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CPP Mediated Insulin Delivery: Current Status and Promising Future

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Abstract: A variety of methods including penetrating enhancers, enzyme inhibitors, as well as cargo mediated drug delivery have been explored to improve the intolerance of parenteral administrated insulin, but little success has been achieved so far. Under this background, cell penetrating peptides (CPPs), with their ability to enhance transport efficiency of macromolecular drugs have been demonstrated to be able to increase insulin bioavailability (BA) in a number of studies, of which a BA up to 50.7% relative to subcutaneously administered insulin could be achieved by nasal route under optimal conditions. Furthermore, CPPs could be conveniently formulated with insulin, or be grafted onto drug-loaded cargoes to facilitate the cargo mediated insulin delivery. Here we reviewed the recent achievements on CPP-mediated insulin transport, and outlined various CPP-based delivery strategies which are expected to show potential in clinical translation in the future.

Keywords: Bioavailability, cell-penetrating peptide, insulin, nonparenteral administration.

1. INTRODUCTION

Type-1 diabetes accounts for approximately 5-10% of the population diagnosed as diabetes. The patient usually lacks pancreatic reserve, thus needs exogenous insulin to sustain life [1]. In clinical practice, insulin is mainly administered via subcutaneous injection which is less tolerable because of pain or associated allergic reactions around the injection site [2, 3]. Other acceptable ways of insulin administration are transdermal, nasal or oral delivery, however, the main limitation in these methods are their poor efficacy which is due to the inability of the macromolecular drugs to cross the physiological barriers [4]. As in the case of oral insulin administration, it is the most popular route when repeated administration of drugs is needed. Furthermore, the orally administrated drugs could be directly delivered to the liver via portal circulation [3]. Since insulin receptors are expressed on the liver cells and fat cells with a greater density compared to the other type of cells [1], and the liver is more sensitive to insulin than the muscle, therefore, it should be a more preferential target for insulin therapy [5].

However, proteins drugs, such as insulin, always suffer from enzymatic degradation in gastrointestinal tract and poor absorption when orally administrated [1]. For most peptides, the oral bioavailability is usually not more than 1%, which is far from satisfactory level when commercially drug is applicable (with bioavailability ranges from 30-50%) [3], thus makes this route quite challenging.

Insulin is a polypeptide hormone composed of two disulfide-bonded amino acid chains (A & B, see Fig. **1A**). It was demonstrated that the amino acid sequences plus their helical structures are essential for maintaining insulin's pharmacological activity [1].

The bright future of non-parenteral administration versus low bioavailability of insulin has inspired worldwide efforts on curbing the challenges of physiological barriers. Those effects mainly focused on three areas: 1) Formulation changes: absorption enhancers which cause tight junctions opening which may facilitate the permeation of water-soluble drugs. Excipients like bile salts, trisodium citrates, and chelating agents like EDTA, labraso which may be helpful to improve the bioavailability of insulin [3]. Anti-proteolytic agents may be used to protect insulin from enzymatic degradation [1]. And novel formulations with superior permeability such as emulsion [7], were utilized to enhance insulin bioavailability. 2) Cargos for enhanced insulin delivery. With the rapid development of nanotechnology, progress has been made with a variety of nanocarriers, such as liposome [8], solid lipid nanoparticle [9], and mucosal adhesive polymer or gels [10]. Such drug carriers may protect drugs from gastrointestinal digestion, enhance permeation across the lipid barrier of GI or skin, and facilitate the absorption of insulin to the intestinal wall. 3) Microneedle

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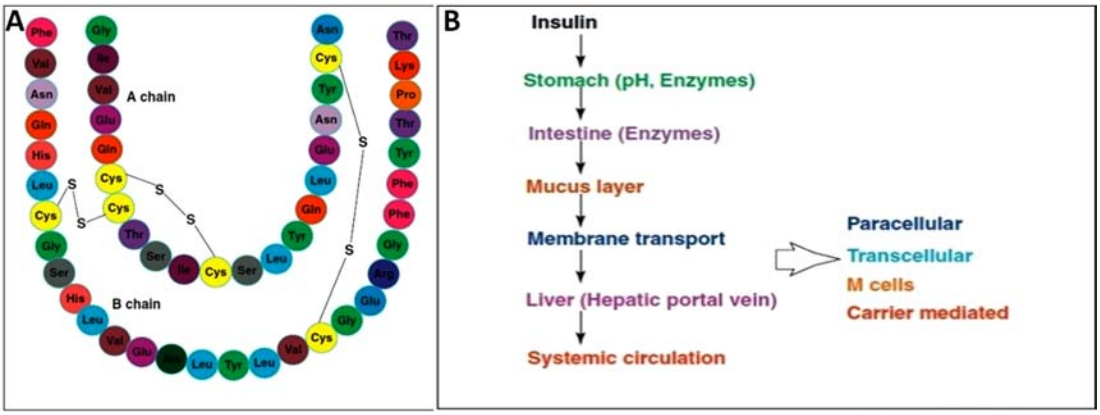

Fig. (1). Structure of insulin and the schematic way of its oral pathway [6]. Reproduced with permission from Elsevier, copyright 2012.

system for minimal invasive transdermal delivery of insulin. The device only penetrates the outermost skin layers, it is superficial enough that it does not touch the nerve receptor in the skin, thus microneedles insertion is perceived to be painless [11].

In spite of the continuous efforts on developing new insulin formulations, the overall outcomes were far from satisfactory level. Epidermis damage caused by microneedle may introduce microparticles into the dermis and initiate immunogenic reactions [12]. Function of protease inhibitors may be attenuated by gastrointestinal secreted pepsinogen. Meanwhile, frequent application of these inhibitors may result in toxicity [13]. Chelators and surfactants may cause gastrointestinal tract damage [1].

In the past few years, a number of reports have been appeared with the effects to enhance insulin delivery via oral route. Various cargoes such as Chitosan nanoparticle [14], nanoemulsion [15], PLGA nanoparticle [16], and polymeric gel [17], have been utilized in insulin delivery. As shown in Table **2**, all cargoes displayed a significant increase of transportation. However, the bioavailability of all the formulations has not exceeded 20% and 10%, respectively [18]. Till now, no oral administration formulation of insulin has been approved yet [1].

The mucosa adhesivity of gel makes it possible to significantly increase gastrointestinal retention time and the subsequent absorption. Compared to other kind of vehicles, polymeric hydrogels seem to be a better candidate for oral insulin delivery [10]. However, efforts on developing more effective polymeric vehicles have been so far met with little improvements. Solid lipid nanoparticle (SLN) is another promising candidate that has been widely applied in oral protein delivery. When insulin was loaded on SLNs, Sarmento *et al*. observed a substantial hypoglycemic effect that lasted for 24 h in diabetic rats. And, relative bioavailability of insulin increased from 1.6% (insulin solution) to 5% (SLN) [13].

2. CPP MAY SERVE AS A NEW KIND OF "MICRONEEDLE" ENABLE PEPTIDE ABSORPTION

Cell penetrating peptides are functional carrier peptides that possess the ability to be efficiently internalized by cells and are considered as a promising tool in drug delivery. Though shared with a common name, CPPs were in fact a large family containing hundreds of peptides which are diverse among size, primary sequences, hydrophilicity, secondary structure, and electric properties. Furthermore, penetration ability among different CPPs is diverse and CPPs may have different affinity to cells of different types [24].

Based on the origin or structure differences, CPPs may be divided into different subgroups. Arginine rich CPPs such as $TAT_{(48\text{-}60)}$ and oligoarginines are linear peptides; amphipathic CPPs (penetratin $(pAntp)_{(43\text{–}58)}$ and pVEC, etc.) are usually presented with its secondary structure (i.e. α-helix or β-sheet) [25]. This structural diversity often leads to different cellular uptake modes and efficiency, whereas uptake of a certain CPP may also be affected by the experimental conditions, as well as the type of the cell lines. However, the universally accepted CPPs may enter the cell by endocytosis and direct translocation through the cellular membrane [26]. Meanwhile, by taking advantage of CPP assisted penetration, membrane integrity may be preserved. Thus, it can be defined as a real non-invasive "needle" for macro-drug delivery [27].

Among the numerous available CPPs, HIV-1 TAT, penetratin and oligoarginine are widely applied for the intracellular delivery of therapeutic proteins. It was expected that CPPs may be helpful to enhance the gastrointestinal absorption of biodrugs [31]. In a series of studies, Morishita *et al*. demonstrated that CPPs, especially octaarginine and penetratin, significantly stimulated the intestinal and nasal insulin absorption following administration of a physical mixture of CPP and the drug. High concentrations (2.0mM) of L-penetratin increased the BA of insulin to 50% after nasal administration. Furthermore, the electrostatic adsorption of R8 onto the membrane proteoglycans would further facilitate subsequent energy-dependent internalization. As a result, mucosal absorption of insulin is enhanced when co-administrated with oligoarginine. On the other hand, penetratin improved absorption as a result of its amphiphilic property in addition to its cationicity [32].

With recent discoveries in exploration novel class of CPPs and the related cell penetrating mechanisms, low permeability of insulin across cellular membrane may be overcome by conjugating the membrane-impermeable large drug compound with a specific cell-penetrating peptide [33].

Table 1. Common cargos mediated insulin delivery.

Name	Dose	AUC uIU·h/L	Formulation details	PA (%)	BA (%)	C_{max}	Outcomes	Ref.
Chitosan/alginate nanoparticle	50	-	-	6.8	-	-	-	[19]
Chitosan/alginate nanoparticle	100	-	-	3.4	-	-	-	[19]
Insulin-chitosan microsphere	-	-	-	15.8	-	-	AUC increased 14.1 fold	[14]
Enteric coated capsule with NPs	30	235.8±30.2	Capsulated NPs, oral	-	20.1±0.3	-	AUC increased	[20]
CS/γPGA-DTPA	30	179.7±32.5	Nanoparticle oral	-	19.7±1.3	39.2±2.8	AUC increased	[21]
Self emulsion	2.5	-	-	-	12.5	-	Papp 20 times than control	[15]
INS-SLN	20	-	SLN oral	5.66-0.53	-	-	PA 113.2	[22]
INS-SLN	25	-	SLN oral	4.28-0.84	-	-	PA 10.2	[23]

-: not available.

Table 2. Commonly used CPPs and their primary sequences.

CPPs	Sequence	Structure	Ref.
Oligoarginine	R6, R8, R10	Random coil/α-helical	[25, 28]
Penetratin	RQIKIWFQNRRMKWKK	α-helical/β-sheet	[25]
TAT(48-60)	GRKKRRQRRRPPQ	Random coil/α-helical	[25]
LMWP	VSRRRRRRGGRRR	Random coil/α-helical	[29]
pVEC	LLIILRRRIRKQAHAHSK	Amphipathic, b-sheet	[25]
L-RRL helix	RRLRRLLRRLRRLLRRLR	α-helix	[30]

On the other hand, negative insulin could electrostatically bind with the positive CPPs and form stable complex, thus can be given with a dose of physical mixture. In the case of drug loaded cargoes, CPPs could be grafted onto the cargoes and facilitate the cargo mediated transport.

2.1. CPP-Conjugate

2.1.1. TAT

$TAT_{(57-60)}$ has been extensively studied in numerous studies for the delivery of functional proteins, peptides, and macromolecules to the cellular target [34]. It was observed that fluorophore-labeled TAT enters the cytoplasm and nucleus of fibroblasts within seconds without cell fixation [35]. Direct penetration or pore formation was believed to be involved in the translocation process [25]. Under physiological pH, hydrogen bonds may be formed between the guanidine head group of arginine and the negatively charged phosphates and sulfates on the plasma membrane, which might initiates the subsequent internalization of CPPs [26]. It was reported that TAT-cargo conjugate could be internalized by cells in an energy-dependent way [36], it remains controversial whether CPP-drug conjugates enter cells by direct translocation or by endocytosis pathways. However, CPPs- mediated delivery also suffered from endosomal entrapment [37].

TAT was often conjugated to the drugs, which may lead to a different internalization pathway [38]. To avoid the inactivation of TAT caused by modification, a spacer (short chain of (PEG)n [39], poly G [35], etc.) is usually introduced as a cross-linker between CPP and drugs to be delivered. We previously prepared covalently bond insulin-CPP hybrids using SMCC as cross-linker. Results from *in vitro* test on Caco-2 cell monolayer showed that by conjugation, the intestinal absorption efficiency of insulin was increased by 6-8 times, and transcytosis-like mechanism seemed to be responsible to insulin-CPP hybrid transportation. Importantly, ELISA assays showed that with the help of CPP, insulin remains intact after passing through the Caco-2 cell monolayer [40]. In another study, insulin-TAT conjugates (molar ratio 1:1) displayed 19 times transporting efficiency increase compared to native INS across the monolayers of primary cultured rat alveolar epithelial cell [41].

2.1.2. LMWP

LWMP, terms low molecular weight protamine, is a 13 residue fragment with heparin-neutralizing ability isolated from protamine by enzymatic digestion [42]. In fact, the full

length protamine had been formulated with insulin as effective excipients in maintaining insulin stability and delayed absorption in subcutaneous injection [43-45]. Despite its universal application, a large population of diabetic patients receiving S.C. administration of classical protamine-insulin formulation is found to be at a high risk to acquire severe protamine response [46]. In contrast, injection of LMWP did not induce detectable antibody products [42]. *In vitro* and *in vivo* tests further confirmed LMWP as a nontoxic heparin antidote [47-49]. Furthermore, LMWP could not react to sera from diabetic patients which received a prior sustained exposure to protamine- containing insulin [46], indicating that it was safer than protamine.

Because LMWP possesses high arginine content and a significant similarity to TAT in sequences, we hypothesized that LMWP would also inherit the similar translocation activity across the cell membrane, which enables any impermeable species to be transduced into the cells. Then we examined, if it has produced any effect to transduce the impermeable protein into the tumor cells by chemical conjugation. Results showed that LMWPs could indeed translocate itself into several mammalian cell lines as efficiently as TAT [50]. In another study, we coupled LMWP to insulin (1:1 ratio) with succinimidyl-[(N-maleimidopropionamido)-polyethyleneglycol] ester (NHS-PEG-MAL) as an intermediate crosslinker [39]. Cell culture studies demonstrated that transport of the insulin-PEG-LMWP conjugated across the intestinal mucosal monolayer was augmented by almost 5-folds compared with native insulin. Furthermore, results from the in situ loop absorption tests in rats showed that systemic pharmacological bioavailability of insulin was significantly enhanced after its conjugation with LMWP (see Fig. **2**). Overall, the presented chemical conjugation with LMWP could offer a reliable and safe mean to improve the intestinal permeability of protein drugs, shedding light to the possibility for their effective oral delivery.

2.1.3. Oligoarginines

Oligoarginine is a kind of chimeric CPPs usually composed of 6 to 12 arginines. Similar to its arginine rich fellows such as TAT, the arginine peptide may cross the membrane by direct penetration of endocytosis [25]. Patel *et al* investigated the membrane permeability and *in vivo* hypoglycemic effect of R9-insulin conjugate on diabetic rats by intratracheal instillation. It was found that transport of insulin by insulin-CPPs across primary cultured rat alveolar epithelial cell monolayers was increased by 27 times. The translocation of insulin-cr9 was temperature- and time- dependent. Covalent conjugation between R9 and insulin was proved to be necessary to enhance transport of INS because unconjugated R9 did not enhance insulin permeation. Furthermore, those diabetic rats which received insulin-R9 had a more sustained serum glucose decrease over time as compared to insulin [41].

In spite of the successful attempts made by insulin-CPP conjugation, there are a number of drawbacks attached to reduced transport efficiency when drugs are covalently coupled to CPPs. In a study, Morishita *et al.* found that covalently attached with R6 does not enhance permeation of le-

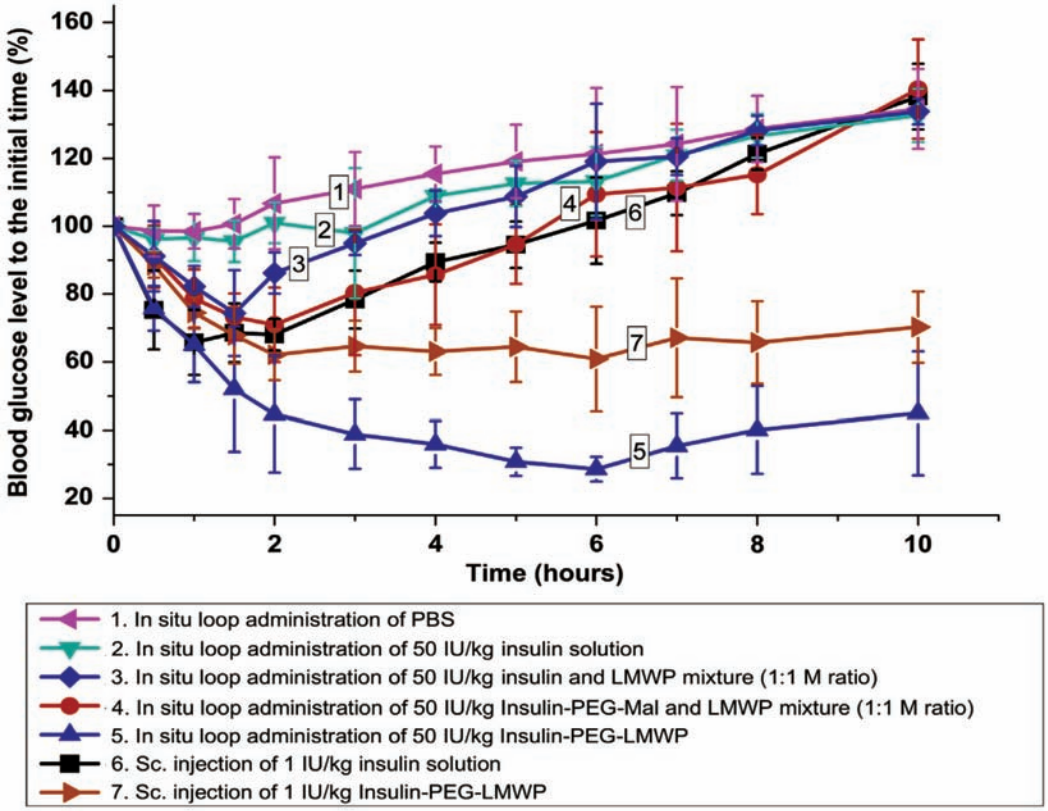

Fig. (2). Plots of the blood glucose level (BGL) against time after in situ loop administration [39]. Reprinted from Huining He, *et al*. The use of low molecular weight protamine chemical chimera to enhance monomeric insulin intestinal absorption. Biomaterials 34 (2013) 7733-7743.

uprolide across rat ileal membrane. In contrast, after the in situ administration, AUC of leuprolide-D-R6 decreased to about 38% of the free leuprolide. Since permeation of leuprolide was not affected by non-conjugated mixture of L- or D-R6, reduced drugs permeation seemed to be the formation of covalent linkeage between drug and CPPs. The authors speculated that permeation ability loss of R8 can be attributed to inability of leuprolide to escape from endosome and be released from basal membrane caused by covalent conjugation with R8 [51]. However, this does not explain why R9-insulin conjugates displayed more enhanced permeation and superior anti-diabetes effects compared to non-conjugated R9-insulin mixture [41]. Furthermore, the fact that leuprolide and R8 were directly linked by amido bond without spacer is another notable point. As we know, the internalization of oligoarginine into the intestinal tissue was mediated by its adsorption to membrane proteoglycans and the subsequent translocation [51]. Direct conjugation may lead to a more strong steric effect that may hinder CPP-proteoglycan interaction and subsequent internalization.

In a word, CPP-insulin conjugation has made some remarkable achievements with improved permeation and bioavailability for non-parenteral insulin administration. However, special care is needed when preparing drug-CPP conjugation, since improper design may lead to loss of CPP function, which is not worth the candle.

2.2. CPP-insulin Mixture

Compared with CPP-insulin conjugation, it is much simple and convenient to incubate certain kinds of CPPs with protein drugs instead of newly synthetized conjugates, and much easier to change the peptide quickly when resistance occurs [52]. Furthermore, unlike covalent binding in which drugs and permeable peptides are usually cross-linked with the molar ratio being fixed at 1:1, permeation ability of CPP-insulin mixtures are diverse with CPP types and doses, thus can be optimized by adjusting the CPP-drug ratios [30].

By using this strategy, Morishita *et al*. have screened a number of CPPs that may enhance insulin absorption via nasal or intestinal administration. Some promising results have been obtained, with the BA of nasally administered insulin can be increased to 50.7% relative to the subcutaneous route which has been achieved in some cases [27], and outlined a bright future for non-parenteral administration of insulin in this area.

2.2.1. TAT

Manosroi *et al*. evaluated the orally administrated insulin-Tat mixture(molar ratio at 1:1, 1:3, 1:6) for their hypoglycemic activity in alloxan-induced diabetic mice, and found that insulin-TAT mixture (1:3) at the dose of 200 IU/kg was most effective and resulted in an FBG reduction of 74.0±10.3% (2.18 folds compared to subcutaneously injected (SC) insulin). Free insulin administered orally did not show any hypoglycemic effect. The prolonged hypoglycemic activity of insulin–TAT mixture can be further developed as an effective mean for oral insulin delivery [53]. However, insulin dose used in the study was too high, which was not economically feasible considering the necessity of frequent administration for blood glucose control.

2.2.2. LMWP

As a cationic CPP, LMWP could form stable complex with negative molecules such as pDNA and siRNA [54, 55]. The complex not only protects the oligo nucleoacids from degradation but also increases transport efficiency to target the cell.

We previously prepared LMWP-insulin complex by co-incubation method. Subcutaneously administrated CPP-drug mixture displayed a more prolonged hypoglycemic effect than free insulin. Besides, LMWP was found to be less toxic than the immunogenic protamine [56]. However, whether mixed LMWP could improve bioavailability of orally administrated insulin remains to be identified.

2.2.3. Oligoarginine

Without containing heterogeneous residues, oligoarginines with higher transport efficiency could be more easily identified by changing the arginine number, configuration, and subsequently determining the permeability in the tests. In a study, Morishita *et al*. found that insulin absorption was significantly increased after co-administration with oligoarginines (R6, R8, R10, L- and D-) without inducing histological damage to the intestinal mucosa. Among oligoarginines composed of 6, 8, or 10 arginine residues, D-R8 displayed the strongest enhancing effects on intestinal permeation of insulin, suggesting that number of arginines for enhancing insulin permeation could be optimized.

Furthermore, since L-type peptides could be digested into intestinal fluid, all D-type oligoarginines displayed a more prominent enhancing effect than their L-types fellows. An interesting fact is that the transport of both positive IFN-β and neutralized FITC–dextran (FD-4) shows little affinity to positively charged oligoarginine, which was not enhanced by CPPs, indicating that electro statistical interaction between CPPs and drugs may be important for oligoarginine assisted transportation [28].

To test this, the authors compared transport efficiencies of R8 mixed with different proteins, along with the intermolecular binding parameters which were obtained by surface plasmon resonance (SPR) based binding assay. Among the 16 peptide drugs that are diverse in isoelectric points, only negative peptides, such as gastrin, insulin and glucagon-like peptide-1 (GLP-1) could bind to D-R8, and their intestinal absorption was subsequently enhanced by co-administration with D-R8, whereas the intestinal absorption of other peptides was not affected following the co-administration with D-R8. This study suggested that intermolecular binding between drug and CPPs plays an important role during intestinal absorption. In addition, binding ratio between insulin and D-R8 is also demonstrated to be important during intestinal insulin delivery, which was in accordance with Manosroi's study [57].

Zhang *et al*. further found that the addition of hydroxypropyl-β-cyclodextrin (HP-β-CD) may protect insulin from degradation by α -chymotrypsin, thus synergistically enhance the insulin permeation across the Caco-2 cell monolayer 8-10 times than that of normal insulin [58].

2.2.4. Penetratin

Penetratin is a 16-amino-acid peptide that is derived from the amphiphilic Drosophila Antennapedia homeodomain. Penetratin could transit the membrane by direct penetration or endocytosis [25]. For insulin delivery, penetratin has made itself an outstanding target to greatly enhance intestinal absorption, which was superior to a list of classical CPPs including R8, R12, Tat (48-60), pVEC, and RRL, etc. [30]. Meanwhile, the permeation enhancing effect of CPPs is dependent on CPP dose, cell type, CPP-drug interaction, as well as amino acid configurations [30, 52]. In an ileal absorption test, Morishita *et al*. found that D-R8 had stronger enhancing ability on insulin absorption than the L-form due to improved stability of D-peptide in the intestinal tract. In contrast, the other three peptides (penetratin, pVEC, and RRL helix) all exerted more significant effects when the L-forms were applied. Furthermore, it was found that penetrating enhancing effect of L-penetratin was increased by increasing CPP concentration (from 0.05 to 2 mM). On the other hand, with increasing the ratio of D-penetratin and insulin, permeation enhancing effect was raised at low CPP concentrations (from 0.05 to 0.2 mM), followed by a dose-dependent decrease with the formation of insulin-CPP aggregates when more CPPs were applied (0.5 to 2 mM) [30].

Morishita *et al*. investigated whether CPPs can enhance the nasally administrated insulin. They found that at high concentration (2 mM, with insulin at the dose of 1 IU/kg), the pharmacological availability (PA) and bioavailability (BA) of insulin could be raised to 76.7% and 50.7% vs. the SC route, respectively. The results suggested that L-penetratin has great potential in insulin transport [27]. While results were promising, CPP concentrations were too high, which made it less attractive when formula cost was taken into account. By sequence modulation based on penetratin template, Morishita *et al*. obtained another potential candidate (shuffle (R, K fix) 2), from 20 analogs of penetratin with various residue properties. The candidate displayed a more effective absorption enhancement of nasally administrated insulin, reaching a relative bioavailability of 1.85 times to penetratin, which was about 16 times to free insulin solution [59].

Similarly, the enhancing effect of co-administered L-penetratin or shuffle (R, K fix) 2 on the nasal absorption of proteins correlated well with the drug-CPP intermolecular binding parameters [60]. The author then analyzed 26 penetratin analogs with self-organizing maps (SOM) in an in situ loop absorption study. It demonstrated that intestinal absorption of insulin was associated with multiple factors (molecular weight, basicity, the lowest unoccupied molecular orbital energy, absolute hardness, and chemical potential of CPPs). Furthermore, the newly synthesized CPPs based SOM clustering analysis displayed significantly greater capacities to enhance insulin absorption than the original penetratin. The results suggesting that the peptide sequence with a specialized structure for insulin delivery can be optimally defined by SOM with a molecular orbital method [61].

2.3. CPP-cargo Systems

CPPs have been utilized to facilitate cellular transport of various cargos such as nanoparticles, liposome, micelles, etc. [62]. Despite the ongoing interests on CPP- insulin coupling strategy, drawbacks often came with low permeation efficiency or inactivation because of enzyme digestion in GI. Instability of insulin could be improved by loading into a cargo which is further modified by CPPs to increase transport efficiency.

2.3.1. Nanoparticle

Nanoparticle is one of the most popular cargos utilized in drug delivery. By modification with CPPs, nanoparticle could display a more efficient transport of chemical drug, DNA, or even magnetic cores [63]; thus induce a stronger physiological effect, compared to unmodified cargos [64, 65]. In a study, LMWP was conjugated PEG-PLA nanoparticles (NP) by maleimide-mediated binding procedure. LMWP-NP was found to be a more preferential uptake than unmodified NP without causing significant cytotoxicity on 16HBE14o-cells. Furthermore, intranasal administrated coumarin-6-loaded LMWP-NP displayed a 2.03-, 2.55-, 2.68- and 2.82- folds increase of $AUC_{0\text{-}8\ h}$ in the rat cerebrum, cerebellum, olfactory tract and olfactory bulb, respectively, compared to that of drugs carried by NP, indicating CPP conjugation facilitates the cargoes across the blood brain barrier [66]. In another study, we prepared a TAT modified PLGA nanoparticles for the nasal delivery of insulin to the brain. Compared to 0.405% of drug disposition in olfactory bulb for the unmodified NPs, the Tat-NPs showed a significant increase. Similar pattern of drug disposition was observed in cerebrum, where the Tat-NPs accumulated at a significantly higher concentration than the unmodified NPs (3.36% vs. 0.95%), indicating its potential to deliver macromolecules to the brain. A completely accumulated dose in the brain (olfactory bulbs and cerebrum) reached 6%, suggesting that it could serve as a good candidate for neurodegenerative diseases [67]. By maleimide-mediated covalent conjugation, Liu *et al*. modified PLGA nanoparticle with poly (arginine) 8 enantiomers (L-R8 and D-R8). It was found that R8-modified nanoparticles displayed increased cellular uptake and transport efficiency in Caco-2 cell monolayer model. Pharmacokinetics and pharmacodynamics studies showed that L-R8 and D-R8 modified nanoparticles increased the relative bioavailability of insulin by 3.2- and 4.4-fold, respectively, compared to the unmodified nanoparticles [68]. The results were in agreement with Morishita's reports, which showed that stable D-R8 has a greater potential to enhance insulin transport efficiency.

Lipid nanoparticle has been verified for having highly efficient membrane transport [69], and widely utilized for various routes of administration [70]. In addition to its hydrophobic nature, Chen *et al*. found that CPPs coating could further increase the transport efficiency of lipid nanoparticles. With $AUC_{(0\text{–}t)}$ of the CT-NLCs being increased to 4.8-fold and 1.5-fold to that of the native drug suspension uncoated NLCs, respectively [71]. Tahara *et al*. prepared a solid in oil O/S nanodispersions containing oligoarginine peptides for topical insulin delivery [72]. It was found that transdermal delivery of insulin by O/S nanodispersion was enhanced by R6 peptides. Insulin formulated as S/O nanodispersion containing R9 peptides penetrated into Yucatan Micropig skin with 25-fold increase, compared to the aqueous solution. Nevertheless, S/O nanodispersion without CPPs only displayed 5.6-fold increase to aqueous solution.

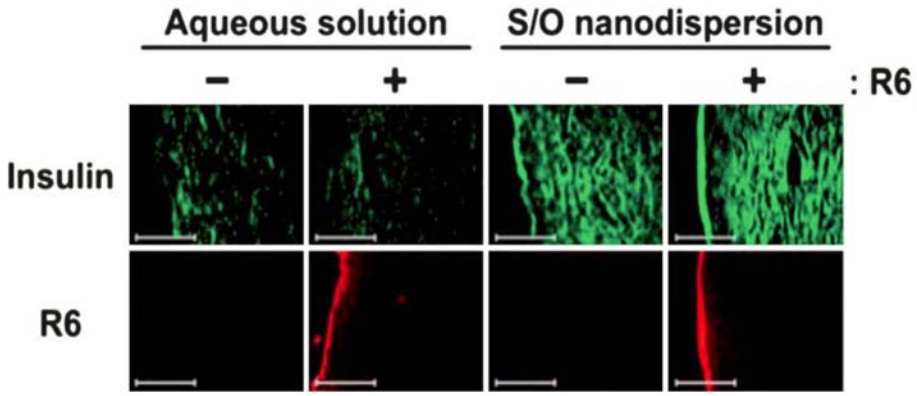

Fig. (3). Fluorescent microscopy images of skin sections treated with samples containing FITC-labeled insulin and rhodamine-labeled R6 peptide visualized at 10 (scale bar, 200 mm) objective lenses after 48 h of application. The skin surface is displayed on the left of the sections. Reprinted with permissions from Royal Society of Chemistry publishing group, copyright 2012.

Fluorescent microscopy images of skin sections showed that the rhodamine labeled CPPs mainly migrated to the skin surface, where it could function by interacting with lipids and destabilize the stratum corneum and tight/adherens-junction proteins on the skin surface [73], thus enhance the skin permeability (see Fig. **3**). Interestingly, among three kinds of oligoarginines (R3, R6, R9) utilized, R6 displayed the most pronounce permeation enhancing effects. The result was inconsistent with Morishita's report, which showed that R8 was more effective than the other CPPs (R6 and R10). The discrepancy may be explained with the physiological differences between the intestinal and skin barriers.

Zhang *et al.* prepared a new kind of solid lipid nanoparticles (SLNs) modified with stearic acid–octaarginine (SA-R8) for oral insulin administration (SA-R8-Ins-SLNs). *In vitro* experiment showed that by formulating with SLNs and SA-R8, insulin could be protected from proteolysis. Besides, cellular uptake of insulin by Caco-2 cell was raised by 18.44 times. Furthermore, a significant hypoglycemic effect on diabetic rats over controls was obtained, with pharmacological availability of SA-R8-Ins-SLN be 13.86±0.79%, suggesting that SA-R8-modified SLNs could enhance the insulin oral absorption [23].

2.3.2. Polymer

Polymer was another cargo superfamily, since monomer varies in origin, size, type, etc., the polymer could be of great diversity and deformability [74]. Malhotra *et al.* prepared a chitosan-PEG-TAT polymer with a bifunctional PEG which serves as a linker for the conjugation of chitosan and TAT. The neutral PEG linker reduced the steric hindrance of the positively charged polymers and thus facilitated the siRNA delivery [75]. Zhu *et al.* conjugated bis-β-cyclodextrin with penetratin, the resulting bis-β-cyclodextrin derivative (P-bis-CD) which could self-assemble into nanocomplex with insulin. The nanocomplex was demonstrated to be able to result in a sustained hypoglycemic effect with maximum inhibitory rate at 60% when intestinally administrated. To be noted, The PA and BA of P-bis-CD nanocomplexes were 10.6% and 7.1%, respectively, which were 3.0- and 2.3- fold higher than that of penetratin nanocomplexes, respectively [76].

Sun *et al.* prepared a block co-polypeptide poly (L-homoarginine) 60-b-poly (L-leucine) 20 (R60L20) [77]. The polymer could self-assemble into versatile vesicles with controllable size and to be served as cargos for loading hydrophilic drugs. Because guanidinium group of arginines could strongly interact with negative membrane (see Fig. **4**), surface located oligoarginine could serve as CPPs and facilitate transport of the cargoes. It was demonstrated that R60L20 vesicles could cross the plasma membrane and deliver the encapsulated drugs to different cell lines, indicating that it may be utilized for insulin delivery.

In spite of the fact that the optimal length of a specific oligoarginine is supposed to contain 6-12 amino acid residues when utilized as effective CPPs. Nemoto *et al.* demonstrated that poly-L-arginine (PLA50, MW 38kDa) could also improve permeation of the hydrophilic FITC-labeled dextran (MW 3800, FD-4) through ocular surface tissues. The permeability coefficient (P_{app}) of FD-4 in the cornea, conjunctiva, and conjunctiva/sclera composite be significantly increased with the addition of PLA50 at 0.1 mg/ml (6.81-, 9.78-, and 7.91-fold, respectively). Besides, a corresponding reduction in TEER was observed in all tissues. However, the reduced TEER in the case of the conjunctiva had recovered to ca. 70% 120 min after replacing the mucosal fluid with fresh bicarbonated Ringer's solution. The result indicated the poly-arginine may enhance the FD-4 transport via different pathways such as increased paracellular permeation induced by dissociation of tight junction assemblies [78].

Seki *et al.* prepared sperminated gelatin (SG), which was ready to be used as a novel arginine-rich absorption enhancer on the absorption of nasally administrated insulin. It was demonstrated that by adding 0.2% SG, the AUC of nasally administrated insulin was increased by 5.3-fold. Besides, by Renkin molecular sieving function analysis, it was found that the pore occupancy/length ratio increased, whereas equivalent cylindrical pore radius is not changed, suggesting that SG addition may transform the true tight junction into pathways for hydrophilic molecules [79].

Later, the authors further compared the absorption enhancing effects of sperminated pullulans (SP) and gelatin (SG) on pulmonary administrated insulin. It was found that

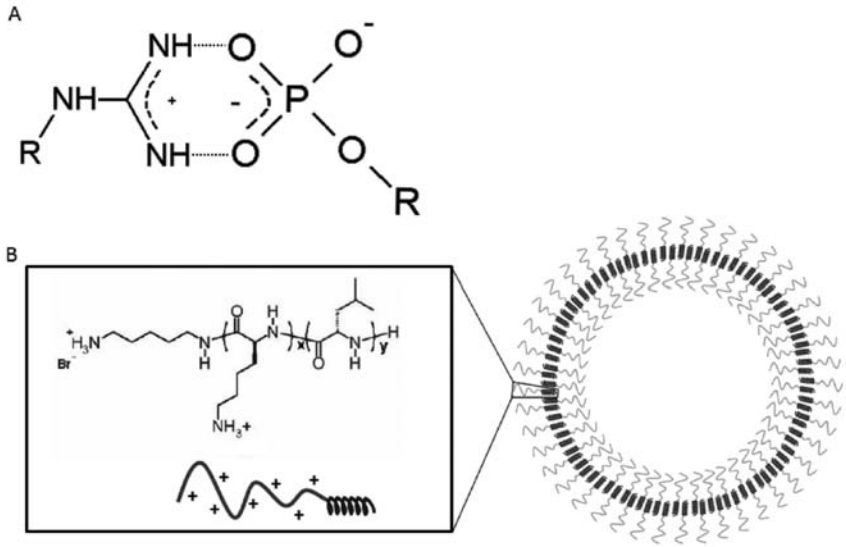

Fig. (4). (**A**) Proposed bidentate H-bonding interactions between guanidinium and phosphate groups. (**B**) Schematic of the K60L20 blocks co-polypeptide and vesicle assembly [77]. Reprinted with permissions from American Chemical Society, copyright 2009.

the enhancing effects of SP and SG correlated well with the amino group contents (with amino group content SG<SP-L<SP-H). The maximum plasma glucose reduction (with glucose concentration about 70.5% relative to control) was observed in the rats treated with SP-H. Furthermore, TEER values of the Calu-3 cell monolayer were also reduced to 75.3%, 61.6% and 17.5% for SG, SP-L and SP-H, respectively. It was suggested the tight junctions were disabled following incubation with the sperminated polymers [80].

Similarly, Sakuma *et al.* grafted oligoarginines onto the backbone of poly (N-vinylacetamide-co-acrylic acid) to form a new polymer (PNVA-co-AA-R8). Mice nasally co-administrated with ovalbumin and D-octaarginine-linked polymer, triggered an enhanced ovalbumin specific immunological effects, compared to the control, indicating PNVA-co-AA-R8 significantly enhanced mucosa transport of OVA [81]. Meanwhile, PNVA-co-AA-R8 (grafting degree at 2%) significantly enhanced the hypoglycemic effect when co-administrated with insulin via nasal route in mice. Similarly, the penetration-enhancing and hypoglycemic function of PNVA-co-AA-R8 increased dramatically with an increase in the grafting degree of D-octaarginine (1001±304 %·min and 5121±552%·min for insulin solution and PNVA-co-AA-R8 (grafting degree at 17%), respectively). It was interesting to note that a PNVA-co-AA-R8 with a grafting degree at 17% enabled fluorescein isothiocyanate-dextran (FD-4) to effectively penetrate the cell membrane, which was less affected in the presence of oligoarginines [82]. It was further demonstrated that co-incubation with PNVA-co-AA-R8 dramatically increased cellular uptake by Caco-2 cell of anionic 5(6)-carboxyfluorescein (CF), cationic atenodol and fluorescein isothiocyanate (FITC)-dextran (FD-4) by 25-, 3.3- and 24-fold, respectively. However, CF uptake was not affected by the individual components, such as D-octaarginine, suggesting that only D-octaarginine anchored the polymeric backbone which had the capacity to promote the CF permeation. With the help of confocal laser scanning microphotograph imaging, it was observed that D-octaarginine-linked polymers bearing rhodamine red could just outline the cell without being engulfed. Furthermore, the polymer enhanced CF uptake could be significantly suppressed by macropinocytosis inhibitor (5-(N-ethyl-N-isopropyl) amiloride), The results indicated that D-octaarginine-linked polymers remained outside the cells, however, the individual D-R8 branches could continuously interact with the membrane which facilitates CF internalization via repeated macropinocytosis. Combining those evidences, the authors proposed that individual CPPs could interact with the cell membrane via the multi-accessible uptake way without being internalized by the cell as a result of conjugation to the polymeric platform. So the CPPs could be "engulfed" by the cells repeatedly, creating an "ever-opening" pathway for molecules in the periphery of D-R8 branches without considering their physicochemical properties [83] (see Fig. **5**). This mechanism could also explain the increased uptake of FD-4 by polyarginine 50 [78] and sperminated gelatin [79].

2.4. Pharmacokinetics Study and Safety Concerns

In spite of the quantitative efforts on enhancing insulin transport the physiological barriers and the related bioavailability, little is known about the ultimate bio fate when CPP-insulin was administrated into the body. Since liver is a more preferential target for insulin therapy, hepatic retention of the delivered drugs may be of obvious benefits for hypoglycemic therapy [5]. However, entrapment of drugs by the reticuloendothelial system (RES) may also contribute to drug accumulation in the liver, which often leads to subsequent elimination from the body, thus has to be avoided [84].

2.4.1. Pharmacokinetic Studies of CPPs

When co-administrated with insulin, CPP may facilitate the encapsulated drugs to be instantaneously removed from blood and distributed into organs owning to rapid uptake by the relevant epithelial tissues [85]. Furthermore, pharmacokinetic behaviors of CPPs may diverse among different

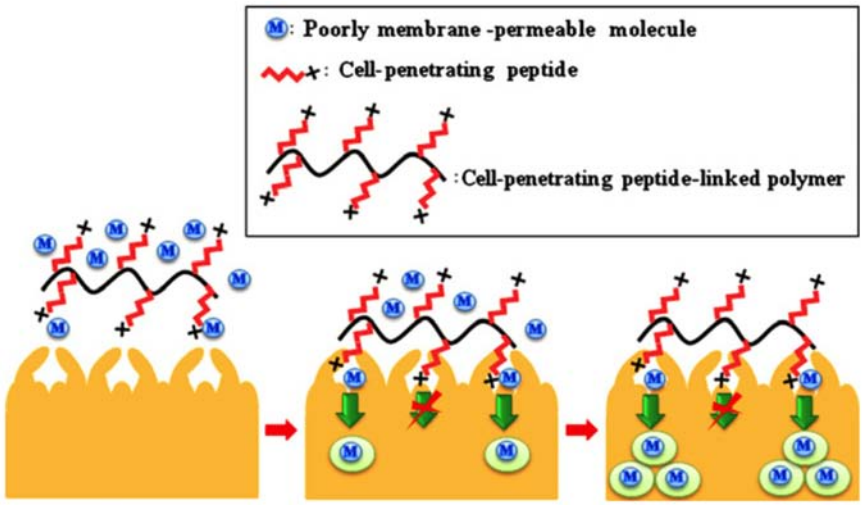

Fig. (5). Schematic representation of the cellular uptake of poor membrane permeable molecules physically mixed with cell-penetrating peptide-linked polymers [83]. Reprinted with permission from Elsevier, copyright 2012.

types, and can be utilized for specialized targeting. In a pharmacokinetic study, Sarko *et al.* investigated the biodistribution and pharmacokinetics of ten different CPPs in Wistar rats after intravenous injection [24]. It was found that with high permeability, all the CPPs could break away from circulation and be internalized by highly perfused organs immediately post i.v. (with blood content less than 1% for all the CPPs in 1 h post i.v.). However, distribution patterns varies among different CPPs (see Fig. **6**): TAT and pVEC tend to accumulate into the kidneys (66.5±9.5% and 32.8±5.4%, respectively, 1 h post i.v.); R9 tends to accumulate in the liver (49.9±2.6%); while the distribution of penetratin in the liver and kidneys is comparable (25.6±0.9% and 22.6±2.5%, respectively).

However, preferential uptake of CPPs by the liver does not always guarantee the enhanced uptake of the target drug by liver to the same extent. With the help of PET imaging, Morishita *et al.* tracked and semi quantified insulin absorption and subsequent distribution after ileal administration [86]. The results showed that the CPPs (especially D-R8 and L-penetratin), significantly increased insulin absorption, with signal intensities of ^{68}Ga-DOTA-insulin in the liver, kidney and circulation being significantly raised. Co-administration of D-R8 or L-penetratin resulted in a 2.18- or 2.06-fold increase in the hepatic accumulation of ^{68}Ga-DOTA-insulin at 120 min, and renal retention was also increased to 3.03- or 3.00-fold in comparison, hepatic or renal accumulation of ^{68}Ga-DOTA-insulin or its metabolite of ileal administrated insulin solution was negligible. It can be observed that the ^{68}Ga-DOTA-insulin passed through the liver rapidly and accumulated in the kidney (see Fig. **7**) after being absorbed from the intestine. In spite of the enhanced permeation with improved hypoglycemic effects, total accumulation of insulin in liver is rather low (see Fig. **7**), where specific receptors for insulin were highly expressed [1].

The high hepatic retention potential of R9 and penetratin seemed to be inconsistent with preferential distribution of insulin to the kidney. However, complex formation with insulin may lead to functional changes and a shifted biodistribution pattern. Considering that CPP was physically mixed with insulin, complex dissociation (for D-R8) and enzyme degradation (for L-penetratin and L-R8) may also contribute to the reduced liver retention. Furthermore, liver retention of non-degraded D-R8-insulin seemed to be treated more than ones with L-R8-insulin or L-penetration-insulin complex, indicating CPP complexes showed minor effects on the insulin distribution patterns (see Fig. **7**). To increase hepatic accumulation, we hypothesized that CPPs may covalently linked with insulin by an inactivated spacer, which could increase intestinal absorption of insulin without affecting the pharmacological effects, at the same time high liver retention is also attained with the help of CPPs. This hypothesis could be supported by Patel's results in which diabetic rats received insulin-R9 complex showed a steady decrease in blood glucose level that was more sustained over time when compared with insulin-R9 mixture [41], as well as our previous report that systemic pharmacological bioavailability of insulin was significantly enhanced after its conjugation with LMWP than the mixture [39].

Amantana *et al.* investigated the pharmacokinetic behavior of a phosphorodiamidate morpholino oligomers (PMO) conjugated to the CPP via an Ahx-β-Ala linker in rats. The results showed that CPP conjugation improved the kinetic behavior of PMO with half-life, volume of distribution increased by 2- and 4-fold, respectively. Furthermore, conjugation to CPP increased the uptake of PMO by most of the tissues except brain, among which greater uptake enhancement occurred in liver, spleen, and lungs [87], and they all are rich in RES systems [88]. Whereas, a remarkable amount of PMO could be detected in the liver even at day 5th after the last dose, indicating that the CPP conjugation has increased PMO retention time in this organ [89]. The result suggested that besides RES entrapment, tissue content of the CPP modified PMO was increased by enhanced tissue permeation. In contrast, the efflux of the accumulated PMO from tissues to the blood is relative slow, while no apparent degradation was observed. Similarly, Gu *et al*. demonstrated that LMWP conjugated PEG-PCL nanoparticles showed higher accumulation in liver, heart, lung and spleen without accelerated degradation, compared with unmodified nanoparticles

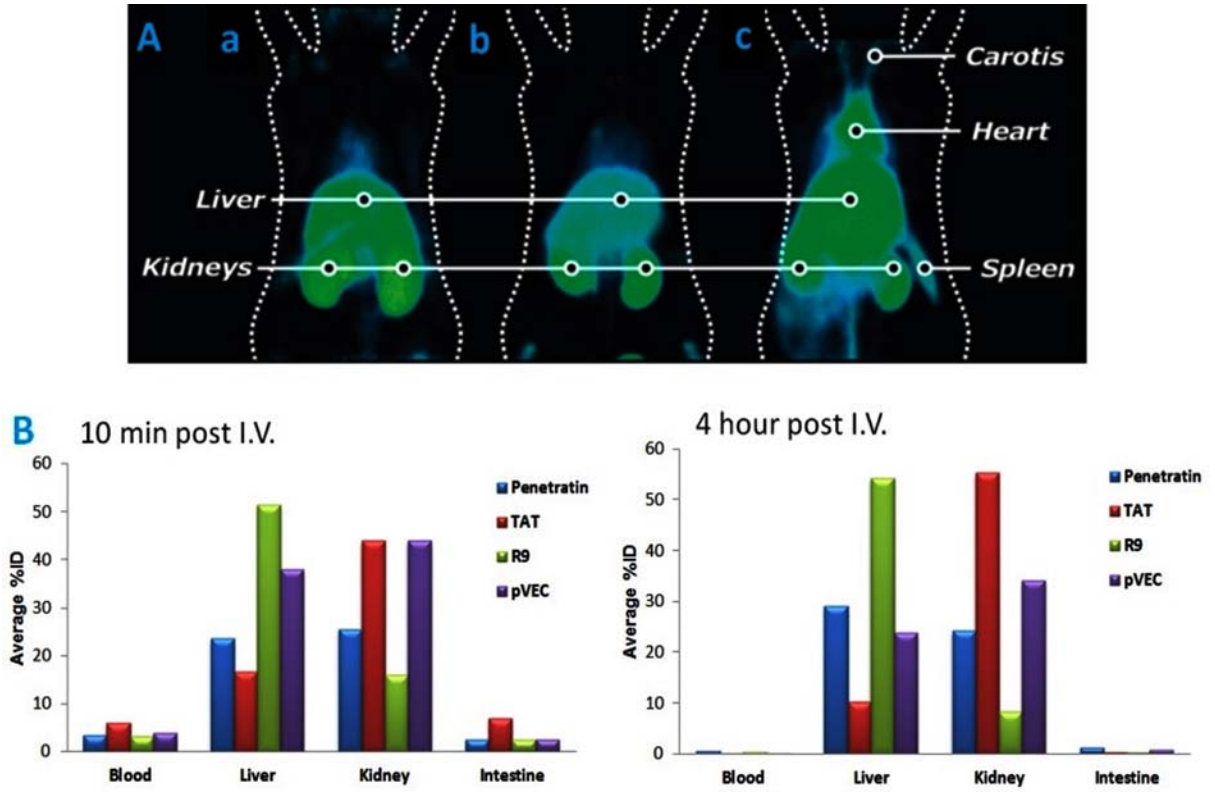

Fig. (6). ***In vivo*** **micro-PET imaging of the biodistribution of three labeled CPPs post intravenous injection.** Wistar rats were injected with ^{68}Ga-labeled penetratin (**a**), Tat (**b**) and TP10 (**c**) via the tail vein and scanned at 1 h post injection. With fig. 6A reprinted from figure 2, and 6B reproduced from tables 3 & 4. Permissions from American Chemical Society, copyright 2010.

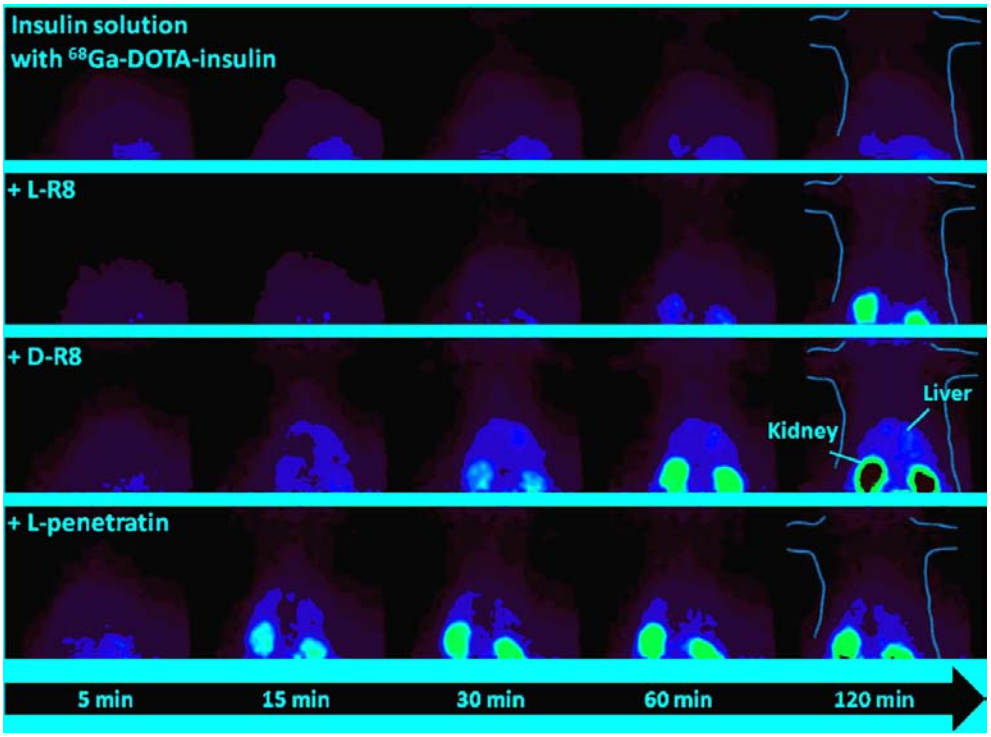

Fig. (7). Time profiles of MIP based on PET imaging following the *in situ* administration of insulin solution with ^{68}Ga-DOTA-insulin into the rat ileal loop in the presence or absence of CPPs [86]. Insulin solution with ^{68}Ga-DOTA-insulin (n=3), +L-R8 (n=3), +D-R8 (n=4), +L-penetratin (n=4). Reprinted with permission from Elsevier, copyright, 2010.

Table 3. CPP mediated insulin delivery.

Name	Dose	Description	AUC uIU·h/L	PA (%)	BA (%)	Outcomes	Ref.
Penetratin-bis-CD-NC	30	Conjugate	279.3±47.7	10.6±2.7	7.1±1.2	AUC increased 2.1-fold	[76]
Insulin-R8 mixture	-	Mixture		-	-	*in vitro* transportation efficiency 5-7 times	[58]
Insulin–HP-β-CD–R8	-	Mixture		-	-	*in vitro* transportation efficiency 8-10 times	[58]
L-R8	10	Mixture CPP at 0.5mM nasal	30.8±24.3	3.0±0.7	5.2±4.1	AUC increased 1.4-fold	[27]
D-R8	10	Mixture CPP at 0.5mM nasal	57.6±36.7	10.4±8.5	9.7±6.2	AUC increased 2.5	[27]
L-penetration	10	Mixture CPP at 0.5mM nasal	197.3±66.8	23.3±6.8	33.3±11.2	AUC increased 8.7	[27]
D-penetration	10	Mixture CPP at 0.5mM nasal	94.1±36.8	14.6±3.5	15.8±6.3	AUC increased 4.1	[27]
L-R8	50	Mixture CPP at 0.5mM in situ admin	4.1±0.3	-	0.1±0.0	AUC increased 0.72	[30]
D-R8	50	Mixture CPP at 0.5mM in situ admin	88.1±49.4	-	3.0±1.7	AUC increased 15.5	[30]
L-penetration	50	Mixture CPP at 0.5mM in situ admin	163.8±27.2	-	5.5±0.9	AUC increased 28.7	[30]
D-penetration	50	Mixture CPP at 0.5mM in situ admin	21.2±9.6	-	0.7±0.3	AUC increased 3.7	[30]
L-Pvec	50	Mixture CPP at 0.5mM in situ admin	121.4±44.7	-	4.1±1.5	AUC increased 21.3	[30]
D-Pvec	50	Mixture CPP at 0.5mM in situ admin	2.1±0.4	-	0.1±0.0	AUC increased 0.37	[30]
L-RRL helix	50	Mixture CPP at 0.5mM in situ admin	33.1±4.6	-	1.1±0.2	AUC increased 5.8	[30]
D-RRL helix	50	Mixture CPP at 0.5mM in situ admin	12.1±5.3	-	0.4±0.2	AUC increased 2.1	[30]
0.5mM L-penetratin	1	Mixture CPP at 0.5 mM nasal	79.3±32.6	-	31.0±12.8	AUC increased 46.4	[92]
0.5mM penetraMax	1	Mixture CPP at 0.5 mM nasal	152.0±13.9	-	59.7±5.4	AUC increased 113.2	[92]
L-penetration	-	Mixture CPP at 0.5 mM nasal	34.9±8.3	-	-	AUC increased 2.87	[97]
D-penetration	-	Mixture CPP at 0.5 mM nasal	9.0±3.2	-	-	AUC increased 0.74	[97]
L-penetration	-	Mixture CPP at 0.5 mM intestinal	11.0±3.4	-	-	AUC increased 13.8	[97]
D-penetration	-	Mixture CPP at 0.5 mM intestinal	0.9±0.7	-	-	AUC increased 1.1	[97]
L-R6	-	Mixture CPP at 2.5mM in situ admin	10.1±2.2	0.0±0.0	0.3±0.1	AUC increased 0.8	[28]
D-R6	-	Mixture CPP at 2.5mM in situ admin	120.9±42.4	2.4±0.8	4.1±1.4	AUC increased 9.6	[28]
D-R8	-	Mixture CPP at 2.5mM in situ admin	417.7±122.1	9.9±1.9	14.1±4.1	AUC increased 33.2	[28]
D-R10	-	Mixture CPP at 2.5mM in situ admin	322.9±106.9	4.4±1.9	10.9±3.6	PA increased 25.6	[28]
Shuffle (R,K fix)2	1	Mixture CPP at 0.5mM	100.9±20.2	46.9±8.8	36.7±7.3	AUC increased 37.4	[59]
SA-R8-INS-SLN	25	SLN nanoparticle oral	-	13.86±0.79	-	PA 33	[23]
L-R8-INS-NP	8.7	Nanoparticle intestinal admin	13570±8059	-	-	AUC increased 2.4	[68]
D-R8-INS-NP	9	Nanoparticle intestinal admin	19145±10876	-	-	AUC increased 3.4	[68]
INS-cr9	-	Conjugate	-	-	-	*in vitro* transportation efficiency 27 times	[41]
INS-cTat	-	Conjugate	-	-	-	*in vitro* transportation efficiency 19 times	[41]
INS-ck9	-	Conjugate	-	-	-	*in vitro* transportation efficiency 4 times	[41]

(Table 3) Contd….

Name	Dose	Description	AUC uIU·h/L	PA (%)	BA (%)	Outcomes	Ref.
TAT-insulin	-	Conjugate	-	-	-	*in vitro* transportation efficiency 6-8 times	[40]
TAT-insulin	100	Mixture oral	-	-	-	FBG reduction vs s.c. 2.18 times	[53]
TAT-insulin	50	Mixture oral	-	-	-	FBG reduction vs s.c. 2.10 times	[53]
L-R8-INS-NP	10	PLGA nanoparticle oral	-	3.9	4.78	AUC increased 9.2	[98]
D-R8-INS-NP	10	PLGA nanoparticle oral	-	8.24	8.39	AUC increased 16.1	[98]
PNVA-co-AA-R8	10	Nasal administration	-	-	-	FBG reduction vs insulin solution 5.1 times	[82]
R8-INS-SLN	20	SLN nanoparticle oral	-	10.39±0.46	-	PA 207.8	[22]

- : not availability; PA: pharmacological bioavailability; BA, bioavailability.

[90]. Hayashi *et al.* took advantage of this to design a new kind of cargo for siRNA delivery to the liver. In this work, lipid nanoparticle was modified with octaarginine (R8). Administration of R8-modified lipid nanoparticles containing SR-BI (a scavenger receptor class B, member 1) siRNA resulted in 75% silencing at 100 μg of siRNA. In contrast, no significantly detectable reduction could be observed when HEPES buffer or luc siRNA loaded nanoparticles were given [91].

2.4.2. Safety Concerns

In spite of the inspiring efforts on CPP mediated insulin transportation, cautions are needed on formula designs because improper formulation may lead to immunogenicity and subsequent safety issues. A number of studies have demonstrated that CPPs alone displayed low toxicity and immunogenicity both *in vitro* and *in vivo* [50, 92, 93]. However, co-formulation with insulin may increase the risk of immunogenicity [94].

Currently, allergic reactions to insulin products appear to be approximately 2%, of which no more than one third of the population was related to the insulin itself. The increased insulin allergenicity may own to structure changes caused by adjuvants such as zinc, protamine, and meta-cresol [94]. Nevertheless, conjugation with nanoparticle may also increase the risk of immunogenicity, it was found that after the treatment with NP-ins (Insulin was conjugated with 50-nm polystyrene NPs), 50% of the transgenic (TG) Balb/c mice were antibody positive, whereas native insulin was negative [95].

However, there are a number of studies which demonstrated that peptide modification does not lead to significant toxicity. In a study, Choi *et al.* found that tumor bearing mice treated with LMWP-siRNA conjugates did not display negligible fluctuations of inflammatory cytokines(IFN-α and IL-12) in serum, indicating that systemic circulated LMWP/siRNA did not potentiate any immunostimulatory effects [55]. Similarly, Hayashi *et al.* proved that R8 modified nanoparticle displayed minimum liver toxicity and immune response [91]. Yang *et al.* demonstrated that conjugation with PEG could increase the size of the protein drugs, while decrease its relevant solvent accessible surface area, thus leads to a sustained circulation despite immunogenic effects [96]. Since increased allergenicity of insulin may be attributed to structure changes in the presence of adjuvants, special cautions are needed to avoid any unfavorable effects when preparing insulin products.

3. PROMISING STRATEGIES FOR THE FUTURE INSULIN DELIVERY

Table **3** demonstrates the CPP mediated insulin delivery similar to the cargo mediated insulin transport. Bioavailability of the insulin could be significantly increased, of which a BA up to 50.7% relative to subcutaneous administrated insulin could be achieved through nasal route under optimal conditions. Furthermore, insulin dose of the optimal formulation was lower than the cargo ones, indicating that CPP possess a greater permeability to enhance potential. It has to be mentioned, on the other hand, that not all the CPPs could display a stronger effect than cargos. However, many CPP-insulin formulations displayed a bioavailability less than 10%, indicating that there is still a long way from oral administration.

Considering the shortcoming of currently utilized CPPs, we hypothesize that more efficient insulin transport could be achieved by the following strategies:

3.1. Pro-drug loading gels

As mentioned above, CPPs insulin permeation may be hampered by L-type peptides degradation in the presence of intestinal enzymes. Meanwhile, the use of D-type CPPs may be more costly and be safety concerned [99]. On the other hand, transport enhanced through other means, such as nanoparticle mediated delivery is found to be unsatisfactory. The pros and cons could be balanced by combining the two together [100]. In this way, CPPs were coupled with insulin and used as “pro-drug”, which were then loaded on a mucosa adhesive nano-cargo. The cargo could protect CPP-insulin from intestinal digestion. Meanwhile, mucosa adhesiveness to GI tract would facilitate insulin-CPP transportation and trigger a rapid response (Fig. **8**).

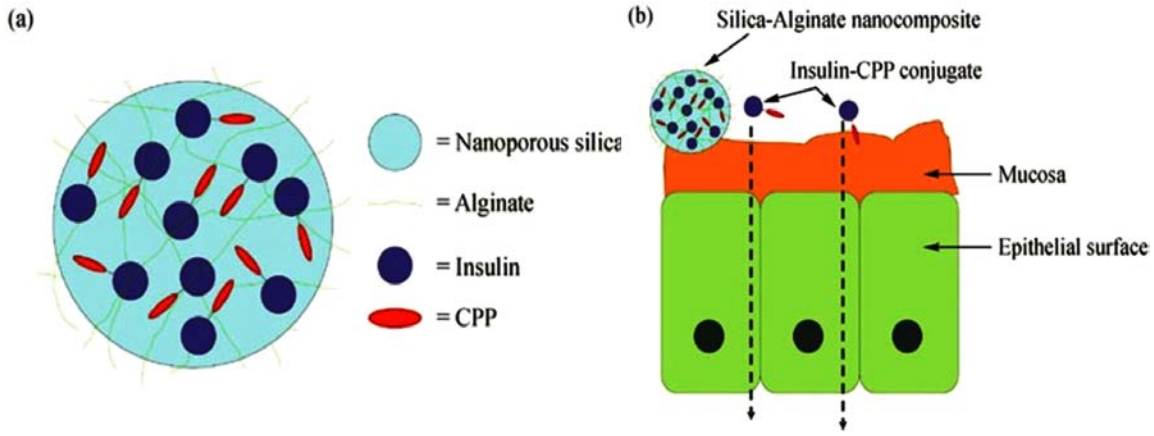

Fig. (8). Schematic diagrams of (**a**) silica/alginate-based mucoadhesive nanocomposite loaded with insulin-CPP conjugates, (**b**) mechanism of action of the proposed oral insulin delivery system in allowing insulin to target intestinal mucosa and cross through the epithelial layer [100]. Reprinting with permissions, copyright 2013.

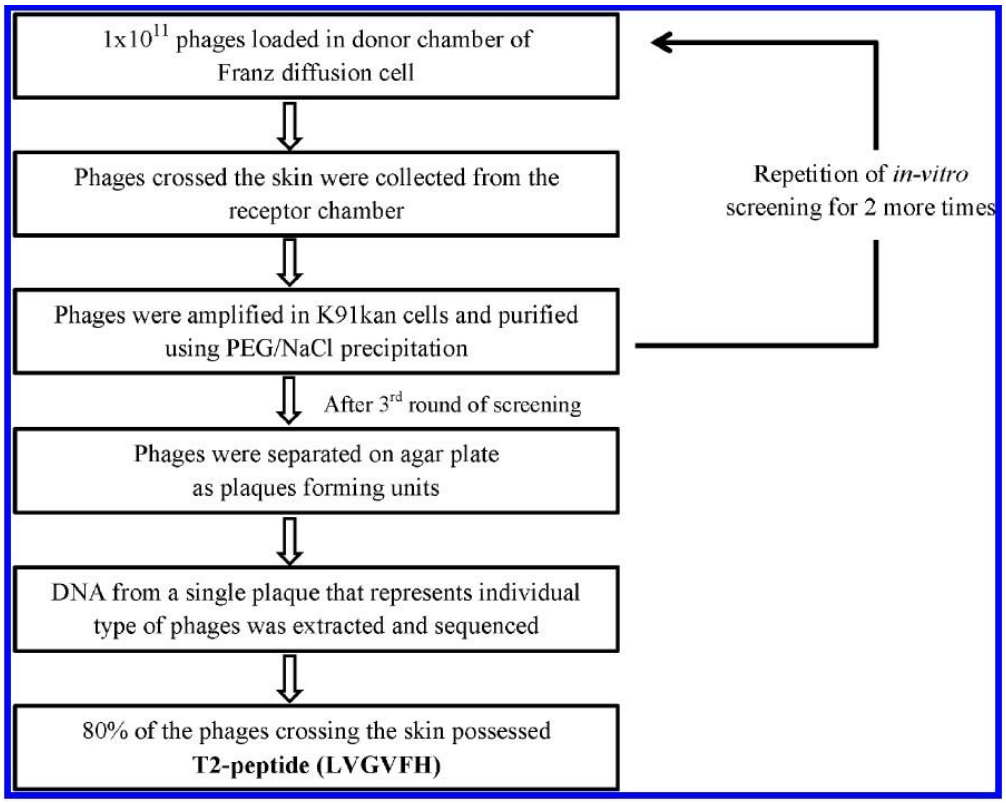

Fig. (9). ***In vitro*** **phage display peptide library (PDL) screening on porcine skin** [102]. Reprinted with permissions from American chemical Society, copyright 2012.

3.2. More Effective CPPs would be Found

Up to now, there are more than 100 CPPs that have been identified and utilized for drug delivery ever since 1988, when the first functional CPP was identified by Frankel and Pabo [26, 101]. But the list is further extending in two directions: 1) by sequence modulation based on the available CPP template and computer assisted analysis, novel functional CPPs with pronounced penetrating ability could be identified [59, 60]. 2) *In vitro* phage display peptide library provides another power tools for CPP screening (see Fig. **9**) [102].

Despite the crowded candidates of CPP family, just a few have been utilized in insulin delivery. Since the CPPs are diverse in size, hydrophobicity, cationic properties, as well as physiological effects among different cell types [52], therefore it is reasonable to speculate that more effective CPPs for insulin delivery will be discovered.

4. CONCLUSION

Non-parenteral administration of insulin has made significant progress with the help of the CPP-coupling technology. In spite of the convenience and significant BA enhanc-

ing potential of manual mixing method, the inability to target hepatic complex has limited further enhancement of pharmacological effects, which may be solved by the conjugation method. However, special care should be taken on the structure and function maintenance of the therapeutic drugs. Furthermore, with the rapid development of protein science, more efficient CPPs would be identified, together with reasonable cargo design, more tolerable insulin formulation will surely be on the way.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

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