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Review Article

Noninvasive transdermal delivery of peptides and proteins

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Summary

Revolutionary advances in biotechnology have given rise to a large number of drugs that are of peptide and protein origin. In order to reap the ultimate therapeutic benefits of these new generation drugs, there is both a commercial and clinical need to develop suitable noninvasive delivery systems. Over the last several decades, the skin has generated a lot of interest as a site for systemic delivery of drugs. However, in order to expand the scope of drugs that can be delivered transdermally, a number of competitive enhancement technologies are under investigation. Iontophoresis using a small electric current offers immense potential for small peptides, while electroporation and sonophoresis can be useful for large peptides. Very large proteins may be delivered by combination of enhancement techniques. Other approaches such as transferosomes and high speed powdered delivery can also be useful for certain therapeutic categories. This article reviews the potential of various strategies for transdermal delivery of peptides and proteins with special emphasis on mechanism of transport and delivery system considerations.

Introduction

Drug discovery and development has undergone a sea of change in the last several years as a result of the significant breakthroughs in biotechnology and greater understanding of disease processes. The recent public availability of the first draft of the human genome has opened up a goldmine for discovery of new therapeutic agents for unmet medical needs (1). Once gene-protein relationships are established for more than 12,000 sequenced genes, the tangible therapeutic benefits will be realized in terms of an unimaginable number of protein drugs. Already there are more than 50 approved peptide and protein (PP) drugs and 350 in various stages of development (2). At the same time, this rapid inflow of PP drugs places tremendous pressure on drug delivery scientists since development of suitable noninvasive delivery systems are required in order to reap the therapeutic benefits of these new generation drugs. Skin, which has primarily evolved as a protective barrier for the outward loss of water and to maintain homeostasis of the body, has been beneficially exploited for the systemic delivery of small molecules having the right combination of physicochemical and pharmacokinetic properties. Transdermal drug delivery systems have matured and emerged as one of the most successful nonoral controlled release systems with a worldwide market share of USD 2 billion, which, interestingly, is shared among only 8 therapeutic molecules since the introduction of the first transdermal patch of scopolamine in the early 80s (3). However, the delivery of molecules with molecular weights above 500 Da without the aid of an enhancement strategy is extremely difficult, if not impossible. In addition to large molecular size and hydrophilicity, PP are extremely sensitive in terms of physical, chemical and biological stability posing unique challenges to the formulation scientist. On the other hand, the low proteolytic activity of the skin (4) coupled with the inherent comforts associated with transdermal drug delivery systems has led to a heightened interest in exploring various enhancement strategies to overcome the remarkable barrier property of skin

and thereby expand the scope of drugs that can be delivered transdermally. According to the recently proposed biopharmaceutic classification system (BCS) (5), established platform technologies are able to meet the peroral delivery requirements of both highly soluble (class I) and highly permeable (class II) drugs, however, limited opportunity exist for poorly permeable PP (class III) and poorly soluble as well as poorly permeable drugs (class IV). This has led to the search for an alternate route of delivery for class III and IV drugs. In light of the above, the present review discusses various enhancement strategies for class III drugs with particular emphasis placed on the mechanisms and system design considerations for transdermal delivery of PP.

Iontophoresis

Iontophoresis is a century old technique that delivers charged molecules through the skin at an enhanced rate in a controllable fashion via application of a small electric current. The PP of interest is placed in the delivery electrode whose polarity is same as the charge of PP and the circuit is completed through a return electrode. Electric current is converted to ionic current at the electrode surface, which in turn delivers PP precisely into the systemic circulation based on the charge, molecular size and ionic mobility of the PP. While, several extensive reviews have appeared throughout the literature on iontophoresis (6-12), the scope of this article includes only the mechanism and issues associated with development of iontophoretic systems for PP delivery.

Mechanistic aspects of peptide and protein transport

The passive delivery of PP has been reported to be very low with the exception of alpha-melanocyte stimulating hormone (α-MSH) and a tetrapeptide, where significant passive flux was observed (7). In contrast to the lipophilic pathway of passive transport, iontophoretic delivery takes place mainly through appendageal routes including the sweat glands and hair follicles although they constitute only 0.1% of the skin surface (13). Iontophoretic transport has been defined mathematically and theoretically using the Nernst-Planck equation with anatomical and physiological data from sophisticated analytical techniques (14, 15). In its simplest form, the Nernst-Planck equation can be represented as J = J(P)+J(ER)+J(EO), where J represents the total flux, J(P) the passive flux, J(ER) the flux due to electrorepulsion and J(EO) the electroosmotic flux. The overall transport is dependent on the permeant's charge, electrode polarity and charge of skin at a given pH. At physiological pH, the skin is negatively charged and therefore is selective to cation permeation which results in an electroosmotic flow predominantly from the anode to cathode direction (16).

Pikal (17) represented the enhancement ratio as $E= J(I)/J(P)=\alpha/1-\exp(-\alpha)$, $\alpha=[F(-\Delta\phi_v)/RT].z_1 + [F(-\Delta\phi_v)/RT].1.5\pi.10^{-3}\, N_0 a r^2_p\, (-z_m)\, C_m G\, (\kappa r_p)$, where the contribution of electroosmotic flow depends upon the sign of the membrane charge (z_m), the concentration of charges in the membrane (C_m), the pore radius (r_p), the stokes radius of the permeant (a), the ionic concentration in the membrane through the inverse Debye length (κ) which is a part of the argument of the function (G), Faraday's constant (F), the potential gradient ($\Delta\phi_v$), Avogadro's number (N_0), the gas constant (R) and temperature (T). The first term in third equation refers to the electric field contribution and the second term to electroosmotic contribution. For high molecular weight proteins, the stokes radius (a) is large, diffusion is slow and the relative contribution of electroosmotic flow to flux enhancement increases resulting in a large enhancement ratio. However, the actual iontophoretic flux is still less since the electrorepulsive contribution to flux decreases with the increasing size of the penetrant. This is evident in the case of the iontophoretic delivery of insulin where reports claim several orders of enhancement compared to passive flux. However, insulin levels achieved *in vitro* are not sufficient to treat diabetes in humans (18).

In an attempt to delineate the role of the 3 transport phenomena, Guy *et al.* (19) have qualitatively correlated the molecular size to flux. As the molecular weight increase, the electrorepulsion falls off sharply and at the same time electroosmosis becomes dominant. However, these theoretical predictions need to be experimentally verified with a range of permeants varying both in molecular size and lipophilicity to arrive at the molecular weight where the mechanism changes from electrorepulsion to electroosmosis. Pikal (20), who has extensively studied the role of electroosmosis in iontophoresis, found that permeation is a complex function of molecular size, membrane pores and an interaction between the mobile species and pores. Since, iontophoretic transport is assumed to take place through aqueous pores, much of the mechanistic work has focused on characterization of pore type, size and density. The skin is made up of heterogeneous pores including large negative pores (average size = 18-20 Å) and smaller neutral and positively charged pores (20). Unlike the preexisting pores, the pores induced at high voltages are negatively charged with no positive or neutral pores (21). Recently, Bath *et al.* (22) have provided direct evidence using scanning electrochemical microscopy that electroosmotic flow originates from the hair follicles and the pH dependency of the acid-base equilibra of amino acid residues located in the epithelial cells lining the hair follicle modulates the electroosmotic flow (15). Although, electroosmotic velocity was constant for small compounds used in the study, this may not hold true for macromolecules which can interact with the epithelial lining cells and modulate the electroosmotic flow (22). In fact, some peptides can modulate their own delivery by influencing the direction of electroosmotic flow. Charro and Guy (23) found that the flux of luteinizing hormone releasing hormone (LHRH) analogs,

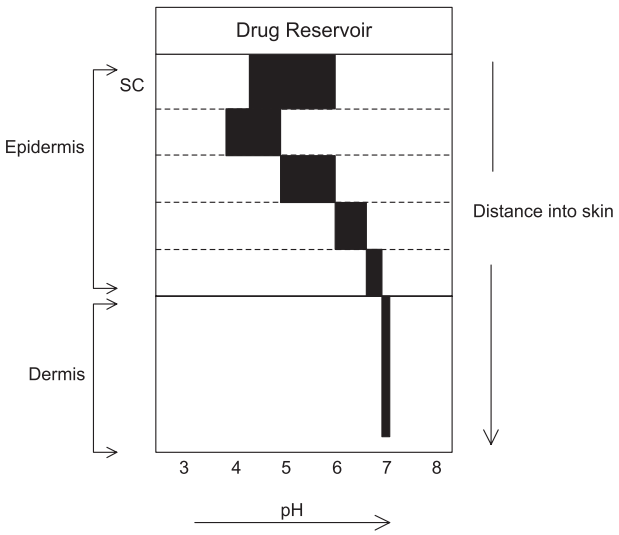

Fig. 1. Schematic representation of varying pH as a function of depth inside the skin. The shaded area qualitatively indicates the range of pH at any particular distance inside the skin and the dotted lines qualitatively indicate the different layers in the skin. (Modified from Sage, initital? *et al. Technological and developmental issues of iontophoretic transport of peptide and protein drugs.* In: Trends and Future Perspectives in Peptide and Protein Drug Delivery. Lee, V.H., Hashida, M., Mizushima, Y. (Eds.). Harwood Academic Publishers: Sydney 1995, 111-134.)

namely nafarelin and leuprolide, was reduced at higher concentrations. This was attributed to the binding of the peptides to the fixed negative charges in the skin, a phenomenon that was not observed with parent LHRH. As a result, the electroosmotic flow was reversed from cathode to anode direction to anode to cathode direction, leading to decreased flux. Thus, driving large positively charged polypeptides into the skin might lead to attenuation of electroosmotic flow which is dictated by molecular size and hydrophobicity (24).

To understand the iontophoretic transport of macromolecules, Ruddy and Hadzija (25) studied the size dependence of polyethylene glycol oligomers and explained their results based on the "hindered transport theory". According to this theory, molecules with stokes radii are of the same magnitude as that of the pore radii undergo restricted mobility or hindered transport. Hence, a large protein would suffer hindered transport or "reflection" from most of the pores, while small ions and water would be transported leading to a marked reduction in transport of the protein (17). The transport of macromolecules is further complicated by their nonspherical and porous structure in addition to their dependence on macromolecule-solvent interactions (26). The use of larger current densities (>0.5 mA/cm^2) over a large electrode surface may aid in the transport of large proteins through current-induced larger pores (27). Therefore, the transport of PP is governed by the complex interplay of a multitude of physicochemical and electronic factors unlike small molecules.

Delivery system considerations

The foremost and fundamental consideration in the delivery of PP is pH which not only determines the charge of protein but also dictates the direction of electroosmotic flow and polarity of delivery electrode. At physiological pH, small cationic peptides are, in general, delivered easily while larger (MW >5000 Da) peptides having low dose requirements can also be delivered albeit at lesser rate (3). The isoelectric point of PP should preferably be either <4 or >7.4 so that they remain charged inside the isoelectric point ranges (28) of human skin (pI ~ 4.8). This is shown in Figure 1 where the pH is depicted as a function of skin depth from the stratum corneum (SC) to the dermis. Thus, for any peptide with a pI between 4-7.4, there is a greater chance of precipitation at a given depth inside the skin that ultimately results in reduced transport into dermis. Table I gives a representative list of the relevant physicochemical and electronic parameters that have been reported in the literature. Some of the proteins that are anionic at physiological pH like insulin, are difficult to deliver since the electroosmotic flow in the reverse direction will reduce the overall flux. However, it is often possible to use tailor-made analogues of peptides for iontophoretic delivery with favorable pIs and high charge to mass ratios (29).

Another important issue in the design of iontophoretic delivery systems for PP is the low efficiency of transport due to the low transference number of the PP as compared to the buffer salts and ions. Increasing the fraction of PP in the formulation or decreasing the number of competing ions may aid in improving the transport efficiency (17). A balance needs to be achieved between the maximum concentration of PP that can be used and the minimum number of ions required for electrical conduction and convective flow to improve the efficiency of iontophoretic delivery of PP (30).

Aspects of device fabrication have been largely restricted to either patent literature or proprietary knowledge. Most of the reported studies for PP have been carried out using the silver-silver chloride electrode (Table I) due to the fact that they do not cause electrolysis of water and need only low potentials to generate electric current (31). However, this type of electrode requires that the PP be in its salt form to provide the necessary fuel for the electrode reaction which may otherwise result in migration of silver into the skin causing discoloration and, in some cases, precipitation of the protein (30). Inert electrodes like platinum overcome the limitations of silver-silver chloride electrode systems but cause the pH of the formulation to shift due to electrolysis of water. Several alternate approaches including ion exchange membranes, use of electroactive polymers (*e.g.*, polyaniline) and intercalation compounds (*e.g.*, sodium tungstate) are being explored to solve the above problems (31). It is important to mention that in the case of insulin, the use of photoetched divided electrodes has been found to efficiently deliver current and reduce skin irritation (32). In selecting the matrix for PP delivery, it is critical to develop

Table I: Phyiscochemical and electronic parameters reported for iontophoresis of PP.

Peptide/Protein	MW (Da)	pI	Electrode	Polarity	Current type
Insulins					
Porcine	5777	5.3	Platinum	Cathode	Continuous/pulsed
Bovine	5733	5.3	Platinum	Anode	Continuous/pulsed
Human	5807	5.3	Platinum	Anode	Continuous/pulsed
Monomeric insulins	~ 5000				
Asp^{B39}(Ser),Glu^{B27}(Thr)		4	Ag/AgCl	Cathode	Continuous
Asp^{B9}(Ser)		4.5	Ag/AgCl	Cathode	Continuous
Asp^{A21}(Asn),Asp^{B9}(Ser),Glu^{B27}(Thr)		3.5	Ag/AgCl	Cathode	Continuous
Asp^{B10}(His)		4.5	Ag/AgCl	Cathode	Continuous
Glu^{B16}(Tyr),Glu^{B27}(Thr)		4	Ag/AgCl	Cathode	Continuous
Glu^{B16}(Tyr)		5	Ag/AgCl	Cathode	Continuous
Asp^{B1}(Phe),Asp^{B4}(Gln),Asp^{B1}0(His),Asp^{B16}(Tyr),Glu^{B27}(Thr)		3	Ag/AgCl	Cathode	Continuous
Gln^{A17}(Glu),His^{B16}(Tyr),Arg^{B27}(Thr),Thr^{B30}-NH_2(Thr)		7	Ag/AgCl	Cathode	Continuous
His^{A8}(Thr),His^{B4}(Gln),Glu^{B10}(His),His^{B27}(Thr)		6	Ag/AgCl	Cathode	Continuous
Arg^{B27}(Thr),Lys^{B30}-NH_2(Thr)		7	Ag/AgCl	Cathode	Continuous
Sulfated, porcine		2.5	Ag/AgCl	Cathode	Continuous
Gonadotropins					
LHRH	1182	>7.4	Ag/AgCl	Anode	Continuous
Leuprolide	1290	>7.4		Anode/cathode	Continuous
Buserelin	1297	>7.4	Platinum	Anode	Continuous/pulsed
Nafarelin	1337	>7.4	Ag/AgCl	Anode/cathode	Continuous/pulsed
Calcitonins					
Salmon	3432	9.7-10.4	Pt-Copper; Ag/AgCl	Anode/cathode	Continuous/pulsed
Human	3418	7.9	Platinum	Anode	Continuous
GRF					
GRF(1-44)	5039	>7.4	Stainless steel	Anode	Pulsed
Ro-23786	3929	8.0	Platinum	Anode	Pulsed
[^{3}H]-SK&F-110679			Ag/AgCl	Anode	Pulsed
GHRP			Ag/AgCl	Anode	Pulsed
Vasopressins					
Arginine	1084	10.9	Platinum; Ag/AgCl	Anode	Continuous/pulsed
Desmopressin	1069	10.9	Platinum	Anode	Pulsed
Desglycinamide, 8-arginine	1028	~10.9	Ag/AgCl	Anode	Continuous
Others					
TRH	362	6.2	Platinum	Anode	Continuous/pulsed
Enkephalins	555	5.8	Ag/AgCl	Anode/Cathode	Continuous
Melanotropin(6-9)	764	10.7-10.8	Platinum	Anode	Continuous
DSIP	849	3-5	Ag/AgCl	Cathode	Continuous
Octreotide	1019	>5.5	Ag/AgCl	Anode	Continuous
Angiotensin II	1046	7.7	Ag/AgCl	Anode	Continuous
CCK-8 1055	1055	<4	Ag/AgCl	Anode	Continuous
hPTH	4118	–	Ag/AgCl	Anode	Continuous

LHRH: leutinizing hormone releasing hormone; GRF: growth hormone releasing factor; GHRP: growth hormone releasing peptide; TRH: thyrotropin releasing hormone; DSIP: delta sleep inducing peptide; hPTH: human parathyroid hormone.

clinically acceptable iontophoretic delivery systems and very few studies address this issue. However, considering the unique stability requirements of PP, hydrogels that can be rehydrated just prior to use would be preferable (18). There are contradictory reports in the literature with regard to the continuous *versus* pulsed current for PP delivery. Most of the studies reported to date show that there is not much difference in the amount of PP transported between continuous and pulsed current, even though skin irritation may be less with pulsed current (17). It is important to note that pulsed iontophoretic delivery is not comparable to pulsatile secretion of hormones, since the pulse length of hormonal secretion may be in the order of hours or longer in contrast to electrical pulses that are only milliseconds or, at the most, minutes apart (7). Furthermore, as pulse frequency increases more of the charge will be delivered to the surface rather than across it thereby resulting in decreased transdermal flux. Considering that current flow is coupled to drug transport which implies constant transference numbers, comparison of the average current at the same time would result in identical amounts of the drug by both pulsed and continuous current (17). However, more experimental evidence is needed to understand the role of pulsed and

continuous current in PP delivery as well as the electrical behavior of skin. The need to preserve the physical, chemical and biological stability and electrochemical stability of PP at every stage of the iontophoretic system design cannot be overlooked. Thus, the iontophoretic system design for PP is influenced by a number of therapeutic, functional and user factors requiring a multidisciplinary approach to thoroughly understand the intricacies as well as unique advantages that it can offer over the existing therapies.

Electroporation

Electroporation is one of the upcoming techniques used to expand the scope of transdermal drug delivery and potentially enable the delivery of macromolecules. Its unique abilities to permeabilize the most impermeable SC layer of the skin makes it a possible technique for the delivery of macromolecules. Although the first demonstration of electroporation of the skin was only in 1993, the technique has been widely used in cell biology for the last 3 decades. Typically electroporation involves the creation of transient aqueous pathways across lipid bilayer membrane by the application of either square wave or exponential high voltage pulses (50-1000 V) of very short duration (mcs to ms). The potential advantages of this method are: dramatically increased flux (up to 4 orders of magnitude); extremely rapid onset of action (compared to any other enhancement method of transdermal drug delivery); temporal control of delivery; and, the possibility of reduced skin irritation (33-35).

The mechanism by which electroporation increases the permeability of the SC is still not completely understood. However, electroporation is thought to involve electrically driven transport (electrophoresis and/or electroosmosis) and diffusion (due to long-lived changes in membrane permeability). Diffusion is dominant for uncharged and weakly charged molecules like mannitol (36) and fentanyl (37) while, on the other hand, electrophoresis is the main driving force for compounds with greater charge and increased hydrophilicity like calcein (38). Molecular transport by electroporation is hypothesized to occur through electrically created new aqueous pathways (pores) mostly through localized transport regions (LTRs). These LTRs where molecular transport is concentrated, are formed away from the appendageal pores. Recent evidence supports the specific hypothesis that high voltage pulses create a direct aqueous pathway mostly within LTRs that perforate the SC lipid bilayers and pass through the interiors of hydrated corneocytes (39). Theoretical attempts to explain this phenomenon consider the role of localized heating and both the stochastic and deterministic model. However, a theory explaining the mechanistic response of the skin to high voltage pulses is far from complete (39).

The technique of electroporation is still in its infancy and not been fully exploited at this point in time. It has been shown that electroporation can dramatically and reversibly increase the flux of a number of small molecules (*e.g.*, fentanyl, alniditan and flurbiprofen) (37, 40, 41), neutral molecules (*e.g.*, mannitol and water) (36), dyes (*e.g.*, calcein and sulphorodamine) (38, 42) and a few macromolecules including peptides (*e.g.*, LHRH, ciclosporin, salmon calcitonin and parathyroid hormone) (43-46) and oligonucleotides (47). Heparin has been delivered transdermally *in vitro* at clinically relevant rates using short (2 ms) high voltage pulses of 150 to 350 V (48) while, LHRH flux from human SC was found to be increased 5- to 10-fold following a single exponential pulse of 1000 V for 5 ms (43). In a separate study, electroporation was shown to repeatedly enhance LHRH transport in a pulsatile manner, thus demonstrating the possibility of achieving programmable delivery of peptides (44).

In vivo data obtained using low duty cycle electric field pulses in hairless rats have especially supported the enhanced transport and rapid onset for delivery of calcein (33, 49). Fentanyl delivered by electroporation exhibited an onset time of few minutes in hairless rats (50). A factorial design shows that the voltage, waveform, duration and number of pulses enabled the control of the quantity of fentanyl transported through the skin *in vitro* and indicated that selection of the optimal electrical parameter (37) can control delivery. An ideal protocol for *in vivo* human studies would be one that maximizes drug delivery without causing pain or damage to skin. The control of delivery will be important for the application of electroporation for delivery of PP as most of these drugs are used for chronic conditions and require complex delivery profiles. Results from animal studies, although limited, suggest that electroporation of the skin is generally safe. Visual scores, histological studies (44, 51) and biophysical methods (52) indicate that skin irritation is mild, short-lived (30 min to a few hours) and dependent on the electrical parameters of the pulses. Experiments with pigs showed that at both the gross and light microscopic levels, electroporation did not result in any changes in skin under the conditions examined (44). An approach to decrease the sensation due to application of electric field is to concentrate the field in the SC by selecting an optimum electrical protocol and improving the electrode configuration (35, 53). The clinical acceptability of this technique still remains to be established, as only sparse human data are available.

It may take a few years before electroporation becomes a commercial reality. Contemporary research upholds its potential to deliver macromolecules based on significantly reduced lag time and a dramatic and reversible increase in flux. The apprehensions that remain are its toxic manifestations on the skin and development of a miniaturized, safe and handy device to meet clinical needs. Most of the information in this regard is a proprietary. There is a developing interest in this area and the progress made will be a key factor in commercialization of this technology.

Sonophoresis

Crystals like quartz and silicon dioxide through what is referred to as a piezoelectric effect (54) generate ultrasonic waves. The recognition that these waves have energy and penetration power led to the use of ultrasonic waves in transdermal drug delivery as a permeation enhancement method. This phenomenon of enhancement of transdermal delivery of drugs is known as sonophoresis. A typical sonophoretic drug delivery system consists of a power source, control system, high frequency generator, acoustic transmission line, ultrasonic transducer and a coupling agent between skin and transducer (55). In diagnostics and therapeutics, currently ultrasonic waves ranging from 1 to 10 MHz in frequency with intensity ranging from 0-4 W/cm^2 are commonly used (55). Low molecular weight drugs (MW < 500 Da; *e.g.*, steroids, nonsteroidal antiinflammatory agents) have been delivered efficiently through transdermal route by therapeutic ultrasonic waves (1-10 MHz) (56). However, this method was not useful for PP. On the other hand, it was found that the PP could be delivered by low-frequency ultrasonic waves since the penetration power of ultrasonic waves is inversely proportional to frequency (57). Enhanced permeation through ultrasonic waves is mainly due to the increased conductivity of skin. Recently, it was observed that a minimum energy of 222 J/cm^2 at 20 kHz was required to increase the transport of mannitol across porcine skin and this threshold energy was a function of intensity, exposure time and duty cycle (58). When skin is exposed to ultrasonic waves, cavitation, acoustic streaming or convection flow and thermal effects are induced which in turn increase the conductivity of skin. Out of these 3 effects, cavitation was found to be the major contributor to enhancement of skin conductivity following ultrasound exposure. The cavitation effect involves generation and oscillation of gaseous bubbles in corneocyte pockets of the SC (59). Oscillation of gaseous bubbles and their collapse at the corneocyte-lipid bilayer interface may be responsible for the disorganization or perturbation in the lipid bilayer of SC resulting in penetration of water in the fatty acyl chain area of the lipid bilayer and thus leading to formation of aqueous channels (60). This ultimately results in increased partitioning and diffusivity of water-soluble drug during sonophoresis. At low frequency (20 kHz) ultrasound, continuous and deep aqueous channels are formed through which high-molecular weight hydrophilic compounds such as insulin, inulin and erythropoietin may efficiently cross the diffusion barrier of SC lipid bilayer and reach systemic circulation. However, in the case of therapeutic ultrasound frequency, channel formation is restricted to the upper few layers as the depth of penetration is very low at this frequency. In addition to cavitation, inhibition of metabolic machinery in the skin may also be one of the mechanisms involved in enhanced permeation of PP by ultrasound exposure. Although there is no evidence of a reduction in enzyme activity of skin in relation to PP metabolism, this has been shown for prednisolone-21-acetate (61). Generation of free radicals was found to be the key mechanism involved in enzyme inhibition (62). Therefore, stability of PP during ultrasound application warrants thorough investigation.

Mitragotri *et al.* conducted an *in vitro* feasibility study of transdermal delivery of PP through human cadaver skin using insulin (MW = ~6000 Da), interferon-γ (IFN-γ; MW = ~17000 Da) and erythropoietin (MW = ~48000 Da) (63). It was observed that a patch area of 40 cm^2 and 1 h sonophoresis (20 kHz, 100 ms pulse applied every second t.i.d.) could achieve therapeutic plasma levels for all 3 proteins. Although, insulin delivery through cadaver skin *in vitro* and in animals were successful (63-65), implementation of this technique in humans to treat diabetes still has a long way to go. The issues of device and safety need to be examined. Miniaturization and fabrication of the device may not be a hurdle since commercially available ultrasonic toothbrushes can be modified for transdermal applications (56). Low-frequency ultrasonic waves are generally believed to induce reversible changes in the SC as assessed by permeation with tritiated water and optical microscopy (66). However, Bommannan and coworkers (67) have shown using electron microscopy that exposure to a frequency of 10-16 MHz for 20 min induced cytotoxic effects on skin.

Combination strategies

Keeping in tune with the everchanging scenario in drug discovery and development, transdermal drug delivery has also evolved new approaches. For very large proteins (MW > 5000 Da), a combination of strategies may be able to achieve meaningful blood levels of drugs and meet therapeutic needs. The various combination strategies investigated in transdermal drug delivery have been recently reviewed (68). Figure 2 shows the possible enhancement strategies and their combinations including the advantages that they can offer for transdermal drug delivery. The basic premise in these combination strategies is to utilize the ‘strengths but at the same time overcome the weaknesses of the enhancement techniques thereby opening up new opportunities for difficult-to-deliver molecules. Although there are a number of reports on various combination techniques, this review presents a brief overview of only those related to PP delivery.

The novel idea of combining chemical enhancers with iontophoresis was demonstrated for the first time by Srinivasan *et al.* (69) where significant enhancement in iontophoretic flux could be achieved for insulin and leuprolide by simple pretreatment with ethanol. Table II lists the literature concerning various chemical enhancers used in combination with iontophoresis for PP. All the chemical enhancers act by one or more mechanisms that includes interaction with lipid bilayers or keratin resulting in alteration of skin resistance when combined with iontophoresis (18). Most importantly, chemical enhancers when combined with iontophoresis may not only show additive or synergistic effect in terms of permeant flux but may also show synergism in disrupting the barrier

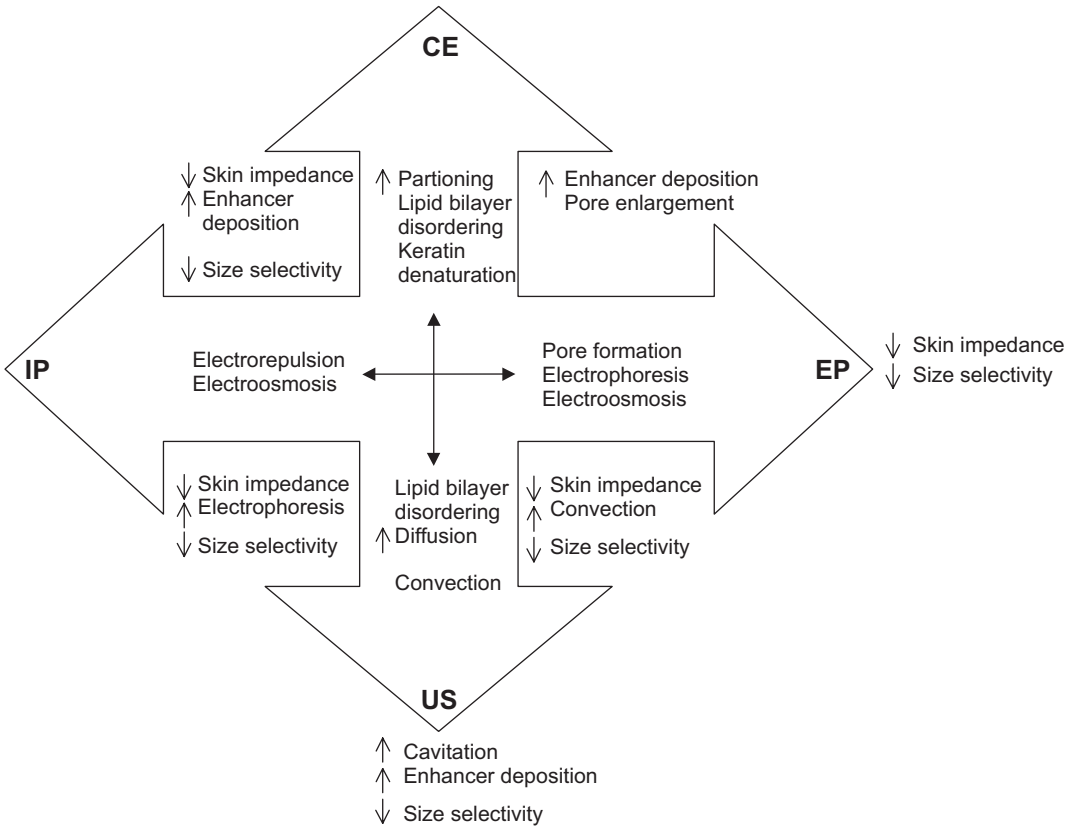

Fig. 2. Possible mechanisms of individaul enhancement strategies (within the box) and their combinations (outside the box). CE: chemical enhancer; IP: iontophoresis; EP: electroporation; US: ultrasound. Upward arrows indicate increase, downward arrows indicate decrease and double-headed arrows indicate combination. Vertical double-headed arrow indicates CE-US combination and its effects given below US. Horizontal double-headed arrow indicates IP-EP combination and its effects given to the right of EP. (Modified from Mitragotri, S. *Synergistic effect of enhancers for transdemal drug delivery*. Pharm Res 2000, 17: 1354-9.)

properties of skin (73). Hence, the enhancement achieved and adverse effects on skin associated with the combination is determined more often than not by the chemical enhancers rather than iontophoretic current (18). Furthermore, the choice of vehicle for chemical enhancers also influences the enhancement as well as the adverse effects on skin (82). Surfactants can interact with the fixed charges in the skin and modulate iontophoretic delivery. Recently dodecyltrimethylammonium bromide was found to enhance the iontophoretic flux of a large anionic molecule, dextran, by neutralizing the negative charge of the skin and increasing the electrorepulsive transport (83). This opens up new possibilities for altering skin charge and thereby aiding in the delivery of large proteins via increases in the contribution of electrorepulsion or electroosmosis to the overall flux. Therefore, the synergistic effect of a combination of chemical enhancers and iontophoresis is dependent on the permeant's charge, membrane charge, enhancer type, duration of treatment and polarity of delivery electrode.

Electroporation followed by iontophoresis may reduce skin impedance and create additional pathways for PP transport. Higher fluxes were achieved for LHRH when initial pulses of electroporation were followed by iontophoresis (43). Further, electroporation shortens the lag time for iontophoretic delivery as was observed in case of calcitonin and parathyroid hormone (46). Interestingly, the creation of new pathways by electroporation may result in an even charge distribution thus reducing skin irritation when higher current densities are used in iontophoresis (18). In order to observe any synergistic effect with iontophoresis, the electroporation pulsing protocol as well as the PP concentration should be optimized (46).

An interesting finding has been reported in literature where macromolecules enhance their own delivery during electroporation (84). Macromolecules including dextran, heparin and poly-L-lysines, are trapped within the skin during electroporation and can hold the pathway open for further transport. Molecules with a greater charge and size were found to be effective as enhancers and they also reduced the number of pulses required for the delivery of macromolecules. The use of keratolytic agents such as sodium thiosulfate and urea create microconduits by interacting with keratin, while electroporation induces

Table II: Combination of chemical enhancers and iontophoresis reported for PP delivery.

Peptide/Protein	Current/ Voltage applied	Chemical enhancer	Effect[a]	Ref.
Porcine insulin	0.25-0.5 V	Ethanol	22	69
	[b]0.25-1 mA/cm^2	30% DMSO	6.7	70
		10% Urea	7.6	
		0.01% PF-68	13.41	
		0.1% STC	19.09	
		0.1% STGC	22.35	
		10% urea + 0.001%PF-68	27.30	
		10% urea + 0.1% STC	31.97	
		10% urea + 0.1%STGC	36.63	
	[b]0.075 mA/cm^2	5-10% urea	Greater reduction in blood glucose	71
	0.25 mA/cm^2	Depilatory lotion	Greater reduction in blood glucose	72
	0.85 mA	0.3 M OA in PG	Greater reduction in blood glucose	73
		0.1 M LA in ethanol	No effect	
	NA	Polaxmer 188 + PG	No effect	74
		Azone + PG	Enhanced transport of insulin	
Bovine insulin	0.44 mA/cm^2	10% v/v OA in ethanol	No effect	75
		1% w/v menthol in 40% v/v ethanol	No effect	75
		Depilatory cream	Greater reduction in blood glucose	75
Human insulin	0.5 mA/cm^2	IPA 70%	No effect	29
		Ethanol 100%	30	
Monomeric insulins	0.5 mA/cm^2	IPA 70%	14-35	29
		Ethanol 100%	1471-2300	
LHRH	0.25 V	Ethanol	4	76
	0.2 mA/cm^2	Ethanol	5-8	77-79
		10% OA in Ethanol	13.13	77
		PG	6	
		10% OA in PG	14.73	
		Capric acid/ethanol	5.30	78
		Lauric acid/ethanol	9.93	
		Myristic acid/ethanol	11.71	
		Palmitic acid/ethanol	16.66	
		Stearic acid/ethanol	8.14	
		Linolenic acid/ethanol	16.4	
		Linoleic acid/ethanol	16.57	
	0.2 mA/cm^2	5% thymol/ethanol	9.8	79
		5% cineole/ethanol	10.1	
		5% carvone/ethanol	11.2	
		5% limonene/ethanol	12.8	
Leuprolide	0.2 mA/cm^2	Methyl acetate	15.41	80
		Ethyl acetate	31.84	
		Propyl acetate	16.24	
		Butyl acetate	13.91	
		Pentyl acetate	19.81	
		Hexyl acetate	16.52	
		Octyl acetate	19.11	
CCK-8	0.2 mA/cm^2	Ethanol	6.14	81
		10% OA/ethanol	12.05	
Calcitonin	[b]0.015 mA/cm^2	10-15% urea	Greater reduction in calcium levels	71

[a]Numbers indicate the number of times the permeability is enhanced by the combination compared to passive permeability, except wherever mentioned. [b]Pulsed current. NA: not available; DMSO: dimethyl sulfoxide; LA: lauric acid; IPA: isopropyl alcohol; OA: oleic acid; PF-68: pluronic F68 (nonionic surfactant); PG: propylene glycol; STC: sodium taurocholate; STGC: sodium tauroglycocholate.

transport pathways in the lipid bilayer (85). Le and coworkers (86) have demonstrated that pretreatment of porcine skin with ultrasound waves followed by iontophoresis resulted in an enhancement of heparin flux 56 times. This combination used lesser ultrasound energy and current density as compared to when either method was used alone. Combination of sodium lauryl sulfate (SLS) with low frequency ultrasound (87) enhanced the dispersion of SLS within the SC and reduced the ultrasound threshold energy for molecular transport. However, this combination is yet to be used for delivery of PP. Similarly, the combination of ultrasound and electroporation can also be investigated for PP delivery based on the synergistic effect found with calcein (88). It would be preferable to select a combination which included methods acting by different rather than similar mechanisms so

Table III: Advantages and limitations of PowderJect system.

Advantages
No upper molecular weight limit
No limit on lipophilicity
Very short lag time, hence rapid therapeutic benefit
Dry powder formulations offer better storage stability
Site specificity and optimal targeting in skin, very suitable for vaccines and allows lower doses because of site specificity
Limitations
Upper dose limit of 6 mg
The potential to cause allergic contact dermatitis
Stability of protein and peptide drugs due to the high shear during operation of the device

as to achieve reasonable enhancement of proteins as well as reduce the skin toxicity associated with each of the strategies. Iontophoresis in combination with chemicals or electroporation offers good promise in terms of both enhancement and commercial viability.

Alternate approaches

Transdermal powdered delivery

High-velocity powder injection is a promising new drug-delivery technique that provides needle- and pain-free delivery of traditional drugs and macromolecules by direct injection of solid powder forms at high velocities. The specific advantages and limitations of the technology are shown in Table III. The principle of the powderject system involves using the energy in a supersonic flow of gas to accelerate and deliver powdered (particulate) drugs into the skin. Fine drug particles of 20-100 μm are accelerated in a transient, supersonic helium gas jet to sufficient velocities (300-900 ms^{-1}) in order to achieve a momentum for penetrating the SC (89, 90). This technology was initially used to deliver DNA to plant cells in order to genetically transform them (91). Now, the delivery of powdered pharmaceuticals into the skin for local or systemic applications is being investigated (92, 93). Depending on the size, density and hardness of the particles, powdered therapeutics can be delivered at a specific depth (*i.e.*, SC) into the epidermis or to the dermo-epidermal junction (94). Particle size distribution, strength and particle density are uniquely important formulation parameters as they control penetration depth and irritation levels. Preclinical and clinical studies indicate that the skin barrier can deflect or stop any typical organic particulate material below about 20 μm in mean mass aerodynamic diameter (94). Such studies revealed that drug penetration across skin exists at 20-100 μm or greater. At the upper limit of the size range, the local biological impact (erythema, tiny subdermal hemorrhages, *etc.*) limits the delivery mode. Physical stability of particles (strength) is also important, as the powder must survive the high stress of gas jet within the device and the ballistic impact with the skin at supersonic velocities. This fact was demonstrated in a preclinical study examining several formulations of salmon calcitonin (sCT). Results showed that the formulation containing a binder enhanced the delivery of sCT by 87% as compared to simple particles and the formulation that contained a waxy component reduced the delivery due to softening of the particles (93). Relatively minor alterations in the sCT formulations significantly affected the maximum serum concentration and delivery of sCT by the dermal powderject system. Controlled release with altered pharmacokinetic parameters can also be achieved by employing bioerodible carriers or slowly dissolving excipient in the formulation to reduce peak blood level, avoid overdose and reduce the frequency of dosing. This type of release also improves tolerability in the local tissue by a gradual dissolution of the drug.

Systemic delivery of sCT and human insulin using the dermal powderject system was demonstrated successfully in preclinical studies. Several pharmacokinetic studies performed in conscious rabbits showed that delivery of powder formulations of sCT was dose-dependent. Significant and rapid reductions in serum calcium were associated with this delivery mode. Another preclinical study demonstrated that delivering human insulin systemically via dermal powderject is reproducible and dose-dependent (95). Despite a number of products under development, this method is still in the very early stages; development of novel testing methods, new methods of powder preparation and handling, unique formulations and refinement of the devices are likely to lead to successful commercialization of a myriad of products, while advances in biotechnology will identify ever more potent and therefore ideal therapeutic entities for delivery by this technique.

Lipid-based delivery systems

One of the more interesting approaches for the transdermal delivery of PP drugs uses liposomes and transferosomes as the delivery agents. Liposomes are thermodynamically stable lipid vesicles with an aqueous core and at least one surrounding bilayer. They are composed of phospholipids and membrane stiffening agents such as cholesterol. Liposomes were shown to facilitate the transdermal transport of some macromolecular drugs such as

interferons (96-98). However use of liposomes in transdermal delivery of macromolecules was not successful to date due to the inability of such vehicles to pass through the narrow (30 nm or less) intercellular passages in the outer skin layers (99). Transferosome carriers are complex, most often vesicular, aggregates capable of crossing barriers and of transferring material between the application and the destination sites. Typically, transferosomes are mixed lipid vesicles that are sufficiently deformable to penetrate pores much smaller than their own size. They are (quasi) metastable, making the vesicle membrane ultraflexible and highly deformable. Transferosomes are different from liposomes in that they are more flexible and more hydrophilic than the latter helping them to avoid the aggregation and fusion commonly observed with liposomes exposed to osmotic stress (99). Similar to liposomes, transferosomes consist of natural amphipaths, most often phospholipids, suspended in a water-based solution which forms a bilayer around an aqueous core. However, unlike liposomes, they incorporate membrane-softening agents thus making the vesicles highly flexible and adaptable. When placed on the skin, water evaporates and the vesicles begin to dry out. Due to the high hydrophilicity of the ingredients and the flexibility of the membrane, the vesicles are driven along the water gradient into the body's interior via intercellular spaces smaller than the vesicle itself. The vesicles can squeeze through these spaces because of their extreme flexibility (100). Once the transferosomes accumulate in the underlying tissue, they can either release the drug or continue to distribute further into the body, depending on formulation characteristics. The added advantage of transferosomes over other transdermal permeation techniques is that without compromising the barrier property of the skin, they enhance transdermal permeation by transiently opening channels between adjoining cells.

Transferosomes permit noninvasive immunization through normal skin giving rise to a similar or higher antibody titre than observed with s.c. injections of the same immunogen formulation. Gap junction proteins (GJP) incorporated into transferosomes and applied to the intact skin surface resulted in specific antibody titres that were slightly higher than those elicited by s.c. injections of GJP alone (101). A pilot clinical study suggested that transdermal insulin in flexible liposomes (Transfersulin) is as effective as injectable insulin but has a more reproducible profile. Transfersulin which contains recombinant human insulin, was compared with a s.c. injection of long-acting insulin in a double-blind, crossover study involving 10 patients with type 1 diabetes. The results showed no significant differences in either mean blood glucose levels or free insulin levels during the 12-h observation period (102). This technology offers long-acting noninvasive delivery of insulin, which compliments the fast release delivery systems and have found to be promising both for type 1 and 2 diabetic patients.

Conclusions

Future drugs are going to be large as well as hydrophilic thus requiring enhancement strategies to solve the permeability dogma. It is of the authors' opinion that investigation should focus mainly on understanding the transport of molecules across biological membranes (like skin), the influence of the physicochemical factors of the drug on transport and the stabilization and delivery of bioactive molecules belonging to class III and IV. The skin, due to its easy accessibility and large surface area, offers tremendous potential for development of noninvasive delivery systems for PP. Although, a lot of excitement is associated with iontophoresis as evidenced from the large number of reports in the literature, other competing technologies like sonophoresis, electroporation and high speed powdered systems, show good promise for certain therapeutic categories such as vaccines. The combination of enhancement strategies can provide new opportunities for delivery of large proteins and also would resolve skin safety issues. However, future development of these technologies will depend on to what extent the initial expectations can be transformed into meaningful therapeutic applications with distinct advantages over existing therapies in terms of safety, cost, efficacy and patient compliance. The above issues will dominate drug delivery in the next 2 decades, with more emphasis placed on the biological/pharmacological aspects of drug delivery. Therefore, development of suitable delivery technologies for PP and validation of those technologies to achieve the desired therapeutic benefits will be challenging in the coming years.

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References

1. Drews, J. *Drug Discovery: A historical perspective.* Science 2000, 287: 1961-4.

2. Drews, J. *Quo vadis, biotech? (Part I).* Drug Discov Today 2000, 5: 547-53.

3. Naik, A., Kalia, Y.N., Guy, R.H. *Transdermal drug delivery: Overcoming the skin's barrier function.* Pharm Sci Technol Today 2000, 3: 318-26.

4. Pannatier, P.J., Testa, B., Etter, J.C. *The skin as a drug metabolizing enzyme.* Drug Metab Rev 1978, 8: 319.

5. Amidon, G.L., Lennarnas, H., Shah, V.P., Crison, J.R. *A theoretical basis for a biopharmaceutic drug classification: The correlation of in vitro drug product dissolution and in vivo bioavailability.* Pharm Res 1995, 12: 413-20.

6. Banga, A.K., Chien, Y.W. *Iontophoretic delivery of drugs: Fundamentals, developments and biomedical applications.* J Control Release 1988, 7: 1-24.

7. Cullander, C., Guy, R.H. *Transdermal delivery of peptides and proteins*. Adv Drug Deliv Rev 1992, 8: 291-329.

8. Singh, D., Maibach, H.I. *Iontophoresis in drug delivery: Basic principles and applications.* Crit Rev Ther Drug Carrier Sys 1994, 11: 161-213.

9. Green, P.G. *Iontophoretic delivery of peptide drugs.* J Control Release 1996, 41: 33-48.

10. Parasrampuria, D., Parasrampuria, J. *Percutaneous delivery of proteins and peptides using iontophoretic techniques.* J Clin Pharmacol Ther 1991, 16: 7-17.

11. Nair, V., Poduri, R., Panchagnula, R. *Transdermal iontophoresis. Part I: Basic principles and considerations*. Methods Find Exp Clin Pharmacol 1999, 21: 139-51.

12. Pillai, O., Nair, V., Pillai, O., Poduri, R., Panchagnula, R. *Transdermal iontophoresis. Part II: Peptide and protein delivery*. Methods Find Exp Clin Pharmacol 1999, 21: 229-40.

13. Cullander, C. *What are the pathways of iontophoretic current flow through mammalian skin?* Adv Drug Del Rev 1992, 9: 119-35.

14. Cullander, C., Guy, R.H. *Sites of iontophoretic current flow into the skin: Identification and characterization with the vibrating probe electrode.* J Invest Dermatol 1991, 97: 55-64.

15. Bath, B.D., White, H.S., Scott, E.R. *Visualization and analysis of electroosmotic flow in hairless mouse skin.* Pharm Res 2000, 17: 471-5.

16. Burnette, R.R., Ongpipattanakul, B. *Characterization of the permselective properties of skin.* J Pharm Sci 1987, 76: 765-73.

17. Pikal, M.J. *Penetration enhancement of peptide and protein drugs by electrochemical means: Transdermal iontophoresis*. In: Trends and Future Perspectives in Peptide and Protein Drug Delivery. Lee, V.H., Hashida, M., Mizushima, Y. (Eds.). Harwood Academic Publishers: Sydney 1995, 83-109.

18. Panchagnula, R., Pillai, O., Nair, V.B., Ramarao, P. *Transdermal iontophoresis revisited.* Curr Opin Chem Biol 2000, 4: 468-73.

19. Guy, R.H., Kalia, Y.N., Charro, M.B.D., Merino, V., Lopez, A., Marro, D. *Iontophoresis: Electrorepulsion and electroosmosis.* J Control Release 2000, 64: 129-32.

20. Pikal, M.J. *The role of electroosmotic flow of transdermal iontophoresis.* Adv Drug Deliv Rev 1992, 9: 201-37.

21. Higuchi, W.I., Li, S.K., Ghanem, A.H., Zhu, H., Song, Y. *Mechanistic aspects of iontophoresis in human epidermal membrane.* J Control Release 1999, 62: 13-23.

22. Bath, B.D., Scott, E.R., Phipps, J.B., White, H.S. *Scanning electrochemical microscopy of iontophoretic transport in hairless mouse skin. Analysis of the relative contributions of diffusion, migration and electroosmosis to transport in hair follicles.* J Pharm Sci 2000, 89: 1537-49.

23. Charro, M.B.D., Guy, R.H. *Characterization of convective solvent flow during iontophoresis.* Pharm Res 1994, 11: 929-34.

24. Hirvonen, J., Guy, R.H. *Transdermal iontophoresis: Modulation of electroosmosis by polypeptides.* J Control Release 1998, 50: 283-9.

25. Ruddy, S.B., Hadzija, B.W. *Iontophoretic permeability of polyethylene glycols through hairless rat skin: Application of hydrodynamic theory for hindered transport through liquid-filled pores.* Drug Des Discov 1992, 8: 207-24.

26. Roberts, M.S., Lai, P.M., Anissimov, Y.G. *Epidermal iontophoresis I. Development of the ionic mobility-pore model.* Pharm Res 1998, 15: 1569-78.

27. Manabe, E., Numajiri S., Sugibayahsi, H., Morimoto, Y. *Analysis of skin permeation-enhancing mechanism of iontophoresis using hydrodynamic pore theory.* J Control Release 2000, 66: 149-58.

28. Marro, D., Guy, R.H., Charro, M.B.D. *Characterization of the iontophoretic properties of human and pig skin.* J Control Release 2001, 70: 213-7.

29. Lankjaer, L., Brange, J., Grodsky, G.M., Guy, R.H. *Iontophoresis of monomeric insulin analogues in vitro: Effects of insulin charge and skin pretreatment.* J Control Release 1998, 51: 47-56.

30. Huang, Y.Y., Wu, S.M. *Transdermal iontophoretic delivery of thyrotropin releasing hormone across rabbit pinna skin.* Drug Dev Ind Pharm 1996, 22: 1075-81.

31. Scott, E.R., Phipps, J.B., Gyory, R., Padmanabhan, R.V. *Electrotransport systems for transdermal delivery: A practical implementation of iontophoresis.* In: Handbook of Pharmaceutical Controlled Release Technology. Wise, D.L. (Ed.). Marcel Dekker: New York 2000, 617-59.

32. Hage, M., Akatani, M., Kikuchi, J., Ueno, Y., Hayashi, M. *Transdermal iontophoretic delivery of insulin using a photoetched microdevice.* J Control Release 1997, 43: 139-49.

33. Prausnitz, M.R., Bose, V.G., Langer, R., Weaver, J.C. *Electroporation of mammalian skin: A mechanism to enhance transdermal drug delivery*. Proc Natl Acad Sci USA 1993, 90: 10504-8.

34. Prausnitz, M.R. *A practical assessment of transdermal drug delivery by skin electroporation.* Adv Drug Del Rev 1999, 35: 61-76.

35. Vanbever, R., Preat, V. *In vivo efficacy and safety of skin electroporation*. Adv Drug Del Rev 1999, 35: 77-88.

36. Vanbever, R., Leroy, M., Preat, V. *Transdermal permeation of neutral molecules by skin electroporation*. J Control Release 1998, 54: 243-50.

37. Vanbever, R., Bouleuge, E., Preat, V. *Transdermal delivery of fentanyl by electroporation I: Influence of electric factors*. Pharm Res 1996, 13: 559-65.

38. Prausnitz, M.R., Pliquett, U., Langer, R., Weaver, J.C. *Rapid temporal control of transdermal drug delivery by electroporation*. Pharm Res 1994, 11: 1834-7.

39. Jadoul, A., Bouwstra, J., Preat, V. *Effects of iontophoresis and electroporation on the SC: Review of the biophysical studies*. Adv Drug Del Rev 1999, 35: 89-105.

40. Jadoul, A., Lecouturier, N., Mesens, J., Caers, W., Preat, V. *Transdermal alnitidan delivery by skin electroporation*. J Control Release 1998, 54: 265-72.

41. Perez, M., Eeckhoudt, S., Verbeeck, R., Preat, V. *Transdermal delivery of flurbiprofen in the rat by iontophoresis and electroporation.* Pharm Res 1997, 11(S14): 309.

42. Chen, T., Segall, E.M., Langer, R., Weaver, J. C. *Skin electroporation: Rapid measurements of the transdermal voltage and*

flux of four fluorescent molecules show a transition to large fluxes near 50 V. J Pharm Sci 1998, 87: 1368-74.

43. Bommannan, D., Tamada, J.A., Leung, L., Potts, R.O. *Effect of electroporation on transdermal iontophoretic delivery of LHRH in vitro*. Pharm Res 1994, 11: 1809-14.

44. Riviere, J.E., Monteiro-Riviere, N.A., Rogers, R.A., Bommannan, D., Tamada, J.A., Potts, R.O. *Pulsatile transdermal delivery of LHRH using electroporation: Drug delivery and skin toxicology*. J Control Release 1996, 36: 229-33.

45. Wang, Su, Kara, M., Krishnan, T.R. *Transdermal delivery of cyclosporin-A using electroporation.* J Control Release 1998, 50: 61-70.

46. Chang, S., Hofmann, G.A., Zhang, L., Deftos, L.I., Banga, A.K. *The effect of electroporation on iontophoretic transdermal delivery of calcium regulating hormones*. J Control Release 1998, 50: 225-35.

47. Zewert, T.E., Pliquett, U.F., Langer, R., Weaver, J.C. *Transport of DNA antisense oligonucleotide across human skin by electroporation.* Biochim Biophys Res Commun 1995, 212: 286-92.

48. Prausnitz, M.R., Edelman, E.R., Gimm, J.A., Langer, R., Weaver, J.C. *Transdermal delivery of heparin by skin electroporation.* BioTechnology 1995, 13: 1205-8.

49. Sharma, A., Kara, M., Smith, F.R., Krishnan, T.R. *Transdermal drug delivery using electroporation I: Factors influencing in vitro delivery of terazosin hydrochloride in hairless pig*. J Pharm Sci 2000, 89: 528-35.

50. Vanbever, R., Langers, G., Montmayeur, S., Preat, V. *Transdermal delivery of fentanyl: Rapid onset of analgesia using skin electroporation*. J Control Release 1998, 50: 225-35.

51. Sharma, A., Kara, M., Smith, F.R., Krishnan, T.R. *Transdermal drug delivery using electroporation II: Factors influencing skin reversibility in electroporative delivery of terazosin hydrochloride in hairless rats.* J Pharm Sci 2000, 89: 536-44.

52. Vanbever, R., Fouchard, D., Jadoul, A., De Moore, N., Preat, V., Marty, P. *In vivo noninvasive evaluation of hairless rat skin after high voltage pulse exposure.* Skin Pharmacol Appl Skin Physiol 1998, 11: 23-34.

53. Prausnitz, M.R., Seddick, D.S., Kon, A.A. et al. *Methods for in vivo tissue electroporation using surface electrodes.* Drug Deliv 1993, 1: 125-31.

54. McDaniel, D.H. *Ultrasound enhancement of percutaneous drug absorption.* US 6030374.

55. Ogden, J.E. *Sonophoretic drug delivery system*. US 5656016.

56. Mitragotri, S., Blankschtein, D., Langer, R. *Transdermal protein delivery using low-frequency sonophoresis.* US 6002961.

57. Gaertner, W. *Frequency dependence of acoustic cavitation*. J Acoust Soc Am 1954, 16: 977-80.

58. Mitragotri, S., Farrell, J., Tang, H., Terahara, T., Kost, J., Langer, R. *Determination of threshold energy dose for ultrasound-induced transdermal drug transport*. J Control Release 2000, 63: 41-52.

59. Mitragotri, S., Edwards, D.A., Blankschtein, D., Langer, R. *A Mechanistic study of ultrasonically enhanced transdermal drug delivery*. J Pharm Sci 1995, 84: 697-706.

60. Johnson, M.E., Mitragotri, S., Blankschtein, D., Langer, R., Pishko, M., Kost, J. *Chemical and physical enhancers and ultrasound for transdermal drug delivery*. US 5947921.

61. Hikima, T., Hirai, Y., Tojo, K. *Effect of ultrasound application on skin metabolism of prednisolone 21-acetate.* Pharm Res 1998, 11: 1680-3.

62. Riesz, P., Kondo, T. *Free radical formation induced by ultrasound and its biological implications.* Free Radical Biol Med 1992, 13: 247-70.

63. Mitragotri, S., Blankschtein, D., Langer, R. *Ultrasound-mediated transdermal protein delivery.* Science 1995, 269: 850-3.

64. Tachibana, K., Tachibana, S. *Transdermal delivery of insulin by ultrasonic vibration.* J Pharm Pharmacol 1991, 73: 270-1.

65. Tachibana, K. *Transdermal delivery of insulin to alloxan-diabetic rabbits by ultrasonic exposure.* Pharm Res 1992, 9: 952-4.

66. Mitragotri, S., Blankschtein, D., Langer, R. *Transdermal drug delivery using low-frequency sonophoresis*. Pharm Res 1996, 13: 411-20.

67. Bommannan, D., Menon, G.K., Okuyama, H., Elias, P.M., Guy, R.H. *Sonophoresis II. Examination of the mechanism(s) of ultrasound-enhanced transdermal drug delivery*. Pharm Res 1992, 9: 1043-7.

68. Mitragotri, S. *Synergistic effect of enhancers for transdermal drug delivery*. Pharm Res 2000, 17: 1354-9.

69. Srinivasan, V., Higuchi, W.I., Sims, S.M., Ghanem, A.H., Behl, C.R. *Transdermal iontophoretic drug delivery: Mechanistic analysis and application to polypeptide delivery.* J Pharm Sci 1989, 78: 370-5.

70. Rao, V.U., Misra, A.N. *A comparative evaluation of the effect of penetration enhancers on passive diffusion and iontophoretic transport of insulin.* Indian Drugs 1995, 32: 437-41.

71. Tomohira, Y., Machida, Y., Onishi, H., Nagai, T. *Iontophoretic transdermal absorption of insulin and calcitonin in rats with newly-devised switching technique and addition of urea.* Int J Pharm 1997, 155: 231-9.

72. Zakewski, C.A., Wasilewski, J., Cawley, P., Ford, W. *Transdermal delivery of regular insulin to chronic diabetic rats: Effect of skin preparation and electrical enhancement.* J Control Release 1998, 50: 267-72.

73. Choi, E.H., Lee, S.H., Ahn, S.K., Hwang, S.M. *The pretreatment effect of chemical skin penetration enhancers in transdermal drug delivery using iontophoresis.* J Control Release 1999, 12: 326-35.

74. Hao, J.S., Zheng, J.M., Yang, W.Z. *Transdermal iontophoresis of insulin: Effect of penetration enhancers on blood glucose level in diabetic rats.* Acta Pharm Sinica 1995, 30: 776-80.

75. Kanikkannan, N., Singh, J., Ramarao, P. *Transdermal iontophoretic delivery of bovine insulin and monomeric human insulin analogue.* J Control Release 1999, 59: 99-105.

76. Srinivasan, V., Su, M.H., Higuchi, W.I., Behl, C.R. *Iontophoresis of polypeptides: Effect of ethanol pretreatment of humna skin.* J Pharm Sci 1990, 79: 588-91.

77. Bhatia, K.S., Gao, S., Singh, J. *Effect of penetration enhancers and iontophoresis on the FT-IR spectroscopy and LHRH permeability through porcine skin.* J Control Release 1997, 47: 81-9.

78. Bhatia, K.S., Singh, J. *Synergistic effect of iontophoresis and a series of fatty acids on LHRH permeability through porcine skin.* J Pharm Sci 1998, 87: 462-9.

79. Bhatia, K.S., Singh, J. *Mechanism of transport enhancement of LHRH through porcine epidermis by terpenes and iontophoresis: Permeability and lipid extraction studies.* Pharm Res 1998, 15: 1857-62.

80. Rastogi, S.K., Singh, J. *Lipid extraction and iontophoretic transport of leuprolide acetate through porcine epidermis*. Int J Pharm 2001, 215: 214-49.

81. Bhatia, K.S., Gao, S., Freeman, T.P., Singh, J. *Effect of penetration enhancers and iontophoresis on the ultrastructure and cholecystokinin-8 permeability through porcine skin.* J Pharm Sci 1997, 86: 1011-5.

82. Jiang, S.J., Hwang, S.M., Choi, E.H., Elias, P.M., Ahn, S.K., Lee, S.H. *Structural and functional effects of oleic acid and iontophoresis on hairless mouse stratum corneum.* J Invest Dermatol 2000, 114: 64-70.

83. Grewal, B.S., Naik, A., Irwin, W.J. et al. *Transdermal macromolecular delivery: Real-time visualization of iontophoretic and chemically enhanced transport using two photon excitation microscopy.* Pharm Res 2000, 17: 788-95.

84. Vanbever, R., Prausnitz, M.R., Preat, V. *Macromolecules as novel transdermal transport enhancers for skin electroporation.* Pharm Res 1997, 14: 638-44.

85. Ilic, L., Gowrishankar, T.R., Vaughan, T.E., Herndon, T.O., Weaver, J. *Spatially constrained skin electroporation with sodium thiosulfate and urea creates transdermal microconduits.* J Control Release 1999, 61: 185-202.

86. Le, L., Kost, J., Mitragotri, S. *Synergistic effect of ultrasound and iontophoresis on transdermal drug delivery: Applications to heparin delivery.* Pharm Res 2000, 17: 1151-4.

87. Mitragotri, S., Ray, D., Farrell, J. et al. *Synergistic effect of ultrasound and sodium lauryl sulfate on transdermal drug delivery*. J Pharm Sci 2000, 89: 892-900.

88. Kost, J., Pliquett, U., Mitragotri, S., Yamamoto, A., Weaver, J., Langer, R. *Enhanced transdermal delivery: Synergistic effect of ultrasound and electroporation.* Pharm Res 1996, 13: 633-8.

89. Bellhouse, B.J., Sarphie, D.F., Greenford, J. *Needleless syringe using supersonic gas flow for particle delivery.* EP 0693119.

90. Bellhouse, B.J., Sarphie, D.F., Greenford, J. *Method of delivering powder transdermally with needleless injector*. US 5630796.

91. Sarphie, D.F. *Gas propulsion of microprojectiles for the transformation of biological cells.* Ph.D. dissertation, University of Oxford, 1992.

92. Sarphie, D.F., Varadi, A., Bellhouse, B.J., Ashcroft, S.J.H. *Transdermal delivery of powdered bovine insulin to diabetic and non-diabetic Wistar rats.* Diabetes 1995, 44(Suppl. 1): 129a.

93. Burkoth, T.L., Bellhouse B.J., Hewson, G., Longridge, D.J., Muddle, A.G., Sarphie, D.F. *Transdermal and transmucosal powdered drug delivery.* Crit Rev Ther Drug Carrier Sys 1999, 16: 331-84.

94. Sarphie, D.F., Greenford, J., Ashcroft, S.J.H., Bellhouse, B.J. *Transdermal powdered delivery (TPD): In vivo determination of particle penetration depth and therpaeutic efficacy,* Proc 4th Percutaneous Penetration Conf 1996, 4B.

95. Hickey, P.L., Longridge, D.J., McCrossin, L.E., Bellhouse, B.J., Ashcroft, S.J.H. *Administration of insulin via dermal PowderJect®: Reproducible and dose-dependent delivery to the anaesthetized rat*. Am Assoc Pharm Sci Conf (Nov, San Francisco) 1998.

96. Foldavari, M., Baca-Estrada, M.E., He, Z., Hu, J., Attah-Poku, S., King, M. *Dermal and transdermal delivery of protein pharmaceuticals: Lipid-based delivery systems for interferon α.* Biotechnol Appl Biochem 1999, 30: 129-37.

97. du Plessis, J., Egbaria, K., Ramachandran, C., Weiner, N. *Topical delivery of liposomally encapsulated γ-interferon.* Antiviral Res 1992, 18: 259-65.

98. Vutla, N.B., Betageri, G.V., Banga, A.K. *Transdermal iontophoretic delivery of enkephalin formulated in liposomes*. J Pharm Sci 1996, 85: 5-8.

99. Cevc, G. *Transferosomes, liposomes and other lipid suspensions on the skin: Permeation enhancement, vesicle penetration, and transdermal drug delivery.* Crit Rev Ther Drug Carrier Syst 1996, 13: 257-388.

100. Cevc, G., Blume, G. *Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force.* Biochim Biophys Acta 1992, 1004: 226-32.

101. Paul, A., Cevc, G., Bachhawat, B.K. *Transdermal immunization with an integral membrane component, gap junction protein, by means of ultradeformable drug carriers, transferosomes.* Vaccine 1998, 16: 188-95.

102. *IDEA obtains promising results in pilot study of transdermal insulin*. DailyDrugNews.com (Daily Essentials) November 4, 1999.