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REVIEW

# Oral Delivery of Protein Drugs Bioencapsulated in Plant Cells

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Plants cells are now approved by the FDA for cost-effective production of protein drugs (PDs) in large-scale current Good Manufacturing Practice (cGMP) hydroponic growth facilities. In lyophilized plant cells, PDs are stable at ambient temperature for several years, maintaining their folding and efficacy. Upon oral delivery, PDs bioencapsulated in plant cells are protected in the stomach from acids and enzymes but are subsequently released into the gut lumen by microbes that digest the plant cell wall. The large mucosal area of the human intestine offers an ideal system for oral drug delivery. When tags (receptor-binding proteins or cell-penetrating peptides) are fused to PDs, they efficiently cross the intestinal epithelium and are delivered to the circulatory or immune system. Unique tags to deliver PDs to human immune or nonimmune cells have been developed recently. After crossing the epithelium, ubiquitous proteases cleave off tags at engineered sites. PDs are also delivered to the brain or retina by crossing the blood–brain or retinal barriers. This review highlights recent advances in PD delivery to treat Alzheimer's disease, diabetes, hypertension, Gaucher's or ocular diseases, as well as the development of affordable drugs by eliminating prohibitively expensive purification, cold chain and sterile delivery.

Peptides or proteins, including hormones, enzymes, ligands, or inhibitors regulate various cellular functions. Therefore, they are useful in the clinic to treat or prevent human disorders by modulating physiological or pathological processes. The use of proteins or peptides for therapeutic purposes will continue to increase in the treatment of cancer, metabolic disorders and neurodegenerative or infectious diseases. In contrast to small-molecule drugs, the high selectivity of peptides or proteins to their targets may reduce side effects and toxicity to host cells.[1] In 2013, cumulative sales of biopharmaceuticals reached $140 billion, and this value is expected to increase steadily in clinics.[2]

Currently, protein drugs are largely manufactured using mammalian, yeast or bacterial cell culture systems. These expressed proteins must be extracted and purified, which requires expensive and complex processes and cold storage and transportation, but they have short shelf-lives.[3,4] Moreover, there is a risk of facility and/or product contamination with toxins or human pathogens.[5] Injectable forms of PDs often require health care personnel for administration, resulting in hospital visits and decreased patient compliance. In addition to issues of high cost, current facilities have limited production capacity. It is estimated that it would require ~77–500 million € and up to 5 years to build a mammalian cell culture production unit,[6] excluding additional years for regulatory approval.

Proteins drugs produced in current manufacturing systems are mostly delivered by injection; oral delivery is not possible due to drug degradation by stomach acids, proteases in the digestive system and the inability to cross intestinal membrane barriers.[7] Attaching molecules like polyethylene glycol,[8] an antibody Fc domain[9] or human serum albumin[10,11] increases peptide stability in serum during circulation. In addition, peptide drugs can be modified to protect from serum proteases and peptidases; such modifications include N-terminal acetylation, C-terminal amidation, the use of non-natural amino acids, and cyclization via disulfide bonds.[12] However, there are still no clinically approved oral peptide drugs.

Plants offer an ideal alternative to conventional manufacturing systems and invasive methods of PD administration. Plants are not hosts for human pathogens. The plant cell wall—heavily packed with lignin and cellulose—provides natural protection for PDs because human enzymes are incapable of breaking down glycosidic bonds of plant cell wall carbohydrates. However, gut bacteria digest the plant cell wall and release bioencapsulated PDs into the gut lumen.[13,14] Plant cells have similar capacity as mammalian cells to produce protein drugs.[15] Antibodies against Ebola virus (ZMapp)[16] or glucocerebrosidase, an enzyme replacement therapy for Gaucher's disease have been produced in tobacco plants[16] or carrot cell suspension cultures.[17] Long shelf life and stability of PDs are critical for successful clinical applications of PDs. Protein drugs made in chloroplasts are stable in lyophilized plant cells when stored at ambient temperature, maintaining their folding and functional efficacy for several years,[18] thereby eliminating expensive cold chain. In addition, the freeze-drying process increases the concentration of PDs and eliminates bacterial contamination.[4] Advantages of plant systems as bioreactors for production of biopharmaceuticals as well as their delivery, and remaining clinical challenges are discussed in this review.

## Chloroplast/Nuclear Production of Biopharmaceuticals

Similar to mammalian cells, plant cells facilitate formation of disulfide bonds, glycosylation, folding, and assembly of PDs.[15,19] The first plant-made pharmaceutical protein, human growth hormone, was made in tobacco and sunflower callus tissue via nuclear transformation and was reported in 1986.[20] First chloroplast-made therapeutic proteins, cholera toxin B subunit (CTB), human serum albumin, and somatotropin were reported in the early 2000s.[21,22,23] Plants stably transformed with transgenes can be easily propagated from seeds. *Agrobacterium tumefaciens* is used to deliver transgenes to the nucleus; whereas a particle delivery system is used to transform plants that are recalcitrant to *A.* mediated transformation.[24] Recombinant proteins that require glycosylation for their functionality are expressed via the nuclear genome and targeted to the endoplasmic reticulum (ER). Targeting recombinant proteins to the ER, apoplast or other subcellular compartments improves yield.[15] Biopharmaceuticals are also produced in plant cell suspension cultures to minimize regulatory concerns and expedite FDA approval.[25] However, despite several decades of research on nuclear transformation, low expression levels have hampered production of industrial-level expression of biopharmaceuticals in plants.[26] There are also risks of dissemination of engineered genes to the environment

via pollen and of contamination of food or feed chains by transgenic seeds.[24]

Chloroplast genomes have also been utilized for stable transformation of numerous heterologous genes since the early 1990s.[27,28,29,30] The most striking feature of the chloroplast genome over the nuclear genome is its high copy number (>10,000 per cell), enabling transgenes to be expressed at up to 70% of total leaf protein.[31] Double homologous recombination and transgene integration at target sites eliminate positional effects. Gene silencing of transgenes has not yet been reported in chloroplasts. In addition, engineering multiple genes into the chloroplast genome is achieved with a single transformation event,[32,33,34,35] facilitating expression of complex proteins. Chloroplasts also minimize the effect of toxic PDs, like CTB, by sequestering transgene products within this compartment.[21,36] Transgene confinement via maternal inheritance and harvesting leaves before flowering offers complete transgene containment.[37]

## Mechanisms of Oral Drug Delivery

### Transmucosal drug delivery across the gut

The large mucosal area of the human intestine ~ 1.8–2.7 $m^2$,[38] offers an ideal surface for drug delivery. Upon oral delivery, the plant cell wall protects PDs from acids and enzymes in the stomach via bio-encapsulation.[39,40] Human digestive enzymes are incapable of breaking down all glycosidic bonds in the plant cell wall. Figure 1b shows intact plant cells expressing GFP in the gut lumen that survived acids and enzymes secreted in the stomach, thereby providing direct evidence for protection of proteins via bioencapsulation. However, when intact plant cells containing PDs reach the gut, commensal microbes digest the plant cell wall and release PDs (Figure 1a).[13,14] Among gut microbes, only Bacteroidetes and Firmicutes can break down the recalcitrant, insoluble plant cell wall.[41] The cellulosome, which is found in anaerobic cellulolytic bacteria (*e.g.*, *Ruminococcus flavefaciens*, a representative of the Firmicutes), is an extracellular enzyme complex that contains catalytic, structural, and cellulose-binding domains. Through the cellulosome, bacteria that retain highly concentrated catalytic activities necessary for cleaving plant cell wall glycosidic bonds on their surface make close contact with cell wall substrates and disrupt plant cells. In addition, specialized groups of bacteria colonize the gut mucus layer. For example, a fluorescence *in situ* hybridization study showed enrichment of Bacteroidetes in the mucous layer. Moreover, mucopolysaccharides in the mucous layer are substrates for gut bacteria. *Bacteroides fragilis* is known to degrade mucin glycoproteins to allow it to penetrate the mucous layer.[42] As seen in Figure 1b, intact plant cells captured by cellulosomes or pilli of bacteria that colonize the mucosal layer undergo cell wall degradation to release GFP. By these mechanisms, bioencapsulated therapeutic proteins are orally delivered. The presence of plant cells expressing GFP in between villi of the ileum offers visible proof of the protection of plant cells from the digestive system and the uptake of proteins by epithelial cells in the upper gut (Figure 1b).[40]

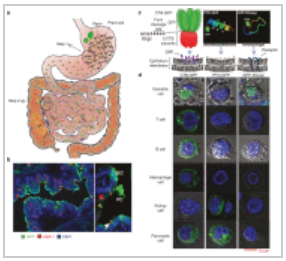

Figure 1

**Mechanism of protein drug (PD) delivery to target cells.** (**a**) Oral delivery of bioencapsulated protein drugs: Step 1, The plant cell wall protects PDs from acids and enzymes in the stomach; Step 2, Digestion of the plant cell wall by gut microbes and release of PDs into the gut lumen (**b**) Intestines of mice fed with lyophilized cells expressing cholera toxin B subunit (CTB)-GFP stained with anti-GFP (green signal; Alexa Fluor 488), UEA-1 (microfold cells (M cells), red signal, rhodamine), and DAPI (nuclear stain, blue). DAPI, 4',6-diamidino-2-phenylindole. Solid arrow, $GFP^+$ M cells; EC, epithelial cells; PC, plant cells. (**c**) Interaction of CTB-GFP with GM1 and predicted 3D structure of protein transduction domain (PTD) and DCpep. Amino acid sequences of PTD and dendritic cell peptide (DCpep) are indicated below 3D structures. (**d**) Uptake of GFP-fused tags by human immune and nonimmune cells. Purified GFP fusion proteins were incubated with human cell lines and cells were stained with DAPI. Images were captured at 100× magnification under a confocal microscope. (**b**), (**c**), and (**d**) are modified from a previous publication.[40]

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When tags (receptor-binding proteins or cell-penetrating peptides) are fused to PDs, they efficiently cross the intestinal epithelium and are delivered to the circulatory or immune system. In our recent study, systemic distribution of GFP was observed in sera and organs including liver, lung, brain, retina, and the tibialis anterior muscle when plant cells expressing CTB-, protein transduction domain (PTD)-, or dendritic cell peptide (DCpep)-fused GFP were orally delivered.[40] To investigate the route of delivery, an Alexa Fluor 488-labeled antibody against GFP was used for immunohistochemical studies on mice intestine that were fed with plant cells expressing GFP-tagged proteins. The intestines were rolled up to observe proximal and distal portions simultaneously. GFP tagged with PTD was observed in gut epithelial cells, whereas more widespread delivery of GFP to epithelial cells was seen with CTB tag. However, GFP was not detected in epithelial cells when DCpep-GFP was orally delivered. All three fusion tags delivered GFP to microfold cells (M cells). Therefore, in addition to the gut-liver axis, systemic delivery of DCpep-GFP was achieved via M cells, whereas CTB- and PTD-GFP reached circulation through both epithelial and M cells.

When tags are used to move proteins across epithelial cells, various endocytic subpathways are employed. CTB can use either clathrin-, caveolar- and nonclathrin/noncaveolar-mediated pathways followed by retrograde transport[43] or transcytosis.[44] In the case of PTD, uptake occurs via electrostatic interactions with the plasma membrane, followed by endocytosis and retrograde transport.[45] The main mechanism for PTD penetrating epithelial cells is through endocytic pathways involving membrane invagination, rather than direct entry via pores, membrane thinning, inverted micelle formation or direct translocation.[46]

DCpep-GFP is exclusively observed in M cells after oral delivery[40] suggesting that the delivery of DCpep-GFP into circulation is possible by transcytosis through M cells, in addition to gut-liver axis.[47] In addition, DCpep fusion proteins can be directly captured in the intestinal lumen by extended dendrites of DCs, thereby facilitating delivery to the immune system.[48] Therefore, DCpep fusion proteins can cross the epithelial barrier through M cells to enter circulation or can be directly taken up by DCs in the intestinal lumen.

In addition to facilitating PD delivery, fusion tags provide N-terminal protection for fused proteins, increasing their stability in sera for several hours over the same proteins after injection.[49,50] After crossing the epithelium, proteases like furin, which is present in all cell types, cleave tags at engineered sites.[51] In the section below, we discuss mechanistic aspects of PD delivery to the circulatory or immune system using different tags.

## Drug delivery to immune or nonimmune cells

Human body is connected to the environment by mucosal systems where nutrient uptake/waste excretion and gas exchange take place; however, these systems are also contact and entry points for pathogens or toxins. So, the mucosal-associated lymphoid tissue is loaded with a large number of immune cells. In particular, the gut-associated lymphoid tissue represents almost 70% of the entire immune system and contains ~80% of immunoglobulin A-bearing cells.[52] Therefore, when therapeutic proteins are orally delivered to treat metabolic diseases, delivery to immune cells, which are heavily represented along the gastrointestinal tract, should be minimized to avoid complications (*e.g.* developing toxic antibodies). Differentiating immune cells from nonimmune cells is therefore important for oral drug delivery. PTD fusion proteins are not delivered to immune cells but are delivered efficiently to nonimmune cells in the pancreas and kidney.[40] This unique selectivity of PTD can be used clinically to treat metabolic disorders such as diabetes or nephropathy by targeting PTD-fused drugs to the pancreas and kidney, respectively.

Likewise, to treat autoimmune diseases or induce specific immune responses, it is important to deliver therapeutic proteins to immune cells. In contrast to splenic DCs, DCs from Peyer's patches induce tolerance to intestinal antigens transported by M cells, rather than prime T-cells as effectors.[48] Indeed, antigen uptake by M cells and transportation to APCs is also considered a prime target to enhance oral vaccine efficacy. Therefore, it is widely accepted that initial systemic priming followed by mucosal boosting with the use of adjuvants is likely to prevent mucosal tolerance induction.[53,54] Therefore, proper targeting of drugs by distinguishing immune and nonimmune cells is very important. DCs deliver GFP only to the immune system and not to any nonimmune cell type.

In addition, different strategies can assure efficient transmucosal delivery of the antigen, once it is released from plant cells. CTB is a highly efficient carrier molecule for transporting PDs across the gut epithelium.[55,56] CTB consists of five identical 11.6-kDa polypeptide monomers that assemble into a stable pentamer ring that binds to the GM1 ganglioside receptor.[57] Up to 15,000 CTB molecules can bind to each intestinal epithelial cell[58] and the GM1 receptor turns over rapidly on the cell surface.[59] CTB-fused proteins form pentamers within chloroplasts irrespective of the size of fused proteins –7 amino acids to >100 kDa.[18,49,50,60,61,62,63,64,65] Pentameric CTB fusion proteins bound to GM1 receptors are transported into gut epithelial cells by two possible pathways: retrograde trafficking and transcytosis. Through retrograde trafficking,[43] the complex traffics to the *trans*-Golgi network and ER via recycling endosomes. Once transported to the ER, unfolding and retro-translocation of the CTB-fused protein occur. Protein drugs are then released into the cytosol from the complex by ER-associated degradation. Transcytosis has been proposed as another pathway for CTB-PDs to cross epithelial cells.[44] A fraction of CTB-PD-GM1 complexes crosses polarized epithelial cells by transcytosis, which requires GM1 receptors with ceramide domains containing short or unsaturated fatty acid chains.

The first direct evidence for oral delivery of a CTB fusion protein produced in plants was provided by Limaye *et al.* (2006).[39] Pentameric assembly of CTB within chloroplasts was confirmed by immunoblot analysis and GM1 binding assays.[21] In mice fed with CTB-GFP-expressing plant cells, fluorescence microscopy showed delivery of GFP to epithelial cells and M cells in the small intestine, as well as the presence of GFP in intestinal mucosa and submucosa, hepatocytes, and spleen cells, indicating successful oral delivery of the protein across the intestinal lumen.[39,40] Inclusion of a furin proteolytic cleavage site assures that CTB is retained in epithelial cells after uptake while the antigen of interest is released, resulting in delivery not only to the immune system but also into systemic circulation. This concept has been successfully applied to several antigens/therapeutic proteins, which could be detected in circulation in mice within 2–5 hours and in some cases as soon as 30 minutes after oral delivery.[18,62,63,64,66]

Fusion of CTB to therapeutic proteins facilitates their effective oral delivery to induce oral tolerance[18,64,65,66,67] or deliver functional proteins to sera[49,50,61] or across blood–brain or retinal barriers.[62,63] However, when primed with adjuvant, CTB is highly immunogenic and diverts the specific response from the antigen of interest.[68,69] In addition, aggregation of protein antigens due to formation of CTB multimers or pentamers is a potential limitation. Determination of antigen dose is another major challenge because it would require complete solubilization of CTB pentamers, which is quite challenging because of the resistance of strongly bound CTB monomers;[70] multimeric forms exist even after treatment with denaturing agents including dithiothreitol, sodium dodecyl sulfate, and boiling.[49,61,62,63] Although pentamer stability is ideal for oral delivery of CTB fusion proteins, quantitation of dose continues to be a major challenge. Therefore, there is a great need to explore other pathways for oral delivery and investigate the point of entry of PDs.

Foreign proteins are also delivered into living cells through fusion with PTDs that do not require specific receptors.[71] PTDs are small cationic peptides of 8–16 amino acids that most frequently function as macromolecule transporters (Figure 1c). PTDs carry molecules into cells by receptor-independent, fluid-phase macropinocytosis, which is a special form of endocytosis. The efficacy of cellular uptake by PTD correlates with the number of basic amino acid residues. Similarly, DCpep is a ligand to mucosal DCs (Figure 1c). This small peptide binds to a DC-specific receptor,[72] facilitating transportation of macromolecules into DCs. These properties may be exploited to deliver efficiently various molecules to DCs for antigen presentation, to block their maturation, or to modulate their functions.

Targeted drug delivery is important to enhance their efficacy at the site of action. Therefore, we have explored targeted drug delivery by oral administration of plant cells expressing GFP fused with different tags in chloroplasts and evaluated their cellular targeting and bio-distribution.[40] PTD was derived from pancreatic and duodenal homeobox factor-1, which induces insulin expression upon protein transduction via macropinocytosis.[45] Delivery of PTD, DCpep, and CTB fusions across the gut epithelium utilized distinct pathways and resulted in systemic delivery, bio-distribution, and most importantly, distinct patterns of uptake by nonimmune or immune modulatory cells. Purified GFP fusion proteins were incubated with cultured human cells. Tested immune cells included blood monocyte-derived mature DCs, T-cells (Jurkat cells), B cells (BCBL1) and differentiated macrophages. Human pancreatic epithelioid carcinoma cells (PANC-1) and kidney cells (HEK293T) were tested in parallel as examples of nonimmune cells. Upon incubation with GFP-DCpep, intracellular GFP was detected only in DCs. PTD-GFP was taken up by pancreatic and kidney cells (or other nonimmune cells) but failed to enter any immune regulatory cells (Figure 1d). Because insulin expression needs to be tightly regulated and is responsive to environmental stimuli, this may in part account for this selectivity.[73] In sharp contrast, GFP was detected in all cell types upon incubation with CTB-GFP, consistent with the ubiquity of GM1 receptors[74] (Figure 1d). These peptides are ideal to deliver therapeutic proteins to sera, immune cells, and nonimmune cells or to specific tissues.

### Drug delivery across blood–brain or retinal barriers

Delivery of PDs from the bloodstream to the brain across the blood–brain barrier (BBB) or blood-retinal barrier (BRB) has long been a major challenge to treat neuronal degenerative disorders or ocular diseases.[62,75] Active and passive immunotherapies have been reported to target amyloid β in clinical studies[76] through immunization, but the recent failure of bapineuzumab,[77] a humanized anti-amyloid antibody, in phase III clinical trials calls for alternative strategies for Alzheimer's drugs and therapies. Restricted delivery of monoclonal antibodies to the brain substantially contributed to the failure of this and other studies.

Current approaches to deliver drugs in the brain are largely categorized into four methods: (i) bypassing the BBB by direct injection such as intracerebral injection or stereotactically guided insertion of a small-caliber catheter; (ii) disrupting the BBB using osmotic shock or ultrasound; (iii) passively diffusing through the BBB using small lipophilic molecules; and (iv) crossing the BBB by transporter- or receptor-mediated delivery. Direct injection of drugs shows poor diffusion efficiency. The disruption method is costly, requires anesthesia, hospitalization, and cause serious side effects after successful disruption, including enhanced tumor dissemination and permanent neuron damage. The efficacy of diffusing through the BBB with small lipophilic molecules could be offset by efflux pumps that can recognize these lipophilic molecules as substrates and return them to the blood.[78]

Because the brain is perfused with capillaries, using transporters or receptors on capillaries should be efficient in delivering drugs. Several studies use amino acid and choline transporters; and insulin, transferrin or low-density lipoprotein receptors for delivering drugs conjugated with ligands to those transporters and receptors. Polymersomes decorated with a peptide that binds GM1 receptors on the BBB effectively cross the BBB by transcytosis after i.v. injection.[79] Likewise, CTB, which has a high binding affinity for GM1, can serve as a carrier protein to deliver protein drugs into the brain in the same way it functions as a mucosal carrier in the intestine.[62]

Indeed, GM1 receptors are present in plasma membranes of the nervous system and retina.[74,80] Likewise, ocular drug delivery, particularly to the posterior segment of the eye, is also a major challenge.[81] Topically administered drugs are inefficiently delivered to the retina or vitreous cavity because of several ocular layers (cornea epithelium, stroma, and endothelium), tear drainage, frontward flow of aqueous humor, and surrounding blood circulation. Intravenous administration is the primary method used to deliver drugs to the posterior part of the eye, but major obstacles include retinal detachment, endophthalmitis, and high intraocular pressure.[82] In specific case studies below, orally delivered plant cells expressing CTB fusion proteins effectively crossed BBB and BRB, and ameliorated the pathological conditions.[40,62,63]

## Case Studies on Oral Delivery of Bioencapsulated Biopharmaceuticals

Although plant-produced biopharmaceuticals such as Elelyso or ZMapp[83,84] showed efficacy or potency, respectively, compared with their competitors, they are injectable PDs, and their production in plants does not reduce their cost because they do not address major challenges of expensive purification, cold chain, and short shelf life. In sections below, we discuss specific case studies.

### Gaucher's disease

This rare inherited metabolic disorder is caused by mutations in glucocerebrosidase, which results in accumulation of glucocerebroside in lysosomes, and causes newborn babies to die within a few months or years unless treated.[85] Before Elelyso, patients depended on costly glucocerebrosidase produced in Chinese hamster ovary or human fibrosarcoma cell lines (sold as Cerezyme or Velaglucerase alfa).[85] However, the annual cost for treatment with PDs is similar due to expensive downstream purification steps; Elelyso costs $324,870, Cerezyme costs $432,978, and Velaglucerase costs $368,550.[86] Therefore, an oral formulation of plant recombinant human glucocerebrosidase (prGCD) has been investigated.[87] Carrot cells expressing prGCD were fed to rats, then levels of active prGCD increased for 6 hours in both the small intestine and plasma, and was detectable in the plasma for at least 24 hours. In biodistribution studies, active prGCD levels peaked at 6 hours, and between 6 and 8 hours postfeeding for rats and pigs, respectively, whereas activity of an i.v. injection formulation peaked at 30 minutes. The active form of prGCD delivered orally remained in the serum much longer than the i.v. injection. However, the efficiency of prGCD uptake in target organs such as liver and spleen was 10-fold < the i.v. formulation, which might be attributed to low-level expression of prGCD via the carrot nuclear genome.

### Diabetes

The number of diabetes patients has steeply risen and is estimated to reach 552 million by 2030.[88] Along with insulin-dependent type I diabetes, one-third of patients with type 2 diabetes, which is responsible for 90% of diabetes cases, require insulin therapy.[89] These diabetes patients may have to inject insulin >60,000 times in their lifetimes.[90] Therefore, oral insulin formulations have been preferentially studied, not only for their simplicity, but also for their physiological activity.[91] Since orally delivered insulin like endogenous insulin, enters circulation through the hepatic portal vein, this method can minimize probable side effects such as hypoglycemia and weight gain, which result from high systemic levels of insulin caused by subcutaneous injection.[91] However, poor biostability from low stomach pH and low bioavailability in the thick mucus layer of intestines hamper the realization of oral insulin. Numerous oral insulin formulations have been under development, such as nanoencapsulated insulin, coated insulin-loaded nanoparticles, hepatic-directed vesicle insulin, and enteric-coated insulin capsules,[91] and are in various phases of clinical trials, but new oral formulations require further improvements due to failure to meet primary efficacy endpoints, unequivocal dose-dependent plasma insulin responses, quicker clearance from circulation than subcutaneous injection, less pronounced metabolic effects, and substantial between-subject variability with regard to the antihyperglycemic effect.[91] In addition, insulin needs cold storage because of its short shelf life at room temperature. Furthermore, current insulin therapy does not include C-peptides, which are released during insulin maturation and can ameliorate diabetes-induced renal and nerve dysfunction and induce disaggregation of hexameric insulin, resulting in an increase of biologically active, monomeric insulin.[92,93]

Therefore, plant-based insulin therapy was designed to release functional C-peptides upon oral delivery.[61] Transplastomic leaves expressing CTB-proinsulin containing 3 furin cleavage sites up to 53% of total leaf protein were fed to mice, and mice showed lowered blood glucose levels within 2 hours, which was similar to the hypoglycemic effect of injected commercial insulin.[61]

A series of PDs, which function as agonists to the glucagon-like peptide-1 receptor has recently been approved by the FDA to treat type 2 diabetes: Dulaglutide, Albiglutide, Bydureon, and Liraglutide. These insulin secretagogues were developed based on glucagon-like peptide-1-based therapy after the great success of their predecessor, exenatide (synthetic peptide of exendin-4, FDA-approved in 2005). These agonists have much longer half-lives in blood than endogenous glucagon-like peptide-1, which has a 2-minutes half-life.[94] Although advances have increased half-lives of these drugs in blood up to 1 week, they still need cold storage and require inconvenient abdominal injections.

The glucose-dependent action of exenatide in circulation can eliminate concerns about hypoglycemia, which can be triggered by an accidental overdose.[94] Recently, two groups evaluated the efficacy of bioencapsulated exenatide.[49,95] They expressed exendin-4 (EX-4) fused to CTB[49] or transferrin[95] in plants to facilitate the translocation of fused proteins across the epithelial barrier via interaction with receptors present in intestinal cells. Lyophilized plant cells expressing CTB-EX4 orally delivered to mice lowered blood glucose levels by 27% over control mice in response to a glucose spike.[49] Similarly, partially purified EX-4-Tf showed glucose-lowering effects when orally delivered to mice.[95] Both plant-derived EX4 fusions had potency similar to subcutaneously injected commercial EX4. In contrast to currently available insulin lacking C-peptides, EX4 releases all insulin peptides.

## Hypertension

Pulmonary arterial hypertension is a fatal disease characterized by remodeling of the pulmonary vasculature and elevated pulmonary vascular resistance and pulmonary artery pressure.[96] Endothelial proliferation, smooth muscle hypertrophy, and adventitial thickening in small muscular pulmonary arteries lead to increase right ventricular afterload, right ventricular dysfunction, and heart failure.[97] The current treatment approach has remained essentially unchanged over past decades, focusing on three pathophysiological pathways: prostacyclin, endothelin, and nitric oxide.[98] Although new formulations, delivery routes, and points of action have emerged, it has been almost a decade since a novel pathway has been successfully targeted by a human therapeutic for PAH. In addition, many of these drugs have inconvenient routes of delivery (continuous i.v., subcutaneous or intermittently inhaled) and bothersome side effects (prostacyclin analogs and phosphodiesterase-5 inhibitors),[99] and all are prohibitively expensive, requiring extensive approval processes and specialty pharmacy distribution to prescribe. Despite these modern, expensive therapies, well-designed epidemiologic studies continue to show a risk of death for PAH patients of ~15% at 1 year and 45% at 3 years.[100]

The renin-angiotensin system (RAS) is the main axis controlling systemic and local blood pressure. Angiotensin-converting enzyme (ACE) generates angiotensin II (AngII), which binds the angiotensin II type I receptor (AT1R), leading to desensitization of the baroreflex, which stimulates water uptake and vasopressin secretion.[101] However, as antihypertension medications, ACE inhibitors and AT1R blockers have had adverse effects, such as reduction in glomerular filtration rate, hyperkalemia, angioedema, inflammation-related pain, hepatotoxicity, and hypotension.[102,103]

However, there was a paradigm shift in hypertension treatment after the serendipitous discovery of angiotensin-converting enzyme 2 (ACE2) in 2000.[104,105] ACE2 counterbalances ACE by converting AngII (1–8) to Ang-(1–7)[106] and an ACE2 deletion study showed adverse ventricular remodeling and worsening ventricular function following myocardial infarction.[107] The beneficial effect of ACE2 on cardiac dysfunction is not only because of the removal of AngII but also because of production of Ang-(1–7), which increased vasodilation and decreased interstitial fibrosis in a hypertension model[108] and prevented ventricular hypertrophy.[109]

In clinical trials of recombinant ACE2 (rACE2, registration number NCT00886353),[110] there was no obvious beneficial effect on cardiovascular function in healthy volunteers, but it showed a therapeutic effect on hypertension patients; Ang-(1–8) levels were suppressed after infusing rhACE2.[110] However, given the requirement for long-term, repetitive delivery of ACE2, a new formulation that can provide protein stability and easy administration, which would improve patient compliance, is desirable. In 2014, oral formulations of ACE2 and Ang-(1–7) were evaluated for their potency against pulmonary hypertension.[50] Transplastomic plants expressing CTB-ACE2 or CTB-Ang-(1–7) were created and fed to monocrotaline-induced pulmonary hypertension rats, which prevented the development of pulmonary hypertension and ameliorated the associated cardiopulmonary pathophysiology, resulting in considerable reductions in right ventricular systolic pressure and right ventricular hypertrophy with decreased right ventricular fibrosis and pulmonary vessel wall thickness. Improvements were coupled with increased circulating levels of Ang-(1–7). Shifting the RAS from vasoconstriction to vasodilation by oral feeding of bioencapsulated ACE2 and Ang-(1–7) suppressed right ventricular hypertrophy and remodeling; reduced collagen deposition and wall thickness of pulmonary arteries; and inhibited proinflammatory cytokines.[50]

Naturally occurring antihypertensive peptides expressed in rice was also investigated for their efficacy against hypertension in an oral formulation. Novokinin (a new ovokinin, RPLKPW, derived from ovalbumin) was expressed in rice seeds as 18 tandems repeats and significantly reduced the systolic pressure of spontaneously hypertensive rats when orally delivered.[111] Binding of novokinin to the AT2 receptor lead to vasodilation, which can help reduce blood pressure.

Hypercholesterolemia is the main cause of atherosclerosis and coronary heart disease, which seriously affect hypertension and diabetes. To prevent or suppress the condition through the daily diet, rice was engineered to express the pentapeptide lactostatin (a novel pentapeptide, IIAEK, derived from bovine milk β),[112] which shows much higher hypocholesterolemic effects than beta-sitosterol, a drug commonly used to treat hypercholesterolemia. Oral administration of rice seeds expressing lactostatin significantly reduced serum low-density lipoprotein cholesterol levels and increased beneficial serum high-density lipoprotein cholesterol.

## Ocular disease

Eye diseases such as uveitis and retinopathy are frequently treated with ocular injections. This painful and scary treatment causes dramatically reduced patient compliance. For retinopathy treatment, monoclonal antibodies, including bevacizumab and ranibizumab, or recombinant proteins including aflibercept,[113] are injected intravitreally, which might be due to the difficulty of high-molecular weight PDs to pass through the BRB. To test the efficient delivery of PDs across the BRB and the therapeutic efficacy of ACE2 and Ang-(1–7) to treat eye disease, formulations of CTB-ACE2 and CTB-Ang-(1–7) were orally delivered using animal models of eye inflammatory disorders such as endotoxin-induced uveitis or experimental autoimmune uveoretinitis.[63] Elevated pro-inflammatory AngII levels, caused by a hyperactive RAS, contribute to ocular inflammation. The vasoconstrictive axis of the RAS, composed of ACE, AngII, and AT1R, is counterbalanced by the protective axis of the RAS involving ACE2/Ang-(1–7)/Mas. After oral delivery of plant cells expressing CTB-ACE2 and CTB-Ang-(1–7) to mice, ACE2 protein was detected in both serum and retina with 20–40% more activity. In mice with endotoxin (lipopolysaccharide)-induced uveitis, oral feeding of ACE2 and Ang-(1–7) decreased not only the infiltration of inflammatory cells but also retinal vasculitis.[63]

## Alzheimer's disease

It is projected that ~65.7 million people will suffer from Alzheimer's disease (AD) by 2030,[114] but the rapid increase in the number of AD patients cannot be slowed with current symptomatic therapy such as acetylcholine esterase inhibitors and *N*-methy-*D*-aspartate receptor antagonists.[115,116] Despite various approaches to stop the underlying decline and death of brain cells, further improvements are required. After terminating active immunotherapy aimed at eliminating amyloid deposition in 2002 due to brain inflammation in patients,[117] passive immunization therapy was extensively clinically tested using humanized monoclonal antibodies (Bapineuzumab, Solanezumab, and

Crenezmab). However, the clinical trial of these antibodies on AD patients did not meet its predefined assessment, showing mild cognitive improvement only in the early stages of AD.[77,118]

In a recent, new plant-based approach, CTB-fused myelin basic protein was expressed in chloroplasts to evaluate its therapeutic potential against AD by the oral route.[62] CTB served as a carrier protein to cross multiple physical barriers, including the intestinal epithelial membrane, the BBB and BRB via interaction with GM1 receptors. Although myelin basic protein is the major structural component of the central nervous system, it has intrinsic protease activity; it binds and degrades amyloid β and inhibits amyloid β fibril formation.[119] Oral administration of CTB- myelin basic protein leaf material caused amyloid β levels to decrease up to 67.3% and 33.4% in the hippocampus and cortex, respectively, in triple transgenic Alzheimer's disease (3×TgAD) mice. In addition, amyloid deposits, which are associated with loss of retinal ganglion cells and reduction of retinal nerve fibers,[120] were dramatically reduced in inner retinas of AD mice.

## Future Perspectives

Plant cells have been approved by the FDA for production of PDs, similar to other cell culture systems. However, these PDs are still purified from plant cells and delivered via injections. Therefore, major cost advantages of plant production—elimination of purification, cold chain and short shelf life—have not yet been realized.[121,122] In this review, we provided several examples of successful oral delivery of PDs to treat AD, diabetes, hypertension, Gaucher's disease or ocular diseases. We also provided examples of commercial-scale cGMP production of human therapeutic proteins and maintenance of protein folding and functional efficacy after storage at ambient temperature for more than 2 years, thereby eliminating the cold chain and short shelf life challenges of current production systems.

However, additional hurdles remain before this concept reaches the clinic. Oral delivery of glucocerebrosidase made in carrot cells is now being tested in human clinical trials, and a few other proteins are entering clinical studies. In planta quantitation of PD, dosage without purification is a critical step. The FDA accepts enzyme-linked immunosorbent assay for quantitation of purified PDs, and western blots are used for qualitative evaluation, especially to detect the presence or absence of cleaved products. However, these methods are not suitable for quantifying PDs from impure extracts due to cross-reacting proteins or autoantibodies[123] or for quantitation of insoluble, multimeric or membrane proteins. Targeted mass spectrometry by parallel reaction monitoring offers a unique concept for absolute quantitation based only on intrinsic properties of the target protein (*i.e.*, protein sequence and specific enzymatic cleavage sites) and can evaluate multiple peptides from the same molecule.[124] Therefore, proteolytic processing in each batch can be monitored by parallel reaction monitoring. We have used this concept to quantify drug dosage in plants for the first time.[125]

Yet another challenge is increasing PD dosage within plant cells to reduce the number or size of capsules filled with lyophilized plant cells. Although transgene expression via the chloroplast genome is widely recognized for its high-level expression of foreign proteins, this is not true for the expression of eukaryotic human genes within prokaryotic chloroplasts. To address this challenge, we have recently developed new codon optimization programs utilizing sequences from a large number (>130) of sequenced chloroplast genomes and a hierarchy of codon usage preferences in the most highly expressed chloroplast gene.[125] This new program helped us identify and eliminate rare codons present in transgenes and increase expression in codon-optimized genes by 5-50-fold. Most importantly, we utilized ribosome profiling studies to identify stalling sites in native genes that were eliminated after codon optimization. In addition, ribosome profiling studies offered insight into tRNA pools and other rate-limiting steps.[125]

One of the current challenges of injectable PDs is their immune response, rendering them less effective or even causing the production of toxic inhibitory antibodies (immunoglobulin E (IgE)), resulting in anaphylaxis or death. Such inhibitory antibodies form in ~25–30% of patients with severe hemophilia A and in ~5% of severe hemophilia B patients.[126] Inhibitors seriously complicate clotting factor replacement therapy and thus increase morbidity and mortality of the disease. Current immune tolerance induction protocols require frequent, high doses of blood clotting factors, often exceeding $1 million, and 30% of patients fail to respond to immune tolerance induction treatment.[127] Oral delivery of plant cells expressing clotting factor VIII or IX are very effective in conferring tolerance in hemophilia A or B animal models.[18,64,66,67] Therefore, oral delivery of plant cells would eliminate potential immune responses to PDs. In addition, the ability to deliver PDs to immune or nonimmune cells offers new approaches to deliver drugs to specific cell types or tissues, thereby eliminating negative immune responses in patients. All these developments augur well for affordable oral delivery of PDs in the near future.

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