

Transdermal Delivery of Antisense Oligonucleotides

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1. Introduction

In recent years, antisense oligodeoxynucleotides (ODNs) have emerged as promising biopharmaceutical therapeutics. These agents specifically target genes or gene transcripts involved in pathogenesis. Several clinical trials have demonstrated the therapeutic value and low toxicity of ODNs (1–5). Because ODNs, like most biopharmaceuticals, are highly susceptible to degradation in the gastrointestinal environment and only poorly absorbed through biomembranes, they have been delivered primarily by parenteral infusion. This route of administration, however, is invasive and inconvenient, and repeated injections or lengthy infusions can be painful and disruptive to normal life. Therefore, transdermal delivery is being explored as a more patient-friendly alternative (6).

Transdermal drug delivery has a long history dating back to the use of plasters and poultices to introduce medicinal compounds into local skin sites or directly underlying tissue. More recently, the potential of the transdermal route for systemic drug delivery has been recognized and exploited. Transdermal delivery offers many of the same advantages as drug delivery by parenteral infusion: both routes of administration can be used in nauseated patients, are minimally affected by intake of food, can be easily interrupted, and allow compounds to escape first-pass metabolism in the liver. Added advantages of transdermal delivery systems over parenteral infusion include noninvasiveness and ease of use.

Transdermal delivery of ODNs is complicated by the presence of nucleases in the skin, which are known to degrade topically delivered ODNs (7). Modification with phosphorothioate (PS) can render an ODN more nuclease-resistant, protecting it from any significant degradation in the skin within a 24-h period (8) and increasing its metabolic half-life in the liver to greater than 24 h (9,10). Structural modification of an ODN at the 2'-position of the carbohydrate moieties or the C(5) position of any pyrimidine moieties can also increase its nuclease resistance (11–14).

The major obstacles to transdermal delivery of ODNs, however, are their large size and their hydrophilicity. Because of its specialized structure, skin is generally only permeable to small, lipophilic drugs (15). This chapter will discuss the various strategies that have been employed to increase permeability of the skin to ODNs without causing undue irritation.

2. Structure and Function of Skin

Skin is composed of two main layers, the inner dermis and the outer epidermis. To become systemically available, a transdermally delivered compound has to traverse the avascular epidermis and reach the dermis, where abundant capillaries provide a vascular surface of approx 1–2 cm² per cm² of skin surface for exchange of substances between blood and skin. In contrast to the largely acellular dermis, the epidermis is composed entirely of cells. Of those cells, 80% are derived from keratinocytes, which owe their name to an intracellular abundance of keratin, a water-insoluble, filamentous protein. Within the epidermis, the keratinocytes are organized into defined strata. The innermost layer of the epidermis, the stratum germinativum, continuously generates new keratinocytes, leading to a renewal of the entire epidermis on average every 14 d. As the keratinocytes move outward from the stratum germinativum, they undergo a series of differentiations. In the stratum spinosum, the flattening cells become filled with cellular organelles (lamellar granules), which extrude their lipid contents into the intercellular space in the next layer, the stratum granulosum. Keratinocytes reach their terminally differentiated state as corneocytes in the stratum corneum, the next and outermost epidermal layer, which represents the main permeation barrier of the skin.

Corneocytes are devoid of all organelles and filled instead with keratin filaments enmeshed in a protein matrix. The normal plasma membrane is replaced by the cornified cell envelope, a unique structure consisting of a highly insoluble, cross-linked protein inner surface and a ω -hydroxyceramide monolayer outer surface that anchors the corneocytes in a lipid matrix of unusual composition. The corneocyte “bricks” are arranged in a regular, interdigitated pattern in this lipid “mortar,” forming a “wall” about 15–20 cell layers deep. This formidable barrier is only disrupted by skin appendages such as hair follicles and sebaceous and sweat glands, which cover less than 1% of the surface area.

The primary function of the skin, the body’s largest organ, is protection from the environment. The physical barrier created by the stratum corneum prevents loss of water and other vital substances from the body and entry of foreign substances or microorganisms into the body. A second line of defense is created by antigen-presenting Langerhans cells in the epidermis and dendritic cells (DCs) in the dermis, which can activate the innate and/or acquired immune system in response to any invading microorganisms or foreign substances (16).

3. Enhancement of Transdermal Transport

In principle, transdermal transport can proceed along three different pathways: the transcellular, intercellular, and appendageal pathways (17). The specialized structure of the stratum corneum, however, limits transcellular and intercellular transport to small and hydrophobic molecules. Hydrophilic macromolecules such as ODNs can only find entry through skin appendages disrupting the stratum corneum.

Chemical additives or mechanical abrasion can be used to disrupt an area of the stratum corneum, allowing ODNs to penetrate into the dermis through the trans- or intercellular routes; this strategy, however, inherently involves the risk of skin irritation. More promising strategies focus on enhancing ODN transport through skin pores, either by increasing the rate of transport through existing skin appendages or by amplifying the number of available skin pores through creation of transient artificial micropores. Because ODNs are highly water-soluble and charged, their flux through

skin pores can be increased by application of a low-level electrical field, a process known as iontophoresis. New conduits for ODN transport can be opened by electroporation and low-frequency ultrasound, which cause a localized and temporary breakdown of the structure of the stratum corneum. Alternatively, artificial aqueous pathways through the superficial layers of the skin can be generated thermally or mechanically using miniaturized wires or precision-fabricated microprojections, respectively.

3.1. Chemical Enhancement

Use of a mixture of polyethylene glycol (PEG) and linoleic acid to reduce the barrier function of the stratum corneum resulted in a marked increase in the passive flux of an uncharged antisense molecule through mouse skin (6). Chemical enhancement of ODN transport could also be achieved through addition of ethanol or 1-menthol or through delipidization of the stratum corneum by solvent treatment (18). However, these approaches may be limited by the irritancy potential of the additives (19).

Mehta et al. (20) formulated a cream containing anti-ICAM-1 phosphorothioate ODNs. At 2% concentration, this cream suppressed about 66% of ICAM-1 expression in the treated skin in a mouse model and lead to a noticeable reduction of ICAM-1 expression in human skin grafted onto severe combined immunodeficient (SCID) mice.

3.2. Iontophoretic Delivery

In iontophoresis, charged molecules are driven into the skin by application of a low level electrical field over long periods of time. Transport of chemical agents across a membrane under the influence of an electrical field is governed by three forces (21): passive diffusion, electromigration, and electro-osmosis. For charged molecules, electromigration, which is believed to proceed predominantly through appendageal pathways (22), represents the major driving force. Electro-osmosis, the convective flow of solvent and species dissolved in it, may become important for the transport of large molecules including ODNs (23). In contrast, the contribution of passive diffusion to the transdermal transport of large, charged molecules such as ODNs is negligible owing to the lipophilic nature of the stratum corneum.

The high water-solubility and charged nature of ODNs characterize them as good candidates for iontophoresis. In vitro iontophoretic ODN delivery through hairless-mouse skin, hairless rat skin, or human skin has been used to determine the influence of pH, salt content, current density, and ODN structure on transport. Oldenberg and colleagues (7) measured the effect of nucleotide composition on the flux of ODNs with a length of 15 nucleotides (15mers). Comparable fluxes were found for oligomers containing only deoxyadenine [(dA)₁₅], only thymidine [(dT)₁₅], or all four nucleotides in a random sequence; in contrast, lower fluxes were recorded for the corresponding (dG)₁₅ or (dC)₁₅ homo-oligomers or for aptamers. This result suggests that the three-dimensional (3D) structure of an ODN plays an important role in iontophoresis. The significance of structure formation for the iontophoretic mobility of ODNs is supported further by the findings that reversing the sequence of bases in an ODN affected the flux (14) and that single nucleotides crossed the dermo-epidermal junction less readily than ODNs did (8).

Brand and Iversen demonstrated that PS modification of ODNs had little or no effect on iontophoretic transport through hairless mouse skin (24). In contrast, 2'-O-methoxyethylation of the carbohydrate moieties in a 20-mer decreased the flux rate to approximately one-fourth of that observed for a PS ODN with the same sequence (un-

published results from authors' lab). In terms of therapeutic value, however, an up to 10-fold increase in potency for the 2'-*O*-methoxyethyl ODN more than compensated for its reduced iontophoretic mobility. A C(5)-propyne modified PS ODN not only showed more than 67-fold higher potency than the unmodified PS ODN, but also exhibited a fivefold higher iontophoretic flux rate (25).

In previous *in vivo* studies in mice (7,8,14,22,25), iontophoresis enhanced the transdermal delivery of ODNs, but did not result in delivery of therapeutically relevant doses. However, the emergence of highly potent modified ODNs with increased iontophoretic flux rates has generated new interest in this mode of transdermal ODN delivery (25).

3.3. Electroporation and Sonophoresis

Electroporation employs brief high-voltage pulses to create highly transient, aqueous pores in the lipid network of the stratum corneum. Upon electroporation, a FITC-labeled anti-c-myc ODN applied to heat-stripped human skin *in vitro* localized in regions approx 30 μm in diameter, demarcating the transient skin pores (26). Regnier and Preat and coworkers examined the parameters controlling transdermal delivery of ODNs through hairless rat skin by electroporation (27) and found an increase in ODN delivery of two orders of magnitude over passive diffusion (28). In addition to its effect on the stratum corneum, electroporation also increased the permeability of cell membranes, resulting in enhanced uptake of topically applied ODN into keratinocytes in the deeper layers of the epidermis (8,29–31). Nuclear localization of ODNs in skin cells after electroporation indicated the feasibility of this method for local skin treatment with ODNs (22). Use of electroporation in the clinic, however, may be limited by patient compliance and device requirement issues.

In sonophoresis, pulses of low-frequency ultrasound are used to increase skin permeability to macromolecules through thermal, mechanical, and cavitation effects on the stratum corneum (32). Experimental results indicate that cavitation of keratinocytes might be the key mechanism in enhancing transdermal transport by low-frequency ultrasound. Use of sonophoresis for transdermal delivery of macromolecules, in particular protein hormones, is under active evaluation (Sontra Medical Corporation, Franklin, MA). Parameters such as frequency, intensity, medium, and time of application affect the efficiency and safety of this method.

3.4. Mechanical Enhancement

Removal of the stratum corneum by either mechanical abrasion or tape stripping significantly enhanced permeation through the skin for a wide range of pharmaceuticals, including PS ODNs (18,21,22). However, practical applications of this method of enhancement have been limited owing to lack of control and reproducibility. In contrast, artificial microchannels or micropores through the stratum corneum can be generated in a highly defined and reproducible manner. These artificial channels form aqueous transport pathways for the passive diffusion or iontophoretic delivery of ODNs. Because the micropores are confined to the epidermis, which does not contain nerve endings or blood vessels, their formation causes little or no pain or bleeding. Depending on their size and whether or not the site is under occlusion, microchannels will self-seal after 1–24 h (33,34).

Two types of devices are currently used to generate micropores through the skin. In the thermal MicroPor™ system (Altea Therapeutics, Tucker, GA), a pulse of thermal

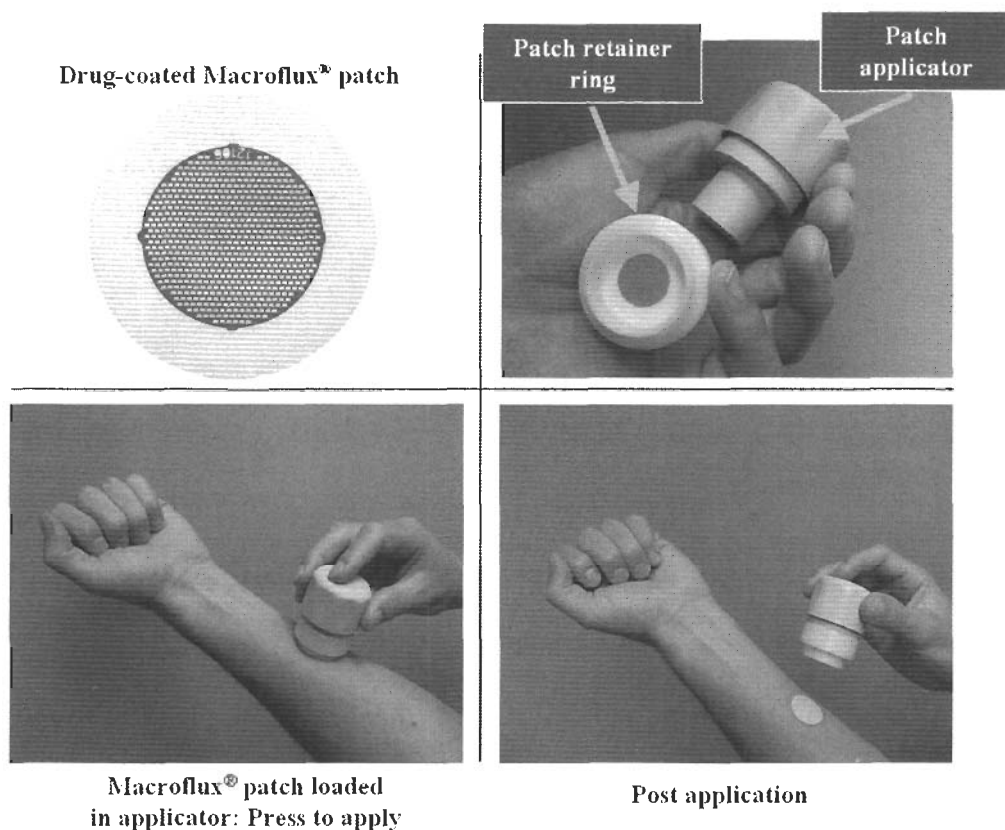


Fig. 1. A prototype Macroflux® transdermal system.

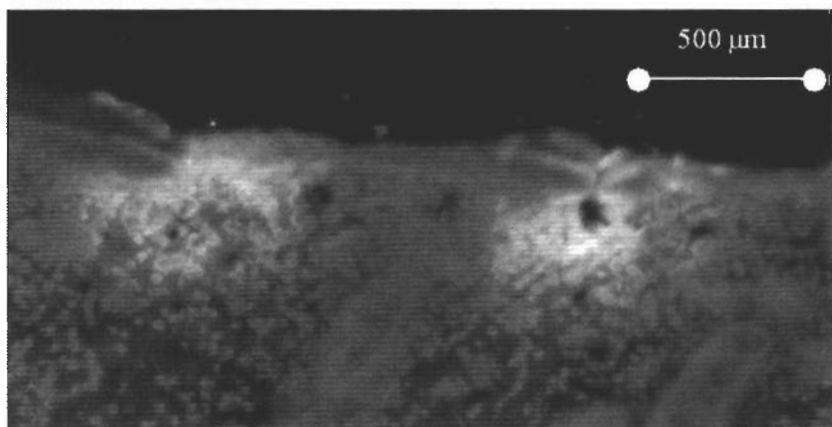
energy delivered from an array of metallic heating filaments placed directly against the skin causes the formation of micropores through the stratum corneum.

Mechanical creation of artificial transport pathways is achieved with microfabricated microprojection arrays of up to 970 projections/cm². The shape of the titanium or silicone microprojections and their angle relative to the skin surface varies according to the application, but is uniform within the array. Microprojections are generally less than 225 μm long to avoid penetration into the dermis. For bolus delivery, drug can be coated directly onto the microprojections (35). Alternatively, the arrays can be integrated with a drug reservoir for passive or iontophoretic delivery (36).

Macroflux® systems (ALZA Corporation, Mountain View, CA) use a spring-loaded, self-actuated device to apply the microprojection array to the skin, resulting in uniform and reproducible penetration (Fig. 1) (34). When the application site of a microprojection array was stained with India ink, the skin surface showed a uniform penetration pattern. Depth analysis revealed that fewer than 30% of the microprojections penetrated deeper than 100 μm , and none went beyond 350 μm . The average depth of penetration was less than 100 μm and thus lay within the 50–150 μm thickness range of both human and hairless guinea pig epidermis (35). As expected, this very shallow microprojection penetration did not result in any topical adverse effects.

We have successfully used the Macroflux® system for *in vivo* transdermal ODN delivery in hairless guinea pigs (36). Iontophoretic flux of an anti-ICAM-1

A One hour passive delivery with Macroflux®



B One hour iontophoretic delivery with Macroflux®

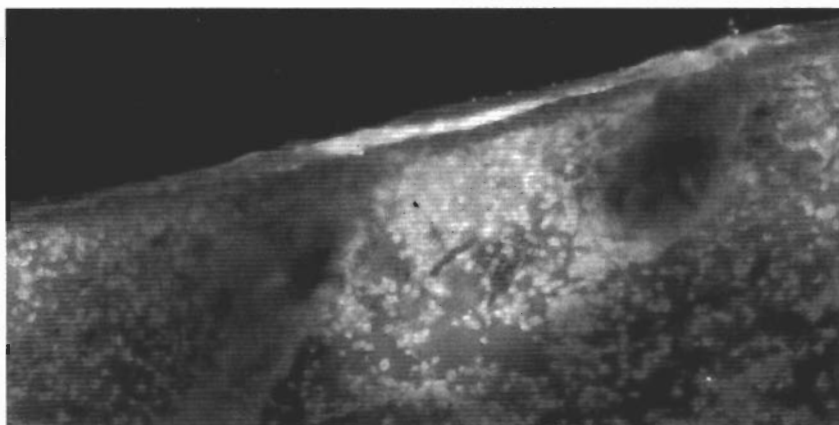


Fig. 2. Distribution of a randomized FITC-labeled phosphorothioate ODN in the skin following transdermal delivery by Macroflux®. Skin samples from hairless guinea pigs were taken after 1 h of Macroflux®-aided delivery with either a passive system (A) or an iontophoretic system (B). Skin samples were also counterstained with propidium iodine (PI), a red fluorescent and DNA-binding dye, to highlight cells in the skin.

phosphorothioate ODN (ISIS 2302) was increased more than 100-fold by pretreatment of the skin with a Macroflux® microprojection array. After Macroflux®-assisted delivery of a randomized FITC-labeled phosphorothioate ODN, fluorescence was found superimposed on the pathways created by the microprojections (Fig. 2); enhanced lateral diffusion in the epidermal layers underneath the stratum corneum was also apparent. In contrast, fluorescence was not observed in areas of intact skin. These results indicate that ODN transport primarily proceeds through the artificial microchannels/micropores created by the microprojection array, whereas skin appendages play only a minimal role in ODN delivery under these conditions. Surprisingly, Macroflux®-assisted iontophoretic delivery of ODNs appeared to be independent of nucleotide sequence or com-

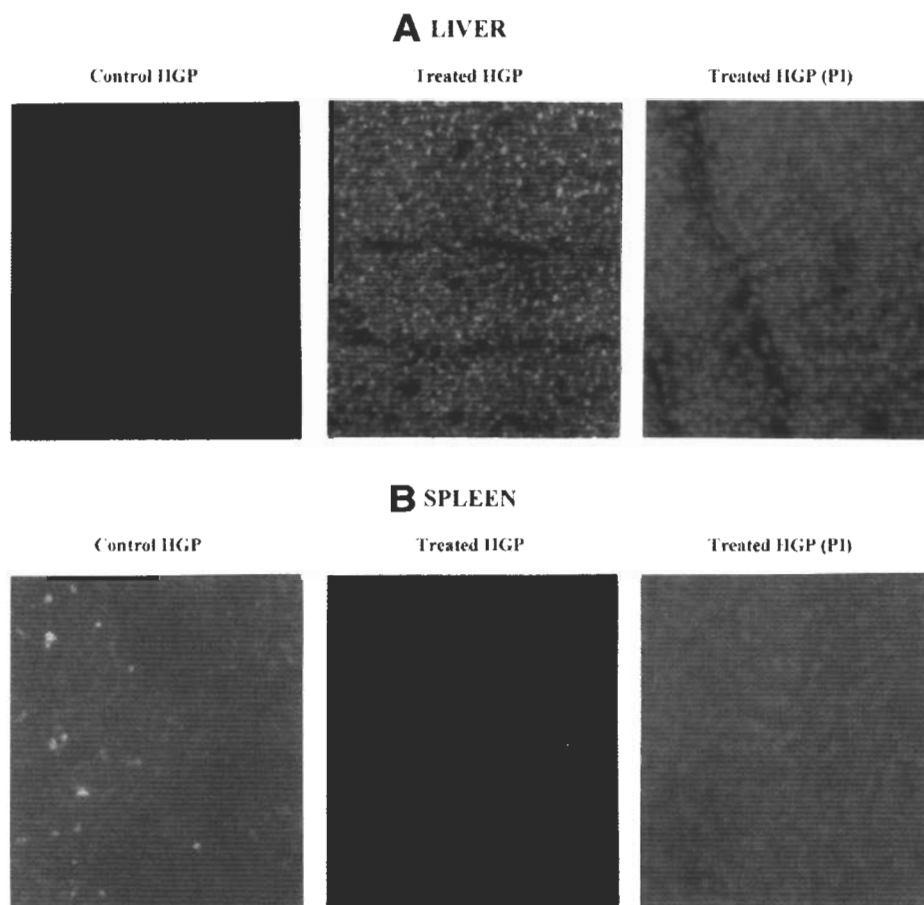


Fig. 3. Systemic localization of a randomized FITC-labeled phosphorothioate ODN following transdermal delivery. Organ samples were taken from hairless guinea pigs 2 h after receiving no ODN (control) or ODN delivered by a Macroflux[®] system. Tissues were immediately frozen, then cryotomed and viewed under a fluorescent microscopy. Some tissue slides were counterstained with PI to reveal cellular nuclei. Green FITC fluorescence appeared to be localized intracellularly in the liver (A), as well as in the spleen (B).

position, which were shown to affect the iontophoretic mobility of ODNs in the absence of microchannels (14). This finding suggests that micropores created by the Macroflux[®] system are less selective than skin appendages, which appear to favor small and positively charged species (23,24).

In skin biopsies from Macroflux[®] application sites, ODN was detected at concentrations of up to 1 mg/cm³ skin (full thickness) (36), a value that is pharmacologically relevant for local therapy (18). Following Macroflux[®] delivery, fluorescence from the randomized FITC-labeled phosphorothioate ODN could also be detected in samples taken from the liver, the spleen, and the duodenum, where it was located mostly intracellularly (Fig. 3). The intensity of the green FITC was rather strong in the liver (Fig. 3A), slightly weaker in the spleen (Fig. 3B), and even weaker in the duodenum (not shown). The quantity of intact phosphorothioate ODN detected by capillary electrophoresis in liver samples after Macroflux[®]-assisted iontophoretic delivery was similar

to that seen after intravenous injections (36). The amount of the ODN in livers of hairless guinea pigs could reach more than 4 mg following transdermal delivery, which is approx 0.2 mg/g tissue. These results suggest that the Macroflux[®] system is effective in both local and systemic delivery of antisense ODNs.

4. Conclusion

Therapeutic use of ODNs would greatly benefit from the availability of a patient-friendly and noninvasive delivery method. Recent advances in increasing the potency of ODNs through structural modifications have made these macromolecules suitable for transdermal delivery. The most promising transdermal delivery method to date is based on the controlled generation of aqueous pathways through the stratum corneum, either mechanically or thermally, to enhance passive or iontophoretic delivery. Using the Macroflux[®] system, a mechanical device for the controlled creation of micropores through the superficial layers of the skin, we demonstrated successful transdermal delivery of antisense ODNs in vivo in hairless guinea pigs. Following Macroflux[®]-assisted transdermal delivery, ODNs were detected both in the skin and systemically distributed to various other organs. At the application site, a pharmacologically relevant concentration of ODN could be achieved. Given the ongoing improvements in ODN chemistry, transdermal or topical delivery of ODNs for therapeutic use is now not only attractive but also feasible.

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