Recent advances in protein drug delivery

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Abstract. Proteins and peptides drugs have been researched for decades and they show therapeutic and medicative function in numerous aspects. However, how to manipulate proteins efficiently to the specific region of the body or even cellular penetrate are the challenges we face. This overview introduces some potential methods of delivering proteins as well as how they act more accurate and controlled in vivo. Simply classify them to five sections which seem different from each other, whereas have possible connections.

1. Introduction

Proteins are the main component of the organisms and play a crucial role in vital process in human body. In other word, play a important role in any vital movement due to its diverse biological functions, include catalyzing biochemical reaction, metabolic regulation to maintain regular vital movement, constituting antibody to participate in immune response, receive and deliver message which can be used to modify drugs so that the drug receptor will combine with ligand to effect therapeutic objective and eventually enhance the bioavailability. As the functions mention above, we know that protein act in a highly specific effects, that’s why protein and peptide drugs are one of the promising and developmental directions with broad market in the pharmaceutical industry. Depending on the high biological activity and diverse biological functions, as the exploitation of peptides has been expended into numerous therapeutic domain, include antitumor, anti-thrombosis, anti-hypertensive, hormone regulation, immune regulation, antibacterial, analgesic, diabetes, vaccine, etc. However, there are also some obstacles in the drug delivery system, such as the instability of protein and peptide due to their surface charge and fragile structure, low permeability of the cell membranes when protein and peptide exert the receptor interaction in vivo, the main barrier is how to enhance the probability of endolysosomal escape which enables proteins and peptides interact with target more efficiently1. The overview will show some updated research and expectations in protein/peptide delivery techniques including liposomes, solid lipid nanoparticles, cell penetrating peptides, polymers and nanoparticles as Figure 1 showes.
2. Liposomes

A liposome is a spherical-shaped vesicle which is composed of phospholipid bilayers, which closely resembles the structure of cell membranes and enables its ability to fuse with cell membrane\(^3\). Biomembranes are mainly composed of phospholipid bilayer and various membrane proteins. The bilayer are typically composed of phospholipid molecules, which has a hydrophilic head group, phosphate, and a lipophilic tail group, hydrocarbon chain. Thus, phospholipid is a member of amphipathic molecules because of the presence of both hydrophilic group and hydrophobic group. When in the water, the phospholipids molecular self-assemble into bilayer driven by the low surface energy because the hydrophilic group surface has lower surface energy when it contacts with polar water molecules. At the same time, the hydrophobic hydrocarbon chains are in low energy state then they are gathering together. Due to the nature of phospholipid molecules mentioned above, the bilayer structure is most common in biomembranes of cells. Because the structure similarity between cell membrane and liposomes, they are able to fuse with each other by phospholipid membrane rearrangement. Thus, the affinity of liposomes to both hydrophilic or lipophilic molecules and ability to fused with cell membrane and deliver encapsulated molecules give it potential to become drug delivery vesicles\(^4\).

Typically, liposomes can be composed of different phospholipids from different source such as phospholipids from egg, phosphatidylethanolamine, or other types of cells\(^5\). What’s more, the liposomes can also contain multiple core-shell structures as a liposome traps another liposome. If the components of liposome are from natural sources, it always has lower inherent toxicity. The scale of liposomes vary in from 0.005 \(\mu \text{m}\) to 5 \(\mu \text{m}\).

Liposome-mediated carrier is an efficient tool for functional protein delivery\(^6\). Normal liposome are usually captured by endosome, as a result, degrade in the lysosome. There are many other drug metabolic systems, For an instance, the reticuloendothelial system (RES) and renal filtration seem to be the soldiers of the body and they get ready to fight with the external matter all the time. That’s why protein drugs are unstable in the body. However, the modifying liposome including target liposome,
which means that liposome can be sent into a specific organ or issue. In this way, protein to a large extent will not be eliminated by the systems. What’s more, the PH-sensitive liposome is a feasible form to assist liposome releasing into the cell\(^7\). When PH-sensitive is relatively low, liposome fuses with the endovascular membranes and make it unstable, so that it can enter the cytoplasm easily. The liposome also can across some main barrier such as blood-brain barrier and can be utilized to deliver drugs to the central nervous system.

3. Solid lipid nanoparticles

Like liposomes, solid lipid nanoparticles are also composed of lipid and are lipid-based submicron colloidal particles. Because of the structural and composition similarity, they share many similarities on properties and so that can be an alternative to liposomes. Different from liposomes, the solid lipid nanoparticles have a more rigid core which stay solid at room temperature\(^8\). Thus, solid lipid nanoparticles are more stable in bodies and for some biological applications than liposomes. To prepare solid lipid nanoparticles, the most widely used methods are the following two methods. (1) Muller and Lucks pioneered a high-pressure homogenization technique. (2) A microemulsion technique explored by Gasco. As for the application, Almeida group demonstrated a method for protein incorporation into solid lipid nanoparticles with lysozyme as a model protein. The result shows that the lysozyme activity is not affected after the solid lipid nanoparticles entrapment. This study proved the feasibility of application of solid lipid nanoparticles as protein/peptide carriers\(^9\).

4. Cell penetrating proteins or peptides(CPPs)

Cell Penetrating Peptides (CPPs), which also named protein transduction domains (PTDs), are short peptides rich in arginine and lysine residues to offer positive charge within the system, which makes them highly cationic and enables their ability to gain access to the interior of almost any eukaryote cells. This property makes them exceptional tools for intracellular delivery\(^10\). They can carry a wide variety of covalently and noncovalently molecules such as proteins, peptides, nucleotides and nanoparticles into the cells which serve as inhibitors, indicators or drugs in the interior of cells. Different from liposomes, the most unique feature of CPPs is their ability to inject themselves and the cargos into cells directly by crossing the plasma membrane, which avoids the cargos getting trapped in endosomes and subsequently degradation. Therefore, the cargoes transported by CPPs is free in cytoplasm and are able to reach their intracellular targets.

The mechanism underlying the unique feature of directly crossing the cell membrane of living cell is still arguing. The core of the puzzle lied in whether this process relies on receptor or just an energy-independent manner. The receptor is not reported and the fact that CPPs are highly cationic thus hydrophilic and lack hydrophobic sequences which allow them to inserted into the hydrophobic part of plasma membrane composed of phospholipid efficiently. Recently, some experiment evidences reveal that the penetration of CPPs is a receptor independent manner\(^11\). In this theory, the guanidinium groups, cell universal components, fatty acids, and the cell membrane pH gradient play central roles in the CPPs penatrating. In the higher extracellular pH environment, the fatty acids on the outer face on cell membrane are deprotonated and interact with positively charged arginine rich peptides through electrostatic interaction. The positive charged CPPs are now neutralized by the fatty acids, which reduce the polarity of CPPs and make them coated with hydrophobic fatty acid shell. This allows the peptides efficiently insert into the hydrophobic part of membrane. This insertion destabilizes the
plasma membrane nucleating a transient toroidal pore. The peptide diffuses in the cell membrane and some contact the interior part where has lower pH. The lower pH protonates the fatty acids and interrupt the electrostatic interaction following by CPPs releasing. The protonated fatty acids remain on plasma membrane and rearrange rapidly to reach equilibrium and ready for another process.

Cell penetrating proteins or peptides is a new method to delivery different kind of molecules into cells by modifying particular proteins or peptides on the membranes of the cells, notably enhance the penetration of cell membranes to macromolecules which can be called translocation property. These peptides can penetrate through low amino acid sequence under 20 but high molecular weights greater than themselves. Thus, this new approach has broken a new ground in the therapeutic applications by aiding the transcellular transport more efficient. TAT (transactivator of transcription) peptide is the first reported CPPs in 1988 and is also the most widely researched CPPs for drug delivery\(^{12,13}\). TAT peptide is successful in delivering heterologous proteins like horseradish peroxidase and RNase A into the cytoplasm efficiently. When deliver b-galactosidase, the drug molecular could be delivered to several different tissues including heart, where highest level is detected, liver, spleen, skeletal muscle as well as little in kidney and brain. TAT-mediated protein delivery showed good potential as a therapeutic vaccine inducing class I-restricted cytotoxic T-lymphocyte response, which is not able for extracellular proteins. After conjugating to TAT peptides, such as TAT ovalbumin conjugate, the antigenic protein was processed by antigen presenting cells, resulting in effective target cells killing.

Another advance of CPPs as a drug deliver system is their capability to incorporated during drug protein synthesis. Some CPPs are concluded in Table 1. The TAT- protein conjugate is convenient to synthesis of using molecular cloning technique an bacterial expression systems. The fusion proteins and TAT peptide sequence were inserted into expression vector, followed by bacterial expression and purification of the fusion protein. The resulting full length TAT fusion proteins ranging in molecular weight from 15 to 115 kDa demonstrated transduction in a variety of cells, such as peripheral blood lymphocytes, all blood cells, bone marrow stem cells.

Besides cationic CPPs, there are two other kinds of CPPs with cell membrane penetrating ability including amphipathic and hydrophobic CPPs. Amphipathic CPPs contain both hydrophilic and hydrophobic regions of amino acids and have similar properties as phospholipid molecular. Besides lysine and arginine, which are distributed throughout the sequence and are polar and positively charged, they are also rich in hydrophobic residues with aliphatic side chain like leucine, isoleucine, valine, Phenylalanine and alanine. Some well-studied Amphipathic CPPs including MPG from HIV glycoprotein 41\(^{14}\), pVEC from vascular endothelial cadherin and ARF from p14ARF protein\(^{15}\). Hydrophobic CPPs contain mainly nonpolar residues, resulting in a low net charge. These hydrophobic sequence have high affinity with the hydrophobic inner part, which is the main barrier for peptides penetrating, of cellular membranes are crucial for cellular internalization. Only a limited number of hydrophobic peptides has been discovered including C105Y from antitrypsin, Pep-7 from CHL8 peptide phage clone\(^{16}\). Hydrophobic CPPs internalization mechanisms have been poorly studied compared with other two CPPs but have been proposed that these peptides cross membranes through energy-independent manner.
Table 1. Examples of CPPs and Their Sequences and their Origins

| CPP name          | Sequence                  | Origin                      | Class     |
|-------------------|---------------------------|-----------------------------|-----------|
| HIV-1 TAT protein, TAT<sub>40</sub> | GRKKRRQRRRPRPPQ         | HIV-1 TAT protein           | Cationic  |
| HIV-1 TAT protein, TAT<sub>45</sub> | RRKRRQRRR              | HIV-1 TAT protein           | Cationic  |
| Penetratin, pAntp<sub>(43–59)</sub> | FKQIKTVFQGRFRMKWKK | Antennapedia Drosophila melanogaster | Cationic  |
| Polymers          | Pin                       | Chemically synthesized      | Cationic  |
| DPV1047           | VKRGLKLFHRPRVTMDV        | Chemically synthesized      | Cationic  |
| MPG               | GALFLGFLGAAGSTMGAWSQPKKRKV | HIV glycoprotein 41/ SV40 T antigen NLS | Amphiphatic |
| Pep-1             | KETWVETTWTEWSQPKKRKV     | Tyrosine-rich cluster/SV40 T antigen NLS | Amphiphatic |
| pVEC              | LIIILRRIPKCAHSHK         | Vascular endothelial cachexin | Amphiphatic |
| ARF(1–22)         | MVRRFVTLRRRACGPRRV      | p14ARF protein              | Amphiphatic |
| BIP(1–29)         | MWSKKGILVLFSWAMSDVGQLCKKFP | N terminus of unprocessed bovine prion protein | Amphiphatic |
| MAP               | KLALKLALKALKAKLA         | Chemically synthesized      | Amphiphatic |
| Transporter       | GWTLNSAGYLLGKINLKAALAKL | Chimeric galarin-metastopan | Amphiphatic |
| p28               | LSTADMQGVTVDGASLGDYKLPDD | Azurin                      | Amphiphatic |
| VT5               | DXPDGDPKVTVTVGTGKDPKD    | Chemically synthesized      | Amphiphatic |
| Bac7 (Bac<sub>1,2</sub>) | RRPRPPLRPRPRPRPLRPRLPRPG | Bacteriace family of antimicrobial peptides | Amphiphatic |
| C105Y             | CSIPPEVKFNKFFYLI     | α1-Antiprotease            | Hydrophobic |
| FFVYLI            | FFVYLI                   | Derived from synthetic C105Y | Hydrophobic |
| Rep-7             | SDLWEMMVWCLACQY         | CHL8 peptide phagocytin clone | Hydrophobic |

5. Polymers

Protein-polymer conjugate is a novelty and special synthetic approach that can be used therapeutically by the conjunct of natural proteins with some specific chemical group, take PEG Conjugates as an example, one of the most promising and heated research at present. This kind of conjugates can dramatically change the whole character of proteins in vivo, like extending the half-life of proteins to prolong the effect of the drug, enhance the stability of proteins and reduce the immunogenicity. Direct attachment of polymers like poly(ethylene glycol) (PEG) to proteins has been extensively studied as the PEG chain greatly increasing the systemic circulation time of exogenous protein and its low cytotoxic. So far, many IgGs and cytokines have been successfully PEGylated to promote circulating time, and some of the polymer-protein conjugate has been approved for clinical use by the U.S. Food and Drug Administration (FDA).18

Besides directly conjugation, physical absorption and interaction forming polymer-protein complex is another wide used protein intracellular delivery method. The major disadvantage of direct
covalent conjugation of carrier and drugs or proteins is the negative affect of the carriers on the cargos as the fact that the cargo is chemically modified and modified with a nonspecific part, which always reduce cargo activities. Alternate, commonly used non-covalent method is self-assembly approach. The assembly is always spontaneous driven by electrostatic forces. Hu group develop pH-responsive nanostructures to serve as vehicles for small molecules and proteins delivery with emulsion polymerization. The pH-sensitive nanoparticles cores were composed of tertiary amine monomers DEAEMA and crosslinkers PEGDMA and the pH-insensitive shell was constructed by adding AEMA. With the SEM characterization, it’s proved that the particles have a diameter of about 205 nm and have good PDI. The diameter of the nanoparticles increased by about 3 fold after the Ph change from 7.4 to 5 at room temperature.

Another method, emulsion-based encapsulation could form pH sensitive particles with sensitive monomers. Frechet group explored many protein intracellular delivery machines using emulsion encapsulation with pH sensitive polymers, which can be degraded in the acidic condition such as in endosomes environment.

However, the non-covalent assembled nanoparticles are always unstable during storage and easily release drugs or proteins in the condition not required. Different from encapsulation through emulsion, in situ polymerization give a new way for encapsulation. The encapsulation occurs near the surface of peptides or proteins resulting in tightly encapsulation. Yan group developed a nanocapsules intracellular delivery strategy recently. In this system, each nanoparticle contains a single protein in the core which is surrounding by very thin polymer layer. Firstly, vinyl groups were covalently modified protein residues; secondly, polymerization is initiated and the monomers and crosslinkers are trapped during the polymerization onto protein core. The polymer shell surrounding the protein can protect the protein in the core from enzyme digestion. The non-degradable or degradable ability is relied on the crosslinkers respectively. TEM result of horseradish peroxidase (HRP) core polymer nanoparticles indicates that these nanoparticles have mean diameter about 15 nm. To ensure the each nanoparticle contains only one protein molecule, the author labeled the core protein with 1.4 nm gold nanoparticle, it could be seen that most nanocapsules observed only contained one single gold nanoparticle. With degradable crosslinkers, the nanocapsules is sensitive in some specific environment such as acidic environment in the endosome, which makes the core protein be released in specific place.

When the polymer chains have charged side chains, like positive charged amino group, they will show some properties like CPPs. Lee group developed a new drug delivery system using polyionic polymer micelles. The micells are formed by mix them with negative charged molecular, during which the two component interact through electrostatic interactions. In this systems, the copolymers contain both ionic and hydrophobic parts were synthesized, which subsequently enable the self-assemble and creating micelles.

6. Nano particles
Nano particles have a range wide include lipid nanoparticles, Polymer nanocarriers and protein-media nanocarriers. In general, it is considered that nanocarrier seem to be a vehicle which protect protein from degradation or endosomal escape. Gold nanoparticles are one of the most widely used nanoparticles in biological applications because of their good biocompatibility, highly tunable and changeable surface structure, high modifications efficiency and mature synthesis techniques.
Rotello group used gold nanoparticles as intracellular delivery carriers for β-galactosidase. 2.5 nm gold nanoparticles were conjugated to ligands with thiol-terminated alkyl chains as an anchor. The protein also has an exterior peptide-tag and a variable part to reduce nonspecific binding. With the positively charged moiety interact with negative charged protein on membrane via electrostatic interaction.

Quantum dots (QDs) have good fluorescent properties and are widely used as fluorescent probes which could show target space, molecular and biological processes. QDs have controllable emission spectra, which is highly related to their size, and good photo and thermos stability compared with organic small molecular fluorescent dyes. Russell group have developed a QDs based delivery method to internalize the exogenous protein into cells. In order to improve endosome escape efficiency, the TAT peptide, a kind of CPP peptides, with a cysteine residue on N-terminal for anchoring was generated. QD coated by target protein was conjugated to TAT peptide. Thus, the internalization of the nanocomposite can be visualized.

Silica nanoparticle materials rich in pores and has large surface areas, which are potential place for small molecular or protein attachment, have great potential to serve as delivery vehicles. Another benefit of silica nanoparticle materials is their relative lower cytotoxic compared with metal nanoparticles. Lin group first utilized silica nanoparticles with about 5.4 nm diameter pore for intracellular delivery of the model protein cytochrome c. The protein encapsulation into the nanoparticles is diffusion-driven that can accelerate the encapsulate speed by increase concentration of protein.

7. Conclusion

The introduction of various delivering proteins and peptides approaches are elaborated in the content such as the membrane-like structure called liposome, cationic peptides to penetrate cells so called CPPs, conjugate chemical groups in order to adapt required is the polymer, diverse nanometer material as a cargo carrier… Some relevant studies are still heated at present, although it has a long way to run before it can be used clinically. More importantly, there are also some deficiencies among these methods such as the safety problem and the development in target protein still need to improve. Nevertheless, protein in therapeutic domain is such a prospective study and we are pretty sure that there will be an outstanding progress in the very near future.

Table 2. Examples of some CPPs and their sequences, physical–chemical properties and applications

| Types of nanocarrier | Physical properties | Delivered protein | Cell lines | Ref. |
|----------------------|---------------------|------------------|------------|------|
| Lipid-mediated       |                     |                  |            |      |
| DOPE                 | Positively charged  | BSA, caspase 3   | HeLa       | 5    |
| DOGS                 | Size: 500-900 nm    | BSA, IgG         | CHO, BHK   | 5    |
| Polymeric-based      |                     |                  |            |      |
| PEI                  | Positively charged  | Rnase, Egfp      | NIH-3T3,   | 23   |
| PPAAc-NH2            | -                   | anti-CD3 antibody| KMS-6      |      |
| Cationic nanogels    | Size: 200-500 nm    | BSA              | Jurkat     | 24   |
| Inorganic-based      |                     |                  |            |      |
| Quantum dots         | Size: about 20 nm   | YFP              | HEK 293T   | 26   |
| Gold nanoparticles   | Size: about 2.5 nm  | β-Galactosidase  | HeLa, COS-1| 27   |
| Silica nanoparticles | Size: about 200 nm  | Cytochrome c     | HeLa       | 28   |
| CPPs mediated        | TAT                 | GFP              | CHO-K1     | 12   |
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