

Non-invasive strategies for protein drug delivery: Oral, transdermal, and pulmonary

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ABSTRACT

Proteins are the building blocks of human life which involve physiological processes such as growth, development, metabolism, and reproduction. Despite its role in various biological processes, recently, the protein's function has been evolving as a promising therapy. The use of protein and peptide as therapeutic agents has several advantages upon small-molecule drugs, such as high specific interaction with its target that is less likely to elicit immune response. Currently, hundreds of protein drugs are available in the market, and this number is expected to increase each year. Consequently, the growth of protein therapeutics requires several improved strategies for drug delivery processes. Generally, protein and peptide drugs are administrated parenterally by conventional injections due to its poor oral bioavailability and limited permeability across epithelial cells in the gastrointestinal tract. However, a high frequency of injections results in decreased patient compliance because of the pain and skin wound. Therefore, a lot of research has been conducted in order to study the non-parenteral route of protein and peptide drug. In this review, we discuss recent findings for non-parenteral administration of protein drugs, for instance, oral, transdermal, and pulmonary route. The recent advancements in protein drug delivery make the non-parenteral route a promising method for protein drug delivery because of the ease of use among patients.

INTRODUCTION

Proteins are complex amino acids, usually containing more than 50 different amino acids, while peptides consist of less than 20 amino acids (Ratnaparkhi *et al.*, 2011). For the last 10 years, the use of therapeutic protein has been increased, and there are hundreds of approved protein therapeutic products in the market (Fosgerau and Hoffmann, 2015). The use of protein and peptide drugs has been attracted to its high selectivity toward the target. However, peptide or protein-based drugs have very low stability and bioavailability, which also require a very high production cost (Craik *et al.*, 2013). Generally, protein and peptide drugs are administrated parenterally (Jitendra *et al.*, 2011) because of their short half-life and low bioavailability issue

(Hamman *et al.*, 2005). Moreover, Peptide-based drug formulation is quite challenging because they are unstable and susceptible to aggregation or oxidation reactions, which subsequently affect the activity of protein-based drugs (Torosantucci *et al.*, 2014). As peptides contain smaller polypeptides, it is difficult for them to form globular structures and tertiary structures. Therefore, peptides tend to be more susceptible to degradation particularly in solution. Compared to protein, the peptide-based drug formulation is harder because of the chemical and physical instability, also because of the tendency of peptides to shape different conformations (Payne and Manning, 2009).

The foremost challenging problem of the parenteral administration is its short half-life, which is related to enzymatic degradation and rapid renal clearance. Besides, this route is administrated frequently, leading to patients' inconvenience and incompliance (Cleland *et al.*, 2001; Schiffter *et al.*, 2011). The challenges that protein and peptide drugs present have encouraged strategies to focus on improving the bioavailability through the delivery system and innovative formulation strategies. These strategies aim to improve protein stability during manufacturing

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and storage until the drug reaches the intended biological target. In this review, we will discuss the recent findings on the non-parenteral administration of protein or peptide drugs, which cover oral, transdermal, and pulmonary routes, and discuss the recent formulation technology in improving protein or peptide drug bioavailability.

PHYSICOCHEMICAL DRUG PROPERTIES OF PROTEINS AND PEPTIDES

Recent advances in the field of genetic engineering and pharmaceutical biotechnology have made it possible to treat various life-threatening diseases using therapeutic proteins. The U.S. Food and Drug Administration Center for Drug Evaluation and Research and the Center for Biologics Evaluation and Research have approved many recombinant therapeutic proteins, consisting of monoclonal antibodies (Mab), coagulation factors, and replacement enzymes, as well as fusion proteins, hormones, growth factors, and plasma proteins (Food and Drug Administration, 2020). Recombinant protein therapy was developed to treat various clinical indications, such as cancer, inflammation, exposure to infectious agents, and genetic disorders (Sauna *et al.*, 2017). In addition, protein has also been proven effective as a vaccine that helps stimulate the body's natural defense mechanism against immunogenic responses (Akash *et al.*, 2015).

Most of the therapeutic proteins are given by parenteral route because of its instability, size, and poor transport (Wagner *et al.*, 2018). Proteins also have a short half-life and high elimination rate; therefore, (Martins *et al.*, 2007). This can be a burden on patients with increased costs and decreased comfort. Giving therapeutic proteins via the oral route can be an alternative to improve patient compliance. However, the unstable nature of proteins in an acidic environment and susceptibility to proteolytic enzymes in the gastrointestinal tract become a challenge in oral administration (Wagner *et al.*, 2018).

Protein solubility is strongly influenced by pH, ions and temperature. At the isoelectric point, the solubility of the protein is very low. Proteins are very hydrophilic with very small partition coefficients in octanol-water solvents. Therefore, protein absorption by passive diffusion needs to be increased by increasing lipophilicity (Ratnaparkhi *et al.*, 2011).

Although generally in the solid phase, peptides and proteins still undergo various degradation reactions, namely chemical and physical degradation (Capelle *et al.*, 2007). Chemical degradation involves a covalent modification of the primary structure of proteins by breaking or forming bonds. While, physical degradation refers to changes in structure that are more structured due to denaturation and aggregation or noncovalent precipitation (Feridooni *et al.*, 2016). The mechanisms of chemical degradation that often occur in the solid phase include deamidation, oxidation, and the Maillard reaction.

Deamidation is the process where amide side chain hydrolysis of glutamine or asparagine residues produce carboxylic acids (Chang and Pikal, 2009). The deamidation reaction that often occurs in proteins in drug formulations is the nonenzymatic intramolecular deamidation reaction of Asn residues. In contrast to the deamidation reaction, the potential for a degradative oxidation reaction can be found at various stages of production, packaging, and storage (Feridooni *et al.*, 2016). For example, peroxide

contamination that has been found in formulation excipients, such as polyethylene glycol and surfactants, which causes oxidation of these products. Activation of molecular oxygen into more reactive species requires light or reducing agents and trace levels of transition metal ions, which can then convert molecular oxygen to more reactive oxidizing species, such as superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals ($\cdot OH$), or hydrogen peroxide (H_2O_2). Transition metal ions are often present in excipients and production processes with stainless steel equipment can cause significant iron contamination (Chang and Pikal, 2009). This potential source can contribute to the degradative oxidation of protein drugs.

After formulation, the selection of the final packaging can also have an effect on drug stability. Research results have shown that overall oxidation in certain products can still occur despite low oxygen levels (1%) on the vial's head (Chang and Pikal, 2009). By understanding the potential sources of contaminants that cause oxidation at all stages of drug production and the mechanism by which oxidation reactions occur, formulation strategies can be designed to minimize these events.

Protein drugs often experience physical changes that can cause changes in pharmacological effects and potential. Physical instability includes changes in the integrity of the three-dimensional conformation of proteins and does not always involve covalent modification (Feridooni *et al.*, 2016). The physical process includes denaturation, aggregation, precipitation, and adsorption on the surface (Lai and Topp, 1999). Protein drugs can undergo these changes during manufacturing, shipping, storage and administration. In recent years, aggregation has become a major problem in therapeutic proteins (Ameri *et al.*, 2009). Protein aggregation is a multilevel process that involves unfolding or misfolding units of protein monomers together with one or more steps of assembling protein monomers to form soluble or insoluble oligomers or aggregates with higher molecular weight (Li *et al.*, 1996). Protein aggregation can be a problem during the drug manufacturing process, especially if the drug is insoluble and tends to experience precipitation. This process usually reduces drug stability and half-life (Weiss *et al.*, 2009). Shear stress, high temperatures, pH changes, and high protein concentrations are factors that trigger protein aggregation (Frokjaer and Otzen, 2005).

BIOLOGICAL BARRIERS OF PROTEIN AND PEPTIDE DRUG DELIVERY

Protein and peptide drug delivery remains challenging due to the biological barriers in human body. The protein and peptide drugs should be sustained under enzymatic, pH changes and the mucosal barrier in the gastrointestinal tract. The presence of proteolytic enzymes in human biological process, such as proteinases, peptidases, and proteases, has an effect on the delivery of protein and peptide drugs. The proteases, generated by human cells, consist of aspartic proteases, threonine, cysteine, serine, and metalloproteinases (Choi *et al.*, 2012). When the protein and peptide drugs reach the colon, the microorganisms in colon may generate peptidases to hydrolyze the peptide bonds. According to the site of actions, proteases are categorized as exopeptidases and endopeptidases. The cleavage of these peptidases through the hydrolysis process is irreversible and leads to protein degradation (Mahato *et al.*, 2003).

Besides the presence of enzymatic reactions, the pH across the gastrointestinal tract also changes the stability of the protein and peptide drugs, for instance, the extreme pH in the stomach may lead to protein hydrolysis. The alteration of pH affects the ionic and hydrogen interaction in the protein, which subsequently transforms the protein conformation and folding (Mahato *et al.*, 2003). The protein folding is important for the biological activity and its misfolding, which causes dysregulation of the protein function (Dobson, 2003). Therefore, the delivery of protein and peptide drugs through oral route faces difficulties due to the different pH conditions in the gastrointestinal tract. The pH in the GI tract is varied from acidic condition (pH 1.2–3.0) to alkaline condition (pH 6.5–8.0) (He *et al.*, 2019).

The most notable biological barriers for protein and peptide drug delivery are presented in the brain and intestine. In the intestine, the barrier for protein and peptide drugs delivery is comprised of epithelial cells, lamina propria, and the muscularis mucosae, while the blood–brain barrier (BBB) is composed of luminal and abluminal membranes of the brain endothelium (Ulapane *et al.*, 2017). Once the protein enters the GI tract, it is destructed into amino acids and is absorbed through the intestinal epithelium. However, the amino acids are not easily absorbed since the brush border (microvilli) in the epithelium contains digestive enzymes. In addition, the existence of glycocalyx and mucus made the absorption through intestinal epithelium even more difficult (Carino and Mathiowitz, 1999). It should be taken into account that there are differences for drug metabolism in different parts of the intestines for both men and women (Iswandana *et al.*, 2018). Subsequently, the regional differences of drug metabolism also affect the protein and peptide drug absorption in different parts of human intestines.

Besides the intestinal epithelium, the delivery of protein and peptide drugs across the BBB drug delivery is hardly successful. In the brain, the protein and peptide drugs are transported across the cerebrovascular endothelium. The endothelium is characterized by very tight junctions. It is challenging for the hydrophilic molecules

to pass the tight junction; in contrast, hormones and peptides were allowed to cross the BBB through the receptor-mediated transcytosis (van Bree *et al.*, 1990). Large molecules, such as proteins, need to be reengineered structurally for crossing the BBB. One of the approaches for BBB delivery is utilizing antibodies that bind to the transferrin receptor (TfR). This molecular tool, namely “Trojan horse” technology, is mainly a peptidomimetic monoclonal antibody or endogenous peptide that passes the BBB through receptor-mediated transport. The transferrin receptor monoclonal antibody a fusion of TfR and Mab, allows the penetration to BBB and acts like the lipophilic small molecules (Pardridge, 2015). However, the weakness of this method is the short half-life of the anti-TfR antibodies. To overcome this limitation, another technology is reported to lengthen the antibodies half-life, called AccumuBrain. The AccumuBrain helps to raise the antibody concentration in the blood by binding to myelin oligodendrocyte glycoprotein, which is present in oligodendrocytes. It was shown that AccumuBrain stimulates the antibody levels ten times higher than the anti-TfR antibodies (Nakano *et al.*, 2019).

NON-PARENTERAL DELIVERY ROUTE

Oral

The oral route of drug administration is desired due to its convenience for the patients (Park *et al.*, 2011). However, one of the problems for oral delivery drugs is the absorption in the gastrointestinal tract, particularly for protein-based drugs that are instable (Schiffter *et al.*, 2011). The degradation of the peptide may be due to biochemical factors, such as the acidic condition in the gastrointestinal tract and the presence of microorganisms that contribute to the metabolism of the peptides (Hamman *et al.*, 2005). Besides biochemical barriers, there are also physical barriers consisting of an unstirred water layer that restricts the transportation of peptides to the epithelial cells, the columnar epithelial cells in intestines, tight junctions, and efflux systems as shown in Figure 1.

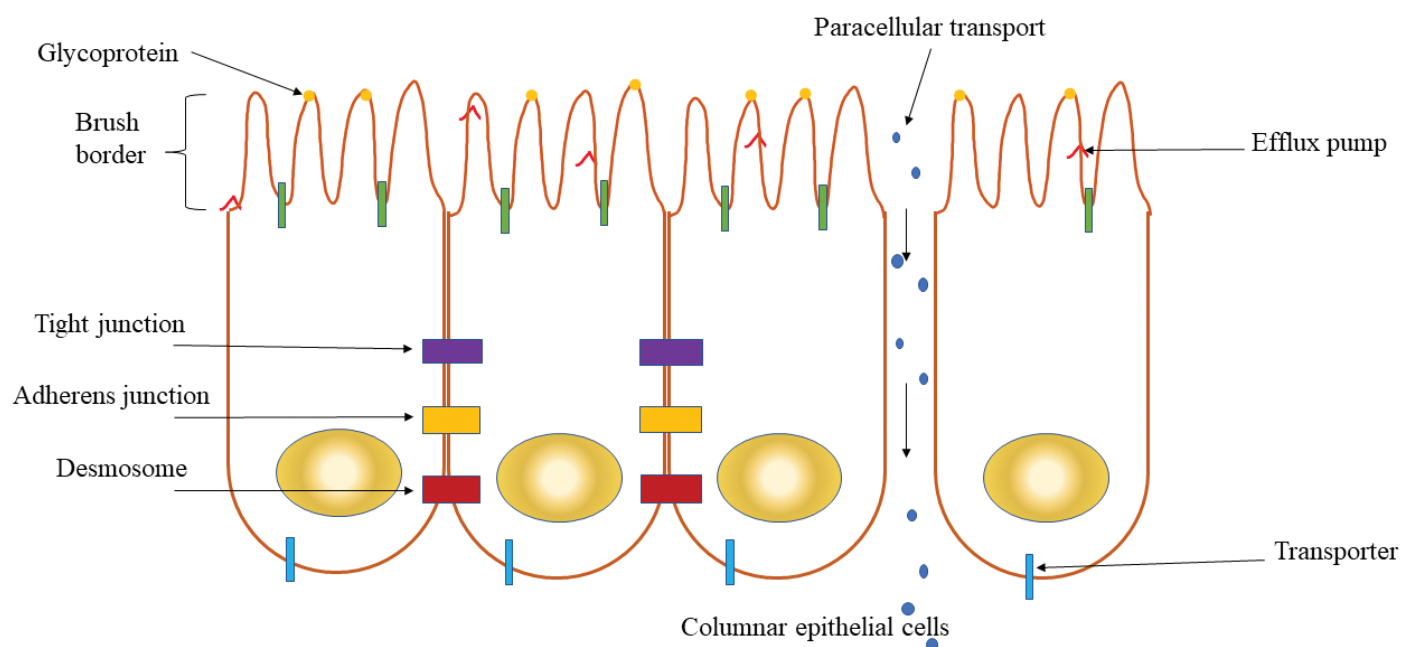


Figure 1. Barriers to peptide delivery in epithelial cells (Adapted from Bruno *et al.*, 2013).

As shown in Figure 2, some mechanisms are possibly involved in the absorption of proteins and peptides in the gastrointestinal tract, such as passive transport through diffusion, active transport, and endocytosis (Bruno *et al.*, 2013). Thus, the protein is transported through transcellular and paracellular pathways (Mnard *et al.*, 2012). In the transcellular pathway, the transport of protein molecules across the cell can be by either passive diffusion or utilizing specific carriers (Mnard *et al.*, 2012). The passive diffusion depends on the characteristics of the drug molecule, including molecular weight and charge, which allows the molecules to travel from a high concentration in the intestinal lumen to a lower concentration in the blood (Zhu *et al.*, 2017). On the other hand, the drug molecules can also be transported using certain carriers, such as a peptide or amino acid transporters, to facilitate the drug across the cells (Zhu *et al.*, 2017). The paracellular pathway requires the transport of small hydrophilic molecules (<200 Da) between the adjacent cells (Antunes *et al.*, 2013).

Some strategies have been developed to obtain the optimal delivery of protein or peptide drugs through oral administration, such as modifying physicochemical structures of the drugs and creating site-specific delivery of peptides (Kumar *et al.*, 2007). Another approach is designing prodrugs, which can be converted to the parent molecules due to the metabolism process (Gangwar *et al.*, 1997). Mainly, formulation technologies have been initiated for enhancing protein and peptide drug bioavailability, including the use of permeation enhancer and protease inhibitors (Choonara *et al.*, 2014). Several small-molecules categories may enhance the permeation of protein and peptide drugs, such as acids and surfactants; also, human bile salts help for the protein and peptide permeation (Brown *et al.*, 2020). Acids, like citric, fumaric, and tartaric acids, help to decrease the adjacent cells integrity by binding to Ca^{2+} which initiate the protein kinase C activation (Tomita *et al.*, 1996; Brown *et al.*, 2020). Surfactants and bile salts are amphiphilic molecules which facilitate the permeation of protein and peptide-based drugs across the paracellular and transcellular routes by altering the membrane integrity (Brown *et al.*, 2020; Maher *et al.*, 2019).

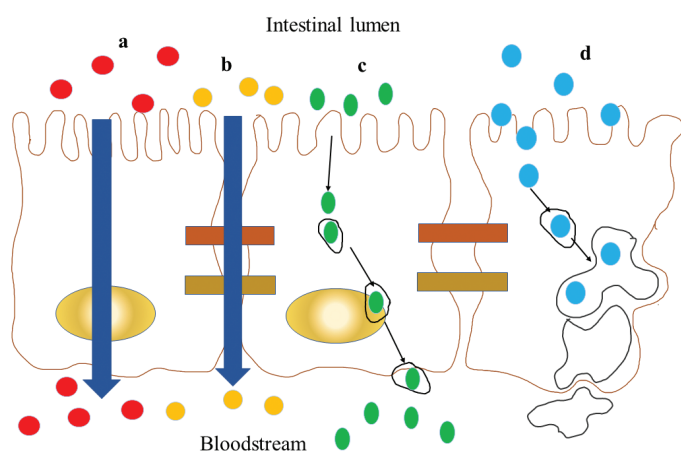


Figure 2. The pathways of protein and peptide drugs in intestinal epithelium: (a) transcellular pathway, (b) paracellular pathway, (c) receptor-mediated endocytosis and transcytosis, and (d) transportation to lymphatic circulation through M-cells of Peyer's patches (Adapted from Goldberg and Gomez-Orellana, 2003).

The use of plant cells is considered as one of the innovative approaches that has been studied to enable the oral administration of protein drugs for several diseases, such as Gaucher's disease, diabetes, Alzheimer's disease, ocular disease, and hypertension (Kwon and Daniell, 2016). The plant cells are used to encapsulate the protein drugs, so they are protected from the acids in the stomach and then digested by the microorganism in the intestines (Kwon and Daniell, 2016). Several examples of technologies that have been developed by pharmaceutical companies for oral protein drug delivery are presented in Table 1.

Transdermal

Transdermal drug administration has been generated recently because it allows the prevention of first-pass metabolism, especially for short half-life drugs. In addition, this type of administration is noninvasive and more convenient for patients (Kalluri and Banga, 2011). Some existing drugs have been delivered by using transdermal route, i.e., nicotine, estrogen, and scopolamine.

Anatomically, the skin is composed of four layers: non-viable epidermis (stratum corneum), viable epidermis, viable dermis (corium), and subcutaneous connective tissue (hypodermis) (Kanikkannan *et al.*, 2012). Among these layers, the stratum corneum is the outermost part of the skin, which contains dead keratinocytes (mainly 75%–85%) and lipids (5%–15%) (Kanikkannan *et al.*, 2012). This layer acts as a barrier for peptide drugs because it limits the absorption of large molecular weight and hydrophilic molecules (Kalluri and Banga, 2011). The viable epidermis is located beneath the stratum corneum, with 50–100 μm thickness and is composed of 90% water (Pathan and Setty, 2009). Just below the viable epidermis, there is the dermis, which is approximately 2–3 mm thick, and contains fibrous protein (Pathan and Setty, 2009). The lower layer of the skin is namely hypodermis that is composed of fibrous connective tissue, sweat gland, and cutaneous nerves where the drug is initiated to enter the circulation system (Pathan and Setty, 2009). When the drug is administrated parenterally, it first enters the outer layer of the skin and penetrates across the stratum corneum. The drug partition is continued to the viable epidermis and then available for systemic absorption when reaching the dermis (Alkilani *et al.*, 2015).

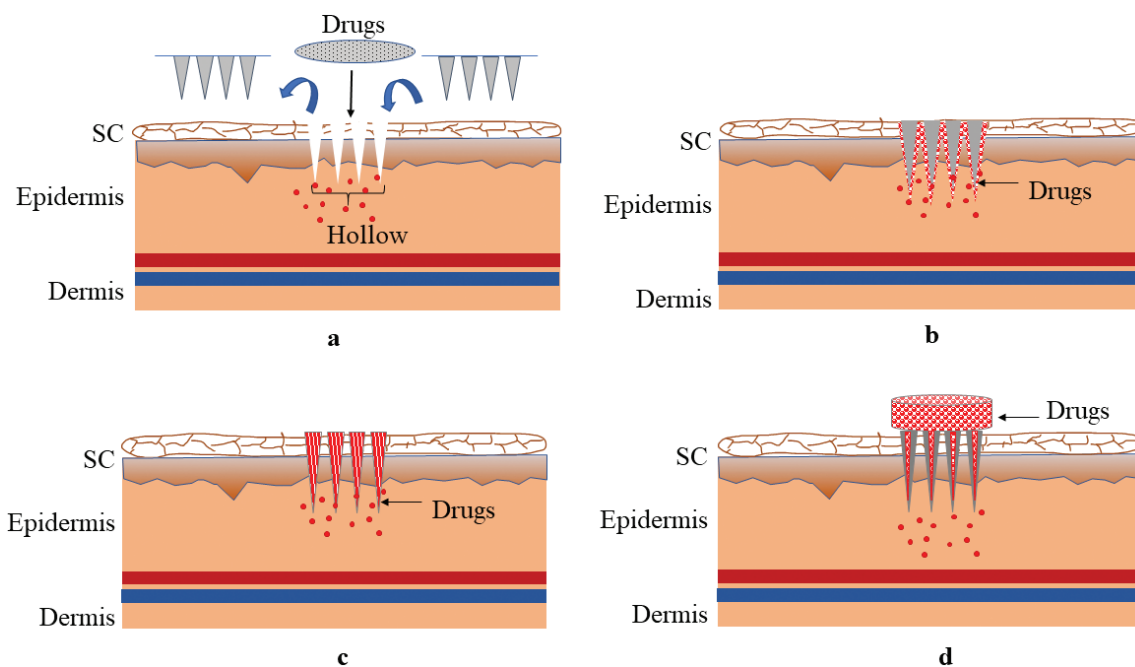
Technology and formulation approaches are initiated to increase the penetration of drugs and overcome the stratum corneum barrier. Some strategies are explored in the formulation of transdermal delivery of protein drugs, such as using chemical enhancers, nanocarriers, and prodrugs (Chaulagain *et al.*, 2018). Generally, the use of a chemical enhancer is mostly used as a formulation approach (Banga *et al.*, 2013). However, more technology techniques are involved in transdermal delivery of protein and peptide drugs, such as microneedles, electroporation, thermal and radiofrequency ablation, sonophoresis, and iontophoresis (Kalluri and Banga, 2011).

Microneedle

Microneedle is one of the major technologies developed to increase penetration. Various types of microneedles are used in protein drug delivery, as shown in Figure 3. The first method is initiated by perforating the skin to make pores and is continued by the application of the drug-loaded patch. The pores allow the

Table 1. Examples of technologies in various administrations of protein drug delivery.

Route of administration	Product name	Company	Biopharmaceuticals	Reference
Oral	Eligen®	Emisphere Technologies, Inc.	Calcitonin, insulin, growth hormone, parathyroid hormone, heparin	(Victor <i>et al.</i> , 2014)
	CLEC (cross-linked enzyme crystal)	Altus Biologics	Calcitonin, lipases, esterases, and proteases	(Sheldon, 2011)
	Hexyl-insulin monoconjugate 2 (HIM2)/IN-105	NOBEX Corp. and Biocon	Insulin and growth hormone, insulin vaccines	(Clement <i>et al.</i> , 2002)
	POD™ technology	Oramed Pharmaceuticals, Inc	Insulin	(Eldor <i>et al.</i> , 2013)
	CODES™ technology	Yamanouchi Pharmaceutical Co., Ltd.	Insulin	(Katsuma <i>et al.</i> , 2004)
	MMX® Technology	Cosmo Pharmaceuticals, Inc.	Heparin	(Sandborn <i>et al.</i> , 2012)
	Peptelligence™ technology	Enteris BioPharma, Inc. (Boonton, New Jersey, United States)	Calcitonin	(Stern <i>et al.</i> , 2013)
Transdermal	PassPort™ System	Altea Therapeutics Corp.	Insulin	(Anhalt and Bohannon, 2010)
	OmniPod®	Insulet Corp.	Insulin	(Anhalt and Bohannon, 2010)
	Solo™	Medingo	Insulin	(Anhalt and Bohannon, 2010)
	Finesse™	Calibra Medical Inc.	Insulin	(Anhalt and Bohannon, 2010)
	ViaDor®	TransPharma Medical Ltd.	Calcitonin	(Kalluri and Banga, 2011)
Pulmonary	Exubera®	Pfizer Inc.	Insulin	(Food and Drug Administration, 2006)
	Afrezza®	MannKind Corporation	Insulin	(Food and Drug Administration, 2014)
	Miacalcin® Nasal Spray	Novartis Pharmaceutical Corporation	Calcitonin	(Food and Drug Administration, 2011)
	Fortical® Nasal Spray	Upsher-Smith Laboratories, Inc.	Calcitonin	(Food and Drug Administration, 2005)

**Figure 3.** Different types of microneedle technologies: (a) porous formation by solid microneedle before the application of drug patch, (b) coated microneedle, (c) dissolving microneedle, and (d) hollow microneedle (Adapted from Kalluri and Banga, 2011).

diffusion of drugs to the lower layer of the skin (Li *et al.*, 2010). The second method is the insertion of microneedles that are covered with drugs (Verbaan *et al.*, 2007). Upon the insertion of microneedles, the drugs are released and dissolved in the skin. The drug-coated microneedles have limitations for the amount of protein that can be used. The third method is called soluble microneedles and they are usually made from biodegradable

excipients, such as carboxymethyl cellulose (Lee *et al.*, 2008). The fourth method is hollow microneedles, where the liquid drugs can be infused from the reservoir. For the last few years, microneedle arrays have gained a lot of interests in their application in delivering proteins or peptides. However, there is truly no microneedle array of products in the market yet (Larrañeta *et al.*, 2016). Several microneedle devices have been developed by companies, such as

Solid Microstructured Transdermal System (3M), Microinfusor (BD technologies), microinjection patch Macroflux® (Alza), Micro-Trans™ Microneedle Array Patch and h-Patch™ developed by Valeritas, and also Soluvia® and Microinjet® which are available in the market (Larrañeta *et al.*, 2016).

Iontophoresis

Iontophoresis technique, in principle, is the use of mild electric current (approximately less than 0.5 mA/cm²) to drive the charged drug molecules into the skin (Gujjar and Banga, 2014). Mainly, the charged molecules are transported through the electro-migration mechanism, while the neutral molecules are delivered by electro-osmosis (Kalluri and Banga, 2011). Besides the current strength and density, other parameters also affect the iontophoretic drug delivery, like the drug features and formulation, patient biological condition, and experimental factors related to the current and electrode material pH, electro-osmosis transport, and patient anatomical factor (Khan *et al.*, 2011). This method has limitations for proteins with a size more than 15 kDa. Thus, the liability of protein to form aggregates should also be considered. The administration of Interferon alpha-2B (hIFN-2b) in hairless rats was improved by using iontophoresis (Badkar *et al.*, 2007).

Electroporation

Electroporation technique is generally used in the transformation method for bacteria cells. The high voltage aims to make the bacteria cell membrane become more permeable for DNA insertion (Miller *et al.*, 1988). The principle in electroporation is used for transdermal drug delivery, where the electric field helps to improve the skin permeability and allows the penetration of protein drugs into the skin (Kalluri and Banga, 2011). The voltage (around 50–500 V) is required to create pores on the skin so that the large molecules will penetrate the skin (Szunerits and Boukherroub, 2018). Electroporation helps the diffusion of insulin delivery on rabbits' skin (Mohammad *et al.*, 2016). The application of both electroporation and iontophoresis resulted in a synergistic effect in transdermal administration of human parathyroid hormone (Medi and Singh, 2003). Moreover, electroporation has been used in combination with a microneedle roller and a flexible interdigitated electroporation array to deliver nucleic acid-based drugs (DNA and siRNA) onto the mouse skin (Huang *et al.*, 2020).

Thermal and radiofrequency ablation

Thermal and radiofrequency ablation utilizes high temperatures to deliver protein or peptide drugs through the disruption of stratum corneum (Aljuffali *et al.*, 2014). The heat creates pores and ablation which help the protein drugs to enter the skin (Szunerits and Boukherroub, 2018). One of the patented products was initiated by Altea Therapeutics (Atlanta, GA), namely PassPort™ patch. This device is ideally applied for proteins and peptides with a molecular weight less than 10 kDa (Banga, 2006).

Pulmonary

Advanced technologies for pulmonary delivery are widely studied in the last two decades since the lungs can be used as a portal for systemic drug delivery. The pulmonary delivery,

such as aerosol, has the advantage of high drug concentration to the airway, which decreases the adverse effect and is painless (Hess, 2008). Moreover, the lack of the first-pass metabolism in the pulmonary route gives a higher possibility for the lung to become an advantageous route of entry for peptide and protein drugs to the body (Agu *et al.*, 2001; Ibrahim *et al.*, 2015). Two different technologies that have been used to deliver drugs through the pulmonary route are pressurized metered-dose inhaler (pMDI) and dry powder inhalers (DPIs) (Ibrahim *et al.*, 2015). Nebulizers consist of jet and ultrasonic nebulizers, which can be differentiated based on the force used to atomize the liquid (Ibrahim *et al.*, 2015). According to Venturi's principle, the fluid pressure in aerosol declines as it moves through a diminishing area (Watts *et al.*, 2008). The challenges in jet nebulizers are the need for compressors to produce the aerosol, the sound that it generates, and the temperature fall because of the liquid evaporation in the nebulized globules (Rubin and Williams, 2014). The sound waves in ultrasonic nebulizers are produced because of the high-frequency vibration from piezoelectric crystals, resulting in crests that split the liquid into small droplets. Ultrasonic nebulizers are costlier compared to jet nebulizers (Dolovich and Dhand, 2011; Ibrahim *et al.*, 2015).

Moreover, this type is not efficient anymore in nebulizing viscous liquids and suspensions because it is less portable due to the need for electricity and it tends to raise the temperature of the nebulized drug solution. Therefore, they are considered inappropriate to nebulize thermolabile peptides or DNA. Generally, nebulizers generate 1–5 µm droplets according to the model and the manufacturer. Nebulizers have advantages over pediatric, geriatric, ventilated, non-conscious patients, or those who cannot use pMDIs or DPIs. Also, nebulizers are potential for administering larger doses than other aerosol devices. However, this will need longer delivery times (Ibrahim *et al.*, 2015).

The accumulation of aerosolized particles in the oropharyngeal domain and upper airways and the lack of synchronization between the device activation and inhalation are the main problems with the use of inhaler devices. In general, pMDIs generate aerosol faster than the patient can inhale. Therefore, children and elderly find it difficult to make a coordination between device actuation and inhalation. On the other hand, the use of DPIs requires the inhalation of the patient to be at maximum power in order to disperse and inhale the powder. However, this requirement is rarely achieved if the patient is not properly trained (Ibrahim *et al.*, 2015). The volume of the drug solution, the viscosity, the airflow and pressure, the tubing, mask, or mouthpiece utilized in the device are some factors that must be considered to get a precise and uniform dose with the nebulizer (Ibrahim *et al.*, 2015). The limited optimization of these variables causes dose variability among the patients. A drawback for nebulizer users is the need for assembling and loading the medication before usage. Also, the users have to de-assemble and clean the device for another usage (Hess, 2008). Insulin and interferon are two examples of protein-based drugs that have been widely studied for pulmonary delivery (Agu *et al.*, 2001; Oleck *et al.*, 2016). When the drug is administered through the pulmonary route, it can be absorbed through the membrane pores, vesicular, intracellular tight junction, and transporter-mediated transport

(Ibrahim and Garcia-Contreras, 2013). However, there are some challenges in protein and peptide-based drug administration, such as the degradation of protein by protease or macrophage; also, the presence of both mucus and surfactant in the alveoli can limit protein absorption (Agu *et al.*, 2001).

Insulin

Insulin is extremely needed in diabetes management, but unfortunately insulin absorption through oral administration is poor. A market innovation in delivering insulin through pulmonary delivery was achieved by the first two rapid-acting inhaled insulins in the market, which are Exubera® in 2006 and Afrezza® in 2014. Inhaled insulin is an advantage for people who have incorrect injection methods or needle phobia. However, that inhaled insulin was withdrawn due to a poor sales volume from low insurance coverage, finding new concern about the adverse effects, and competition from other insulin alternatives. As a pulmonary delivery, contraindications would include smokers and respiratory diseases like asthma due to a change in pulmonary lung function. Besides, the risk of respiratory adverse effects was also increased, such as cough, pharyngitis, rhinitis, and respiratory infection (Banga, 2015).

Interferon

Interferon is another type of drug that is potentially administered through the lungs. A study from Jaffe *et al.* (1991) compared aerosol and subcutaneous injection in humans delivering recombinant interferon- γ (rINF- γ) to activate alveolar macrophages for cytokine therapy (Jaffe *et al.*, 1991). Compared to parenteral drugs that usually have a systemic side effect, the inhalation drug delivery had a better acceptance with no side effects. The lower significant systemic concentrations rINF- γ may be due to high drug deposition in the lung and reduced inhalation absorption rate. Another interferon study from Dai *et al.* (1987) with 7 years of clinical study in China showed INF- α aerosol treatment as an effective and safe therapy for viral diseases, including asthma, asthmatic bronchitis, influenza, bronchiolitis, mumps, and recurrent upper respiratory tract infections in children (Banga, 2015, Dai *et al.*, 1987).

FORMULATION TECHNOLOGY APPROACHES

Despite the efforts to administer peptides and proteins through a noninvasive delivery system, the instability of protein drugs due to enzyme degradation, pH, low bioavailability, and toxicity are still the foremost challenging problem. Several novel strategies in the formulation of protein or peptide drugs have been developed to face these challenges. The examples of technologies that have been developed by the pharmaceutical companies for oral protein drug delivery are presented in Table 1. Chemical modification and development of colloidal carriers are the formulation approaches that can be applied for nasal, transdermal, and pulmonary delivery (Bajracharya *et al.*, 2019), and these approaches are discussed in this article.

Chemical modification

Since the manipulation of the peptide and protein structure is less feasible to increase half-life time, the current

approach in chemical modification goes to the addition of covalent conjugation of the polymer, such as mannosylation, PEGylation, and hyperglycosylation. Mannosylation of protein is endowed to target the mannose receptor cells, which are highly expressed by macrophages, dendritic cells, hepatic, and lymphatic endothelial cells. The *in-vivo* study showed that mannosylated protein therapeutics result in a better therapeutic outcome as reported in the enhancement of Antigen-specific antibody and T lymphocyte response after the administration of mannosylated mucin-type immunoglobulin fusion protein (Ahlén *et al.*, 2012).

PEGylation is a protein modification that conjugates to polyethylene glycol (PEG) in order to enhance protein delivery. To provide a suitable conjugation site, PEG is usually conjugated to amine terminal which allows the suitable conjugation site (Parveen and Sahoo, 2006; Ryan *et al.*, 2008). The addition of PEG alters the solubility and steric hindrance of proteins, resulting in better stability, increased half-life time, and optimal pharmacokinetic. The steric hindrance ability of higher PEGylation causes the reduction of contact with the active site. However, it shields the protein from enzymatic degradation and reduces contact with the antigen-presenting cell. Consequently, it increases the systemic circulation and therapeutic outcome (Patel *et al.*, 2014).

The physicochemical alteration of PEGylated protein and peptide because of PEG characteristic can improve the systemic circulation and reduce renal filtration (Parveen and Sahoo, 2006). PEG has been successful in generating a market for protein therapeutics as seen in the first Food and Drug Administration (FDA) approved PEGylated-protein drug in 1990, namely adenosine deaminase, Adagen®, which contains an enzyme for severe combined immunodeficiency disease. This successful production was followed by other drugs such as doxorubicin liposomal, Doxil® as an antineoplastic drug, PEGinterferon alfa-2a PEGasys®, as anti-hepatitis B and C, an opioid antagonist for opioid addiction Movantik® (Bailon *et al.*, 2001).

The success of previous PEGylated-protein drugs has proved to increase half-life, the stability of protein-based therapeutics and enhance peptide, and protein delivery (AlQahtani *et al.*, 2019). They are thus triggering further development of protein therapeutics through PEGylation, such as for filgrastim (methionyl human granulocyte colony-stimulating factor, rh-met-G-CSF) produced by recombinant DNA technology. Filgrastim has a function to regulate the production of neutrophils within the bone marrow (Welte *et al.*, 1987). In order to treat neutropenia, filgrastim has to be administered every 24 hours continuously for 11–20 days to maintain steady-state serum concentrations. The current research showed that enzymatic and nonenzymatic PEGylated of filgrastim prolonged the stability and plasma half-life *in vitro* (Scaramuzza *et al.*, 2012). However, further preclinical and clinical trials of this enzymatic PEGylated filgrastim are needed.

Hyperglycosylation is a co- or post-enzymatic process which conjugate protein, or other organic molecules with the polysaccharide. Hyperglycosylation improves the pharmacokinetic profile of peptide and protein therapeutics. There are two types of hyperglycosylations: *in-situ* chemical reaction and site-directed mutagenesis, which can result in N-linked or O-linked protein glycosylation. The N-linked

is specifically attached to asparagine. Meanwhile, O-linked oligosaccharide is not site-specific, but is generally found binding to serine or threonine (Pisal *et al.*, 2010). The addition of carbohydrates may stabilize protein by the formation of hydrogen bonds with polypeptide backbone or surface hydrophilic amino acid and steric interaction with the adjacent residues (Patel *et al.*, 2014). Hyperglycosylation may also work to hinder the human immune system, such as polysialic acid (PSA), which are available at different sizes of molecules. Also, PSA is also able to control the clearance rate of conjugated proteins or peptides. The terminal ends of every glycan added for hyperglycosylation usually contains a functional structure, such as phosphate, sulfates, and carboxylic acids, which can alter the protein surface charge, isoelectric point, and increase half-lifetime of circular hyperglycosylated protein (Solá and Griebenow, 2010). Notably, another advantage of hyperglycosylation is the nature of its biodegradability in the human body. Examples of FDA-approved hyperglycosylated proteins are Cerezyme®, Fabrazyme®, and Naglazyme®.

Mannosylation of protein is endowed to target the mannose receptor cells, which are highly expressed by macrophages, dendritic cells, hepatic, and lymphatic endothelial cells. The *in-vivo* study showed that mannosylated protein therapeutics result in a better therapeutic outcome as reported in the enhancement of Antigen-specific antibody and T lymphocyte response after the administration of mannosylated mucin-type immunoglobulin fusion protein (Ahlén *et al.*, 2012).

Colloidal carrier

Colloidal carrier, a lipid-based formulation, has been widely used to overcome delivery problems of peptides and protein drug. This carrier can protect the drug against degradation *in vitro* and *in vivo*, modify the release rate, as well as target specifically in the body (Martins *et al.*, 2007). The simple approaches of colloidal

carrier are nanoemulsions (NEs), microemulsions (MEs), and nanogels (NGs).

NEs are a colloidal dispersion system consisting of two immiscible liquids (water and oil), in which one liquid is dispersed in the other by means of an appropriate surfactant/co-surfactant mixture, forming oil-in-water (o/w) or water-in-oil (w/o) nanodroplet systems, with droplets of 20–200 nm in size. NEs are a very cost-effective technique due to high storage stability and ease of preparation. Despite the similarities of NEs and MEs in terms of their physical appearance, components, and preparation techniques, NEs are kinetically stable and thermodynamically metastable, while MEs are thermodynamically stable (Shaker *et al.*, 2019).

NGs are nanosized, three-dimensionally, cross-linked, hydrophilic polymeric networks that are composed of hydrogel particulate entities with a nanometer-sized space; so it has the features of hydrogel (high water content and versatile mechanical properties) and nanoparticles at the same time. Dimensions, less than 200 nm in diameter, facilitate cellular uptake through receptor-mediated endocytosis, making NGs suitable carriers for the peptide drugs. NGs may have a role as chaperones in preventing denaturation or aggregation of proteins, promoting refolding, and controlling the release rate. When proteins are encapsulated within the cross-linked polymer matrix of NGs, higher stability is reported even at temperatures above the physiological values and in the presence of organic solvents (Grimaudo *et al.*, 2019; Zhang *et al.*, 2016).

Other colloidal carriers that can be used to deliver protein and peptide drugs are liposomes, microparticles carbon nanotubes (CNTs), and nanoparticles (polymeric nanoparticles, solid lipid nanoparticles (SLNs), micelle, and CNTs (Bajracharya *et al.*, 2019; Patel *et al.*, 2014). The structures of each colloidal carrier are shown in Figure 4. Compared to chemical modification

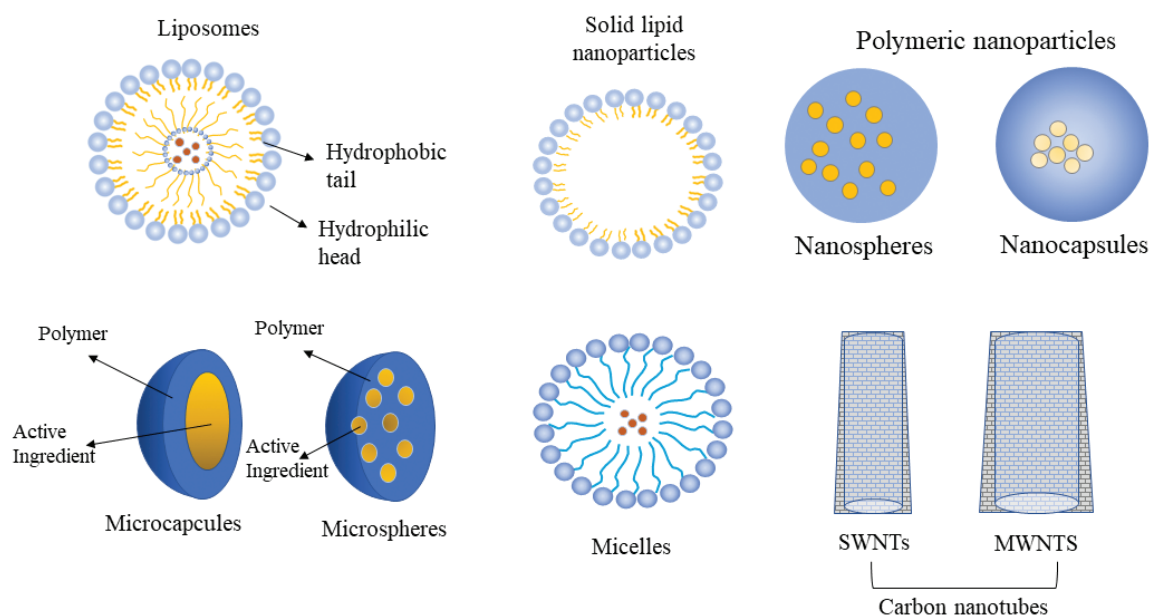


Figure 4. Different types of microneedle technologies: (a) porous formation by solid microneedle before the application of drug patch, (b) coated microneedle, (c) dissolving microneedle, and (d) hollow microneedle (Adapted from Kalluri and Banga, 2011).

approaches, colloidal carriers exhibit the ability to protect sensitive proteins and prolonged release.

Compared to the liposome, which are vesicular nanostructures made of phospholipid and amphipathic lipid, microparticles and nanoparticles have a better kinetic morphology and rigid structure (Battaglia and Ugazio, 2019). Microparticle biodegradable polymers are extensively studied to provide controlled release over months (Shi and Li, 2005). Polymer types as a coating material, fabrications method, and formulation are identified as important factors for microparticles. The coating material needs to have the biodegradable ability as they can break into nontoxic material in the body and can easily (Fredenberg *et al.*, 2011). Three major subsets of polymers are natural, semi-synthetic, and synthetic, which are known to be used as coating material such as starch, alginate, collagen, chitosan, lecithin, ethyl-cellulose, cellulose acetophthalate, polyesters poly(glycolic acid), poly(D,L-lactic acid), and poly(D,L-lactic co-glycolic acid) (PLGA) (Kamaly *et al.*, 2016; Saez *et al.*, 2007). Due to the biocompatibility and capacity to achieve different drug release, PLGA and lactic acid homopolymers are mostly employed as coating material (Saez *et al.*, 2007). The success of PLGA as a microencapsulated polymer was shown with the availability of marketed drugs, such as Nutropin® Depot, as a treatment for growth disorder pediatric patients with monthly administration, Trelstar® Depot and Plenaxi®, as a treatment for advanced prostatic cancer (Patel *et al.*, 2014; Saez *et al.*, 2007). Even though it advances in control drug release, there is a major concern related to the possible initiation of immunological response, which can be triggered by degraded protein because of the fabrication process (Van De Weert *et al.*, 2000). Thus, fabrication and formulation methods become important. Although the development of better methods and formulations are ongoing, solvent evaporation (single and double emulsion process), phase separation (coacervation), spray-drying emulsion techniques are being used to enhance protein stability (Makadia and Siegel, 2011). Meanwhile, various additions of excipients can be added to sustain the release of the drug, such as the addition of alginate, chitosan, and caffeic acid-grafted PLGA (Han *et al.*, 2016; Selmin *et al.*, 2015; Zheng and Liang, 2010).

Two types of nanoparticles – polymeric and SLNs – have been widely investigated. Both of them are alternative carrier systems, not only for hydrophilic but also insoluble and labile compounds. In addition, they are also able to deliver the drugs in a sustained release manner and reduce the degradation of labile compounds. These systems have less toxicity compared to other because the matrix is biodegradable and well tolerated in the human body (Campos *et al.*, 2015).

Polymeric nanoparticles are colloidal carriers that have 1–1,000 nm in size. The extreme biocompatibility of polymeric nanoparticle makes it an efficient nanocarriers in the medical field. Two types of polymeric nanoparticles are nanocapsules (a polymeric membrane containing protein/peptide), and nanospheres (protein/peptide are well distributed into the polymeric matrix). Both of these can be generated via fabrication methods. The above-described preparation of microparticles can be employed for polymer nanoparticles preparation. The most widely used technique for hydrophobic encapsulation is solvent evaporation techniques (Makadia and Siegel, 2011; Mao *et al.*, 2007). Nanoparticles and salting-out (w/o/w) are the alternative

techniques for nanoparticle encapsulation (Hans and Lowman, 2002; Kwon and Daniell, 2016; Lamprecht *et al.*, 2000). On the other hand, the active compound loading into nanoparticles can be done by two methods, by incorporating during nanoparticles production or incubating the nanoparticles with concentrated active compounds (Yih *et al.*, 2006). Since the active component was encapsulated or well distributed in its polymeric matrix, the release of active compounds follow the diffusion process with three step, which are matrix swelling, the rubbery matrix formation and active compound diffusion through rubbery matrix (Jawahar and Meyyanathan, 2012).

Although polymeric nanoparticles are the site-specific target and control the drug release, polymer internalization in the cells gives the possibility of cytotoxic induction (Smith and Hunneyball, 1986; Wissing *et al.*, 2004). Therefore, SLNs can be an alternative for nanoparticles with better cell tolerability. SLNs are lipid nanoparticle systems that have a size less than 1,000 nm. They are stabilized by an emulsifier together with tolerated lipid contents, such as triglycerides, diglycerides, monoglycerides and fatty acids (Abhishek *et al.*, 2019). SLNs are very useful carriers for active compounds and the mobility was restricted by lipid; therefore, it can lead a modified release profile (Wissing *et al.*, 2004).

However, SLNs still have disadvantages related to the loading capacity of the drug (25%) and the formation of aggregates (Wissing *et al.*, 2014). A combination of suitable preparation techniques might help in reducing those disadvantages (Abhishek *et al.*, 2019). A derivate of SLNs is introduced to overcome the disadvantages of conventional SLNs, such as nanostructured lipid carriers and Lipid protein conjugate. Some of the techniques can be used for SLNs and its derivative fabrication, such as High-pressure homogenization, MEs, solvent emulsification–evaporation or diffusion, high-speed stirring, ultrasonication, and double emulsion method (Cortesi *et al.*, 2002).

Furthermore, another nanopolymeric carrier, called dendrimers, has attracted the interest of scientists in delivering therapeutics, targeting, and diagnostic agents together in a single system. Dendrimers are interesting for biomedical applications because of their properties, including hyperbranching, well-defined globular structures, excellent structural uniformity, multivalency, variable chemical composition, and high biological compatibility (Noriega-Luna *et al.*, 2014). Previously, Ciolkowski *et al.* (2012) showed the influence of dendrimers' surface modification on the strength of interaction with proteins. This study was performed using poly (propylene imine) G4 and G3.5 polyamidoamine dendrimers as a drug carrier and a model protein from hen egg white lysozyme. Moreover, Liu *et al.* (2019) reported a boronic acid-rich dendrimer for cytosolic delivery of native proteins. This system could deliver 13 cargo proteins into the cytosol of living cells and maintained their bioactivities after cytosolic delivery.

Other colloidal carriers that contribute to peptide delivery are micelles and CNTs. Micelles have advantages over others on its particle size which ranges from 10 to 100 nm (Zhang *et al.*, 2014). PEG as a hydrophilic segment and lipid as core segment is usually used for its amphiphilic block. In protein delivery, water-in-oil-in-water micelles are more preferable, since the proteins will be entrapped in an aqueous chamber of micelles. Recently, micelles have been known as first-line drug delivery

because of their size and large manufacturing feasibility (Kim *et al.*, 2010). The stability of micelles has become one of the factors that make it feasible for manufacturing. In order to maintain its stability, some techniques, such as shell cross-linking (covalent bond between shellcore) and noncovalent cross-linking (static electric interaction), have been used widely (Lu *et al.*, 2018). For example, micelles technology has been applied for insulin to make slower degradation and controlled release (Li *et al.*, 2016).

One type of micelles technology is polyion complex micelles (PIC) that can also be used to deliver organic solvent-sensitive therapeutic agents, such as proteins and nucleic acid, which are naturally occurring polyelectrolytes (Chen and Stenzel, 2018). Harada and Kataoka (1998) prepared a PIC from chicken egg white lysozyme and poly(ethylene glycol)-poly(aspartic acid) block copolymer through electrostatic interaction in aqueous medium. Their study showed that the PIC was expected to be useful as functional materials including carrier systems in drug delivery applications and a nanometric-scale reactor for enzymes. Furthermore, Wakebayashi *et al.* (2004) produced PIC in an aqueous solution by using an acetal-poly(ethylene glycol)-poly(2-(dimethylamino)ethyl methacrylate) (acetal-PEG-PAMA) block copolymer spontaneously associated with plasmid DNA (pDNA). They showed that the pDNA in the micelle was adequately protected from DNase I attack. The transfection ability of the PIC micelles toward 293 cells was remarkably enhanced with an increasing the residual molar mixing ratio as high as 25.

CNTs, large molecules with a cylindrical shape, have been known to not only carry small molecules but also large molecules such as protein (Elhissi *et al.*, 2012; Zhang *et al.*, 2011). The walls of the CNTs in graphene sheets have two types, single-walled and multi-walled. These sheets impact their size length. Despite their variation in physical length (hundred nanometers to micrometer), CNTs is also be used not only in noninvasive administration but also in invasive route (Yang *et al.*, 2010). A large surface area of CNTs is important for their ability to conjugate with various molecules and also to penetrate the target area; thus, surface modification is usually performed for the effective delivery (Elhissi *et al.*, 2012). Previous research has showed that bovine serum albumin (Huang *et al.*, 2002), DNA (Singh *et al.*, 2005), and other proteins can be bind to CNTs. Some of the proteins that have been immobilized onto CNTs through covalent linkages include chymotrypsin, ferritin (Lin *et al.*, 2004), fibrinogen, hemoglobin (Wei *et al.*, 2010), and streptavidin (Elhissi *et al.*, 2012).

Protein-functionalized CNTs have shown their advantages in drug delivery, i.e., by having high pay loads, long release rates, retaining their biological function, and relatively easily enter the cells than free proteins (Nagaraju *et al.*, 2015). Despite its beneficial properties, further research is needed to understand the safety aspect of CNTs before and after functionalization with proteins since there were some specific studies which showed cytotoxic effects of CNTs (Sun *et al.*, 2011).

FUTURE PROSPECTIVE

The drawbacks of invasive delivery of protein drugs, like the inconvenience, high price, hydrophilic properties, and high molecular weight, have encouraged more studies to invent non-parenteral administration. Furthermore, the high inventions

of protein-based drugs require more research on designing the appropriate delivery system and technologies. In the last decades, some advanced technologies have been developed by pharmaceutical companies to assist protein drug delivery through oral, transdermal, and pulmonary routes. With these new technologies, the advancement of protein drug delivery systems will increase shortly.

In addition to advancing the technology aspects, formulation modification was generated to improve drug carrying due to the limitation of technologies to tackle several barriers to protein drug delivery. Therefore, both technologies and formulation approaches for protein-based drugs complement each other and address patient questions and concerns in drug administration. Challenges in the future will be to find a better formulation or specific dosage form to obtain an effective therapy and safety.

Furthermore, *in vitro* and *in vivo* evaluation of protein or peptide drug delivery also play a prominent role in the development of effective therapy. To establish *in vitro/in vivo* correlations, it seems that more than one testing method should be applied. It is necessary to characterize the drug release and justify the system design according to the real condition. This limitation gives challenges to pharmaceutical scientists to develop a method that brings about a better understanding for evaluation of protein or peptide drug delivery and also which is feasible to be used routinely in the industry setting.

Taken together, noninvasive strategies for protein drug delivery will attract more attention from the public in the near future. New technologies, formulation modification, and better understanding for evaluations are required in order to develop a dosage form that is therapeutically viable in the market.

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AUTHORS CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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