like fluorescein isothiocyanate (FITC), and tetramethylrhodamine isothiocyanate (TRITC) proved to be efficient and less time-consuming for neuronal detection by fluorescence microscopy (Dederen et al., 1994). Notably, Alexa Fluor conjugates of the toxin have found application in recent years to identify neuroanatomical connections in animals (Conte et al., 2009; Fasanella et al., 2008). Furthermore, CTxB conjugated to biotin was investigated for application in trans-neuronal labeling, being able to label interneurons in the rat spinal cord when injected into the sciatic nerve (Lai et al., 2015). A recent enhancement in CTxB conjugates comes from a new construct incorporating non-canonical azido amino acid (azido-CTxB) capable of further conjugation by click-chemistry, with possibilities and uses as both tracing and delivery tools (Haigh et al., 2020; Dommerholt et al., 2016). The purified azido-CTxB, as well as CTxB-functionalized nanoparticles, were shown to be effective for targeting the hypoglossal nucleus in the brain stem following tongue injections in mice.

3.3. Vaccine and adjuvant design

3.3.1. Adjuvant design

Bacterial toxins and their carbohydrate-binding B-subunit are often the subjects of vaccine research, frequently when recombinantly linked to immunogenic epitopes (Lu et al., 2020; Ishikawa et al., 2003; Zhu et al., 2008; Kerner et al., 2015; Pan et al., 2018). For example, the cholera toxin holotoxin has proven to have a strong adjuvant potential, targeting bacterial and viral pathogens (Stratmann, 2015). The leading example of bacterial toxins used in vaccines is DUKORAL®, recommended by the World Health Organisation (WHO) for vaccination against *V. cholerae*. It contains inactivated whole cells of *V. cholerae* O1 with the addition of recombinant CTxB with protective efficacy of up to 85% (Jelinek and Kollaritsch, 2008). One dose contains 1 mg of recombinant CTxB,[1] so the production demands require large scale industrial production. Examples of other vaccines protective against *V. cholerae* infection have been reviewed by Shaikh et al., 2020.

The ability of CTxB to elicit mucosal humoral immunity has led to the targeting of mucosally-transmitted viruses. This resulted in vaccine development research against, for example, the human immunodeficiency virus (HIV-1), where CTxB was used as an adjuvant in DNA- or protein-based vaccines (Boberg et al., 2008; Hou et al., 2014). Boberg et al., 2008, investigated whether CTxB can be used as an effective intramuscular adjuvant for an HIV-1 DNA vaccine by fusing recombinant CTxB to HIV peptide, both in vitro and in vivo. During in vitro studies, CTxB induced the activation of mice bone marrow-derived dendritic cells (BMDCs) and the secretion of pro-inflammatory cytokines and chemokines. After intramuscular administration of the DNA vaccine in mice, CTxB induced more robust HIV Env-specific cellular and humoral immune responses compared with DNA alone, suggesting its effectiveness as a mucosal adjuvant also for DNA-based vaccination. More recently, Hou et al., 2014, fused CTxB to mutant epitopes derived from HIV-1 enzymes in an attempt to induce cellular responses against-resistant variants of the virus. By combining HIV-1 epitopes representing drug resistance mutations with recombinant CTxB, they showed that the immune response against the different peptides was strongly enhanced by their fusion to the toxin, thereby revealing a long-lasting immune response in the animals.

Ohmura-Hoshino et al., 2004, described the non-toxic STx derivatives from *E. coli* possessing adjuvant activity for mucosal immunity. In the study, the group used ovalbumin as an antigen and non-toxic STx-1 as an adjuvant for nasal immunization of adult mice. Choi et al., 2005, linked the STx-1B-subunit to a rotavirus immunodominant antigen NSP4 for STx-1B mediated delivery of the virus antigen to the gut-associated lymphoid tissues (GALT) in mice. The fusion protein, expressed in *E. coli*, enhanced humoral and cellular responses against rotavirus gastroenteritis, thereby proving the STx-1B efficiency as a carrier protein for rotavirus antigen.

3.3.2. Vaccine design

In many studies, chimeric CTxB has been administered via intranasal or oral routes to elicit strong mucosal immunogenicity in several animal models. For example, CTxB was used as a mucosal adjuvant in an epitope vaccine against *Helicobacter pylori*, combined with the epitope urease A-subunit of *H. pylori* in a complex named CTxB-UA (Guo et al., 2011). The complex, expressed in *E. coli*, was used for intraperitoneal immunization experiments in mice, where it showed good immunoreactivity and inhibition of the enzymatic activity of *H. pylori* urease.

STx-1B also appears to represent an attractive candidate for the development of therapeutic vaccines (Sadraeian et al., 2013; Adotevi et al., 2007). Human dendritic cells (DCs) are considered the most potent antigen-presenting cells (APCs) for the induction of cytotoxic T lymphocytes (CTLs), which are critical players in the control of virus-infected cells and tumor growth. Several studies have confirmed the potential of STx in targeting these professional APCs. Indeed, the B-subunit of STx-1 was proven to elicit immunity when fused to exogenous cancer and viral antigens, targeting the selective expression of Gb3 on DCs. The STx-1B was conjugated to a tumor antigen and showed efficiency in activating and expanding $CD8^+$ CTL without the use of adjuvants (Haicheur et al., 2000). Immunization of mice with STx-1B resulted in specific strong CTL responses via the major histocompatibility complex (MHC) class I pathway in a receptor-dependent manner. Also, the reported improvement of STx-1B-based vaccines combined with adjuvants like α-galactosylceramide resulted in a long-lasting T cell response, suggesting the success of the STx-1B vector for improvement of the antiviral immunity (Adotevi et al., 2007).

PTx, from *B. pertussis*, a human-specific pathogen and the causative agent of whooping cough, is another candidate for the development of subunit vaccines (Gregg and Merkel, 2019). Here, PTx is subjected to inactivation and stabilization methods before final formulation. For example, it has been suggested that PTx inactivated with hydrogen

[1] www.medicines.org.uk/emc/files/pil.5087.pdf

peroxide, rather than formaldehyde, can improve the overall protective immune response (Di Tommaso et al., 1994; Heron et al., 1999). *B. pertussis* strain can also be modified to produce genetically inactive PTx by two critical amino acid substitutions in the *S1*-subunit (Nikbin et al., 2020). Modified *s1* gene was genome integrated and introduced back into its location replacing the temporarily placed chloramphenicol gene.

The toxin can be modified chemically, by heat, or genetically, thus making PTx a suitable antigen in the final vaccine. The conformational changes of the subunit vaccine achieved during the formulation process affect how immunoglobulins (Igs) recognize the antigens, process them, and present their target epitopes. Formulated PTx subunit vaccine recognized by Igs can activate helper CD4 and killer CD8 T cells. The effectiveness of the PTx subunit vaccine can also be achieved by selecting a delivery method. Zhu et al., 2019 presented an innovative approach where dissolvable microneedles deliver the subunit vaccine intracutaneously. These minimal invasive patches injectable directly to the patient's skin activates both cellular and humoral immunity.

The most effective vaccine formulation for PTx is achieved by tetanus and diphtheria toxins called the DPT toxoid vaccine. DPT often replaces a vaccine that contains an addition of acellular pertussis with chemically or genetically detoxified pertussis toxin, filamentous haemagglutinin (FHA), outer-membrane pertactin, and fimbrial-2 and fimbrial-3 antigens (https://www.cdc.gov/vaccines/hcp/vis/vis-statements/tdap.html). The final immunological boost of the formulated pertussis vaccine is created by selecting the right adjuvant, for example, recombinant CTxB (Isaka et al., 2003), the fusion of CTxB and fimbrial-2 protein (Oliveira et al., 2014), or CTxB with LTxB hybrids (Bowman and Clements, 2001).

The examples presented in Section 3 have demonstrated that bacterial toxins and their non-toxic B-subunit can be used as promising innovative tools in different biomedical applications. There is space for further investigations in targeted cancer therapy, tumor imaging, and vaccine developments, which render the large-scale production and purification of these toxins an essential requisite.

4. Fermentative expression of carbohydrate-binding toxins

As scientists started to acknowledge the importance of carbohydrate-specific binding proteins for the biopharmaceutical industry, the development of lectin production processes on a large scale became a new concept practiced by specialist companies. Initially, scientists focused on applications of plant lectins (carbohydrate-binding proteins that are neither enzymes nor antibodies), which are often glycosylated, making them unsuitable for microbial production. An even larger obstacle for recombinant expression is the frequency of disulfide bonds common in plant-based toxins. Therefore, plant lectins are usually extracted from plants with an extremely slow, and cumbersome production process with a low yield for industrial-scale protein production. The breakthrough began when bacterial lectins, including bacterial toxins, became recognized for their ability to bind selectively to specific protein glycosylation patterns (Lebens et al., 1993). Along with this adjustment came the driving force for making toxins on a much larger scale and more customized bioprocesses, eventually leading scientists to microbial expression in the bioreactor. Toxin-based vaccine technology led to toxoid production in high yield expression in a bioreactor. The best example of large-scale vaccine production is the WHO pre-qualified oral cholera vaccines (OCV) DUKORAL® (SBL Vaccine Technology (Hsiao et al., 2017). More than 20 million doses of OCVs have been used in mass vaccination campaigns. This includes areas experiencing an outbreak or populations living in highly endemic areas, recognized as "hotspots".

4.1. Small-scale fermentation

Small-scale fermentation begins with the design of a toxin construct and understanding its secretory pathways after expression. In *V. cholerae* LTx expresses A- and B-subunit as the precursors (pA and pB) that pass through the cytoplasmic membrane and get released into the periplasmic space where their signal peptides are removed to give the mature subunits. As described in Section 2, the B-subunit assembles into a pentamer (B5) and associates with A-subunit to form a holotoxin (AB_5). Often the production of carbohydrate-binding toxins focuses on the expression of only a carbohydrate-binding domain without the toxic A1-domain of the AB_5 toxin (Zhang et al., 2017; Sharma et al., 2012). In either case, the holotoxin or B-pentamer can be translocated across the outer membrane by a toxin secretory apparatus (TSA) and released into the extracellular milieu (Hirst et al., 1984). Periplasmic expression mediated by a signal peptide is an essential step for assembly of the toxin as the bacterial protein disulfide isomerase DsbA is required for the correct formation of disulfide bonds as a precursor for effective folding and assembly (Freedman et al., 1994; Sospedra et al., 2018).

The small-scale fermentations described in Table 1 show that toxin expression can already be sufficient for research purposes in shaker flask format, e.g. CTx reaching 20 mg/L (Zhang et al., 2017). In other cases, shake flask production is performed for media optimisation essential for fed-batch cultivation (Sharma et al., 2012). The final yield of biologically active STxB can be increased 6-fold, from 5.1 mg/L in shaker flask to 30 mg/L in fed-batch fermentation. Media optimization for upscaling purposes can be improved by installing statistical methods to vary media components in shaker flask, as presented by Yari et al., 2010. They were able to achieve 52 mg/L of soluble recombinant BoNT/A-Hc after their optimization. Another focus of optimization at the shaker flask level is to maximize specific toxin yields (mg toxin per g cell dry) by varying not only media components but as well fermentation condition (temperature, iron-limitation, production strain), which can be used then for large scale fermentation (Thalen et al., 2006b). This approach shows that most processes can be significantly optimised by taking simple

Table 1
The summary of small-scale fermentations of carbohydrate-binding toxins.

Acronym	Full name	Glycan specificity	*E. coli shake flask* expression	Reference
VCC	*Vibrio cholerae cytolysin/ hemolysin*	Complex N-glycans	Expression of VCC in *Origami E. coli*	Olson and Gouaux, 2005
VVH	*Vibrio vulnificus hemolysin*	Preference for *N*-acetyl-D-galactosamine and *N*-acetyl-D-lactosamine.	Expression of native and recombinant VVH in BL21 *E. coli*	Song et al., 2016 and Choi et al., 2002
CTx	*Vibrio Cholerae toxin*	GM1 ganglioside	Recombinant expression of CTxB co-expressed with SKP chaperones in BL21 *E. coli*	Zhang et al., 2017
LTx	*Enterotoxigenic Escherichia coli (ETEC) strains producing heat-labile toxin*	GM1 ganglioside	Expression of LTx in ETEC strains	Sospedra et al., 2018
STx	*Shiga toxin from Shigella dysenteriae and Shiga toxins that are produced by EHEC strain*	Gb3	Recombinant expression of STxB in K12 strain	Sharma et al., 2012
BoNT	*Clostridium neurotoxin complex*	gangliosides	Recombinant expression of BoNT/A-H in BL21 *E. coli*	Yari et al., 2010
PTx	*Pertussis Toxin from Bordetella toxin*	GTP	Expression of PTx in *Bordetella pertussis*	Thalen et al., 2006b

measures on the level of process parameters.

The industrial production of full toxin or only recombinant carbohydrate-binding domain for a drug delivery system will require larger-scale production. Consequently, the next step is to upscale the production of these toxins, as described in the chapter below.

4.2. Large-scale fermentation

Successful small-scale fermentation is scaled up to large 1 L–20 L fermenters. As described in Fig. 4, different protein production strategies can be employed during the scale-up procedures. The general approach begins with batch fermentation and later proceeds with fed-batch or continuous fermentation. Scale-up-related problems arise often from reduced mixing quality, enhanced stress exposure, plasmid loss, metabolic shift, and misincorporation of amino acids (Schmidt, 2005). Optimal fermentation conditions maintained in the bioreactor can resolve those problems and ensure a consistent increase in protein yields and final product quality. Other problems, such as excess glucose concentration, acetate overproduction, reduced growth, and reduced productivity by high-density culture or product toxicity at high yields, can be resolved by switching production strategy from batch to fed-batch or continuous fermentation (Mears et al., 2017; Berteau et al., 2019). In this section, we describe examples of homologous and heterologous carbohydrate-binding toxin production on a large-scale with different types of fermentation processes and diverse bioreactors systems.

4.2.1. Homologous production

A primary concern for CTxB large-scale production was the influence of different media components and fermentation parameters (van de Walle et al., 1990). 3 L to 5 L reactors cultivating *Vibrio cholerae* regulated the pH at different strengths from 6.5 to 9, maintained the temperature between 25 °C to 37 °C, and dissolved oxygen level at 30% saturation by adjusting the agitation and aeration. Under defined conditions of high salt and high amino acids concentration in the media, called AGM4, CTxB production at pH 8 and 30 °C was 36 mg/L. Holotoxin production, as well as CTxB, can be stimulated by the addition of extra asparagine, serine, arginine and glutamic acid into the media. The reason for this is that CTxB synthesis was proven to be related to serine metabolism. Homologous CTxB expression in *V. cholerae* was then scaled-up to 6-L reactors to increase the overall dry cell weight, 20–23 g/L in 12 h (De Maré et al., 2003). *V. cholerae* growth rate is similar to *E. coli,* and with the ability to export proteins extracellularly, it is an attractive host for large-scale fermentation. Growth rate can be influenced by both glucose excess that leads to organic acid production or glucose limitations that lead to cell starvation. Therefore, the probing feeding algorithm was used to adjust the feed to avoid the acetic acid formation and maintain a high growth rate. With a simple sensor and pulse response system, maximal glucose uptake was maintained with minimal acetate formation. Acetic acid exerts a toxic effect at concentrations as low as 0.5% (*w/v*) and causes a decrease in intracellular pH and metabolic disturbance, among other deleterious effects.

One of the last attempts to improve homologous CTxB production was done on a much smaller scale in the 2 L bioreactor with the final yield of 19 mg/L at the end of purification (Jang et al., 2009a, 2009b). The purity of protein reached 92% with only one purification step, Cation Exchange Chromatography (CEX), which was performed directly after the production. This simple process allows for safer and more affordable vaccine production.

Similar to CTxB, PTx's large-scale production begun with defining the media components for homologous production in *Bordetella pertussis* strain (Thalen et al., 1999). A balanced medium with lactate and glutamate as the main component was determined in a simple 0.5 L

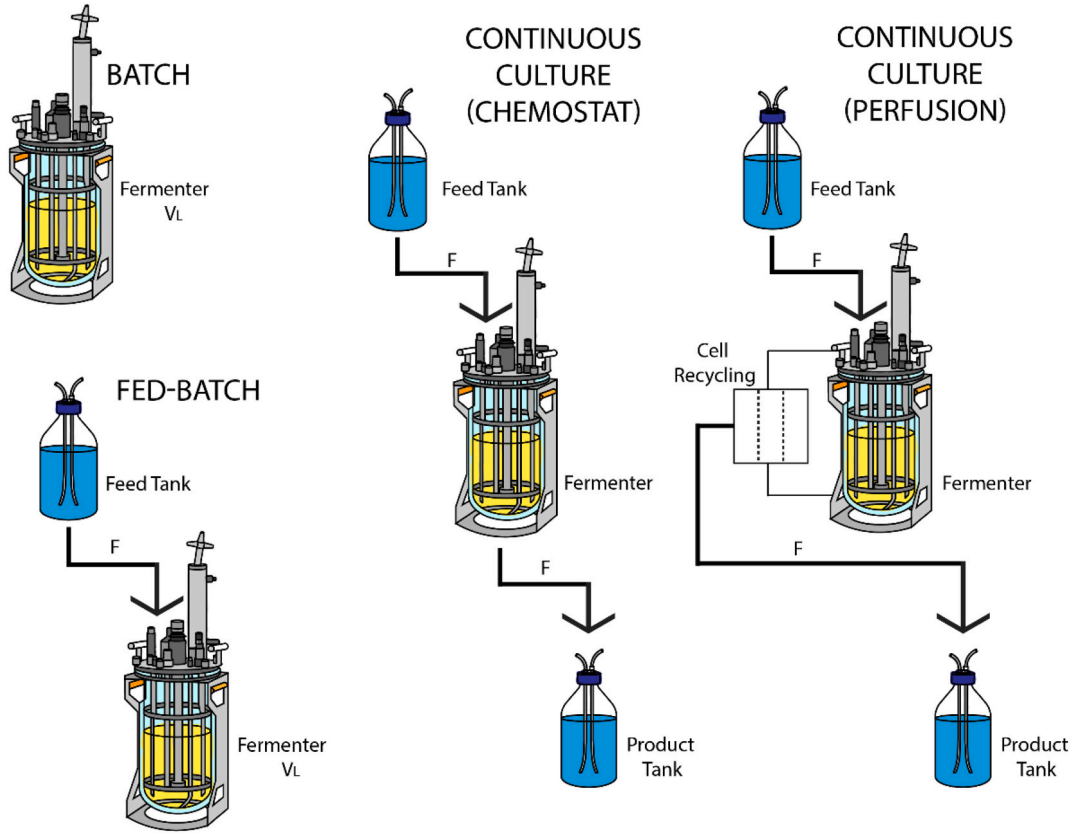

Fig. 4. Different types of fermentation units are divided into three categories: batch, fed-batch, and continuous process. The batch process is completely closed during fermentation; the fed-batch is connected to the feed tank enabling linear or exponential fermentation after the batch is finished and the chemostat with and without perfusion system maintaining a steady-state in the bioreactor.

shake flask experiment. During the process, ammonium did not accumulate and polymer Polyhydroxybutyrate (PHB) is formed but no β-hydroxybutyrate is excreted. The right quantity of PHB present in the bacterium is converted into biomass. Increased PHB level at which β-hydroxybutyrate is formed at a high rate, partially oxidize β-hydroxybutyrate to acetoacetate instead of generating biomass. Those preliminary experiments were then upscaled. Thalen et al., 2006a reported on a 3 L PTx fed-batch fermentation using the strain *B. pertussis* 509. They investigated exponential (μ ranging from 0.11 h^{-1} to 0.07 h^{-1}) and constant (10 mmol C L^{-1} h^{-1} to 45 mmol C L^{-1} h^{-1}) feed profiles and identified the addition of glutamate and lactate to the feed media as key components for preventing the accumulation of the microorganic stress PHB. The inhibition of PHB production provided a 7-fold increase in biomass formation (6,5 g/L cell dry mass) and consequently an overall volumetric PTx yield of 12 mg/L. The experimental findings acquired from PTx production on a large-scale were implemented in *B. pertussis* whole-cell production on a large-scale (Streefland et al., 2007; Thalen et al., 2008).

4.2.2. Heterologous production

STx-1B is one of the most studied carbohydrate-binding toxins characterized by successful scale-up in heterologous systems. STx-1B expression in K12 *E. coli* strain began with an initial test in batch-type reactors and evolved to a more controlled fed-batch 5 L bioreactor with a final yield of 30 mg/L in 11.38 g/L dry cell weight (Sharma et al., 2012). In comparison, shake flask, batch cultivation reached only 5.1 mg/L.

Substantially more research was conducted on the structurally- and functionally-related toxins CTxB, in both homologous and heterologous expression. The heterologous fed-batch CTxB production in MC1061 strain was performed with a final 58 g/L dry cell weight. The final target protein yield was 4.7 g/L in the intracellular fraction, 0.96 g/L in the periplasm, and 0.27 g/L in the supernatants produced in *E. coli* (Mendoza-Vega et al., 1995).

Non-ribosylating BoNT/A Heavy chain (BoNT/A- Hc) ganglioside specific toxin has been produced using high cell density cultivation (Table 2). It is a vaccine candidate for the prevention of botulism and a cheaper replacement for the toxin-inactivated vaccine. This recombinant toxin was expressed heterologously with *E. coli* BL21 (DE3) in a 5 L bioreactor under batch and fed-batch conditions (Yari et al., 2012). Unique for this process development was integrating the Taguchi statistical design method (Davis and John, 2018) for final yield estimation in each fermentation, maximization toxin expression, and optimizing final IPTG inducer concentration (1 mM). This statistical method improved the overall productivity by comparing the effect of medium component and process parameters. Besides, it serves as a screening filter that examines the effects of process variables that have major effects on the process. With a 2 L working volume they produced 62 mg/L in batch culture and 486 mg/L in fed-batch culture (3 h post-induction) of BoNT/A- Hc carbohydrate-binding toxin. A high-density fermentation was achieved by feed control (to avoid an excess of glucose in the growth media) and dissolved oxygen above 40% saturation (to prevent oxygen limitation and anaerobic fermentation conditions). These conditions prevent overflow metabolism and consequently acetate formation during oxygen-limiting conditions. The fed-batch production resulted in a fixed growth rate at 0.2 h^{-1} and decreased acetate concentration to 5 g/L that reduced toxicity for cell growth and increased biomass final yield. The selection of *E. coli*, along with fermentation that maintains the exponential feeding rate during fed-batch, improved the final productivity of the recombinant BoNT/A- Hc. The exponential feeding method allowed cells to grow at specific growth rates below the critical value of acetate formation. (See Table 3.)

PTx subunit vaccines require either strong adjuvants or the addition of other potent antigens to elicit potent immunity. One way to overcome this limitation is to generate PTx fusion proteins with other strong *B. pertussis* antigens. Jinyong et al., 2011 described fusion of mutant pertussis toxin *S1*-subunit with a truncated peptide from filamentous hemagglutinin (FHA) type I immunodominant domain. This fusion protein was expressed heterologously in *E. coli* BL21(DE3) in 2 L batch fermentation with a final volumetric yield of 10 mg/L. Another approach, presented by Koopaei et al., 2018, made use of a tripartite fusion protein of N-terminal fragment (1–179 aa) of the *S1*-subunit of pertussis toxin, the full length genetically detoxified diphtheria toxin (CRM197), and the 50 kDa tetanus toxin fragment C. They used response surface methodology to optimize fusion protein yields at 3 L batch scale and were finally able to improve yields 1.9-fold to 0.45 μg/L, still not applicable for industrial-scale production. However, generating genetic fusion proteins could someday replace the classical DPT vaccines

Table 2
Bioprocess of carbohydrate-binding toxins.

Process	Key benefits	Examples
Batch	Facilitate preliminary experiments on expression host and protein production. Allow for the parallel fermentations.	CTxB expression in a 2 L bioreactor with the final yield of 19 mg/L of the product (Jang et al., 2009a, 2009b).
Fed-batch	Enable linear or exponential post-batch fermentation. Suitable for finding optimal conditions for scale-up. Substantial increase in biomass- comparing to simple batch fermentation.	STxB fed-batch production in 5 L bioreactor with a final yield of 30 mg/L in 11.38 g/L dry cell weight (Sharma et al., 2012).
Chemostat	Increase in space-time yield and better scale-up strategy for the plant, the possibility of continuously removing unstable products from the reaction medium, and the increased operational flexibility.	Continuous production of LTx Enterotoxin in chemostat culture with a final yield of 3 mg LT l^{-1} h^{-1} and mg LT g cell dry $weight^{-1}$ h^{-1} (Lloyd et al., 1997).
Chemostat with perfusion	Reducing the volume of the bioreactor and enable high space-time yields and can be easier coupled to downstream systems. The perfusion system is perfect for the integration of additional biosensors.	Multi modular perfusion bioreactors are operated parallel for the continuous culture of biologicals production, such as *Botulinum* neurotoxin (Berteau et al., 2019).
Hollow-fiber	Generate high concentration of product in 3-dimensional cell culturing system based on hollow fibers. Hollow fiber is composed of small, semi-permeable capillary membranes arranged in a parallel array and allow for the passage of low molecular weight nutrients and wastes.	The reactor system operated in the transverse mode, where growth medium is passed over immobilized *Vibrio sp. 60*, and used metabolite and waste products collected after passage through the semi-permeable fibre's membrane (Lloyd and Bunch, 1996).

Table 3
Main purification methods for carbohydrate-binding toxins.

Carbohydrate-binding toxins	Chromatography	References
Cholera toxin and its B-subunit	Spherosil-DEAE-dextran-LysoGM1 D-galactose-agarose column Ni2+ nickel column	Tayot et al., 1981 Yasuda et al., 1998 Dertzbaugh and Cox, 1998
Heat-labile enterotoxin and its B-subunit	D-galactose-agarose column Agarose A-5 m	Uesaka et al., 1994 Clements and Finkelstein, 1979
Shiga toxin, STx-I, STx-II, and their B-subunits	Hydatid cyst P1(gp) column	Donohue-Rolfe et al., 1989
Botulinum neurotoxin (BoNT)	*p*-aminophenyl-β-D-thiogalactopyranoside-Sepharose 4B	Moberg and Sugiyama, 1978
Pertussis Toxin (PTx)	Capto SP ImpRes ion exchange chromatography resin	https://cdn.cytivalifesciences.com

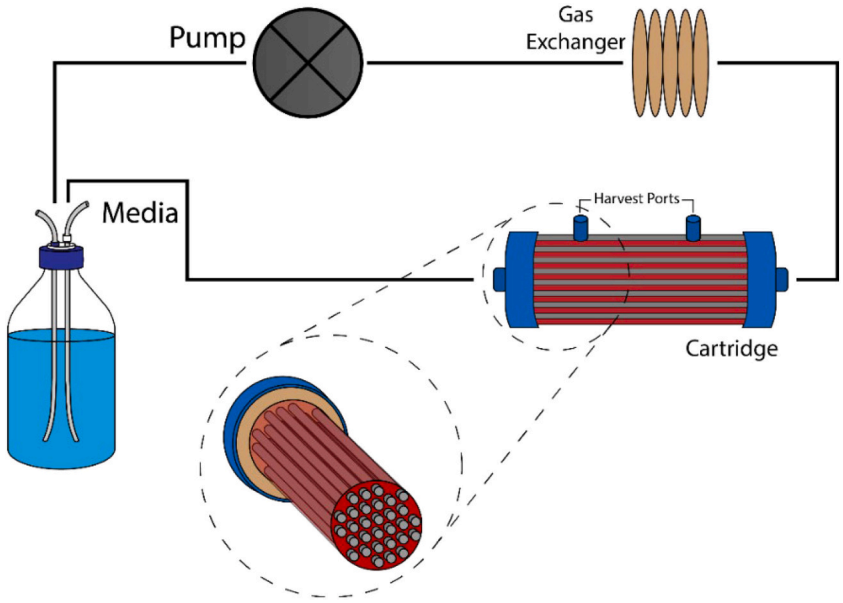

Fig. 5. Design of a hollow-fiber reactor. This reactor is a cell culturing system based on hollow fibers with capillary membranes connected to the reservoir bottle that contains medium. A perfusion pump circulates the medium through the closed circuit. Fresh medium perfuses through the hollow fibers to nurture the cells growing within the cartridges.

generated by formulating cellular and acellular components, by convenient single-fermentation production.

Hollow-fiber bioreactors were designed by Lloyd and Bunch, 1996 and included immobilized cells on a fiber membrane with growth media passing through the fiber lumen (transverse mode) (Fig. 5). The hollow-fiber strategy prevented excessive biomass formation and shifted the supply of oxygen towards disulfide bond formation for protein folding. In hollow-fiber reactors, the product can be obtained through batch or continuous harvesting. Large-scale production of LTx-IB from *E. coli* was conducted in batch, chemostat, and Hollow-fiber bioreactor by the marine *Vibrio sp. 60* (Table 2).

The growth of *Vibrio sp. 60* immobilized on different quality fiber lumen gave the potential to select fibers with permeability properties that could help improve the purity of the LTx-IB (Lloyd et al., 1997). Volumetric productivity per hour showed final production to be 6.3 mg/L in batch, 3 mg/L in a chemostat, and 9.4 mg/L in a hollow-fiber bioreactor. Accordingly, specific production per hour was 2 mg, 1.2 mg, and 1.2 mg per g dry cell weight. The hollow-fiber reactor dominates in volumetric yields over other reactors, and it can maintain LTx-IB productivity for over 50 h. The selection of the right fiber material increases the overall productivity of the process by sustaining the integrity and facilitating the clear passage of a final toxoid product. The reactor with Amicon P100–20 fibers reached its highest productivity with partial loss of system integrity. The extended time of LTx-IB production was achieved with Amicon P100–43 fibers that can be improved by omitting the glucose as a C-source. This, however, decreases the overall volumetric productivity. The reactor productivity was regulated by the physical characteristics of the fiber used. It maintained extended process times over 100 h and stability of semi-permeable membranes of fibers (Fig. 5). The authors observed loss of the recombinant plasmid during hollow-fiber bioreactor cultivation after induction with IPTG. Consequently, antibiotics have been supplemented during continuous chemostat fermentation runs to improve plasmid retention and stabilize toxoid productivity. This research aimed to select the right manufacturing strategies for LTx-IB toxoid and present bioprocess differences of chemostat and Hollow-fiber reactors.

5. Purification of carbohydrate-binding toxins

Purification processes are often divided into two categories, traditional and simplified. The first one refers to the purification train that consists of Anion Exchange and/or Cation Exchange Chromatography (AEX and/or CEX), Hydrophobic Interaction Chromatography (HIC), and Size Exclusion Chromatography (SEC) steps (Zhao et al., 2019 and Łącki and Riske, 2020). The second one has implemented an affinity chromatography step with specific interactions between the target protein and the affinity ligand. Simplified purification eliminates the need for multiple unit operations to achieve desired purity. Amongst exemplified separation techniques, affinity chromatography is a preferable choice for the purification of the carbohydrate-binding toxin. Carbohydrate being the affinity ligand selectively targets protein of interest and improves productivity and flexibility of the process. Affinity chromatography is also considered the first step to assess if the protein was produced soluble and active. Many of that affinity chromatography were engineered quickly after identifying the correct carbohydrate ligand. However, challenges can arise due to the design and manufacturing of ideal ligands or high-productivity chromatography media. In the section below, we describe the rise of the first carbohydrate-binding toxin's chromatography and purification techniques which are often used in modern manufacturing facilities.

5.1. Purification of cholera and heat-labile toxin

Finkelstein et al., 1966 purified CTx from *Vibrio cholera* strain 569B through a combination of ion-exchange, size-exclusion, and agarose-affinity chromatography. This method's yield of purified protein was substantially greater than in previous studies (Finkelstein et al., 1964 and 1966), making it possible for researchers to work on CTx's structural and biological activities, mode of action, and immunogenic potential. In previous studies, purification was carried out using gel filtration and ion-exchange chromatography (Coleman et al., 1968). The same authors further enhanced the purification process by replacing ammonium-phosphate-induced precipitation with physical membrane filtration – a less laborious and higher-yielding technique (Finkelstein and LoSpalluto, 1970). One point of note is that although the initial CTx purification involved an agarose column, it was more generally used for size

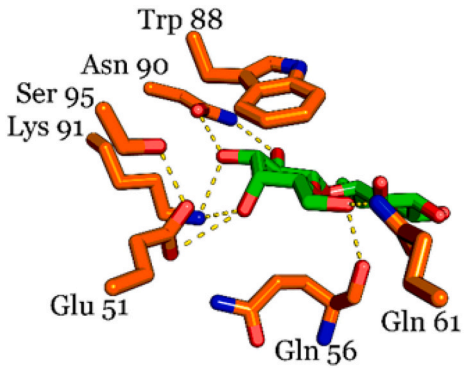

Fig. 6. Cartoons of heat-labile enterotoxin I (LTx-I) with lactose bound to the B-pentamer (PDB: 1LTT).

exclusion purposes than as an affinity column. However, the movement of CTx through agarose is slowed down, and later researchers realized that the analogous *E. coli* protein LTx-I binds to the column tightly (Clements and Finkelstein, 1979). Agarose is a polymer with a repeating unit of agarobiose (4-O-β-D-galactopyranosyl-3,6-anhydro-L-galactose) disaccharide of which galactose is a constituent. Despite galactose-binding sites in both CTx and LTx-I having been confirmed (Merritt et al., 1994a; Merritt et al., 1994b), the structure for their interaction with agarose has not been determined, although it presumably involves a terminal galactose residue, in analogy with the terminal galactosyl residue in ganglioside GM1 (Merritt et al., 1994a, Merritt et al., 1994b). The difference in affinity between these toxins was explained when the X-ray crystal structure of LTx-I interacting with lactose was published (Sixma et al., 1992). Lactose is the receptor analog of GM1 terminal galactose and the glucose in lactose is replaced by *N*-acetyl-galactosamine in GM1. The residues in the galactose binding sites are nearly identical between LTx-I and CTx, with the only difference lying in Ser 95 and Arg 13 of LTx-I. Ser 95 forms a hydrogen bond with Glu 51 and makes van der Waals interactions with Trp 88 (Fig. 6). Trp 88, on the other hand, has extensive hydrophobic interaction with galactose. Hence the substitution of Ser 95 to alanine might influence the local binding sites and explains the weaker galactose-binding affinity of CTx. In contrast, recent work by Heggelund et al., 2019, highlights residue 94 as being the most important for distinguishing the ability of LTxh and LTxp to bind to Lactose.

Purifying CTx on a large scale remained a problem until a receptor-specific affinity column based on lyso$_{GM1}$-derivatized beads was described by Tayot et al., 1981. This method enabled one to purify CTx from 1000 L of culture with 1 kg of resin, achieving 95% final purity. The beads were manufactured by coupling hydrolyzed GM1 (lyso$_{GM1}$) to DEAD-dextran coated on spherosil particles. Although difficult to synthesize, the beads could be re-used and provided good purification capacity. However, the elution buffer required a pH of 2.8, which caused partial dissociation of CTx, even with quick neutralization. The separation of intact and disassembled CTx could be achieved with size-exclusion or ion-exchange chromatography. The latter was demonstrated by Mekalanos, 1988, who exploited the different cationic charges distributed on the surface between CTx and CTxB. Whereas CTxB could still bind to the column under pH 8.3, CTx was detached and washed out under the same condition. The process was then improved by Jang et al., 2009a, 2009b with a 30-kDa crossflow ultrafiltration step (10 mM phosphate buffer, pH 7.6). 3-fold concentrated CTx was purified with pre-packed mono S 5/50 GL cation exchange column with a salt gradient of phosphate buffer, pH 7.0, and containing a high salt concentration of 1.0 M NaCl. The purity was determined to be 92%.

In 1994, Merritt noted that the terminal galactose in GM1 participated more extensively in the interaction with CTxB and LTx-I than with the rest of GM1 sugars (Merritt et al., 1994a, Merritt et al., 1994b). Soon after, Uesaka et al., 1994 employed immobilized D-galactose-agarose column as a simple one-step purification method for both CTx and LTx-I, although with different elution conditions (1 M and 0.3 M D-galactose in TEAN buffer, respectively) and recovery (34% and 59%, respectively). Interestingly, four years later, the Yasuda group applied the same type of column to isolate a recombinant CTxB (Yasuda et al., 1998), and they were able to elute the CTxB from the column with just 0.4 M D-galactose, and a yield of more than 90%.

Yasuda et al., 1998 published an article focused on the interaction between CTxB and metal ions and discovered that CTx could bind to Ni^{2+}, while LTx-Ip, which only obtains one histidine residue on position 57 per monomer, could not. After comparing the nickel-binding ability of recombinant CTxB with mutations in the three surface-exposed histidine residues, the predominant nickel-binding site was revealed to be His-13. While a single histidine residue does not usually give sufficient affinity for a nickel column, as there are 5 copies of His-13 arranged on the same face of the CTxB pentamer, and efficient multivalent interaction can arise of comparable affinity to a regular His-tag. His-94, on the other hand, could only participate in binding in the presence of His-13-nickel combination. Similarly, LTx-Ih expressed from *E. coli* H74–114 isolate of human origin has the prominent His-13, which ensures its binding capacity with nickel column. This research provided an alternative pathway for purifying CTx and LTx-Ih by nickel chelation chromatography (Guimaraes et al., 2011; Okuno et al., 2013; Ross et al., 2019). Compared to the LysoGM1-based column, the nickel columns were more commercially available, and the elution condition was milder – 42 mM imidazole in the experiment operated by Dertzbaugh and Cox, 1998 for eluting CTxB at near-neutral pH. Additionally, nickel resin could be used to isolate LTxh or CTxB based on its endogenous nickel-binding property and facilitate the purification for LTxh or CTxB carrying polyhistidine tags (Liu et al., 2016; Areas et al., 2002; Gong et al., 2009; Dakterzada et al., 2012).

Accumulating CTxB in the cytoplasm as inclusion bodies instead of transporting it to periplasm offers an alternative strategy for isolation and purification stages. If the signal peptide sequence is deleted, a denaturation-renaturation step with 6.5 M urea with β-mercaptoethanol is required for recovering the CTxB pentameric structure (L'hoir et al., 1990). The refolded CTxB could be further purified by nickel column either through its inherent nickel-binding ability or through a fused His-tag (Areas et al., 2002; Areas et al., 2004).

Successful purification of LTx-I traces back to 1979 when the utilization of agarose-affinity chromatography was proposed. LTx-I expressed from *E. coli* 711 (F_1LT) stain was able to bind to an Agarose A-5 m column with strong affinity and could be eluted by free galactose (Clements and Finkelstein, 1979). This was not surprising, since GM1 ganglioside – the LTx-I ligand – contains a terminal galactose residue, as does agarose. Later experiments demonstrated that this purification procedure was also suitable for LTx-I expressed from other enteropathogenic strains of *E. coli* (Clements et al., 1982; Geary et al., 1982). Interestingly, other agarose-derivative columns, such as Affigel 202 which possess free carboxyl groups at the end, had been applied even earlier to purify LTx-I (Evans Jr. et al., 1976), but their use did not become widespread, probably due to too-high affinity of LTx-I for the resin, making it difficult to elute the proteins (Clements and Finkelstein, 1979). Since LTx-I was proven to be able to bind GM1 ganglioside (Holmgren, 1973), a type of GM1 ganglioside-coupled cellulose was created to purify LTx-I from both human and porcine origin (Hirst et al., 1984). The release of bound toxin needs to boil the sample in the buffer containing sodium dodecyl sulfate, which might lead to partial dissociation of A- and B-subunit and hence be less user-friendly compared to D-galactose-agarose column. Apart from affinity columns, the pairing of hydrophobic chromatography (norleucine-Sepharose 4B column) and hydroxylapatite chromatography was also used to purify LTx-I (Kunkel and Robertson, 1979). Alternatives, such as controlled pore glass (CPG), were devised to separate LTx-I from the periplasm (Pronk et al., 1985) and later used to purify a detoxified LTx (Pizza et al., 1994). CPG is form of chromatography used for batch extraction processes where the main

advantages of porous glass is a homogeneous pore size, high mechanical stability and chemical surface variability. The purification of LTx-II is trickier because GM1 ganglioside is a poor ligand for it. Likewise, until 1986 efforts to purify LTx-II from *E. coli* SA53 suffered from a high level of impurities (Holmes et al., 1986). This problem was solved by transforming an LTx-II-harbouring plasmid into *E. coli* HB101 (pCP3837) and the expressed toxins were purified by combining multiple purification strategies, including Affi-Gel Blue, chromatofocusing, and size exclusion (Holmes et al., 1986). Affi-Gel Blue affinity gel is a beaded, crosslinked agarose gel with a covalently attached dye that purifies an extensive range of proteins of different sizes and origins. An alternative has been to express LTx-IIB as inclusion bodies and refold it to give the soluble pentameric B-subunit (Zalem et al., 2016).

Similarly, LTx-IB could be induced as the inclusion body to aggregate in cells rather than in extracellular media for a more convenient harvest (Ma et al., 2010). Moreover, the recombinant LTxB contained a His-tag to facilitate purification through the nickel column, which efficiently increases purity and makes the preparation scheme more economic compared with using the LysoGM1-based column and hydrophobic column mentioned above.

5.2. Purification of Shiga toxin

Several methods were proposed to purify STx on a small scale during the 1980s, including the application of an acid-treated chitin column (Olsnes and Eiklid, 1980) and antitoxin affinity column chromatography (O'Brien et al., 1980). STx binds to oligosaccharides derived from chitin (Keusch and Jacewicz, 1977). The antitoxin-based column was prepared with the rabbit antiserum against partially purified STx, and it showed better selectivity than the chitin column. However, either technique could achieve no more than 5% recovery. The first successful large-scale purification of Shiga toxin was in 1984, utilizing chromat0focusing as the principal operation (Donohue-Rolfe et al., 1984). In 1986, Jacewicz et al. found that STx could bind to glycolipids extracted from rabbit jejunal mucosa and Hela cells, and further identified the relevant component as Gb_3 (Jacewicz et al., 1986). Moreover, they demonstrated that the P1 antigen presented in human erythrocyte glycolipid extracts could also interact with STx. Considering that Gb_3 and P1 antigen share a terminal Galα1 → 4Gal disaccharide (galabiose), the P1 antigen could be exploited for specific purification of STx. Indeed, towards the end of the 1980s, Ryd et al. covalently linked a trisaccharide Galα1 → 4Galβ1 → 4Glc (globotriose) to Fractogel and achieved a simple and specific one-step purification (Ryd et al., 1989). In parallel, Donohue-Rolfe et al. coupled hydatid cyst (fluid-filled sac of larval form of tapeworm)P1 glycoprotein (P1gp) to Sepharose 4B matrix, which became a popular column for one-step purification of both STx, STx-1, and STx-2 holotoxins (Donohue-Rolfe et al., 1989) and their B-subunits (Acheson et al., 1993a; Acheson et al., 1993b), including some of their mutants. At last, the purification yield and final purity were improved by constructing the Globotriaosylceramide-Conjugated Octyl Sepharose CL-4B (Nakajima et al., 2001).

Other than relying on the native affinity of Shiga B-subunits for sugars, the Shiga toxins could also be purified by expressing them as fusion proteins to glutathione S-transferase (Gunzer and Karch, 1993) and maltose-binding protein (Acheson et al., 1995), which permitted glutathione- or amylose-affinity chromatography, respectively. Similar to LTx or CTxB, STx can be also purified with Ni-NTA column (Sharma et al., 2012; Arimitsu et al., 2013a). Affinity chromatography can also be replaced or complemented with modern cation exchangers, hydrophobic interaction columns, anion exchangers, and gel filtration (Skinner et al., 2013).

A large study was conducted by Arimitsu et al., 2013a, 2013b on STx-2e variant and their affinity to D-galactose, lactose, and *N*-acetylgalactosamine as ligands for affinity purification with each sugar immobilized to the resin. D-galactose resin was the only one successfully purifying STx2e variant and thereof selected for 1 L large scale purification. Further investigation to D-galactose based resin has shown that α-D-galactose immobilized on agarose resin via a divinyl sulfone linker is a key to successful purification. A possible substitution to this affinity chromatography can also be thiophilic adsorbent resin containing β-mercaptoethanol immobilized on agarose resin via a divinyl sulfone, which shows equivalent results to the α-D-galactose.

5.3. Purification of other carbohydrate-binding toxins

The widely adopted purification method for BoNT/A is ion-exchange chromatography (Dasgupta et al., 1966; Knox et al., 1970), which could separate BoNT/A-hemagglutinin from the impurities, chief of which was hemagglutinin. Single-step affinity chromatography, enabled by coupling *p*-aminophenyl-β-D-thiogalactopyranoside to CH-Sepharose 4B, was also used to purify BoNT/A and achieved a 50–60% recovery with more than 99% purity (Moberg and Sugiyama, 1978). This affinity column utilized the binding capacity between the ligand and hemagglutinin under pH 6.3 and dissociation under pH 7.9 to achieve the separation. Both ion-exchange chromatography (Jacobson et al., 2011; Eisele et al., 2011; Schmidt and Siegel, 1986) and affinity chromatography (Pellett et al., 2018) are commonly used to purity BoNT/A and still work as a powerful tool to isolate subtypes for diverse purposes.

GE Healthcare presented an efficient purification strategy of pertussis antigens developed for DaPT.[2] PTx and FHA were purified with Capto SP ImpRes ion-exchange chromatography with sulfonate group as the ligand. Another example of PTx purification was presented by Lee et al., 2002 where recombinant PTx and FHA were fused, named S1S3FHA. The affinity chromatography was conducted with a monoclonal anti-FHA immunoglobulin G (IgG) column. The column was prepared by cross-linking the anti-FHA monoclonal 9F IgG to Affi-Gel protein A agarose.

6. Conclusion

Microbial carbohydrate-binding toxins have been successfully implemented in many biopharmaceutical applications, from cancer-targeting to vaccine development. Glyco-selectivity of bacterial toxins has been widely studied in vitro and animal models, showing high potential in targeting glyco-surfaces for drug delivery or vaccine technology. Given the circumstances that the dosage of vaccines or drugs per patient can be high (e.g., 1 mg of recombinant CTxB for the vaccine DUKORAL®), large-scale production is often required for it to become a practical therapeutic option. Selecting a well-defined and optimized fermentation regime can improve overall productivity. Production supported by optimized glycan-based affinity chromatography further enables effective bacterial toxin purification. Finding the right production regime of microbial toxins is thus considered important to provide cost-effective manufacturing of vaccines and diagnostic or therapeutic applications. Carbohydrate-binding toxins have been studied for decades. With the future rise of glycobiology the use of these important multi-functional protein complexes will become more and more state-of-the-art and with the advent of *in-silico* protein design and engineering the carbohydrate-binding functionalities can be crafted on promising effector proteins for cancer cell killing or drug delivery.

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[2] https://cdn.cytivalifesciences.com/dmm3bwsv3/AssetStream.aspx?mediaformatid=10061&destinationid=10016&assetid=18267

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