Peptides as Skin Penetration Enhancers for Low Molecular Weight Drugs and Macromolecules

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21.1 Introduction

The skin is the largest and most easily accessible organ of the human body for drug delivery (Zaffaroni 1991), and as a route of drug administration, it offers many advantages such as ease of application, patient compliance, absence of first-pass metabolism, and reduced side effects (Scheindlin 2004). For the aforementioned reasons, transdermal drug delivery (TDD) has received a lot of attention and emerged as one of the most successful non-oral controlled delivery option. Despite its success in terms of market demand and wide acceptance by patients, till today only a handful of drugs are marketed as TDD systems. The fundamental reason limiting the transdermal access to many drugs is the impermeable nature of the outermost layer of the skin, stratum corneum (SC), which prevents the permeation of most compounds. Nevertheless, many other drugs and/or drug candidates lie outside the gamut of physicochemical requirements for sufficient skin permeation which will benefit if the SC properties are altered and/or manipulated

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transiently. A number of physical and chemical skin penetration enhancement approaches have been tested in the past to promote the drug delivery through the skin (Kanikkannan et al. 2000; Barry 2004; Rizwan et al. 2009). For example, chemical enhancers such as alcohols, terpenes, fatty acids, surfactants, pH variations, and physical approaches such as microneedles, tape stripping, ultrasonic waves, and iontophoresis have been used to enhance transdermal drug delivery (Kanikkannan et al. 2000; McAllister et al. 2000; Mitragotri et al. 2000; Barry 2004; Narishetty and Panchagnula 2004, 2005; Nanda et al. 2006; Rizwan et al. 2009). Ideally, skin permeation enhancers are expected to be pharmacologically inert, nontoxic, nonirritating, cost-effective, and must not permanently compromise the barrier function of the skin. However, most of the reported approaches have one or more drawbacks, to list few: irritation potential of chemical enhancers at high concentration, invasiveness of microneedles or tape stripping, destabilization of protein or peptide drugs by organic solvent-based enhancers such as alcohol or DMSO, and high cost and complex production process of the equipment for physical enhancement techniques such as ultrasonic waves and iontophoresis. Recently, peptides are gaining popularity as non-invasive and safe skin penetration enhancers for both small molecular drugs and macromolecules such as proteins or short-interfering ribonucleic acids (siRNAs) (Rothbard et al. 2000; Lopes et al. 2005; Chen et al. 2006; Kim et al. 2007; Desai et al. 2010; Hsu and Mitragotri 2011; Uchida et al. 2011; Cohen-Avrahami et al. 2012; Kumar et al. 2012; Shah et al. 2012). This chapter is focused on the discovery of various classes of peptides as skin permeability enhancers and their mechanism of permeation enhancement.

21.2 Peptides as Skin Penetration Enhancers

Over the last decade, several peptides have been identified and tested for their abilities to enhance the penetration of small/large molecules into and across the skin (Rothbard et al. 2000; Chen et al. 2006; Cohen-Avrahami et al. 2010, 2012; Kumar et al. 2012; Shah et al. 2012). These peptides are called skin penetration enhancement peptides (SPEPs). SPEPs offer number of advantages for TDD; they are safe, cost-effective, noninvasive in application, and thus better compliance from patients. SPEPs can be broadly classified into three major categories based on their origin of discovery:

1. Cell-penetrating peptides (CPPs)
2. Antimicrobial peptides (AMPs)
3. Phage peptides (peptides identified from phage display library technology)

21.3 Cell-Penetrating Peptides (CPPs)

21.3.1 Overview

Cell-penetrating peptides (CPPs), also known as protein transduction domains (PTDs), are short peptides (up to 30 amino acids in length) with an ability to translocate through the plasma membrane of a cell and enter the cytoplasm (Patel et al. 2007; Madani et al. 2011; Baoum et al. 2012). Usually, CPPs are cationic or amphipathic at physiological pH (Lindberg et al. 2011; Madani et al. 2011; Nakase et al. 2012). Although numerous CPPs have been reported, some of the most commonly used CPPs include transactivator of transcription (TAT) peptide, YARA, polyarginine, penetratin, transportan, and Pep-1 (Chauhan et al. 2007; Jones and Sayers 2012). The major advantage of CPPs is that they penetrate the cell membrane at low micromolar concentrations *in vivo* and *in vitro* without causing significant membrane damage or toxicity to the cell (Chauhan et al. 2007; Madani et al. 2011; Jones and Sayers 2012; Nakase et al. 2012). In addition to entering into the cells by themselves, CPPs can also carry associated cargo molecules into the cells with high efficiency (Jones and Sayers 2012). CPPs have been used to deliver several cargo molecules such as proteins, oligonucleotides, solid lipid nanoparticles, and liposomes into the cells (as reviewed in (Chauhan et al. 2007; Patel et al. 2007; Lindberg et al. 2011; Bechara and Sagan 2013). Considering

the similarity in the nature and barrier properties of the cell membrane and the skin's outermost layer, in recent years, CPPs have been tested to enhance the topical/transdermal delivery of the above cargo molecules (Rothbard et al. 2000; Lim et al. 2003; Hou et al. 2007; Shah et al. 2012).

The studies on CPPs as skin penetration enhancers can be broadly classified into two major categories based on their interaction with the cargo molecules: (1) cargos delivered through covalent conjugation with the CPPs and (2) cargos delivered by incubating the skin along with CPPs without any chemical attachment between CPPs and cargos.

21.3.2 Conjugated CPPs

In initial studies, CPPs were tested for their ability to deliver macromolecules such as proteins or peptides into and/or across the skin (Jin et al. 2001; Lim et al. 2003). Topical delivery of peptides and proteins has become an attractive field for both pharmaceutical and cosmetic applications (Ma et al. 2002; Partidos et al. 2002; Gorouhi and Maibach 2009; Sundaram et al. 2009). In addition, delivery of several peptide antigens through the dermis was investigated for the development of needle-free topical vaccines (Schutze-Redelmeier et al. 2004; Itoh and Celis 2005; Frankenburg et al. 2007). However, the delivery of macromolecules either into or across the skin is very challenging due to barrier functions of the skin (Langer 2004). CPPs have been used to deliver various kinds of conjugated proteins or peptides, as small as 3 amino acids long to as large as 527 amino acids long, across the skin (Jin et al. 2001; Lim et al. 2003). CPPs have been linked to therapeutic peptides/proteins either by chemical conjugation or synthesized together as a single peptide chain (for small peptides) or genetically fused and expressed together as a single protein (for large peptides/proteins) (Jin et al. 2001; Zhao et al. 2012). These fusion proteins/peptides have been successfully tested for their delivery into or across the skin after topical application (Jin et al. 2001; Zhao et al. 2012).

The first study to test the ability of a known CPP to cross the SC of the skin was reported by Rothbard et al., in the year 2000 (Rothbard et al. 2000). They have shown that an established CPP, polyarginine (R7), could penetrate through the SC and enter into the epidermis of the mouse skin within 2 h of application (Rothbard et al. 2000). The amount of peptide penetrated and the depth of skin penetration were dependent on the concentration of the peptide and the time of incubation (Rothbard et al. 2000). More importantly, R7 peptide delivered a macromolecule, cyclosporin A (CsA) (Mol. Wt. 1202.61 Da), across the cutaneous barrier of mouse and human skin, when conjugated together (R7-CsA) (Rothbard et al. 2000). Cyclosporine was delivered into the skin to inhibit inflammation in the mouse models of contact dermatitis. After topical application, enough amount of R7–CsA has reached dermal T lymphocytes to inhibit cutaneous inflammation (Rothbard et al. 2000). More importantly, the amount of R7-CsA conjugate delivered into the skin and the free drug released from the conjugate was sufficient enough to elicit therapeutic response in the mouse model of contact dermatitis (Rothbard et al. 2000).

One of the most well-studied and efficient CPP is the amino acid residue 47–57 (YGRKKRRQRRR) of human immunodeficiency virus-1 transactivator of transcription protein (HIV-1 TAT), which is popularly known as the TAT peptide. A conjugate of TAT peptide with a tripeptide glycine-lysine-histidine (GKH) was successfully used to deliver GKH into the deeper layers of the skin for cosmetic purposes (lipolysis). GKH is derived from parathyroid hormone and is involved in lipolysis (Lim et al. 2003). Conjugation with the TAT peptide enhanced the penetration of GKH into excised hairless mice skin by more than 36 times (Lim et al. 2003). In addition to small peptides, CPPs have also delivered large therapeutic proteins, such as catalase with 527 amino acids (a.a.), into the skin after topical application (Jin et al. 2001). In this study, CPPs were associated with large proteins through genetic fusion. The gene fragments coding for several CPPs (TAT; polyarginine, R9; and polylysine; K9) were fused with

the genes of large therapeutic proteins (catalase, 527 a.a.; and CuZn SOD1, 154 a.a.), and the fused gene is expressed to produce a single fusion protein. In this fusion protein, the sequence of CPPs are present at the N-terminal part of the therapeutic protein (Jin et al. 2001). With the presence of CPP sequences at the N-terminal, these fusion proteins have gained the ability to penetrate deep into the viable epidermis and dermis layers of the mouse skin (Jin et al. 2001). A fusion protein with R9 (RRRRRRRRR) at the N-terminus was more efficient in skin penetration than fusion proteins with Tat (RKKRRQRRR) peptide (Jin et al. 2001). Similarly, TAT peptide enhanced the penetration of another protein, epidermal growth factor (53 amino acids), into the epidermis and hair follicles of the porcine skin when associated together as fusion protein (Zhao et al. 2012).

By molecular modification and optimization of TAT peptide, more efficient cell-transducing peptide, YARA (YARAAARQARA) peptide, was discovered (Ho et al. 2001). Lopes et al. have compared the skin penetration ability of the YARA peptide with the TAT peptide and a non-transducing peptide (YKAc) (Lopes et al. 2005). Interestingly, after 4 h of application, the amount of YARA peptide entered into the epidermis and dermis layers of the skin was not significantly different than that of the TAT peptide (Lopes et al. 2005). However, both peptides entered the skin in higher amounts than the nontransducing YKAc peptide (Lopes et al. 2005). In addition to entering into the skin, YARA and TAT peptides, when conjugated, carried a 13-amino-acid-long hydrophilic peptide P20 (derived from heat shock protein 20) into viable layers of the porcine ear skin. However, the transdermal delivery was negligible after 8 h of incubation (Lopes et al. 2005). Although YARA peptide is more efficient than TAT peptide in cell transduction; the efficiency of these two peptides to carry P20 into the skin was comparable (Lopes et al. 2005).

It is very well established that the skin is an attractive target for topical immunization because of the presence of antigen-processing immune cells (Langerhans and dendritic cells) in the epidermis and dermis layers of the skin (Celluzzi and Falo 1997; Banchereau and Steinman 1998; Campton et al. 2000; Seo et al. 2000). Antigens or pathogens entering topically into the viable layers of the skin are engulfed by antigen-presenting cells (APCs) such as Langerhans and dendritic cells, residing locally at the epidermis and dermis. Once receiving the antigen, APCs migrate to the lymph node, where they interact with the T cells and B cells for the generation of immune response against the antigen. However, most of the antigens used for immunization are either small peptides or proteins, which cannot penetrate into the viable layers of the skin after topical application. Schutze-Redelmeier et al. have reported that antennapedia transduction sequence peptide (ANTP) could deliver an eight-amino-acid-long antigenic peptide originated from ovalbumin protein ($OVA_{257–264}$) into the mouse skin after topical application (Schutze-Redelmeier et al. 2004). After topical application, significantly higher amount of ANTP-conjugated $OVA_{257–264}$ peptide was found in the epidermis and dermis layers of the skin as compared to unconjugated $OVA_{257–264}$ peptide (Schutze-Redelmeier et al. 2004). In addition, topical application of ANTP-conjugated $OVA_{257–264}$ peptide also resulted in the generation of significantly stronger immune response as compared to free $OVA_{257–264}$ peptide (Schutze-Redelmeier et al. 2004). The immune response was strong enough, to generate therapeutically effective anti-tumor immunity in mice with the help of proper vaccine adjuvants (Schutze-Redelmeier et al. 2004). This study showed the feasibility of topical delivery of vaccine antigens with conjugated CPPs as penetration enhancers.

Although conjugation of macromolecules with CPPs enhances their penetration into the skin, degradation and rapid clearance of drug molecules from the skin has always been a concern. One way to overcome this challenge is by encapsulating the drug molecules inside a particulate carrier, which not only protects the drug from degradation but also releases the drug in a controlled manner for prolonged period of time (Teeranachaideekul et al. 2008; Desai et al. 2010; Pople and Singh 2010). Although particulate delivery systems offer the above advantages,

irrespective of the material used, rigid particles above 10 nm in diameter do not penetrate the intact skin (Souto and Muller 2008). To address this problem, Singh et al. have conjugated CPPs to nanoparticles and tested their ability to permeate through SC (Patlolla et al. 2010; Shah et al. 2012). Transducing TAT peptide or a non-transducing YKA peptide (YKALRISRKLAK) was conjugated onto the surface of a fluorescent dye (DID oil, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate) and encapsulated nano-lipid crystal nanoparticles (FNLCN). Subsequently, their ability to penetrate into viable layers of the skin was tested (Patlolla et al. 2010). After 24 h of incubation, nonconjugated and YKA peptide-conjugated nanoparticles were localized in the appendages of the skin. However, TAT-conjugated nanoparticles penetrated into the deeper layers of the skin such as the epidermis (Patlolla et al. 2010). When a drug molecule, celecoxib (CXb), was encapsulated inside these nanoparticles, not only the drug was delivered into the skin, but also the release of Cxb from the nanoparticles was sustained for prolonged period of time (Patlolla et al. 2010). The presence of more arginines and thus more positive charge on TAT peptide (six arginines) compared to YKA peptide (two arginines) might have played a role in the superior ability of TAT peptide in mediating skin penetration of nanoparticles.

In a follow up study, Singh et al. have compared the ability of CPPs with different number of arginines present in their sequence (polyarginines: R8 (8 arginines), R11 (11 arginines), and R15 (15 arginines) and TAT peptide (6 arginines)) in enhancing the penetration of nanostructured lipid carriers (NLCs) into the skin (Shah et al. 2012). NLCs conjugated with a CPP containing 11 arginine groups (R11) have shown higher amounts of penetration into the skin and also into deeper layers of the skin compared to NLCs conjugated with other peptides tested (Shah et al. 2012). The results have suggested that the ability to enhance the skin penetration was increased when arginine number in the CPP was increased from 6 and 8 to 11 (Shah et al. 2012). However, further increase in arginine number from 11 to 15 had resulted in reduction in the ability to enhance the skin penetration (Shah et al. 2012). It could be due to the saturation effect or steric hindrance caused by long chain length of the arginine peptides (Shah et al. 2012). This study indicates that there may be an optimum positive charge, which is required to enhance the interaction of the CPPs with the skin (Shah et al. 2012). Similarly, a polyarginine with nine arginines (R9) has been shown to be more efficient in enhancing the penetration of sodium diclofenac-containing reversed hexagonal liquid crystals ($H_{II}LC$) when conjugated on the surface in comparison to RALA peptide containing five arginines (RALARALARALRALAR) on the surface (Cohen-Avrahami et al. 2010, 2012). The ability to enhance the skin penetration of $H_{II}LC$ by R9 was lost, when R9 was present inside the $H_{II}LC$ instead of conjugated onto the surface. Therefore, it is important for CPPs to be on the surface for proper interaction with the skin.

Overall, these studies suggest that CPP conjugation to the cargo molecules (macromolecules and nanoparticles) could enhance their skin penetration.

21.3.3 Nonconjugated CPPs

The previous section has emphasized the role of CPPs in delivering covalently attached therapeutic moieties or nanoparticles into the skin. However, covalent attachment of CPPs to cargo molecules may pose several challenges, such as high cost and extra time for synthesis and purification, loss of activity after conjugation, and challenges associated with purification and stability. CPPs such as Pep-1, MPG, and Pep-2 are reported to deliver cargos, such as peptides, proteins, and antibodies into different cells both *in vitro* and *in vivo*, without conjugation to cargos (Morris et al. 1997, 2001, 2007; Simeoni et al. 2003, 2005; Crombez et al. 2007). Similar to these intracellular delivery studies, Wang et al. have shown that arginine-rich peptides (R9, TAT) could promote the entry of the green fluorescent protein (GFP) into the deeper layers of the skin when incubated together on the mouse skin (*in vivo*). After 20 min of incubating skin with CPPs

and GFP, strong green fluorescence was observed among the epidermis, dermis, panniculus adiposus, and even hypodermis (Wang et al. 2006). Interestingly, when incubated together (co-incubated), R9 peptide resulted in penetration of GFP into the skin within 1 min of incubation and reached maximum after 20 min (Wang et al. 2006). Prolonged incubation with the R9 peptide resulted in further enhancement in skin penetration of GFP. Similarly, TAT peptide has also been shown to enhance the penetration of GFP across the rat skin without conjugation (Lohcharoenkal et al. 2011).

In another study, Lopes et al. have tested the abilities of YARA and WLR (WLRRIKAWLRRIKAWLRRIKA) peptides as skin penetration enhancers without conjugating them to the cargo molecule (Lopes et al. 2008). The skin penetration of P20 peptide alone was increased by two- to threefold when incubated together with YARA and WLR peptides, respectively (Lopes et al. 2008). The WLR peptide was more efficient (2.8-fold) in enhancing the penetration of P20 into the epidermis and dermis layers of the skin as compared to YARA peptide (2.3-fold) (Lopes et al. 2008). This result could be attributed to the higher positive charge of the WLR peptide (six arginines) as compared to YARA peptide (three arginines) (Lopes et al. 2008). Interestingly, the ability of YARA peptide to enhance the skin penetration of P20 peptide was significantly higher when these two peptides were covalently conjugated (5–7 times) in comparison to when they were incubated together as independent peptides (co-incubation) (2.3 times). However, co-incubation offers several advantages as mentioned earlier in this section.

The skin penetration of sodium diclofenac (Na-DFC) embedded in reversed hexagonal mesophases was enhanced when co-incubated with CPPs such as RALA peptide (RALARALARALRALAR) and penetratin (RQIKIWFQNRRMKWKK) (Cohen-Avrahami et al. 2010, 2012). Both the peptides enhanced the skin permeability coefficient of Na-DFC without changing its lag time (Cohen-Avrahami et al. 2010, 2012). Hexagonal mesophase serves as an excellent gel matrix for drug solubilization (Cohen-Avrahami et al. 2010, 2012). ATR-FTIR analysis suggests that penetratin accelerated the structural transition of skin lipids from hexagonal to liquid and thus enhanced the diffusion rate of Na-DFC through the SC (Cohen-Avrahami et al. 2012).

In addition to small molecular drugs, proteins, and peptides, CPPs have also been shown to enhance the delivery of siRNAs into the skin after topical application. Topical delivery of siRNAs has great therapeutic significance for treating various skin diseases, transcutaneous vaccination, and improving skin properties (Partidos et al. 2003; Lopes et al. 2005). However, transdermal delivery of siRNA is also limited due to its low permeability through various skin barriers such as the SC (multiple lipid bilayers) and viable epidermal layers (paracellular connection by tight junctions) (Guan et al. 2008; Uchida et al. 2011). Uchida et al. in 2011 have successfully delivered siRNAs deeper into the skin layers (viable epidermis) and hair follicles by incubating together with TAT peptide and tight junction-modulating peptide (AT1002, amino acid sequence: FCIGRL), to alter SC and tight junction barriers, respectively. In contrast, siRNAs were localized only to hair follicles when given with TAT peptide alone (Uchida et al. 2011).

In summary, CPPs have applications in noninvasive topical skin delivery of drugs and nanoparticles. The CPP-mediated skin delivery of cargo molecules (drugs, proteins, peptides, siRNAs, and nanoparticles) depends on the nature of CPP, conjugation method (covalent or noncovalent type of attachment), and characteristics of the cargo protein.

21.4 Antimicrobial Peptides (AMPs)

21.4.1 Overview

Antimicrobial peptides (AMPs), which are involved in host innate immunity, share many structural similarities with CPPs (Splith and Neundorf 2011). AMPs are short peptides, usually less than 60 amino acids, and consists of

blocks of cationic (+4 to +6 charge) and hydrophobic amino acids that are spatially separated. This provides amphipathic properties to the AMPs (Hancock 1997; Powers and Hancock 2003). Cationic charge and amphipathic arrangement are the basis of their interaction with anionic lipid bilayers of the microbial cellular membranes (Splith and Neundorf 2011). Considering the presence of anionic lipid bilayers in SC of the human skin, AMPs have been hypothesized to interact with the skin lipids and thereby alter the skin permeability to drugs.

21.4.2 Magainin

Magainin is the most studied AMP for altering the permeability of the skin to various drugs (Kim et al. 2007, 2008a, b, 2010). Magainin is a 23-amino-acid-long AMP (GIGKFLHSAKKFGKAFVGEIMNS) isolated from the skin of the African frog (*Xenopus laevis*) (Zasloff 1987). Magainin is known to form pores in the bacterial cell membranes and hence also called as pore-forming peptide (Matsuzaki et al. 1994; Matsuzaki 1998). It has a net +4 charge and binds to negatively charged phospholipid membranes with the aid of electrostatic interactions and permeabilizes the lipid bilayers (Matsuzaki et al. 1997; Kim et al. 2008a). Considering the ability of magainin to interact with the lipid membranes, its potential utility as a skin penetration enhancer was evaluated by Prausnitz et al. group (Kim et al. 2007, 2010).

21.4.3 Magainin as Skin Penetration Enhancer

Magainin increases the membrane permeability in bacteria by creating pores in the lipid membrane, and this often leads to cell lysis (Matsuzaki et al. 1994; Matsuzaki 1998). Kim et al. have shown that magainin could also disrupt the monolayer of synthetic vesicles made up of lipids with similar biophysical characteristics to the lipids present in the human SC (Kaushik et al. 2001). However, treating the skin with magainin alone could not enhance the penetration of a small molecule, fluorescein (323 Da), into the skin (Kim et al. 2007). Interestingly, the addition of magainin to other skin penetration enhancers (N-lauroyl sarcosine (NLS) in Ethanolic solution) significantly improved their ability to enhance the penetration of fluorescein into the skin (Kim et al. 2007). This may be due to the fact that SC consists of multiple lipid bilayers in comparison to monolayered lipid vesicles tested in the previous study (Kim et al. 2007). To disrupt multiple lipid bilayers, magainin may require the help of other chemical skin penetration enhancers (Kim et al. 2007). This theory was further supported by the observation that magainin alone (rhodamine labeled magainin) showed negligible penetration into the SC; however, when incubated together with NLS and ethanolic solution, it could penetrate into deeper layers of SC (10–15 μm) (Kim et al. 2007). Magainin was proposed to form angstrom (A^0)-scale pores in the lipid layers of the skin (Kim et al. 2008a). Angstrom-scale pores are known to be large enough only to transport small molecules. In consistent with this hypothesis, magainin enhanced the transport of only small molecules such as fluorescein (323 Da), which has radius of approximately $5A^0$ (Prausnitz and Noonan 1998), but not the penetration of larger molecules such as calcein (623 DA, radius = $6A^0$) or FITC-dextran (3000 Da, radius = $16A^0$) (Oliver et al. 1992; Edwards et al. 1995).

In further studies, interaction of magainin with either skin lipids or drug molecules was optimized to increase its skin penetration enhancement ability. Increasing the concentration of magainin (up to 1 mM) or its time of incubation with the skin (up to 12 h) enhanced the penetration of fluorescein molecules into the skin (Kim et al. 2008b). In addition to interaction of magainin with negatively charged skin lipids, its interaction with the drug molecule also seems to be important factor for its ability to increase skin penetration of drugs. Magainin acquires positive, neutral, and negative charges at pH 7.4, 10, and 11, respectively (Kim et al. 2008a). At pH 7.4, positively charged magainin electrostatically interacts with negatively charged fluorescein molecules and skin lipids. However, at pH 11, these interactions are

disturbed (Kim et al. 2008a). The observation that magainin enhanced the penetration of fluorescein at pH 7.4 but failed at pH 11.0 suggests that the electrostatic interaction of magainin with drug molecule and/or skin lipids may be vital for its skin penetration enhancement abilities (Kim et al. 2008a). The importance of magainin interaction with the drug molecule for its skin penetration ability is confirmed by the observation that magainin could not enhance the penetration of positively charged molecule (granisetron) into the skin, even at pH 7.4.

The role of the electrostatic interactions of magainin in skin penetration enhancement was further confirmed by the following two studies. Neutralizing the charge of magainin by incubating it with increasing salt concentration or replacement of positively (+4) charged magainin with negatively charged anti-magainin (−4) peptide decreased the magainin's ability to enhance the skin penetration of negatively charged fluorescein molecule (Kim et al. 2008a, 2010). Although several modifications of the magainin such as increase in positive charge or hydrophobicity, or change of certain amino acids, etc., have been shown to improve its antimicrobial activity, these modifications could not further enhance its ability to increase skin permeability (Kim et al. 2010). These studies clearly suggest that in addition to the net charge on the magainin, the appropriate amino acid composition is also critical for its ability to change skin permeability of certain drugs (Kim et al. 2010).

In summary, magainin peptide could enhance the skin penetration of small molecules when used in combination with other chemical skin penetration enhancers. The amino acid composition of the magainin and its interaction with the charged drug molecules are crucial for its effect on skin penetration.

21.5 Phage Peptides

21.5.1 Overview

Several peptides such as CPPs and AMPs were shown to act as skin penetration enhancers (Lopes et al. 2005; Wang et al. 2006; Kim et al. 2007, 2008b). But majority of these peptides were large in size (12–23 amino acids) and hence costly and difficult to synthesize. Moreover, some of them, such as magainin, may need the help of other chemical skin penetration enhancers (Kim et al. 2007) to enhance the permeability of the skin. Therefore, it was of great significance to find smaller peptides (five to six amino acids), which could enhance the skin penetration. Phage display technology enabled us to identify such smaller peptides (Chen et al. 2006; Hsu and Mitragotri 2011; Kumar et al. 2012).

21.5.2 Phage Display Technology

Bacteriophages are bacterial viruses, which usually cannot penetrate through biological barriers, such as the skin (Haq et al. 2012). Phage display technology is based on screening a library of bacteriophages (billions of them), where each bacteriophage expresses unique random peptide on its surface, to identify small peptide sequences that could carry bacteriophages across the biological barriers such as the skin (Haq et al. 2012). This technique has also been used successfully to identify peptides that could cross other biological barriers such as the blood-brain barrier and intestine (Duerr et al. 2004; Chen et al. 2006; Wan et al. 2009).

21.5.3 SPEPS Identified by Phage Display Peptide Library Screening

To identify skin penetration-enhancing peptides, the screening of phage display peptide libraries was performed both *in vivo* and *in vitro* on mouse and pig skins (Chen et al. 2006; Hsu and Mitragotri 2011; Kumar et al. 2012). In these screenings, phage particles were applied onto the skin and were allowed to penetrate into and across the skin. Phages that have penetrated were collected, amplified, and again applied onto the skin (Chen et al. 2006; Hsu and Mitragotri 2011; Kumar et al. 2012). After few rounds of screening,

the sequence of the peptide present on the surface of the bacteriophages that were consistently penetrating into or through the skin was identified by genomic DNA sequencing of the penetrated phages. The identified peptides were synthesized and further investigated as skin penetration enhancers.

By screening cyclic peptide (C7C) displayed phage library by using mouse skin (*in vivo*), Chen et al. have identified the TD-1 peptide (ACSSSPSKHCG), which not only enhanced the penetration of phages across mouse skin when expressed on a phage surface coat protein but also significantly increased the permeation of insulin through the skin when incubated together with insulin (Chen et al. 2006). Most importantly, insulin delivered with the help of TD-1 peptide resulted in significant reduction of serum glucose levels in rats (Chen et al. 2006). The effect of TD-1 peptide on the delivery of insulin was dependent on the amount of the peptide applied (Chen et al. 2006). In other studies, TD-1 peptide was also shown to deliver other macromolecules such as human growth hormone (hGH) and siRNAs into the skin (Zhang et al. 2010; Lin et al. 2012). When TD-1 peptide was incubated together with siRNAs, it enhanced the penetration of topically applied siRNAs into the epidermis and subcutaneous tissues of the skin and resulted in efficient downregulation of the target protein expression (GAPDH). In contrast, scrambled peptide failed to enhance the penetration of siRNAs (Lin et al. 2012). Although TD-1 peptide is not expected to deliver 100 % of the applied siRNAs, the functional efficiency of siRNAs delivered topically using TD-1 peptide was comparable to the efficiency of siRNAs delivered by intradermal injection (Lin et al. 2012) in inhibiting the target protein expression. This may be due to the large surface area of the skin that was exposed to siRNAs after topical application as compared to restricted area exposed by intradermal injection (Lin et al. 2012). Skin penetration enhancement mediated by TD-1 peptide is highly sequence specific. Even a single amino acid substitution in the TD-1 peptide resulted in significant loss in its ability to enhance the skin penetration (Chen et al. 2006).

TD-1 peptide was discovered by using mouse system. It enhanced the skin penetration of insulin mainly through hair follicles (Chen et al. 2006). Although the discovery of TD-1 peptide as a skin penetration enhancer was an exciting advancement, its use in humans is limited because of the fact that the hair follicle density in mice or rats is several times higher than in humans and also mouse skin is more permeable than human skin (Priborsky and Muhlbachova 1990; Ghosh et al. 2000; Prausnitz 2006). Therefore, mouse/rat skin may not be a clinically relevant model for human TDD application.

To address this issue, in later studies, screening of PDLs was performed using porcine skin. This is because porcine skin is more close to human skin in terms of both lipid composition and hair follicular density (Dick and Scott 1992; Hammond et al. 2000). By screening PDL using porcine skin, Hsu et al. have identified SPACE (skin permeating and cell entering) peptide (ACTGSTQHQCG), which could cross human, porcine, as well as mouse skin (Hsu and Mitragotri 2011). Importantly, SPACE peptide delivered macromolecular cargos across porcine SC (Hsu and Mitragotri 2011). For example, when conjugated to SPACE peptide, streptavidin (159 amino acid protein) penetrated into the epidermis and dermis of porcine skin (Hsu and Mitragotri 2011). SPACE peptide-mediated skin penetration enhancement of cargo molecules was size dependent. Increase in cargo size affected skin penetration enhancement effect of SPACE peptide (Hsu and Mitragotri 2011). Interestingly, SPACE peptide could also cross the cellular membranes, similar to CPPs. SPACE peptide could penetrate into different cell lines such as keratinocytes, fibroblasts, endothelial cells (HUVECs), and breast cancer cells (MDA-MB-231) (Hsu and Mitragotri 2011). Considering the ability of SPACE peptide to cross the skin and cellular barriers, it has been used for topical delivery of siRNAs. SPACE peptide conjugated to siRNAs enhanced the delivery of siRNAs into the skin (Hsu and Mitragotri 2011). Importantly, these conjugated siRNAs enter the cell and are efficient in downregulating the target protein expression (GFP in endothelial

cells and GAPDH and IL-10 in mice skin) (Hsu and Mitragotri 2011). The efficiency of SPACE peptide-mediated siRNA delivery was dependent on the amount of the peptide applied and the time of incubation on the mice skin. Higher dose of the peptide and longer application time resulted in higher knockdown of the target genes (Hsu and Mitragotri 2011).

SPACE peptide was found primarily effective in enhancing skin penetration of only conjugated molecules (Hsu and Mitragotri 2011). Considering the limitation associated with the conjugation technique, Kumar et al. have identified a novel peptide (T2 peptide: LVGVFH) that enhanced the penetration of small molecules across the porcine skin without the need of conjugation (Kumar et al. 2012). T2 peptide is a linear peptide unlike TD-1 or SPACE peptides. T2 peptide enhanced the penetration of bacteriophages across porcine and mouse skin (Kumar et al. 2012). Pretreatment of the skin with synthetic T2 peptide at pH 4.5 resulted in significant enhancement in the permeability of the skin to several small molecules with different lipophilicities, such as fluorescein isothiocyanate (hydrophobic), rhodamine-123 hydrochloride (hydrophilic), and 5-fluorouracil (5-FU) (hydrophilic) across the skin (Kumar et al. 2012). The major challenge in the skin penetration of hydrophilic drugs such as 5-FU is their partitioning into the lipid layers of SC (Kumar et al. 2012). T2 peptide enhanced the partitioning of both hydrophilic (5-FU and rhodamine 123 hydrochloride) and hydrophobic (fluorescein isothiocyanate) molecules into the lipid layers of SC by altering the SC lipid structures (Kumar et al. 2012). Pretreating the skin with T2 peptide increased the steady-state flux (J_{ss}), permeability coefficient (K_p), and cumulative amount (Q_{24}) of 5-FU crossing the skin by three- to fourfold in comparison to untreated skin (Kumar et al. 2012).

In summary, PDL technology has been successfully employed to discover small peptides which enhanced the delivery of small and macromolecules into and across the skin without the need of any other penetration enhancers.

21.6 Mechanism of Peptides as Skin Penetration Enhancers

Introduced just a decade ago, SPEPs are still evolving as a new class of skin penetration enhancers. Although many studies have been reported on SPEPs, the common mechanism by which these peptides enhance the skin penetration is yet to be elucidated. More than one mechanism may be involved for the enhancement of skin penetration by SPEPs (Chen et al. 2006; Kim et al. 2007; Hsu and Mitragotri 2011; Kumar et al. 2012). In general, drugs may penetrate through the skin via intracellular pathway, intercellular lipid pathway, intercellular pathway through appendages, and/or through hair follicles (Prausnitz et al. 2004). SPEPs have been shown to act on one or more of these pathways to enhance skin penetration.

TD-1 peptide, identified via PDL screening using mouse skin, penetrates and delivers associated cargos (insulin) into the hair follicles of the mouse/rat skin (Chen et al. 2006), and insulin diffuses from there into deeper layers of the skin (Chen et al. 2006). Similarly, polyarginine (a CPP) delivered the conjugated drug-loaded nanoparticles into the hair follicles, and the drug released from these nanoparticles diffuses into the different layers of the skin or across the skin (Shah et al. 2012). Although skin penetration via hair follicles delivers the cargo molecules into the skin, it may not be relevant to human application because of the variability in the hair follicle density in different species or between individuals. However, this may be advantageous in delivering therapeutic molecules specifically targeted to hair follicles.

Permeation of molecules through intercellular lipids (lipid bilayer) represents the classical mechanism for transdermal delivery (Hsu and Mitragotri 2011). Some SPEPs such as penetratin, magainin, and T2-peptide have been reported to alter the lipid bilayer structure of the skin (Kim et al. 2007; Cohen-Avrahami et al.

2012; Kumar et al. 2012), and this was proposed as a possible mechanism for their ability to enhance the skin penetration. Alteration in lipid acyl chain, after treatment with these peptides, was indicated either by shift in lipid peaks of SC to higher wave numbers (C-H stretching absorbance) or by increase in the height and area of these lipid peaks (Kim et al. 2007; Cohen-Avrahami et al. 2012; Kumar et al. 2012), observed via Fourier transform infrared (FTIR) spectroscopy. This alteration suggests a change in the molecular conformation of the skin lipids, which may cause fluidization of the lipid bilayers and thus results in the enhancement of skin permeability (Cornwell et al. 1994; Kim et al. 2007; Cohen-Avrahami et al. 2012; Kumar et al. 2012). Instead of interacting with SC lipids, some SPEPs are shown to interact with the proteins present in the SC. SPACE peptide interacts with keratin, a corneocyte protein, to enhance the skin permeability (Hsu and Mitragotri 2011). Similarly, magainin was shown to alter the secondary structures of the proteins present in SC from α-helix to β-sheet structure (Kim et al. 2007). Magainin alters the structural arrangement of both lipids and proteins present in the SC to enhance skin penetration (Kim et al. 2007).

Most of the SPEPs discovered so far are positively charged at pHs that they were successfully used (such as magainin, TAT, Pep-1, YARA, etc.) This suggests that the cationic nature of the SPEPs (Table 21.1) may play a very important role in their interaction with the anionic lipids of the SC or in their skin penetration enhancement ability (Kim et al. 2007, 2008b, 2010; Kumar et al. 2012). For example, a peptide with 11 arginines enhances the skin penetration more efficiently than peptides with six or eight arginines. However, additional increase in arginines to 15 did not result in further increase in skin penetration enhancement (Jin et al. 2001; Lopes et al. 2008). Similarly, increase in charge of the magainin peptide from +4 to +5 did not result in further skin penetration enhancement (Kim et al. 2010). The above studies suggest that an optimum positive charge may be necessary for these peptides to function as skin penetration enhancers.

In addition to the charge on the peptide, many other physicochemical properties of the SPEPs may also play a critical role in their function. For example, even a single amino acid substitution in TD-1 peptide eliminates its skin penetration enhancement ability (Chen et al. 2006). In case of T-2 peptide, it only enhances the skin permeability at pH 4.5, but not at pH 7.4. It was proposed that the histidine present at the C-terminal part of the T-2 peptide acquires a positive charge at pH 4.5, and this charge may be necessary for its interaction with the skin lipids. When histidine in T2 peptide was replaced with alanine (H6A peptide), the peptide lost its ability to enhance the skin penetration. This suggests that the histidine present at the C-terminal part of the T-2 peptide is critical for its function. Interestingly, SPEPs identified by multiple random PDL screenings also have histidines at the C-terminal part of the peptide. This suggests that the amino acid histidine may be involved in the interaction with the anionic lipids of the SC. The total or local charge on the peptide, the secondary structure of the peptide, the rigidity of the peptide, and the availability of particular amino acids for interaction with the skin vary when the SPEPs are conjugated or nonconjugated to the cargo molecules. The above properties of the SPEPs may also alter depending on the type of cargo molecule and the linker used during conjugation and transport. Therefore, more studies are warranted to further understand on how the physicochemical properties of the peptides influence their interaction with the skin.

Overall, the interaction of SPEPs with the skin and their function as skin penetration enhancers depend on multiple factors such as the physicochemical properties of the peptide, type of the skin (mice, porcine, or human), buffer conditions, time of the treatment (pretreatment vs. co-treatment), type of cargo molecules, and the type of interaction with the cargo molecules (conjugated vs. nonconjugated).

Table 21.1 List and sequence of various skin penetration-enhancing peptides studied in the literature

S.No.	Name	Sequence	Category	Application	Conjugation	Species	Reference
1	Arginine-rich intracellular delivery (AID) peptides (R9)	RRRRRRRRR	CPP	*In vitro*	Nonconjugated	Mouse	Wang et al. (2006)
2	Antennapedia transduction sequence (ANTP)	RQIKIWFQNRRMKWKK	CPP	*In vivo*	Conjugated	Mouse	Schutze-Redelmeier et al. (2004)
3	Arginine oligomers (R7)	RRRRRRR	CPP	*In vivo*	Conjugated	Mouse and human	Rothbard et al. (2000)
4	Polyarginines	R8: RRRRRRRR R11: RRRRRRRRRRR R15: RRRRRRRRRRRRRRR	CPP	*In vitro*	Conjugated	Rats	Shah et al. (2012)
5	Penetratin	RQIKIWFQNRRMKWKK	CPP	*In vitro*	Nonconjugated	Porcine	Cohen-Avrahami et al. (2012)
6	RALA	RALARALARALRALAR	CPP	*In vitro*	Nonconjugated	Porcine	Cohen-Avrahami et al. (2010)
7	Protein transduction domain (PTD)	YARA: YARAAARQARA TAT: YGRKKRRQRRR	CPP	*In vitro*	Conjugated	Porcine	Lopes et al. (2005)
8	Protein transduction domain (PTD)	YARA: YARAAARQARA WLR: WLRRIKAWLRRIKAWLRRIKA	CPP	*In vitro*	Nonconjugated	Porcine	Lopes et al. (2008)
9	Tight junction modulator	AT1002: FCIGRL		*In vivo*	Nonconjugated	Mice	Uchida et al. (2011)
10	YKA and TAT peptides	YKA: YKALRISRKLAK TAT: YGRKKRRQRRR	CPP	*In vitro*	Conjugated	Rats	Patlolla et al. (2010)
11	Magainin	GIGKFLHSAKKFGKAFVGEIMNS	AMP	*In vitro*	Nonconjugated	Porcine	Kim et al. (2007)
12	TD-1	ACSSSPSKHCG	Phage peptides	*In vivo*	Nonconjugated	Mouse/rat	Chen et al. (2006)
13	TD-1	ACSSSPSKHCG	Phage peptides	*In vivo*	Nonconjugated	Porcine	Zhang et al. (2010)
14	TD-1	ACSSSPSKHCG	Phage peptides	*In vivo*	Nonconjugated	Rat	Lin et al. (2012)

15	SPACE	ACTGSTQHQCG	Phage peptides	*In vitro*	Conjugated	Porcine/mouse/ human	Hsu and Mitragotri (2011)
16	T2	LVGVFH	Phage peptides	*In vitro*	Nonconjugated	Porcine	Kumar et al. (2012)

Abbreviations: *CPP* cell-penetrating peptide, *SC* stratum corneum, *TAT* transactivator of transcription, *R9* nine arginine amino acid residues (RRRRRRRRR), *R7* seven arginine amino acid residues (RRRRRRR), *K9* nine lysine amino acid residues (KKKKKKKKK), *siRNA* short-interfering ribonucleic acids, *OVA* ovalbumin, *CsA* cyclosporin A, *GKH* glycine-lysine-histidine, *CuZn SOD1* copper-zinc superoxide dismutase-1, *ANTP* antennapedia transduction sequence, *APCs* antigen-presenting cells, *FNLCN* fluorescent dye encapsulated nano-lipid crystal nanoparticles, *Cxb* celecoxib, *NLCs* nanostructured lipid carriers, *($H_{II}LC$)* hexagonal lyotropic liquid crystals, *Na-DFC* sodium diclofenac, *ATR-FTIR* attenuated total reflectance Fourier transform infrared spectroscopy, *IL-10* interleukin 10, *GFP* green fluorescent protein, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

Amino acid abbreviations: *A* alanine, *P* proline, *Q* glutamine, *C* cysteine, *R* arginine, *D* aspartate, *S* serine, *E* glutamate, *T* threonine, *F* phenylalanine, *G* glycine, *V* valine, *H* histidine, *W* tryptophan, *I* isoleucine, *Y* tyrosine, *K* lysine, *L* leucine, *M* methionine

Conclusion

Transdermal drug delivery offers several advantages and has great clinical implications both in pharmaceuticals and cosmetics. Delivering active drugs through the skin is a challenging aspect. Further, drug delivery through innovative methods has been a great strategy to enhance the efficiency of both existing and new drugs. Recently, peptides have been explored to enhance the delivery of active molecules into and/or across the skin without causing any significant skin damage. Especially, these peptides (SPEPs) have been used to deliver larger cargos such as proteins, siRNAs, or nanoparticles into the skin. Although this will open a new avenue for successful percutaneous/transdermal delivery of small and macromolecules, the field is still at very early stage. More studies are needed to understand the interaction of these peptides with the skin and its short-term and long-term implications. And, there is also a need to develop more of small SPEPs (five to ten amino acids), which offer several advantages compared to large peptides, such as more cost-effective due to smaller in size, easy to synthesize, and less likely to develop an immune response against the peptide. With more efficient SPEPs at disposal and with the understanding of the molecular mechanism for their penetration enhancement, SPEPs offer an exciting future for dermal/transdermal delivery of drug molecules and dermal delivery of cosmetics.

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